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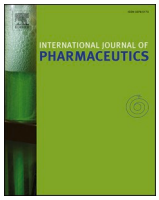

Excipient effect on phenol-induced precipitation of human growth hormone and bovine serum albumin

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ABSTRACT

The aim of this study was to investigate the impact of phenol on the precipitation of bovine serum albumin (BSA) and human growth hormone (hGH), in the presence of other excipients frequently used in biological drugs for parenteral delivery. The focus of the study lieson incompatibilities observed in multidose formulations containing non-ionic surfactants and preservatives. Previous research has shown that above a critical concentration, phenol reduces the cloud point of polysorbate surfactants to room temperature or lower. Here, it is demonstrated that for BSA-polysorbate solutions, phenol-induced incompatibility is primarily controlled by this depression of the surfactant cloud point, resulting in turbidity and/or precipitation. However, for formulations with human growth hormone (hGH) in isotonic salt solutions, the precipitation mechanism is instead driven by protein-phenol interactions. The precipitation is affected by the concentration of sodium chloride and at low salt concentrations the incompatibility is again controlled by depression of the surfactant cloud point. The concentration of salt needed for protein induced precipitation seems to follow the Hofmeister series, with sodium chloride and sodium sulphate inducing precipitation at a lower salt concentration than sodium nitrate. Notably, non-ionic tonicity agents, such as glucose and mannitol, which are known to impact the surfactant cloud point depression of phenol, do not induce precipitation of hGH in the presence of phenol. In the system containing polysorbate, phenol and hGH, salt-triggered protein precipitation occurs at slightly higher sodium chloride concentrations than in solutions without polysorbate. This indicates a stabilizing effect of polysorbate on hGH below the cloud point. However, the stabilising effect is surfactant dependent, and in the presence of dodecyl maltoside, hGH precipitation occurs at much lower sodium chloride concentrations than for solutions with polysorbates. This illustrates the complexity of the interplay of excipients with each other and with the active ingredient (the protein) in the development of multidose pharmaceutics.

1. Introduction

In parenteral pharmaceutical products intended for multiple injections, the addition of preservatives assures the microbiological safety of the product (Hodges and Hanlon, 2000). However, it is well known that for formulations containing polysorbate surfactants, there is an incompatibility between these surfactants and the most commonly used preservatives (Ford et al., 2023; Gilbert et al., 2022; Rios, 2006; Rowe et al. 2006a; Stroppel et al., 2023; Zhi Chen, 2015). We have shown in previous work that this is mainly due to a cloud point depression of the surfactant (Wahlgren et al. 2024). This, in turn, means that there is a temperature and concentration range where the system does not cloud, where it is possible to formulate a stable product with acceptable preservative function. For example, systems that become cloudy at room temperature can be reversed by lowering the temperature through refrigeration. However, other excipients like salts and polyols also affect the clouding phenomenon. This is further complicated by the fact that when the phenolic preservative concentration is high enough, proteins are known to aggregate, independent of the presence of polysorbate (Arora et al., 2017; Bis and Mallela, 2014; Bis et al., 2015; Maa and Hsu, 1996, 2004 #62; Thirumangalathu et al., 2006). This aggregation has been linked to perturbation of the tertiary structure of the protein

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without secondary structure changes (Bis et al., 2015; Hutchings et al., 2013; Thirumangalathu et al., 2006; Zhang et al., 2004). Further, this perturbation does not lead to aggregation if the protein can be stabilised through electrostatic repulsion (Thirumangalathu et al., 2006). Maa and Hsu have shown that a range of phenolic compounds cause aggregation of human growth hormone (hGH) at a preservative concentration of 10 mg/ml (Maa and Hsu, 1996).

In this work two proteins are studied, bovine serum albumin (BSA) and human growth hormone (hGH). Bovine serum albumin was selected as it is a well-known protein used in many biotechnological applications (Xu et al., 2023). It is also known to interact with preservatives such as sodium benzoate and could thus potentially show incompatibility with phenol (Yu et al., 2019). Human growth hormone was investigated as it is commercially available and commonly used as a multidose product containing phenol or metacresol as preservatives. The preservative used is phenol. It is a common preservative for multidose presentations of proteins. It has a solubility in water around 8 wt% (Hill and Malisoff, 1926) which is well above the concentrations where it is used as a preservative which is 0.15–0.5 wt% (Rowe et al., 2006b).

Recombinantly produced human growth hormone is used in treatment for both adults and children. For children, it is used to increase growth when the child has conditions that affect normal growth and development (Hindmarsh and Dattani, 2006; Mehta and Hindmarsh, 2002). For adults, it has been used to treat HIV patients to increase body weight and physical endurance (Benedini et al., 2008), as well as to treat short bowel syndrome in adults who are receiving additional nutrition or fluids from intravenous (IV) therapy (Barahona-Garrido et al., 2009). The protein is given as a multidose presentation. There are several hGH products on the market. Most of these contain a combination of a preservative and a surface-active agent such as polysorbate 20 and 80 or poloxamer 188. The presentations also contain buffer salts and tonicity agents. The most commonly used preservatives in hGH products are phenol and metacresol. hGH is a small protein with 191 amino acid residues and a molecular weight of 22 kDa. It has an isoelectric point around pH 5 (Aloj and Edelhoch, 1972)). The structure of hGH from the Protein Data Bank (PDB) is given in Fig. 1.

Serum albumin is the most abundant protein in blood, and both human and bovine serum albumin have numerous uses in biomedical sciences (Xu et al., 2023) such as tissue engineering (Yuan et al., 2020), microspheres and nanospheres for drug delivery (Jun et al., 2011; Sokolik et al., 2018; Zhao et al., 2010), cryopreservation (Riel et al., 2011), and as surface active agent for blocking non-specific binding of other proteins to surfaces (Baldo et al., 1986). Serum albumin binds to a large range of molecules including drugs (Banerjee et al., 2017; Behera et al., 2023), fatty acids and surfactants (Banerjee et al., 2017). BSA contains 583 amino acids and have a molecular weight of 66 kDa (Riel et al., 2011). It has an isoelectric point of around 5 (Guo et al., 2016).

This study aims to investigate the (in)-compatibility of preservatives, in formulations of protein and surfactants, containing other common excipients like salts. Ion effects can be due to both screening of electrostatic forces and specific ion effects. To investigate this, the effects of salt concentration and type of ion were studied. The Hofmeister series of salts has, since the late 19th century, been used to study the specific ion effect (Hofmeister, 1888). It ranks ions based on their effect on macromolecules, primarily proteins. With chloride ions in the middle, the ions are divided into kosmotropes and chaotropes. The kosmotropes show a salting-out effect on proteins, increasing their stability, while chaotropes have a salting-in effect, increasing the protein solubility (Zhang and Cremer, 2006). Chaotropes have shown a destabilising effect on proteins which is linked to preferential binding and exclusion of salts from the protein-solution interface (Broering and Bommarius, 2005). The Hofmeister series has shown a systematic effect on several phenomena, such as the distribution of phenol into ionic liquids, (Asrami and Saien, 2018) and the clouding of surfactants (Dave and Joshi, 2018, Schott, 1984). The aim is to better understand the incompatibility seen in multidose formulations of proteins containing surfactants and preservatives (Stroppel et al., 2023).

2. Material and methods

2.1. Material

Recombinant human growth hormone (hGH) used in this study was a kind gift from Ferring Pharmaceuticals and polysorbate 80 (superrefined) was a gift from Croda (UK). BSA was supplied by Sigma Aldrich (>98 %) and was used without further purification. All other chemicals were of *Pro Analysis* grade and the water used was of Millipore grade.

Protein concentration was 10 mg/ml. The buffer used was 10 mM phosphate with a pH of 7.0 prepared with sodium phosphate monobasic and dibasic (Reag. Ph Eur, Sigma-Aldrich, US) and sodium azide (Reag. Ph Eur, Scharlab, Spain). This gave a pH of around 7.3 for the hGH solution and 7.0 for BSA. The surfactant concentration was if nothing else is stated, 0.1 wt%. The standard titrant was 1 M NaCl in 10 mM phosphate buffer.

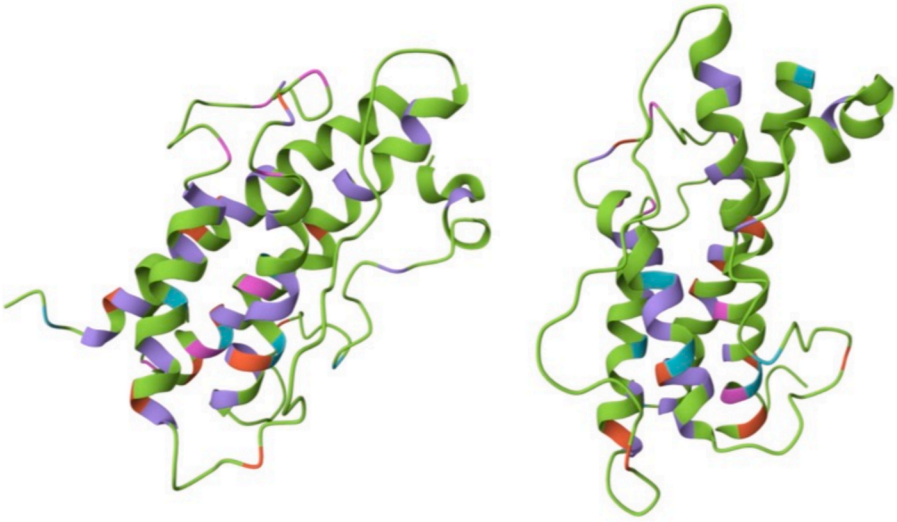

Fig. 1. Two different views of the PDB model of the hGH structure (Chantalat et al., 1995). Sites of valine residues are shown in pink, leucine in purple, isoleucine in blue, and arginine in red.

2.2. Probe drum

The studies were conducted using titration in Probe Drum equipment (Labbot, Lund Sweden) to determine the cloud point. Probe Drum allows for automatic concentration titration on a microliter scale, with temperature control, and mixing of the sample, whilst measuring laser-based light scattering at a 90° angle to the sample (red laser with the detector at 637 nm). For all experiments, two replicates were performed.

The effect of the titration volume of sodium chloride was investigated to ensure that this does not affect the results, see Fig. S1. A titration volume of 50 µl leads to an overshoot of sodium chloride, seen as a late onset of precipitation. There are small differences between 20 µl and 5 µl, but as a precaution, the selected titration volume was 5 µl, see Fig. S1.

In these experiments, the initial content of the cuvettes was 1000 µL and a total titrant volume of 240 µL was added. The measurements were conducted at a temperature of 25 °C, with intermittent stirring. The stirring was set at speed setting 4 out of 7 available for the ProbeDrum instrument. After each titration step, the sample was equilibrated for 60 s before being measured for 40 ms. Clouding/precipitation was determined based on the point where scattering started to increase. The pH of the hGH solution was 7.3, and the final pH after salt titration in the presence of phenol was 7.0. This was observed regardless of the pH of the salt solution (pH 7.3, 7.0 and 6.4 were tested). Simultaneous measurements of fluorescence were conducted using an excitation wavelength of 280 nm with a 4 s excitation time.

2.3. NMR spectroscopy

NMR experiments were performed at the NMR centre at Gothenburg University, Sweden, on a Bruker Advance III HD 800 MHz spectrometer equipped with a 5 mm TXO cold probe at 37 °C. $^{1}H–^{13}C$ SOFAST-HMQC spectra were acquired with a ^{1}H spectral width of 13 ppm using 1040 and 256 points in direct and indirect dimensions, respectively. $^{1}H–^{1}H$ TOCSY spectra were acquired with a ^{1}H spectral width of 18 ppm using 8192 and 1024 points in direct and indirect dimensions, respectively. The protein concentration was 0.35 mM (8 mg/mL). The buffered solvent contained 5 % v/v of D_2O for locking purposes and 100 µM DSS. Spectra were processed using the nmrPipe software suite (Delaglio et al., 1995).

3. Results and discussion

The effect of preservatives on aqueous solutions of polysorbate surfactants has previously been investigated, and it was shown that the incompatibility observed in these systems is due to clouding (Wahlgren et al. 2024). However, it is also known that preservatives such as phenol can induce aggregation of proteins (Arora et al., 2017; Bis and Mallela, 2014; Bis et al., 2015; Hutchings et al., 2013; Maa and Hsu, 1996; Thirumangalathu et al., 2006; Zhang et al., 2004). It is thus of interest to understand which of these phenomena dominates the incompatibility often seen in multidose protein formulations. In this work, we conduct a systematic study of the effect of phenol on protein/surfactant containing formulations for two model proteins BSA and hGH.

In Fig. 2, the effect of the addition of BSA to a polysorbate 80/phenol mixture in 10 mM phosphate buffer pH 7 with 0.15 M sodium chloride was investigated. As can be seen, the effect of the protein on the cloud point is minor or non-existent. Thus, it can be concluded that for the BSA/phenol/polysorbate 80 system, the incompatibility seen is probably triggered by the surfactant cloud point depression and not by the precipitation of the protein.

Quite surprisingly, when conducting the same investigation with hGH, there was an immediate precipitation when phenol was added to hGH and PS20 at 0.1 % polysorbate 20 in a 10 mM phosphate buffer containing 0.15 M sodium chloride, thus the incompatibility is due to protein-phenol interactions and not cloud point depression. Whereas, in the absence of salt, a system of hGH/phenol/polysorbate 20 in 10 mM phosphate buffer, pH 7.0, showed no difference between the clouding with and without protein (SI Fig. S2). Indicating that in the absence of salt, the clouding dominates the incompatibility. To better understand the observed incompatibility seen for hGH, the effect of salt and surfactant was investigated. This was done by titrating a hGH/phenol solution with sodium chloride in the presence and absence of polysorbate 20 and polysorbate 80.

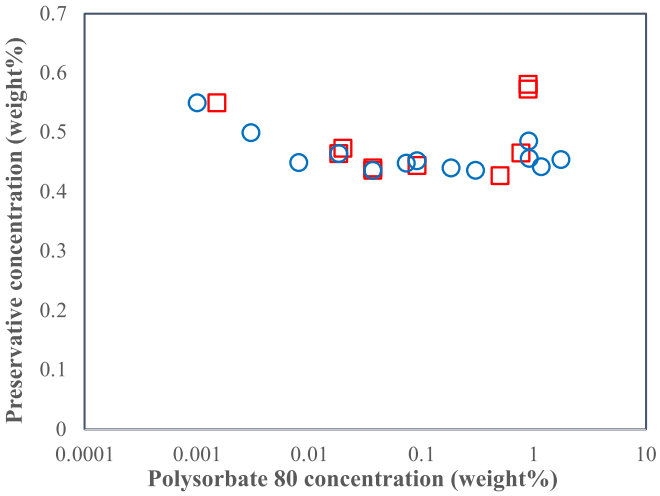

Fig. 2. Cloud point diagram of phenol, polysorbate 80, and BSA at 25 °C with 0.15 M NaCl and 10 mM phosphate buffer pH 7.0 (red squares), and without BSA (blue circles).

3.1. Salt-induced precipitation of hGH and phenol

Initially, the degree of mixing order when preparing the protein-phenol solution was investigated to ensure that this does not affect the interpretation of the results, see Fig. 3. As can be seen, the degree of scattering is affected by the mixing order. Although the concentration when sodium chloride induces scattering is the same, the scattering is substantially higher when phenol is added to a protein solution than vice versa. The degree of scattering can be affected both by the size and the number of scattering particles and the technique does not allow for differentiation between these. One reason for the larger scattering

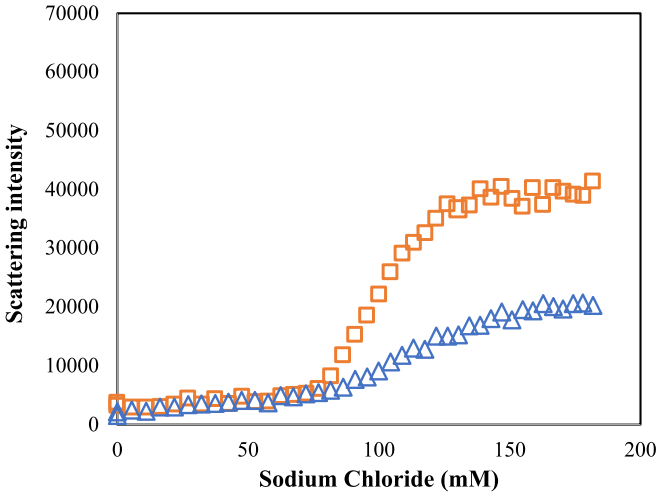

Fig. 3. Effect of the mixing order for 10 mg/ml hGH and phenol 0.36 % titrated with 1 M NaCl in 10 mM sodium phosphate in 5 µl addition steps. Mixing order; phenol added to protein (orange squares) or protein added to phenol (blue triangles).

observed when phenol is added to the protein solution could be that the addition of phenol stock solution gives a brief, locally high phenol concentration before the system is fully mixed. This could lead to local hGH-phenol interactions that can function as nucleation points for further precipitation when sodium chloride is added. As shown in Fig. 4 the degree of scattering upon sodium chloride addition is strongly affected by the concentration of phenol. The effect of the mixing order suggests that interactions between locally high phenol concentrations and hGH are not fully reversible within the experiment's time frame. It also indicates that phenol interacts with hGH before precipitation is seen, but a specific sodium chloride concentration is required for detectable precipitation to be observed. To eliminate the effect of mixing order between experiments, the protein was consistently added to the phenol solution in all experiments.

To further investigate the effect of sodium chloride on hGH precipitation in the presence of phenol, four phenol concentrations were studied (0.36, 0.44, 0.68 and 1.0 wt%), see Fig. 4. As can be seen, the scattering increases around the same concentration of sodium chloride but the degree of scattering is much higher at 1 wt% phenol than for the lower phenol concentrations. The solutions at 1 wt% phenol also have visible particles at the end of the titration, as seen by the photographs in Fig. 4, indicating that the aggregates formed are larger than for the lower concentrations of phenol. This is, to some extent, in line with what was seen for the mixing order of protein and phenol. High phenol concentrations seem to increase scattering intensity, indicating a rise in either the quantity or size of protein aggregates. However, the concentration at which sodium chloride induces precipitation, as shown by increased scattering, is not lowered by higher phenol concentrations. The precipitation could be due either to solid–liquid phase separation or aggregation triggered by irreversible changes in structure of the protein (Raut and Kalonia, 2016). Visual inspection of how the system separates especially when large flakes are seen in the cuvette makes it highly unlikely that it is a solution-solution phase separation. However, it has not been possible to separate solid −liquid phase separation from aggregation in this system.

A change in the pH of the solution was also observed upon titration. Although both the sodium chloride solution and the hGH solution had a pH of 7.3 ± 0.04 when starting the titration, the final pH was 7.0 ± 0.05 (n = 10), probably due to a salt effect on the buffer system (Robinson, 1929). The starting point of 7.3 was chosen to end the titration at 7.0 a pH where we know that the protein in itself does not precipitate in at 0.15 M NaCl and 10 mM phosphate buffer.

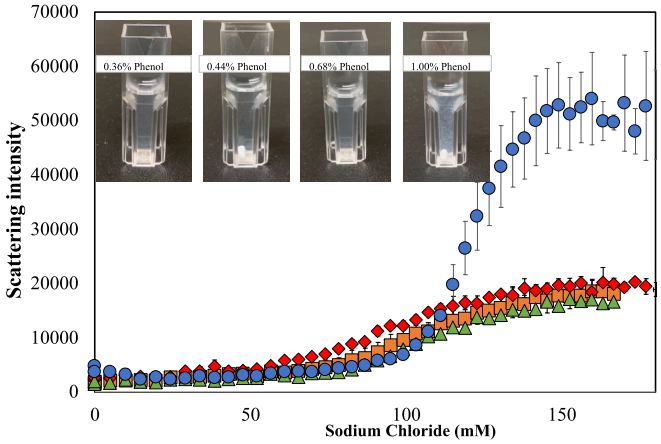

Fig. 4. 10 mg/ml hGH and phenol of varying concentration titrated with 1 M NaCl in 10 mM sodium phosphate in 5 µl addition steps. The curves show phenol concentrations of 036 wt%, (orange squares), 0.44 wt% (red diamonds), 0.68 wt% (green triangles) and 1 wt% (blue circles). The data show 90° scattering at 637 nm. The insert shows photographs of the cuvettes after the final addition of NaCl.

To further investigate the effect of phenol on the structure of hGH, 2D HMQC NMR measurements were performed for hGH with and without phenol, see Fig. 5. As can be seen in the figure, no major change occurs in the overall spectra. This shows that the higher-order structure of hGH in the presence of phenol is intact. If phenol induced a more general effect on hGH, such as unfolding or aggregation, all peaks in the spectra would shift. There are, however, chemical shift changes for specific amino acids as can be seen for the circled regions of the spectra in Fig. 5. Thus, phenol likely binds to one or more distinct sites on hGH given that a select number of peaks are shifted by the addition of phenol. In comparison, the structure of hGH does not change at all in the presence of 0.1 wt% polysorbate 80 or 0.15 M sodium chloride (see Fig. S3). This binding could potentially lead to local changes in structure that could induce aggregation when the colloidal stability is decreased due to addition of salt. An alternative explanation could be that binding does not change the structure but could lead to phase separation of the protein for example due to increased hydrophobicity or anionic or cationic PI interactions.

The type of amino acid, for some of the isolated peaks in the 2D HMQC spectra in Fig. 5, can be identified using a TOCSY spectrum, by comparing observed chemical shifts with tabulated values for the various types of amino acids that contain methyl groups. The identified residues are two leucine's, one isoleucine and one valine, however there are clear shifts for other methyl groups which cannot be easily identified using the TOCSY spectrum to spectral overlap. This suggests that these residues are in a well-formed secondary and tertiary structure environment, and not in a loop region. There are at least three surface-accessible valine residues in hGH and there are leucine residues in the vicinity of these, while isoleucine residues are slightly more buried. Residues close to the binding site(s) of phenol is likely to have a large chemical shift change, while residues further away from the binding site(s) are likely to have smaller shifts. These results are in line with previous observations, by Maa et al, that the presence of phenol reduces the thermal stability of hGH [10]. There is, in at least one of these combinations of amino acids with a proximity to arginine and thus one possible interaction for the phenol could be the formation of a cation-pi bond with arginine. In other projects, we have seen that positively charged proteins have been especially sensitive to phenol.

3.2. *Effect of type of salt on precipitation*

The effect of sodium chloride on the precipitation could either be a specific ion effect, or purely due to a decrease in electrostatic repulsion when the ionic strength of the system increases. To investigate this, three salts from different parts of the Hofmeister series were investigated (Fig. 6).

Sodium chloride is in the middle of the Hofmeister series, while sodium nitrate is a salting-in salt and sodium sulphate a salting-out salt. The results follow the Hofmeister series with a ranking of the salt concentrations triggering precipitation according to $SO_2^{-2} < Cl^- < NO_3^-$. As can be seen, the effect of ionic strength is not consistent between the salts. Thus, the observed effects are not only related to the change in ionic strength. Before the onset of substantial precipitation, the scattering intensity curves seem to fall on top of each other showing a slight increase in scattering with the addition of salt. This could be due to an increase in electrostatic shielding. However, both sodium sulphate and sodium chloride show an abrupt increase in scattering at a specific salt concentration. This is not the case for sodium nitrate, for which a gradual increase in scattering is observed, in the investigated concentration range.

All investigated salts are dissociating salts. However, the conclusions can be highly relevant also for buffer system selection, as salts are important as buffer components. However, the buffer concentrations used in the formulation of protein drugs are generally lower than the salt concentrations needed to trigger precipitation in this study. All the same, it could be prudent to use buffer salts that are not too far on the

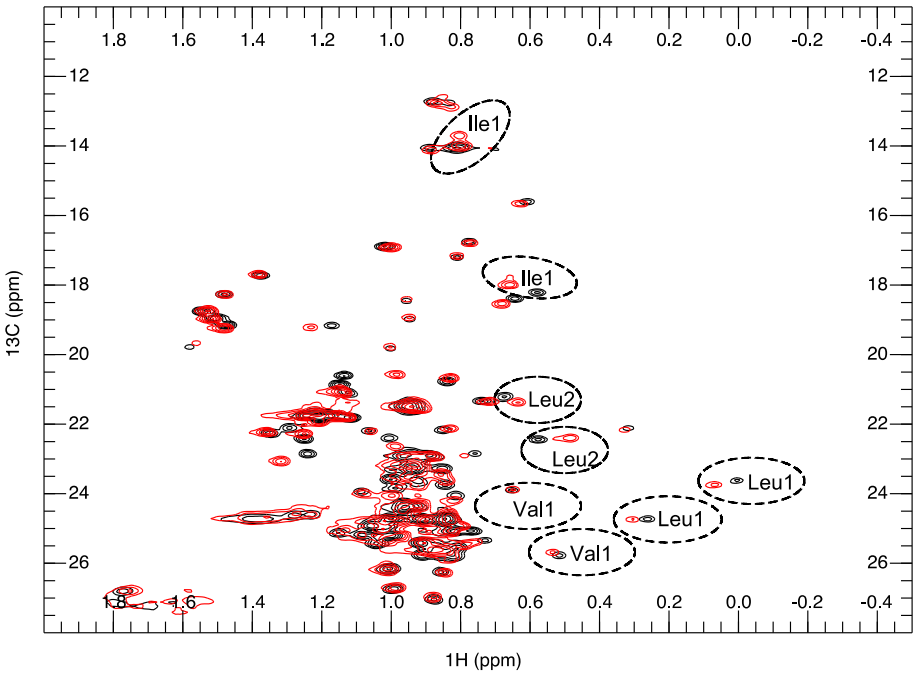

Fig. 5. 2D HMQC NMR spectra of hGH (black) and hGH and 0.68 wt% phenol in 10 mM phosphate buffer (red).

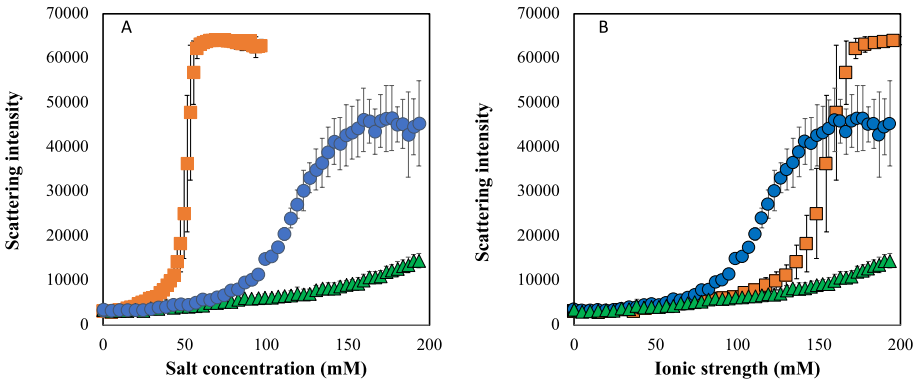

Fig. 6. 10 mg/ml hGH in phosphate buffer and 1 wt% phenol titrated with 1 M NaCl (blue circles), 1 M $NaNO_3$ (green triangles) and 0.5 M Na_2SO_4 (orange squares). The left panel (A) displays scattering as a function of salt concentration and the right panel (B) as a function of ionic strength. The titration was done in steps of 5 µl. The data show 90° scattering at 637 nm.

salting-out side of the Hofmeister series when formulating proteins containing phenolic preservatives, especially if high buffer concentrations are required.

Moreover, sodium chloride is a common tonicity agent and normal concentrations used, i.e., around 0.15 M, will lead to precipitation. To understand if similar effects are observed with other tonicifiers, the effect on hGH precipitation by common tonicity agents such as mannitol and glucose was investigated. In this case, hGH in the presence of the highest concentration of phenol, 1 wt%, was titrated with the tonicifiers but no precipitation was detected (SI Fig. S4). This indicates that the precipitation observed for the salts can be avoided if other tonicifiers are used. However, one should be aware that while no effect is seen in a system containing hGH and phenol, most formulations also contain surfactants. In previous studies, we have shown that polysorbate surfactants cloud in the presence of phenolic preservatives but that there is a region where the preservatives will not cloud and that this region, to some extent, encompasses the region where the preservatives are active (Wahlgren et al. 2024). In this case, both the addition of salt and polyols, such as mannitol, decreases the cloud point and thereby the tonicifier/phenol/surfactant concentration ranges possible to use.

3.3. *Effect of type of nonionic surfactant on precipitation*

Mixtures of phenol and the surfactant dodecyl maltoside (DDM) do not precipitate at phenol concentrations where clouding of equivalent mixtures with polysorbate occurs. The effect of different surfactants in a hGH/phenol system with gradually increasing sodium chloride concentration was investigated. The effect of polysorbate 20, polysorbate 80, or DDM in hGH and phenol containing solutions, when titrated with sodium chloride is shown in Fig. 7.

The onset of scattering for polysorbate 20 and 80 is similar. This is in line with previous results for these surfactants, where the clouding due to the presence of phenolic preservatives was observed at similar preservative and surfactant concentrations. In these experiments, however, the polysorbate concentration is below the concentration where clouding would be expected. Compared to hGH/phenol without surfactant, the salt concentration needed to trigger scattering is slightly higher with polysorbate. Thus, it could be expected that the precipitation observed in the hGH/phenol/polysorbate system is due to hGH and that the additional presence of surfactants lowers the concentration of free phenol in the solution and thus increases the onset of precipitation.

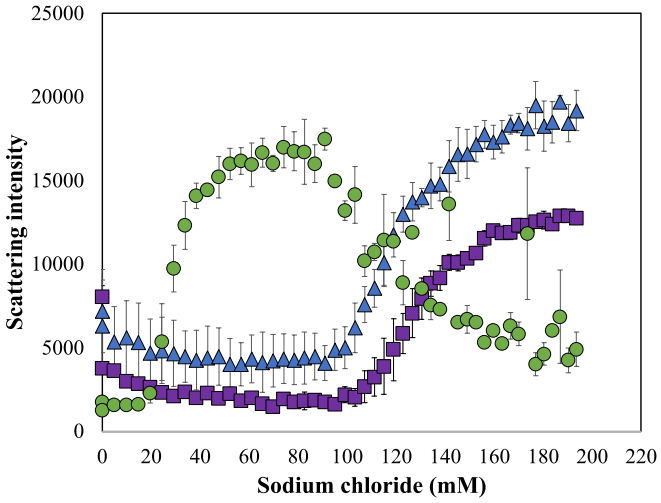

Fig. 7. Titration of 10 mg/ml hGH, 0.44 wt% phenol and different surfactants at a concentration of 1 mg/ml in 10 mM sodium phosphate, with 1 M NaCl. The surfactants are polysorbate 20 (purple squares), polysorbate 80 (blue triangles) and β-DDM (green circles). The titration was done as steps of 5 μl. The data show 90° scattering at 637 nm.

The presence of DDM triggers precipitation at a much lower sodium chloride concentration than the presence of polysorbates. This is quite surprising since DDM has not been shown to interact with the protein alone, and DDM does not have a cloud point that is decreased by phenol. To investigate if the observed precipitation is specific for hGH, a BSA/phenol/DDM solution was titrated with salt. No precipitation was observed in the interval investigated (SI Fig. S5).

We have previously seen that in mixed micelle systems, DDM interacts with hGH in the presence of SDS, but this is primarily attributed to mixed surfactant aggregates interacting with the protein. It is, however, possible that due to the conformational changes induced by the specific binding of phenol, DDM can interact with hGH. This would be in line with previous observations that phenolic preservatives perturb the structure of proteins (Bis et al., 2015; Hutchings et al., 2013; Thirumangalathu et al., 2006; Zhang et al., 2004). Furthermore, it has been shown that phenol is adsorbed in the palisade layer of maltoside surfactants, which results in changes in the morphology of the micelles, forming wormlike micelles (Lu and Somasundaran, 2007).

It is also known that properties such as micelle structure can differ for the isomers of maltoside surfactants (Dupuy et al., 1997; Larsson et al., 2021; Larsson et al., 2019). To further investigate the effect of DDM on precipitation, the α– and β– isomers of the surfactant were investigated, see Fig. 8. As can be seen, the difference in scattering onset between the α– and β-isomers is larger than the difference between polysorbate 20 and 80. In the presence of α-DDM, the precipitation occurs at much higher sodium chloride concentrations than in the presence of the β-isomer. In the phenol concentration range investigated, the critical sodium chloride concentration, that triggers precipitation, is linearly dependent on phenol concentration. This is not the case for the protein system without surfactants. Thus, it is likely that micelle interactions play a part in the induced precipitation.

In summary, this paper shows how complex the interplay of excipients can be, even for protein formulations that may seem simple at first glance, containing only preservatives, surfactants, and some type of buffer or salt. It is important to keep in mind how individual components, in these colloidal systems, can interact with each other to destabilise the system. In previous work, we have shown that precipitation can be due to the clouding of surfactants. However, as shown here, for some protein and excipient combinations, preservative-induced precipitation of protein is observed instead. The phenomenon that comes to dominate depends on protein properties, and how these in turn change in the presence of excipients like preservatives, salt, and surfactants.

4. Conclusions

In this work, we have shown the complex behaviour of protein formulations containing surfactants and the preservative phenol. The results show that for some proteins, like BSA, the incompatibility with phenol is controlled by the cloud point depression of polysorbates and that changes due to excipients are primarily related to their effect on cloud point depression. For hGH, on the other hand, the incompatibility in the presence of phenol shifts from being a result of cloud point depression to being triggered by protein precipitation when salt concentration increases. Furthermore, even though DDM and hGH are generally thought not to interact, precipitation was observed for the phenol/hGH/DDM systems at a 25 mM sodium chloride concentration, which is significantly lower than the concentration required for salt trigged precipitation in the polysorbate systems.

The type of salt has also been observed to strongly affect the onset of hGH/phenol precipitation, while polyols do not induce precipitation. Thus, using polyol as an osmolality giver will increase the range where the hGH/phenol system is stable. However, it will have a similar effect as salt on the cloud point depression of polysorbates. Thus, the benefit of changing tonicity agent will depend on what effect dominates, surfactant clouding or protein-preservative precipitation.

These findings can be used to better understand how to design protein multidose formulations containing preservatives. Further, the

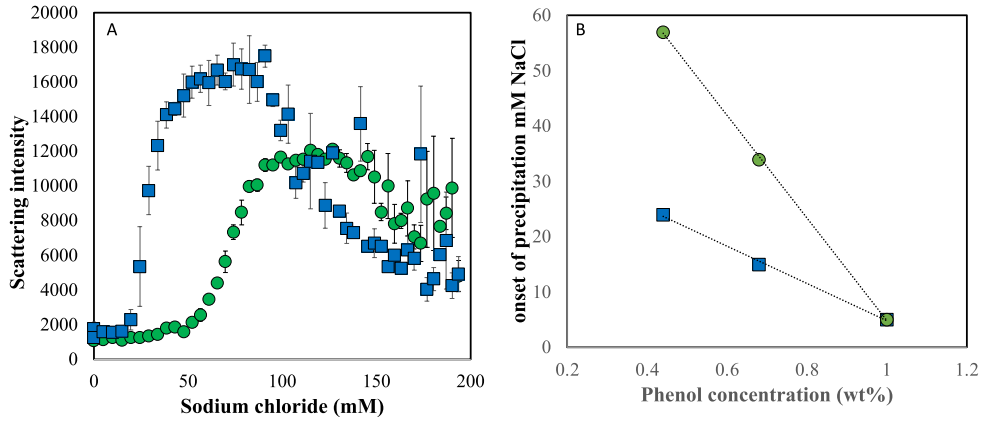

Fig. 8. The left panel (A) displays the effect of surfactant type on the precipitation of hGH, β-DDM (green circles) and α-DDM (blue squares), titration of 10 mg/ml hGH, 0.44 wt% phenol and different surfactants with 1 M NaCl. The right panel (B) displays the NaCl concentrations for the onset of scattering for titrations at three different phenol concentrations. The titrations were done in steps of 5 μl. The data show 90° scattering at 637 nm.

findings also illustrate how complex the compatibility between proteins, preservatives and surfactants can be.

CRediT authorship contribution statement

Johanna Hjalte: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Anna-Maria Börjesdotter:** Investigation. **Carl Diehl:** Writing – review & editing, Methodology, Investigation, Conceptualization. **Stefan Ulvenlund:** Writing – review & editing, Conceptualization. **Marie Wahlgren:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Conceptualization. **Helen Sjögren:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpharm.2025.125624.

Data availability

Data will be made available on request.

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