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Physical and biochemical characterization of five commercial resins for immunoaffinity purification of factor IX

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ABSTRACT

The American Red Cross has developed an immunoaffinity chromatography method to purify human coagulation factor IX (FIX) to homogeneity using monoclonal antibodies (MAb) that bind FIX in the presence of divalent cations. The MAb is immobilized on Sepharose CL2B, a soft gel with a low pressure tolerance as well as poor large-scale performance characteristics, including low reusability, and resin crumbling and deterioration. In this study, we examined several commercially available resin supports. Aside from Sepharose CL2B, we studied two other cross-linked agaroses, as well as two synthetic polymer supports. Immobilization chemistries included cyanogen bromide activation of agarose, 2-fluoro-2 methylpyridinium toluene-4-sulfonate activation of one of the synthetic polymer as well as aldehyde group reduction by $NaCNBH_3$ to form secondary amine linkages on one of the cross-linked agaroses. To determine the feasibility of using the resins in large-scale immunoaffinity chromatographic purification of FIX, we studied physical and biochemical properties of the resins. The physical characteristics studied included the crushability of the resins under pressure as well as ability to support increasing flow-rates at increasing pressures. The biochemical examination of the various resins focused on efficiency of antigen capture by the immobilized antibody ligand and the effect of flow-rate on MAb efficiency, where we found that very low flow-rates slightly increased the capacity of the MAb.

The results demonstrate a straightforward method of assessing the feasibility of using particular resins in large-scale affinity purification.

INTRODUCTION

The separation of proteins from complex mixtures using immunoaffinity chromatography is being increasingly applied to large-scale processes [1]. In the development of these large-scale processes, it is important to select a resin that effectively separates the desired protein while being capable of supporting high flow-rates and resisting extensive crushing.

The American Red Cross has developed a large-scale immunoaffinity process [2] based on the use of a divalent cation-dependent monoclonal antibody (MAb) [3] coupled to Sepharose CL2B (Pharmacia, Uppsala, Sweden) to produce highly pure human coagulation factor IX (FIX), an essential protein involved in the blood clotting cascade. This resin support is also being utilized in the development of various other immunoaffinity-purified plasma protein products. In the course of the process devel-

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opment, we discovered that the CL2B resin was not capable of supporting a high flow-rate without developing a significant back pressure. We also observed that the maximum pressure that the CL2B resin could withstand without being crushed was less than 0.3 bar. To operate the process at this low pressure involved an unacceptable lengthening of the process time and attendant decreases in process efficiency. The CL2B resin performed well as far as the ability to bind high levels of FIX and the purity of the product obtained. In earlier studies, for example, comparing hydrazide and CNBr-activated immunosorbents [4], the CNBr-CL2B resin performed as well or better than the other resins tested.

However, in order to decrease process time while maintaining the purity of the FIX produced, we explored the use of several other MAb support resins, two other agarose-based resins as well as two synthetic polymer supports. In this paper, we report the data from our investigations.

MATERIALS AND METHODS

Resins

The resins used in the flow and compression studies as well as the immunoaffinity chromatography studies are listed in Table I. They have been identified by letter to facilitate labeling and their major characteristics are listed, including pore exclusion limit, pressure limit and the maximum supportable linear flow-rate. Manufacturer's of the resins are also identified.

Pressure/flow experiments

Fig. 1 illustrates the experimental apparatus used to measure resin compression and the relationship between pressure and flow for each of the resins tested. In each experiment, the resin was packed into the column (C10/20, 200 mm × 10 mm I.D., adjustable bed depth, Pharmacia) and allowed to settle under gravity. The upper flow adapter was then inserted to the upper level of the resin bed and then the pressure source connected. Pressure was increased in the feed source tank and flow-rate and resin bed height were measured. After reaching the pressure limit of the column (2 bar), the pressure was decreased and the flow-rate and column height were measured again. The volumetric flow-rate was divided by the column cross sectional area (0.7854 cm^2) and expressed as a linear flow-rate (cm/min).

TABLE I

RESINS COMPARED IN FLOW AND GEL COMPRESSION STUDIES AND FACTOR IX AFFINITY CHROMATOGRAPHY

Resin label	Resin type	Exclusion[a] limit (dalton)	Pressure[a] limit (bar)	Maximum[a] linear flow-rate (cm/min)	Coupling chemistry	MAb density immobilized (mg MAb/ml resin)	Manufacturer
A	2% Cross-linked agarose	$20 \cdot 10^6$	<0.1	0.25	CNBr activation	1.0	Pharmacia (Piscataway NJ, USA)
B[b]	6% Cross-linked agarose	$4 \cdot 10^6$	1.0	>35	NC	–	Pharmacia
C	6% Cross-linked agarose	$6 \cdot 10^6$	2	50	Aldehyde reduction	1.0, 0.5[a]	Sterogene (Arcadia, CA, USA)
D	Azlactone copolymer		>3.5	>32	Amino group reduction	0.96[a]	3M (St. Paul, MN, USA)
E[c]	Poly-acrylate	$10 \cdot 10^6$	6.9	2.5	FMP activation	1.55[a]	Bioprobe (Tustin, CA, USA)

[a] Data reported by manufacturer.
[b] Resin B not tested in affinity studies.
[c] Resin E not tested in flow studies.

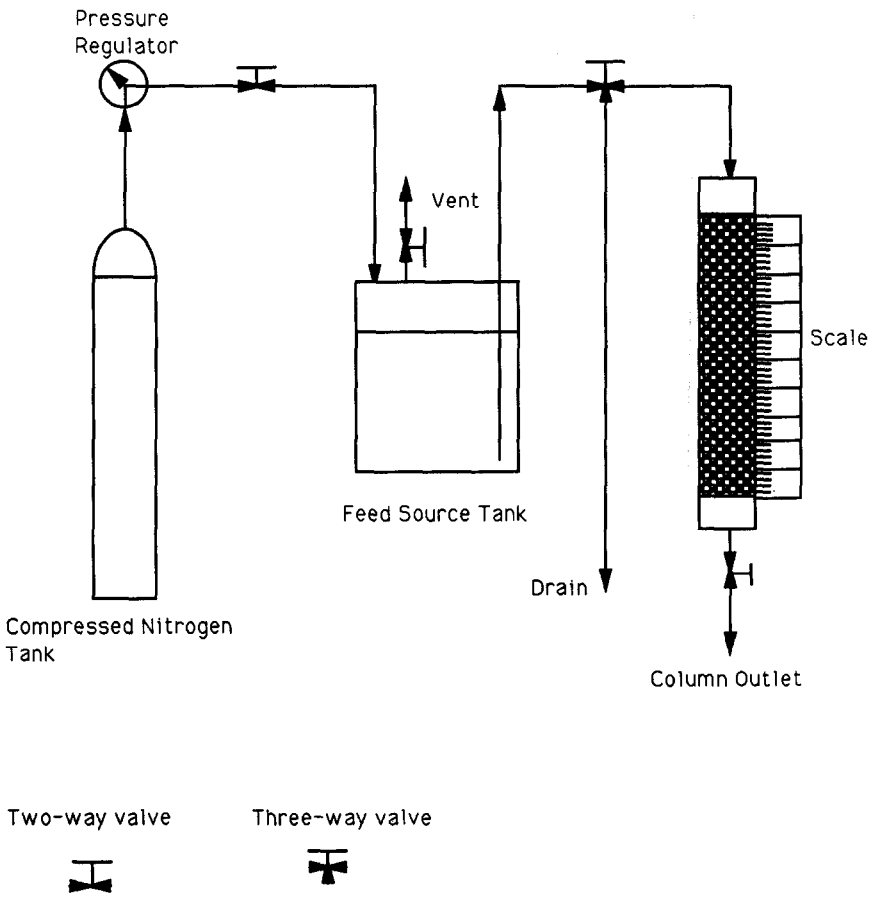

Fig. 1. Experimental apparatus for flow-rate and resin compression studies.

The change in bed height was expressed in terms of the percent change in resin bed volume.

Factor IX binding and immunoaffinity chromatography

The experimental apparatus used for the FIX binding and chromatography studies is illustrated in Fig. 2. The various resins were obtained from the respective manufacturers with MAb coupled at the densities indicated in Table I. The experimental conditions employed are listed in Table II. The resins were packed into a column (G10 × 150, 150 mm × 10 mm I.D., adjustable bed depth, Amicon, Danvers, MA, USA) and allowed to settle under gravity. The upper flow adapter was inserted to the upper level of the resin bed and the resin was then equilibrated with 10 m*M* $MgCl_2$, 100 m*M* NaCl, 20 m*M* imidazole, pH 7.2. The load material was immunoaffinity-purified FIX [5]. $MgCl_2$ was added to this to a final concentration of 40 m*M* and then it was filtered through a 0.2 μm filter (Sterivex, Millipore, Bedford, MA, USA) and loaded onto the column. The column was then washed with 1.0 *M* NaCl, 20 m*M* imidazole, 10 m*M* $MgCl_2$, pH 7.2 until the absorbance at 280 nm was <0.01. The bound proteins were then eluted with 20 m*M* sodium citrate, 110 m*M* NaCl, pH 6.8. Elution was considered complete after the absorbance reached the baseline. The column was regenerated with 2.0 *M* NaCl, 100 m*M* sodium citrate, pH 7.2. The flow-through material from the load, wash, elution and regeneration steps was collected in pools and as-

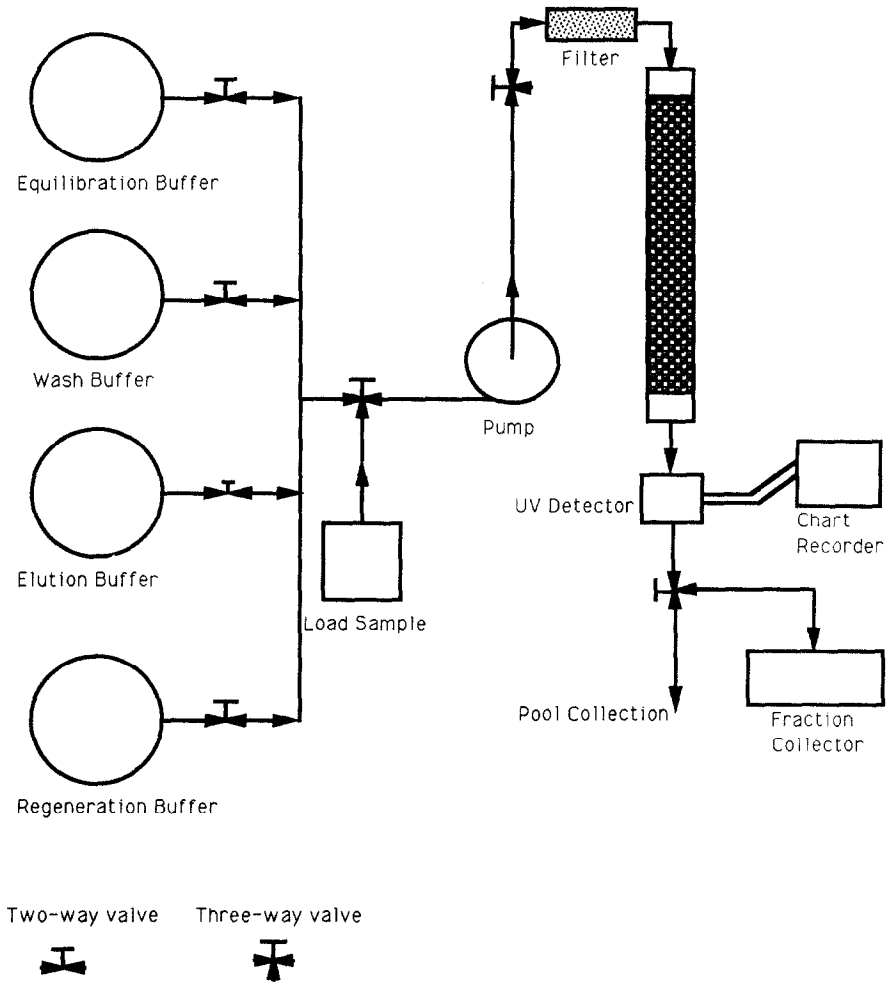

Fig. 2. Experimental apparatus for immunoaffinity purification studies.

sayed for FIX activity and protein content. All chemical were reagent grade from Sigma (St. Louis, MO, USA).

To study the effect of flow-rate on MAb capacity and efficiency, resin C was chosen at a MAb density of 1.0 mg MAb/ml. In these experiments, the volumetric flow-rates were varied from 0.35 to 5.0 ml/min. Using similar methods as described above, the capacity of the MAb was calculated as well as the recovery of FIX.

Factor IX Activity assay

FIX activity was measured using a one-stage coagulation assay [6] of the sample against a pooled plasma standard. Details of the assay procedure are available from Menache *et al.* [7].

Protein assay

Total protein was calculated from the absorbance at 280 nm, after subtracting scatter at 320 nm, assuming an extinction coefficient of 13.3 [8]. It was possible to calculate the protein mass balance as the starting material was essentially a single protein of highly pure FIX.

RESULTS AND DISCUSSION

In Fig. 3A, the effect of pressure on flow-rate is

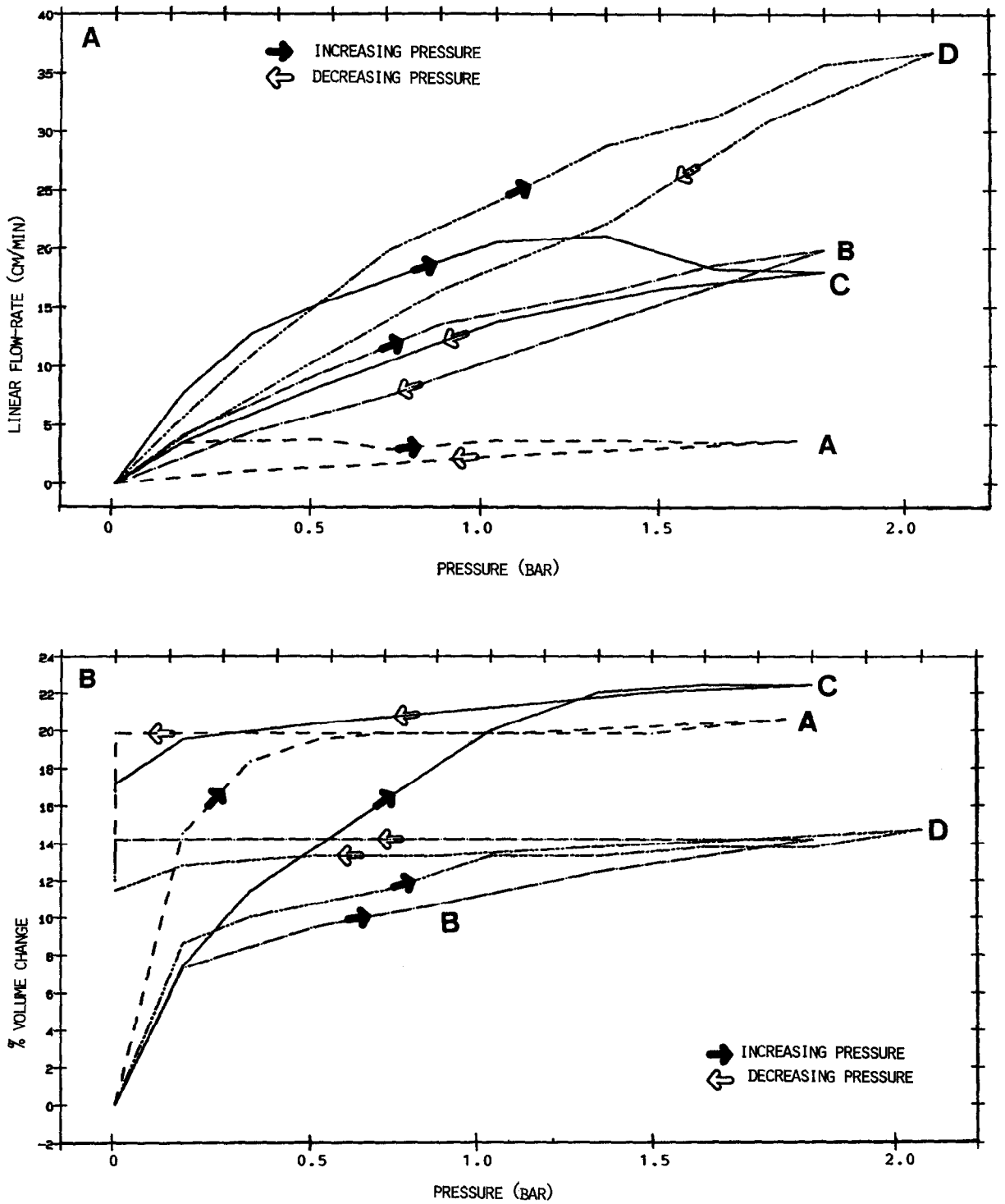

Fig. 3. Comparison of (A) linear flow-rate and (B) volume change in resins A, B, C and D.

shown. As the pressure on the resins is increased the flow-rate increases upto a certain point. This trend is common to all the resins studied. In the case of resin A (a 2% cross-linked agarose), the increase in flow-rate levels off after 0.136 bar. The pressure was then decreased within an hour of reaching this maximum point. As the pressure decreases, the flow-rate demonstrates hysteresis-type behaviour, suggesting that the structure of the resin may have been altered under pressures greater than the specified pressure limit (see Table I). This structural alteration may include irreversible deformation of the resin that results in a configuration that offers greater resistance to flow, hence the lower flow-rate at similar pressures for the decreasing pressure regime. Resins B and C (both 6% cross-linked agaroses) and resin D (a synthetic polymer) demonstrate a greater ability to support higher flow-rates at greater pressure, a consequence of their ability to support higher pressure drops (Table I). However, each resin demonstrated hysteresis behaviour for the flow-rate when the pressure was decreased, even though the maximum pressure limit was not reached. This would suggest that (1) all the soft resins undergo some irreversible compression upon the application of pressure, and (2) the synthetic polymers undergo some degree of compaction. For all the resins tested in this manner, the resins were allowed to "relax" under atmospheric conditions after the entire experiment, and then pressure was applied again. Although quantitative data was not recorded, we observed that the resins supported lower flow-rates for a given pressure drop, suggesting again that some irreversible deformation may have occurred during the first application of pressure. A more thorough evaluation of this phenomenon would require studies that explicitly compare the time-based behaviour of the elasticity of the resins. Resin D shows the least resistance to flow, probably due to its synthetic polymeric structure. However, it is interesting to note that even in this case hysteresis in flow-rate is observable. Resin E was not tested in flow studies.

Fig. 3B shows the volume change that the resins undergo upon application of pressure. The change in volume is a measure of the compression of the resins under pressure. Comparison of Fig. 3A and B suggests that there is no direct correlation between the effect of pressure on flow-rate and resin volume. For example, one of the 6% cross-linked agaroses (C) and the 2% cross-linked agarose (A) undergo

TABLE II

IMMUNOAFFINITY PURIFICATION EXPERIMENTAL CONDITIONS

Experimental condition	Resin					
	A	C	C'[a]	D	D'[b]	E[c]
Column volume[d] (ml)	5.0	5.5	5.1	2.0	2.5	4.3
MAb density (mg MAb/ml resin)	1.0	1.0	0.5	0.97	0.97	1.55
FLow-rate[e] (ml/min)	1 ± 0.1	0.99 ± 0.1	0.97 ± 0.1	0.31 ± 0	0.96 ± 0.05	0.95
Feed concentration of FIX[e] (units/ml)	108 ± 9	111 ± 3	111 ± 3	110 ± 3	104 ± 5	115
FIX/MAb load ratio[e] (units FIX loaded/ mg MAb immobilized)	101 ± 9	104 ± 2	108 ± 6	112 ± 3	100 ± 0.5	108

[a] Resin C' was the same as resin C except the density of MAb on C' was halved.
[b] Resin D' is the same as resin D, but the flow-rate in experiments with D' were increased by a factor of three.
[c] Mean and standard deviation were not calculated for resin E.
[d] The cross-sectional area of the column was 0.7854 cm^2.
[e] Mean ± standard deviation (n = 4).

similar changes in volume, although demonstrating very different flow-rate capacities. This suggests that the compression of the resin, the reduction in supportable flow-rate and the irreversible deformation of the resin are interconnected but not in a straightforward manner. Studies specifically targetted at the relationship between volume change and supportable flow-rate for each resin are indicated.

Table III shows the results of the binding and elution studies. Since all the resins were challenged with the same ratio of FIX to immobilized MAb, the percent of FIX protein not bound is an indication of the ability of the immobilized MAb to bind FIX. Resin A demonstrates the highest capacity for FIX. Resin C and C′ perform similarly, the main difference being in the percent of MAb that is active. As resin C′ contains half the amount of MAb as C, these results suggest that at lower immobilization densities less MAb is available for FIX to bind to. Interestingly, previous work in this laboratory demonstrated that at resin densities ranging from 1.0 to 9.7 mg MAb/ml, the higher MAb densities bound less FIX. Working with other antibodies and ligands, Eveleigh and Levy [9] demonstrated that higher ligand densities lead to lower capacity, and suggested that steric hinderances at high ligand densities decreased the accessibility of the ligand. The results obtained here suggest that there may be a threshold density at which the benefits of more immobilized MAb are offset by increasing steric or diffusional hinderances that result in lower MAb utility and activity. At the very low immobilization densities of 0.5 to 1.0 mg MAb/ml, however, the more MAb immobilized the more FIX bound.

Resin D and E did not perform well in comparison to A and C (with the highest amount of active MAb), suggesting that the MAb immobilized on these resins may have lost activity during the immobilization step. The synthetic polymers tested demonstrated the poorest activity of MAb. It should be pointed out that the coupling chemistries utilized for MAb immobilization (listed in Table I) were different for each resin studied. The chemistries employed were those recommended as the optimum choice by the respective manufacturer. Coupling chemistry has a definite impact on the efficiency of affinity purification using immobilized MAbs. Different coupling chemistries have given rise to variation in the ability of ligand to bind ligate [9] and in the stability of immobilized ligands [11]. There is an

TABLE III

MAb UTILITY AND CAPACITY AND FACTOR IX BINDING AND ELUTION TO RESINS

	Resin					
	A	C	C′	D	D′	E[b]
% of load FIX protein unbound[a]	13±3	21±5	24±10	52±4	25±6	95
% of load FIX protein eluted[a]	65±3	52±14	65±3	39±3	53±9	13[c]
Utility of FIX binding to MAb[a,d] (units FIX bound/ mg MAb immobilized)	93±8	95±4	79±5	12±0.1	51±2	1.5
% of MAb active[e]	69±6	71±3	59±4	9±0.08	38±1.5	1.1

[a] Means ± standard deviation (n = 4).

[b] Mean and standard deviation were not calculated for resin E. Although only one experiment is reported, resin E repeatedly did not bind FIX. The data reported is the best of all experiments performed.

[c] The summed recovery of protein in the case of resin E was > 100%, possibly because of UV absorbing material leaching from this resin.

[d] MAb binding efficiency is calculated by subtracting the total unitage of FIX that does not bind to the affinity resin from the total FIX unitage loaded.

[e] The percent of active MAb is calculated assuming that a theoretical maximum (100%) of two moles of FIX binds per mole of MAb.

TABLE IV

PURITY OF FACTOR IX PRODUCED BY DIFFERENT RESINS

The specific activity of the product was calculated by dividing the measured activity by the calculated protein concentration. A 100% pure solution of Factor IX has a theoretical specific activity that can range between 200 and 250 units/mg [9].

Resin	Specific activity (units FIX/mg protein) (mean ± S.D., $n=4$)
A	182 ± 15
B	252 ± 24
C	148 ± 3
D	69 ± 2
D′	243 ± 25
E	28[a]

[a] Mean and standard deviation were not calculated for resin E. Although only one experiment is reported, the data reported are the best of all experiments performed with resin E.

optimum coupling chemistry for each resin used, one that is usually recommended by the resin manufacturer after extensive testing. However, the specificity of coupling protocol to resin most likely extends to a specificity for different ligands as well. Thus, what works best for one ligand–resin couple may not be optimum for another ligand on the same resin. It is likely that these factors affected our results and lead to the wide variation in MAb utility and activity.

Table IV shows FIX specific activity, which is a measure of the purity and activity of the FIX recovered from each immunoaffinity resin. Resins A, C and D′ demonstrate that they are capable of producing very pure FIX products. Due to difficulties inherent in assaying very pure FIX preparations [12], specific activities around 200 units FIX/mg are considered to be around or at the maximum possible [10]. Resin E consistently did not perform well according to any of the criteria.

Fig. 4 shows the effect of flow-rate on MAb ca-

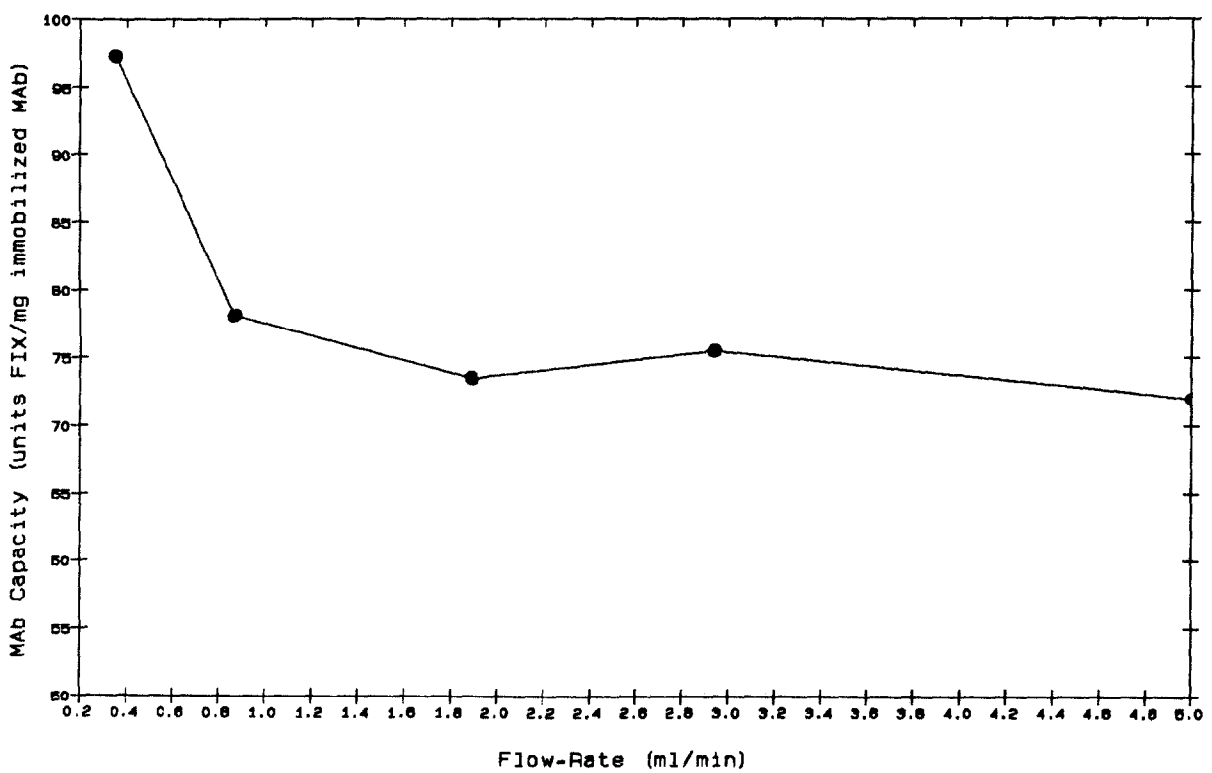

Fig. 4. Effect of flow-rate on the capacity of MAb immobilized on resin C at 1.0 mg/ml resin.

pacity (defined as the units of FIX bound per mg of immobilized MAb) for resin C. There is a slight decrease in the capacity of the MAb as the flow-rate is increased from 0.35 to 1.0 ml/min. After this point, further increases in flow-rate do not demonstrate a significant decrease in the amount of FIX bound by the MAb. The small decrease in capacity suggests that slight kinetic or diffusive effects may influence the binding of FIX. As the flow-rate increases, residence time for the FIX in the immunoaffinity resin bed decreases and thus the time available for the FIX to migrate (diffusion) to MAb immobilized inside the bead and bind (kinetics) to it decreases. Earlier work [2] suggested that FIX purification on Sepharose CL2B was not affected by increases in flow-rate. The lower limit of the flow-rate in those experiments [2] (in an identical column) was 1.0 ml/min. When the flow-rate is decreased below 1.0 ml/min, it is likely that the process approaches a batch load, thus increasing time for FIX binding and concomittantly increasing MAb capacity. Another factor may have been the resin used. In the earlier studies [2], the resin used was 2% cross-linked agarose, instead of the 6% cross-linked agarose (resin C) used here. Increased cross-linking may magnify diffusive resistance and thus increase the effect of flow-rate as shown by the slight increase demonstrated here. Further research will focus on investigating the effect of the degree of cross-linking of the resin substrate on immobilized MAb capacity.

For resin D, the results obtained at a reduced flow-rate was the reverse of those obtained with resin C, as shown in Table III. A three-fold decrease in flow-rate (D′) resulted in a four-fold decrease in MAb utility and % MAb active. This difference in response may be caused by the different coupling chemistries employed (Table I). The exact reason is not clear and a thorough study of flow-rate effects on resin D will yield more comprehensive results.

From these experimental comparisons, it appears that of all the resin tested, the 6% cross-linked agarose would be the most amenable to larger scale purification of FIX using this MAb. It can support a high pressure and flow-rate while not undergoing extensive compression. At the same time, it yields an extremely pure FIX preparation as demonstrated by the specific activity of the recovered FIX. It should be stressed that these results obtain for the ligand–ligate pair of this MAb and FIX. As has been emphasized by numerous investigators, these results cannot be extended to other ligand–ligate–resin systems.

CONCLUSIONS

All the resins tested consistently bound FIX, except for E which was as consistent in its inability to bind FIX. Although resin A performs well according to FIX binding, elution and purity criteria, its ability to support high flow-rates is severely restricted at higher pressures. Resins C and D, a 6% cross-linked agarose and a synthetic polymer, respectively, both performed well according to the physical criteria. Resin C is, however, better in the efficiency with which it binds FIX. Resin E did not perform satisfactorily. Studies on the effect of flow-rate demonstrated a slight increase in the capacity of MAb on resin C at very slow flow-rates.

The conclusions reached in this study cannot be directly extended to other resin–ligand systems. However, they do illustrate a systematic evaluation of the suitability of various resins for large-scale immunoaffinity purification processes.

REFERENCES

1 D. C. Yanis, *Bio/Technology*, 5 (1987) 1290.
2 J. Tharakan, F. Highsmith, D. Clark and W. Drohan, W., *J. Chromatogr.*, 522 (1990) 153–162.
3 H.-L. Wang, J. Steiner, F. Battey and D. Strickland, *Fed. Proc.*, 46B (1987) 2119.
4 R. D. Madurawe, C. L. Orthner, J. Tharakan, F. A. Highsmith, W. N. Drohan and W. H. Velander, *J. Chromatogr.*, 558 (1991) 55.
5 J. P. Tharakan, S. I. Miekka, H. E. Behre, B. D. Kolen, D. M. Gee, W. N. Drohan and D. B. Clark, *Throm. Haem.*, 62 (1989) 56.
6 R. Biggs (Editor), *Human Blood Coagulation Haemostasis and Thrombosis*, Blackwell Scientific, Oxford, 1st Ed., 1972, p. 64.
7 D. Menache, H. E. Behre, C. L. Orthner, H. Nunez, H. D. Andersen, D. C. Triantaphyllopoulos and D. P. Kosow, *Blood*, 64 (1984) 1220.
8 H. A. Liebman, S. A. Limentani, B. C. Furie and B. Furie, *Proc. Natl. Acad. Sci. U.S.A.*, 82 (1985) 3879.
9 J. W. Eveleigh and D. E. Levy, *J. Solid-Phase Biochem.*, 2(1) (1977) 45.
10 J. Tharakan, D. Strickland, W. Burgess, W. N. Drohan and D. B. Clark, *Vox Sang.*, 58 (1990) 21.
11 H. Sato, T. Kidaka and M. Hori, *Appl. Biochem. Biotech.*, 15 (1987) 145.
12 S. I. Miekka, *Thromb. Haem.*, 58 (1987) 349.