# 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

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**ABSTRACT:** An observation of visible particulate matter was made during formulation development of a mAb and investigations initiated to understand the colloidal instability of the formulation. It was observed that there was a loss of polysorbate in the IgG formulation and concurrent hydrolysis of polysorbate 80 (PS80) into fatty acid, polyethylene glycol (PEG), and pegylated sorbitan in the presence of mAb A. This observation was confirmed with two other mAb development programs (mAb B and mAb C) that used PS80 and formed particulate, but was absent in any placebo sample tested. Comparative analysis to acid, base and esterase hydrolysis, and exposure to the oxidation reagents Iron(II) and *tert*-Butyl hydroperoxide demonstrates that the observed reaction is reproduced by a biologic (enzymatic) mechanism. Monooleates of PS80, including the sorbitan and PEG oleates, are hydrolyzed first, showing a slower reaction with higher-order oleates. This leads to a change in the composition of the formulation over time where PS85 becomes the predominant component of the original surfactant remaining in solution. Data suggest that there is a lipid-specific mechanism rather than a general biologic hydrolysis mechanism that hydrolyzes oleate esters of PS80 increasing the risk that colloidal IgG particles will form in mAb drug product. © 2014 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 103:2268–2277, 2014

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## INTRODUCTION

Using surfactant in large molecule formulations is the current *de facto* way to stabilize the large molecule for the prevention of adherence to surfaces, avoidance of turnover at the air–water interface, and limiting self-association events that would otherwise lead to aggregation. The interaction of proteins and surfactants has become a field unto itself because of the increasing value of biopharmaceuticals.[1–6] The most common surfactants used are polysorbate 20 and 80 (PS20 and PS80), respectively, each having advantages and disadvantages to use: decreased surface turnover of protein,[1,2] prevention of protein self-association,[4,5] filter adsorption,[7] color, critical micelle concentration value, heterogeneity,[8–10] and real or perceived impurities in the raw material.[11–15] All of these issues add time and cost to drug product development programs and in turn the eventual cost of a biopharmaceutical.

Autooxidation and residual manufacturing impurities, including hydroperoxide and reactive aldehyde/ketone, are known risks to the development of drug products that can be tested for in raw materials.[12,13] Issues brought about by the use of polysorbate that has not been properly handled, which leads to possible accelerated degradation as a result of using higher concentrations of surfactant in a formulation, are not addressed here. It is assumed that formulation development uses at minimum polysorbate that meets EU, USP, and JP specification and has been stored and used properly during scientific studies.

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Of lesser known outcome is the stability of polysorbates after the compounding process, fill-finish manufacturing and final real-time stability over the shelf-life of the drug product.[2,16] Reported here is the observation and initial characterization of the ester hydrolysis of PS80 by a biologic-based mechanism, similar in appearance to a proposed lipase enzyme mechanism.[17–21] The mechanism appears to be unique to biopharmaceuticals, being different from acid and base-catalyzed hydrolysis, and is not representative of reaction with Iron(II) or hydroperoxide in solution. A 12-week study indicated that the hydrolysis of PS80 positively correlates to IgG concentration and the percentage of hydrolysis negatively correlates with increasing PS80 concentration.

The appearance of visible protein particulate matter in varying amounts led to testing that revealed the degradation of PS80 in some instances. The PS80 in solution is first chemically altered to be more representative of PS85, an emulsifier, followed by slower hydrolysis of the higher-ordered oleates to a complete loss of polysorbate material and the continued accumulation of hydrolysis products: fatty acids, polyethylene glycol (PEG), and pegylated sorbitan (PEG–sorbitan). One hypothesis of the origin of the effect in this case is the putative presence of triacylglycerol lipase[17–24] (EC 3.1.1.3), a ubiquitous eukaryotic and prokaryotic enzyme commonly called tweenase,[24] as a small but significant portion of residual host cell protein (HCP) in high-concentration drug products. At low mAb concentration, the actual quantity of HCP that contains tweenase in a formulation may be low, leading to no observation of appreciable loss of PS80 during real-time analysis. At high-protein concentration, the actual ng/mL concentration of a tweenase molecule may become significant as a portion of total HCP in the drug product, and the hydrolysis of the oleate ester becomes

appreciable, with the hypothesized risk for aggregate formation increased. Multiple modes of aggregation may be involved related to the products of the surfactant hydrolysis; however, the best path to avoid the issue of ester hydrolysis and eliminating the risk of degraded surfactant in biopharmaceuticals may be the use or creation of surfactants that do not contain ester bonds.

## MATERIAL AND METHODS

### Materials

All water used in the study is 18MOhm or equivalent. Acetonitrile, hydrochloric acid, trifluoroacetic acid (TFA), and PS80 (Crillet 4 HP stored at 2°C–8°C) were produced by JTBaker (Phillipsburg, New Jersey). Methanol was obtained from Alfa Aesar (Ward Hill, New Jersey). Histidine hydrochloride was produced by EMD (Gibbstown, New Jersey). Sodium hydroxide, porcine liver esterase, lipase immobilized on polystyrene beads (Fluka), PEG monooleate, and ammonium Iron(II) sulfate hexahydrate were produced by Sigma–Aldrich (St. Louis, Missouri). Histidine was produced by Anjinomoto (Raleigh, North Carolina) and sucrose was produced by Ferrro Pfanstiehl (Waukegan, Illinois). Waters OASIS HLB SPE columns are from Waters Corporation, Milford, Massechusetts and Agilent Zorbax columns are from Agilent Technologies, Santa Clara, California. All mAbs were produced by Janssen Supply Chain, LLC (Horsham, Pennsylvania) using a series of chromatography and filtration steps. All assay reagents were of ACS grade unless otherwise specified, and all formulation components were multicompendial grade.

### Particulate Counting

Samples of mAb A were analyzed on a HIAC Royco Model 9703 Liquid Particle Counting instrument (Beckman Coulter, Brea, California) without dilution of the sample. The instrument was prepared by flushing and testing with 3 × 5 mL of 18 MΩ water to certify the absence of particulate in the instrument. Certification of instrument performance was performed using 3 × 5 mL of 15, 30, and 70 μm polystyrene bead particulate standards at a level of 1000, 250, and 100 particles/mL, respectively, and conformed to a ±25% count range for the designated particle level. Samples were analyzed 3 × 4 mL to obtain an average particle count for each sample in the ≥10 μm and ≥25 μm count level.

### Polysorbate Extraction and Isolation

Samples of polysorbate were separated and isolated using solid phase extraction on Waters Oasis HLB columns, part number WAT094225, in a similar manner as Nayak.[25] Columns were prepared for use by washing with 1.0 mL of the following reagents in order: water, methanol, 5% methanol in water, and lastly water. Samples for extraction were adjusted to a final concentration of 100 μg/mL PS80 and 1.0 mL of sample was drawn through the column. Samples less than 100 μg/mL PS80 had a sufficient volume of sample passed through the column to obtain a 100 μg/mL load on the column. Columns were then washed with 1.0 mL of the following reagents in order: water, 3 M guanidine hydrochloride, and lastly water. Samples were eluted from the columns using 2 × 1.0 mL of acetonitrile and collected in 12 × 75 mm disposable glass tubes. Each sample was dried under a gentle stream of dry nitrogen gas.

Samples were then resuspended by vortex in 1.0 mL of water for 30 s. Dichloromethane was added to each tube (1.0 mL), the tube capped and a vigorous vortex applied for 45–60 s to perform a liquid–liquid extraction. Each sample was then centrifuged for 5 min at 500*g* to obtain two separate layers in the tube. The bottom (dichloromethane) layer was collected in a clean 12 × 75 mm glass tube and dried under a stream of dry nitrogen gas. Once dry, the residue was reconstituted in 100 μL of 5% acetonitrile in water by vortex for 30 s and used for analysis of the PS80 residuals.

### Polysorbate Quality Analysis

The analysis of polysorbate residuals isolated from mAb formulations was accomplished using a Waters 2695 HPLC equipped with a 2995 photodiode array detector and a Polymer Labs PL-2100 (Agilent 1260, Agilent Technologies, Santa Clara, California) evaporative light scattering detector (ELSD) to perform the chromatographic separation and detection. Fractionation of the heterogeneous PS80 was accomplished on either an Agilent Zorbax SB-Cyano 4.6 × 150 mm or an Agilent Zorbax SB-Cyano 4.6 × 50 mm, 3 μ particle column using water with 0.1% TFA as mobile phase A and acetonitrile with 0.1% TFA as mobile phase B (MPB). The 150-mm column uses a two-part linear gradient of 15%–40% MPB over 4 min followed by 40%–90% MPB over the following 45 min at a flow rate of 1.0 mL/min and a temperature of 30°C. The optimized method for the 50 mm column uses a two-part linear gradient of 10%–45% MPB over 4 min followed by 45%–95% MPB over the following 11 min at a flow rate of 1.0 mL/min and a temperature of 30°C. Both columns provide equivalent results, with the optimized method requiring only a total of 24.5 min run time for each sample compared with 70 min for the 150-mm column. UV detection of PS80 is accomplished at 237 nm; the maximum wavelength for the PS80-related response. ELSD detection settings is a nitrogen gas flow of 1.6 SLM, nebulizer temperature of 50°C and evaporator temperature of 50°C.

### Mechanistic Hydrolysis Reactions

Esterase reactions were performed at 1.0 U/mL with dilutions calculated from the actual suspension volume for the specific lot of reagent manufactured on the material label. As an example, a 5000U porcine liver esterase (PN E2884-5KU, lot 129K7010, 154 U/mg) was purchased with a total volume of 0.91 mL affording a suspension with 5.5 U/μL. One microliter of this suspension was suitable to digest a solution of 3% PS80 with a total volume of 5.5 mL. Samples of PS80 with esterase were analyzed directly and the time course of all these reactions was followed using the polysorbate quality analysis method using the Agilent Zorbax SB-Cyano 150 mm column.

Lipase immobilized on polystyrene beads (Fluka PN 73940, lot 1241096) was 2.0 U/mg of beads. Two milliliters of 3% PS80 solution was digested using 1 mg of beads, adjusting the final volume based on the total milligram of beads dispensed. Aliquots of digested PS80 solution were removed and filtered to remove any beads or particulate before analysis. The time course of all these reactions was followed using the polysorbate quality analysis method using the Agilent Zorbax SB Cyano 150 mm column.

Hydrolysis using acid or base was accomplished using 10, 20, or 50 mM of HCl or NaOH, respectively, in 3% PS80 in water at 37°C. Ester hydrolysis by esterase enzyme was accomplished