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Single-step purification of immunotoxins containing a high ionic charge ribosome inactivating protein clavin by carboxymethyl high-performance membrane chromatography

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Abstract

High-performance membrane chromatography (HPMC) and HPLC hydroxyapatite chromatography were compared for their efficiency in purifying immunotoxins (ITs) containing the ribosome-inactivating protein clavin, which is characterized by a high anionic charge and a low molecular mass. Both methods efficiently removed unreacted clavin from the conjugate crude mixture, but only the cation-exchange HPMC allowed efficient single-step separation of the unreacted monoclonal antibody (mAb) from ITs obtained by different coupling procedures.

Keywords: Membranes; Immunotoxins; Toxins; Clavin; Proteins; Monoclonal antibodies

1. Introduction

Immunotoxins (ITs) are hybrid proteins composed of a monoclonal antibody (mAb) coupled to a toxic molecule for delivery to target cell populations [1]. The high activity and specificity of ITs has made them promising reagents in therapeutic applications for hematological malignancies [2] and for breast and ovarian carcinomas overexpressing the HER-2 oncogene [3,4].

The ribosome-inactivating protein (RIP) clavin, derived from *Aspergillus clavatus*, recently cloned and expressed in *E. coli*, is highly active and induces low and transient systemic toxicity in mice [5]. This protein was used to selectively target breast and ovarian carcinoma cells by conjugation with an IgG mAb (Mgr6) that recognizes an epitope of the $gp185^{HER\text{-}2}$ extracellular domain [6]. The distribution of $gp185^{HER\text{-}2}$ in normal human tissues is restricted, and overexpression of the HER-2 oncogene is associated with poor prognosis in breast and ovarian carcinoma patients [7–9]. We recently prepared a series of clavin–Mgr6 ITs with altered linkage properties to improve in vivo stability, pharmacokinetic behavior and, thus, antitumor activity [10].

However, while available chemical methods to prepare ITs allow modulation of the length and linkage between RIP and an mAb, the homogeneity of the conjugate preparation and the purification steps remain suboptimal, especially when compared

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with the results obtained by biotechnological approaches [11]. The purification procedure is particularly important in producing highly active ITs suitable for clinical trials since unreacted mAb remains in the circulation longer than toxin-conjugated mAb and can compete for target cell binding, thus reduce the anti-tumor effect of the conjugate. Several methods for IT purification based on different techniques for removing free antibody from the preparations have been described [12–17]. Gel filtration used alone [12], efficiently removed unreacted RIP (molecular mass 17 000 to 30 000) but was not suitable for complete separation of free antibody from ITs, because of the similarity in molecular mass. Gel filtration combined with affinity chromatography using dye-linked phases (Blue Sepharose CL-6B or Affi gel Blue) was suitable for purification of basic RIP and ricin toxin A chain [13,14]. Immunoaffinity has been used to efficiently remove immunotoxin from the antibody [15], although this procedure has limited applicability since the gel used is not commercially available and the conditions required to elute the IT from the gel may cause a decrease in cytotoxic activity. Another approach involves a combination of gel filtration or protein A Sepharose chromatography (able to link the mAb Fc portion) and ion-exchange chromatography [16,17].

Dhanabal et al. [18] prepared recombinant toxins containing 10 consecutive histidine residues at the amino terminus and chemically linked them to mAb, exploiting the high affinity of consecutive histidine residues for nickel-based resin to purify IT from unreacted mAb.

In the present study, we describe a method for the single-step purification to homogeneity of an IT preparation. Comparison of two ionic interaction methods, hydroxyapatite (HPHT) and ion-exchange high-performance membrane chromatography (HPMC), for purification efficiency, indicated superiority of the latter in removing unreacted mAb from the IT.

2. Experimental

ITs composed of mAb Mgr6 and clavin linked by a disulfide bond, a steric hindered disulfide bond, or a thioether linkage were prepared as described [10]. Fig. 1 shows the structure of the ITs synthesized.

2.1. Chromatography

The crude reaction mixture after conjugation (6–8 mg/ml) was centrifuged at 3500 *g* for 5 min to remove aggregates, filtered on a 0.22-μm Millex filter (Millipore, Bedford, MA, USA), and chromatographed at 20°C on hydroxyapatite Bio-Gel HPHT (100×7.8 mm), 10 μm (Bio-Rad, Hercules, CA, USA) and a cation exchanger (CM MemSep 1010 chromatography cartridge, bed volume, 4.9 ml; bed height, 1 cm) (Millipore). All chromatograms were generated on a Merck–Hitachi 655A-12 liquid chromatograph equipped with L5000 LC controller (Merck, Milan, Italy) and the eluting fractions were monitored at 280 nm using an L4000 UV detector. Peak heights and areas were recorded and processed on a CBM-10A Shimadzu interface (Shimadzu, Milan, Italy).

2.2. HPHT

IT samples were diluted at 2 mg/ml with 10 m*M* sodium phosphate buffer (PBS), pH 6.8, and 300 μl were applied on a Bio-Gel HPHT column. The column was equilibrated with the same buffer containing 0.01 m*M* calcium chloride (mobile phase A) The elution was then accomplished by an increasing gradient of buffer B (350 m*M* PBS, pH 6.8, containing 0.01 m*M* calcium chloride). A first steep linear gradient of ionic strength (from 10 to 220 m*M*) was applied in the first 20 min with an elution flow-rate of 0.5 ml/min, then followed by a slower gradient increase (up to 350 m*M*) with a flow-rate of 0.25 ml/min. Fractions in the relevant peaks were collected, an aliquot run on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and pooled from the corresponding volumes: 17.5–19, 19–20, 20–22.5 and 25.9–28.2 ml. Non-eluted material was removed by washing with a gradient of 10 m*M* PBS, pH 6.8, containing up to 0.5 *M* NaCl.

2.3. Cation-exchange HPMC

IT samples of the crude mixture were diluted to 5 mg/ml with 20 m*M* acetate buffer, pH 5.5, and 400

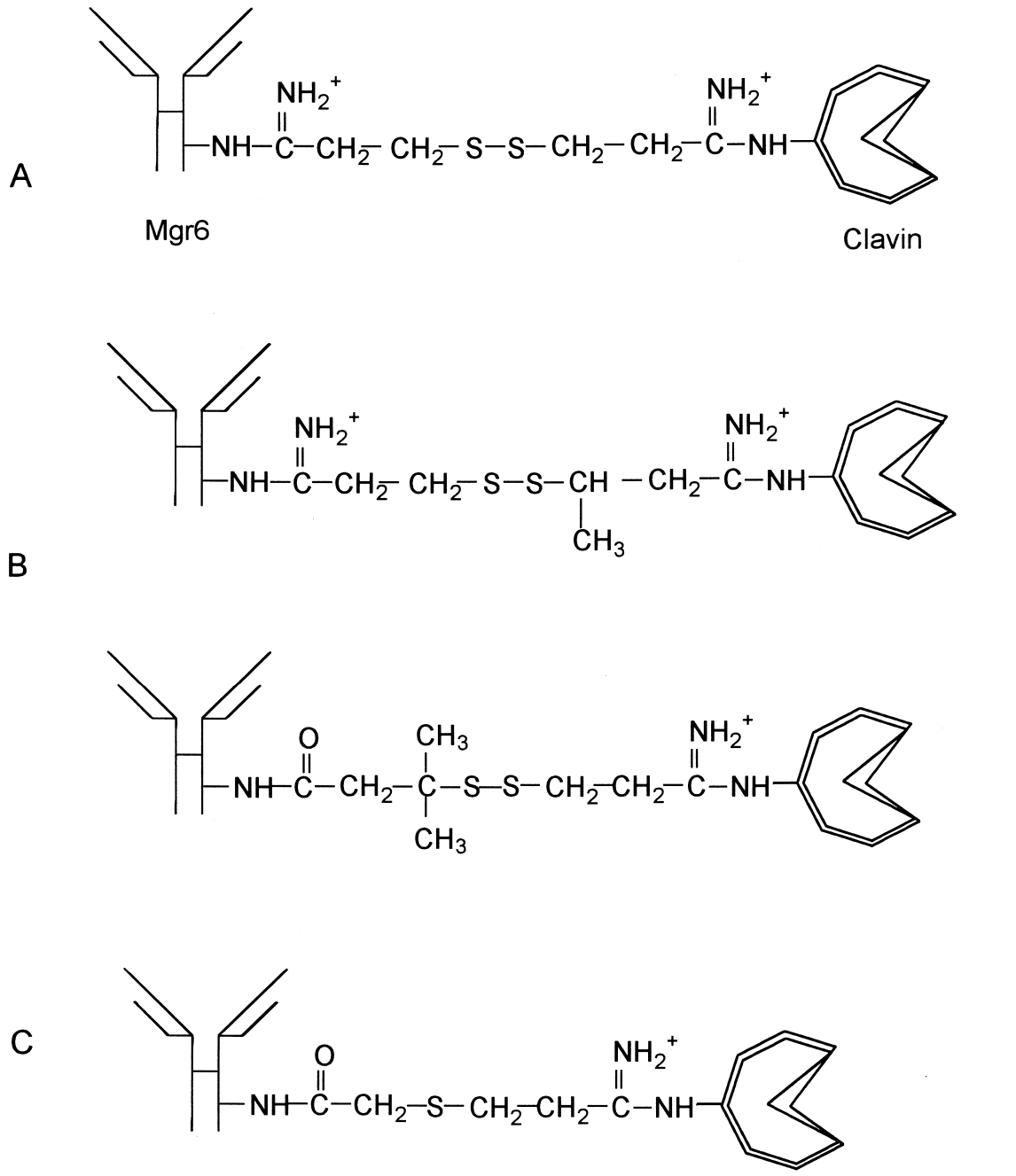

Fig. 1. Structure of immunoconjugates synthesized. (A) Disulfide linkage; (B) sterically hindered disulfide linkage; (C) thioether linkage.

μl were applied on a chromatographic cartridge CM Mem-Sep 1010. The column was eluted with the same buffer (mobile phase A) and then with a linear gradient of the mobile phase B (20 m*M* acetate buffer, pH 5.5, with 1 *M* NaCl). Buffers were filtered on a 0.22-μm Millex filter. As a safety device a Millex LCR4 or GV filter was fitted in the inlet tube. To increase adsorption of mixture, the starting flow-

rate was 0.5 ml/min for the first 12 min and 2 ml/min thereafter. Collected fractions were immediately brought to pH 7.0 with 1 *M* PBS, pH 8.2, and then dialyzed and concentrated. Fractions in the relevant peaks were collected and, after SDS–PAGE analysis, were pooled from the corresponding volumes: 22–36.4, 36.4–41.6, 41.6–48.6, 48.6–68.6 and 80–100 ml.

After three purification passages, the column was reversed and cleaned by sequential a flushing with 40 ml of 0.5 *M* NaOH (5 ml/min) ultrapure water to pH 8.0, 40 ml of 0.5 *M* HCl solution, and ultrapure water to pH>4. The column was then returned to the normal flow direction and equilibrated with starting buffer.

2.4. Electrophoresis

Fractions from both chromatographic procedures were analyzed by SDS–PAGE in precast slab gels (Phastgel gradient 4–15% Pharmacia, Uppsala, Sweden) under nonreducing conditions, using the automated microprocessor-driven Phastsystem according to the manufacturer's suggestion. The same apparatus was used for isoelectrofocusing (IEF) analysis with the Isoelectric Calibration Kit (isoelectric point (p*I*) range 3.5–9.3, Pharmacia) as IEF standard. Coomassie blue staining was used to visualize proteins.

3. Results and discussion

We previously described a cross-linking method that produces a good yield of homogeneous IT composed of clavin and mAb Mgr6 [10]. We have also studied different methods to improve the purification of IT in an effort to reduce the cost and increase the production rate of these reagents.

Usually two purification steps are required for ITs: the first removes the free toxin, exploiting the difference in molecular mass, while the second separates unreacted mAb from IT. The methods generally used in the purification of ITs containing RIPs extracted from fungi (α-sarcin, mitogillin, restrictocin), which are highly basic proteins with low molecular mass, consist in gel filtration alone or followed by a dye–ligand chromatography [19–21].

Although widely used, the dye–ligand interaction presents three important drawbacks: (1) the interaction of RIP with the dye is variable and depends on the ionic strength and pH; (2) at least for α-sarcin and mitogillin, the binding is not complete (range of the protein total recovery 65–90%) [22]; and (3) some mAbs have a slight affinity interaction with the dye and co-elute with IT (our unpublished results).

We used two separation procedures based on the large difference in the p*I* of clavin (more than 10) and mAb (p*I* 6.2). The different p*I* values (7.1–7.3) of the ITs compared to unconjugated antibody (Fig. 2), together with the low molecular mass (17 000) of clavin, favoured the purification process. We therefore analyzed the charge interaction of clavin and ITs on HPHT, which is characterized by a negatively charged phosphate matrix (Fig. 3). HPHT bound clavin tightly (eluted at PBS concentration 300–320 m*M*) and allowed its separation from IT and mAb in a single step (Fig. 4, lane 7). At low ionic strength, a non-retained fraction (low molecular mass products or derivatives of the conjugation process) was eluted in the first 7 ml. Subsequently, mAb Mgr6 eluted in

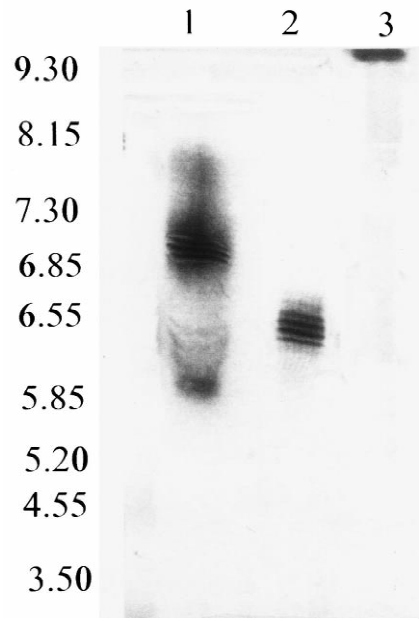

Fig. 2. IEF of Mgr6–clavin immunoconjugate (lane 1), Mgr6 (lane 2) and clavin (lane 3) in precast mini-slab gels. Calibration was obtained with IEF standards (p*I* range 3.5–9.3).

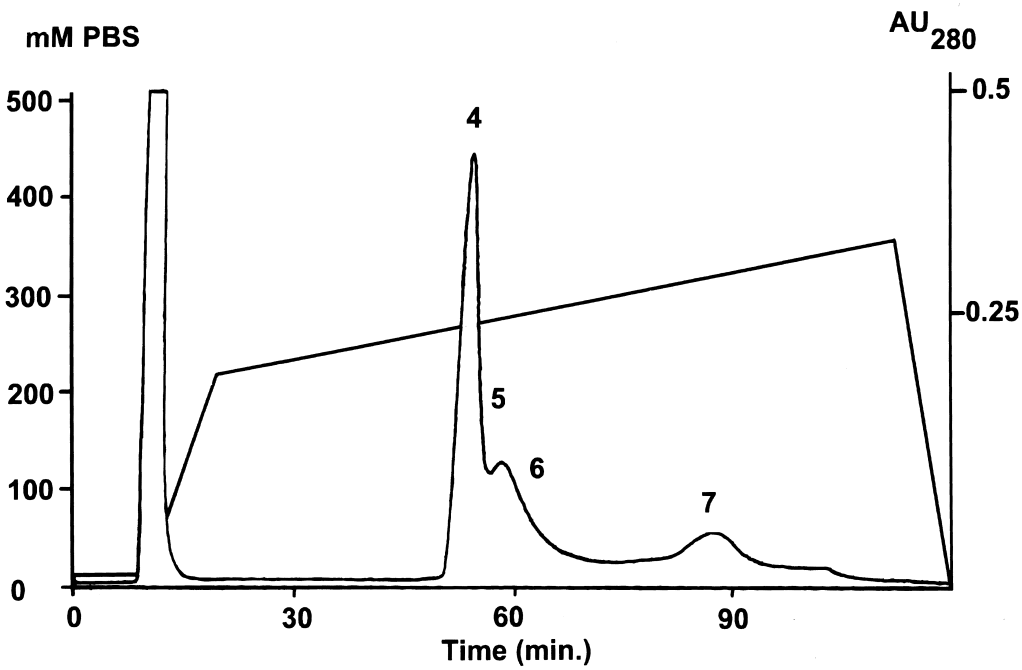

Fig. 3. Elution profile of 0.6 mg of crude mixture of IT preparation on Bio-Gel HPHT column. Eluent A: 10 m*M* PBS, pH 6.8; eluent B: 350 m*M* PBS, pH 6.8; both containing 0.01 m*M* $CaCl_2$. Flow-rate was 0.5 ml/min for 20 min and 0.25 ml/min for the next 100 min. Numbers refer to lanes in Fig. 4.

a single peak together with a significant amount of IT (Fig. 4, lane 4). A slow increase in gradient concentration (210 to 350 m*M* of phosphate in 98 min) did not result in a satisfactory separation between IT and MAb (Fig. 4, lanes 5 and 6), although a progressive increase in IT was observed and the fraction collected at 58–65 min contained less than 10% of unconjugated antibody.

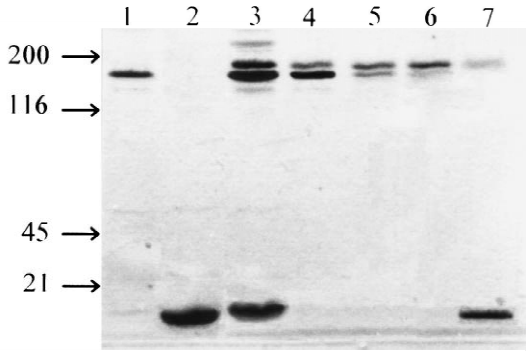

Fig. 4. Electrophoretic profile of fractions eluted from a Bio-Gel HPHT column and resolved on a SDS–polyacrylamide gradient gel (4–15%). Lanes: (1) Mgr6; (2) clavin; (3) crude conjugate mixture; (4) fraction 50–56 min; (5) fraction 56–58 min; (6) fraction 58–65 min; (7) fraction 83–93 min. Molecular masses are expressed in kilodaltons.

The ionic exchange method has been used to separate ITs prepared with basic RIPs of molecular mass of 30 000 such as gelonin and saporin under low pressure [16,17]. The HPMC cartridge (Mem-Sep) we used provides a macroporous (1.2 μm) network of pure regenerated cellulose in a packed-bed column configuration, exhibiting very low non-specific binding properties, so that binding occurs via the immobilized ion-exchange groups. A flow-rate of 2 ml/min, combined with a stepwise NaCl gradient, allowed elimination of the unbound material (low molecular mass products), followed by elution of mAb Mgr6 in a sharp peak (Fig. 5). SDS–PAGE confirmed the low amount of IT co-eluted with mAb (Fig. 6, lane 3). The IT eluted in multiple peaks when a gradient of 60–100 m*M* NaCl was applied. Increased IT purity was observed in fractions eluted

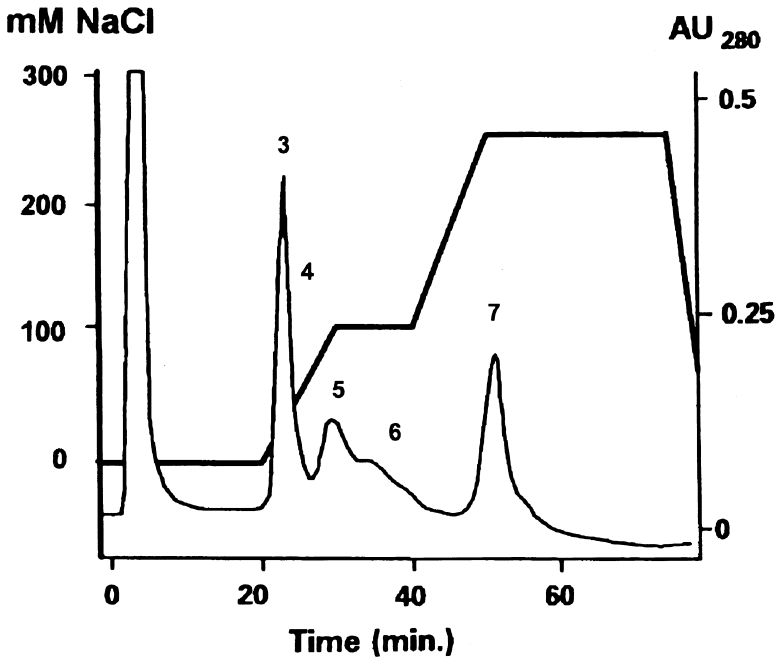

Fig. 5. Elution profile of 2 mg of crude mixture of IT preparation on MemSep 1010 membrane chromatography cartridge. Eluent A: 20 m*M* acetate buffer, pH 5.5; eluent B: 20 m*M* acetate buffer, pH 5.5, with 1 *M* NaCl. Flow-rate was 0.5 ml/min for the first 12 min and 2 ml/min thereafter. The numbers refer to lanes in Fig. 6.

from 27 to 40 min (Fig. 6, lanes 4–6). Clavin was completely removed from the column at 250 m*M* NaCl.

Reduction of the flow-rate in the first 12 min increased the loading capacity and binding of IT on the exchanger resulting in complete absorption of up to 2.5 mg of crude mixture. In comparison, the HPHT column maintained good separation with up to 0.7 mg of loaded sample.

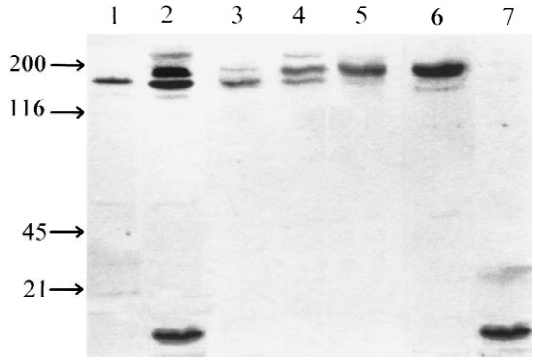

Fig. 6. Electrophoretic profile of fractions eluted from a MemSep 1010 membrane chromatography cartridge and resolved on a SDS–polyacrylamide gradient gel (4–15%). Lanes: (1) Mgr6; (2) crude conjugate mixture; (3) fraction 20–27 min; (4) fraction 27.2–30.5 min; (5) fraction 30.5–33.3 min; (6) fraction 33.3–43.3 min; (7) 48–59 min. Molecular masses are expressed in kilodaltons.

The yield of pure IT (i.e., containing less than 5% of Mgr6) from HPHT purification never exceeded 20% of the total IT, while yield from the CM-HPMC procedure was at least 60% of pure IT. Although the total yield of IT varied according to the synthetic procedure used, the results with the different purification procedures are similar.

The different purified ITs all showed specific cytotoxicity on ErbB2-positive cell lines, but ITs purified to completely remove free mAb Mgr6 showed greater in vivo stability verified after i.v. bolus administration in Balb/c mice given [10].

In conclusion, we applied a cation exchange on HPMC to efficiently purify in a single step a homogeneous population of IT, composed of an mAb and a toxin with low molecular mass and high ionic charge (pI>10). The loading capacity and purification yield are high and the purification is

faster than with HPHT. Furthermore, the use of an HPMC cartridge, for the bulk production of ITs intended for clinical use, allows depyrogenation with bleach, steam, strong acids, and bases.

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