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THE SUBTLE CHANGE IN THE ANTIVENOM PURIFICATION PROTOCOL AFFECTS DIFFERENTLY DOWNSTREAM INTERMEDIATES AND THE FINAL PRODUCT

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INTRODUCTION

Animal-derived antibody therapeutics are widely used in medicine for viral and toxin neutralization. Although the various effective manufacturing methodologies have already been implemented into commercial scale production, due to more demanding regulatory requirements there is the constant need for their improvement. One of the major challenges in optimization concerns achieving the final product of high purity in order to ensure its safety and clinical efficiency. Recently, we have established the high yielding protocol for the preparation of snake venom-specific $F(ab)_2$ fragments from hyperimmune horse plasma. The process itself consists of two main phases: caprylic acid (CA) selective precipitation of majority of contaminating proteins and pepsin fragmentation of IgG molecules preserved in solution. In the previous research, we have noticed that the most variable part of our refining scheme was caprylic acid fractionation step, thus affecting the purity of the IgG intermediate preparation. Despite the fact that we have succeeded to obtain the $F(ab)_2$ -based final product of 100% purity, we aimed for further process improvement regarding its stability by optimizing this critical step.

AIM

Our goal was to investigate the possible beneficial impact of higher caprylic acid concentration on the IgG fraction composition in order to establish less variable and more reproducible manufacturing process. Here we present the results indicating that even small variations in operating parameters could cumulatively affect refinement strategy for immunotherapeutics preparation, influencing consequently the quality and, particularly, safety of the final product.

RESULTS

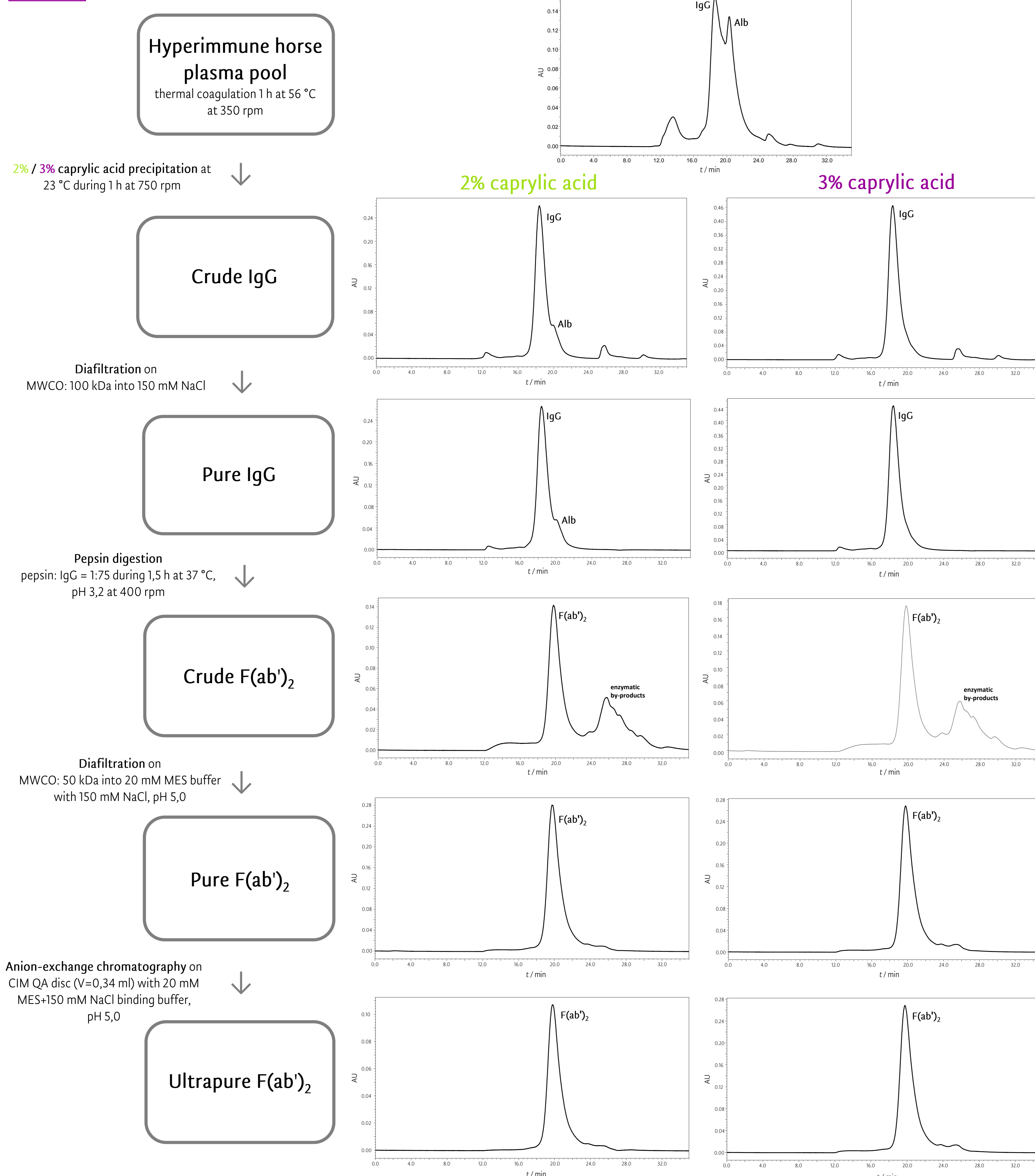


Figure 1. Schematic presentation of the antivenom purification process protocol and corresponding chromatograms of the samples from each step showing the difference in purity when 2 % and 3 % caprylic acid was employed.

The selective protein precipitation step proved to be critical in the snake antivenom preparation scheme and sensitive even to the fine change in the CA concentration. Comparison of the purity and the yield of the samples in each phase of the process showed different impact on the downstream products depending whether 2% or 3% CA was employed.

- CA in 2% concentration leads to the lower purity of the IgG product, in comparison with 3% CA, where the purity of the same product is almost complete (Figure 1; Table 1). Higher CA concentration precipitates more contaminating proteins from plasma giving sample of the higher purity.
- Yield of the IgG obtained with 3% CA concentration is slightly lower then the yield obtained with 2% CA concentration (Figure 1; Table 1). Besides contaminating protein in plasma, caprylic acid in higher concentration probably precipitates certain amount of IgG too, causing its loss in this and the subsequent steps as well.
- On the $F(ab)_2$ level both, purity and yield, are higher after fractionation of plasma with lower CA concentration. Final $F(ab)_2$ product of 100% purity was achieved as 2 % CA leaves all the IgG content intact in the solution and thus accessible for the limited hydrolysis, subsequently performed by pepsin digestion. As a result, whole content of IgG population is being cleaved into $F(ab)_2$ fragments (Figure 1; Table 1).
- Despite the fact that IgG product obtained after precipitation with 3% CA was highly pure, there was a decrease in purity of its final $F(ab)_2$ product when compared with the one obtained after CA fractionation in concentration of 2% . It could be presumed that, by pepsin digestion, not only Fc fragments of immunoglobulins were cleaved, but also non-specific cleavage of the $F(ab)_2$ fragments occurred. The resulting by-products of the enzymatic cleavage could therefore negatively affect both, the purity and the yield of the $F(ab)_2$ product.

CONCLUSION

In a cascade procedure of the therapeutic antivenoms production, even the slight variation of one parameter can have cumulative impact on the quality and safety of the final products.

Although fractionation with 3% of caprylic acid increases the purity of the IgG level in comparison with 2%, in the subsequent step of the antivenom preparation process, its seemingly positive effect reverses resulting in both, lower purity and lower yield of the $F(ab)_2$ product.

REFERENCES

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Halassy et al. (2018) J. Pharmaceut. Biomed., in press



Long-nosed viper
(*Vipera ammodytes ammodytes*)

MATERIALS AND METHODS

The protein concentrations in hyperimmune horse plasma, all intermediates and the final product were measured spectrophotometrically by using Ehresmann's method (Ehresmann et al., 1973). The appropriate dilution of each sample was prepared and measured three times in order to obtain the mean value of the concentration calculated afterwards by using following equation: $(A_{228.5\text{ nm}} - A_{234.5\text{ nm}}) \times 0.2553 = [\text{mg mL}^{-1}]$. The measured concentrations were further used for calculating yield and purity.

The concentration of venom-specific IgGs for yield and purity calculation was determined by ELISA with sample-specific correction of results (Halassy et al., 2018). The microtiter plate was coated with 100 μL /well of venom coating solution (1 mg/mL) in 50 mM carbonate buffer, pH 9.6, and left overnight at room temperature (RT). After blocking with 0.5 % (w/v) BSA in PBS/T (0.05% (v/v) Tween 20 in PBS) buffer at 37 °C for 2 h, the samples were added in two-fold serial dilutions in duplicates and left overnight at RT. In the subsequent steps, incubation with HRP-anti-horse $F(ab)_2$ (25 000-fold diluted) at 37 °C for 2 h occurred, followed by the addition of OPD (0.6 mg/ml solution) in citrate phosphate buffer, pH 5.0. After 30 min of incubation in the dark, the enzymatic reaction was stopped with 1 M H_2SO_4 and the absorbance at 492 nm was measured. The antibodies isolated from hyperimmune plasma by protein A affinity chromatography were used as a standard. The correction of results was included based on the internal sample-specific reference.

The concentration of venom-specific $F(ab)_2$ fragments for yield and purity calculation was determined by analogous ELISA method using $F(ab)_2$ purified from European viper venom antiserum (Zagreb antivenom, Institute of Immunology, Inc., Croatia) by diafiltration as a standard.

The purity of IgG and $F(ab)_2$ was additionally monitored by the size-exclusion chromatography (SEC HPLC) using TSK-Gel G3000SWXL (7.8 x 300 mm) column at a flow rate of 0.5 mL min^{-1} at room temperature. The absorbance of the samples was monitored at 280 nm. The running buffer was 0.1 M phosphate sulphate buffer, pH 6.6. Standard proteins used for molecular weight determination were tyroglobulin (Mr 669000), γ -globulin (Mr 150000), ovalbumin (Mr 43000), and ribonuclease A (Mr 13700).

Efficiency of the purification process was characterized by two parameters in every step of the process:

- Yield was calculated using the following equations :

$$\left[\frac{\chi(\text{IgG})}{\chi(\text{IgG in starting material})} \times \text{dilution factor} \times 100\% \right]$$

$$\left[\frac{\chi(F(ab)_2)}{\chi(\text{IgG in starting material} \times 0,64)} \times \text{dilution factor} \times 100\% \right]$$
- Purity was expressed as:

$$\frac{\chi(\text{IgG})}{\chi(\text{protein})} \times 100\%.$$

$$\frac{\chi(F(ab)_2)}{\chi(\text{protein})} \times 100\%.$$

Table 1. Comparison of purities and yields of intermediates and final product obtained after 2% and 3% caprylic acid (CA) fractionation of hyperimmune horse plasma. Results are given as +/- 95% CI.

	2% CA purity (%)	3% CA purity (%)	2% CA yield (%)	3% CA yield (%)
crude IgG	82,2±1,4 (n=7)	93,7±1,1 (n=6)	88,2±3,3 (n=8)	84,8±5,6 (n=6)
pure IgG	87,7±1,4 (n=8)	97,7±1,2 (n=6)	91,0±2,1 (n=8)	86,7±6,5 (n=6)
crude $F(ab)_2$	57,2±0,9 (n=8)	58,6±0,7 (n=5)	82,4±8,3 (n=9)	76,9±4,1 (n=6)
pure $F(ab)_2$	98,1±1,8 (n=8)	92,6±1,1 (n=6)	78,0±2,6 (n=8)	69,9±2,7 (n=4)
ultrapure $F(ab)_2$	100,0±0,0 (n=8)	93,3±0,8 (n=5)	77,8±2,1 (n=8)	66,2±5,0 (n=5)



ACKNOWLEDGMENTS

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