COMPARISON OF FIVE PROTOCOLS FOR THE WHOLE IgG SNAKE ANTIVENOM PURIFICATION IN TERMS OF STABILITY, PURITY AND IMMUNOGLOBULIN SUBCLASS COMPOSITION

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INTRODUCTION & AIM

Besides therapeutic action, the whole IgG antivenoms obtained from the hyperimmune animal plasma still commonly cause clinical side effects attributable to contaminating proteins and / or aggregates.[1] There has been a hypothesis in the literature that some purification procedures (precipitation of IgGs, low pH-mediated elution of IgGs in chromatography), which induce transient conformational changes of IgG molecules, make their structure less stable and more prone to aggregation, in contrast to others that leave them in solution throughout (caprylic acid precipitation of non-IgG plasma proteins).[1,2,3,4,5] Our goal was to verify this hypothesis by comparing IgGs isolated by five different commonly employed protocols from the same plasma in terms of stability, purity and immunoglobulin subclass composition. The fractionation methods were: ammonium sulphate precipitation (ASP), anion- (AEC) and cation-exchange chromatography (CEC), affinity chromatography (AC) and caprylic acid precipitation (CAP).

EXPERIMENTAL

The *V. a. ammodytes* venom-specific IgG subclass distribution within IgG purified fractions was determined by the respective in-house ELISA for IgGa, IgGb and IgG(T) subclass detection as reported.[6] $A_{492\ nm}$ to IgG concentration of tested samples gave curves described either by 2nd step polynom or natural logarithm that were used for determination of IgG concentration giving absorbance at 492 nm of 1.0 value in each subclass ELISA.

The protein concentration was measured spectrophotometrically by use of the equation γ (mg/mL) = ($A_{228.5\ nm} - A_{234.5\ nm}$) × f × dilution factor, where Ehresmann's factor "f" for equine IgG of 0.2553 was used.[7]

The purity was examined by **SDS-PAGE** on 4-12% Bis-Tris gel with MOPS as running buffer under non-reducing conditions and CBB R250 staining.

The impact of different refinement protocols on the IgG thermal stability was determined by **thermal shift assay (TSA)** as described.[8] The IgG samples (1 mg/mL in 0.2 M phosphate buffer, pH 6.0) with or without 2 M sorbitol as stabilising agent, were analysed, prior and after one-month storage at 37 °C.

Size-exclusion-HPLC was performed on TSK Gel G3000SW column (7.5 × 600 mm) with 0.1 M phosphate-sulphate running buffer, pH 6.6, at a flow rate of 0.8 mL/min. The absorbance was monitored at 280 nm.

Hyperimmune horse plasma refinement protocols

The starting material for each purification procedure was hyperimmune horse plasma (HHP) thermally treated for 1 h at 56 °C.

C**aprylic acid precipitation (CAP)** was performed by adding caprylic acid (CA) to the HHP so that final concentration of 3% (*V/V*) in 2-fold diluted reaction mixture was achieved. Following vigorous stirring (750 rpm for 1 h at 23 °C) and centrifugation (2800 x g, 45 min), IgG enriched supernatant (CAP) was obtained.

Anion-exchange chromatography (AEC) was performed in batch mode. HHP, 5-fold diluted with 25 mM Tris/HCl binding buffer, pH 8.0, containing 35 mM NaCl was incubated with Toyopearl SuperQ-650S stationary phase for 1 h at 23 °C. Following centrifugation, IgG enriched supernatant (AEC1) was collected. The remaining impurities were removed by 0.5% CA (*V/V*) precipitation and the pure IgG fraction (AEC2) was obtained.

Cation-exchange chromatography (CEC) was performed by loading HHP, 10-fold diluted with 20 mM MES binding buffer, pH 6.0 (14 mL/run) to CIMultus SO3-1 column at a flow rate of 1 mL/min. The bound material was eluted with binding buffer containing 1 M NaCl and diafiltrated into 50 mM MES buffer, pH 5.5 using 100 kDa MWCO membrane. After precipitation of non-IgG proteins from eluted fraction (CEC1) with 1% CA (*V/V*), pure IgG sample (CEC2) was obtained.

Protein A based **affinity chromatography (AC),** was performed by applying 2-fold diluted HHP (7 mL/per run) on MabSelect columns (*V* = 2 x 1 mL) with 20 mM Tris/HCl binding buffer, pH 7.4, at a flow rate of 1 mL/min. The bound antibodies were eluted with 20 mM citric acid and the pure IgG sample was obtained (AC).

Ammonium sulphate precipitation (ASP) protocol was followed as described.[9] The precipitate was dissolved in 50 mM MES buffer, pH 5.5. The obtained IgG-enriched preparation (ASP1) was diafiltrated into the same buffer using 100 kDa MWCO membrane and further purified with 2% CA (*V/V*) precipitation (ASP2).

Each IgG-enriched fraction was diafiltrated into 200 mM phosphate buffer, pH 6.0 using 100 kDa MWCO membrane.

RESULTS

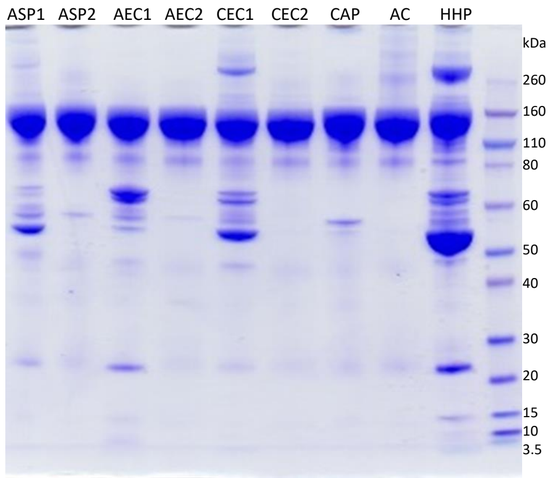

Figure 1. SDS-PAGE analysis of IgG fractions' purity under non-reducing conditions obtained by ASP, AEC, CEC, CAP and AC refinement protocols. Samples denoted with ″2″ were obtained by additional caprylic acid precipitation step. The standard is shown on the right side of the gel.

Table 1. SEC analysis of aggregates content in IgG preparations obtained by ASP, AEC, CEC, CAP and AC purification protocols. Samples denoted with ″2″ were aditionally purified by caprylic acid precipitation step.

IgG purification method	Protein aggregates (%)
ASP1	2.83
ASP2	1.31
AEC1	0
AEC2	0.43
CEC1	3.97
CEC2	1.25
CAP	2.03
AC	8.77

Table 2. SEC determination of protein aggregates in pure IgG fractions obtained by ASP, AEC, CEC, CAP and AC purification protocols after one-month storage at 37 °C with or without sorbitol as stabilising agent.

IgG purification method	Protein aggregates after one-month at 37 °C (%)	
	without sorbitol	with sorbitol
ASP2	1.56	1.54
AEC2	0	0
CEC2	1.54	1.13
CAP	2.31	1.89
AC	6.88	5.26

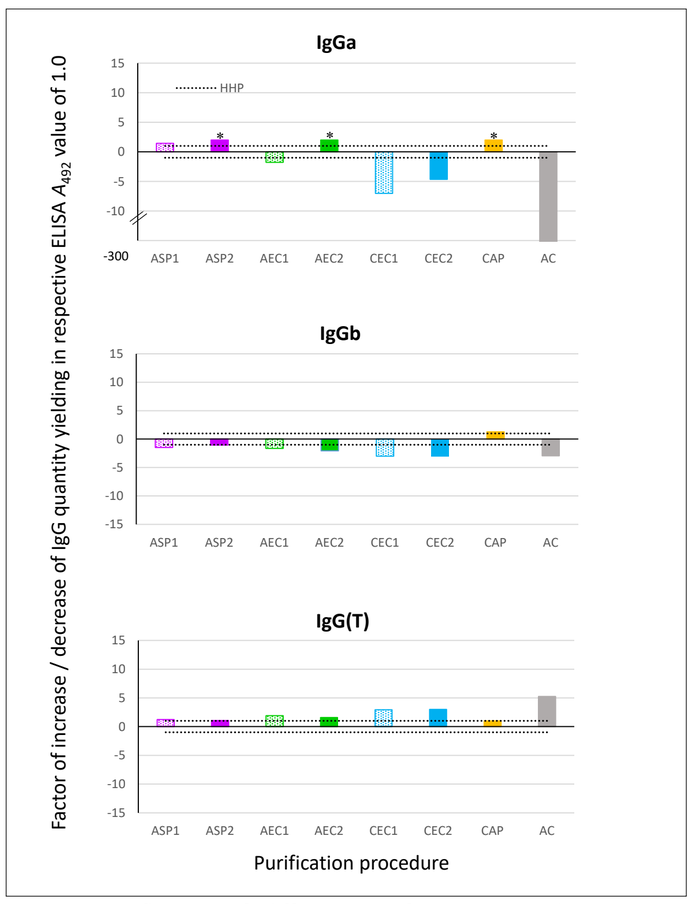

Figure 3. Influence of different purification procedures on IgG subclass composition in IgG fractions determined by respective ELISA and expressed as factor of increase / decrease of IgG quantity required to yield absorbance of value 1.0 at 492 nm. IgG quantity in samples was calculated by extrapolation from $A_{492\ nm}$ to IgG concentration curve. For each purification protocol the factor was calculated relative to the IgG quantity in HHP (hyperimmune horse plasma). Increase of the IgG by certain factor value demonstrates the loss of respective subclass, while the decrease indicate the enrichment of the sample with respective IgG subclass. In samples denoted by asterisk, the endpoint value of the factor indicating the IgGa loss could not be reached due to ELISA limitation.

- The highest purity was achieved by AC and CAP (Fig. 1), while the highest aggregates content was observed in samples from AC, CEC1 and ASP1 (Table 1).
- Additional purification step by CAP performed on IgG fractions of lower purity (ASP1, AEC1 and CEC1) reduced aggregates content in preparations obtained by ASP and CEC (Table 1).
- In samples from all studied protocols, melting temperature (*Tm*) of IgGs was around 70 °C, except for the one obtained by AC (Fig. 2), which was slightly decrased. That might be the consequence of its different subclass composition (Fig. 3) as well as the influence of conditions during the process of purification. Addition of sorbitol improved IgG thermal stabilitity in all samples (Fig. 2).
- Aggregates content (Table 2) and *Tm* of analysed preparations (Fig. 2) remained without significant change during one-month stability study.
- AC had the greatest impact on the subclass composition since it caused substantial loss of IgG(T) (by factor 5) and significant enrichment of IgGa subclass (by factor 332) (Fig. 3).
- CEC procedure showed the same phenomenon although to a lesser extent (Fig. 3).
- Other purification protocols did not have substantial impact on subclass composition, even though some loss of IgGa has been observed in samples gained by ASP, AEC and CAP. This loss might not be highly relevant for IgG function, since IgGa is one of the minor IgG subclasses in horse plasma (Fig. 3).

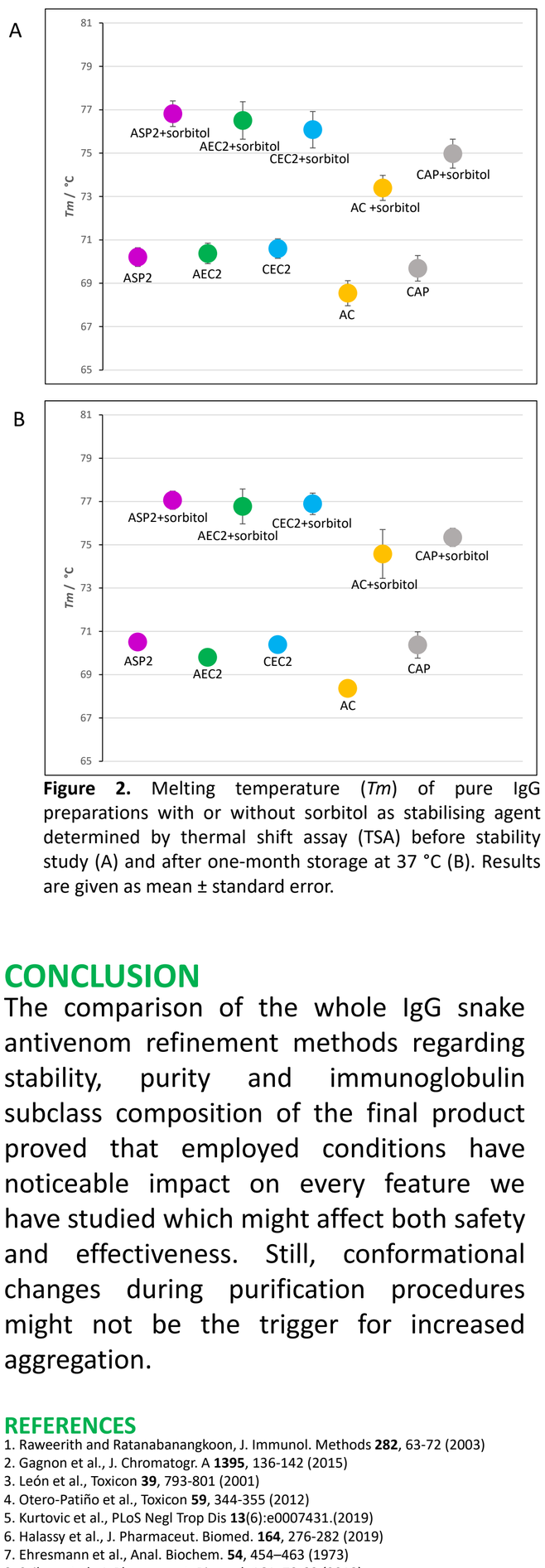

Figure 2. Melting temperature (*Tm*) of pure IgG preparations with or without sorbitol as stabilising agent determined by thermal shift assay (TSA) before stability study (A) and after one-month storage at 37 °C (B). Results are given as mean ± standard error.

CONCLUSION

The comparison of the whole IgG snake antivenom refinement methods regarding stability, purity and immunoglobulin subclass composition of the final product proved that employed conditions have noticeable impact on every feature we have studied which might affect both safety and effectiveness. Still, conformational changes during purification procedures might not be the trigger for increased aggregation.

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