

# Isolation, Purification and Characterisation of Proteins from Plants

# In vivo Study

Cellular localization of protein

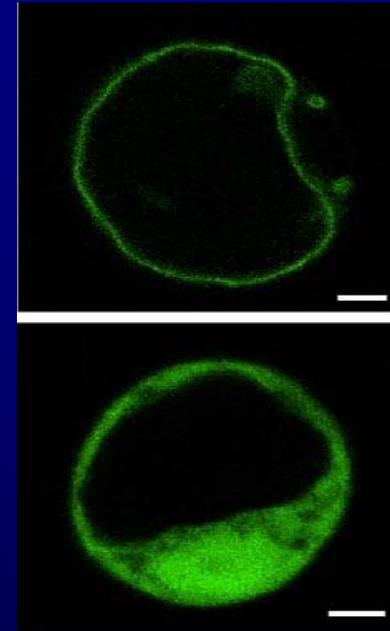
Activity e.g. detection of metal transport by specific fluorescent dyes or patch clamping

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



# 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 homogenisation

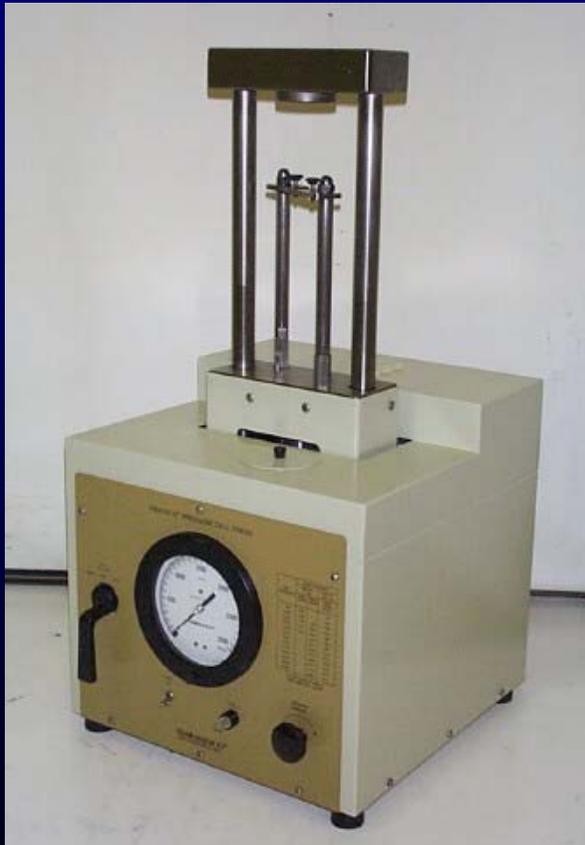
- Used for soft tissues like some leaves, and a post-treatment after grinding
- Does not require freezing and thus may avoid artefacts of freezing, but may cause artefacts by heating of the sample
- Requires expensive (usually several thousand €) machinery



# 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](http://www.diversified-equipment.com)

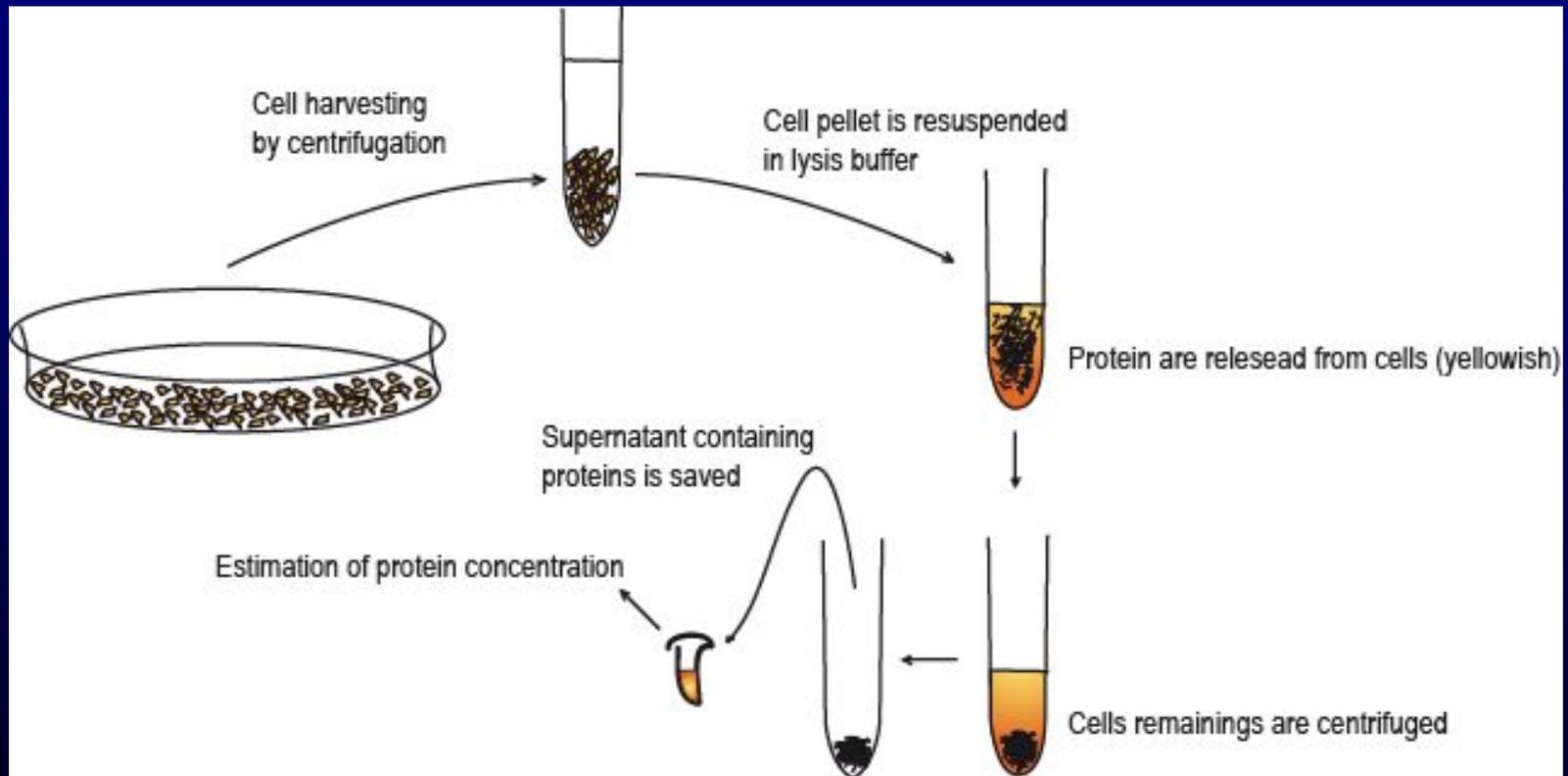


From: [www.pegasusscientific.com](http://www.pegasusscientific.com)

# 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...](http://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**

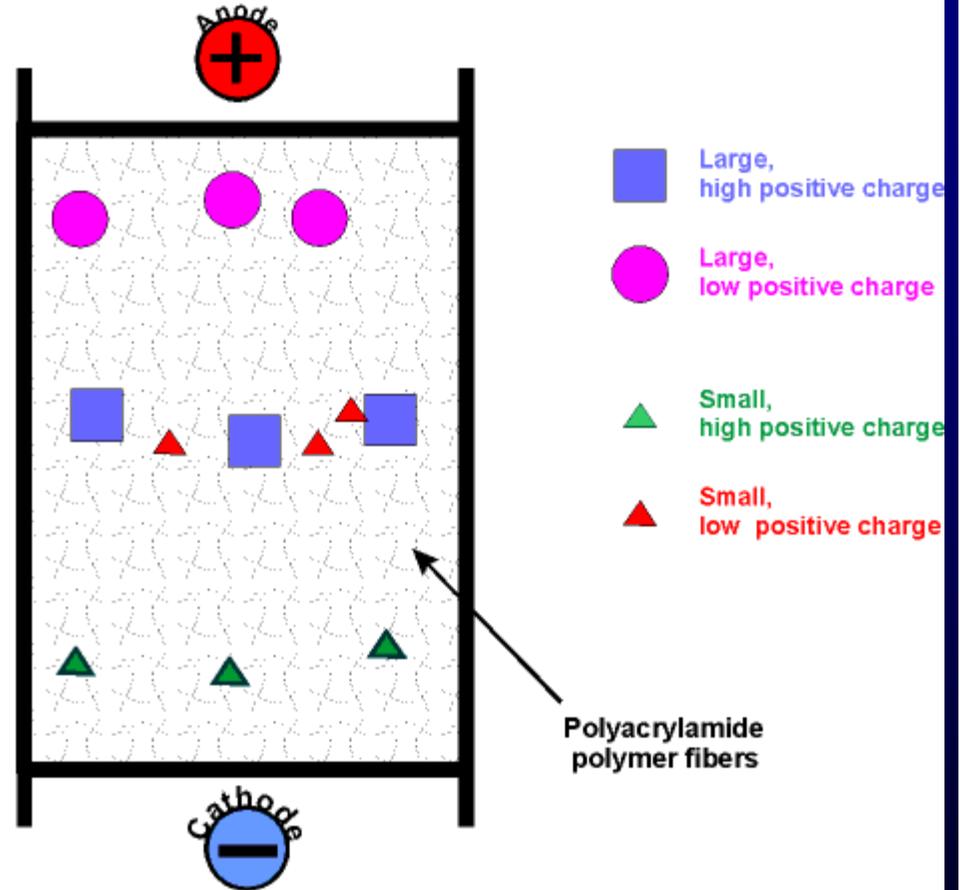
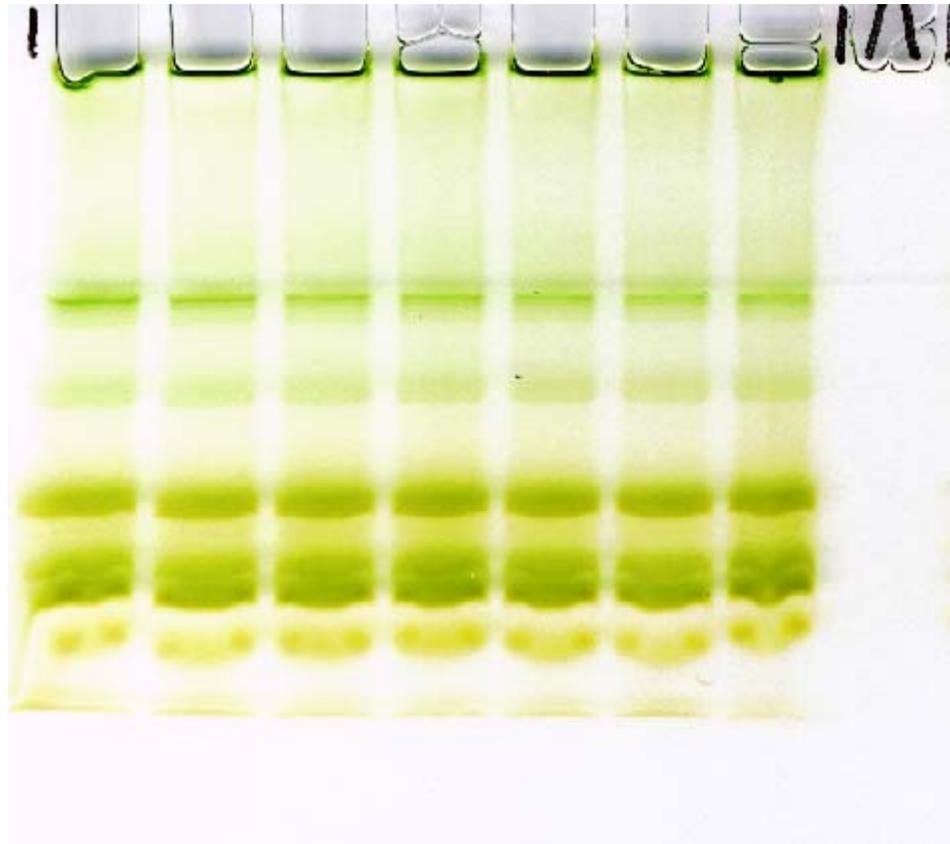


foto of a green gel (Küpper et al., 2003, Funct Plant Biol)

From: [www.columbia.edu/.../c2005/lectures/lec6\\_09.html](http://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

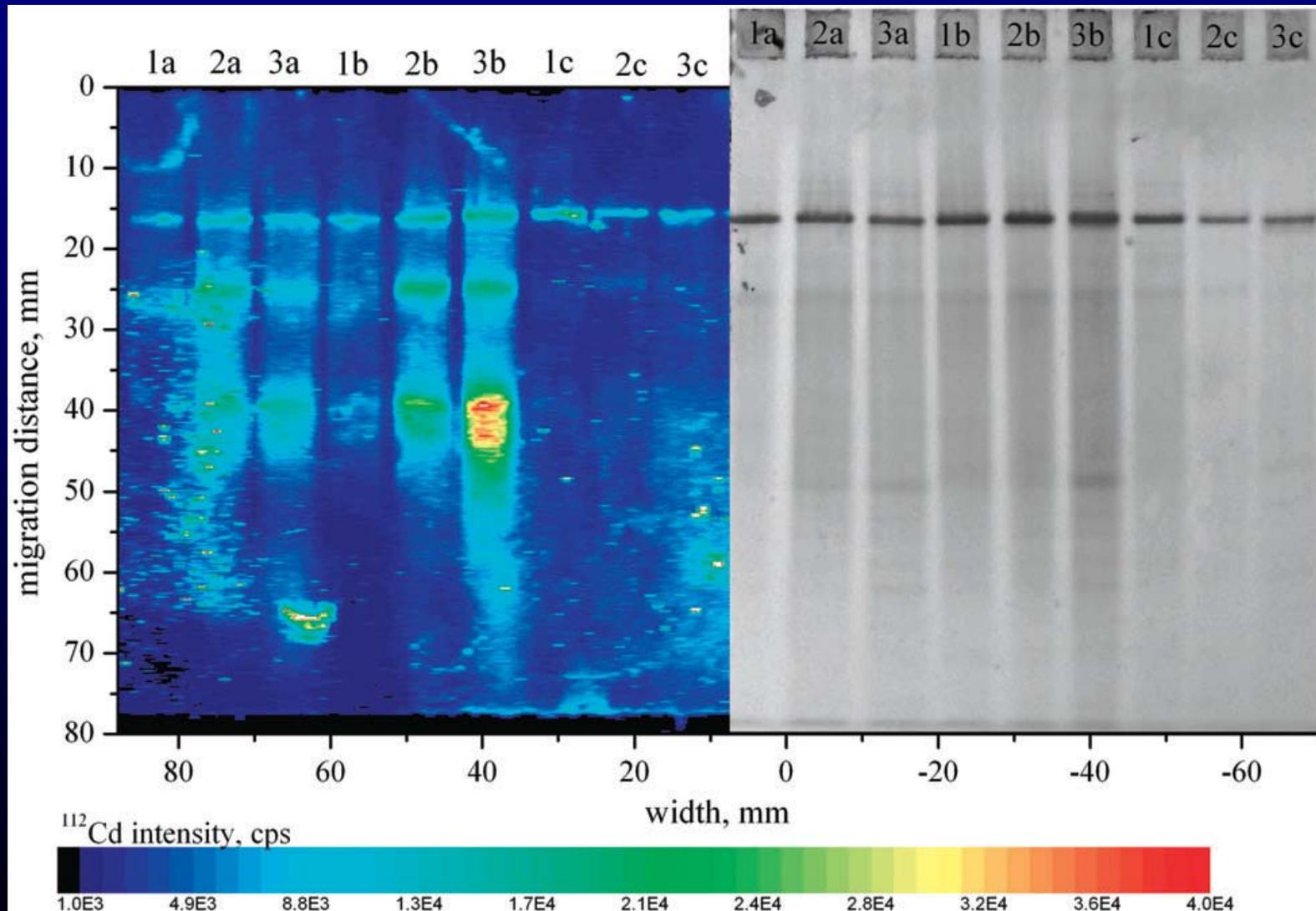
- Fragmentation of the protein, identification of fragment sizes, and subsequent comparison to a library of known fragmentation patterns

## N-terminal Sequencing (Edman degradation)

- Sequential chemical removal of individual amino acids from the N-terminus

### III) Protein Identification by assays in native gels

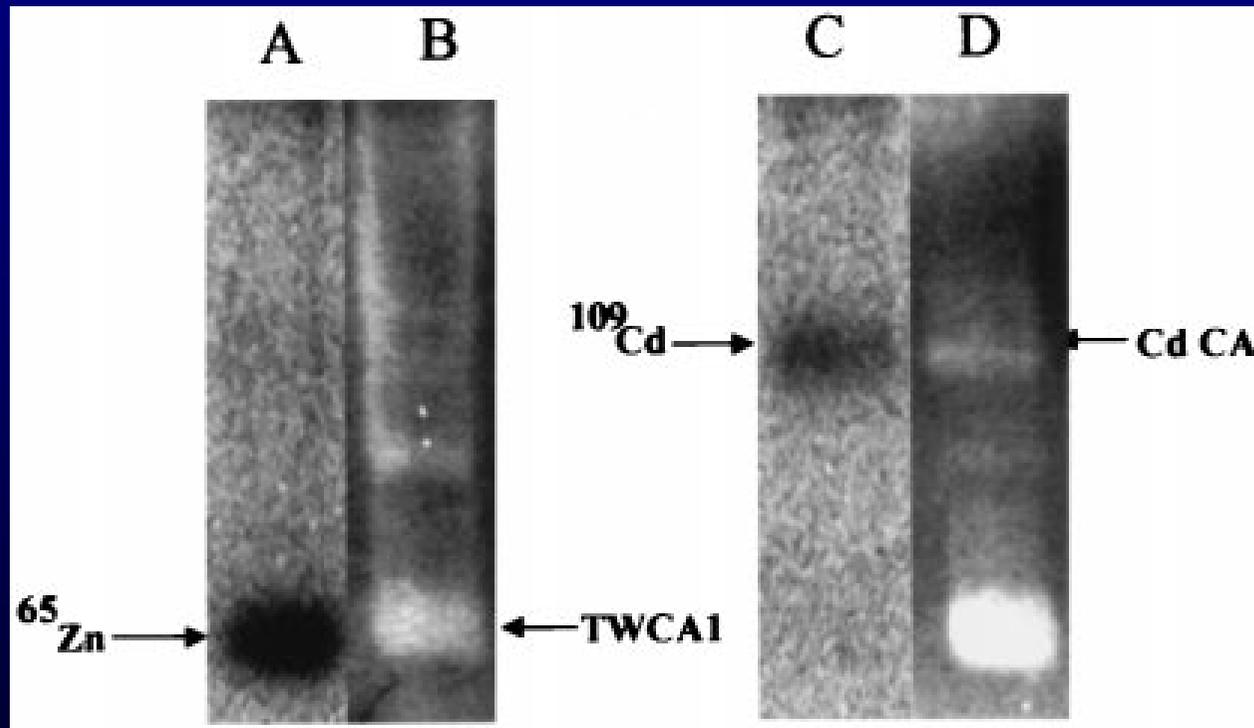
- Example of a metalloprotein identification in native gels: Scanning of the gels with laser ablation inductively coupled mass spectrometry (LA-ICP-MS) for obtaining information about metal binding to individual bands of a gel



from: Polatajko A, Azzolini M, et al (2007) JAnalAtomSpect 22, 878-887

### 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

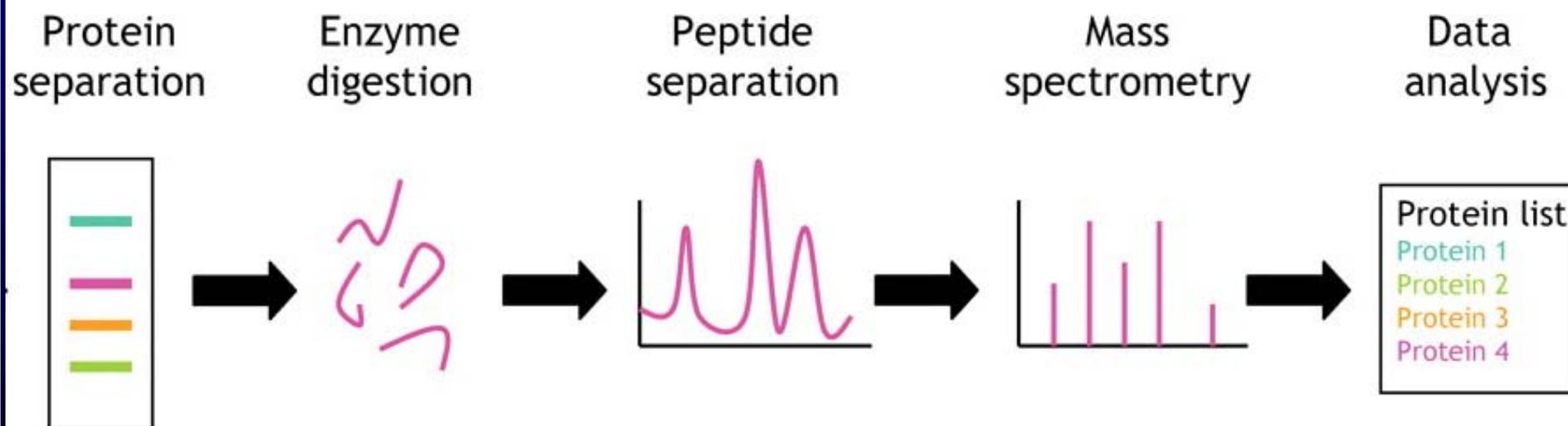


from: Lane TW, Morel FMM (2000) PNAS97, 4627-4631

## III) Protein Identification: Mass Spectrometry

### Principle

- 1) protein band is cut out of 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

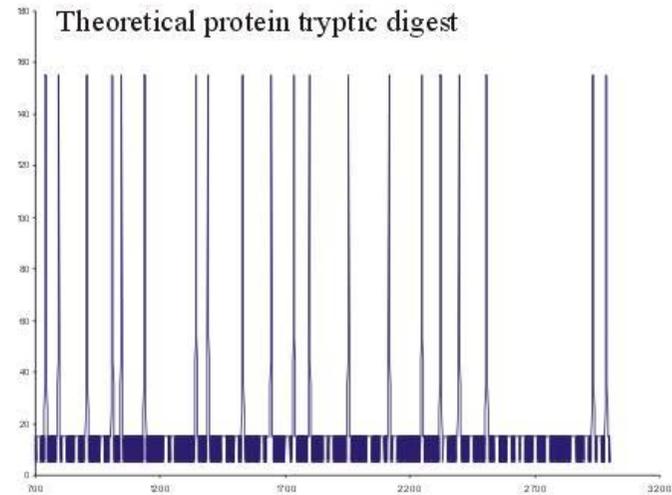
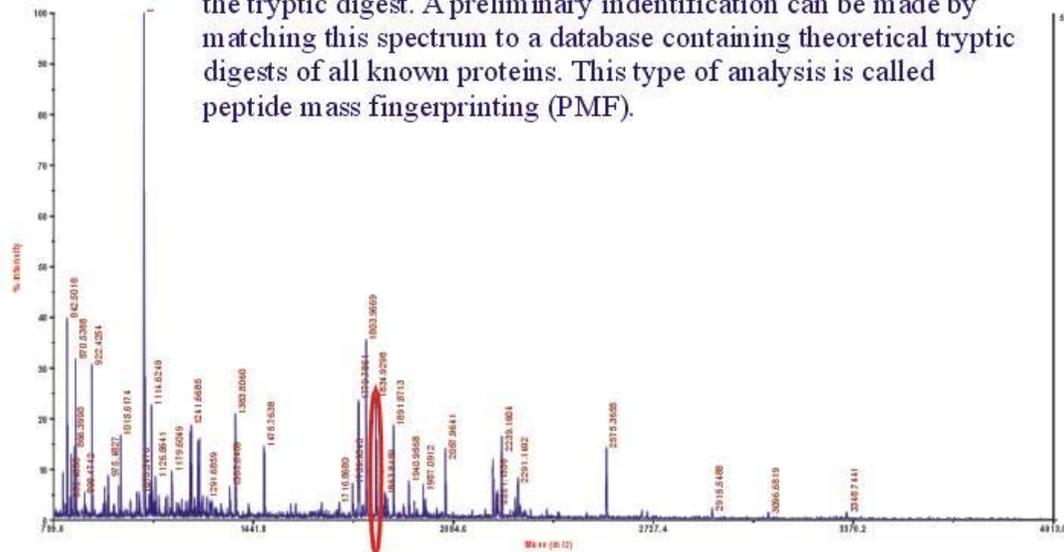


from: Gingras AC et al, 2004, J Physiol 563, 11-21

# III) Protein Identification: Mass Spectrometry

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

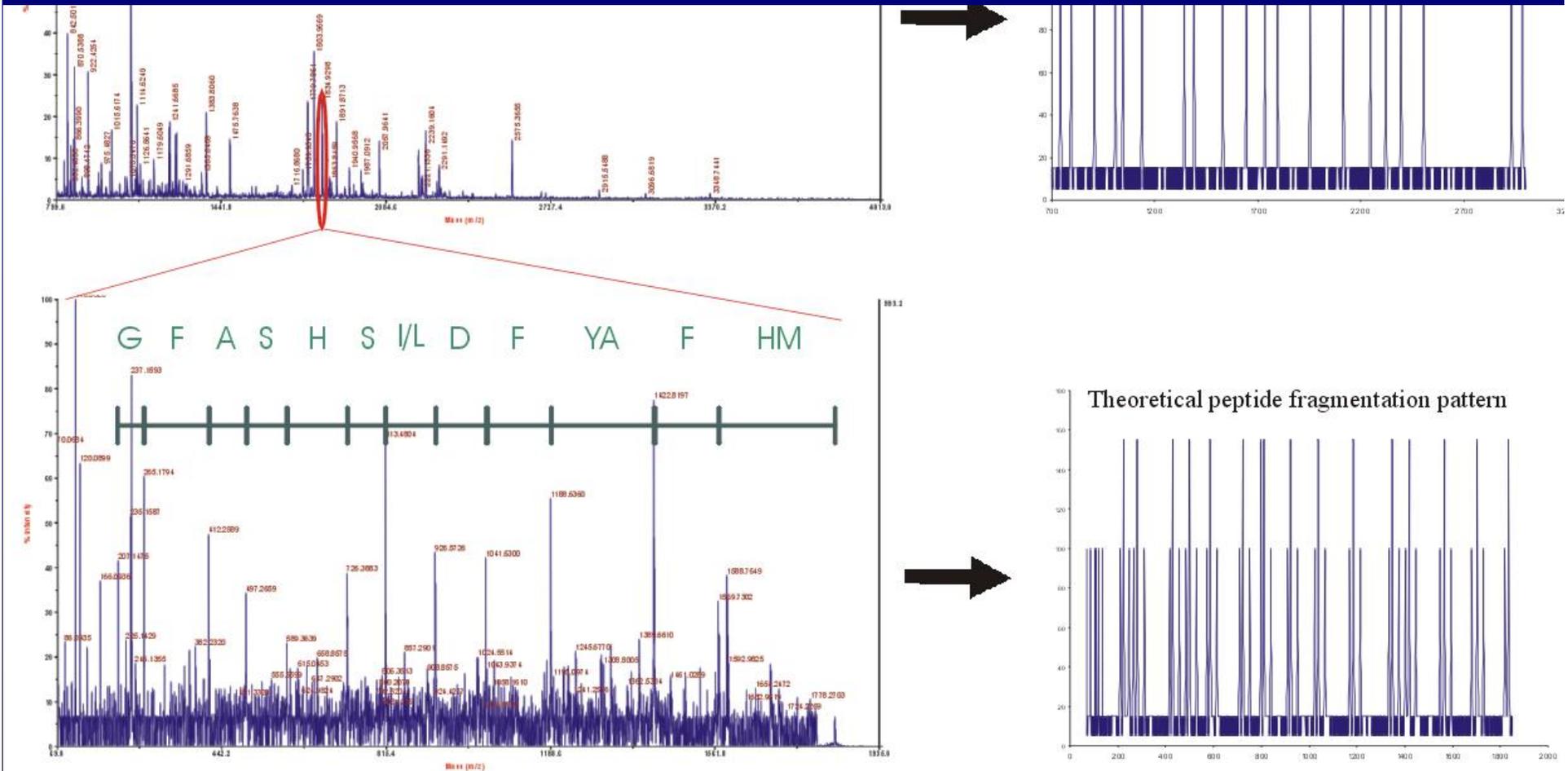
The peaks in the mass spectrum each represent a peptide present in the tryptic digest. A preliminary identification can be made by matching this spectrum to a database containing theoretical tryptic digests of all known proteins. This type of analysis is called peptide mass fingerprinting (PMF).



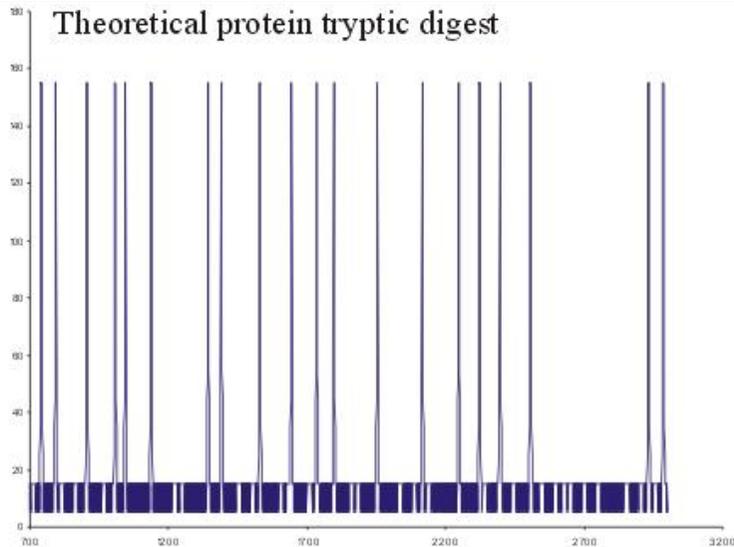
from: <http://www.med.monash.edu.au/biochem/facilities/proteomics/maldi.html>

# III) Protein Identification: Mass Spectrometry

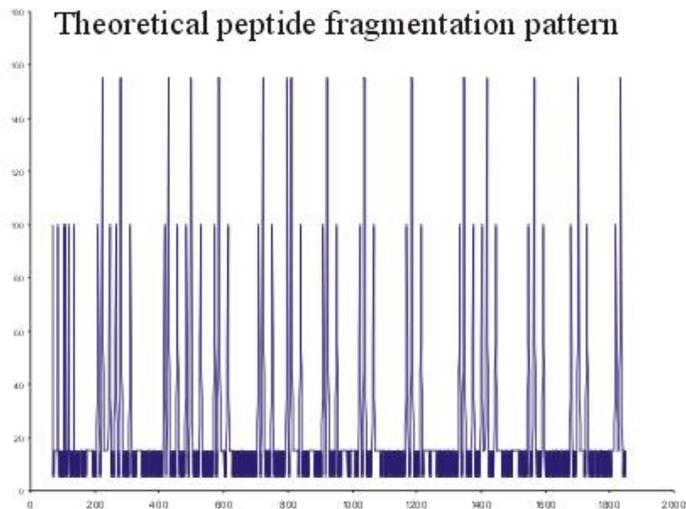
- 3a) further fragmentation of one of the fragments from the digest in the mass spectrometer, then again mass spectrometry (MSMS)
- 4a) comparison of the fragment sizes with a database



# III) Protein Identification: Mass Spectrometry



- 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



GPS Explorer TM Software - Results Browser

MSMS screen

### Mascot Search Results

User: |  
Email: |  
Search title: SampleSetID: 63, AnalysisID: 115, MaldiWellID: 2699, SpectrumID: 11198, Path:\dav  
Database: SwissProt 041223 (167089 sequences; 60988342 residues)  
Taxonomy: Homo sapiens (human) (11777 sequences)  
Timestamp: 14 Mar 2005 at 04:53:23 GMT  
Top Score: 293 for **AIAT\_HUMAN**, (P01009) Alpha-1-antitrypsin precursor (Alpha-1 protease inh

#### Probability Based Mowse Score

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

Protein Summary Report

[Switch to Peptide Summary Report](#)

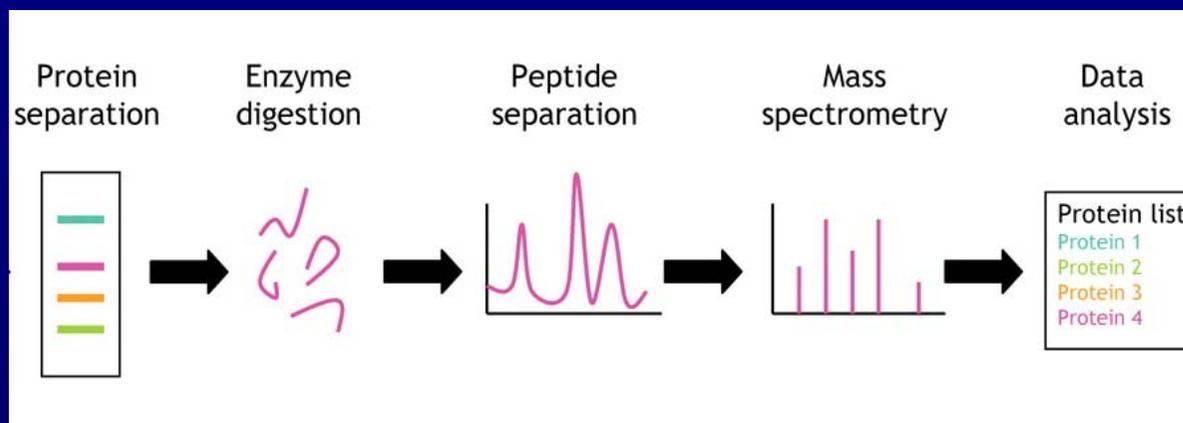
To create a bookmark for this report, right click this link: [Protein Summary Report \(SampleSetID: 63, AnalysisID: 115, MaldiWellID: 2699, SpectrumID: 11198\)](#)

Re-Search All Search Unmatched

#### Index

Accession	Mass	Score	Description
1. <a href="#">AIAT_HUMAN</a>	46878	293	(P01009) Alpha-1-antitrypsin precursor (Alpha-1 protease inh
2. <a href="#">ZAP5_HUMAN</a>	40216	56	(Q14320) ZAP-5 protein (RRC-26 protein)
3. <a href="#">FABP_HUMAN</a>	37266	54	(Q6ET52) Protein FABP4B
4. <a href="#">TAG3_HUMAN</a>	22629	51	(Q9UI15) Transgelin-3 (Neuronal protein NP25) (Neuronal prote
5. <a href="#">SPECP_HUMAN</a>	272496	45	(O15020) Spectrin beta chain, brain 2 (Spectrin, non-erythro

### III) Protein Identification: Mass Spectrometry



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

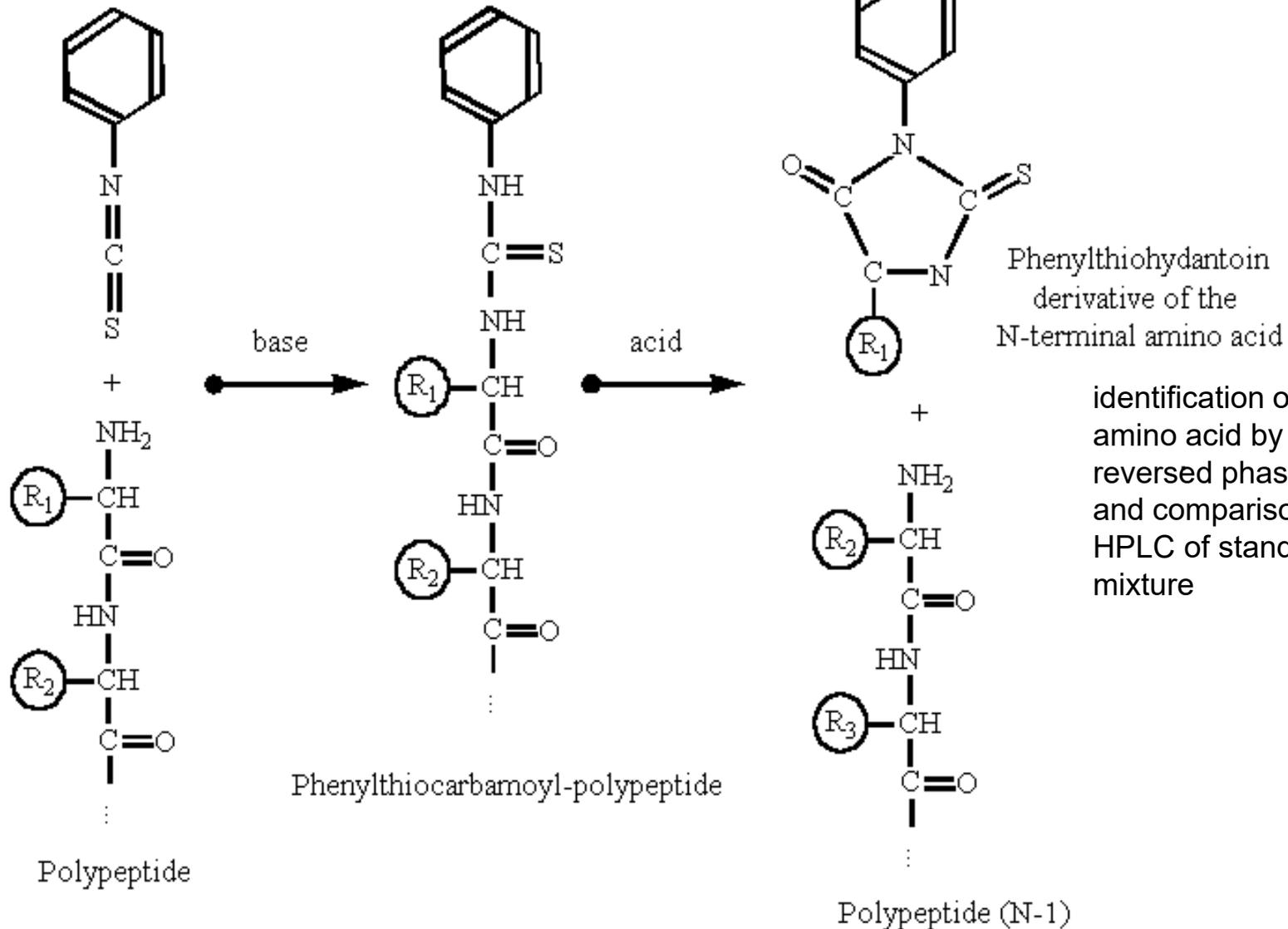
#### Limitations and artefacts

- 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

# III) Protein Identification: N-terminal sequencing

## Principle

Phenylisothiocyanate



# 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

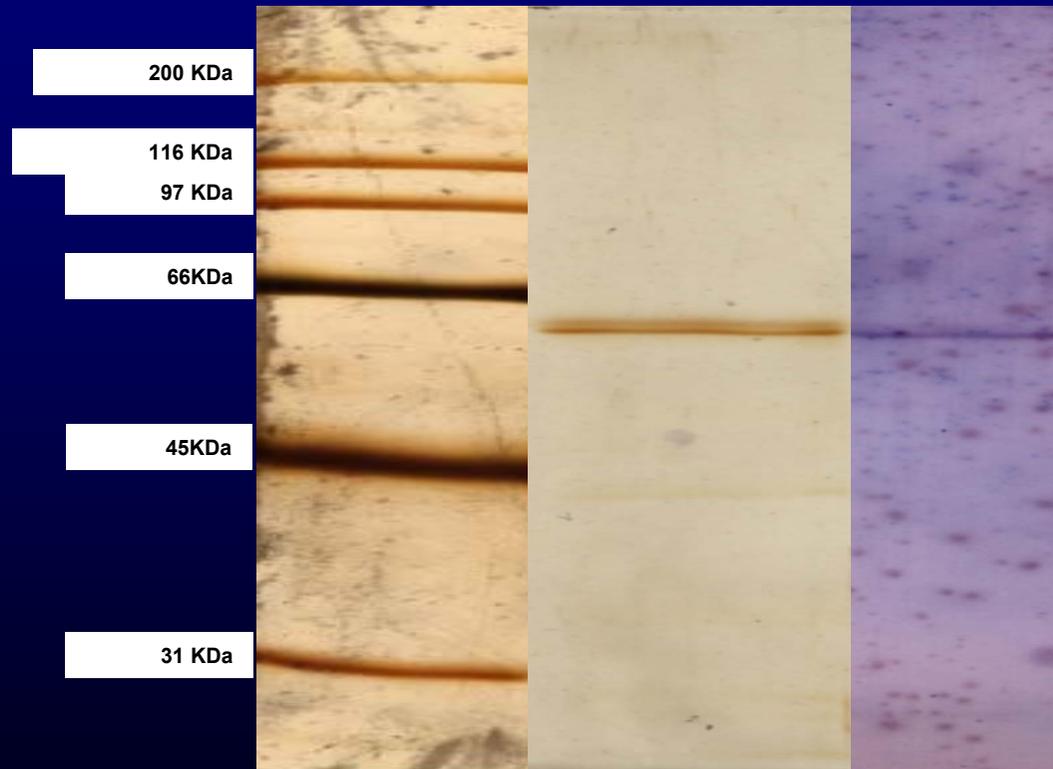
- problems with contaminations
- in eukaryotic proteins, the N-terminus is often blocked (e.g. by methylation), which required complicated de-blocking procedures. Also non-polymerised acrylamide remains in the gel can cause blocking, so let the gel polymerise over night
- Cys residues cannot be detected,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



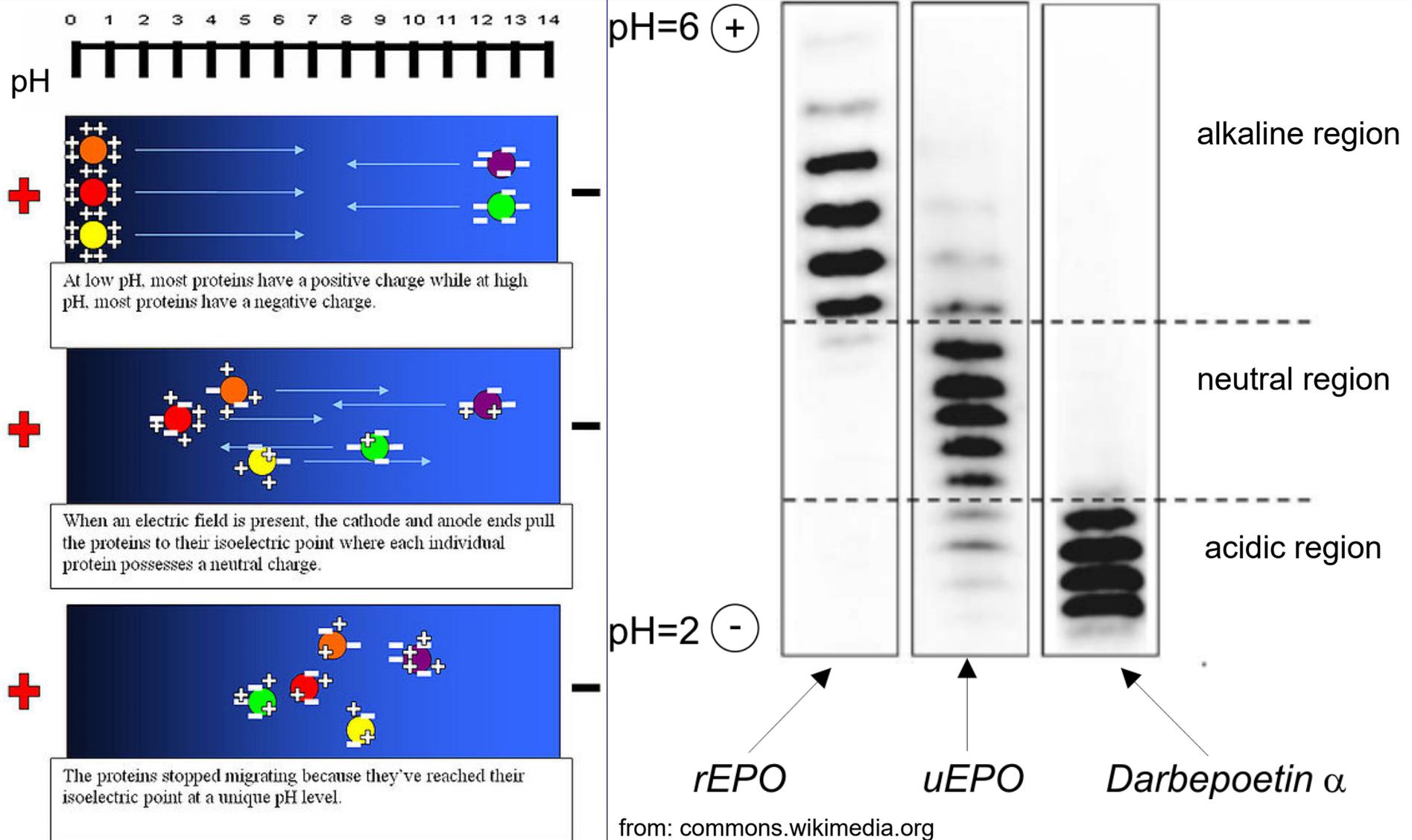
SDS gel and  
Western blot of  
TcHMA4

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

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

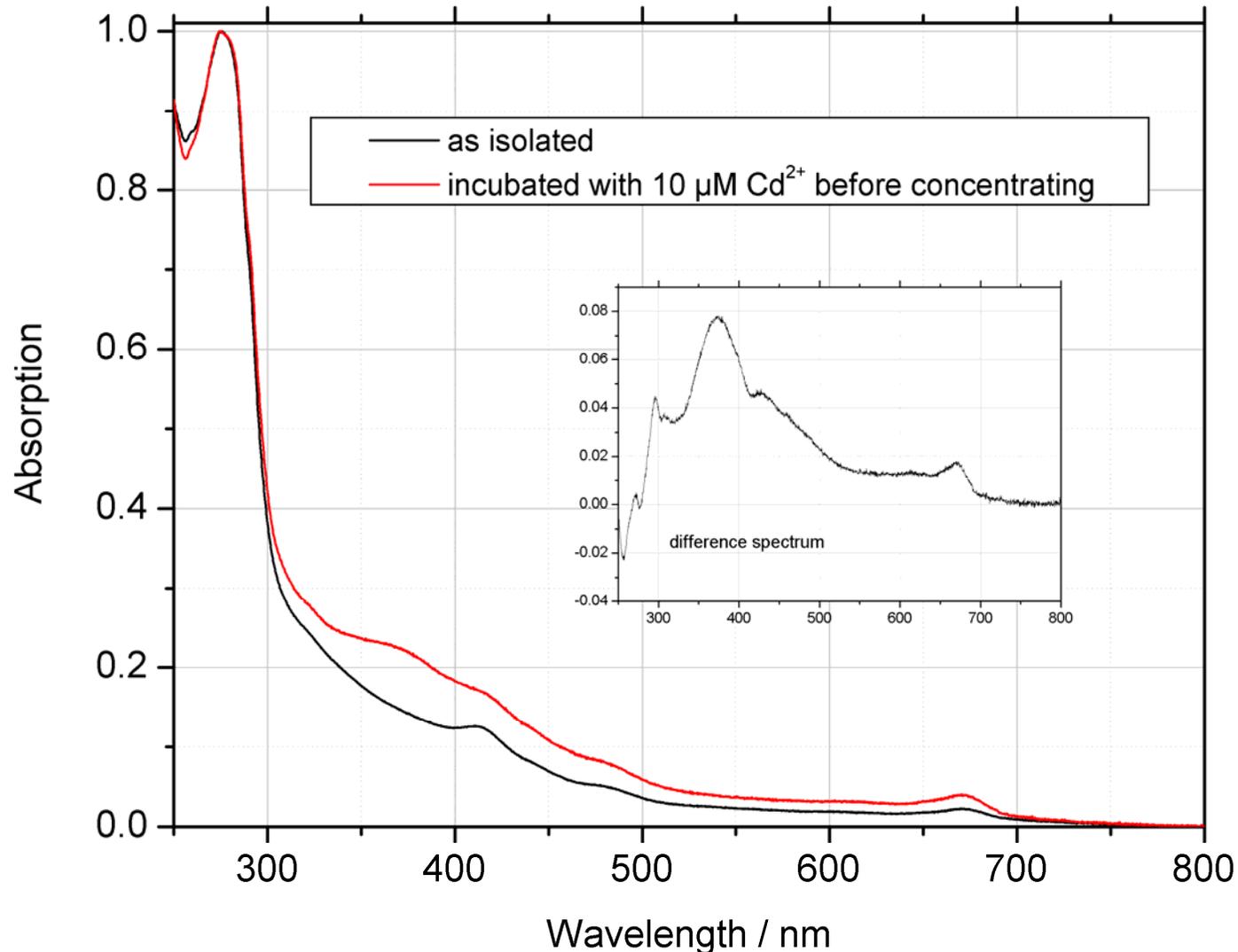
## IV) Protein Characterisation: Charge determination

- 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



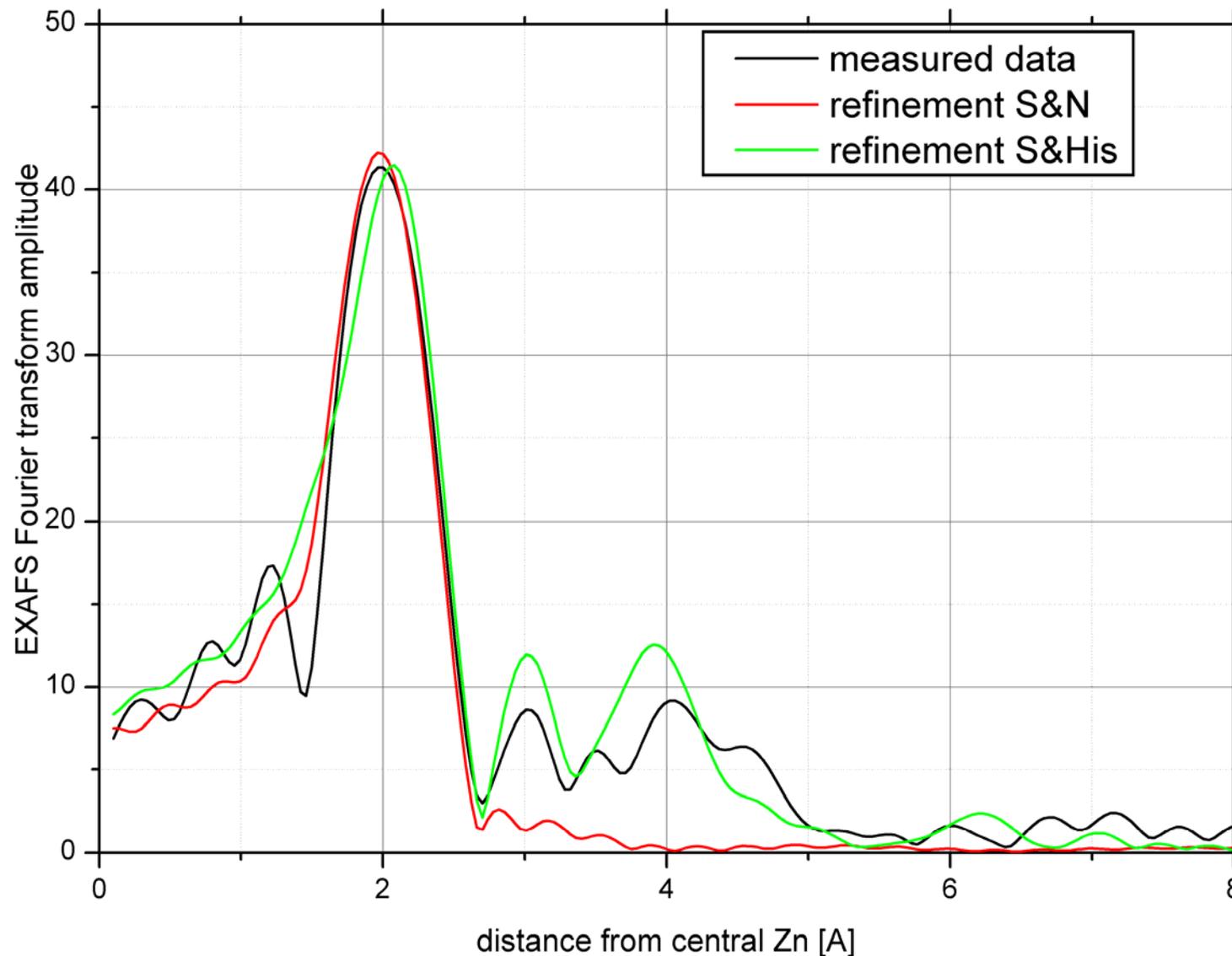
## IV) Protein Characterisation: Analysis of cofactors

- 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



## 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 strengths)



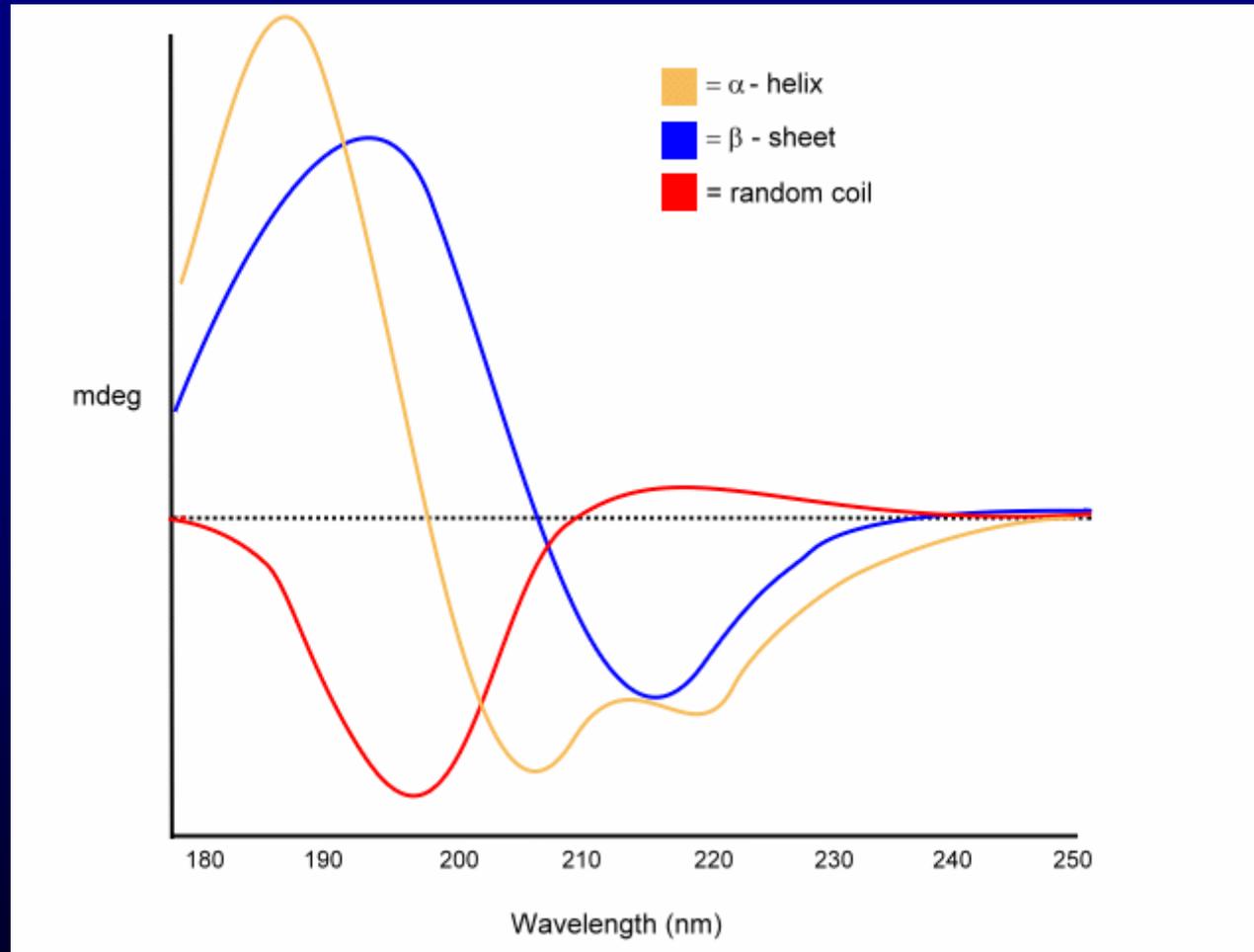
EXAFS spectrum of Zn-TcHMA4

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

(Leitenmaier and Küpper, unpublished)

# IV) Protein Characterisation: Analysis of the 3D Structure

## Circular Dichroism (CD) Spectroscopy



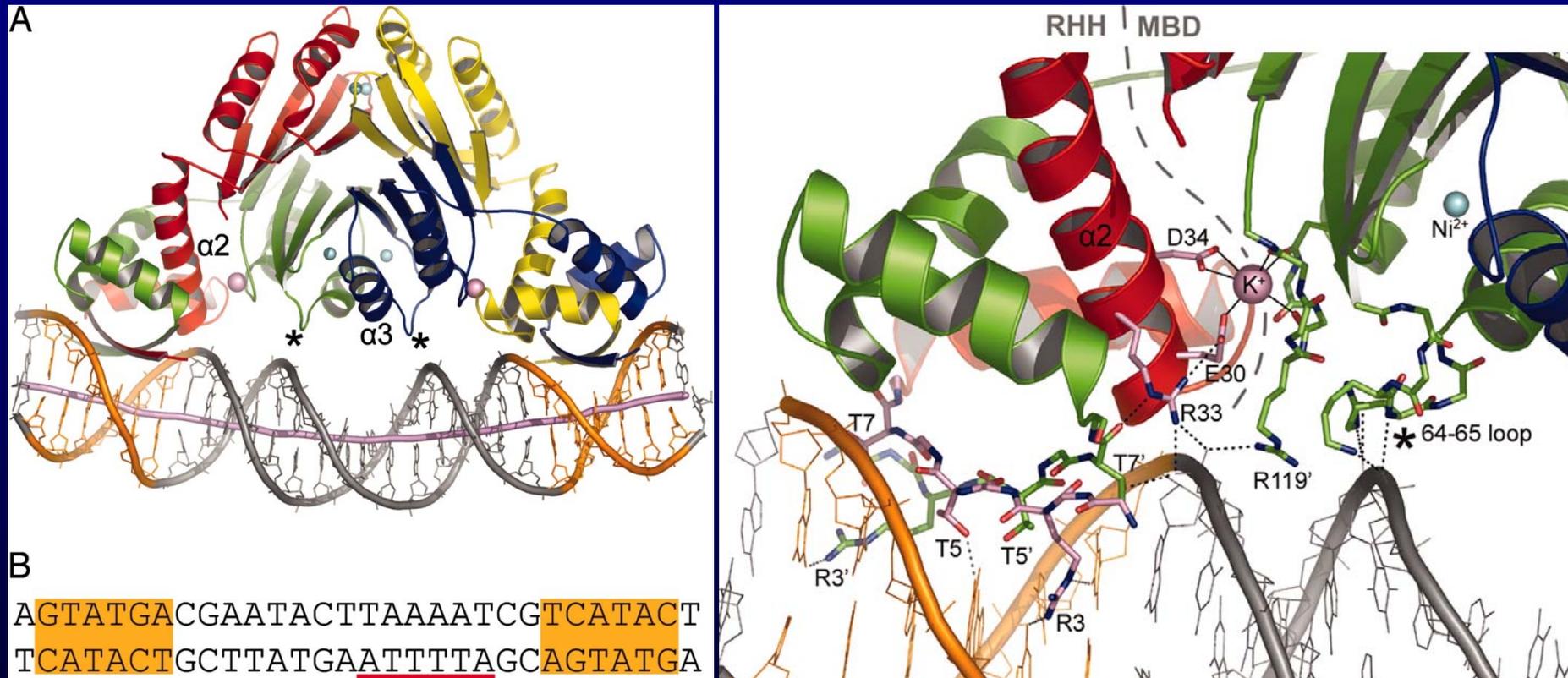
From: [www.proteinchemist.com/cd/cdspec.html](http://www.proteinchemist.com/cd/cdspec.html)

- 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

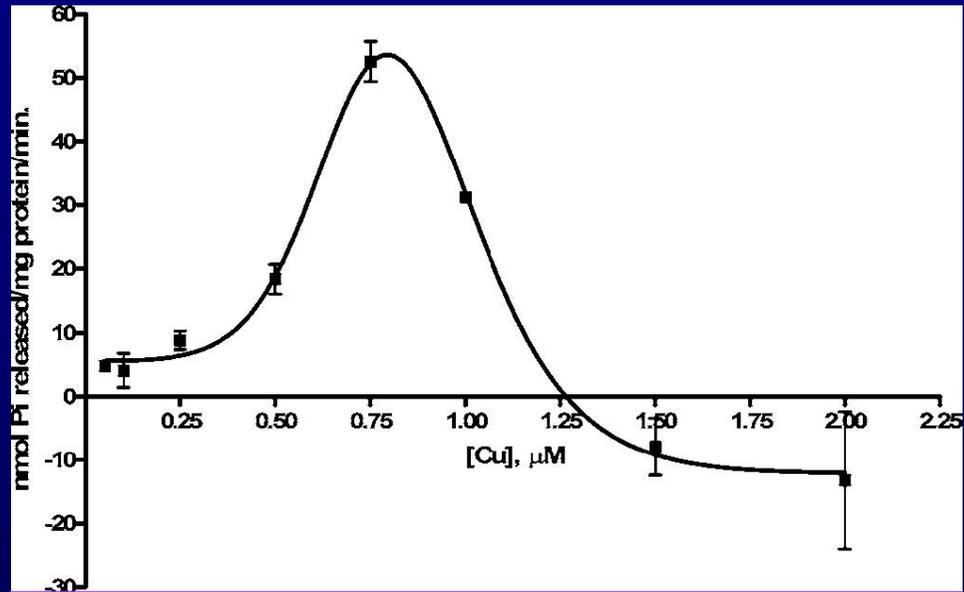


From: Schreiter ER et al., 2006, PNAS103, 13676-81

- Ni binding causes RHH domains to rotate about the flexible interdomain linkers to orient their antiparallel  $\alpha$ -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

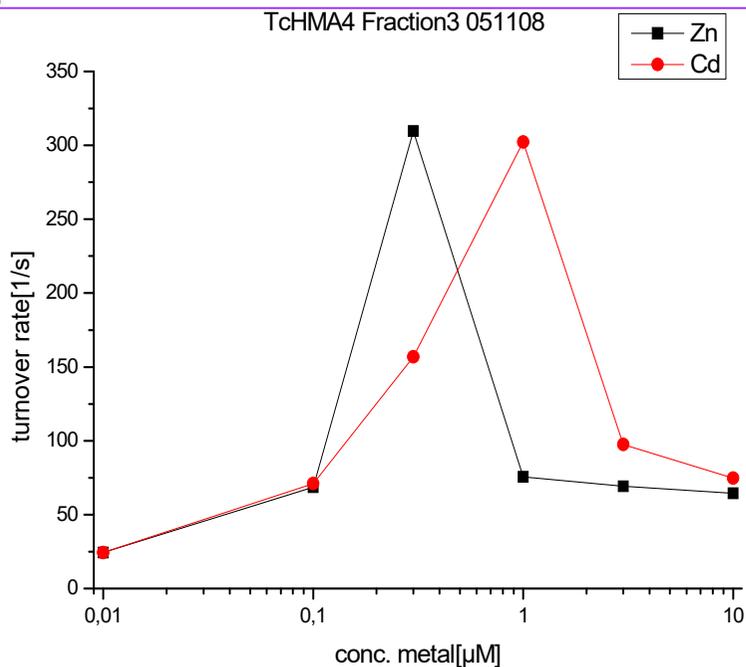
Titration of an enzyme with its substrate(s) reveals binding constants and possible substrate inhibition



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

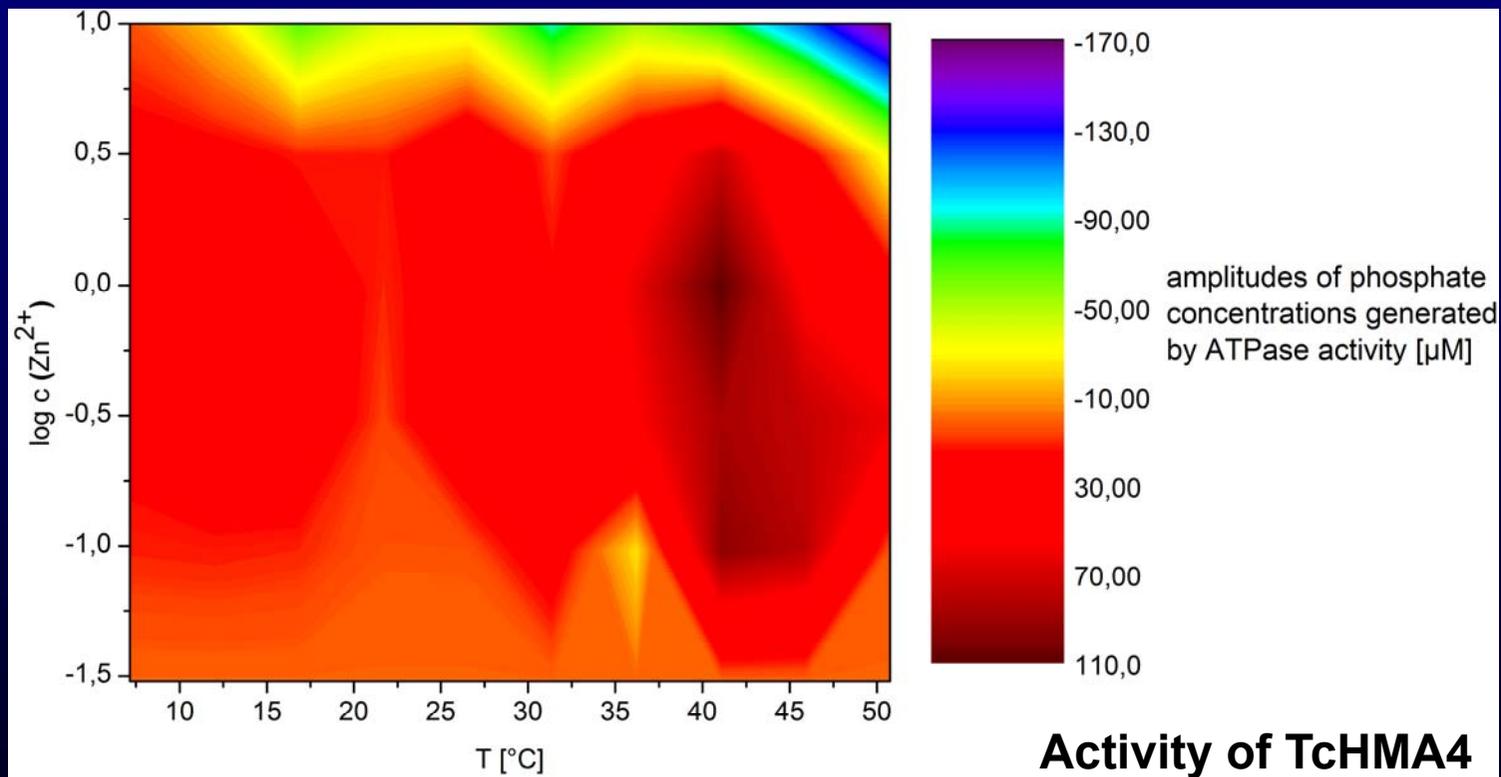


Activity of TcHMA4

(purified by Leitenmaier and Küpper)

## 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



(Leitenmaier et al (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

Grind the frozen roots with isolation buffer in liquid N<sub>2</sub>, thaw

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

Centrifuge for removing insoluble residue, collect solubilised protein

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  
(→ 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](http://webserver.umbr.cas.cz/~kupper/AG_Kuepper_Homepage.html)