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Isolation and characterization of SAP and CRP, two pentraxins from *Pangasianodon* (*Pangasius*) *hypophthalmus*

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ABSTRACT

From the serum of *Pangasianodon hypophthalmus*, two proteins were isolated by affinity chromatography on Sepharose and phosphorylcholine–Sepharose. Their binding on the affinity matrices critically depends on the presence of Ca^{2+} ions. N-terminal sequencing and sequencing of internal tryptic peptides identified the proteins as pentraxins and from their binding properties they are identified as SAP (serum amyloid P component) and CRP (C-reactive protein). Per ml serum, 36 μg SAP and 56 μg CRP was purified. Upon gel filtration, both the SAP and CRP elute as trimers of respectively 24 kDa and 28 kDa subunits. Both proteins are devoid of inter-chain disulfide bonds. Both SAP and CRP are glycosylated and agglutinate rabbit erythrocytes and pathogenic bacteria *Edwardsiella ictaluri* and *Aeromonas hydrophila*, but not *Micrococcus lysodeikticus* or *Escherichia coli*. Haemagglutination of SAP and CRP is inhibited by galactose (MIC = 1 mM) and by phosphorylcholine (MIC = 1–2 mM), respectively. Circular dichroism studies revealed that antiparallel β-pleated sheets are dominating the secondary structure. Upon removing the Ca^{2+} ions by EDTA, slight structural changes are observed by CD spectroscopy in the near-UV region. Immunodiffusion shows that *P. hypophthalmus* SAP and CRP do not cross-react.

1. Introduction

Today, about 30% of all fish and shellfish produced for human consumption is grown in aquaculture [1]. Concomitant with the intensification of fish production in culture, there is a growing risk of infection and stress that adversely affect productivity and sustainability of fish farming.

The diverse series of reactions that are initiated as a response to infection, injury or trauma is called the acute phase response. These reactions aim at eliminating infective organisms and preventing further tissue damage, but also at restoring the host's normal functions [2]. Some plasma proteins such as pentraxins are known to undergo notable changes in concentration during an acute phase response. Positive or negative acute phase proteins where originally defined as proteins of which the concentration increases, respectively decreases by at least 25% during disorder [3]. Pentraxins [4] constitute a superfamily of serum proteins that can be classified as either short pentraxins, such as C-reactive protein (CRP) and serum amyloid P component (SAP), or long pentraxins such as TSG-14/PTX3. While the former are produced in the liver of many organisms [5–7], the latter, comprising a C-terminal domain related to CRP/SAP and an unrelated N-terminal domain, are expressed in different tissues [8,9]. CRP was first discovered in human serum in 1930 by Tillet and Francis [10], while SAP was identified as a plasma component of amyloid in 1967 by Cathcart et al. [11]. In mammals they are pentameric proteins with identical subunits that are often glycosylated. CRP and SAP share extensive sequence homology, indicating that they evolved from a common ancestor protein [12]. For example, human CRP and SAP show nearly 50% sequence identity. Especially in mammals, the pentraxins are well characterized, both structurally and functionally. They are implicated in pathogen recognition, clearance of tissue debris, regulation of the inflammatory reaction, prevention of autoimmunization and they are involved in the activation of the classical complement system [13]. During infection, human CRP serum levels can increase a 1000-fold and as

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such it is an excellent marker of the acute phase [14]. While CRP is the main positive acute phase protein in *e.g.* human, rat and pig, SAP is the positive acute phase protein in mice, but neither CRP nor SAP seem to be induced upon infection and/or stress in guinea pig [4]. These differences in the regulation of expression result from changes in genetic elements that belong to regions on the DNA that are critical for the acute phase response [4].

Both proteins are however not only confined to mammals but homologous proteins have also been found in the serum of birds and amphibians, *e.g. Xenopus laevis* [15], and in the haemolymph of some molluscs, *e.g. Achatina fulica* [16], and arthropods, *e.g.* horseshoe crabs *Limulus polyphemus*, *Tachypleus tridentatus* and *Carcinosporus rotundicauda* [17–20] and in fish. SAP and/or CRP have been isolated and characterized from several teleost fish species [21–42], but also from cartilaginous fish [41,43,44]. In spite of their substantial sequence conservation throughout evolution, pentraxins vary considerably in terms of ligand-binding specificity, oligomerization, serum concentration, complement activation capacity and acute phase behaviour [5]. Only in some fish species they have been proven to be acute phase proteins whose level in the serum changes, though much less pronounced than in mice and man, upon infection, inflammation, injury or abiotic stress situations such as heavy metal pollution or other environmental pollutants (*e.g.* [22,23,25,28,29,35,45]).

Pentraxins are currently intensively used as markers of the acute phase, both in human and veterinary medicine [14,46]. As a prelude to investigate whether CRP and/or SAP of *Pangasianodon hypophthalmus* can be used as a biomarker to monitor the health status of this fish, both proteins were purified and characterized. SAP and CRP are generally isolated by affinity chromatography based on their substrate selectivity. CRP mainly recognizes the phosphate ester of phosphorylcholine, a common component of fungal and bacterial polysaccharides and cell membranes [47], while the main ligands of SAP are polysaccharides and cellular matrix components including heparin, mannose-6-phosphate, 3-sulphated saccharides, 4,6-cyclic pyruvate acetal of galactose [48], glycosylaminoglycan [49], bacterial endotoxic lipopolysaccharide (LPS) and phosphoethanolamine [50,51]. SAP shares the latter binding capacity with CRP. Both CRP and SAP require Ca^{2+} for ligand-binding activity.

In this paper, we describe the purification and characterization of SAP and CRP from the serum of *P. hypophthalmus*, an economically important fish that is especially cultured in the Mekong delta in Vietnam. This fish is grown in cages or pens in the Mekong river, or in ponds in the country side. Since 2001, the production of *P. hypophthalmus* increased from 100,000 tons to 1,200,000 tons in 2007 [52]. Except from being a local rather cheap protein source for the Vietnamese, about half of the production is exported, mainly to EU, Russia and other Asian countries, thereby generating an important income for the country (http://www.globefish.org/).

2. Materials and methods

2.1. Collection of serum samples

Blood was collected from adult *P. hypophthalmus* that were slaughtered for consumption (Mekong Fisheries Enterprise, Tra Noc Industrial Zone, Can Tho City). The blood was allowed to clot for 2 h at 4 °C and then centrifuged at 4 °C during 20 min at 650 *g* (Rotanta 46R centrifuge, Hettich). The serum obtained was stored frozen (at −80 °C) until use.

2.2. Purification of serum amyloid P component (SAP)

SAP was purified by affinity chromatography making use of its known Ca-dependent binding to Sepharose [53]. To 50 ml of thawed serum was added a protease inhibitor cocktail (Sigma Chemical Company, St. Louis, MO, USA) consisting of 1 mM phenylmethylsulfonyl fluoride (PMSF; from a freshly prepared stock solution of 40 mM in absolute ethanol), 1 μM leupeptin (from a 1 mM stock solution in bidistilled water) and 1 μM pepstatin (from a 1 mM stock solution in methanol). It was then dialyzed 2 h against TBS (20 mM Tris–HCl buffer + 150 mM NaCl) containing the same protease inhibitors and 10 mM $CaCl_2$, pH 7.5. The serum sample was clarified by centrifugation (20 min at 27,000 *g*) and applied, at a flow rate of 0.5 ml min^{-1}, to a 20 ml Sepharose-4B (GE Healthcare) column (1.6 × 10 cm) that was equilibrated with the same buffer. The column was washed with the same buffer until the absorbance at 280 nm of the effluent was zero, and SAP was eluted with TBS buffer containing 10 mM EDTA, pH 7.5. The fraction obtained was extensively dialyzed against TBS buffer pH 7.5 containing 10 mM $CaCl_2$ and stored at −20 °C until further use.

2.3. Purification of C-reactive protein (CRP)

To the unbound fraction recovered from the previous chromatography with TBS containing protease inhibitors and 10 mM $CaCl_2$ was again added 1 mM PMSF. The fraction was then applied, at a flow rate of 0.5 ml min^{-1}, to a 5 ml Sepharose-PC (phosphorylcholine) column (Pierce, Perbio Science Belgium) (1 × 6.5 cm) that was equilibrated with the same buffer. Unbound proteins were removed by washing with the same buffer, and CRP was eluted with TBS buffer containing 10 mM EDTA, pH 7.5. The fraction obtained was extensively dialyzed against TBS buffer pH 7.5 containing 10 mM $CaCl_2$ and stored at −20 °C until further use.

2.4. Concentration of protein solutions

Protein solutions were concentrated by centrifugation in Vivaspin tubes (VivaScience, Hannover Germany) with a molecular weight cut-off of 3500.

2.5. Protein concentration measurements

Protein concentrations were determined using bicinchoninic acid [54] with bovine serum albumin as standard protein. The assays were performed in flat-bottom ELISA plates (Nunc, Maxisorp) that were read at 595 nm in a BioRad 3550 microplate reader. Each sample was assayed in quadruplicate.

2.6. Agglutination and inhibition of agglutination

Haemagglutination assays were performed using rabbit erythrocytes. The cells were washed three times by centrifugation (15 min at 400 *g*) with saline–azide (150 mM NaCl containing 0.02% w/v NaN_3) and resuspended in saline–azide to obtain a final concentration of 4% (v/v). Protein samples were serially diluted in U-shaped microtiter plates with equal volumes of saline–azide to give a final volume of 25 μl in each well. To each dilution were then added 50 μl of the erythrocyte suspension, and the plates were incubated at room temperature for 1 h. The titer is defined as the reciprocal of the highest dilution causing complete agglutination of the erythrocytes.

For haemagglutination-inhibition assays, the ligands to be tested were serially diluted with saline–azide and an equal volume (25 μl) of agglutinin-containing sample was added that was properly diluted to give a titer of four in the absence of the inhibitor. The mixture was incubated for 15 min at room temperature before adding 50 μl of the erythrocyte suspension. Results are expressed as the final ligand concentration inhibiting four units of the agglutinin.