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Evaluation of the polyethylene glycol–KF–water system in the context of purifying PEG–protein adducts

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Abstract

Covalent binding of PEG to proteins leads to conjugates widely investigated in several biotechnological processes. Their use as pharmaceuticals requires both careful purification and proper characterization. In this context, this paper examines the potentialities offered by hydrophobic interaction chromatography and compares aqueous potassium fluoride and ammonium sulfate as the eluents. Relative contribution of the various forces which dictate the chromatographic behaviour of PEG–protein adducts on Fractogel TSK–Butyl 650 is discussed.

Keywords: Aqueous two-phase system; Hydrophobic interaction chromatography; Salting out; Partition

1. Introduction

The covalent modification of proteins by polyethylene glycol (PEG) chains leads to adducts which, upon delivery to living organisms, exhibit extended in vivo residence time. It is thus not a surprise if grafting PEG to pharmacologically active polypeptides has become today a technique currently evaluated for improving therapeutical indexes of protein drugs (Nucci et al., 1991; Zalipsky and Lee, 1992; Katre, 1993; Veronese, 1994). However, before any drug development, an important aspect of the PEG-technology which should inevitably be addressed concerns purification and chemical characterization of PEG–protein conjugates (Hoyle, 1991). This point thus deserves sustained attention.

Ion-exchange chromatography has been successfully used to separate monopegylated proteins from their unmodified counterparts. Some reported examples include the proteins: interleukin-

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2 (Goodson and Katre, 1990; Sato et al., 1996), papain (Paul et al., 1994), caricain (Musu et al., 1996), glycyl endopeptidase (Azarkan et al., 1996b) and human granulocyte colony-stimulating factor (Kinstler et al., 1996). S-Sepharose Fast Flow still provides a nice separation between unmodified chymopapain and the two PEG–chymopapain conjugates containing respectively one and two PEG chains of $M_r = 5$ kDa (Azarkan et al., 1996a). As expected however, bad chromatographic separations on ion-exchange supports result from a further increase of the rate of proteins modification. This is illustrated, e.g. in the recent paper dealing with pegylation of human growth hormone (Clark et al., 1996). It may also be anticipated that ion-exchange chromatography will fail to fractionate PEG–protein conjugates when neutral proteins ($6 < pI < 8$) are implicated in these adducts.

To overcome such limitations, we recently investigated hydrophobic interaction chromatography (HIC) which separates substances in solution as a result of their varying degrees of hydrophobic interaction with the hydrophobic groups on a gel matrix. According to the manufacturer, Fractogel TSK–Butyl 650 used in our studies is prepared by introducing butyl groups, via ether linkage, to a semi rigid copolymer of polyethylene glycol, glycidyl methacrylate and pentaerythrol-dimethylacrylate. The presence of ether linkage and of hydroxyl groups in the polymer leads to gel particules with a strongly hydrophilic surface. When ammonium sulfate solutions were used as eluents, efficient separations of PEG–papaya proteinases adducts from unmodified proteinases have only been observed if the native enzyme exhibited very strong affinity for the HIC support (e.g. papain) or, at the other extreme, a very weak binding capacity to the gel (e.g. caricain). Contrasting with such a situation, when proteins (e.g. chymopapain) exhibit moderate affinity for the gel, no separation could ever be observed. Furthermore, unattached PEG and PEG–papaya proteinase adducts (whatever which proteinase and whatever the ratio of PEG to the proteinase) are eluted from the column under very similar (about 0.6 M $(NH_4)_2SO_4$) conditions (Azarkan et al., 1996b).

A recent report by Oscarsson (1995) prompted us to investigate potassium fluoride solutions as the eluent in order to circumvent some of the drawbacks encountered with ammonium sulfate.

2. Materials and methods

2.1. Materials

Aldrich-Chemie (Steinheim, Germany) provided PEG 4600, ammonium sulfate and potassium fluoride. Alizarine complexone, bovine serum albumin (BSA; lot 15H0425) and bovine β-lactoglobulin (BLG; lot 100H8185) were supplied by Sigma (St Louis, MO). Cerium nitrate and Fractogel TSK–Butyl 650 were from Merck (Darmstadt, Germany) and fluorescein as the disodium salt from Janssen Chemical (Geel, Belgium). Hen egg white lysozyme (EC 3.2.1.17; HEWL) was purchased from Belovo (Bastogne, Belgium). The cysteine proteinase, caricain (EC 3.4.22.30), was extracted from the laticifers of *Carica papaya*. Purification of this enzyme to homogeneity was obtained after its conjugation to a 5-kDa functionalized mPEG as a mixed disulfide derivative (PEG–proteinase) (Musu et al., 1996). Removal of the thiopegyl group and substitution by methylthiogroup afforded the proteinase in the form of its *S*-methylthioconjugate as well. PEG–BLG was prepared in a similar way as described previously (Nijs et al., 1997).

2.2. Analytical methods

Conductivities (mS) were measured, at constant temperature, with a Radiometer conductivity meter CDM3 equipped with a Radiometer measurement cell type CDC314. Salt concentrations were calculated from conductivities measurements by means of appropriate reference curves.

Fluoride ions were determined spectrophotometrically at 625 nm (by interpolation on a standard curve) as described previously (Yamamura et al., 1962). It was assessed that PEG 4600 did not interfere with the test.

PEG 4600 was accurately determined after oxidation with potassium bichromate in H_2SO_4 using

the micro COD digestion tests commercially available from the Hach Company. The method developped in our laboratory (Guermant et al., manuscript in preparation) was adapted from Jirka and Carter (1975). For determination of PEG in chromatographic fractions, the method based on fluoresceine color fading was used (Guermant et al., 1995).

Absorbances were measured with a Varian DMS 300 spectrophotometer and pHs with the pHM-84 meter.

2.3. Determination of the binodal curve characterizing the system PEG 4600–KF–H_2O at 30°C

The biphasic systems constituting the phase diagram were prepared by weighing appropriate amounts of PEG, potassium fluoride and water. The solution was magnetically stirred for one hour after which it was transferred into a polypropylene tube. The tube was tightly capped and allowed to equilibrate for 48 h at 30 ± 0.1°C in a water bath. The top phase was then collected with the use of a pasteur pipet while the lower phase was drained from the polypropylene tube by piercing through its bottom. The composition of each phase was determined on the basis of analyses of their PEG and fluoride ion content (the water content being thus obtained by substraction). The phase compositions were plotted, as shown in Fig. 1. The tie lines were determined by connecting each corresponding set of total, bottom and top phase points. The binodal curve was drawn through the top and the bottom phase points and estimated near the critical point on the basis of location of single phase points.

2.4. Partition experiments

Phase systems with the required compositions (PEG 4600–KF–H_2O: 18/9.5/72.5 mass%; PEG 4600–$(NH_4)_2SO_4$–H_2O: 20/11/69 mass%) adjusted at a pH around 7, were made up in separation funnels and allowed to equilibrate at 30°C in a water bath. After incubation for 48 h, the demixed phases were separated and protein powders (2–10 mg) introduced either in the PEG rich phase or in the salt rich phase depending on the protein under study in order to reach a $A_{280\,nm}$ value around 1. For each protein, a series of biphasic systems (of constant final mass) were then reconstituted, in polypropylene tubes, by mixing top and bottom phases in various proportions. The tubes were tightly capped and incubated for 48 h, at 30°C. The protein content in each phase was then determined and the partition coefficient calculated as the ratio of protein concentrations in the upper and bottom phases.

2.5. Protein solubility measurements

Aqueous solutions of the proteins under study were diluted in salts solutions of known concentrations and pH (~ 7) and preincubated at 25°C. While diluting the protein solutions, the test tubes content was mixed by vortexing. The resulting protein solutions or suspensions, after equilibration, were submitted to centrifugation ($2700 \times g$; 30 min.; 25°C). The protein contents in the supernatants were determined and thereof, the fraction of the protein remaining in each solution was calculated.

2.6. HIC experiments

Analytical chromatographies were performed at 25°C on a (5×1.6 cm, I.D.) column of Fractogel TSK–Butyl 650 preequilibrated in the start eluent (unless otherwise stated: 1.5 M KF or 2.0 M $(NH_4)_2SO_4$, pH ajusted around 7). After the sample has been loaded onto the column, the start eluent (50–100 ml) was pumped through followed by a linear gradient (total volume: 700 ml) from 1.5 to 0 M of KF or from 2.0 to 0 M of $(NH_4)_2SO_4$. Elution was performed at 25°C at a flow rate of 35 ml/h. Fractions of 7 ml were collected. Each chromatographic fraction was analysed for conductivity and either for $A_{280\,nm}$ or for PEG content.

3. Results and discussion

As compared to ion-exchange chromatography, the fundamentals involved in protein separation

by HIC are less understood. Oversimplifying, the driving forces implicated in HIC may be regarded as resulting from the interplay of interactions between protein surface groups and hydrophobic ligands on the HIC gel. The role of salts present in the eluent consists in modulating these interactions. In the case of PEG–protein adducts, the PEG moiety can further interact with each one of the above mentioned actors as well as with the hydrophilic matrix of the HIC gel.

It is expected that the hydrophilic character of the matrix of a HIC support (which is the case of Fractogel TSK–Butyl 650) constitute an element which disfavours binding of a PEG–protein conjugate as compared to the native protein. Incompatibility between PEG and other hydrophilic polymers is indeed a fairly well-known phenomenon to polymer chemists (Harris, 1992). Biochemists also have used aqueous two-phase systems constituted for example of PEG and dex-

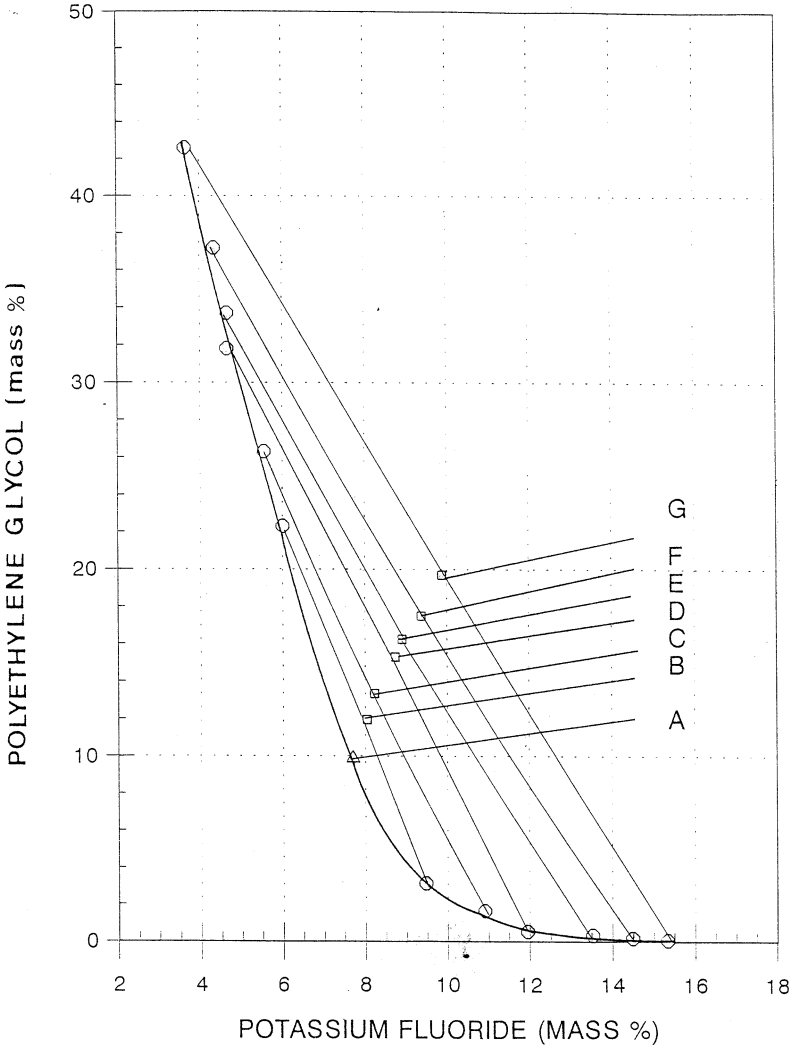

Fig. 1. Phase diagram of potassium fluoride–PEG 4600–water at 30°C, pH 7, showing experimentally determined tie lines. The composition (mass% of PEG 4600 and KF, respectively) of the phase systems A–G were: A (10.00, 7.70), B (11.96, 8.04), C (13.35, 8.22), D (15.30, 8.73), E (16.25, 8.90), F (17.50, 9.37) and G (19.70, 9.87).

tran for the separation of proteins (Asenjo et al., 1994) and other biomolecules. The PEG–dextran biphasic system has even been exploited to determine the extent of pegylation of proteins. This determination is based on the observation of a linear relationship between the logarithm of the partition coefficient of PEG–protein adducts and the degree of pegylation of the protein moiety (Delgado et al., 1990, 1994, 1997). Experimentally, the contribution of incompatibility exhibited by PEG towards the hydrophilic matrix of Fractogels has been pointed out on several occasions. Fractogel media has been found more suitable (less nonspecific interactions) than others for the molecular weight determination of PEG (Van Dam and Daenens, 1993). Also, the *S*-monopegylthioderivative of papain binds less strongly to Fractogel TSK–Butyl 650 than papain (Azarkan et al., 1996b). However, an inverse relationship is observed when affinities of pegylated chymopapain, glycyl endopeptidase and caricain are compared to those of the parent papaya proteinases showing that other contributing forces may be preponderant. Inorganic electrolytes are long known to affect solubility of PEG which results in depressing the cloud point of aqueous solutions of the polymer (Bailey and Callard, 1959) and their ability to induce formation of aqueous two-phase systems. Both properties do nicely correlate and show that the fundamental forces involved in processes of biphasic formation and clouding are the same (Ananthapadmanabhan and Goddard, 1986). The cloud point precipitation of ethoxylated materials has been attributed to dehydration, at high temperature, of the ethylene oxide units. Ionic species which strongly hydrate will evidently induce the dehydration of ethylene oxide units at a lower temperature. The formation of PEG–salt aqueous two-phase systems also involve dehydration of the polymer even if dehydration is only partial. All experimental evidences obtained so far indicate that the order of effectiveness of various salts to depress the cloud point of PEG and to simultaneously govern formation of two-phase systems precisely corresponds to the well-known Hofmeister series (Ananthapadmanabhan and Goddard, 1986). This means that lyotropic series may be used as a predictive tool for anticipating the chromatographic behaviour of PEG on HIC supports (Melander and Horvath, 1977; Absolom and Barford, 1988).

Although it only modestly depresses the clouding point of PEG (Bailey and Callard, 1959), KF, as shown in Fig. 1, is still able to induce formation of a biphasic system with PEG 4600. When compared to the effectiveness of potassium phosphate and ammonium sulfate to do so, higher concentrations of potassium fluoride are required to achieve formation of a two-phase system (see Fig. 2, as well as Snyder et al. (1992) and Hassinen et al. (1994)). These observations do strictly conform to predictions based on the lyotropic series. Noteworthy, KF is also much less efficient than $(NH_4)_2SO_4$ to promote binding of PEG to Fractogel TSK-Butyl 650. As shown in Figs. 3 and 4, desorption and elution of PEG 4600 from the HIC column is effected at 1.10 M KF and 0.65 M $(NH_4)_2SO_4$, respectively. Altogether, these observations strongly suggest that salting out effects on PEG 4600 may constitute a determining factor dictating the behaviour of the polymer on the gel (this hypothesis will appear more evident in the forthcoming paragraphs).

Comparison of the effects of potassium fluoride and ammonium sulfate was extended to three proteins. Those proteins, namely caricain, hen egg white lysozyme and bovine serum albumin, outcoming from different origins (plant, bird and mammal, respectively), have been chosen because they have different structures and exert quite different functions. The behaviour of those selected proteins in solution has been studied under conditions promoting precipitation, binding to hydrophobic adsorbents and partition.

As shown in Fig. 2, all three proteins are more soluble in KF solutions than in $(NH_4)_2SO_4$ solutions. This is particularly pronounced in the case of bovine serum albumin. Thus, as already observed in the case of PEG, the effectiveness of KF to promote salting out of proteins is less than that of $(NH_4)_2SO_4$ in agreement with the respective numbers of fluoride and sulfate anions in the lyotropic series.

The partitioning of the three proteins in the PEG 4600–KF or $(NH_4)_2SO_4$–H_2O systems has also been studied. The results of this investigation

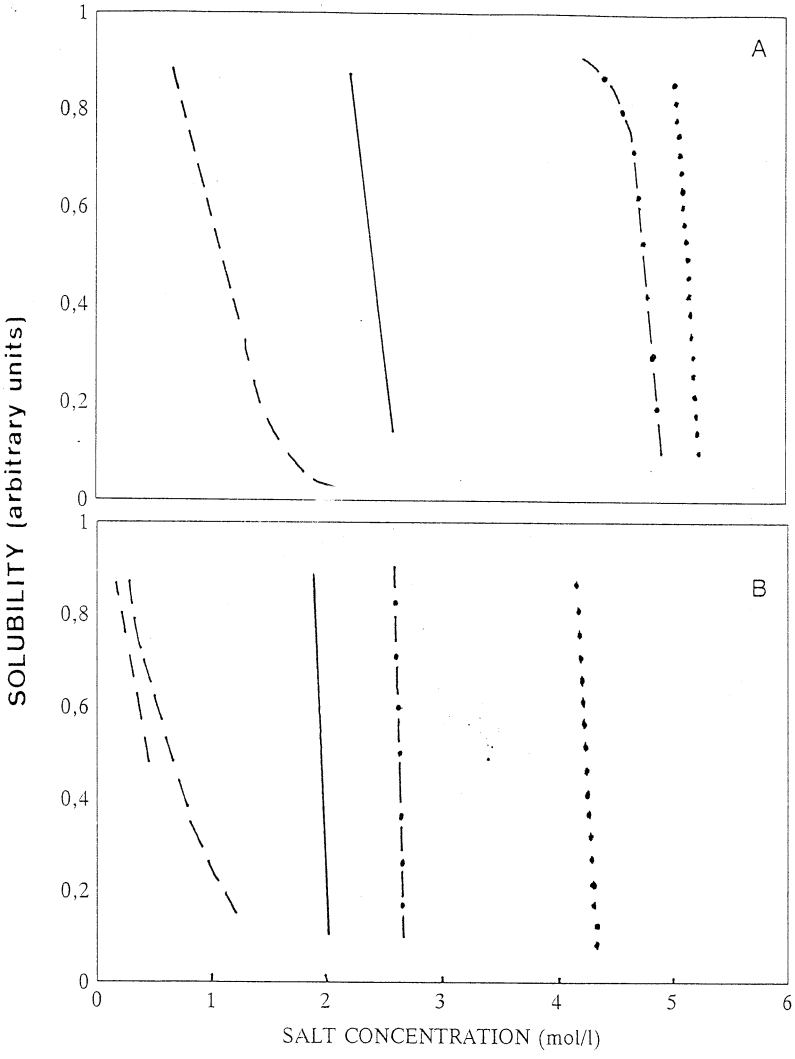

Fig. 2. Phase diagram showing the solubility in potassium fluoride (A) and in ammonium sulfate (B) of PEG (long dashed), hen egg white lysozyme (solid line), BSA (dash–dot) and caricain (dot). Results from Fig. 1 and from the paper by Snyder et al. (1992) were used to draw the PEG 4600 (A), PEG 1000 (lefthand in B) and PEG 8000 (righthand in B) curves. Details are given in the text.

are reported in Table 1. In the PEG 4600–KF–H_2O system, lysozyme shows a marked preference for PEG phase in contrast to caricain and bovine serum albumin, the presence of which is confined in the salt phase. In the PEG 4600–$(NH_4)_2SO_4$–H_2O system, all three proteins show strong preference for the salt phase. Under the experimental conditions used (see Section 2), KF and $(NH_4)_2SO_4$ concentrations found in the salt phases approximate 3 and 1.3 M respectively. Reporting these values in Fig. 2 lets appear that partition of caricain, bovine serum albumin and lysozyme may well be dictated by their solubility in salt solutions. Results shown in Table 1 and Fig. 2 thus support previously reported observations indicating that salting forces increasingly influence protein partition at longer tie line lengths (Huddleston et al., 1994). Fig. 3 illustrates the elution patterns of caricain, bovine serum albumin and hen egg white lysozyme on the Frac-

togel TSK–Butyl 650 column. When compared to ammonium sulfate solutions as the eluting system, potassium fluoride solutions obviously reinforce interactions between these proteins and the HIC support. All three proteins indeed (the case of caricain being particularly striking) are eluted from the HIC column at much lower concentrations than expected from considering the lyotropic series only. In marked contrast, as shown in Fig. 4, bovine β-lactoglobulin elutes on line with the molal surface tension increments afforded by fluoride and sulfate anions (Melander and Horvath, 1977; Absolom and Barford, 1988). In this respect, bovine β-lactoglobulin behaves quite similarly to PEG 4600. The exact reason why such a rather chaotic situation is observed here and else-

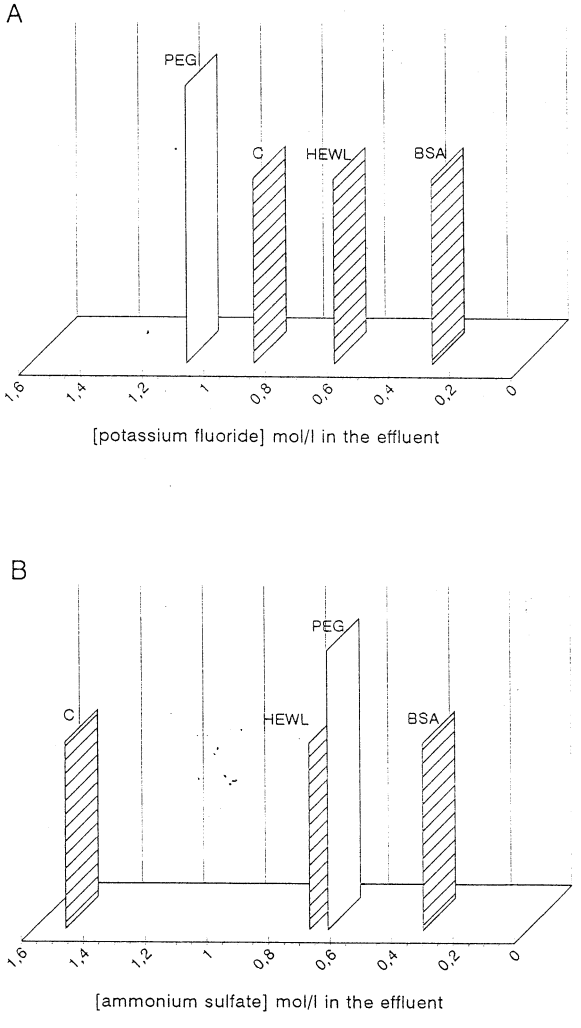

Fig. 3. Schematic representation of elution profiles of PEG 4600 and of selected proteins as a function of mobile-phase potassium fluoride (A) and ammonium sulfate (B) concentration on a Fractogel TSK–butyl 650 column. Only the position of the maximum of the eluted peak is shown. Abbreviations: C, caricain; HEWL, hen egg white lysozyme; BSA, bovine serum albumin. Full details are given in the experimental section.

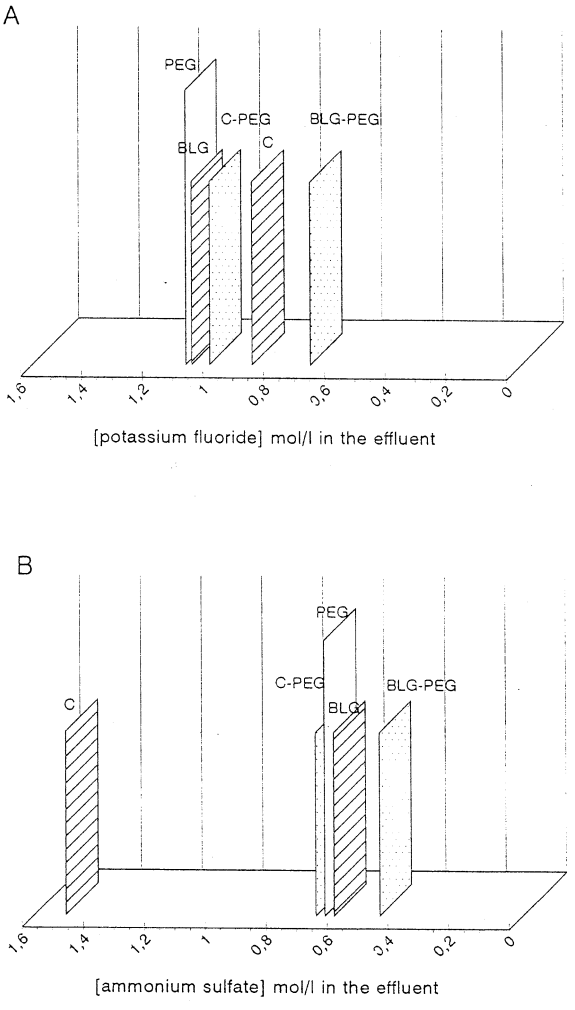

Fig. 4. Schematic representation of elution profiles of PEG 4600 and of selected proteins (caricain and BLG and their pegylated form) as a function of mobile-phase potassium fluoride (A) and ammonium sulfate (B) concentration on a Fractogel TSK–butyl 650 column. Abbreviations: BLG, bovine β-lactoglobulin. Details as in Fig. 3.

Table 1
Partition, at 30°C, of lysozyme, caricain and bovine serum albumin (BSA) in PEG 4600–KF and PEG 4600–$(NH_4)_2SO_4$ aqueous two-phase systems

Protiens	Two-phase systems	pH		K
		PEG phase	Salt phase	
Lysozyme	PEG–KF	7.6 ± 0.1	7.8 ± 0.1	>100
	PEG–$(NH_4)_2SO_4$	7.6 ± 0.1	7.3 ± 0.1	0.150 ± 0.06
Caricain	PEG–KF	6.97 ± 0.07	7.09 ± 0.05	0.150 ± 0.012
	PEG–$(NH_4)_2SO_4$	7.09 ± 0.04	7.06 ± 0.02	0.020 ± 0.008
BSA	PEG–KF	7.6 ± 0.1	7.8 ± 0.1	<0.05
	PEG–$(NH_4)_2SO_4$	7.4 ± 0.1	7.3 ± 0.1	<0.05

where (Oscarsson, 1995; Huddleston et al., 1996) is not precisely known. Some recent investigations however have suggested that conformational changes of proteins at interfaces may be an important parameter involved in protein interaction with amphiphilic adsorbents (Oscarsson, 1995; Billsten et al., 1997). Keeping this possibility in mind, the chromatographic elution patterns of pegylated proteins such as those shown in Fig. 4, should be cautiously interpreted. The elution pattern from Fractogel TSK–Butyl 650 of the cysteine proteinase caricain is greatly modified after its derivatization into the S-monopegylthioconjugate. Whatever the type of salt (KF in Fig. 4A or $(NH_4)_2SO_4$ in Fig. 4B) used as the eluent, the PEG–caricain adduct elutes at a position which is intermediate between PEG 4600 and native caricain but much closer to PEG 4600. This type of chromatographic behaviour outlines the prevailing influence that the PEG moiety can exert on the surface properties of some enzymes. The case of bovine β-lactoglobulin (a protein containing two disulfide bonds and a free thiol function) is also reported in Fig. 4. Coincidently, the native protein coelutes with PEG 4600 on Fractogel TSK–Butyl 650 whatever the nature of the salt used in the eluent. *S*-pegylation of the fully reduced protein led to a conjugate containing five PEG chains per protein monomer. As shown in Fig. 4, this conjugate exhibits stronger affinity than either PEG 4600 or native β-lactoglobulin for Fractogel TSK–Butyl 650. In this case it must be noted that the *S*-pegylated protein is no more close to its native form and that its chromatographic behaviour may thus be better interpreted as the result of an enhanced susceptibility of the protein surface to conformational changes. As a consequence, its purification by HIC is based on the exploitation of variations of protein surface deformability. In agreement with previous observations (Oscarsson, 1995) this may be best achieved by using salts such as KF as the eluents.

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