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Robust quantitation of basic-protein higher-order aggregates using size-exclusion chromatography

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ABSTRACT

Detection of higher-order aggregates (HOA) using size-exclusion chromatography (SEC) was found to be variable for a basic protein, using exposed-silanol or diol-silica-based SEC columns. Preparations of the tetrameric biopharmaceutical enzyme *Erwinia chrysanthemi* L-asparaginase (ErA), which has an isoelectric point of 8.6, were analysed using a diol-silica SEC column. Although the proportions of ErA main peak and octamer species were unaffected, HOA recovery and detection were extremely variable and had poor agreement with an orthogonal measurement technique, analytical ultracentrifugation (AUC). The observation that only HOA was selectively affected by non-specific silanol interactions was unexpected, so alternatives were sought. Coated-silica SEC columns improved the resolution and reproducibility of HOA detection for this alkaline-pI protein, and improved the agreement of HOA with the AUC method. Basic proteins, such as ErA, should be thoroughly evaluated in SEC method development, to ensure that resolution of larger aggregate species is not compromised.

1. Introduction

Size-exclusion chromatography (SEC) is a widely-used technique for the measurement of soluble protein aggregates [1–3], particularly in a biopharmaceutical manufacturing context. In SEC, analyte species are fractionated according to size, due to the relative degree of access any one size species has to the pores in the chromatography matrix. SEC is particularly useful in the study of protein aggregation, as size differences among protein aggregate species (monomer, dimer, etc) usually exist as discrete multiples which are easily resolved during chromatography. SEC is only one technique for measuring the degree of soluble protein aggregation, but it is widely used, as it is more convenient than many alternative techniques such as analytical ultracentrifugation (AUC), field-flow fractionation (FFF), light-scattering, fluorescence spectrophotometry and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). However, in addition to acknowledging the relative convenience of SEC, it should be noted that due to the features of available commercial column matrices (e.g., pore size, particle size, etc) SEC is mainly useful for small protein aggregates (ca. 1–50 nm diameter) [4], and not for larger aggregates, such as subvisible particulates (SbVP) in the 2–10 μm range.

Protein aggregates may exist in a range of sizes, from dimeric (two protein units) to trimeric and higher-order aggregate (HOA) forms. Protein aggregates may be transient in nature or may be temporally stable, and can be formed by hydrophobic, electrostatic, and covalent (e.g., disulphide) interactions. The uncertainty around the nature and stability of protein aggregates may lead to difficulties in design of a robust analytical method for measuring them. Measurement and control of the formation of protein aggregates are important, as aggregates may be implicated in the undesired immunogenicity of biopharmaceuticals [5]. Protein aggregates, especially HOA and larger multi-unit aggregates, may present a more ordered array of epitopes, including the possibility of newly-created foreign epitopes, to the immune system. Part of the undesired immune response caused by protein aggregates may include anti-drug antibody (ADA) formation [6]. Therefore, it may be critical to detect, control and understand protein aggregation in biopharmaceuticals.

As a biopharmaceutical product, protein aggregate measurement is important for the enzyme L-asparaginase (EC 3.5.1.1). The biological function of L-asparaginase is to convert L-asparagine to L-aspartic acid. Biopharmaceutical-grade L-asparaginase is available commercially for the treatment of acute lymphoblastic leukaemia (ALL) [7–9]. Clinically, the enzyme deprives circulat-

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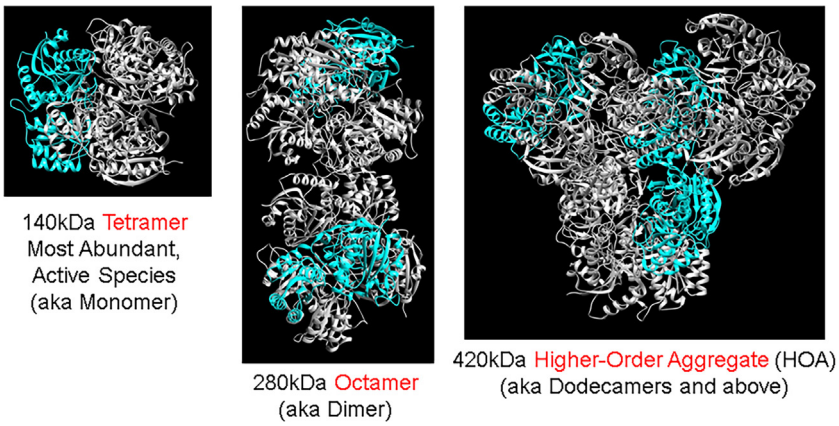

Fig. 1. Schematic Representation of ErA Soluble Aggregates. The images were created with the Chimera molecular modelling software package and PDB entry 1O7J. Note that the interfaces between tetramers in the 280 kDa and 420 kDa aggregates are arbitrary for illustration purposes only.

ing ALL lymphoblasts of L-asparagine, a nutrient essential to ALL cell proliferation. Clinical preparations of L-asparaginase are based on the enzyme from two organisms, *Erwinia chrysanthemi* (ErA) and *Escherichia coli* (EcA) [10]. ErA, marketed as Erwinase® or Erwinaze® [9], is given to those patients who first are treated with EcA, but develop a hypersensitivity reaction. ErA exists as a 140 kDa tetramer composed of four identical 35 kDa subunits. The tetramer is the active form of the enzyme and has a diameter of roughly 84 Å, as determined using the published crystal structures [11–13]. The active tetramer in native form has an alkaline isoelectric point of 8.6, making ErA a basic protein.

The aggregation behaviour of ErA has been studied previously. The propensity of ErA to aggregate after repeated freeze-thaw cycles was described by Jameel and co-workers [14] to be related to dissociation of the tetramer into the 35 kDa subunits, with subsequent cleavage of the subunit leading to aggregation. More recently, the SbVP profile of ErA drug product was studied using flow-imaging microscopy [15] and the presence of large quantities of SbVP was found to have little relation to the clinical manifestation of hypersensitivity.

Recently, the manufacturing process for ErA was studied in detail [16]. During this endeavour, new analytical methods were developed, including a method for aggregate determination by SEC. This SEC method was adequate for the determination of the tetrameric and octameric (Fig. 1) ErA species. However, with increasing manufacturing experience using this SEC method, the quantitation of higher-order aggregates (HOA, three or more ErA tetramers bound together) was found to be extremely variable. The aim of the present study was to develop a robust SEC method for ErA, capable of quantitation of the tetrameric, octameric and HOA species.

2. Materials and methods

All reagents were from Sigma (Dorset, UK) unless otherwise indicated.

2.1. Source of enzyme

Samples of ErA Drug Product (DP) were provided by Porton Biopharma Limited, Porton Down, UK. The composition of ErA DP is approximately 10 mg protein per vial with 0.5 mg sodium chloride and 5 mg glucose monohydrate.

2.2. Protein analyses

The protein content of ErA samples was determined using the UV absorbance at a wavelength of 280 nm and a mass extinction coefficient for ErA of 0.747 $(mg/mL)^{-1}\ cm^{-1}$.

2.3. Size exclusion chromatography of ErA

SEC analyses of ErA were conducted as follows. Vials of lyophilised ErA drug product (DP) were used as the starting material. A vial of ErA DP was reconstituted in sodium phosphate buffer (pH 7.2, 0.1 M) and diluted to the desired protein concentration. Chromatography was conducted on a Waters Alliance 2695 HPLC workstation (Elstree, UK) using either a TSKgel G3000SWXL (7.8 mm x 300 mm, 5 μm, 250 Å) column (Tosoh Bioscience GmbH, Stuttgart, Germany) or a ProSEC 300S (7.5 mm x 300 mm, 5 μm, 300 Å) column (Agilent Technologies, Wokingham, UK). The separation was conducted using isocratic conditions, and the mobile phase consisted of sodium phosphate buffer (pH 7.2; 0.1 M) containing sodium chloride (0.1 M). The flow rate through the column was 0.5 mL/min unless otherwise specified in the text, and the absorbance was monitored at 220 nm or 280 nm. Unless otherwise specified, the ProSEC column injection volume was 20 μL and the injection protein concentration 10 mg/mL.

2.4. Analytical ultracentrifugation (AUC) of ErA

AUC analyses of ErA were conducted at Solvias AG (Basel, Switzerland) as follows. Vials of ErA DP were reconstituted in water to a target 5 mg/mL protein concentration and further diluted to 0.5 mg/mL using phosphate-buffered saline for AUC analysis. AUC was conducted on an Optima XL-A ultracentrifuge (Beckman Coulter, High Wycombe, UK) using a buffer reference. The separation temperature was 22 °C and the monitoring wavelength 280 nm. Each sample was run in triplicate. Data were processed using Sedfit [17].

2.5. Molecular viewing of ErA

Molecular graphics were generated with the UCSF Chimera package [18] and PDB entry 1O7J [12]. Chimera is developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by NIGMS P41-GM103311).