

Monitoring Proteolytic Digestion using the Fluorophore Epicocconone.

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Introduction

Proteolytic digestion prior to MS-analysis for identification of proteins through MALDI PMF or HPLC-ESI-MS is central to contemporary proteomics. Most digests take between 1 and 18 hours to complete. Ensuring digestion is complete and over-digestion is avoided is important before submitting samples for MS-analysis. Failure, partial digestion or over digestion can waste valuable MS-time and make the results difficult to interpret. Methods for monitoring proteolytic digestions include time consuming gel electrophoresis, HPLC or circular dichroism. Epicocconone, a natural product from the fungus *Epicoccum nigrum*, reversibly reacts with proteins to form an internal charge transfer complex that is highly fluorescent in the hydrophobic environment around proteins. This unique staining mechanism prompted an investigation into the use of epicocconone, formulated as a kit called LavaDigest, to tracelessly monitor the proteolysis in real-time.

Methods

A simple, 3-step, real-time method to monitor proteolysis *in situ* using epicocconone was developed. Tryptic digestion of bovine serum albumin (BSA), casein, apo-transferin (ApoT) and carbonic anhydrase (CA) was monitored by adding epicocconone to the reaction at a range of trypsin: protein ratios. The progress of these digestions was monitored using fluorescence and independently verified by SDS-PAGE. Digestion of BSA with different proteases (chymotrypsin, papain Lys-C) was similarly monitored. The results from the epicocconone assay were used to determine the enzyme kinetics of the different proteases, with different proteins and different protein: protease ratio. Samples were analysed by MALDI-MS and Mascot scores to determine if epicocconone interferes with subsequent identification. LavaDigest was applied to monitor the digestion of complex proteomes (eg serum and *E. coli*) and to achieving a consistent partial digestion for peptide mapping.

Results

The results obtained using the epicocconone assay closely correlate with those obtained using 1D-gel electrophoresis for all combinations of proteins and proteases tested. Epicocconone did not interfere with proteolysis by a range of different proteases when compared to proteolysis validated by SDS-PAGE (Fig.1), enables monitoring proteolysis in real-time.

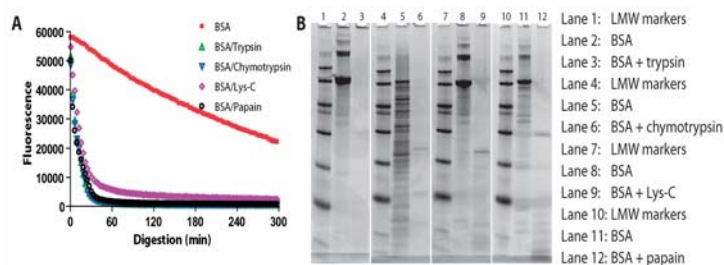


Fig.1. Real-time monitoring of BSA digestion with trypsin, chymotrypsin, Lys-C and papain (A) and SDS-PAGE validation (B).

The epicocconone assay was found to be suitable for the calculation of pseudo-first order rate constants of proteolytic digestion. With varying substrate (BSA) to enzyme (trypsin) ratio it was found that when digestion is complete can easily be identified (Fig. 2). The lines of best fit are single exponential decays showing a good fit to pseudo-first order kinetics for all ratios except 1:30 (green line). In cases where the enzyme concentration approaches the substrate concentration deviation from first order kinetics is observed and a two-phase exponential decay (blue line) better fits the data. The half-life of proteolysis can be determined from the pseudo-first order rate constant (0.69/K).

Rate constants determined from real-time LavaDigest assays were found to vary considerably for different proteins and different enzymes.

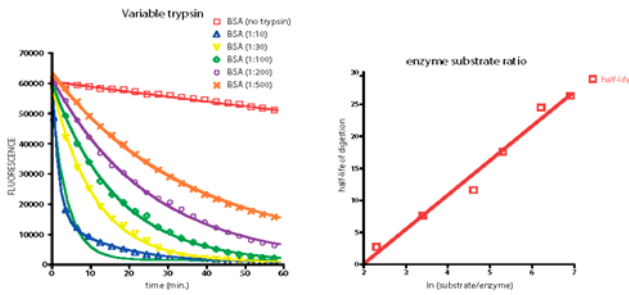


Fig.2. Real time digestion of BSA with different ratios of trypsin (left) progress curves of BSA digested with different ratios of trypsin to substrate (BSA). (B) Shows the plot of reaction half-lives (from A and figure 7 for 1:1000) plotted against the natural log (ln) of the substrate/enzyme ratio. The linear correlation indicates that the progress curves are exponentially related to the enzyme concentration and thus evidence that the observed progress curves follow the rate of hydrolysis of the BSA.

Epicocconone was found not to interfere with PMF and no statistical difference in coverage was observed between samples with or without epicocconone added (table 1). Epicocconone can be used to optimize digestion condition or to reliably identify the stage of digestion facilitating consistent partial digestion of proteins. This may be useful in HPLC-MS-MS peptide mapping of new proteins.

Number of peptides for BSA identification				
Time of Digestion (min)	with LavaDigest	Percent Coverage	without LavaDigest	Percent Coverage
0	0	0	0	0
15	17	39	18	33
30	16	31	22	40
60	19	35	18	35
180	21	38	27	49
360	28	50	24	41
overnight	35	54	46	64

Table 1. MS analysis of the sub-samples of BSA with and without epicocconone solution

Epicocconone was applied to monitoring the digestion of entire proteomes (Fig. 3) and to identify the status of digestion, e.g. partial digestions (Fig. 4).

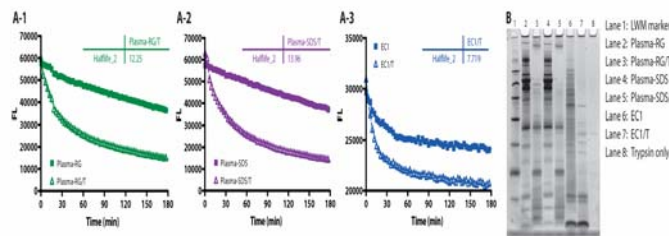


Fig. 3. Kinetics of trypsin-driven hydrolysis of complex proteins: A-1 (with RapiGest) and A-2 (with SDS) for plasma, A-3 for *E.coli* proteome; B, SDS-PAGE validation.

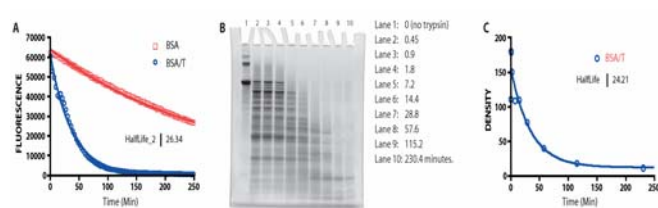


Fig. 4. Use of epicocconone for identify the status of digestion. A, real-time data; B, SDS-PAGE validation; C, kinetics of B. The ratio of trypsin and BSA was 1:1000 in both epicocconone assay (A) and SDS-PAGE validation (B). C represents kinetics derived from SDS-PAGE validation (B).

Conclusion

Epicocconone (LavaDigest) provides a simple traceless approach to monitor tryptic and other proteolytic digestions in real-time that is suitable for defined proteins and entire proteomes. The assay does not interfere with proteolytic activity and is compatible with commonly used chemicals found in protein preparations. The unique features of this method are that it can be used to achieve reliable partial digestions for LC-MS-MS analysis, is fully compatible with mass spectrometry and can be used to calculate the pseudo-first order rate constants for proteolysis. The assay replaces and expensive and time consuming gel electrophoresis, HPLC or CD for validation of tryptic digestion and is suitable for high throughput analyses in 384 well plates.