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Polysorbates 20 and 80 Degradation by Group XV Lysosomal Phospholipase A_2 Isomer X1 in Monoclonal Antibody Formulations

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ABSTRACT

Decreases in polysorbate (PS80) content were observed while evaluating the long-term storage stability of Chinese hamster ovary derived, purified monoclonal antibodies. It was determined that polysorbate had been enzymatically degraded; therefore, studies were performed to identify and characterize the protein(s) responsible. Polysorbate degrading activity was enriched from Chinese hamster ovary media leading to the identification of group XV lysosomal phospholipase A_2 isomer X1 ($LPLA_2$) by L shotgun proteomics Recombinant $LPLA_2$ was over expressed, purified, and functional integrity confirmed against a diheptanoyl phosphatidylcholine substrate. Incubation of recombinantly produced $LPLA_2$ with PS20 and PS80 resulted in hydrolysis of PS20 and PS80 monoester but a much slower rate was observed for higher order PS80. Endogenous $LPLA_2$ was detected and quantitated at less than 1 ppm in 3 formulated antibodies while $LPLA_2$ was not detected (or less than 0.1 ppm) in a fourth formulated antibody. Furthermore, antibodies with detectable quantities of endogenous $LPLA_2$ demonstrated polysorbate hydrolysis while in contrast the antibody without detectable $LPLA_2$ did not show polysorbate hydrolysis. Comparison of polysorbate degradation products generated from the formulated antibody and samples of polysorbate incubated with recombinant $LPLA_2$ resulted in similar elution profiles by liquid chromatography–mass spectrometry. These results suggest that $LPLA_2$ may play a key role in polysorbate degradation in some antibody preparations.

Introduction

Biocompatible, nonionic detergents known as polysorbates are frequently included in large molecule formulations to improve protein stability during manufacture, shipment, and storage.[1] Polysorbates 20 and 80, the most commonly used, are composed of fatty acid esters of polyoxyethylene (POE) sorbitan with either the monolaurate or monooleate as a main component, respectively.[2,3] These amphipathic nonionic detergents reduce adherence to surfaces,[4] limit self-association,[5] protect against freeze-thaw,[6] and agitation-induced aggregation.[7,8] However, these surfactants are prone to degradation which can lead to undesirable chemical modification of proteins and diminished protein protection.[9,10]

Polysorbate degradation can occur through different mechanisms including autoxidation, cleavage at the ethylene oxide subunits, and hydrolysis of the fatty acid ester bond.[11] Chemical stability of the detergents can be influenced by hydroperoxide formation and consequently lead to oxidation of proteins.[12,13] In addition, acid and base can hydrolyze the ester bond but proceed through separate and identifiable mechanisms.[14] Hydrolysis of the fatty acid ester bond releases the long-chain fatty acids, and these degradation rates are influenced by temperature, pH, and presence of micelles, oxygen, peroxides, heat, UV light, and metal ions.[15] Furthermore, free fatty esters can form insoluble particulates resulting in visible turbidity of the formulate.[16] Fortunately, proper storage of polysorbate can minimize, if not prevent, many of these degradation processes.

Enzymes identified or isolated from soil flora, fungi, and animals have also demonstrated an ability to hydrolyze polysorbate.[17-21] The lipase, LipHim1, was identified from a metagenomics library constructed from DNA isolated from soil samples and was shown to process Tween 60.[17] Mycobacterial cutinase, a serine esterase, can hydrolyze fatty acid moieties of polysorbates 20, 40, 60, and 80.[18,19] Cutinases are members of the alpha-beta hydrolase fold superfamily which is structurally similar to various lipases, also known to release free fatty acids.[20,21] For example, pancreatic lipases which

Abbreviations used: $rLPLA_2$, recombinant group XV lysosomal phospholipase A_2 isomer X1; PS80, polysorbate 80; PS20, polysorbate 20; CHO, Chinese hamster ovary; LC-MS, liquid chromatography–mass spectrometry; LC-MS/MS, liquid chromatography-tandem mass spectrometry.

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play a critical role toward the breakdown of dietary fat degrade PS20, PS80, CremophorEL, and RH40.[22,23] Loss of mono-oleates by an apparent enzymatic-like mechanism was recently described in an antibody formulation but the enzyme, believed to be a lipase, was not identified.[14] Degradation of polysorbate by chemical and physical mechanisms in protein formulations has been thoroughly examined; however, enzymatic degradation of polysorbate is less understood, and the enzyme(s) involved have not been isolated and identified.

During standard development storage stability studies, enzymatic hydrolysis of polysorbate was observed using a formulated monoclonal antibody purified from a Chinese hamster ovary (CHO)-based cell manufacturing process. The enzymatic activity was enriched from CHO media, and the isolated proteins subjected to a shotgun proteomic approach identifying the presence of group XV phospholipase A_2 isomer X1. Recombinant group XV lysosomal phospholipase A_2 isomer X1 ($LPLA_2$) was expressed, purified, and shown to hydrolyze both PS20 and PS80 in a concentration and time-dependent manner. In addition, endogenous $LPLA_2$ was identified in 3 purified antibodies that demonstrated polysorbate degradation while an antibody without detectable $LPLA_2$ did not show polysorbate hydrolysis.

Materials and Methods

Materials

Phospholipase A_2 assay kit was purchased from Abcam, Cambridge, MA and nickel-nitrilotriacetic acid Superflow resin was purchased from Qiagen (Hilden, Germany). Diethylaminoethyl Sepharose FF, SP Sepharose HP, and Superdex 75 resin were obtained from GE Healthcare Bio-Sciences (Uppsala, Sweden). Imidazole, sodium chloride, and trizma (Tris) were purchased from Sigma Chemical Company (St. Louis, MO). Amicon centrifugal filters were purchased from Millipore Corporation (Billerica, MA). Polysorbates 80 and 20 were purchased from T.J. Baker (Phillipsburg, NJ). Lysyl endopeptidase was from Wako Chemicals (Richmond, VA), and sequencing grade modified trypsin from Promega (Madison, WI). HPLC grade acetonitrile and water were purchased from Honeywell (Morristown, NJ) and trifluoroacetic acid (TFA) from Thermo Scientific (Pittsburgh, PA). All monoclonal antibodies were prepared at Eli Lilly and Company (Indianapolis, IN). The PLRS reversed phase column was purchased from Varian (Palo Alto, CA) and the Acquity ultra performance liquid chromatography (UPLC) BEH C18 column purchased from Waters (Milford, MA). SeeBlue Plus2 prestained molecular weight standards were from Life Technologies (Grand Island, NY).

Liquid Chromatography–Mass Spectrometry Assay to Detect Polysorbate Degradation

Polysorbate 20 (PS20) or 80 (PS80) degradation was analyzed by liquid chromatography–mass spectrometry (LC-MS) injecting 1 μL of undiluted solution or 5 μL of diluted solution. A Waters Acquity UPLC coupled to Thermo Fisher Velos Orbitrap Elite mass spectrometer was applied for all stability sample analysis. Separations were performed on a Varian PLRS reversed-phase column (1 × 50 mm, 1000Å, 5 μm) at 80°C using 0.05% TFA in water as mobile phase A and 0.04% TFA in acetonitrile as mobile phase B. The column was equilibrated at 8% mobile phase B for 1 min, linearly increased from 8% to 100% in 14 min, held at 100% for 1.5 min then reequilibrated at 8% mobile phase B. Eluting peaks separated at 0.2 mL/min between 2 and 16.5 min for the stability samples without antibody or between 2 and 4.5 min and between 6.2 and 16.5 min for the samples with antibody were analyzed using an electrospray ionization (ESI) source operating at positive model, scan range of 160-2000 amu, Fourier transform mass spectrometry resolution at 120,000, spray voltage of 4.0 kV, capillary temperature of 275°C, sheath gas of 40°C, and source induce collision of 50 V.

PS20 or PS80 in the antibody samples was quantitated by LC-MS based on the understanding that dioxalanylium ion intensities are proportional to the amount of intact polysorbate. Peak area of each POE sorbitan fatty acid ester was obtained from the extracted ion chromatogram of its dioxalanylium ion and added to give the peak area of intact PS20 or PS80. The relative percent of intact PS20 or PS80 was calculated by comparing its peak area with that of the sample at the time zero (100%) or the control sample (only buffer and PS20 or PS80) at each time point (100%). Relative percent of each individual fatty acid ester or different order ester, such as monoester or higher order ester (di or more ester), could be calculated similarly.

Stability Samples of PS20 or PS80 in CHO Cell-Free Media or Formulated Antibody

PS20 or PS80 stability samples with CHO cell-free media were prepared by mixing 990 μL of CHO cell-free media flow through from a protein A capture column with 10 μL of 3% PS20 or PS80 in water then incubated at 37°C for 0, 10, 30, 60, 120, 240, 450 min, and 24 h. Samples (100 μL) with PS20 or PS80 were acidified with 5 μL of 5% TFA in water to quench polysorbate hydrolysis before LC/MS analysis. Aliquots (990 μL) of antibody 1, 155 mg/mL, in 10 mM citrate, 150 mM NaCl buffer, pH 6, were mixed with 10 μL of 2% PS20 or PS80 in water and aliquots (100 μL) transferred into HPLC glass vials. The vials were incubated at 37°C for 0, 1, 2, 4, 7, 14, 28 days then stored at −70°C until LC/MS analysis.

Enrichment of Polysorbate Degrading Activity From CHO Cell-Free Media

Clarified CHO cell culture medium from an antibody-producing cell line was charged using an AKTA avant 150 onto a 72-mL Mab Select protein A column preequilibrated in 50-mM Tris, pH 8.0. Flow through (120 mL) was collected, diluted approximately 2-fold using 20-mM Tris (pH 7.0), and loaded onto a 20-mL diethylaminoethyl Sepharose FF column at 5 mL/min. The column was washed with 2 column volumes of 20-mM Tris (pH 7.0) containing 50-mM NaCl and protein eluted using a 5%-35% 20-mM Tris (pH 7.0) containing 1-M NaCl linear gradient over 30 column volumes run at 5 mL/min while collecting 14 mL fractions. Fractions that contained peak activity, as determined using an LC-MS method, were pooled, the pH lowered to 5.0 using 20-mM citrate (pH 3.0), and the sample allowed to sit at room temperature for 1 h. The turbid sample was clarified using a 0.22-μm filter and diluted approximately 2-fold with 20-mM Na acetate (pH 5.0) to lower conductivity. This was loaded onto a 15-mL SP Sepharose FF column at 10 mL/min and the flow through collected. Tris base (1 M) was added to the flow through pool to raise the pH to 7.3, diluted 2-fold with 20-mM Tris (pH 7.0) to lower conductivity, then loaded onto an 8-mL MonoQ column at 2.5 mL/min. The column was washed using 2 column volumes of 20-mM Tris (pH 7.0) containing 50-mM NaCl and protein eluted using a 5%-35% 20-mM Tris (pH 7.0) containing 1-M NaCl linear gradient over 30 column volumes run at 2.5 mL/min while collecting 8 mL fractions. Fractions which contained peak activity were concentrated to 100 μL using an Amicon-10 centrifugal filter. This was loaded onto a Superdex 200 (10/300) column run in 20-mM Tris (pH 8.0) containing 250-mM NaCl at 0.50 mL/min collecting 1-mL fractions. Fractions containing activity were pooled, aliquoted, and stored at −70°C.