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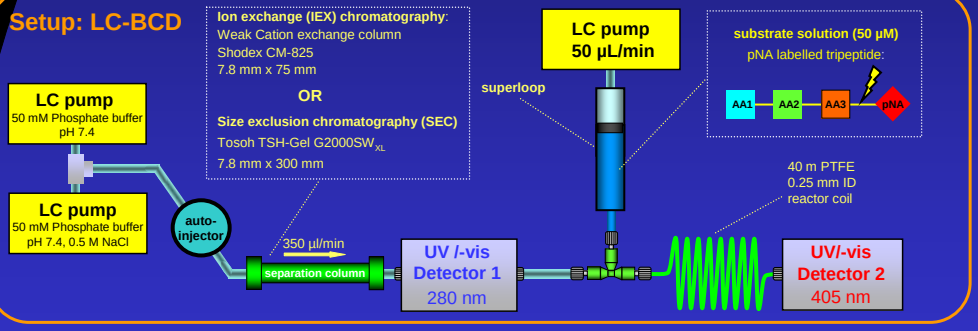
Screening for Proteases by means of LC with Biochemical Detection (BCD)

Background:

Proteases are important catalysts used in industry and are involved in many regulatory physiological reactions. Prominent examples are well characterized proteases like thrombin and coagulation factors, playing a key role in the regulation of blood coagulation. Other proteases are of clinical importance as well, e.g. characteristic protease activity in tissue can be used as a cancer biomarker or an increase in the proteolytic activity of serine-protease in the digestive system can be correlated to the irritable bowel syndrome (IBS). On this poster, we present a new methodology to screen for protease activities. This is based on a continuous flow assay serving as BCD, known from inhibitor-screening methods [1, Poster T-LB-13]. This rapid and accurate technique is a promising screening tool for the analysis of proteolytic activities in complex biological mixtures as shown for snake venom and amoeba cultures.

Setup: LC-BCD



Approach:

- Non-denaturing separation of proteins by ion exchange (IEX) chromatography
- Post-column addition of *p*-nitroaniline (pNA)-labeled tripeptides as substrates to the eluate
- Reaction of eluting enzymes and added substrate in the capillary
- A knotted capillary allows 5.0 minutes of reaction time while causing only moderate peak broadening
- The monitoring of released pNA by detector 2 serves as BCD for proteolytic activity (Fig.1)
- The substrate for BCD is identified by an improved multisubstrate flow-injection (FIA)-MS/MS assay [2] detecting the conversion of 8 tripeptides in parallel (Fig.2)

Characterization of the BCD signal

- FIA-analysis of trypsin and chymotrypsin

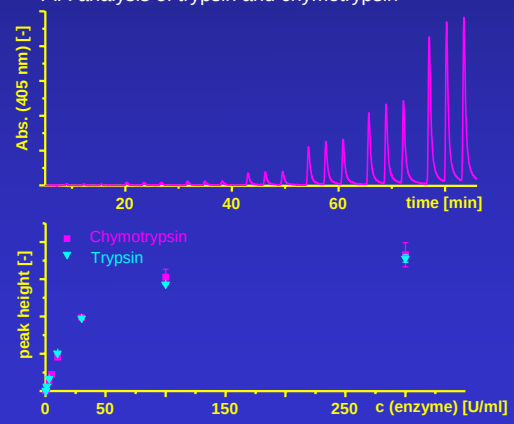


Fig. 1: BCD signal (substrate VII) for trypsin and chymotrypsin of tripple injections (50 µL) in flow-injection analysis (FIA) mode. Top: Triple injections (50 µL) of 0, 0.1, 0.3, 1, 3, 5, 10 U/mL and the corresponding signal curve (bottom).

- Saturated signal curve due to complete conversion
- Lineare range: 0.3 – 30 U/mL (R>0.99)
- LOD 0.1 U/mL → 0.5 ng protein injected for trypsin
  → Similar results after IEX separation (not shown)

FIA-MS/MS multisubstrate assay

- **Aim:** Finding of a tripeptide suitable as substrate for LC-BCD for unknown proteases:
  Approach: Incubation of a the sample with a multisubstrate mixture and monitoring of the reaction by FIA-MS/MS

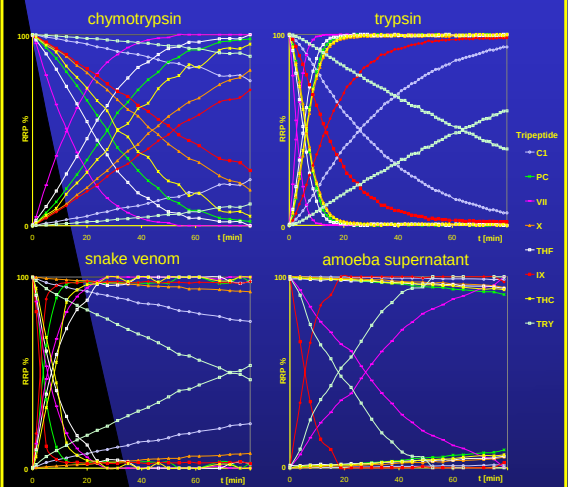


Fig. 2: Substrate specificity pattern of chymotrypsin, trypsin, the crude venom of *Bothrops moojeni* venom and supernatant of *Acanthamoeba castellanii*. 50 µl sample protease solution was added to 950 µl multisubstrate solution (50 µM) and incubated at room temperature. Aliquotes (5 µL) were periodically analyzed by FIA-MS/MS. Shown is the relative reaction progress (RRP%) for substrate consumption and product formation (RRP% = $signal_{product}$ / ($signal_{product}$ + $signal_{substrate}$) x 100)

- Broad activity pattern for trypsin, chymotrypsin and snake venom

→ Low specificity of pNA-tripeptides for proteases

- Characterization of the protease only possibly by their activity pattern

LC-BCD screening for proteases in snake venom and amoebe culture

- Crude *Bothrops moojeni* venom and supernatant of *acanthamoeba castellanii* cultures was screened with the developed LC-BCD method (Fig.3)

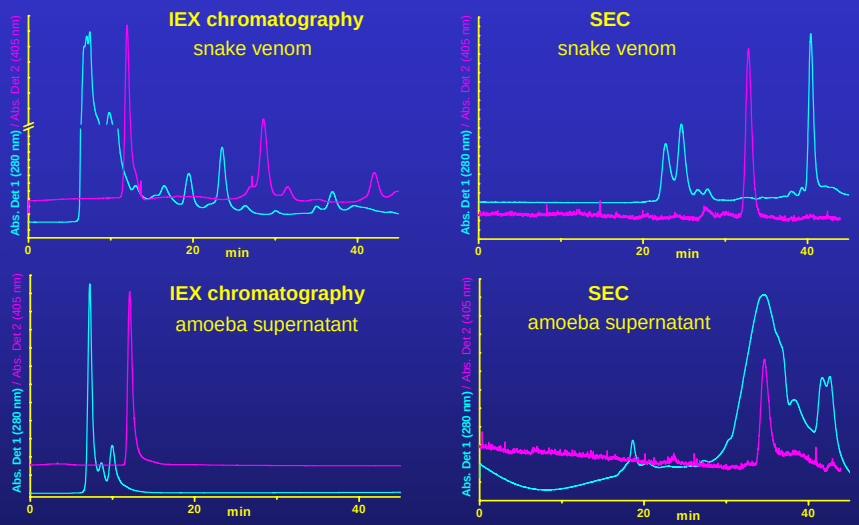


Fig. 3: LC-BCD screening of snake venom (top) and amoeba supernatant (bottom) for proteolytic activity. The tripeptide IX was used as substrate which was converted most rapidly in FIA-MS/MS assay (Fig. 2). **On the left** the non-denaturing separation is carried out on a cation exchange column applying a gradient from 0 to 0.5 mM NaCl. **On the right** SEC was used for separation running isocratically with phosphate buffer containing 0.3 mM NaCl. The cyane line shows the unspecific monitoring of eluting proteins by UV detector 1 at 280 nm, whereas the magenta curve is the signal of UV/-vis detector 2 representing the proteolytic activity.

- Direct identification of active fractions in snake venom and amoeba extract after IEX and SEC separation by BCD signal (Fig.3)
- SEC of both samples show one distinct activity peak at molecular weight of about 30-40 kD
- Amoeba protease is not retained in IEX, whereas snake venom samples shows 3 activity peaks
  → Active fractions can be collected for further characterisation of the active enzymes

Literature:

[1] N.H. Schebb, F. Heus, T. Saenger, U. Karst, H. Irth, J. Kool (2008) Anal Chem, in press.
[2] A. Liesener and U. Karst (2005) Analyst 130, 850-854.

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