Isolation, Purification and Characterisation of Proteins from Plants

In vivo study of proteins
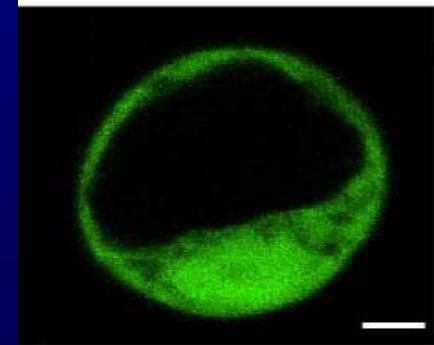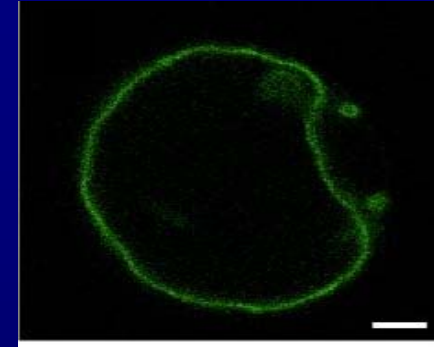
Cellular localization of protein

Activity e.g. detection of metal transport by specific fluorescent dyes or patch clamping
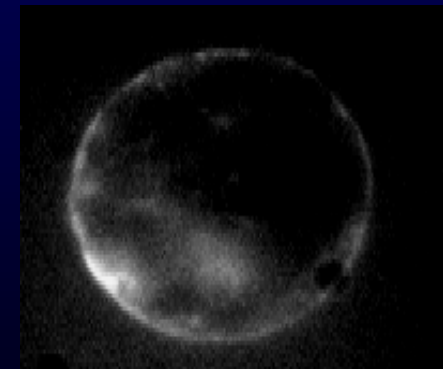
Limitations :
No structural determination

No analysis of binding constant

No analysis of catalytic mechanism

Kobae et al., 2004, PlantCell Physiol 45, 1749-58

Leitenmaier B, Küpper H (2011) Plant Cell & Environment 34, 208-219

I) Protein Isolation: Sources (expression) of plant proteins

Native expression (i.e. in intact organs or in tissue culture)

➢ Advantages:
- Correct folding
- Correct post-translational processing (e.g. insertion of active centre, phosphorylation, glycosylation, cleavage, ...)
- No cloning needed

➢ Disadvantages
- Usually lower yield
- Often many similar proteins in the same organisms → difficulties with purification

root freezing in liquid nitrogen and hydroponic plant cultivation in Küpper lab

I) Protein Isolation: Sources (expression) of plant proteins

Heterologous expression (e.g. in bacteria, yeast or insect cells)

➢ Advantages:
  - Usually higher yield
  - Specific expression of one protein in large amounts and possibly with specific tag (e.g. His-tag) facilitates purification

➢ Disadvantages
  - Often problems with folding, in particular in the case of large proteins and integral membrane proteins
  - Insertion of complicated cofactors (e.g. iron-sulfur clusters) is difficult or impossible, other post-translational modifications may be missing or different compared to the native protein → possibly wrong conclusions about functions/mechanisms

I) Protein Isolation: Methods for isolation

Grinding in liquid nitrogen

➢ Used for hard tissues and cells like roots, stems, but also for hard-walled cells like some algae and cyanobacteria

➢ Low temperature protects the proteins during grinding

➢ Time consuming (manual grinding) or requiring suitable machinery (expensive solution: lab mill, several thousand €, cheap solution: wheat mill, about 200 €)


Liquid nitrogen cooled wheat mill in Küpper lab

I) Protein Isolation: Methods for isolation

Ultrasound homogenization ... Can cause artefacts of denaturation

Many peaks of metalloproteins were strongly reduced and/or moved to low MW, showing severe protein denaturation, already by „gentle" ultrasound treatment of protein extracts

Küpper H, Hussain Bokhari SN, Jaime Perez N, Lyubenova L, Ashraf N, Andresen E (2019) Analytical Chemistry 91, 1710961-10969
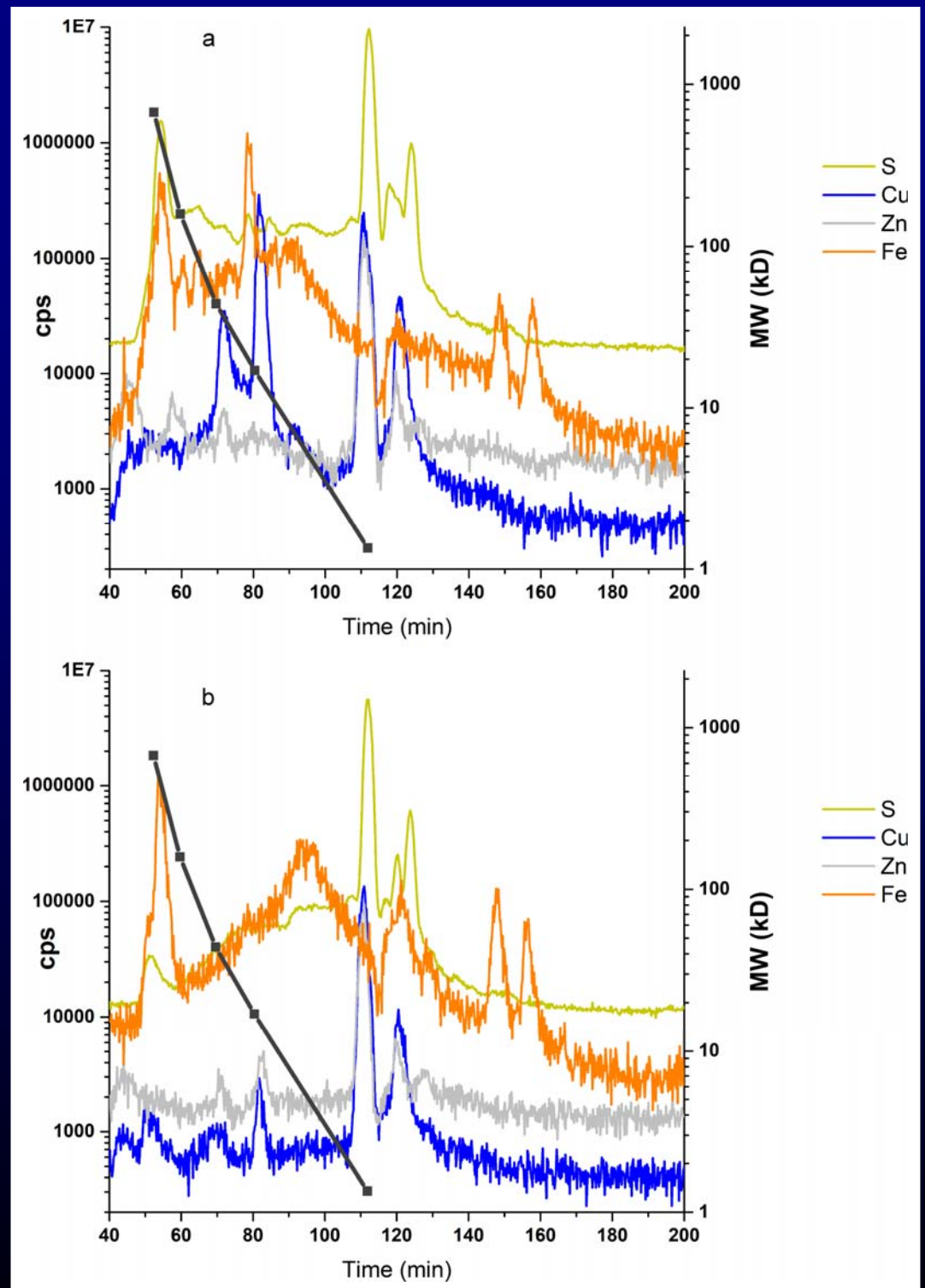
I) Protein Isolation: Methods for isolation

French Press

➢ Used for individual cells (plant cell culture, algae or bacteria) without or with soft walls
➢ Does not require freezing and thus may avoid artefacts of freezing
➢ Requires very expensive (usually many thousand €) machinery

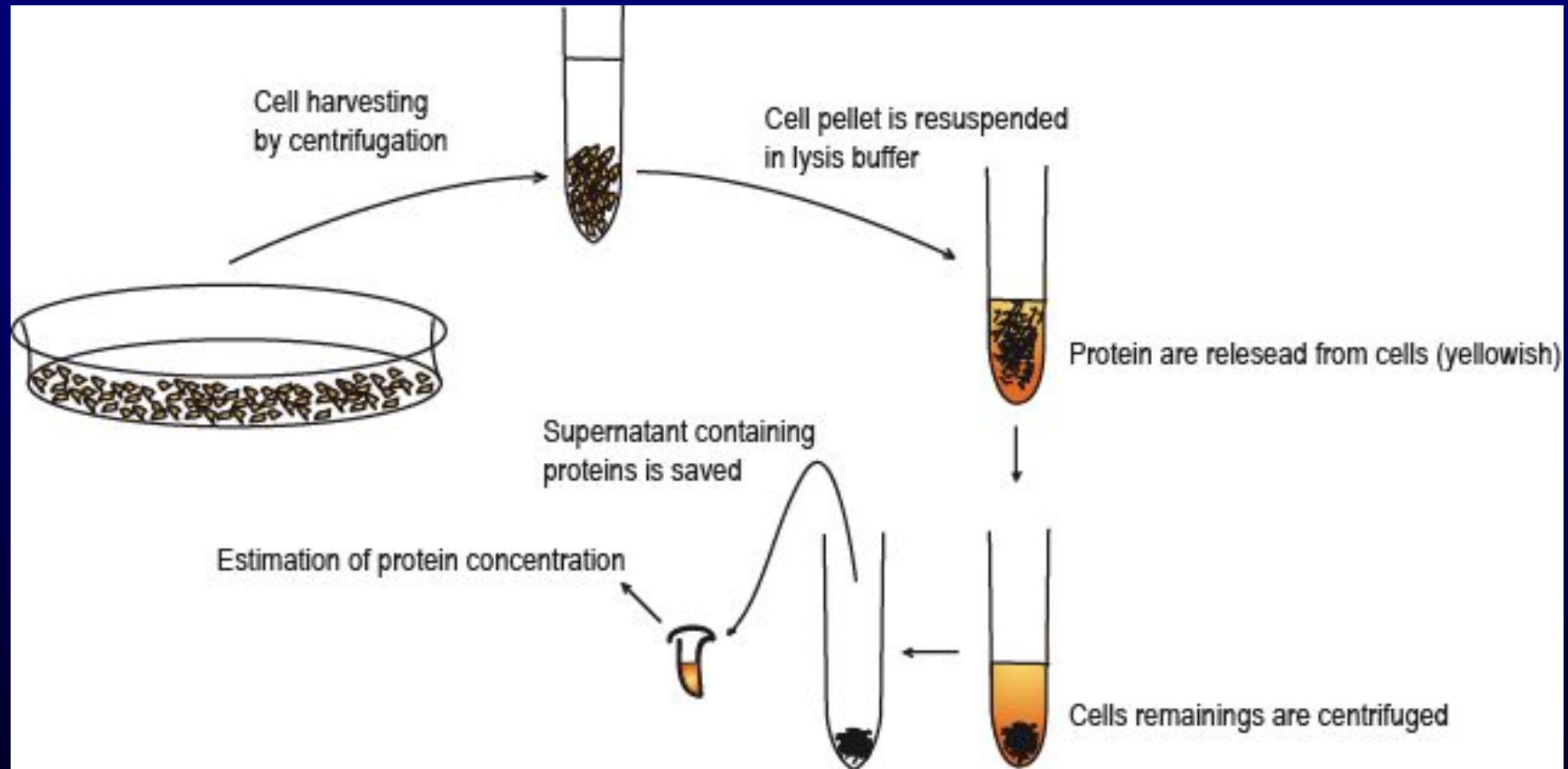

From: www.diversified-equipment.com

From: www.pegasusscientific.com

I) Protein Isolation: Methods for isolation

Lysis buffer

➢ Used only for bacteria or animal cells
➢ May cause degradation
➢ No machinery needed

From: bio-ggs.blogspot.com/2009/11/ggs-live-western...

II) Protein Purification: Overview of Principles

Separation by expression site
- Selective use of tissues or organelles
- Separation of soluble from membrane proteins by centrifugation

Separation by size
- Ultrafiltration
- Size exclusion chromatography
- Preparative native gel electrophoresis

Separation by charge
- Ion exchange chromatography
- Isoelectric focussing (as chromatography, in solution or in gel electrophoresis)

Separation by specific binding sites
- Metal affinity chromatography using natural or artificial metal binding sites
- Immuno-Chromatography using immobilised antibodies
- Magnetic separation using magnetically tagged antibodies

II) Protein Purification: Separation by size in native gels

➢ Principle: Small proteins are less retained by the fibers of the gel than large proteins, so that small proteins migrate **faster**
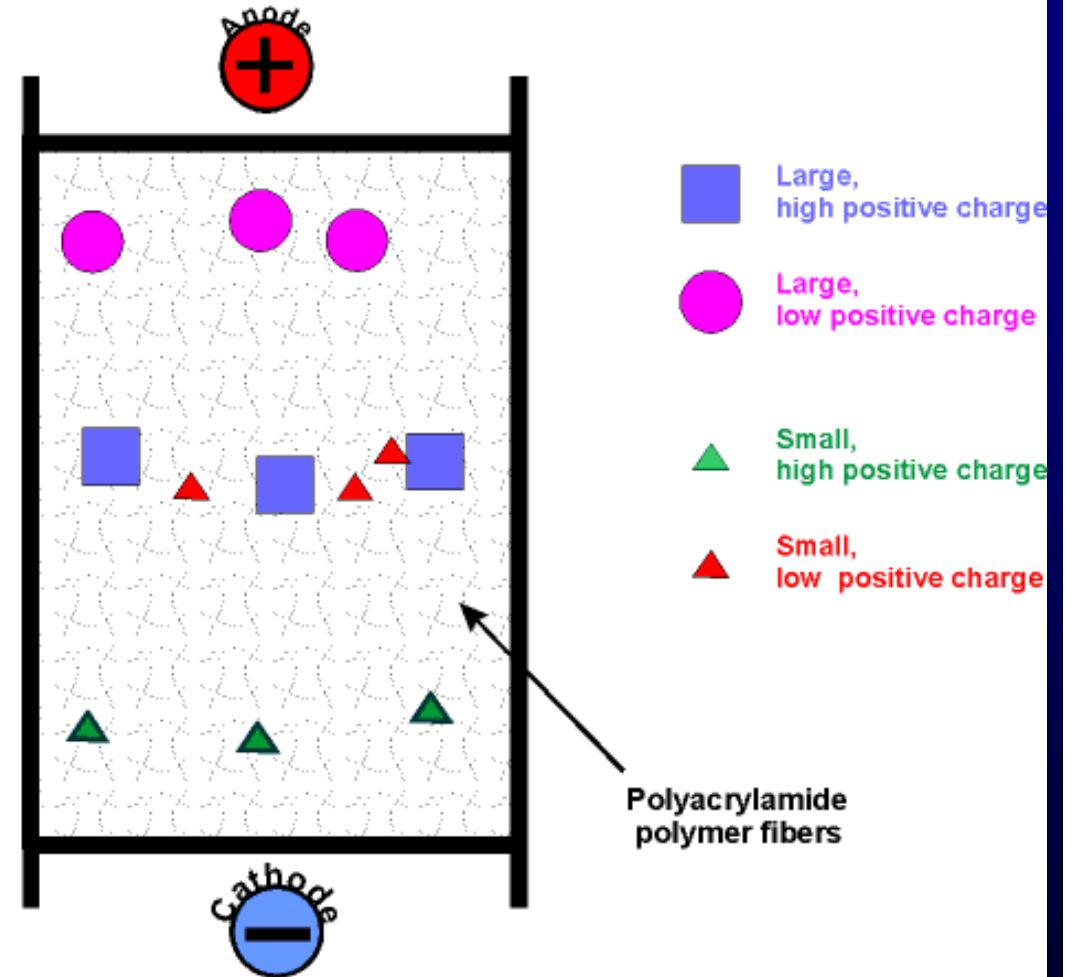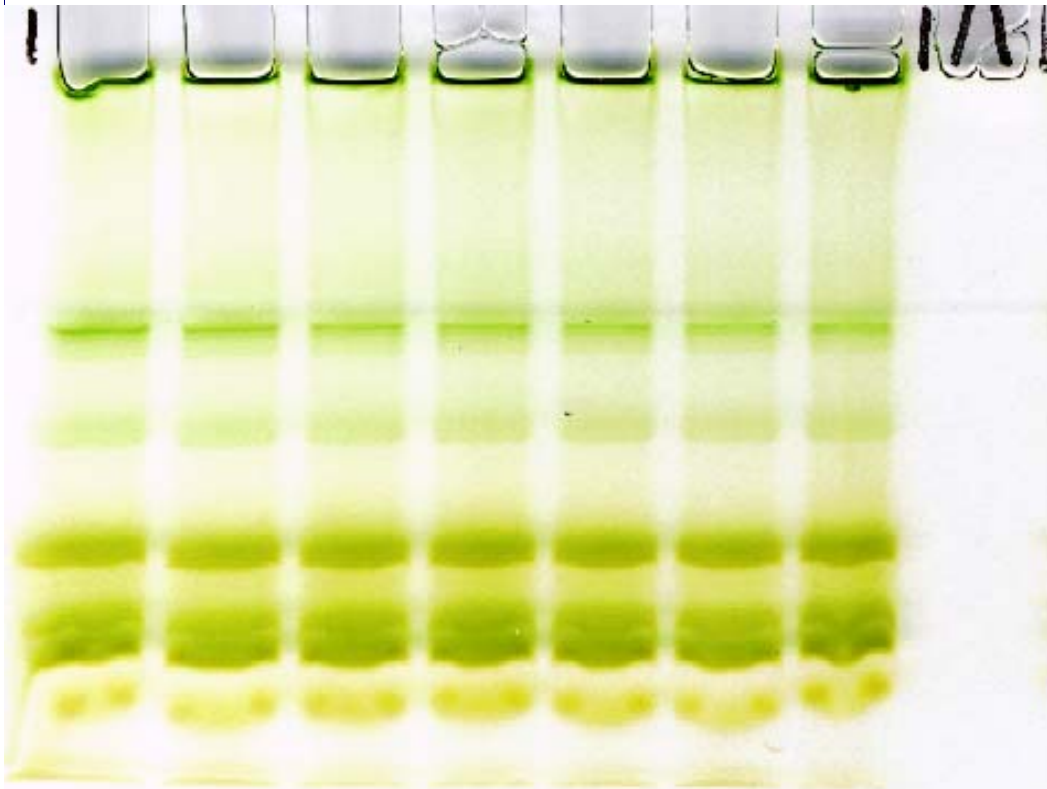

foto of a green gel (Küpper et al., 2003, Funct Plant Biol)

From: www.columbia.edu/.../c2005/lectures/lec6_09.html

III) Protein Identification: Overview of Principles

Size determination
➢ Size exclusion chromatography or SDS PAGE
➢ Comparison with expected size of protein (known e.g. from reference or cDNA)

Western Blotting
➢ Binding to specific primary antibody, detected via labelled or enzymatically active secondary antibody

Biochemical assays in native gels
➢ Identification of enzymes by their characteristic activity
➢ Identification of metalloproteins by their metal content
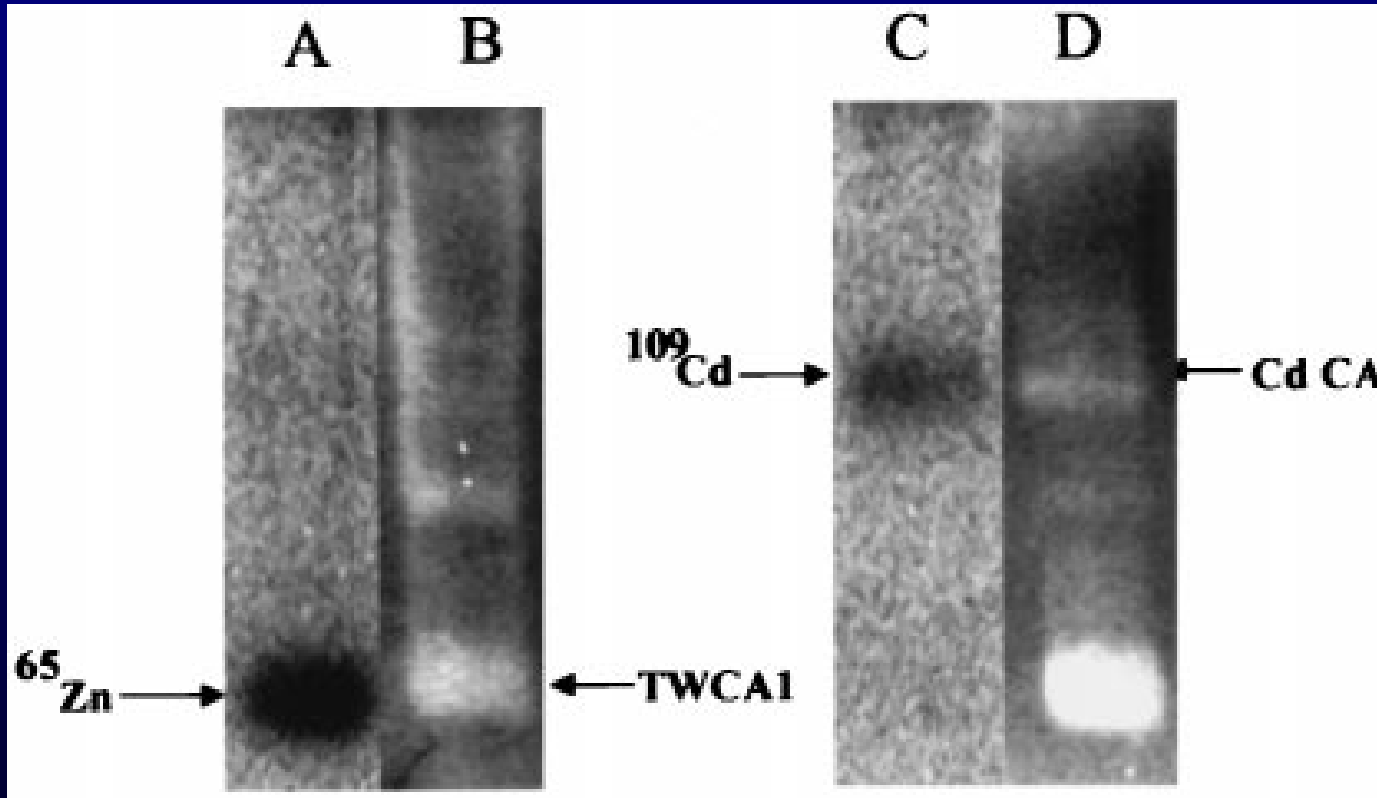
Mass Spectrometry
➢ Fragmentation of the protein, identification of fragment sizes, and subsequent comparison to a library of known fragmentation patterns

N-terminal Seqencing (Edman degradation)
➢ Sequential chemical removal of individual amino acids from the N-terminus
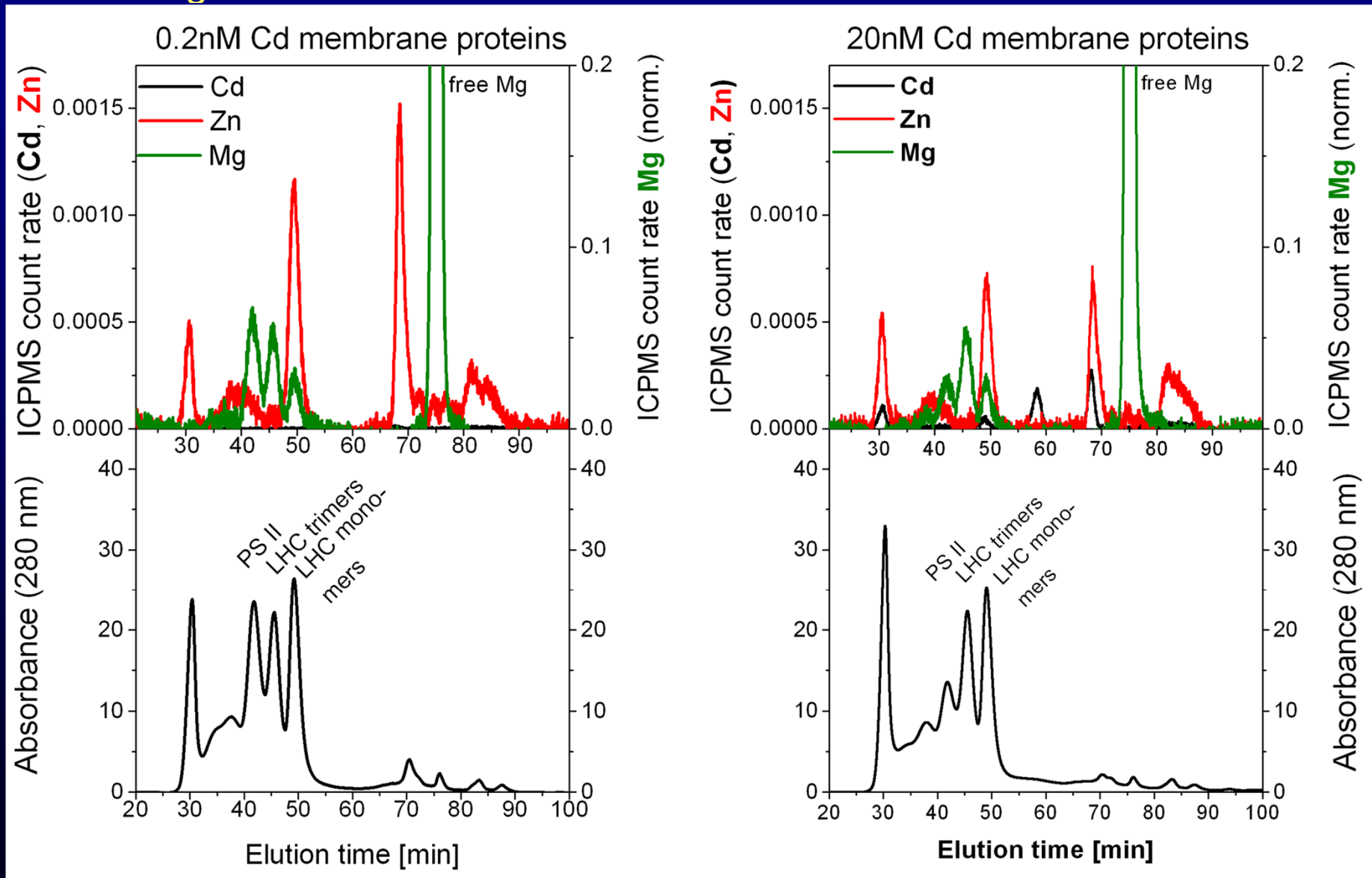
III) Protein Identification by assays in native gels

➢ Example of a biochemical assay in native gels: identification of Cd-Carboanhydrase by its metal content combined with an in-gel carboanhydrase assay

from: Lane TW, Morel FMM (2000) PNAS97, 4627-4631
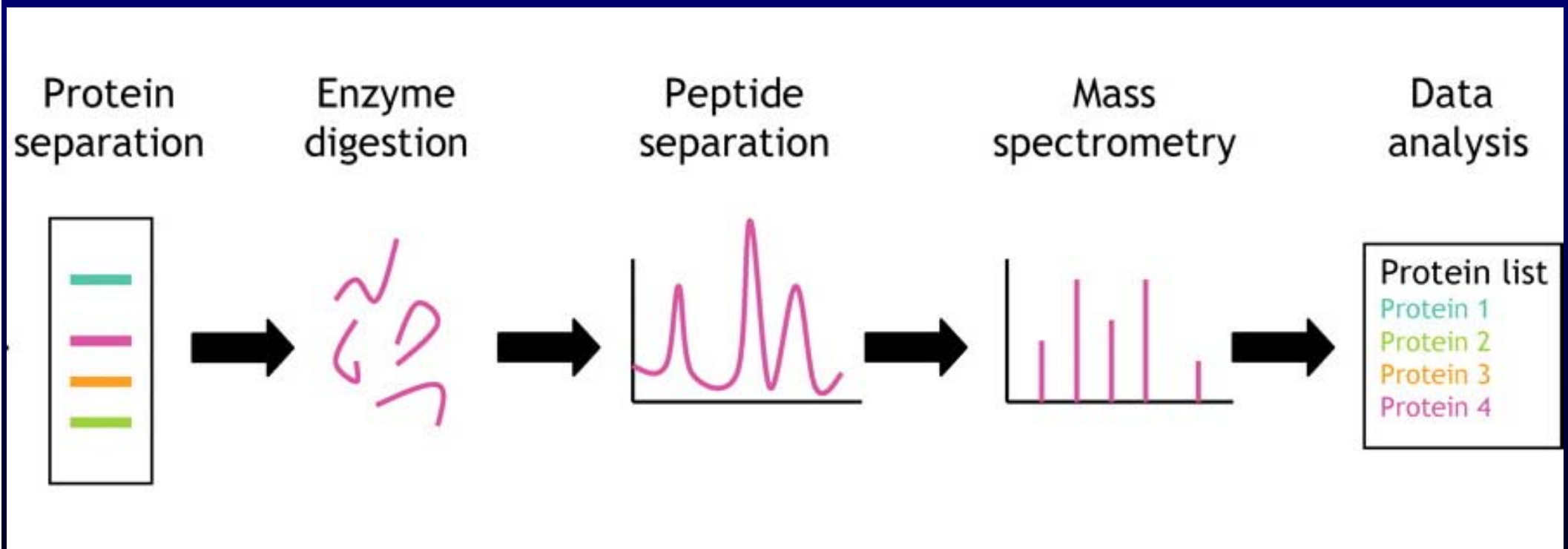
III) Protein Identification by HPLC-ICPMS

➢ Example of a metalloprotein identification by HPLC-ICPMS → Cd binding to main light harvesting antenna LHCII

Andresen E, Kappel S, Stärk HJ, Riegger U, Borovec J, Mattusch J, Heinz A, Schmelzer CEH, Matoušková Š, Dickinson B, Küpper H (2016) New Phytologist 210, 1244-1258.

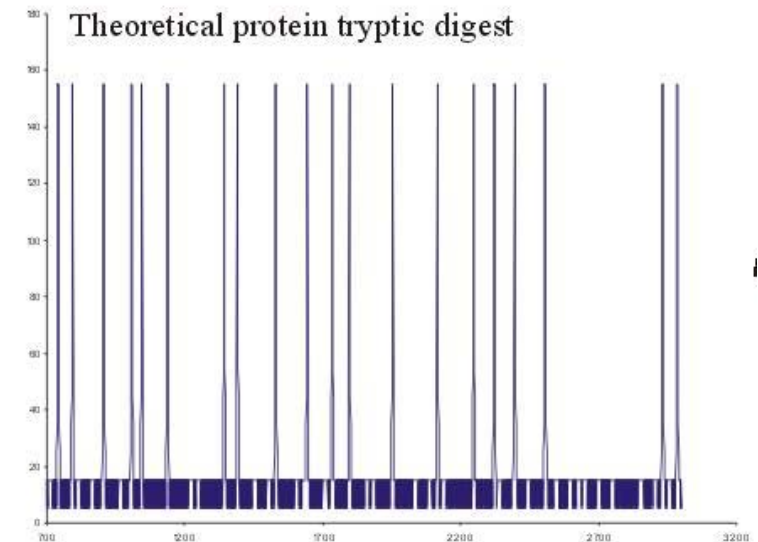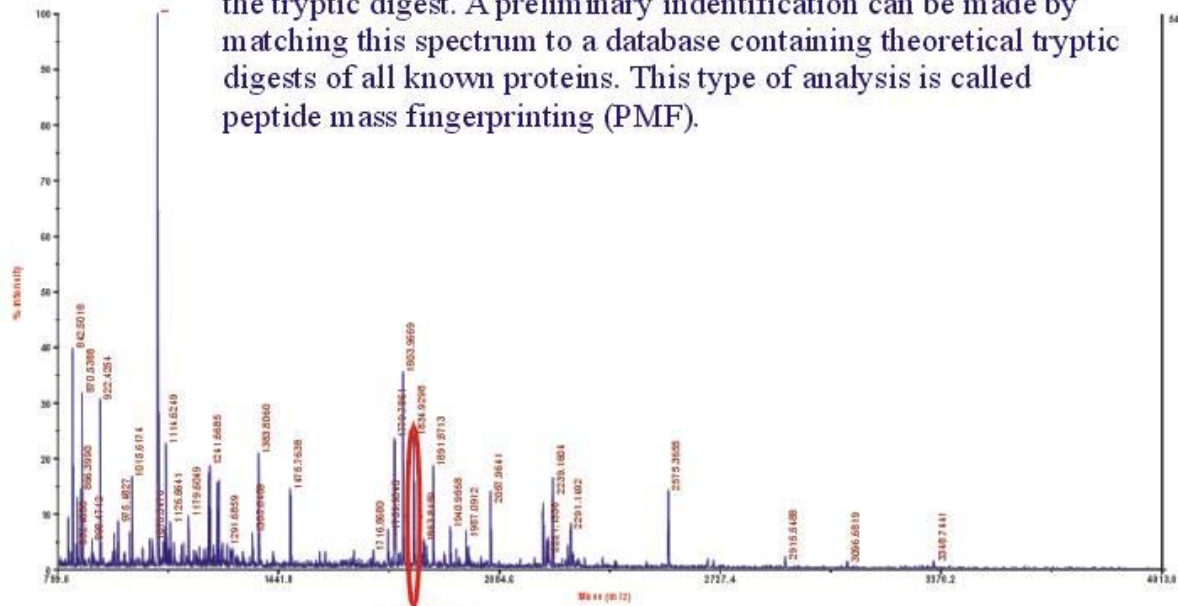# III) Protein Identification: Mass Spectrometry
Principle

➢1) protein band is cut of out gel

➢2) protein is digested into peptides

➢3) mass spectrometry

➢4) comparison of the fragment sizes with a database

➢5) assignment of likely sequences to fragments

➢6) comparison of the fragment sequences with a database

➢7) result: list of proteins from the database that have a similar fragmentation pattern

III) Protein Identification: Mass Spectrometry

➤ 3) mass spectrometry
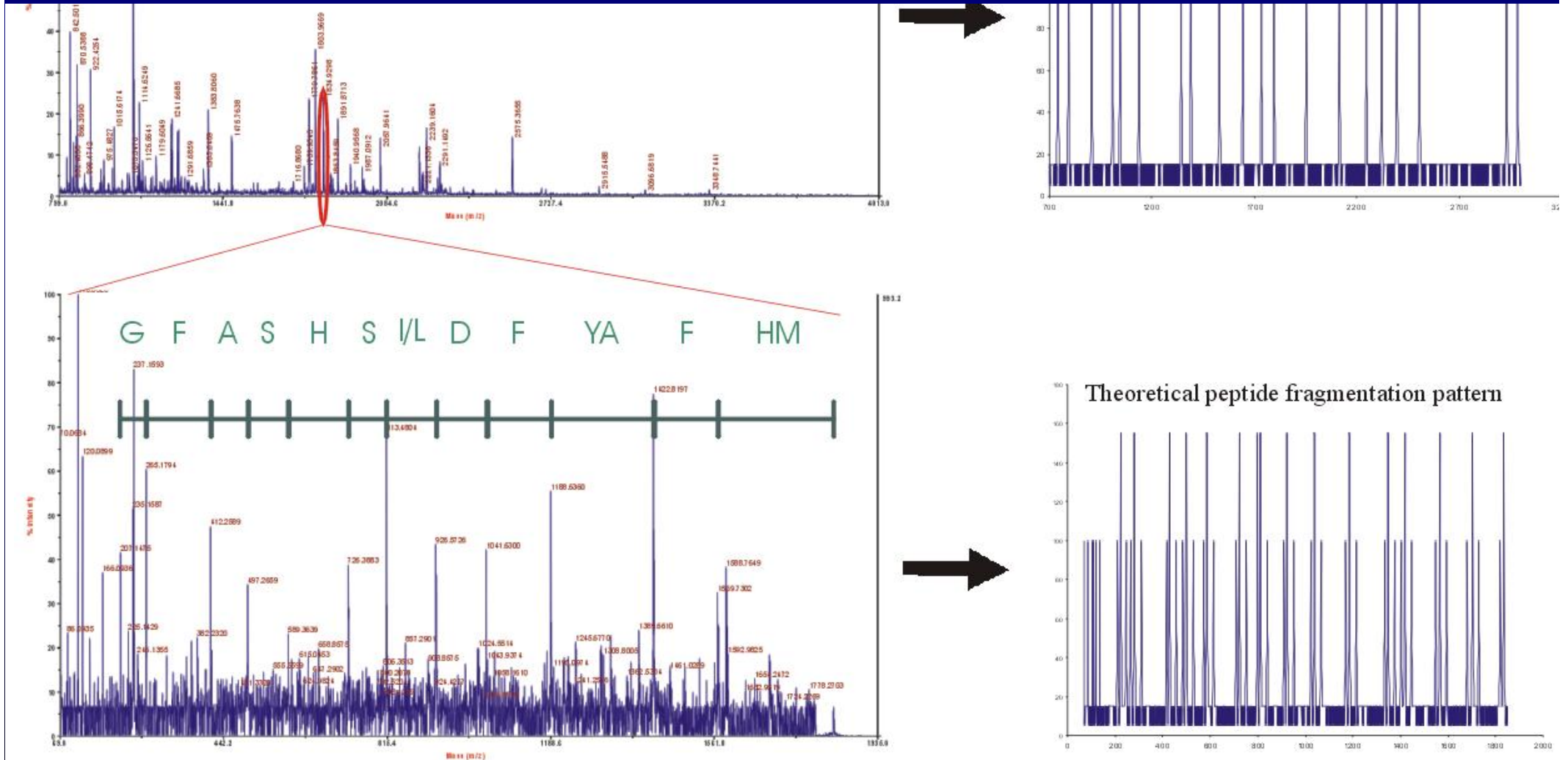➤ 4) comparison of the fragment sizes with a database

The peaks in the mass spectrum each represent a peptide present in the tryptic digest. A preliminary indentification can be made by matching this spectrum to a database containing theoretical tryptic digests of all known proteins. This type of analysis is called peptide mass fingerprinting (PMF).
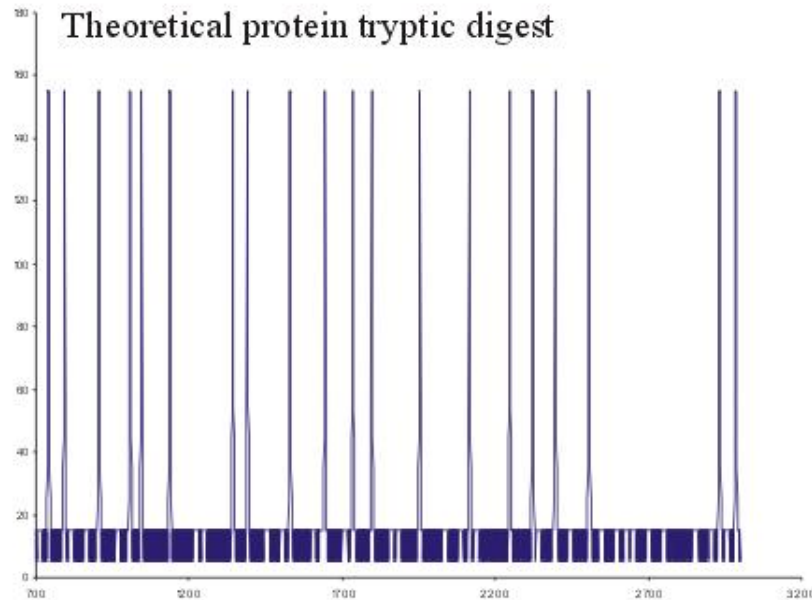
Theoretical protein tryptic digest
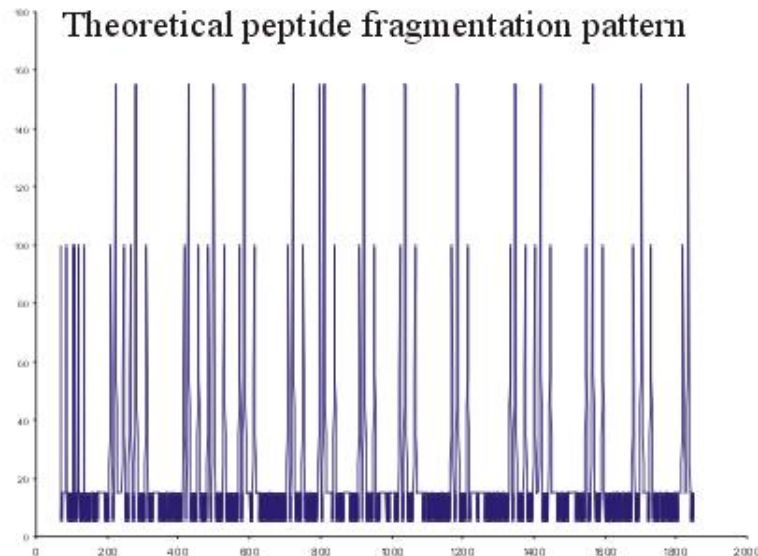
III) Protein Identification: Mass Spectrometry

➢ 3a) further fragmentation of one of the fragments from the digest in the mass spectrometer, then again mass spectrometry (MSMS)

➢ 4a) comparison of the fragment sizes with a database

G F A S H S I/L D F YA F HM

Theoretical peptide fragmentation pattern

III) Protein Identification: Mass Spectrometry

Theoretical protein tryptic digest

GPS Explorer TM Software - Results Browser
File Edit View Help

Prev Next Up Projects Properties Refresh Spotline Plate Protein Spectrum Prev Spec Next Spec DB Result Help

MSMS screen

{MATRIX SCIENCE} **Mascot Search Results**

User	:
Email	:
Search title	: SampleSetID: 63, AnalysisID: 115, MaldiWellID: 2699, SpectrumID: 11198, Path=\dav
Database	: SwissProt 041223 (167089 sequences; 60908342 residues)
Taxonomy	: Homo sapiens (human) (11777 sequences)
Timestamp	: 14 Mar 2005 at 04:53:23 GMT
Top Score	: 293 for A1AT_HUMAN, (P01009) Alpha-1-antitrypsin precursor (Alpha-1 protease inhi

Probability Based Mowse Score

Score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 53 are significant (p<0.05).

- 5) assignment of likely sequences to fragments
- 6) comparison of the fragment sequences with a database
- 7) result: list of proteins from the database that have a similar fragmentation pattern

Theoretical peptide fragmentation pattern

Protein Summary Report

Switch to Peptide Summary Report

To create a bookmark for this report, right click this link: Protein Summary Report (SampleSetID: 63, AnalysisID: 115, Mald&WellI

Re-Search All   Search Unmatched

Index

	Accession	Mass	Score	Description
1.	A1AT_HUMAN	46878	293	(P01009) Alpha-1-antitrypsin precursor (Alpha-1 protease inhi
2.	XAP5_HUMAN	40216	56	(Q14320) XAP-5 protein (HXC-26 protein)
3.	F43B_HUMAN	37266	54	(Q6ZTS2) Protein FAM43B
4.	TAG3_HUMAN	22629	51	(Q9UI15) Transgelin-3 (Neuronal protein NP25) (Neuronal prote
5.	SPCP_HUMAN	272496	45	(O15020) Spectrin beta chain, brain 2 (Spectrin, non-erythroi

Ready

Start   GPS Explorer TM Soft...   Untitled - Notepad

III) Protein Identification: Mass Spectrometry
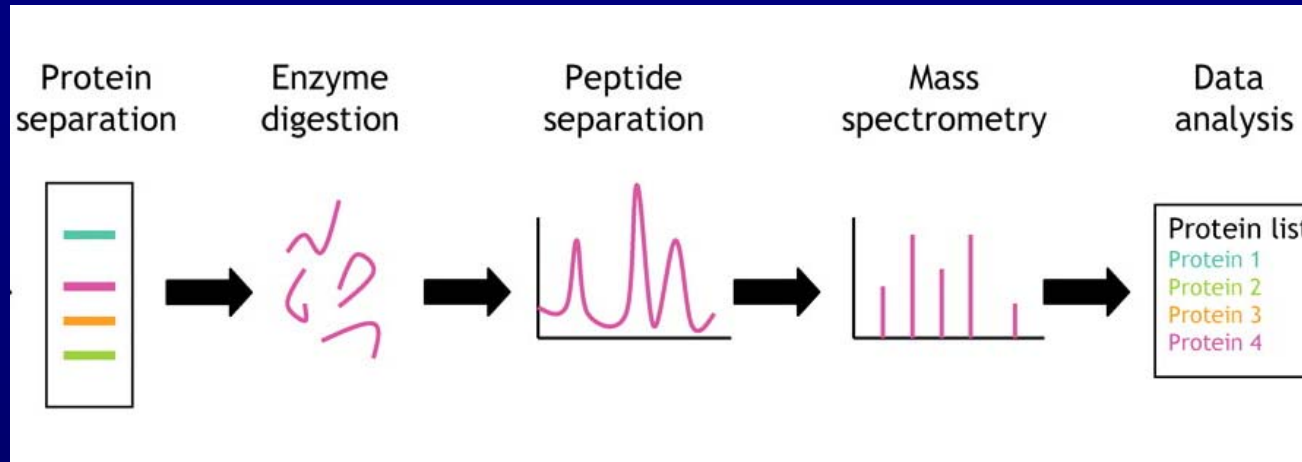

from: Gingras AC et al, 2004, J Physiol 563, 11-21

Limitations and artefacts

➢ contamination of gel bands with other proteins → identification of the contamination (very common: keratin from skin!) instead of the protein of interest

➢ not all proteins have the same detection efficiency (problems e.g. if many cysteines present) → even small contaminations sometimes lead to wrong identifications

➢ not all proteins are in the databases → database may show results that have a similar fragmentation pattern, but are otherwise unrelated

Principle

Phenylisothiocyanate

base → acid →

Phenylthiohydantoin derivative of the N-terminal amino acid
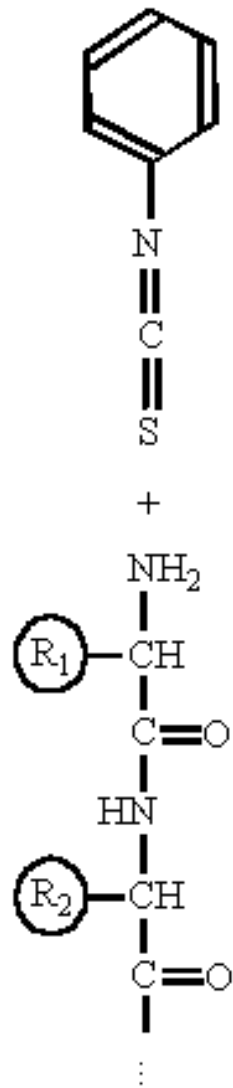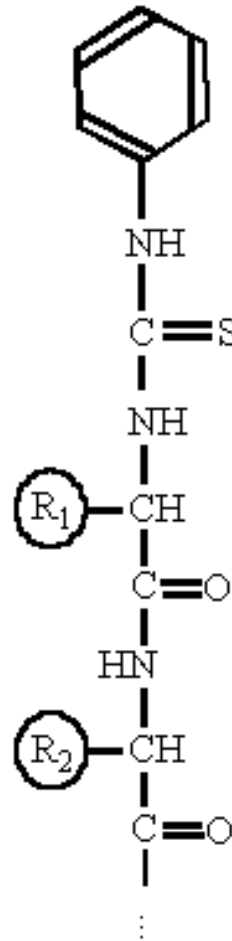
Phenylthiocarbamoyl-polypeptide

Polypeptide

Polypeptide (N-1)

identification of the amino acid by reversed phase HPLC and comparison with HPLC of standard mixture

III) Protein Identification: N-terminal sequencing

Sample preparation

- 1) run acrylamide gel (denaturing or native)
- 2) blot onto PVDF (NOT nitrocellulose) membrane using glycine-free buffer
- 3) stain the membrane with ponceau red (CBB and other stains also work)
- 4) submit for sequencing

Limitations and artefacts

- problems with contaminations
- in eukaryotic proteins, the N-terminus is often blocked (e.g. by methylation), which required complicated de-blocking procedures. Also non-polymerised acrylamide remains in the gel can cause blocking, so let the gel polymerise over night
- Cys residues cannot be detected, and also glycosylated redidues may appear as blank spaces in the sequence

IV) Protein Characterisation: Overview of Principles

➢ **Size determination**

➢ **Charge determination**

➢ **Analysis of cofactors**

➢ **Analysis of the 3-dimensional Structure**

➢ **Activity tests**

IV) Protein Characterisation: Size determination

Comparison with predicted size by native and denaturing gel electrophoresis and by size exclusion chromatography can show native oligomerisation, post-translational modification but also artefactual degradation/aggregation
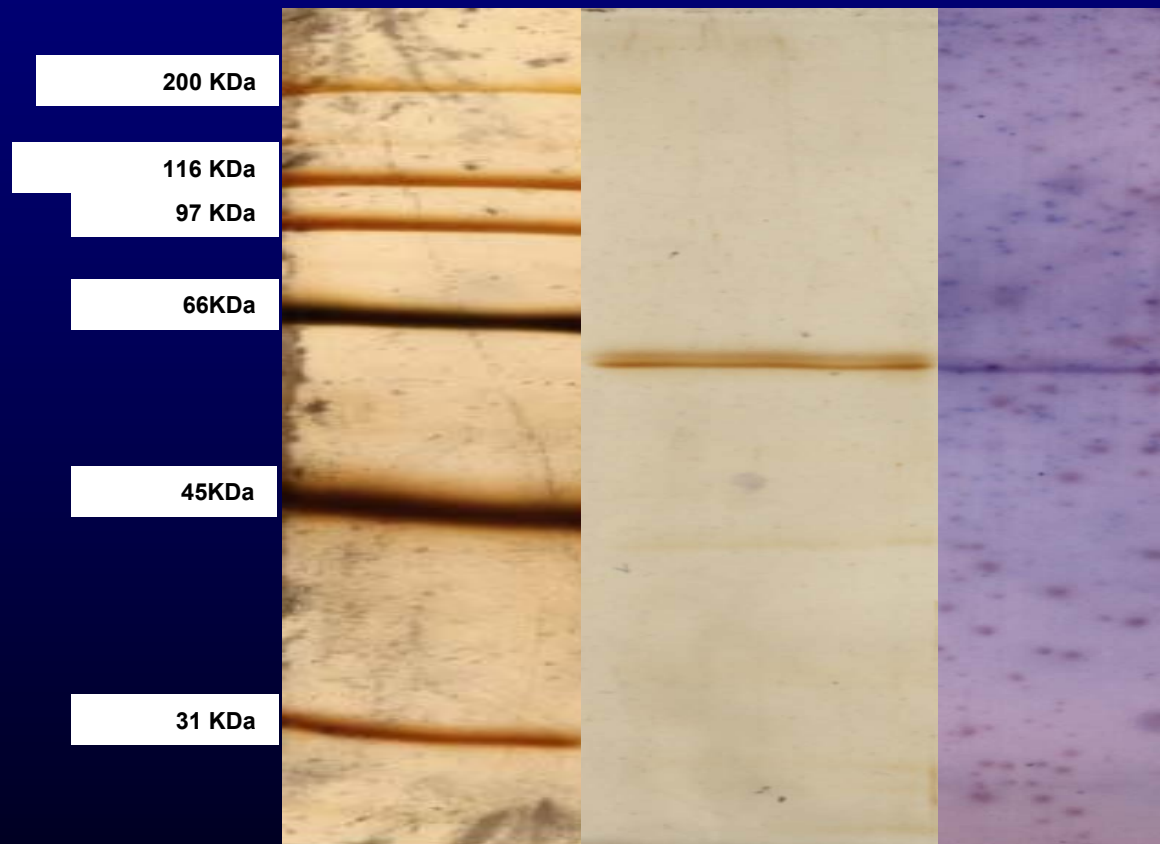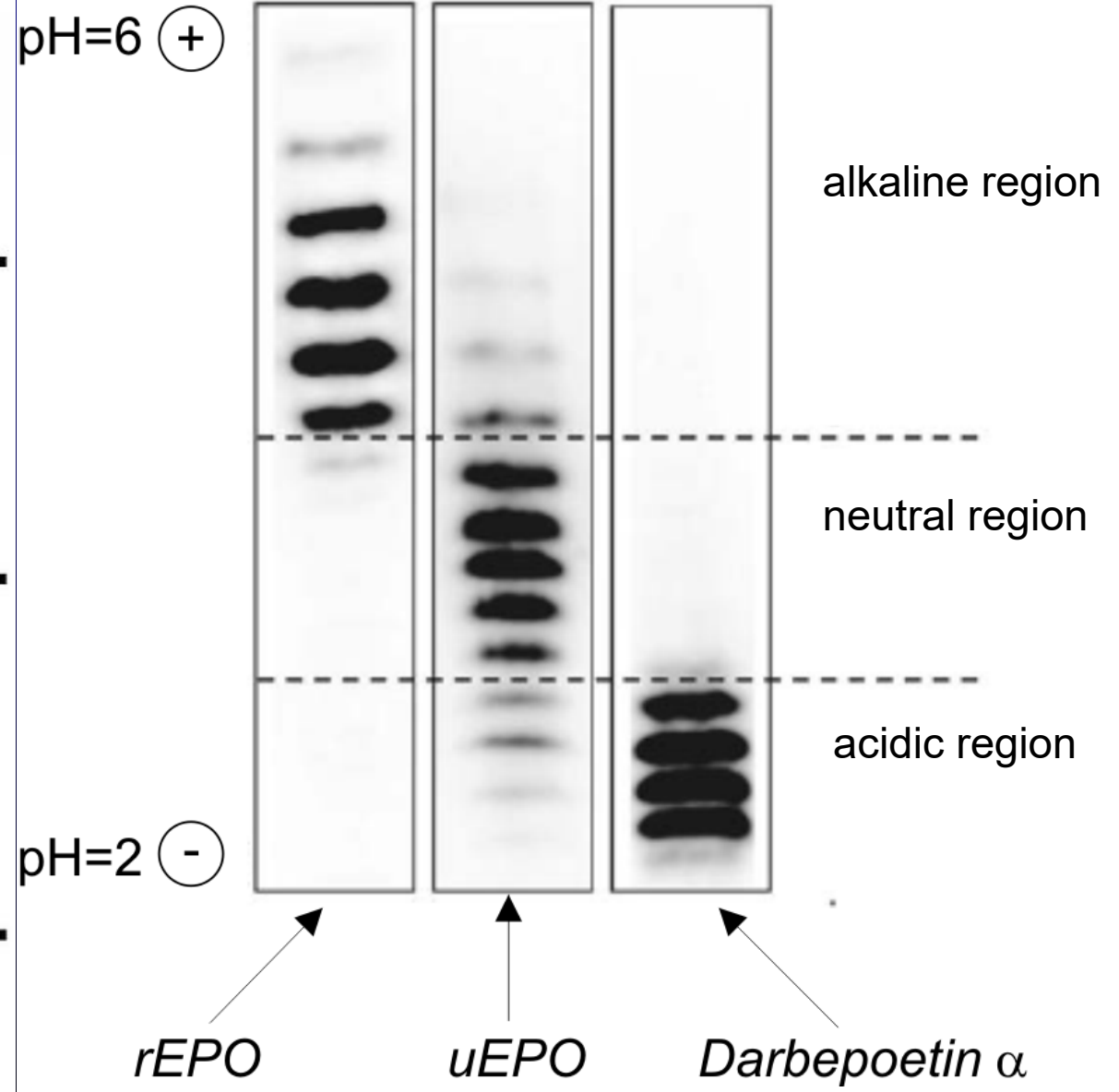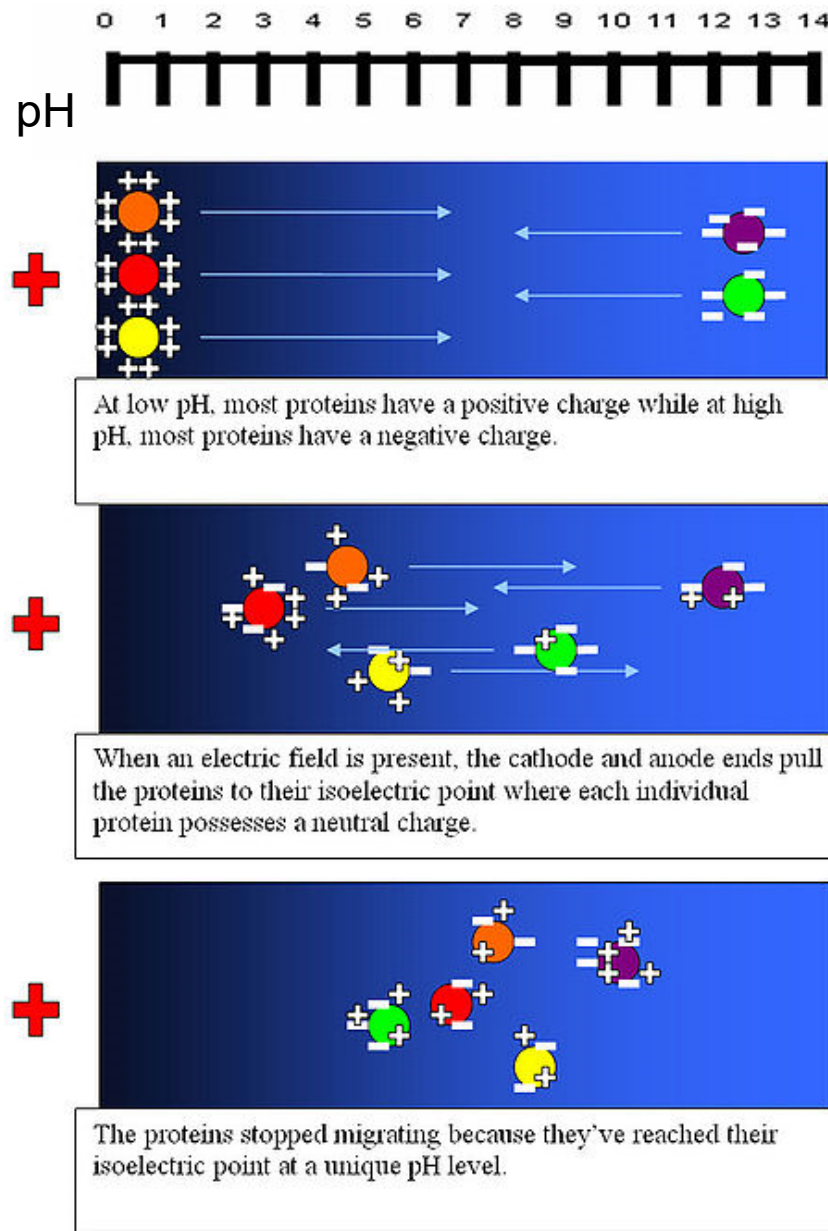

SDS gel and Western blot of TcHMA4

→ size shows post-translational processing as cDNA sequence predicts 128kDa

(Parameswaran, Leitenmaier et al., 2007, BBRC)

200 KDa
116 KDa
97 KDa
66KDa
45KDa
31 KDa

IV) Protein Characterisation: Charge determination

➢ Isoelectric focussing (IEF) can reveal the isoelectric point where the net charge of the protein is zero, i.e. balance between protonation of carboxyl groups and deprotonation of amino groups is achieved

At low pH, most proteins have a positive charge while at high pH, most proteins have a negative charge.

When an electric field is present, the cathode and anode ends pull the proteins to their isoelectric point where each individual protein possesses a neutral charge.

The proteins stopped migrating because they've reached their isoelectric point at a unique pH level.

pH=6 (+)   alkaline region

neutral region

acidic region

pH=2 (-)

rEPO   uEPO   Darbepoetin α

from: commons.wikimedia.org

IV) Protein Characterisation: Analysis of cofactors

➢ Identification and reactivity: UV/VIS Absorption and fluorescence Spectroscopy
➢ Metal content (about 30% of all proteins are metalloproteins!): AAS, ICP-MS/OES, EDX/PIXE/XRF
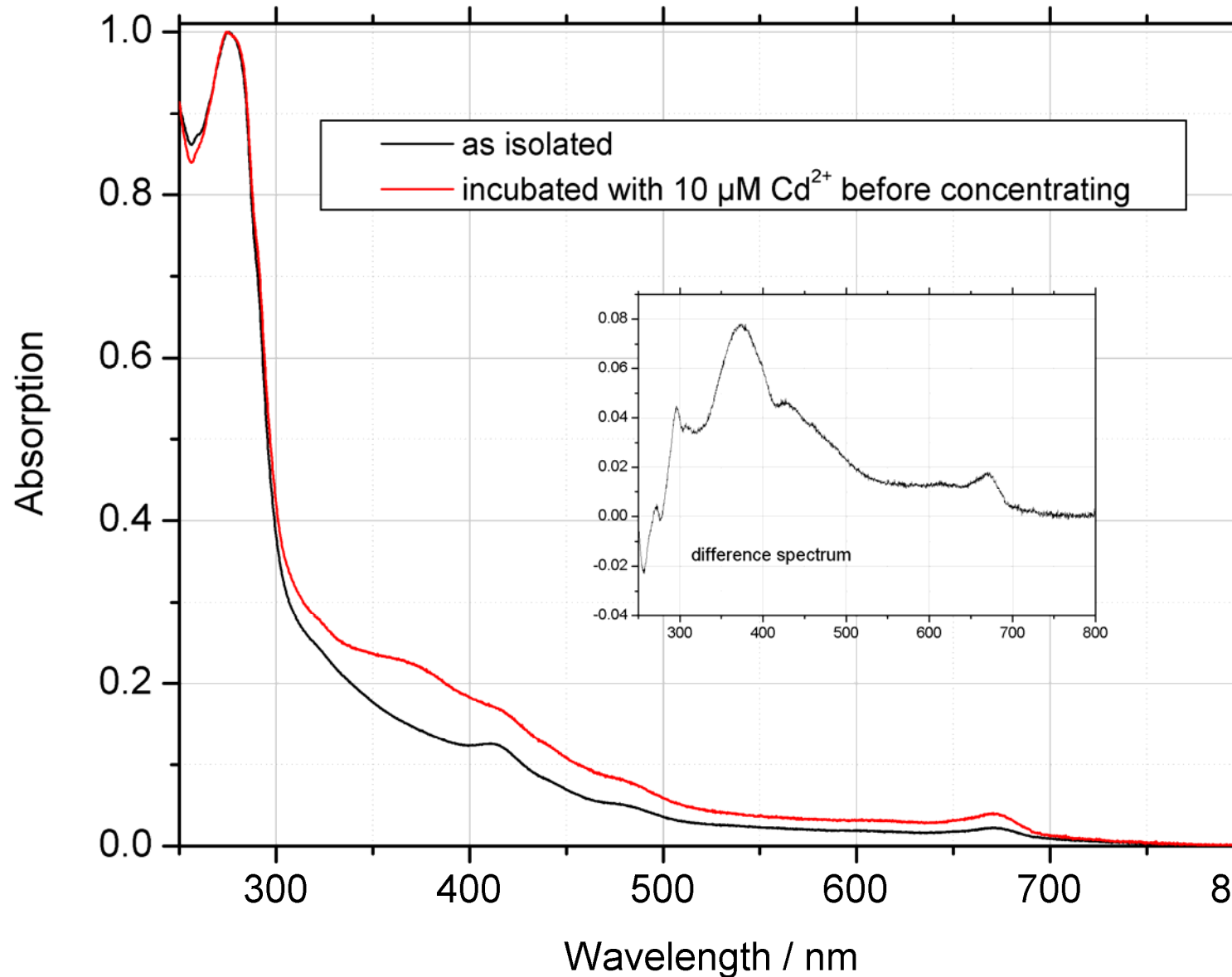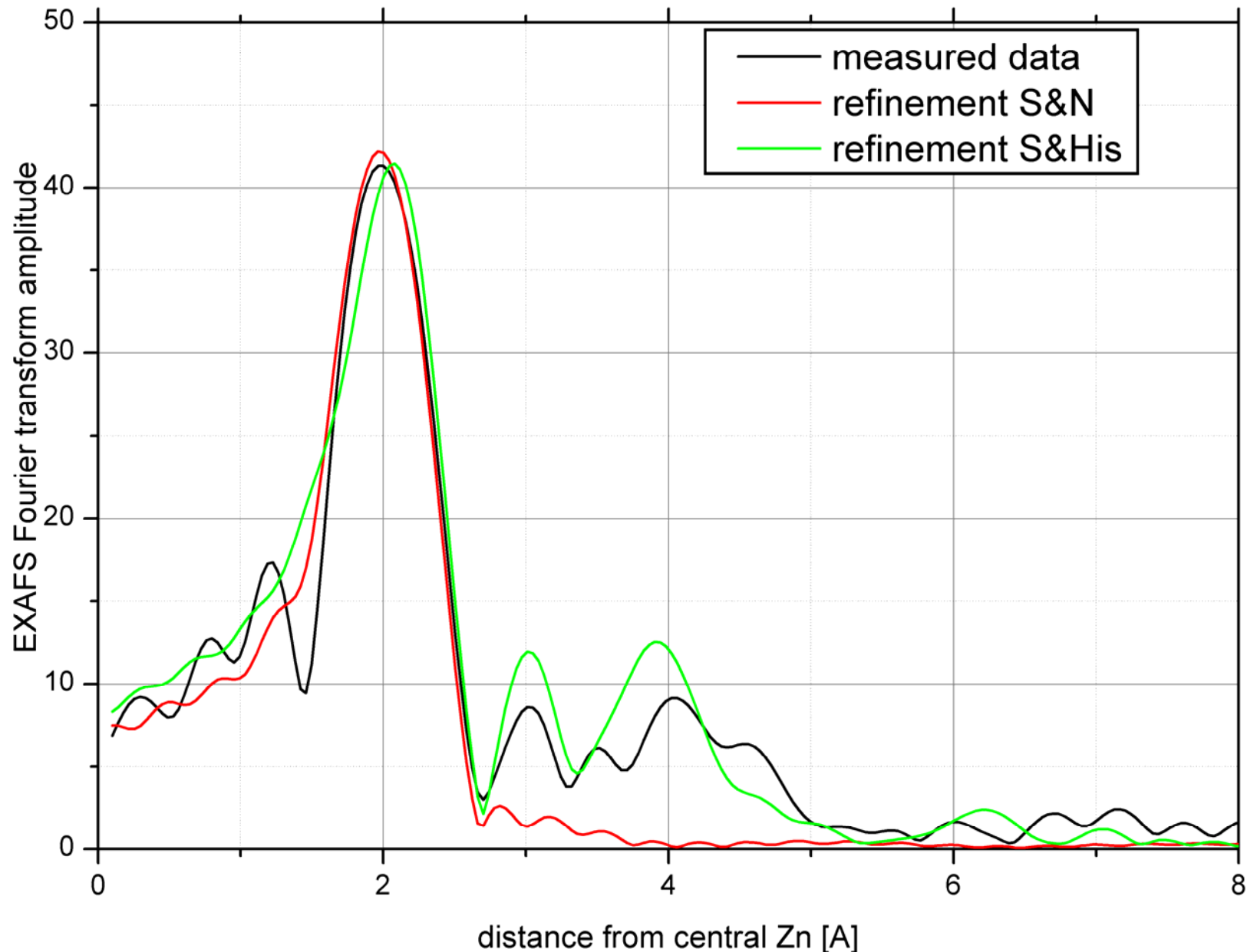

UV/VIS spectrum of TcHMA4

→ Cadmium binding causes ligand-metal-charge-transfer (LMCT) bands indicating cysteine ligation

Leitenmaier B, Witt A, Witzke A, Stemke A, Meyer-Klaucke W, Kroneck PMH, Küpper H (2011) Biochimica et Biophysica Acta - Biomembranes 1808, 2591-2599.

IV) Protein Characterisation: Analysis of cofactors

Metal ligands: EXAFS, EPR, heteronuclear NMR provide information about ligand types and their spatial arrangement around the active site (each of these techniques has different strenghts)
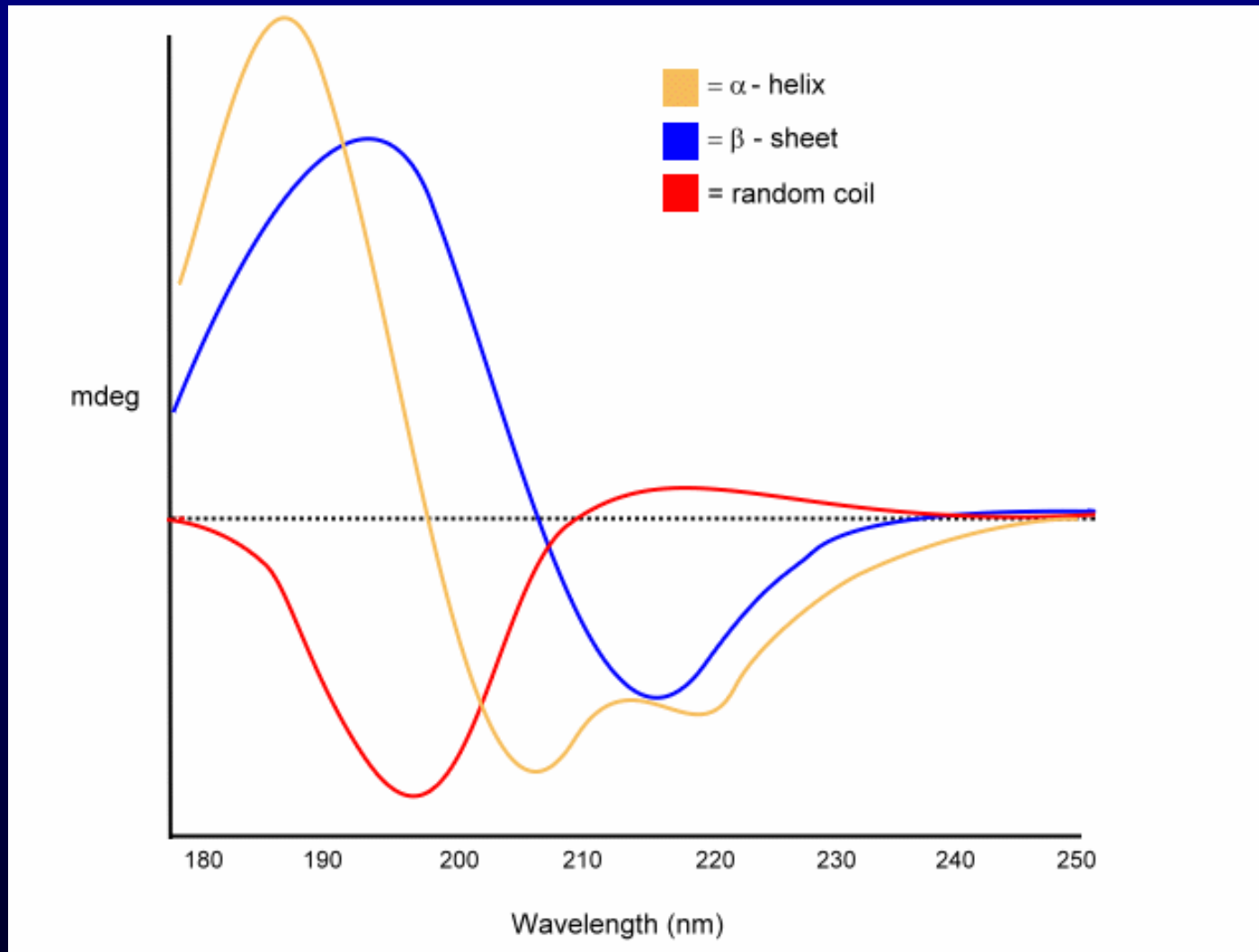

EXAFS spectrum of Zn-TcHMA4

→ mixed S/N ligands, multiple scattering shows histidine contribution

(Leitenmaier B, Witt A, Witzke A, Stemke A, Meyer-Klaucke W, Kroneck PMH, Küpper H (2011) Biochimica et Biophysica Acta - Biomembranes 1808, 2591-2599.)

IV) Protein Characterisation: Analysis of the 3D Structure

Circular Dichroism (CD) Spectroscopy

From: www.proteinchemist.com/cd/cdspec.html

➢ information about proportions of secondary structure types in a protein
➢ particularly useful when X-ray crystallography and NMR are not applicable

IV) Protein Characterisation: Analysis of the 3D Structure
X-ray crystallography
Example: in the nickel-binding transcription factor NikR, the mechanism was concluded from the X-ray structure
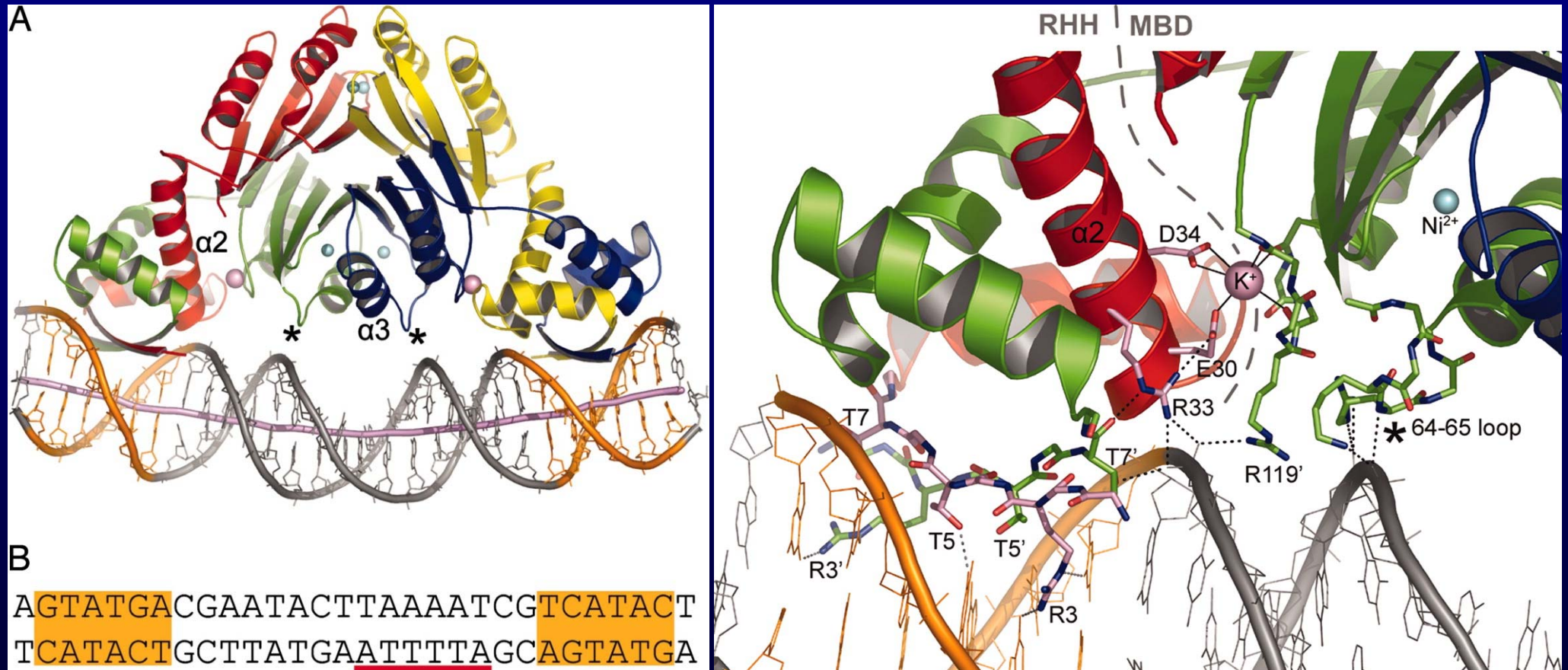

From: Schreiter ER et al., 2006, PNAS103, 13676-81

➢ Ni binding causes RHH domains to rotate about the flexible interdomain linkers to orient their antiparallel -strands toward the same face of the repressor, allowing each to occupy the DNA major groove of an operator palindrome half-site

➢ Binding of Ni creates a surface of the MBD suitable for interacting with DNA by stabilization of a helix and a loop

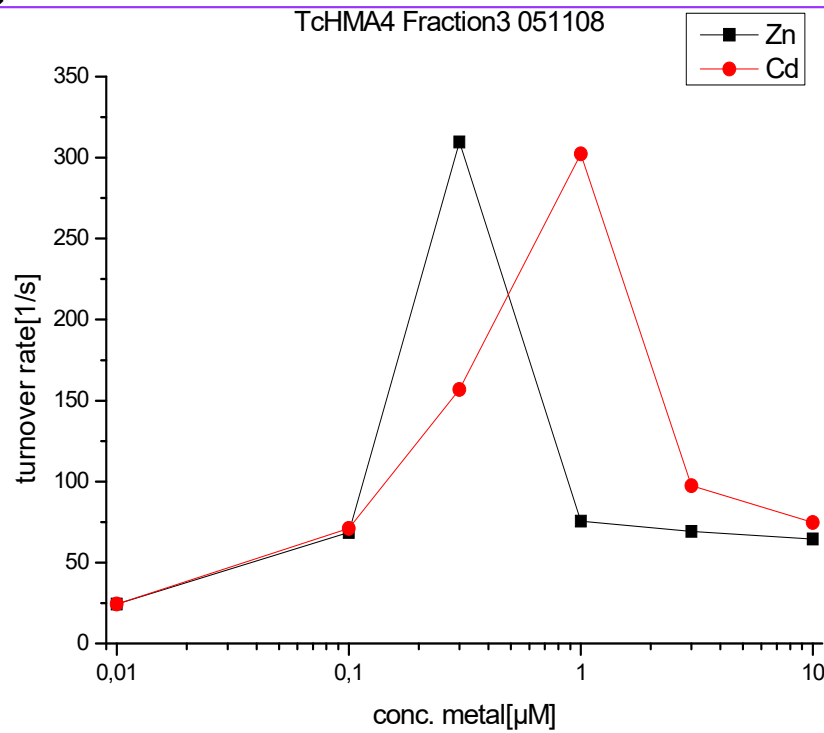# IV) Protein Characterisation: Activity tests

Titration of an enzyme with its substrate(s) reveals binding constants and possible substrate inhibition
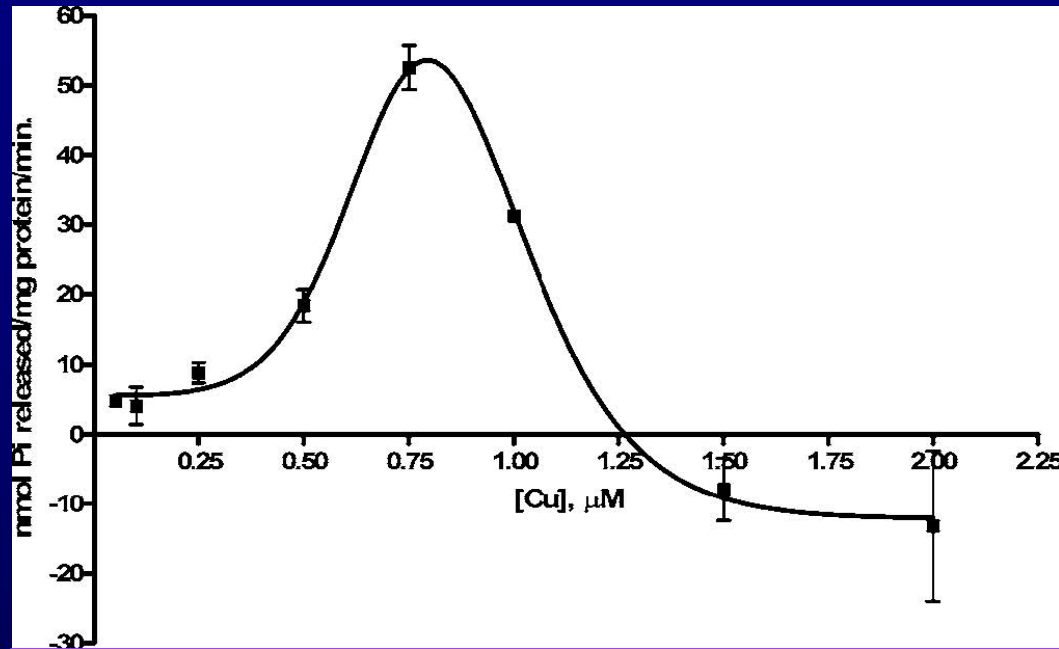
Activation of a human Cu-ATPase

Hung et al. Biochem.J. 2007, 401

Both are P_{1B}-Type ATPases showing the same activation pattern by „their" metal after reconstitution into artificial lipid vesicles

Activity of TcHMA4

(Leitenmaier B, Witt A, Witzke A, Stemke A, Meyer-Klaucke W, Kroneck PMH, Küpper H (2011) Biochimica et Biophysica Acta - Biomembranes 1808, 2591-2599)

IV) Protein Characterisation: Activity tests

Two-dimensional data, e.g. substrate concentration and temperature reveal insights into interactions between factors that are decisive for enzyme activity, e.g. temperature dependence of substrate binding and influence of the substrate on the thermostability of the enzyme
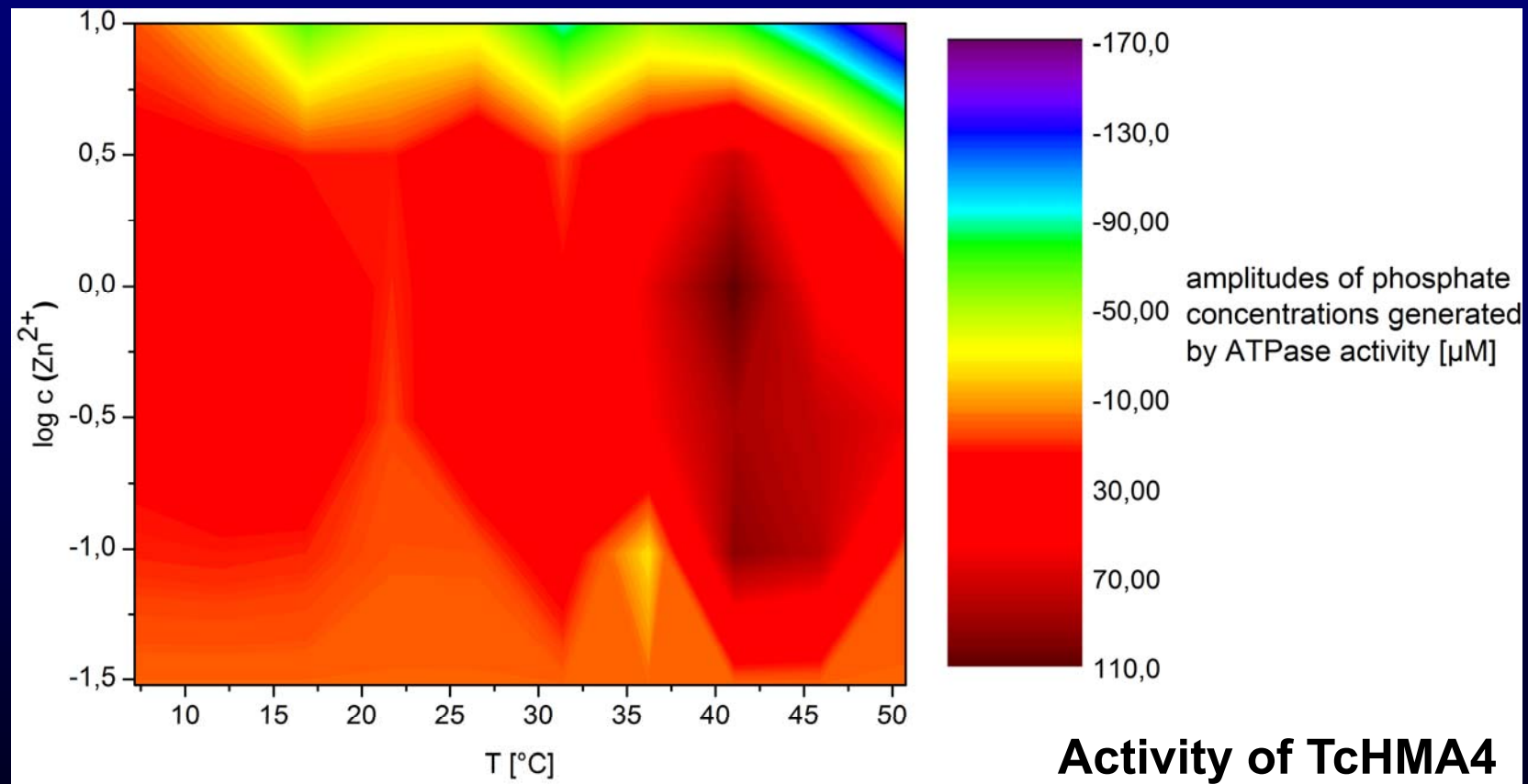

Activity of TcHMA4

(Leitenmaier B, Witt A, Witzke A, Stemke A, Meyer-Klaucke W, Kroneck PMH, Küpper H (2011) Biochimica et Biophysica Acta - Biomembranes 1808, 2591-2599)

Example: Flowchart of expression, isolation, purification and characterisation of the Cd/Zn-ATPase TcHMA4

Grow plants expressing TcHMA4 hydroponically, harvest and freeze roots

$\downarrow$

Grind the frozen roots with isolation buffer in liquid N_2, thaw

$\downarrow$

Centrifuge, discard the supernatant (soluble proteins) and resuspend the pellet (membrane proteins) with solubilisation buffer

$\downarrow$

Centrifuge for removing insoluble residue, collect solubilised protein

$\downarrow$

Immobilized Metal Affinity Chromatography on Ni-IDA column

| Identify: Western-Blotting, Edman Sequ. | Quantify: In SDS gel via fluorescent dye | Metal binding: AAS/ICP, EXAFS, UV/VIS | Activity tests ($\rightarrow$ catalytical properties) |

**All slides of my lectures can be downloaded
from my workgroup homepage**

Biology Centre CAS → Institute of Plant Molecular Biology → Departments
→ Department of Plant Biophysics and Biochemistry,
or directly
http://webserver.umbr.cas.cz/~kupper/AG_Kuepper_Homepage.html