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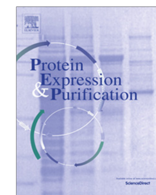

Application of coupled affinity-sizing chromatography for the detection of proteolyzed HSA-tagged proteins

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ABSTRACT

Coupled affinity liquid chromatography and size exclusion chromatography (ALC–SEC) is a technique that has been shown to successfully report product quality of proteins during cell expression and prior to the commencement of downstream processing chromatography steps. This method was applied to monitoring the degradation and subsequent partial remediation of a HSA-tagged protein which showed proteolysis, allowing for rapid cell line development to address this product quality dilemma. This paper outlines the novel application of this method for measuring and addressing protease-induced proteolysis.
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Introduction

Coupled affinity liquid chromatography and size exclusion chromatography (ALC–SEC)[1] was introduced in 2013 [1] as a method to track or observe antibody aggregation in cell culture supernatants. This is a time saving approach; widely accepted methods for protein quality detection such as western blotting of supernatant and analytical size exclusion chromatography (SEC) of purified protein have their drawbacks – they are often not quantitative and time consuming (western blotting) and occur later in the purification process (analytical SEC). Coupled ALC–SEC can avoid some of these issues and provide information regarding protein quality early in the cell culture expression stage of production; strong correlations have been shown between the ALC–SEC reported aggregate content of unpurified material and that of the measured SEC values of the corresponding purified material. For this study, the technique was modified by replacing the affinity column of the ALC–SEC setup with a HSA-Select column rather than the previously characterized Protein A column in order to track the quality of a HSA-tagged small protein.

The HSA-tagged protein, from now on referred to as "Protein X", was found to be clipped post-HSA Select capture, as detected by both analytical SEC and mass spectroscopy analysis. Closer evaluation by mass spectroscopy revealed that the proteolytic site was between an arginine and asparagine residue, separating the HSA tag from the remainder of the protein (Fig. 1). This site was hypothesized to be a possible serine protease cleavage site, and it was thought that by introducing a serine protease inhibitor, aprotinin, the proteolysis would be reduced in the culture, thus preserving the structure of the Protein X. The modification of the ALC–SEC method would allow for the online monitoring of the effectiveness of the protease inhibitor, thereby reporting the quality of the Protein X sooner, allowing for development to continue. In addition, if the proteolysis was detectable in culture, it would rule out harvest and purification approaches as the cause of the protein degradation.

Materials and methods

Affinity liquid chromatography HPLC (ALC–HPLC)

For the affinity HPLC (POROS® CaptureSelect® HSA (Cat. No. 4469151)), mobile phase A (adsorption buffer), consisting of 10 mM sodium phosphate, 150 mM sodium chloride, adjusted to pH 7.5 with 1 M sodium hydroxide, was prepared. Further, mobile phase B (elution buffer) consisting of 10 mM hydrochloric acid, 150 mM sodium chloride, adjusted to pH 2.0 with 1 M hydrochloric acid and mobile phase C consisting of 20% acetic acid were prepared. The method was as follows: 100% mobile phase A 0–0.49 min, 100% mobile phase B 0.50–0.89 min, followed by

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[1] *Abbreviations used:* ALC–SEC, coupled affinity liquid chromatography and size exclusion chromatography; ALC–HPLC, affinity liquid chromatography HPLC; SEC–HPLC, Size-exclusion HPLC; ALC–SEC HPLC, coupled affinity and sizing HPLC; dPBS, Dulbecco's Phosphate Buffered Saline.

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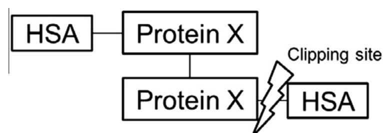

Fig. 1. Schematic showing the site of clipping in HSA-tagged Protein X. The protein naturally occurs as a dimer.

100% mobile phase C from 0.90 to 0.99 min to strip the column of any remaining protein before re-equilibrating the affinity column for the next injection with 100% mobile phase A from 1.00 to 2.50 min at a flow-rate of 2 mL/min. A detection wavelength of 280 nm and a sample injection volume of 50 µL were used for all samples. The column temperature was kept constant at 20 °C and the sample tray was kept cooled at 5 °C to prevent an early fouling of feed samples. For each sequence, the relative area under the peak was integrated using Chromeleon Software version 6.8.

Size-exclusion HPLC (SEC–HPLC)

For the SEC HPLC, mobile phase A (adsorption buffer) consisting of 150 mM potassium phosphate, adjusted to pH 6.5 with phosphoric acid, was prepared. The SEC column was pre-equilibrated at a flow rate of 0.4 mL/min with mobile phase A for 30 min prior to sequence initiation. An isocratic mobile phase elution with a run time of 35 min was used, allowing all of the sample components to elute from the SEC column (TSKGel G3000SWXL Tosoh #808541; 5 µm; 7.8 mm × 300 mm). A detection wavelength of 210 nm and a sample injection volume of 30 µL equivalent to a column load of 5 µg were used for all samples. The column temperature was kept constant at 30 °C and the sample tray was kept cooled at 5 °C. For each sequence, the relative area under the peak was integrated using Chromeleon Software version 6.8.

Coupled affinity and sizing HPLC (ALC–SEC HPLC)

The instrumental set-up for the two columns, HSA-Select and SEC, was performed as follows: the affinity column was connected to the SEC column (Sepax Technologies part number 213300–7815, 7.8 mm × 150 mm) via a capillary with fittings of the following dimensions; 0.17 I.D. mm × 9 cm. After injection of the sample, all unbound material flows through the HSA-Select and SEC columns with mobile phase A (adsorption buffer: 10 mM sodium phosphate, 150 mM sodium chloride, adjusted to pH 7.5 with 1 M sodium hydroxide). Once the last peak corresponding to the unbound material passes through the SEC-column, the mobile phase B (elution buffer: 10 mM hydrochloric acid, 150 mM sodium chloride, adjusted to pH 2.0 with 1 M hydrochloric acid) releases the bound protein from the affinity column and it enters the SEC column. The protein molecules are now separated based on molecular weight and are directed to the UV-detector where the peak can be analyzed. The instrumental method was as follows: 100% mobile phase A 0–4.49 min, 100% mobile phase B 4.50–5.19 min and kept at 100% mobile phase A from 5.20 to 10 min to re-equilibrate the HSA-Select cartridge for the next injection at a general flow-rate of 2 mL/min. Sample injection volumes or loading volumes were adapted to compensate for samples showing lower titer values to produce reliable profiles, with the target amount of 40 µg being loaded onto the column. The column temperature was kept constant at 20 °C and the sample tray was kept cooled at 5 °C. A detection wavelength of 210 nm was used and for each sequence, the relative area under the peak was integrated using Chromeleon Software version 6.8.

Batch binding capture

The harvested supernatants from cell lines expressing HSA-Protein X were purified using microcentrifuge tubes containing 0.2 mL of HSA-Select resin. The resin was equilibrated with 2 mL 1× Dulbecco's Phosphate Buffered Saline (dPBS), and then 1.8 mL of the sterile filtered cell supernatant (clarified harvest) was loaded on the resin. The mixture was left to rotate at room temperature for one hour. The tubes were then centrifuged at 500 g for 1 min to remove the supernatant and fresh 1× dPBS was added. The tubes were mixed by inverting and later centrifuged to remove the supernatant. The wash step with 1× dPBS was repeated and the supernatant was discarded. 500 µL of elution buffer containing 0.2 M glycine–HCl at pH 2.5 was added and the tubes were rotated for 5 min at room temperature. The tubes were then centrifuged and the supernatant containing the released protein was carefully dispensed in 65 µL of neutralization buffer containing 1 M Tris-HCl at pH 9.

Cellular expression of Protein X

A Novartis-proprietary cell line derived from CHOK1 cells was grown in proprietary media supplemented with L-glutamine (Invitrogen). Following expansion in culture flasks the CHO seed culture of 12.5 mL was transferred into 87.5 mL of preheated media to a final volume of 100 mL for a final starting cell density of 0.5×10^6 cells/mL and a cell viability of ~100%. The protease inhibitor aprotinin (Alfa Aesar, product # J63039) was reconstituted in 1× dPBS to a concentration of 1 mg/mL (1,53,583 nM), and added to the CHO cultures for a final concentration 750 nM and 1000 nM (4.9 and 6.9 mg/L, respectively). For the 750 nM and 1000 nM aprotinin cultures, aprotinin was dosed daily. A control culture was also run that had no aprotinin addition. All three conditions were run in triplicate. Culture conditions were maintained at 5% CO2, shaker speed of 120 rotations per minute and a temperature of 36.5 °C for a total of 9 days. Samples were taken daily to monitor viable cell density and viability (NOVA Bioflex). After 9 days, cells were harvested by centrifugation and 0.2 µm filtration.

Discussion

A previous study which introduced the concept of coupled affinity and sizing chromatography [1] highlighted the ability of monitoring antibody aggregation while still in the cell culture phase of development. After early quality data of purified protein samples revealed proteolysis of Protein X (Fig. 2) resulting in only 53% intact protein after affinity capture, the coupled affinity-sizing method was modified to track the quality of the protein while in culture. After replacement of the Protein A column with the HSA-Select column, it was immediately observed that proteolysis was occurring in culture, upstream of process-scale purification (Fig. 3).

After closer analysis of the clip by mass spectroscopy, it was found that the clipping site was located between an arginine and asparagine residue, classic behavior of a serine protease [2]. With that in mind, it was hypothesized that the addition of a serine protease inhibitor, such as aprotinin, would have an effect on the degree of proteolysis. Aprotinin is a well-known protease inhibitor that has historically been used in cardiac surgery to induce platelet agglutination [3–5], but there are limited studies [6,7] that have shown its ability to limit protein degradation in cell culture. With this in mind, a study was designed to monitor the effect of aprotinin in culture in regards to the proteolysis of Protein X, and monitored with the ALC–SEC method for rapid results.

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