Comparison of two downstream processes for efficient and sustainable antivenom preparation in terms of yield, final product purity and virus-inactivating potential

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INTRODUCTION AND AIM

Snakebite envenoming has recently been recognized by a WHO as a highly relevant public health issue. The only validated treatment is immunotherapy with animal-derived antivenoms. Since these life-saving medications have been out of the mainstream of pharmaceutical development/manufacture for decades, number of drawbacks pertaining to their availability, safety and efficacy is becoming increasingly evident. One of the main reasons is associated with low sustainability of current productions and technological innovation is of great need. We have developed two highly efficient antivenom downstream processing strategies at the laboratory scale. Both involve caprylic acid fractionation of hyperimmune horse plasma (HHP) for purification of IgGs, their pepsin-mediated digestion and final polishing by flow-through chromatography, but differ in number of processing steps which reflects on performance complexity. Namely, in the first one digestion follows after precipitation (2-step procedure). Another, more compressed process includes simultaneous precipitation and digestion (1-step procedure). Here we provide their comparison in terms of the final product yield and quality as well as virus-inactivating potential.

METHODS

MS ANALYSIS. As the first dimension of 2D gel electrophoresis, a ZOOM IPGRunner Mini-Cell was used in combination with immobilised pH gradient (IPG) strip (7 cm long, linear pH 3-10) rehydrated with $F(ab')_2$ sample (350 µg). For the 2nd dimension, 4-12% Bis-Tris gel was used after reduction (20 mM DTT) and alkylation (125 mM IAA). CBB R250-stained protein spots were excised and analysed by MALDI MS/MS. Proteins were considered to be confidently identified after submitting peptide sequencing data to Mascot and receiving statistically significant scores for at least two peptides per protein.

SIZE-EXCLUSION CHROMATOGRAPHY for purity profiling and aggregate monitoring was performed on TSK-Gel G3000SWXL column (7.8 × 300 mm) with 0.1 M phosphate-sulphate running buffer, pH 6.6, at a flow rate of 0.5 mL min^{-1}.

YIELD was calculated as: [($\gamma(F(ab')_2)$ × dilution factor) / (γ(IgG) in HHP × 0.67)] × 100%. IgG and $F(ab')_2$ concentrations were measured by the respective ELISA assays.

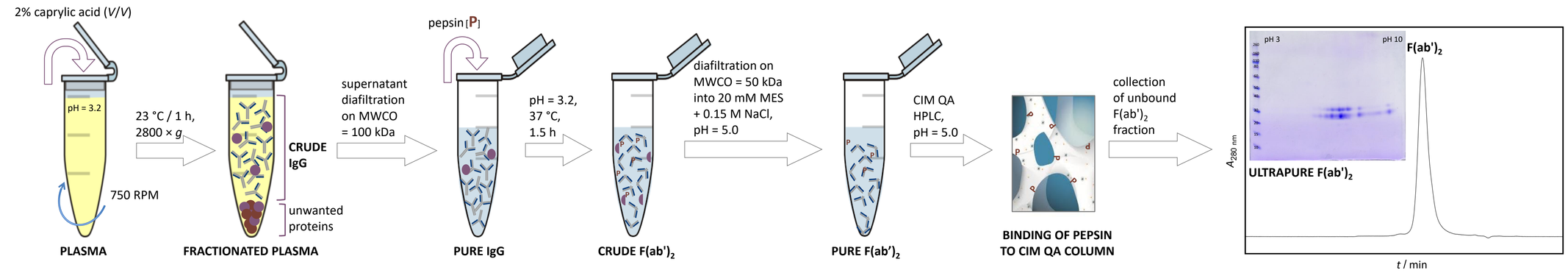
PURITY was calculated as: [$\gamma(F(ab')_2)$ / γ(protein)] × 100%. Total protein concentration was determined spectrophotometrically according to equation: ($A_{228.5\ nm} - A_{234.5\ nm}$) × f × dilution factor, where analytically determined Ehresmann's factor "f" for horse IgG of 0.2553 was used.

PURIFICATION FACTOR was calculated as ratio of specific activities (LD_{50} mg^{-1}) of $F(ab')_2$ product and HHP. Specific activity was expressed as: R / γ(protein), where R represents the number of LD_{50} venom doses that can be neutralised by 1 mL of sample *in vivo*.

VIRUS INACTIVATION ASSESMENT. The process was conducted routinely until before the chosen step when the sample was spiked with one of the tested model viruses (measles or mumps). Following incubation under standardised conditions, the product was immediately evaluated by $CCID_{50}$ test, comparatively with the starting virus material used for spiking. Reduction in virus titer was expressed as: ($CCID_{50}$ dose in starting virus material – $CCID_{50}$ dose in sample after the evaluated step).

RESULTS

2-STEP PROTOCOL



1-STEP PROTOCOL

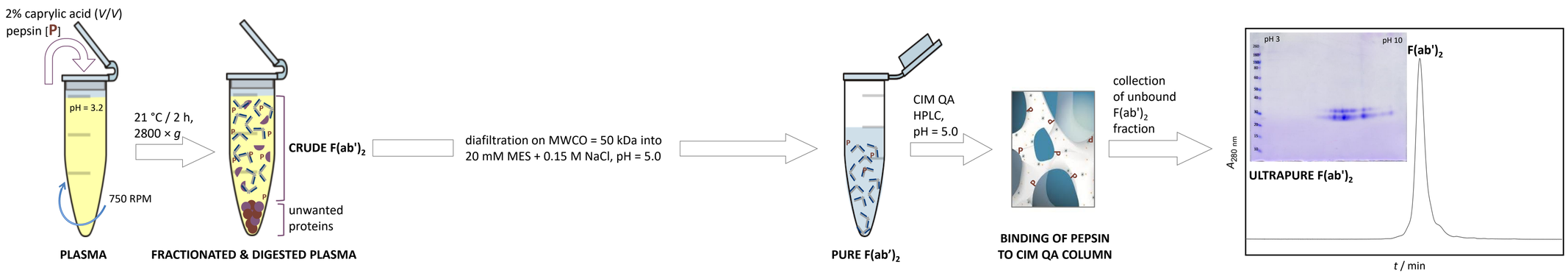


Figure 1. Schematic presentation of two antivenom downstream processing strategies. Two-step (or "first precipitation than digestion") procedure includes removal of unwanted proteins by caprylic acid-mediated fractionation of horse plasma, depletion of precipitating agent from the IgG-enriched fraction, pepsin digestion, diafiltration of the $F(ab')_2$-based preparation and its final polishing by flow-through chromatography (upper scheme). One-step procedure is based on precipitation simultaneously performed with digestion and polishing of $F(ab')_2$-based preparation by diafiltration and flow-through chromatography (bottom scheme).

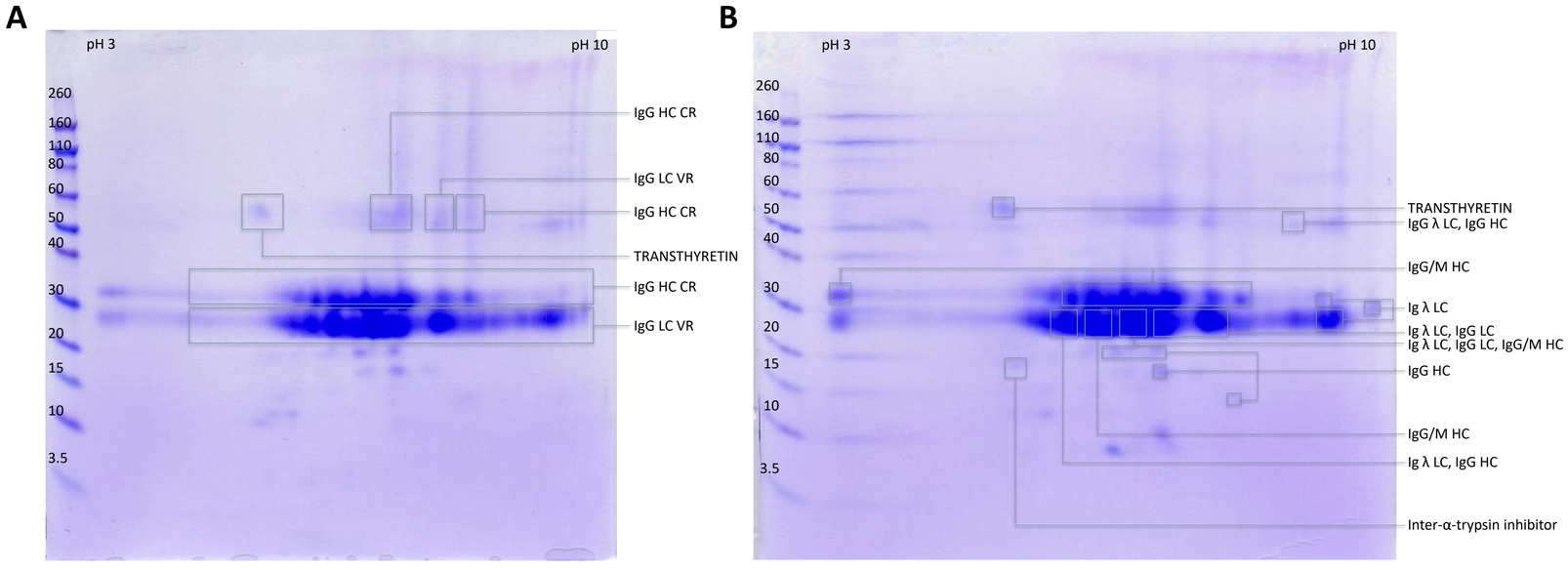


Figure 2. 2D gel electrophoresis of venom-specific $F(ab')_2$ final product, intentionally overloaded with sample for even minor impurities to occur, obtained by 2-step (A) or 1-step procedure (B) with proteins identified by MS/MS analysis.

Table 1. Comparison of two refinement strategies concerning yield, final product's purity, aggregate content, purification factor and virus inactivating potential.

		2-step procedure			1-step procedure
Yield [%]		77.0 ± 2.3 (n = 6)			74.3 ± 5.9 (n = 8)
$F(ab')_2$ (g) / HHP (L)		11.7 - 15.3*			11.3 - 14.7*
Purity [%]		100.0 ± 2.8 (n = 6)			97.3 ± 4.0 (n = 8)
Aggregates		not detected			not detected
Purification factor		3.6			3.9
		Processing step			
Virus titer reduction [log]	**Model virus**	Caprylic acid precipitation	Pepsin digestion	Cumulative effect	Precipitation with digestion
	Measles	> 5.43	4.45	> 9.88	> 5.06
	Mumps	> 4.35	2.61	> 6.96	> 4.77

* Depending on IgG concentration in starting HHP pool.

CONCLUSIONS

Both $F(ab')_2$-based antivenoms were of high purity (100 and 97%) (Figure 1, Table 1), proving comparable to the purest currently available on the regulated market. Each preparation was free from aggregates. Both processes yield between 11 and 15 g of active principle from 1 L of HHP (Table 1). As contaminants, minor traces of IgM, transthyretin and inter-alpha-trypsin inhibitor were identified, mostly in antivenom prepared by 1-step procedure (Figure 2). Prevention of viral transmission as safety assuring requirement was assessed on mumps and measles vaccine strains. Their infectivity was reduced by more than 5 log during caprylic acid-involved processing steps (Table 1). Additional inactivation was achieved by digestion step, which appeared more efficient for measles (4.5 log) than for mumps virus (2.6 log).

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