1. MONITORING PROTEOLYTIC DIGESTIONS IN REAL-TIME

Proteolytic digestion prior to MS analysis has become central to proteomics for identification through MALDI peptide mass fingerprinting or HPLC-ESI-MS. Most in-solution tryptic digests take between 1 and 18 hours to complete, depending on the protein, enzyme activity and reaction conditions (pH, temperature, buffer etc). Ensuring that digestion is complete and over-digestions is not produced is important before submitting samples for PMF or HPLC analysis. Failure or partial digestion can waste valuable instrument time and make results difficult to interpret and over-digestion can produce peptides too small to positively identify. These problems are even more important when translated to high throughput methods. HPLC, circular dichroism, SDS-PAGE and MS have been used to access protein denaturation\(^1\). However, these techniques are slow, may require expensive instrumentation, can be inaccurate and/or are unsuitable for real-time monitoring and not suitable for high throughput. Here we describe the use of a natural fluorophore, epicocconone\(^6\) formulated as a kit called LavaDigest to tracelessly monitor the proteolysis in real-time.

2. METHODS

Proteolysis can be monitored using LavaDigest with a simple 3-step procedure.

1. Digestions were carried out in a 96-well plate incubated in a fluorimeter.
2. Fluorescence was measured at regular intervals, e.g. every 3 minute.
3. Fluorescence decay was plotted, and the kinetics of proteolysis could be determined from the loss of fluorescence.

![Figure 1](image1.png)

Figure 1. A 3-step real-time monitoring of proteolytic digestion using the fluorescent reporter.

3. RESULTS

3.1 Real-time monitoring of digestion of BSA with different ratios of trypsin

Real time assay (Fig. 2) revealed complete digestion of BSA with trypsin can be achieved with variable ratios of protein to enzyme.

![Figure 2](image2.png)

Figure 2. Real-time digestion of BSA with different ratios of trypsin. (A) progress curves of BSA-digested with 1:30 to 1:900 ratios of trypsin to substrate (BSA). 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 any proteolysis can be determined as \(0.693/K\), the pseudo-first order rate constant. (B) Shows the plot of reaction half-lives (from A and figure 8 for 1:1000) plotted against the natural log (\(\log_{10}\)) 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.

3.2 Real-time digestion of BSA with other proteases

LavaDigest was suitable for a range of different proteases tested. These results were validated by SDS-PAGE (Fig. 3B). Protease:protein ratio of 1:30 (w/w) was used.

![Figure 3](image3.png)

Figure 3. Real-time digestion of BSA with different proteases (A) and SDS-PAGE validation of the digests (B).

3.3 Determination of Rate Constants

![Figure 4](image4.png)

Figure 4. Kinetic analysis of proteolytic digestion of proteins using LavaDigest.

Trypsin and Lys-C hydrolysis of BSA (A, E), o-casein (B, F), Apo-transferrin (C, G) and carbonic anhydrase (D, H) under identical conditions except the protein:trypsin ratio was 1:30 and the protein to Lys-C ratio was 1:20.
3.4 MS compatibility

BSA digests with and without LavaDigest were analyzed by MALDI-MS and Mascot (Matrix Science Ltd) scores determined. Sub-samples at all time points were identified as bovine serum albumin (P02769) with sequence coverage from 31-64% (Table 2 and Fig. 5). There was no statistical difference between coverage with or without LavaDigest added to the samples.

<table>
<thead>
<tr>
<th>Number of peptides for BSA identification</th>
<th>With LavaDigest</th>
<th>Without LavaDigest</th>
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<tr>
<td>Time of Digestion (min)</td>
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<td>Percent Coverage</td>
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<tr>
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<td>35</td>
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<tr>
<td>overnight</td>
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</tr>
</tbody>
</table>

Table 1. MS analysis of sub-sampled BSA trypptic digests with and without the inclusion of LavaDigest from time 0 (no trypsin added) to overnight (18 hrs).

3.5 Limit of detection and interfering compounds

Detection limit of LavaDigest in monitoring in-solution trypptic digestion in real-time is 1.5 µM for BSA protein tested. The hydrolysis of the BSA sample with trypsin at a concentration of 0.15 µM (Fig. 6C) was not be detected by LavaDigest assay.

LavaDigest is compatible with chemicals commonly used in in-solution digestion. Table 2 shows acceptable maximum level of chemicals for LavaDigest kit. Human plasma sample (A-1 and 2) was digested with trypsin at a ratio of 80:1 (w/w) and E. coli proteins (A-3) at a ratio of 40:1. For testing compatibility of RapiGest with LavaDigest, RapiGest was mixed with the plasma sample at a final concentration of 0.1% and 10-fold diluted plasma sample used for LavaDigest assay. Inserted is half life of trypsin-driven hydrolysis of each sample tested. B represents independent SDS-PAGE validation of LavaDigest assays tested.

3.6 Applications of LavaDigest

1) Monitoring of digestion of complex proteome samples

LavaDigest can monitor trypsin-driven hydrolysis of complex proteomes as well as pure proteins, e.g. human plasma and E. coli proteome (figure 7).

LavaDigest is also compatible with RapiGest™ SF (Waters) a detergent, used to accelerate trypptic digestions, particularly of insoluble membrane proteins. Using the LavaDigest assay with RapiGest does not accelerate proteolysis of human plasma proteome (Fig. 7A-2), as evidenced by the kinetics of hydrolysis. Independent validation of these results was obtained by SDS-PAGE (Fig. 7B, lane 3).

2) Use of LavaDigest for achieving a partial digestion

LavaDigest can be used to reliably identify the stage of digestion enabling researchers to arrest proteolysis at a consistent, pre-defined level. Figure 8A shows a time course of trypsin-driven hydrolysis of BSA measured by LavaDigest (A) and SDS-PAGE (B and C). The half life measured by both techniques was around 25 minutes, demonstrating that LavaDigest can be used to reliably identify the stage of digestion.

4. CONCLUSION

LavaDigest is based on epicocconone, a fluorophore that reacts reversibly with proteins to form an internal charge transfer complex by a novel mechanism that is highly fluorescent in the hydrophobic environment around proteins1. The unique mechanism allows epicocconone to follow the rate of proteolysis in real time without interfering with the hydrolysis reaction. The rate of trypptic digestion of pure protein, complex proteomes can be followed and the samples are suitable for downstream processing such as PMF. The limit of detection is 1.5 µM protein for BSA.

LavaDigest:

- Provides a simple traceless approach to monitor trypctic and other proteolytic digestions in real-time
- Is suitable for defined and complex protein samples tested
- Does not interfere with proteolytic activity and is compatible with commonly used chemicals (including RapiGest™ SF) for In-solution digestion
- Can be used to achieve reliable partial digestions for LC-MS-MS analysis
- Is fully compatible with MS analysis
- Replaces expensive/time consuming gel electrophoresis, HPLC or CD for validation of trypptic digestion
- Is suitable for high throughput analyses in 384 well plates
- Can be used to calculate pseudo-first order rate constants of proteolytic digestion on the actual substrate of interest rather than a model substrate

5. REFERENCE


*Lava is a registered trademark of Fluorotechnics

www.fluorotechnics.com.au