Protein Mixtures
Using Two-Dimensional Chromatography and Ion Trap MS

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Introduction

Studying proteomes is of central interest in life sciences, because expression and degradation of proteins are highly dynamic processes, which admit cells to adapt to changing conditions like drugs, temperature changes etc. In this respect, e.g. the discovery of proteins with unknown functionality or the investigation of metabolic pathways are main topics.

Proteins with extremes in pH and molecular mass, low abundance proteins and membrane associated proteins are rarely resolved by 2D PAGE. Moreover, this procedure is highly demanding and time-consuming.

The classic approach to study proteomes is to separate proteins by 2D gel electrophoresis, to excise spots, to cleave the proteins enzymatically and to investigate the peptides by mass spectrometry. However, proteins with extremes in pH and molecular mass, low abundance proteins and membrane associated proteins are rarely resolved by 2D PAGE. Moreover, this procedure is highly demanding and time-consuming.

As an alternative separation technique, we used 2D liquid chromatography prior to mass spectrometric investigation. Ribosomal proteins from *Azoarcus*-like strain EbN1 served as a model system of limited complexity (<200 proteins). The digested proteins were separated in two dimensions. One digested non-bacterial protein (Enolase, *baker's* yeast) was added to an aliquot of the ribosomal protein mixture, and both samples were analysed by electrospray ion trap analysis. This approach focused on the identification of the non-bacterial protein and to show an instrumental setup for promising proceeding analyses.

Experimental

Sample preparation: The denitrifying bacterium *Azoarcus*-like strain EbN1 (b-Proteobacteria) has been isolated from anoxic freshwater mud sampled in Bremen, Germany (Rabus and Widdel 1995, "Anaerobic degradation of ethylbenzene and other aromatic hydrocarbons by new denitrifying bacteria". Arch Microbiol. 163:96-103). Strain EbN1 was grown in an ascorbate-reduced medium with nitrate (10 mM) and benzoate (4 mM) (Rabus and Widdel 1995). Cells were harvested during the exponential growth phase as described before and stored at −80°C.

Ribosomal proteins from strain EbN1 were disrupted with the PlusOne Sample Grinding Kit (Amersham, Freiburg, Germany), and cell debris was removed by centrifugation (30 000 × g, 30 min). The supernatant was subjected to an ultra-centrifugation at 250 000 × g for 2 h. The pellet was resuspended and further purified by centrifugation through a sucrose cushion (19 h, 900 000 × g) yielding in a pellet of highly enriched ribosomes. To remove ribosomal RNA proteins were extracted with phenol. The purified ribosomal proteins thus obtained were reduced with DTT and alkylated with vinylpyridine prior to digestion with trypsin (Roche) following standard protocols. The other protein digests were performed according to standard in-solution digest protocols.

2D HPLC

Ion-exchange chromatography: The digested protein mixture was bound to a strong cation exchange column (BioX-SCX 500 µm, 15 mm) at pH 2.5. Salt plugs of increasing concentrations were injected applying a step gradient, which makes MS-data sets easier to handle during post-processing. Nine peptide fractions were eluted (Microflow, 30 µl/min with 125 µm capillaries) for further separation by RP-chromatography.

Trapping peptides and desalting: The trapping column was used for concentrating and desalting the eluate from the IEX. The use of microflow (30 µl/min with 125 µm capillaries) allowed fast salt steps in the first dimension, and large sample volumes. When

Figure 1: Mascot search result of the 11 protein mixtures. All proteins were identified unambiguously.

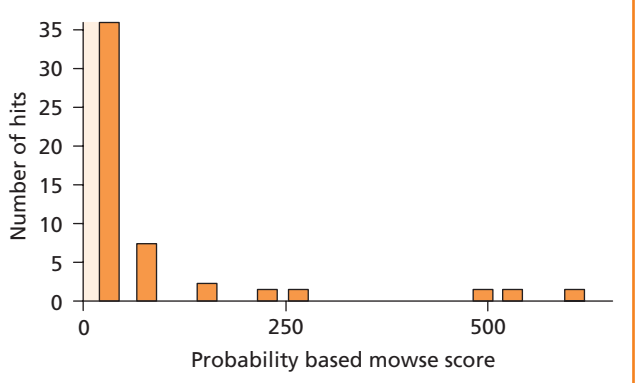
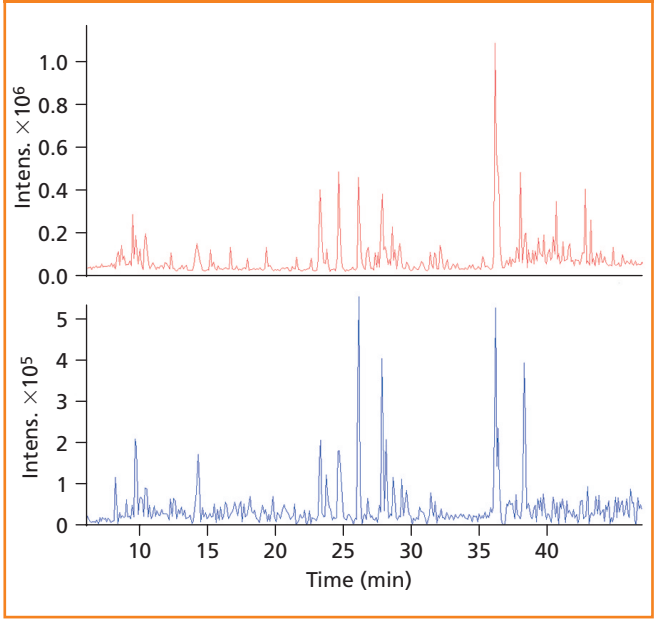


Figure 2: Ultrasensitive Analyses: base peak chromatogram and total ion chromatogram MS(n)-trace of 1 fmol enolase (gradient time 30 min, standard RP-column).



the valves change their position the trapping column gets in line with the RP-column. The gradient flushed the peptides from the trapping column onto the RP-column. The IEX column was disconnected from the loading flow, until the next salt injection.

RP-chromatography

Nanoflow:	200 nl/min, 20-30 µm capillaries
Column:	PepMap™ C18, 75 µm, 15 cm (LC-Packings)

1-D HPLC

Nanoflow:	200 nl/min, 20-30 µm capillaries
Column:	PepMap™ C18, 75 µm, 15 cm (LC-Packings)
Capillary flow:	2.5 µl/min,
Column:	PS-DVB monolithic columns, 200 µm ID, 5 cm, 60 °C (LC-Packings)

ESI MS: esquire$_{3000}^{plus}$

The ribosomal protein LC-MS/MS data were acquired on an esquire$_{3000}^{plus}$ (Bruker) with a Bruker on-line nanospray source (inner needle tip diameter 10 µm, New Objective; stainless steel union). HyStar™ 2.3 was used to control 2D chromatography and data acquisition by esquire 5.1™. The data were processed using DataAnalysis 3.1™ and BioTools 2.2™.

ESI MS: esquire$_{HCT}$

The in-solution digest LCMSMS data were acquired on an esquire$_{HCT}$ (High Capacity Trap).

Results

Comparing complex protein mixtures

2D HPLC MS-data were acquired for two samples:

1. ribosomal proteins from EbN1
2. ribosomal proteins from EbN1 plus 100 fmol yeast enolase.

A software tool which was used to compare the Total Ion Chromatograms for each salt concentration (both samples) extracted a list of masses which was used as a Preferred Mass List for setting up a subsequent LC-MS/MS run (ribosomal proteins plus enolase) in the esquire™ software. The added enolase could be identified unambiguously.

Outlook

To further speed up analysis time, the use of fast monolithic RP-columns can be an option. However, the MS instrument must have a duty cycle fast enough to cope with coeluting peptides showing peak widths of 3-4 sec. This is given by the esquire$_{HCT}$ with its fast scan speed (ultrascan, 26000 u/sec) allowing MSMS cycle times of 0.7 sec (Figure 1). For very sensitive measurements with high resolution and accuracy the slightly slower peptidescan™ (8100 u/sec for MS, 26000 u/sec for MSMS) shows better results (Figure 2).

As 2D-chromatography of complex mixtures requires a high dynamic range, the improved ion storage capacity of the esquire$_{HCT}$ in combination with an intelligent spectra dependant ion charge control (smart-ICC™) appears to be particularly beneficial.

Summary/Conclusion

MS-chromatogram traces of two medium complex protein mixtures were compared. Submitting the resulting masses to MS/MS-fragmentation the additional protein was identified unambiguously. Further, an instrumental setup is presented which promises a significant progress in the analysis of complex protein mixtures due to high speed and sensitivity.

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