

COMPARATIVE STUDY OF DIFFERENT WHOLE IgG SNAKE ANTIVENOM REFINEMENT STRATEGIES AND THEIR IMPACT ON THE PURIFIED IgG FEATURES



Sanja Mateljak Lukačević¹, Tihana Kurtović¹, Marija Brgles¹, Stephanie Steinberger², Martina Marchetti-Deschmann² and Beata Halassy¹

¹University of Zagreb, Centre for Research and Knowledge Transfer in Biotechnology, Rockefellerova 10, HR-10 000, Zagreb, Croatia

²Institute of Chemical Technologies and Analytics, TU Wien, Getreidemarkt 9, AT-1060 Vienna, Austria



INTRODUCTION & AIM

The whole IgG antivenoms are obtained from the hyperimmune animal plasma by various refinement strategies. Besides their therapeutic effect, the „refined“ antivenoms 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} The goal of this study was to verify this hypothesis by comparing IgGs isolated by five different commonly employed purification protocols from the same hyperimmune horse plasma in regard 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 $A_{492\text{ nm}}$ 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) = $(A_{228.5\text{ nm}} - A_{234.5\text{ nm}}) \times f \times \text{dilution factor}$, where Ehresmann's factor "f" for equine IgG of 0.2553 was used.^{6,7}

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. Protein bands were excised, *in-gel* digested by trypsin and the proteins identified by MALDI-TOF/TOF-MS (peptide mass fingerprinting and peptide sequencing).

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.

Caprylic 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 × 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 diafiltered 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 × 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 diafiltered into the same buffer using 100 kDa MWCO membrane and further purified with 2% CA (V/V) precipitation (ASP2).

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

RESULTS

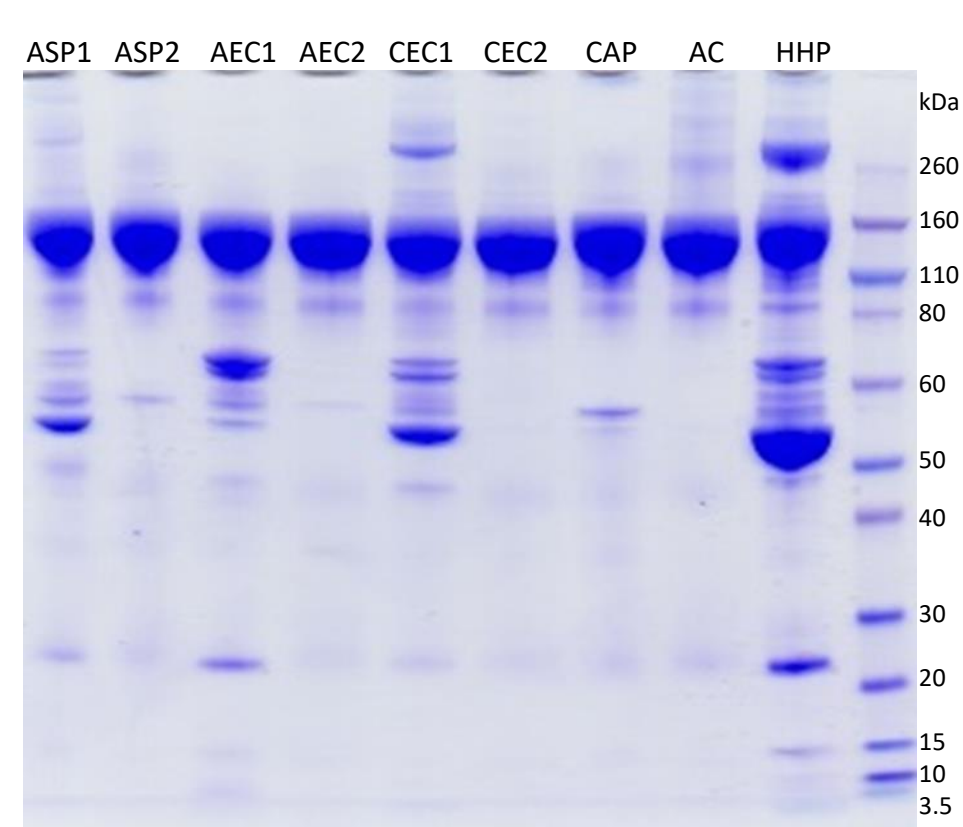


Figure 1. SDS-PAGE analysis of IgG fractions' purity under non-reducing conditions obtained by ASP, AEC, CEC, CAP and AC refinement protocols. Samples denoted with "2" were additionally purified by caprylic acid precipitation step. The standard is shown on the right side of the gel.

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 additionally 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

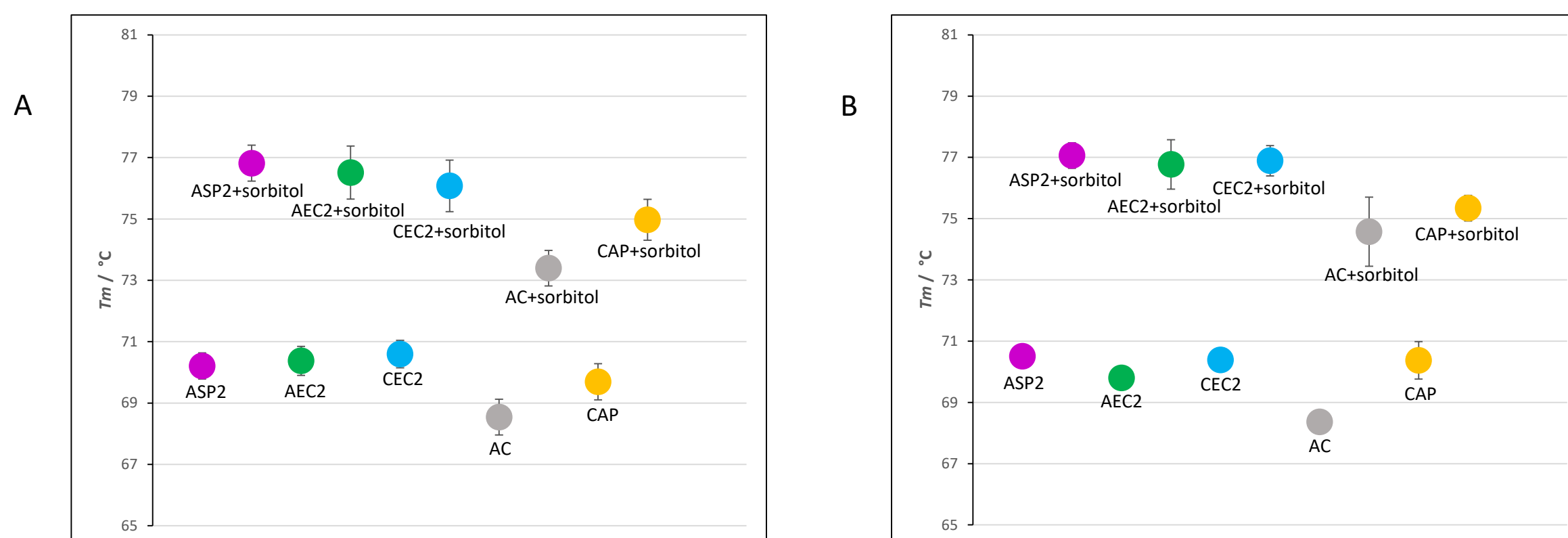


Figure 4. Melting temperature (T_m) of pure IgG preparations with or without sorbitol as stabilising agent determined by thermal shift assay (TSA) before stability study (A) and after one-month storage at 37 °C (B). Results are given as mean ± standard error.

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

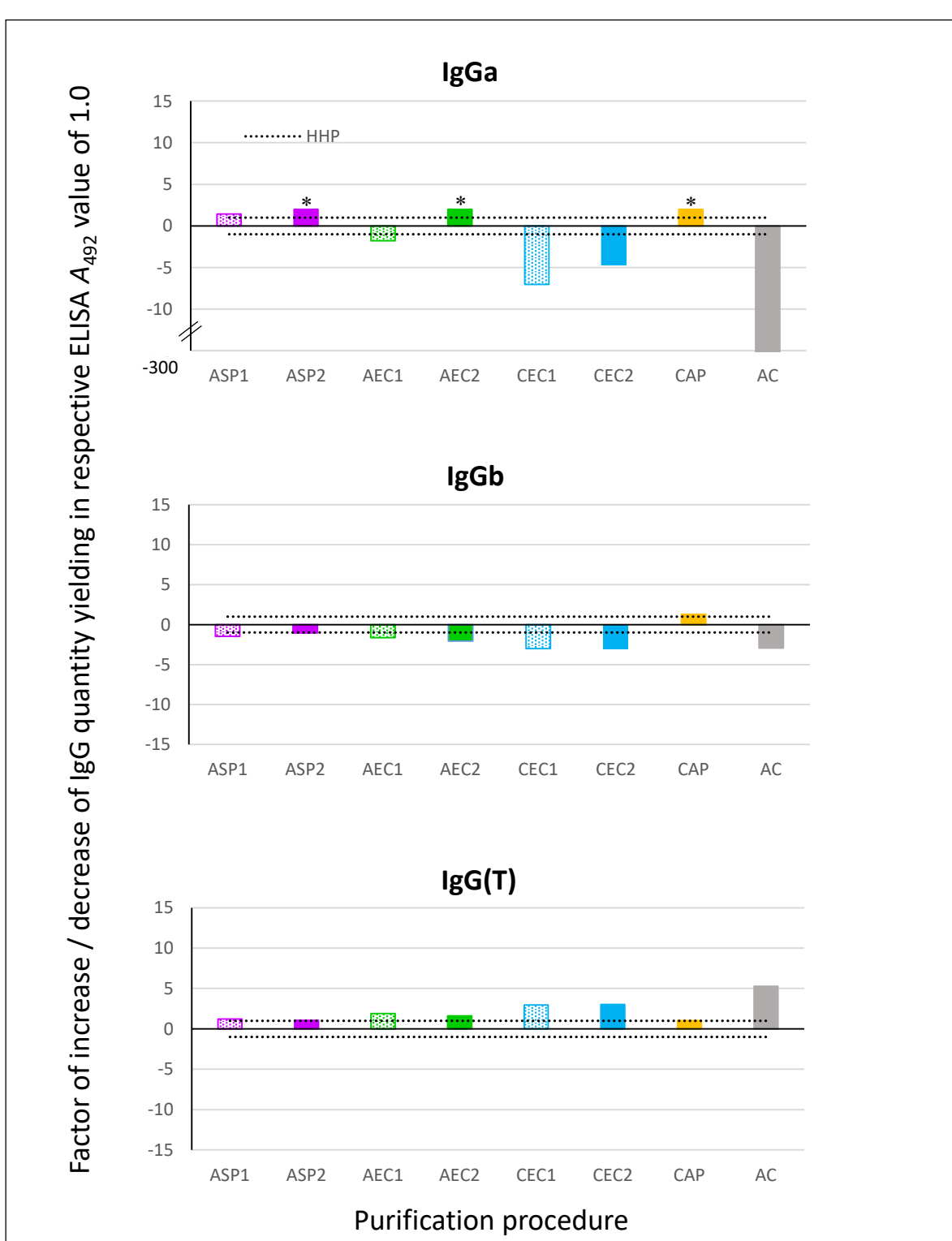


Figure 3. Influence of different purification procedures on IgG subclass composition in IgG fractions determined by respective ELISA and expressed as factor of increase / decrease of IgG quantity required to yield absorbance of value 1.0 at 492 nm. IgG quantity in samples was calculated by extrapolation from $A_{492\text{ nm}}$ 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.

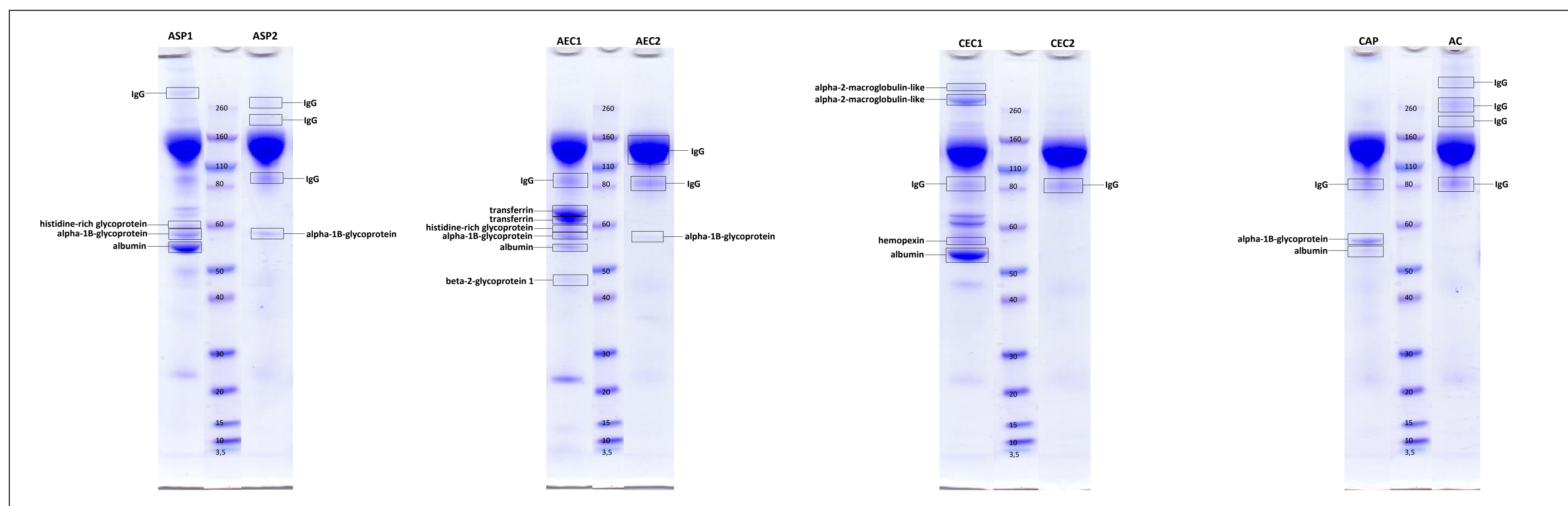


Figure 2. MALDI-TOF/TOF-MS analysis of protein bands from purified IgG fractions obtained by ASP, AEC, CEC, CAP and AC. Samples denoted with "2" were additionally purified by caprylic acid precipitation step.

- 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).
- Albumin was the main contaminating protein in IgG preparations obtained by ASP and CEC, while transferrin dominantly contaminated IgG sample purified by AEC. Alpha-1B-glycoprotein was present in CAP IgG fraction as well as in pure ASP2 and AEC2 samples (Fig. 2).
- 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).
- In samples from all studied protocols, melting temperature (T_m) of IgGs was around 70 °C, except for the one obtained by AC (Fig. 4), which was slightly decreased. 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 stability in all samples (Fig. 4).
- Aggregates content (Table 2) and T_m of analysed preparations (Fig. 4) remained without significant change during one-month stability study.

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.

ACKNOWLEDGMENT

This work was fully financially supported by Croatian Science Foundation (IP-2014-09-4915). Any opinions, findings or recommendations expressed in this material are those of the authors and do not necessarily reflect the views of Croatian Science Foundation

REFERENCES

- Raweerith and Ratanabanangkoon, J. Immunol. Methods **282**, 63-72 (2003)
- Gagnon et al., J. Chromatogr. A **1395**, 136-142 (2015)
- León et al., Toxicon **39**, 793-801 (2001)
- Otero-Patiño et al., Toxicon **59**, 344-355 (2012)
- Kurtovic et al., PLoS Negl Trop Dis **13**(6):e0074311 (2019)
- Halassy et al., J. Pharmaceut. Biomed. **164**, 276-282 (2019)
- Ehresmann et al., Anal. Biochem. **54**, 454-463 (1973)
- Sviben et al., J. Pharmaceut. Biomed. **161**, 73-82 (2018)
- Rojas et al., Toxicon **32**, 351-363 (1994)

