

Ion-exchange chromatography using CIM QA as a final polishing step in horse F(ab')₂-based immunotherapeutics preparation

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INTRODUCTION

Antivenoms (based on immunoglobulins or their F(ab')₂/Fab fragments) obtained from hyperimmune animal plasma are the only specific therapeutics for rapidly counteracting post-snakebite pathological manifestations. Design of ideal process preceding their commercial scale production should be guided by the tendency to refine IgGs from residual plasma proteins in only a few easy, simple and effective purification steps, providing active principle of retained potency and reduced adverse side effects-inducing potential. Purification steps that minimise the risk of provocation of instability of antibodies should be sought for incorporation into downstream processing protocol, since any conformational or structural change can trigger their degradation or aggregation, leading to the product of lower quality and, particularly, safety. Production of F(ab')₂-based antivenoms includes step of pepsin digestion for removal of immunogenic Fc portion from IgG molecule. Any traces of pepsin in the final product could compromise the stability and its elimination should be guaranteed.

AIM

Our study investigated the possibility of employing anion-exchange chromatography using CIM QA disk as a method of choice for the final polishing – removal of any processing by-product traces, particularly pepsin, in equine F(ab')₂-based antivenom preparation. The goal was to design chromatographic protocol which will bind impurities to the column, while leaving equine F(ab')₂ fragments in the flow through fraction, thus minimising the risk of triggering any change in their structure. Effective anion-exchange chromatographic protocol using Q-Sepharose has already been described for pepsin removal from ovine F(ab')₂ fragments (Jones and Landon 2002, 2003).

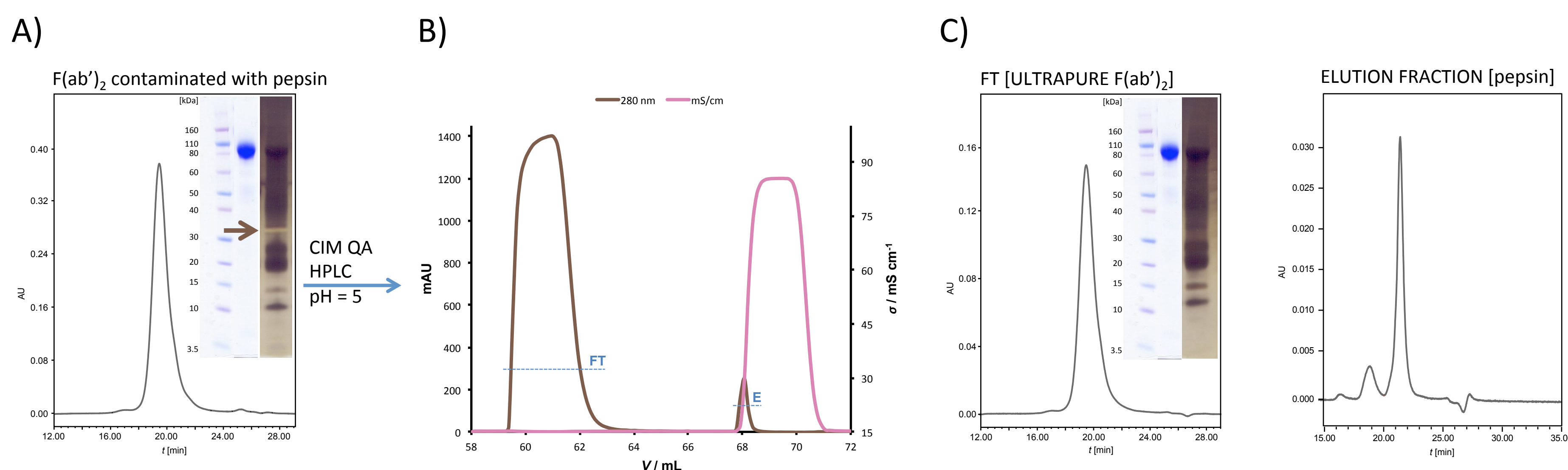


FIGURE 3. Final polishing of the F(ab')₂ preparation. (A) SEC analysis of the F(ab')₂ sample on TSK-Gel G3000SWXL column (7.8 × 300 mm) with 0.1 M phosphate-sulphate running buffer, pH = 6.6, at a flow rate of 0.5 mL / min. Inset indicates SDS-PAGE analysis of the F(ab')₂ preparation as starting material for CIM QA chromatography stained with CBB R250 and silver – “negatively” stained band corresponding to pepsin is marked by arrow. (B) Anion-exchange chromatography of F(ab')₂ sample on CIM QA disk (V = 0.34 mL) with MES + 0.15 M NaCl buffer, pH = 5, at a flow rate of 2 mL / min as conditions which allowed binding of pepsin traces and other residual acidic impurities, keeping the active principle in solution. (C) Size-exclusion chromatography of anion-exchange fractions. Inset indicates SDS-PAGE analysis of the flow through fraction containing ultrapure F(ab')₂ stained with CBB R250 and silver – no pepsin could be detected. Detection: UV at 280 nm.

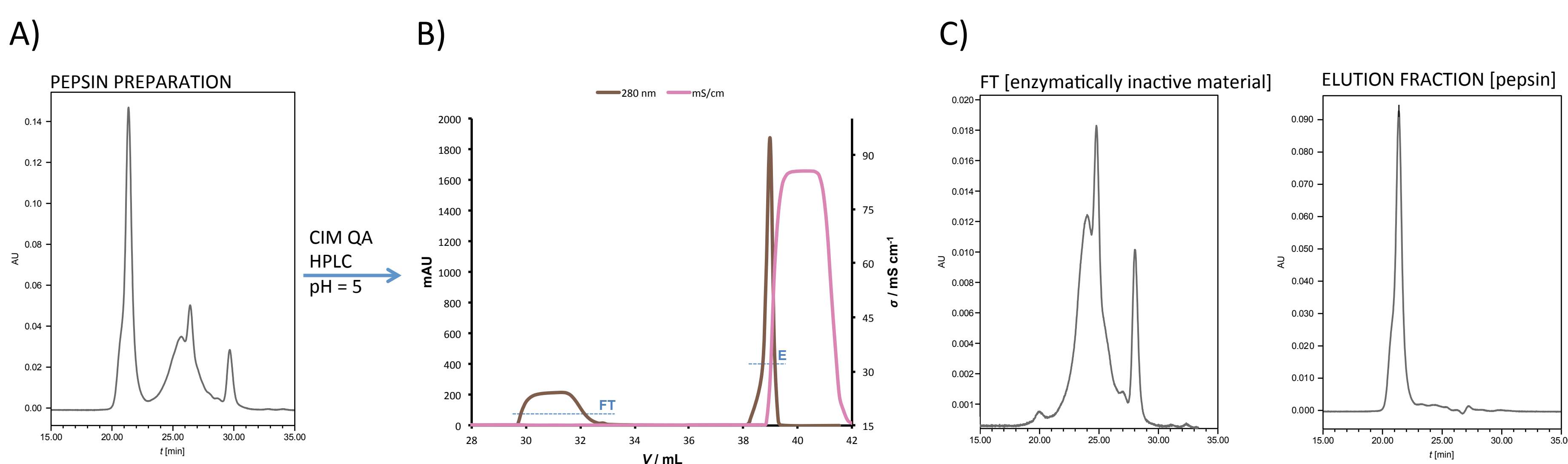


FIGURE 4. Verification of effective pepsin binding by the final polishing procedure. (A) SEC analysis of pepsin sample on TSK-Gel G3000SWXL column (7.8 × 300 mm) with 0.1 M phosphate-sulphate running buffer, pH = 6.6, at a flow rate of 0.5 mL / min. (B) Anion-exchange chromatography of pepsin sample on CIM QA disk (V = 0.34 mL) with MES + 0.15 M NaCl buffer, pH = 5, at a flow rate of 2 mL / min. The whole proportion of the enzymatically active material was retained on the column and it was subsequently eluted with 100% yield. Manufacturing by-products that affect pepsin purity lacked capability of binding. (C) Size-exclusion chromatography of anion-exchange fractions. Detection: UV at 280 nm.

RESULTS

Optimisation of the chromatographic separation of pepsin from F(ab')₂ fragments revealed pH = 5 as the most convenient for achieving balance between its removal by adsorption to anion exchange support and retention of the active principle in solution (Figure 1, Table 1) so it was further used for the final polishing step.

Dynamic binding capacity of CIM QA disk for pepsin at 10% breakthrough (RT, flow rate 2 mL / min) was 17.8 ± 0.5 mg (Figure 2).

Ion-exchange polishing enabled complete binding of pepsin to CIM QA disk (Figure 3). Also, it produced completely pure F(ab')₂-based preparation without losses, according to ELISA based calculation. The yield was 100.0 ± 2.5% (n = 23).

The potential of CIM QA disk for the pepsin binding was verified by applying the enzyme preparation under final polishing conditions, but in the higher quantity (Figure 4). According to SEC profile, commercially available preparation contains 41% of pepsin. The whole proportion of the active material was retained on the column and it was subsequently eluted with 100% yield. Namely, in comparison to the enzymatic activity of the sample applied to column (9.54 ± 0.47 PU / g), that of the bound fraction was fully preserved (9.15 ± 1.16 PU / g). The remainder proteins lacked capability of binding. So, described procedure was also shown to be applicable for further purification of commercial pepsin preparation that is often contaminated with manufacturing by-products.

MATERIALS AND METHODS

Optimisation of chromatographic conditions

Chromatographic separation of pepsin from F(ab')₂ fragments was optimised on UNOsphere Q media in batch mode with 20 mM MES + 0.15 M NaCl as binding buffer under varying pH conditions (from 4 to 6). Elution was performed with 1 M NaCl in binding buffer. The starting material was F(ab')₂ preparation obtained by pepsin digestion of IgGs previously fractionated from hyperimmune equine plasma by caprylic acid precipitation.

The binding potential of CIM QA disk (V = 0.34 mL) for removal of pepsin was verified by applying the enzyme preparation in quantity 10 times higher than that present in the digestion reaction mixture (2 mL / run). The binding buffer was 20 mM MES + 0.15 M NaCl, pH = 5. The elution buffer was 1 M NaCl in loading buffer. The experiment was performed on ÄKTA HPLC system at room temperature. The flow rate was 2 mL / min. The proteins were monitored at 280 nm. The enzymatic activity of collected fractions was measured spectrometrically on haemoglobin as substrate (Ryle, 1970).

Dynamic binding capacity of CIM QA disk for pepsin was determined under above described conditions, excepting volume load of 90 mL / run and starting sample concentration which varied in every of three independently performed analyses.

Final polishing of F(ab')₂ preparation

F(ab')₂ preparation was loaded to the CIM QA disk under conditions utilised for pepsin characterisation. After collecting the flow through fraction, the bound components, including pepsin and other acidic impurities, were subsequently eluted from the column. For purity and yield calculation total F(ab')₂ content (γ(F(ab')₂)) in flow through fraction was estimated by ELISA determining the venom-specific antibodies in which F(ab')₂ from European viper venom antiserum served as standard. Purity was additionally monitored by size-exclusion HPLC 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. The effluent was monitored at 280 nm. Total protein concentration (γ(protein)) was estimated spectrophotometrically (Ehresmann *et al.*, 1973).

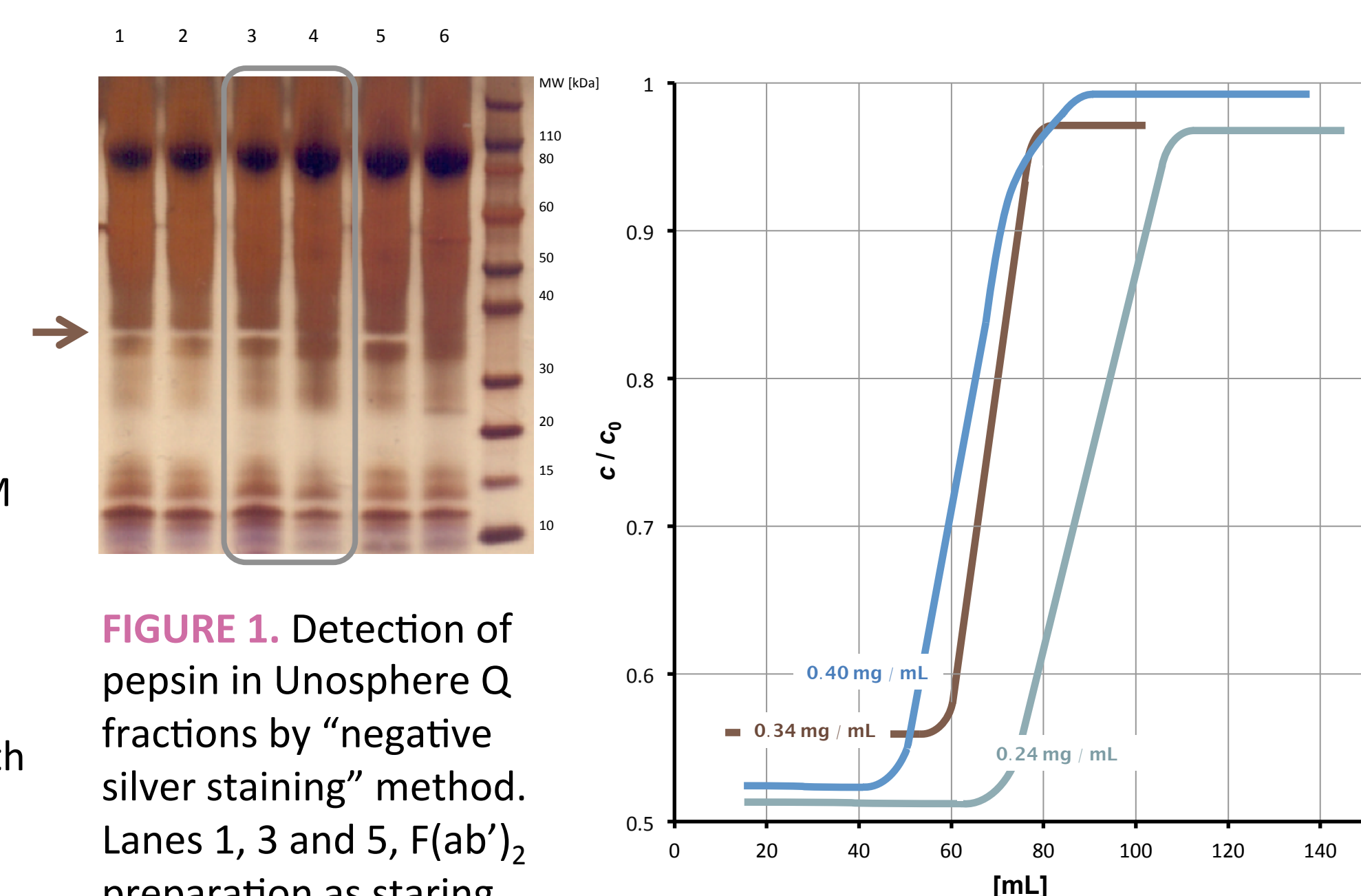


FIGURE 1. Detection of pepsin in Unosphere Q fractions by “negatively” stained silver staining” method. Lanes 1, 3 and 5, F(ab')₂ preparation as starting material; lanes 2, 4 and 6, unbound fraction at pH 4, 5 and 6, respectively. “Negatively” stained bands corresponding to pepsin are marked by arrow.

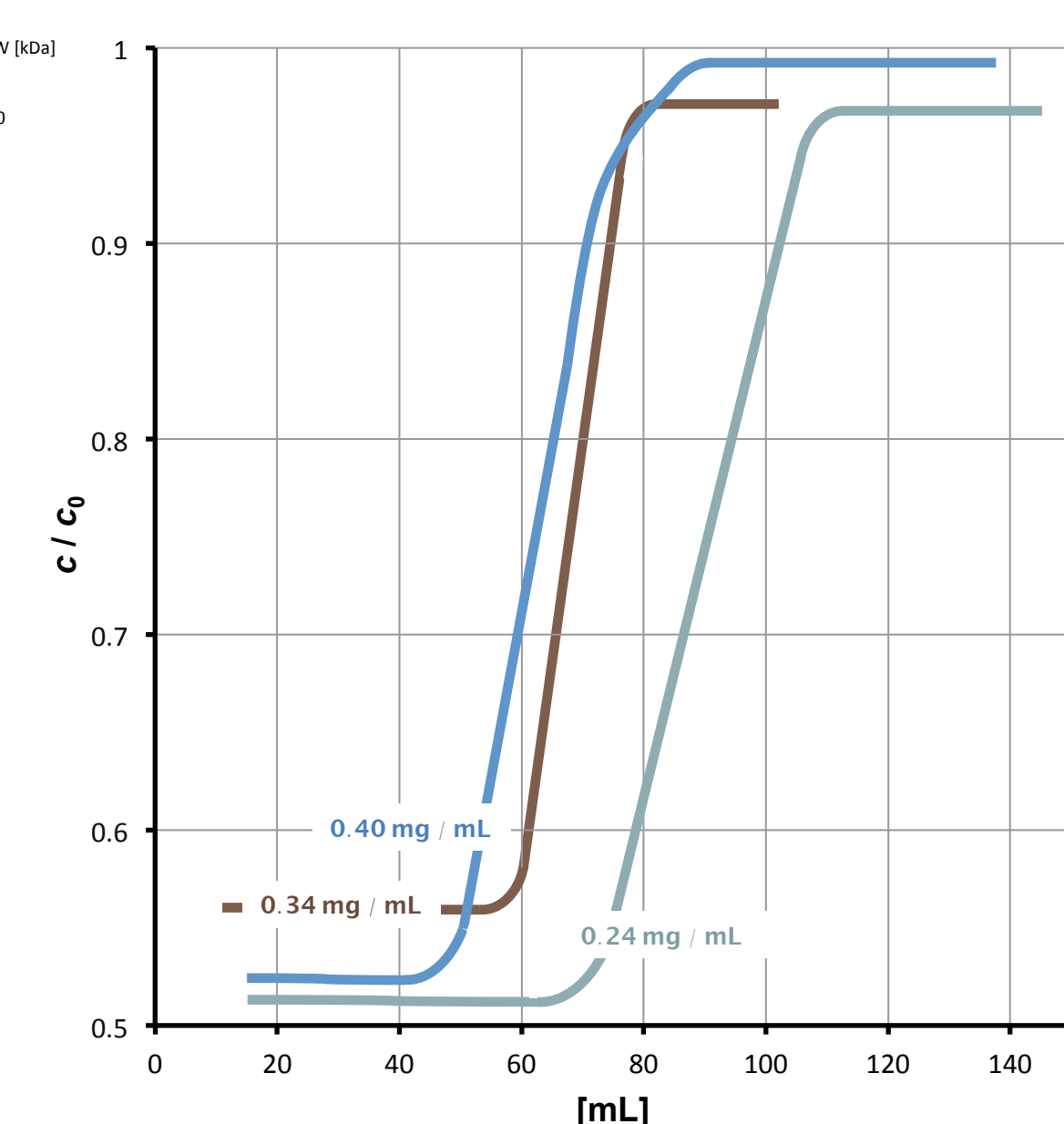


FIGURE 2. Breakthrough curves for pepsin samples (n = 3) on CIM QA disk (V = 0.34 mL) with MES + 0.15 M NaCl, pH = 5 as running buffer at RT. Starting sample concentrations are denoted. Flow rate was 2 mL / min. Detection: UV at 280 nm.

TABLE 1. Characterisation of unbound fractions following incubation of F(ab')₂ preparation containing pepsin with UNOsphere Q matrix under various pH conditions – detection of pepsin traces by SDS-PAGE and active principle yield calculated according to ELISA results. Optimal pH is framed.

pH	PEPSIN IN UNBOUND FRACTION	F(ab') ₂ yield [%]
4	+	97.6
5	–	89.2 - 93.0
6	–	86.2 - 91.3

CONCLUSION

Through pepsin removal potential and preservation of the active principle yield CIM QA chromatography performed at pH = 5 imposes as a desirable final polishing step suitable for achievement of completely pure F(ab')₂-based product of improved stability and/or safety.