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Non-Denaturating Separation and Detection of Proteases by LC-Post-Column Reaction UV/vis

Background:

Proteases are important catalysts used in industrial processes. Furthermore, they are potential pharmaceuticals and are applied for diagnostic purposes. Online methods that have been established for inhibitor screening combine the separation of substances and detection of their biological activity in one experimental step [1]. This rapid and accurate technique is therefore a promising screening tool for proteolytic activities in biological samples like snake venom or bacteria.

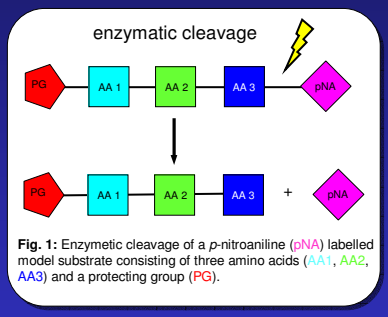


Fig. 1: Enzymetic cleavage of a *p*-nitroaniline (pNA) labelled model substrate consisting of three amino acids (AA1, AA2, AA3) and a protecting group (PG).

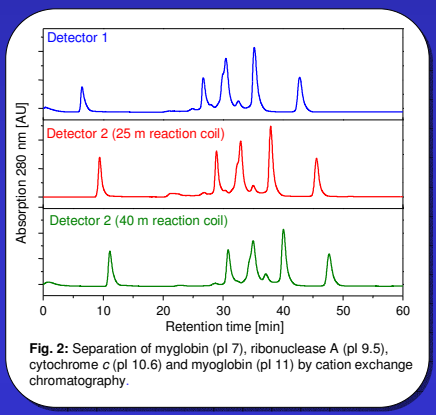
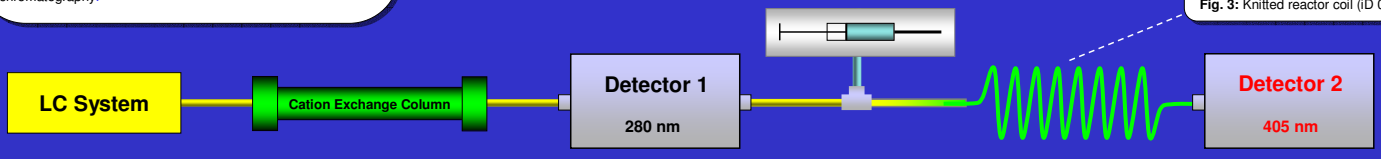


Fig. 2: Separation of myglobin (pI 7), ribonuclease A (pI 9.5), cytochrome *c* (pI 10.6) and myoglobin (pI 11) by cation exchange chromatography.

Approach:

- Non-denaturating separation of proteins by ion exchange chromatography (IEC) followed by UV-detection (detector 1) .
- Post-column addition of *p*-nitroaniline (pNA)-labelled substrate (Fig. 1) to enzyme fractions eluting from the IEC column.
- Reaction of eluting enzymes and added substrate in the capillary.
- Knitted capillary (Fig. 3) allows several minutes of reaction time, causing only moderate peak broadening (Fig. 2) [2] .
- Monitoring of released pNA (Fig. 1) in detector 2 (Fig. 5-6).

Fig. 3: Knitted reactor coil (iD 0.25 mm).



Results:

- Excellent separation of a mixture of different model proteases by IEC.
- Successful detection of the eluting proteases based on enzymatic activity.
- Increased sensitivity of the biological assay compared to direct UV-detection (280 nm), e.g. 100 times for trypsin (Fig. 5).
- Identification of active fractions by correlating both detector signals.
- Semi-quantitative determination of enzymatic activity owing to steady-state of the reaction in the flow-system.
- Characterisation of substrate specificity, using different substrates.

Example: Separation of trypsin, chymotrypsin, and pronase E by a weak cation exchange column (75 x 4.6 mm) in phosphate buffer 20 mM pH 7.5, using a linear gradient from 0 to 0.5 M NaCl at a flow rate of 0.5 mL/min in 60 min. 1 mL/h of the substrate solution (1 mM) was delivered from the syringe pump.

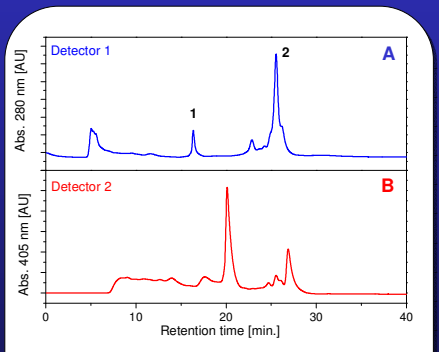


Figure 5: Separation of trypsin (1) and chymotrypsin (2). Chromatogram A shows the signal of detector 1 at 280 nm (120 U trypsin, 2.3 U chymotrypsin). Chromatogram B is the signal of detector 2 at the end of the 25 m reaction coil at 405 nm (1.2 U trypsin, 2.3 U chymotrypsin). In contrast to direct UV-detection of trypsin (Chromatogram A), this biological assay provides singnificantly lower LODs.

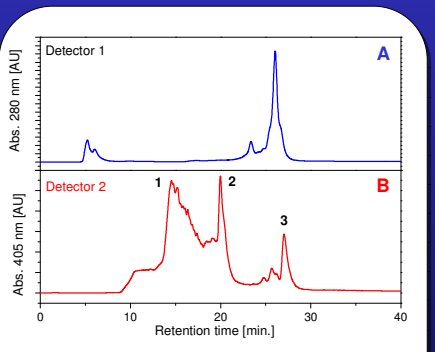


Figure 6: Separation of pronase A (1), trypsin (2) and chymotrypsin (3). Chromatogram A is the signal of detector 1, where the signal of trypsin (1.1 U) and pronase (0.01 U) is below LOD, only the peak of chymotrypsin (2,3 U) is clearly observed. Chromatogram B shows the signal of detector 2 at 405 nm of the same run.

Outlook:

- Application of the screening method to snake venom.
- Use of SEC and anion exchange chromatography as separation techniques.

Literature:

[1] C. F de Jong, R. J. Derks, B. Bruyneel, W.Niessen, and H. Irth, (2006) J Chromatogr. A **1112,** 303-310.

[2] H. Engelhardt and B. Lillig, (1985) J. High Resol. Chromatogr. **8,** 531-534.

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