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Hydrolysis-Kinetic Study of AEBSF, a Protease Inhibitor Used during Cell-Culture Processing of the HIV-1 Broadly Neutralizing Antibody CAP256-VRC25.26

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Abstract

One approach to mitigate product clipping during HIV mAb CAP256-VRC26.25 cell-culture development is the addition of the protease inhibitor 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF) to the cell-culture media. AEBSF can undergo hydrolysis to form an inactive compound, 4-(2-aminoethyl) benzenesulfonic acid. Using mass-spectrometry detection, a kinetic profile of AEBSF hydrolysis was generated for conditions simulating those of cell culture at pH 7.0 and 37 °C. It was found that increasing the pH or the temperature could accelerate AEBSF hydrolysis. The kinetic-study results in this report provide an analytical characterization and guidance when optimizing an AEBSF-addition strategy for product-clipping control during cell-culture development and offer an alternative approach for AEBSF-related clearance studies post protein production.

Graphical Abstract:

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ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: [10.1021/acs.anal-chem.7b05316](https://doi.org/10.1021/acs.anal-chem.7b05316).
Five-parameter logistic regression equation under different hydrolysis conditions (PDF)

Notes

The authors declare no competing financial interest.

The proteolytic degradation of recombinant proteins is occasionally observed during protein production.^{1–4} During the development of a highly potent, HIV-1 broadly neutralizing monoclonal antibody, CAP256-VRC26.25,⁵ it was detected that this molecule is vulnerable to protease clipping, which significantly reduced the molecular potency. One of the mitigation approaches to reducing clipping involves the addition of a protease inhibitor, specifically 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF),^{1,6} which binds to the protease to form an inactive sulfonyl enzyme derivative.⁷ It was demonstrated that the harvested CAP256-VRC26.25 clipping level was significantly reduced with the addition of AEBSF to the cell culture. However, AEBSF was reported to undergo hydrolysis upon its reaction with hydroxyl ions, yielding an inactive form and diminishing its inhibition activity over time at pHs above 5.⁸ Therefore, understanding the hydrolysis kinetics of AEBSF is important and may provide guidance on AEBSF-supplementation strategies to ensure effective reductions in clipping throughout the durations of cultures. In addition, AEBSF-hydrolyzed-product tracking is reported for the first time in this scientific field and can be monitored in future processes for clearance during purification steps to ensure product purity and safety.

EXPERIMENTAL SECTION

Four vials of 500 μ M 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF) were prepared in 50 mM ammonium bicarbonate solutions, two at pH 7.0 and the other two at pH 8.6. For each pH condition, one tube was incubated at 25 °C, and the other tube was incubated at 37 °C. Aliquots (10 μ L) from each of the four tubes were sampled at the designated time points within 0–17 640 min (12 days). The samples were diluted to 20 μ M using 50% water/acetonitrile (v/v) containing 0.1% formic acid and analyzed immediately by direct infusion into the heated electrospray-ionization source of a Thermo Q-Exactive HF mass spectrometer at flow rate of 10 μ L/min. The mass-detection range was 50–500 Da, the spray voltage was 3.5 kV, and the capillary temperature was 320 °C. Mass Tune software was applied to capture the MS data and the MS/MS data with the collision energy at 35 eV for the precursor ions. To analyze the cell-culture samples with minimal interference in the mass measurement, the harvest was mixed with acetonitrile at the ratio of 1:100 to precipitate the protein components and then was spun down. The supernatant was diluted 10 times using 50% water/acetonitrile (v/v) containing 0.1% formic acid before the mass spectrometry analysis.

RESULTS AND DISCUSSION

AEBSF was incubated at pH 7.0 and 37 °C in 50 mM ammonium bicarbonate buffer, a mass-spectrometry-friendly buffer for making pH adjustments and simulating cell-culture conditions. Mass-spectrometry (MS) data were acquired at different hydrolysis time points in order to monitor the kinetic process. The MS full scan showed three major peaks at 201.063, 202.047, and 204.043 Da (Figure 1a), which were referred to as the 201, 202, and 204 Da peaks, respectively.

Fragment ions of MS/MS spectra confirmed that the peak at 201 Da corresponds to the amine-substituted product of AEBSF, 4-(2-aminoethyl) benzenesulfonamide (Figure 1b); the

peak at 202 Da corresponds to the hydrolysis product of AEBSF, 4-(aminoethyl) benzenesulfonic acid (Figure 1c); and the peak at 204 Da corresponds to AEBSF (Figure 1d). The peaks were identified with MS full scans (Table 1) and the MS/MS spectra.

When hydrolysis was performed at 1440 min (24 h) at pH 7.0 and 37 °C in 50 mM ammonium bicarbonate buffer, it was observed that the AEBSF peak intensity at 204 Da decreased with time, and the intensities of the degraded AEBSF peaks at 201 and 202 Da increased. Figure 2 displayed an overview of the major peaks observed at the designated hydrolysis durations. The overlaid traces of the mass full-scan spectra represented the degradation products and the original form of AEBSF at pH 7.0 and 37 °C for 0, 40, 80, 120, 240, 360, 720, and 1440 min.

To confirm the identity of the degradation product, hydrolysis experiments were also performed under similar conditions at pH 7.0 and 37 °C, using Tris buffer (data not shown). The samples, hydrolyzed with ammonium bicarbonate or Tris buffer, were analyzed using reverse-phase UPLC-UV-MS. For the samples hydrolyzed with ammonium bicarbonate, three peaks at 201, 202, and 204 Da were detected at different retention times. For the Tris-buffer-hydrolyzed samples, only two peaks, 202 and 204 Da, were detected, and no peak at 201 Da was observed. The results further proved that 4-(2-aminoethyl) benzenesulfonamide was the result of an amine-substitution, and only existed when ammonium bicarbonate was present in the hydrolyzing buffer. With similar rates being observed for both the amine- and hydroxyl-substituted AEBSF in aqueous solutions, the amine-substituted peak was ruled out of this kinetic calculation, and only the AEBSF peak at 204 Da and the hydrolyzed-AEBSF peak at 202 Da were selected for hydrolysis-kinetic monitoring. In addition, spot-checking at the half-life time (around 6 h) with reverse-phase UPLC-UV demonstrated the correlation between the hydrolysis-rate results obtained by UV and MS detection (data not shown). It further validates the effectiveness of the kinetic monitoring approach using the two mass-peak intensities. A kinetic profile of AEBSF hydrolysis was generated on the basis of the relative intensities of the peaks at 204 and 202 Da versus time. The result was modeled with a five-parameter logistic regression,⁹ shown in Figure 3, which indicated that the AEBSF-hydrolysis rate increased significantly around 3 h, reached 50% at around 6 h, and had equilibrated by 12 h. The effects of varying the temperature and pH on the rate of AEBSF hydrolysis were also investigated (Figure 4). The half-life, $t_{1/2}$, was determined as the time at which the relative intensity was half that of the initial AEBSF. The $t_{1/2}$ values for the hydrolysis were 141 min at pH 8.6 and 37 °C, 339 min at pH 7.0 and 37 °C, 544 min at pH 8.6 and 25 °C, and 1597 min at pH 7.0 and 25 °C.

After the proof-of-concept kinetic-study design was successfully achieved under cell-culture-like conditions, the next step was to confirm the same AEBSF-degradation pathway in real cell-culture media. To minimize the interference in the mass-spectrometer detection, the proteins from the cell-culture harvest were precipitated with acetonitrile, and the supernatant was analyzed. Neither AEBSF (the starting form) nor its amine-substitution form (from the application of ammonium bicarbonate) was detected in the cell-culture harvests. However, a major fragment ion at 185 Da was detected as the 202 Da precursor ion (the hydrolyzed AEBSF form) in the cell-culture product when AEBSF was added during the cell-culture process (Figure 5). This further proves that the formation of 4-(aminoethyl)

benzenesulfonic acid is the hydrolysis pathway in the real cell-culture process. Thus, the presented AEBSF-degradation kinetic model is supportive of the actual inhibition process that occurs during cell culture.

CONCLUSION

Under cell-culture-like conditions (pH 7.0 and 37 °C) AEBSF is rapidly hydrolyzed, with a half-life around 6 h. The temperature and pH conditions were observed to have significant effects on the rate of AEBSF hydrolysis. Higher pHs and temperatures accelerate the hydrolysis reaction. Therefore, the pH and temperature should be carefully controlled in cell cultures during application of AEBSF. The kinetic profiles in this report provide significant guidance for AEBSF-addition strategies during cell culture to mitigate product clipping. The final AEBSF-addition strategy should also take into account the manufacturing feasibility and resulting product quality.

This study will also benefit purification clearance checks regarding in-process-related impurity monitoring for residual AEBSF and its degradant. It proved that the hydrolyzed AEBSF form would be the major degradation product of AEBSF in cell-culture harvests. After the purification process, monitoring both the residual AEBSF and its hydrolyzed product is required to ensure product safety and purity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(a) MS full-scan spectrum of AEBSF hydrolyzed for 4 h at pH 7.0 and 37 °C showing three major peaks at 201, 202, and 204 Da. The structures were proposed according to the MS/MS spectra for the peaks at (b) 201, (c) 202, and (d) 204 Da.

Figure 2.

Mass-spectra overlay of the hydrolyzed AEBSF samples in ammonium bicarbonate buffer at pH 7.0 and 37 °C at different time points (0, 40, 80, 120, 240, 360, 720, and 1440 min).

Figure 3.

AEBSF-hydrolysis kinetics in cell-culture-like conditions (pH 7.0 and 37 °C). The relative peak intensities at 202 Da (hydrolyzed AEBSF) and 204 Da (AEBSF) from the MS detection have been plotted against time. Details can be found in the Supporting Information (SI).

Figure 4. AEBsf hydrolysis at different pH values and temperatures. $t_{1/2}$ indicates the time at which the intensity is half that of the initial AEBsf. Details can be found in the SI.

Figure 5. MS/MS spectra of the 202 Da precursor ion from the cell-culture-harvest samples. The fragment ion at 185 Da, related to the AEBSF-hydrolysis product 4-(aminoethyl) benzenesulfonic acid, was observed in the cell-culture media (a) when AEBSF was added but was not observed (b) when AEBSF was not added (negative control).

Table 1.
Measured Masses and Proposed Structures for AEBSF and Its Degradation Products from Figure 1

product	AEBSF	hydrolyzed product	amine substitution
formula	$[\text{C}_8\text{H}_{10}\text{FNO}_2\text{S}]^+ \text{H}^+$	$[\text{C}_8\text{H}_{11}\text{NO}_3\text{S}]^+ \text{H}^+$	$[\text{C}_8\text{H}_{12}\text{N}_2\text{O}_2\text{S}]^+ \text{H}^+$
name	4-(aminoethyl) benzenesulfonyl fluoride	4-(aminoethyl) benzenesulfonic acid	4-(aminoethyl) benzenesulfonamide
theoretical $[\text{M} + \text{H}]^+ \text{ (Da)}$	204.050	202.054	201.070
observed $[\text{M} + \text{H}]^+ \text{ (Da)}$	204.043	202.047	201.063