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Phenol-Protein Solution Stability

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1 Introduction

Formulation of intravenous and parenteral protein solution is increasingly common in the pharmaceutical industry as proteins are an increasing percentage of the medical arsenal. As the vast majority of proteins cannot be administered orally, they have to be produced as solutions which are more sensitive than their solid counterpart. Solutions necessitate the addition of preservatives to inhibit microbiological growth. Determining how preservatives affects a protein solution allows for more optimized designs that minimize waste and costs while improving shelf life. This work aims to determine what concentration of phenol is reasonable for a specific protein solution and aims to characterize the interactions between the protein and phenol and the surfactant polysorbate 80.

2 Aim

The aim of this project is to study the effects of phenol on various therapeutic protein solutions. Through determining the concentration of phenol that turns a protein solution turbid further understanding of a frequently used pharmaceutical preservative can be achieved. As salt is common in protein solutions all tests will be done for both a solution with salt and without to investigate what effect salt has on the protein solutions. In addition to this various other tests to investigate specific characteristics of a protein have been conducted.

3 Theory

Proteins are macromolecules consisting of at least 100 amino acids that are derived from mammalian sources, bacteria or yeast. As a group they are very target specific and versatile, but they can also be very sensitive to heat, pH and other disruptions. Their specificity is a major advantage for pharmaceuticals as that allows for specific targeting with a minimized amount of side-effects. As a type of biomolecule, the human body can break them down easily which is both an advantage and a disadvantage. Being easy for the body to break down means that they have minimized side effects, but it also means that most protein products have to be parenteral to avoid the digestive system. Transport of protein-based drugs requires more care than oral medications as they commonly are produced as solutions that require refrigeration. Being liquid, they also typically have short shelf lives compared to solids. As protein-based drugs are a rising percentage of drugs this research will minimize waste and make such medications more economically viable.

The future of pharmaceuticals is increasingly protein based as they allow for a specificity that is difficult to replicate with smaller molecules. Proteins also have the advantage of not needing synthetic chemistry to create as they can be grown instead. This minimizes the use of chemicals and avoids issues with yield from repeated synthesis.

4 Solution Stability

4.1 Protein instability

Protein solutions are vulnerable to numerous different types of degradation as they are complex molecules in a liquid environment with several excipients. As all solutions in this project were titrated with phenol until turbidity occurred aggregation from chemical degradation and unfolding are the two likeliest degradation pathways [10]. Phenol can cause both soluble and insoluble aggregates to form [7] and is incompatible with some proteins (albumin, gelatin [8]). Aggregates and incompatibilities will be caught by routine testing but could present problems during development. Temperature and pH can also cause similar degradation of the solution, but aggregation caused by phenol is the focus in this project.

Misfolded proteins can cause serious side effects or at least render a solution useless and must therefore be screened for and avoided. While some aggregates can be soluble, they are frequently insoluble, and the presence of aggregates is likely to mean that there is a problem with the solution beyond the formation of said aggregates. Therefore, careful design is required to produce a solution that can withstand transport and storage.

4.2 Cloud point

As the surfactant chosen for this project is a nonionic surfactant the cloud point of the solution is highly relevant. Above a certain temperature the surfactant separates from the rest of the solution resulting in a cloudy solution instead of a transparent one. While temperature changes haven't been tested the cloud point is sensitive to the addition of other component meaning that with the addition of enough phenol the cloud

point will be lower than room temperature. As the added components interfere with the interaction between protein and surfactant the critical micelle concentration changes [11] as well as the micelle size and phase behavior. This means that at a certain phenol concentration some of the components will start leaving the solution. This is where turbidity develops and where the titration curve shows a break point.

5 Proteins

The proteins chosen for this project and their basic physical characteristics are shown in the table below. β-lactoglobulin and Lysozyme were purchased from Sigma-Aldrich. GA-Z and Sobi Lipase were kindly gifted by SOBI. Trastuzumab was a kind gift from Bernt Nilsson at LTH.

Table 1 Isoelectric point, molar weight and molar extinction coefficient for the included proteins

Protein	**Isoelectric point**	**Molar weight (kDa)**	**Molar extinction coefficient**
β-lactoglobulin	5	18	17600
Lysozyme	11	14.3	38940
Trastuzumab	8.7	148	317400
GA-Z	4.5	13.58	13580
Palifermin	9.47	16.193	22900
Sobi Lipase	5	84.1	101300

The proteins selected are intended to be a representative list of proteins that cover most extremes in size, characteristics and isoelectric point. Lysozyme and β-lactoglobulin are chosen because they are ubiquitous and extensively studied. Palifermin, Trastuzumab, GA-Z and Sobi Lipase are pharmaceutical proteins or attempts at designing such and they are therefore fitting for a pharmaceutical project.

5.1 Molar Extinction Coefficient

A molar extinction coefficient was either found in literature (β-lactoglobulin, Lysozyme) or calculated by determining the absorbance of the protein solution at different concentrations and then plotting the molar concentration against the absorbance which gives the molar extinction coefficient as the angle of the resulting line. The accuracy of this method seems to be sufficient as the resulting coefficient gave an accurate concentration for the stock solutions with a known concentration.

5.2 β-lactoglobulin

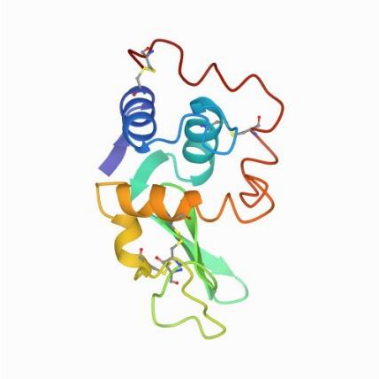


Figure 1 β-lactoglobulin

β-lactoglobulin is the main whey protein in cow and sheep's milk. It has the ability to bind fatty acids which means it can potentially interact with the surfactant PS80. It also exists as both a monomer and a dimer [1] depending on pH but at pH 7 the dimer is the main species.

The structure of β-lactoglobulin consists of 162 residues folded into a β-barrel consisting of 8 strands with a 3-turn α-helix on the outside with a ninth β-strand next to the first strand. It is a spherical protein of about 3.6 nm in diameter.[1]

5.3 Lysozyme

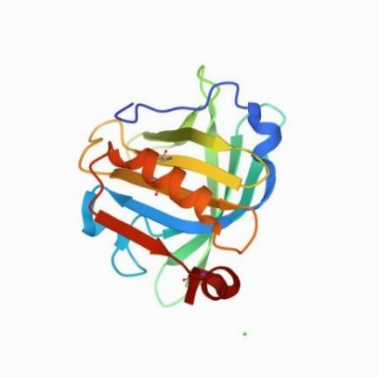


Figure 2 Lysozyme

Lysozyme is an enzyme with low molecular weight that is commonly derived from egg whites. It contains five β-sheet regions and five helical α-helical regions that are linked by β-turns and random coils It is a small single chain protein consisting of 129 residues that has antibacterial properties.[2] It takes the shape of a prolate ellipsoid with a maximum length of 9 nm and a diameter of 1.8 nm.

5.4 Trastuzumab

Trastuzumab is a large monoclonal antibody that has a diameter of 13-14 nm. [3]. It is used to treat her2 (Human epidermal growth factor) positive breast cancer and is by a factor of almost two the heaviest protein included in this project.

5.5 GA-Z

GA-Z is a small pharmaceutical protein that is predominantly dimeric at pH 7 and has a R_h of 2.8 nm [4]. It has a tendency to chemically degrade in aqueous solutions but that is acceptable in this project as degradation is the main focus.

5.6 Palifermin

Figure 3 Palifermin [5]

Palifermin is a recombinant keratinocyte growth factor used to counteract inflammation in patients receiving treatment for leukemia and lymphoma. It is a globular protein with a 1.55 nm radius of gyration.

5.7 Sobi Lipase

A glycosylated version of Human Bile-salt-stimulated Lipase, or hBSSL. hBSSL is a digestive enzyme secreted from the pancreas and is the second biggest protein at 84.1 kDa. The glycosylation is intended to raise stability while not affecting activity. As a lipase, it is expected that it interacts strongly with the surfactant PS80.

6 Excipients

6.1 Buffer solution

A 10 mM phosphate buffer at pH 7 was used. It consisted of sodium dihydrogenphosphate and disodium hydrogenphosphate. Sodium Azide (0.01 wt%) was added as a preservative and one of the two buffer solutions had 150 mM Sodium Chloride added to it. Afterwards the pH was checked with a pH-meter and acid or base was added to achieve a pH of 7.0. All other solutions (Phenol, proteins and surfactant) were made with this buffer. A second batch of buffer solution was made during the end of the project.

At any time this report refers to a salted version of a solution it refers to the use of the buffer solution with 150 mM Sodium Chloride.

6.2 Phenol

Phenol is an aromatic compound that is one of the most common preservatives [7] for parenteral solutions. As a preservative it has the advantage of being stable in an aqueous solution and potent enough that side effects are unlikely due to the low amount of phenol required (1% w/v) [8] to achieve a bacteriostatic solution.

Phenol has a tendency to cause aggregation in protein solutions [8] which may cause issues depending on how well the protein and surfactant used handle the presence of phenol. Some proteins and polypeptides are incompatible with phenol such as albumin and gelatin.

6.3 Phenol Solution

Two 3 wt% phenol solutions were used as the titrant in the ProbeDrum titrations. One was made with the salt free buffer and the other with the 150 mM NaCl buffer solution. These were sonicated for 30 minutes at 7 days intervals (minimum) to keep the titrant bubble free. As the titration volume is quite small at 2 μl per injection a bubble could reduce the injected volume by a significant percentage which would result in reduced accuracy of the titration program. The three percent concentration was chosen due to the low solubility of phenol in water but also due to the low amount of phenol required (and permitted) for a pharmaceutical product.

6.4 PS80

Polysorbate 80 or PS80 is the most commonly used non-ionic surfactant for protein-based pharmaceuticals. [8] It is an ethoxylated sorbitan monooleate with the advantages of having a low toxicity, high biocompatibility as well as a small critical micelle concentration (CMC) at 0.0017 wt% [8]. Potential by-

products can form due to hydrolysis or auto-oxidation of the polyoxyethylene side chain meaning that using the lowest concentration possible is advantageous as it minimizes the potential side-effects [8].

A standard starting concentration of 0.1 wt% PS80 was used for all titrations. The standards of the U.S Food and Drug Administration allow up to 0.5 wt% for intravenous injections and 0.4% for intramuscular injections which these solutions are well below while still being well above the CMC of the surfactant

7 Instruments and Methods

7.1 ProbeDrum

The ProbeDrum (PD) is a titration and spectrophotometry machine that titrates according to a set program while measuring the absorbance and fluorescence of the solution. Mixing is done with a magnetic stir bar and the temperature of the cuvette is controlled.

The PD was set to measure absorbance and fluorescence in three different ways but the main one for this project is a red laser at 636 nm. The laser gives the graphs presented in this thesis while the other two are largely not used as the behavior of the graphs don't give any relevant information about the system. The laser however gives a measure of when the solution turns turbid that is more accurate than a visual inspection. The decision of what value means that the solution is turbid is somewhat arbitrary but visual inspections after titration programs shows that the number selected ($35*10^3$) is at least close to when turbidity occurs.

7.2 NanoDrop

As protein powders can be highly hygroscopic a more exact method of determining the concentration of a protein solution is required. Lysozyme and β-lactoglobulin were used in powdered form and determining the concentration by weight was shown to be insufficient. The other four proteins started out in solutions of a known concentration but mixed with a different buffer, necessitating a buffer exchange. As this process is not perfect the concentration of the protein cannot be calculated with acceptable precision.

Every protein solution was tested with the NanoDrop that uses spectroscopy to determine the absorption of light at 280 nm. Via the absorption, the molar weight of the protein and the molar extinction coefficient a concentration can be calculated using Beer Lamberts Law.

$$c = \frac{A}{\varepsilon * l}$$

Equation 1 Beer Lamberts Law

Where c is the concentration of proteins in mg/ml, A is the absorbance at 280 nm, ε is the molar extinction coefficient and l is the path length in cm.

8 Experimental Setup

The titrations were carried out using the ProbeDrum. A standard titration run consists of preparing the sample by mixing a 900 µl cuvette and preparing a buffer cuvette for zeroing the spectrometer. All titrations were done at 25 °C. The sample cuvette consists of enough protein solution to reach 1 wt% protein, 0.1 wt% PS80 and buffer to reach 900 µl total volume. The titration results in a lowered concentration of both protein and PS80 but the total added amount was less than 200 µl in nearly every run meaning that the effect on the concentration is minimized. The same titrations were carried out with both buffer solutions but with different programs to account for the varying breakpoints. To determine roughly where the breakpoint is located a faster titration program was carried out for all protein solutions before doing the slower program. The faster titration program consisted of 5µl injections every 30 seconds compared to the slower program of 2 µl every three minutes. All proteins with the one exception of Sobi Lipase have had two runs done with breakpoints that fall within 5 µl. If the results were not within the same 5 µl window another run was done. Sobi Lipase is an outlier in that it is several times more resistant to phenol compared to any other protein included in this project and therefore the program was changed to 2 µl every 30 seconds to avoid 8 hour titration programs.

After determining where the breakpoint is located an addition program was run to add phenol to about 50 µl before the breakpoint. The addition program consisted of 5 µl injections every minute. Manual addition was tried but quickly abandoned as it creates a concentration gradient that can cause turbidity before titration. Other tests were carried out with no addition program but at a faster rate of 4 µl per minute divided into additions of 2 µl every 30 seconds. Adding the phenol solution faster gave lower breakpoints as the system didn't have time to adapt. Insufficient stirring resulting in a concentration gradient of phenol in the top of the cuvette is also possible.

The effect of the rate of addition was tested early in the project and 2µl every 3 minutes was found to be a safe rate that didn't negatively impact the test without being slow to the point of impracticality. 2 µl every 4 minutes was tested but found to not impact the breakpoint of the protein solution any more than every 3 minutes did.

The breakpoint of a solution is defined as the amount of phenol solution required to turn the solution turbid. Turbidity in the ProbeDrum is determined to be at 35000 intensity and above. This usually comes after a sudden sharp increase in intensity and the start of the increase is chosen as the breakpoint. As there are some exceptions to this behavior a set number is required as all titrations do not have a clear breakpoint as defined above.

9 Results

Table 2 below shows the breakpoints in µl phenol solution, weight percentage and mM. As PS80 is included in all solutions it is presented first as a comparison to the other results. The titration curve starts where the main titration was started i.e., after the addition program was run to add the starting amount of phenol. Figure 4 and 5 shows the titration graphs for both the salted and non-salted solutions.

Table 2 Breakpoints for proteins and surfactant with and without salt. Compare to a maximum allowed concentration of 0.5 wt% Phenol allowed by the FDA.

Surfactant	**Breakpoint (µl)**	**Wt%**	**mM**	$\frac{m_{Phenol}}{m_{Surfactant}}$
PS80	130	0.39	40.14	4.46
PS80 with salt	115	0.32	33.86	3.60
Protein	**Breakpoint (µl)**	**Wt%**	**mM**	$\frac{m_{Phenol}}{m_{Protein}}$
Lysozyme	160	0.45	42.35	0.49
Lysozyme with salt	140	0.40	37.64	0.44
β-lactoglobulin	180	0.50	53	0.6
β-lactoglobulin with salt	180	0.50	53	0.6
GA-Z	130	0.39	40.14	0.45
GA-Z with salt	118	0.35	36.86	0.40
Trastuzumab	176	0.49	52.05	0.58
Trastuzumab with salt	180	0.54	57.81	0.65
Palifermin	135	0.39	41.47	0.45
Palifermin with salt	115	0.34	36.03	0.38
Sobi Lipase	1100	1.65	175	1.83
Sobi Lipase with salt	1000	1.57	167	1.66

9.1 Protein Categories

The proteins can be divided in two main categories:

1. The proteins that don't raise the breakpoint compared to only PS80
2. The proteins that do raise the breakpoint

Both versions of Lysozyme, GA-Z and Palifermin solutions belong to category one. Their breakpoints almost entirely overlap with the breakpoint for just PS80 which means that they do not affect the stability of the solution much.

Category two is populated by β-lactoglobulin, Trastuzumab and Sobi Lipase. The reasons for this increase in stability likely varies between the three proteins as their curves and breakpoints don't overlap much, when considering that Sobi Lipase breaks at a concentration several times higher than any other protein solution.

Below are the titration graphs (fig 4 and 5) that show the titrations for all proteins except Sobi Lipase.

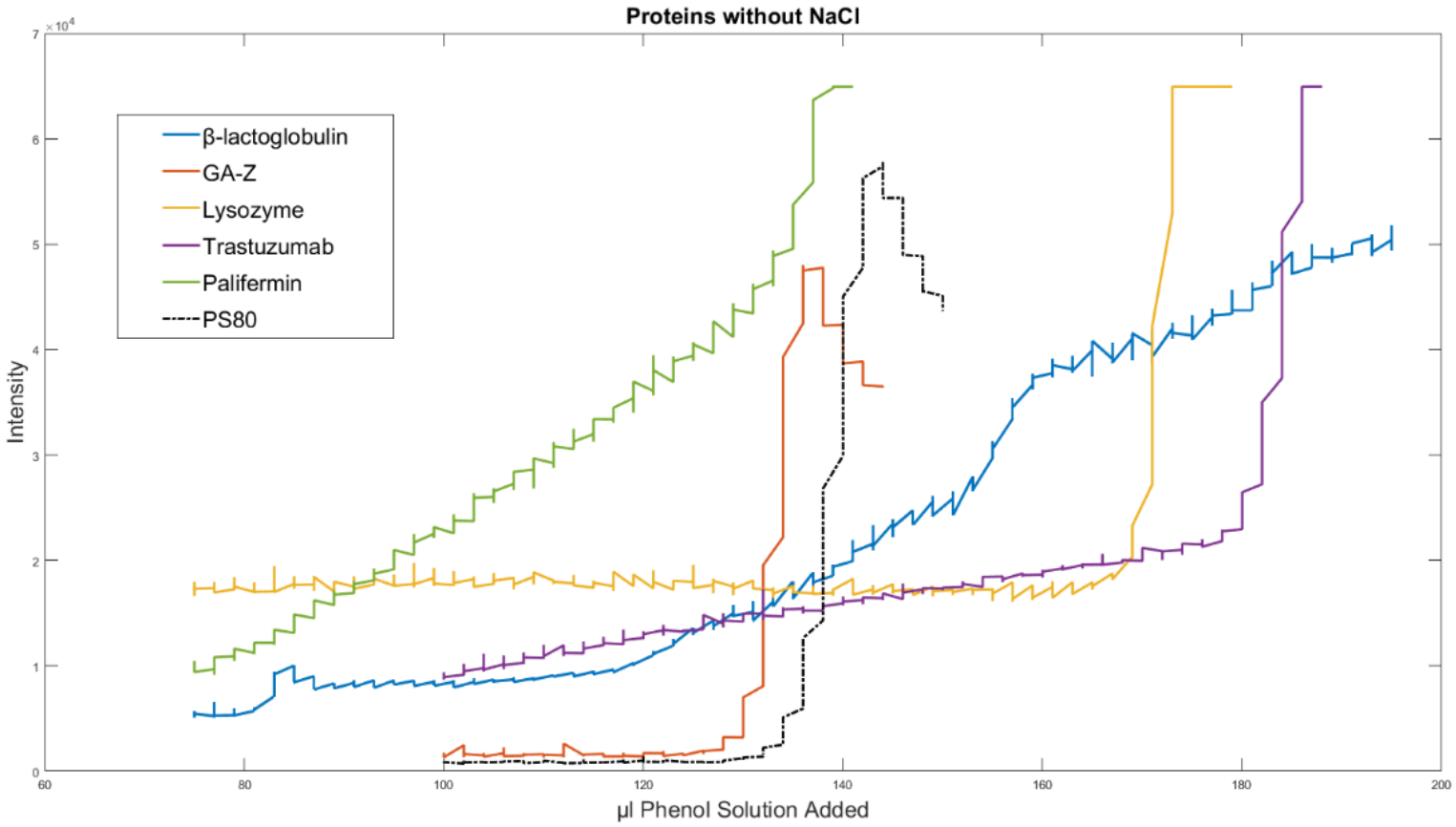


Figure 4 ProbeDrum Titrations for proteins without salt

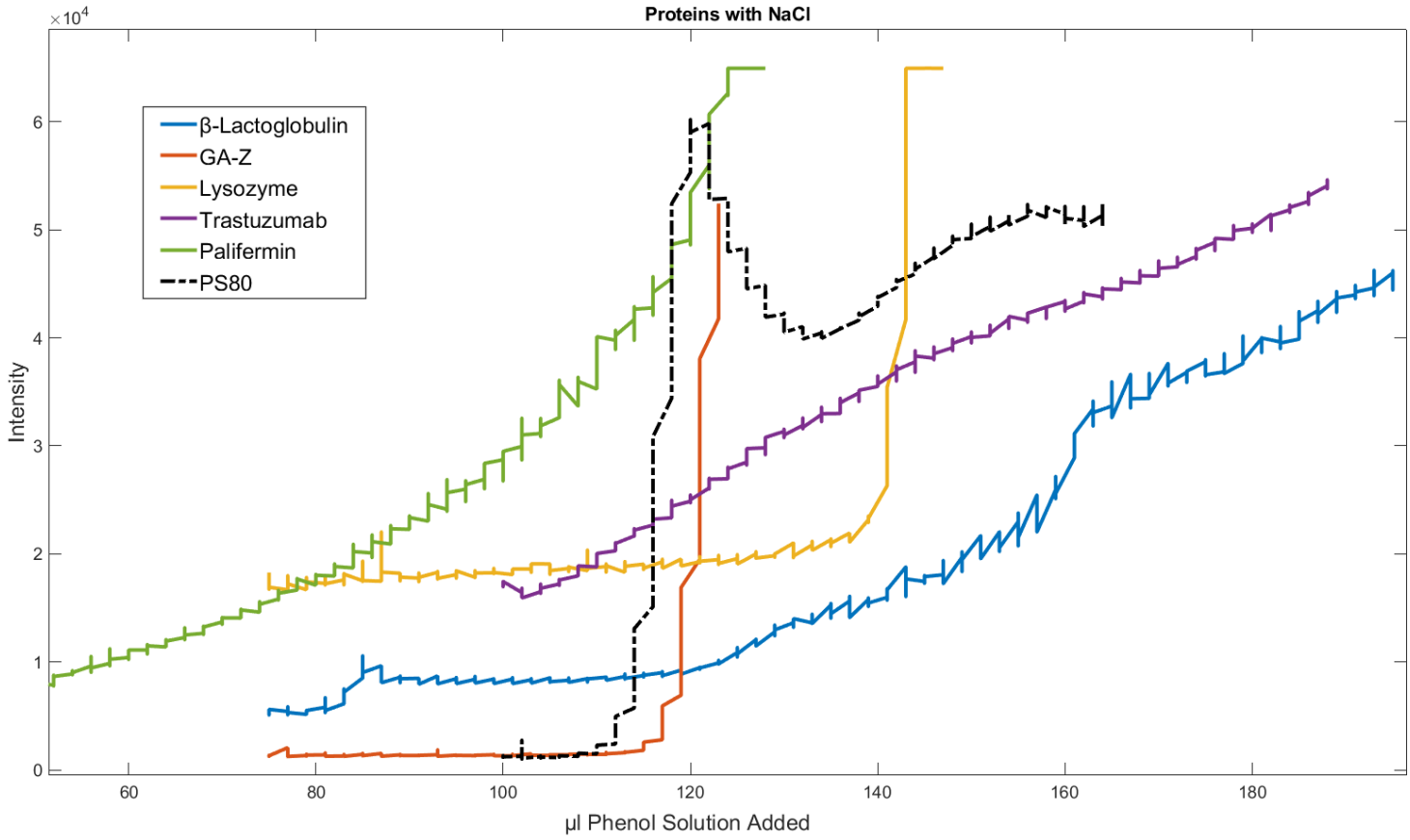


Figure 5 ProbeDrum titrations for proteins with salt

Some trends can be observed, such as that all protein solutions with one exception (TRZ with salt) all breakpoints are higher in µl phenol solution for the solutions without salt. This is likely due to the salt interfering with the stability of the Protein-PS80 system by lowering the cloud point for the surfactant resulting in turbidity earlier in the titration program.

9.2 Polysorbate 80

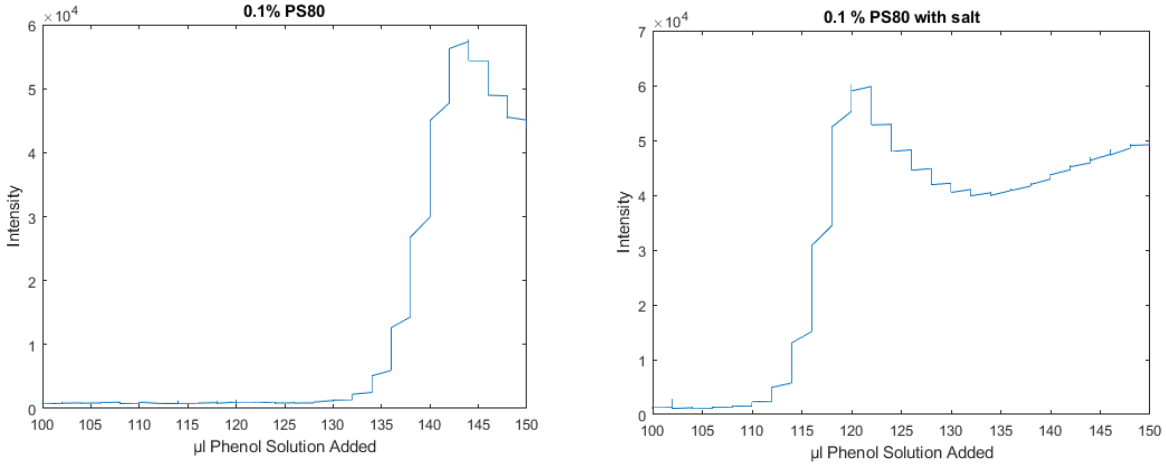


Figure 6 Titration graphs for PS80 with and without salt

Since Polysorbate 80 is present in all tests and turns turbid due to the addition of phenol it is considered the base against which all proteins must be compared. PS80 is less resistant to phenol (see 9.9) than all proteins meaning that it is likely the surfactant that causes turbidity in the regular titrations. The salt titration breaks 20 µl earlier than the non-salted version which is a behavior that almost all other protein solutions follow. Salt being a destabilizing factor is expected as it interferes with the surfactant.

9.3 Lysozyme

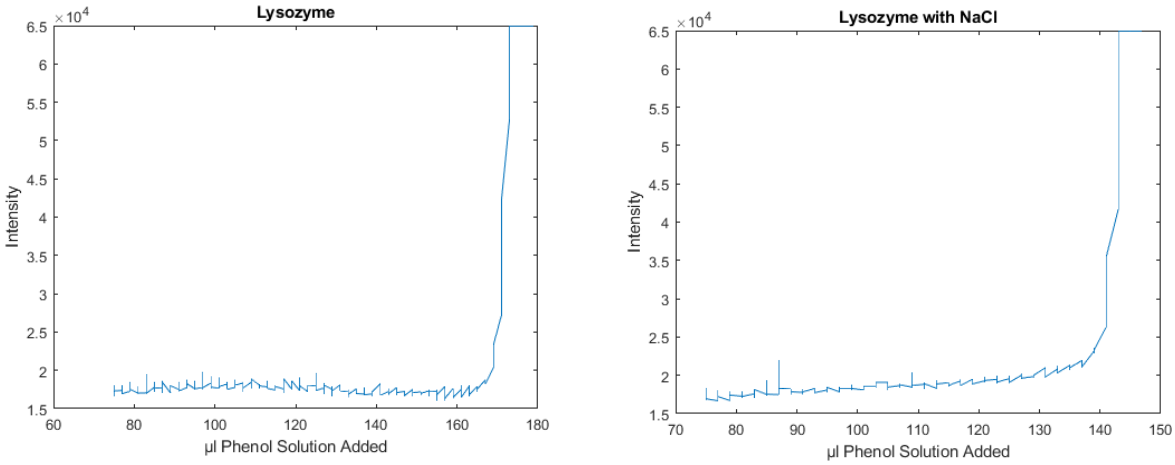


Figure 7 Titration graphs for Lysozyme with and without salt

Lysozyme gives a good example of an ideal curve where the curve shows a clear breakpoint. There is no transition to speak of before the curve turns sharply upwards. Lysozyme raises the breakpoints for both solutions by about 30 µl compared to a solution with only surfactant which means that the protein is reducing the impact of the Phenol somewhat.

9.4 Palifermin

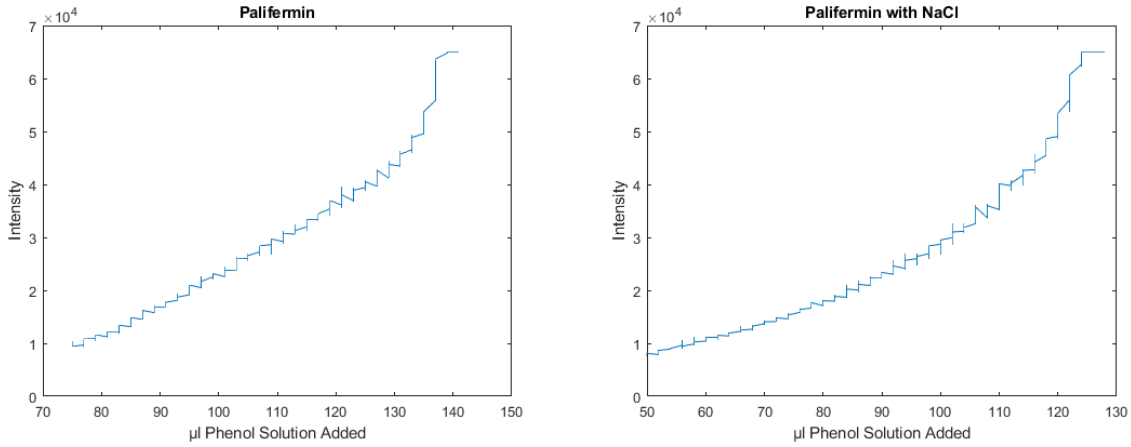


Figure 8 Titration graphs for Palifermin with and without salt

Palifermin is an exception to the rule as the curve has no sharp breakpoint but gradually climbs towards turbidity instead. This is why a set number for turbidity is decided on, but the decision is an educated guess. It is likely that there is an incompatibility between the protein and the buffer solution that gives this result or that Palifermin is negatively affected by coming into contact with oxygen at the surface.

As Palifermin presents an odd curve a test was done where a standard cuvette with 1 wt% Palifermin, 0.1 wt% PS80 both with and without phenol was prepared and tested with the PD to see if it would reach turbidity without the addition of any (more) phenol solution. The results are shown in figure 9 below.

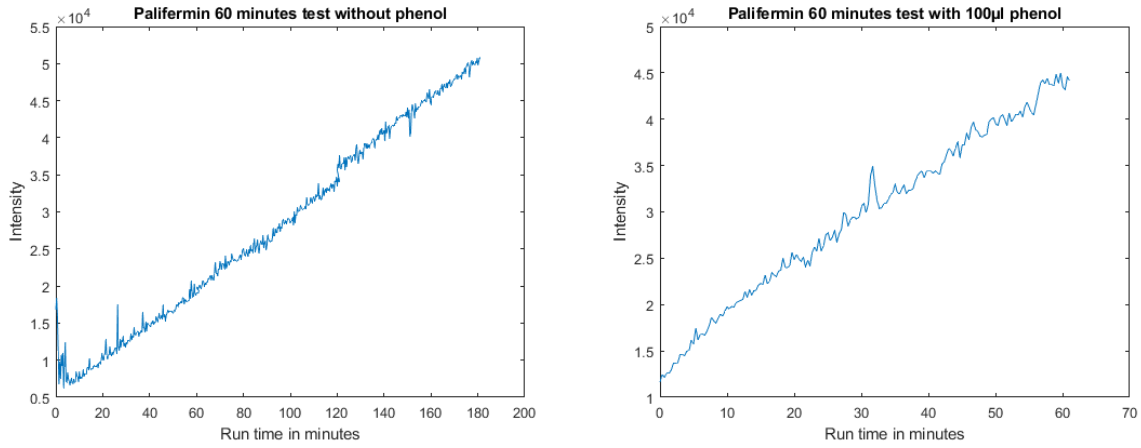


Figure 9 Time test for Palifermin with and without phenol

Both the cuvette with 100µl phenol solution added and the cuvette without reached turbidity but the cuvette with phenol solution reached turbidity in one hour while it took three hours for the cuvette with no phenol. This shows that phenol does play a part in turning the solution turbid but the Palifermin-PS80 system is also capable of turning turbid without any phenol.

In all cases except possibly Palifermin the mixing time is an advantage but since Palifermin can turn turbid over time it is difficult to determine a breakpoint that isn't affected by the 90+ minutes long titration program. What could be considered breakpoint for other proteins is here a combination of how fast the protein turns turbid by itself reduced by the influence of the phenol solution. It is evident that there is a factor that only seems to apply to Palifermin that gives inaccurate results. It is possible that Palifermin is significantly sensitive to oxygen compared to the other proteins or that there is some interaction with the surfactant except for the expected behavior. As the breakpoints are almost identical to a protein-free solution this is the less likely option of the two.

9.5 Trastuzumab

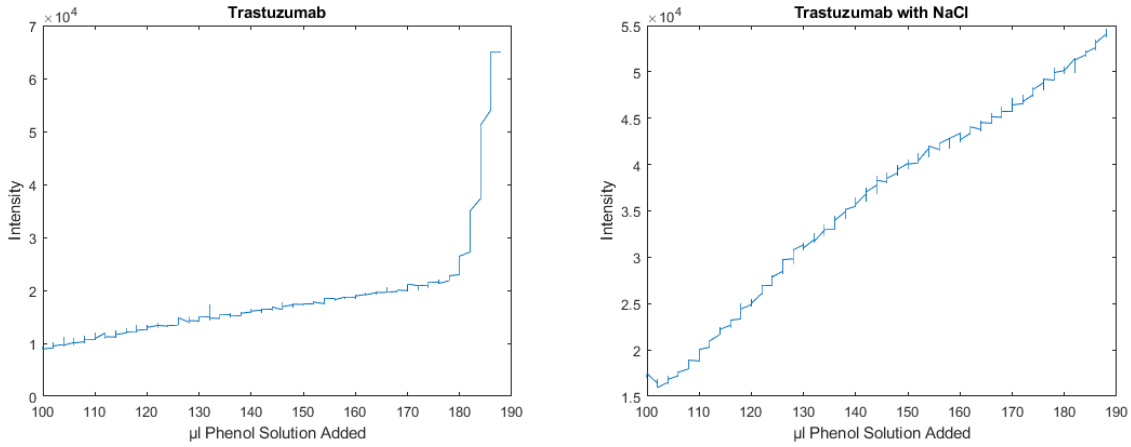


Figure 10 *Titration graphs for Trastuzumab with and without salt*

Trastuzumab follows the standard behavior of having a well-defined breakpoint, but the salt solution climbs slowly like Palifermin and presents no clear breakpoint. While salt is a destabilizing component in all other solutions tested in this project for TRZ it actually makes the system marginally more resistant to phenol or at the very least does not seem to lower the breakpoint. As the curve doesn't behave as most others do the breakpoint is somewhat arbitrary.

As TRZ with salt behaves in a way similar to Palifermin the same test where a standard cuvette was mixed and put into the PD for 1 hour was conducted. The test showed that while the TRZ curve does climb gradually without the addition of phenol it climbs even slower than Palifermin without phenol. It is possible that TRZ with salt will reach turbidity with just mixing but the test indicates that the process would take significantly more time than the three hours it takes for Palifermin.

9.6 GA-Z

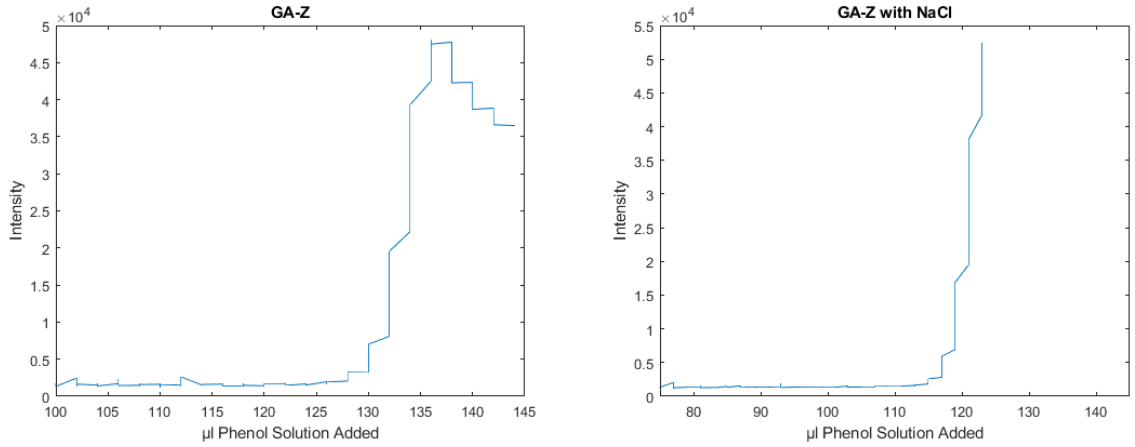
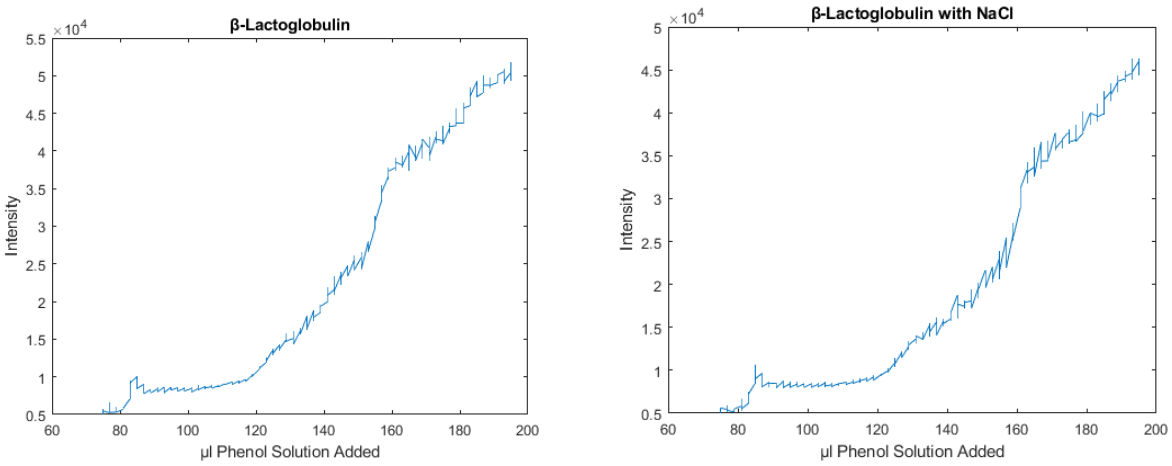


Figure 11 Titration graphs for GA-Z with and without salt

GA-Z behaves in a way that is very similar to only PS80 with almost identical results. This indicates that there is no specific interaction between the surfactant and protein other than the usual behavior for the surfactant. The two curves are almost identical meaning that the solution is dominated by the behavior of the surfactant.

The breakpoints and the curve for GA-Z are almost identical to the ones observed for a solution with only PS80 which indicates that GA-Z has a minimal effect on the stability of the solution. As GA-Z itself is more resilient to phenol than the surfactant this is expected. The degree to which the GA-Z curve overlaps with the surfactant curve is surprising but can be attributed to GA-Z having an unusually weak response to the laser at 636 nm.

9.7 β-lactoglobulin



β-lactoglobulin develops an unusual curve that seems to indicate more interaction between the protein and the surfactant.. As a large part of the surfactant PS80 is a fatty acid this fits with expected behavior. The two curves are almost identical as are their breakpoints. Only β-lactoglobulin and Trastuzumab display this indifference to salt. The identical graphs with a characteristic appearance shows that some effect caused by the protein is the dominating factor in these solutions. At two points there is a change in angle that may reflect a change in the solution. These are just after 80 µl and again at 120 µl.

It is possible that the sudden jumps at 80 and 120 µl are due to protein conformational changes in the solution as β-lactoglobulin has a tendency to form dimeres or oligomeres which could affect its resistance to phenol.

9.7.1 β-lactoglobulin 1% PS80 test

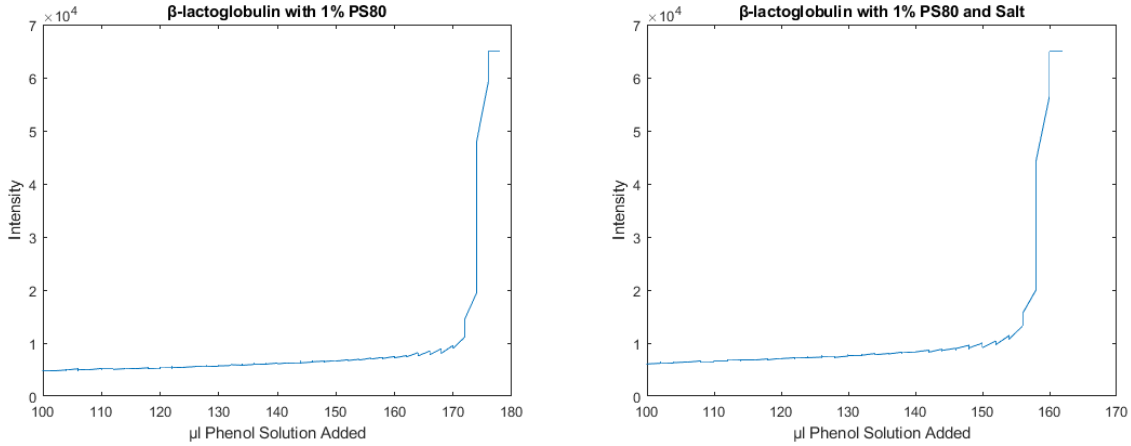


Figure 12 Titration graphs for β-lactoglobulin with 1 wt% PS80 with and without salt

To further investigate this behavior identical solutions with significantly more PS80 were tried in the PD. These solutions had 1% PS80 instead of the usual 0.1% and show that the curves are again basically identical but the breakpoints no longer overlap. The appearance of the curve is no longer particularly interesting as it simply behaves like lysozyme and comes to a clear breakpoint.

The fact that the breakpoints no longer overlap means that the presence of too much PS80 outcompetes the effects of β-lactoglobulin on the solution and makes the solution behave like the others where the protein does not interact as strongly with the surfactant.

The breakpoints for β-lactoglobulin with 1% PS80 overlaps with the breakpoint for only PS80 at 1 wt% meaning that at 1 wt% there is too much surfactant in the solution for the proteins binding tendencies to show but at 0.1 wt% there is a noticeable effect that changes the curve from the norm. The protein still presents a clear breakpoint and turns turbid like other proteins.

9.8 Sobi Lipase

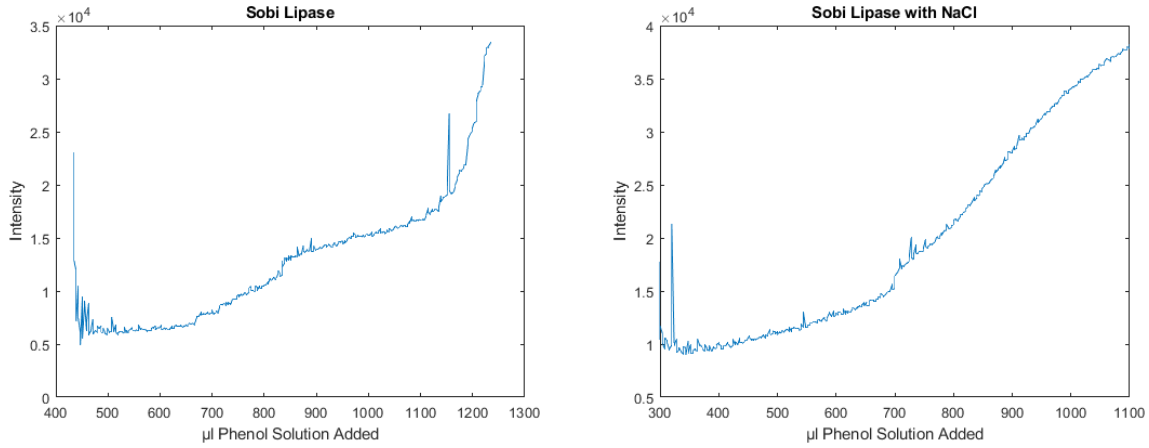


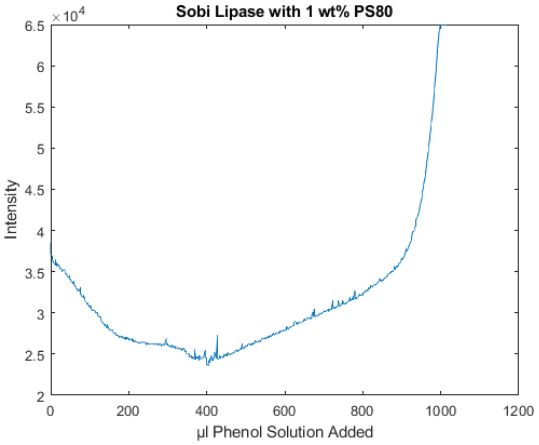
Figure 13 Titration graphs for Sobi Lipase with and without salt

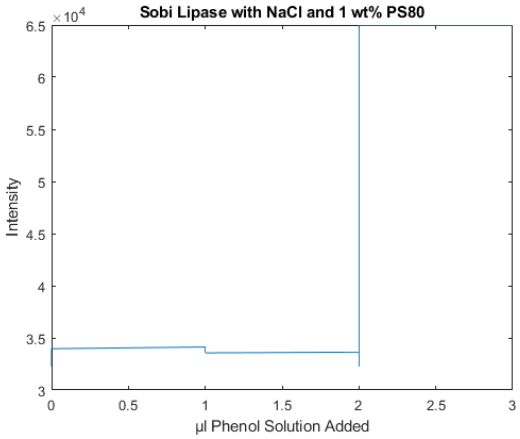
Sobi Lipase presents the most unusual graph as both solutions are very resistant to phenol.It takes roughly five times the amount of phenol before the solution turns turbid compared to any other protein. This means that Sobi Lipase can tolerate an amount of phenol that is significantly above what would be allowed in a parenteral solution. The solution with salt is again less stable than the solution without.

9.8.1 Sobi Lipase 1% PS80 test

When tested at 1 wt% PS80 the behavior of the solution with salt changes drastically. The Lipase solution with salt turns turbid at the slightest addition of phenol. While the exact interaction between PS80 and Sobi Lipase isn't known at this time it is likely that as a lipase it interacts very strongly with the fatty acid part of the

surfactant. At 1 wt% surfactant this interaction disappears behind the effects of the surfactant that isn't interacting as strongly with the protein. The solution without salt is still unusually resilient to phenol.





9.8.1 Sobi Lipase Time Test

Because Sobi Lipase with salt shows high instability when titrated with phenol a stability test was conducted to see if the addition of phenol is required for the solution to turn turbid or if time and oxygen is enough.

A standard cuvette of 900 µl was mixed with Sobi Lipase with salt, PS80 and the salt buffer and put in the ProbeDrum for 60 minutes. After an hour the solution had not turned turbid and showed no tendency to do so if left for longer.

9.8.2 Sobi Lipase Salt titration

As Sobi Lipase shows unusually strong interactions with salt a salt titration was tested to determine how sensitive the protein is. A standard cuvette with 1 wt% protein, 0.1 wt% PS80 and buffer to reach 900 µl was mixed and titrated with a 1 M NaCl buffer solution.

This titration went to 0.24 M NaCl and caused no clouding in the solution. This concentration is significantly higher than the 0.15 M used in all other salt solutions used in this project and shows that while the Lipase PS80 system is sensitive to salt it is not so sensitive that an elevated salt concentration is enough to force the surfactant out of solution.

9.8.3 Sobi Lipase pH test

As Sobi Lipase is indeed a lipase it is possible that it would attack PS80 and thereby create more fatty acid fragments which in turn would lower the pH of the solution. To test this a Sobi Lipase solution was left in the PD for 60 minutes with stirring and a pH electrode (Thermo Scientific Orion Star A215).

The pH stabilized within a few minutes at a pH of 6.9 after an initial reading of 6.96. The obvious problem with this test is that all solutions used are buffered which means that the solution would have to become acidic enough to overcome the buffer before anything of note is measured. As such this result does not have much meaning but is something that could be explored further in the future.

9.9 Manual Test without Phenol

To further investigate the stability of the proteins a manual test was conducted where a 900 µl solution of 1 wt% protein but no surfactant had phenol solution added to it until they turned turbid. As this test was mainly conducted to investigate the proteins by themselves it was only done with the non-salted versions. Phenol solution was added in 30-50 µl intervals and the vials were shaken for 30 seconds and checked visually for turbidity before further additions. The accuracy of this test is not the same as the PD tests but work as a way to estimate the ability of the proteins to resist phenol addition.

Protein	Breakpoint
Lysozyme	170
β-lactoglobulin	930
GA-Z	2000
Trastuzumab	270
Sobi Lipase	420
Palifermin	200*

Table 3 Breakpoints for protein solutions without surfactant

What this shows is that most proteins are significantly more resilient to phenol than PS80 is meaning that in most if not all cases it is the surfactant that leaves the solution first. Lysozyme is close to its breakpoint with PS80 but it is the only protein where this is true. β-lactoglobulin and GA-Z are vastly more resistant than PS80 to the point where they are functionally immune because no pharmaceutical solution would ever contain enough phenol to force them out of solution. This is likely not entirely relevant for β-lactoglobulin as it isn't a pharmaceutical protein but GA-Z on the other hand is.

Sobi Lipase is less stable than with PS80 which is unusual but not entirely unexpected as it is incredibly stable when PS80 is present. This further supports the assumption that Sobi Lipase and PS80 interact very strongly because it can accept more than twice the amount of phenol in the solution with PS80 present than without.

Trastuzumab is more resistant to phenol than PS80 is but not that much more compared to β-lactoglobulin and GA-Z.

Palifermin has a well deserved asterisk next to its breakpoint for this test as Palifermin has proved itself to be unstable in the buffer with no phenol added. The addition of phenol speeds up the development of turbidity but isn't necessary for it to occur. The breakpoint for Palifermin in this test is not a set breakpoint as much as it is a combination of the time it took to add the phenol and the mixing time combined with the reduced stability from more phenol. The other breakpoints are fixed but Palifermin is unstable in the solution it was tested.

9.10 DLS

To further investigate what happens at high phenol concentration DLS tests (Dynamic light scattering) was done for three of the six proteins. These three, β-lactoglobulin, Sobi Lipase and Trastuzumab were selected because they have the most interesting interactions with phenol. They also give a decent representation of the various sizes of protein included in this study.

Two 10 ml solutions were prepared for each protein containing 0.1 wt% protein, phenol and buffer. The amount of phenol added before titration varied between protein, but all are around 50 % of the phenol required to reach turbidity for the protein without surfactant. The phenol was added just before the start of titration to minimize the aggregates formed before measuring. Every run consists of three measurements after each injection of phenol and the three proteins were tested twice each.

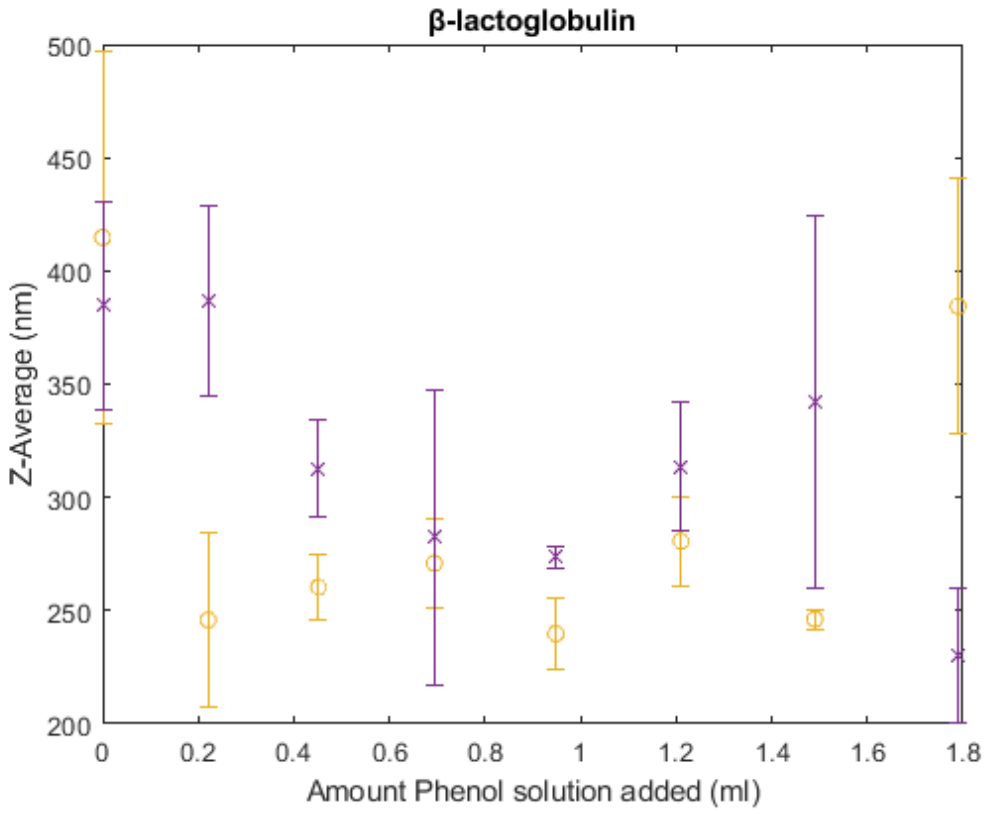


Figure 14 DLS graph for β-lactoglobulin. The two tests are shown as x and o with their standard deviations marked.

β-lactoglobulin shows no consistent increase or decrease in the average sizes of particles in solution. It is by far the smallest protein of the three so it having a smaller z-average is expected due to the aggregates formed being smaller too. There is no consistent change in size of the average which can either mean that there is no aggregation occurring or that aggregation is done at the time the sample was measured. It is possible that β-

lactoglobulin falls out of solution faster than the other two proteins and is worse at gathering into large aggregates due to the size difference between β-lactoglobulin and the other two proteins.

β-lactoglobulin is very resistant to phenol compared to other proteins without surfactant which means that the phenol content in the β-lactoglobulin samples were significantly higher than in the others (see table 3). The addition of phenol to the sample was likely done too fast resulting in a higher local concentration of phenol that started aggregation before titration. The addition of phenol was done in the same way for all three proteins but since β-lactoglobulin started with roughly twice the amount of phenol in the sample before titration it is likely that β-lactoglobulin received the biggest chock of the three.

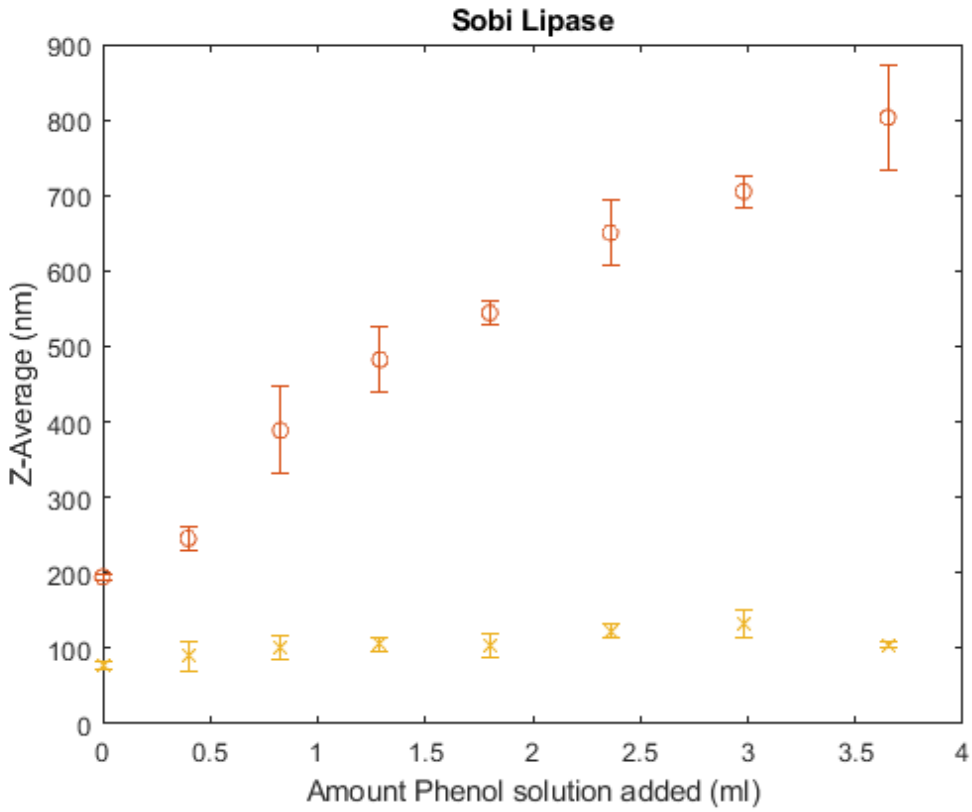


Figure 15 DLS graph for Sobi Lipase. The two tests are shown as x and o with their standard deviations marked.

One of the Sobi Lipase titrations shows a consistent increase in average size which means that the sample is forming larger aggregates with the addition of more phenol. The lower titration (marked with x) does not show an increase in average size but has formed aggregates as the average is around a 100 nm. It is possible that larger aggregates did form but sedimented out before being measured. As was the case for β-lactoglobulin it is possible that all Sobi Lipase had already left solution at the time of measurement or that the protein does not necessarily form a consistent size of aggregate. If aggregation occurs during the titration the stirring would help push aggregates together which would give a consistent increase in size during the titration.

However, the titration during DLS was significantly faster than the titrations carried out in the ProbeDrum which means that the sample spends a shorter amount of time exposed to phenol compared to the usual titrations. While a PD titration consists of 2 µl every 3 minutes the DLS titration was 500-600 µl every 3 minutes. Therefore, it is difficult to determine if aggregation reaches its maximum for a given amount of phenol or if the effects of previous injections are still occurring while more phenol is being added. It is likely that aggregation would continue without the addition of more phenol and that there is less of a set level of phenol where aggregation starts but instead there is a phenol dependent gradient of how fast the protein leaves solution.

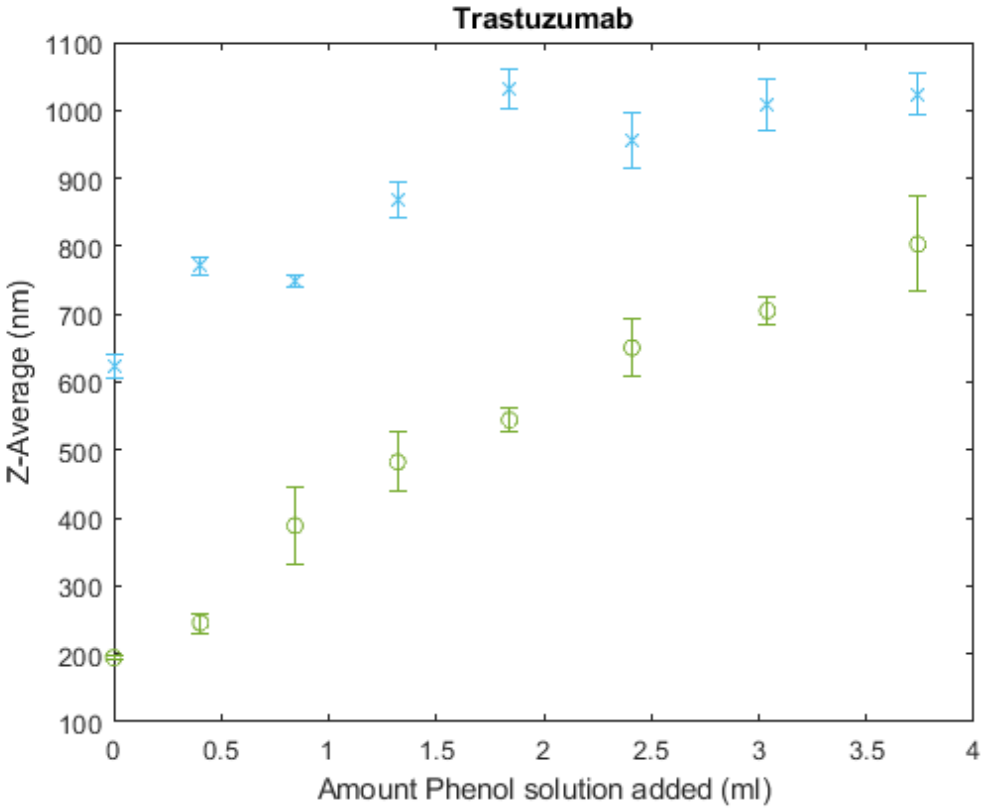


Figure 16 DLS graph fpr Trastuzumab. The two tests are shown as x and o with their standard deviations marked.

Trastuzumab behaves much like Sobi Lipase which is expected as they are the two largest proteins included in this project. Both titrations show a consistent increase in size meaning that aggregation is still taking place during the titration. There is a consistent increase in size with the addition of more phenol, but it is again likely that this increase would continue without additional phenol. The fact that there is no obvious point were the phenol content crosses the limit (table 3) means that aggregation starts before what has been determined to be the critical phenol content that forces the protein out of solution.

9.11 CoSAXS

To study the effects of phenol on the shape and size of the proteins SAXS measurements (Small angle X-ray Scattering) were taken. By determining the angle of the X-rays scattered by a protein it is possible to determine the general shape and size of the tested protein and thereby determine the changes induced by phenol.

The radius of gyration was determined for each protein with and without phenol but all tests were done without surfactant as a micelle forming excipient would interfere with the measurements. Salt was excluded due to time constraints as including it would have doubled the number of tests. Phenol concentrations were set to roughly 90 % of the concentrations determined in manual tests without surfactant (9.9) in order to study what happens when the phenol concentration is close to when turbidity occurs.

Below are figures 17-22 [12] that show the radius of gyration for the six proteins.

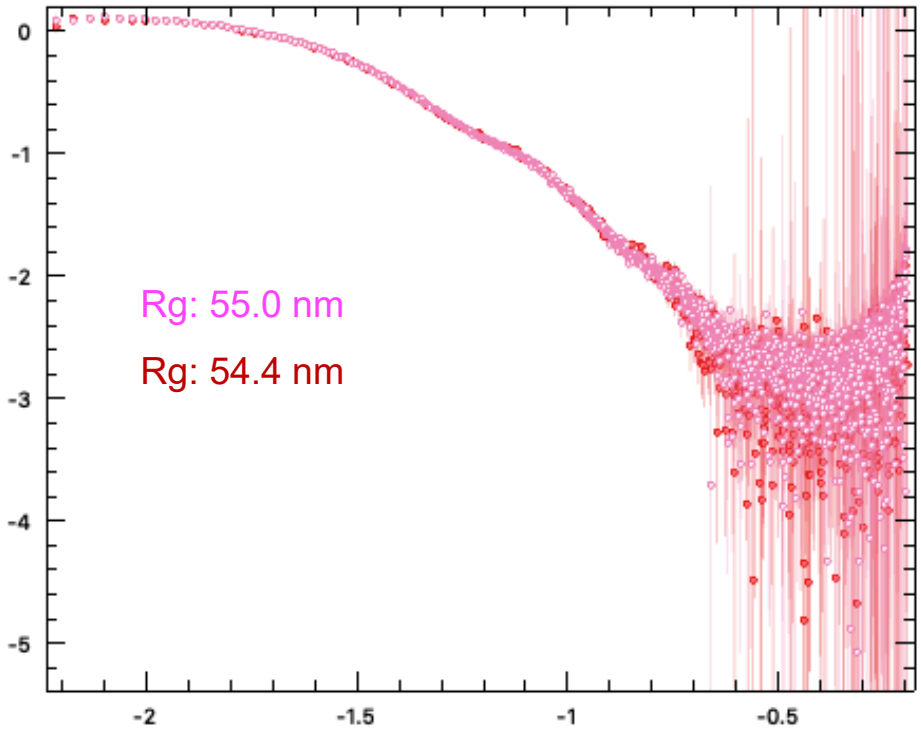


Figure 17 Trastuzumab without phenol (red) and with 0.47 wt% phenol (pink)

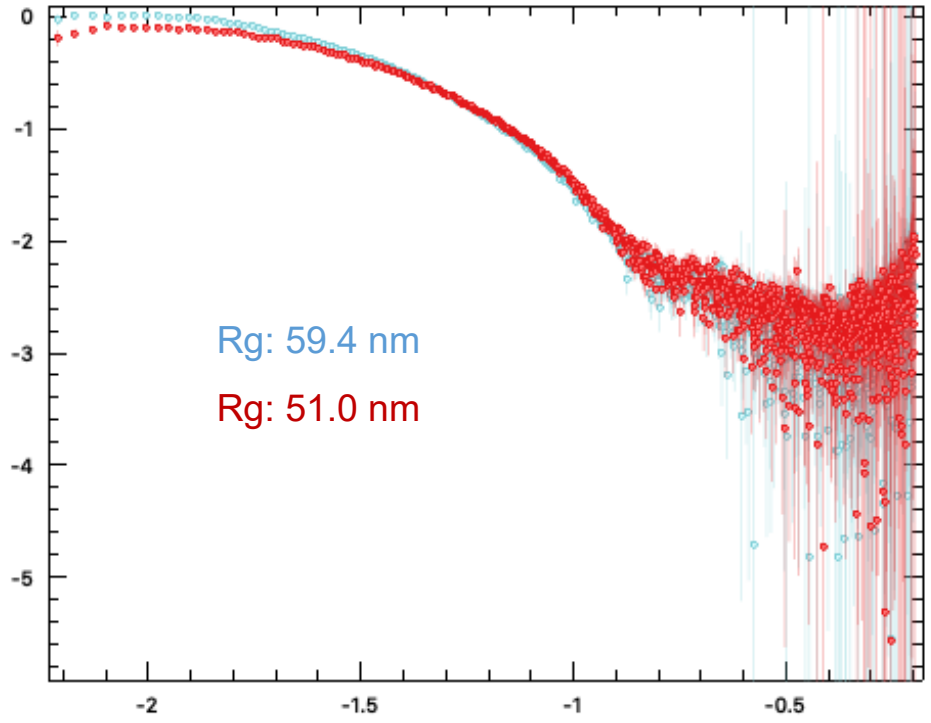


Figure 18 SOBI Lipase without phenol (red) and with 0.83 wt% phenol (blue)

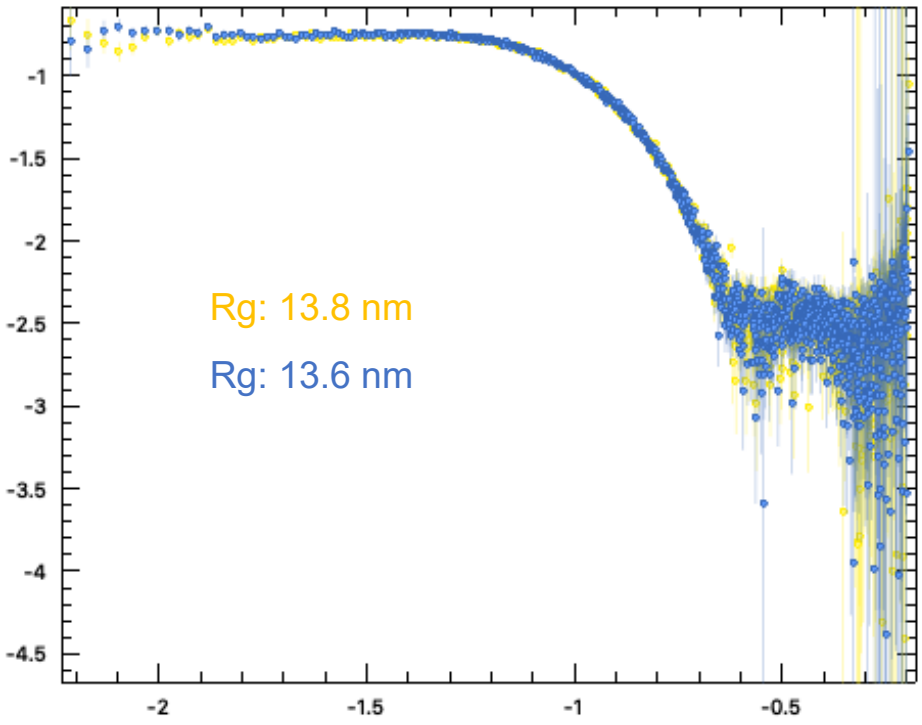


Figure 19 Lysozyme without phenol (blue) and with 0.43 wt% phenol (yellow)

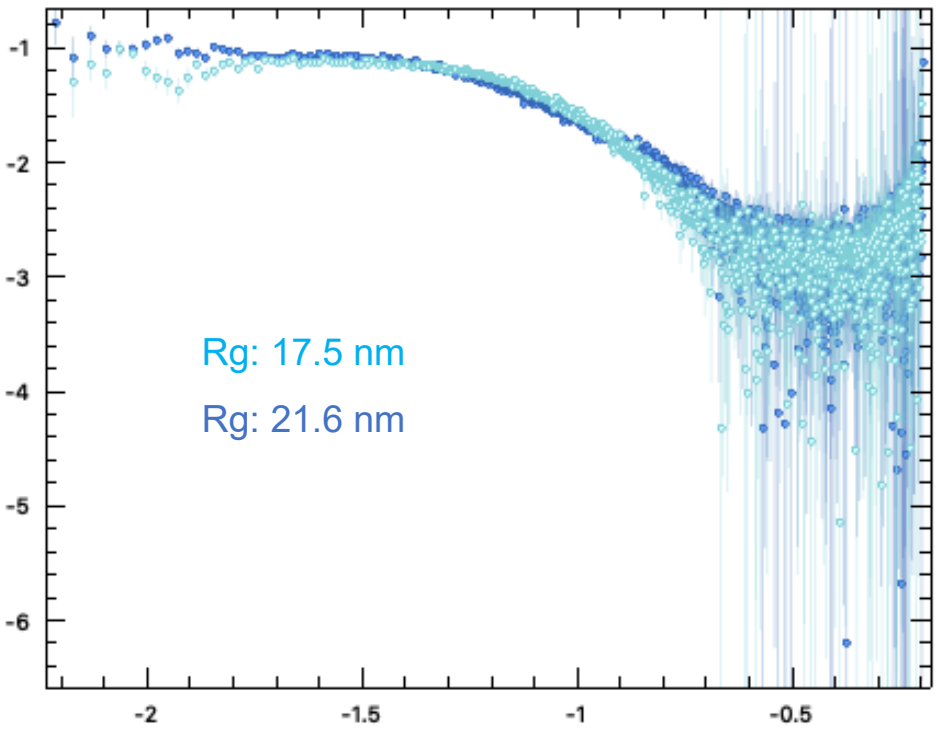


Figure 20 GA-Z without phenol (blue) and with 1.43 wt% phenol (teal)

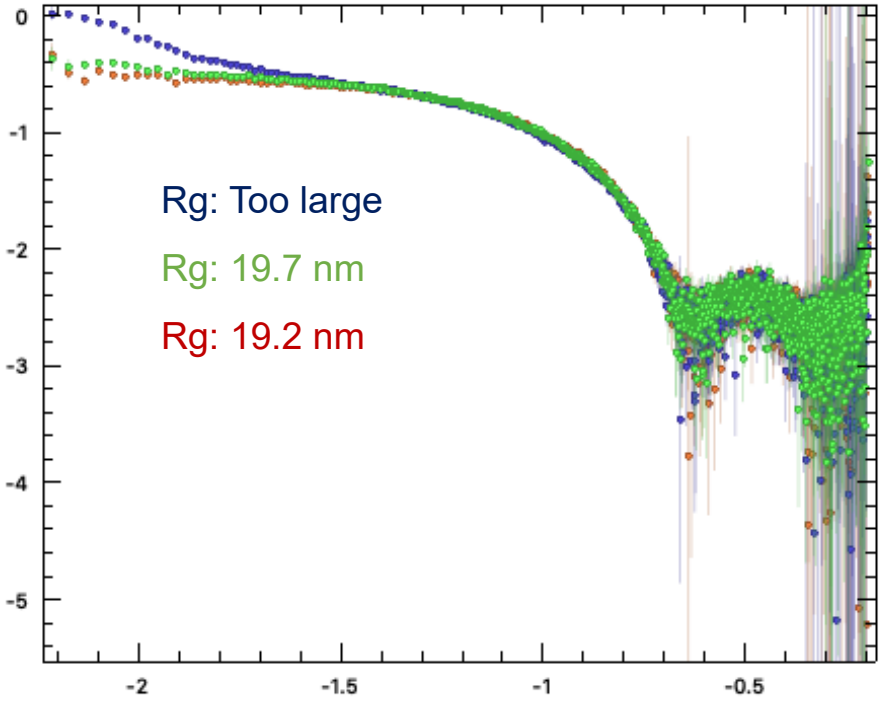


Figure 21 Palifermin without phenol (red) with 0.15 wt% phenol (green) and 0.47 wt%

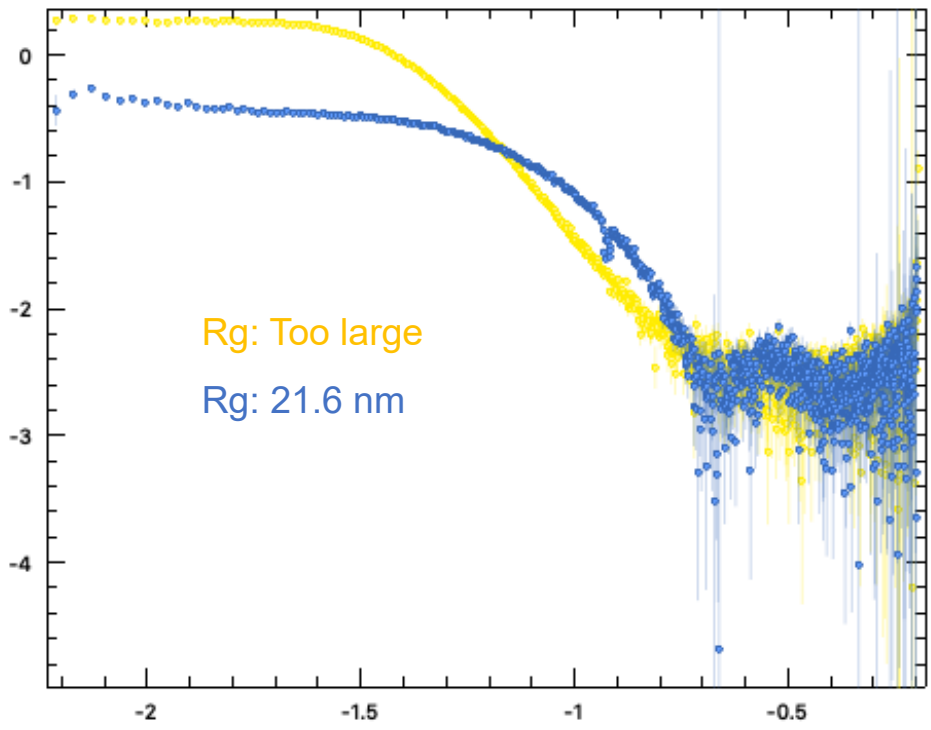


Figure 22 β-lactoglobulin without phenol (blue) and with 1.35 wt% phenol (yellow)

Trastuzumab (fig 17) does not seem to change much in either shape or size when the phenol concentration is raised as the radius of gyration barely changes. There is a slight increase in size which could indicate some unfolding but as the increase is so minor it seems that Trastuzumab is not very affected by phenol before the point it is forced out of solution.

Sobi Lipase (fig 18) shows a bigger difference between the two measurements as the radius of the protein with phenol has increased by 8.4 nm. Sobi Lipase is the most phenol resistant protein when the surfactant is involved but without surfactant it is significantly less resistant than both GA-Z and β-lactoglobulin. From the size increase it seems that phenol causes the protein to unfold which may result in a nonfunctional protein should the unfolding go too far. The concentration tested here is significantly above the 0.55 wt% phenol that is allowed in protein solutions however meaning that a lowering of the phenol concentration would be required in an actual pharmaceutical product.

Figure 19 shows the graph for Lysozyme with and without phenol. There is a miniscule change in R_g indicating that the protein barely changes shape or size in the presence of phenol. As Lysozyme is one of the proteins with very standard titration graphs (9.3) that don't seem to show any kind of specific interaction between the preservative and the protein a very minor change seems reasonable.

GA-Z in figure 20 shows a large decrease in R_g indicating that the protein contracts when the phenol concentration is raised. GA-Z is also interesting because it is the only protein of the six that decreases in size with increasing phenol concentration. It is also by far the most phenol resistant protein when the surfactant is not involved as well as being the smallest protein in this project. Contracting in the presence of phenol may add to the proteins resistance but further study is required.

Palifermin in figure 21 was tested at two different concentrations as it was difficult to determine an accurate breakpoint. The lower of these two phenol concentrations results in a small increase in radius whereas the higher concentration results in too much aggregation to measure properly. As has been mentioned previously Palifermin is unstable in the buffer used which means that the measurements aren't entirely reliable. It seems that the protein follows the standard behavior of becoming slightly larger when the phenol concentration is increased.

β-lactoglobulin in figure 22 seems to have aggregated too quickly to determine a radius when phenol is present. This may be due the high phenol concentration which could cause aggregates to form faster. β-lactoglobulin is the second most phenol resistant protein after GA-Z but seems to increase in size rather than decrease as GA-Z does. Whether or not this is true is difficult to determine from these measurements however and further study is required to accurately determine the actual changes taking place.

10 Discussion

All protein solutions without surfactant are more resistant phenol than solutions containing both. This indicates that in all cases it is the surfactant that leaves the solution first. Some of the proteins improve the solutions resistance to phenol however, resulting in breakpoints at higher phenol concentrations than with only surfactant. The specific stabilizing behaviors has not been studied in detail but there is a clear difference in the behavior of a Sobi Lipase or Trastuzumab solution as compared to a GA-Z solution that breaks at the same time as a solution with only surfactant.

Lysozyme, β-lactoglobulin, Trastuzumab and Sobi Lipase without salt all break later than just PS80 which indicates some stabilizing behavior from the proteins. The curves for these vary in appearance meaning that they likely do not stabilize the solution in the same way. GA-Z has almost the exact same titration curve as just the surfactant meaning that the protein does little to affect the behavior of that solution. Palifermin also has the same breakpoints as PS80 but the titration curve it produces is different from all other titrations (except TRZ with salt). As Palifermin seems to be affected by a buffer incompatibility it seems reasonable that it doesn't aid the stability of the solution.

Salt is consistently a destabilizing factor which is expected as it interferes with the surfactant resulting in a less stable solution. All salted solutions break 10-20 µl earlier than their non-salted counterparts with the one exception being Trastuzumab with salt. Trastuzumab with salt shows similar behavior as Palifermin does but doesn't seem to be as unstable. The two proteins have somewhat similar isoelectric point, but they differ in weight by a factor of ten meaning that the two are generally dissimilar. As no two proteins are overly similar different stabilizing behaviors for each protein is likely.

11 Conclusion

The various proteins included in this study show a number of different responses to phenol. Half of the proteins (Lysozyme, GA-Z and Palifermin) do not seem to raise the solutions resistance to phenol where as the other three (β-lactoglobulin, Trastuzumab and Sobi Lipase) do. However, all of them can work in a solution with an amount of phenol that is reasonable for a pharmaceutical product. The mechanisms through which the stability of the solution is affected are mostly beyond this report, but some educated guesses have been made.

In the future any of the pharmaceutical proteins could be singled out for closer study to determine the actual degradation pathways. Furthermore, the use of other preservatives, surfactants and buffer solutions could be explored as it is unlikely that the configuration tested here is the optimal version. Significant work could be done with further DLS studies or ITC to study the binding interactions between protein and surfactant.

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