have been used in previous purifications of SAA across several different species, and some protocols have even been proposed to be useful for large-scale purification [13]. Ultra-centrifugation is a useful method for separation of lipoproteins [17], and ultracentrifugation and hydrophobic interaction chromatography [18] have both been used as important initial steps in several protocols for SAA purification, including a protocol for purification of equine SAA [5]. When such procedures are followed by additional purification steps such as gel-filtering, SAA purities of up to 98% have been demonstrated [18]. However, the usefulness of the purified products depends on the aim of the purification. The antigenic properties of SAA can, thus, be affected by some purification procedures [14], and immuno-based SAA assays are, consequently, often based on calibration and standards consisting of recombinant SAA [19], heterologous SAA [20], or pooled acute phase serum [14,21], rather than being based on purified species-specific SAA. Even though such heterologous calibrated assays are commercial available for diagnostic measurements of equine SAA [22-24], species-specific calibration material, consisting of the native purified protein is, however, needed to obtain precise measurements of SAA across different analytical methods and different laboratorial settings [23,25,26]. However, such native protein material is currently not commercially available [22].

Based on the known biochemical properties of SAA, size-exclusive chromatography (SEC) and preparative isoelectric focusing (IEF) have been used as main procedures in previous purifications [5,13,27], but because of the lipophilic nature of SAA, and the tight association to HDL, previous purifications have always been preceded by delipidation steps [5,27,28] like ultracentrifugation [17] or hydrophobic interaction chromatography (HIC) [18].

A recent report has suggested that porcine SAA has less lipophilic properties than previously believed [29]. If this is also true for other species, the general approach to SAA purification should probably be altered and large scale purification of SAA could maybe be possible without initial denaturing delipidation or other steps which could potentially affect the antigenic properties of the purified product. The use of more gentle protocols for SAA purification could potentially facilitate a commercial production of species specific calibration material for veterinary SAA assays, but further knowledge about the biochemical properties of SAA will be needed before such protocols can be established.

The aim of the study was, therefore, to investigate the solubility as part of the biochemical properties of equine SAA and the potentials for purification of SAA using size-exclusion chromatography (SEC) and preparative isoelectric focusing (IEF) without initial delipidation.

Methods

Pooled equine acute phase serum was used in the study, based on individual samples from client owned horses remaining after diagnostic analyses at the Central Laboratory, Department of Veterinary Clinical and Animal Sciences, University of Copenhagen, Denmark. The inclusion of samples in the present study was approved by the local ethical committee at the Department of Veterinary Clinical and Animal Sciences, University of Copenhagen, Denmark. The concentration of SAA in the serum pool was measured to 1600 mg/L, using a commercial available turbidimetric immunoassay previously validated for diagnostic measurements of equine SAA by members of our group [24]. Fifty millilitres of the serum pool was freeze dried from −4°C to room temperature at 10 mbar. An overview of the analytical and preparative procedures is given in Figure 1. The presence and composition of SAA was detected in serum and preparations by IEF, electroblotting (Amersham Pharmacia Biotech), and immunostaining with anti-SAA antibodies (Tri-delta Development Ltd), as previously done in several studies of SAA executed by our group [6,30,31].

Freeze dried serum was suspended in 70% 2-propanol, 8 M urea, and Milli-Q water, respectively, in a concentration of 10 mg freeze dried serum per milliliter solvent. Supernatants were analyzed for presence of dissolved SAA isoforms.

Supercritical fluid extraction (SFE) was performed using 600 mg freeze-dried serum in a Speed SFE system (Applied Separations). The extraction was performed at 50 mPa with a flow of 4 L CO_2 per minute at 40°C for 30 minutes. Extracts were collected in glass vials and the extraction was repeated using 96% ethanol as modifier (1.0 mL/min). Extracts and remains were air dried, diluted in 8 M urea, and analyzed for the presence of SAA.

SEC was performed by fast polymer liquid chromatography (FPLC-SEC) using a Superdex™75 10/300 GL column (GE Healthcare) and a buffer containing 20 mM sodium dihydrophosphate and 50 mM sodium chloride, pH 6.9. A gel filtration standard containing thyroglobulin (660 kDa), gammaglobin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and cyanocobalamin (1.35 kDa) (Bio-Rad) was used to make a standard curve for estimating molecular weights of different FPLC-fractions. Two-hundred microliter of the filtered supernatant resulting from the suspension of freeze-dried serum in Milli-Q water (described above) was injected to the column and separated at a flow of 1 ml/min yelding a pressure of 1.5 mPa. The molecular weight of equine SAA was verified by Sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE) in Phast Gel System (Amersham Pharmacia Biotech) followed by electroblotting and immunostaining as described above. A molecular

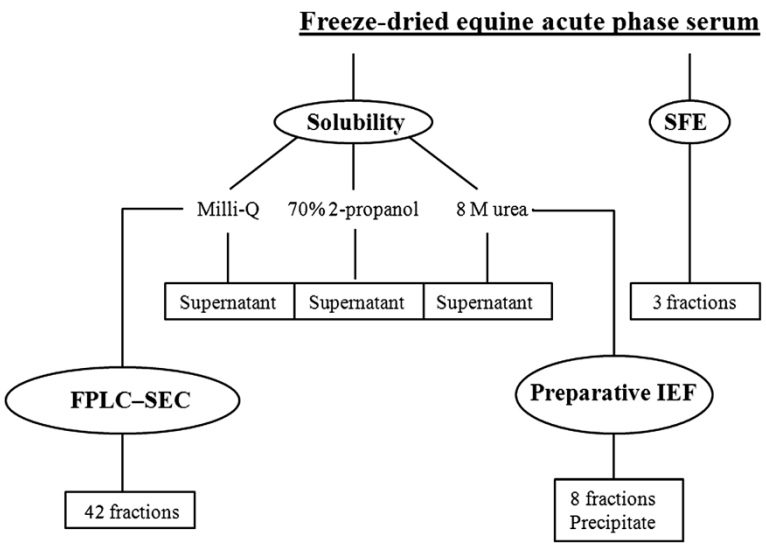

Figure 1 Overview of the procedures included in the study. Applied methods (encircled) and obtained products analyzed for SAA content and impurities (outlined in boxes) in the attempt to purify equine serum amyloid A (SAA). Isoforms of SAA were detected in supernatants, fractions, and precipitate using isoelectric focusing, electroblotting, and immunostaining. Purity of detected SAA was assessed by sodium dodecyl sulphate polyacryl amide gel electrophoresis (SDS-PAGE), followed by staining of serum proteins with silver nitrate. SFE: Supercritical fluid extraction with CO_2. FPLC-SEC: Fast polymer liquid chromatography size-exclusive chromatography. IEF: Isoelectric focusing.

weight marker (Bio Rad) was separated prior to blotting and stained with silvernitrate (Pharmacia LKB Biotechnology) [32] and used as standard.

Preparative IEF was performed in Hoefer IsoPrime IEF Purification Unit, (Amersham Pharmacia Biotech) [33,34]. Seven buffered polymembranes with pH 5, 6, 7, 7.5, 8, 8.5, and 9 were prepared with acrylamidobuffers (Fluka BioChemika and GE Healthcare Biosciences), in gels consisting of 30% acrylamide/bis-acrylamide (Sigma Aldrich), Tetramethylethylenediamine (Pharmacia Biothech) and 40% ammonium persulphate (Bio-Rad). Whatman glass microfiber filters GF/D 47 mm were encased in the membranes. Chambers were filled with Milli-Q water according to recommendations from the manufacturer, and 5 ml of the supernatant resulting from the suspension of freeze dried serum in 8 M urea (described above) was applied in separation chamber 2 (between the gels with pH 5 and 6). Separation was performed over 24 hours at a constant power of 4 W. Preparations were air dried and dissolved in 8 M urea prior to SAA detection. Visible precipitates were solubilized in 8 M urea overnight at room temperature, and the solute was investigated for presence of SAA.

Serum proteins in supernatants, fractions, and precipitate were stained with silvernitrate [32] following SDS-PAGE in Phast Gel System (Amersham Pharmacia Biotech) to assess purity of preparations, and a molecular weight marker was used as standard (Bio Rad).

Results

Equine SAA was detected in supernatants of freeze dried serum solubilized in 70% 2-propanol, 8 M urea, and Milli-Q water. At least 3 isoforms of SAA were detected in each solvent, but the isoform pattern in serum dissolved in 8 M urea resembled the pattern of isoforms detected in untreated equine serum most accurately (Figure 2).

Three fractions were obtained by SFE (Figure 1); a lipophilic fraction (extracted in pure CO_2), a lipophilic/ampophilic fraction (extracted in CO_2 modified with ethanol), and a non SFE-extractable fraction (remains). SAA was only detectable in the non SFE-extractable fraction (Figure 3).

Forty-two fractions were obtained by FPLC-SEC of freeze dried serum diluted in Milli-Q water (Figure 1). SAA was detected in fraction 9 of the SEC (Figure 4), corresponding to an estimated molecular weight of 237 kDa, even though the molecular weight of SAA in equine serum was confirmed to be 10–15 kDa through SDS-PAGE (Figure 5). The isoform pattern of SAA in the FPLC-SEC-fraction (Figure 4) seemed to be incomplete compared to the material injected to the column (the supernatant obtained by the suspension of freeze-dried serum in Milli-Q water, Figure 2, lane 3), and several other proteins were detected in the SDS-PAGE analysis of the fraction, showing impurity of the separated SAA (Figure 4). No SAA was observed in the remaining 41 FPLC fractions.