Journal of Chromatography A, 1217 (2010) 4696–4703

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Solubility and binding properties of PEGylated lysozyme derivatives with increasing molecular weight on hydrophobic-interaction chromatographic resins

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ARTICLE INFO

Article history:
Received 3 February 2010
Received in revised form 30 April 2010
Accepted 7 May 2010
Available online 16 May 2010

Keywords:
PEG lysozyme
Hydrophobic-interaction chromatography of PEG-proteins
Separation of PEG-proteins
Binding capacity of HIC resins for PEG-proteins
Repulsion of PEG-proteins from HIC resins in NaCl

ABSTRACT

Dynamic binding capacities and resolution of PEGylated lysozyme derivatives with varying molecular weights of poly (ethylene) glycol (PEG) with 5 kDa, 10 kDa and 30 kDa for HIC resins and columns are presented. To find the optimal range for the operating conditions, solubility studies were performed by high-throughput analyses in a 96-well plate format, and optimal salt concentrations and pH values were determined. The solubility of PEG-proteins was strongly influenced by the length of the PEG moiety. Large differences in the solubilities of PEGylated lysozymes in two different salts, ammonium sulfate and sodium chloride were found. Solubility of PEGylated lysozyme derivatives in ammonium sulfate decreases with increased length of attached PEG chains. In sodium chloride all PEGylated lysozyme derivatives are fully soluble in a concentration range between 0.1 mg protein/ml and 10 mg protein/ml. The binding capacities for PEGylated lysozyme to HIC resins are dependent on the salt type and molecular weight of the PEG polymer. In both salt solutions, ammonium sulfate and sodium chloride, the highest binding capacity of the resin was found for 5 kDa PEGylated lysozyme. For both native lysozyme and 30 kDa mono-PEGylated lysozyme the binding capacities were lower. In separation experiments on a TSKgel Butyl-NPR hydrophobic-interaction column with ammonium sulfate as mobile phase, the elution order was: native lysozyme, 5 kDa mono-PEGylated lysozyme and oligo-PEGylated lysozyme. This elution order was found to be reversed when sodium chloride was used. Furthermore, the resolution of the three mono-PEGylated forms was not possible with this column and ammonium sulfate as mobile phase. In 4 M sodium chloride a resolution of all PEGylated lysozyme forms was achieved. A tentative explanation for these phenomena can be the increased solvation of the PEG polymers in sodium chloride which changes the usual attractive hydrophobic forces in ammonium sulfate to more repulsive hydration forces in this hydrotrophic salt.

1. Introduction

PEGylation is a procedure of increasing importance for enhancing the therapeutic potential of biologically active proteins and polypeptides. Properly PEGylated proteins maintain their biological function, such as enzymatic activity or receptor binding and antigen recognition. On the other hand, poly (ethylene) glycol (PEG) conjugation masks the surface and increases the molecular size of the original protein. It results in reduction of many negative biological features such as its immunogenicity and sensitivity to proteolytic degradation. In most cases PEGylation also reduces proteins renal ultrafiltration, resulting in much longer half-life of the modified therapeutics [1–5]. When PEG is covalently linked to a protein it also modifies many of its physicochemical properties, such as biodistribution, thermal stability and solubility. PEGylation also increases water solubility of biologically active macromolecules, resulting in significant improvement of their administration [6].

The change of physicochemical properties of PEGylated proteins also influences their behavior during the whole purification process [7–9]. More specifically, proteins whose molecules have been modified by covalently attaching PEG show altered size, charge, hydrophobicity, metal chelation, and generally interaction with other molecules. It resulted in the change in their behavior in practically all modes of chromatographic separation such as size-exclusion chromatography (SEC), ion-exchange chromatography (IEX), hydrophobic-interaction chromatography (HIC), immobilized metal affinity chromatography (IMAC), and affinity chromatography (AC) [7,8]. Other important separation methods frequently used for down-stream processing, e.g. filtration, are also affected [9]. Changes of protein behavior during all kinds of electrophoretical separations (SDS-PAGE, isoelectric focusing, 2D electrophoresis and different modes of capillary electrophoresis) can also be expected [10].

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0021-9673/$ – see front matter © 2010 Published by Elsevier B.V.
doi:10.1016/j.chroma.2010.05.016

Purification of a PEGylated protein involves removal of all molecular species that are not part of the product. PEGylation of proteins creates two basic challenges for purification. The first involves separation of PEGylated proteins from other reaction products including non-reacted PEG and protein. The second is the sub-fractionation of PEGylated proteins on the basis of their degree of PEGylation (PEGamer resolution). The chromatographic methods used for the purification of PEGylated proteins are mainly ion exchange (e.g. cation exchange), size-exclusion chromatography [10], and reversed-phase chromatography, the last one used for PEGylated peptides [11]. Ultrafiltration and diafiltration were less frequently applied [10]. PEGylation affects the protein hydrophobicity, and consequently, HIC can be considered to be used as an additional method for separation of PEG modified proteins. Surprisingly, HIC is rather rarely applied for the separation of PEGylated proteins. Clark et al. [12] used HIC for the separation of PEGylated growth hormone, Vincentelli et al. [13] have separated PEGylated bovine β-lactoglobulin from PEG and native protein and Cisneros-Ruiza et al. [14] have investigated separation of mono- and di-PEGylated forms from unmodified form of ribonuclease A.

2. Theory

2.1. Interaction of PEG with water, salt and proteins

Poly (ethylene) glycols (PEG) are water-soluble synthetic polymers with a general formula HO–$(CH_2CH_2O)_n$–H or CH_3O–$(CH_2CH_2O)_n$-reactive group (e.g. aldehyde, NHS) have been utilized in various applications in the biotechnology industry. PEG and methoxy-PEG (MPEG) are widely used as precipitants and crystallization agents for proteins and as chemical agent for PEGylation of proteins [15].

Polyethylene glycol itself is an amphiphatic molecule. Such a polymer is neutral, hydrophilic and soluble in various aqueous solutions, as well as hydrophobic and soluble in the organic solvents. PEG also tends to behave like a weak detergent [16,17].

In aqueous solutions PEG can be classified as electron donor or Lewis base because of the two lone pair electrons on the oxygen atom. Hydrogen bonds between oxygen electrons and water hydrogen result in a negative enthalpy of interaction [18]. Because of the strong interaction with water, PEG polymers are strongly hydrated in aqueous solutions. The Lewis base character of the ether bond is important for the PEG structure and function in electrolyte solutions. Takeuchi et al. [19] demonstrated that multivalent cations bound on the oxygen atoms from PEG chains by ion–dipole interactions lend them to anion-exchange sites. Structural changes of the PEG polymer in higher concentrated electrolyte solutions are reported in the literature [20]. Neutron scattering studies and measurements of the intrinsic viscosities have shown that some anions like nitrate or chloride do not change the coiled PEG structure, whereas phosphate, carbonate and sulfate ions increases the PEG tendency to aggregate [21]. These structural changes of PEG polymers in salt solutions are expected to influence the adsorption properties of PEG-proteins on HIC resins.

There is currently no theory which describes the solubility of PEGylated proteins and adsorption to HIC resins, but the PEG is frequently used in protein purification.

Precipitation of proteins by adding PEG is broadly described in the literature by experimental values and by statistical models [22–24]. Usually the addition of PEG decreases the solubility of proteins in water. In general, the increase of the molecular weight of the PEG has a greater effect on the solubility than the addition of electrolytes which either increase or decrease solubility according to their position in the lyotrophic series [25].

Precipitation of proteins in the presence of PEG has been described by a statistical–mechanical perturbation theory with a volume-exclusion potential from Asakura and Oosawa [26]. According to this theory, protein molecules are excluded from the regions of the solvent occupied by PEG molecules. As a result, protein is concentrated and precipitates out when its solubility limit is exceeded. In this theory, a linear trend was found of the logarithm protein solubility against added PEG [23].

Aqueous PEG electrolyte mixtures were also extensively used as components for liquid–liquid partitioning (LLP) systems for protein purification [27]. Here, the logarithm of the distribution coefficients for the partitioning of biomolecules between two phases is proportional to the molecular weight of the molecule being partitioned [28].

Despite some analogies found for the solubilities of PEGylated proteins the above presented theories describing interactions between PEG and proteins in aqueous solutions are not applicable, because PEG is not covalently linked to the protein.

Because there is still no molecular theory that describes the interaction of PEGylated protein with water and electrolytes, the empirical solubility models might be applicable. The solubility of macromolecules in electrolyte solutions can be often successfully described by the empirical Cohn's [29] or Setschenow's equation [30].

$$\text{Log } S = \beta' - K' \cdot \left(\frac{\Gamma}{2}\right) \quad (1)$$

where S is the solute concentration at the experimental salt concentration, g/L; β' is the extrapolated solubility intercept which represents the logarithm of the hypothetical concentration of the solute at zero ionic strength; $\Gamma/2$ is the ionic strength, mol/L ($\Gamma/2 = (1/2)\sum z_i^2 c^2$); Z_i is the valences of cationic and anionic species; c is the molar concentration of cationic and anionic species, mol/L, for ammonium sulfate $\Gamma/2 = 3c$ and K' is the constant obtained from slope of solubility curve.

The slope of Eq. (1) can be considered as the difference between "salting out" and "salting in" effect [31]:

$$K' = K_{Si} - K_{So} \quad (2)$$

where K_{Si} is the "salting in" constant and K_{So} is the "salting out" constant.

2.2. PEGylated model proteins

The influence of PEGylation on solubility and binding properties of modified proteins in HIC was investigated with hen egg white lysozyme as model substance. This protein was used by Yamamoto et al. [3,8] for binding studies of PEGylated forms onto ion-exchanger resins. Lysozyme is also often used as a standard protein for characterization and binding capacity studies in HIC. Consequently, binding and elution conditions for this protein in HIC mode are thoroughly investigated [32].

Hen egg white lysozyme is well characterized and is available in large quantities. It contains a total of six lysine residues. Only three of the residues: 33, 97 and 116 are surface accessible for large molecules and can be PEGgylated [43]. Lee and Park have found following reactivity increments for Lys 33 > Lys 97 > Lys 116 were found if amino reactive coupling methods for protein PEGylation were applied [33]. Because of the three reactive lysine residues, in random PEGylation three mono-PEGylated, three di-PEGylated and one tri-PEGylated forms of this protein are expected. The number of PEGylation products is relatively small, and individual species can be easily separated and isolated by cation-exchange chromatography [3,8].

There is no systematic study available for describing the influence of the size of PEG residues on the solubility and binding

Table 1
Specification of various HIC and IEX resins.

Resin	Mode	Particle size (μm)	Pore size (nm)	Mean binding capacity (mg/ml)
Toyopearl TSKgel SP 5PW	Cation exchange	20	100	20–40 (lysozyme)
Toyopearl GigaCap S-650M	Cation exchange	75	100	156 ± 20 (human γ-globulin)
Toyopearl Butyl-650M	HIC	65	100	40 ± 10 (lysozyme)
Toyopearl Butyl-600M	HIC	65	75	50 ± 10 (human γ-globulin)
Toyopearl Phenyl-600M	HIC	65	50	55 ± 10 (lysozyme)
Toyopearl PPG-600M	HIC	65	50	>25 (human γ-globulin

properties of PEGylated proteins neither on cation exchange nor on HIC resins. In the present study, 5 kDa, 10 kDa and 30 kDa mono-PEGylated lysozyme derivatives were synthesized and used as model proteins for solubility studies and characterization of HIC resins.

3. Experimental

3.1. Reagents

5 kDa, 10 kDa, 30 kDa methoxy-PEG (MPEG) aldehyde were from NOF Corp. (Shibuya-kju, Japan), sodium cyanogen borhydride, sodium dihydrogenphosphate, sodium tricitrate, ammonium sulfate, and hen egg white lysozyme were from Sigma–Aldrich (Munich, Germany).

3.2. Columns

TSKgel G3000 SWXL column (7.8 mm × 300 mm) and TSKgel Butyl-NPR column (4.6 mm × 35 mm) were all from TOSOH Bioscience GmbH (Stuttgart, Germany).

3.3. Resins

Toyopearl TSKgel SP 5PW, Toyopearl GigaCap S-650M, Toyopearl Butyl-650M, Toyopearl Butyl-600M, Toyopearl PPG-600M, Toyopearl Phenyl-600M were products from TOSOH Bioscience GmbH (Stuttgart, Germany).

Selected properties of HIC resins are summarized in Table 1.

3.4. Stock salt solutions

For the solubility measurements two stock salt solutions were prepared:

(A) 0.1 M sodium phosphate and 5 M sodium chloride, pH 7
(B) 0.1 M sodium phosphate and 4 M ammonium sulfate, pH 7

3.5. Instruments

The analytical SEC, ion-exchange and HIC separations were performed on a "Prominence" HPLC system (Shimadzu, Duisburg, Germany). The preparative purification of the PEG products and the dynamic binding capacity determination were performed on Äkta Explorer HPLC from GE Healthcare (Freiburg, Germany).

For diafiltration a VIVAFLOW 50 from Stedim Sartorius (Göttingen, Germany) was used.

3.6. Preparation and purification of PEGylated lysozyme model protein

The reaction was, slightly modified, performed according to a previously described procedure by Kim [34] methoxy-PEG (MPEG) aldehyde and lysozyme at a (w/w) ratio of 2:1 were dissolved in a 25 mM phosphate buffer, pH 6, followed by the addition of 20 mM sodium cyanogen borhydride for the reduction of Schiff's bonds, and the reaction was performed overnight at 15 °C. The reaction degree was monitored by an analytical TSKgel 3000 SWXL column. The reaction mixtures were fractionated on a 2.6 cm × 26 cm Superformance glass column [TOSOH Bioscience] packed either with Toyopearl TSK SP 5PW (particle size 20 μm) or with Toyopearl GigaCap S-650M (particle size 75 μm). 150 ml reaction mix from the PEGylation reaction contained 3–7 g of product. After a 1:1 dilution with water the solution was applied to the column. (The dilution was necessary to prevent a too early breakthrough of the product.)

The products were separated and fractionated by a linear sodium chloride gradient. After fractionation, the solution was concentrated and desalted using the VIVAFLOW 50 membrane filter and freeze dried. The overall yield was 40 ± 10% for the mono-PEGylated lysozymes. The three mono-PEGylated lysozymes were pooled and not further fractionated. Separation of the PEGamers is possible with resins with smaller particle size than Toyopearl GigaCap S-650M as described in [35]. The final product purity was routinely controlled by analytical SEC with a TSKgel G3000 SWXL column, SDS-PAGE and for selected samples, by laser light scattering with a MiniDawn (Wyatt Dernbach, Germany) for the presence of aggregates. The MALDI-TOF mass spectrometry was used for determination of molecular weights of separated components [35]. The purity of PEGylated lysozymes was greater than 95%.

3.7. Analytical separation with the TSKgel Butyl-NPR column

The separations were performed with the Shimadzu "Prominence" HPLC.

Buffer A: To 0.1 M phosphate buffer, the adequate amount of salts (ammonium sulfate or sodium chloride) were added and the pH was adjusted at 7.0
Buffer B: 0.1 M phosphate buffer, the pH was adjusted
Column: TSKgel Butyl-NPR column (4.6 mm × 35 mm)
Sample: 1 mg/ml single compound was dissolved in buffer A
Sample amount: 5 μl
Flow: 1 ml/min
Monitor: 280 nm
Linear gradient from A to B in 10 min

3.8. Solubility measurements

The solubility measurement was performed in 96-well plates with an optical reader at a wave length of 620 nm by recording the absorbance. $100 - x$ μl of 0.1 M phosphate buffer, pH 7, containing the PEG-protein variants were placed in each well and mixed with $(100 + x)$ μl concentrated stock salt solutions (sodium chloride and ammonium sulfate) to the final concentrations. The concentrations of sodium chloride and ammonium sulfate were varied in 0.05 M steps and the measurements repeated two times. The plates were placed in an orbital shaker for mixing. The mixing time was 20 min. Due to optical inspections no precipitation was observable till 0.02 AU. Slight precipitation was observed between 0.02 AU and 0.1 AU. The salt concentration at 0.02 AU was taken as the limiting solubility value. The standard deviation in molar salt concentration of the method was determined to be ±0.05 M. The

absorption value was determined immediately after mixing and again after 24 h. The solubility of the PEGylated derivatives and of native lysozyme remained constant, indicating an equilibrium value in precipitation. In contrast to that, some re-solubility of the MPEG was observed. To be more relevant for practical applications, the solubility data directly after mixing for the MPEG were used for further evaluation.

3.9. Dynamic binding capacities

The capacity determinations were determined either in Omnifit glass columns 18 mm × 6.6 mm ID or in ToyoScreen plastic columns 30 mm × 6.4 mm ID (TOSOH Bioseparations). The protein was loaded at a concentration of 1 mg/ml and run at a linear flow rate of 150 cm/h in an appropriate salt concentration. If not otherwise stated a 0.1 M sodium phosphate buffer, pH 7, was used. The breakthrough point was determined at 10% UV absorption at 280 nm compared to the absorption value for the bypassed protein solution.

4. Results and discussion

4.1. Solubility in ammonium sulfate

The solubility studies were performed by high-throughput screening according to Kramarczyk et al. [36]. This setup was chosen for future implementation on a robotic system for industrial applications, and to monitor the change of the solubility properties by an increasing degree of PEG modification. The data obtained in the 96-well plate format may not fully agree with equilibrium solubility data because under the chosen conditions the complete equilibrium was not always achieved and, solutions were not centrifuged before analyzing the supernatant.

In most applications for protein purification the concentration of a target protein is in the range from 0.1 mg/ml to 10 mg/ml. For that reason the concentrations of PEGylated lysozymes of 0.125 mg/ml, 0.5 mg/ml, 1 mg/ml, 5 mg/ml and 10 mg/ml were used as experimental data points for solubility studies.

The solubility data obtained for lysozyme, 5 kDa mono-PEGylated lysozyme, 10 kDa mono-PEGylated lysozyme and 30 kDa mono-PEGylated lysozyme are shown in Fig. 1. The logarithms of the protein concentration were plotted against the ionic strength of ammonium sulfate. The experimental data for the molar concentrations are summarized in Table 2. For a protein concentration of 0.125 mg/ml, the maximum ammonium sulfate concentration for the native lysozyme is 2.4 M. After modification with 5 kDa, 10 kDa and 30 kDa PEG, the maximum salt concentrations at the same protein concentration decreased to 1.8 M, 1.5 M and 1 M respectively. For the higher protein concentration of 10 mg/ml the maximum concentrations of ammonium sulfate at precipitation points are 0.8 M, 0.9 M and 0.75 M and thus in the same order of magnitude,

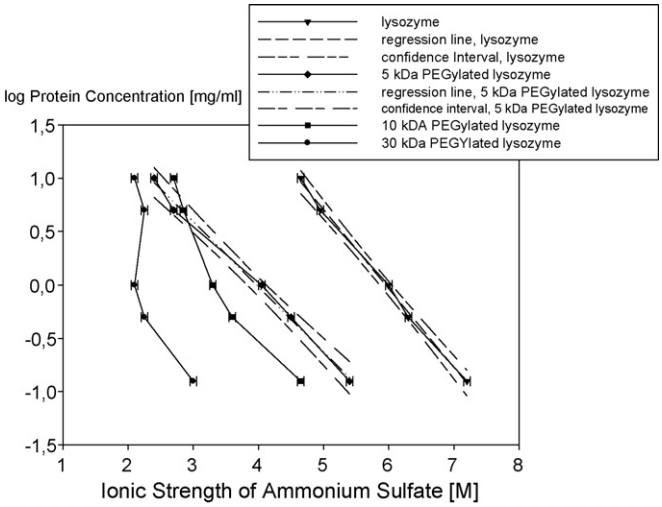

Fig. 1. Plot of the logarithm of solubility for native lysozyme, 5 kDa mono-PEGylated lysozyme, 10 kDa mono-PEGylated lysozyme and 30 kDa mono-PEGylated lysozyme against ionic strength of ammonium sulfate. Linear regression line was found for lysozyme and 5 kDa mono-PEGylated lysozyme (buffered with 0.1 M sodium phosphate, pH 7, room temperature).

but still significantly lower than the value of 1.45 M, that is the maximum concentration of this salt for precipitation of native lysozyme. The plot of the logarithm of the solubility against the ionic strength for lysozyme gave a straight line (see Fig. 1). Eq. (1) was used to fit the solubility data for lysozyme. Intercept β and slope K' were 4.4 and −0.74 respectively with a correlation coefficient of 0.99. For the 5 kDa mono-PEGylated lysozyme, it is still a straight line with an intercept and slope of 2.42 and −0.61 respectively and with a correlation coefficient of 0.99. The value for the intercept with the y-axis is different than for the native lysozyme, but the slope is similar.

Stronger deviations from the lysozyme solubility plot were observed for 10 kDa mono-PEGylated lysozyme and 30 kDa mono-PEGylated lysozymes especially for protein concentrations higher than 0.25 mg/ml (see Fig. 1). The ionic strength necessary for precipitation is essentially constant and the solubility curves nearly parallel to the y-axis.

For comparison, the solubility for the activated methoxy (polyethylene) glycol in ammonium sulfate was determined. The results are shown in Fig. 2 and in Tables 2 and 3. The solubility is a decreasing function of the polymer molecular weight ranging from 1.8 M, 1.2 M and 0.8 M for the ammonium sulfate precipitation concentration for the 5 kDa, 10 kDa and 30 kDa MPEG respectively. The MPEG solubility in the considered concentration range of ammonium sulfate is not a function of salt concentration, because only smaller change was observed for the different MPEG concentration.

Table 2
Solubility data of lysozyme and PEGylated lysozyme forms in concentrated ammonium sulfate and sodium chloride solutions (buffered with 0.1 M sodium phosphate, pH 7, room temperature).

Protein concentration (mg/ml)	Lysozyme	5 kDa mono-PEGylated Lysozyme	10 kDa mono-PEGylated Lysozyme	30 kDa mono-PEGylated Lysozyme
	Ammonium sulfate concentration for precipitation (M)			
0.125	2.4	1.8	1.55	1
0.5	2.1	1.5	1.2	0.75
1	2	1.35	1.1	0.8
5	1.65	0.9	0.95	0.7
10	1.55	0.8	0.9	0.8
	Sodium chloride concentration for precipitation (M)			
0.125	Soluble	Soluble	Soluble	Soluble
0.5	Soluble	Soluble	Soluble	Soluble
1	4.7	Soluble	Soluble	Soluble
5	3	Soluble	Soluble	Soluble
10	2.4	Soluble	Soluble	Soluble

Table 3
Solubility of methoxy (polyethylene) glycol in ammonium sulfate (buffered with 0.1 M sodium phosphate, pH 7, room temperature).

Protein concentration (mg/ml)	Ammonium sulfate concentration for precipitation (M)		
	5 kDa MPEG	10 kDa MPEG	30 kDa MPEG
0.125	1.8	1.2	0.8
0.5	1.8	1.2	0.75
1	1.8	1.2	0.8
5	1.7	1.2	0.7
10	1.7	1.20	0.8

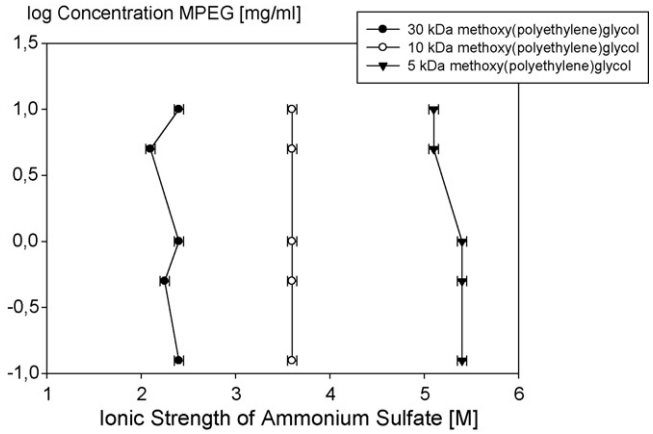

Fig. 2. Plot of the logarithm of solubility for 5 kDa, 10 kDa and 30 kDa methoxy (polyethylene) glycol (MPEG) as function of the ionic strength from ammonium sulfate (buffered with 0.1 M sodium phosphate, pH 7, room temperature).

The solubility curves are parallel to the *y*-axis as for the PEGylated lysozyme with 10 kDa and 30 kDa PEG and nearly linear.

The independency of the solubility for MPEG and PEGylated proteins with higher PEG molecular weight from the salt concentration can be tentatively explained by the amphiphatic character of the PEG. The solubility constant K' in Eq. (2) is the difference between "salting in" and "salting out" constant. For salting out, the constant K is negative and has a positive value for salting in precipitation phenomena [37].

In the transfer region the solubility should be independent from the salt concentration as found for the MPEG and for the PEGylated lysozymes with higher PEG content. It can be concluded that for proteins with large PEG to protein ratio the solubility in ammonium sulfate is independent from the protein concentration. The above hypothesis was raised for PEGylated lysozymes only, and has to be confirmed for other PEGylated proteins.

4.2. Influence of the pH value on solubility of 5 kDa-mono-PEGylated lysozyme and 30 kDa mono-PEGylated lysozyme

The solubility at the pH values of 5, 6, 7, and 8 was investigated for 5 kDa-mono-PEGylated lysozyme and 30 kDa mono-PEGylated lysozyme at a concentration of 1 mg/ml. For this concentration the solubility in ammonium sulfate did not depend on the pH value. Consequently, the non-electrostatic nature of the PEG moiety suppresses the pH dependency on the PEGylated protein solubility. These results are in accordance with the findings of Bailey and Callard [21] about pH dependency of PEG solubility in electrolyte solutions.

4.3. Solubility in sodium chloride

All PEGylated forms from lysozyme and also the MPEGs were fully soluble in sodium chloride at room temperature at pH 7 in the concentration range of 0.125–10 mg/ml until the solubility limit of sodium chloride of 5.3 M. In contrast, native lysozyme is fully soluble for concentrations only below 1 mg/ml. For the lysozyme concentration of 1 mg/ml, 5 mg/ml and 10 mg/ml, the solubility limits are 4.7 M, 3 M and 2.4 M sodium chloride respectively.

The solubilities of PEGylated lysozyme forms in sodium chloride are thus different than their solubilities in ammonium sulfate (see Table 2). According to their position in the Hofmeister series [38], ammonium sulfate has an "salting out" effect whereas sodium chloride has an "salting in" effect on PEGylated forms of this protein. It points out the ambivalent nature of the PEG modification. Bailey and Callard [21] and Florin et al. [39] described the effect of a lyotrophic salt like ammonium sulfate on PEG aqueous solutions as a solubility depressant, while hydrotrophic salts like sodium chloride may cause an increase in the solubility or behave indifferent [21,40].

A possible explanation for higher solubility of the PEG polymers in sodium chloride could be a repulsive hydration force resulting from different interactions between salt ions and the PEG. This existence of a repulsive interaction between different anions and PEG in the presence of nonbonding cations like K^+, NH_4^+ and Na^+ was reported by Ananthapadmanabhan and Goddard [40]. In neutron scattering experiments the PEG chains were found to be aggregated in lyotrophic salts, but stretched out in hydrotrophic salt solutions [20]. As a result, PEGylated proteins are getting aggregated in the presence of ammonium sulfate whereas sodium chloride keeps them in solution.

4.4. Dynamic binding capacities

As described above, the solubility in ammonium sulfate is a function of the molecular weight of the PEG residue. For determination of the dynamic binding capacities salt concentrations close to the solubility limits of modified and unmodified lysozyme were chosen. The concentration choices were arbitrary and might not provide the maximum possible dynamic capacity values. For lysozyme, 5 kDa mono-PEGylated lysozyme, 10 kDa mono-PEGylated lysozyme and 30 kDa mono-PEGylated lysozyme maximal concentrations of ammonium sulfate of 1.8 M, 1.2 M, 1 M and 0.6 M were used respectively. For the capacity determination in sodium chloride for native and PEGylated lysozyme, a protein concentration of 1 mg/ml and salt concentration of 4 M for sodium chloride was used and thus staying well below the precipitation concentration of 4.7 M for lysozyme. All experimental conditions are summarized in Table 4.

Following HIC resins were used for capacity determinations:

(1) Toyopearl Butyl-650M
(2) Toyopearl Butyl-600M
(3) Toyopearl PPG-600M
(4) Toyopearl Phenyl-600M

Results of these experiments are shown in Fig. 3. The binding capacities found for non-modified lysozyme are higher for Toyopearl PPG-600M (poly (propylene) glycol as ligand) and Toyopearl

Table 4
Chromatographic conditions for the breakthrough experiments.

Protein	Concentration ammonium sulfate	Concentration sodium chloride
Lysozyme	1.8 M + 0.1 M phosphate buffer, pH 7	4 M + 0.1 M phosphate buffer, pH 7
5 kDa PEGylated lysozyme	1.2 M + 0.1 M phosphate buffer, pH 7	4 M + 0.1 M phosphate buffer, pH 7
10 kDa PEGylated lysozyme	1.0 M + 0.1 M phosphate buffer, pH 7	4 M + 0.1 M phosphate buffer, pH 7
30 kDa PEGylated lysozyme	0.6 M + 0.1 M phosphate buffer, pH 7	4 M + 0.1 M phosphate buffer, pH 7

Phenyl-600M type resins than for the two resins containing butyl residues as ligands, Toyopearl Butyl-650M and Toyopearl Butyl-600M.

The experimental binding capacity for 5 kDa PEGylated lysozyme exceeds the binding capacity for lysozyme with all resins which might be the result of the increased hydrophobicity of the PEG residue, also confirmed by lower solubility of this molecule (see Fig. 1). However, all investigated resins had lower capacity for 30 kDa-PEGylated lysozyme than for the 5 kDa PEGylated lysozyme. This result is unexpected, due to the higher hydrophobicity of the 30 kDa PEGylated lysozyme compared to the 5 kDa PEGylated lysozyme. The reason for this discrepancy might be the lower accessibility of the inner pore space of the resin because of the different "fictious" molecular weight of the PEGylated lysozymes. In terms of size, a PEG molecule has a much larger hydrodynamic radius than a protein with a similar molecular weight. The hydrodynamic radius and the corresponding molecular weights for the PEGylated lysozyme were calculated according to an equation from Fee and Van Alstine [10]. According to this equation, the "fictious" molecular weight for the 5 kDa mono-PEGylated lysozyme is 50 kDa and 450 kDa for the 30 kDa mono-PEGylated lysozyme.

The binding capacities for both butyl type resins are lower than the PPG type resin. A possible reason might be explained by the similarity in ligand structure between PPG and PEG modification which could result in enhanced interaction energy and lead to increased binding capacity.

The highest binding capacities for PEGylated lysozyme in ammonium sulfate were found with Toyopearl Phenyl-600M and Toyopearl PPG-600M (see Fig. 3). These resins were also selected for further investigation of dynamic binding capacities in 4 M sodium chloride.

The binding capacities in sodium chloride are summarized in Fig. 4. In general, binding capacities were higher when compared to ammonium sulfate. For the 5 kDa PEGylated lysozyme the binding capacity is higher than 100 mg/ml and for the 30 kDa PEGylated lysozyme the binding capacity is higher than 50 mg/ml. The decrease in binding capacity for the 30 kDa PEGylated lysozyme might also be a pore space accessibility problem. The relatively high binding capacities are to a certain extent unexpected because the PEGylated lysozymes were fully soluble in the concentrated sodium chloride solutions. This result seems to be in contradiction to Melander's and Horvath's theory about protein precipitation and retention in HIC [41]. According to this theory, decreasing protein solubility at high salt concentrations corresponds to high binding capacities (retention). Low solubility in a buffer–salt system is one of the prerequisite for high binding capacity on a HIC resin. Consequently, at the precipitation point or close to it, hydrophobic interactions are enhanced and lead to protein aggregation. On the other hand, hydrophobic forces also increase tendency of interaction between the protein and hydrophobic ligands on the resin, resulting in higher binding capacity.

4.5. Selectivity

According to Fee and Van Alstine [10], the most difficult task for separation of PEGylated proteins is the PEGamer resolution. Monkarsh et al. [42] were able to separate 11 positional isomers of mono-PEGylated α-interferon using cation-exchange chromatography. However, this separation was achieved only at an analytical scale, and no scaling-up experiments were performed. Differences between the PEGamers regarding their isoelectric points, surface charge distributions and relative hydrophobicities are likely to be very small and it is doubtful whether this kind of separation can be exploited at the preparative scale. PEGamer fractionation may require additional separation methods such as very long linear salt gradients and the use of small particles in ion-exchange chromatog-

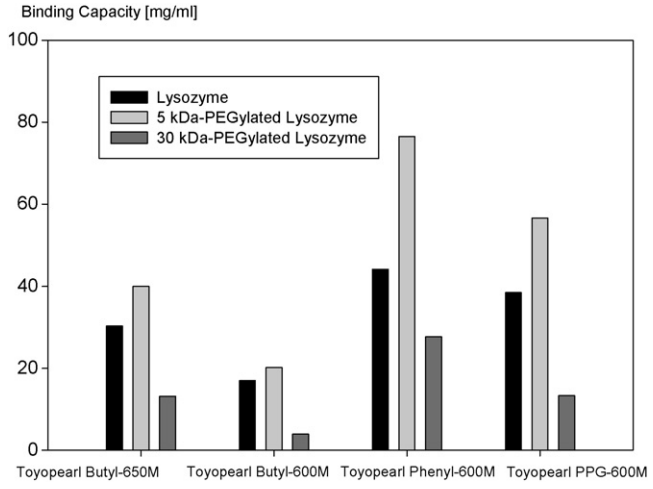

Fig. 3. Dynamic binding capacities of lysozyme, 5 kDa mono-PEGylated lysozyme and 30 kDa mono-PEGylated lysozyme for various HIC resins in ammonium sulfate. Loading: 1 mg protein/ml 0.1 M in phosphate buffer, pH 7, containing 1.8 M ammonium sulfate for lysozyme, 1.2 M ammonium sulfate for 5 kDa mono-PEGylated lysozyme, and 0.6 M ammonium sulfate for 30 kDa mono-PEGylated lysozyme. Flow: 150 cm/h; Column: 6.6 mm × 18 mm glass column, breakthrough at 10% max. AU.

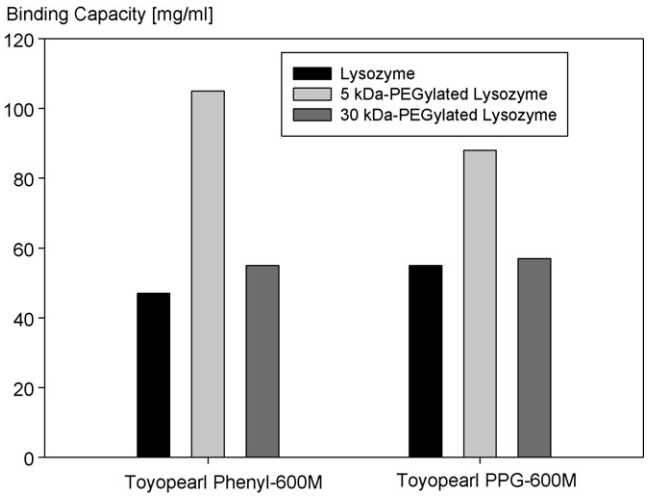

Fig. 4. Dynamic binding capacities of native lysozyme, 5 kDa mono-PEGylated lysozyme and 30 kDa mono-PEGylated lysozyme for Toyopearl Phenyl-600M and Toyopearl PPG-600M in sodium chloride. Loading: 1 mg protein/ml in 0.1 M phosphate buffer, pH 7, containing 4 M sodium chloride. Flow: 150 cm/h; Column: 6.6 mm × 18 mm glass column, breakthrough at 10% max. AU.

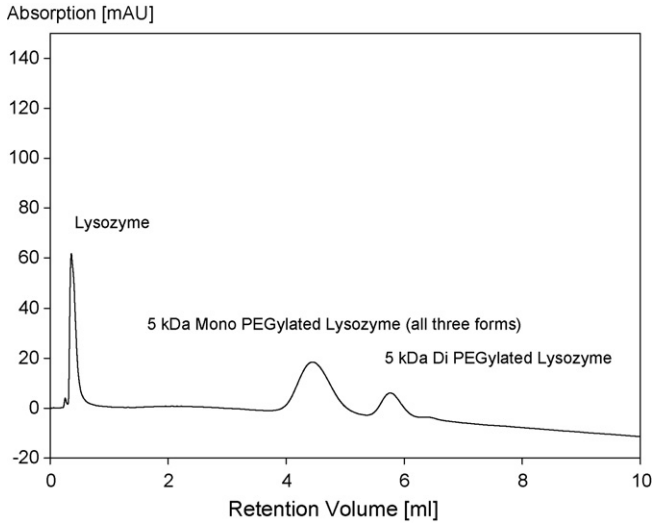

Fig. 5. HIC separation of native lysozyme, 5 kDa mono (1)-PEGylated lysozyme, 5 kDa mono (2)-PEGylated lysozyme, 5 kDa mono (3)-PEGylated lysozyme and 5 kDa di-PEGylated lysozyme on a TSKgel Butyl-NPR column in ammonium sulfate. Conditions: Buffer A: 0.1 M phosphate buffer, pH 7, containing 1.2 M ammonium sulfate; Buffer B: 0.1 M phosphate buffer, pH 7; Column: TSKgel Butyl-NPR (4.6 mm × 35 mm); Sample: 1 mg/ml of each single compound dissolved in buffer A; Sample amount: 5 μl; Flow: 1 ml/min; Monitor: 280 nm; Linear gradient from A to B in 10 min.

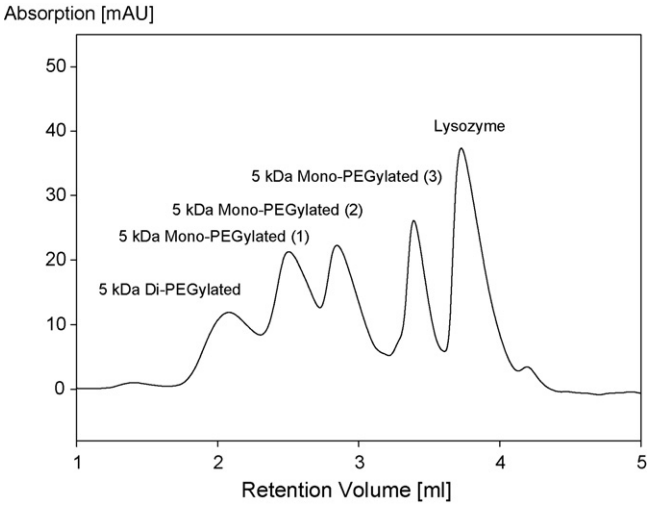

Fig. 6. HIC separation of native lysozyme, 5 kDa mono (1)-PEGylated lysozyme, 5 kDa mono (2)-PEGylated lysozyme, 5 kDa mono (3)-PEGylated lysozyme and 5 kDa di-PEGylated lysozyme on a TSKgel Butyl-NPR column in sodium chloride. Conditions: Buffer A: 0.1 M phosphate buffer, pH 7, containing 3.5 M sodium chloride; Buffer B: 0.1 M phosphate buffer, pH 7; Column: TSKgel Butyl-NPR (4.6 mm × 35 mm); Sample: 1 mg/ml of each single compound dissolved in buffer A; Sample amount: 5 μl; Flow: 1 ml/min; Monitor: 280 nm; Linear gradient from A to B in 10 min.

raphy, or a different separation mode such as high resolution HIC. However, until now the PEGamer resolution has only been reported for the IEX mode. The reason for better separation efficiency in IEX is not clear yet, but it might be the result of change in charge distribution by random PEGylation of the positive charged lysine residues in the protein molecule. This change is more drastic than other physicochemical properties such as relative hydrophobicities. That makes HIC separation of PEGamers a challenging task.

To achieve a good performance, a column packed with the high resolution TSKgel Butyl-NPR 2.5 μm non-porous particles was used for analytical scale separation experiments of the PEG lysozymes. Separations were performed on a mixture of lysozyme, the three different 5 kDa mono-PEGylated lysozymes (PEGamers) and the 5 kDa di-PEG lysozyme (all as a mixture of the pure compounds) in parallel experiments with either ammonium sulfate or sodium chloride as mobile phase. In Figs. 5 and 6 the separation in ammonium sulfate and in sodium chloride respectively is shown. The elution order in ammonium sulfate is lysozyme (1), mono-PEGylated lysozyme (2) and di-PEGylated lysozyme (3). As shown in Fig. 5, the three mono-PEGylated forms were not separated.

In contrast to this poor resolution in ammonium sulfate, a very good resolution in 4 M sodium chloride solution was achieved. Here, the elution order is reversed and di-PEGylated lysozyme is the first peak followed by mono-PEGylated lysozymes and native lysozyme. All three mono-PEGylated lysozyme forms are fully separated (identified by single injections, see Fig. 6).

The change in elution order for the different salt types can be explained by the different solubilities of the PEGylated lysozymes in the two different salt solutions. In ammonium sulfate, lysozyme is the protein with the highest solubility and elutes first, whereas mono-PEGylated and di-PEGylated forms are less soluble and elute later. In sodium chloride the solubility of lysozyme is lower, whereas the solubilities for the PEGylated lysozyme derivatives are higher and consequently they elutes faster. The better resolution of the lysozyme PEGamers is more difficult to interpret, but may be in general related to the change of the solvation state of the PEG polymer.

The extended length of the PEGylated polymer in sodium chloride enables a better contact between the surfaces of PEG-protein and the hydrophobic ligands on the column surface. This might evolve in a general better resolution in HIC separation in sodium chloride as a mobile phase than in ammonium sulfate.

5. Conclusions

The modification of native hen egg white lysozyme with different molecular size PEGs changes the solubility and binding properties of this protein in HIC. PEGylation decreases lysozyme solubility in ammonium sulfate. On the other hand the PEGylated lysozyme has an increased solubility in sodium chloride. Due to the non-electrostatic nature of PEG the solubility of PEGylated lysozyme is less concentrated and pH dependent especially for higher PEG to protein ratios and it deviates from Melander's and Horvath's solvophobic theory [41]. The dynamic binding capacities of various HIC resins are maximum for the 5 kDa PEG lysozyme with binding capacities of 80 mg/ml in ammonium sulfate and more than 100 mg/ml in sodium chloride. Binding capacities for the 30 kDa PEG lysozyme were found to be lower. This behavior can be explained by the large size of the hydrated molecule with a "fictious" molecular weight of more than 450 kDa [10].

Compared to the separation in ammonium sulfate, the protein selectivity was reversed in sodium chloride. A good selectivity, with PEGamer separation, was observed only in sodium chloride as a result of a still unknown supporting separation mechanism.

In summary PEGylation changes the hydrophobic properties of proteins in a way that is difficult to predict. High binding capacities and high resolution can be achieved by selecting appropriate conditions. For the time being, these conditions can be determined only empirically.

References

[1] F.M. Veronese, Biomaterials 22 (2001) 405.
[2] A.P. Chapman, Adv. Drug Deliv. Rev. 54 (2002) 531.
[3] Y. Yamamoto, Y. Tsutsumi, Y. Yoshioka, T. Nishibata, K. Kobayashi, T. Okamoto, Y. Mukai, T. Shimizu, S. Nakagawa, S. Nagata, T. Mayumi, Nat. Biotechnol. 21 (2003) 543.

[4] K. Yang, A. Basu, M. Wang, R. Chintala, M.-C. Hsieh, S. Liu, J. Hua, Protein Eng. 16 (2003) 761.
[5] A. Abuchowski, J.R. McCoy, N.C. Palczuk, T. van Es, F.F. Davis, J. Biol. Chem. 252 (1977) 3582.
[6] C. Monfardini, F. Veronese, Bioconjug. Chem. 9 (1998) 418.
[7] J.E. Seely, C.W. Richey, J. Chromatogr. A 908 (2001) 235.
[8] S. Yamamoto, S. Fujii, N. Yashimoto, P. Akbarzadehlaleh, J. Biotechnol. 132 (2007) 196.
[9] B. Kwon, J. Molek, A.L. Zydney, J. Membr. Sci. 319 (2008) 206.
[10] C.J. Fee, J.M. Van Alstine, Chem. Eng. Sci. 61 (2006) 924.
[11] Y.S. Youn, D.H. Na, S.D. Yoo, S.-C. Song, K.C. Lee, J. Chromatogr. A 1061 (2004) 45.
[12] R. Clark, et al., J. Biol. Chem. 271 (1996) 969.
[13] J. Vincentelli, C. Paul, M. Azarkan, C. Guermant, A. El Moussaoui, I. Looze, Int. J. Pharm. 176 (1999) 241.
[14] M. Cisneros-Ruiza, K. Mayolo-Deloisaa, T.M. Przybycien, M. Rito-Palomaresa, Sep. Purif. Technol. 65 (2009) 105.
[15] A. McPherson, Methods Enzymol. 14 (1985) 120.
[16] S.R. Sheth, D. Leckband, Proc. Natl. Acad. Sci. U.S.A. 94 (1997) 8399.
[17] G. Karlström, J. Phys. Chem. 89 (1985) 4962.
[18] R. Kjellander, E. Florin, J. Chem. Soc., Faraday Trans. 77 (1981) 2053.
[19] T. Takeuchi, B. Oktavia, l.W. Lim, Anal. Bioanal. Chem. 393 (2009) 1267.
[20] P. Thiyagarajan, D.J. Chaiko, R.P. Hjelm Jr., Macromolecules 28 (1995) 7730.
[21] F.E. Bailey Jr., R.W. Callard, J. Appl. Polym. Sci. 1 (1959) 56.
[22] H. Mahadevan, C.K. Hall, Fluid Phase Equil. 78 (1992) 297.
[23] H. Mahadevan, C.K. Hall, AIChE J. 36 (1990) 1517.
[24] K.C. Ingham, Arch. Biochem. Biophys. 186 (1978) 106.
[25] M.J. Garvey, I.D. Robb, J. Chem. Soc., Faraday Trans. 75 (1975) 993.
[26] S. Asakura, F. Oosawa, J. Polym. Sci. 33 (1958) 183.
[27] H. Walter, D.E. Brooks, D. Fisher, Partitioning in Aqueous Two-phase Systems: Theory, Methods, Uses and Applications in Biotechnology, Academic Press, Orlando, 1985.
[28] J.N. Bronstedt, Z. Phys. Chem. Abt. A 155 (1931) 257.
[29] E.J. Cohn, Physiol. Rev. 5 (1925) 349.
[30] J. Setschenow, Z. Phys. Chem. 4 (1889) 117.
[31] J.G. Kirkwood, in: E.J. Cohn, J.T. Edsall (Eds.), Proteins, Amino Acids, and Peptides, Reinhold, New York, 1943 (Chapter 12).
[32] R. Hahn, K. Deinhofer, C. Machold, A. Jungbauer, J. Chromatogr. A 790 (2003) 99.
[33] H. Lee, T.G. Park, J. Pharm. Sci. 92 (1) (2003) 97.
[34] E. Kim, Poster, ISPPP Meeting Orlando, 2007.
[35] A. Moosmann, J. Christel, H. Boettinger, E. Mueller, J. Chromatogr. A 1217 (2010) 209.
[36] J.F. Kramarczyk, B.D. Kelley, J.L. Coffman, Biotechnol. Bioeng. 100 (4) (2008) 707.
[37] M.R. Ladisch, Bioseparations Engineering, Principles, Practice, and Economics, Wiley-Interscience, New York, 2001, p. 117.
[38] F. Hofmeister, Arch. Exp. Pathol. Physiol. 24 (1888) 247.
[39] E. Florin, R. Kjellander, J.C. Eriksson, J. Chem. Soc., Faraday Trans. 80 (1984) 2889.
[40] K.P. Ananthapadmanabhan, E.D. Goddard, Langmuir 3 (1987) 25.
[41] W. Melander, C. Horvath, Arch. Biochem. Biophys. 183 (1977) 200.
[42] S.P. Monkarsh, et al., in: J.M. Harris, S. Zalipsky (Eds.), Poly(ethylene glycol) ACS Symposium Series, vol. 680, American Chemical Society, Washington, DC, 1997, p. 207.
[43] C.M. Smales, C.H. Moore, L.F. Blackwell, Bioconjug. Chem. 19 (1999) 693.