

METHODS OF REDUCING POLYSORBATE DEGRADATION IN DRUG FORMULATIONS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority to U.S. Provisional Patent Application No. 62/982,346, filed February 27, 2020, U.S. Provisional Patent Application No. 63/021,181, filed May 7, 2020 and U.S. Provisional Patent Application No. 63/073,125, filed September 1, 2020, the contents of which are incorporated herein by reference in their entirety.

FIELD

[0001] The present invention generally pertains to compositions with reduced amount of certain lipases, methods of making such compositions and methods of reducing polysorbate degradation due to the presence of such lipases. In particular, the present invention generally pertains to compositions and methods of making compositions with reduced presence of liver carboxylesterase-B1-like protein and liver carboxylesterase-1-like protein.

BACKGROUND

[0002] Among drug products, protein-based biotherapeutics are an important class of drugs that offer a high level of selectivity, potency and efficacy, as evidenced by the considerable increase in clinical trials with monoclonal antibodies (mAbs) over the past several years. Bringing a protein-based biotherapeutic to the clinic can be a multiyear undertaking requiring coordinated efforts throughout various research and development disciplines, including discovery, process and formulation development, analytical characterization, and pre-clinical toxicology and pharmacology.

[0003] One critical aspect for a clinically and commercially viable biotherapeutic is stability of the drug product in terms of the manufacturing process as well as shelf life. This often necessitates appropriate steps to help increase physical and chemical stability of the protein-

based biotherapeutics throughout the different solution conditions and environments necessary for manufacturing and storage with minimal impact on product quality, including identifying molecules with greater inherent stability, protein engineering, and formulation development. Surfactants, such as, polysorbate are often used to enhance the physical stability of a protein-based biotherapeutic product. Over seventy percent of marketed monoclonal antibody therapeutics contain between 0.001% and 0.1% polysorbate, a type of surfactant, to impart physical stability to the protein-based biotherapeutics. Polysorbates are susceptible to auto-oxidation and hydrolysis, which results in free fatty acids and subsequent fatty acid particle formation. The degradation of polysorbate can adversely affect the drug product quality since polysorbate can protect against interfacial stress, such as aggregation and adsorption. Presence of some lipases can be a likely cause of degradation of polysorbates in a formulation. Thus, such lipases in drug products need to be detected, monitored and reduced.

[0004] Direct analysis of lipases can require isolation of the product in a sufficiently large amount for the assay, which is undesirable and has only been possible in selected cases. Hence, it is a challenging task to determine the workflow and analytical tests required to characterize lipases responsible for polysorbate degradation in a sample. In addition to detecting the lipases responsible for polysorbate degradation, the drug product must be obtained by purification methods that remove or reduce such lipases.

[0005] It will be appreciated that a need exists for methods for depleting lipase from a formulated drug product.

SUMMARY

[0006] Maintaining stability of drug formulations, not only during storage but also during manufacturing, shipment, handling and administration, is a significant challenge. Among drug products, protein biotherapeutics are gaining popularity due to their success and versatility. One of the major challenges for protein biotherapeutics development is to overcome the limited stability of the protein and excipients in the products, which can be affected by the presence of lipases (present as host-cell proteins). Evaluation of its effect on the drug formulation and reduction of such lipases can be an important step in drug formulation development, followed by

methods to prepare the drug formulation so as to have reduced lipases and increased stability owing to the reduced lipases.

[0007] In one exemplary embodiment, the disclosure provides a method of depleting lipase from a sample comprising contacting the sample including lipase with a probe, said probe capable of binding to the lipase to form a complex and separating the complex from the sample to thereby deplete the lipase from the sample. In one aspect, the sample can comprise a protein of interest. In one aspect, the sample can comprise a polysorbate excipient. In a specific aspect, the polysorbate excipient can be selected from polysorbate-20, polysorbate-60, polysorbate-80 or combinations thereof. In yet another specific aspect, the polysorbate excipient is polysorbate-80.

[0008] In one aspect, the lipase is liver carboxylesterase-B1-like protein. In another aspect, the lipase is liver carboxylesterase-1-like protein.

In one aspect, the lipase is capable of degrading the polysorbate in the sample. Thus, the method of this embodiment reduces the degradation of polysorbates by depleting the sample of the lipase.

[0009] In one aspect, the probe can be capable of being linked to a solid support. In a specific aspect, the solid support can be agarose beads or magnetic beads.

[0010] In one aspect, the probe can be attached to a solid support using a ligand. In a specific aspect, the ligand can be an indicator, biotin molecule, a modified biotin molecule, a nuclei, a sequence, an epitope tag, an electron poor molecule or an electron rich molecule.

[0011] In one aspect, the method can further comprise recovering the lipase from the complex.

[0012] In one exemplary embodiment, the disclosure provides a method of purifying a sample having a protein of interest and a lipase, comprising contacting the sample with a probe, said probe capable of binding to the lipase to form a complex and separating the complex from the sample. In one aspect, the lipase is liver carboxylesterase-B1-like protein. In another aspect, the lipase is liver carboxylesterase-1-like protein.

[0013] In one aspect, the sample comprises a polysorbate excipient. In a specific aspect, the polysorbate excipient can be selected from polysorbate-20, polysorbate-60, polysorbate-80 or combinations thereof. In yet another specific aspect, the polysorbate excipient can be polysorbate-80.

[0014] In one aspect, the probe can be capable of being linked to a solid support. In a specific

aspect, the solid support can be agarose beads or magnetic beads.

[0015] In one aspect, the probe can be attached to a solid support using a ligand. In a specific aspect, the ligand can be an indicator, biotin molecule, a modified biotin molecule, a nuclei, a sequence, an epitope tag, an electron poor molecule or an electron rich molecule.

[0016] In one exemplary embodiment, the disclosure provides a method of decreasing degradation of polysorbate in a sample, comprising contacting the sample including lipase and polysorbate with a probe, said probe capable of binding to the lipase to form a complex and separating the complex from the sample to thereby decrease degradation of polysorbate in the sample.

[0017] In one aspect, the lipase is liver carboxylesterase-B1-like protein. In another aspect, the lipase is liver carboxylesterase-1-like protein.

[0018] In one aspect, the sample can comprise a protein of interest. In one aspect, the sample can comprise a polysorbate excipient. In a specific aspect, the polysorbate excipient is selected from polysorbate-20, polysorbate-60, polysorbate-80 or combinations thereof. In yet another specific aspect, the polysorbate excipient is polysorbate-80.

[0019] In one aspect, the probe can be capable of being linked to a solid support. In a specific aspect, the solid support can be agarose beads or magnetic beads.

[0020] In one aspect, the probe can be attached to a solid support using a ligand. In a specific aspect, the ligand can be an indicator, biotin molecule, a modified biotin molecule, a nuclei, a sequence, an epitope tag, an electron poor molecule or an electron rich molecule.

[0021] In one exemplary embodiment, the disclosure provides a composition comprising a protein of interest purified from mammalian cells and a residual amount of liver carboxylesterase-B1-like protein. In one aspect, the residual amount of liver carboxylesterase-B1-like protein is less than about 5 ppm. In another aspect, the composition can further comprise a surfactant. In yet a further aspect, the surfactant can be a hydrophilic nonionic surfactant. In another aspect, the surfactant can be a sorbitan fatty acid ester. In a specific aspect, the surfactant can be a polysorbate. In another specific aspect, the concentration of the polysorbate in the composition can be about 0.01% w/v to about 0.2% w/v. In a further specific aspect, the surfactant can be a polysorbate 80. In one aspect, the mammalian cells can include a CHO cell.

[0022] In one aspect, the liver carboxylesterase-B1-like protein can cause degradation of

polysorbate 80.

[0023] In one aspect, the composition can be a parenteral formulation.

[0024] In one aspect, the protein of interest can be a monoclonal antibody, a polyclonal antibody, a bispecific antibody, an antibody fragment, a fusion protein, or an antibody-drug complex. In one aspect, the concentration of the protein of interest can be about 20 mg/mL to about 400 mg/mL.

[0025] In one aspect, the composition can further comprise one or more pharmaceutically acceptable excipients. In another aspect, the composition can further comprise a buffer selected from a group consisting of histidine buffer, citrate buffer, alginate buffer, and arginine buffer. In one aspect, the composition can further comprise a tonicity modifier. In yet another aspect, the composition can further comprise sodium phosphate.

[0026] In one exemplary embodiment, the disclosure provides a composition comprising a protein of interest purified from mammalian cells and a residual amount of liver carboxylesterase-1-like protein. In one aspect, the residual amount of liver carboxylesterase-1-like protein is less than about 5 ppm. In another aspect, the composition can further comprise a surfactant. In yet a further aspect, the surfactant can be a hydrophilic nonionic surfactant. In another aspect, the surfactant can be a sorbitan fatty acid ester. In a specific aspect, the surfactant can be a polysorbate. In another specific aspect, the concentration of the polysorbate in the composition can be about 0.01 %w/v to about 0.2% w/v. In a further specific aspect, the surfactant can be a polysorbate 80. In one aspect, the mammalian cells can include a CHO cell.

[0027] In one aspect, the liver carboxylesterase-1-like protein can cause degradation of polysorbate 80.

[0028] In one aspect, the composition can be a parenteral formulation.

[0029] In one aspect, the protein of interest can be a monoclonal antibody, a polyclonal antibody, a bispecific antibody, an antibody fragment, a fusion protein, or an antibody-drug complex. In one aspect, the concentration of the protein of interest can be about 20 mg/mL to about 400 mg/mL.

[0030] In one aspect, the composition can further comprise one or more pharmaceutically acceptable excipients. In another aspect, the composition can further comprise a buffer selected from a group consisting of histidine buffer, citrate buffer, alginate buffer, and arginine buffer. In

one aspect, the composition can further comprise a tonicity modifier. In yet another aspect, the composition can further comprise sodium phosphate.

[0031] In one exemplary embodiment, the disclosure provides a method of detecting a lipase in a sample. In one aspect, the lipases can be liver carboxylesterase-1-like protein or liver carboxylesterase-B1-like protein. In one aspect, the method of detecting a lipase in a sample can comprise contacting the sample with a serine hydrolase probe. In one aspect, the method of detecting a lipase in a sample can comprise contacting and incubating the sample with a serine hydrolase probe to form a complex of lipase and serine hydrolase probe. In a further aspect, the method of detecting a lipase in a sample can comprise filtering out the serine hydrolase probe that does not form the complex of lipase and serine hydrolase probe.

[0032] In one aspect, the method of detecting a lipase in a sample can further comprise contacting the sample with magnetic beads having an ability to bind to the serine hydrolase probe to such that magnetic beads are bound to the complex of lipase and serine hydrolase probe. The magnetic beads bound to the complex of lipase and serine hydrolase probe can be further removed from the sample and washed with a buffer.

[0033] In another aspect, the method can further comprise removing the magnetic beads which are bound to the complex of lipase and serine hydrolase probe to form a solution of enriched lipases.

[0034] In one aspect, the method can further comprise adding hydrolyzing agent to the solution to obtain digests. In a specific aspect, the hydrolyzing agent can be trypsin. In one aspect, the method can further comprise analyzing the digests to detect the lipases. In one aspect, the digests can be analyzed using a mass spectrometer. In a specific aspect, the mass spectrometer can be a tandem mass spectrometer. In another specific aspect, the mass spectrometer can be coupled to a liquid chromatography system. In yet another specific aspect, the mass spectrometer can be coupled to a liquid chromatography - multiple reaction monitoring system.

[0035] In one aspect, the method can further comprise adding protein denaturing agent to the solution. In a specific aspect, the protein denaturing agent can be urea. In one aspect, the method can further comprise adding protein reducing agent to the solution. In a specific aspect, the protein reducing agent can be DTT (dithiothreitol). In one aspect, the method can further comprise adding protein alkylating agent to the solution. In a specific aspect, the protein

alkylating agent can be iodoacetamide.

[0036] These, and other, aspects of the invention will be better appreciated and understood when considered in conjunction with the following description and the accompanying drawings. The following description, while indicating various embodiments and numerous specific details thereof, is given by way of illustration and not of limitation. Many substitutions, modifications, additions, or rearrangements may be made within the scope of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

[0037] FIG. 1 shows chemical structures of major species in polysorbates. Polysorbates are mainly composed of fatty acid esters sharing a common sorbitan POE, isosorbide POE or POE head group, with oleic acid as the main fatty acid for PS80. The right panel A shows a total ion current (TIC) chromatogram of PS80 in mAb formulation by online 2D-LC/MS analysis. The identity of the labeled peaks are: (1) POE- POE isosorbide- POE sorbitan, (2) POE sorbitan monolinoleate, (3) POE sorbitan monooleate, (4) POE isosorbide monooleate and POE monooleate, (5) POE sorbitan linoleate/oleate diester, (6) POE sorbitan di-oleate, (7) POE isosorbide di-oleate and POE di-oleate, (8) Probably POE isosorbide/POE linoleate/oleate diester as mass spectra are too complicated to interpret, (9) POE sorbitan mixed trioleate and tetraoleate. The right panel B shows a CAD chromatogram showing the separation and detection of PS80 in mAb formulation by online 2D-LC/CAD analysis.

[0038] FIG. 2 shows a chromatogram of 0.1% PS80 in 50 mg/mL mAb-1 incubated at 5°C in 10mM histidine, pH 6 for 0 hours and 36 hours according to an exemplary embodiment. Peaks eluted between 11 to 17.5 minutes were POE, POE isosorbide and POE sorbitan.

[0039] FIG. 3 shows a chart of the percentage of PS80 remaining plotted against incubation time, where the original mAb-1, mAb-1 mixed with 0.125 μ M, 0.5 μ M and 2 μ M FP probe are indicated by filled circle with black solid line, filled diamond with red dotted line, filled square with orange dashed line and filled triangle with blue dotted line.

[0040] FIG. 4 shows a schematic diagram of the lipase(s) depletion experiment according to an exemplary embodiment. Streptavidin dynabeads magnetic beads were coupled with

desthiobiotin-FP probe and used for lipase(s) depletion. The original mAb-1 and flow through mAb-1 as well as process control mAb-1 were incubated with 0.1% PS80 at 5°C for 36 hours and subjected to PS degradation measurement. The enriched lipase(s) are subjected to digestion and HCP analysis using mass spectrometry.

[0041] FIG. 5 shows a chart of percentage of PS80 remaining in original mAb-1, process control and lipase(s) depleted mAb-1, where the original mAb-1, process control and lipase(s) depleted mAb-1 are indicated by filled diamond with black solid line, filled square with blue dotted line and filled circle with orange dashed line.

[0042] FIG. 6A shows a chromatogram of 0.1% PS80 in 20 µg/mL commercial rabbit liver esterase incubated at 5°C in 10mM histidine, pH 6 for 0 hours, 1.5 hours and 8 hours according to an exemplary embodiment.

[0043] FIG. 6B shows a chromatogram of 0.1% PS80 in 100 µg/mL commercial human liver carboxylesterases 1 incubated at 5°C in 10mM histidine, pH 6 for 0 hours, 5 hours and 18 hours according to an exemplary embodiment.

[0044] FIG. 6C shows a chromatogram of 0.1% PS80 in 50 mg/mL mAb-1 incubated at 5°C in 10mM histidine, pH 6 for 0 hours, 18 hours and 36 hours according to an exemplary embodiment.

[0045] FIG. 6D shows the sequence alignment of Liver Carboxylesterase B-1-like (A0A061I7X9), Liver Carboxylesterase 1-like (A0A061FE2) and Human liver carboxylesterase (hCES-1).

DETAILED DESCRIPTION

[0046] Host cell proteins (HCPs) are a class of impurities that must be removed from all cell-derived protein therapeutics. The FDA does not specify a maximum acceptable level of HCP, but HCP concentrations in final drug product must be controlled and reproducible from batch to batch (FDA, 1999). A primary safety concern relates to the possibility that HCPs can cause antigenic effects in human patients (Satish Kumar Singh, *Impact of Product-Related Factors on*

Immunogenicity of Biotherapeutics, and 100 JOURNALS OF PHARMACEUTICAL SCIENCES 354–387 (2011)). In addition to adverse health consequences for the patient, enzymatically active HCPs can potentially affect product quality during processing or long-term storage (Sharon X. Gao *et al.*, *Fragmentation of a highly purified monoclonal antibody attributed to residual CHO cell protease activity*, 108 BIOTECHNOLOGY AND BIOENGINEERING 977–982 (2010); Flavie Robert *et al.*, *Degradation of an Fc-fusion recombinant protein by host cell proteases: Identification of a CHO cathepsin D protease*, 104 BIOTECHNOLOGY AND BIOENGINEERING 1132–1141 (2009)). HCPs may present the greatest risk for persisting through purification operations into the final drug product. During long-term storage, the critical quality attributes of the product molecule must be maintained and degradation of excipients in the final product formulation must be minimized.

[0047] Several drug formulations on the market comprise polysorbate as one of the most commonly used nonionic surfactants in biopharmaceutical protein formulation that can improve protein stability and protect drug products from aggregation and denaturation (Sylvia Kiese *et al.*, *Shaken, Not Stirred: Mechanical Stress Testing of an IgG1 Antibody*, 97 JOURNAL OF PHARMACEUTICAL SCIENCES 4347–4366 (2008); Ariadna Martos *et al.*, *Trends on Analytical Characterization of Polysorbates and Their Degradation Products in Biopharmaceutical Formulations*, 106 JOURNAL OF PHARMACEUTICAL SCIENCES 1722–1735 (2017)). Polysorbate 20 (PS20) and polysorbate 80 (PS80) are the most commonly used nonionic surfactants in biopharmaceutical protein formulation that can improve protein stability and protect drug products from aggregation and denaturation. Typical polysorbate concentrations in drug products range can be between about 0.001% to about 0.1% (w/v) to provide sufficient efforts on protein stability.

[0048] Polysorbates, however, are liable to degradation that can drive undesired particulate formation in the formulated drug substances. Polysorbates are known to degrade in two main pathways: auto-oxidation and hydrolysis. Oxidation was found to be more likely to take place in PS80 due to the high content in unsaturated fatty acid ester substituents, whereas in PS20, oxidation was believed to take place on ether bond in polyoxyethylene chain that is not frequently observed (Oleg V. Borisov, Junyan A. Ji & Y. John Wang, *Oxidative Degradation*

of Polysorbate Surfactants Studied by Liquid Chromatography–Mass Spectrometry, 104 JOURNAL OF PHARMACEUTICAL SCIENCES 1005–1018 (2015); Anthony Tomlinson *et al.*, *Polysorbate 20 Degradation in Biopharmaceutical Formulations: Quantification of Free Fatty Acids, Characterization of Particulates, and Insights into the Degradation Mechanism*, 12 MOLECULAR PHARMACEUTICS 3805–3815 (2015); Jia Yao *et al.*, *A Quantitative Kinetic Study of Polysorbate Autoxidation: The Role of Unsaturated Fatty Acid Ester Substituents*, 26 PHARMACEUTICAL RESEARCH 2303–2313 (2009)). In addition, polysorbates can also undergo hydrolysis by breaking the fatty acid ester bond. The particulates originating on degradation of polysorbates can form visible or even sub-visible which can raise the potential for immunogenicity in patients and may have varying effects on the drug product quality. One such possible impurity could be fatty acid particles that are formed during manufacture, shipment, storage, handling or administration of drug formulations comprising polysorbate. The fatty acid particles could potentially cause adverse immunogenic effects and impact shelf life. Additionally, the degradation of polysorbates can also cause reduction in the total amount of surfactant in the formulation affecting the product's stability during its manufacturing, storage, handling, and administration.

[0049] Typically, polysorbate degradation can only be observed in drug products after a fairly long storage time. However, PS80 degradation was observed in case of one monoclonal antibody (mAb) within 24 hours at 4 °C although no obviously high concentrated lipase was detected, suggesting unfamiliar lipase(s) existed in this drug substance. It is imperative to detect and reduce concentration(s) of such lipase(s) in order to maintain the stability of the drug formulation.

[0050] Putative phospholipase B-like 2 (PLBD2) was the first host cell protein that was proposed to cause an enzymatic hydrolysis of PS20 (Nitin Dixit *et al.*, *Residual Host Cell Protein Promotes Polysorbate 20 Degradation in a Sulfatase Drug Product Leading to Free Fatty Acid Particles*, 105 JOURNAL OF PHARMACEUTICAL SCIENCES 1657–1666 (2016)). Porcine liver esterase was reported to be able to specifically hydrolysis of polysorbate 80 (not PS20) and lead the formation of PS85 over time in mAb drug product (Steven R. Labrenz, *Ester Hydrolysis of Polysorbate 80 in mAb Drug Product: Evidence in Support of the Hypothesized Risk After the*

Observation of Visible Particulate in mAb Formulations, 103 JOURNAL OF PHARMACEUTICAL SCIENCES 2268–2277 (2014)). Group XV lysosomal phospholipase A₂ isomer X1 (LPLA₂) demonstrated the ability to degrade PS20 and PS80 at less than 1 ppm (Troii Hall *et al.*, *Polysorbates 20 and 80 Degradation by Group XV Lysosomal Phospholipase A 2 Isomer X1 in Monoclonal Antibody Formulations*, 105 JOURNAL OF PHARMACEUTICAL SCIENCES 1633–1642 (2016) and Ying Cheng *et al.*, *A Rapid High-Sensitivity Reversed-Phase Ultra High Performance Liquid Chromatography Mass Spectrometry Method for Assessing Polysorbate 20 Degradation in Protein Therapeutics*, 108 JOURNAL OF PHARMACEUTICAL SCIENCES 2880–2886 (2019)).

[0051] Recently, a range of carboxyesters, including pseudomonas cepacia lipase on immobead 150 (PCL), candida antarctica lipase B on immobead 150 (CALB), thermomyces lanuginosus lipase on immobead 150 (TLL), rabbit liver esterase (RLE), Candida antarctica lipase B (CALB) and porcine pancreatic lipase type II (PPL), were selected to study the hydrolysis of two unique PS20 and PS80 which contained 99% of laurate and 98% oleate esters, respectively. Different carboxyesters showed their unique degradation patterns, indicating that degradation pattern can be used to differentiate enzymes that hydrolyze polysorbates (A. C. Mcshan *et al.*, *Hydrolysis of Polysorbate 20 and 80 by a Range of Carboxylester Hydrolases*, 70 PDA JOURNAL OF PHARMACEUTICAL SCIENCE AND TECHNOLOGY 332–345 (2016)). It can be essential to evaluate the effect of a host-cell protein co-purified with a drug product on polysorbates to ensure stability of the drug formulation. This can require identification of the host-cell protein and its ability to degrade polysorbates. Identification of host-cell proteins can be particularly challenging since the presence of HCPs is generally in ppm range, which makes the isolation and identification of the HCP difficult.

[0052] The present invention discloses improved compositions comprising polysorbate with reduced level of host-cell proteins that can degrade polysorbate(s), methods for detection of such host-cell proteins and methods for depleting such host-cell proteins.

[0053] Unless described otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can

be used in the practice or testing, particular methods and materials are now described. All publications mentioned are hereby incorporated by reference.

[0054] The term “a” should be understood to mean “at least one”; and the terms “about” and “approximately” should be understood to permit standard variation as would be understood by those of ordinary skill in the art; and where ranges are provided, endpoints are included.

[0055] In some exemplary embodiments, the disclosure provides a composition comprising a protein of interest, polysorbate, and a residual amount of a lipase.

[0056] As used herein, the term “composition” refers to an active pharmaceutical agent that is formulated together with one or more pharmaceutically acceptable vehicles.

[0057] As used herein, the term “an active pharmaceutical agent” can include a biologically active component of a drug product. An active pharmaceutical agent can refer to any substance or combination of substances used in a drug product, intended to furnish pharmacological activity or to otherwise have direct effect in the diagnosis, cure, mitigation, treatment or prevention of disease, or to have direct effect in restoring, correcting or modifying physiological functions in animals. Non-limiting methods to prepare an active pharmaceutical agent can include using fermentation process, recombinant DNA, isolation and recovery from natural resources, chemical synthesis, or combinations thereof.

[0058] In some exemplary embodiments, the amount of active pharmaceutical agent in the formulation can range from about 0.01 mg/mL to about 600 mg/mL. In some specific embodiments, the amount of active pharmaceutical agent in the formulation can be about 0.01 mg/mL, about 0.02 mg/mL, about 0.03 mg/mL, about 0.04 mg/mL, about 0.05 mg/mL, about 0.06 mg/mL, about 0.07 mg/mL, about 0.08 mg/mL, about 0.09 mg/mL, about 0.1 mg/mL, about 0.2 mg/mL, about 0.3 mg/mL, about 0.4 mg/mL, about 0.5 mg/mL, about 0.6 mg/mL, about 0.7 mg/mL, about 0.8 mg/mL, about 0.9 mg/mL, about 1 mg/mL, about 2 mg/mL, about 3 mg/mL, about 4 mg/mL, about 5 mg/mL, about 6 mg/mL, about 7 mg/mL, about 8 mg/mL, about 9 mg/mL, about 10 mg/mL, about 15 mg/mL, about 20 mg/mL, about 25 mg/mL, about 30 mg/mL, about 35 mg/mL, about 40 mg/mL, about 45 mg/mL, about 50 mg/mL, about 55

mg/mL, about 60 mg/mL, about 65 mg/mL, about 70 mg/mL, about 5 mg/mL, about 80 mg/mL, about 85 mg/mL, about 90 mg/mL, about 100 mg/mL, about 110 mg/mL, about 120 mg/mL, about 130 mg/mL, about 140 mg/mL, about 150 mg/mL, about 160 mg/mL, about 170 mg/mL, about 180 mg/mL, about 190 mg/mL, about 200 mg/mL, about 225 mg/mL, about 250 mg/mL, about 275 mg/mL, about 300 mg/mL, about 325 mg/mL, about 350 mg/mL, about 375 mg/mL, about 400 mg/mL, about 425 mg/mL, about 450 mg/mL, about 475 mg/mL, about 500 mg/mL, about 525 mg/mL, about 550 mg/mL, about 575 mg/mL, or about 600 mg/mL.

[0059] In some exemplary embodiments, pH of the composition can be greater than about 5.0. In one exemplary embodiment, the pH can be greater than about 5.0, greater than about 5.5, greater than about 6, greater than about 6.5, greater than about 7, greater than about 7.5, greater than about 8, or greater than about 8.5.

[0060] In some exemplary embodiments, the active pharmaceutical agent can be a protein of interest.

[0061] As used herein, the term "protein" or "protein of interest" can include any amino acid polymer having covalently linked amide bonds. Proteins comprise one or more amino acid polymer chains, generally known in the art as "polypeptides." "Polypeptide" refers to a polymer composed of amino acid residues, related naturally occurring structural variants, and synthetic non-naturally occurring analogs thereof linked via peptide bonds, related naturally occurring structural variants, and synthetic non-naturally occurring analogs thereof. "Synthetic peptides or polypeptides" refers to a non-naturally occurring peptide or polypeptide. Synthetic peptides or polypeptides can be synthesized, for example, using an automated polypeptide synthesizer. Various solid phase peptide synthesis methods are known to those of skill in the art. A protein may contain one or multiple polypeptides to form a single functioning biomolecule. A protein can include any of bio-therapeutic proteins, recombinant proteins used in research or therapy, trap proteins and other chimeric receptor Fc-fusion proteins, chimeric proteins, antibodies, monoclonal antibodies, polyclonal antibodies, human antibodies, and bispecific antibodies. Another exemplary aspect, a protein can include antibody fragments, nanobodies, recombinant antibody chimeras, cytokines, chemokines, peptide hormones, and the like. Proteins may be produced using recombinant cell-based production systems, such as the insect baculovirus

system, yeast systems (*e.g.*, *Pichia* sp.), mammalian systems (*e.g.*, CHO cells and CHO derivatives like CHO-K1 cells). For a recent review discussing biotherapeutic proteins and their production, see Ghaderi *et al.*, "Production platforms for biotherapeutic glycoproteins. Occurrence, impact, and challenges of non-human sialylation," (Darius Ghaderi *et al.*, *Production platforms for biotherapeutic glycoproteins. Occurrence, impact, and challenges of non-human sialylation*, 28 BIOTECHNOLOGY AND GENETIC ENGINEERING REVIEWS 147–176 (2012)). In some embodiments, proteins comprise modifications, adducts, and other covalently linked moieties. These modifications, adducts and moieties include for example avidin, streptavidin, biotin molecule, a modified biotin molecule, glycans (*e.g.*, N-acetylgalactosamine, galactose, neuraminic acid, N-acetylglucosamine, fucose, mannose, and other monosaccharides), PEG, polyhistidine, FLAGtag, maltose binding protein (MBP), chitin binding protein (CBP), glutathione-S-transferase (GST) myc-epitope, fluorescent labels and other dyes, and the like. Proteins can be classified on the basis of compositions and solubility and can thus include simple proteins, such as, globular proteins and fibrous proteins; conjugated proteins, such as, nucleoproteins, glycoproteins, mucoproteins, chromoproteins, phosphoproteins, metalloproteins, and lipoproteins; and derived proteins, such as, primary derived proteins and secondary derived proteins.

[0062] In some exemplary embodiments, the protein of interest can be an antibody, a bispecific antibody, a multispecific antibody, antibody fragment, monoclonal antibody, fusion protein, and combinations thereof.

[0063] In a particular aspect, the protein of interest can aflibercept (see, US 7,279,159, the entire teaching of which is incorporated herein by reference).

[0064] The term "antibody," as used herein includes immunoglobulin molecules comprising four polypeptide chains, two heavy (H) chains and two light (L) chains inter-connected by disulfide bonds, as well as multimers thereof (*e.g.*, IgM). Each heavy chain comprises a heavy chain variable region (abbreviated herein as HCVR or V_H) and a heavy chain constant region. The heavy chain constant region comprises three domains, C_{H1} , C_{H2} and C_{H3} . Each light chain comprises a light chain variable region (abbreviated herein as LCVR or V_L) and a light chain constant region. The light chain constant region comprises one domain (C_{L1}). The V_H and V_L

regions can be further subdivided into regions of hypervariability, termed complementarity determining regions (CDRs), interspersed with regions that are more conserved, termed framework regions (FR). Each V_H and V_L is composed of three CDRs and four FRs, arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, and FR4. In different embodiments of the invention, the FRs of the anti-big-ET-1 antibody (or antigen-binding portion thereof) may be identical to the human germline sequences or may be naturally or artificially modified. An amino acid consensus sequence may be defined based on a side-by-side analysis of two or more CDRs.

[0065] The term "antibody," as used herein, also includes antigen-binding fragments of full antibody molecules. The terms "antigen-binding portion" of an antibody, "antigen-binding fragment" of an antibody, and the like, as used herein, include any naturally occurring, enzymatically obtainable, synthetic, or genetically engineered polypeptide or glycoprotein that specifically binds an antigen to form a complex. Antigen-binding fragments of an antibody may be derived, *e.g.*, from full antibody molecules using any suitable standard techniques such as proteolytic digestion or recombinant genetic engineering techniques involving the manipulation and expression of DNA encoding antibody variable and optionally constant domains. Such DNA is known and/or is readily available from, for example, commercial sources, DNA libraries (including, *e.g.*, phage-antibody libraries), or can be synthesized. The DNA may be sequenced and manipulated chemically or by using molecular biology techniques, for example, to arrange one or more variable and/or constant domains into a suitable configuration, or to introduce codons, create cysteine residues, modify, add or delete amino acids, *etc.*

[0066] As used herein, an "antibody fragment" includes a portion of an intact antibody, such as, for example, the antigen-binding or variable region of an antibody. Examples of antibody fragments include, but are not limited to, a Fab fragment, a Fab' fragment, a F(ab')₂ fragment, a scFv fragment, a Fv fragment, a dsFv diabody, a dAb fragment, a Fd' fragment, a Fd fragment, and an isolated complementarity determining region (CDR) region, as well as triabodies, tetrabodies, linear antibodies, single-chain antibody molecules, and multi specific antibodies formed from antibody fragments. Fv fragments are the combination of the variable regions of the immunoglobulin heavy and light chains, and ScFv proteins are recombinant single chain

polypeptide molecules in which immunoglobulin light and heavy chain variable regions are connected by a peptide linker. In some exemplary embodiments, an antibody fragment contains sufficient amino acid sequence of the parent antibody of which it is a fragment that it binds to the same antigen as does the parent antibody; in some exemplary embodiments, a fragment binds to the antigen with a comparable affinity to that of the parent antibody and/or competes with the parent antibody for binding to the antigen. An antibody fragment may be produced by any means. For example, an antibody fragment may be enzymatically or chemically produced by fragmentation of an intact antibody and/or it may be recombinantly produced from a gene encoding the partial antibody sequence. Alternatively, or additionally, an antibody fragment may be wholly or partially synthetically produced. An antibody fragment may optionally comprise a single chain antibody fragment. Alternatively, or additionally, an antibody fragment may comprise multiple chains that are linked together, for example, by disulfide linkages. An antibody fragment may optionally comprise a multi-molecular complex. A functional antibody fragment typically comprises at least about 50 amino acids and more typically comprises at least about 200 amino acids.

[0067] The phrase “bispecific antibody” includes an antibody capable of selectively binding two or more epitopes. Bispecific antibodies generally comprise two different heavy chains, with each heavy chain specifically binding a different epitope—either on two different molecules (*e.g.*, antigens) or on the same molecule (*e.g.*, on the same antigen). If a bispecific antibody is capable of selectively binding two different epitopes (a first epitope and a second epitope), the affinity of the first heavy chain for the first epitope will generally be at least one to two or three or four orders of magnitude lower than the affinity of the first heavy chain for the second epitope, and vice versa. The epitopes recognized by the bispecific antibody can be on the same or a different target (*e.g.*, on the same or a different protein). Bispecific antibodies can be made, for example, by combining heavy chains that recognize different epitopes of the same antigen. For example, nucleic acid sequences encoding heavy chain variable sequences that recognize different epitopes of the same antigen can be fused to nucleic acid sequences encoding different heavy chain constant regions, and such sequences can be expressed in a cell that expresses an immunoglobulin light chain.

[0068] A typical bispecific antibody has two heavy chains each having three heavy chain CDRs, followed by a C_H1 domain, a hinge, a C_H2 domain, and a C_H3 domain, and an immunoglobulin light chain that either does not confer antigen-binding specificity but that can associate with each heavy chain, or that can associate with each heavy chain and that can bind one or more of the epitopes bound by the heavy chain antigen-binding regions, or that can associate with each heavy chain and enable binding of one or both of the heavy chains to one or both epitopes. BsAbs can be divided into two major classes, those bearing an Fc region (IgG-like) and those lacking an Fc region, the latter normally being smaller than the IgG and IgG-like bispecific molecules comprising an Fc. The IgG-like bsAbs can have different formats, such as, but not limited to triomab, knobs into holes IgG (kih IgG), crossMab, orth-Fab IgG, Dual-variable domains Ig (DVD-Ig), Two-in-one or dual action Fab (DAF), IgG-single-chain Fv (IgG-scFv), or $\kappa\lambda$ -bodies. The non-IgG-like different formats include Tandem scFvs, Diabody format, Single-chain diabody, tandem diabodies (TandAbs), Dual-affinity retargeting molecule (DART), DART-Fc, nanobodies, or antibodies produced by the dock-and-lock (DNL) method (Gaowei Fan, Zijian Wang & minutes gju Hao, *Bispecific antibodies and their applications*, 8 JOURNAL OF HEMATOLOGY & ONCOLOGY 130; Dafne Müller & Roland E. Kontermann, *Bispecific Antibodies*, HANDBOOK OF THERAPEUTIC ANTIBODIES 265–310 (2014)).

[0069] The methods of producing BsAbs are not limited to quadroma technology based on the somatic fusion of two different hybridoma cell lines, chemical conjugation, which involves chemical cross-linkers, and genetic approaches utilizing recombinant DNA technology. Examples of bsAbs include those disclosed in the following patent applications, which are hereby incorporated by reference: U.S. Ser. No. 12/823838, filed June 25, 2010; U.S. Ser. No. 13/488628, filed June 5, 2012; U.S. Ser. No. 14/031075, filed September 19, 2013; U.S. Ser. No. 14/808171, filed July 24, 2015; U.S. Ser. No. 15/713574, filed September 22, 2017; U.S. Ser. No. 15/713569, filed September 22, 2017; U.S. Ser. No. 15/386453, filed December 21, 2016; U.S. Ser. No. 15/386443, filed December 21, 2016; U.S. Ser. No. 15/22343 filed July 29, 2016; and U.S. Ser. No. 15/814095, filed November 15, 2017. Low levels of homodimer impurities can be present at several steps during the manufacturing of bispecific antibodies. The detection of such homodimer impurities can be challenging when performed using intact mass analysis due to low abundances of the homodimer impurities and the co-elution of these

impurities with main species when carried out using a regular liquid chromatographic method.

[0070] As used herein “multispecific antibody” or “Mab” refers to an antibody with binding specificities for at least two different antigens. While such molecules normally will only bind two antigens (*i.e.*, bispecific antibodies, BsAbs), antibodies with additional specificities such as trispecific antibody and KIH Trispecific can also be addressed by the system and method disclosed herein.

[0071] The term “monoclonal antibody” as used herein is not limited to antibodies produced through hybridoma technology. A monoclonal antibody can be derived from a single clone, including any eukaryotic, prokaryotic, or phage clone, by any means available or known in the art. Monoclonal antibodies useful with the present disclosure can be prepared using a wide variety of techniques known in the art including the use of hybridoma, recombinant, and phage display technologies, or a combination thereof.

[0072] In some exemplary embodiments, the protein of interest can have a pI in the range of about 4.5 to about 9.0. In one exemplary specific embodiment, the pI can be about 4.5, about 5.0, about 5.5, about 5.6, about 5.7, about 5.8, about 5.9, about 6.0, about 6.1, about 6.2, about 6.3, about 6.4, about 6.5, about 6.6, about 6.7, about 6.8, about 6.9, about 7.0, about 7.1, about 7.2, about 7.3, about 7.4, about 7.5, about 7.6, about 7.7, about 7.8, about 7.9, about 8.0, about 8.1, about 8.2, about 8.3, about 8.4, about 8.5, about 8.6, about 8.7, about 8.8, about 8.9, or about 9.0.

[0073] In some exemplary embodiments, the types of protein of interest in the compositions can be at least two. In some specific embodiments, one of the at least two protein of interest can be a monoclonal antibody, a polyclonal antibody, a bispecific antibody, an antibody fragment, a fusion protein, or an antibody-drug complex. In some other specific embodiments, concentration of one of the at least two protein of interest can be about 20 mg/mL to about 400 mg/mL. In some exemplary embodiments, the types of protein of interest in the compositions are two. In some exemplary embodiments, the types of protein of interest in the compositions are three. In some exemplary embodiments, the types of protein of interest in the compositions are five.

[0074] In some exemplary embodiments, the two or more protein of interest in the composition

can be selected from trap proteins, chimeric receptor Fc-fusion proteins, chimeric proteins, antibodies, monoclonal antibodies, polyclonal antibodies, human antibodies, bispecific antibodies, multispecific antibodies, antibody fragments, nanobodies, recombinant antibody chimeras, cytokines, chemokines, or peptide hormones.

[0075] In some exemplary embodiments, the composition can be a co-formulation.

[0076] In some exemplary embodiments, the protein of interest can be purified from mammalian cells. The mammalian cells can be of human origin or non-human origin can include primary epithelial cells (*e.g.*, keratinocytes, cervical epithelial cells, bronchial epithelial cells, tracheal epithelial cells, kidney epithelial cells and retinal epithelial cells), established cell lines and their strains (*e.g.*, 293 embryonic kidney cells, BHK cells, HeLa cervical epithelial cells and PER-C6 retinal cells, MDBK (NBL-1) cells, 911 cells, CRFK cells, MDCK cells, CHO cells, BeWo cells, Chang cells, Detroit 562 cells, HeLa 229 cells, HeLa S3 cells, Hep-2 cells, KB cells, LSI80 cells, LS174T cells, NCI-H-548 cells, RPMI2650 cells, SW-13 cells, T24 cells, WI-28 VA13, 2RA cells, WISH cells, BS-C-I cells, LLC-MK2 cells, Clone M-3 cells, 1-10 cells, RAG cells, TCMK-1 cells, Y-1 cells, LLC-PKi cells, PK(15) cells, GH_i cells, GH₃ cells, L2 cells, LLC-RC 256 cells, MHiCi cells, XC cells, MDOK cells, VSW cells, and TH-I, B1 cells, BSC-1 cells, RAf cells, RK-cells, PK-15 cells or derivatives thereof), fibroblast cells from any tissue or organ (including but not limited to heart, liver, kidney, colon, intestines, esophagus, stomach, neural tissue (brain, spinal cord), lung, vascular tissue (artery, vein, capillary), lymphoid tissue (lymph gland, adenoid, tonsil, bone marrow, and blood), spleen, and fibroblast and fibroblast-like cell lines (*e.g.*, CHO cells, TRG-2 cells, IMR-33 cells, Don cells, GHK-21 cells, citrullinemia cells, Dempsey cells, Detroit 551 cells, Detroit 510 cells, Detroit 525 cells, Detroit 529 cells, Detroit 532 cells, Detroit 539 cells, Detroit 548 cells, Detroit 573 cells, HEL 299 cells, IMR-90 cells, MRC-5 cells, WI-38 cells, WI-26 cells, Midi cells, CHO cells, CV-1 cells, COS-1 cells, COS-3 cells, COS-7 cells, Vero cells, DBS-FrhL-2 cells, BALB/3T3 cells, F9 cells, SV-T2 cells, M-MSV-BALB/3T3 cells, K-BALB cells, BLO-11 cells, NOR-10 cells, C3H/IOTI/2 cells, HSDMiC3 cells, KLN205 cells, McCoy cells, Mouse L cells, Strain 2071 (Mouse L) cells, L-M strain (Mouse L) cells, L-MTK' (Mouse L) cells, NCTC clones 2472 and 2555, SCC-PSA1 cells, Swiss/3T3 cells, Indian muntjac cells, SIRC cells, Cn cells, and Jensen cells, Sp2/0, NS0, NS1

cells or derivatives thereof).

[0077] In some exemplary embodiments, the composition can be stable. The stability of a composition can comprise evaluating the chemical stability, physical stability or functional stability of the active pharmaceutical agent. The formulations of the present invention typically exhibit high levels of stability of the active pharmaceutical agent.

[0078] In terms of protein formulations, the term “stable,” as used herein refers to the protein of interest within the formulations being able to retain an acceptable degree of chemical structure or biological function after storage under exemplary conditions defined herein. A formulation may be stable even though the protein of interest contained therein does not maintain 100% of its chemical structure or biological function after storage for a defined amount of time. Under certain circumstances, maintenance of about 90%, about 95%, about 96%, about 97%, about 98% or about 99% of a protein's structure or function after storage for a defined amount of time may be regarded as “stable”.

[0079] Stability can be measured, inter alia, by determining the percentage of native protein(s) that remain in the formulation after storage for a defined amount of time at a defined temperature. The percentage of native protein can be determined by, inter alia, size exclusion chromatography (*e.g.*, size exclusion high performance liquid chromatography [SE-HPLC]), such that native means non-aggregated and non-degraded. An “acceptable degree of stability,” as that phrase is used herein, means that at least 90% of the native form of the protein can be detected in the formulation after storage for a defined amount of time at a given temperature. In certain embodiments, at least about 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% of the native form of the protein can be detected in the formulation after storage for a defined amount of time at a defined temperature. The defined amount of time after which stability is measured can be at least 14 days, at least 28 days, at least 1 month, at least 2 months, at least 3 months, at least 4 months, at least 5 months, at least 6 months, at least 7 months, at least 8 months, at least 9 months, at least 10 months, at least 11 months, at least 12 months, at least 18 months, at least 24 months, or more.

[0080] Stability can be measured, inter alia, by determining the percentage of protein that forms

in an aggregate within the formulation after storage for a defined amount of time at a defined temperature, wherein stability is inversely proportional to the percent aggregate that is formed. This form of stability is also referred to as “colloidal stability” herein. The percentage of aggregated protein can be determined by, *inter alia*, size exclusion chromatography (*e.g.*, size exclusion high performance liquid chromatography [SE-HPLC]). An “acceptable degree of stability,” as that phrase is used herein, means that at most 6% of the protein is in an aggregated form detected in the formulation after storage for a defined amount of time at a given temperature. In certain embodiments an acceptable degree of stability means that at most about 6%, 5%, 4%, 3%, 2%, 1%, 0.5%, or 0.1% of the protein can be detected in an aggregate in the formulation after storage for a defined amount of time at a given temperature. The defined amount of time after which stability is measured can be about at least 2 weeks, at least 28 days, at least 1 month, at least 2 months, at least 3 months, at least 4 months, at least 5 months, at least 6 months, at least 7 months, at least 8 months, at least 9 months, at least 10 months, at least 11 months, at least 12 months, at least 18 months, at least 24 months, or more. The temperature at which the pharmaceutical formulation may be stored when assessing stability can be any temperature from about -80°C to about 45°C, *e.g.*, storage at about -80°C, about -30°C, about -20°C, about 0°C, about 4°C, about 5°C, about 25°C, about 35°C, about 37°C or about 45°C. For example, a pharmaceutical formulation may be deemed stable if after six months of storage at 5°C, less than about 3%, 2%, 1 %, 0.5%, or 0.1 % of the protein is detected in an aggregated form. A pharmaceutical formulation may also be deemed stable if after six months of storage at about 25°C, less than about 4%, 3%, 2%, 1 %, 0.5%, or 0.1 % of the protein is detected in an aggregated form. A pharmaceutical formulation may also be deemed stable if after 28 days of storage at 45°C, less than about 6%, 5%, 4%, 3%, 2%, 1%, 0.5%, or 0.1% of the protein is detected in an aggregated form. A pharmaceutical formulation may also be deemed stable if after three months of storage at -20°C, -30°C, or -80°C less than about 3%, 2%, 1 %, 0.5%, or 0.1 % of the protein is detected in an aggregated form.

[0081] Stability can also be measured, *inter alia*, by determining the percentage of protein that forms in an aggregate within the formulation after storage for a defined amount of time at a defined temperature, wherein stability is inversely proportional to the percent aggregate that is formed. This form of stability is also referred to as “colloidal stability” herein. The percentage

of aggregated protein can be determined by, *inter alia*, size exclusion chromatography (*e.g.*, size exclusion high performance liquid chromatography [SE-HPLC]). An acceptable degree of stability,” as that phrase is used herein, means that at most about 6% of the protein is in an aggregated form detected in the formulation after storage for a defined amount of time at a given temperature. In certain embodiments an acceptable degree of stability means that at most about 6%, 5%, 4%, 3%, 2%, 1%, 0.5%, or 0.1% of the protein can be detected in an aggregate in the formulation after storage for a defined amount of time at a given temperature. The defined amount of time after which stability is measured can be about at least 2 weeks, at least 28 days, at least 1 month, at least 2 months, at least 3 months, at least 4 months, at least 5 months, at least 6 months, at least 7 months, at least 8 months, at least 9 months, at least 10 months, at least 11 months, at least 12 months, at least 18 months, at least 24 months, or more. The temperature at which the pharmaceutical formulation may be stored when assessing stability can be any temperature from about -80°C to about 45°C, for example, storage at about -80°C, about -30°C, about -20°C, about 0°C, about 4°-8°C, about 5°C, about 25°C, about 35°C, about 37°C or about 45°C. For example, a pharmaceutical formulation may be deemed stable if after six months of storage at about 5°C, less than about 3%, 2%, 1%, 0.5%, or 0.1% of the protein is detected in an aggregated form. A pharmaceutical formulation may also be deemed stable if after six months of storage at about 25°C, less than about 4%, 3%, 2%, 1%, 0.5%, or 0.1% of the protein is detected in an aggregated form. A pharmaceutical formulation may also be deemed stable if after about 28 days of storage at 45°C, less than about 6%, 5%, 4%, 3%, 2%, 1%, 0.5%, or 0.1% of the protein is detected in an aggregated form. A pharmaceutical formulation may also be deemed stable if after three months of storage at about -20°C, -30°C, or -80°C less than about 3%, 2%, 1%, 0.5%, or 0.1% of the protein is detected in an aggregated form.

[0082] Stability can be also measured, *inter alia*, by determining the percentage of protein that migrates in a more acidic fraction during ion exchange (“acidic form”) than in the main fraction of protein (“main charge form”), wherein stability is inversely proportional to the fraction of protein in the acidic form. While not wishing to be bound by theory, deamidation of the protein may cause the protein to become more negatively charged and thus more acidic relative to the non-deamidated protein (see, *e.g.*, Robinson, N. (2002) “Protein Deamidation” PNAS, 99(8):5283-5288). The percentage of “acidified” protein can be determined by, *inter alia*, ion

exchange chromatography (*e.g.*, cation exchange high performance liquid chromatography [CEX- HPLC])). An “acceptable degree of stability,” as that phrase is used herein, means that at most 49% of the protein is in a more acidic form detected in the formulation after storage for a defined amount of time at a defined temperature. In certain exemplary embodiments, an acceptable degree of stability means that at most about 49%, 45%, 40%, 35%, 30%, 25%, 20%, 15%, 10%, 5%, 4%, 3%, 2%, 1%, 0.5%, or 0.1% of the protein can be detected in an acidic form in the formulation after storage for a defined amount of time at a given temperature. The defined amount of time after which stability is measured can be about at least 2 weeks, at least 28 days, at least 1 month, at least 2 months, at least 3 months, at least 4 months, at least 5 months, at least 6 months, at least 7 months, at least 8 months, at least 9 months, at least 10 months, at least 11 months, at least 12 months, at least 18 months, at least 24 months, or more.

[0083] The temperature at which the pharmaceutical formulation may be stored when assessing stability can be any temperature from about -80°C to about 45°C, *e.g.*, storage at about -80°C, about -30°C, about -20°C, about 0°C, about 4°-8°C, about 5°C, about 25°C, or about 45°C. For example, a pharmaceutical formulation may be deemed stable if after three months of storage at -80°C, -30°C, or -20°C less than about 30%, 29%, 28%, 27%, 26%, 25%, 24%, 23%, 22%, 21%, 20%, 19%, 18%, 17%, 16%, 15%, 14%, 13%, 12%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, 0.5% or 0.1% of the protein is in a more acidic form. A pharmaceutical formulation may also be deemed stable if after six months of storage at 5°C, less than about 32%, 31%, 30%, 29%, 28%, 27%, 26%, 25%, 24%, 23%, 22%, 21%, 20%, 19%, 18%, 17%, 16%, 15%, 14%, 13%, 12%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, 0.5% or 0.1% of the protein is in a more acidic form. A pharmaceutical formulation may also be deemed stable if after six months of storage at 25°C, less than about 43%, 42%, 41 %, 40%, 39%, 38%, 37%, 36%, 35%, 34%, 33%, 32%, 31%, 30%, 29%, 28%, 27%, 26%, 25%, 24%, 23%, 22%, 21%, 20%, 19%, 18%, 17%, 16%, 15%, 14%, 13%, 12%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, 0.5% or 0.1% of the protein is in a more acidic form. A pharmaceutical formulation may also be deemed stable if after 28 days of storage at 45°C, less than about 49%, 48%, 47%, 46%, 45%, 44%, 43%, 42%, 41%, 40%, 39%, 38%, 37%, 36%, 35%, 34%, 33%, 32%, 31%, 30%, 29%, 28%, 27%, 26%, 25%, 24%, 23%, 22%, 21%, 20%, 19%, 18%, 17%, 16%, 15%, 14%, 13%, 12%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, 0.5% or 0.1% of the protein can be detected in a more acidic

form.

[0084] Other methods may be used to assess the stability of the formulations of the present invention such as, for example differential scanning calorimetry (DSC) to determine thermal stability, controlled agitation to determine mechanical stability, and absorbance at about 350 nm or about 405 nm to determine solution turbidities. For example, a formulation of the present invention may be considered stable if, after 6 or more months of storage at about 5°C to about 25°C, the change in OD405 of the formulation is less than about 0.05 (*e.g.*, 0.04, 0.03, 0.02, 0.01, or less) from the OD405 of the formulation at time zero. Measuring the biological activity or binding affinity of the protein to its target may also be used to assess stability. For example, a formulation of the present invention may be regarded as stable if, after storage at *e.g.*, 5°C, 25°C, 45°C, etc. for a defined amount of time (*e.g.*, 1 to 12 months), the protein contained within the formulation binds to its target with an affinity that is at least 90%, 95%, or more of the binding affinity of the protein prior to said storage. Binding affinity may be determined by *e.g.*, ELISA or plasmon resonance. Biological activity may be determined by a protein activity assay, such as for example, contacting a cell that expresses the protein with the formulation comprising the protein. The binding of the protein to such a cell may be measured directly, such as, for example, via FACS analysis. Alternatively, the downstream activity of the protein system may be measured in the presence of the protein and compared to the activity of the protein system in the absence of protein.

[0085] In some exemplary embodiments, the composition can be used for the treatment, prevention and/or amelioration of a disease or disorder. Exemplary, non-limiting diseases and disorders that can be treated and/or prevented by the administration of the pharmaceutical formulations of the present invention include, infections; respiratory diseases; pain resulting from any condition associated with neurogenic, neuropathic or nociceptive pain; genetic disorder; congenital disorder; cancer; herpetiformis; chronic idiopathic urticarial; scleroderma, hypertrophic scarring; Whipple's Disease; benign prostate hyperplasia; lung disorders, such as mild, moderate or severe asthma, allergic reactions; Kawasaki disease, sickle cell disease; Churg-Strauss syndrome; Grave's disease; pre-eclampsia; Sjogren's syndrome; autoimmune lymphoproliferative syndrome; autoimmune hemolytic anemia; Barrett's esophagus; autoimmune

uveitis; tuberculosis; nephrosis; arthritis, including chronic rheumatoid arthritis; inflammatory bowel diseases, including Crohn's disease and ulcerative colitis; systemic lupus erythematosus; inflammatory diseases; HIV infection; AIDS; LDL apheresis; disorders due to PCSK9-activating mutations (gain of function mutations, "GOF"), disorders due to heterozygous Familial Hypercholesterolemia (heFH); primary hypercholesterolemia; dyslipidemia; cholestatic liver diseases; nephrotic syndrome; hypothyroidism; obesity; atherosclerosis; cardiovascular diseases; neurodegenerative diseases; neonatal Onset Multisystem Inflammatory Disorder (NOM ID/CINCA); Muckle-Wells Syndrome (MWS); Familial Cold Autoinflammatory Syndrome (FCAS); familial Mediterranean fever (FMF); tumor necrosis factor receptor-associated periodic fever syndrome (TRAPS); systemic onset juvenile idiopathic arthritis (Still's Disease); diabetes mellitus type 1 and type 2; auto-immune diseases; motor neuron disease; eye diseases; sexually transmitted diseases; tuberculosis; disease or condition which is ameliorated, inhibited, or reduced by a VEGF antagonist; disease or condition which is ameliorated, inhibited, or reduced by a PD-1 inhibitor; disease or condition which is ameliorated, inhibited, or reduced by a Interleukin antibody; disease or condition which is ameliorated, inhibited, or reduced by a NGF antibody; disease or condition which is ameliorated, inhibited, or reduced by a PCSK9 antibody; disease or condition which is ameliorated, inhibited, or reduced by a ANGPTL antibody; disease or condition which is ameliorated, inhibited, or reduced by an activin antibody; disease or condition which is ameliorated, inhibited, or reduced by a GDF antibody; disease or condition which is ameliorated, inhibited, or reduced by a FcγRI antibody; disease or condition which is ameliorated, inhibited, or reduced by a CD antibody; disease or condition which is ameliorated, inhibited, or reduced by a C5 antibody or combinations thereof.

[0086] In some exemplary embodiments, the composition can be administered to a patient. Administration may be via any route acceptable to those skilled in the art. Non-limiting routes of administration include oral, topical, or parenteral. Administration via certain parenteral routes may involve introducing the formulations of the present invention into the body of a patient through a needle or a catheter, propelled by a sterile syringe or some other mechanical device such as a continuous infusion system. A composition provided by the present invention may be administered using a syringe, injector, pump, or any other device recognized in the art for parenteral administration. A composition of the present invention may also be administered as

an aerosol for absorption in the lung or nasal cavity. The compositions may also be administered for absorption through the mucus membranes, such as in buccal administration.

[0087] As used herein, “polysorbate” refers to a common excipient used in formulation development to protect antibodies against various physical stresses such as agitation, freeze-thaw processes, and air/water interfaces (Emily Ha, Wei Wang & Y. John Wang, *Peroxide formation in polysorbate 80 and protein stability*, 91 JOURNAL OF PHARMACEUTICAL SCIENCES 2252–2264 (2002); Bruce A. Kerwin, *Polysorbates 20 and 80 Used in the Formulation of Protein Biotherapeutics: Structure and Degradation Pathways*, 97 JOURNAL OF PHARMACEUTICAL SCIENCES 2924–2935 (2008); Hanns-Christian Mahler *et al.*, Adsorption Behavior of a Surfactant and a Monoclonal Antibody to Sterilizing-Grade Filters, 99 Journal of Pharmaceutical Sciences 2620–2627 (2010)) and can include a non-ionic, amphipathic surfactant composed of fatty acid esters of polyoxyethylene-sorbitan. The esters can include polyoxyethylene sorbitan head group and either a saturated monolaurate side chain (polysorbate 20; PS20) or an unsaturated monooleate side chain (polysorbate 80; PS80). In some exemplary embodiments, the polysorbate can be present in the formulation in the range of 0.001% to 2% (weight/volume). Polysorbate can also contain a mixture of various fatty acid chains; for example, polysorbate 80 contains oleic, palmitic, myristic and stearic fatty acids, with the monooleate fraction making up approximately 58% of the polydisperse mixture (Nitin Dixit *et al.*, *Residual Host Cell Protein Promotes Polysorbate 20 Degradation in a Sulfatase Drug Product Leading to Free Fatty Acid Particles*, 105 JOURNAL OF PHARMACEUTICAL SCIENCES 1657–1666 (2016)). Non-limiting examples of polysorbates include polysorbate-20, polysorbate-40, polysorbate-60, polysorbate-65, and polysorbate-80.

[0088] A polysorbate can be susceptible to auto-oxidation in a pH- and temperature-dependent manner, and additionally, exposure to UV light can also produce instability (Ravuri S.k. Kishore *et al.*, *Degradation of Polysorbates 20 and 80: Studies on Thermal Autoxidation and Hydrolysis*, 100 JOURNAL OF PHARMACEUTICAL SCIENCES 721–731 (2011)), resulting in free fatty acids in solution along with the sorbitan head group. The free fatty acids resulting from polysorbate can include any aliphatic fatty acids with six to twenty carbons. Non-limiting examples of free fatty acids include oleic acid, palmitic acid, stearic acid, myristic acid, lauric acid, or combinations

thereof.

[0089] In some exemplary embodiments, the polysorbate can form free fatty acid particles. The free fatty acid particles can be at least 5 μm in size. Further, these fatty acid particles can be classified according to their size as visible ($> 100 \mu\text{m}$), sub-visible ($< 100 \mu\text{m}$, which can be subdivided into micron (1-100 μm) and submicron (100 nm-1000 nm)) and nanometer particles ($< 100 \text{ nm}$) (Linda Narhi, Jeremy Schmit & Deepak Sharma, *Classification of protein aggregates*, 101 JOURNAL OF PHARMACEUTICAL SCIENCES 493–498). In some exemplary embodiments, the fatty acid particles can be visible particles. Visible particles can be determined by visual inspection. In some exemplary embodiments, the fatty acid particles can be sub-visible particles. Subvisible particles can be monitored by the light blockage method according to United States Pharmacopeia (USP).

[0090] In some exemplary embodiments, the concentration of polysorbate in the composition can be about 0.001 %w/v, about 0.002 %w/v, about 0.003 %w/v, about 0.004 %w/v, about 0.005 %w/v, about 0.006 %w/v, about 0.007 %w/v, about 0.008 %w/v, about 0.009 %w/v, about 0.01 %w/v, about 0.011 %w/v, about 0.015 %w/v, about 0.02 %w/v, 0.025 %w/v, about 0.03 %w/v, about 0.035 %w/v, about 0.04 %w/v, about 0.045 %w/v, about 0.05 %w/v, about 0.055 %w/v, about 0.06 %w/v, about 0.065 %w/v, about 0.07 %w/v, about 0.075 %w/v, about 0.08 %w/v, about 0.085 %w/v, about 0.09 %w/v, about 0.095 %w/v, about 0.1 %w/v, about 0.11 %w/v, about 0.115 %w/v, about 0.12 %w/v, about 0.125 %w/v, about 0.13 %w/v, about 0.135 %w/v, about 0.14 %w/v, about 0.145 %w/v, about 0.15 %w/v, about 0.155 %w/v, about 0.16 %w/v, about 0.165 %w/v, about 0.17 %w/v, about 0.175 %w/v, about 0.18 %w/v, about 0.185 %w/v, about 0.19 %w/v, about 0.195 %w/v, or about 0.2 %w/v.

[0091] In some exemplary embodiments, the polysorbate can be degraded by the lipase(s) present in the composition. These lipase(s) can be a process-related impurity which can be derived from the manufacturing process and can include the three major categories: cell substrate-derived, cell culture-derived and downstream derived. Cell substrate-derived impurities include, but are not limited to, proteins derived from the host organism and nucleic acid (host cell genomic, vector, or total DNA). Cell culture-derived impurities include, but are not limited to, inducers, antibiotics, serum, and other media components. Downstream-derived

impurities include, but are not limited to, enzymes, chemical and biochemical processing reagents (*e.g.*, cyanogen bromide, guanidine, oxidizing and reducing agents), inorganic salts (*e.g.*, heavy metals, arsenic, nonmetallic ion), solvents, carriers, ligands (*e.g.*, monoclonal antibodies), and other leachables.

[0092] In one aspect, the lipase can be a serine hydrolase. In a specific aspect, then lipase can be carboxylesterase B-1-like protein (A0A061I7X9). In another specific aspect, the lipase can be liver carboxylesterase 1-like protein (A0A061IFE2). In yet another specific aspect, the lipase can be both carboxylesterase B-1-like protein and liver carboxylesterase 1-like protein.

[0093] The effect of lipases on degradation of polysorbate was identified by using detecting methods according to some exemplary embodiments.

[0094] Having identified lipases that can degrade polysorbates in certain protein preparations, it would be highly advantageous and desirable to have reagents, methods, and kits for the specific, sensitive, and quantitative determination and/or depletion of such lipase levels, as well as to develop methods of preparing compositions with low levels of lipases.

[0095] In some exemplary embodiments, the disclosure provides compositions which comprises less than about 5 ppm of carboxylesterase B-1-like protein and/or liver carboxylesterase 1-like protein.

[0096] In some exemplary embodiments, the residual amount of carboxylesterase B-1-like protein and/or liver carboxylesterase 1-like protein in the composition can be less than about 5 ppm. In some specific exemplary embodiments, the residual amount of carboxylesterase B-1-like protein and/or liver carboxylesterase 1-like protein is less than about 0.01 ppm, about 0.02 ppm, about 0.03 ppm, about 0.04 ppm, about 0.05 ppm, about 0.06 ppm, 0.07 ppm, 0.08 ppm, 0.09 ppm, about 0.1 ppm, about 0.2 ppm, about 0.3 ppm, about 0.4 ppm, about 0.5 ppm, about 0.6 ppm, 0.7 ppm, 0.8 ppm, 0.9 ppm, about 1 ppm, about 2 ppm, about 3 ppm, about 4 ppm, or about 5 ppm.

[0097] In some exemplary embodiments, the disclosure provides various methods of preparing a composition having a protein of interest which comprises less than about 5 ppm of

carboxylesterase B-1-like protein and/or liver carboxylesterase 1-like protein.

[0098] The disclosure also provides a method of preparing a composition having a protein of interest with less than about 5 ppm of carboxylesterase B-1-like protein and/or liver carboxylesterase 1-like protein comprising forming a sample with the protein of interest and the lipase, contacting the sample with a probe, said probe capable of binding to the lipase to form a complex and separating the complex from the sample.

[0099] In some exemplary embodiments, the sample can be obtained from any step of the bioprocess, such as, culture cell culture fluid (CCF), harvested cell culture fluid (HCCF), process performance qualification (PPQ), any step in the downstream processing, drug solution (DS), or a drug product (DP) comprising the final formulated product. In some other specific exemplary embodiments, the sample can be selected from any step of the downstream process of clarification, chromatographic purification, viral inactivation, or filtration. In some specific exemplary embodiments, the drug product can be selected from manufactured drug product in the clinic, shipping, storage, or handling. In some other specific exemplary embodiments, the drug product can comprise polysorbate(s).

[0100] In some exemplary embodiments, the method of preparing a composition having a protein of interest with less than about 5 ppm of carboxylesterase B-1-like protein and/or liver carboxylesterase 1-like protein can also include further chromatographic steps.

[0101] In some exemplary embodiments, method of preparing a composition having a protein of interest with less than about 5 ppm of carboxylesterase B-1-like protein and/or liver carboxylesterase 1-like protein can further include filtering one or all of the following: sample, eluate from one or more of the chromatographic steps, and/or flow-through from one or more of the chromatographic steps.

[0102] As used herein, “viral filtration” can include filtration using suitable filters including, but not limited to, Planova 20N™, 50 N or BioEx from Asahi Kasei Pharma, Viresolve™ filters from EMD Millipore, ViroSart CPV from Sartorius, or Ultipor DV20 or DV50™ filter from Pall Corporation. It will be apparent to one of ordinary skill in the art to select a suitable filter to obtain desired filtration performance.

[0103] In some exemplary embodiments, method of preparing a composition having a protein of interest with less than about 5 ppm of carboxylesterase B-1-like protein and/or liver carboxylesterase 1-like protein can further include performing UF/DF on one or all of the following: sample, eluate from one or more of the chromatographic steps, and/or flow-through from one or more of the chromatographic steps.

[0104] As used herein, the term “ultrafiltration” or “UF” can include a membrane filtration process similar to reverse osmosis, using hydrostatic pressure to force water through a semi-permeable membrane. Ultrafiltration is described in detail in: LEOS J. ZEMAN & ANDREW L. ZYDNEY, MICROFILTRATION AND ULTRAFILTRATION: PRINCIPLES AND APPLICATIONS (1996). Filters with a pore size of smaller than 0.1 μm can be used for ultrafiltration. By employing filters having such small pore size, the volume of the sample can be reduced through permeation of the sample buffer through the filter while antibodies are retained behind the filter.

[0105] As used herein, “diafiltration” or “DF” can include a method of using ultrafilters to remove and exchange salts, sugars, and non-aqueous solvents, to separate free from bound species, to remove low molecular-weight material, and/or to cause the rapid change of ionic and/or pH environments. Microsolutes are removed most efficiently by adding solvent to the solution being ultrafiltered at a rate approximately equal to the ultrafiltration rate. This washes microspecies from the solution at a constant volume, effectively manufacturing the retained antibody. In certain embodiments of the present invention, a diafiltration step can be employed to exchange the various buffers used in connection with the instant invention, optionally prior to further chromatography or other purification steps, as well as to remove impurities from the antibody preparation.

[0106] In some exemplary embodiments, the probe can be capable of being linked on a solid support. The solid support may be any of the well known supports or matrices which are currently widely used or proposed for immobilisation, separation etc. These may take the form of particles, sheets, gels, filters, membranes, fibres, capillaries, or microtitre strips, tubes, plates or wells etc. Conveniently the support may be made of glass, silica, latex or a polymeric material.. Particulate materials, for example, beads are generally preferred due to their greater binding capacity, particularly polymeric beads. A particulate solid support used according to the invention will comprise spherical beads. Non-magnetic polymer beads suitable for use in the

method of the invention are available from Dyno Particles AS (Lillestrøm, Norway) as well as from Qiagen, Pharmacia and Serotec.

[0107] However, to aid manipulation and separation, magnetic beads are preferred. The term “magnetic” as used herein means that the support is capable of having a magnetic moment imparted to it when placed in a magnetic field, and thus is displaceable under the action of that field. In other words, a support comprising magnetic particles may readily be removed by magnetic aggregation, which provides a quick, simple and efficient way of separating the particles following the nucleic acid binding step, and is a far less rigorous method than traditional techniques such as centrifugation which generate shear forces which may degrade nucleic acids. Thus, using the method of the invention, the complex formed between the probe and lipase may be removed by application of a magnetic field, for example, using a permanent magnet. It is usually sufficient to apply a magnet to the side of the vessel containing the sample mixture to aggregate the particles to the wall of the vessel and to pour away the remainder of the sample. In some specific aspects, the superparamagnetic particles can be used, for example those described by Sintef in EP-A-106873, as magnetic aggregation and clumping of the particles during reaction can be avoided, thus ensuring uniform and nucleic acid extraction. The well-known magnetic particles sold by Dynal AS (Oslo, Norway) as DYNABEADS, are particularly suited to use in the present invention. Further, beads, or other supports, may be prepared having different types of functionalised surface, for example positively charged or hydrophobic. Weakly and strongly positively charged surfaces, weakly negatively charged neutral surfaces and hydrophobic surfaces e.g. polyurethane-coated have been shown to work well.

[0108] In some exemplary embodiments, the probe can be capable of being linked on a solid support using a ligand. Non-limiting examples can include an indicator, biotin molecule, a modified biotin molecule, modified biotin molecule, a modified biotin molecule, a nucleic acid, a protein sequence, an epitope tag, an electron poor molecule or an electron rich molecule. Specific examples of ligands can include, but are not limited to, biotin molecule or a modified such as deiminobiotin molecule, desthiobiotin molecule, vicinal diols, such as 1,2-dihydroxyethane, 1,2-dihydroxycyclohexane, etc., digoxigenin, maltose, oligohistidine, glutathione, 2,4-dinitrobenzene, phenylarsenate, ssDNA, dsDNA, a peptide or polypeptide, a

metal chelate, a saccharide, rhodamine or fluorescein, or any hapten to which an antibody can be generated. Examples of ligands and their capture reagents include but are not limited to: dethiobiotin or structurally modified biotin-based reagents, including deiminobiotin molecule, a modified biotin molecule, which bind to proteins of the avidin/streptavidin family, which may, for example, be used in the forms of strepavidin-Agarose, oligomeric-avidin-Agarose, or monomeric-avidin-Agarose; any 1,2-diol, such as 1,2-dihydroxyethane ($\text{HO}-\text{CH}_2-\text{CH}_2-\text{OH}$), and other 1,2-dihydroxyalkanes including those of cyclic alkanes, for example, 1,2-dihydroxycyclohexane which bind to an alkyl or aryl boronic acid or boronic acid esters, such as phenyl-B(OH)₂ or hexyl-B(OEthyl)₂ which may be attached via the alkyl or aryl group to a solid support material, such as Agarose; maltose which binds to maltose binding protein (as well as any other sugar/sugar binding protein pair or more generally to any ligand/ligand binding protein pairs that has properties discussed above); a hapten, such as the dinitrophenyl group, for any antibody where the hapten binds to an anti-hapten antibody that recognizes the hapten, for example the dinitrophenyl group will bind to an anti-dinitrophenyl-IgG; a ligand which binds to a transition metal, for example, an oligomeric histidine will bind to Ni(II), the transition metal capture reagent may be used in the form of a resin bound chelated transition metal, such as nitrilotriacetic acid-chelated Ni(II) or iminodiacetic acid-chelated Ni(II); glutathione which binds to glutathione-S-transferase.

[0109] The disclosure also provides a method of detecting a liver carboxylesterase-1-like protein or liver carboxylesterase-B1-like protein in a sample by contacting the sample with a serine hydrolase probe. In one aspect, the method of detecting a lipase in a sample can comprise contacting and incubating the sample with a serine hydrolase probe to form a complex of lipase and serine hydrolase probe. In a further aspect, the method of detecting a lipase in a sample can comprise filtering out the serine hydrolase probe that does not form the complex of lipase and serine hydrolase probe.

[0110] In some exemplary embodiments, the method of detecting a lipase in a sample can further comprise contacting the sample with magnetic beads having an ability to bind to the serine hydrolase probe such that magnetic beads are bound to the complex of lipase and serine hydrolase probe.

[0111] In some specific exemplary embodiments, the magnetic beads bound to the complex of

lipase and serine hydrolase probe can be further removed from the sample and washed with a buffer.

[0112] In some exemplary embodiments, the method of detecting a lipase in a sample can further comprise removing the magnetic beads, which are bound to the complex of lipase and serine hydrolase probe to form a solution of enriched lipase.

[0113] In some exemplary embodiments, the method of detecting a lipase in a sample can further comprise adding hydrolyzing agent to the solution to obtain digests.

[0114] As used herein, the term “hydrolyzing agent” refers to any one or combination of a large number of different agents that can perform digestion of a protein. Non-limiting examples of hydrolyzing agents that can carry out enzymatic digestion include protease from *Aspergillus Saitoi*, elastase, subtilisin, protease XIII, pepsin, trypsin, Tryp-N, chymotrypsin, aspergillopepsin I, LysN protease (Lys-N), LysC endoproteinase (Lys-C), endoproteinase Asp-N (Asp-N), endoproteinase Arg-C (Arg-C), endoproteinase Glu-C (Glu-C) or outer membrane protein T (OmpT), immunoglobulin-degrading enzyme of *Streptococcus pyogenes* (IdeS), thermolysin, papain, pronase, V8 protease or biologically active fragments or homologs thereof or combinations thereof. Non-limiting examples of hydrolyzing agents that can carry out non-enzymatic digestion include the use of high temperature, microwave, ultrasound, high pressure, infrared, solvents (non-limiting examples are ethanol and acetonitrile), immobilized enzyme digestion (IMER), magnetic particle immobilized enzymes, and on-chip immobilized enzymes. For a recent review discussing the available techniques for protein digestion see Switazar *et al.*, “Protein Digestion: An Overview of the Available Techniques and Recent Developments” (Linda Switazar, Martin Giera & Wilfried M. A. Niessen, *Protein Digestion: An Overview of the Available Techniques and Recent Developments*, 12 JOURNAL OF PROTEOME RESEARCH 1067–1077 (2013)). One or a combination of hydrolyzing agents can cleave peptide bonds in a protein or polypeptide, in a sequence-specific manner, generating a predictable collection of shorter peptides.

[0115] The ratio of hydrolyzing agent to the lipase and the time required for digestion can be appropriately selected to obtain a digestion of the lipase. When the enzyme to substrate ratio is unsuitably high, the correspondingly high digestion rate will not allow sufficient time for the peptides to be analyzed by mass spectrometer, and sequence coverage will be compromised. On

the other hand, a low E/S ratio would need long digestion and thus long data acquisition time. The enzyme to substrate ratio can range from about 1:0.5 to about 1:200. As used herein, the term “digestion” refers to hydrolysis of one or more peptide bonds of a protein. There are several approaches to carrying out digestion of a protein in a sample using an appropriate hydrolyzing agent, for example, enzymatic digestion or non-enzymatic digestion.

[0116] In some exemplary embodiments, the method of detecting a lipase in a sample can further comprise adding protein denaturing agent to the solution.

[0117] As used herein, “protein denaturing” can refer to a process in which the three-dimensional shape of a molecule is changed from its native state without rupture of peptide bonds. The protein denaturation can be carried out using a protein denaturing agent. Non-limiting examples of a protein denaturing agent include heat, high or low pH, or exposure to chaotropic agents. Several chaotropic agents can be used as protein denaturing agents. Chaotropic solutes increase the entropy of the system by interfering with intramolecular interactions mediated by non-covalent forces such as hydrogen bonds, van der Waals forces, and hydrophobic effects. Non-limiting examples for chaotropic agents include butanol, ethanol, guanidinium chloride, lithium perchlorate, lithium acetate, magnesium chloride, phenol, propanol, sodium dodecyl sulfate, thiourea, N-lauroylsarcosine, urea, and salts thereof. In a specific aspect, the protein denaturing agent can be urea.

[0118] In some exemplary embodiments, the method of detecting a lipase in a sample can further comprise adding protein denaturing or reducing agent to the solution.

[0119] As used herein, the term “protein reducing agent” refers to the agent used for reduction of disulfide bridges in a protein. Non-limiting examples of the protein reducing agents used to reduce the protein are dithiothreitol (DTT), β -mercaptoethanol, Ellman’s reagent, hydroxylamine hydrochloride, sodium cyanoborohydride, tris(2-carboxyethyl)phosphine hydrochloride (TCEP-HCl), or combinations thereof. In one aspect, the protein reducing agent can be DTT (dithiothreitol).

[0120] In some exemplary embodiments, the method of detecting a lipase in a sample can further comprise adding protein alkylating agent to the solution.

[0121] As used herein, the term “protein alkylating agent” refers to the agent used for alkylation certain free amino acid residues in a protein. Non-limiting examples of the protein alkylating agents are iodoacetamide (IOA), chloroacetamide (CAA), acrylamide (AA), N-ethylmaleimide (NEM), methyl methanethiosulfonate (MMTS), and 4-vinylpyridine or combinations thereof.

[0122] In some exemplary embodiments, the method of detecting a lipase in a sample can further comprise analyzing the digests to detect the lipases. In one aspect, the digests can be analyzed using a mass spectrometer. In a specific aspect, the mass spectrometer can be a tandem mass spectrometer. In another specific aspect, the mass spectrometer can be coupled to a liquid chromatography system. In yet another specific aspect, the mass spectrometer can be coupled to a liquid chromatography - multiple reaction monitoring system.

[0123] As used herein, the term “mass spectrometer” includes a device capable of identifying specific molecular species and measuring their accurate masses. The term is meant to include any molecular detector into which a polypeptide or peptide may be eluted for detection and/or characterization. A mass spectrometer can include three major parts: the ion source, the mass analyzer, and the detector. The role of the ion source is to create gas phase ions. Analyte atoms, molecules, or clusters can be transferred into gas phase and ionized either concurrently (as in electrospray ionization) or through separate processes. The choice of ion source depends heavily on the application.

[0124] As used herein, the term “tandem mass spectrometry” includes a technique where structural information on sample molecules is obtained by using multiple stages of mass selection and mass separation. A prerequisite is that the sample molecules can be transferred into gas phase and ionized intact and that they can be induced to fall apart in some predictable and controllable fashion after the first mass selection step. Multistage MS/MS, or MS^n , can be performed by first selecting and isolating a precursor ion (MS^2), fragmenting it, isolating a primary fragment ion (MS^3), fragmenting it, isolating a secondary fragment (MS^4), and so on as long as one can obtain meaningful information, or the fragment ion signal is detectable. Tandem MS has been successfully performed with a wide variety of analyzer combinations. What analyzers to combine for a certain application can be determined by many different factors, such as sensitivity, selectivity, and speed, but also size, cost, and availability. The two major

categories of tandem MS methods are tandem-in-space and tandem-in-time, but there are also hybrids where tandem-in-time analyzers are coupled in space or with tandem-in-space analyzers. A tandem-in-space mass spectrometer comprises an ion source, a precursor ion activation device, and at least two non-trapping mass analyzers. Specific m/z separation functions can be designed so that in one section of the instrument ions are selected, dissociated in an intermediate region, and the product ions are then transmitted to another analyzer for m/z separation and data acquisition. In tandem-in-time, mass spectrometer ions produced in the ion source can be trapped, isolated, fragmented, and m/z separated in the same physical device.

[0125] The peptides identified by the mass spectrometer can be used as surrogate representatives of the intact protein and their post translational modifications. They can be used for protein characterization by correlating experimental and theoretical MS/MS data, the latter generated from possible peptides in a protein sequence database. The characterization includes, but is not limited, to sequencing amino acids of the protein fragments, determining protein sequencing, determining protein de novo sequencing, locating post-translational modifications, or identifying post translational modifications, or comparability analysis, or combinations thereof.

[0126] As used herein, the term “database” refers to bioinformatic tools which provide the possibility of searching the uninterpreted MS-MS spectra against all possible sequences in the database(s). Non-limiting examples of such tools are Mascot (<http://www.matrixscience.com>), Spectrum Mill (<http://www.chem.agilent.com>), PLGS (<http://www.waters.com>), PEAKS (<http://www.bioinformaticssolutions.com>), Proteinpilot (<http://download.appliedbiosystems.com/proteinpilot>), Phenyx (<http://www.phenyx-ms.com>), Sorcerer (<http://www.sagenresearch.com>), OMSSA (<http://www.pubchem.ncbi.nlm.nih.gov/omssa/>), X!Tandem (<http://www.thegpm.org/TANDEM/>), Protein Prospector (<http://www.prospector.ucsf.edu/prospector/mshome.htm>), Byonic (<https://www.proteinmetrics.com/products/byonic>) or Sequest (<http://fields.scripps.edu/sequest>).

[0127] In some exemplary embodiments, the mass spectrometer can be coupled to a liquid chromatography system.

[0128] As used herein, the term “chromatography” refers to a process in which a chemical mixture carried by a liquid or gas can be separated into components as a result of differential distribution of the chemical entities as they flow around or over a stationary liquid or solid phase. Non-limiting examples of chromatography include traditional reversed-phased (RP), ion exchange (IEX) and normal phase chromatography (NP). Unlike RP, NP and IEX chromatography, in which hydrophobic interaction, hydrophilic interaction and ionic interaction respectively are the dominant interaction modes, mixed-mode chromatography can employ a combination of two or more of these interaction modes. Several types of liquid chromatography can be used with the mass spectrometer, such as, rapid resolution liquid chromatography (RRLC), ultra-performance liquid chromatography (UPLC), ultra-fast liquid chromatography (UFLC) and nano liquid chromatography (nLC). For further details on chromatography method and principles, see Colin *et al.* (COLIN F. POOLE *ET AL.*, LIQUID CHROMATOGRAPHY FUNDAMENTALS AND INSTRUMENTATION (2017)).

[0129] In some exemplary embodiments, the mass spectrometer can be coupled to a nano liquid chromatography. In some exemplary embodiments, the mobile phase used to elute the protein in liquid chromatography can be a mobile phase that can be compatible with a mass spectrometer. In some specific exemplary embodiments, the mobile phase can be ammonium acetate, ammonium bicarbonate, or ammonium formate, or combinations thereof.

[0130] In some exemplary embodiments, the mass spectrometer can be coupled to a liquid chromatography - multiple reaction monitoring system.

[0131] As used herein, “multiple reaction monitoring” or “MRM” refers to a mass spectrometry-based technique that can precisely quantify small molecules, peptides, and proteins within complex matrices with high sensitivity, specificity and a wide dynamic range (Paola Picotti & Ruedi Aebersold, *Selected reaction monitoring-based proteomics: workflows, potential, pitfalls and future directions*, 9 NATURE METHODS 555–566 (2012)). MRM can be typically performed with triple quadrupole mass spectrometers wherein a precursor ion corresponding to the selected small molecules/ peptides is selected in the first quadrupole and a fragment ion of the precursor ion was selected for monitoring in the third quadrupole (Yong Seok Choi *et al.*, *Targeted human cerebrospinal fluid proteomics for the validation of multiple Alzheimers disease biomarker*

candidates, 930 JOURNAL OF CHROMATOGRAPHY B 129–135 (2013)).

[0132] In some exemplary embodiments, the mass spectrometer can be coupled to a liquid chromatography – selected reaction monitoring system.

[0133] It is understood that the present invention is not limited to any of the aforesaid, chromatographic resin(s), excipient(s), filtration method(s), hydrolyzing agent(s), protein denaturing agent(s), protein alkylating agent(s), instrument(s) used for identification, and any chromatographic resin(s), excipient(s), filtration method(s), hydrolyzing agent(s), protein denaturing agent(s), protein alkylating agent(s), instrument(s) used for identification can be selected by any suitable means.

[0134] Various publications, including patents, patent applications, published patent applications, accession numbers, technical articles and scholarly articles are cited throughout the specification. Each of these cited references is incorporated by reference herein in its entirety and for all purposes,.

[0135] The present invention will be more fully understood by reference to the following Examples. They should not, however, be construed as limiting the scope of the invention

EXAMPLES

Materials.

[0136] Dynabeads MyOne Streptavidin T1 was purchased from Invitrogen of Thermo Fisher Scientific (Waltham, MA). ActivX Desthiobiotin-FP Serine Hydrolase Probe, Formic acid, acetonitrile, Dithiothreitol (DTT) and 1-step ultra TMD-blotting solution were purchased from Thermo Fisher Scientific (Waltham, MA). Acetic acid, 10X Tris buffered saline (TBS), Iodoacetamide (IAM), bovine serum albumin (BSA) and urea were purchased from Sigma-Aldrich (St. Louis, MO). HEPES buffered saline with EDTA and 0.005% v/v Surfactant P-20 (HBS-EP) was purchased from GE (Boston, MA). Monoclonal antibody drug substance was made at Regeneron Pharmaceutical Inc. Polysorbate 80 were purchased from Croda (East Yorkshire, UK). Rabbit rLE was purchased from Sigma Aldrich (St. Louis, MO). Human CES-1

was purchased from Abcam (Cambridge UK). Sequencing Grade Modified Trypsin was purchased from Promega (Madison, WI). Oasis Max column (2.1x20 mm, 30 µm) and Acquity UPLC BEH C4 column (2.1x50 mm, 1.7 µm) were purchased from Waters (Milford, MA). Acclaim PepMap 100 C18 analytical column (0.075x250 mm, 3 µm) and Acclaim PepMap 100 C18 trap column (0.075x20 mm, 3 µm) were purchased from Thermo Fisher Scientific (Waltham, MA). DPBS (10x) was purchased from Gibco (Thermo Fisher Scientific, Waltham, MA) and Tween20 was purchased from J.T. Baker (Phillipsburg, NJ). Q-Exactive Plus with electrospray ionization (ESI) source was purchased from Thermo Fisher Scientific (Waltham, MA).

Two-Dimensional Liquid Chromatography- Charged Aerosol Detection (CAD)/ Mass Spectrometry (MS) Method to Analyze Polysorbate Degradation.

[0137] The degradation of PS20 and PS80 in CHO cell-free media or formulated antibody were analyzed by two-dimensional HPLC-CAD/ MS method as previously described by Genentech (Yi Li *et al.*, *Characterization and Stability Study of Polysorbate 20 in Therapeutic Monoclonal Antibody Formulation by Multidimensional Ultrahigh-Performance Liquid Chromatography–Charged Aerosol Detection–Mass Spectrometry*, 86 ANALYTICAL CHEMISTRY 5150–5157 (2014)). Polysorbates were first separated from formulated mAb using Oasis MAX column (2.1x20 mm, 30 µm) pre-equilibrated with 99% solvent A (0.1% formic acid in water) and 1% solvent B (0.1% formic acid in acetonitrile). Post sample injection, the equilibration gradient was held for 1 minute, followed by a linear increase of Solvent B to 15% in 4 minutes to separate polysorbate from mAb. The eluted polysorbates were then diverted to Acquity BEH C4 column (2.1x50 mm, 1.7µm) using a switch valve for reversed phase chromatography-based separation. At the start of the separation, solvent B was quickly increased to 20% in 1.5 minutes, then gradually increased to 99% at 45 minutes and held for 5 minutes, followed by an equilibration step of 1%B for 5 minutes. The flow rate was kept at 0.1 mL/min and column temperature at 40°C.

[0138] The 2D-LC system was set up with Thermo UltiMate 3000 and coupled with Corona Ultra CAD detector, operating at nitrogen pressure of 75 psi for quantitation. Chromeleon 7 was used for system control and data analysis. Q-Exactive Plus with ESI source was coupled with

the 2D-LC system for characterization only. The instrument was operated in a positive mode with capillary voltage at 3.8kV, capillary temperature at 350°C, sheath flow rate at 40, and aux flow rate at 10. Full scan spectra were collected over the m/z range of 150-2000. Thermo Xcalibur software was used to collect and analyze MS data.

[0139] Peak area of each ester was obtained from the CAD chromatogram and added up to account for intact PS80. The remaining percentage of PS80 after degradation was calculated by comparing the sum of the peak area of monoester eluting between 25 minutes and 30 minutes at each time point to the sum of peak areas at time zero. Relative percentage of different order ester or total esters can be calculated similarly.

PS80 Degradation Assay with Human CES-1, Rabbit LES and Formulated Antibody.

[0140] The effect of human CES-1 and rabbit LES on PS80 was examined by mixing 2 μ L 0.1 mg/mL human CES-1 or 0.02 mg/mL rabbit LES with 2 μ L 1% PS80 in 16 μ L 10 mM histidine buffer, pH 6.0, followed with incubation at 4°C for 1.5, 8 and 18 hours, respectively. One aliquot (3 μ L) of each solution was diluted 25 times by adding 72 μ L 10 mM histidine, pH 6.0, before the LC-CAD analysis.

[0141] The hydrolysis of PS80 in formulated mAb was examined by mixing 18 μ L 50 mg/mL mAb (buffer exchange to 10 mM histidine, pH 6.0) with 2 μ L 1% PS80 then incubated at 5°C for 18, 24 and 36 hours. One aliquot (3 μ L) of each solution was diluted 25 times with 10mM histidine, pH 6.0 before the LC-CAD analysis.

Inhibition of Lipases from CHO-Derived Antibodies.

[0142] ActivX Desthiobiotin-FP serine hydrolase probe were diluted in DMSO to 0.1 mM as stock solution. Aliquoted 1.25 μ L, 5 μ L and 20 μ L probe stock solution each was mixed with 5 mg mAb in 1 X PBS in a final volume of 1 mL, followed by gentle rotating at room temperature for 1 hour. Each mixture was then buffer exchanged into 10 mM histidine, pH 6.0 to remove the free probes, and the mAb concentration was adjusted to 50 mg/mL. Each buffer exchanged sample was incubated with 0.1% PS80 at 5°C followed by LC-CAD PS80 degradation assay.

Depletion of Lipases from CHO-Derived Antibodies.

[0143] Lipases depletion experiment was performed by using immobilized ActivX Desthiobiotin-FP serine hydrolase probe. To immobilize the probe, 35 μ L ActivX Desthiobiotin-FP serine hydrolase probe (0.1 mM stock solution in DMSO) was first coupled with 2 mg Streptavidin Dynabeads to a final volume of 1 mL in 1 X PBS by gentle rotating at room temperature for 2 hours. Process control sample was prepared by mixing 35 μ L DMSO with 2 mg Streptavidin Dynabeads to a final volume of 1 mL in 1 X and gentle rotating at room temperature for 2 hours. The beads were washed by 1 X PBS 3 times and then resuspended into 800 μ L 1 X PBS. 5 mg mAb sample was then added into the FP probe-coupled Streptavidin Dynabeads and incubated at room temperature with gentle rotation for 1 hour. The supernatant was buffer exchanged into 10 mM histidine, pH 6.0, and the mAb concentration adjusted to 50 mg/mL. The buffer-exchanged supernatant samples were then incubated with 0.1% PS80 at 5°C followed by LC-CAD PS80 degradation assay.

Detection of Host Cell Proteins (HCPs) in CHO-Derived Antibodies with ABPP.

[0144] ActivX Desthiobiotin-FP serine hydrolase probe were diluted in DMSO to 0.1 mM as stock solution. Aliquoted 20 μ L probe stock solution was first mixed with 5 mg mAb in 1 X PBS to a final volume of 1 mL, followed by gentle rotating at room temperature for 1 hour. Free probes were removed by filtration and protein was recovered by 5 M urea in PBS. 2 mg Streptavidin Dynabeads was added to the solution and incubated by gentle rotating at room temperature for 2 hours. After removing the supernatant, Dynabeads were collected by magnet, and washed by 5 M urea in PBS and then resuspended into 5 M urea/50 mM tris solution with 5 mM TCEP. The proteins were denatured and reduced at 55°C for 30 minutes and then incubated with 10 mM iodoacetamide for 30 minutes in dark. Alkylated proteins were diluted 5 times and digested with 1 μ g trypsin at 37°C for overnight. Dynabeads were removed by magnet and the supernatant with peptide mixture was acidified by 5 μ L of 10% FA, desalted using GL-TipTM SDB desalting tip (GL science, Japan) and resuspended into 40 μ L 0.1% FA. 15 μ L were transferred to Eppendorf tubes for Nano LC-MS/MS analysis and the rest were stored at -80°C. Negative control was performed by heating mAb sample at 80°C for 5 minutes first to denature all proteins to prevent host cell proteins from binding to the ActivX Desthiobiotin-FP serine

hydrolase probe.

LC-MS/MS Analysis.

[0145] The peptide mixture was dissolved in 40 μ L of 0.1% formic acid (FA) and 10 μ L was first loaded onto a 20 cm x 0.075 mm Acclaim PepMap 100 C18 trap column (Thermo Fisher Scientific) for desalting and later separated on a 250 mm x 0.075 mm Acclaim PepMap 100 C18 analytical column in an UltiMate 3000 nanoLC (Thermo Fisher Scientific). The mobile phase A was made of 0.1% FA in ultra-pure water and mobile phase B was made of 0.1% FA in 80% ACN. The peptides were separated with a 150 minute linear gradient of 2%-32% of buffer B at flow rate of 300 nL/min. The UltiMate 3000 nanoLC was coupled with a Q-Exactive HFX mass spectrometer (Thermo Fisher Scientific). The mass spectrometer was operated in the data-dependent mode in which the 10 most intense ions were subjected to higher-energy collisional dissociation (HCD) fragmentation with the normalized collision energy (NCE) 27%, AGC 3e6, max injection time 60 ms for each full MS scan (from m/z 375-1500 with resolution of 120,000) and AGC 1e5, max injection time 60 ms for MS/MS events (from m/z 200-2000 with resolution of 30,000).

mAb-1 Direct Digestion.

[0146] 100 μ g of mAb-1 was dried with speed vacuum, then re-constituted with 20 μ L 8 M urea containing 10 mM DTT. The protein was denatured and reduced at 55°C for 30 minutes, and then incubated with 6 μ L of 50 mg/mL iodoacetamide for 30 minutes in dark. Alkylated protein was digested with 100 μ L 0.1 μ g/ μ L trypsin at 37°C for overnight. The peptide mixture was acidified by 5 μ L of 10% TFA. The sample was diluted to 0.4 μ g/ μ L and 2 μ L was injected onto the column for LC-MS/MS analysis.

PRM analysis of CES-B1L and CES-1L in mAb-1.

[0147] Direct digestion of samples (0.8 μ g) were loaded onto a 20 cm x 0.075 mm Acclaim PepMap 100 C18 trap column (Thermo Fisher Scientific) for desalting and later separated on a 250 mm x 0.075 mm Acclaim PepMap 100 C18 analytical column in an UltiMate 3000 nanoLC (Thermo Fisher Scientific). The column was preequilibrated with 98% mobile phase A (made of

0.1% formic acid in water) and 2% mobile phase B (made of 0.1% formic acid in 80% ACN) at a flow rate of 300 nL/min. Post sample injection a linear gradient from 2% to 37% mobile phase B was applied over 100 minutes to separate the peptides. Mass spectrometry data were acquired by parallel reaction monitoring (PRM) targeting 3 peptides LNVQGD²⁺TK [m/z 437.7351²⁺], AISESGVILVPGLFTK [m/z 815.9744²⁺] and ENHAFVPTVLDGVLLPK [m/z 925.0145²⁺] from CES-1L, 3 peptides APEEILAEK [m/z 500.2715²⁺], DGASEEETNLSK [m/z 640.2861²⁺] and IRDGVLDILGDLTFGIPSVIVSR [m/z 819.1355³⁺] from CES-B1L, and 3 peptides GPSVFPLAPCSR [644.3293²⁺], LLIYDASNRPTGIPAR [586.3283³⁺] and STSESTAALGCLVK [712.3585²⁺] from mAb-1. In all experiments, a full mass spectrum at 120,000 resolution relative to m/z 200 (AGC target 1e6, 60 ms maximum injection time, m/z 350–2000) was followed by time scheduled PRM scans at 30,000 resolution (AGC target 1e5, 100 ms maximum injection time). Higher energy collisional dissociation (HCD) was used with 27eV normalized collision energy and an isolation window of 2 m/z for MS/MS analysis.

Example 1. Polysorbate in mAb Formulation Detected by 2D-LC-CAD/MS

[0148] Polysorbate in formulated mAbs was separated, identified, and quantitated by 2D-LC-CAD/MS following slightly modified method by Yi Li *et al.*, *supra* and Oleg V. Borisov *et al.*, *Toward Understanding Molecular Heterogeneity of Polysorbates by Application of Liquid Chromatography–Mass Spectrometry with Computer-Aided Data Analysis*, 83 ANALYTICAL CHEMISTRY 3934–3942 (2011). The first dimensional LC by Oasis Max column was designed to remove mAb, and the second dimensional reversed phase chromatography was implemented to separate the remaining POE and POE esters based on their fatty acid content and type. The PS80 species eluted in the order of POE, POE isosorbide, POE sorbitan, monoesters, diesters, triesters and tetraesters (FIG. 1, right panel). The structure of each ester was elucidated by mass spectrometry based on the chemical formula of the polymer and dioxolanylium ion generated by in-source fragmentation. FIG. 1 right panel A is the representative total ion current (TIC) chromatogram of PS80 with major peaks labeled, in the eluting order of POE- POE isosorbide- POE sorbitan, POE sorbitan monolinoleate, POE sorbitan monooleate, POE isosorbide monooleate and POE monooleate, POE sorbitan linoleate/oleate diester, POE sorbitan di-oleate, POE isosorbide di-oleate and POE di-oleate, Probably POE isosorbide/POE linoleate/oleate

diester and POE sorbitan mixed trioleate and tetraoleate. It should be noted that peak 8 in figure 1 was labeled as possible POE isosorbide/POE linoleate/oleate diester mixer as the mass spectra were too complicated to interpret. Quantitation of polysorbates was determined by charged aerosol detection (CAD) chromatography analysis (FIG. 1, right panel B).

Example 2. Rapid PS80 Degradation in mAb-1 Formulation

[0149] Rapid PS80 degradation was observed in mAb-1 during storage at 5°C for 36 hours. Significant decreases occurred in peaks eluting between 25 and 30 minutes, representing POE monoesters, *i.e.*, POE sorbitan monolinoleate, POE sorbitan monooleate, POE isosorbide monooleate and POE monooleate, while POEs eluting between 10 and 18 minutes showed significant increases (FIG. 2). There were no changes on POE di-, tri- and tetra-esters eluting between 32-45 minutes. This unique degradation pattern suggests that it is more likely one family of lipase/esterase responsible for PS80 degradation. This family of hydrolases can only degrade the monoester part of PS80 while leaving higher order esters untouched. If more than one type of hydrolase was involved in the degradation, the degradation pattern would be more complex.

Example 3. Inhibition of Lipases by Desthiobiotin- Fluorophosphonate (FP) Probe Results in a Vanished PS80 Degradation

[0150] Because most of the lipases that have been reported to degrade polysorbates belong to the family of serine hydrolases, we carried out inhibition experiments using the FP probe. This experiment enables the identification and distinction of enzymatically active hydrolyses from other inactive hydrolyses either in their zymogen form or with endogenous inhibitors. The rationale of this experiment is that if there is any active serine hydrolase, adding its inhibitor would stop the enzyme from functioning, in our case, degrading PS80. Desthiobiotin-FP probe is one of the commercially available serine hydrolase probes that contain the reactive fluorophosphonate group which forms covalent bond with Ser at the catalytic center of the serine type hydrolase and blocks its enzymatic activity. The inhibition experiment clearly demonstrated that by adding as little as 0.125 μM of the FP probe, the enzymatic activity was completely stopped (FIG. 3). This experiment also demonstrated that only the serine type of lipase is

presented in the formulated drug substance as the desthiobiotin-FP probe is specific to serine hydrolase.

Example 4. Depletion of Lipases by Desthiobiotin-Fluorophosphonate (FP) Probe Results in a Diminished PS80 Degradation in formulated mAb

[0151] We then performed depletion of lipases using immobilized ActivX FP serine hydrolase probe. The biotin part of the probe can be captured and immobilized on Streptavidin surface, allowing enrichment and purification of the captured serine hydrolases. The design of the depletion experiment serves two goals: 1) if PS80 degradation is caused by lipase(s) belonging to the serine hydrolase family, depletion will result in a diminished PS80 degradation; 2) the lipase(s) captured on the Desthiobiotin-FP probe can be further identified by mass spectrometry analysis.

[0152] The depletion experiment was performed as outlined in the Material and Methods section with depletion scheme for mAbs shown in FIG. 4. Desthiobiotin-FP probe was coupled to Streptavidin Dynabeads for depletion of lipases. As shown in FIG. 5, prior to lipase depletion, approximately 44.7% of PS80 monoester degradation in mAb-1 was observed after 18 h incubation at 5°C. Additional 18 h incubation led to complete PS80 monoesters loss. After lipase depletion, less than 8% PS80 degradation was observed after either 18 h or 36 h incubation. The depletion results demonstrated that the lipase(s) that degraded PS80 in mAb-1 was removed by the desthiobiotin-FP probe. To ensure that it is the probe rather than streptavidin magnetic beads that interacted with the lipases, the experiments were performed by introducing a process control sample. The process control sample was produced by mixing mAb-1 with streptavidin magnetic beads only without adding desthiobiotin-FP probe. Approximately 29% and 86% of PS80 degradation in mAb1 was observed after 18 h and 36 h 5°C incubation, respectively, indicating that there were certain non-specific interactions between lipases and magnetic beads. Compared to the FP probe, the lipase removed by the non-specific interactions was significantly less, therefore, the majority of the lipase was removed by specific binding between the immobilized FP probe and lipase.

Example 5. Liver Carboxylesterases were Identified in the FP Probe-Enriched Fraction

from mAb-1

[0153] The host cell proteins captured by the desthiobiotin-FP probe were subject to tryptic digestion and mass spectrometry analysis as described in material and method section. Among the 15 host cell proteins identified (Table 1), CES-B1L and CES-1L were identified for the first time. By comparing with the denatured control sample (Table 2), it was concluded that both proteins were captured by specific binding to the FP probe. CES-1L was only identified in the active form but not in the denatured form suggesting that it was biologically active in mAb-1. CES-B1L was identified in the active form with 13 unique peptides while only 2 unique peptides in the denatured forms, suggesting a small amount of CES-B1L protein was able to bind non-specifically to the magnetic beads, and the results were in line with process control results in the depletion experiment (FIG. 5). Nevertheless, the much higher number of unique peptides identified in the active forms suggests that CES-B1L is also responsible for PS80 degradation. For other identified host cell proteins, most can be easily determined as non-active enzymes as they were found in both conditions with a similar number of unique peptides, for example, cullin-9-like protein and ceruloplasmin. Anionic trypsin-2 was identified in the active form of mAb-1 as it is also a serine protease, however, it can be excluded owing to its function as a protease irrelevant to PS80 degradation. The other two co-captured proteins, actin and annexin, can also be excluded from degrading PS80 due to their lack of enzymatic functions.

[0154] To determine the abundance of the newly identified lipases, CES-B1L and CES-1L, PRM analysis were performed. The concentrations of CES-B1L and CES-1L were determined to be 9.6 and 9.0 ppm, respectively. The relatively low abundance of these two lipases suggests that their enzymatic activity for degrading PS80 is strong.

Table 1. Enriched host cell protein by FP probe identified in native mAb-1

Protein in mAb-1 (native)	Accession #	# Uniq. Peps.
Liver carboxylesterase B-1-like protein	A0A061I7X9	13
Liver carboxylesterase 1-like protein	A0A061IFE2	7
Peroxiredoxin-1	Q9JKY1	7
Cullin-9-like protein	A0A061IMU7	7
Junction plakoglobin	G3HLU9	6
Transthyretin	G3I4M9	6

Glyceraldehyde-3-phosphate dehydrogenase	A0A061IDP2	3
Ceruloplasmin	A0A061INJ7	3
Annexin	G3IG05	3
Anionic Trypsin-2	G3HL18	3
Vitamin D-binding protein	G3IHJ6	3
Ubiquitin-40S ribosomal protein S27a-like protein	A0A061IQ58	3
Desmocollin-2-like protein	A0A061IEG0	2
tyrosine-protein kinase receptor	G3HG67	2
Actin, aortic smooth muscle	G3HQY2	2

Table 2. Enriched host cell protein by FP probe identified in denatured mAb-1

Protein in mAb-1 (denatured)	Accession #	# Uniq. Peps.
Cullin-9-like protein	A0A061IMU7	7
Transthyretin	G3I4M9	4
Ubiquitin-40S ribosomal protein S27a-like protein	A0A061IQ58	3
Glyceraldehyde-3-phosphate dehydrogenase	A0A061IDP2	3
Peroxiredoxin-1	Q9JKY1	3
Vitamin D-binding protein	G3IHJ6	3
Ceruloplasmin	A0A061INJ7	2
tyrosine-protein kinase receptor	G3HG67	2
Desmocollin-2-like protein	A0A061IEG0	2
Liver carboxylesterase B-1-like protein	A0A061I7X9	2
Junction plakoglobin	G3HLU9	1

Example 6. PS80 Degradation Pattern with human Liver Carboxylesterase-1 and Rabbit Liver Esterase

[0155] One common and important practice in HCP analysis is to validate the function of lipase activities. The inhibition and depletion experiments have provided strong evidence that CES-B1L and CES-1L are most likely the lipases responsible for PS80 degradation. However, considering the FP probe that was used is not specific to a single protein but to a family of proteins, it is possible that other lipase(s) presented in the drug product that may also play a role in the degradation pathway. A spiked-in experiment can offer the essential verification on whether the suspected lipases are the root cause of PS80 degradation. If the spiked-in lipase can generate exactly the same degradation pattern as the endogenous lipase, the identified lipase can then be confirmed as the key element for PS degradation. This confirmation is usually difficult

due to the lack of available active lipases. To further confirm the role of these two newly identified lipases, a BLAST search was performed. The search results suggested commercially available rabbit liver esterase and human liver carboxylesterase 1 are functionally similar with each having 56.0% and 69.7% sequence homology to the first segment of CES-B1L and CES-1L, respectively (FIG. 6D). Both human liver carboxylesterase 1 and rabbit liver esterase were chosen to compare the PS80 degradation pattern as mAb-1. FIG. 6 showed that both human and rabbit liver esterase exhibited an equivalent degradation pattern as that showed in mAb-1 (FIG. 6, A-C). In all three samples, the rapidly degraded components of PS80 were monoesters, which eluted between 25 to 30 minutes, while di-, tri- and tetra-esters which eluted after 32 minutes remained unchanged. The signature degradation pattern experiments by the known lipases verified that CES-B1L and CES-1L were responsible for PS80 degradation in mAb-1.

What is claimed is:

1. A method of depleting lipase from a sample, comprising:

 contacting the sample including lipase with a probe, said probe capable of binding to the lipase to form a complex; and

 separating the complex from the sample to thereby deplete the lipase from the sample
2. The method of claim 1, wherein the sample comprises a protein of interest.
3. The method of claim 1, wherein the sample comprises a polysorbate excipient.
4. The method of claim 3, wherein the polysorbate excipient is selected from polysorbate-20, polysorbate-60, polysorbate-80 or combinations thereof.
5. The method of claim 1, wherein the lipase is liver carboxylesterase – B1-like protein.
6. The method of claim 1, wherein the lipase is liver carboxylesterase – 1-like protein.
7. The method of claim 1, wherein the probe is capable of being linked to a solid support.
8. The method of claim 7, wherein the solid support is agarose beads.
9. The method of claim 7, wherein the solid support is magnetic beads.
10. The method of claim 1, wherein the probe is attached to a solid support using a ligand.
11. The method of claim 10, wherein the ligand can be an indicator, biotin molecule, a modified biotin molecule, a nuclei, a sequence, an epitope tag, an electron poor molecule or an electron rich molecule.
12. The method of claim 1 further comprising recovering the lipase from the complex.
13. A method of a method of purifying a sample having a protein of interest and a lipase, comprising:

contacting the sample with a probe, said probe capable of binding to the lipase to form a complex; and

separating the complex from the sample to thereby purify the protein of interest in the sample.

14. The method of claim 13, wherein the sample comprises a polysorbate excipient.
15. The method of claim 14, wherein the polysorbate excipient is selected from polysorbate-20, polysorbate-60, polysorbate-80 or combinations thereof.
16. The method of claim 13, wherein the lipase is liver carboxylesterase – B1-like protein.
17. The method of claim 13, wherein the lipase is liver carboxylesterase – 1-like protein.
18. The method of claim 13, wherein the probe is capable of being linked to a solid support.
19. The method of claim 18, wherein the solid support is agarose beads.
20. The method of claim 18, wherein the solid support is magnetic beads.
21. The method of claim 13, wherein the probe is attached to a solid support using a ligand.
22. The method of claim 21, wherein the ligand can be an indicator, biotin molecule, a modified biotin molecule, a nuclei, a sequence, an epitope tag, an electron poor molecule or an electron rich molecule.
23. The method of claim 13 further comprising recovering the lipase from the complex.
24. A method of decreasing degradation of polysorbate in a sample, comprising

contacting the sample including lipase and polysorbate with a probe, said probe capable of binding to the lipase to form a complex; and

separating the complex from the sample to thereby decreasing degradation of polysorbate in the sample.

25. The method of claim 24, wherein the sample further comprises a protein of interest.
26. The method of claim 24, wherein the polysorbate is selected from polysorbate-20, polysorbate-60, polysorbate-80 or combinations thereof.
27. The method of claim 24, wherein the lipase is liver carboxylesterase – B1-like protein.
28. The method of claim 24, wherein the lipase is liver carboxylesterase – 1-like protein.
29. The method of claim 24, wherein the probe is capable of being linked to a solid support.
30. The method of claim 29, wherein the solid support is agarose beads.
31. The method of claim 29, wherein the solid support is magnetic beads.
32. The method of claim 24, wherein the probe is attached to a solid support using a ligand.
33. The method of claim 32, wherein the ligand can be an indicator, biotin molecule, a modified biotin molecule, a nuclei, a sequence, an epitope tag, an electron poor molecule or an electron rich molecule.
34. The method of claim 24 further comprising recovering the lipase from the complex.
35. A composition having a protein of interest purified from mammalian cells, surfactant and a residual amount of liver carboxylesterase B-1-like protein, wherein the residual amount of liver carboxylesterase B-1-like protein is less than about 5 ppm.
36. The composition of claim 35, wherein the surfactant is polysorbate 80.
37. The composition of claim 36, wherein the liver carboxylesterase B-1-like protein causes degradation of the polysorbate 80.
38. The composition of claim 35, wherein the composition is a parenteral formulation
39. The composition of claim 36, wherein a concentration of the polysorbate in the composition is about 0.01% w/v to about 0.2% w/v.

40. The composition of claim 35, wherein the protein of interest is selected from a group consisting of a monoclonal antibody, a polyclonal antibody, a bispecific antibody, an antibody fragment and an antibody-drug complex.
41. The composition of claim 35 further comprising one or more pharmaceutically acceptable excipients.
42. The composition of claim 35 further comprising a buffer selected from a group consisting of histidine buffer, citrate buffer, alginate buffer, and arginine buffer.
43. The composition of claim 35 further comprising a tonicity modifier.
44. The composition of claim 35, wherein concentration of the protein of interest is about 20 mg/mL to about 400 mg/mL.
45. A composition having a protein of interest purified from mammalian cells, surfactant and a residual amount of liver carboxylesterase 1-like protein, wherein the residual amount of lysosomal acid lipase is less than about 5 ppm.
46. The composition of claim 45, wherein the surfactant is polysorbate.
47. The composition of claim 46, wherein the surfactant is polysorbate 80.
48. The composition of claim 47, wherein the liver carboxylesterase 1-like protein causes degradation of the polysorbate 80.
49. The composition of claim 46, wherein the composition is a parenteral formulation
50. The composition of claim 46, wherein concentration of the polysorbate in the composition is about 0.01% w/v to about 0.2% w/v.
51. The composition of claim 45, wherein the protein of interest is selected from a group consisting of a monoclonal antibody, a polyclonal antibody, a bispecific antibody, an antibody fragment and antibody-drug complex.

- 52. The composition of claim 45 further comprising one or more pharmaceutically acceptable excipients.
- 53. The composition of claim 45 further comprising a buffer selected from a group consisting of histidine buffer, citrate buffer, alginate buffer, and arginine buffer.
- 54. The composition of claim 45 further comprising a tonicity modifier.
- 55. The composition of claim 45, wherein concentration of the protein of interest is about 20 mg/mL to about 400 mg/mL.