Establishment of bioanalytical workflows for the quality assessment of formulated proteins

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Abstract

Proteins and peptides contribute to a significant class of therapeutic agents in the pharmaceutical market. Over the last decades, they gained growing interest due to their safety and specificity compared to small molecule drugs. Around 20% of the approved moieties by the Food and Drug Administration (FDA) in the last decade are of biological origin, such as antibodies, enzymes, peptides, and amino acids. One of the factors to be considered during the handling of proteins and peptides, especially on a large scale, is their stability. The sensitive nature of these biomolecules could lead to a loss of activity in cases of improper handling or storage. Therefore, biotechnologists focused on developing different formulation techniques to provide adequate protection and stabilization for the target protein or peptide. The design of a pharmaceutical formulation is a tailored mission according to the nature of the targeted protein, route of administration, and required mode of release. Furthermore, testing of the formulated protein is required to investigate the original characteristics of the protein and its stability after the formulation process. Thus, numerous analytical techniques and methods were developed and optimized for protein and peptide analysis. In the present thesis, the analysis of formulated proteins by employing different techniques and assessment of their stability was executed.

The first aim of the present work was to develop bioanalytical workflows for the analysis of formulated proteins. Lysozyme was selected as a model protein in different formulations that were based on hot-melt extrusion as one of the appropriate methods for protein stabilization in the solid state. Analytical characterization of the lysozyme extrudates was performed to test identity, purity, and biological activity based on chromatographic, electrophoretic, and spectrofluorimetric methods. These validated methods were adapted into a workflow that was followed to test the stability of lysozyme hot-melt extrudates over six months of storage according to the guidelines of the International Council for Harmonization (ICH) Topic Q1A (R2).

The second objective was focused on analysing the stability and structural integrity of the tested protein which is directly linked to its function. Thereby, the disulfide connectivity, one of the most important posttranslational modifications, in a protein or a peptide plays an essential role in maintaining the correct folding and, in turn, activity. In order to evaluate the protein's folding state, validated methods are required, however, a lack of bioanalytical standards to test conformational isomerism based on disulfide bonds in a protein structure was obvious. Therefore, a series of standards were designed and validated according to the ICH M10 and Q14 guidelines and model peptides were generated to evaluate the developed bioanalytical standards. These standards were further applied for the elucidation of the connectivity in selected disulfide-rich peptides and proteins by application of optimized workflows and protocols using the developed standards.

The objectives are extensively described and studied in the following chapters including the results (the respective manuscripts are enclosed in the appendix), which can be considered as a milestone in the development of bioanalytical methods for testing protein characteristics to evaluate their sequential, structural, and functional integrity in the pure and formulated state.

Zusammenfassung

Proteine und Peptide stellen eine bedeutende Klasse von Therapeutika auf dem Arzneimittelmarkt dar. In den letzten Jahrzehnten haben sie aufgrund ihrer Sicherheit und Spezifität im Vergleich zu Arzneimitteln mit kleinen Molekülen zunehmend an Interesse gewonnen. Etwa 20% der in den letzten zehn Jahren von der Food and Drug Administration (FDA) zugelassenen Bestandteile sind biologischen Ursprungs wie Antikörper, Enzyme, Peptide und Aminosäuren. Einer der Faktoren, die beim Umgang mit Proteinen und Peptiden, insbesondere in großem Maßstab, zu berücksichtigen sind, ist ihre Stabilität. Die empfindliche, instabile Natur dieser Kategorie biologischer Verbindungen könnte bei unsachgemäßer Handhabung oder Lagerung zum Verlust der gewünschten Aktivität führen. Daher konzentrieren sich die Biotechnologie auf die Entwicklung verschiedener Formulierungstechniken, um einen ausreichenden Schutz und eine ausreichende Stabilisierung für das gewünschte Protein oder Peptid zu gewährleisten. Die Entwicklung einer pharmazeutischen Formulierung ist eine maßgeschneiderte Aufgabe, die von der Art des Zielproteins, dem Verabreichungsweg und der gewünschten Art der Freisetzung abhängt. Darüber hinaus ist eine Prüfung des formulierten Proteins erforderlich, um die ursprünglichen Eigenschaften des Proteins und seine Stabilität nach dem Formulierungsprozess zu untersuchen. Daher wurden zahlreiche analytische Techniken und Methoden für die Protein- und Peptidanalyse entwickelt und optimiert. In der vorliegenden Arbeit wurde die Analyse von formulierten Proteinen mit Hilfe verschiedener Techniken und die Bewertung ihrer Stabilität durchgeführt.

Das erste Ziel der vorliegenden Arbeit bestand darin, bioanalytische Arbeitsabläufe für die Analyse von formulierten Proteinen zu entwickeln. Lysozym wurde in der Regel als Modellprotein für die Entwicklung und das Design von Formulierungen ausgewählt. Daher wurde es als Modellprotein für die Formulierung durch Heißschmelzextrusion ausgewählt, die Berichten zufolge eine angemessene Stabilisierung für Proteine im festen Zustand bietet. Die analytische Charakterisierung der Lysozym-Extrudate wurde durchgeführt, um Identität, Reinheit und biologische Aktivität auf der Grundlage chromatographischer, elektrophoretischer und spektrofluorimetrischer Methoden zu prüfen. Diese validierten Methoden wurden in einen Arbeitsablauf integriert, mit dem Lysozym-Extrudate über einen Zeitraum von 6 Monaten gemäß den Richtlinien des International Council of Harmonization (ICH) Topic Q1A (R2) getestet wurden.

Dies führt zum zweiten Ziel der vorliegenden Arbeit, nämlich der Prüfung der sequenziellen Stabilität eines zu untersuchenden Proteins. Die Disulfidverbrückung in einem Protein oder Peptid spielt eine wichtige Rolle bei der Aufrechterhaltung der korrekten Faltung und damit der Aktivität. Um die Unversehrtheit des Proteins und seinen Faltungszustand zu bewerten, sind validierte Methoden erforderlich, jedoch mangelt es an bioanalytischen Standards zur Prüfung von Isomerisierungs-bedingten Konformationsänderungen in der Proteinstruktur. Aus diesem Grund wurden eine Reihe von Standards entwickelt und gemäß den ICH-Richtlinien M10 und Q14 validiert. Darüber hinaus wurden Modellpeptide generiert, um die entwickelten bioanalytischen Standards zu bewerten. Diese Standards

wurden dann zur Aufklärung der Disulfidverbrückung in ausgewählten disulfidreichen Peptiden durch Anwendung optimierter Arbeitsabläufe und Protokolle eingesetzt.
Diese Ziele werden in den folgenden Kapiteln einschließlich der Ergebnisse ausführlich beschrieben und analysiert (die entsprechenden Manuskripte befinden sich im Anhang). Diese können als Meilenstein in der Entwicklung bioanalytischer Methoden zur Prüfung der Eigenschaften von Proteinen betrachtet werden, um ihre strukturelle, funktionelle und sequenzielle Integrität im reinen und formulierten Zustand zu bewerten.

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1. Introduction and thesis outline

The therapeutic significance of proteins and peptides has grown significantly over the last decades concerning the treatment and control of different pathophysiological disorders. The number of approved peptide drugs by the Food and Drug Administration (FDA) steeply increased and the share of biologics and peptides in the sales of the pharmaceutical market reached ~25% (US$250 billion)[1–6]. Biologics and peptides share the drawback of instability in the liquid form[3] and different formulation techniques were developed and designed to provide more stabilization for the therapeutic active agent[7,8]. Sources of instability could emerge during stages of preparation of the active agent or its storage[9] and are outlined in this thesis. In this context, physical and chemical characterization of the formulated protein or peptide is essential to ensure product quality in terms of identity, purity, and activity[10]. Agencies such as the FDA and the International Council of Harmonization (ICH)[11–13] set guidelines for analyzing biotechnological products and specifying the criteria for therapeutics analysis.

Various analytical techniques were developed for protein and peptide analysis[14], and in this work validation of different methods was performed in order to set up bioanalytical workflows for testing formulated proteins. Moreover, in the present thesis sequence and folding stability of the proteins were evaluated using a set of bioanalytical standards developed to characterize and elucidate the disulfide connectivity in peptides and proteins. Therefore, researchers will be provided with validated workflows and protocols to be followed to assess protein integrity, particularly after formulation.

1.1 Therapeutic proteins and peptides

Our cells contain a plethora of proteins with versatile functionalities in our body, including receptors, transporters, hormones, antibodies, enzymes, etc. These proteins regulate essential biological reactions in the human body such as metabolism, immune function, cellular signaling and death, as well as homeostasis[15]. In cases of deficiencies or malfunction, the administration of extracellular agents is required to regain the normal functionality of the cell. Therefore, biologics (proteins) and peptides have been introduced as therapeutic agents, especially since the synthesis of the first therapeutic peptide, insulin, was achieved in 1921[3,16,17]. The nature of biologics and peptides in contrast to small molecule drugs was always under discussion. Proteins (>50 amino acids) and peptides (2-50 amino acids) have common biological advantages, i.e. high specificity, potency, and safety, over small molecules. However, the drawback of their low stability and short half-life was faced[3]. Protein-based therapeutics have been developed over the last decade upon the improvement of extraction methods for the active proteins and antibodies from animal tissues or cell cultures. Monoclonal antibodies (mAb), coagulation factors, hormones, and cytokines are the common classes of therapeutic proteins. Numerous proteins were meanwhile approved by the FDA for clinical conditions such as cancer and metabolic, endocrine, and hematological disorders (Figure 1A)[16]. Peptides, however, gained a wide range of interest, due to their advantage over proteins, such as low immunogenicity, high affinity, and better membrane permeability[3]. Since then, approval of more peptide drugs worldwide has been achieved, and the

development of peptide drugs has thus become one of the attractive topics in pharmaceutical research as shown in Figure 1B. Bioactive peptides commonly act as hormone agonists or antagonists, enzyme inhibitors, growth factors, ion channel ligands, or anti-infective agents[2,3,5]. From an economic point of view, therapeutic protein and peptide-based drugs represent 20-25% of the pharmaceutical market (~US$250 billion in 2019) and this share is growing substantially over the years[18].

Between 2016 and 2023, 21 proteins, 72 mAb, and 34 peptides gained approval from the FDA after the successful clinical trials were executed and their pharmacokinetics were studied (Figure 1C) showing the high potential of these bioactive molecules to be involved in the routine therapy for several health conditions[5].

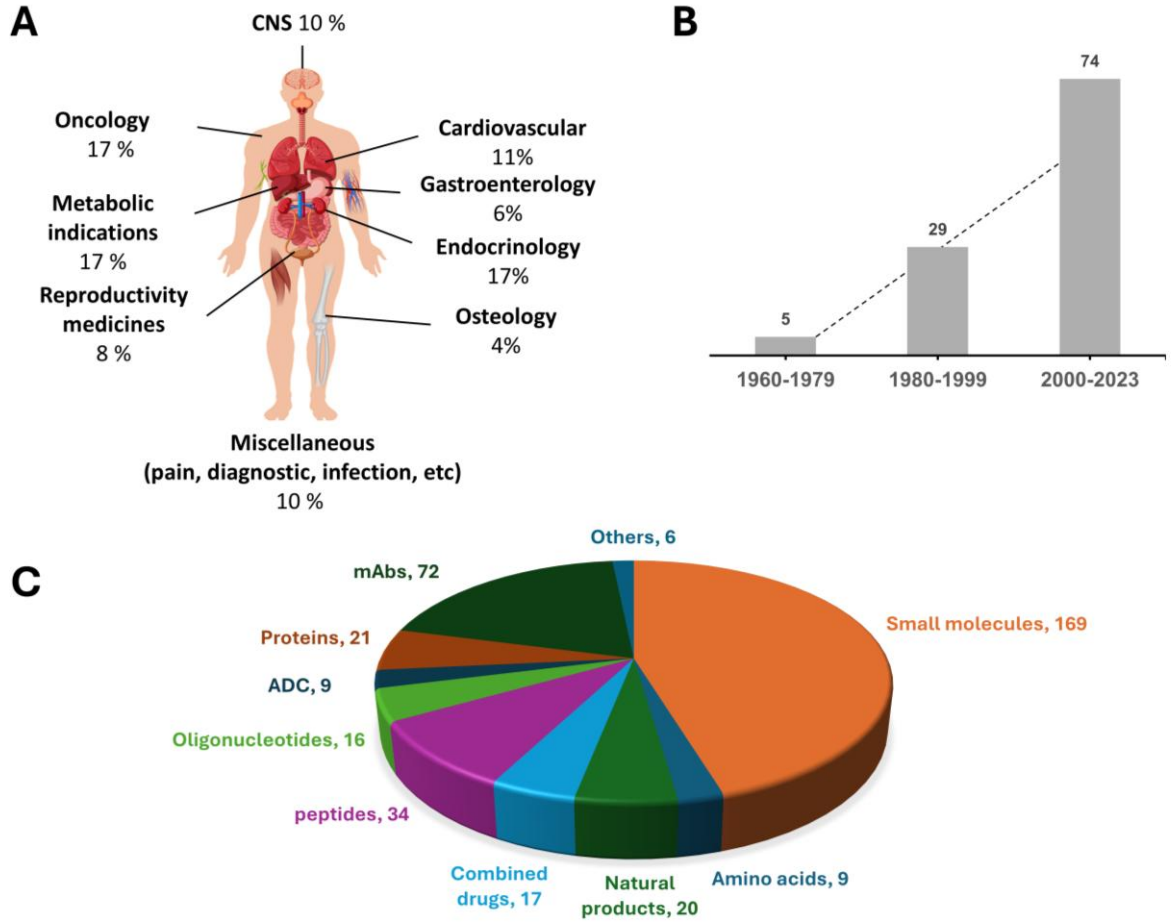


Figure 1: Global pharmaceutical market of biological drugs. A) Distribution of therapeutic indications of FDA-approved peptides and proteins (2000-2023). B) Number of FDA-approved peptides over the last six decades. C) A total of 373 new drugs were approved by the FDA between 2016-2023. CNS is central nervous system, mAbs: stands for monoclonal antibodies, and ADC: antibody-drug conjugates. The figure was modified from Muttenthaler *et al*[1] and *Al Shaer et al*[5].

However, the challenging characteristic of proteins and peptides, their physical and chemical instability affected the pharmaceutical handling of their active products, therefore numerous strategies were developed and adapted to overcome these stability issues[19]. The following subchapters outline the factors affecting the stability of proteins and peptides and the efforts executed for their stabilization.

1.1.1 Stability of proteins and peptides

Proteins and peptides consist of repetitive unit blocks which are the amino acids connected by peptide bonds into chains. They can be arranged in a single chain or a polypeptide with chains interconnected by additional bonds like disulfide bridges[20]. Atoms of the polypeptide backbone and amino acid side chains are also attracted with weak noncovalent bonds (hydrogen bonds, ionic bonds, and van der Waals attractions) and then rearranged into a secondary structure of α-helixes and β-sheets that provide higher folding stabilization[21]. The nonpolar (hydrophobic) side chains of amino acids in a protein (i.e., phenylalanine, leucine, valine, and tryptophan) tend to knot into the interior of the protein molecule in order to decrease the contact with the exterior atmosphere leading to the least energy conformation, abundant native active form. The tertiary structure defines the final tridimensional shape of the protein based on the hydrogen bonds, hydrophobic or hydrophilic interactions, electrostatic forces, and disulfide bridges. Supermolecules such as enzymes or protein filaments can be an assembly of subunits noncovalently clustered to form a giant final structure. Finally, the quaternary structure is defined by the spatial arrangement of different subunits of a protein. The final structures of each protein are specific and vital for its biological activity inside the body[22,23].

Different circumstances could lead to physical or chemical changes in the protein structure (involving the loss of the secondary or tertiary structure) such as changes in temperature, pH, ionic strength, or interfacial exposure between the biological molecule and surrounding medium[24]. These changes could take place during preparation (synthesis or purification), transfer, storage, or other processes applied to the protein/peptide molecules. Figure 2 lists the forms of proteins and peptides instability. Physically, proteins upon exposure to the aforementioned factors can lose their native folding (unfolding), degrade, or get aggregated. The resulting protein aggregation can be reversible or irreversible and the formed aggregates can be soluble/insoluble based on non-covalent/covalent formed bonds. [27] The non-native irreversible aggregation leads to denaturation of the protein and could end with fibril formation, which raises unfavored immunological responses inside the human body[25,26]. Protein aggregation can occur through chemical as well as physical degradation.

Chemical degradation-based aggregation takes place through chemical modification of the protein, including oxidation, deamidation, hydrolysis, and disulfide bond shuffling[27,28]. Oxidation of the amino acid residues in the protein chain could take place due to exposure to light, oxidizing agents (oxygen, peroxides), free radicals, or metal ions. The most susceptible amino acids for oxidation are not only methionine and cysteine but also histidine, tyrosine, tryptophan, and phenylalanine[29,30]. The location of amino acids affects liability for oxidation, as deep core amino acids are less exposed compared to terminal amino acids. Methionine residues in globulins and unpaired cysteines are sites of possible oxidation and degradation[31]. Free cysteines can be oxidized and form disulfide bonds or lead to thiol-disulfide exchanges resulting in protein aggregation as in the basic fibroblast growth factor (bFGF) as reported by Wang et al[32,33]. The rate of side oxidation reactions increases with increasing the pH of the surrounding phase. Even without the presence of free cysteine, disulfide scrambling may take place.

Disulfide bridge breakage and/or isomerization are common degradation and are reported to be the most common chemically induced aggregation pathway[34,35], especially with the crucial role of disulfide bridges in protein folding[36].

On the other side, deamidation of amino acids and loss of ammonia changes the nature of the protein in terms of hydrophobicity, charge, and mass. Asparagine and glutamine deamidation were reported at neutral or alkaline pH[31,37–39]. Asparagine-glycine, asparagine-serine, and asparagine-threonine have also been identified as amino acids prone to deamidation, in contrast to glutamine, which is susceptible to a lesser extent[28].

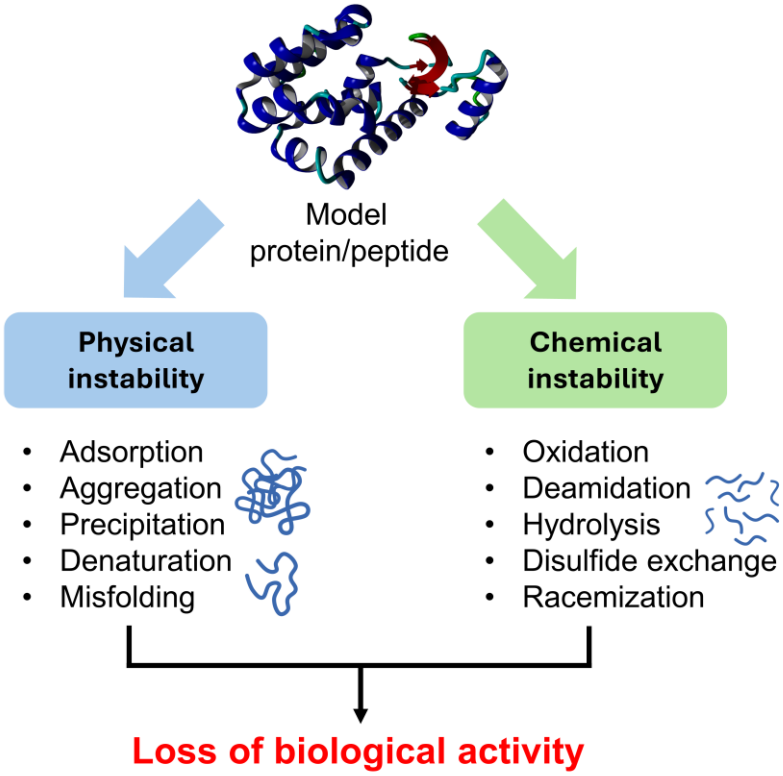


Figure 2: Physical and chemical influences on proteins and peptides leading to conformational changes or degradation.(Inspired from references [24,40,41])

Hydrolysis of a protein is a sort of severe degradation that takes place in highly acidic or basic conditions. Asparagine is one of the labile sites for hydrolysis in the protein sequence[28]. Protein hydrolysis is not expected under normal conditions of preparation or storage but should be considered as a control factor for the protein handling conditions[28]. Proteins are also susceptible to adsorption on surfaces leading to loss of availability and denaturation. Selection of containers is as crucial as selection of the solvents for the bioactive agent. Table 1 summarized the possible reasons and sites of protein denaturation emphasizing the importanance of the proper monitoring of the conditions and the duration of contact as numerous possible stress factors occur during exposure of the protein raw material in handling, storage, shipping and transfer, as well as manufacturing[10]. With the knowledge gained about the instability of proteins, especially in the liquid form, researchers aimed to develop protective techniques, to facilitate handling with proteins and ensure their integrity and consequently desired bioactivity.

Table 1: Common stability problems observed for proteins, their possible causes, and sites of contact with stressful conditions. (modified from Chang *et al*[10])

Stability problem	Causes	Stage of possible contact	Solutions
Non-covalent aggregation (precipitation or aggregation)	Structural changes, solubility, mechanical shear, impurities, heat, humidity	Storage, shipping, handling, delivery, container integrity, lyophilization, accidental freezing	Temperature control, Raw material purity, pH optimization, ionic additives
Covalent aggregation	Disulfide scrambling	pH changes at any stage of handling	pH optimization, protein denaturants
Deamidation	pH<5.0 or >6.0	pH changes at any stage of handling	pH optimization
Cleavage and hydrolysis	Highly acidic or alkaline pH, protease impurities	pH changes, impurities	pH optimization, product purity
Oxidation	Active oxygen species, free radicals, metal impurities, light	Light exposure during storage, excipients instability or impurity	Free radical scavengers, active oxygen scavengers
Surface denaturation	Protein hydrophobicity, specific affinity to surfaces	Container incompatibility, impurities	Surfactants, container material selection

1.1.2 Stabilization of proteins and peptides

With the known nature of proteins and peptides and their susceptibility to degradation (denaturation) from subchapter 1.1.1, pharmaceutical biotechnologists invested their knowledge to develop different strategies and delivery systems to fulfill the needs of functional therapeutic agents for appropriate administration[40]. Considering that protein therapeutics act mainly on extracellular targets which depend on protein or peptide size and amino acid composition[42]. For better control of certain medical conditions, intracellular delivery is highly recommended, therefore, the design of more efficient therapeutic agents was progressively needed. A new milestone in the development of therapeutic proteins and peptides has been achieved by the introduction of sequence modification and engineering (chemically or biologically) to improve the pharmacological proprieties of the bioactive moiety (higher potency or fewer side effects) and enhance stability[17].

The strategies adapted to produce engineered proteins and peptides are genetic, covalent, or non-covalent[9]. Genetic engineering includes site-specific mutagenesis and the creation of more stable analogs with desirable pharmacokinetic profiles. Human recombinant insulin was subjected to a wide range of genetically substituted amino acid analogs (with various stability and state of activity) providing

personalized diabetic therapy[43–45]. Covalent fusion of the protein to other molecules was reported as an effective protein engineering strategy, too. Fc-fusion (fusion with the Fc part of an antibody) increased the stability and half-life of the targeted protein. One of the famous Fc-fused proteins is the etanercept protein, which used in the treatment of rheumatic diseases and was obtained from the extracellular domain of p75 tumor necrosis factor receptor (TNFR) fused to the Fc part of an IgG antibody. Interestingly, it showed a longer half-life in circulating blood than the unfused form. Fusion and increasing size led to endosomal recycling and a longer duration of activity[46,47]. Similar results were observed by covalent linking of a polymer, sugar or lipid to a target molecule. The covalent linkage depends on the functional groups of the protein's N- or C-termini, existing thiol groups, tyrosine or lysine residues exposed for chemical modification. Most importantly, these functional groups should not be involved in the active site of the protein or the receptor binding interface in order to maintain its therapeutic activity[48]. PEGylation (conjugation with polyethylene glycol, PEG) is one prominent example for modification of therapeutic proteins. More than 40 FDA protein drugs were conjugated with PEG and showed higher stability in the digestive system[9]. Acylation (lipidation) of the peptide backbone can provide impressive stability for the target molecule and may enhance bioavailability. Liraglutide[49] and insulin detemir[50] are among the most successful lipidated peptides known today. Cyclization and insertion of unnatural amino acids[17], in addition to disulfide mimetics and stapling by intrachain bridges[51–53] are other sorts of modifications applied mainly on peptides to increase their stability. Glycosylation can modify the protein's stability, solubility, and activity, as the glycosylated protein can form additional hydrogen bonds with the hydrophilic residues on the polypeptide backbone. Glycosylated p-succinamidophenylgluco-pyranoside-insulin conjugate (SAPG-insulin) was found with a reduced tendency for dimer or fibril formation[54].

Non-covalent engineering of therapeutic proteins and peptides takes place through formulation which is investigated on a wide scope to preserve the integrity of the therapeutic agent during the storage and handling stages until administration by the patient[55]. The incorporation of excipients is designed according to the nature of the carried drug molecule. Therefore, solvents, excipients, and formulation techniques should be selected wisely to obtain and/or maintain the desired pharmacological and pharmaceutical properties[41,56].

In comparison to conventional small molecule pharmaceuticals, the development of a stable protein formulation requires more resources and effort due to the complex nature and delicate structural stability of proteins[57]. Pre-formulation starts with obtaining the native form of the protein in its highest purified quality. The structural, physical, and biological properties of the target molecule should be investigated. Besides the structural information (primary, secondary, tertiary, and quaternary structures), the physicochemical properties of the therapeutic proteins (molecular weight, solubility, extinction coefficient, stability, aggregation tendency, and native post-translational modifications (i.e. glycosylation)) should be studied[10,40]. Additionally, the biological activity, affinity to substrate or receptor (in vivo or in vitro models), and pharmacokinetic profile of the therapeutic moiety are required to be available before formulation[40]. Taking into consideration that the therapeutic activity of proteins and peptides is primarily dependent on their conformational structure. These preliminary data will support

the design and development of the formulation in order to maintain the structural integrity of the proteins in the native form.

Pre-purification steps (precipitation or separation from a biological mixture) may be required before formulation based on the quality of the supplied protein[24]. Furthermore, the selection of added excipients is a crucial step[41]. Qualified excipients are mainly involved in the development, including a complete profile about their origin, structure, physicochemical properties, function, stability, and compatibility with other excipients and the active ingredients[41,58,59]. Indication of the formulation and targeted route of administration will guide the selection of the excipients in addition to their stabilization potential for the pharmaceutical product. Different classes of excipients, listed in Table 2, were described according to their effectiveness and stabilization effect[41].

Table 2: Classes of excipients used in peptide and protein pharmaceutical formulations. (modified from Jorgensen *et al*[41])

Excipient		Aimed formulation effect
Type	**Example**	
Surfactant	Poloxamer[60] Polysorbate 20 and 80[61]	Anti-adsorption, cryoprotectants, lyoprotectants
Polymers	Dextran[62] Cyclodextrin[63,64] Polyethylene glycol (PEG)[65–67] PVP[65,66,68] PLGA[69–71]	Anti-adsorption, stabilizers
Sugars	Glucose[72] Sucrose[65,66,68,70,72] Trehalose[65,66,73,74]	Stabilizers, cryoprotectants, lyoprotectants
Polyols	Glycerol[65,66,75] Mannitol[65,66,76] Sorbitol[59,65,66,75]	Stabilizers, cryoprotectants, lyoprotectants
Antioxidants	Ascorbic acid[65,77] Ectoine[59,78] Glutathione[65] Monothioglycerol[79] Vitamin E[80,81]	Oxidation protection
Chelating agents	Citric acid[80,81] EDTA[77,82] Thioglycolic acid[80,81]	Oxidation protection
Buffer salts	Phosphate, bicarbonate, sulphate, nitrate, acetate, chloride, pyruvate[80,83]	pH control, stabilizer, tonicity modifier
Antacids	$Mg(OH)_2$ [70] $ZnCO_3$[71]	pH control
Amino acids	Alanine[65,66], Arginine[65,71,84] Aspartic acid[65], Glycine[65,66] Histidine[85], Lysine[65] Proline[65,66,86]	Stabilizer, solubilizer
Ligands	Phenol[87] Zinc[88]	Stabilizer
others	HSA, BSA[89]	Stabilizer, anti-adsorption

BSA: bovine serum albumin; HSA: human serum albumin; PEG: polyethylene glycol; PVP: poly(vinyl pyrrolidone) PLGA: poly(lactic-co-glycolic-acid), EDTA: ethylenediamine tetra acetic acid

Due to the nature of proteins and peptides, they are usually prepared in a lyophilized form ready for reconstitution prior to administration, mainly as an injectable dosage form (intramuscular, intravenous or subcutaneous) and under refrigerated storage (2 to 8°C). Incorporation of numerous excipients such as polymers, sugars, and cyclodextrins facilitates the injection-free administration i.e., transdermal, pulmonary, ocular, nasal, rectal, and vaginal routes[90–93]. Moreover, the oral administration of proteins and peptides gained an increasing interest over the years for the favor of patient convenience and compliance. Gastrointestinal tract stability is a critical concern for the technologists; therefore, many approaches were optimized for protecting and stabilizing the administrated protein/peptide and, at the same time, ensuring their biological activity. This includes namely cyclization, PEGylation, attachment to cell penetrating peptides, and prodrug formation[94–99]. Particulate formulation systems were developed as a carrier system for proteins and peptides, such as emulsion, micro- and nanoparticles, solid core particles, and liposomes, and they have been successfully modulated the release rate and targeting of the formulated protein, in addition to the possibility of oral delivery[94,100,101]. Specific medical devices were fabricated for controlled targeted release of therapeutic proteins and peptides, such as biodegradable microneedle-based delivery system[102], ingestible self-orienting system,[103] and intestinal mucoadhesive patches[104].

1.1.3 Stabilization of proteins and peptides in solid state

Formulation of the proteins intended for any route starts with handling of the raw material, followed by numerous processing steps which could also cause protein or peptide denaturation[57]. Therefore, the environment of protein handling during formulation needs to be adjusted for stabilization of the formulated active agent, and factors affecting stability of the protein should be closely controlled[30]. Among these factors that may lead to process-related degradation are purity of the used solvent and excipients, moisture content, pH of the formulation, sterility if required and uncontrolled temperature. Additionally, water plays a critical role in protein degradation, and thus, preparation of a solid protein formulation is commonly prepared in the first place by removal of water through evaporation and atomization (e.g., spray drying) or sublimation (e.g. freeze drying or spray freeze drying) of liquid protein solutions[30,105,106].

Upon exposure of the protein to atomization, dehydration, freezing, interfacial and/or shear stress during drying, removal of water could raise the possibility of some process-related degradation to the protein, including aggregation, unfolding, oxidation and/or deamination[107] and eventually the proteins can lose their structure and functionality [30,99,108] Another mechanism of protein stabilization in its solid state is by formation of an amorphous glass matrix composed of polyols, sugar and polymer, in which the protein molecules are immobilized and embedded in the glass matrix[109]. This technique is the basis of hot-melt extrusion (HME), a novel stabilization technique for embedding drug molecules that dramatically decrease the mobility and tendency of protein degradation, unfolding or interaction with surrounding media,leading to an increased stability of the extruded protein or peptide[106,110]

HME of a protein starts with filling a preheated ram extruder with a physical mixture of powdered protein and polymer via a cylindrical barrel, which then gets mixed and molten. Finally, the molten mixture

pressurized with a driven pistol through a die and the blend is transformed into rods of a desired size[111] as represented in Figure 3.

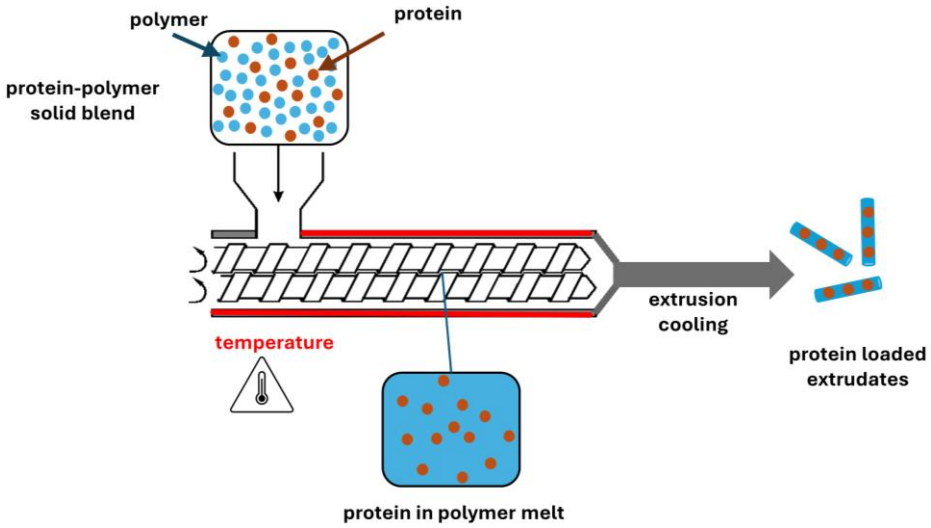


Figure 3: Schematic representation of the hot-melt extrusion procedure of proteins. (Inspired from references[111,112])

Generally, HME offers various advantages in formulating proteins in a solid dosage form with a solvent-free technique cutting the need for removal of water or organic solvents, the feasibility of handling highly potent proteins in a closed system with less excipients and the potential for easier up-scaling to production scale[113]. Additionally, advantages of taste masking, improved bioavailability and controlled and targeted release of the biological drug from the matrix make HME an appealing formulation technology for protein delivery systems[111,114,115]. However, due to the heated system, the formulation components must be thermally stable at the melting and extrusion temperature, in addition to the large batch size of the required drug substances and excipients, that increases the development costs, respectively[116–118]. In this thesis, the stabilization potential of HME should be assessed in comparison to the process-related protein degradation that could take place due to the heating and shear process. Proper analytical characterization of the treated protein will provide a better evaluation of the stability of the active ingredient. Generally, all the developments in protein delivery systems depend on the availability of the biological drug molecules in the pure active state. Therefore, validated characterization methods for monitoring their physicochemical properties are crucial and further described in the following chapter.

1.2 Characterization of proteins and peptides

In the previous chapter (1.1), the significance of therapeutic proteins and peptides was highlighted, and their complex nature was considered during storage and handling followed by the launched stabilization strategies. That raises the need for proper characterization of the physicochemical properties of the therapeutic molecule and to monitor their integrity and stability. Various analytical techniques were performed and applied for protein analysis, including the identification of the structure, post-translational modifications, charge, folding state, purity, bioactivity, and the concentration of the protein or

peptide[119,120]. Additionally, protein degradation products should be determined by analytical methods that differentiate between structurally related impurities that could have no, differing or even opposite biological activity[9]. The quality of the raw material or the final product should be assessed and evaluated. Therefore, the characterization should be performed at the different stages of handling of the protein molecule. The analytical techniques are classified according to the measured analytical feature by the instrument and the physical/chemical stability problem[28,121–125]. An overview of the applied analytical techniques for characterizing the protein formulations is presented in Table 3.

Table 3: Different analytical techniques applied for the characterization of proteins and peptides and their involvement in quality control (QC) routine analysis of pharmaceuticals. (modified from references[9,10,28,121–125]).

Stability parameter	Analytical technique	Analyzed output	QC method
Structural and conformational changes	DSC, DSF	Thermal parameters (melting point T_m)	No
	ED	Primary structure, disulfide connectivity	No
	LC-MS/MS	Primary structure, disulfide connectivity	No
	CD	Secondary and tertiary structures	No
	Fluorescence spectroscopy	Tertiary statures	No
	UV/visible spectrometry	Tertiary structure	Yes
	Raman spectroscopy, Infrared	Secondary structure/ chemical characterization	No
	NMR	Secondary and tertiary structures	No
Aggregation tendency	SDS-PAGE	Molecular weight	Yes
	CE	Molecular weight	Yes
	AUC	Molecular weight/shape	Yes
	MS	Molecular weight/charge/aggregates	No
	SEC	Hydrodynamic size	Yes
	DLS, NTA	Hydrodynamic size	No
	Optical microscopy	Size/morphology	No
	Fluorescence microscopy	Size/morphology/aggregates	No
Chemical changes	RP-HPLC	Hydrophobicity/purity/rotein degradation/concentration	Yes
	LC-MS	Molecular weight	No
	Ion exchange chromatography (IEC)	Charge	Yes
	IEF	Charge	Yes
	Zeta potential	Charge	No
Activity assay	ELISA (Visible)	Specific binding sites/concentration	Yes
	EPR	Binding to ligand	No
	Refractometry (SPR)	Binding to ligand	No

Abbreviations: DSC: differential calorimetry, DSF: differential scanning fluorimetry, ED: Edman degradation, LC: liquid chromatography, MS: mass spectrometry, CD: circular dichroism, UV: ultraviolet, NMR: nuclear magnetic resonance, SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis, CE: capillary electrophoresis, AUC: analytical ultracentrifugation, SEC: size-exclusion chromatography, DLS: dynamic light scattering, NTA: nanoparticle tracking analysis, RP-HPLC: reversed-phase high-performance liquid chromatography, IEF: isoelectric focusing, IEC: ion exchange chromatography, ELISA: enzyme-linked immunosorbent assay, EPR: Electron paramagnetic resonance, SPR: surface plasmon resonance.

Changes in protein or peptide thermodynamic properties can be assessed by differential calorimetry (DSC)[126] or scanning fluorimetry (DSF)[127] based on measuring the melting unfolding temperature (T_m) of the protein molecule, reflecting the stability of the protein structure after formulation procedures[128]. Moreover, the structural information of protein's primary structure can be gained by Edman degradation (ED)[129] and tandem mass spectrometry (MS/MS)[130] in order to confirm the mutation points and/or post-translational modification such as disulfide bridges or glycosylation sites in the amino acid sequence[131–133]. Peptide mapping or sequencing would be informative about the chance of chemical degradation of isomerization instability. Moreover, secondary and tertiary structures have been reported to be characterized and evaluated by spectroscopic methods such as circular dichroism (CD)[134,135], fluorescence, Fourier transform infrared (FTIR)[136], X-ray crystallography[137] and Raman and nuclear magnetic resonance (NMR)[138]. In cases of unfolding, degradation, aggregation or hydrolysis, changes in the outputs of the native protein will be observed and both chemical and physical stability would be evaluated. Different chromatographic techniques are highly involved in protein analysis, based on the hydrophobicity, mass and charge of the biological molecule. Signs of degradation could be assessed by reversed phase high pressure liquid chromatographic (RP-HPLC) separation and quantification of the active therapeutic protein from other impurities or degradants can be achieved[139,140]. Size exclusion and ion exchange chromatography offers proper evaluation for the protein quality with the aid of different instrumental detectors, either UV, fluorescence or refractive which have been applied to numerous proteins and peptides[141]. Therefore, not only qualitative, but also quantitative assessment of the stability will be achieved by applying the chromatography-based techniques[95–99].

Electrophoresis methods, such as sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)[142], native PAGE[143], isoelectric focusing (IEF)[144] and capillary electrophoresis (CE)[145–147] are usually applied for the quality control of proteins based on the charged ionization nature of the protein molecule and its behavior in ionic medium[141]. Degradation products or aggregates would be differentiated from the native molecule because of altered charge and/or size. Further assessment can be performed using light scattering techniques. By measuring light intensities as turbidity indicator, dynamic light scattering (DLS)[148,149] and nanoparticle tracking analysis (NTA)[150] can be applied to assess the reformed particle size, and/or molecular weight of unstable molecules. Last, but not least specific analysis for maintenance of the functional activity would be necessary for the final characterization of a protein or peptide[148] including identifying the ligand molecule and testing the binding upon the administration of the therapeutic biological drug. In vivo or in vitro testing is designed and performed in cultures or on chips, thus the binding affinity and capacity of the peptide or protein to its specific receptor or ligand is evaluated properly[151]. Using electron paramagnetic resonance (EPR)[152] and surface plasmon resonance (SPR)[153,154] spectroscopy the ligand binging is analyzed by interaction of the ligand with the receptor molecule which is immobilized on a metal surface (e.g., gold). Reflection of the light on the metal surface in a distinct angle is corresponding to the mass increase upon ligand-protein interaction[151]. Moreover, enzyme-linked immunosorbent assays (ELISA) are pretty popular for testing the binding of proteins to a specific antibody. Binding site specificity plays a big role in designing

and developing ELISA assays, antibody-treated plates, and enzyme-labeled antibodies for feasible detection[155,156].

The most commonly used enzyme labels are Horseradish peroxidase (HRP) or alkaline phosphatase (AP), which catalyze oxidation reactions of a substrate into a colored product, which is then measured spectrophotometrically which in turn is used to evaluate and quantify the binding[9,21,33].

By application of various analytical techniques, a complete characterization of a protein or peptide can be achieved, especially in the case of newly explored biomolecules[120].

1.2.1 Guidelines for analysis of biological products

Biologics are regulated by both the Center for Biologics Evaluation and Research (CBER) and the Center for Drug Evaluation and Research (CDER) at the Food and Drug Administration (FDA) and the European Medical Agency (EMA)[13]. Various regulatory guidelines were released for biological drug analysis, and the criteria that should be met were specified for biological products for human or animal use as a new drug entity, including their stability testing or the performance of clinical studies[157]. Industrial guidelines are also available to control the packaging material[158], and impurities in the final products[159]. Listed below are the guidelines drafts for biological products to be followed by the researchers and industrial technologists upon the development of a new or a modified biological product intended for human use:

- Guideline for Submitting Documentation for the Stability of Human Drugs and Biologics, FDA (1987)[160]
- Demonstration of Comparability of Human Biological Products, Including Therapeutic Biotechnology-Derived Products, FDA (1996)[161]
- Guidance for Industry: Changes to an Approved Application: Specified Biotechnology and Specified Synthetic Biological Products, FDA (1997)[158]
- ICH Topic Q1B Photostability testing of new active substances and medicinal products. EMEA (1998)[162]
- ICH Q6B specifications: Test procedures and acceptance criteria for Biotechnological/Biological Products, EMEA* (1999)[13]
- Guidance for Industry. For the Submission of Chemistry, Manufacturing and Controls and Establishment. Description Information for Human Plasma- Derived Biological Products, Animal Plasma or Serum-Derived Products, FDA (1999)[163]
- Development of Pharmaceutics for Biotechnological and Biological Products. Annex to Note for Guidance on Development Pharmaceutics (CPMP/BWP/328/99), EMEA* (1999)[159]
- ICH Q1A (R2) Stability testing of new drug substances and products-Step 5, EMEA* (2003)[12]
- ICH Q3B (R2) Impurities in new drug products, EMEA* (2006)[11]
- Guidance for Industry. ANDAs: Impurities in Drug Products, FDA (2010)[164]
- Note for guidance on minimizing the risk of transmitting animal spongiform encephalopathy agents via medicinal products, EMA (2011)[165]

- Note for guidance on plasma-derived medicinal products, EMA (2011)[166] (*since 2010 EMA)

The aforementioned guidelines specify the parameters to be tested during the handling of a biological product in all stages of development[13], including:
(1) Raw materials: purified protein and qualified excipients,
(2) Manufacturing facility: Calibrated equipment and Good Manufacturing Practice (GMP) certification,
(3) Characterization laboratory: Structural analysis, chromatography/electrophoresis, and bioassays,
(4) Stability studies: Stability champers and tested temperatures, humidity, and light.
ICH Q6B specifications[13] represent the reference guidelines for biological products that describe the essential acceptance criteria to be tested and evaluated. ICH Q6B describes (a) visual inspection of appearance and the identification of the biological substance by characterization of the physicochemical properties including the structural information and post-translational modifications (PTMs), b) biological activity assays and how potency is to be evaluated, c) immunochemical proprieties of antibodies, d) purity testing and analytical considerations of product- and process-related impurities, and e) the quantification procedures for protein content in correlation to biological assays. ICH Q1A (R2)[12] describes the stability testing of the biological products in line with the Q6B guidelines[13]. Accelerated stability studies investigate the shelf life and simulate long-term storage of drug substances or products at regular storage conditions. Stress stability conditions[12] are temperature, humidity, and photolysis liability, and they are selected according to the tested storage (long, intermediate, or accelerated to drug substance or product). Biopharmaceuticals are recommended to be stored at 2-8°C, therefore testing intervals are regulated accordingly, as shown in Table 4. If the drug substance is intended to be stored at -20°C, then only long-term storage will be evaluated at -20°C ± 5°C for 12 months.

Table 4: Different analyzed conditions during stability studies of biological substances or products. (source: ICH Q1A (R2)[12] guidelines)

Study	Storage conditions	Minimum storage time period
Long term	5°C ± 3°C	12 months
Intermediate	25°C ± 2°C/60% RH ± 5% RH	6 months
Accelerated	40°C ± 2°C/75% RH ± 5% RH	6 months

RH refers to relative humidity

The stability of the protein across a wide range of pH values should be evaluated when it is anticipated to be used in the liquid form[12]. Moreover, the stability of the container and package material is included in the stress stability studies of the final biological product[13]. These stress tests can help in the identification of degradation products and clarify the degradation pathways for each active ingredient or excipients and validate the analytical methods[12].

Pharmacopeial specifications (monograph) are documented for each drug, and the analytical procedures for the drug substance or the drug product are included[167]. Additionally, the acceptance criteria of the analytical procedures can be altered according to the nature of the drug and the analysis matrix. The described analytical procedures should be proven to be validated and fulfill the guidelines for validation of analytical procedures ICH Q2(R2)[168]. For the analysis of proteins and peptides as well as biological matrices, the validation parameters have been adapted and suitable bioanalytical methods were introduced in ICH M10[169]. The validation parameters of an analytical method according to ICH M10[169] are defined and summarized in Table 5. These parameters are generally applied to analytical techniques; however, chromatographic and ligand binding assays are mainly used for the quantification of biological products due to their sensitivity and selectivity. Each case should be evaluated separately, and either full, partial or cross-validation is needed, depending on the changed analytical parameters compared to already validated methods and the influence of these changes on the analytical response. Furthermore, the stability-indicating properties of the analytical method have been specified in the ICH guidelines by testing the response of the degradation products as well. Recent release and update in quality assurance guidelines for validation of analytical procedures ICH Q14[170] has been incorporated in the recent drafts of ICH Q2(R2)[168] and ICH M10[169] (2022), thus, the whole process of method validation and application procedures in quality control of biopharmaceutics is up to date.

Recent advances in instrumental developments helped in the establishment of numerous analytical techniques and the validation of bioanalytical methods for protein characterization. The analytical comparison needs consideration of the availability of reference standards with high purity and quality that can be used as a guidance for method evaluation[171].

Table 5: Validation parameters of bioanalytical methods. (source: ICH Q2(R2)[168] and ICH M10[169] guidelines)

Validation parameter	Definition	Conduction
Selectivity/ Specificity	Ability of an analytical method to differentiate and measure the analyte in the presence of potential interfering substances as impurities, metabolites and structurally related degradants	Observation of the response of the interfering substances in comparison to the analyte
Range	Regression model describes the range of relationship between the nominal analyte concentration and the response of the analytical platform to the analyte. (linear, non-linear or multivariant)	Calibration curve of at least 6 levels of concentrations, including zero (blank sample), lower limit of quantification (LLOQ) and upper limit of quantification (ULOQ), repeated three independent times on at least 2 days. Signal to noise ratio can be used for calculation of detection limit and quantification limit
Accuracy	Comparison of the measured results with expected values and represents the repeatability of the response compared to concentration that evaluate intra-day or within run precision	Levels of quality controls are selected, and each is measured for 3-5 replicates. Standard deviation from expected values is calculated.
Precision	Degree of closeness of the measured results and represents Reproducibility of the response compared to concentration, evaluates inter-day or between run precision	Same levels of accuracy are measured on 3 different sets on at least on 2 days. Percent coefficient of variation (%CV) is calculated.
Carry-over	Expresses the alteration of a measured concentration due to residual analyte from a preceding sample that remains in the analytical instrument.	Assessed by analysing blank samples after the calibration standard at the ULOQ.
Dilution integrity	Assessment of the sample dilution procedures	Dilution quality control is prepared by dilution of a stock solution of a concentration higher than ULOQ and measured at least 5 replicates.
Robustness	Evaluate the effects of minor changes in the analytical parameters on the response.	Modifications in the analytical parameters as pH, temperature, flow rate are done and standard deviation of the measured values than expected is evaluated.
Stability	Evaluate of sample preparation or storage does not affect the quality of the analyte.	Different storage conditions are tested: stability of stock and working solutions, bench-top stability, freeze-thaw stability (at least 3 cycles of freezing of at least 12 h and thawing) and long-term stability.
Reinjection reproducibility	Evaluate reproducibility of analyte response in cases of repeated analysis of the samples (e.g. equipment failure)	Same samples and reinjected and analyzed at different concentration levels (min. 5 replicates)

In order to evaluate a newly developed formulation, a series of validated analytical methods should be tested and optimized initially on the pure biological substance and then applied to the formulated product. Interference by other ingredients with the analysis procedures should be removed by physical means that do not alter the physicochemical properties of the active ingredients[169,171]. Prior sample preparation may be necessary to be performed[172]. Finally, bioanalytical workflows and protocols are described according to the tested formulation.

As mentioned in section 1.1.3, newly developed formulation technologies such as hot melt extrusion claimed to offer higher stabilization for the extruded protein. In order to test the stabilization potentials of HME, the establishment of a bioanalytical workflow for the analysis of extruded proteins was anticipated. Lysozyme (N-acetylmuramide glycanhydrolase or muramidase, a protein carrying four disulfide bridges) was reported in numerous studies[105,108,113,173–175] as a model protein formulated using several techniques and different excipients. Therefore, it was adapted in this study to be formulated using HME (based on RAM extrusion) of lysozyme with polyethene glycol 20,000 as polymer, as detailed in Chapters 2 and 3.

1.2.2 Characterization of disulfide-rich proteins

A major class of proteins and peptides carries one or more disulfide bridges that play an important role in their conformational structure, which is necessary for their biological activity[176–180]. Disulfide-rich proteins and peptides are interesting and promising therapeutic agents as they have potent activity as e.g., toxins and hormones such as oxytocin, vasopressin, angiotensin, insulin, and conotoxins[181–183]. Uniquely, the pattern of cysteine pairing provides the correct oxidative fold and constrained flexibility[178] that is specific for receptor binding and activity. Disulfide connectivity also plays a significant role in stabilizing the therapeutic agent[176]. Various approaches were reported correlating the mutation in cysteine residues with loss of folding stability with signs of aggregation and immunological responses[184]. Cyclization of peptides by forming disulfide bridges[51,53] is one of the strategies adapted for stabilizing peptides as mentioned in section 1.1.2. The secondary structure will be more defined than the linear form and stable against proteases[183]. Disulfide bridges were also inserted as cleavable linkers to enhance the cellular permeability of the peptides[185]. Therefore, disulfide bridges are a major structural prerequisite for therapeutic activity as well as stabilization technologies.

For such an important class of therapeutic agents, structural characterization is paramount, and the confirmation of the native connectivity and folding should be included in the drug analysis and quality control procedures. As previously stated in Table 3, spectroscopic and spectrometric methods were reported for structure analysis. Investigation of the secondary structure of disulfide-bonded peptides and proteins can be performed using CD[135], Raman spectroscopy[135], and NMR[186] which provide an overview of the folding state of the biological substance and its composition of the α-helixes and β-sheets. NMR spectroscopy and X-ray crystallography[137] provide general information about disulfide bonds at the molecular level and require comparable higher amounts of highly pure samples. Still, due to their low throughput of solving structures, these methods are not typically used for disulfide bond mapping and to identify specifically the connected cysteines. Tandem mass spectrometric method (MS/MS)[187], and Edman degradation (ED) were described for disulfide bridge localization[14,188], as going to be discussed

in the next section. It is worth mentioning that formulating disulfide-rich peptides can have an influence on the disulfide connectivity due to the stressful processing. Therefore, the development of suitable workflows for disulfide connectivity elucidation is crucial for investigating any alterations in the native protein or peptide connectivity due to processing- or storage-related factors.

1.2.3 Elucidation of the disulfide connectivity in disulfide-rich proteins and peptides

A pair of cysteines in a peptide connected with a disulfide bond is responsible for its characteristic spatial conformation[189]. More than one pair of disulfide bridges raises the probability of forming various isoforms with different profiles of activity, including the potential of higher toxicity, and specificity (Figure 4)[190]. The disulfide linkages in a protein cannot sourly be predicted from the amino acid sequence and the differentiation between the isoforms is not an easy assignment, especially if the analytical tools, protocols, and standards are unavailable. Great advances in instrumentation, automation, and detection techniques over the last decades enhanced the analytical capabilities and applications[191]. Mass spectrometry has been widely applied for peptide mapping and structural elucidation based on the digestion (enzymatic or chemical) of the peptide or protein, chromatographic separation of the digest-derived fractions, identification of the disulfide-carrying fractions, and finally, reduction and/or derivatization of these fractions for disulfide elucidation[192,193]. After the complete analysis of all peptide fractions in different conditions, the disulfide connectivity can be determined.

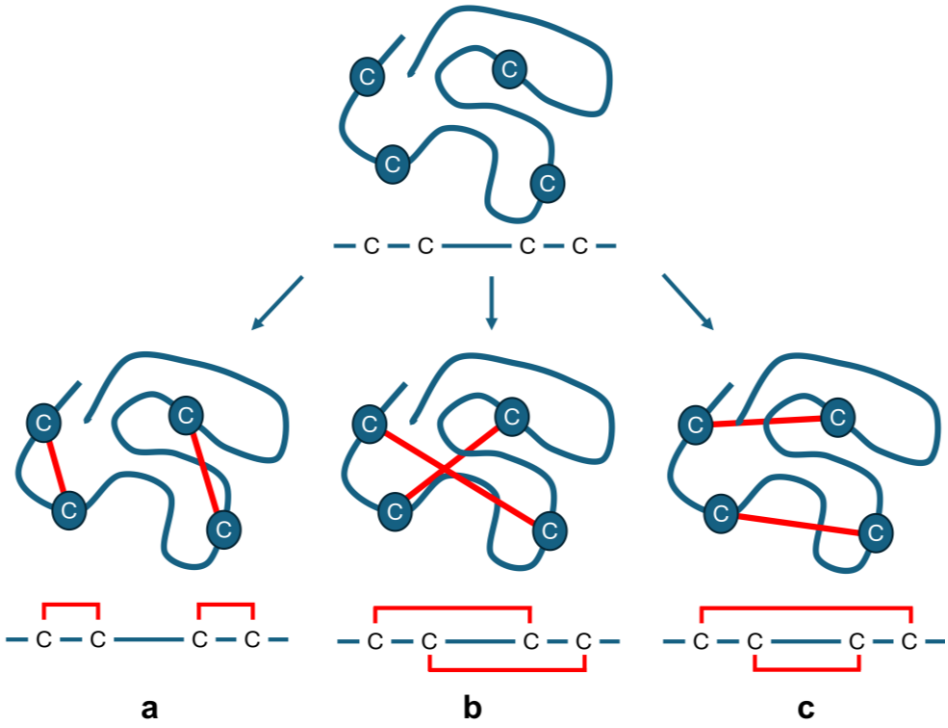


Figure 4: Different possible isomers of 2-disulfide-bonded proteins or peptides. a: beads form, b: globular form, c: ribbon form. (inspired from reference[194])

The presence of available cleavage sites for digestive enzymes (Arg/Lys for trypsin, Phe/Tyr/Trp for chymotrypsin, Ser/Ala/Val for elastase) in the protein or peptide sequence of interest is required, otherwise, MS application will be abundantly limited[195,196]. MS connected to online electrochemical cells offers an alternative in case of the absence of cleavage sites, based on different responses of the isoforms to the applied electric current[197–200].

Tandem mass spectrometry (MS/MS) using different fragmentation pathways has been commonly used for disulfide elucidation as an alternative to the sophisticated top-down MS approaches[189]. Collision-induced dissociation (CID) or high-energy collision dissociation (HCD) MS/MS fragmentations induce not only the cleavage of the peptide backbone but also fragmentation of the amide bonds included in the disulfide bridge loop, resulting in the identification of cysteine residues in the fragments[201,202]. Electron-mediated dissociation techniques [electron-capture dissociation (ECD) and electron-transfer dissociation (ETD)] were also widely employed as MS/MS fragmentation methods for multiple disulfide-bonded peptides and proteins based on the electron affinity of the disulfide bond[203–206]. A combination of CID and ETD has been applied to complicated disulfide patterns of recombinant proteins[192,207]. Additionally, recent developments in fragmentation pathways such as ion mobility and trapped ion mobility enhanced the mass detection of fragment ions compared to electron spray ionization (ESI)[208–211]. The isomers are expected to show different flight paths and collision cross sections (CCS) in a magnetic field upon fragmentation, and, thus, could possibly be sufficiently different for analysis. Furthermore, various modes of fragmentation are described in the literature such as laser-induced dissociation (LID), and in-source decay in the reflector mode of the mass analyzer (reISD) for matrix-assisted laser-desorption/ionization time-of-flight/time-of-flight mass spectrometer with LIFT technique (MALDI LIFT-TOF/TOF MS). These methods provide clear fragmentation patterns with lower noise for ion families[212–214].

The major problem that arises during the protein digestion, MALDI analysis, or fragmentation procedures is the disulfide scrambling, especially at neutral or alkaline pH and at elevated temperatures, high energy levels, or in the presence of free thiols after partial hydrolysis of the protein[189,215–219] that leads to the formation of non-native disulfide patterns and incorrect assessment. Therefore, proper control of the digestion parameters is required, and monitoring of collision-based fragmentation parameters is considered very critical[220]. Interpreting the mass spectra and fragments ions is usually done by software trained on peptide models and standard structural models. Still, this is not applicable in all cases, and manual interpretation is imperative. Despite the high resolution and new advances in MS analysis, certain challenges limit the MS application for analyzing disulfide bonds in proteins and peptides including the complex nature of fragmentation patterns, which require both time and expertise for accurate evaluation[221]. Moreover, the risk of loss of critical fragments for elucidation, especially if the samples are diluted, and the need for a standard validated pattern for evaluation hinders the data validity[194,222,223]. However, despite the wide application of MS for structural elucidation and peptide mapping, it still does not provide sufficient differentiation between all isomers of multiple disulfide-rich peptides, especially in the case of vicinal cysteines[182,190,208,224,225]. This emphasizes the need for the

incorporation of other methods to provide better analytical elucidation and differentiation between disulfide isomers.

The gold standard “Edman degradation”, developed and published by Pehr Edman in 1950[129], is the frequently applied method for the determination of the primary amino acid sequence of a protein or a peptide based on N-terminal sequencing and coupling with phenyl isothiocyanate in an alkaline medium. The conversion of the N-terminal free amino acid (aa_n) into a phenyl thiohydantoin amino acid derivative is followed by cleavage in an acid medium and eluted into a chromatographic system to identify the amino acid. The remaining protein (aa_{n-1}) is again subjected to sequencing and the next N-terminal amino acids react in subsequent cycles releasing the PTH-amino acid derivatives. Finally, the amino acid sequence is detected using chromatographic identification of the PTH-amino acids. The mechanism of Edman degradation and intermediate derivatives[188] are shown in Figure 5. Among the amino acids, detection of cysteine using traditional N-terminal sequencing is not possible due to the instability of the formed PTH-cysteine derivative and conversion into PTH-dehydroalanine derivative that is released with Edman chemistry byproduct dithiothreitol (DTT)[226]. Besides the formation of PTH-cystine in the case of a disulfide bridge was reported to be not reproducible or quantitative[181].

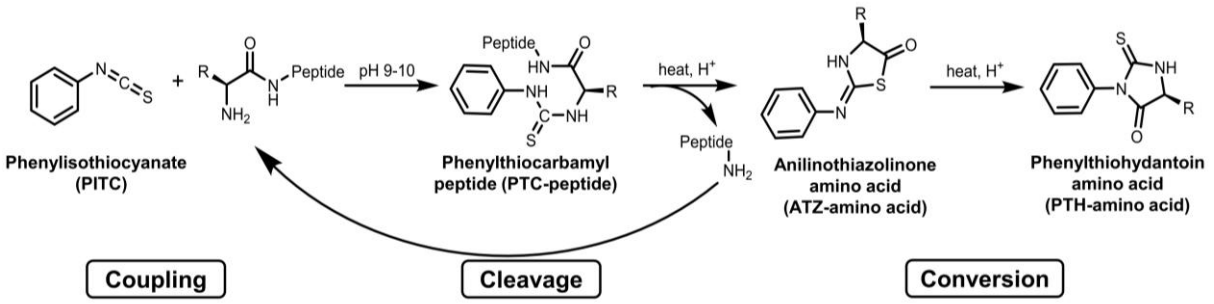
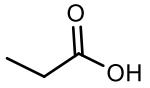
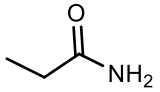
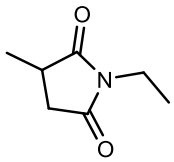
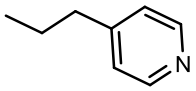
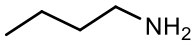
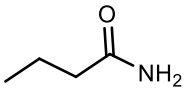


Figure 5: Reaction mechanism of Edman sequencing for proteins and peptides. (Modified from reference[188])

Modification approaches for cysteine were established and various cysteine alkylating reagents were utilized to modify the free cysteine, either natively present or released after partial or full reduction of disulfide bonds[227]. Edman degradation of modified cysteines forms a range of PTH-S-cysteine derivatives that facilitate the detection of cysteine residues. It starts with the cleavage of the disulfide bridges with reducing agents such as dithiothreitol (DTT) and tris-(2-carboxyethyl)phosphine) (TCEP) followed by reaction with an excess of alkylating agent[228,229]. The final step is the quenching of the unreacted alkylating agent and the removal of salts and reaction byproducts. A list of alkylating agents and the produced tagged cysteine products are presented in Table 6[230]. Iodoacetic acid and iodoacetamide are commonly applied for cysteine alkylation, however, low stability was stated for their adduct was under acidic conditions[227,231,232]. Haloacyl, N-ethylmaleimide (NEM), and 4-vinylpyridine alkylation are involved in various applied alkylation approaches[229,230]. N-ethylmaleimide was reported to be reactive with amino groups and histidine, and its adduct was found unstable[230]. Despite the usage of 4-vinylpyridine has been reported for sequencing, its derivative was susceptible to the ionic strength of the chromatographic solvents, resulting in altered retention time and decreased reproducibility[233].

Table 6: Common reagents for cysteine modification[230].

Alkylating agent	Molecular weight	Reaction product	Structure of S-added group (S-R)
Iodoacetic acid	186	S-carboxymethyl cysteine	
Iodoacetamide	185	S-carbamidomethyl cysteine	
Halocyl (iodide derivative) R-I	R- dependent	S-alkylated cysteine	R-
N-ethylmaleimide	125	S-(ethylsuccinimido) cysteine	
4-Vinylpyridine	105	S-pyridylethyl cysteine	
3-Bromopropylamine	138	S-aminopropyl cysteine	
Acrylamide	71	Cysteine-S-β- propionamide	

Moreover, modification using acrylamide into cysteine-S-propionamide that coelutes with (2,6-dimethyl-phenyl)thiourea (DMPTU), a byproduct of ED, also limit the usage of acrylamide besides its toxicity[234,235]. Ethylenimine and 2-Bromopropylamine corresponding cysteine derivatives were observed to elute close to PTH-lysine, i.e. are not detected properly[236,237]. Considering all the previously mentioned aspects, there is still no commercially available standard PTH-cysteine derivative for cysteine detection or quantification that can be introduced into the standard PTH-amino acid mixture for sequence evaluation in the first place and for disulfide bridge elucidation as well. In the following Chapters 3 and 4, the development of standard PTH-cysteine derivatives was described, in addition to an optimized workflow using the standardized derivatives that can be applied for elucidation of disulfide connectivity in disulfide-rich peptides and proteins, such as human insulin and members of conotoxins family using Edman degradation and mass spectrometric methods.

2. Bioanalytical workflow for qualitative and quantitative assessment of hot-melt extruded lysozyme formulations

Research article

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2.1 Introduction

Proteins and peptides have gained increasing interest as therapeutic agents over the last decades for treating and management of pathophysiological disorders such as cancer, and haematological diseases, and to date, more than 20% of the pharmaceutical marketed agents are biological medications[2,5,18,238]. However, due to the sensitive nature of biological moieties and their susceptibility to aggregation and denaturation, the development of a stable protein dosage form is needed and challenging[19]. Factors affecting the structural and functional integrity of the proteins during formulation should be closely monitored, especially on the large-scale transfer[239]. Therefore, various biological formulation techniques have been developed, such as hot-melt extrusion (HME) and were tested for their effects on active ingredient characteristics[240,241]. As a model protein, lysozyme has been selected to be formulated by different techniques and its stability is generally used as an indication of the influence of the formulation parameters on the carried active pharmaceutical ingredient (API)[242–244]. Additionally, for proper assessment of the formulations, bioanalytical methods are required for qualitative and quantitative analysis of the API in different stages of formulation as well as storage[239,245]. Thus, the objective of this article[246] is to establish workflows to evaluate lysozyme in its pure and hot-melt formulated form.

2.2 Summary and outlook

A hot-melt extruded formulation of lysozyme with polyethylene glycol (PEG) 20,000 as a biodegradable polymer is prepared in collaboration with AK Wagner (Pharmaceutical Technology, University of Bonn), and the extrudates were analyzed to evaluate the technique's impact on the biomolecule. A bioanalytical workflow was described and optimized for the analysis of lysozyme to assess its characteristics before and after formulation. The workflow starts with grinding the extrudates and extraction of PEG 20,000 prior to lysozyme analysis (due to interference), followed by a pre-analysis spectrophotometric quantification at 280 nm[247]. Extracted samples are quantified accurately by a validated RP-HPLC method (modified from Noda *et al*[248]) and the mass of lysozyme was confirmed using SDS-PAGE[249] (using 15% acrylamide gel) and MALDI-TOF mass spectrometry (using 2,5-dihydroxybenzoic acid[250] as a matrix in the presence of sodium trifluoroacetic acid) techniques, and the corresponding mass and band for lysozyme (~14.3 KDa) was detected. Moreover, the activity of Lysozyme was assessed using

the traditional turbidimetric method[251] and fluorogenic assay using 4-methylumbelliferyl-*β*-*D*-*N,N′,N″*-triacetylchitotrioside as substrate hydrolyzed by lysozyme into 4-methylumbelliferone[252]. The linearity range of the fluorescent product was scanned, and kinetic parameters were calculated. Higher reproducibility and lower standard deviation (1.9%) were observed compared to the bacterial turbidimetric assay (18.2%), therefore was incorporated in the workflow. Hot-melt extrudates containing 20% w/w lysozyme and 80% PEG 20,000 (H-Lyso/PEG20) and an untreated physical mixture of the protein and polymer (P-Lyso/PEG20) as well as pure lysozyme (P-Lyso) were assessed using the optimized workflow. Recovery rates of lysozyme in the studied extrudates showed full recovery (99.9 ± 3.6%, n = 3) of the claimed contents and retained specific activity of lysozyme (100.3 ± 4.1%, n = 3), revealing maintained stability after the HME procedures.

The analytical workflow was not only applied for the assessment of the lysozyme/PEG20 extrudates but also to test their stability during storage at 4, 25, and 40 °C for six months through an accelerated long-term stability study following the ICH Topic Q 1 A (R2)[12] guidelines. Samples at different time intervals (day 0, week 2, week 4, month 3, and month 6) were withdrawn and analyzed. Compared to the P-lyso and P-Lyso/PEG20, the identity of extruded lysozyme was confirmed without evidence of degradations, and the findings (recovery rates and specific activity) confirmed the preserved quality throughout the study. Therefore, the established workflow can be applied for qualitative and quantitative analysis of hot-melt extruded lysozyme formulations that revealed not only the good stability of lysozyme upon storage at high temperatures (40 °C for six months) but also after exposure to hot-melting extrusion procedures (temperature up to 65 °C, shear mixing and pressing). The established workflow can be adapted into the routine analysis of lysozyme analysis. Future protein candidates can be treated with HME for stabilization, and whether to be analyzed with the established workflow with refinement needs to be investigated.

2.3 Author contribution

Diana Imhof and Karl G. Wagner designed the experimental studies with the help of the author Yomnah Y. Elsayed, Dr. Katharina Dauer, and Dr. Toni Kühl. Dr. Katharina Dauer formulated lysozyme into hot-melt extrudates or physical mixtures. The optimization for the analytical experiments was performed by the author Yomnah Y. Elsayed with the help of Alina Sayin. The author executed the analyses of lysozyme samples (including stability samples) and evaluated the data together with Prof. Dr. Diana Imhof and Dr. Toni Kühl. All authors discussed the results and contributed to writing the final manuscript. *The author, Yomnah Y. Elsayed, and Dr. Toni Kühl contributed equally.

3. Innovative pharmaceutical stabilization and formulation processes for protein drugs and their impact on sequence and structure. Part: Analytics

Book chapter

Authors: **Yomnah Y. Elsayed**, Toni Kühl, and Diana Imhof.

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3.1 Introduction

Therapeutic peptides and proteins contribute as a vital class of biopharmaceutical agents for controlling various pathophysiological disorders[2,5,238]. Their instability and high tendency for aggregation and hydrolysis raise the concern of biotechnologists to design and develop protective dosage forms that match the route of administration[10]. Innovative formulation techniques were elaborated, including hot-melt extrusion, referring to its stabilization potential for proteins in a solid state[253], besides the other advantages of enhanced bioavailability and drug targeting[111]. Analytical characterization of the cargo protein is required to ensure structural, functional, and sequential integrity, including essential post-translational modifications such as disulfide bridges, which play a crucial role in the protein's conformational folding and biological activity[176,193]. From this aspect, preserving the proteins' characteristics upon handling, processing, storage, or formulation is one of the primary targets of quality assurance procedures applied to therapeutic products[239]. Optimized and validated analytical methods are incorporated into bioanalytical workflows applied in order to assess the quality of the biomolecule and its disulfide connectivity[254,255]. A Plethora of peptides and proteins are disulfide-rich and active as important therapeutic agents, such as insulin (metabolic regulation[3]) and linaclotide (three-disulfide bonded peptide for irritable bowel syndrome[256]). Edman sequencing for proteins was described in this book chapter as an analytical procedure applied to determine the disulfide connectivity in peptides and proteins. Furthermore, suitable standards are needed to calibrate the analytical method for proper data evaluation, which was applied for Edman degradation for cysteine detection[36] and described in the chapter in detail.

3.2 Summary and outlook

Hot melting extrusion (HME) was evaluated using lysozyme extrudates with PEG20 000 described in Chapter 2[246] and its structural and functional properties were assessed by established workflow for qualitative and quantitative analysis of lysozyme after extracting PEG 20,000 following ICH guidelines Topic Q1A (R2)[12]. Starting with PEG extraction and further analysis by a RP-HPLC method, the recovery of samples was calculated. SDS-PAGE and MALDI-TOF techniques were applied to confirm the

structural integrity of lysozyme based on the observed band and mass[246]. Finally, the activity of lysozyme was evaluated using a fluorogenic assay[252] using 4-methylumbelliferyl-*β-D-N,N′,N″*-triacetylchitotrioside as substrate hydrolyzed into a fluorescent product correlated to lysozyme specific activity by quantitative correlation to found lysozyme content. The quality of lysozyme was observed to be maintained without signs of degradation or aggregation showing the conservation of characteristics after HME procedures (heating, shear mixing, and pressing). Moreover, accelerated stability study for lysozyme hot-melt extrudates was performed by storage at different temperatures (4, 25, and 40 °C for six months) according to ICH Topic Q1A (R2)[12] for stability studies of biotechnological products. Samples were withdrawn at different intervals and analyzed using the established protocol. Findings indicated retained lysozyme stability and activity over the storage time highlighting the stabilization chance offered by HME for the formulated protein[246]. Further application using HME can be an alternative stabilization technique for protein drugs and the presented bioanalytical workflow would be applied for assessment of lysozyme formulations qualitatively as well as quantitatively.

Analysis of disulfide connectivity in the studied biomolecules was performed using various mass spectrometric techniques that mainly employed enzymatic digestion and compared reducing and non-reducing conditions to elucidate fractions carrying cysteine residues[236]. Extensive data analysis of the digested fragments requires experienced labour and dedicated time for proper evaluation, with the risk of losing significant fragments, negatively affecting the evaluation accuracy[194]. Edman degradation was proposed in the manuscript[36] and this chapter as an analytical technique to be combined with MS techniques to provide complete elucidation and sequence confirmation. Edman sequencing has the drawback of indetectable PTH-cysteine (unstable[226,235,257]) and prior modification by an alkylating agent was suggested for its detection[230]. None of the reported modified PTH-Cys derivatives has been validated as a standard for cysteine detection and quantification. Herein, two PTH-Cys derivatives, PTH-S-methylated cysteine (PTH-Cys(Me)) and PTH-S-carbamidomethylated cysteine (PTH-Cys(Cam)) were suggested and examined for their characteristics as an Edman sequencing reference standard for the detection and quantification of cysteine in a peptide sequence. The two derivatives were synthesized, purified analytically characterized, and validated according to the ICH guidelines ICH M10[169] and Q14[170] for bioanalytical method validation. Both PTH-Cys derivatives fulfilled the requirements for a reference standard, however, close elution of PTH-Cys(Me) to the PTH-DTh (a byproduct of threonine sequencing) limited the quantification accuracy in the case of a former threonine residue in the amino acid sequence[36].

Next, a series of model peptides were designed to carry cysteine, threonine, and modified cysteines surrounded with the close-eluted amino acids, synthesized, and characterized followed by Edman sequencing, where the cysteine or modified cysteine cycles were observed. Results of sequencing the model peptides revealed clear detection and resolution of PTH-Cys(Cam) in comparison to PTH-Cys(Me) that got affected by PTH-DTh, suggesting that both derivatives can be applied for cysteine detection and PTH-Cys(Cam) is more favoured for quantification purposes. In-house produced analog of coagulation factor XIIIa inhibitor, tridegin[254] (66-mer with two disulfide bridges) was examined for its

disulfide connectivity using tris(2-carboxyethyl) phosphine (TCEP) as a reducing agent followed by derivatization of the released cysteines with iodoacetamide into carbamidomethyl cysteine quantitatively[190]. ED was applied on oxidized and fully reduced tridegin, and cysteine cycles were observed for the detectability of cysteine in C5, C17, C31, and C37, which was only possible in reduced tridegin.

Another example of multiple bridged proteins is the whey milk protein β-lactoglobulin (BLG)[258,259] where traditional MS protocols failed to ultimately determine the disulfide connectivity in different variants of BLG due to lost fragments in addition to diluted fragments led to inadequate elucidation. BLG was exposed to physical conditions such as irradiation by UV B light, mutations, or various production approaches[223,260]. Such treatment stimulated structural changes and altered connectivities[212], and the presence of a free cysteine Cys 121 in the BLG structure elevated the risk of disulfide scrambling upon digestion[190,193]. ED provided data about specific fractions to provide supporting information about structural changes for BLG sequence that emphasized the valuable contribution of ED in the complete analysis of BLG without the need for sophisticated crystallographic or NMR analyses[212]. The BLG analysis was performed by Dr. Toni Kühl in collaboration with PD Dr. Rebekka Biedendieck and Prof. Dr. Rainer Krull (Technische Universität Braunschweig), Prof. Dr. Julia Keppler (Wageningen University & Research) and Prof. Dr. Karin Schwarz (Christian-Albrechts-Universität zu Kiel).

3.3 Author contribution

The author, Yomnah Y. Elsayed, optimized the analytical experiments and executed the analyses of lysozyme samples (including stability samples). The author validated the PTH-Cys derivatives and sequenced model peptides and tridegin under the supervision of Diana Imhof. The synthesis of PTH-cysteine derivative and model peptides was technically supported by M.Beck (acknowledged).
Dr. Toni Kühl performed BLG analysis in collaboration with Rebekka Biedendieck, Rainer Krull, Julia Keppler, and Karin Schwarz. The author and Toni Kühl prepared the figures, and all authors carried out the data analysis. The book chapter was written through the contribution of all authors.

4. Edman degradation reveals unequivocal analysis of the disulfide connectivity in peptides and proteins

Research article

Authors: **Yomnah Y. Elsayed**, Toni Kühl, and Diana Imhof.

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4.1 Introduction

A plethora of peptides and proteins are characterized by their disulfide connectivity, which is essential for their desired folding, which provides proper ligand binding and retains their biological activity. Disulfide-rich proteins contribute as bioactive therapeutic agents for the management of various health conditions such as pain management (ziconotide), gastric disorders (linaclotide, plecanatide) and diagnostics, besides the vital role of hormones (insulin, angiotensin, and oxytocin) in metabolic regulation and homeostasis [3,187,261]. Their conformational and functional integrity highly depends on correct disulfide bridging within the protein sequence[13,254]. Recent advances in MS fragmentation and detection approaches, such as trapped ion mobility and source decay, were developed[208–211]; however, the risk of losing significant fragments or incomplete fragmentation/digestion, especially in low concentrations, limits the applicability for complete elucidation[194,222,223]. MS may not be the proper technique to elucidate all isomers of multiple bridged peptides; therefore, other analytical methods were involved. The manuscript[36] presented simple procedures to elucidate disulfide connectivity in proteins and peptides without complicated data evaluation or the need for digestion by incorporating Edman degradation (ED) with MS. Cysteine modification is required to facilitate the detection of cysteine residues by ED due to unstable PTH-Cys[226,257] and non-quantitative formation of diPTH-Cystine in case of disulfide bridge[181,231,237,262]. Numerous derivatizing agents for cysteine modification were suggested in the literature[230]. However, none was considered a standard for cysteine detection and quantification. Herein, two PTH-Cys derivatives were developed and validated as ED cysteine standards, and a series of model peptides were designed and sequenced to test the developed standards. Moreover, a Partial reduction protocol was established and applied to determine the disulfide connectivity in native disulfide-bonded peptides such as CCAP-vil, *μ*-KIIIA and human insulin, independently from MS analysis.

4.2 Summary and outlook

Two PTH-Cys derivatives (PTH-Cys(Me) and PTH-Cys(Cam)) were herein synthesized, purified and characterized using TLC, HPLC, ESI-MS, elemental analysis, and ^{1}H- and ^{13}C-NMR spectroscopy to ensure their analytical grade. Moreover, their elution pattern on the automated Edman sequencer Shimadzu PPSQ-53A was measured and validated according to ICH guidelines M10[169] on bioanalytical method validation in agreement with Q14[170] guidelines for quality assurance in analytical method development. The following validation parameters were examined for both derivatives as mentioned in

Table 5: 1) Selectivity and specificity: to ensure lack of interference with the solvents system and the other standard PTH-mixture used for ED sequencing calibration. 2) Calibration curve and linearity range of their peak area in correlation to concentration in pmol/50µl to obtain the corresponding regression equations and lower and upper limits of detection were specified. 3) Accuracy and precision: repeatability and reproducibility of obtained concentration at 4 levels of quality controls (QC) selected according to the linearity range for each PTH derivative. 4) Carry-over: the tendency for carry over was tested by injection of concentration exceeding the upper limit of quantification and observing a following blank cycle. 5) Dilution integrity: the effect of dilution on the repeatability was evaluated and 6) Stability: aliquots of low and high QC were prepared and stored at different conditions according to the tested stability. A) stock and working solutions were tested daily to check their stability at -20 °C. B) benchtop stability was checked by analyzing QC samples kept at RT for 30 minutes. C) Freeze thaw stability was evaluated by analyzing QC samples stored at -20 °C for at least 12 hrs and subjected to cycles of thawing and refreezing. D) Long-term stability was assessed by analyzing QC aliquots stored at -20 °C monthly in comparison to a freshly prepared set of QC samples.

Both PTH-cysteine derivatives showed good agreement over a practical linear range and proved to be an acceptable reference for cysteine detection and quantification, however, a close elution of PTH-Cys(Me) to the derivative of DTh (a byproduct of threonine sequencing) was observed that had an impact on quantification accuracy and was further investigated. Additionally, the stability of PTH-Cys(Cam) derivative was reported to be critical and upon executing the stability study, the derivative was found adequately stable for two months equivalent to the recommended storage period for the standard PTH-amino acid mixture.

In order to investigate the applicability of the developed standards, a series of model peptides (of length 5-9 amino acids) was designed to carry cysteine or modified cysteine residues surrounded with the close eluted amino acids (i.e. threonine, serine) in different positions referring to the elution pattern of the standard PTH-amino acid mixture chromatogram. Model peptides were sequenced in their bead-bound (on PVDF membranes) and free forms (on glass fiber disk) and the yield for the PTH-cysteine derivative confirmed the full sequence identification in both cases. The overlay of PTH-DTh and PTH-Cys(Me) because the close elution was confirmed by sequencing of model peptides carrying threonine before the modified cysteines led to slight interference with the quantification, however, the detection was feasible. That was not the case for PTH-Cys(cam), as threonine and its byproduct were resolved from its peak. Therefore, findings from sequencing the model peptides verified the applicability of the developed PTH-cysteine derivatives as standards for cysteine detection (both PTH-Cys(Me) and PTH-Cys(Cam)) and quantification (mainly PTH-Cys(Cam)). The latter was incorporated into a protocol for disulfide connectivity elucidation.

The manuscript outlined a protocol of partial reduction using TCEP as a mild reducing agent followed by derivatization using iodoacetamide[190,263]. The reduction was time-controlled by sample withdrawal at different time intervals (30 s up to 15 min) according to the nature of the disulfide-rich peptide. Chromatographic separation of the derivatized peptide at each time point was executed and the stage

of carbamidomethylation for separated fractions was confirmed by MALDI/TOF-MS or by LC-ESI-qTOF-MS. Two additional carbamidomethyl groups per open bridge (2 x 57 Da) have been observed, and later followed by N-terminal sequencing for the fractions of mass correlated to partially reduced peptide and thus, by detection of modified cysteines, the determination of the disulfide connectivity was facilitated.

CCAP-vil[264] is a 10-mer one-disulfide-bonded cyclic peptide sequenced in its oxidized and reduced form; the detection of PTH-Cys(Cam) in the cysteine cycles of the reduced CCAP-vil enabled the detection of cysteine positions in the peptide sequence which was not feasible in the oxidized form. A more complicated example is conotoxin *μ*-KIIIA[177], a 16-mer three-disulfide-bonded peptide, and with the successive reduction of the three disulfide bridges, various partially reduced peptide forms had been formed. After the collection of fractions, mass confirmation revealed apart from the oxidized peptide (M, peak I), the formation of partially reduced peptide with one open bridge (M +2 times Cam, peak II), partially reduced peptide with two open bridges (M +4 times Cam, peak III) and fully reduced peptide with three open bridges (M +6 times Cam, peak IV). Edman sequencing for the partially reduced peptide (peak II) and detection of PTH-Cys(Cam) in cycles 1 and 15 indicated a bridge between C1 and C15; while the other bridges were intact. Moreover, sequencing of peak III with two additional detected PTH-Cys(Cam) peaks in cycles 4 and 16 indicated a second reduced bridge between C4 and C16. The fully reduced *μ*-KIIIA with six carbamidomethylated cysteines (peak IV) showed three open bridges, and the last bridge was concluded to be between C2 and C9.

Furthermore, human insulin as a polypeptide with two chains A and B connected with two interchain and one intrachain disulfide bridges was analyzed by the suggested protocol. Collected fractions with masses corresponding to partially reduced human insulin (M +2times Cam) and (M +4times Cam) were subjected to Edman sequencing and the detection of PTH-Cys(Cam) indicated the intrachain connectivity in chain A C6-C11 and the interchain connectivity C7-C7 in both chains A and B. Full reduction of human insulin released two reduced and modified chains indicating a third bridge between C20 (chain A) and C19 (chain B) matching the human insulin's disulfide connectivities in literature[180,265]. Schemes for sequencing results of *μ*-KIIIA and human insulin were discussed in the manuscript to facilitate results evaluation and decision of the disulfide connectivity in the peptide. In the future, the above-suggested protocol can be further applied to multiple bridges, longer peptides and proteins (more than three bridges).

4.3 Author contribution

The author, Yomnah Y. Elsayed, analyzed the literature, validated the PTH-cysteine derivatives, sequenced the model peptide, and optimized and performed the partial reduction protocol on CCAP-vil, *μ*-KIIIA and human insulin. The author prepared the figures and wrote the main text and supplementary information under the supervision of Prof. Dr. Diana Imhof and Dr. Toni Kühl. The data evaluation and writing the manuscript were carried out by all authors.

5. Summary

Therapeutic proteins and peptides represent an essential branch of biopharmaceuticals, contributing to ~25% of the pharmaceutical market, reflected by the increasing number of approved biopharmaceutical agents by the FDA annually for the treatment of various pathological conditions[1–6]. However, proteins and peptides possess the advantage of higher receptor specificity, potency, and safety profile, the drawback of their instability is challenging during preparation, handling, and storage[3]. The physical and chemical forms of protein degradation affect its structure and conformation[24,40,41], and respectively, the activity; therefore, stabilization techniques such as hot-melt extrusion were developed in order to protect the active biological molecule against surrounding stressful conditions[10]. Moreover, the application of heat and shear pressure during extrusion could be applied to thermal stable molecules, and these factors' impact on the protein characteristics was questionable[66,111]. Consequently, to assess the stability of proteins, analytical workflows and protocols are needed for proper characterization to confirm the integrity of the therapeutic biomolecule's necessary sequential and structural properties[10,123]. Besides, the demand for validated standards for method calibration is a prerequisite for quality control regulations highlighted by international organizations such as the FDA and EMA through the ICH guidelines[12,13,266] for analytical procedures, which was addressed in the thesis chapters.

In the second chapter, lysozyme was selected as a test protein mixed with PEG 20,000 as a biodegradable polymer and treated with HME to evaluate the technique's influence on the target molecule[111]. The manuscript[246] described a bioanalytical workflow applied for the analysis of lysozyme in its untreated and hot-melt form in order to assess its identity, purity, biological activity and stability before and after formulation. The preparation of extrudates was designed and optimized in collaboration with Prof. Dr. Karl G. Wagner and Dr. Katharina Dauer (Pharmaceutical Technology, University of Bonn). Analysis for lysozyme characterization was performed under Prof. Dr. Diana Imhof's supervision. First, the extraction of PEG 20,000 from ground extrudates was performed, followed by a pre-analysis spectrophotometric quantification assay for lysozyme. The applied workflow is based on a validated RP-HPLC method for quantitative analysis of lysozyme in the analysed sample. Preservation of molecular properties was evaluated using SDS-PAGE and MALDI-TOF mass spectrometry techniques and characteristic mass and band for lysozyme (~14.3 KDa) was detected. Furthermore, activity assays for lysozyme were reviewed using the commonly used turbidimetric method[251] in comparison to fluorogenic assay using 4-Methylumbelliferyl-*β-D-N,N′,N″*-triacetylchitotrioside as substrate[252]. The latter was found to show higher reproducibility and lower standard deviation (1.9%) compared to the bacterial turbidimetric assay (18.2%). Therefore, it was adapted to the suggested workflow.

The established workflow was applied to analyze pure lysozyme (P-Lyso), hot-melt extrudates containing 20% w/w lysozyme and 80% PEG 20,000 (H-Lyso/PEG20) as well as an untreated physical mixture of the protein and polymer (P-Lyso/PEG20). Data evaluation has been executed to examine the samples' quality and the obtained recovery rates of lysozyme in the studied extrudates showed 99.9 ±

3.6% (*n* = 3) of the claimed contents, and the specific activity of lysozyme was observed to be retained 100.3 ± 4.1% (*n* = 3) after the HME procedures, that indicated maintained characteristics.

The analytical workflow was applied to assess the extrudates' quality after storage at 4, 25, and 40 °C for six months through an accelerated long-term stability study following the ICH Topic Q1A (R2) guidelines[12]. Samples at different time intervals (up to six months) were withdrawn and analyzed. Observed findings (recovery rates and specific activity) for the stored extrudates confirmed the preserved quality throughout the study. Therefore, the established workflow can be applied for qualitative and quantitative assessment of hot-melt extruded lysozyme formulations that revealed an accepted stabilization effect for lysozyme after formulation (up to 40 °C for six months or at 65°C for minutes during HME).

The validated bioanalytical workflow was also reported in a book chapter (Chapter 3) to highlight the potential of hot-melt extrusion as an innovative pharmaceutical stabilization and formulation technique. Relatively significant is the presence of analytical tools to evaluate the technique in order to investigate the impact of formulation on the sequence and structure of protein drugs. As aforementioned, lysozyme was formulated with PEG 20,000 using HME (AK Wagner, University of Bonn), and the quality of lysozyme (purity, identity and activity) was assessed by application of the optimized workflow. Structural and functional integrity of lysozyme were preserved after formulation and on storage up to 40 °C for six months, as observed in Chapter 2. Furthermore, the impact on the sequence was studied, particularly on disulfide connectivity as an essential post-translational modification in proteins[267,268]. A plethora of disulfide-rich peptides and proteins contribute to vital functions in the human body (e.g. insulin) and used as medical agents to cure chronic conditions (e.g. linaclotide 3-disulfide bonded peptide for irritable bowel syndrome [256,269]), and their activity is mainly dependent on the correct protein conformation and folding, i.e. correct disulfide bonding[176]. Methodologies to elucidate the disulfide connectivity in a protein and peptides are developed to detect cysteine residues in a connected bridge, including mass spectrometry (MS) and tandem MS (MS/MS) based analysis depending on protein digestion followed by detection of molar masses corresponding to the digested fragments[189,270]. Data evaluation and elucidation of fragment patterns is challenging, especially in the case of complex patterns or unavailable calibration modules, in addition to the extensive time and expertise required for fragment elucidation [194]. Moreover, the risk of disulfide scrambling associated with digestion or the absence of available digestion sites hinders the elucidation of disulfide connectivity in some cases. Other than MS, Edman degradation (ED) was used for sequence determination; however, it failed to detect cysteine residues or disulfide bonds[181,231,237,257,262]. Neither technique, MS or ED, provides full and adequate recognition of complex sequences, raising the need to combine both for comprehensive sequence confirmation and determination of disulfide connectivity in the biomolecule.

As clarified from the chemical mechanism of ED[188] (Figure 5), it is based on the subsequent formation and release of phenylthiohydantoin (PTH) derivative of the N-terminal amino acids in a protein followed by chromatographic elution and detection of the amino acid sequence that fails to give a quantitative

product in the case of cysteine residues[209]. Therefore, protocols for cysteine modification are adapted in order to enable the detection of cysteine residues using a range of derivatizing agents[230] (Table 6). There is no commercially available validated standard for cysteine detection and quantification. In Chapters 3 and 4, two PTH derivatives of modified cysteines were selected and studied for their analytical characteristics as bioanalytical standards for cysteine after Edman sequencing for a protein or peptide, namely PTH derivative of methylated and carbamidomethylated cysteine, abbreviated as PTH-Cys(Me) and PTH-Cys(Cam). The PTH-derivatives were herein synthesized, purified, characterized and validated following the ICH M10[169] and Q14[170] guidelines for bioanalytical methods.

The following validation parameters were examined for both derivatives as aforementioned in Table 5: i) selectivity and specificity, ii) calibration curve and linearity range, iii) accuracy and precision, iv) carry-over effect, v) integrity of response after dilution and vi) stability. The following stability parameters were studied: stability of stock and working solutions at -20 °C, benchtop stability, freeze-thawing, and Long-term stability were assessed by analyzing stored QC aliquots compared to a freshly prepared set of QC samples. Both PTH-cysteine derivatives fulfilled the criteria as an acceptable cysteine detection and quantification reference. However, a close elution of PTH-Cys(Me) to the derivative of DTh (a byproduct of threonine sequencing) was observed and further investigated. Additionally, the stability of PTH-Cys(Cam) derivative was found to be adequately stable for two months equivalent to the recommended storage period for the standard PTH-amino acid mixture.

In order to investigate the applicability of the developed standards, a series of model peptides (of length 5-9 amino acids) was designed to carry cysteine or modified cysteine residues surrounded with the close eluted amino acids (i.e. threonine, serine) in different positions referring to the elution pattern of the standard PTH-amino acid mixture chromatogram. The Model peptides were sequenced in their bead-bound and free forms, and the yield for the PTH-cysteine derivative confirmed the full sequence identification in both cases. The overlay of PTH-DTh and PTH-Cys(Me) was confirmed by sequencing of model peptides carrying threonine before the modified cysteines led to slight interference with the quantification; however, detection of cysteine was possible. That was not the case for PTH-Cys(cam); therefore, findings from sequencing the model peptides verified the applicability of the developed PTH-cysteine derivatives as standards for cysteine detection (both) and quantification (mainly PTH-Cys(Cam)), thus involved in the analysis of native peptides.

Based on validation results, PTH-Cys(Cam) was selected as a modified cysteine derivative to be elaborated into the protocols applied for disulfide connectivity elucidation in disulfide-rich peptides. In chapter 3, coagulation factor FXIII inhibitor tridegin, a 66mer peptide modified to carry two disulfide bridges, between C5 - C37 and C17 - C31[271]. N-terminal sequencing was applied to the oxidized and fully reduced tridegin obtained after the reaction with tris(2-carboxyethyl) phosphine (TCEP), present in excess, followed by derivatization using excess iodoacetamide that derivatized the free thiol groups in the released cysteines after breakage of disulfide bridges with added carbamidomethyl groups[190].

Sequencing analysis of cysteine cycles in the oxidized and reduced tridegin revealed the quantitative conversion of cysteines into carbamidomethylated cysteines, and detection was successfully enabled.

Besides validation of the PTH-cysteine derivative as a bioanalytical standard according to ICH guidelines and testing their applicability for cysteine detection in the model peptides, the involvement of these standards in a protocol for disulfide connectivity elucidation in native peptides and proteins by merging Edman sequencing was additionally described as an objective in Chapter 4. The manuscript[36] outlined a protocol of partial reduction using TCEP as a mild reducing agent followed by derivatization using iodoacetamide that was applied for analysis of disulfide connectivity in the cardioactive peptide CCAP-vil, *μ*-conotoxin KIIIA and human insulin. Different samples were withdrawn at certain time intervals (30 s up to 15 min) according to the nature of the disulfide-rich peptide. Chromatographic separation of the derivatized peptide was executed, followed by confirmation of the stage of carbamidomethylation by MALDI/TOF-MS using HCCA as a matrix or by LC-ESI-qTOF-MS. By observing additional carbamidomethyl groups (2 x 57 Da per open bridge), the fractions of mass correlated to partially reduced peptide were selected for Edman sequencing. By detecting modified cysteines, the determination of the disulfide connectivity was facilitated.

CCAP-vil[264] is a 10-mer one-disulfide-bonded cardioactive cyclic peptide reduced using TCEP and derivatized, and PTH-Cys(Cam) peak was observed at cysteine cycles in reduced CCAP-vil. It enabled the detection of cysteine residues in the peptide sequence, which was not the case in the oxidized form. Conotoxin *μ*-KIIIA[177], a 16-mer three-disulfide-bonded peptide, was subjected to the partial reduction protocol. Upon the successive reduction of the three disulfide bridges, various partially reduced peptide forms were formed. Mass analysis of collected fractions revealed the oxidized peptide (M), partially reduced peptide with one open bridge (M +2 times Cam), partially reduced peptide with two open bridges (M +4 times Cam) and fully reduced peptide with three open bridges (M +6 times Cam). Edman sequencing for the peptide fraction of mass M +2 times Cam revealed PTH-Cys(Cam) in cycles 1 and 15, indicating a bridge between C1 and C15, and the other bridges were intact. Moreover, the fraction with mass M +4 times Cam showed an additional two PTH-Cys(Cam) peaks in cycles 4 and 16, indicating a second reduced bridge between C4 and C16. The fully reduced *μ*-KIIIA with mass M +6 times Cam showed three open bridges, indicating the last bridge between C2 and C9.

With the same concept, human insulin, as a polypeptide carrying three (intra- and interchain) disulfide bridges was analyzed by the suggested protocol. Again, the collected fractions with masses corresponding to partially reduced human insulin (M +2times Cam) and (M +4times Cam) were subjected to Edman sequencing and the detection of PTH-Cys(Cam) indicated the connectivities between C6-C11 in chain A and C7-C7 in both chains A and B, respectively. Complete reduction of human insulin released two reduced and modified chain A (mass of chain A + 4times Cam) and B (mass of chain B + 2times Cam), confirming a third bridge between C20 (chain A) and C19 (chain B) matching the reported human insulin's disulfide connectivities in literature[180,265]. Additionally, the manuscript

outlined detection schemes for evaluating sequencing results of *μ*-KIIIA and human insulin in order to facilitate the final elucidation of the disulfide connectivity in the peptide.

Taken together with the aforementioned findings, the present dissertation encloses bioanalytical workflows and protocols for the quality assessment of formulated peptides and proteins. The influence of processing parameters on the stability of the biomolecule can be deduced based on the validated methodologies and standards. Lysozyme formulation using hot-melt extrusion using PEG 20,000 adequately maintained their characteristics that extends the hot-met extrusion stabilization potential for other therapeutic agents. The structural and sequential integrity of the studied biomolecules has been investigated by Edman degradation adjacent to the MS analysis with the aid of an analytical protocol for determining the disulfide connectivity involving a validated PTH-cysteine reference derivative. The applicability of the established protocol confirmed the unequivocal elucidation of disulfide connectivity in model and native (up to three disulfide bridges) peptides without the need for enzymatic digestion or complicated data evaluation. Future applications to investigate sequence changes of formulated peptides and proteins can be fulfilled using the suggested protocol with additional refinements to study larger and richer (more than three disulfide bridges) proteins.

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List of abbreviations

aa	amino acid
BLG	β-lactoglobulin
Cam	carbamidomethyl
CD	circular dichroism
CPP	cell-penetrating peptide
Cys	cysteine
ED	Edman degradation
et. al.	*et alii*
FDA	Food and Drug Administration
GFD	glass fiber disk
HME	hot-melt extrusion
HPLC	high-performance liquid chromatography
ICH	International Conference of Harmonization
MALDI-TOF	matrix-assisted laser desorption ionization – time-of-flight
MS	mass spectrometry
NMR	nuclear magnetic resonance
PEG	polyethylene glycol
PTH	phenylthiohydantoin
PTM	post-translational modification
PVDF	polyvinylidene difluoride
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TCEP	tris(2-carboxyethyl) phosphine

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Appendix

This section of the thesis includes the full-length papers (main text), published and submitted within the conduction time of the doctoral studies. The respective introductions to the papers are found in chapters 2 – 4, in the same order: appendix A “Bioanalytical Workflow for Qualitative and Quantitative Assessment of Hot-Melt Extruded Lysozyme Formulations”, followed by appendix B “Innovative pharmaceutical stabilization and formulation processes for protein drugs and their impact on sequence and structure. Part: Analytics”, and appendix C “Edman Degradation Reveals Unequivocal Analysis of the Disulfide Connectivity in Peptides and Proteins”. The copyright of the papers belongs to the respective publishers of the journals, as indicated by copyright statements displayed before each paper (when applied).

Appendix A: 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. Bioanalytical Workflow for Qualitative and Quantitative Assessment of Hot-Melt Extruded Lysozyme Formulations . *ACS Omega.* (**2022**), 7(45), 40836–40843, doi: 10.1021/acsomega.2c03559.

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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*

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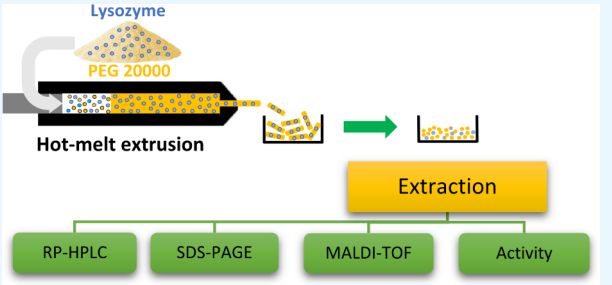
ABSTRACT: Structural and functional integrities of formulated proteins are key characteristics that provide a better understanding of influencing factors and their adjustment during formulation development. Here, the procedures commonly used for protein analysis were applied and optimized to obtain a 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

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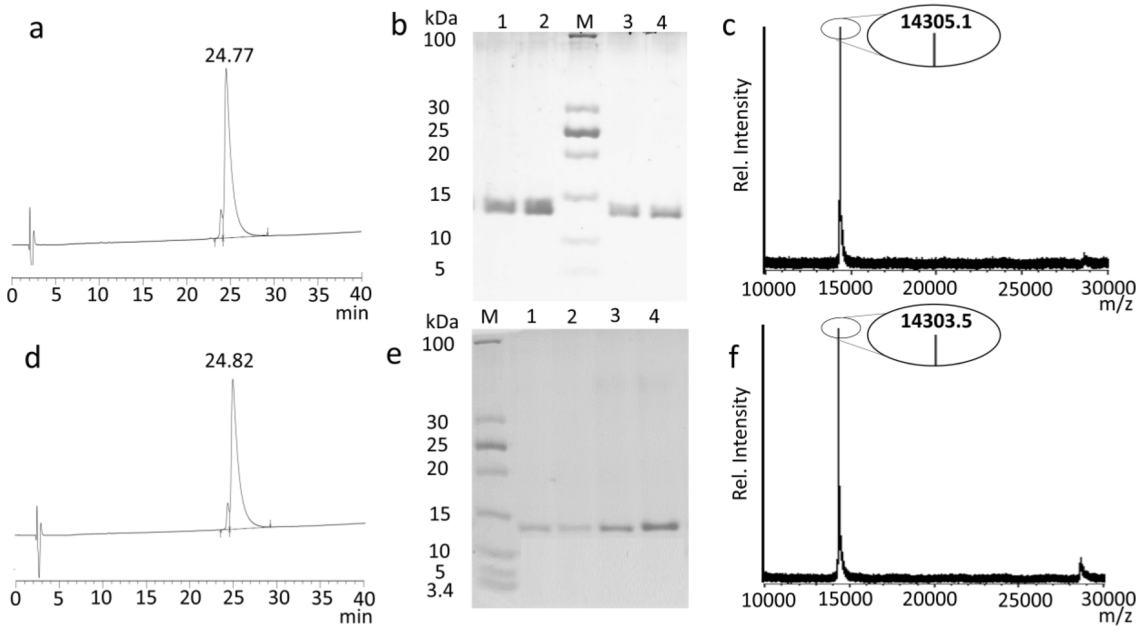

Figure 1. (a) HPLC chromatogram of 150 $\mu g \cdot mL^{-1}$ lysozyme in phosphate buffer (pH 5.2) using, as the mobile phase, acetonitrile/water /0.1% TFA (each) 20 to 60% for 40 min (b) SDS-PAGE analysis of 1 μg pure lysozyme under nonreducing conditions (lanes 1, 2) and reducing conditions (lanes 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

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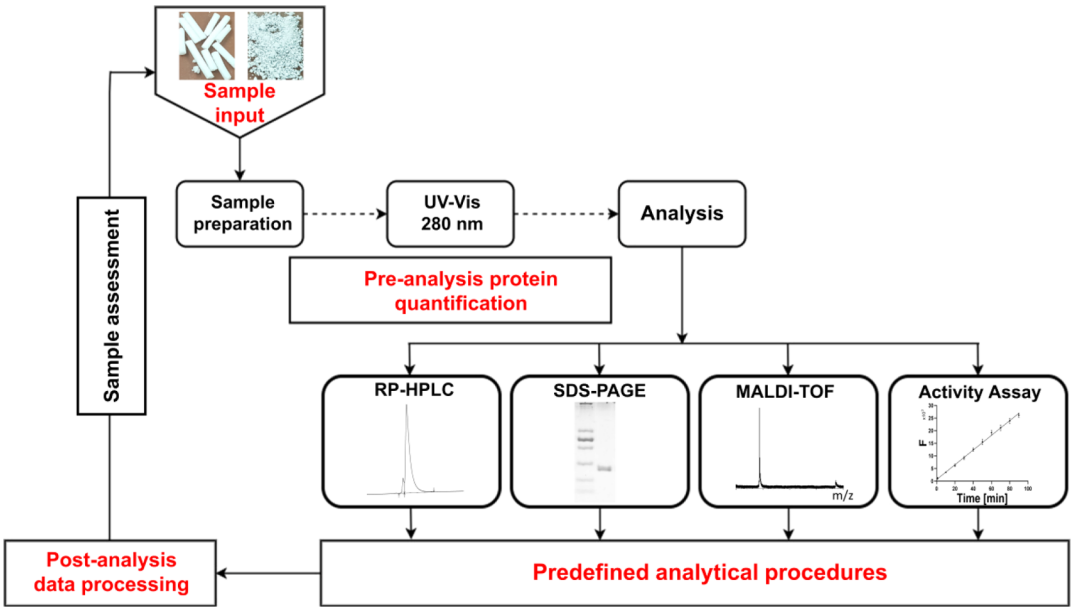
Scheme 1. Workflow for the Applied Analysis Methods on Hot-Melt Extrudates.



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.

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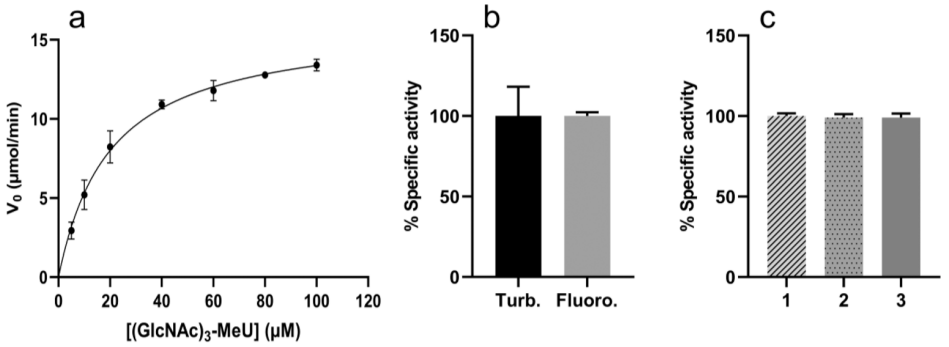

Figure 2. (a) Michaelis–Menten kinetics diagram for lysis of $(GlcNAc)_3$-MeU by lysozyme in 0.1 M sodium phosphate buffer, pH = 5.2 at 42 °C using GraphPad Prism 7 software (n = 3, $K_M = 2.02 \times 10^{-5}$ M). (b) % Specific activity of pure unextracted lysozyme samples by turbidimetric (n = 12)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

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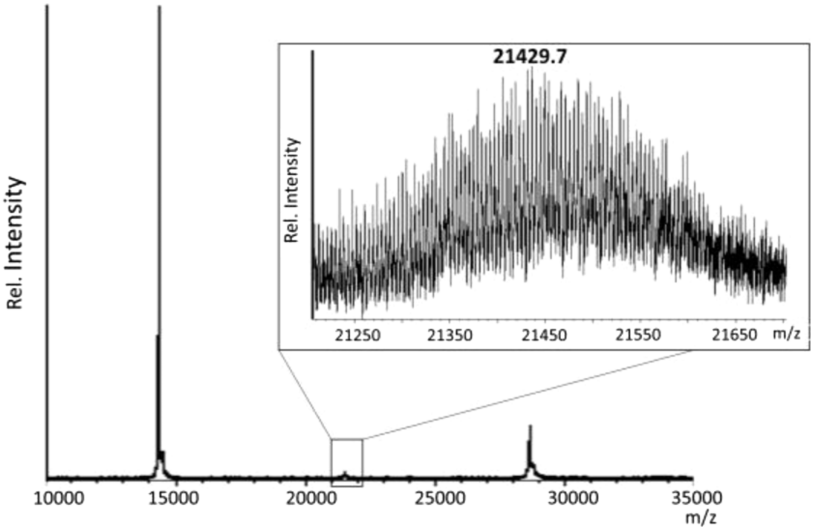

Figure 3. MALDI-TOF mass spectra of unextracted H-Lyso/PEG20 sample showed a lysozyme peak around 14 310 Da, PEG 20 000 was observed around 21 500 Da using 2,5-DHB as the matrix.

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

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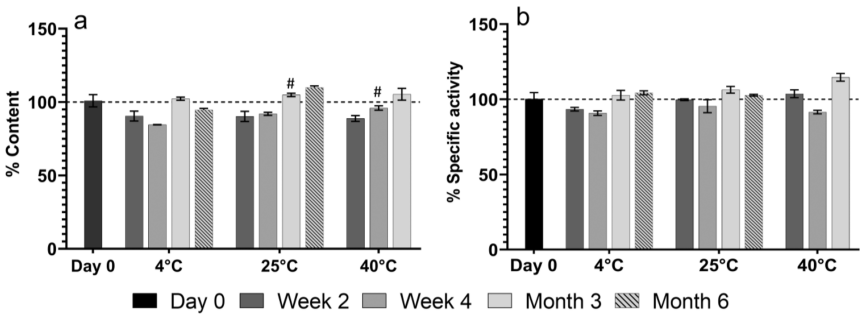

Figure 4. Content recovery rates (a) and % specific activities (b) of extracted H-Lyso/PEG20 extrudates stored at 4, 25, and 40 °C. Samples were analyzed at the following time points: day 0, week 2, week 4, month 3, and month 6 using the optimized RP-HPLC and the fluorogenic activity assay (n = 3 and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

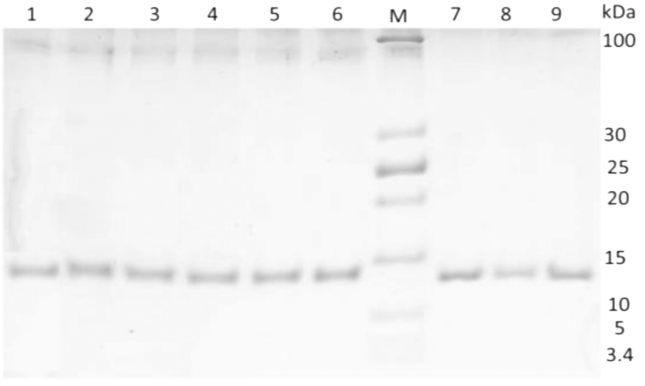

Figure 5. SDS-PAGE gel for 3 months-extracted H-Lyso/PEG20 samples at 4, 25, and 40 °C (lanes 1,2 and 3, respectively), extracted P-Lyso/PEG20 at 4, 25, and 40 °C (lanes 4, 5, and 6, respectively), and extracted P-Lyso samples at 4, 25, and 40 °C, (lanes 7, 8, and 9, respectively) showing a band for 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.

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■ 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)

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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.

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Notes

The authors declare no competing financial interest.

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Appendix B: Innovative pharmaceutical stabilization and formulation processes for protein drugs and their impact on sequence and structure. Part: Analytics

Yomnah Y. Elsayed, Toni Kühl, and Diana Imhof. Innovative pharmaceutical stabilization and formulation processes for protein drugs and their impact on sequence and structure. Part: Analytics (**2024**).

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Innovative pharmaceutical stabilization and formulation processes for protein drugs and their impact on sequence and structure. Part: Analytics

Yomnah Y. Elsayed, Toni Kühl, and Diana Imhof

Abbreviations

ED	Edman degradation
GFD	Glass fiber disk
HPLC	High performance liquid chromatography
HME	Hot-melt extrusion
SDS-PAGE	Sodium dodecyl sulfate–polyacrylamide gel electrophoresis
MALDI-TOF	Matrix-assisted laser desorption ionization – time-of-flight
PTH-AA	Phenyl thiohydantoin amino acid derivative
PVDF	Polyvinylidene fluoride membrane
BLG	β-lactoglobulin

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Abstract

Peptide and protein design for biopharmaceutical and food applications gained considerable attention in recent years. Numerous peptides and proteins are approved every year, however, their quality and long-term stability as e.g., a pharmaceutical product is still a matter of concern. Innovative methods in drug formulation were developed, including hot-melt extrusion and spray freeze drying, which have been tested to study their potential for protein stabilisation. Since the formulation procedures could also influence the features of the carried entity, it is also required to provide appropriate analysis methods to test stability, structural integrity, and activity of the peptide or protein cargo after formulation. In addition, many proteins carry post-translational modifications, such as disulfide bridges, which are crucial for the protein's fold and specificity and should thus be sustained throughout the formulation process. To address both issues, the development of a bioanalytical workflow for the analysis of hot-melt lysozyme extrudates as well as an Edman sequencing protocol for determining the disulfide connectivity in peptides and proteins were investigated and reported in this chapter. The latter study included the establishment of suitable cysteine standards for sequencing and their examination in the context of suitable model peptides before investigating relevant functionally active representatives.

Introduction

Over the last decade, proteins and peptides gained higher contribution in the pharmaceutical market (about 25%), which is also reflected by an increasing number of approved biological products every year for the treatment of various disorders.[1–3] However, the formulation of such entities is still challenging due to the well-known physical and chemical features, including instability, of proteins. A high tendency for aggregation or denaturation could affect the quality of the final product and activity on top.[4] Consequently, many formulation techniques were developed and studied in order to provide higher stability and protection of the protein or peptide cargo, especially before large-scale production of such valuable products is set up.

Hot-melt extrusion (HME) emerged as an interesting formulation technology that was applied in the pharmaceutical industry as a convenient and robust manufacturing tool for the preparation of solid dosage forms.[5] It provides advantage of taste masking, higher bioavailability, and the possibility of drug targeting,[6] and thus was used as an innovative

stabilization technique for proteins. Through HME, the target is mixed with selected polymers and plasticizers, and heated in a stepwise manner then extruded into a final homogeneous solid rod. Cossé et al.[7] confirmed the applicability of the hot melt extrusion process to prepare homogeneous solid dispersions of different proteins in a PLGA-matrix.

The first part of our studies thus focussed on the development of a general procedure for analysing proteins from HM-extrudates [a corresponding link to Lamprecht/Wagner chapter in same book]. Thereby, lysozyme (N-acetylmuramide glycanhydrolase or muramidase) was selected as a model protein because it was already used in earlier studies employing techniques such as freeze drying,[8, 9] hot-melt extrusion,[10, 11] or other polymer-based formulation strategies.[12–14] A model HME formulation of lysozyme with polyethylene glycol (PEG20.000) for further analysis was provided by project part process [a corresponding link to Lamprecht/Wagner chapter] and examined thoroughly for its structural and functional properties before and after formulation following the workflow shown in Fig. 1A.[15] Evaluation of the model HME formulation was performed following the international guidelines for identity, purity, and activity of the International Council of Harmonization ICH Topic Q1A (R2).[16] The guidelines for biotechnological products stating the parameters to be tested were followed to examine the stability and activity of lysozyme after exposure of lysozyme to hot-melt extrusion and long-term storage of the formulation. As a result, the analysis data of the hot-melt extruded lysozyme should allow for judging the extent how the additives or applied procedures, in particular mechanical stress, and temperature changes, could impair with the proteins' properties during processing and the work-up procedure. The presence of such stability evaluation protocols is urgently needed to support the development of formulation strategies for protein drugs.

Continuing this line, the development of proper analysis workflows and protocols for protein drugs needs to be established as well, however, may require even more customized strategies due to the diversity and individual complexity of the protein of interest (Fig. 1B). Among others, proteins are subjected to post-translational modifications that are significant for maintaining their structural integrity and functional activity.[17] The formation of disulfide bonds often leads to a well-defined 3-dimensional structure responsible for highly specific interactions and thus certain tasks in the context of the cell, tissue or even the entire organism.[18]. In nature, there is a plethora of disulfide-rich peptides and proteins occurring, which fulfil very different tasks and functions, ranging from regulation of metabolic reactions (e.g., insulin)[19] to management of chronic diseases such as plecanatide and linaclotide (2- and 3-disulfide bonded peptides for chronic idiopathic constipation and irritable bowel syndrome, respectively)[20, 21] or diagnostics such as

^{68}Ga-dotatoc[22]. The correct disulfide bond connectivity is highly essential for proper folding. Different analytical techniques were developed to determine the disulfide connectivity of a peptide or protein, primarily employing enzymatic digestion and applying reducing or non-reducing conditions including derivatization of the released cysteine of the digest.[23]

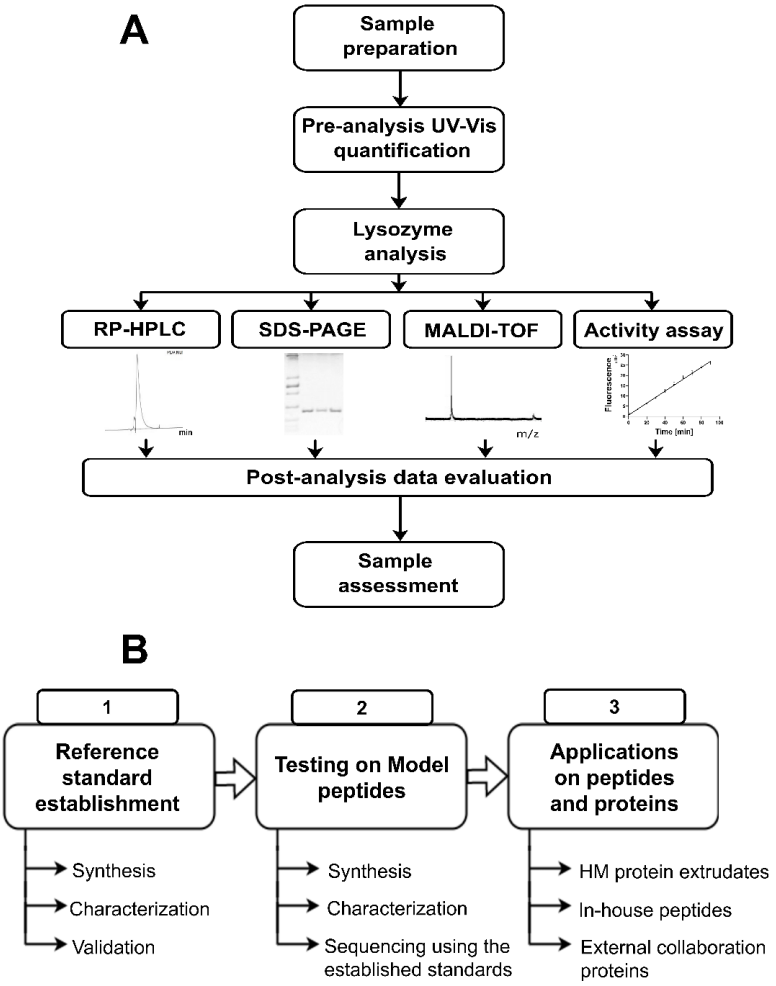

Fig . 1. A) Schematic representation of the workflow for the analysis of the lysozyme hot-melt extrudates (modified from [15], further permissions related to the material excerpted should be directed to the ACS), B) Workflow for establishing a strategy for the elucidation of the disulfide bond connectivity using automated Edman sequencing.

This is followed by an intense analysis using mass spectrometric (MS) and MS/MS methods to investigate the molar masses obtained for the digested fragments and perform sequencing of these peptides.[24] Apart from MS, also automated Edman degradation was used, however, fails to adequately determine cysteine or disulfide bonds. So far, both techniques are not approved for full and unequivocal recognition of a complex modified protein sequence,[25] proving the necessity of combining Edman degradation and MS

methods for complete sequence confirmation and elucidation of the disulfide bond connectivity in peptides and proteins.

Therefore, in the second part of our studies, we focussed on the assessment of the earlier established MS methods and protocols for disulfide bond analysis[26, 27] as well as the revival of the method of Edman degradation to check its applicability for the same scientific question. Edman degradation (ED) is an N-terminal sequencing technique introduced in the 1950ies that is based on the stepwise release of the N-terminal amino acid after modification with phenyl isothiocyanate, subsequent cyclization and cleavage of the amino acid as phenyl thiohydantoin (PTH) derivative.[28] The released PTH-amino acid derivative is eluted and compared with the characteristic elution pattern of a PTH-amino acid standard. After cleavage and release of the N-terminal amino acid, the remaining protein is recycled, and the next amino acid can undergo the same procedure. One major drawback of ED is the incomplete analysis and detection of cysteines due to incomplete conversion or cleavage of cysteine.[29] One of the attempts investigated in the present study was to develop suitable standards of modified cysteines that could be applied for cysteine detection in protein samples to minimize or bypass digestion with a maximum of accuracy concerning connectivity preservation and detection. The bioanalytical method development according to ICH M10[30] and Q14[31] was applied on the PTH cysteine standards synthesized herein in order to test their applicability as reference standard. Additionally, incorporation of the developed standards into model peptides and establishment of protocols for partial reduction followed by an alkylation step were undertaken to facilitate future application on peptides and proteins. In parallel, the earlier introduced MS protocols were applied to a selection of β-lactoglobulin (BLG) proteins from natural sources or recombinant expression,[32–34] Since BLG is a suitable example of a 2-disulfide bonded miniprotein, which has a special feature, namely an additional free cysteine. Although intensive studies have been carried out to recombinantly produce and modify BLG and thereby change its physicochemical properties, continuous analysis and monitoring of changes in disulfide connectivities have not been primarily focussed on yet.[32, 34] This becomes even more interesting when modifications, such as mutations, are introduced or specific treatments, such as chemical reduction or UV light irradiation, are applied in which the structural features, i.e. the disulfide bond patterns of the resulting BLG versions, are changed.

Material and Methods

Chemicals

Freeze dried chicken egg white lysozyme hydrochloride, Tris(2- carboxyethyl)phosphine (TCEP), *N,N′*-methylene-bisacrylamide, Ammonium persulphate (APS) and Coomassie brilliant blue G-250 dye were purchased from AppliChem GmbH, Darmstadt, Germany. Polyethylene glycol 20,000 were obtained from Carl Roth, Karlsruhe, Germany, while acrylamide, tris base/HCl, sodium dihydrogen phosphate, disodium hydrogen phosphate, glycine, EDTA, sodium citrate, citric acid, trifluoroacetic acid (spectroscopic grade) were purchased from Merck KGaA (Darmstadt, Germany), and *N,N,N′,N′*-tetraacetylethylenediamine (TEMED), HPLC grade acetonitrile and reagents for solid phase peptide synthesis (piperidine, N,N-dimethylformamide, and N-methylmorpholine) from VWR (Germany). Sodium dodecyl sulphate (SDS), 4-methyl-umbelliferyl *β*-D-*N,N′,N″*-triacetylchitotrioside, 4-methyl-umbelliferone, 2,5 dihydroxybenzoic acid (2,5-DHB), 2,5-dihydroxyacetophenone (2,5-DHAP) and α-cyano-4-hydroxycinnamic acid (HCCA), phenyl isothiocyanate (8.36M, 99%), 2-iodoacetamide, Sequa-brene, S-methyl cysteine hydrochloride and Fmoc-S-carbamidocysteine were obtained from Sigma-Aldrich, GmbH, Germany, sodium trifluoroacetate (98%) and 2-iodoacetamide were obtained from Thermo Fisher Scientific (Germany).

Reagents for the Edman protein sequencer (phenyl isothiocyanate (5% in n-heptane), acetonitrile (37% (v/v)), ethyl acetate, n-chlorobutane, trifluoroacetic acid, triethylamine, TFA treated glass fiber disks (GFD), polyvinylidene fluoride (PVDF) membranes, standard phenyl thiohydantoin (PTH) amino acid derivative mixture and PTH-amino acids mobile phase for isocratic elution were purchased from FUJIFILM, Wako Pure Chemical Corporation, Japan. The 2-disulfide bonded Tridegin was produced in-house as earlier described.[18, 35]

Instrumentation

Shimadzu LC-8A chromatograph (Duisberg, Germany) equipped with a Knauer 300-5 reversed phase C18 column (particle size 5 µm, 300 Å pore size, 32 x 250 mm) at 25 °C was used for preparative separation and purifications. Shimadzu LC-10AT chromatograph equipped with a Vydac 218TP54 reversed phase C18 column (particle size 5 µm, 300 Å pore size, 4.6 x 250 mm) was used for purity confirmation of synthesized PTH-Cys

derivatives and model peptides. Shimadzu LC-20AD chromatograph equipped with a Vydac 218TP54 reversed phase C18 column (particle size 5 µm, 300 Å pore size, 4.6 x 250 mm) at 25 °C was used for quantitative analysis of lysozyme and fraction collection of Tridegin samples after partial reduction and alkylation. Spectrofluorimeter FP-8300 (Jasco, Germany) was used for fluorescence measurements. Mass spectrometers: UltrafleXtreme TOF/TOF mass spectrometer (Bruker Daltonics GmbH, Bremen, Germany) was used for MALDI-TOF analysis and for LC-ESI-MS measurement, micrOTOF-Q III spectrometer (Bruker Daltonics GmbH, Bremen, Germany) was used. The NMR experiments for the ^{1}H and ^{13}C chemical shift assignments were performed on an Avance III HD 700 MHz NMR spectrometer (Bruker Daltonics GmbH, Bremen, Germany). Protein sequencing was executed using Shimadzu PPSQ-53A isocratic protein sequencer (Japan) equipped with Wakopak® Wakosil PTH-II column 4.6 mm x 250 mm (S-PSQ).

Hot-melt extruded lysozyme formulation and analysis

Effect of hot melt extrusion on lysozyme was studied by mixing lysozyme with PEG20,000 and as described earlier in chapter [a corresponding link to Lamprecht/Wagner chapter],[15]. 20% Lysozyme/PEG20,000 hot melt extrudates were prepared (referred as H-Lyso/PEG20) and a physical mixture of 20% lysozyme with PEG20,000 (referred as P-Lyso/PEG20) was prepared and tested to evaluate the effect of ram extrusion and heating on lysozyme characteristics. Both H- and P-Lyso/PEG20 as well as the untreated lysozyme (referred as P-Lyso) were stored at 4, 25 and 40 °C at 65% relative humidity (RH) ± 5% RH and their stability was evaluated according to ICH guidelines [16] at first day of formulation and after 2 weeks, 4 weeks, 3 months and 6 months. Four techniques were presented in our study to evaluate the quality of lysozyme in the accelerated storage samples.[15] Initially, extraction procedures of lysozyme from formulation were optimized to provide quantitative analysis of lysozyme, that was confirmed with RP-HPLC analysis of extracted samples using elution system of A: 0.1% TFA in water and eluent B:0.1% TFA in acetonitrile in gradient of 20 to 60% eluent B in 40 minutes and detection was at 220 nm. 50 µl samples were injected and peak area of lysozyme peak observed at 24.7 min was recorded. Concentration of lysozyme was determined using established calibration curve and regression equation over 50-500 µg/ml. Activity of lysozyme in samples was measured fluorometrically using 4-methyl-umbelliferyl *β*-D-*N,N′,N″*-triacetylchitotrioside as substrate releasing 4-methylumbelliferone that was quantitatively correlated to lysozyme content. SDS-PAGE was performed using 15% acrylamide gels for lysozyme bands and

indication of degradation products. MALDI-TOF mass spectrometric analysis was executed using 2,5-DHB matrix in presence of sodium trifluoroacetic acid to confirm sample identity and stability as well. The established workflow was applied on lysozyme and the tested formulations at the selected temperatures and specified time intervals. Quantitative and qualitative data evaluation for samples was implemented for assessment of their stability.

Edman degradation of cysteine-containing sequences

PTH amino acids were synthesized according to an earlier reported protocol,[36–38] purified and chemically analyzed using TLC, HPLC, ESI-MS, ^{1}H- and ^{13}C-NMR as described for PTH-derivatives.[39–41] The synthesized PTH-derivatives were validated according to ICH M10 for chromatographic methods. Model pentapeptides containing cysteine or modified cysteine in different positions and amino acid environment were automatically synthesized by solid-phase peptide synthesis following a standard Fmoc-protocol, purified and characterized using amino acid analysis, TLC, RP-HPLC and MALDI-TOF-MS using HCCA as the matrix.[42, 43] Subsequently, the model peptides were sequenced, and differential chromatograms were established for data evaluation by means of the developed standards. Edman sequencing was performed on an automated Edman protein sequencer PPSQ-53A using PTH-amino acid mobile phase as provided by FUJIFILM, Wako Pure Chemical Corporation, Japan at flow rate of 1 ml/min at 40 °C. Analysis was done by a PDA detector at 269 nm. For validation of PTH-Cys derivatives, they were directly eluted over the chromatographic system without prior chemical treatment by the sequencing unit. For sequencing of model peptides, peptide solutions were applied on GFD (200 pmol) and Edman degradation was executed. Each released N-terminal PTH-derivative was detected by the aforementioned isocratic HPLC system and identified based on a comparison to standard mixture of PTH derivatives of all proteinogenic amino acids (excluding cysteine, including dehydrothreonine).

As an example for an active peptide containing disulfide bridges, Tridegin was selected and subjected to full reduction and alkylation using 2-iodoacetamide in order to modify the reduced cysteines to carbamidomethyl cysteine. Separation of oxidized and fully reduced fractions was performed using HPLC. Fractions were subjected to Edman degradation by applying GFD membranes, the modified cysteines will be released as PTH-Cys(Cam) and detected during the ED analysis.

Results and Discussions

Qualitative and quantitative characterization of hot-melt extruded lysozyme by applying methods for protein bioanalysis

Analytical methods reported for hen egg white lysozyme characterization were re-evaluated to develop a proper analysis protocol for formulated lysozyme over a storage time of 6 months. The established analysis procedure based on RP-HPLC, SDS-PAGE, MALDI-TOF MS, and a fluorescence activity assay were tested initially on pure lysozyme (control) and then applied on lysozyme hot-melt extrudates.

For chromatographic analysis by RP-HPLC, the establishment of a calibration curve for untreated lysozyme was required. Serial dilutions of lysozyme (in 0.1 M sodium phosphate buffer pH 5.20, 50-500 µg/ml) were prepared, injected and their peak area (at t_R= 24.7 ± 0.2 min) was correlated to the corresponding concentration to establish the calibration curve that was used later for lysozyme quantification. Accuracy samples of unextracted lysozyme samples were prepared and tested. The recovery rate of the found lysozyme concentrations was 99.7 ± 1.56% (n = 6), that indicated applicability of the procedure for lysozyme quantification. In addition, identification of lysozyme in the pre-formulated state was performed using reducing and non-reducing conditions for SDS-PAGE on a 15% acrylamide resolving gel,[44] and molar mass was confirmed with MALDI-TOF mass spectrometric analysis of the protein samples. All protocols were proven useful for lysozyme identification and quantification and later used for the hot-melt extrudates (see below).

In order to test the lysozyme hydrolytic activity, the reported turbidimetric and fluorogenic assays were used and evaluated. The conventional turbidimetric assay[45] is based on incubation of lysozyme with bacterial culture. Changes in the culture's optical density are monitored and specific activity of lysozyme is then calculated as normalized density reduction to culture biomass. This method suffers from a high degree of deviation, low robustness and reproducibility (RSD = 18.2%, n=12), thus another assay was evaluated for quantitative activity assessment using a fluorogenic substrate, namely 4-methyl-umbelliferyl β-D-*N,N′,N″*-triacetylchitotrioside (in 0.1 M sodium phosphate buffer pH 5.2).[46] The substrate was hydrolyzed by lysozyme releasing 4-methyl-umbelliferone (4-MU), a fluorescent product measured at an excitation wavelength of 355 nm and an emission wavelength of 460 nm. The release kinetics parameters of 4-MU were studied by the measurement of the released 4-MU using different substrate concentration and a fixed

lysozyme concentration. The K_M value was found to be 20.2 μM that matched the reported value of 15.9 μM.[46] The activity of lysozyme (control) was thus determined and calculated using the fluorogenic method since it provided more accurate and reproducible results (RSD = 1.9%, n = 6). This fluorogenic assay was adapted and applied for the analysis of formulated lysozyme (Fig. 2).[15]

Initially, the effect of PEG20,000 on the aforementioned selection of methods for lysozyme analysis needed to be evaluated as the formulation of lysozyme by hot-melt extrusion was carried out using PEG20,000 as the polymer. It turned out that presence of PEG20,000 interfered with analysis of lysozyme, i.e., mass spectrometry analysis was impossible. Therefore, lysozyme extraction was required for analysis of formulated extrudates. Solubility of lysozyme and PEG20,000 were tested using different commonly used solvents such as acetonitrile, ethanol, methanol, acetone, diethylether and ethylacetate. Differential solubility was observed with acetonitrile, that solubilizes only PEG20,000 and for maximizing the extraction procedure. Hot-melt extruded lysozyme rods were ground to fine particles, so higher surface area exposure as well as complete precipitation was achieved. The precipitated lysozyme was collected by centrifugation and supernatants containing PEG20,000 were discarded, while lysozyme pellets were dried for further analysis. A suitable diluent was selected for each method; 0.1 M sodium phosphate, pH 5.2 was used for activity and chromatographic assays, while pellets were dissolved in double distilled water for SDS-PAGE runs followed by freeze drying and redissolution in 30% acetonitrile/0.1%TFA (v/v) for MALDI-TOF analysis.[15]

The established analytical methods and extraction protocols were applied on hot-melt extrudates (H-Lyso/PEG20), physically mixed lysozyme with PEG20,000 (P-Lyso/PEG20) and pure, untreated lysozyme (P-Lyso). P-Lyso samples were considered as controls to be tested along with each time point's sample set. Analysis of freshly formulated extrudates (Day 0) matched with the analysis of pure lysozyme and showed a good recovery rate of 99.9 ±3.6%, n = 3 of the claimed content (Fig. 2A). The obtained specific activities for the extrudates (100.3 ± 4.1%, *n* = 3) emphasized that lysozyme maintained its activity upon undergoing the process of hot-melt extrusion (Fig. 2B) that correlated with HPLC (Fig. 2C), SDS-PAGE (Fig. 2D) and MALDI-TOF-MS (Fig. 2E) findings verifying mass purity, compound identity and absence of aggregates or degradation products.[15]

The suggested workflow was subsequently applied to test the stability and lysozyme properties of extruded lysozyme after storage over different conditions as specified by ICH guidelines. Contents and specific activities were correlated to Day 0 results (Fig. 2A, B). Over a period of 6 month obtained contents for lysozyme were 84.6-110.0% ± 4.2% (n = 3

at each tested temperature and time point) and specific activities were 97.7-104.9% ± 4.7% (n = 3) indicating no significant activity changes upon storage. All SDS-PAGE gels and MALDI-TOF mass spectra also showed no change in lysozyme characteristics. For P-Lyso/PEG20 and P-Lyso full activity with 98.0−105.1% (RSD ± 3.1%, *n* = 3) and 90.2−110.6% (RSD ± 4.3%, *n* = 3), respectively, was observed.[15]

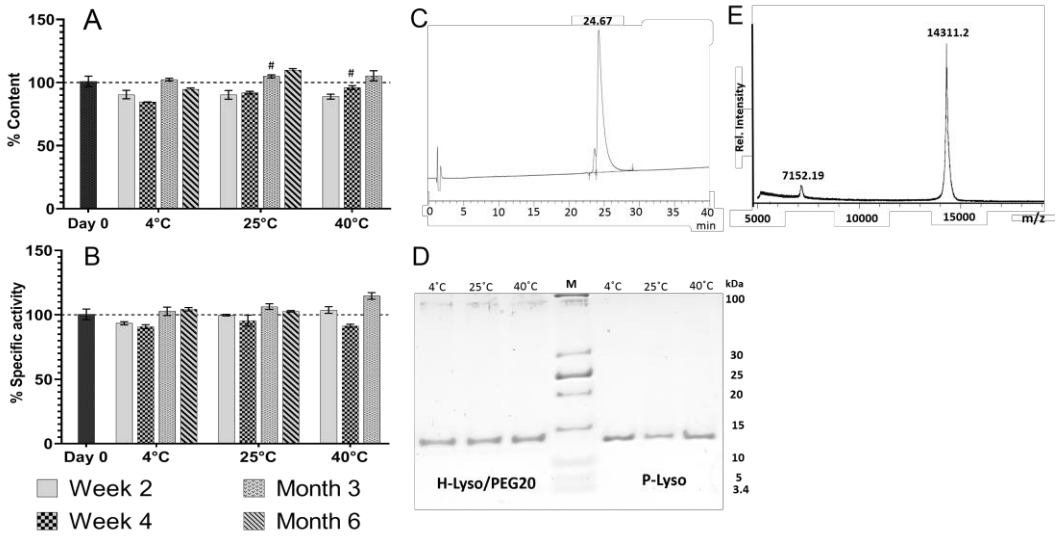

Fig . 2. A) Protein content analysis and B) specific activities results of extracted samples from 6-months stored H-Lyso/PEG20 at 4, 25, and 40 °C (n=3, # n=2). C) HPLC chromatogram of extracted H-Lyso/PEG20 (stored for 6 months at 25 °C, in phosphate buffer, pH 5.2), D) Reducing SDS-PAGE gel for extracted P-lyso and H-Lyso/PEG20 (stored for 6 months at 4, 25, and 40 °C), where M is the protein ladder, E) MALDI-TOF mass spectrum for extracted H-Lyso/PEG20 (stored for 6 months at 25 °C) using 2,5-DHB as matrix, modified from reference [15], further permissions related to the material excerpted should be directed to the ACS.

Establishment of a strategy to analyze free cysteines and disulfide bonds in peptides by automated Edman degradation

Analysis of cysteines and elucidation of the correct disulfide bond connectivity in a peptide or protein is essential for confirming their correct folding and active or functional state. This could, however, be affected during different processing procedures, such as formulation or handling of large quantities. Cysteine detection during ED is possible after modification of the thiol moiety and applying suitable PTH-cysteine standards for unequivocal identification. So far, the latter are missing, in particular if considering quantification of the sequencing steps. We herein established an in-depth analysis of two PTH-Cys derivatives to examine their applicability for proper detection and quantification.[47] Both, the PTH-derivative of methylated cysteine, i.e. PTH-Cys(Me), and the PTH-derivative of carbamidomethylated cysteine, PTH-Cys(Cam), were

investigated for their use as PTH-Cys standards in automated ED.[47] These derivatives were synthesized by the reaction of cysteine with phenyl isothiocyanate (PITC) in triethylamine and extraction of the mixture with ethyl acetate. After purification, the PTH-cysteine standards were purified and chemically characterized using TLC, HPLC, LC-ESI-MS, ^{1}H and ^{13}C-NMR spectroscopy to confirm purity and identity before use for Edman sequencing.[47]

Validation of the PTH-Cys derivatives as ED standards was performed to investigate their analytical properties according to the ICH guidelines ICH M10 and Q14. Therefore, stock solutions of both derivatives were prepared, diluted to obtain test samples and stored for different time periods before analysis on an automated protein sequencer accordingly. The elution of the respective peaks was monitored and the peak areas were recorded to evaluate the following parameters:[30, 31] i) selectivity of analysis in 37% acetonitrile, ii) specificity compared to other PTH-amino acid derivatives, iii) linearity range for the peak area related to the concentration of the PTH-amino acid derivative, iv) accuracy and precision, v) presence of carry-over effects during the sequencing cycles, vi) integrity of the response after dilution, and vii) stability. The stability was studied concerning the following criteria: short-term storage, room temperature, freezing-thawing, and long-term storage at -20 °C.[48] Both PTH-Cys standards displayed adequate characteristics as bioanalytical standard, however, PTH-Cys(Me) showed an elution time close to PTH-DTh (PTH-dehydrothreonine) that affected quantification accuracy to a certain extent (data not shown).[47]

Next, a series of model peptides (**1**-**12**) of different length containing cysteine, threonine and modified cysteines, respectively, were synthesized, chemically characterized and analyzed by ED (Tab. 1). For the selection of the peptide sequences we were considering the sequence environment in proteins as well as the contamination from adjacent amino acids eluting close to the PTH-Cys standards: While PTH-Cys(Cam) eluted next to threonine, quantification of PTH-Cys(Me) could be influenced by its close proximity to DTh, which is a product that could be formed upon sequencing of threonine. Therefore, model peptides containing threonine in relevant positions (1-2 previous cycles) to the modified cysteines were selected (Tab. 1). The peptide length varied between 5 and 9 amino acids with the residue of interest, i.e., threonine, cysteine or modified cysteine derivatives in the central position. Longer model peptides (9 amino acids) were sequenced to test the accessibility and yield of the modified PTH-Cys derivatives after sequencing of PTH amino acid derivatives with close by retention times such as Ser, Thr, His or DTh.[47]

Upon sequencing of the model peptides, each cycle was observed for characteristic peaks. Differential chromatograms were generated by subtraction of the previous cycle's chromatogram, which minimized background noise and provided an increased resolution of the obtained results. For model peptide **1**, peaks for PTH-threonine and dehydrothreonine derivatives could be observed, while detection of the unmodified cysteine in peptide **2** (Fig. 3) was not possible, which is in agreement with the literature.[49] Thr and DTh in peptide **1** were detected at 4.30 and 9.15 min, respectively.

Table 1. Sequences of selected model peptides for analysis in automated Edman degradation. All peptides were synthesized as C-terminal amides and as resin-bound compounds.

No.	Peptide sequence
1	AA**C**AA
2	AA**T**AA
3	AA**C (Me)** AA
4	**T**A**C (Me)** AA
5	A**TC (Me)** AA
6	AYR**C (Me)** MVAA
7	AYR**TC (Me)** MVAA
8	AA**C (Cam)** AA
9	**T**A**C (Cam)** AA
10	A**TC (Cam)** AA
11	NQS**C (Cam)** HGAA
12	NQS**TC (Cam)** HGAA

The PTH-Cys derivatives PTH-Cys(Me) and PTH-Cys(Cam) could also be clearly observed with an appropriate resolution to still distinguish Cys sufficiently from surrounding byproduct peaks as can be seen from peptides **3** and **8** (Fig. 3). However, a high degree of overlay was observed between PTH-DTh and PTH-Cys(Me) (9.10 min) (Fig. 3).[47]

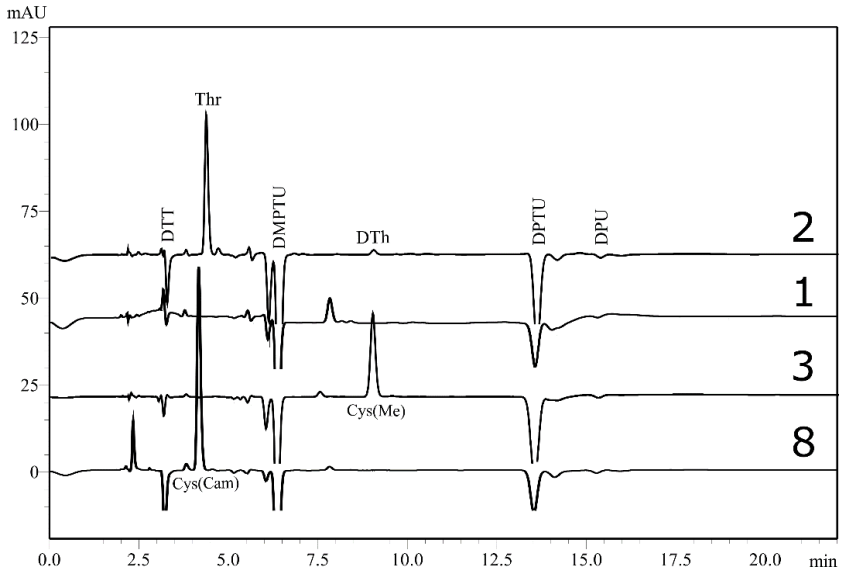

Fig . 3. N-terminal Edman degradation sequence analysis: Differential chromatograms of cycle 3 for peptides **1** (AACAA), **2** (AATAA), **3** (AAC(Me)AA), and **8** (AAC(Cam)AA) displaying the peaks of PTH-Cys(Me) and PTH-Cys(Cam) (modified from [47]).

Analysis of peptides **4** and **5** with threonine at positions -1 or -2 relative to the modified cysteine revealed that DTh was produced in a minor amount in all cycles of peptides that contained Thr and accumulated in the next cycle as a broad, though, small peak. The latter was also confirmed by sequencing of longer peptides **6**, **7**, **11**, and **12** (8-9 amino acids, Fig. 4). The maximum effect for the accumulation was found in peptides containing threonine in the immediate prior cycle to a cycle, in which a cysteine derivative was supposed to occur. However, detection of cysteine was still feasible and identification of DTh from Cys(Me) could be achieved due to the significant intensity differences between PTH-DTh and PTH-Cys(Me) in peptides containing threonine.[47]

The proximity of the peaks for Thr (4.30 min) and Cys(Cam) (4.18 min) was evaluated with the model peptides **8**-**12**. Here, identification and quantification of the Cys(Cam) derivative peaks were achieved without interference from Thr (Fig. 4 top).[47]

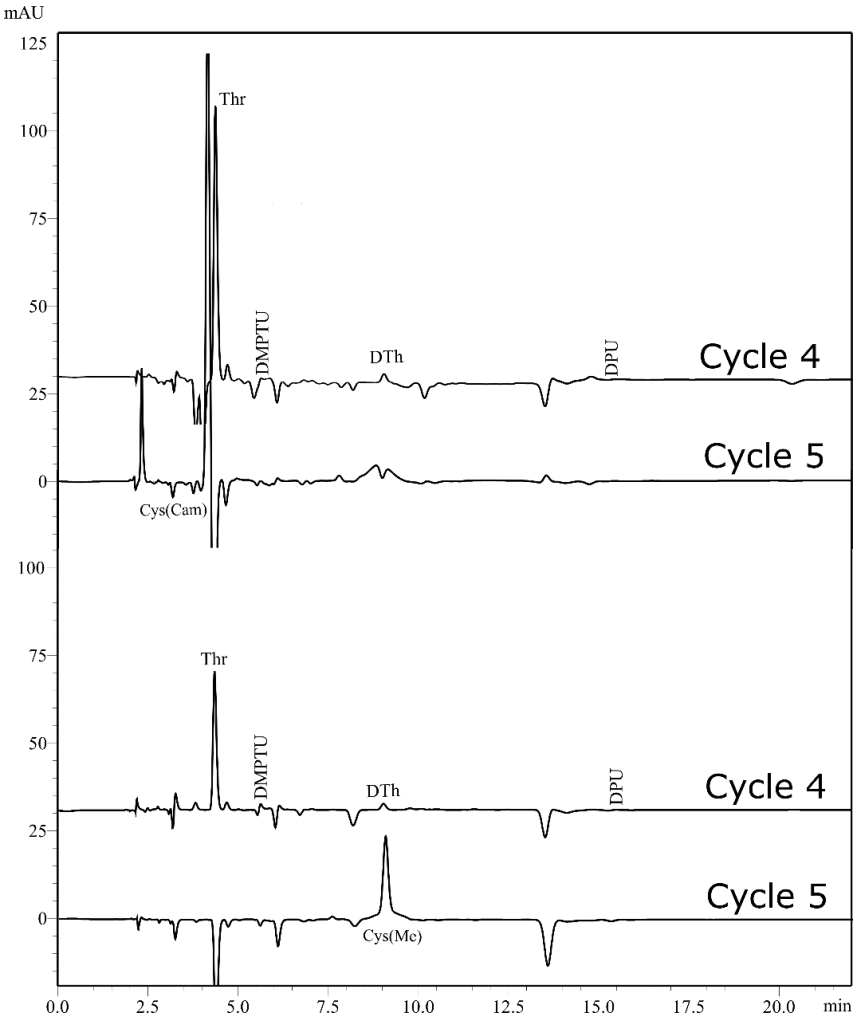

Fig. 4. N-terminal Edman degradation sequence analysis chromatograms of cycles covering Thr4 and Cys5 for peptides **12** NQS**TC(Cam)**HGAA (top) and **7** AYR**TC(Me)**MVAA (bottom) displaying the peaks of PTH-Cys(Cam) (cycle 5, top) and PTH-Cys(Me) (cycle 5, bottom).[47]

Taken together, although there is some interference between PTH-DTh and PTH-Cys(Me), which will slightly affect the quantification of the PTH-Cys(Me) derivative, in standard proteins DTh appears very rarely and will, thus, not significantly affect standard analyses procedures in which modified cysteines such as PTH-Cys(Me) or PTH-Cys(Cam) are produced and analyzed. The information about the herein described PTH-Cys derivatives obtained from the sequencing of the model peptides thus confirms applicability of the derivatives for cysteine detection (for both) and quantification (for Cys(Cam) with higher accuracy).[47]

In the next step, natural disulfide-containing peptides should be examined to check the applicability of the established PTH-Cys standards. An in-house produced analog of the

leech-derived compound tridegin was selected for the determination of the disulfide connectivity. This tridegin derivative is a 66mer 2-disulfide-bonded peptide inhibitor of coagulation factor XIIIa that was synthesized according to an orthogonal protecting group strategy to selectively form disulfide bridges between C5 and C37 as well as C17 and C31 (Fig. 5A).[41] Edman sequencing was implemented as a tool to confirm formation of disulfide bridges in tridegin compared to the completely reduced compound. In order to perform the analysis, the oxidized and the fully reduced peptide obtained after treatment with an excess of TCEP have been applied to excess concentrations of iodoacetamide for derivatization of available free thiol groups.[47] As expected, the analysis revealed that complete reduction and cleavage of disulfide bridges followed by quantitative conversion of free cysteines into carbamidomethyl cysteine (Cys(Cam)) occurred at all available positions 5, 17, 31, and 37 (Fig. 5B-E).

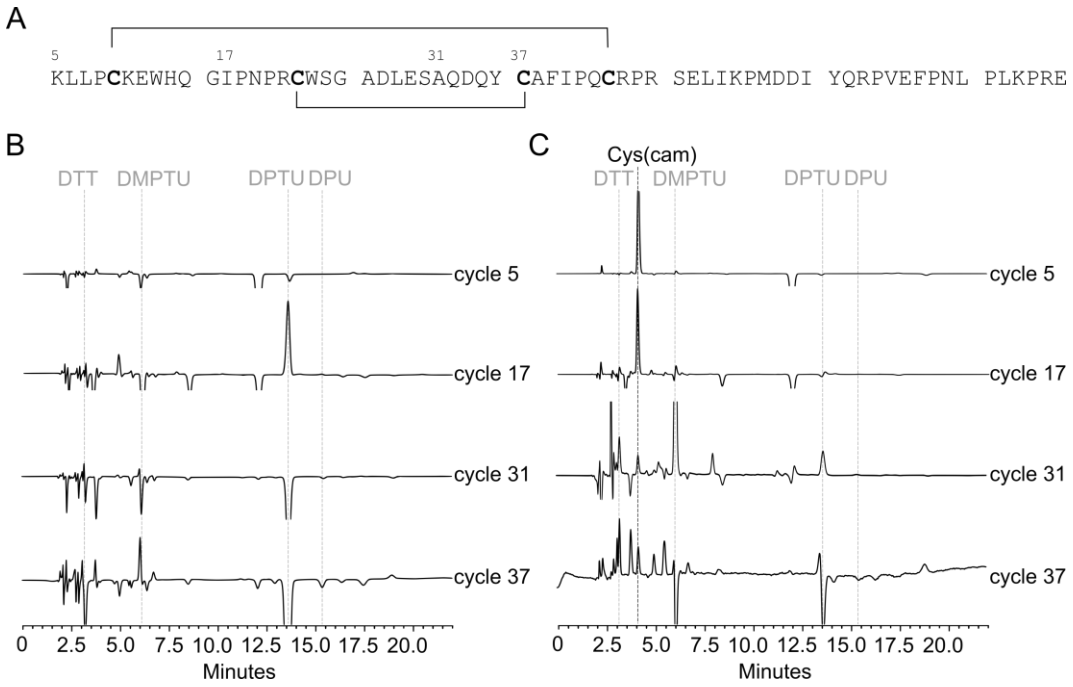

Fig. 5. A) Sequence of the 2-disulfide bonded tridegin isomer used in this study, B, C) N-terminal sequencing chromatograms of cycles Cys5, Cys17, Cys31, and Cys37 in oxidized tridegin (B) and completely reduced, carbamidomethylated tridegin (C), where all cysteines are modified as Cys(Cam). The background noise increases at higher cycle numbers due to a higher degree of magnification of the y-axis as a result of lower yields of PTH-amino acid derivatives.

Analysis of technologically treated proteins by tandem mass spectrometry and Edman degradation

Optimization, production, and treatment of proteins for industrial applications require varying parameters and procedures the protein is exposed to. For cysteine-containing proteins, this represents a special challenge as highlighted in the aforementioned section. Another molecule of interest in our studies, which had been performed in close collaboration with Rebekka Biedendieck and Rainer Krull [chapter 6] and Julia Keppler and Karin Schwarz [a link to the corresponding chapter of Julia Keppler and Karin Schwarz and to the corresponding chapter of Rebekka Biedendieck and Rainer Krull] represented the milk whey protein β-lactoglobulin (BLG), which is a significant component in many foods and important for the texture of several of them. [32, 50] This protein consists of 162 amino acids. Two variants, A and B, that differ in two positions (64, 118) only are well described in the literature (Fig. 6A). [33, 51] Apart from this, BLG contains 5 cysteines at positions 66, 106, 119, 121, and 160, which were found to form two intramolecular disulfide bonds (Cys66-Cys160 and Cys106-Cys119) according to previously described structures derived from X-ray crystallography and NMR spectroscopy.[52–55] However, the correct disulfide connectivity and the question, which of the cysteines remains in its reduced state, was a matter of debate for several decades.[56, 57] In a recent approach, the role of the reduced Cys121 in the folding process was discussed to essentially contribute to the formation of properly formed disulfide bonds.[58]

The determination of the disulfide connectivity in multiply bridged peptides and proteins is difficult per se, however, is particularly complicated by potential disulfide scrambling and the occurrence of disulfide isomers in the sample.[18, 59] Especially when reduced cysteines are present in the sequence there is a high chance of disulfide shuffling in the process of protein folding. The risk for this phenomenon to occur increases when proteins are expressed in non-native environment and organisms or when mutations are introduced. In case of BLG, the successful expression of the variant B in *E. coli* Origami B along with its crystal structure was an important step to proof the formation of correctly folded protein in non-native settings.[32–34] In addition, proper formation of disulfide bridges in BLG may significantly affect its physicochemical properties as well as its aggregation behavior as outlined by others in this edition (a link to the corresponding chapters as outlined before chapter 6 and 7). Thus, the analysis of the disulfide bonds and the changes in the sequence of the BLG are essential for understanding the changes associated with mutations or modifications by physical or chemical treatment. Herein, a combined approach applying tandem mass spectrometry and N-terminal Edman sequencing was used to evaluate the

different BLGs provided by Brune and coworkers and Fitzner and coworkers (Fig. 6B).[60][61] In addition to the recombinantly produced BLG variant B (rBLG), different mutants thereof were produced with alanine mutations at either position Cys121 (rBLG-SH), Cys66 and Cys160 (rBLG-SS), or at all cysteine positions at the same time (rBLG-C). Initially, the number of reduced cysteines was investigated by mass spectrometry after carbamidomethylation of the protein.[60] These data revealed different numbers of disulfide bridges depending on the cysteine that was mutated. For bovine BLG (BLG AB, wild-type) and rBLG a single carbamidomethylation could be observed consistent with the presence of the single reduced Cys121. Accordingly, mutation of Cys121 in rBLG-SH abolished the occurrence of reduced cysteines completely, while mutations in rBLG-SS maintained the occurrence of one reduced cysteine. No modifications could be found for the cysteine-deficient mutant rBLG-C.

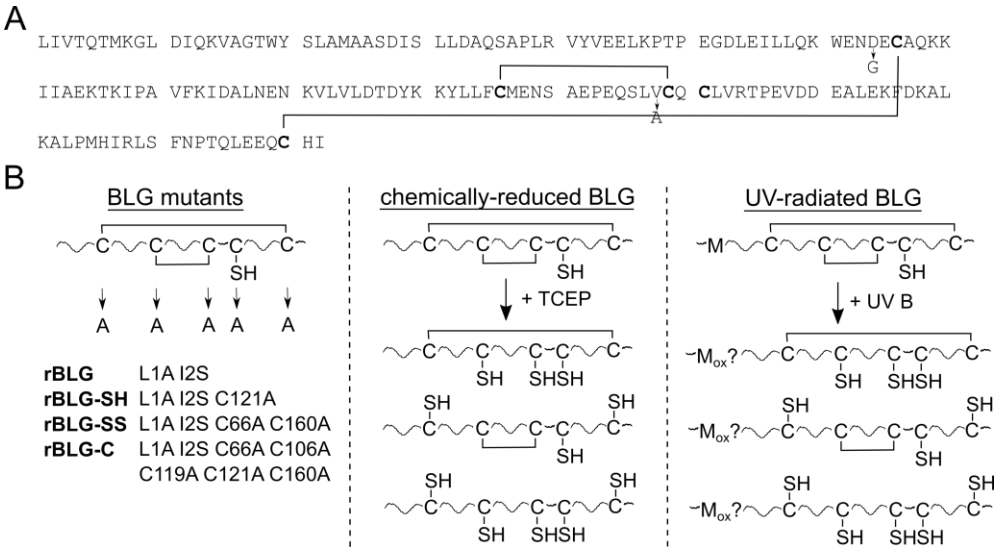

Fig. 6. A) Sequence of BLG AB. Variants A and B differ in position 64 and 118 (indicated by arrows). Cysteines are shown in bold and disulfide bridges are represented by connecting lines. B) Schematic overview of analyzed BLGs and their different treatments.

Subsequently, analysis of BLG wild-type (BLG AB) after tryptic digest was performed.[60] The resulting peptide fragments were separated by HPLC and analyzed by LC-ESI-MS/MS or Edman degradation. Successful annotation of the corresponding HPLC peaks to specific peptide fragments yielded a complete sequence coverage of the BLG wild type protein (Fig. 7A). A comparative analysis finally allowed for the precise annotation of a disulfide bond between Cys66 and Cys160 (outer disulfide bridge). Comparison of the chromatograms of the different BLG mutants to BLG AB or rBLG revealed significant changes in the retention times for fragments containing cysteine, i.e. loss of disulfide

bridges due to mutation of the involved cysteine residues (Fig. 7A). The latter could be annotated to the corresponding mutated sequence as can be seen from their MS- and MS/MS-spectra (Fig. 7B) as well as their chromatograms from Edman sequencing (Fig. 7C). Specifically, disulfide bridge Cys66-Cys160 could be preserved in BLG AB, rBLG, and rBLG-SH, while loss of the outer bridge was detected for rBLG-SS and rBLG-C. Within their studies, Creamer *et al.* described the absence of oxidized fragment 102-124 covering disulfide bridge Cys106-Cys119 (inner disulfide bridge) which only could be observed when the protein was reduced.[62] The same was observed in our studies, i.e. no peak for the desired oxidized fragment only occurred in the chromatogram upon separation for BLG AB, rBLG, rBLG-SS. Only upon reduction with TCEP or when Cys121 was mutated as in fragment 102-124 of rBLG-SH, a major peak in the chromatogram appeared and could be assigned accordingly. Consequently, formation of the inner disulfide bridge as in BLG AB was derived for rBLG, rBLG-SS and rBLG-SH.

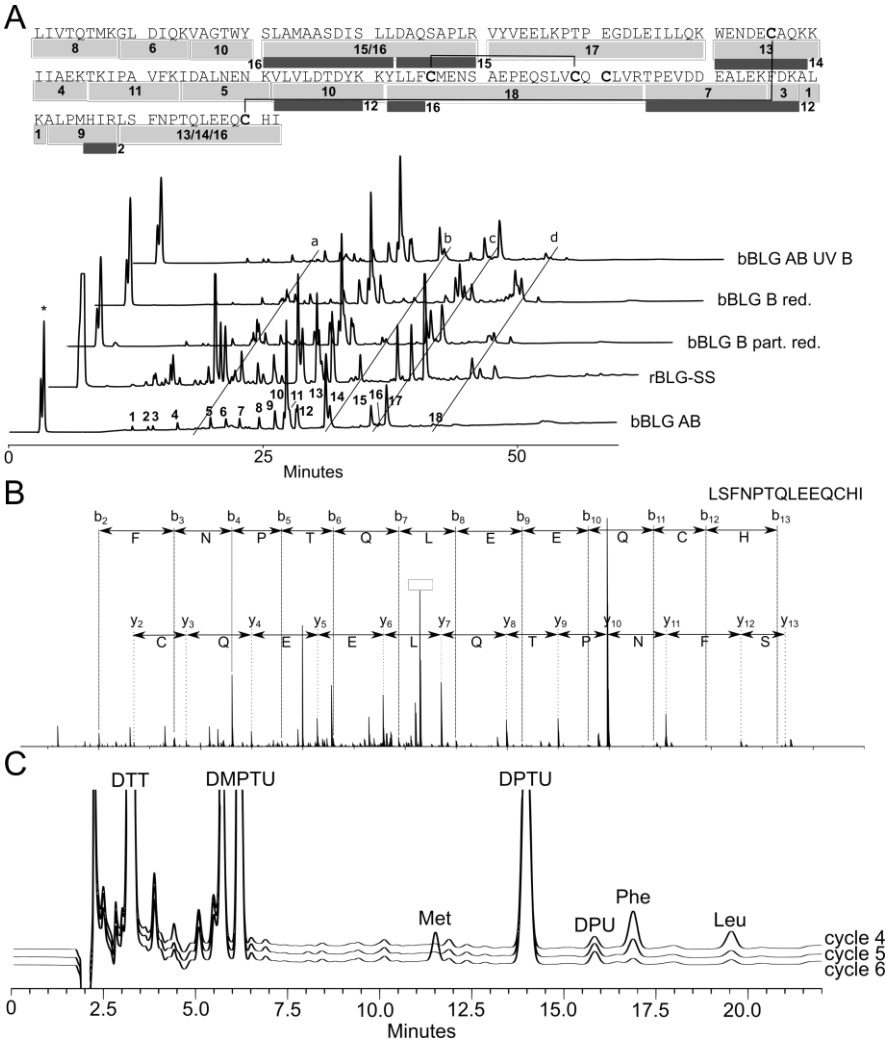

Fig 7. Analysis of the tryptic digest of BLG AB. (A) RP-HPLC profiles of bBLG AB, rBLG-SS, bBLG B partially and completely chemically reduced and UV B-treated bBLG AB after tryptic digest. Corresponding peaks that were collected and selected for further analysis by MS and MS/MS are labeled with numbers for bBLG AB and annotated to the sequence as depicted by the light and dark grey bars below the BLG sequence. Diagonal lines indicate positions of peaks containing fragments with C66 (a, fragment 61-70), C160 (c, fragment 149-162), the disulfide-linked fragment C66-C160 (b, fragment 61-70 linked to fragment 149-162) and the fragment covering cysteines 106, 119, and 121 (d, fragment 102-124). Asterisk marks the injection peak. B) LC-ESI-QTOF-MS/MS spectrum of m/z 829.9028 (2+) from the MS spectrum of peak No. 16 of reduced bBLG B representing the reduced fragment 149-162. C) Cycle runs of N-terminal sequencing covering amino acids at positions 4-6 of peak 18 displaying an increase in the intensity of the corresponding PTH-derivatives that are representative for the occurrence of the amino acids phenylalanine, cysteine (no peak increase) and methionine, respectively. Occurrence of residual PTH-leucine from cycle 3 that was not cleaved in that cycle can be still detected in cycle 4. The total run for this peptide was performed for 10 cycles confirming the existence of peptide sequence YLLFCMENSA that allowed for further determination in combination with the observed mass detected by MS and MS/MS analysis. C is taken from [60], licensed under the Creative Commons Attribution 4.0 International license (CC BY 4.0)

Analysis of the BLG mutants represented an ideal start to continue investigations on chemically and physically treated BLGs. For chemical reduction, BLG variant B (BLG B) was reduced by TCEP and analyzed by LC-ESI-MS/MS.[60] Using the same methodology as for the analysis of BLG mutants, two major observations were made compared to the investigated mutant proteins: 1. a peak for fragment 102-124 could be clearly observed after complete reduction of the protein, and 2. reduction of the outer disulfide bridge occurred before reduction of the inner disulfide bridge (Fig. 7A). The latter could be detected by analysis of partially reduced samples of BLG B. Only minor amounts of the reduced fragment 102-124 were observed for partially reduced BLG B, while the fraction containing the disulfide-connected fragments 61-70 and 149-162 disappeared nearly completely and two new peaks for single fragments 61-70 and 149-162 emerged.

In addition to controlled reduction approaches, physical treatment of BLG AB by UV B light was more challenging. Radiation with UV B light may lead to a breaking of the disulfide bonds but can also cause further oxidative damage of the protein. To analyze the effect of UV B light, BLG AB was treated accordingly and the induced damages and/or structural changes were investigated by the aforementioned approach for sequence analysis (Fig. 7A).[61] Interestingly, only two major changes could be observed: 1. oxidation of Met7 indicated by a shift of the corresponding HPLC peak and a mass increase in the MS/MS spectrum at the corresponding position and 2. reduction of the signal intensity for the HPLC peak representing the disulfide-linked fragments 61-70 and 149-162 and a concomitant increase of a peak for a modified fragment 149-162. Thus, radiation with UV

B light induced opening of the outer bridge as well as oxidation of Met7 in the protein, which both may lead to significant structural and physicochemical changes if the radiation process lasts longer.

The analysis of disulfide-linked BLG from different production and treatment approaches using mild chemical as well as harsh physical conditions was successfully performed and elucidation of disulfide connectivities in the corresponding samples yielded further impetus on how structural changes may influence protein properties as well as aggregation behavior. However, it became evident that in some cases LC-MS/MS analysis is not sufficient for a proper analysis of peptide sequences, especially when intensities of the relevant compounds had been very low as was found for fragment 102-124. In these cases supporting analysis by N-terminal Edman sequencing was a valuable additional tool to complete the analysis of model protein BLG.

Conclusions

The studies presented in this chapter first explored analytical methods applied for lysozyme analysis in innovative formulations, e.g. in hot-melt extrudates prepared with PEG20,000. The extrudates were analyzed for their lysozyme content using an optimal RP-HPLC method. Activity of lysozyme in the extrudate was examined using a fluorogenic method and showed preferable accuracy and reproducibility compared to a turbidimetric method that was reported earlier. SDS-PAGE and MALDI-TOF-MS methods were established for mass analysis. Extrudates were analyzed following the workflow described and compared to pure lysozyme and a physical mixture of the protein with PEG20,000 as controls including stability studies of the extrudates following ICH Topic Q1A (R2) guidelines. Extrudates succeeded to retain lysozyme characteristics over 6 months, indicating suitability of hot-melt extrusion as formulation technique for stabilization of the applied protein.

Additionally, conservation of essential post-translational modifications in a protein or peptide was examined and Edman degradation chemistry was adapted to study disulfide connectivity. Suitable PTH-standards were established and validated for the detection of cysteine and its quantification. Evaluation of the standards was performed by sequencing selected model peptides containing modified cysteines, that revealed applicability of the standards. Elucidation of disulfide bridge formation in a model miniprotein containing two disulfide bonds that was derived from the FXIIIa inhibitor tridegin was performed employing the examined PTH-Cys derivatives, PTH-Cys(Me) and PTH-Cys(Cam).

Application of our standards for the qualitative determination of modified cysteines and, thus, changes in the formed disulfide bridges before and after complete reduction was possible and allowed for a distinct differentiation between reduced and oxidized cysteines. The established PTH-Cys derivatives are also in agreement with the ICH Q14 guidelines as bioanalytical standard compounds and can be involved for free cysteines and disulfide bridges assessment.

Moreover, a combined technology applying mass spectrometry and N-terminal Edman sequencing turned out as a useful approach to completely elucidate and identify disulfide connectivies in a tryptic digest of differently produced and treated BLGs. This allowed for complete annotation and identification of disulfide bridges and also their changes in connectivities in BLGs which represents valuable information to clearly identify structural changes in the future without a need of sophisticated crystallographic or NMR analyses.

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Appendix C: Edman Degradation Reveals Unequivocal Analysis of the Disulfide Connectivity in Peptides and Proteins

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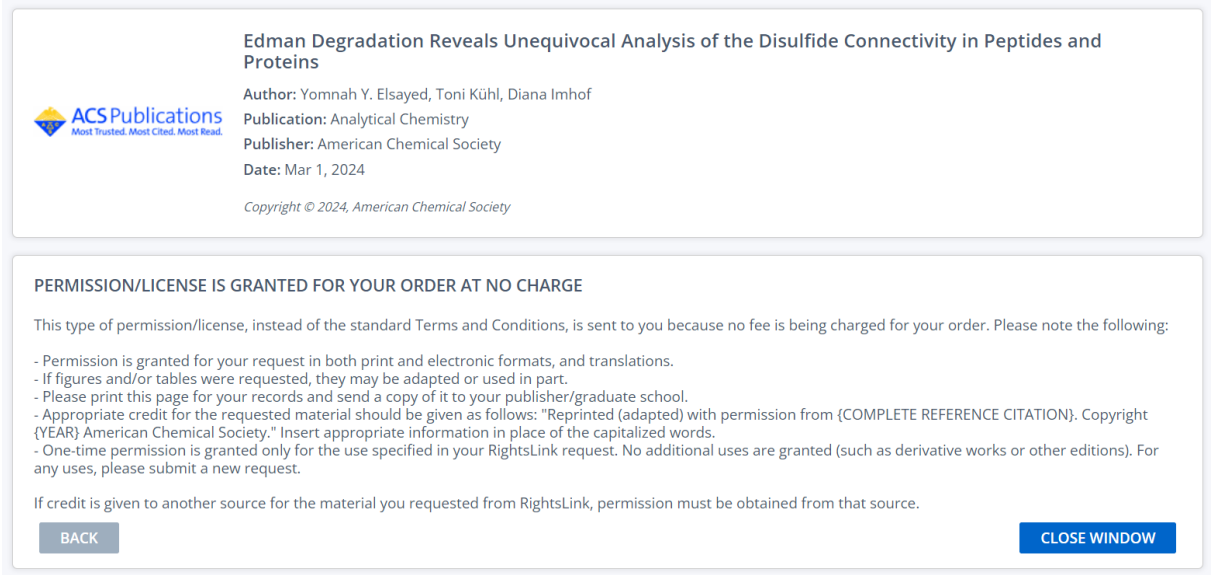

Edman Degradation Reveals Unequivocal Analysis of the Disulfide Connectivity in Peptides and Proteins

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Edman Degradation Reveals Unequivocal Analysis of the Disulfide Connectivity in Peptides and Proteins

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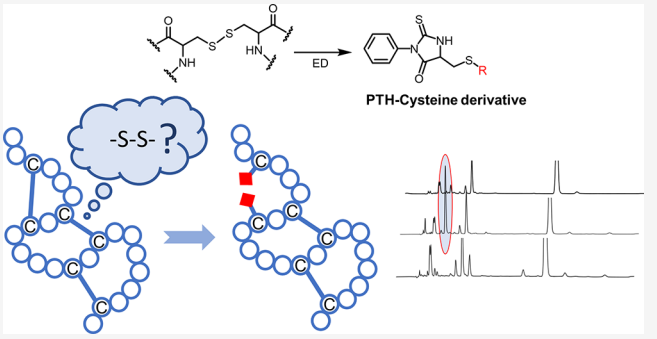
ABSTRACT: Disulfide bridges in peptides and proteins play an essential role in maintaining their conformation, structural integrity, and consequently function. Despite ongoing efforts, it is still not possible to detect disulfide bonds and the connectivity of multiply bridged peptides directly through a simple and sufficiently validated protein sequencing or peptide mapping method. Partial or complete reduction and chemical cysteine modification are required as initial steps, followed by the application of a proper detection method. Edman degradation (ED) has been used for primary sequence determination but is largely neglected since the establishment of mass spectrometry (MS)-based protein sequencing. Here, we evaluated and thoroughly characterized the phenyl thiohydantoin (PTH) cysteine derivatives PTH-S-methyl cysteine and PTH-S-carbamidomethyl cysteine as bioanalytical standards for cysteine detection and quantification as well as for the elucidation of the disulfide connectivity in peptides by ED. Validation of the established derivatives was performed according to the guidelines of the International Committee of Harmonization on bioanalytical method validation, and their analytical properties were confirmed as reference standards. A series of model peptides was sequenced to test the usability of the PTH-Cys-derivatives as standards, whereas the native disulfide-bonded peptides CCAP-vil, μ-conotoxin KIIIA, and human insulin were used as case studies to determine their disulfide bond connectivity completely independent of MS analysis.



INTRODUCTION

Biological products are increasingly employed for pharmaceutical applications over the past 10 years and meanwhile account for approximately 25% of the global pharmaceutical market (approximately US$300 billion in 2019).[1] Numerous peptide hormones, e.g., insulins and GLP-1 (glucagon-like peptide 1 receptor agonist) analogs, monoclonal antibodies, vaccines, and other peptides derived from nature or drug design, were developed and approved by the FDA.[2,3] Among them, disulfide-rich peptides and proteins have emerged as valuable therapeutic or diagnostic tools for controlling different medical conditions, such as pain management (ziconotide), gastric disorders (pembrolizumab, linaclotide, plecanatide), and tumor diagnostics (^{64}Cu oxodotreotide).[1,3,4] For approval of a developed peptide or protein, analysis of its chemical and biological characteristics needs to be performed in order to ensure its structural and functional integrity.[5,6] Nondestructive (NMR, X-ray crystallography) and destructive (mass spectrometry (MS)) methods were developed for peptide and protein sequence and structure elucidation. MS is predominantly used for peptide mapping and identification of cysteine residues and disulfide bonds after enzymatic digestion, reduction, and derivatization.[7] MS, however, has limitations in determining the proper disulfide connectivity when suitable cleavage sites of digestive enzymes such as Arg/Lys for trypsin, Phe/Tyr/Trp for chymotrypsin, and Ser/Ala/Val for elastase are absent.[8,9] Other approaches, which do not require enzymatic digestion such as online electrochemical cells connected to MS, exist and were also reported to be used for disulfide bridge elucidation.[10−13] Recent improvements in MS fragmentation and detection approaches such as ion mobility and trapped ion mobility as well as in source decay were developed and also involved in disulfide bridge identification, especially for vicinal cysteines.[14−17] The elucidation of the disulfide connectivity then requires an accurate evaluation of the fragment pattern and the contained disulfide bonds.[2,7,18] In addition, MS data evaluation of complex samples or unknown sequences could be a difficult task due to the high number of fragments that may require specific expertise and extensive time for correct evaluation.[6,19−21] The loss of significant fragments being involved in a disulfide bridge, especially in case of low concentrated

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samples, can dramatically affect the final assessment.[22−24] Finally, although MS is a widely used tool for elucidating posttranslational modifications, it may not provide adequate differentiation between all possible isomers of a multiple disulfide-bonded peptide.[7,14,21,25,26] Therefore, all analytical methods available should be exhausted to determine the correct disulfide connectivity and also to provide quantitative information as required. Edman degradation (ED), which was introduced in the 1950s, is a method developed for the analysis of the primary amino acid sequence of a peptide (aa_n) (for a comparison between tandem MS and ED regarding the determination of the disulfide connectivity in disulfide-rich peptides and proteins see Table S1).[27] Thereby, the N-terminal amino acid (aa_1) is coupled to a chemical reagent (phenyl isothiocyanate) to produce a phenyl thiohydantoin (PTH) derivative that is eluted and detected in a chromatographic system. The remaining peptide (aa_{n-1}) is subjected to the reaction process again, and the next N-terminal amino acid is converted and released.[28] In our study, we were interested in developing a fast and reliable method for the analysis of cysteine-containing peptides and proteins by exploiting ED as performed earlier more frequently.[29−31] Therefore, PTH-cysteine standards that provide reliable detection and quantification of cysteine residues are required. Cysteine is known as a critical amino acid in ED as it does not react quantitatively due to its acid lability.[32] The diPTHcystine derivative was reported for the cleaved disulfide bridge, but it suffers from inadequate detection and reproducibility.[30,33−35] To overcome this issue, chemical modification of cysteine has been described in different studies to improve its detection and quantification in the form of a suitable PTH-S-cysteine derivative.[30,35−37] Many such derivatives were developed and suggested over the years as summarized in Figure 1, with select examples applied for thin layer chromatographic detection and quantification.[38] Derivatization using 4-vinylpyridine was reported to be sensitive to the ionic strength of the HPLC solvents, affecting its retention time and leading to a loss of reproducibility.[39] Acrylamide was used for cysteine modification to obtain cysteine-S-propionamide that coelutes with (2,6-dimethylphenyl)thiourea (DMPTU), a byproduct of ED.[40] 2-Bromopropylamine and ethylenimine were used for the modification as well, but the corresponding cysteine derivatives elute close to PTH-lysine and thus could not be detected properly.[34] Iodoacetamide, *N*-methyl iodoacetamide, and iodoacetic acid were the most common agents for the alkylation of disulfide-containing proteins.[33,41−44] Thereby, cysteine is converted into S-carbamidomethyl (Cam), *N*-methyl-S-carbamidomethyl, or S-carboxymethyl cysteine, respectively. PTH-S-carboxymethyl cysteine, however, suffers from close elution to PTH-serine and a lack of reproducibility that affected quantification,[33,45] whereas modification to S-carbamidomethyl was reported to be complete and quantitative but suggested to be impaired by partial instability.[33,46]

Despite all these efforts, there are still no suitable standard compounds for the analysis of Cys-containing peptides and proteins available today, and only mixtures of the PTH derivatives of all 19 proteinogenic amino acids (excluding cysteine) can be obtained commercially. We thus investigated PTH-S-carbamidomethyl cysteine (PTH-Cys(Cam)) and PTH-S-methyl cysteine (PTH-Cys(Me)) thoroughly concerning their characteristics to be reestablished for automated ED analysis. The suitability of these PTH-modified cysteines as bioanalytical standards was evaluated following the guidelines

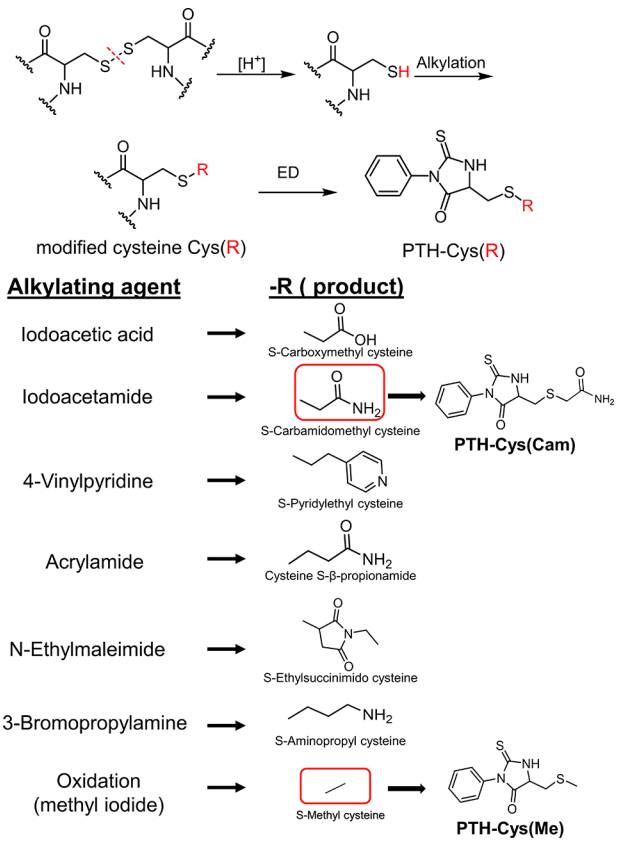

Figure 1. Overview of cysteine modifications in disulfide-containing peptides or proteins showing the modifying agents and resulting S-cysteine derivatives. The selected derivatives for this study are highlighted in red, and their corresponding PTH derivatives PTH-Cys(Me) and PTH-Cys(Cam) are depicted.

for analytical reference standards described by the International Committee of Harmonization (ICH) M10 on bioanalytical method validation[47] in agreement with the Q14 guidelines for quality assurance in analytical method development.[48] Model peptides containing modified cysteines were especially designed for the evaluation of the PTH derivatives. Ultimately, the validated PTH derivatives and the established ED method were successfully applied for the analysis of disulfide connectivities in cysteine-containing natural peptides, i.e., μ-conotoxin KIIIA,[49] cardioactive conopeptide CCAP-vil,[50] and human insulin, whose analysis by a combination of different MS/MS techniques is quite laborious and tedious, especially with respect to data evaluation.[49]

EXPERIMENTAL SECTION

Materials. The chemicals used were of analytical grade quality and described in Supporting Information Text S1.

PTH-Cys Reference Standards. The synthesis of PTH-Cys(Me) and PTH-Cys(Cam) (Scheme 1) has been performed according to earlier reports and is, together with purification and chemical characterization, briefly described in the Supporting Information (Supporting Information Text S2, Tables S2, and S3).

For ED, stock solutions of the PTH-cysteine derivatives (100 μM in 37% acetonitrile) were used to prepare the working solutions by 1:20 dilution with 37% acetonitrile in compliance with the PTH-AA standard mixture provided by FUJIFILM Wako Pure Chemical (Osaka, Japan).[51] The

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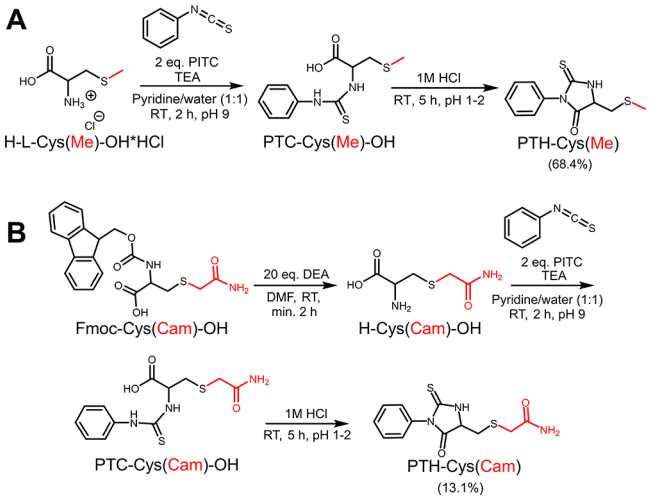
Scheme 1. Chemical Synthesis of PTH-Cys(Me) (A) and PTH-Cys(Cam) (B)



standard mixture of the common 19 PTH-amino acids and PTH-delta-threonine provided was dissolved and diluted with 37% acetonitrile to get a final concentration of 25 pmol/50 μL for each amino acid. In general, samples of 80−90 μL were prepared, from which 50 μL was automatically injected in the PPSQ-53A protein sequencer. The working solution of each PTH-Cys derivative was used to establish the calibration standards, quality controls, or tested samples as required in the corresponding steps of validation (Supporting Information Text S3). Stock and working solutions were stored at −20 °C.

Automated Edman Sequencing. An automated protein sequencer PPSQ-53A (Shimadzu) was used for N-terminal sequencing which was connected to a Wakosil PTH-II, 4.6 × 250 mm (S-PSQ) column for isocratic elution at 40 °C (column oven). The PTH-amino acid mobile phase (40% acetonitrile and 0.1% acetic acid) from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan) was used at a flow rate of 1 mL/min and UV detection at 269 nm. For the direct analysis of PTH-amino acids, the corresponding derivatives and standards were directly injected in the chromatographic system. For the analysis of proteins and peptides, samples were dissolved in 37% acetonitrile (or a suitable solvent) and then applied on a TFA-treated glass fiber disk (GFD) or poly(vinylidene difluoride) (PVDF) membrane for longer proteins, dried under nitrogen stream, and sequenced. The GFDs were initially treated with sequa-brene according to the manufacturer's instructions to increase the sequencing efficiency and optimize the amino acid yield for shorter peptides. Identification and quantification of PTH-amino acid derivatives were performed according to comparison with either the PTH-amino acid standard mixture or the prepared and validated PTH-cysteine standard derivatives.

Cys-Containing Model Peptides and Native Disulfide-Bonded Peptides. Synthesis, purification, and characterization of the model peptides **1**−**12** (Table 1) are described in the Supporting Information (Text S4 and Table S4). The model peptides were subjected to ED in the free or bead-bound state for a number of cycles according to the peptide size. A single bead of each resin-bound peptide was applied on a PVDF membrane, while solutions of free peptide (200 pmol) were applied on TFA-treated GFDs (pretreated with sequa-brene) and dried. Different disulfide-bonded functional

Table 1. Sequences of the Model Peptides

peptide no.	sequence
1	A AC A A
2	A A **T** A A
3	A A **C(Me)** A A
4	T A **C(Me)** A A
5	A T **C(Me)** A A
6	A Y R **C(Me)** M V A A
7	A Y R T **C(Me)** M V A A
8	A A **C(Cam)** A A
9	T A **C(Cam)** A A
10	A T **C(Cam)** A A
11	N Q S **C(Cam)** H G A A
12	N Q S T **C(Cam)** H G A A

peptides were selected as target peptides for the evaluation of ED as analytical tools for determining the disulfide connectivity. The cardioactive conopeptide CCAP-vil,[50] a 10-mer one-disulfide-bonded cyclic peptide, was initially tested in the oxidized and reduced state (Cam derivative). For sample preparation, procedures of partial reduction of disulfide bridges of the oxidized peptide followed by alkylation[21,52] were performed by directly dissolving the oxidized CCAP-vil in citrate buffer (0.05 M, pH 3.0) containing 20 mM tris(2-carboxyethyl) phosphine (TCEP) acting as mild reducing agent to obtain a final concentration of 125 μM. Solutions were kept for different time intervals (30 s−5 min) at room temperature followed by alkylation with 3 volume equiv of 1.1 M iodoacetamide (in 0.5 M Tris-acetate pH 8.0, 2 mM EDTA) to facilitate carbamidomethylation of free thiol groups. After 5 min, the mixture was acidified with 1 equiv of 10% trifluoroacetic acid (TFA) to stop the alkylation reaction. Samples were stored on dry ice until further separation on an LC-20AT chromatograph equipped with an analytical Vydac 218TP54 column (C18, 4.6 mm × 25 mm, 5 μm particle size, 300 Å pore size) using an elution gradient of 0% of eluent B (0.1% TFA in acetonitrile) in eluent A (0.1% TFA in water) for 10 min followed by a linear increase of eluent B up to 60% for another 60 min at a flow rate of 1 mL/min and UV detection at 220 nm. Fractions were collected and subjected to MALDI/TOF MS using HCCA as the matrix or LC-ESI-qTOF to prove carbamidomethylation of the compounds.

Fractions were lyophilized, redissolved in 50% acetonitrile, 0.1% TFA, applied on sequa-brene pretreated GFDs, and subjected to ED. Sequencing results for fractions corresponding to oxidized and alkylated reduced CCAP-vil were assessed, and cycles in which cysteine or modified cysteine residues have been expected were compared to evaluate the corresponding disulfide connectivities. Since CCAP-vil carries only one disulfide bridge, it was fully reduced after TCEP treatment. The more complex three-disulfide-bonded peptides human insulin (Merck KGaA, Darmstadt, Germany) and μ-conotoxin KIIIA[49] were subjected to partial reduction followed by alkylation with iodoacetamide as described above. Samples were reduced over a time interval (30 s−10 min for KIIIA and 30 s−15 min for insulin), and at each tested time point, alkylation took place for 5 min at room temperature and was stopped by the addition of 10% TFA as described for CCAP-vil. After fractionation by HPLC under the same conditions described above and confirmation of carbamidomethylation by MALDI/TOF-MS using HCCA as matrix or by LC-ESI-qTOF MS, the different fractions corresponding to partially reduced

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peptides were subjected to ED sequencing and evaluated as aforementioned. MALDI-LIFT tandem mass spectrometric analysis was performed for μ-KIIIA for comparison as described earlier.[21]

RESULTS AND DISCUSSION

Selection and Synthesis of PTH-Cysteine Derivatives. The potential of automated ED is not completely exhausted and could be developed further beyond the basic application for sequence determination,[28,43] for example, by using this technique to disclose the connectivities of disulfide-rich peptides and proteins.[35,37,53] Within such an analysis, cysteine modification is essentially required due to the reduced stability of the PTH-cysteine derivative toward acidic conditions and, thus, would enable its detection within such an approach.[32,36] In addition, there was still no quantitation possible due to the lack of validated cysteine standards. From a selection of possible modifications used for cysteine such as carboxymethylation, carbamidomethylation,[33,41−44] methylation,[38] and pyridylethylation,[39] as well as modifications upon treatment with N-ethylmaleimide,[54] 3-bromopropylamine,[34] and acrylamide[40] (Figure 1), we decided to proceed with PTH-Cys(Cam) and PTH-Cys(Me) in our study. Modification with iodoacetamide was widely used for cysteine alkylation in proteins for the detection of the corresponding PTH-S-carbamidomethyl cysteine derivative (PTH-Cys(Cam)) during ED.[41,55,56] But degradation to PTH S-carboxymethyl cysteine during sequencing was reported which might hamper its applicability for quantification.[45] S-methyl cysteine is frequently used for cysteine protection in the peptide synthesis field.[57] The corresponding PTH derivative PTH-Cys(Me) was reported to show good stability compared to PTH-cysteine for detection of cysteines in ED[58] and was thus selected in our study to investigate its characteristics as a reference for modified PTH-cysteine derivatives.

For both, PTH-Cys(Cam) and PTH-Cys(Me), we intended to assess their applicability as bioanalytical standards for qualification and quantitation in ED according to the ICH guidelines for the development of bioanalytical standards, which was, to the best of our knowledge, not performed so far.[47,48] Initially, the synthesis of both derivatives was required, as shown in Scheme 1. Coupling of S-methyl cysteine or S-carbamidomethyl cysteine (obtained from deprotection of Fmoc-S-carbamidomethyl cysteine) with PITC under basic conditions was performed followed by cyclization of the produced thiocarbamoyl (PTC) derivative into anilinothiazolinone (ATZ) derivatives. Finally, a decrease of the pH led to spontaneous conversion and formation of the desired PTH-cysteine derivatives.[59,60]

Validation of PTH-Cysteine Derivatives. In order to achieve cysteine identification in samples, the selected PTH-Cys derivatives need to meet the analytical criteria for reference standards before possible integration into the existing PTH-AA standard mixture (Figure 2D). In our study, we followed the ICH guidelines for bioanalytical methods M10 and Q14 guidelines for analytical method development.[47,48] The ICH stated the following criteria for a bioanalytical standard of a chromatography-based method to be tested: selectivity, specificity, linearity range, accuracy and precision, matrix effect, carry over and dilution effect, and stability. Thus, these criteria were studied extensively for PTH-Cys(Me) and PTH-Cys(Cam), a full description can be found in the Supporting Information (Text S3, Figure S1, Tables S5, and S6). For the ED analysis, initially a direct run for the PTH-cysteine derivatives was performed that showed no interference with the blank solvent (Figure 2A−C), and the PTH-amino acid standard mixture was analyzed with both derivatives to also investigate possible interferences (Figure 2D,E). PTH-Cys(Me) was observed to be eluted at 9.12 min, which is close to PTH-delta-threonine (9.07 min) that is produced as a byproduct during ED of threonine (Figure 2D). The close retention time may therefore affect the detection and quantification of methylated cysteine if a threonine is present in the immediately preceding degradation cycle (Thr-Cys) and vice versa (Cys-Thr). PTH-Cys(Cam), however, was eluted at t_R = 4.22 min between PTH-serine (4.04 min) and PTH-threonine (4.40 min) as shown in Figure 2E with proper detection and identification. The observed elution pattern for PTH-Cys(Cam) is in agreement with an earlier study performed by Shimadzu researchers.[61] To the best of our knowledge, a comparison for PTH-Cys(Me) applying the same setup was not performed so far.

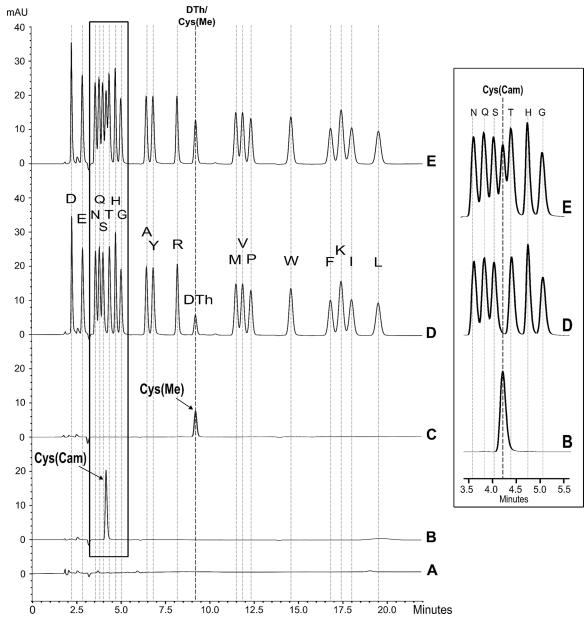

Figure 2. Direct N-terminal sequencing runs of (A) 37% acetonitrile as blank, (B) PTH-Cys(Cam) (25 pmol/50 μL), (C) PTH-Cys(Me) (25 pmol/50 μL), (D) PTH-AA standard mixture (25 pmol/50 μL for each amino acid), and (E) PTH-AA standard mixture (25 pmol/50 μL) spiked with PTH-Cys(Me) (25 pmol/50 μL) and PTH-Cys(Cam) (25 pmol/50 μL). A zoom-in for chromatograms B, D, and E to show the PTH-Cys(Cam) peak and nearby eluted PTH amino acid derivatives is shown in the box. Samples for B−E were dissolved in 37% acetonitrile.

PTH-Cys(Me) and PTH-Cys(Cam) showed a good linearity range for quantification (25−250 and 25−300 pmol/50 μL, respectively), accuracy, and precision (Table S5 and Text S3). No matrix effect was detected. Dilution integrity and carryover showed satisfactory results for both compounds, too. Concerning storage, the solid state of PTH-cysteine derivatives is highly recommended for long-term storage up to at least 3 months, while solutions of PTH-Cys(Cam) showed some degradation after 1 month of storage time (Figure S1, Table S6, and Text S3). Solutions of PTH-Cys(Me) were found to be more stable and showed reproducible results, even after 3 months of storage.

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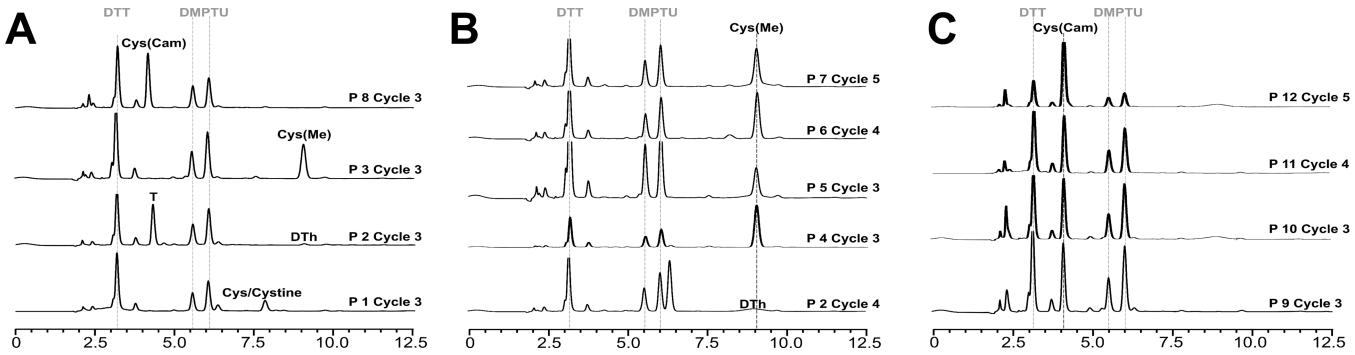

Figure 3. (A−C) Chromatograms of N-terminal sequencing of the following peptides: (A) **1**: AA**C**AA, and **2**: AA**T**AA, **3**: AA**C**(**Me**)AA and **8**: AA**C**(**Cam**)AA, (B) S-methyl cysteine-containing peptides **4**: TA**C**(**Me**)AA, **5**: AT**C**(**Me**)AA, **6**: AYR**C**(**Me**)MVAA and **7**: AYRT**C**(**Me**)MVAA (fourth cycle of peptide **2**: AA**T**AA was also evaluated to show residual PTH-DTh), and (C) S-carbamidomethyl cysteine-containing peptides: **9**: TA**C**(**Cam**)AA, **10**: AT**C**(**Cam**)AA, **11**: NQS**C**(**Cam**)HGAA and **12**: NQST**C**(**Cam**)HGAA.

Overall, validation results for PTH-Cys(Me) and PTH-Cys(Cam) confirmed acceptable quality as standards for cysteine detection and quantification and indicated suitability of both derivatives as bioanalytical reference standards according to ICH guidelines M10 and Q14.[47,48] However, it needs to be considered that the applicability of PTH-Cys(Me) as a standard might be influenced by the occurrence of PTH-DTh during prior threonine cycles. To elucidate and determine the influence of such effects, a set of model peptides was designed and analyzed accordingly.

Edman Sequencing of Model Peptides. Sequences of model peptides (Table 1) were selected according to the eluted PTH-amino acid derivatives that were closest to the two suggested PTH-Cys standards in the standard mixture (Figure 2E) and consequently subjected to ED. Two control peptides carrying cysteine (peptide **1**) and threonine (peptide **2**) were used as negative controls for the assessment of the released amounts of PTH-Cys/diPTH-cystine and PTH-Thr/PTH-DTh, respectively. Upon sequencing of peptide **1**, no peak for PTH-Cys was observed in the third cycle; however, a peak of diPTH-cystine was detected at 7.80 min overlapping with PTH-arginine and lacking reproducibility (Figure 3A). The third cycle of peptide **2** showed characteristic peaks for PTH-Thr and its byproduct PTH-DTh as confirmed by comparison to the standard mixture (Figure 3A).

During sequencing of the model peptide carrying S-methylcysteine (peptide **3**), the corresponding PTH-Cys(Me) peak was identified, which showed a close overlap with PTH-DTh, also detected in peptide **2**, while PTH-Cys(Cam) in the corresponding peptide **8** could be unequivocally annotated (Figure 3A). All peptides were analyzed in the free (solution) and resin-bound forms, revealing identical results (data not shown).

In order to evaluate interferences of nearby PTH-amino acid derivatives, further model peptides containing S-methyl or S-carbamidomethyl cysteine were designed. In these peptides, an amino acid was placed in the immediately preceding cycle to the modified cysteine that would yield a PTH-amino acid with a retention time directly adjacent to the PTH-cysteine derivative (peptides **4**−**7**, **9**−**12**, Table 1). For S-methyl cysteine-containing peptides **5** and **7**, released PTH-Cys(Me) was found to overlap with PTH-DTh as described above. In comparison to peptides **4** and **6** that do not possess threonine directly before the cysteine cycle, the residual PTH-DTh may induce a broadening of the peak base that could be confirmed by analysis of, e.g, peptide **2** as shown in Figure 3B. Consequently, threonine N-terminal to a modified cysteine in a peptide or protein may distort the PTH-Cys(Me) peak baseline in the following cycle. In contrast, for carbamidomethylated cysteine converted to PTH-Cys(Cam) in peptides **9**−**12**, adequate resolution and detection of PTH-Ser and PTH-Thr could be accomplished and both can be properly distinguished from PTH-Cys(Cam) (Figures 3C and S2).

The analysis of the bead-bound model peptides showed complete identification up to the last amino acid in good yield in comparison to the general drop in yield observed in the ED of the free peptides. Thus, complete differentiation of PTH-Cys(Cam) from other PTH-amino acids was possible. In comparison to PTH-Cys(Me), there is no overlap with retention times of other amino acids, and therefore, PTH-Cys(Cam) was employed for the development of a fast and convenient protocol for the determination of disulfide connectivities in cysteine-rich peptides.

Determination of Disulfide Connectivities in Cysteine-Rich Peptides by ED. To evaluate the applicability of the validated PTH-Cys(Cam) standard in actual peptides, three naturally occurring representatives with different numbers of disulfide bridges were analyzed, i.e., cardioactive decapeptide CCAP-vil (1 disulfide-bridge), μ-conotoxin KIIIA, and human insulin (both three disulfide bridges). Concerning the choice of μ-KIIIA and insulin, it is important to note that the three disulfide bonds in the latter peptide are not only intramolecular bonds but also one intermolecular bond, which may change the sequencing results and thus had been included for reasons of comparison. In detail, each peptide was subjected to (partial) reduction and stepwise cleavage of the disulfide bonds followed by alkylation of the reduced cysteine residues after different time points to form S-carbamidomethyl cysteine (Scheme 2).

Chromatographic fractionation was performed to separate the different partially reduced peptide variants, and subsequently ED analysis of relevant fractions was performed. A similar protocol had been established earlier by Young et al.,[35] however, their report lacks information about how the existence of specifically modified cysteines in their analyses was verified. In addition, after *N*-ethylmaleimide alkylation of their compounds collected after partial reduction, the authors performed a complete reduction and alkylated all remaining cysteines with iodoacetic acid. However, modification with *N*-ethyl-maleimide already led to the occurrence of several peaks for a single fraction which was explained by the formation of different diastereomers that resulted from the introduction of a

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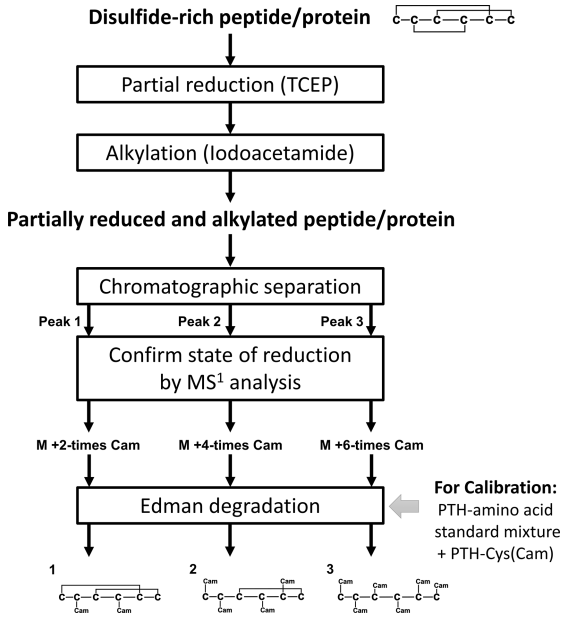
Scheme 2. Workflow of Disulfide Connectivity Elucidation for Disulfide-Rich Peptides/Proteins



new chiral center in the maleimide ring of the modified cysteines.[35,37] This can be exemplified with peptide sr5a, that contains 4 cysteines and revealed more than 10 peaks following their protocol, which made the analysis of the disulfide connectivity unnecessarily complex. This is, however, not the case for our approach that allows for a fast and unambiguous determination of disulfide connectivities, as explained in the following. The decapeptide CCAP-vil carries one disulfide bridge and upon reduction both cysteines were modified in comparison to the oxidized form, as shown in Figure 4, enabling detection of the cysteine residues.

Similar results have been recently reported by Kuriki[61] for the nonapeptide oxytocin that also contains one disulfide bridge which was identified by carbamidomethylation,

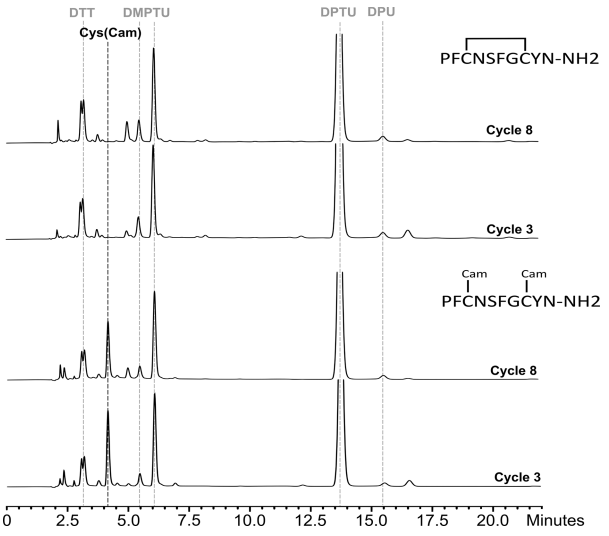


Figure 4. Sequence of oxidized (top) and reduced carbamidomethylated CCAP-vil (bottom) and the corresponding cycles 3 and 8 obtained by N-terminal sequencing, indicating bridge closure and opening upon reduction.

carboxymethylation, or pyridylethylation of the reduced cysteines.[61,62] However, data on the application of PTH-cysteine standards for comparison were not given and identification of the respective PTH-cysteine derivative could only be derived from the appearance of a new peak in the chromatogram in comparison to the unmodified peptide.[61,62]

A more complex sample represents μ-conotoxin KIIIA carrying three disulfide bridges in a distinct pattern (C1−C15, C2−C9, C4−C16, Figure 5A) which are crucial for appropriate folding and activity.[49] To confirm the expected fold, partial reduction for up to 10 min and subsequent alkylation using iodoacetamide and ED were performed as described above (Figure 5A−C).

Peaks for the oxidized (I), partially reduced (II and III, observed to possess two and four carbamidomethylated cysteines, respectively), and fully reduced μ-KIIIA (IV) were analyzed by ED after confirmation of alkylation by MS (Figures 5A−C, S3, and Table S7). While no peaks for PTH-Cys(Cam) could be detected in the oxidized compound (peak I, Figure S3A), a clear increase for PTH-Cys(Cam) in cycles 1 and 15 became apparent during the ED of peak II (Figure 5B). No PTH-amino acids showed up in any of the other cycles for cysteines, indicating the opening of the disulfide bridge C1−C15 and leaving the other two disulfide bonds intact.

Additional detection of PTH-Cys(Cam) in cycles 4 and 16 for peak III (Figure 5C) identified the occurrence of four reduced cysteines and confirmed the opening of a second disulfide bridge between C4 and C16. Residual amounts of PTH-Cys(Cam) in cycle 2 of peak III emerged from incomplete cleavage of carbamidomethylated cysteine in the previous cycle which is well described for ED in which the yield for each cycle is known to only show an average of approximately 92−94%.[63]

This can also be confirmed by sequencing of the carbamidomethylated, fully reduced peptide (peak IV, Figure S3B) in which a significantly increased concentration of PTH-Cys(Cam) in cycle 2 as well as cycle 9 was detected. In addition to the occurrence of PTH-Cys(Cam) in cycles 1, 4, 15, and 16, this confirms the opening of all three disulfide bridges. Thus, bridge opening for this peptide occurred in the order (1) C1−C15, (2) C4−C16, and (3) C2−C9 (in accordance with the highlighted determination route in Scheme S1). This confirmed the expected pattern for disulfide bridges known for μ-KIIIA.[64,65] As compared to tandem mass spectrometric analysis, especially direct and fast assignment as well as the continuation of sequencing beyond a disulfide-bridged cysteine present a huge advantage for the analysis of this compound by ED. In contrast, in tandem MS disulfide bridges of μ-KIIIA lead to abrogation of fragmentation and to a significant drop in intensities of fragment ions beyond that cysteine within the bridge (Figure S4) as was also observed in previous studies.[21,24] This was especially aggravated by the vicinal cysteines in both terminals of μ-KIIIA that prevented sufficient assignment of fragment ion peaks in the oxidized form (Figure S4A). Thus, only analysis by ED would reveal the correct and completely assigned disulfide pattern for this peptide after partial reduction without any ambiguities and without great effort.

The workflow mentioned above was also applied to human insulin as a second example for a peptide carrying three disulfide bridges. However, analysis is more complicated as it consists of two peptide chains (chain A with 21 aa and chain B with 30 aa, Figure 5D) in which these disulfide bridges are

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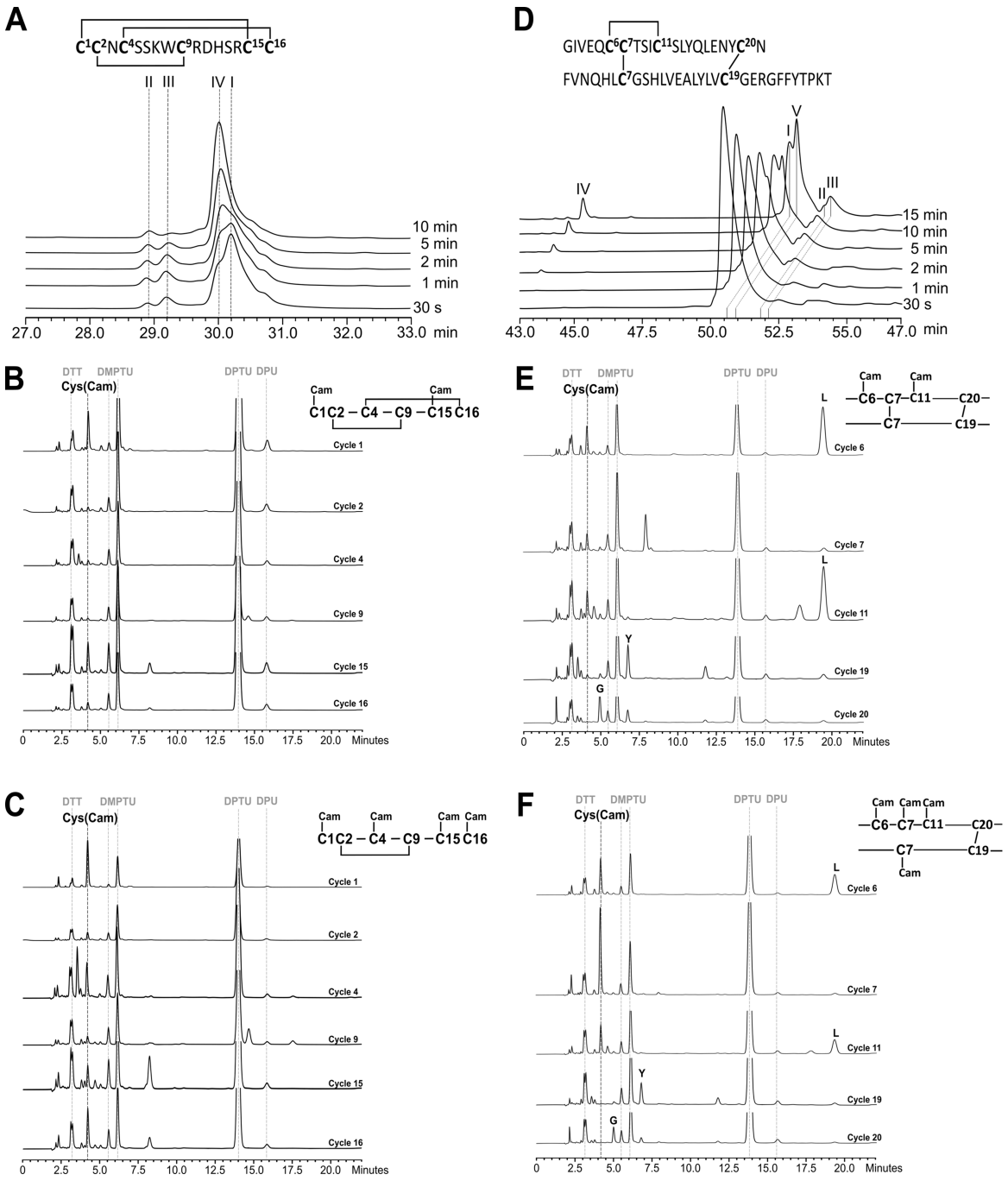

Figure 5. (A) Overlay of HPLC chromatograms for partially reduced μ-KIIIA variants obtained from treatment with 20 mM TCEP followed by alkylation with iodoacetamide at different time points. (B, C) Chromatograms of cycles 1, 2, 4, 9, 15, and 16 obtained from N-terminal sequencing of peaks II (B) and III (C). (D) Overlay of HPLC chromatograms for partially reduced human insulin using 20 mM TCEP followed by alkylation with iodoacetamide. (E, F) Chromatograms of cycles 6, 7, 11, 19, and 20 for N-terminal sequencing of peaks II (E) and III (F). Dashed lines in the chromatograms for N-terminal sequencing indicate the retention time of PTH-Cys(Cam).

reported to be formed between C6(A)−C11(B), C7(A)−C7(B), and C20(A)−C19(B). Thus, two simultaneous N-terminal sequencing routes have to be considered during analysis, which increases the complexity of the results (Scheme S1).

Upon treatment of insulin for up to 15 min with TCEP followed by subsequent carbamidomethylation, a stepwise reduction of the three bridges could be observed by the appearance of peaks with masses corresponding to completely oxidized insulin (peak I), insulin with one open bridge (2× carbamidomethylated, peak II), and insulin with two open bridges (4× carbamidomethylated, peak III) as well as the fully reduced peptide, i.e., chains A (4× carbamidomethylated, peak IV) and B (2× carbamidomethylated, peak V) (Figure 5D and Table S7).

N-terminal sequencing was performed for all peaks to determine the sequential opening of the disulfide bridges and, consequently, the connectivities of the cysteines within human insulin used. While no modified cysteine residue, i.e., carbamidomethylated cysteine was observed for the oxidized

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human insulin (Figure S5A), partially reduced insulin fractions (peaks II−V) showed PTH-Cys(Cam) appearance in a distinct pattern (Figures 5E,F and S5B,C). Sequencing of peak II containing one opened disulfide bridge showed carbamidomethylation of cysteines in cycles 6 and 11, while the other disulfide bridges were still intact; i.e., no increase for PTH-Cys(Cam) in the respective cycles could be detected (Figure 5E).

In addition to modifications in cycles 6 and 11, in cycle 7 of peak III, a significant increase in released PTH-Cys(Cam) that was approximately twice as high (57.8 pmol) as the detected concentration of PTH-Cys(Cam) in cycle 6 (30.6 pmol) revealed the existence of 2 carbamidomethylated cysteines at that position of insulin (Figure 5F). This indicated cleavage of the second disulfide bridge between C7(A) and C7(B), while cysteines 20 of chain A and 19 of chain B still remained intact. Opening of the third bridge was not detected in any of the peaks I−III. It could only be identified from the detection of fully reduced human insulin peptide chains A and B showing two separate fully alkylated reduced compounds (peaks IV and V) with PTH-Cys(Cam) in any of the cycles for cysteine upon application of the herein described protocol (Figure S5B,C).

It should be noted that for cycle 7 a peak for a PTH derivative at 7.80 min could be detected for the completely oxidized as well as the partially reduced insulin with one bridge opened (Figures 5E and S5A), which was described by others to appear due to formation of diPTH cystine.[53] This is described as a special feature for this specific cycle in insulin due to the appearance of two adjacent cysteines that form individual disulfide bridges at the N-terminus of both peptide chains.[53] This decreased significantly as soon as the second bridge (C7(A)−C7(B)) was opened (Figure 5F).

Consequently, as observed for μ-KIIIA, the expected disulfide connectivity, i.e., C6(A)−C11(B), C7(A)−C7(B), and C20(A)−C19(B) as described for native human insulin could be confirmed without any ambiguities (Scheme S1) simply based on a partial reduction and alkylation approach followed by N-terminal Edman sequencing in which the validated standard compound PTH-Cys(Cam) turned out as a valuable reference compound for the identification of the obtained alkylated cysteines.

CONCLUSIONS

N-terminal ED is a technique that is well-established and was frequently used in the 1970s−1990s.[66−68] Nowadays, its applications have been reduced due to the rise of MS as an alternative approach for sequence elucidation of peptides and proteins. However, scientists still struggle with applications that can be solved only by N-terminal sequencing using ED. With this study, we present the development and application of a protocol for expeditious analysis of disulfide connectivities in peptide and protein sequences. An optimized workflow, subsequently applying partial reduction, alkylation, fractionation, and ED, was established and successfully applied for the confirmation of disulfide connectivities in peptides of different complexity (1−3 disulfide bridges, 1−2 peptide chains), i.e., CCAP-vil, μ-KIIIA, and human insulin. The requirement of suitable standard compounds for the detection of modified PTH-cysteine derivatives within this analysis was solved by synthesizing and validating PTH-Cys(Cam) and PTH-Cys-(Me) as standards for the ED of cysteine. By applying and standardizing beneficial approaches for the application of ED, we aim at relaunching this technique as an alternative to tandem MS analysis, which involves tedious data analysis and evaluation of disulfide-rich peptides and proteins.

The establishment of easy-to-use protocols, such as the protocol presented herein, for the rapid and straightforward detection of disulfide connectivities will be a pivotal step in restoring the attractiveness of this technique to the scientific community as well as the respective drug design and development processes in the future.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.analchem.3c04229.

Detailed information for PTH-Cys derivative analysis and validation is described in the Supporting Information, as are the scheme for potential products of partial reduction (Scheme S1), chromatograms and sequencing results for oxidized and fully reduced μ-KIIIA, MS/MS analysis of μ-KIIIA as well as sequencing results of oxidized human insulin in addition to the fully reduced alkylated human insulin chain A and chain B (Figures S1−S5). Supporting tables for comparison beween ED and tandem MS, analytical characterization of PTH-Cys derivatives, validation results, characterization of model peptides, analytical data for μ-KIIIA and partial reduction fractions of human insulin and a summarizing table for comparison of ED and MS/MS are included as well (Tables S1−S7) (PDF)

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Author Contributions

The manuscript was written through contributions of all authors. D.I. and T.K. designed the experimental studies with the help of Y.Y.E. The optimization for the analytical experiments was performed by Y.Y.E. and data were analyzed together with D.I and T.K. All authors discussed the results and contributed to the final manuscript.

Notes

The authors declare no competing financial interest.

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Publications

Peer-reviewed papers:

Elsayed, Y. Y., Kühl, T., Imhof, D., "Regulatory guidelines for the analysis of therapeutic peptides and proteins" J. Pept. Sci. (2025) accepted.

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Book contributions:

Elsayed, Y.Y., Kühl, T., Imhof, D. (2024) "Innovative pharmaceutical stabilization and formulation processes for protein drugs and their impact on sequence and structure. Part: Analytics", In: Dispersity, Structure and Phase Changes of Proteins and Bio Agglomerates in Biotechnological Processes: Analytics, (Springer, Cham., Eds. Kwade, A., Kampen, I.), *157–179.*

Poster presentations:

Elsayed, Y.Y., Kühl, T., Imhof, D. (2023) "Edman sequencing as a relaunched tool for elucidation of disulfide connectivity", *16th German Peptide Symposium, Jena, Germany*.

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Abdel-Ghany, M.F., Abdel-Aziz, O., **Mohammed, Y.Y.**, (2016) "Two different techniques for simultaneous determination of Domperidone and Omeprazole in pharmaceutical formulation", *2nd International Conference on Research in Science and Technology, Kuala Lumpur, Malaysia.*