ACS OMEGA

http://pubs.acs.org/journal/acsodf

Article

Bioanalytical Workflow for Qualitative and Quantitative Assessment of Hot-Melt Extruded Lysozyme Formulations

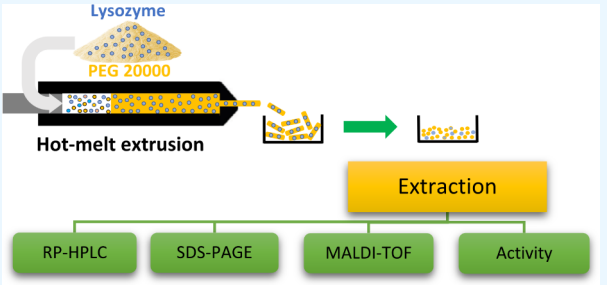
Yomnah Y. Elsayed, Toni Kühl, Katharina Dauer, Alina Sayin, Karl G. Wagner, and Diana Imhof*

Cite This: *ACS Omega* 2022, 7, 40836−40843

Read Online

ACCESS | Metrics & More | Article Recommendations | Supporting Information

ABSTRACT: Structural and functional integrities of formulated proteins are key characteristics that provide a better understanding of influencing factors and their adjustment during formulation development. Here, the procedures commonly used for protein analysis were applied and optimized to obtain a higher degree of accuracy, reproducibility, and reliability for the analysis of lysozyme extracts from hot-melt extrudates (HME). The extrudates were prepared with polyethylene glycol 20 000. The test lysozyme HMEs were subjected to extraction procedures and analytical methods following the International Council of Harmonization guidelines for testing the active protein ingredient Q 1 A (R2) in its pure and formulated form. Therefore, reversed-phase high-pressure liquid chromatography, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, matrix-assisted laser desorption ionization mass spectrometry, and fluorescence-based activity measurements were applied to study lysozyme stability and function after formulation. Long-term accelerated stability studies were performed for the pure and formulated protein. Our findings revealed a high degree of stability for lysozyme toward different temperatures and storage times, confirming that HME is a suitable formulation alternative that preserves lysozyme's properties and stability. The presented methods and workflow are recommended to be exploited for further protein drugs to assess usability and compatibility concerning different pharmaceutical applications.



INTRODUCTION

The protein therapeutics market has been growing for years, and the number of approved peptides and proteins has dramatically increased over the last decade.[1−3] The formulation of protein drugs, however, is still highly challenging because of the sensitive nature of proteins and their high propensity to denaturation and aggregation.[4] Upon formulation, multiple factors need to be considered in order to maintain the proteins' stability, that is, structural and functional integrity. Before expanding to large-scale processing, several bench-scale studies are required. Therefore, many proteins were selected and tested as models to study the effect of different formulation techniques and their process parameters (such as temperature, applied pressure, or processing time) on protein stability, properties, and activity.[5]

Lysozyme, that is, *N*-acetylmuramide glycanhydrolase or muramidase, is an enzyme that is commonly used for pharmaceutical formulations by different techniques such as freeze drying,[6,7] hot-melt extrusion,[8,9] and numerous other polymer-based techniques as a control.[10−12] Lysozyme cleaves *β*-1,4-glycosidic bonds between *N*-acetylmuraminic acid and *N*-acetylglucosamine. Testing of the enzyme activity at different stages of processing has been reported to provide information about the effects of formulation additives, preparation methods, or manufacturing procedures on the quality of the formulated active ingredient,[13−16] and its stability was considered as an indicative parameter for formulation evaluation. Analysis data for lysozyme allow for a better understanding of the extent to which the additives or applied procedures could impair the proteins' properties during the processing pathway. Therefore, the presence of stability evaluation protocols is urgently needed to support the development of formulation strategies for protein drugs.

In this study, we investigated the impact of the process of hot-melt extrusion (HME) using polyethylene glycol (PEG) 20 000 on the quality attributes of lysozyme and optimized the recovery and activity-testing methods. To test lysozyme formulation, the HME technique was used as a technology that is used in the pharmaceutical industry as a continuous and robust manufacturing tool for the preparation of solid dosage forms.[17] It is advantageous concerning taste masking, enhanced bioavailability, variability of medical applications, and drug targeting options.[18] For HME, the target is mixed with certain polymers and optionally plasticizers, and heated at

Received: June 7, 2022
Accepted: September 7, 2022
Published: November 1, 2022

https://doi.org/10.1021/acsomega.2c03559
ACS Omega 2022, 7, 40836−40843

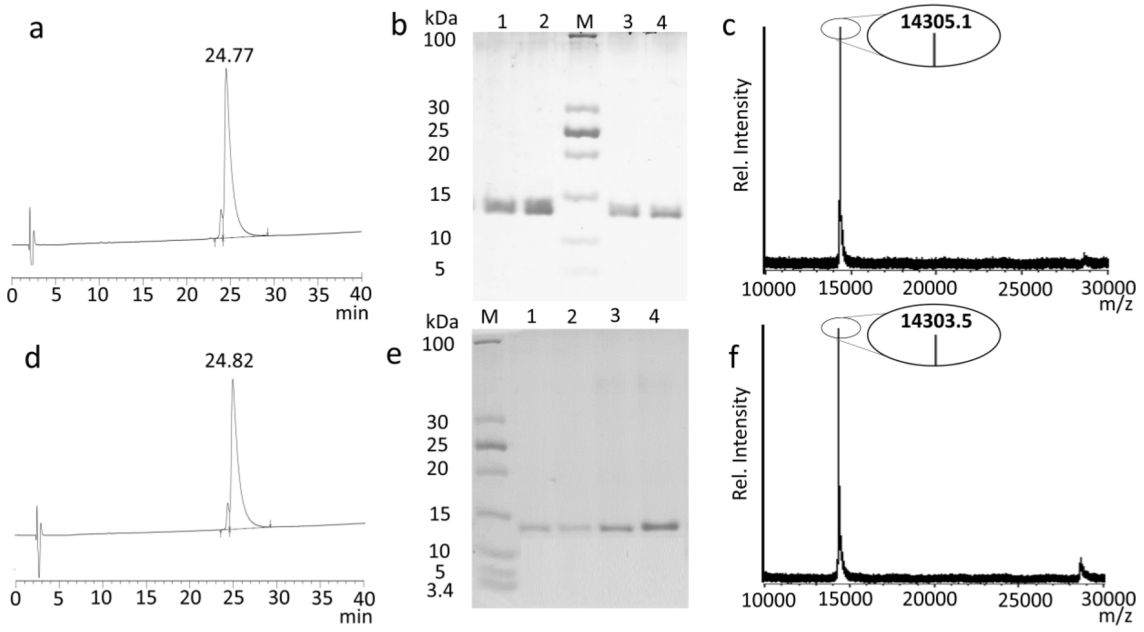


Figure 1. (a) HPLC chromatogram of 150 $\mu g \cdot mL^{-1}$ lysozyme in phosphate buffer (pH 5.2) using, as the mobile phase, acetonitrile/water /0.1% TFA (each) 20 to 60% for 40 min (b) SDS-PAGE analysis of 1 μg pure lysozyme under nonreducing conditions (lanes 1, 2) and reducing conditions (lanes 3, 4). M is the protein marker covering 3.4−100 kDa. (c) MALDI-TOF mass spectrum of pure lysozyme using 2,5-DHB as the matrix. (d) HPLC chromatogram of extracted H-Lyso/PEG 20 extrudates in phosphate buffer (pH 5.2, ∼50 μM) using the same mobile phase as in (a). (e) SDS-PAGE analysis of 1 μg extracted lysozyme (lanes 1, 2) and H-Lyso/PEG 20 extrudates (lanes 3, 4). M is the protein marker covering 3.4−100 kDa. (f) MALDI-TOF mass spectrum of extracted H-Lyso/PEG 20 extrudates using 2,5-DHB as the matrix.

different stages to be molten and mixed properly. The preparation of implants for sustained release of proteins by HME is described in previously published works[19,20] Cossé et al.[19] confirmed the applicability of the HME process to prepare homogeneous solid dispersions of lysozyme, BSA, or a monoclonal antibody in a PLGA-matrix. Here, we used lysozyme-loaded extrudates to establish methods and a workflow for protein stability assessment.

For analysis, the enzyme HMEs were extracted and examined for protein stability and activity over different storage periods and conditions. In accordance with the guidelines of the International Council of Harmonization (ICH), it is required to test the active ingredients of a protein drug formulation in their pure and formulated form. We followed the recommendations of ICH Topic Q 1 A (R2)[21] to examine the stability of lysozyme after exposure to different stress conditions either by the formulation process or the long-term storage conditions.

International organizations such as Food and Drug Administration (FDA) and European Medicines Agency stated different guidelines to control the quality of formulated biological products. According to the recent draft of ICH Q 14,[22] the established procedures could be optimized to fulfill the regulatory requirements. FDA guidances[23,24] described purity, identity, and biological activity as the main parameters for the evaluation of a biopharmaceutical product. Therefore, the selected parameters to test the stability, yet structural and functional integrity of lysozyme before and after formulation were evaluated using electrophoretic [sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)] and chromatographic [reversed-phase high-pressure liquid chromatography (RP-HPLC)] techniques, as has been successfully applied for lysozyme quantification in the past[8,9,16,25] and well known for their robustness. Furthermore, the commonly used turbidimetric assay for determining lysozyme activity with *Micrococcus lysodeikticus*[26] was examined for its validity in the approach. However, due to inconsistency of the data, it was finally substituted by a herein-adapted spectrofluorimetric assay using a chemical probe as the substrate with the aim to ensure robustness, reproducibility, and reliability of the activity measurements. The preservation of other protein characteristics such as molar mass was concurrently studied employing matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry. This study provides a suitable workflow for the qualitative and quantitative assessment of a protein following its processing by an established drug formulation technique. It has the potential to be exploited in a similar way for other protein drugs in the future, yet demonstrating the urgent need for suitable and adapted test systems for activity measurements of a specific protein to be formulated.

EXPERIMENTAL SECTION

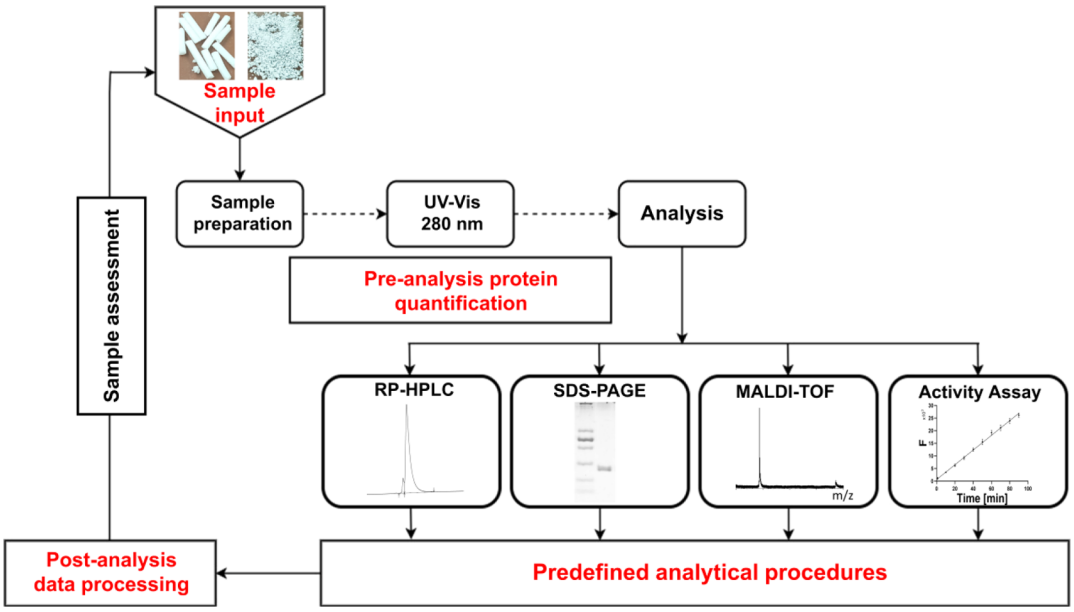
Materials. The applied chemicals were of analytical grade quality. Further details about the chemicals are given in Supporting Information S1.

Formulation by Hot-Melt Extrusion. Lysozyme was formulated as described in Supporting Information S2.

Extraction Procedures. Different solvents (water, acetonitrile, ethanol, *n*-propanol, isopropanol, acetone, chloroform, dichloromethane, and diethylether) were used to test lysozyme and PEG 20 000 solubilities (Table S1). Acetonitrile was then selected for extraction. A suitable volume of acetonitrile (approx. 500 μL per 5 mg HME or 1 mg lysozyme) was added to the samples, mixed, and centrifuged for 10 min at 13 000 rpm at room temperature. The pellets (if available) were washed 2 times with the solvent and finally dried on a rotary vacuum concentrator (SpeedVac) at 30 °C for 30 min. The

https://doi.org/10.1021/acsomega.2c03559
ACS Omega 2022, 7, 40836−40843

Scheme 1. Workflow for the Applied Analysis Methods on Hot-Melt Extrudates.



dried lysozyme samples were then dissolved in 0.1 M sodium phosphate buffer pH 5.2 to give a concentration of 120 μM.

The lysozyme concentration was pre-estimated spectrophotometrically by measuring the absorbance at 280 nm using an extinction coefficient of 2.69 mL mg^{-1} cm^{-1}[27] using a Multiskan Go spectrophotometer (Thermo Scientific). Further dilution to 50 μM working solution was performed with buffer. These solutions were used for HPLC content quantification as well as for the activity assay.

HPLC Quantification. Analysis of lysozyme concentrations from solutions was performed on a Shimadzu LC-20AD chromatograph equipped with a Vydac 218TP54 reversed-phase C18 column (particle size 5 μm, 300 Å pore size, 4.6 × 250 mm) at 25 °C. Lysozyme was eluted using a linear gradient from 20−60% of solvent B [0.1% trifluoroacetyl (TFA) in acetonitrile] in solvent A (0.1 % TFA in water) for 40 min at a flow rate of 1 mL/min. Detection was performed at 220 nm, and the injection volume was 50 μL. A major peak was observed (Figure 1a) for lysozyme (original sample), and the sum of the peak areas of the observed peaks was calculated.

Amino Acid Analysis. The protein content of samples was confirmed by amino acid analysis as described in earlier studies.[28,29]

Activity Assay. The activity of lysozyme was evaluated using two methods: turbidimetric and fluorogenic assays. The turbidimetric assay was performed as described by the manufacturers' procedure (Merck) based on Shugar[26] and The lytic activity of lysozyme on *Micrococcus lysodeikticus* was determined by scanning the optical density changes of the bacterial culture over 3 min at 450 nm after lysozyme addition at 25 °C (dissolved in 0.1 M phosphate buffer pH 6.2), then the specific activity was calculated accordingly (One unit is defined as the change of 0.001 in absorbance per minute).

The fluorogenic assay was performed according to Yang Hamaguchi.[27] 4-Methylumbelliferyl-β-D-*N*,*N*′,*N*″-triacetylchitotrioside ($(GlcNAc)_3$-MeU) was used as a substrate for lysozyme. For Michaelis−Menten kinetics, a substrate stock solution of 500 μM $(GlcNAc)_3$-MeU in 0.1 M sodium phosphate buffer pH 5.2 was initially prepared and diluted correspondingly. A fixed final concentration of lysozyme of 10 μM was incubated at 42 °C with different concentrations of substrate (5−100 μM). Reactions were stopped by the withdrawal of aliquots from the mixtures and a 1:6 dilution with stopping buffer (0.1 M glycine buffer pH 12.0) after different reaction times (1−60 min). Samples were transferred into a cuvette and fluorescence was measured at an excitation wavelength of 380 nm and an emission wavelength of 460 nm using a Spectrofluorimeter FP-8300 (JASCO, Germany). The obtained fluorescence values were converted into product concentrations based on a calibration curve that was obtained upon measurement of a serial dilution of 4-methylumbelliferone in glycine buffer pH 12.0 prior to the experiments (Figure S1). Data were plotted to standard Michaelis−Menten kinetics using GraphPad Prism 7.0.

For the activity determination, samples, as well as controls, were applied in a similar setup with slight modifications. A substrate concentration of 100 μM and a lysozyme concentration of 5 μM were used for the activity assay. Diluted aliquots of enzyme−substrate mixtures were withdrawn and diluted directly with the stopping buffer (ratio 1:6) at different time intervals (1−90 min), then 200 μL were transferred into an Optiplate 96 well plate. Fluorescence was recorded on a PerkinElmer Victor 3 1420 multilabel counter fluorimeter at an excitation wavelength of 355 nm and emission wavelength of 460 nm. Fluorescence values were converted into product concentrations and plotted against time for activity determination.

Subsequent determination of the sample's specific activity in U/mg was performed in correlation to contents obtained by the HPLC method. As controls, aliquots of lysozyme stored at −20 °C were treated using the same procedures as the tested samples, and the results of each storage time point were compared to those of the controls and set as 100%.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis. One dimensional SDS-PAGE and following Coomassie blue staining was performed as described by the Laemmli gel method.[30,31] A description of gel casting can be found in Supporting Information S3 and Table S2.

https://doi.org/10.1021/acsomega.2c03559
ACS Omega 2022, 7, 40836−40843

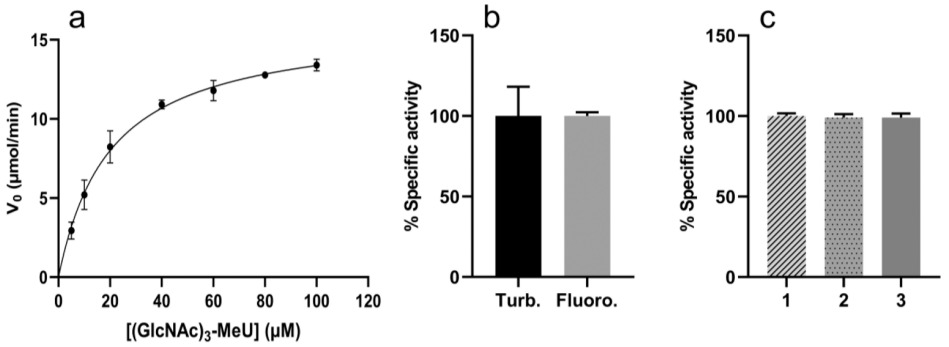


Figure 2. (a) Michaelis–Menten kinetics diagram for lysis of $(GlcNAc)_3$-MeU by lysozyme in 0.1 M sodium phosphate buffer, pH = 5.2 at 42 °C using GraphPad Prism 7 software (n = 3, $K_M = 2.02 \times 10^{-5}$ M). (b) % Specific activity of pure unextracted lysozyme samples by turbidimetric (n = 12) and fluorogenic (n = 6) methods. (c) % Specific activity of pure, unextracted (1) considered as 100%; pure, extracted (2); and formulated, extracted (3) lysozyme samples, (n = 6, each) using the fluorogenic activity assay.

MALDI-TOF Mass Spectrometry. The molecular masses of lysozyme samples were analyzed using saturated 2,5-dihydroxybenzoic acid (2,5-DHB) mixed with sodium trifluoroacetate (1 mg/mL) as the matrix. The dried crystallized samples were analyzed by an UltrafleXtreme TOF/TOF mass spectrometer (Bruker Daltonics GmbH, Bremen, Germany). Details for sample and matrix preparation are given in Supporting Information S4.

RESULTS AND DISCUSSION

Method Selection and Optimization. Typically, standard procedures for the analytical characterization of proteins cover methods such as SDS-PAGE, mass spectrometry, various chromatographic and spectroscopic methods, and functional studies among others.[5,32] For our study, to address the FDA guidelines, we aimed to utilize robust and reproducible techniques to evaluate parameters such as purity, identity, quantity, and activity for lysozyme before and after formulation as well as for stored formulated lysozyme samples. For this reason, we investigated two different activity assays, that is a turbidimetric assay and a fluorogenic assay, as well as SDS-PAGE and RP-HPLC analysis for purity determination. The latter was concomitantly used for quantification of the protein concentration. Finally, the molecular weight of lysozyme was determined by MALDI-TOF mass spectrometry. The workflow established in this study is shown in Scheme 1. Although the aforementioned methods sufficiently cover the bioanalytical characterization of sequence identity, purity, quantity, and activity of lysozyme, the introduction of forced degradation studies would allow for polishing the complex methodological validation of the herein-described methods and will be recommended for less stable proteins.

Chromatographic elution of lysozyme by RP-HPLC was already studied earlier,[13] and provided comparable results to our approach. The main peak at 24.7 ± 0.2 min representing lysozyme as confirmed by MALDI-TOF mass spectrometry (Figure 1a,c), could be quantified accordingly (Figure S2). The quantification range was found to be linear over a concentration range of 50–500 $\mu g \cdot mL^{-1}$, which matched the reported methods.[25] The method's accuracy was tested using pure lysozyme samples and provided a protein recovery rate of 99.7 ± 1.56% (n = 6) for pure unextracted lysozyme. All data were obtained after correction of the weighed mass by the protein content determined by amino acid analysis for the applied lysozyme batch. Regarding MALDI-TOF MS measurements, matrix selection was based on the requirement for simultaneous detection of the protein and the polymer for formulated samples. 2,5-DHB was suggested in many studies as a promising matrix for the detection in presence of contaminants,[33,34] while the addition of sodium trifluoroacetate was described for the analysis of polymers, such as PEG, in formulated protein samples.[35]

Analysis by SDS-PAGE (15% w/v acrylamide in the resolving gel) at reducing and nonreducing conditions always yielded a clear single band for each sample between 10 and 15 kDa (~14 kDa) without indication of impurities (Figure 1b). The reducing conditions showed linear lysozyme and were adapted in this study following a literature report.[32]

The lysozyme activity was tested using the common turbidimetric assay,[26] in which a bacterial culture is incubated with the enzyme solution. The hydrolytic activity was monitored by changes in the optical density of the bacterial culture. The average specific activity obtained was 15836.5 ± 2885.4 U/mg (RSD = 18.2%, n = 12). However, high standard deviations were observed frequently for the bacterial assay method and led to unreliable, unsuitable activity assessments. Besides the biological hazard, observed data fluctuations affected the analysis robustness, and the turbidimetric assay would not be effectively extrapolated for the extrudate analysis. The findings from the bacterial assay raised the need for a more stable assay method. Therefore, the fluorogenic activity assay earlier suggested by Yang et al.[27,36] was taken into consideration. A recent study applied it for lysozyme activity evaluation,[37] and it was adapted to the present study. The assay was performed using the substrate 4-methylumbelliferyl-β-D-N,N',N''-triacetyl-chitotrioside ($(GlcNAc)_3$-MeU) at pH 5.2 to maximize substrate binding to lysozyme.[36] Initially, the kinetics for the hydrolysis of $(GlcNAc)_3$-MeU was studied (Figure 2a). The Michaelis–Menten plot provided a K_M value of 20.2 ± 1.2 μM for this conversion, as shown in Figure 2a, which is in good agreement with previously reported data (K_M = 15.9 μM).[36] For the following activity determination studies, a concentration of 100 μM of $(GlcNAc)_3$-MeU (5× K_M) was applied to assure sufficient substrate excess in all experiments. To assess the stability of the fluorogenic activity assay, the average specific activity for pure lysozyme was measured as 2565.5 ± 48.8 U/mg (RSD = 1.9%, n = 6). The obtained results showed a significantly higher degree of reproducibility indicated by a lower standard deviation (1.9%) compared to the aforementioned bacterial turbidimetric assay (18.2%) (Figure 2b). Not only did it present a more accurate alternative for lysozyme activity determination, but additionally

https://doi.org/10.1021/acsomega.2c03559
ACS Omega 2022, 7, 40836–40843

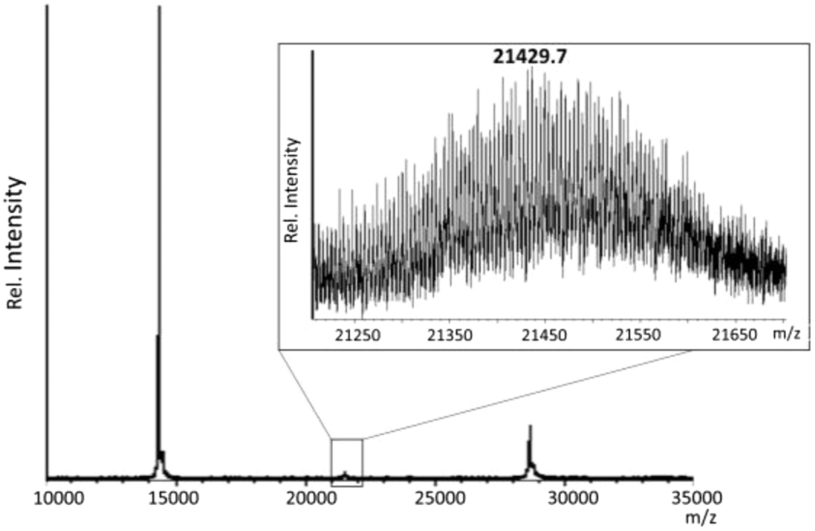


Figure 3. MALDI-TOF mass spectra of unextracted H-Lyso/PEG20 sample showed a lysozyme peak around 14 310 Da, PEG 20 000 was observed around 21 500 Da using 2,5-DHB as the matrix.

eliminated the need for preparing a nonhomogenous bacterial cell solution. Consequently, for all further analyses in this study, only the fluorogenic assay was applied. Besides, the effect of PEG 20 000 on the lysozyme response was evaluated, and no significant influence was found (Figure S3).

Analysis of Formulated Lysozyme Samples before Storage. The established preparative and analytical procedures were applied to analyze hot-melt extrudates which contained 20% (w/w) lysozyme and 80% (w/w) PEG 20 000 (abbreviated as H-Lyso/PEG20) as well as an untreated physical mixture of lysozyme and the polymer (abbreviated as *P*-Lyso/PEG20).

As a control, pure lysozyme was always analyzed in parallel for the activity studies (abbreviated as P-Lyso). For analytical characterization of the samples, in particular, if applying fluorometric, MALDI-TOF, or SDS-PAGE, it is required to remove the polymer to the extent possible by extraction of lysozyme after proper grinding of the sample batch. Different solvents were tested for lysozyme extraction and acetonitrile provides reproducible good recoveries; therefore, it was selected as the solvent of choice (Supporting Information S5). Following the extraction of lysozyme with acetonitrile from the extrudates, lysozyme pellets were dried to evaporate residual acetonitrile to receive lysozyme ready for further analysis. According to the method to be applied, the corresponding solvent was selected, for example, 0.1 M sodium phosphate buffer (pH 5.2) for HPLC content analysis and following fluorogenic activity assay (performed with the same sample), double distilled water for SDS-PAGE and MALDI TOF MS, with the sample for the latter being freeze-dried and taken up with 30% acetonitrile/0.1% TFA prior to measurements. This procedure was chosen in order to avoid salt contamination, for example, MS analysis. The results of the chromatographic runs of extrudates at time zero (Figure 1d) showed the same elution pattern as pure lysozyme (Figure 1a), the resulting data was thus used for content and recovery determination. The obtained recovery rate for the extrudates was 99.9 ± 3.6% (n = 3) of the expected contents. SDS-PAGE gels showed a single band around 14 kDa as well as a peak around 14 300 Da in MALDI-TOF mass spectra (Figure 1e,f), indicating the identity of lysozyme. Unextracted H-Lyso/PEG20 extrudates were tested by MALDI-TOF and showed an additional peak around 21 500 for PEG 20 000 (Figure 3). The presence of polymer led to interference in MALDI-TOF analysis of many unextracted lysozyme extrudates that emphasized the importance of enzyme extraction before further analysis. Concomitantly, the specific activity of the enzyme was observed to be retained after formulation and extraction (100.3 ± 4.1%, n = 3, Figure 2c, column 3) compared to unextracted and extracted control lysozyme (P-Lyso, Figure 2c, column 1 and 2, respectively).

In addition, a mixture of lysozyme and PEG 20 000 (P-Lyso/PEG20) was prepared (physically mixed without further processing by hot-melt extrusion) to test formulation effects on lysozyme characteristics against unformulated lysozyme. Aliquots of P-Lyso/PEG20 were analyzed using the established methods and the results were compared to H-Lyso/PEG20 and extracted control lysozyme (P-Lyso). Comparable results for content recovery, quality, and activity were observed for P-Lyso/PEG20 and P-Lyso in comparison to H-Lyso/PEG20 (Figures S4 and S5), as well as a mass band around 14 kDa in SDS-PAGE (data not shown) and a peak at around 14 300 Da in the MALDI-TOF mass spectra (Table S3). Expected results for purity, identity, quantity, and activity for all measured samples were determined and confirmed for lysozyme in the investigated samples. Thus, the analysis results demonstrate the reliability of the methods and the established workflow for application to the lysozyme formulations. Hot-melt lysozyme extrudates were shown to have the same characteristics as pure lysozyme or physical mixtures thereof with polymer without interference from stress parameters during the extrusion, mixing, and/or extraction process.

Application of the Bioanalytical Workflow to Stored Extrudates (Accelerated Stability Studies). To evaluate the long-term effects on lysozyme upon formulation by hot-melt extrusion, H-Lyso/PEG20 and P-Lyso/PEG20 samples were stored simultaneously with pure untreated lysozyme (P-Lyso). The ICH guidelines state three temperatures (4, 25, and 40 °C) for accelerated stability studies for a duration of 6 months that were adapted and applied to hot-melt extrudates of lysozyme and PEG20 as described above.[21] The selected time intervals for sample evaluation were day 0, week 2, week

https://doi.org/10.1021/acsomega.2c03559
ACS Omega 2022, 7, 40836−40843

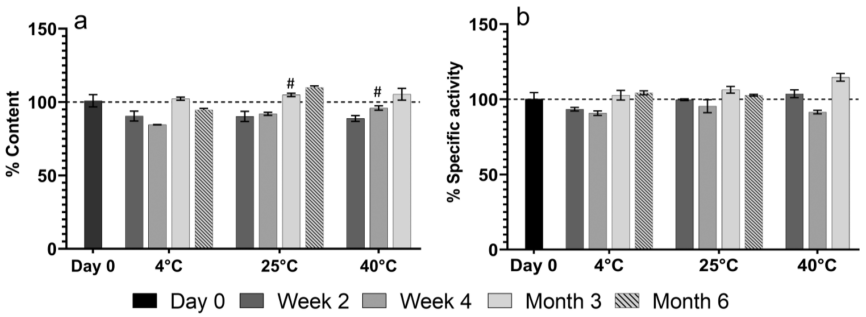


Figure 4. Content recovery rates (a) and % specific activities (b) of extracted H-Lyso/PEG20 extrudates stored at 4, 25, and 40 °C. Samples were analyzed at the following time points: day 0, week 2, week 4, month 3, and month 6 using the optimized RP-HPLC and the fluorogenic activity assay (n = 3 and # n = 2). Specific activity was calculated as % to that of pure lysozyme stored at −20 °C.

4, month 3, and month 6. At each time point, the samples from each temperature approach were withdrawn and subjected to subsequent analysis by the presented methods and workflow (Scheme 1). Through the storage time interval, the quality of the extrudates H-Lyso/PEG 20 was evaluated versus the control P-Lyso and the physical mixture P-Lyso/PEG20. Recovery rates of extrudates were determined over the 6 months storage period at the three selected temperatures to be 84.6−110.0, RSD ±4.2% (n = 3 at each sample point) (Figure 4a). Standard deviation in this range of the lysozyme determined can be justified upon consideration of the salt content and the hygroscopic nature of lysozyme that may affect the original preparation of the extrudates. Activity assays for the same set of samples showed full activity over the tested storage time (97.7−104.9, RSD ±4.7%, n = 3) (Figure 4b). P-Lyso and P-Lyso/PEG20 were additionally stored and tested at the same time and temperature points. Full activity of 98.0−105.1 RSD ±3.1% (n = 3) and 90.2−110.6, RSD ± 4.3% (n = 3) was observed for these samples (Figures S4 and S5, respectively) without any significant changes in the recovery rates. All SDS-PAGE gels for the H-Lyso/PEG20, P-Lyso/PEG20, and P-Lyso samples showed the lysozyme band in its molecular mass range without extra bands for degraded fragments of the protein (Figure 5). Mass spectra for the H-Lyso/PEG20, P-Lyso/PEG20, and P-Lyso control analysis by MALDI-TOF MS were obtained as described above. No changes in mass could be observed for any of the samples.

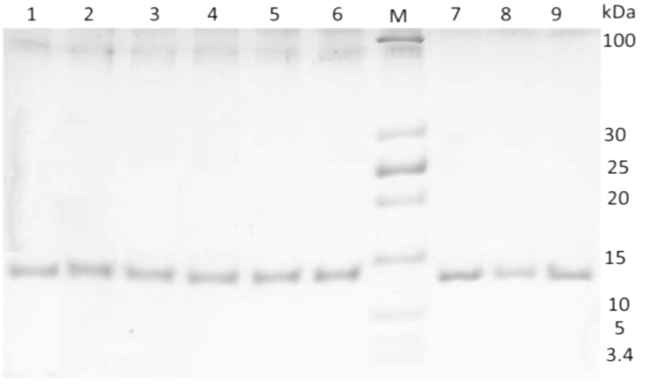


Figure 5. SDS-PAGE gel for 3 months-extracted H-Lyso/PEG20 samples at 4, 25, and 40 °C (lanes 1,2 and 3, respectively), extracted P-Lyso/PEG20 at 4, 25, and 40 °C (lanes 4, 5, and 6, respectively), and extracted P-Lyso samples at 4, 25, and 40 °C, (lanes 7, 8, and 9, respectively) showing a band for lysozyme around 14 kDa, M is the protein marker covering a mass range of 3.4−100 kDa.

Findings for pure, hot-melt, or physically mixed lysozyme demonstrated maintenance of characteristics over the storage interval without evidence of degradation or alteration in enzyme activity.

Consequently, lysozyme stability in its pure or hot-melt extruded form with PEG 20 000 at different temperatures could be confirmed according to ICH Topic Q 1 A (R2) guidelines. It was observed that not only lysozyme as a pure protein has good stability up to 40 °C for 6 months but also external influences due to hot-melt extrusion (including temperatures up to 65 °C) as well as different storage conditions did not affect the stability of the protein.

CONCLUSIONS

The present study investigated the methods used for standard lysozyme analysis in pharmaceutical formulations and optimized them for better accuracy, robustness, and reproducibility. The procedures were applicable for hot-melt extruded formulations of lysozyme as a negative control prepared to test the effects of extrusion parameters such as shear stress or heating as well as storage at different temperatures on the target protein characteristics. Four analysis methods were selected as suitable techniques in terms of the determination of purity, identity, quantity, and activity of lysozyme, that is, RP-HPLC, SDS-PAGE, MALDI-TOF mass spectrometry, and a fluorogenic activity assay. The latter test system proved to be favorable compared to the unreliable turbidity assay. The presented workflow and methods can be considered a good analytical toolbox for reliable evaluation of lysozyme stability under stressful conditions. Although the described methodological setup analyzes most of the standard features of proteins such as identity, purity, quantity, and activity, structural changes of lysozyme during the treatment cannot be excluded. Additional techniques such as CD spectroscopy, NMR spectroscopy, crystallography, and/or a careful analysis of disulfide bonds should be included to cover this aspect in the future, too.

The treatment of the herein-investigated lysozyme by hot-melt extrusion was verified to be harmless for lysozyme over the studied storage conditions and provided stable formulations over their shelf lives. This study presents a routine for the analytical workflow of hot-melt extruded lysozyme. Whether this can be applied to other hot-melt extruded pharmaceutically relevant protein drug candidates remains elusive and will be investigated in the future.

https://doi.org/10.1021/acsomega.2c03559
ACS Omega 2022, 7, 40836−40843

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.2c03559.

Detailed information of used chemicals and materials, formulation procedures, SDS-PAGE gel preparation, MALDI-TOF sample application, extrudates extraction procedures, calibration curves for HPLC and fluorogenic methods, response curve for PEG 20 000, analysis results of *P*-Lyso and *P*-Lyso/PEG 20, solubility testing for PEG 20 000 and lysozyme, SDS gels and buffers composition, and summary of MALDI masses for all analyzed samples (PDF)

■ AUTHOR INFORMATION

Corresponding Author

Diana Imhof − *Department of Pharmaceutical Biochemistry and Bioanalytics, Pharmaceutical Institute, University of Bonn, Bonn D-53121, Germany;* orcid.org/0000-0003-4163-7334; Email: dimhof@uni-bonn.de

Authors

Yomnah Y. Elsayed − *Department of Pharmaceutical Biochemistry and Bioanalytics, Pharmaceutical Institute, University of Bonn, Bonn D-53121, Germany; Department of Pharmaceutical Analytical Chemistry, Faculty of Pharmacy, Ain Shams University, Cairo 11566, Egypt*

Toni Kühl − *Department of Pharmaceutical Biochemistry and Bioanalytics, Pharmaceutical Institute, University of Bonn, Bonn D-53121, Germany;* orcid.org/0000-0002-6822-1585

Katharina Dauer − *Department of Pharmaceutics, Pharmaceutical Institute, University of Bonn, Bonn D-53121, Germany;* orcid.org/0000-0002-2661-1083

Alina Sayin − *Department of Pharmaceutical Biochemistry and Bioanalytics, Pharmaceutical Institute, University of Bonn, Bonn D-53121, Germany*

Karl G. Wagner − *Department of Pharmaceutics, Pharmaceutical Institute, University of Bonn, Bonn D-53121, Germany*

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.2c03559

Author Contributions

Y.Y.E and T.K contributed equally. The manuscript was written through the contributions of all authors. D.I. and K.G.W. designed the experimental studies with the help of Y.Y.E, K.D., and T.K. The optimization for the analytical experiments was performed by Y.Y.E and A.S. K.D. formulated lysozyme into hot-melt extrudates or physical mixtures. Y.Y.E executed the analyses of stability of the samples and analyzed the data together with D.I and T.K. All authors discussed the results and contributed to the final manuscript.

Funding

The work was supported by The Deutsche Forschungsgemeinschaft (DFG) within priority program SPP 1934 under project number 425781873 (to D.I., T.K., and K.G.W.).

Notes

The authors declare no competing financial interest.

■ REFERENCES

(1) Leader, B.; Baca, Q. J.; Golan, D. E. Protein Therapeutics: A Summary and Pharmacological Classification. *Nat. Rev. Drug Discovery* **2008**, *7*, 21−39.

(2) Al Musaimi, O.; Al Shaer, D.; Albericio, F.; de la Torre, B. G. FDA TIDES (Peptides and Oligonucleotides) Harvest. *Pharmaceuticals* **2021**, *14*, 145.

(3) Mullard, A. FDA Drug Approvals. *Nat. Rev. Drug Discovery* **2021**, *20*, 85−90.

(4) Manning, M. C.; Matsuura, J. E.; Kendrick, B. S.; Meyer, J. D.; Dormish, J. J.; Vrkljan, M.; Ruth, J. R.; Carpenter, J. F.; Sheftert, E. Approaches for Increasing the Solution Stability of Proteins. *Biotechnol. Bioeng.* **1995**, *48*, 506−512.

(5) Tabbassum, M.; Zeeshan, F. Effects of Formulation Development Methods on the Stability of Model Protein Pharmaceuticals Embedded in Solid Lipid Matrices. *Pharm. Dev. Technol.* **2019**, *24*, 649−662.

(6) Liao, Y. H.; Brown, M. B.; Martin, G. P. Investigation of the Stabilisation of Freeze-Dried Lysozyme and the Physical Properties of the Formulations. *Eur. J. Pharm. Biopharm.* **2004**, *58*, 15−24.

(7) Ferrati, S.; Wu, T.; Fuentes, O.; Brunaugh, A. D.; Kanapuram, S. R.; Smyth, H. D. C. Influence of Formulation Factors on the Aerosol Performance and Stability of Lysozyme Powders: A Systematic Approach. *AAPS PharmSciTech* **2018**, *19*, 2755−2766.

(8) Ghalanbor, Z.; Körber, M.; Bodmeier, R. Improved Lysozyme Stability and Release Properties of Poly(lactide-co-glycolide) Implants Prepared by Hot-Melt Extrusion. *Pharm. Res.* **2010**, *27*, 371−379.

(9) Farinha, S.; Moura, C.; Afonso, M. D.; Henriques, J. Production of Lysozyme-PLGA-Loaded Microparticles for Controlled Release Using Hot-Melt Extrusion. *AAPS PharmSciTech* **2020**, *21*, 274.

(10) Srinivasan, C.; Katare, Y. K.; Muthukumaran, T.; Panda, A. K. Effect of Additives on Encapsulation Efficiency, Stability and Bioactivity of Entrapped Lysozyme from Biodegradable Polymer Particles. *J. Microencapsul.* **2008**, *22*, 127−138.

(11) Gaudana, R.; Gokulgandhi, M.; Khurana, V.; Kwatra, D.; Mitra, A. K. Design and Evaluation of a Novel Nanoparticulate-Based Formulation Encapsulating a HIP Complex of Lysozyme. *Pharm. Dev. Technol.* **2013**, *18*, 752−759.

(12) Jiang, G.; Woo, B. H.; Kang, F.; Singh, J.; DeLuca, P. P. Assessment of Protein Release Kinetics, Stability and Protein Polymer Interaction of Lysozyme Encapsulated Poly(D,L-Lactide-Co-Glycolide) Microspheres. *J. Controlled Release* **2002**, *79*, 137−145.

(13) Noda, S.; Kanai, H.; Oshima, T.; Sagara, K.; Yoshida, T. Determination of Lysozyme Chloride in Drug Products by High-Performance Liquid Chromatography. *Anal. Chem.* **1988**, *37*, 570−574.

(14) Strang, R. Purification of Egg-White Lysozyme by Ion-Exchange Chromatography. *Biochem. Educ.* **1984**, *12*, 57−59.

(15) Satish, L.; Millan, S.; Sahoo, H. Sustained Activity and Stability of Lysozyme in Aqueous Ionic Liquid Solutions Containing Carboxymethylcellulose and Polyethylene Glycol. *J. Mol. Liq.* **2019**, *278*, 329−334.

(16) Kerkaert, B.; Mestdagh, F.; De Meulenaer, B. Detection of Hen's Egg White Lysozyme in Food: Comparison between a Sensitive HPLC and a Commercial ELISA Method. *Food Chem.* **2010**, *120*, 580−584.

(17) Breitenbach, J. Melt Extrusion: From Process to Drug Delivery Technology. *Eur. J. Pharm. Biopharm.* **2002**, *54*, 107−117.

(18) Patil, H.; Tiwari, R. V.; Repka, M. A. Hot-Melt Extrusion: From Theory to Application in Pharmaceutical Formulation. *AAPS PharmSciTech* **2016**, *17*, 20.

(19) Cossé, A.; König, C.; Lamprecht, A.; Wagner, K. G. Hot Melt Extrusion for Sustained Protein Release: Matrix Erosion and In Vitro Release of PLGA-Based Implants. *AAPS PharmSciTech* **2017**, *18*, 15−26.

(20) Even, M. P.; Bobbala, S.; Gibson, B.; Hook, S.; Winter, G.; Engert, J. Twin-Screw Extruded Lipid Implants Containing TRP2 Peptide for Tumour Therapy. *Eur. J. Pharm. Biopharm.* **2017**, *114*, 79−87.

(21) *ICH Topic Q 1 A (R2). Stability Testing of New Drug Substances and Products Step 5 Note for Guidance on Stability Testing: Stability Testing on New Dug Substances and Products*; European Medicines Agency, 2003. .
(22) *ICH Guideline Q14 on Analytical Procedure Development.* European Medicines Agency 2022.
(23) *Fda; Cder; Beers; Donald. Analytical Procedures and Methods Validation for Drugs and Biologics Guidance for Industry*, 2015. .
(24) *ICH Topic Q 6 B Specifications:Test Procedures and Acceptance Criteria for Biotechnological/Biological Products Step 5 Note for Guidance on Specifications: Test Procedures and Acceptance Criteria for Biotechnological/Biological Products*; European Medicines Agency, 1999. .
(25) Liao, Y.-H.; Brown, M. B.; Martin, G. P. Turbidimetric and HPLC Assays for the Determination of Formulated Lysozyme Activity. *J. Pharm. Pharmacol.* **2001**, *53*, 549−554.
(26) Shugar, D. The Measurement of Lysozyme Activity and the Ultra-Violet Inactivation of Lysozyme. *Biochim. Biophys. Acta* **1952**, *8*, 302−309.
(27) Yang, Y.; Hamaguchi, K. Hydrolysis of 4-Methylumbelliferyl N-Acetyl-Chitotrioside Catalyzed by Hen and Turkey Lysozymes: Ph Dependence of the Kinetic Constants. *J. Biochem.* **1980**, *87*, 1003−1014.
(28) Heimer, P.; Tietze, A. A.; Böhm, M.; Giernoth, R.; Kuchenbuch, A.; Stark, A.; Leipold, E.; Heinemann, S. H.; Kandt, C.; Imhof, D. Application of Room-Temperature Aprotic and Protic Ionic Liquids for Oxidative Folding of Cysteine-Rich Peptides. *ChemBioChem* **2014**, *15*, 2754−2765.
(29) Kühl, T.; Wißbrock, A.; Goradia, N.; Sahoo, N.; Galler, K.; Neugebauer, U.; Popp, J.; Heinemann, S. H.; Ohlenschläger, O.; Imhof, D. Analysis of Fe(III) Heme Binding to Cysteine-Containing Heme-Regulatory Motifs in Proteins. *ACS Chem. Biol.* **2013**, *8*, 1785−1793.
(30) Laemmli, U. K. Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. *Nature* **1970**, *227*, 680−685.
(31) Goldring, J. P. D. Protein Quantification Methods to Determine Protein Concentration Prior to Electrophoresis. *Methods Mol. Biol.* **2012**, *869*, 29−35.
(32) Wulandari, Z.; Fardiaz, D.; Budiman, C.; Suryati, T.; Herawati, D. Purification of Egg White Lysozyme from Indonesian Kampung Chicken and Ducks. *Media Peternak* **2015**, *38*, 18−26.
(33) Strupat, K.; Karas, M.; Hillenkamp, F. 2,5-Dihydroxybenzoic Acid: A New Matrix for Laser Desorption—Ionization Mass Spectrometry. *Int. J. Mass Spectrom. Ion Process.* **1991**, *111*, 89−102.
(34) Grant, D. C.; Helleur, R. J. Simultaneous Analysis of Vitamins and Caffeine in Energy Drinks by Surfactant-Mediated Matrix-Assisted Laser Desorption/Ionization. *Anal. Bioanal. Chem.* **2008**, *391*, 2811−2818.
(35) Yoo, C.; Suckau, D.; Sauerland, V.; Ronk, M.; Ma, M. Toward Top-Down Determination of PEGylation Site Using MALDI In-Source Decay MS Analysis. *J. Am. Soc. Mass Spectrom.* **2009**, *20*, 326−333.
(36) Yang, Y.; Hamaguchi, K. Hydrolysis of 4-Methylumbelliferyl N-Acetyl-Chitotetraoside Catalyzed by Hen Lysozyme. *J. Biochem.* **1980**, *88*, 829−836.
(37) Radi, L.; Fach, M.; Montigny, M.; Berger-Nicoletti, E.; Tremel, W.; Wich, P. R. Methods of Protein Surface PEGylation under Structure Preservation for the Emulsion-Based Formation of Stable Nanoparticles. *Medchemcomm* **2016**, *7*, 1738−1744.

https://doi.org/10.1021/acsomega.2c03559
ACS Omega 2022, 7, 40836−40843