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Why working with porcine circulating serum amyloid A is a pig of a job

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HIGHLIGHTS

- The conformation of pig circulating SAA was described.
- The unfruitful attempts of purifying porcine SAA were detailed.
- An *in silico* analysis of pig SAA structure and properties was performed.
- Pig systemic acute phase SAA showed the structural properties of locally produced SAA.
- Pig circulating SAA showed a complex conformation and peculiar biochemical properties.

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ABSTRACT

Serum amyloid A (SAA) is a major acute phase protein in most species, and is widely employed as a health marker. Systemic SAA isoforms (SAA1, and SAA2) are apolipoproteins synthesized by the liver which associate with high density lipoproteins (HDL). Local SAA (SAA3) isoforms are synthesized in other tissues and are present in colostrums, mastitic milk and mammary dry secretions. Of systemic SAA the bulk is monomeric and bound to HDL, and a small proportion is found in serum in a multimeric form with a buried HDL binding site. In most species, systemic SAA could easily be studied by purifying it from serum of diseased individuals by hydrophobic interaction chromatography methods. For years, we were not able to isolate systemic pig SAA using the latter methods, and found that the bulk of pig SAA did not reside in the HDL-rich serum fractions but in the soluble protein fraction mainly as a multimeric protein.

Based on these surprising results, we analysed *in silico* the theoretical properties and predicted the secondary structure of pig SAA by using the published pig primary SAA amino acid sequence. Results of the analysis confirmed that systemic pig SAA had the highest homology with local SAA3 which in other species is the isoform associated with non-hepatic production in tissues such as mammary gland and intestinal epithelium. Furthermore, the primary sequence of the pig SAA *N*-terminal HDL binding site did differ considerably from SAA1/2. Secondary structure analysis of the predicted alpha–helical structure of this HDL binding site showed a considerable reduction in hydrophobicity compared to SAA1/2. Based on these results, it is argued that systemic acute phase SAA in the pig has the structural properties of locally produced SAA (SAA3). It is proposed that in pig SAA multimers the charged *N*-terminal sequence is buried, which would explain their different properties.

It is concluded that pig systemic SAA is unique compared to other species, which raises questions about the proposed importance of acute phase SAA in HDL metabolism during inflammation in this species.

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1. Introduction

Serum amyloid A (SAA) refers to a family of differentially expressed proteins that comprises two isotype groups: acute phase SAA and constitutive SAA. Acute phase SAA expression is dramatically induced after inflammation or tissue damage either in liver (SAA1 and SAA2, known as the systemic, circulating isoforms) or in other tissues (SAA3, known as the local isoform), while constitutive SAA (SAA4) expression is independent of the acute phase reaction (Uhlar and Whitehead, 1999). Within the acute phase isotypes, the circulating isoforms SAA1 and SAA2 share common features, such as a neutral IEP and a high degree of homology, whereas local SAA3 is highly alkaline and share less homology with the circulating forms (Uhlar and Whitehead, 1999). SAA4 is a more divergent relative, whose main differences with acute phase isoforms are the presence of an octapeptide insert and a glycosylation site (de Beer et al., 1995).

Circulating SAA is a small protein (12–15 kDa, depending on the species) often regarded as an apolipoprotein, since in human serum the majority is bound to HDL (Uhlar and Whitehead, 1999). Although it's exact structure has yet to be elucidated, analysis of human and murine circulating SAA primary sequences has revealed that its a hydrophobic, amphipathic protein consisting of a *N*-terminal helical bundle that comprises the 80% of the protein and a remaining potentially disordered *C*-terminus that might serve as ligand binding region (Stevens, 2004). However, it is known that human and murine HDL-free circulating SAA can also self-aggregate to form different order multimers, possibly by the above mentioned *C*-terminal region (Wang et al., 2002). Interestingly, the SAA HDL binding site, identified as the 1–11 amino acid *N*-terminal peptide, seems to be buried within the hexamer of mouse SAA 2.2 (Wang and Colón, 2004). Furthermore, the 1–11 amino acid *N*-terminal peptide has been identified as essential for AA-amyloid fibril formation, and deletions or residue substitutions in this peptide prevented AA-fibrillogenesis (Röcken and Shakespeare, 2002; Patel et al., 1996; Westermark et al., 1992).

The sequence of SAA proteins is highly conserved through the evolution of eutherian mammals, and SAA can be found not only in a wide range of vertebrates like mammals, marsupials, fish, birds, and also invertebrates like the sea cucumber (Santiago–Cardona et al., 2003). Considering this, together with the wide range of tissues where SAA is expressed, and the dramatic induction of SAA production shortly after tissue damage, it seems evident that SAA plays an essential protecting role in the organism. Nonetheless, the range of SAA functions is still unclear. Proposed functions of circulating SAA include a major role in recycling and reusing cholesterol from damaged cells (Kisilevsky and Manley, 2012), immunomodulatory functions on polymorphonuclear cells (Badolato et al., 2000; Song et al., 2009) and antimicrobial properties (Shah et al., 2006). However, it is generally considered that such a high number and variety of functions seem improbable for a protein of this size. In fact, the functionality of this protein has been recently related to its aggregation state and the conformational changes in SAA provoked by ligand binding (Wang et al., 2002; Raynes, 2006; Björkman et al., 2010; Wang et al., 2011).

Although SAA has been extensively studied in humans and some other species, little is known about porcine SAA, although a number of peculiarities have been described in this species. Pigs, as rats, show an extraordinary resistance to the development of AA-amyloidosis (Niewold et al., 2005) and their circulating SAA sequence show the properties of local SAAs, in contrast with humans and other species (Soler et al., 2011). These results suggest that the apparent resistance of both species to amyloidosis is due to the exclusive expression of the more alkaline local-like SAAs. This also implies pig circulating SAAs to be less hydrophobic and not bound to HDL (Kisilevsky and Manley, 2012). If so, it is predicted that HDL-free SAA would self-aggregate by their C-terminal end in the form of different-order multimers (Stevens, 2004).

The main objective of the present study was to contribute to the better understanding of pig systemic SAA, which appeared to have peculiar properties and in the authors' experience shows also erratic, unpredictable behaviour, unlike systemic SAA from other species. Here we applied different traditional isolation methods based on SAA hydrophobicity and HDL binding to pig serum to identify the fraction in which pig SAA resides. These results were compared to the theoretical properties calculated from the pig SAA published primary sequences available in UniProtKB database and the consensus SAA sequences calculated previously (Soler et al., 2011).

2. Materials and methods

2.1. Sample collection

All the procedures involving animals were approved by the Murcia University Ethics Committee. The study population consisted in 7 pigs, conventional Duroc × (Landrace × Large White) from the experimental farm of the University of Murcia. Blood samples were taken from finishing male pigs suffering different active inflammatory processes including tail biting, abscesses and arthritis ($n=5$; SD) as well as from healthy pigs of similar age, sex breed and origin ($n=2$; SH). Blood was collected by jugular venipuncture using single-use blood collection tubes without any additive (Vacutainer, BectonDickinson) and allowed to clot for 1 h at room temperature prior to centrifugation at 2000 g for 15 min to obtain serum. The content of SAA in the studied samples was determined with a commercial enzyme-linked immunoassay following the manufacturer's instructions (Tridelta Development Plc). Samples were kept at −20 °C until time of analysis. For purification purposes, a pool of serum was prepared by mixing 2 mL of serum from the five diseased pigs and kept at −20 °C until time of analysis.

2.2. Immunodetection of pig SAA in different body fluids

Recombinant porcine SAA (rSAA) was produced as formerly described (Soler et al., 2011) and employed as positive control in immunoblotting.

A total amount of 15 μg (serum) or 5 μg (rSAA) of protein were run in 4–12% SDS-PAGE gels under reducing conditions according to standard protocols (Laemmli, 1970) using a vertical electrophoretic chamber (Mini-protean 3 electrophoresis cell, Bio-Rad). Separated proteins were transferred onto PVDF membranes by using a Semi-Dry trans-blot (Bio-Rad) and SAA was identified with an in-house produced rat anti-pig SAA biotin-labelled mAb (Soler et al., 2011), followed by streptavidin-HRP conjugate (GE Healthcare Life Sciences). For detection, chemifluorescent signal detection (ECL Plus, GE Healthcare Life Sciences) was used and for imaging, a Typhoon scanner was employed (GE Healthcare Life Sciences). All the images were digitalized and analyzed by ImageQuant TL (GE Healthcare Life Sciences). Lanes containing molecular weight markers were used as standards to calculate the molecular weight of unknown bands.

2.3. Isolation of pig SAA by Hydrophobic interaction chromatography

1 mL of pooled serum was dialyzed (Nap-10, GE Healthcare Life Sciences) against the corresponding wash buffer and filtered through a 0.22 μm filter unit (Millex, Millipore) prior to loading to the hydrophobic interaction (HIC) column (OctylSepharose FF, GE