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Lysozyme - Lysozyme self-interactions as assessed by the osmotic second virial coefficient: Impact for physical protein stabilization

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Keywords: Protein-protein interaction; B_{22}, self-interaction chromatography, osmotic second virial coefficient; Lysozyme; stabilisation

List of abbreviations: osmotic second virial coefficient (B_{22}), self-interaction chromatography (SIC)

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The purpose of the presented study is to understand the physicochemical properties of proteins in aqueous solutions in order to identify solution conditions with reduced attractive protein-protein interactions, to avoid the

Biotechnology Journal

formation of protein aggregates and to increase protein solubility. This is assessed by measuring the osmotic second virial coefficient (B_{22}), a parameter of solution non-ideality, which is obtained using self-interaction chromatography. The model protein is lysozyme. The influence of various solution conditions upon B_{22} was investigated: protonation degree, ionic strength, pharmaceutical relevant excipients and combinations thereof. Under acidic solution conditions B_{22} is positive, favoring protein repulsion. A similar trend is observed for the variation of NaCl concentration, showing that with increasing the ionic strength protein attraction is more likely. B_{22} decreases and becomes negative. Thus solutions conditions are obtained favoring protein-protein interactions. The B_{22} parameter also reflects, in general, the influence of the salts of the Hofmeister series with regard to their salting-in/salting-out effect. It is also shown that B_{22} correlates with protein solubility as well as physical protein stability. This is shown.

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1 Introduction

Currently most protein therapeutics are applied via the parenteral route [1, 2]. Among the parenteral routes of administration, the development efforts for subcutaneous injection systems have increased in the last years. This is due to the fact that, especially for immunoglobulin therapeutics, relatively high doses are necessary, requiring high protein concentrations up to 100 mg/ml and higher. Furthermore, high protein concentrations are encountered in the downstream processing for protein purification. For such highly concentrated protein solutions, protein self-interactions and the formation of protein aggregates, reversible or irreversible, are critical issues [3]. The formed protein aggregates often lack bioactivity, may be immunogenic, can have altered half-lives and may serve as nucleation activators. Therefore, various excipients are added to the protein solution to mitigate or avoid protein aggregation [4]. Up to now, it is often unclear how the different excipients and especially combination thereof affect physical protein stability and protein solubility [5, 6].

Protein-protein interactions are often more pronounced if irreversible alterations in protein secondary structure are observed [7, 8], with the transition from alpha-helix to beta-sheet structure being the most prominent change. Such changes reduce protein activity and solubility [3].

Up to now it is still under debate whether further structural perturbations occur after protein self-association and aggregation [9], thus increasing protein aggregation kinetic.

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Therefore, it is important to characterize and understand protein-protein interactions and to assess them already during the early pre-formulation development phase. Different solution conditions have a strong impact on protein-protein interactions [10-13] and parameters that minimize protein-protein interactions have to be identified in order to avoid the potential formation of protein aggregates [14-16].

A number of different techniques are available for the investigation of protein-protein interactions and protein self-association [17-22], however, most techniques lack the possibility being used as a high throughput assay [23-25].

Various chromatography based techniques have emerged in the last years in order to characterize protein-protein interactions [17, 26-31]. For examples, Stevens [32] proposed the use of small-zone size exclusion chromatography for investigating protein-protein association and Wen et al. [33] showed the benefit of the additional combination of various detection methods for analyzing protein-protein interaction (see also [17]). Lemque et al. [34] used ion-exchange chromatography for analyzing self-association of beta-lactoglobulin B. In 1996, Patro and Przybycien [28] introduced the application of self-interaction chromatography as a protein characterization tool for the rapid screening of protein formulation additives against aggregation or for protein purification [35]. However, in the presented study they did not derive from their results the osmotic second virial coefficient. The study published by Bloustine et al. [36] showed how to calculate form the SIC results the osmotic second virial

coefficient. Winzor et al. [21] have even proposed a new thermodynamic approach supporting a simplified procedure for determining the osmotic second virial coefficient by SIC using a reduced number of parameters. Thus, there are a lot of activities in order to introduce and improve self interaction chromatography for measuring the osmotic second virial coefficient.

The concept behind the above described approach is related to studies and observations made many years ago. In 1946, Bruno Zimm published a paper entitled “application of the methods of molecular distribution to solutions of large molecules” [37], where he stated that the objective of statistical mechanics is to link the knowledge of solution thermodynamics to the properties of the molecules that compose the solution. Zimm recognized that the large deviations from Raoult’s law, which are especially observed for “large” solutes like polymers and proteins, are related to non-ideal properties of the solutes and this can be accounted for by means of methods using continuous molecular distributions functions. The outcome of his investigation was the ability to interpret and better understand thermodynamic data derived from protein solutions. The thermodynamic property examined by Zimm was the osmotic pressure, and he considered the different molecular interactions between the solutes in solution. Zimm analyzed the dependency of the concentration with regard to the osmotic pressure. 1945 William McMillan and Joseph Mayer [38] derived a series of expansion for the osmotic pressure (Π) in terms of concentration. Under the consideration that the higher order terms can be neglected, the non-ideality of Π is given as:

$$\Pi = R \cdot T \cdot c_p (1/M_w + B_{22} \cdot c_p) \quad (1)$$

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with R is the gas constant, T is the absolute temperature, c_p is the protein concentration in mass units, M_w is the molecular weight of the protein and B_{22} the osmotic second virial coefficient. The parameter B_{22} reflects the extent and direction of the non-ideal solution property, and thus protein-protein interactions.

Various investigations have given evidence that the osmotic second virial coefficient is a valuable parameter for the optimization of crystallization solution conditions which are prone to the formation of crystals [39-44]. Such solution conditions bear a slightly negative B_{22} value, i.e. conditions denoting weak attractive interactions [45-46]. Various authors have shown that supersaturated protein solutions primary form crystals when the corresponding equilibrium solubility is such that the resulting data for the osmotic second virial coefficient is between $-1 \cdot 10^{-4}$ and $-8 \cdot 10^{-4}$ $mol \cdot ml \cdot g^{-2}$ [46-50].

When the negative B_{22} value further decreases, solution conditions are obtained enhancing particle-particle attractions, and as a result in a number of cases amorphous protein precipitation is observed [51-53].

This concept was applied for the prediction of solubility activity. Within the framework of the sticky hard sphere model [54], Rosenbaum and colleagues [55, 56] showed that the experimentally measured B_{22} can be correlated with solubility data [50, 57-60]. This signifies that short range attractive interactions are the main interactions that describe the phase properties and behavior of a colloidal system [61].

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Based on these considerations, the relation between B_{22} and the solubility S is given by:

$$B_{22} = (-\Delta\mu_2 / R{\cdot}T) \cdot (1 / 2{\cdot}M_2{\cdot}S) - (\ln S / 2{\cdot}M_2{\cdot}S) \quad (2)$$

With $\Delta\mu_2 = \mu_2^0$ (solution) - μ_2^0 (solid), μ_2^0 (solution) is the chemical potential of the protein in solution, μ_2^0 (solid) is the chemical potential of the protein in a solid form (e.g. crystal), R is the gas constant, T is the absolute temperature, M_2 is the molecular weight of the protein (denoted as 2) and S is the solubility expressed in $g{\cdot}ml^{-1}$. This approach clearly shows that the second virial coefficient and the solubility are correlated [42, 50]. Ruckenstein and Shulgin [62] have recently reviewed the correlation between aqueous protein solubility and the osmotic second virial coefficient in the presence of salts, where good agreement were obtained between theoretical predictions and experimental results. When the cosolvent is an organic component, the situation is not so clear. Last year, Shulgin and Ruckenstein [63] presented a study using the Kirkwood-Buff fluctuation theory [64] to elucidate the effects of various contributions to the osmotic second virial coefficient in protein-water-cosolvent solutions and how to interpret this (for more details see reference).

Various molecular theories of fluids can be used to develop a mean force expression for describing protein-protein interactions. The application of chromatography methods like self-interaction chromatography (SIC) [21, 65, 66] requires the correlation of the osmotic coefficient of diluted to concentrated aqueous solution conditions [67].

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Rosenberger and coworkers [68] have shown by means of static and dynamic light scattering for undersaturated and supersaturated lysozyme solutions that B_{22} values measured at low solution concentrations, which are prone for the formation of crystals, are indicative for supersaturated solution conditions [55, 56, 68-72].

In contrast to the typical B_{22} target, i.e. the optimization of protein crystallization solution conditions, in the presented study, solution conditions that minimize attractive protein-protein interactions have to be identified, in order to avoid protein precipitation and aggregation. The procedure for the determination of the osmotic second virial coefficients as derived from SIC can be briefly summarized as follow. First, the protein of interest is covalently immobilized on chromatography particles. Then, a pulse of the protein of interest, free in solution, is injected and passed through the chromatography column filled with the chromatography beads carrying the protein on their surface. The obtained elution profile reflects the interaction of immobilized protein with protein that is free in solution, under the assumption that the immobilized protein retains its native three-dimensional and secondary structure (this will be addressed in a subsequent paper, which is in preparation: comment of the authors). Furthermore, it is assumed that the protein is immobilized to the chromatography particles in a broad range of orientations, avoiding a side specific interaction, which will not be representative for the interaction between two protein molecules, both free in solution. Under these conditions the measured protein-protein interaction reflects the ensemble

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average interaction energy between two protein molecules under the investigated solution conditions.

The influence of various excipients with regard to protein-protein interactions can be screened using an automated chromatography system. Thus, the effectiveness of formulation solutions, containing e.g. different excipients at varying concentrations, can easily be assessed. Solution conditions reducing attractive protein-protein interactions are characterized by a reduction of the retention volume, whereas the opposite is the case for solution conditions favoring attractive protein-protein interactions.

The following solution conditions are investigated with regard to lysozyme-lysozyme interactions:

- protonation degree
- ionic strength
- temperature
- salts of the Hofmeister series
- pharmaceutical excipients (sucrose, glycerol)
- PEGs (poly-ethylene glycol) of different molecular weights

In addition, the effect of chromatography bead surface coverage as well as storage stability of the functionalized chromatography particles with regard to the osmotic second virial coefficient was studied. B_{22} is also correlated with protein solubility and protein physical stability.

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2 Materials and methods

2.1 Materials

Lysozyme from chicken egg white (135500 U/mg cryst.) was obtained from Serva (Heidelberg, Germany), Toyopearl AF Formyl 650M from Tosoh Bioscience (Stuttgart, Germany), potassium phosphate, sodium chloride, magnesium chloride, ammonium chloride, glacial acetic acid and sodium cyanoborhydride from Merck (Darmstadt, Germany), PEG 4000 and PEG 6000 from Merck-Schuhardt (Hohenbrunn, Germany), potassium chloride from Caelo (Hilden, Germany), PEG 400 from Fluka (Buchs, Switzerland), ethanolamine from VWR Prolabo (Fontenay sous bois, France), citric acid monohydrate from Roth (Karlsruhe, Germany), ammonium sulfate and glycerol from Grüssing Diagnostika Analytika (Filsum, Germany), sodium sulfate from Riedel-de-Häen, Seelze, Germany), potassium sulfate from J.T. Baker (Deventer, Netherlands) and BCA-assay Uptima from Interchim (Montluçon, France).

The pH of the solutions was adjusted using hydrochloric acid or sodium hydroxide and measured with a pH meter Inolab level 1 from WTW (Weilheim, Germany). The protein concentrations were evaluated with an UV-photometer UV1 from Thermo Spectronic (Dreieich, Germany).

2.2 Lysozyme immobilization

3 ml Toyopearl AF-Formyl 650M particles were washed on a glass frit with a 0.2 µm hydrophilic polyethersulfone membrane filter with first 250 ml de-ionized water and secondly with 50 ml of 0.1 M potassium phosphate

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buffer pH 7.5. The washed particles were recovered and mixed to 10 ml lysozyme solution (6.5 mg/ml in 0.1 M potassium phosphate buffer pH 7.5) and 90 mg sodium cyanoborhydride used as activator of protein binding. The suspension was mixed over night (≈12 h) on a rotary mixer. At the end of the coupling reaction the particles were washed with 200 ml of 0.1 M potassium phosphate buffer. After recovery they were added to 15 ml of 1 M ethanolamine pH 8.0 and 20 mg sodium cyanoborhydride to cap the remaining matrix reactive groups. The suspension was mixed on a rotary mixer during 4 h. At the end of the reaction the particles were washed with 200 ml of 1 M sodium chloride solution pH 7.0 to remove any unbound material. The amount of bound protein was determined by analyzing the absorbance of the initial protein solution and the wash solutions (A280), and by determining the protein quantity immobilized on the matrix by BCA (bicinchoninic acid) assay.

The chromatography particles were prepared in the form of a 50%-slurry in 1 M NaCl, 5 mM acetic acid solution pH 4.5. Approximately 2.5 ml slurry was packed in a Tricorn® 5/50 column (GE Healthcare, Uppsala, Sweden) with the same buffer 1 M NaCl, 5 mM acetic acid buffer pH 4.5 at a flow rate of 3 ml/min during 15 min using a FPLC system (Äkta Purifier, GE Healthcare, Uppsala, Sweden). At the end of the packing procedure the flow-rate was maintained at 0.75 ml/min during at least 30 min. The column integrity was tested by injecting 50 µl of 1 v% acetone solution. Columns were stored at 4 °C in a 5 mM sodium phosphate buffer pH 7.0 containing 0.05 w% sodium azide.

Biotechnology Journal

2.3 Determination of the osmotic second virial coefficient B_{22}

All mobile phase solutions were buffered with 5 mM acetic acid at pH 3.0 and 4.5 or 5 mM sodium phosphate at pH 6 - 8. Lysozyme was dissolved in the studied solutions at 20 mg/ml. B_{22} measurements were realized with a FPLC Äkta Purifier system equipped with an UV detector (A280). Before each run the column was equilibrated with 10 ml of protein free mobile phase. The column dead volume was determined with the 1 v% acetone solution injection. All experiments were carried out at 25 °C and at a flow rate of 0.75 ml/min. 10 µl sample was injected; each sample was measured 6 times. Chromatogram peaks were analyzed with the UNICORN® software (GE Healthcare, Uppsala, Sweden). The retention volume was determined at the peak maximum. The retention measurements were used to calculate the retention factor *k'* (equation 3) that measures the strength of interaction between the mobile phase protein and non-interacting species:

$$k' = \frac{V_o - V_r}{V_o} \qquad (3)$$

V_r is the volume required to elute the protein in the mobile phase and V_o the retention volume of non-interacting species (e.g. acetone). B_{22} is related to the retention factor as follows [37, 66]:

$$B_{22} = B_{HS} - \frac{k'}{\rho_s \cdot \Phi} \qquad (4)$$

$$B_{HS} = \frac{16}{3} \pi \cdot r^3 \cdot \frac{N_A}{M_2^2} \qquad (5)$$

Biotechnology Journal

ρ_s is the number of immobilized molecules per unit area, Φ the phase ratio, which is the total available surface available to the mobile phase protein, r the protein radius, N_A Avogadro's number and M_2 the protein (index 2) molecular weight.

2.4 Determination of lysozyme charges

Lysozyme molecule charges were calculated for the amino acid sequence of lysozyme from white chicken egg (EC 3.2.1.17) with the EMBOSS-software [73] based on single chain and 4 disulfide bonds.

2.5 Determination of protein solubility

350 µl of buffer solution were added to 100 mg lysozyme powder. The samples were first continually stirred during 24 h at 25°C to facilitate the powder dissolution and then centrifuged at 20,000 g during 1 h. After correction of the pH shift, the samples were stirred a second time 12 h at 25°C and finally centrifuged at 20,000 g during 1 h and pH controlled again. Lysozyme concentration was measured by UV spectroscopy at 280 nm with an extinction coefficient of 2.63 ml · mg^{-1}· cm^{-1}. Each determination was carried out in triplicate.

2.6 Determination of protein physical stability via stirring stress

Stirring stress was performed using 10 R glass vials (Schott, Mainz, Germany) filled with 5 ml of 10 mg/ml lysozyme solution at 25 °C. The Teflon coated stirring bar (Carl Roth GmbH & Co., Karlsruhe) has the

Biotechnology Journal

dimension of: length = 12 mm, diameter = 4.5 mm. Stirring was performed on a RT 10 power Ikamag multiple-stirrer (IKA® Werke GmbH & Co. KG, Staufen, Germany) at 1200 rpm (pounds per minute). After defined time points, a vial was removed and analyzed for protein aggregation by turbidity as described previously [74]. The experiments were performed at room temperature. As control experiments the pure buffer solutions were stirred, as well as the different protein formulations were stored at 25 °C (non stirred).

Biotechnology Journal

3 Results and Discussion

The surface charge of a protein, and related effects e.g. protein hydration, has a strong influence on protein-protein interactions. Therefore, the solubility of proteins is minimal at pH solution conditions close to their pI. Lysozyme is a protein with an extremely basic pI (pI ≈11) [75-77]. Its titration curve (in absence of any excipient, i.e. ion binding is not considered that may affect the surface charge in solution) was calculated using the EMBOSS-software [73]. At pH 4.5 lysozyme is positively charged, with 11 positive elementary charges. At pH 7.5 lysozyme is still positively charged, with 8 positive elementary charges. Charge neutralization is given for pH 11.

3.1 Correlation between protein surface coverage and B_{22}

As described above, the approach using SIC for the determination of B_{22} based on the consideration of Zimm [37] takes account of an anisotropy interaction energy of a two-body interaction (i.e. interaction of two protein molecules). This means that the potential mean force for the description of the interaction depends on the separation of the two bodies and is a function of all orientations. Therefore, the concept just considers the interaction of a free protein molecule in solution with only one protein molecule immobilized on the chromatography particles. The interaction of e.g. a free protein molecule in solution with two immobilized protein molecules, i.e. a three-body interaction, is not considered (see equation 1). Therefore, the validity of the applied method depends on the

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chromatography particle surface coverage of the immobilized protein [66]. This has also to be considered for the interaction in solution of the free protein molecules, namely interactions of free protein molecules with each other in solution should be strongly reduced. This can easily be realized using relatively diluted protein solutions. Thus, the free protein in solution interacts just with one immobilized protein molecule.

Three different degrees of protein surface coverage were tested: 18 mg/g, 21 mg/g and 56 mg/g (mg protein per g chromatography particles). The experiment with 21 mg/g was repeated with a similar surface coverage of 22 mg/g. The protein surface coverage was determined using UV difference measurement and via the BCA assay (see section Materials and Methods).

B_{22} was measured, for a constant protein surface coverage at constant temperature T = 25°C, 5 mM acetic acid pH = 4.5, as a function of ionic strength. The NaCl concentration was varied between 0 M to 0.8 M. Figure 1 shows exemplarily elution profiles of lysozyme at four different ionic strengths. The area under the curve (AUC) remains mainly unchanged. The retention volume increases with increasing ionic strength, reflecting changes in B_{22}.

The data reported in Figure 2 represent the average data of 6 independent experiments with their standard error of deviations.

As can be seen from Figure 2 an increase of the ionic strength induces a decrease of B_{22}. At low ionic strength B_{22} values are positive, and a change in sign is observed at a NaCl concentration of ≈0.6 M. Above 0.6

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M NaCl B_{22} data are negative. Thus, at higher NaCl concentrations attractive protein-protein interactions are favored, because the protein charges become strongly screened with higher amounts of present NaCl [78, 79].

With regard to the protein surface coverage (Figure 2) between 18 – 56 mg/g the obtained B_{22} data are quite similar with a slight trend of decreasing values for 56 mg/g. This is in accordance with literature [66]. The comparison of two independent preparations of protein immobilization to the chromatography particles (21 and 22 mg lysozyme / g chromatography particles) shows a rather good reproducibility of B_{22} values of $\pm\, 0.1 \cdot 10^{-4}$ mol · ml · g^{-2} at different NaCl concentrations.

Based on the derived theoretical approach, the osmotic second virial coefficient should be independent of protein concentration. This is the case when assuming no changes in protein conformation as well as neglecting higher-order processes [53]. Using relative low protein surface coverages, Teske et al. [80] were able to show a good correlation between their results of osmotic second virial coefficient and results derived from static light scattering. Large deviations, compared to the results obtained by static light scattering, were observed for protein surface coverage up to 33 %. This implies multiple body interactions occur with increasing protein surface coverage. The presented result, is however not in accordance to the study presented by Tessier et al. [45] , who found that a coverage close to 33 % is required.

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The data presented in this study are in accordance to the SIC results presented by Tessier et al. [66]. They have also shown a quantitative agreement between virial coefficients measured by SIC and by static light scattering. This is also evident from other studies [53, 81-82]. These examples show that the SIC method is a reliable technique for measuring virial coefficients.

3.2 Effect of temperature

The effect of temperature with regard to B_{22} depends on the overall solution condition properties. Figure 3A shows the variation of B_{22} as a function of temperature in the range between 5 and 35 °C at a NaCl concentration of 800 mM (pH = 4.5). The investigated temperature interval is well below the denaturation temperature of lysozyme [7]. Thus the interactions between native structures are investigated. Under these solution conditions and in the investigated temperature interval all B_{22} data are negative and lie between -7 and $-1 \cdot 10^{-4}$ $mol \cdot ml \cdot g^{-2}$. These are solution conditions favoring crystallization [83-87].

With increasing temperature repulsive interactions intensify. However, this trend depends on the overall solution conditions. Reducing the ionic strength from 0.8 M NaCl to 0.3 M NaCl, the temperature effect is rather small (Figure 3B) with a slight decrease in B_{22}. Under these solution conditions B_{22} is positive with values between 2.5 and $3.5 \cdot 10^{-4}$ $mol \cdot ml \cdot g^{-2}$.

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Valente et al. [53] also observed the osmotic second virial coefficient becoming more positive as the temperature increases, which can be correlated with increasing protein solubility with increasing temperature. Antipova et al. [88] reported an opposite effect, namely a decrease in the osmotic second virial coefficient as the temperature was increased up to 50 °C. Indeed, when the temperature increases, proteins begin to partially unfold, exposing hydrophobic regions that lead to more attractive interactions [53]. One important factor governing the trend of the osmotic second virial coefficient as a function of temperature increase is due to the fact whether the present excipients are able to solubilise or even stabilize the protein against thermal induced protein denaturation. This means, the comparison of such studies is only possible, when exactly the same formulations are used and tested. Tiny changes in the formulation composition seems to have a large effect on the osmotic second virial coefficient.

3.3 Effect of the protonation degree

Lysozyme has a strongly alkaline isoelectric point of ≈11 [75-77]. Choosing pH conditions close to the pI would reduce protein solubility (see below) and thus favor protein precipitation. Increasing the pH substantially decreases B_{22} (Figure 4). Under acidic pH conditions B_{22} is positive, whereas at pH ≈7 B_{22} becomes negative. Experiments for pH > 8 were not followed, because strongly alkaline solution conditions would damage the chromatography particles. In the presented case the dependency on pH

Biotechnology Journal

with regard to B_{22} is linear. At pH 11 one would obtain a B_{22} of $-3 \cdot 10^{-4}$ mol · ml · g^{-2} (data derived from extrapolation), reflecting conditions prone for precipitation. The explanation for the pH dependency with regard to B_{22} is the change in protonation degree of lysozyme accompanied by changes of protein hydration. With decreasing pH the positive surface charge increases, i.e. the zetapotential and thus the solubility [58, 89]. These results are in accordance with Dumetz et al. [85]. Elcock and McCammon [14] have shown how to calculate and consider the pH dependency of the osmotic second virial coefficient by taking into account protein-protein interaction energies related to (a) electrostatic interactions based on the use of effective charges, (b) electrostatic desolvations occurring when charged groups are buried by an approaching protein partner and (c) van der Waals and hydrophobic interactions. The proposed calculations seems to be adequate for the description of weak protein-protein interactions.

In this context, one has to be aware that lysozyme has then tendency to self-associated depending on the pH of the solution [90-93].

3.4 Correlation between B_{22} and protein solubility

For protein formulation, solution conditions favoring protein solubility are of interest in order to stabilize the protein solution. Figure 5A, represents the correlation between B_{22} and protein solubility. The lowest protein solubility was set to 1, thus relative solubilities are reported in order to directly compare the impact on the formulation change. Protein solubility is the

Biotechnology Journal

lowest under alkaline conditions because of the strong alkaline pI of lysozyme. Under these conditions negative B_{22} are determined, which indicate a predominance of attractive protein-protein interactions and thus reduced solubility. Under acidic conditions, B_{22} becomes positive and corresponds to an increase in solubility by a factor of 7. Increasing the ionic strength (Figure 5B) reduces strongly the protein solubility; B_{22} is below $-2 \cdot 10^{-4}$ $mol \cdot ml \cdot g^{-2}$. A removal of NaCl from the formulation induces a protein solubility increase of a factor of 20 (see Figure 5B).

The correlation between B_{22} and solubility as a function of pH was recently presented by Payne et al. [65] for a 36 amino acid therapeutic peptide. A good correlation between peptide solubility and B_{22} was found in the pH range of 6 to 10, emphasizing the validity of the method (see equation 2, which relates solubility to B_{22}).

Thus, the assay allows the identification of solution conditions leading to highest protein solubility.

3.5 Effect of ionic strength and various salts of the Hofmeister series

For more than 120 years (1888) it has been known from the pioneering work of Franz Hofmeister [94] that the presence of some salts is able to precipitate proteins, whereas other salts exert a salting in effect. The salt concentrations applied for e.g. protein precipitation by $(NH_4)_2SO_4$ are often very high (up to 3 M). Especially at these high salt concentrations the effects and trends of the Hofmeister series become relevant [95].

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However, for pharmaceutical applications such high salt concentrations are of minor importance. Therefore, the presented investigations are focused on the presence of lower salt concentrations. A parameter not further considered by Hofmeister was the pH of the solution, as the concept of pH was not known at that time. The concept of pH was introduced about 20 years later by Sørensen [96]. However, the solution pH and thus the overall surface charge of a particle have a strong impact on its interaction characteristics, and have to be considered. As ammonium sulphate has the property of being kosmotropic at high salt concentration (4 M), which favors protein precipitation [97], increasing the $(NH_4)_2SO_4$ concentration should decrease B_{22}. Figure 6A shows the variation of B_{22} as a function of $(NH_4)_2SO_4$ concentration between 0 and 0.8 M. Even if B_{22} decreases by increasing salt concentration, B_{22} data are still positive for the investigated $(NH_4)_2SO_4$ salt concentrations. Negative B_{22} could only be obtained when the $(NH_4)_2SO_4$ concentration exceeds concentration of at least 3 M.

Considering the B_{22} data of two additional sulphate salts, namely K_2SO_4 and Na_2SO_4, it can be seen that the B_{22} data are very similar for salt concentrations between 0 and 0.3 M. Differences are observed for salt concentrations larger than 0.6 M, with the sodium salt resulting in more negative B_{22} values.

As well as sulphate salts, the chloride salts of Na^+, K^+ and Mg^{2+} have a higher effect at large salt concentration, with $MgCl_2$ having the largest salting-in effect (Figure 6B1). At 0.8 M salt concentration one observed:

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$B_{22}(MgCl_2)$ > $B_{22}(KCl)$ > $B_{22}(NaCl)$. At high ionic strength, the observed cation effect follows the trend of the Hofmeister series (see below).

With increasing the $MgCl_2$ concentration, up to a concentration of approximately 0.3 M, first a decrease in B_{22} is observed. However, further increasing the salt concentration above 0.3 M, induces an increase of B_{22} (B_{22} > 0) (see Figure 6B2), which is representative for repulsive interactions. This trend has also been described by Tessier et al. [66]. The reason for this behavior is due to binding of the divalent magnesium cation to the acidic residues of lysozyme. This effect depends on the pH of the solution.

3.6 Effect of various pharmaceutical excipients (sucrose and glycerol)

In the presence of glycerol in the concentration range from 0 to 6 w% and in the presence of sucrose up to a concentration of 20 w%, the B_{22} data are all strongly positive and nearly independent of excipient concentration (Figure 7). The reported data are between 8 and $10 \cdot 10^{-4}$ $mol \cdot ml \cdot g^{-2}$. Thus, the presence of e.g. sucrose favors repulsive interactions between lysozyme molecules. The additional presence of high concentrations of NaCl (0.8 M) induces a strong decrease of B_{22}. However, the presence of high sugar concentrations (up to 10 w% of sucrose) overcompensates the charge screening effect of the salt, which leads to a salting-out effect. Thus the overall solution condition properties become positive in the sense of favoring protein solubility at sucrose concentration larger than 10 w%.

Biotechnology Journal

The presence of glycerol also induces an increase of B_{22} in presence of NaCl in protein solution (see Figure 7).

Valente et al. [13] have reported that sugars (e.g. sucrose) influence the conformational dynamics of proteins, favoring the most compact conformation within the native-state ensembles. This reduction of protein volume is accompanied by a concomitant reduction in the hydrophobicity of the protein surface, thus reducing the tendency for protein-protein self association [13]. Kaushik and Bhat [98] have pointed out to another possible stabilization mechanism that has to be considered. They explained the stabilization effect of certain polyols and sugars being related to the surface tension increase (for more details see [98]).

3.7 Effect of PEG molecular weight

Under the tested solution conditions, the B_{22} values are all positive for the three tested PEG qualities with molecular weights of 6000, 4000 and 400 g/mol (Figure 8A). Under these conditions protein precipitation is unlikely. The trend for the high molecular weight PEG is very similar. Increasing the PEG concentration even leads to a salting-in effect, indicative of an increased and positive B_{22}. The presence of PEG 400 shows basically no effect on the B_{22} data, which lie between 7 and $10 \cdot 10^{-4}$ mol · ml · g^{-2} for the concentration range from 0 to 24 w% PEG 400. Depending on the overall solution conditions, PEG can also act as a precipitation reagent as shown by Tessier et al. [44]. For Ribonuclease A (pH 8 and 50 mM NaCl) with increasing the concentration of PEG 3350 g/mol B_{22} decreases

Biotechnology Journal

strongly and in the presence of 15 w% PEG values of $-18 \cdot 10^{-4}$ $mol \cdot ml \cdot g^{-2}$ are determined [44].

3.8 Storage stability of the functionalized chromatography column

Using the presented method as a screening assay, it is crucial that the column shows certain stability in order to be able to perform and evaluate various solution conditions with regard to B_{22}. Therefore, the storage stability at 4 °C of the functionalized column was investigated (Figures 9). The column was stored in a 5 mM sodium phosphate buffer at pH 7.0 containing 0.05% sodium azide at 4 °C up to 307 days. Figure 9A shows that the obtained B_{22} data are very similar showing that the column still remains its functionality. However, analysis of the chromatograms (Figure 9B) shows the appearance of a shoulder at about 2.3 ml. This is especially observed after the column has been stored for 161 days. Based on the obtained data, stability of the lysozyme functionalized column, under the chosen storage conditions, is given for 3 months, which is in accordance to Tessier et al. [66]. This allows SIC as being used as a high throughput screening method for the evaluation of a large number of solution conditions.

3.9 Physical protein stability via stirring stress

The physical protein stability was investigated under rapid stirring (1200 rpm) of 10 mg/ml lysozyme formulations in 300 mM NaCl at 25 °C as a function of pH. The stability of the different formulations was assessed by

Biotechnology Journal

turbidity measurement. The turbidity of the freshly prepared lysozyme formulations lies between 1.0-1.3 FNU, whereas the corresponding buffers show slightly reduced turbidities between 0.4-1.0 FNU. During the stress time of the experiment the turbidity of the buffer solutions is unchanged. For the stressed lysozyme formulations a strong increase of turbidity is observed that is intensified by increasing the solution pH. After 24 h stirring of the formulation at pH 8, the turbidity reached values of nearly 24 FNU. Furthermore visible, insoluble particles are observed. As further reference samples, lysozyme formulations at pH 3, 6, and 8 were stored at room temperature (no stirring) for 24 h. The turbidity was mainly unchanged with values between 1-2 FNU during the storage of 1 day at 25 ℃.

Thus, stirring induces the formation of protein aggregates, which is more pronounced with increasing pH. The figures 4 and 5A represent the B_{22} data as a function of pH. Under acidic conditions positive B_{22} data are measured, indicating solution conditions that favor protein-protein repulsion, whereas under alkaline pH conditions B_{22} becomes negative. As stronger protein-protein repulsive interactions would be expected at high pH, the physical stability of the lysozyme correlates to B_{22} data at high pH. This is in accordance with the study presented by Valente et al. [99] for the physical stabilization of *Pseudomonas* amylase. Their conclusions was that self interaction chromatography measurements of total soluble amylase and enzymatic activity measurements correlated qualitatively with

Biotechnology Journal

trends in the osmotic second virial coefficient except near the pI of the protein amlylase, where physical stability was minimal [99].

Biotechnology Journal

4 Conclusions

The overall protein solution conditions are dependent on a number of factors of the solution (e.g. pH, temperature, ionic strength, osmolarity) and the presence of excipients and cosolutes. The presence of various ions/excipients may have a more or less pronounced impact on the protein solution and stability conditions, depending on whether unspecific or specific interactions are involved. The interactions of ions with proteins are governed by electrostatic interactions, but the influence of solvation (and solvation forces) also has to be considered [100, 13]. The interactions of protein molecules in an aqueous environment are mediated by water molecules, and excipients and cosolutes can influence the protein surface characteristics, as well as the structure of the liquid water (kosmotropic and chaotropic agents). These effects on the water/protein interface influence directly the interfacial energies involved in the interaction and these interfacial energies can not be predicted so easily.

As described above, the effect of salts on the protein stability characteristics depends on the overall solution conditions. Yamasaki et al. [101] have shown for BSA with regard to the thermally induced denaturation, that in the presence of kosmotrope salts, the protein structure is stabilized, whereas it becomes destabilized in the presence of chaotrope salts. This is especially the case at high ionic strengths, but a reversal of the stability effect is observed at low ionic strength (0.01-0.1 M). The reason for such a behavior lies in the screening of the electrostatic repulsions via the close interaction/binding of the anion to the

Biotechnology Journal

protein, and this effect is stronger with chaotrope than with kosmotrope ions.

In this study, it was shown that the effect of different salts/ions with respect to increasing protein solubility, reflected in an increase of B_{22}, does not necessarily correlate with the Hofmeister series. In some cases a reverse effect is even observed. This was also described by Riès-Kautt & Ducruix [102]. The reason for this effect was attributed to the effectiveness of anions to promote protein crystallization, which is dependent on the net charge of the protein. Therefore, for acidic proteins the salting-in/salting-out behavior of ions follows the Hofmeister series, whereas the effect and order are reversed for basic proteins like lysozyme.

A strong impact on B_{22} was observed as a function of the nature of the anion in solution.

The presented study shows that self-interaction chromatography can be used as a rapid development tool for biopharmaceuticals. It has to be emphasized that the used methods are based on the characterization of protein-protein interactions in their native state [9]. The second osmotic virial coefficient, which is derived from self-interaction chromatography, is a parameter of solution non-ideality that is useful for the prediction of solution conditions minimizing protein aggregation or solution conditions promoting protein solubility (see Figure 5). SIC allows rapid determination of B_{22} under high throughput conditions using automated systems. A recent study [103] has presented a high-throughput self interaction chromatography application combined with chemometry (ANN: artifical

Biotechnology Journal

neural network) in order to predict protein-protein interactions based on the osmotic second virial coefficient. Additional efforts were undertaken to miniaturize the SIC technique. Deshpande et al. [104] showed a SIC microchip application, which allows a rapid screening of various solution conditions with a strongly reduced protein consumption.

Self interaction chromatography can be used for screening evaluations, with column stability given for at least 3 months stored at 4℃ (for lysozyme). The analysis of B_{22} determines protein-protein interactions and is an indicator for physical protein stability (see Figure 10) [9, 105, 106]. Thus, for formulation and/or purification development, the determination of the second osmotic virial coefficient allows the identification of solution conditions promoting protein solubility and reducing the formation of aggregation due to protein-protein interactions. For the development of acceptable formulations one has additionally to consider solution conditions that are well tolerated for the intended medical application. For example, the pH of subcutaneous formulation should be close to the physiological pH, whereas the pH of infusion formulation can be much more acidic (pH of approximately 5). Thus, using additional excipients, for a given (acceptable) pH, one has to identify solution conditions leading to repulsive interactions between the protein molecules in order to avoid protein aggregation. It has to be mentioned excipients reducing the conformational stability of the protein in solution, should be avoided.

In future investigations, the presented method will be used for the analysis of protein-protein interactions of therapeutic proteins, e.g. antibodies, in

Biotechnology Journal

order to generalize the findings described in this study and to verify the value of the second osmotic virial coefficient for the identification of formulations favoring protein colloidal stability.

Biotechnology Journal

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Biotechnology Journal

Figures

Figure 1.

Elution profiles of lysozyme as a function of NaCl concentration in presence of 5 mM acetic acid at pH 4.5 at a loading of 18 mg/g: no salt (—), 100 mM NaCl (- - -), 300 mM NaCl (- - -) and 800 mM NaCl (—) (Injection of 10 µl lysozyme solution at 20 mg/ml).

Figure 2.

Effect of surface coverage (mg lysozyme / g particles) on lysozyme B_{22} values as a function of NaCl concentration in presence of 5 mM acetic acid at pH 4.5: 18 mg/g (■), 21 mg/g (□), 22 mg/g (▲), 56 mg/g (△).

Figure 3.

Effect of temperature from 5 to 35°C on lysozyme B_{22} data as a function of NaCl concentration in presence of 5 mM acetic acid at pH 4.5 at a loading of 22 mg/g.

(A) NaCl concentration = 0.8 M,

(B) NaCl concentration = 0.3 M.

Figure 4.

Effect of pH on lysozyme B_{22} values at a loading of 51 mg/g in presence of 0.3 M NaCl and 5 mM acetic acid at pH 3.0 and 4.5, and 5 mM sodium phosphate at pH 6.0, 7.0 and 8.0.

Biotechnology Journal

Figure 5.

Correlation between B_{22} and lysozyme relative solubility under different conditions.

(A) Influence of pH (3.0, 4.5, 6.0 and 8.0) at a constant NaCl concentration of 300 mM, (B) Influence the ionic strength (NaCl concentration of 0, 300 and 800 mM) at a constant pH of 4.5. The buffer conditions for B_{22} determination and protein solubility were identical.

Figure 6.

Effect of ionic strength on lysozyme B_{22} values at a loading of 18 mg/g in presence of (A) sulfate salts and 5 mM acetic acid at pH 4.5: $(NH_4)_2SO_4$ (■), K_2SO_4 (□),Na_2SO_4 (●) (The sulfate concentration corresponds to the cation concentration),

(B) chloride salts cation and 5 mM acetic acid at pH 4.5: $MgCl_2$ (■), KCl (□), NaCl (●) (plotted as a function of (B1) cation concentration, (B2) chloride concentration),

(C) chloride salts and 5 mM sodium phosphate at pH 7.4: NH_4Cl (■), NaCl (□).

Figure 7.

Effect of sucrose and glycerol in presence of 5 mM acetic acid at pH 4.5 on lysozyme B_{22} values at a loading of 18 mg/g: glycerol (□), sucrose (■), glycerol + 0.8 M NaCl (O), sucrose + 0.8 M NaCl (●).

Biotechnology Journal

Figure 8.

Effect of PEG on lysozyme B_{22} values at a loading of 18 mg/g:

(A) influence of PEG molecular weight in presence of 5 mM acetic acid at pH 4.5: PEG 6000 (■), PEG 4000 (□), PEG 400(●),

(B) influence of pH in presence of PEG 6000: pH 4.5 (■) with 5 mM acetic acid, pH 7.4 (□) with 5 mM sodium phosphate.

Figure 9.

Column stability at a loading of 22 mg/g:

(A) lysozyme B22 values as a function of NaCl concentration in presence of 5 mM acetic acid at pH 4.5 after 21 days (■), 30 days (□), 45 days (●), 86 days (O), 161 days (▲); 307 days (△),

(B) Elution profiles of lysozyme with 0.8 M NaCl and 5 mM acetic acid at pH 4.5 after 21 days (—), 45 days (- - -), 86 days (- - -) and 161 days (—). (Injection of 20 µl lysozyme solution at 20 mg/ml, storage at 4°C in 5 mM sodium phosphate buffer pH 7.0 containing 0.05% sodium azide).

Figure 10.

Influence of stirring stress (1200 rpm) upon lysozyme (10 mg/ml) physical stability in 300 mM NaCl at pH 3 (■), 6(●), and 8 (▲) at room temperature. Protein stability is assessed by turbidity and expressed in FNU (Formazin nephelometric units). As reference the pure solutions at different pH were stirred (open symbols).

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