Short communication

Is Apolipoprotein A-I a regulating protein for the complement system of cod (Gadus morhua L.)?

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Apolipoproteins are a heterogenic class of lipid-associated proteins found in the plasma and other body fluids of vertebrates. Apolipoprotein is a constituent of the hydrophilic coat that surrounds the lipids and different types are characteristic for different lipoprotein densities. Apolipoprotein A-I (ApoLP A-I) is the major protein component of high density lipoproteins (HDL) [1–3].

ApoLP A-I is an activator of the lecithin-cholesterol-acyl-transferase (LCAT), a plasma enzyme involved in cholesterol metabolism [1]. Several other functions have been attributed to ApoLP A-I. It is, for example, involved in the binding of LPS (lipopolysaccharide) [4], antiviral activity has been described [5,6] and ApoLP A-I isolated from carp (Cyprinus carpio) has been shown to have heparin binding activity implicated in nerve regeneration processes [7]. A regulatory role in the complement system has also been discovered, ApoLP A-I primarily acting as an inhibitor of the membrane attack complex, C5b-9 [8–11].

The complement system of cod (Gadus morhua L.) has been studied at our institute in recent years [12,13]. The initial emphasis was on isolating and characterizing the complement component C3, the central component of the three complement pathways, and on the production of a specific anti-C3 antibody [13]. This was tackled in three ways:

1. Purification of C3 was attempted, based on the well documented ability of C3 to bind to zymosan, an insoluble preparation of yeast (Saccharomyces cerevisiae) [14] and to MacroGard (MG), an insoluble yeast cell wall, beta-1,3-and beta-1,6-linked glucan.

For the preparation of zymosan-absorbed serum proteins, zymosan prepared at our laboratory was used, washed in complement fixation test buffer (prepared from tablets, Oxoid, UK) containing 0.1% gelatine (CFT-G). This was then mixed with cod serum and the suspension agitated overnight at 4 °C. After centrifugation, the supernatant was discarded, the zymosan was washed with CFT-G buffer and bound proteins eluted with distilled water. The eluted proteins were collected after centrifugation and concentrated by filter centrifugation. The protein yield was about 50–100 µg ml⁻¹ serum.

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For the preparation of MG-absorbed serum proteins, MG (from Biotec Pharmacon Asa, Norway, provided by Jarl Bøgwald, University of Tromsø, Norway), washed in CFT-G buffer, was mixed with cod serum and incubated with agitation at room temperature for 1 h. The suspension was centrifuged, washed and resuspended in PBS.

Polyclonal antibodies against the zymosan-absorbed and eluted proteins and the MG suspension were produced in mouse ascites according to Overkamp et al. [15] and are referred to as anti-C3/Z and anti-C3/MG antibodies respectively.

(2) Cod serum samples, showing either the characteristic heat stable and EDTA enhanced high haemolytic activity of cod sera [12] or no haemolytic activity, were separated on Superose 6 gel filtration column and a single 1 ml fraction (about 180–200 kDa) showing haemolytic activity or/and reacting with the anti-C3/Z and anti-C3/MG antibodies was collected from individual serum separations. The fraction from a haemolytic serum was further purified using MonoQ ion exchange column and the final product used to produce polyclonal antibody in mouse ascites. This antibody is referred to as anti-Haem antibody.

(3) The third approach was conventional chromatographic methods for C3 purification described by Lange et al. [13]. This involved PEG precipitation of cod plasma, the supernatant then being sequentially passed through ion exchange, gel filtration and ion exchange columns. Antibody against the purified C3 component was produced in mouse ascites and is referred to as anti-C3 antibody [13].

Fig. 1 shows Western blotting analysis of cod serum, the zymosan-absorbed and eluted serum proteins and purified C3 using these four antibodies. As well as detecting the \(\alpha-\) and/or \(\beta-\) chains of C3 (verified by monospecific anti-\(\alpha-\) and anti-\(\beta-\) chain antibodies) all the antibodies detected a 30 kDa band in all preparations (lanes 1–3). The reaction was, however, very faint with the anti-Haem antibody (Fig. 1c). An approximately 11 kDa band was also detected in all the preparations by all the antibodies except the anti-C3/Z antibody. Analysis using monospecific antibody against the \(\alpha-\) chain suggests that the other protein bands, for example seen in Fig. 1d, lane 3, may be breakdown fragments of the \(\alpha-\) chain, which is relatively unstable [13].

SDS–PAGE analysis of the haemolytic fraction or anti-C3/Z/anti-C3/MG reactive fraction from the Superose 6 column is shown in Fig. 2, showing a silver stained gel. The most noticeable feature is the apparent absence of the 30 kDa band in the fraction from the haemolytic serum and its strong presence in the fraction from the non-haemolytic serum. However, the anti-C3/Z and anti-C3/MG antibodies detected a faint band in this area indicating the strong immunogenic nature of this protein.

The zymosan-absorbed and eluted serum proteins were separated by SDS–PAGE under reducing conditions and transferred to a polyvinylidene difluoride (PVDF) membrane. After staining with amido black the 30 kDa band was excised and N-terminal amino acid analysis carried out. This work was kindly done by K. Bailey, Department of Biochemistry, University of Nottingham UK, using an Applied Biosystems 473A automated protein sequencer. The sequence obtained for the first 10 amino acids was DAPSQLAHIR. This sequence showed 70% homology with the N-terminal sequence of Apolipoprotein A-I (ApoLP A-I) from rainbow trout (Oncorhynchus mykiss) [3].

The anti-C3/MG antibody was used to screen a cDNA library (prepared from cod liver) for the complement component C3. After three consecutive immunoscreenings, two positive clones were identified and sequenced. From the positive insert in the plasmid, 399 bp were sequenced and 133 N-terminal amino acids were deduced. The sequence was checked in Fasta search (European Bioinformatics Institute) and a 50% similarity was found to Apolipoprotein A-I precursor from rainbow trout. The sequence alignment between the 133 N-terminal amino acids of cod and rainbow trout ApoLP A-I precursor is shown in Fig. 3.

ApoLP A-I is synthesized in the liver as a preproapolipoprotein. The pre-segment is cleaved cotranslationally inside the cells and the proApoLP A-I released into the plasma. The pro-segment is then cleaved by a specific plasma protease and converted into the mature ApoLP A-I [16]. In all species so far described the pre-segment consists of 18 amino acids whereas the pro-segment consist of 5 amino acids in fish and 6
amino acids in higher vertebrates [3,17,18]. The ApoLP A-I of cod according to the present results appears to have a longer pre-segment than previously described in other species or 27 amino acids instead of 18 (Fig. 3).

The association of the 30 kDa ApoLP A-I with the approximately 190 kDa complement component C3 in cod serum withstood extensive purification of C3 by repeated ion exchange and gel filtration chromatography. The link was maintained in an aqueous phase in a relatively high salt concentration (during ion exchange chromatography) but was dissociated by SDS. This suggests a hydrophobic association of the two molecules rather than covalent bonding. Analysis under non-reducing conditions showed that ApoLP A-I was a monomeric structure.
This close association of ApoLP A-I with the C3 component and the reduced presence in cod serum showing high haemolytic activity lead to the speculation that ApoLP-A-I might be an inhibitor of the haemolytic activity and have a regulatory role in the complement system of cod.

Steps are being taken to purify ApoLP A-I from cod serum to investigate its role further. In the meantime the effects of human ApoLP A-I, (from Sigma, USA, cat. no. 0722), on the spontaneous haemolytic (SH) activity of four cod sera were examined. The haemolytic assay has been described before [12]. The results showed that the human ApoLP A-I (5.5 mg ml⁻¹ serum for 2 h at 4 °C) significantly reduced the haemolytic activity from SH₅₀% 664 ± 254 (mean and standard deviation) to 384 ± 134, the level of significance being $P=0.026$.

Fig. 2. SDS–PAGE (silver staining) analysis of a single fraction showing maximum haemolytic activity or maximum reactivity with anti-C3/Z antibody: (a) fraction from serum showing high spontaneous haemolytic activity (b) fraction from serum showing no haemolytic activity. Lane 1: protein molecular weight standards as shown, lane 2: the serum fraction.

| Cod         | GTRLHQTNMTKFAALALALLAVGSHAAAMQSDAPSQALHIRSTAVGMYLD | 50 |
| Rainbow trout | MQFLALALTILLAAATQAVPMQADAPSQLEHVKVAMMEYMA         | 41 |

| Cod         | QVKDSXARSLDHLGTEYESYKAQLATSDLQASIKTAQAGAEPYTDAA | 100 |
| Rainbow trout | QVKETAQRSIDHLDTEYKEYKVQLSQSLODNLQQYAQATASESLAPSEAI | 91 |

| Cod         | VAQVMEATTEVRASIMADIETLRTXPFPQS---K | 133 |
| Rainbow trout | GQVLTEATAAVRAEVMKDVELRSQLEPKRAELKEVLKDHIYKRLEP | 141 |

Fig. 3. Comparison of 133 and 141 (of 262) N-terminal amino acids of preproapolipoprotein A-I from cod and rainbow trout respectively. The pre-segment is indicated by a solid line and the pro-segment by a dotted line. The N-terminal amino acid sequence of the mature protein is shown in bold typescript.
Although this association between ApoLP A-I and C3 has not been described before, examination of data from other species indicates in some instances the presence of an unidentified 30 kDa protein during the purification process of C3 [19].

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References

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