Effects of mixed surfactant system on chemical and physical stability of human growth hormone

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EXECUTIVE SUMMARY

In protein formulation, addition of surfactants is commonly executed to enhance product stability and shelf-life. The reason is that surfactants can protect proteins against surface-induced aggregation. However, the interaction between surfactant and protein depends on the type of surfactant used. Anionic surfactants can unfold proteins and form complexes in equilibrium manner. The complex structure depends on the ratio of anionic surfactant-to-protein. On the other hand, nonionic surfactants can weaken interactions between anionic surfactants and proteins. Here, human growth hormone (hGH) is used. Sodium dodecyl sulfate (SDS) in the SDS-hGH complex was slowly extracted to form mixed micelles with n-dodecyl-β-D-maltopyranoside (DDM). This gentle process allows the protein to refold back to its native form with less aggregation. From previous studies, the DDM/SDS ratio is pivotal for the protein refolding procedure using a nonionic surfactant, regardless of mixing order. This study is aimed at assessing the stability of refolded SDS-unfolded hGH after incubating at 37°C for 42 days. Regarding hGH properties, deamidation seems to be dominant in chemical degradation. One of the formulations was designed for less deamidation, at pH 5.4, to eliminate other destabilizing effects compared to pH 7. In the physical stability aspect, the SDS-hGH formulation at pH 5.4 had the lowest stability of refolded hGH, as seen in the results from FlowCAM, DLS, and Probe Drum. However, the refolded hGH structure in this formulation was the most chemically stable as seen from HPLC analysis. Nuclear magnetic resonance is needed to assess the complex conformation in this formulation.

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PROJECT AIM

General objective:

This project was conducted to study effects from different surfactant and pH on human growth hormone (hGH) stability. Different surfactant types interacted with protein differently. The use of nonionic surfactant seems to be able to remove ionic surfactant by forming as mixed micelles. hGH structure that lost ionic surfactant from its complex could refold to its native structure. Here, the effect of nonionic and ionic surfactant would be investigated in both physical and chemical stability aspects.

The stability of human growth hormone in formulations with ionic or nonionic surfactant for forty-two days at 37°C would be established. hGH formulation with the ionic surfactant sodium dodecyl sulfate (SDS) would be formulated in pH 5.4 to decelerate deamidation reaction. Another formulation with same components was also prepared in pH 7 to be a control formulation to investigate pH effect. The formulation with n-dodecyl-β-D-maltopyranoside **(**DDM) was prepared to assess the stability with different type of surfactants. Lastly, a control without surfactants (hGH in buffer pH 7) was included in the study.

Specific objectives:

- Assess the reversibility when refolding SDS-unfolded hGH using DDM
- Determine physical stability of refolded hGH in pH 7 and 5.4 using Fluorescence Spectroscopy, Flow Imaging Spectroscopy, and Dynamic Light Scattering
- Evaluate chemical stability of refolded hGH in pH 7 and 5.4 using reversed-phase High-Performance Liquid Chromatography

INTRODUCTION

Biopharmaceutical products

In 2014, the number of approved biologics by the FDA reached double digits for the first time. The highest number was reached in 2018. The 96 approved biologics during 2014–2021 represent 27% of the approved drugs by the FDA (Figure 1).[1] Protein-based therapeutics provide an advantage with great specificity against their target sites. They can be used as a treatment for cancer, autoimmune disorders, infectious diseases, etc.[2] In this study, human growth hormone (hGH) is the protein of interest that is also classified as a biological product. It has been approved for several indications, for instance, childhood growth hormone deficiency, chronic renal insufficiency, Turner syndrome, adult growth hormone deficiency, HIV wasting syndrome, etc.[3]

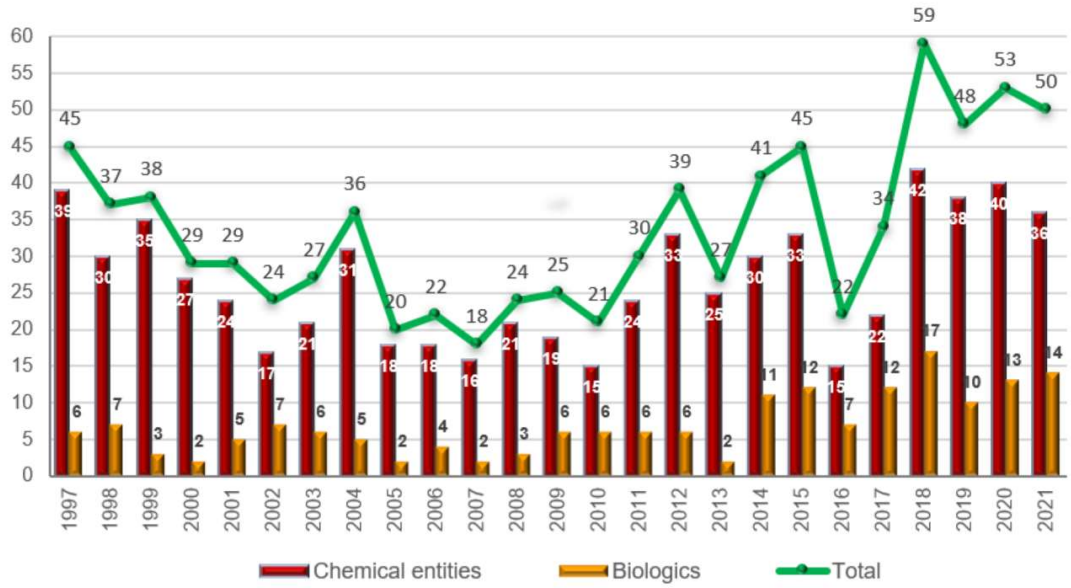

Figure 1. *Drugs (New Chemical Entities and Biologics) approved by the FDA[1]*

Developing therapeutic proteins requires an understanding of their physicochemical properties, immunogenicity, and pharmacokinetic properties.[4] Once a protein is subjected to external factors like production, formulation, and product handling, it can be degraded chemically and physically, which eventually affects biological activity.[2] So, the optimization of external conditions such as pH, temperature, surface interaction, and excipients requires critical attention during the developing phase.[4]

Interaction between surfactant and protein

The addition of surfactant to a formulation is commonly done to enhance the formulated product's stability profile and shelf-life.[5] The idea is to stabilize interfaces between air and liquid, protecting proteins from surface-induced aggregation.[6] Interactions between surfactant and protein have been studied.[5, 7] Different surfactant types interact differently with different protein.[7] Anionic surfactants are capable of binding to globular proteins, resulting in denatured and unfolded proteins as equilibrium structures[5, 7, 8], while nonionic surfactants are known for weakening interactions between anionic surfactants and proteins by forming mixed micelles.[7] Kaspersen et al.[7] found that the refolding protein process was driven by enough extraction of sodium dodecyl sulfate (SDS) from SDS-protein complexes. As SDS has

both electrostatic and hydrophobic interactions with proteins, gentle extraction of SDS allows the protein to refold with less aggregation. By achieving this, the process required enough n-dodecyl-β-D-maltopyranoside **(**DDM) to form mixed micelles. The addition of DDM to a system of proteins and SDS diminishes the rate of protein unfolding. If the system contains a sufficient amount of DDM, the SDS-unfolded protein can refold into its native conformation. Nonionic surfactants generally do not bind to globular proteins. The refolding procedure using nonionic surfactants has demonstrated an advantage over other developing refolding mechanisms, like using cyclodextrin, with a slower and more gentle procedure.[7]

Native hGH is a globular protein with a tryptophan residue in the hydrophobic region.[5] Once hGH alters its conformation, altered fluorescence spectra of hGH can be observed corresponding to environmental changes in the hydrophobic core. Interaction between hGH and SDS changes the protein conformation depending on the surfactant-to-protein ratio as seen in Figure 2.[5] The interaction seen here was in equilibrium and could be reversed.[5, 7] When the system consists of a low concentration of SDS, Stage a, SDS molecules adsorb onto the protein without major changes to its tertiary structure.[5] SDS alters local environment in the core through hydrophobic interactions between the hydrophobic tail of SDS and hydrophobic amino acids. Moreover, the SDS could interact with hGH via electrostatic interaction through ionic surfactant head and positive charge amino acid.[5, 8] These interactions together result in the adsorption of SDS at low SDS concentrations. Further addition of SDS, Stage b, introduces greater changes in the local environment as surfactant forms as a cluster on the complex. The conformation of hGH at this stage evolves into unfolded form and starts altering its tertiary structure. This structure is known as a molten globule. Once the tertiary structure is completely disrupted, Stage c, the protein secondary structure is disrupted and remained on a complex interface. The unstructured cluster evolves into a decorated micelle. The higher SDS concentration, Stage d, does not introduce any more change in either secondary or tertiary protein conformation.[5]

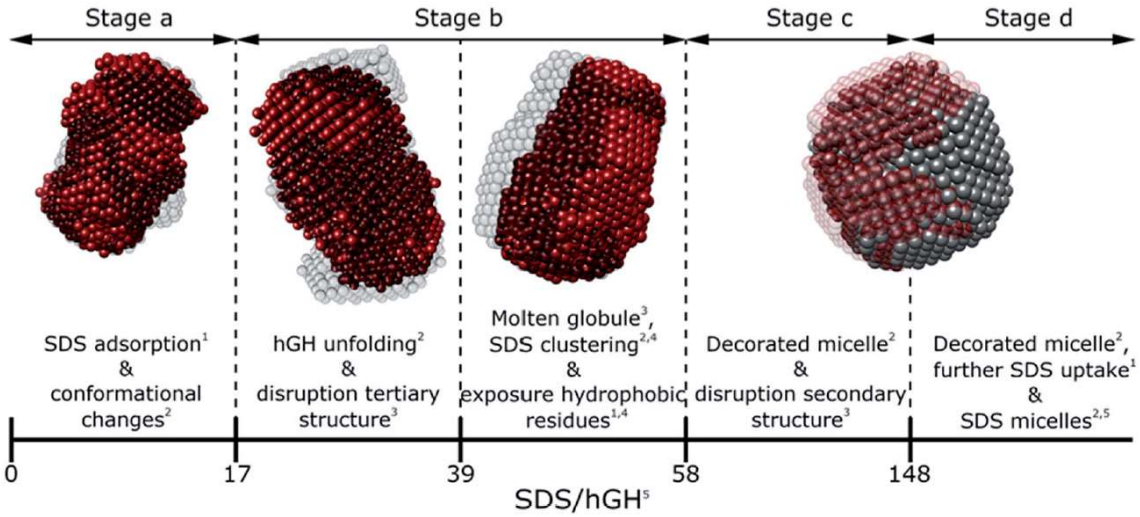

Figure 2. *The dynamic interaction and conformation of SDS-hGH complex in different SDS/hGH ratio[5]*

There is a benefit in refolding proteins using nonionic surfactants. hGH can refold to its native structure in an equilibrium manner via a triplex structure of SDS, DDM, and hGH (personal communication Johanna Hjalte 2023). As mentioned previously, SDS binding to hGH affected the protein local environment of a fluorophore which can be tracked by measuring intrinsic fluorescence. While adding DDM alone, there were no changes observed on hGH fluorescence spectra. The addition of DDM to SDS-unfolded hGH structure had potential to refold the unfolded-hGH structure. However, it was proven that order of mixing did not affect outcomes. The ratio of DDM/SDS showed as a crucial factor for the refolding approach and drove the process. In other words, this refolding procedure requires enough amount of nonionic surfactant, in relation to ionic surfactant. The long-term stability profile, when implementing this method, still needs to be established to prove if DDM could refold hGH to its native structure after being unfolded by SDS for long time.

Protein physical degradation

In terms of physical instability, it includes partial unfolding, aggregation, adsorption, and precipitation. The environmental pH has effects on both the chemical degradation process and charges of amino acid side chains resulting in alteration of electrostatic interaction and conformational stability. From the point of view of protein intrinsic stability, the protein aggregation process usually relates to conformational changes through forming molten globule intermediate; however, that is not the only possible pathway. Protein could also undergo an aggregation mechanism through the initial formation of nucleated species as a multi-stage process during storing. (Figure 3) Moreover, another well-known factor for destabilizing protein is temperature. The increased temperature alters protein in the level of secondary, tertiary, and quaternary structure which results in conformation changes exposing hydrophobic regions to an unfavored environment. Unfolded proteins tend to aggregate to lower this unfavored interaction. Once partially unfolded protein has formed, the protein at this stage can either reverse toward native structure or form as irreversible aggregates as shown in Figure 3. If the net effect of all protein-protein interactions is attractive forces, this implies the tendency for protein aggregation dominates. The aggregated molecule can either be in native or non-native form. In other words, protein aggregation is an amalgamation of conformational, colloidal, and nucleation effects. Nevertheless, one of them might be noted as a dominating process due to a particular destabilizing factor.[2]

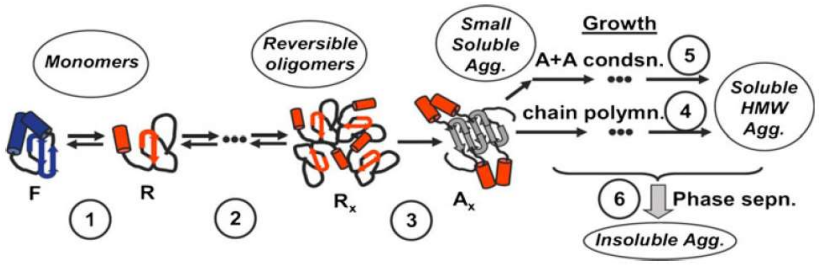

Figure 3. *Protein aggregation[2]*

There are many high-throughput techniques used for physical stability assessment. Here, only the chosen techniques are mentioned. Intrinsic fluorescence spectroscopy provides information about internal fluorophores of protein based on its aromatic side chain. Protein conformational changes could be detected as changes in intensity and a line shape shift of emission peak maximum. Dynamic Light Scattering (DLS) is often used for monitoring aggregation and size measurement.[2] The DLS principle is based on intensity changes of scattered light in suspension. The fluctuations of scattered light intensity, as a result of Brownian

motion, is used to determine particle size. The intensity corresponds to the square of particle volume[2]and the power of six to particle diameter. The analysis of sample containing aggregated products is weighted toward larger sizes.[9] Therefore, DLS is sensitive to big particles even in small amounts, including large aggregated products and dust, interfering with particle size determination if the majority is smaller in size.[10] Even though this technique can offer lots of data with a small amount needed[2], DLS is limited with intensity-biased detection.[10] Flow imaging microscopy is another useful technique to characterize small transparent particles like proteins. This technique works with a high magnification camera to capture images when the solution passes through a flow cell and particles are detected based on decreased refractive index compared to the background signal. These captured images were used for analyzing particle size, particle count, and particle morphology.[11]

Protein chemical degradation

Chemical degradation of proteins is important since it could also destroy protein function.[12] From production to storage, the important degradative pathway is non-enzymatic post-translational modification.[8] The two most common chemical degradation pathways are asparagine (Asn) deamidation and aspartate (Asp) isomerization.[12] These reactions initially start with the Asn and Asp residues, which form an intra-molecular succinimide intermediate. (Figure 4) [8, 12] At this point, the intermediate could form several variants afterward leading to heterogenicity of the product. The resulting variants are crucial for product safety and efficacy. The heterogenicity is problematic for the consistency of product production.[8] In addition, the rate of deamidation and isomerization is affected by physical properties of the surrounding conditions. These two chemical degradative reactions also induce physical degradation pathways via protein aggregation leading to an undesired immunogenicity potential.[12]

In the formulation aspect, pH is an important factor since it has an impact on the degradation pathway. Formulation of proteins is usually prepared within the pH range of 5-8, to avoid protein unfolding. Within a pH of 5-8, the Asn deamidation reaction rate is increased correlated to higher pH. On the contrary, Asp isomerization reaction rate is increased if the formulation is more acidic. Thus, potential degradation is taken into consideration during formulation.[8] At the isoelectric point (pI), the net charge of protein surface is neutral. In the study done by Ablinger et al., the deamidation rate of solubilized hGH in formulation with protamine was studied at pH 5.4, which is close to its pI of 5.3. The deamidation was followed by quantifying deamidated products at 0, 1, and 3 months in comparison with the formulation of hGH without protamine at pH 7. At the initial point, no significant difference could be observed; however, the significantly higher level of succinimide variant and lower level of the main deamidated product were measured in the pH 5.4 sample after storing for 3 months. The deamidation reaction in pH 5.4 was found as retarded at succinimide variant to 3 months of storing.[13] Even though the formulation was different from our study, pH 5.4 was selected to minimize deamidation reaction. So, our samples are formulated at pH 7 and 5.4, presenting as pH for known stable condition and less deamidation condition respectively.

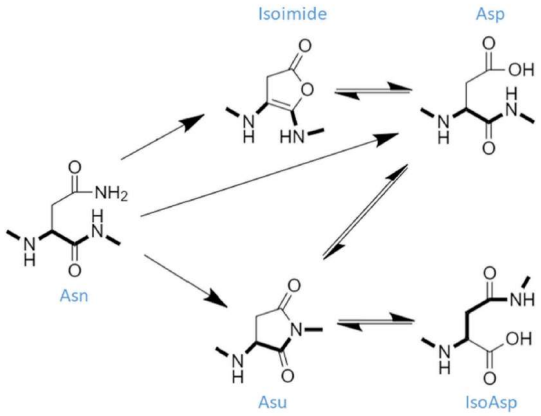

Figure 4. *Deamidation/Isomerization reaction[14]*

hGH formulation development

For this study, the amount of SDS required to provide enough physical stabilizing effect was tested. This was done through a series of proteins formulated with increasing amounts of SDS, from 1 to 10 mM. The requirement was for the formulation to not show visible signs of instability over 24 hours at room temperature and for a concentration of SDS as low as possible was selected. With more SDS, a higher amount of DDM will be needed during the SDS removal step resulting in a higher number of total surfactants to be removed. DDM titration was done to determine the appropriate DDM/SDS ratio for refolding hGH in this study. Based on the study, 3 mg/ml hGH was formulated with 5 mM SDS with a DDM/SDS ratio of 16. The SDS removal approach was also tested to ensure protocol appropriateness.

The study aims to investigate the refolding ability of hGH using nonionic surfactant at different pH, and whether changing pH in a formulation can minimize the effect of deamidation. So, one of the formulations with SDS would be prepared at pH 5.4. Another formulation with SDS was in pH 7, to compare the pH effect on physical and chemical stability. As a control, hGH was also formulated with DDM at pH 7. Lastly, the formulation of hGH alone was needed to investigate hGH stability in pH 7 without any surfactant. Citric acid/Na_2HPO_4 buffer was chosen in this study to maintain a stable pH throughout the selected range, in addition, they are approved for pharmaceutical use. For citric acid, it was also listed as one of the excipients in the growth hormone formulation commercialized as Nutropin AQ©.[15] The addition of 150 mM NaCl was needed to balance different ionic strengths between buffer pH 7 and 5.4 to equalize the effect of ionic strength.

METHODS

Materials and Reagents

The protein and other reagents used were provided by NextBioForm consortium. The human growth hormone, Somatropin, was generously supplied by Ferring Pharmaceuticals A/S (Kastrup, Denmark) in lyophilized powder form. Prior to analysis, hGH powder and DDM (≥99.9%, Anatrace, US) were stored at -20ºC. SDS (≥99.5%, Sigma Aldrich, US), sodium chloride (≥99.5%, Sigma Aldrich, US), citric acid (≥99.5%, Sigma Aldrich, US) and Na_2HPO_4 (Anhydrous, Sigma Aldrich, US) were kept in the designated cupboard. All related chemicals were ensured with sufficient purity. Pre-sterilized microcentrifuge tubes, 0.2 μm syringe filters and silicon free syringes were used in sample preparation.

Sample Preparation

The hGH samples were prepared to evaluate the stability of different formulations and environmental conditions. All four samples contained 3 mg/ml hGH. The differences among formulations were surfactant and pH. The first formulation (F1) contained 3 mg/ml hGH, 150 mM NaCl, and 1.18 mM citric acid/10.98 mM Na_2HPO_4 buffer at pH 7. The second formulation (F2) consisted of 3 mg/ml hGH, 82.22 mM DDM, 150 mM NaCl, and 1.18 mM citric acid/10.98 mM Na_2HPO_4 buffer at pH 7. The third formulation (F3) consisted of 3 mg/ml hGH, 5 mM SDS, 150 mM NaCl, and 1.18 mM citric acid/10.98 mM Na_2HPO_4 buffer at pH 7. Lastly, the fourth buffer (F4) consisted of 3 mg/ml hGH, 5 mM SDS, 150 mM NaCl, and 2.95 mM citric acid/17.43 mM Na_2HPO_4 buffer at pH 5.4. (Table 1)

The hGH powder was weighed separately into four centrifuge tubes at approximately 120 mg. SDS and DDM were prepared as stock solutions with the corresponding buffer at concentrations of 30 mM and 500 mM, respectively. The buffers were prepared as pre-made buffers with the surfactant, if needed, and autoclaved one day before. Then, the sterile buffers were added to the protein tube to achieve an hGH concentration of 3 mg/ml under a laminar air flow cabinet with aseptic technique. The solution was filtered into new pre-sterilized centrifuge tubes using a 0.2 μm PES filter with silicon-free syringes. To control the volume in each tube, the hGH solutions were pipetted into the corresponding microcentrifuge tube. The samples were then incubated at 37ºC for 6 weeks. Usually, the storage condition recommended for a Somatropin product is in a refrigerator (2–8 ºC). The accelerated stability testing for this kind of drug products is suggested to be conducted at 25ºC ± 2ºC, 60%RH ± 5%RH for 6 months according to ICH Q1A (R2).[16] Thus, a stress temperature of 37ºC is high, but this temperature was chosen to see large effects. At each time point, HPLC samples were directly stored at -80ºC to stop the degradation process for performing HPLC analysis afterward.

Table 1. *hGH formulations*

Formulation	pH	Surfactants
F1	7	No
F2	7	DDM
F3	7	SDS
F4	5.4	SDS

SDS removal method

Prior to performing analysis, SDS in the formulation of F3 and F4 was removed using DDM. To 2 ml F4 samples, 189 µl 0.2 M Na_2HPO_4 was added at once to bring the pH up to 7, followed by the addition of DDM. To the F3 samples, only DDM was added. The total amount of DDM was calculated based on the ratio of 16 DDM/SDS and the addition was divided into three steps with gentle tilting and swirling of the samples in between.

Flow Imaging Spectroscopy

The particles in each formulation were visualized and quantified using FlowCAM® with a 10X lens, which could capture both subvisible and visible particles. Each run was performed with approximately 500 µL solution and at least 2 runs per formulation were performed. The sampling plan for analysis with FlowCAM® was set on days 0, 7, 14, 28, and 42. The analysis was performed on the same day as the sampling day.

Dynamic Light Scattering

The particle size distribution was studied using Dynamic Light Scattering (DLS) with Dynamic V7 analyzing program. The samples were loaded in triplicate. The size distribution was distributed by percentage of number. The sampling plan for DLS analysis was set on days 0, 7, 14, 28, and 42. The samples were stored at 2°C before performing analysis on the day after the sampling day.

Fluorescence spectroscopy

The intrinsic fluorescence spectroscopy was done on Probe Drum at 280 nm to follow hGH conformation. The sampling plan for measuring intrinsic fluorescence was set on days 0, 7, 14, 28, and 42. The sample was directly kept at 2ºC for performing intrinsic fluorescence spectroscopy on the day after. On the analysis day, the sample was diluted by half with buffer to lower the concentration of hGH to be within the detection limit of the instrument.

Sample preparation for HPLC analysis

The samples for all four formulations were centrifuged to separate big particles from the solution. The supernatant was gently loaded into the Pierce™ detergent removal spin column to remove all detergents before HPLC analysis. The surfactant-removed sample is diluted with 10%ACN, MQ as a final concentration of 0.33 mg/ml.

High-Performance Liquid Chromatography

Prior to the analysis, HPLC method for hGH was optimized from the established method done by Emmanuel Deniola[17] to improve the peak separation. The Shimadzu LC-20AD system was used in combination with a DAD UV detector, and a BioResolve reversed-phase column (Polyphenyl, 450 Å, 2.7 µm, 3 x 150 mm. Waters, MA, USA). In this study, HPLC was done with gradient elution technique with mobile phase A (Milli-Q water with 0.08% formic acid and 0.02% trifluoroacetic acid) and mobile phase B as LC grade acetonitrile with 0.08% formic acid and 0.02% trifluoroacetic acid. The injection volume was 10 µL, 1.0 ml/min flow rate, and the gradient was optimized to achieve a better chromatogram resolution. For the developed method, mobile phase B was increased from 10-45.1% during the first 24 minutes, then 45.1-52% in 24-47 minutes, and finally 52-95% during 47-50 minutes.

In this study, the optimization was focused to separate the native peak from the others. Additionally, this study is aimed to mainly quantify the amount of native hGH in each sample and the total amount of degraded product. So, this optimized method would be feasible for measuring the amount of native hGH only.

RESULTS AND DISCUSSION

Physical stability

Overview of hGH physical stability

In general, the particle size measured by DLS for all four formulations had shown that the longer storage time resulted in larger particles. (Figure 5) The particle kept growing until day 28, then became smaller. As seen in Figure 5, all of them had no significant changes in average particle diameter during the first 7 days. Since day 14, all four formulations started showing a big difference in particle size compared to day 0 and 7.

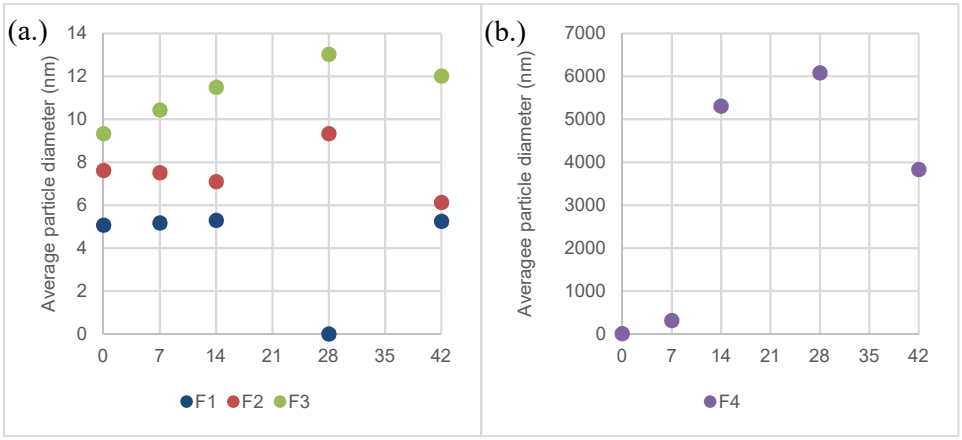

Figure 5. *Average particle diameter measured by DLS (a. – F1-3, b. – F4) The average particle diameter was calculated from the average among three measurement of DLS. F1 represents the formulation of hGH alone in pH 7. F2 represents the formulation of hGH with DDM in pH 7. F3 represents the formulation of hGH with SDS in pH 7. F4 represents the formulation of hGH with SDS in pH 5.4.*

The trend in particle number observed from FlowCAM analysis was contradictory to the trend of particle size from DLS analysis. At an initial point, the formulation of hGH alone (F1), showed the highest particle number, followed by the hGH formulation with DDM (F2). The formulations with SDS (F3 and F4) showed the lowest particle number in FlowCAM analysis on day 0. (Table 2) The number of particles dramatically decreased for the formulation of hGH alone (F1) after 7-day storing even though no visible particles were observed. This could indicate that the sample was not fully dissolved on the first day.

In summary, hGH alone had the highest particle number with the smallest particle size on day 0, followed by the formulation of hGH with DDM, which contained fewer particles of a bigger size. Then, the hGH formulation with SDS in pH 7 had a lower particle number and bigger particle size than the formulation with DDM. Finally, the formulation of hGH with SDS in pH 5.4 had the lowest particle number and the largest particle size. Thus, the formulation with a higher particle number seems to have smaller particles. However, this is not the case on day 42. The order seen from day 0 still occurs for F1-3. However, F4 developed the most particles with the largest size, by far. None of the samples had visible particles when

they were brought out from the incubator. The results indicate that a change in pH, from 7 to 5.4, had a significant effect on particle size and number.

Table 2. *Average particle number from FlowCAM measurement on day 0 and 42. The average particle number was calculated from particle/ml. F1 represents the formulation of hGH alone in pH 7. F2 represents the formulation of hGH with DDM in pH 7. F3 represents the formulation of hGH with SDS in pH 7. F4 represents the formulation of hGH with SDS in pH 5.4. 1.18 mM citric acid/10.98 mM Na_2HPO_4 pH 7.*

Formulation	Particle number/ml on day 0		Particle number/ml on day 42	
	Average	STD	Average	STD
hGH alone (F1)	1.1×10^5	1.4×10^4	4.7×10^4	1.6×10^4
hGH with DDM, pH 7 (F2)	3.1×10^4	7.5×10^3	1.6×10^4	5.2×10^3
hGH with SDS, pH 7 (F3)	1.6×10^4	1.9×10^3	9.4×10^3	8.68×10^2
hGH with SDS, pH 5.4 (F4)	1.3×10^4	1.9×10^3	7.0×10^6	1.3×10^6
Buffer pH 7	N/A	N/A	2.0×10^2	7.8×10^1

Physical stability of hGH alone and in formulation with DDM

After day 14, the particle number was slightly increased in the FlowCAM result for hGH alone. The particle size measured by DLS remained approximately the same throughout 42 days. It was not possible to measure particle size with DLS for F1 on day 28. However, the particles in F1 day 28 were seen in the FlowCAM measurement. Additionally, the tendency of particle size for this hGH alone as seen in Figure 5a from the other days was quite stable in size, so the particle diameter on day 28 was probably 5 nm as well. The intrinsic fluorescence spectra remained the same throughout the study indicating hGH remained in its native structure during storage in citric acid/Na_2HPO_4 buffer, 150 mM NaCl, pH 7 for 42 days. (Figure 6b, d)

From FlowCAM result, the number of particles in hGH formulation with DDM (F2) reached the maximum on day 7 and then decreased until day 42. The particle diameter, according to DLS, of 14-day storing was 7 nm in average. The particle in F2 evolved to the biggest size on day 28, which had a diameter as 9 nm. Then, the particle was in the smallest size, as 6 nm. Although there was an evolution of particle size, the difference was around 3 nm, which was relatively small compared to F4 as discussed below. However, it is worth noting that DLS measurement is based on the light scattering on particles in the solution. So, both DDM micelles and hGH contributed to the result.

The intrinsic fluorescence spectra of hGH in the formulation with DDM were overlaid perfectly until day 28. A small change was observed on the spectra of day 28 and 42, as decreased and increased intensity respectively. This could be resulted from different hGH content in these two samples, as seen in Figure 10b the hGH content in these two samples was a little higher and lower than the other three samples. The line shape of the spectra of each sample remained approximately the same as on day 0. Here, this could imply that the structure of hGH remained as day 0 throughout the study. Moreover, the addition of DDM did not affect the physical stability of hGH even after storing for 42 days. (Figure 6a, c, e)

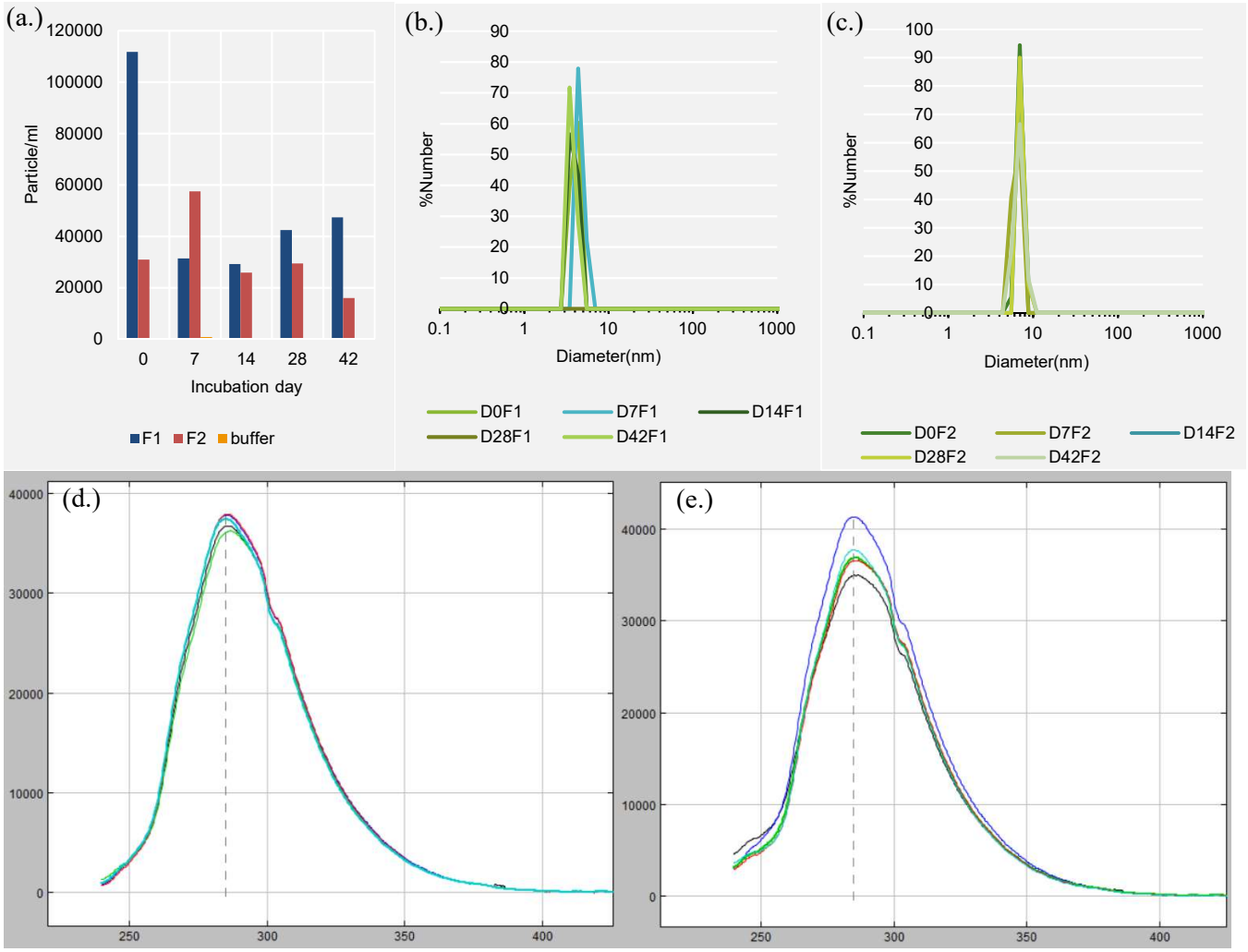

Figure 6*. Particle counted in F1 and F2 using FlowCAM (a.), Particle size measurement using DLS (b. for F1 and c. for F2), and Intrinsic fluorescence spectra using ProbeDrum at 280 nm (d. for F1 and e. for F2) (blue – day 0, red – day 7, green – day 14, dark blue – day 28, black – day 42) F1 represents the formulation of hGH alone in pH 7. F2 represents the formulation of hGH with DDM in pH 7.*

Physical stability of hGH in formulation with SDS at different pH

The number of particles, according to FlowCAM analysis, in formulation with SDS in pH 7 (F3), after adding DDM, reached the lowest number on day 7, then increased until day 28. On day 42, the number dropped. Even though the number of particles changed during the storage time, the trend was constant as seen in Figure 7b. The largest particles detected were 13 nm in diameter on day 28. (Table 4, Appendix 2) The particle had approximately the same diameter, as seen in Figure 8a, throughout the 42 days. In the same way as the formulation with DDM, the difference in particle size was around 4 nm. However, it was also possible that DLS measured several species; DDM-SDS-hGH complexes, DDM-SDS mixed micelles, and free hGH. The intrinsic fluorescence spectra indicated that hGH remained in the same conformation until day 7 with its native structure. In the sample for day 14, the intrinsic spectra had slightly lower intensity with a similar overall shape. (Figure 8c) On the sample for day 28 and 42, the spectra had shifted toward a lower wavelength while the curve shape remained as it was on day 0. This implies that hGH had slightly changed its conformation on day 28, at the same time, the alteration probably did not have much impact on the hydrophobic pocket environment as seen as a slight change in intrinsic fluorescence spectra. However, further conformational analysis is needed.

From FlowCAM analysis, the hGH formulation with SDS in pH 5.4 (F4) had the lowest particle number on day 0 when compared to the other three formulations (Table 2), then extremely increased to millions since 7-day incubation. (Figure 7c) The numbers continued increasing until day 28 after which it began to decrease, as seen on day 42. Even though the sample did not contain any visible particles when it was taken from the incubator, the solution became cloudy after adding DDM except for the sample from day 0. As seen in Figure 8b, the average particle size measured by DLS for this formulation drastically changed during the incubation. The average particle diameter was around 10 nm on day 0, then grew larger by incubation time. (**Table 4**, Appendix 2) The particle size measured by DLS was reported as the largest on day 28 with a diameter of 6088 nm. During day 0-7, the number of particles became higher while these particles were getting smaller. This made it likely that they were degraded into smaller pieces. After that, these small fragments agglomerated and became larger in size which were eventually seen as white particles and detected as changes toward larger diameters of particles in DLS measurement.

During the study, the observed white particles on day 7 led to additional measurements both before and after SDS removal step. As seen in Figure 7a, before removing SDS, the particle number for hGH formulation with SDS in pH 7 was slightly higher than the formulation in pH 5.4, on day 14 and 42. After removing SDS, the formulation with SDS in pH 7 had an excessively decreased number of particles while F4 behaved in the opposite way. The vastly increased particle number in the formulation with pH 5.4 to millions shed the light on pH effect. In the study done by Sanchez-Fernandez et al., SDS-hGH complex conformation in pH 7 was illustrated[5], which further proved by Johanna Hjalte that the refolding mechanism was able to successfully refold hGH back to its native structure. However, the structure of the SDS-hGH complex in pH 5.4 had not been previously studied. As the pI of hGH is 5.3, hGH in pH 5.4 would have a neutral surface while it had a net negative charge in pH 7. Due to its distinct electrostatic surface properties, the SDS-hGH complex in pH 5.4 might have formed in a different shape and conformation. SDS could interact with net neutral hGH via the hydrophobic part leading to unexpected conformation and interactions.

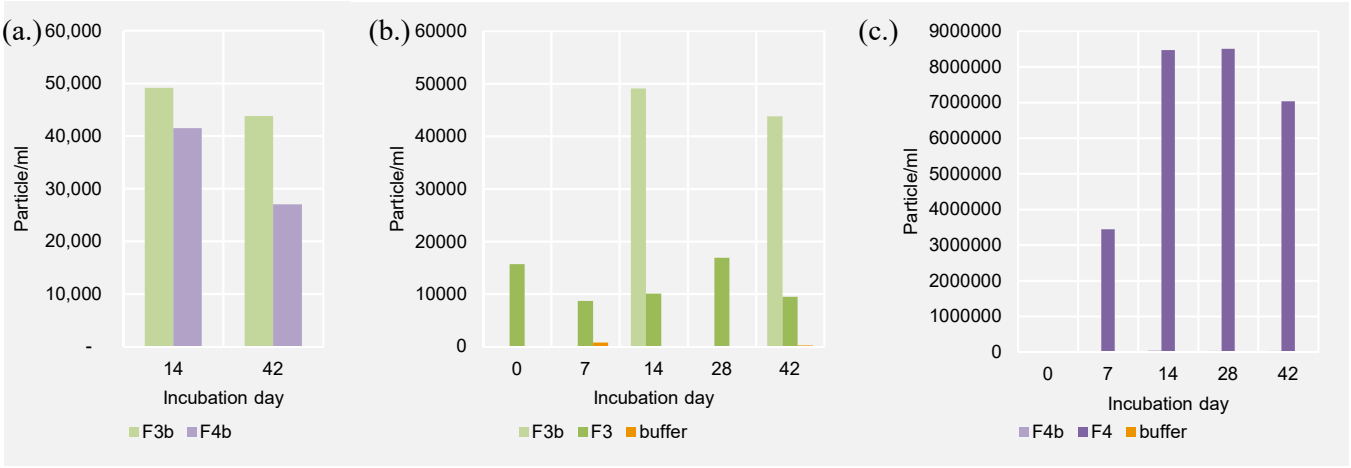

Figure 7. *Particle counted in F3 and F4 before removing SDS using FlowCAM (a.). The comparison of particle number in F3 and F4 between before (F3b and F4b) and after removing SDS. (F3 and F4) (b. – F3, and c. – F4) F3 represents the formulation of hGH with SDS in pH 7. F4 represents the formulation of hGH with SDS in pH 5.4.*

In Figure 8d, the intrinsic fluorescence spectra of hGH in formulation with SDS pH 5.4 were completely different from day 0. The spectra for day 7-42 had lost an s-shape on the left side of the curve, which was used to indicate hGH native conformation. Other spectra from hGH in pH 7 formulation, with or without surfactant added, were able to retain their native curve shape throughout the study. At this point, the

intrinsic fluorescence of hGH in SDS formulation proved that hGH in complexation with SDS at pH 7 was capable of refolding into the same structure as day 0 even after 42-day incubation with the use of DDM. On the contrary, the intrinsic fluorescence of hGH in SDS formulation at pH 5.4 could not recover the native fluorescence pattern already since day 7. At this point, it was clear that the hGH structure in the formulation with SDS at pH 5.4 was already changed since day 7. From a physical stability point of view, the refolding procedure using a nonionic surfactant cannot reverse hGH fully back to its native form, if it was stored in pH 5.4 as seen from F4. However, in pH 7, hGH can fold back to its native form after storing for 42 days with a slowly increased number of aggregated protein particles as seen in F3.

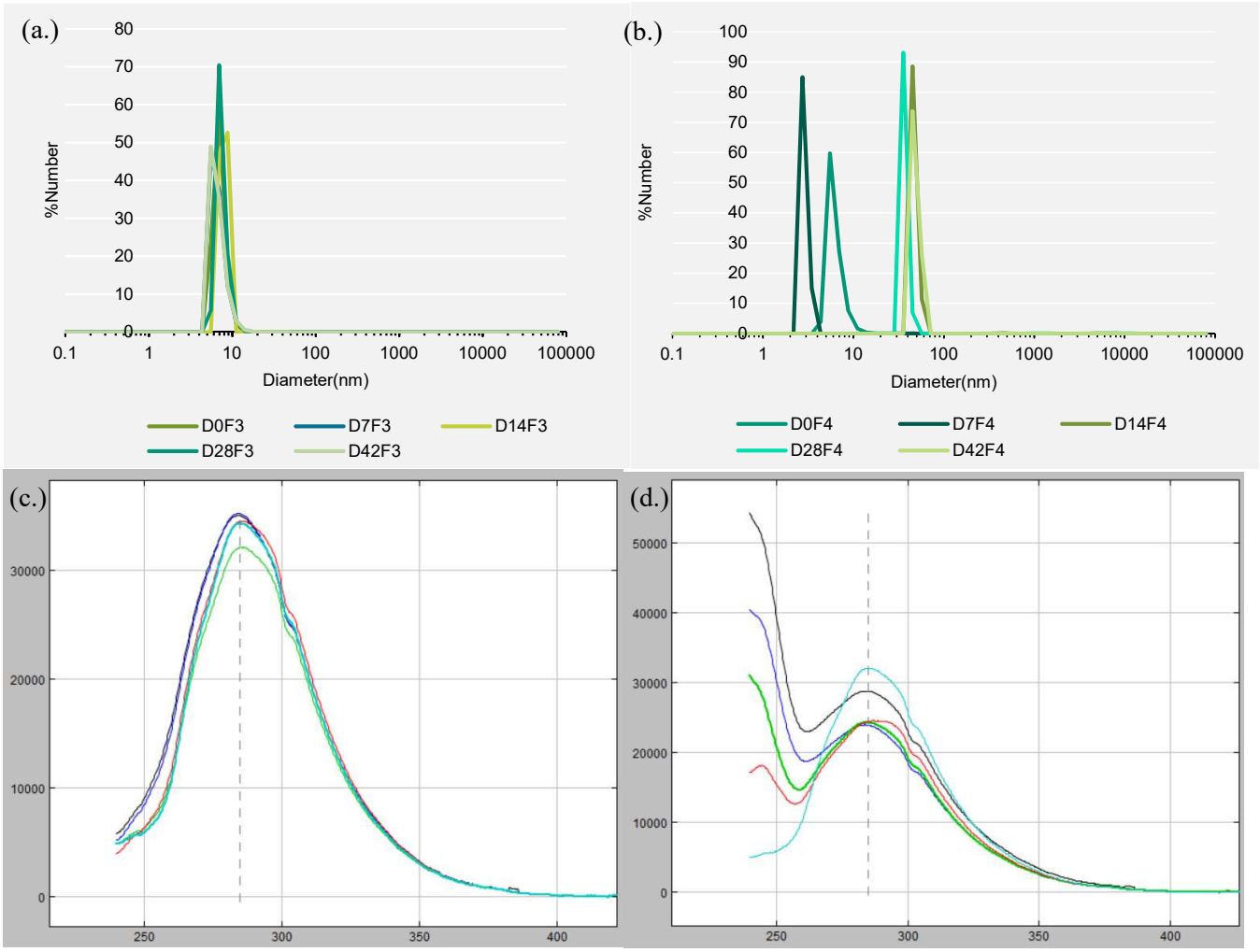

Figure 8. *Particle size measurement using DLS (a. for F3 and b. for F4), and Intrinsic fluorescence spectra using ProbeDrum at 280 nm (c. for F3 and d. for F4) The particle had approximately the same size until day 42 in pH 7 formulation while the particle in pH 5.4 was fragmented into small pieces and became larger since day 28. The intrinsic fluorescence in pH 5.4 also indicated changes in local hydrophobic pocket environment and could not regain the native fluorescence pattern. Moreover, the fluorescence detected in ProbeDrum was probably affected from big particle. (blue – day 0, red – day 7, green – day 14, dark blue – day 28, black – day 42) F3 represents the formulation of hGH with SDS in pH 7. F4 represents the formulation of hGH with SDS in pH 5.4.*

Chemical stability

Human growth hormone chemical stability

Overall, hGH was degraded chemically over time in any formulation. Figure 9 shows the proportion of native hGH out of hGH in all species. The sharpest decline occurred during the first 7 days. This is commonly observed for deamidation and isomerization reactions. For example, in the same way as hGH, the steepest decrease of the RNase A fraction was seen during the early phase of the reaction, related to the increase of isomerized and deamidated products.[18] As seen in Figure 9, all pH 7 formulations, regardless of surfactant, had similar amounts of native hGH throughout the study. The duplicates were in line with the trend in almost all time points. The sharpest decrease for formulations in pH 7 can be observed during the first 7 days, at approximately 25%. After that, chemical degradation was getting slower over time following the kinetics of deamidation and isomerization.[19]

The formulations of hGH alone and with DDM had the same degradation profile, as seen by the fact that the trends of these two formulations were overlaid by each other. Starting from day 14, the trend of native hGH proportion in the formulation with SDS at pH 7 laid above the formulation of hGH alone and with DDM, but on day 7, it had the least percentage of native hGH. This could result from more degradation, or one of the points was an outlier at 61%. According to the trend, it is reasonable to expect the data from day 7 to also be a little higher than F1 and F2. Due to the similar trend observed for the formulations in pH 7, the proportion of native hGH on day 7 for the SDS-hGH formulation in pH 7 (F3), seen in Figure 9, was probably less than expected, which might be around 70–75%. The variation was probably caused by a pipetting error. In comparison with the formulation in pH 5.4 (F4), the trend of native hGH proportion in pH 5.4 was approximately 5% higher than at pH 7. At this point, it was obvious that the formulation in pH 5.4 showed a higher chemical stability than in pH 7 until the last day of incubation. This implied that the chemical stability of hGH was affected by pH, not surfactants.

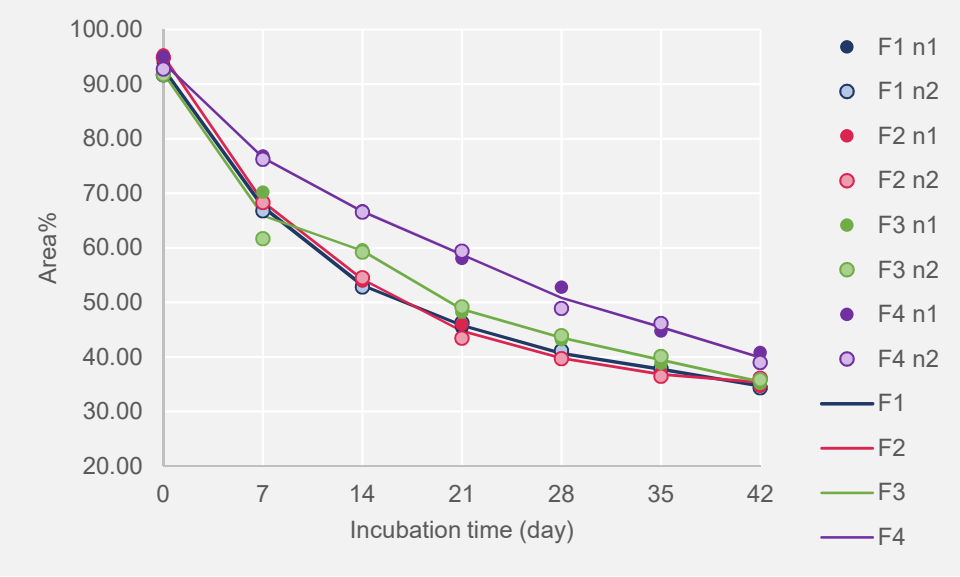

Figure 9. *Percentage area of native peak representing the amount of hGH that was formed in native conformation measuring by HPLC method. The proportion of native hGH compared to all hGH species shows as Area%. The formulation with pH 5.4 (F4) had the highest proportion of native hGH while the other three formulations in pH 7 (F1-3) had approximately the same proportion of native hGH in the samples. F1 represents the formulation of hGH alone in pH 7. F2 represents the formulation of hGH with DDM in pH 7. F3 represents the formulation of hGH with SDS in pH 7. F4 represents the formulation of hGH with SDS in pH 5.4.*

Figure 10 provides a comparison of the amount of native hGH to the total amount of hGH species, in all formulations. Overall, hGH was degraded with a longer incubation time. The total area under the hGH peaks for the formulation of hGH alone and with DDM demonstrated a constant trend during the incubation, as seen in Figure 10a-b. This implies that the degraded products did not separate from the solution of the formulation of hGH alone and with DDM.

The total area for the formulation of hGH with SDS in pH 7 showed a slightly decreasing trend over incubation time. (Figure 10c) This means there was hGH aggregated and separated out from the formulation during incubation. As discussed above, for F3, a small number of particles were generated. Thus, it can be concluded that the hGH loss demonstrated by HPLC results was physically degraded products.

In the case of hGH formulation with SDS in pH 5.4, the total area was decreased over time indicating a loss of hGH from solution during incubation. This means there was hGH degraded and separated out from the solution. The hGH loss as seen in Figure 10d was in line with the results from FlowCAM and DLS analysis. The longer incubation time resulted in a higher particle number, larger particle size, and more hGH loss from the sample. As discussed above, the hGH formulation in pH 5.4 elaborated an advantage over formulations in pH 7 as providing the highest proportion of native structure of hGH compared to other formulations. However, this was concluded from a relative number as a percentage of native structure out of injected sample, which was already removed the white particles that formed after the SDS removal step. If compared with other formulations based on the total hGH that was initially added, the proportion of native hGH could be lower than illustrated in Figure 9. This implies that hGH loss from the formulation in pH 5.4 were physically degraded products. As this formulation had the largest amount of precipitated protein, there is a chance that a part of the precipitated protein was also chemically degraded. However, the extent of degraded protein lost to precipitation was unknown. Here, it was shown that storing SDS-hGH complexes at pH 5.4 could provide an advantage in chemical stability for unprecipitated hGH.

At this point, the results reflected the chemical degradation theory. The deamidation reaction was slower in more acidic pH; on the contrary, the isomerization reaction would be enhanced with more acidic pH. In the previous study[17], an hGH chromatogram had been identified with native hGH and related products. The peaks during 18-21 min were found as a product of the hydrolysis reaction. The peaks surrounding the native peak resulted from deamidation/isomerization.[17] From the chromatogram of hGH alone (Appendix 3, Figure 13) , native peak height was decreased by incubation time indicating that native hGH contained in the formulation of hGH alone was lower along the incubation. The hydrolyzed product, as detected at 18.5-19 min, and deamidated products, as seen as the peaks surrounding the native peak, were increasing along with incubation time.

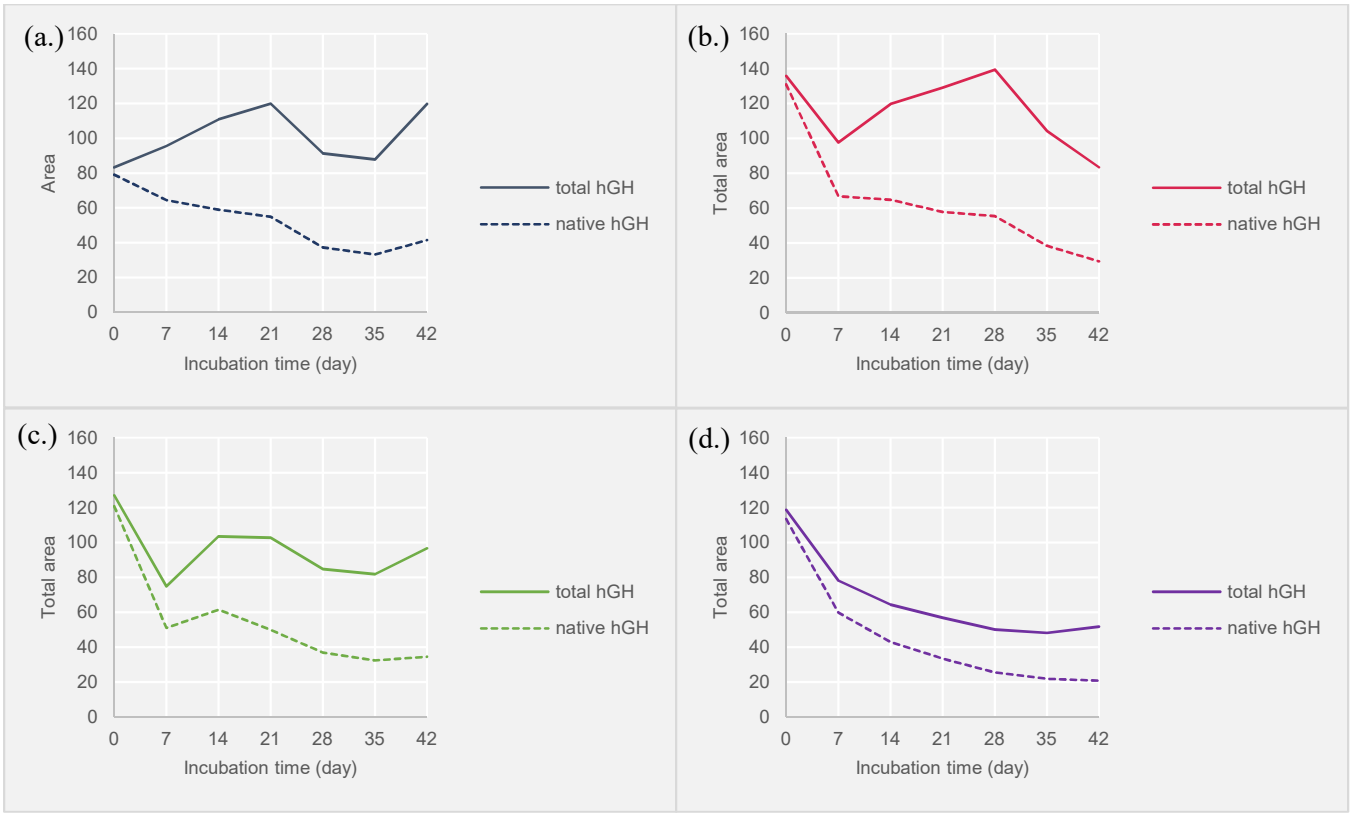

Figure 10. *Total Area under the hGH peaks with area under the native hGH peak measured by HPLC with UV detector at 280 nm from (a.) F1, (b.) F2, (c.) F3, and (d.) F4. F1 represents the formulation of hGH alone in pH 7. F2 represents the formulation of hGH with DDM in pH 7. F3 represents the formulation of hGH with SDS in pH 7. F4 represents the formulation of hGH with SDS in pH 5.4.*

However, the peak of hydrolyzed product became smaller for the hGH formulation with SDS in pH 7 and disappeared for the hGH formulation with SDS in pH 5.4. (Figure 11(b.)) This indicated that SDS could enhance hGH stability in the formulation with SDS in pH 7. However, there was even less hydrolyzed product as seen that the absence of a peak at pH 5.4. This probably resulted from the pH effect.

After storing for 42 days (Figure 11(a.)), a chromatogram for pH 5.4 consisted of 2 main peaks of degraded products, while the chromatograms for pH 7 formulations consisted of 3 main peaks of degraded products. At this point, it was obvious that the factor determining the degradation pattern was the pH of formulation. Due to the different components among pH 7 formulations, it was proven that the formulation, either with or without surfactant, did not change the pattern of hGH degradation in chemical stability aspect. Moreover, it was clear that multiple peaks during 27.5-29.0 min, which were deamidated/isomerized products, could not be detected in pH 5.4. The peak after the native peak was excessively lower in height compared to other formulations in pH 7. This was likely a consequence of a more acidic formulation that decreased the rate of deamidation. On the contrary, for the peak before native peak, it was taller than other formulations in pH 7 implying that this was increased from degraded products from isomerization.

(a.)

(b.)

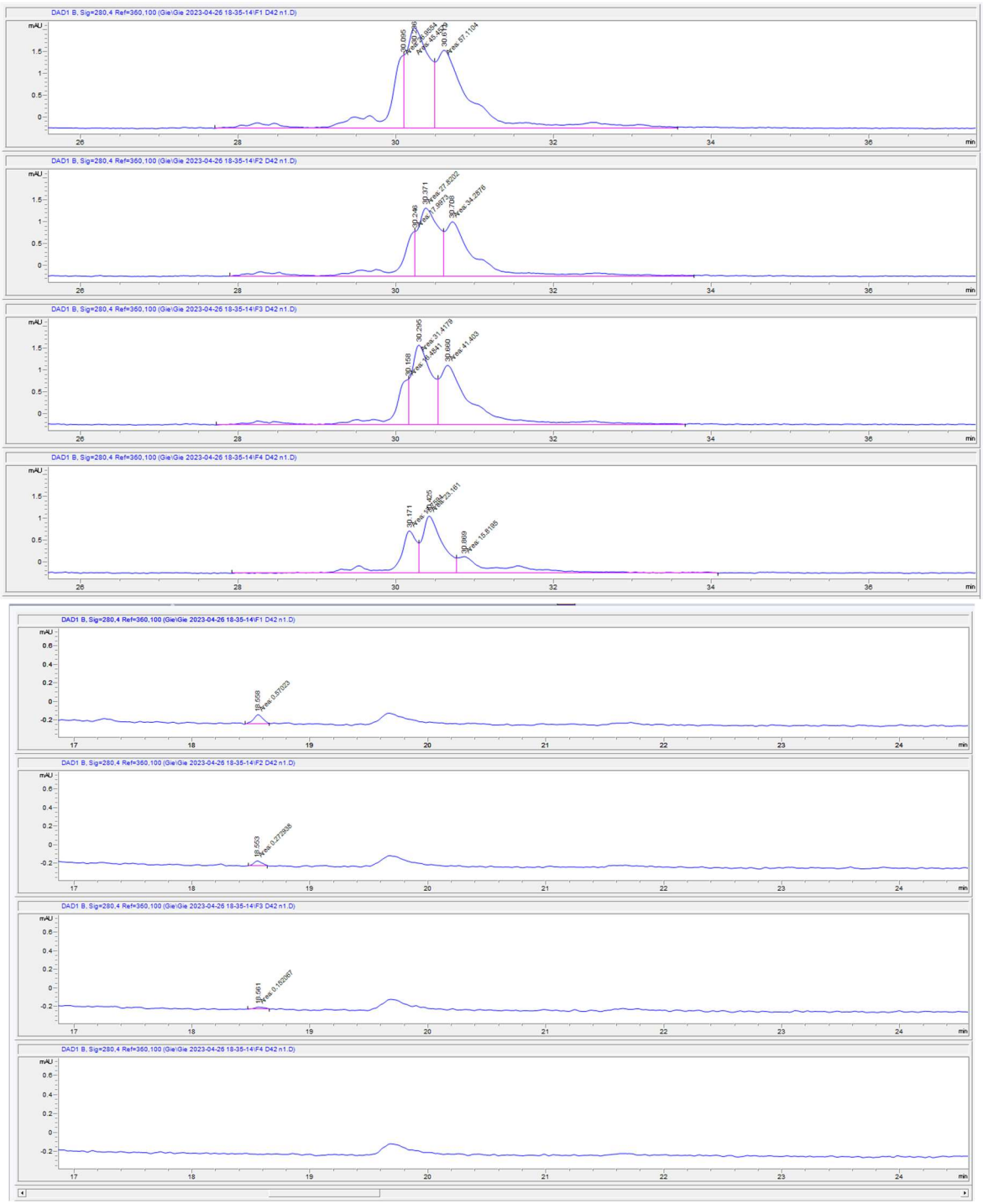

Figure 11. *Chemical degradation of hGH in different formulation detected with UV at 280 nm after incubation at 37°C for 42 days. F1 represents the formulation of hGH alone in pH 7. F2 represents the formulation of hGH with DDM in pH 7. F3 represents the formulation of hGH with SDS in pH 7. F4 represents the formulation of hGH with SDS in pH 5.4. The chromatogram pattern are the same for all formulations in pH 7; however, the pattern is different in pH 5.4. (a.) showed the peak of hGH native form as a middle area surrounded by deamidated/isomerized products. F4 had different pattern of peaks compared to other formulations. The peaks of deamidated/isomerized product during 28-29 minutes was obviously smaller than other formulations. (b.) showed hydrolyzed product. F1 had the largest peak area of hydrolyzed product, then F2 and F3 respectively. This peak was vanished in the case of F4.*

Table 3. *Summary of physical and chemical analysis*

	hGH alone in pH 7 (F1)	hGH with DDM in pH 7 (F2)	hGH with SDS in pH 7 (F3)	hGH with SDS in pH 5.4 (F4)
Visible particle	No	No	No	The solution became cloudy after adding DDM since day 7
Particle number from FlowCAM analysis	In the range of 29000 – 48000 particles/ml, except day 0	In the range of 25000 – 58000 particles/ml	In the range of 8000 – 17000 particles/ml	Extremely increased since day 7 (12669 particles/ml for day 0, then reached 3.4 million particles/ml on day 7)
Particle size from DLS analysis	Around 5 nm until day 42	6-9 nm until day 42	9-13 nm until day 42	10 nm on day 0 then increased 30 times during the first 7 days and reached the largest size on day 28
Line shape of Fluorescence spectrum	Same as day 0	Same as day 0	Same as day 0	Different since day 7
Native hGH proportion from HPLC analysis	Approximately the same as other pH 7 formulations*	Approximately the same as other pH 7 formulations*	Approximately the same as other pH 7 formulations*	The highest native proportion
hGH loss from HPLC analysis	No	No	Observed as a slight decrease	Clearly seen

**Approximately the same as other pH 7 formulations was defined from Area% of native hGH of all pH 7 formulations in each time point, as seen in Figure 9 for F1-3. The average of native hGH proportion were 94.92% ± 1.724% for day 0, 67.28% ± 2.939% for day 7, 55.58% ± 3.035% for day 14, 46.40% ± 2.067% for day 21, 41.28% ± 1.827% for day 28, 38.00% ± 1.294% for day 35, and 35.15% ± 0.671% for day 42.*

CONCLUSIONS

The stability of hGH with refolding procedure was established. The hGH was inherently physically stable in the formulation with citric acid/Na_2HPO_4 buffer at pH 7; however, chemical degradation of hGH in this formulation was observed, as 35% of hGH remained in its native structure after 42 days of storage at 37ºC. From a chemical stability point of view, DDM shows no effect on protecting hGH from deamidation or isomerization. With DDM, hGH had the same stability profile as hGH alone in both physical and chemical stability aspects. The refolding procedure using DDM on SDS-unfolded hGH successfully retrieved the intrinsic emission fluorescence spectrum of the native protein at pH 7. After this procedure, the formulation in pH 7 had mostly the same chemical degradation profile as other formulations at pH 7. For the formulation at pH 5.4, hGH was the most chemically stable. However, white particles were observed after adding DDM. The structure could not regain the native fluorescence spectra implying that hGH could not refold to the native structure. A lower amount of chemically degraded product was observed in pH 5.4 as less deamidation occurred, but more hGH was lost as precipitation. The different degradation patterns observed were caused by the change in pH.

FUTURE DIRECTIONS

This study is aimed to elaborate on the stability in the scale of weeks. The results show dramatic changes during the first 7 days, so it is recommended to investigate the stability of hGH during this first week at 25ºC to gain more details on this effect. As seen from both physical and chemical analysis, the most extensive difference was observed during this time frame. Thus, the stability test with a more frequent sampling time could provide more details. In addition, Nuclear magnetic resonance (NMR) spectroscopy should be done to study the electrostatic properties and conformation of the SDS-hGH complex in pH 5.4 in order to clarify the driving force of the white particle growing in F4 after removing SDS.

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APPENDICES

Appendix 1 Sample preparation protocol development

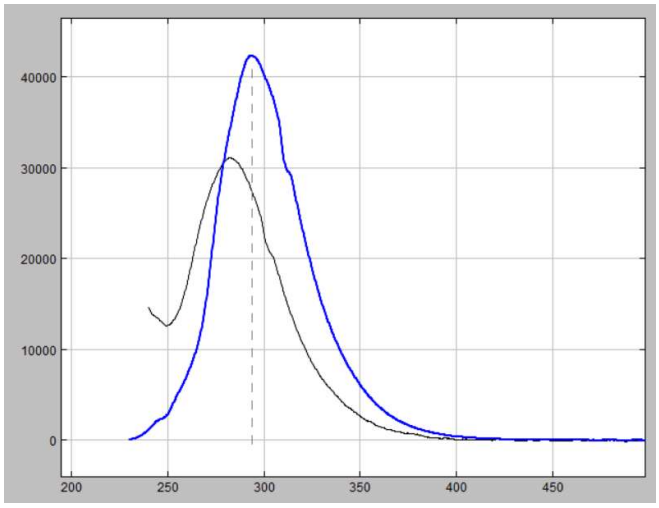

Figure 12. *Intrinsic Fluorescence spectra of hGH in a formulation with 5 mM SDS at pH 7 adding DDM solution to a final DDM concentration of 72.98 mM as direct addition (black) and titration addition (blue). The spectrum indicated a formation of large particle while this was not shown in the case of titration addition.*

Appendix 2 Average diameter measured by DLS

Table 4. Average particle diameter measured by Dynamic Light Scattering method in nm

	F1	F2	F3	F4
Day 0	5.06665	7.61551	9.34317	10.5115
Day 7	5.17549	7.52172	10.4324	312.145
Day 14	5.3027	7.09811	11.5006	5311.44
Day 28	NA	9.33073	13.0326	6087.62
Day 42	5.25115	6.13107	12.0198	3834.77

Appendix 3 Chromatogram

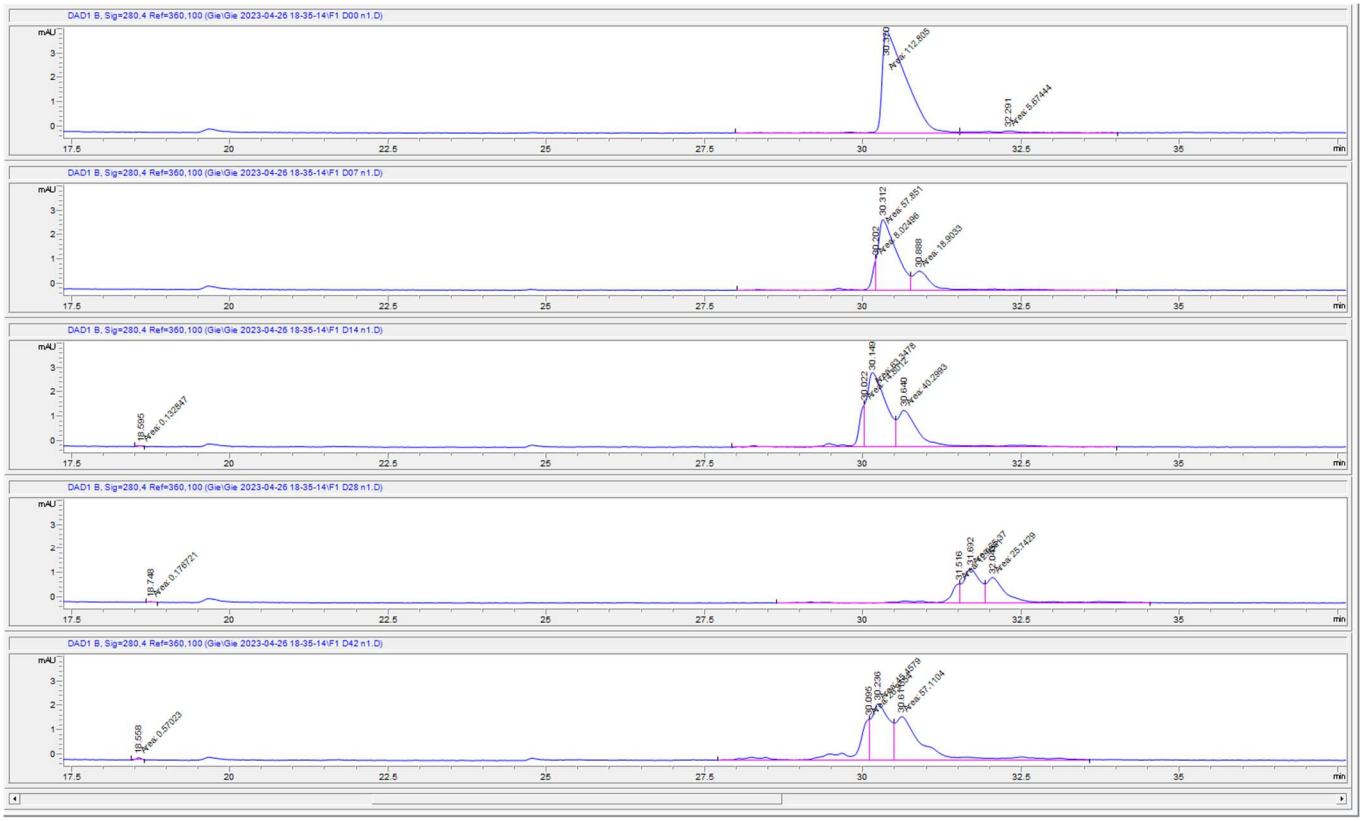

Figure 13. *Chemical degradation of hGH in F1 detecting with UV detector at 280 nm after incubation at 37°C on day 0, 7, 14, 28, and 42*

POPULAR LANGUAGE SUMMARY

The protein-based treatment provides an advantage with great specificity. This kind of product has been increasing in the pharmaceutical market recently. In the development of protein formulations, the addition of surfactants is commonly executed to enhance product stability and shelf-life. The reason is that surfactants can protect proteins against surface-induced aggregation. However, the interaction between surfactant and protein depends on the type of surfactant used. Anionic surfactants, like sodium dodecyl sulfate (SDS), can unfold proteins and form as complexes in equilibrium. The complex structure depends on the ratio of anionic surfactant to protein. On the other hand, nonionic surfactants, like n-dodecyl-β-D-maltopyranoside (DDM), can weaken interactions between anionic surfactants and proteins. Here, human growth hormone (hGH) was used. SDS in the SDS-hGH complex was slowly extracted to form mixed micelles with DDM. This gentle process allows the protein to refold back into its native form. From previous studies, the DDM/SDS ratio was important for the protein refolding procedure using a nonionic surfactant, regardless of mixing order. This study was aimed at assessing the stability of refolded hGH after being unfolded at 37°C for 42 days. Regarding hGH properties, deamidation was dominant in chemical degradation. One of the formulations was designed for less deamidation, at pH 5.4, to minimize other destabilizing effects compared to pH 7. After removing SDS, particle size and particle number were determined. The conformation of hGH was tracked by measuring the fluorescence spectra. In the physical stability aspect, the hGH formulation with SDS at pH 5.4 had the lowest stability of refolded hGH as it developed the most particle number and had the largest particle size. Moreover, the fluorescence spectra were different from the initial point. The trend of particle number was contradictory to particle size in pH 7 formulations. The chemical stability was evaluated by separating the native form of hGH from degraded products, which were then detected with an ultraviolet detector. By comparing the proportion of the native form, the formulation at pH 5.4 had the highest percentage. However, this formulation developed white particles after adding DDM during the SDS removal step.

In conclusion, the unfolded hGH can retrieve its native structure by using this refolding procedure at pH 7, but not in pH 5.4. The pH of the formulation had an impact on its physical and chemical stability. The more acidic pH reduces the deamidation rate, as seen in the chemical stability result. In the future, the stability study is recommended to be conducted at 25°C for 7 days to gain the most useful and informative data. Lastly, the complex structure of hGH with SDS in pH 5.4 should be investigated to better understand how pH affects degradation.