ELSEVIER

Journal of Chromatography A, 743 (1996) 51–55

JOURNAL OF CHROMATOGRAPHY A

Method for obtaining unique selectivities in ion-exchange chromatography by addition of organic polymers to the mobile phase

Pete Gagnon[a,*], Bill Godfrey[b], Don Ladd[b]

[a]*Validated Biosystems, 5800 North Kolb Road, Suite 5127, Tucson, AZ-85750, USA*

[b]*Becton Dickinson Immunocytometry Systems, 2350 Qume Drive, San Jose, CA-95131, USA*

Abstract

We have observed that the addition of poly(ethylene glycol) (PEG) to the mobile phase systematically and significantly alters the retention behaviour of proteins in ion-exchange chromatography. The magnitude of the effect is proportional to the molecular mass and concentration of the added PEG. Retention of most proteins is increased, with fair correlation between protein size and degree of enhancement, but with significant influence by variations in protein surface chemistry. The charge on the exchanger and the native (nonenhanced) retention characteristics of the proteins appear to have no effect. Because of its independence from the native selectivity of the ion-exchanger, mobile phase polymer addition creates unique compound selectivites. Addition of PEG also increases viscosity, with the attendant affects of reducing flow-rate and dynamic binding capacity, while increasing eluted peak width.

Keywords: Mobile phase composition; Selectivity; Proteins; Poly(ethylene glycol)

1. Introduction

The addition of organic polymers has been reported previously to increase protein partition coefficients in size-exclusion and protein A affinity chromatography [1–7]. In the case of protein A, the addition of PEG-6000, has the same ability as added salt to enhance the binding of mouse IgG_1, with the degree of enhancement directly dependent upon the concentration of added polymer [3]. In size-exclusion, partition coefficients are likewise increased in proportion to the polymer concentration, with larger proteins generally being more affected than smaller ones. Most size-exclusion studies have attributed these effects to steric exclusion of the protein by the polymer. This is consistent with the mechanism as it has traditionally been described for fractional precipitation with PEG [8–11]. More recently, this view has been challenged on the basis of studies demonstrating preferential exclusion of PEG from protein surfaces. This is hypothesized to elicit an energetically favourable sharing of the cosolvent exclusion (hydration) shells surrounding the proteins and chromatography media, and hence to elevated partition coefficients [12–14].

We evaluated the behaviour of model proteins in PEG solutions of various concentrations on ion-exchangers. The results indicated that although PEG increases retention, it acts independently from ion-exchange and thereby produces potentially useful compound selectivities. Preliminary experiments showed the effects of PEG to be superficially consistent with the steric exclusion hypothesis. How-

*Corresponding author.

0021-9673/96/$15.00
PII S0021-9673(96)00125-2

ever additional data invalidated this model while supporting the cosolvent exclusion model.

Although the combination of PEG and ion-exchange produced unique selectivities, the preparative potential of this approach is limited. The increase in mobile phase viscosity also restricted flow-rate, depressed dynamic binding capacity and caused substantial peak broadening. In this study we report our findings and suggest preparative applications that may benefit from them.

2. Experimental

SOURCE 15Q and 15S ion-exchangers (1 ml prepacked, 30×6.4 mm) were obtained from Pharmacia Biotech (Piscataway, NJ, USA). Detailed physical and chemical descriptions of these media can be found in ref. [15]. Buffers, salts and PEGs of different molecular masses were purchased from Sigma (St. Louis, MO, USA). Purified transferrin (TRF, $M_r \approx 76 \cdot 10^3$), bovine serum albumin (BSA, $M_r \approx 68 \cdot 10^3$), α-chymotrypsin (ACT, $M_r \approx 25 \cdot 10^3$) and lysozyme (LYS, $M_r \approx 14 \cdot 10^3$) were also purchased from Sigma. R-phycoerythrin (RPE, $M_r \approx 260 \cdot 10^3$) was purchased from QuantaPhy (Santa Cruz, CA, USA). Mouse IgG monoclonal antibodies (MAb, $M_r \approx 155 \cdot 10^3$) were obtained from Becton Dickinson Immunocytometry Systems (San Jose, CA, USA).

Anion-exchange experiments were conducted in a base binding buffer of 0.05 *M* Tris, pH 8.6, and eluted in a linear gradient to 0.05 *M* Tris, 1.0 *M* sodium chloride, pH 8.6. The base buffers for cation-exchange were 0.05 *M* MES, pH 6.0 and 0.05 *M* MES, 1.00 *M* sodium chloride, pH 6.0. Series of experiments were conducted with PEG formulated into the base buffers at various incremental levels: from 0–15% for PEG-6000; and 0–37.5% for PEG-400. The run format used through the study was to equilibrate the column with 10 column volumes (CV) of binding buffer, inject 20 μl of purified protein at 1 mg/ml, wash with 2 CV binding buffer, elute in a 30 CV linear gradient ending at 1 *M* sodium chloride and strip with an additional 5 CV of the high salt buffer. Experiments with 0%, 5%, 10% and 15% PEG-6000 were conducted at eluent velocities of 940, 750, 565 and 375 cm/h respectively. These values correspond to 5, 4, 3 and 2 ml/min. Experiments with 12.5%, 25% and 37.5% PEG-400 were conducted at eluent velocities of 750, 565 and 375 cm/h. All experiments were conducted at room temperature (20–22°C). Protein elution positions were expressed as the salt concentration at peak centre.

The effects of mobile phase viscosity on eluted peak width were determined at 565 cm/h in 0%, 5% and 10% PEG-6000. Measurements were taken at 10% peak height.

3. Results and discussion

Fig. 1 compares anion-exchange results obtained from RPE with PEG-6000 and PEG-400 (average molecular masses of 6000 and 400, respectively). Protein retention was enhanced with increasing PEG concentration for both additives, but dramatically more so and at lower concentrations with PEG-6000. These data are consistent with the observed effectivity of different PEGs in fractional precipitation of proteins [8–11].

Fig. 2 illustrates the anion-exchange results from BSA, TRF and a MAb at varying levels of PEG-6000. The shapes of the response curves were generally similar but the degree of binding enhancement varied with the protein. Elution order was

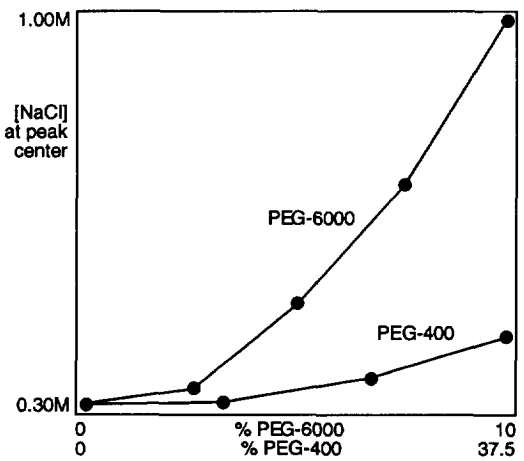

Fig. 1. Enhancement of R-phycoerythrin retention in anion-exchange as a function of PEG polymer size and concentration. See Section 2 for materials and methods.

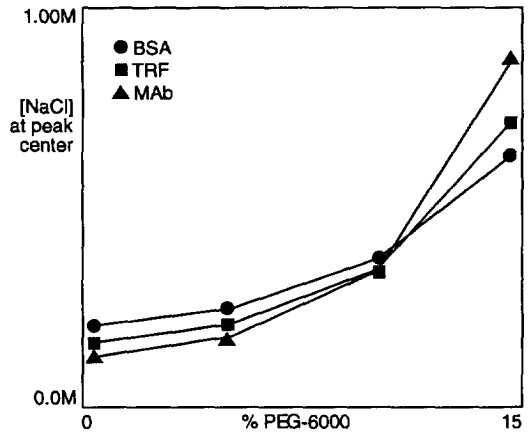

Fig. 2. Enhancement of anion-exchange retention for BSA, TRF and MAb as a function of PEG-6000 concentration. BSA=bovine serum albumin, TRF=human transferrin and MAb=mouse monoclonal IgG. See Section 2 for materials and methods.

completely reversed at 15% PEG. Fig. 3 illustrates the cation-exchange results from LYS, ACT and the same MAb at the same levels of PEG. The magnitude of the MAb response in cation-exchange was nearly identical to the response on anion-exchange. ACT showed virtually no response up to 10% PEG, and only a minor positive response at 15%. LYS retention diminished with increasing PEG. Had native retention behaviour been a positive factor for either anion- or cation-exchange, then the original elution orders would have been maintained and enhancement would have been greatest for the proteins with the strongest initial retention characteristics. Data to the contrary confirmed that the effects of PEG were independent of ion-exchange.

Linear regression of percent enhancement at 10% PEG, versus log molecular mass for the six proteins in Figs. 1–3 revealed a fair correlation (r=0.964, y=0.005x+4.348). Although this was consistent with the steric exclusion model and with reported results of fractional precipitation with PEG, it was not clear whether it reflected a causal relationship or whether it reflected secondary correlation to an alternative mechanism. The effect of PEG-6000 on six different mouse IgG_1 monoclonal antibodies was measured in order to evaluate the relative contribution of protein surface chemistry (Fig. 4). The magnitude of the response was highly variable from one MAb to another; generally on a par with the range of differences observed with proteins of different size. Reminiscent results have been reported in size-exclusion chromatography with three proteins of $M_r \approx 25 \cdot 10^3$ [4]. While these data do not eliminate a contributory role by protein size, they severely discredit the steric exclusion model of PEG action.

Published data from studies of fractional precipitation with PEG further compromise the steric exclusion model. Like Fig. 4, precipitation studies have noted variation in the behaviour of proteins of similar size [8–11]. They have also reported varia-

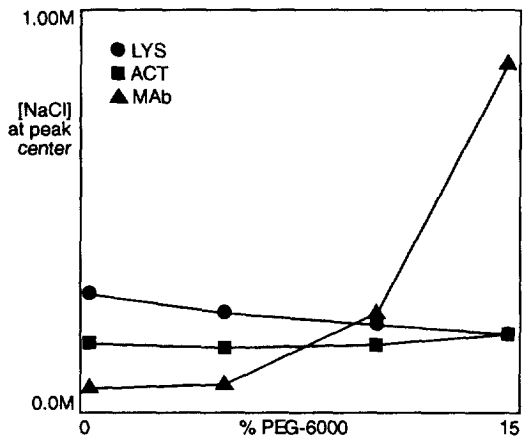

Fig. 3. Enhancement of cation-exchange retention for LYS, ACT and MAb as a function of PEG-6000 concentration. LYS=lysozyme, ACT=α-chymotrypsin and MAb=mouse monoclonal IgG. See Section 2 for materials and methods.

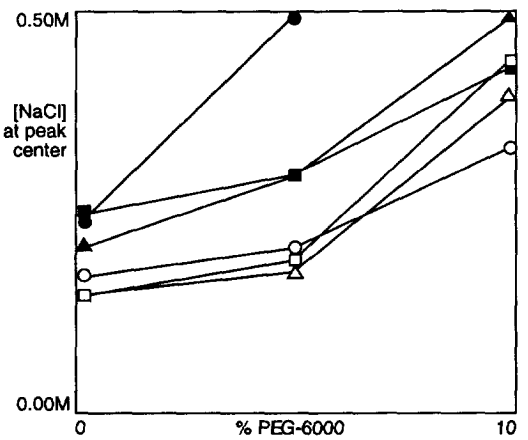

Fig. 4. Differential enhancement of mouse IgG_1 monoclonal antibodies as a function of PEG-6000 concentration. See Section 2 for materials and methods.

tions with respect to pH and ionic strength [8,16]. All of these findings are contrary to the expectations of the steric exclusion hypothesis. However they are fully consistent with the cosolvent exclusion model, which suggests that PEG response should be influenced by protein surface chemistry, to the extent that it determines hydratability; and by protein size, to the extent that it reflects hydratable surface area.

While the foregoing results confirmed that PEG could be used in combination with ion-exchange to produce unique compound selectivities, other data suggested that the range of practical preparative applications would be limited. Dynamic binding experiments with MAb and ACT at 0 and 10% PEG-6000 showed approximately a 60% loss of capacity for ACT versus a 5% increase for MAb. Given that ACT retention was essentially level across this PEG concentration range, we attributed the reduction in capacity to the viscosity of the PEG solution. This is consistent with the observation that increased viscosity depresses diffusivity [17]. Given that enhancement of retention for the MAb was about 300% versus only a 5% increase in capacity, we interpreted the data as indicating that even though the retention enhancing effect of PEG on a relatively large protein was able to prevent viscosity-mediated capacity reduction, viscosity nevertheless had a depressive effect. This interpretation is consistent with related observations in hydrophobic interaction chromatography where dynamic capacity increases with increasing surface tension despite the depressive effects of mobile phase viscosity [18]. Eluted peak width also increased substantially with PEG concentration (Fig. 5). As with retention, larger proteins were affected more than smaller ones. These results predict that among proteins of similar size in general, and among large proteins in particular, the potential benefits of altered selectivity may be sacrificed to peak broadening.

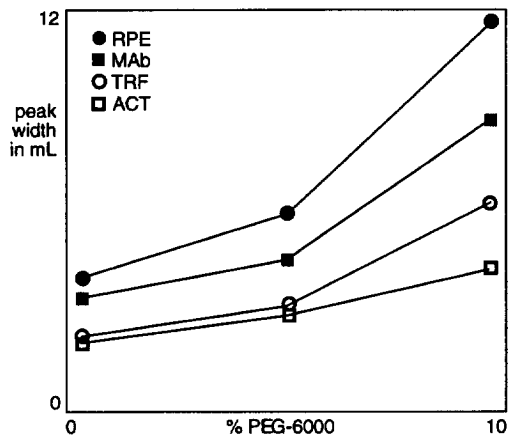

Fig. 5. Peak broadening as a function of PEG-6000 concentration. See Section 2 for materials and methods. BSA=bovine serum albumin, TRF=human transferrin, MAb=mouse monoclonal IgG, LYS=lysozyme, ACT=α-chymotrypsin and RPE=R-phycoerythrin.

4. Conclusions

Increases in the retention of proteins on ion-exchangers as a function of PEG concentration appeared to result from the sharing of cosolvent exclusion shells between the protein and chromatography matrix. Larger size classes of PEG were stronger effectors. Differences in both protein size and surface chemistry significantly influenced the degree to which any given protein was affected.

Although addition of PEG produced unique compound selectivities, the secondary effects of viscosity severely limit the preparative potential for this technique. Elevated viscosity required reduction of flow-rate. It severely depressed dynamic binding capacity for small proteins, even though capacity appeared to be maintained or slightly increased for larger ones. It also substantially increased peak width. Nevertheless, among coeluting proteins of significantly dissimilar size, addition of PEG may have useful preparative applications. Under favourable circumstances PEG enhancement of ion-exchange separations may prove more effective than size-exclusion chromatography. This should be especially true in situations where the smaller protein elutes from an ion-exchanger in advance of the larger. The application areas where this technique is most likely to be useful include purifications of large protein fragments from enzyme digests, such as FAb or $F(ab)_2'$; purification of protein conjugates; and fractionation of aggregates or polymers from monomeric protein.

References

[1] S.-C. Yan et al., Anal. Biochem., 138 (1984) 137.
[2] C. De Ligny et al., J. Chromatogr., 294 (1984) 223.
[3] P. Gagnon, Multiple mechanisms for improving binding of IgG to Protein A, Program of contributed posters, BioEast '92, Washington, D.C. (1992).
[4] J. Brewer and L. Soderberg, in R. Epton (Editor), Chromatography of Synthetic and Biological Polymers, Ellis Horwood, Chichester, 1977, Vol. 1. p. 285.
[5] K. Hellsing, J. Chromatogr., 36 (1968) 170.
[6] J. Giddings and D. Dahlgren, Sep. Sci., 5 (1970) 717.
[7] T. Laurent and J. Killander, J. Chromatogr., 14 (1964) 317.
[8] K. Ingham, Methods Enzymol., 182 (1990) 301.
[9] P. Foster et al., Biochim. Biophys. Acta, 317 (1973) 505.
[10] A. Polson et al., Biochim. Biophys. Acta, 82 (1964) 463.
[11] I. Juckes, Biochim. Biophys. Acta, 229 (1971) 535.
[12] J. Lee et al., Biochemistry, 18 (1979) 5518.
[13] J. Lee et al., J. Biol. Chem., 256 (1981) 625.
[14] T. Arakawa, Anal. Biochem., 144 (1984) 267.
[15] P. Gagnon, E. Grund and T. Lindbäck, BioPharm, 8 (1995) 21.
[16] P. Albertsson, Partition of Cell Particles and Macromolecules, Almqvist and Wiksell, Stockholm, 1960.
[17] R. Chicz and F. Regnier, Methods Enzymol., 182 (1990) 392.
[18] P. Gagnon and E. Grund, BioPharm, 9 (1996) 34.