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PURIFICATION OF SPECIFIC HETEROLOGOUS $F(ab)_2$ FRAGMENTS WITH DEAE-ZETA-PREP® CARTRIDGES FOR ION-EXCHANGE CHROMATOGRAPHY

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SUMMARY

A simple two-step procedure for purifying $F(ab)_2$ fragments of horse immunoglobulins is described. In the first step, the horse plasma is diluted, made up to 12% (w/v) with ammonium sulphate and digested with pepsin. In the second step, the previously dialyzed solution is chromatographed. Instead of a normal ion-exchange resin, a DEAE-cellulose, covalently linked to a synthetic vinyl polymer, was used (DEAE-Zeta-Prep®). With this assembly it is possible to perform chromatography at a high flow-rate without the problems related to the use of large columns. The yield and purity of the final product are satisfactory. This method has been scaled up for industrial application.

INTRODUCTION

The purification of immunoglobulins or $F(ab)_2$ fragments with ion-exchange resins, in particular with diethylaminoethyl (DEAE-) cellulose, has been described in many papers[1–3]. Although this method gives highly purified immunoglobulins, we have found that its adaptation to an industrial scale is difficult due to the need for large columns and the long adsorption and elution times owing to the compaction of the resins. This problem can be eliminated by using ion-exchange cartridges in which the ion-exchange resin is bound to a solid support that allows high rates of adsorption and elution. In this paper we describe the purification of $F(ab)_2$ fragments of horse immunoglobulins, digested with pepsin, with DEAE-Zeta-Prep® (LKB, Bromma, Sweden) ion-exchange cartridges. As different cartridge sizes are available, this method can easily be scaled up simply by using larger Zeta-Prep 3200 cartridges.

MATERIALS AND METHODS

DEAE-Zeta-Prep cartridges were obtained from LKB. The dimensions were 7 cm × 6.7 cm for Zeta-Prep 250, 24 cm × 12 cm for Zeta-Prep 3200.

Digestion of plasma with pepsin

Plasma from immunized horses was diluted in 2 volumes of water, adjusted to

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pH 4, and digested with 1 g pepsin 2000 FIP (Fédération International Pharmaceutique) U/g (E. Merck, Darmstadt F.R.G.) per litre of undiluted plasma at 42°C for 18 h under continuous agitation in the presence of 12% (w/v) ammonium sulphate[4,5]. The suspension containing $F(ab)_2$ fragments was then filtered through Duplex paper (Galvani, Siena, Italy) and CW 19 filters (Millipore, Bedford, MA, U.S.A.). Digested specific antidiphtheria, antitetanus and anti-snake bite immunoglobulins were studied.

Dialysis

Different ultrafiltration systems were employed depending on the consistency of the digested lots: a Minitan and Pellicon cassette system (Millipore) or two process ultrafiltration cartridges (Millipore). The membranes used had a molecular weight cut-off of 10 000. Dialysis was performed with a constant volume, after the pepsin-digested solution had been concentrated *ca.* 10 times at least 8 volumes of 0.01 *M* phosphate buffer (PB) (pH 6.5) were exchanged. The dialyzate, diluted to 3% in 0.01 *M* PB (pH 6.5), was filtered through CW 19 filters before chromatography.

Chromatography

DEAE-Zeta-Prep cartridges were equilibrated according to the instructions of the manufacturer. The final equilibration buffer was 0.01 *M* PB (pH 6.5). Chromatography was performed at a flow-rate of 40 ml/min for the Model 250 cartridges and 520 ml/min for 3200 cartridges. Under these conditions, $F(ab)_2$ fragments are not bound by DEAE whereas most of the impurities are. The cartridges were therefore eluted at the same flow-rate with 0.01 *M* PB (pH 6.5) in order to recover the $F(ab)_2$, until the eluate had an absorbance of about 0.1 at 280 nm. Then 0.9% sodium chloride was added to the eluate, which was concentrated by ultrafiltration through a Minitan or Pellicon system, until the protein concentration was between 5 and 12%. Purified and concentrated $F(ab)_2$ fragments were filtered in a sterile manner using a Durapore (Millipore) 0.22-μm filter after the addition of 0.3% (w/v) *m*-cresol. Finally the Zeta-Prep cartridges were treated with 1 *M* sodium chloride to wash out the bound impurities, and they were subsequently regenerated according to the instructions of the manufacturer.

Analytical methods

Electrophoresis[6] was performed in 0.5 *M* veronal–EDTA buffer (pH 9.0) on strips of cellulose acetate (14 cm × 5.7 cm), Cellogel (Chermetron Chimica, Milan, Italy), at 200 V for 30 min. Following staining with Coomassie Blue, the percentages of albumin α-, β- and γ-globulins (immunoglobulins) were determined by densitometry. The *in vivo* antibody titer was evaluated in mouse and guinea pigs according to the European Pharmacopoeia[7]. Proteins were determined by the method of Lowry *et al*[8]. Pepsin was determined by counter immunoelectrophoresis (CEP)[9]. The analysis was performed with a plastic plate support, covered with 1% agarose in veronal buffer (pH 8.8) in 3 mm diameter wells, at 50 V/cm for 2 h. Rabbit antipepsin serum was placed in an anodic well and the sample to be analyzed in a separate cathodic well. Another series of wells was filled with antipepsin serum and with standard solutions of pepsin at various dilutions as controls.

High-performance liquid chromatography (HPLC) was performed with a Waters (Millipore, Bedford, MA, U.S.A.) system consisting of a Model 510

solvent-delivery system, a Model 480 absorbance detector and a Model 680 automated gradient controller, with a 600 mm × 7.5 mm TSK G3000SW column (LKB). The eluent was 0.2 *M* phosphate buffer (pH 6.7) containing 0.05% sodium azide. A 10-μl volume sample with a protein content of *ca.* 2% was injected. The toxicity was determined on guinea pigs according to the European Pharmacopoeia[10].

RESULTS

Part of the contaminating proteins in the native plasma were precipitated by addition of 12% ammonium sulphate. The paper- and CW 19-filtered solution was dialyzed to remove the ammonium sulphate and part of the low-molecular-weight proteins, yielding a quantitative recovery of $F(ab)_2$ fragments. Fig. 1 shows the chromatograms of the sample before and after concentration and dialysis. It is obvious that many low-molecular-weight materials were removed. The dialyzed solution containing the $F(ab)_2$ fragments was applied to the ion-exchange cartridges. In a preliminary test it was found that Zeta-Prep 250 cartridges cannot be loaded with amounts of protein exceeding 20 g without loss of large amounts of α-globulins. About 90% of the $F(ab)_2$ was unbound and recovered in 0.01 *M* PB eluate. About 10% of the $F(ab)_2$ was bound to DEAE together with the impurities that are bound under these conditions. The purified solution (90–98%) of $F(ab)_2$, concentrated by ultrafiltration, was free from albumin and pepsin ($< 10^{-4}$ mg/ml) and contained only slight traces of β-globulins. Ion-exchange chromatography increases significantly the purity of the

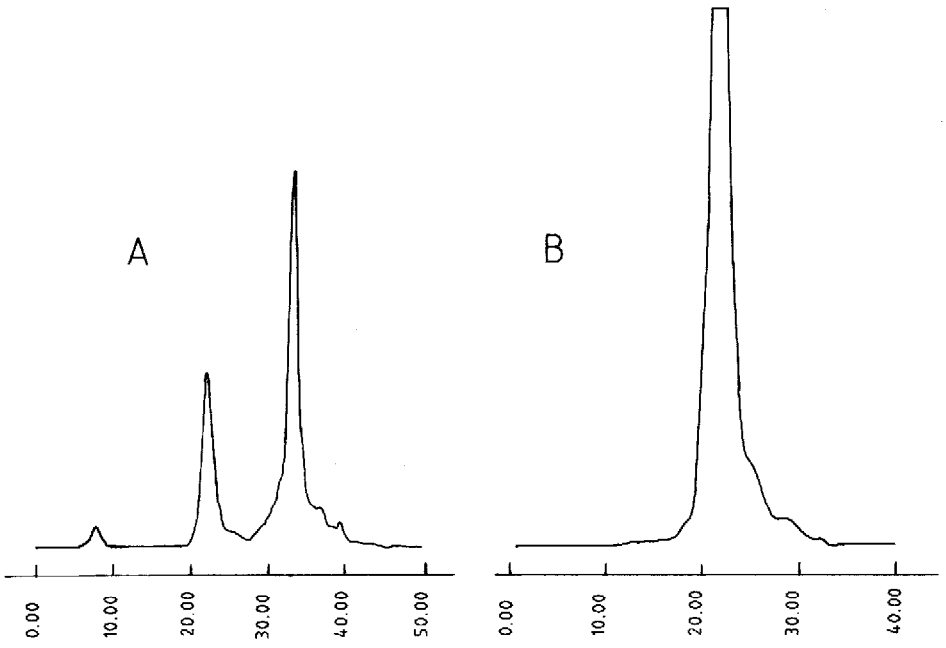

Fig. 1. Chromatograms of (A) digested horse plasma, and (B) digested horse plasma after dialysis. Column: TSK G3000SW (600 mm × 7.5 mm). Eluent: 0.2 *M* phosphate buffer (pH 6.7) containing 0.05% sodium azide. Detection: 280 nm. Time scale in min.

TABLE I

PURIFICATION OF $F(ab)_2$ FRAGMENTS WITH THE ZETA-PREP 250 CARTRIDGE

Chromatography was performed with 0.01 *M* PB (pH 6.5) except where indicated.

Initial plasma			*Final product*							
Lot	*Amount (ml)*	*Titer (I.U./ml)*	*Volume (ml)*	*Protein (g/l)*	*Titer (I.U./ml)*	*Yield (%)*	*Composition (%)**			
							Albumin	*α-Globulins*	*β-Globulins*	*γ-Globulins*
2272	230	450	205	14.8	325	64.4	0	0	2.2	97.8
298A	230	450	170	13.3	350	57.7	0	0	1.5	98.5
298B**	230	450	150	18.7	450	62.5	0	0	5.9	94.1
239A	230	450	180	13.9	350	61.2	0	0	2.5	97.7
239B**	230	450	215	13.7	325	67.5	0	0	6.5	93.5
1791	216	1000	210	13	650	62.3	0	0	1.2	98.8

* By electrophoresis on cellulose acetate.

** 0.03 *M* PB (pH 6.5).

TABLE II

PURIFICATION OF F(ab)$_2$ FRAGMENTS WITH ONE OR MORE ZETA-PREP 3200 CARTRIDGES

Chromatography was performed with 0.01 *M* PB (pH 6.5).

Initial plasma				*Final product*							
Lot	*Amount (l)*	*Titer (I.U./ml)*	*Zeta-Prep cartridges used*	*Volume (l)*	*Protein (g/l)*	*Titer (I.U./ml)*	*Yield (%)*	*Composition (%)**			
								Albumin	*α-Globulins*	*β-Globulins*	*γ-Globulins*
135	3.05	575	1	0.61	70	1750	60.8	0	1.0	8.5	90.5
205	6	209	1	0.7	82.4	739	41.2	0	0.6	11.8	86.8
225	6.3	550	1	1	54	2150	62	0	0	10.3	89.7
110	62	125	3	2.7	128	1500	53.3	0	0	6.4	93.6
610	64	400	3	5.5	75	2500	53	0	2.4	9.6	88
910	53	600	5	7	95	3000	66	0	1.5	5.9	92.6
1510	65	400	4	3.75	131	3500	51.3	0	0.7	5.6	93.7

* By electrophoresis on cellulose acetate.

final product as the solution containing the $F(ab)_2$ fragments has an electrophoretic purity not exceeding 78% before chromatography. The yield of the entire purification process was *ca.* 60–65% (Table I). The losses were due to chromatography, various filtrations and mainly the digestion with pepsin. The yield increased when chromatography was performed with 0.03 *M* PB (pH 6.5), but the purity of the final product was decreased.

Scale-up

For the industrial application of this method we used the larger Zeta-Prep cartridges (3200) allowing work with amounts of horse plasma of 1 l or more. Our results are shown in Table II. A test similar to the one performed earlier with Zeta-Prep 250 allowed us to establish the sample capacity of the cartridge as 220–250 g. It was possible to treat even larger quantities of plasma by arranging many Zeta-Prep 3200 cartridges in parallel. The results are shown in Table II, where the volumes of plasma (up to 60 l) were purified by maintaining a constant protein load for each cartridge.

DISCUSSION

Many purification systems have been used to obtain intact or proteolyzed immunoglobulins suitable for therapeutic use. Ion-exchangers give satisfactory results, but this approach is complicated by the excessive adsorption and elution times needed for processing large volumes of starting materials. The method described combines ultrafiltration with the use of an ion exchanger, supported on a rigid matrix, which allows rapid and simple purification of $F(ab)_2$ fragments. The purity of the product obtained depends on the amount of sample and the dimensions of the cartridge used. The purity of $F(ab)_2$ fragments obtained by using Zeta-Prep 250 is about 98% as determined by electrophoresis. With the larger cartridges we were unable to obtain purity levels higher than 92%. The yields obtained (expressed by *in vivo* antibody titer) were in the range of 50–60%, comparable or superior to those obtained by precipitation with ammonium sulphate. This method can easily be scaled up to an industrial level. The final product is non-toxic and apyrogenic, and thus suitable for human use.

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