Oddělení funkční genomiky a proteomiky
Národní centrum pro výzkum biomolekul
Přírodovědecká fakulta MU

Charakterizace proteinů hmotnostní spektrometrií

Bi7050

Část IV

Zbyněk Zdráhal

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Oddělení funkční genomiky a proteomiky, NCBR PřF
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MS charakterizace modifikací

posttranslačních

Fosforylace

Protein Phosphorylation is of Fundamental Importance in Biological Regulation

cca 10-30% of all proteins are phosphorylated

- S, T, Y 1800 : 200 : 1 *www.protein.sdu.dk*
- H ???

*Whereas phosphorylation of **serine, threonine or tyrosine** results in the formation of **a phosphoester linkage**, phosphorylation of **histidine** residues occurs **on nitrogen atoms**, producing a phosphoramidate bond. Phosphohistidines have a large standard free energy of hydrolysis making them **the most unstable** of any known phosphoamino acid.*

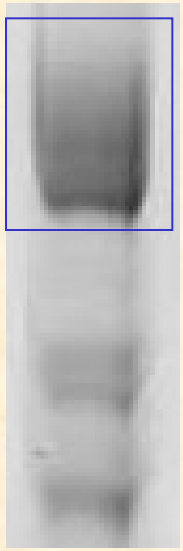
Klumpp et al, *Eur. J. Biochem*. **269**, 1067-1071 (2002)

Phosphorylation sites db: http://phospho.elm.eu.org

Fosfoproteom - Potížista

- potlačení signálu v MS
  - pouze malá část z celkového počtu proteinů je fosforylovaná
  (přednostní ionizace nemodifikovaných peptidů)
- většina signálních proteinů je v buňce v nízkých koncentracích
- protein se může vyskytovat v různých fosfo formách
- proteiny mohou být během zpracování defosforylovány fosfatázami

úprava vzorku
obohacení



Specifické barvení fosforylovaných proteinů, 2D GE

(from the Molecular Probes website)

phosphoproteins
(Pro-Q Diamond , **blue**)
proteins
(SYPRO Ruby, **red**).

alternativa
Metabolické značení ^{32}P
měření radioaktivity

immunoblotting

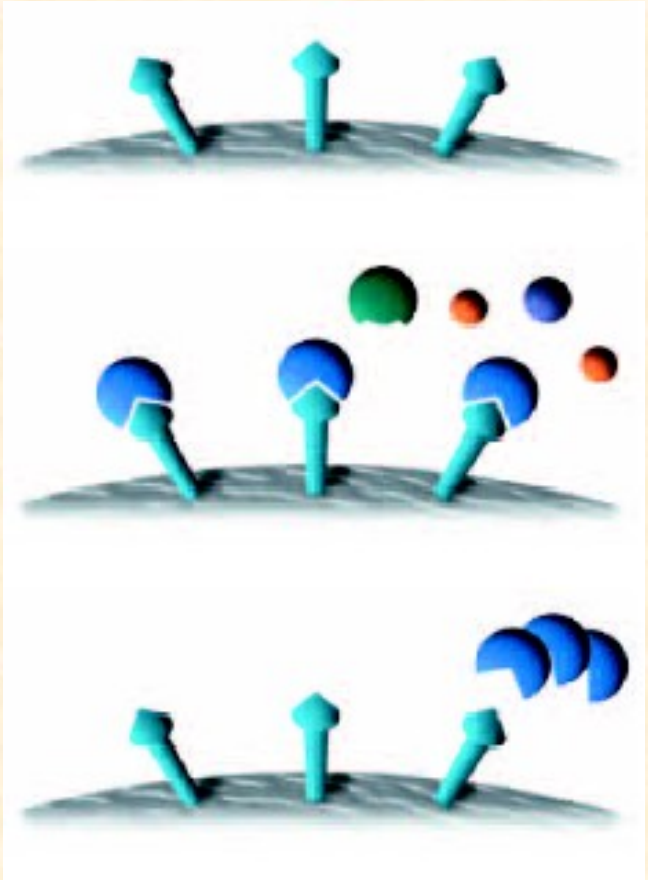
phosphatase treatment
phosphoproteins display a basic shift in their pI after the dephosphorylation.
comparison 2D gels

Immobilized metal affinity chromatography (IMAC)

charging

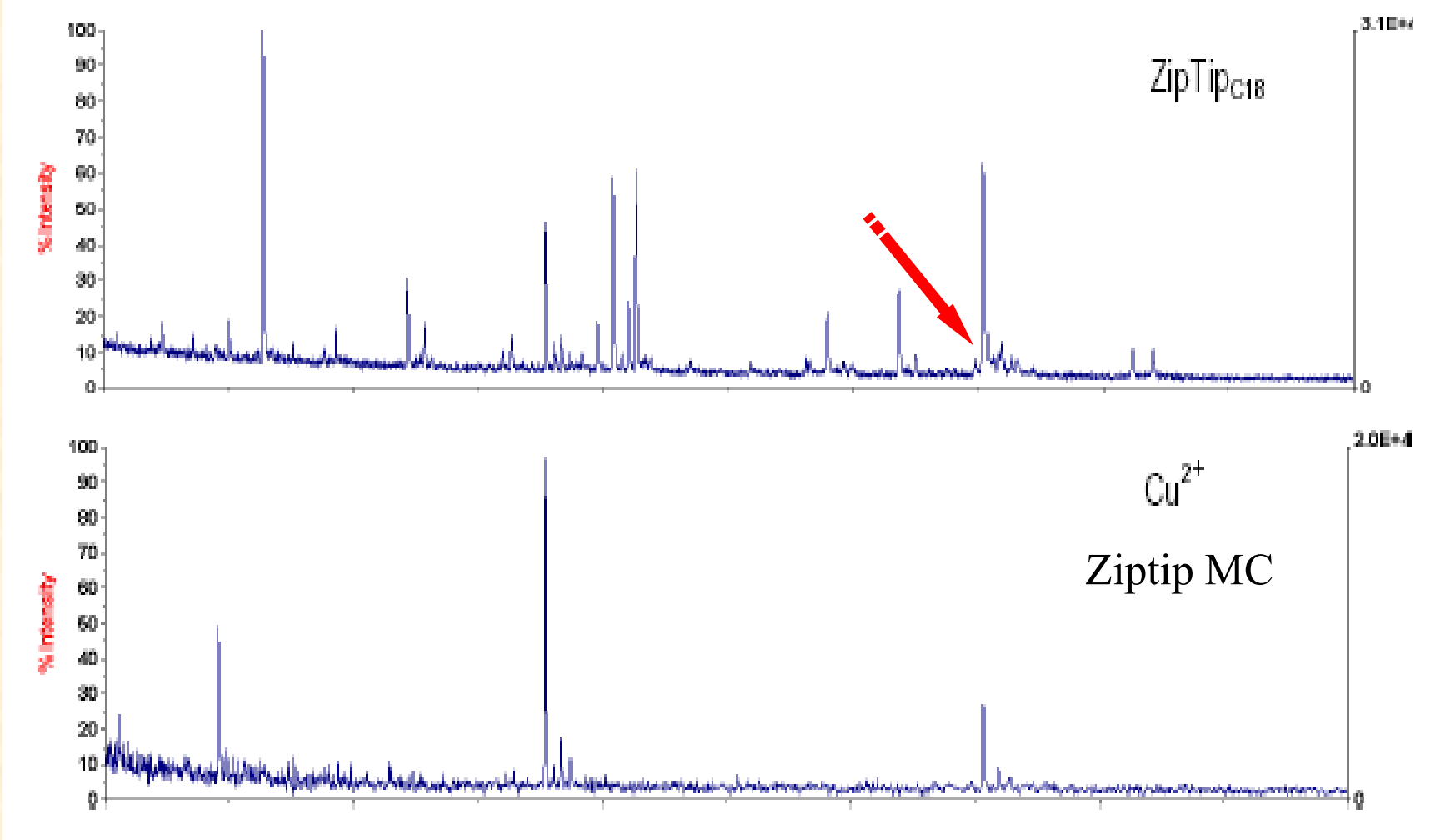
specific binding

eluce



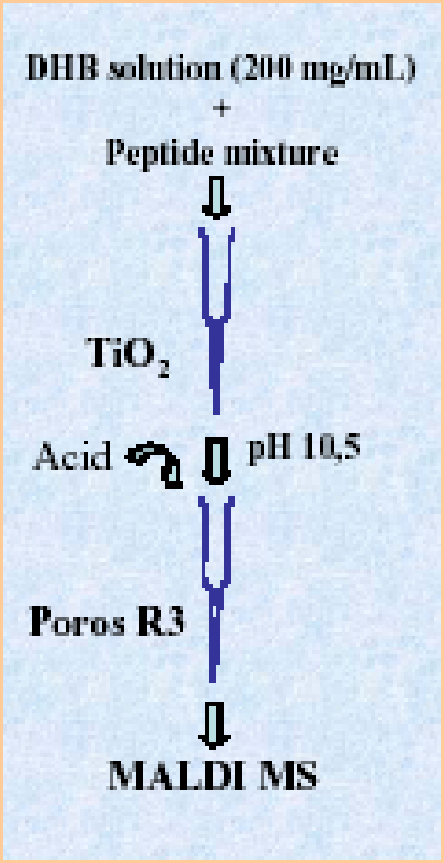
(Cu^{2+}, Fe^{3+}, Ga^{3+})

IMAC enrichment of -Casein phosphopeptides (1 pmol of tryptic digest)

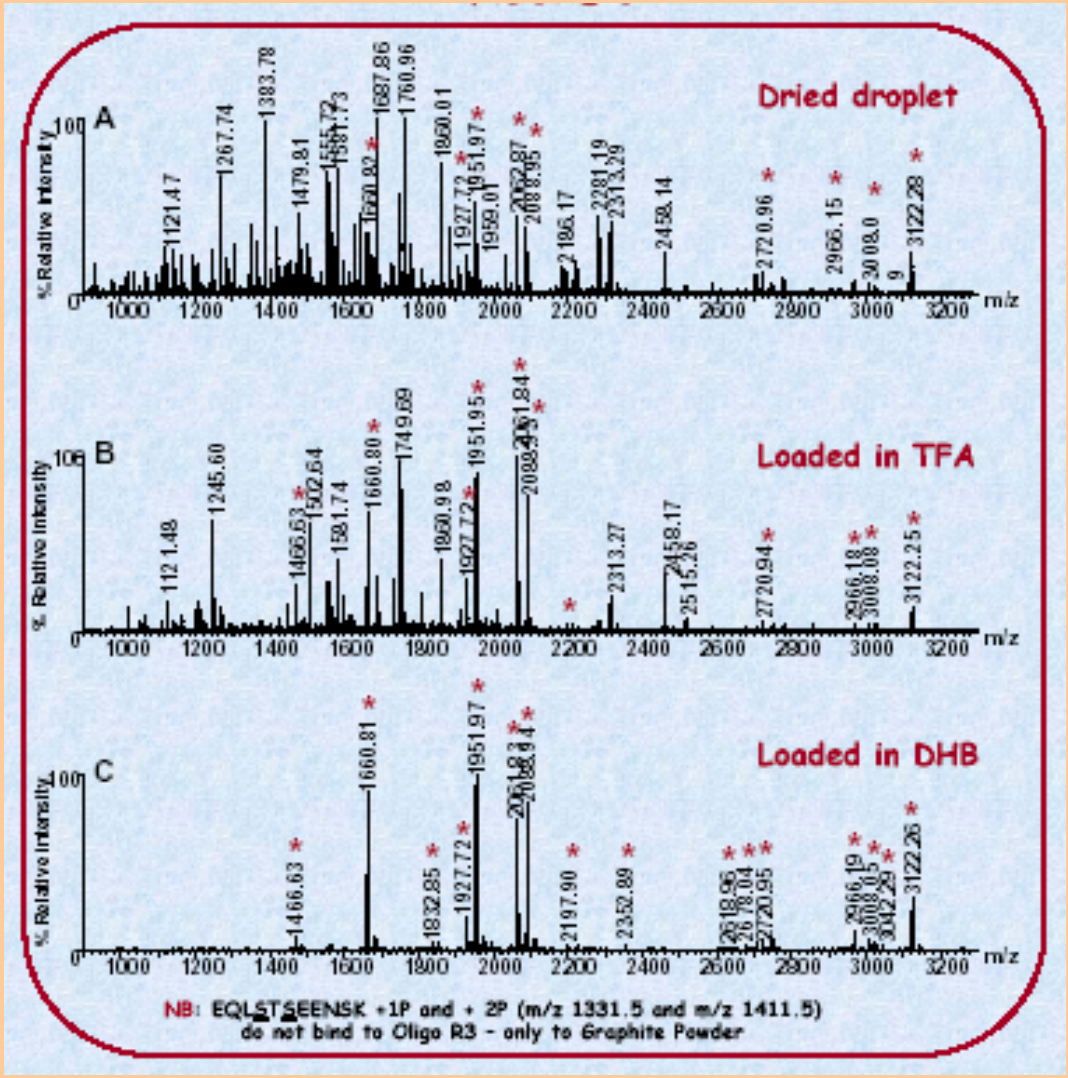


Technical note - Millipore

Enrichment of phosphopeptides by TiO_2



eluce změnou pH



z posteru M.R. Larsena, HUPO, 2005

Kasein (1 ug) po digesci trypsinem

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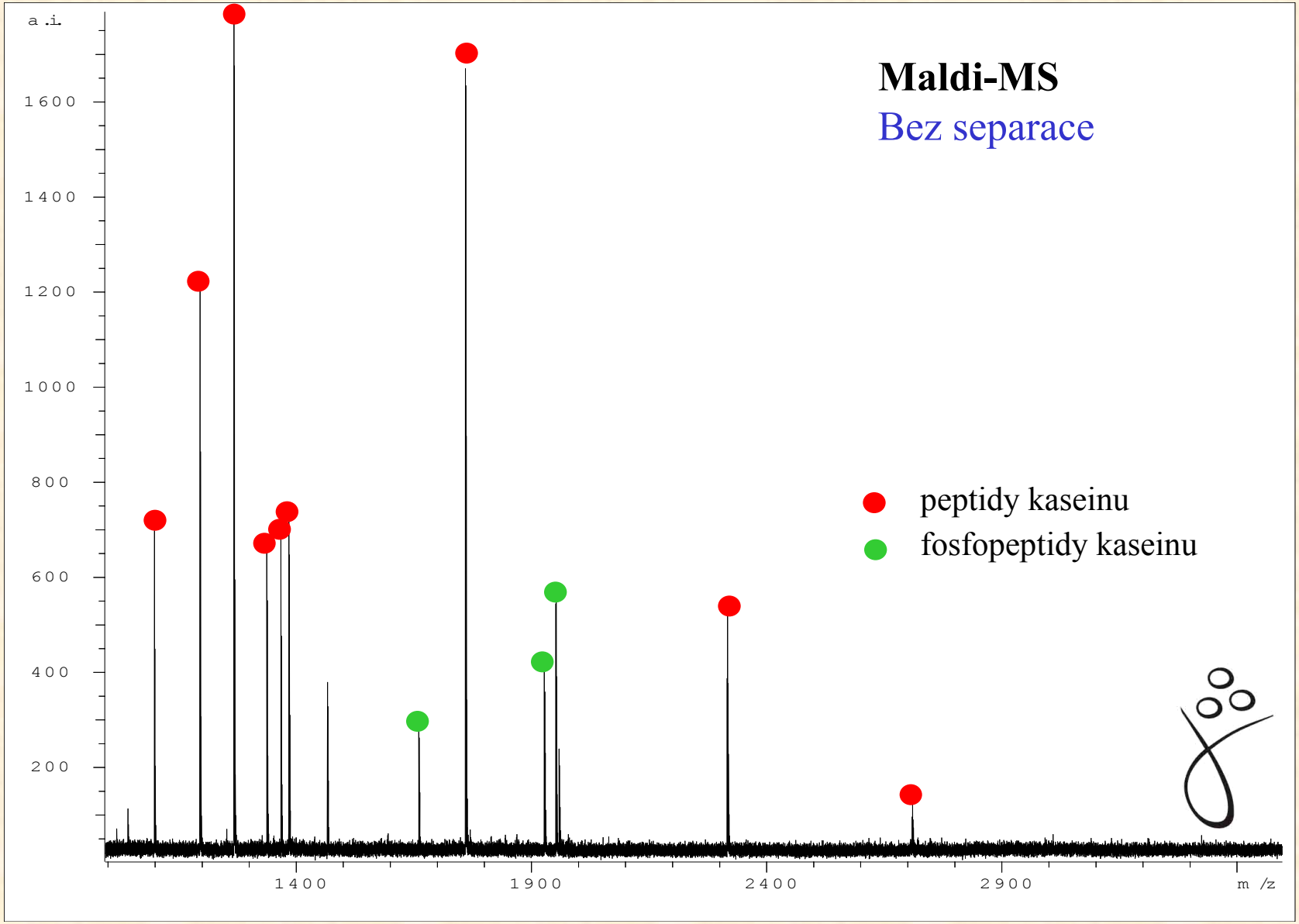

Kasein (1 ug) po digesci trypsinem

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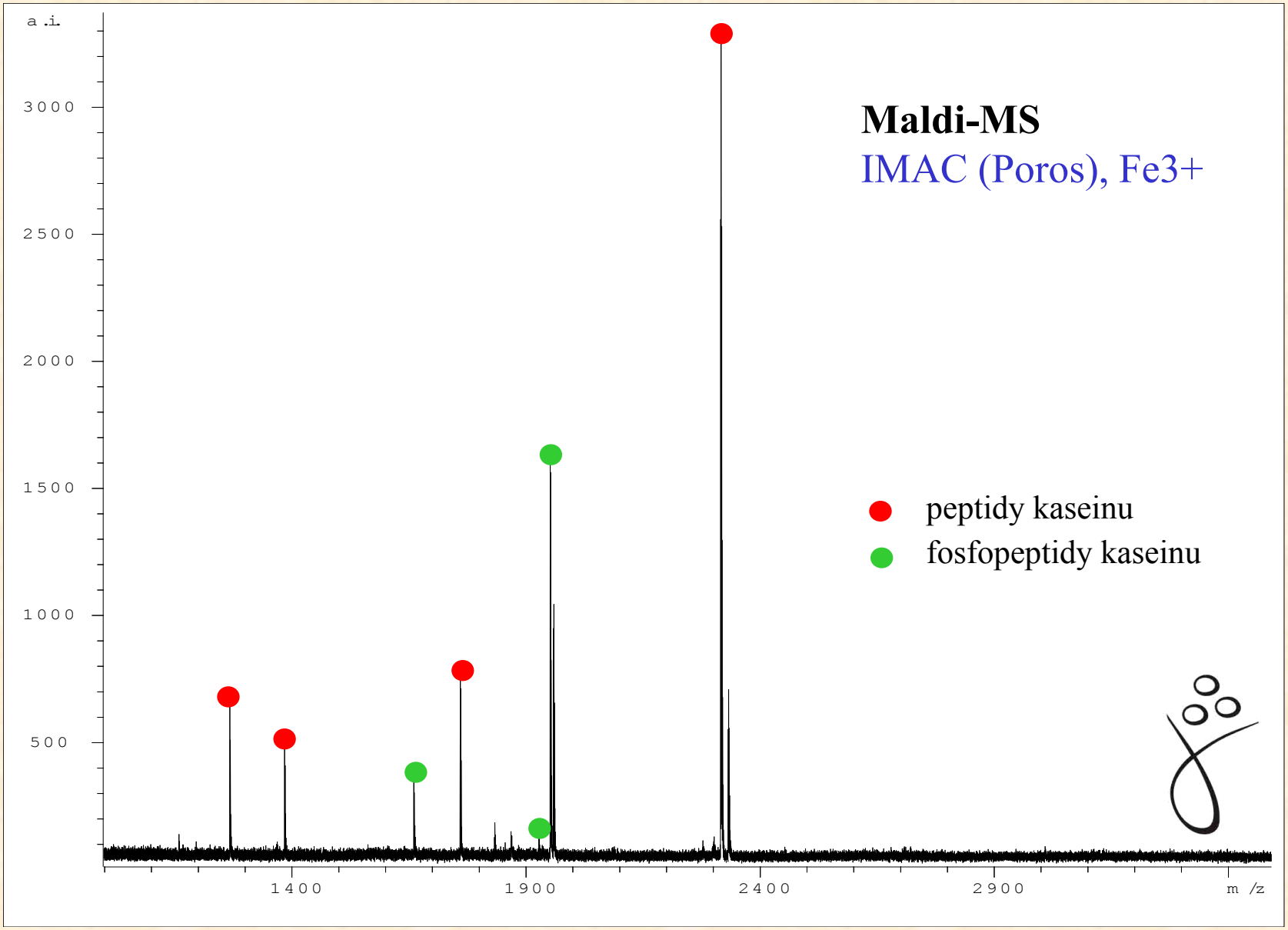
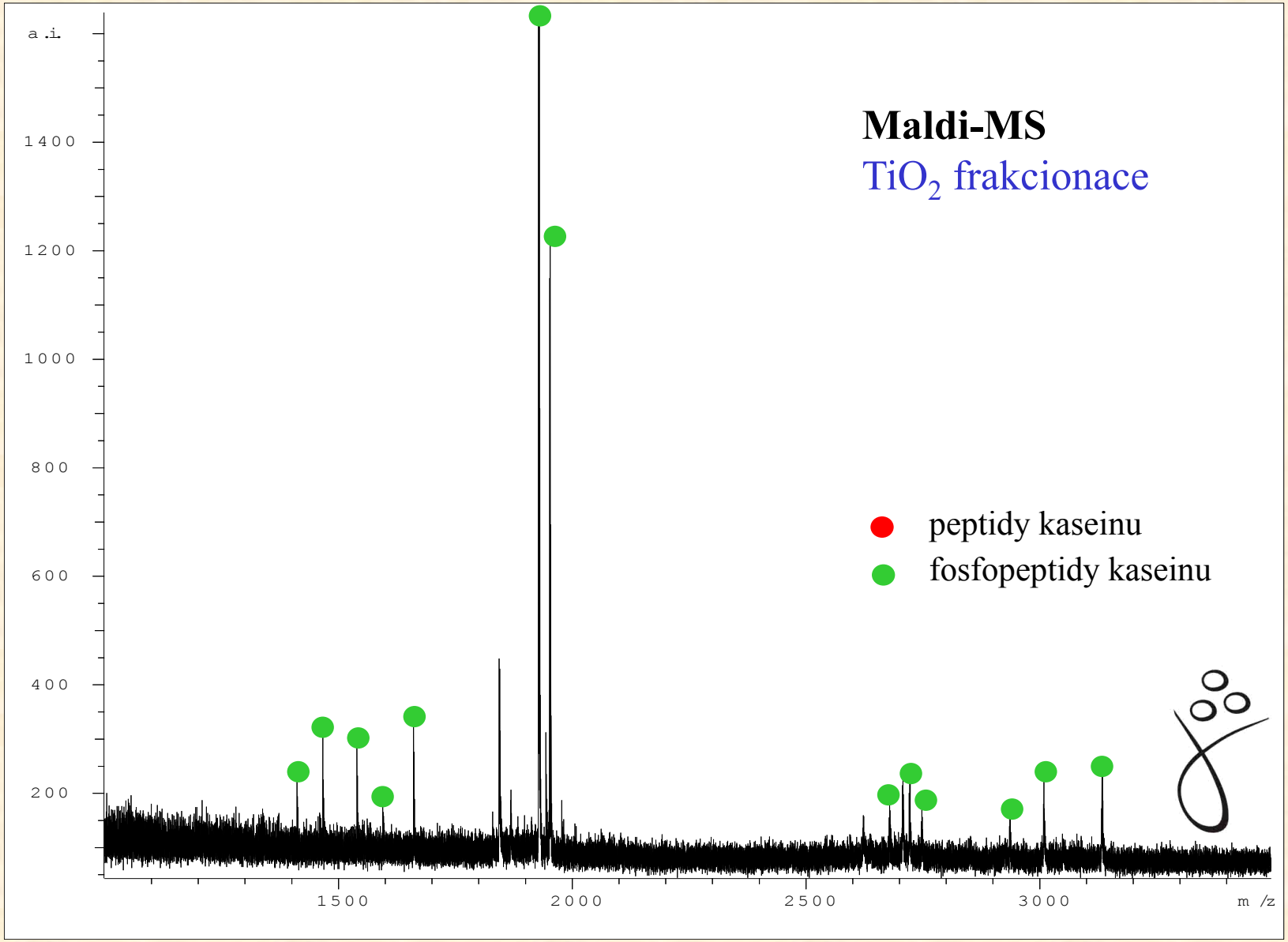
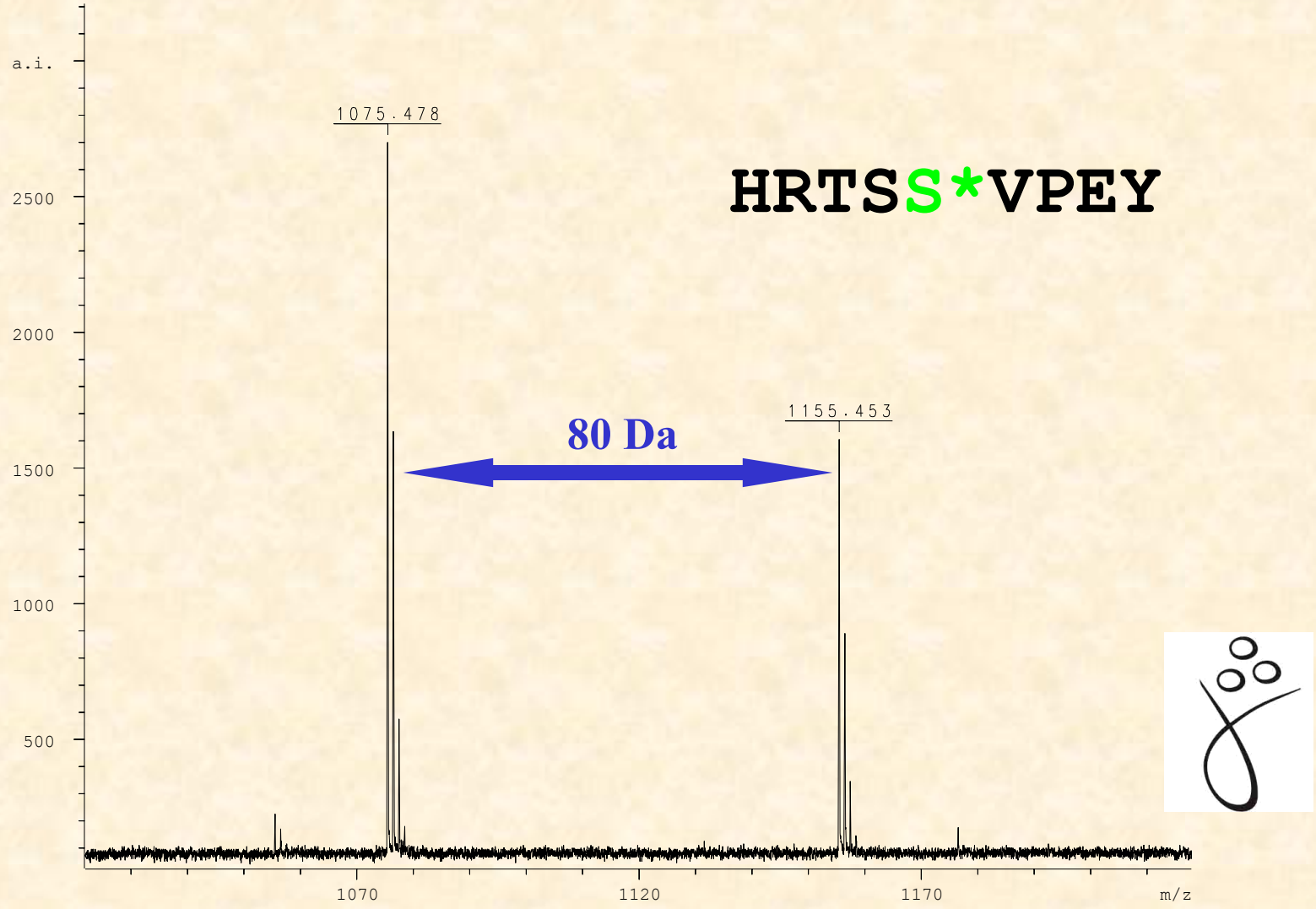


Kasein (1 ug) po digesci trypsinem

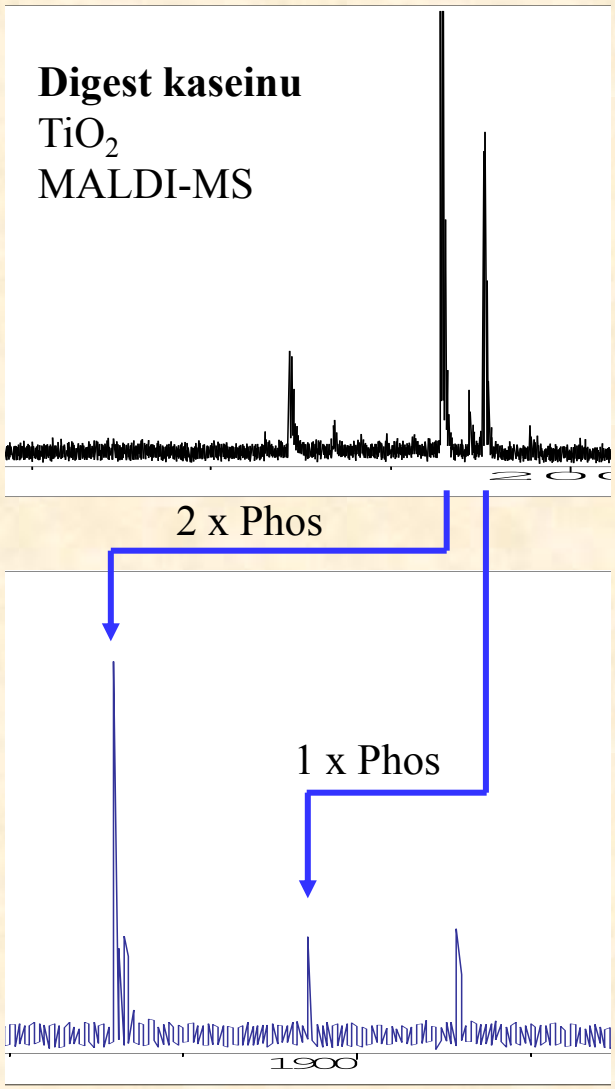
Bi7050



MALDI–MS spektrum peptidu bez a s fosforylací



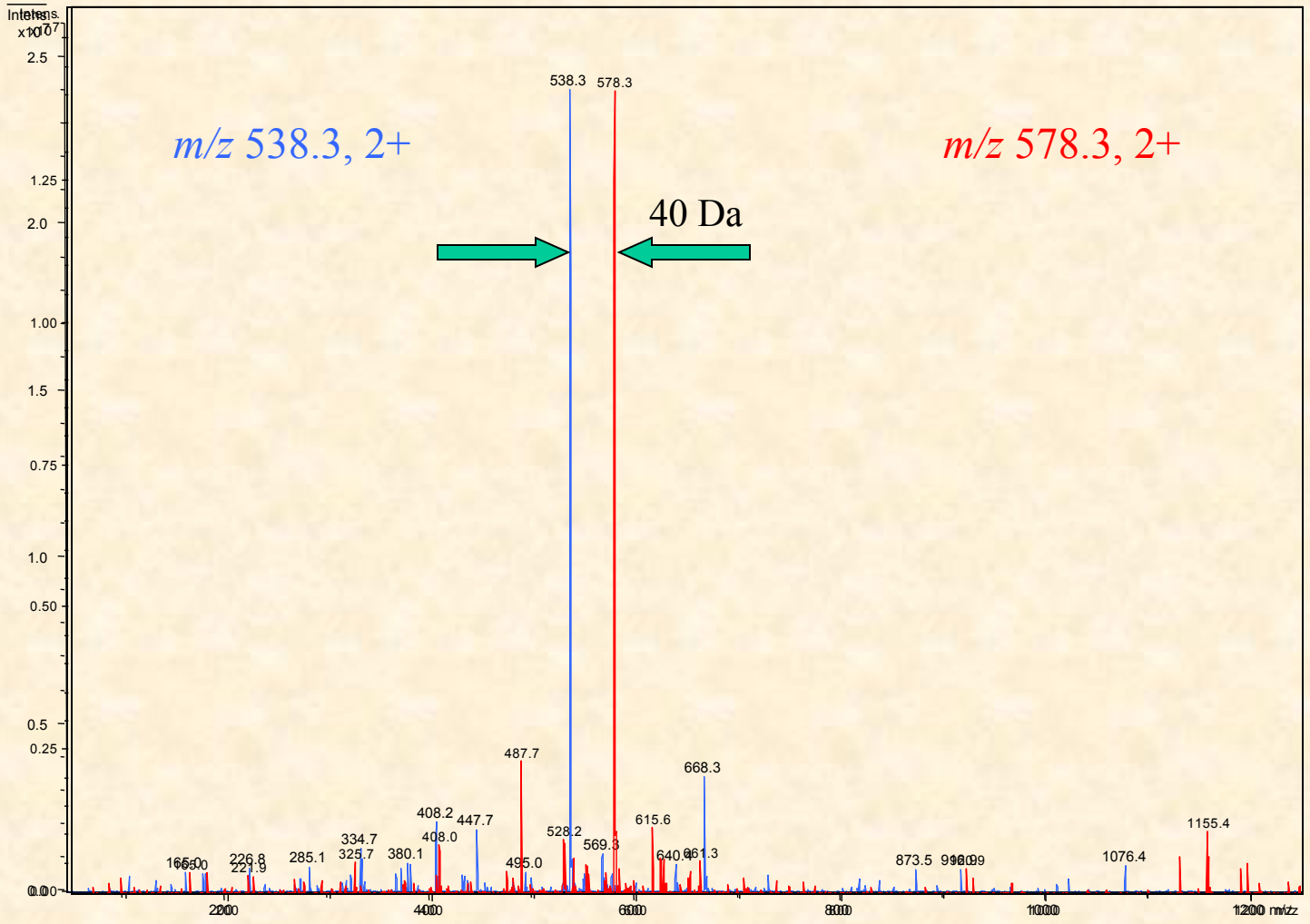
Potvrzení fosforylace pomocí alkalické fosfatázy



ESI–MS (IT) spektrum peptidu bez a s fosforylací

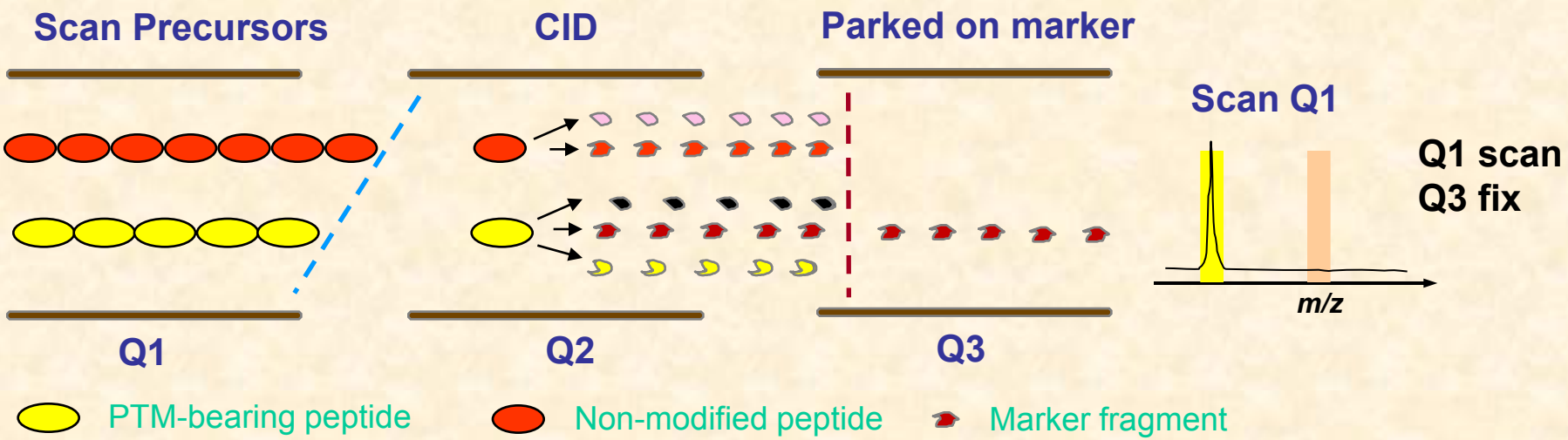
Bi7050

positive mode



Precursor Ion Scan

Sken prekurzorů



- kvadrupol **Q1** analyzuje všechny ionty prekursorů, zaznamenány jsou jen prekurzory, z kterých vznikl fragmentací v kolizní cele (Q2) vybraný fragment s hledaným rozdílem v m/z
- kvadrupol **Q3** propouští na detektor pouze vybraný fragment (marker)

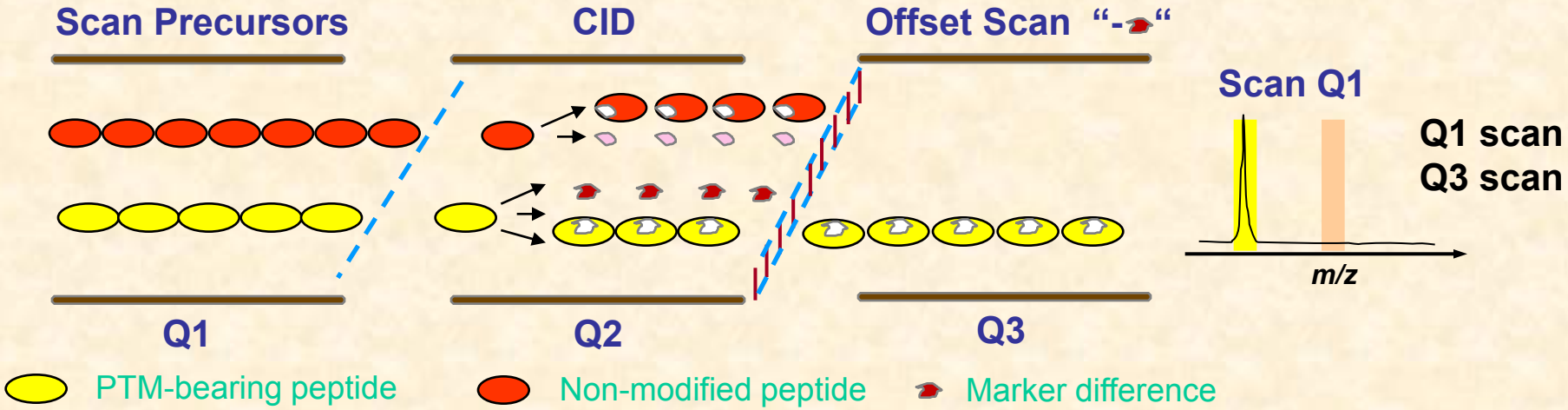
Marker fragment examples

- *-79 for phosphorylation (PO_3^-)*
- *+204 for N-glycosylation (HexNAc)*
- *+216 for Phosphotyrosine (pY immonium ion)*

upraveno z materiálů ABI

Constant Neutral Loss Scan

Sken neutrální ztráty



- kvadrupol **Q1** analyzuje všechny ionty prekursorů, zaznamenány jsou jen prekurzory, z kterých fragmentací v kolizní cele (Q2) vzniká fragment s odpovídající neutrální ztrátou (tj. nejčastěji $M_{prekurzor} - M_{neutrální\ molekula}$)
- kvadrupol **Q3** oproti **Q1** skenuje s posunem odpovídajícím hmotnosti vybrané neutrální ztráty

Constant Neutral Loss Scanning examples

- *Δ 49 for doubly charged phosphopeptides* ($Peptide^{2+}$-H_3PO_4)
- *Δ 32.7 for triply charged phosphopeptides* ($Peptide^{3+}$-H_3PO_4)

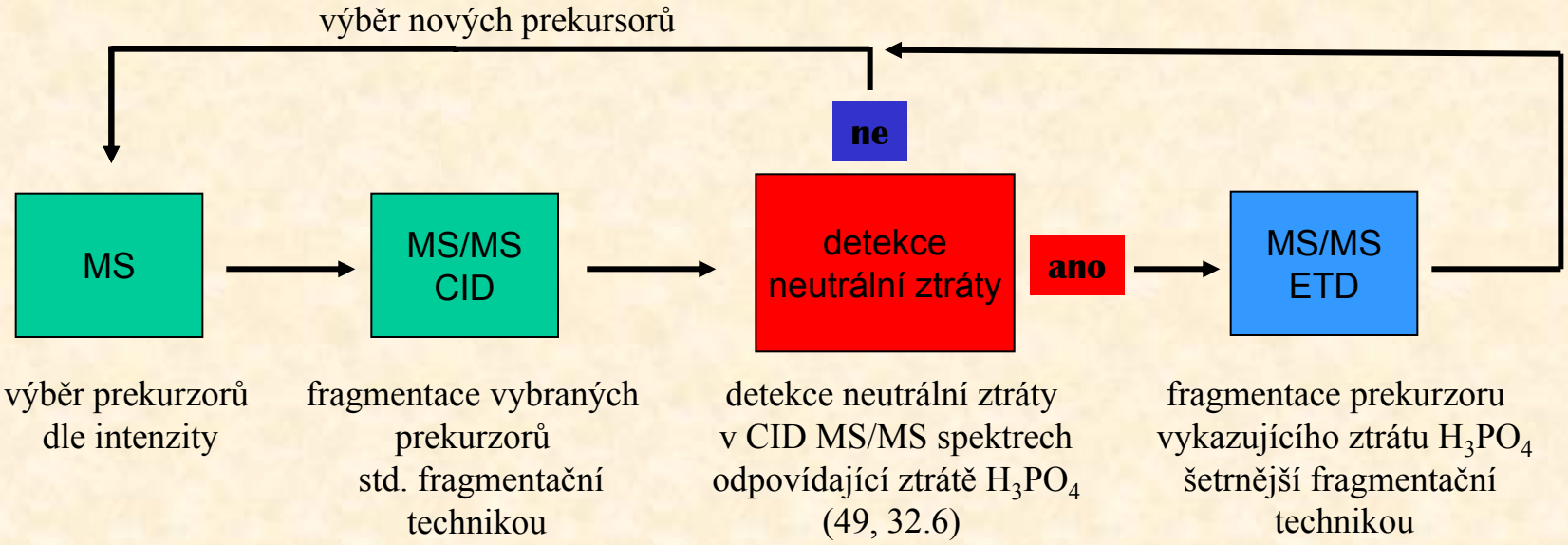
upraveno z materiálů ABI

Fosforylace histonu H4 kinázou Aurora B

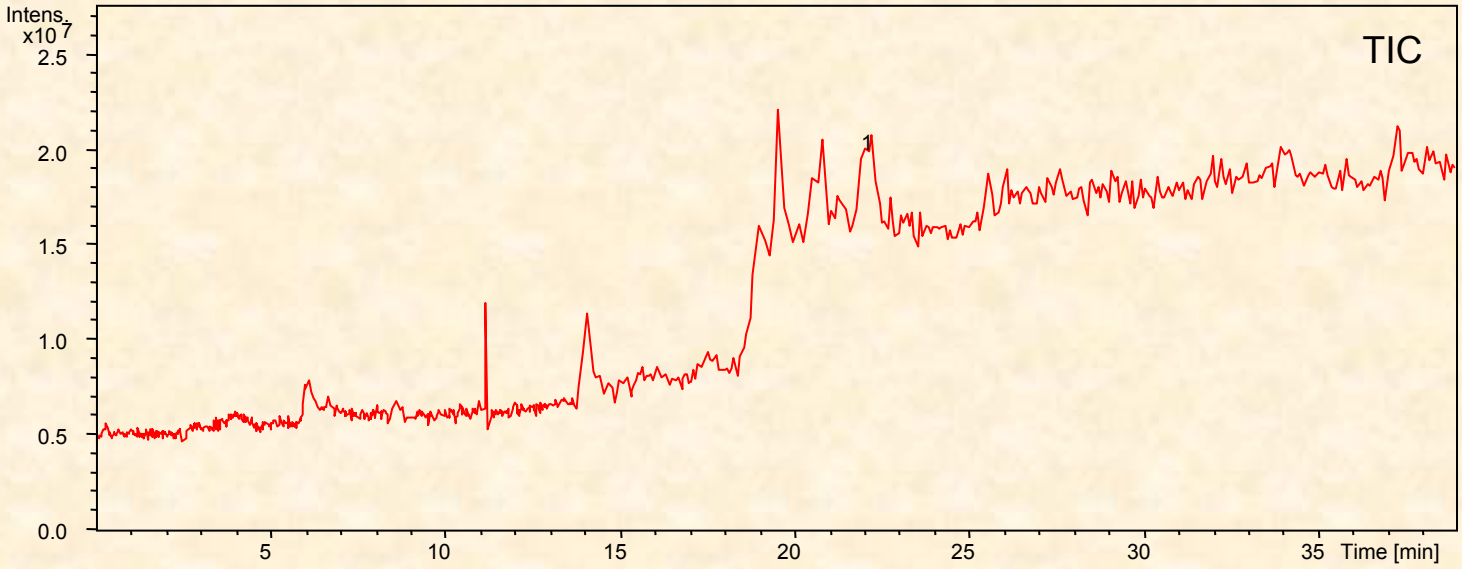
(K. Nováková)

- fosforylace
- proteolytické štěpení trypsinem
- frakcionace fosfopeptidů na TiO_2
- LC-MS/MS analýza – sken neutrální ztráty (ETD)

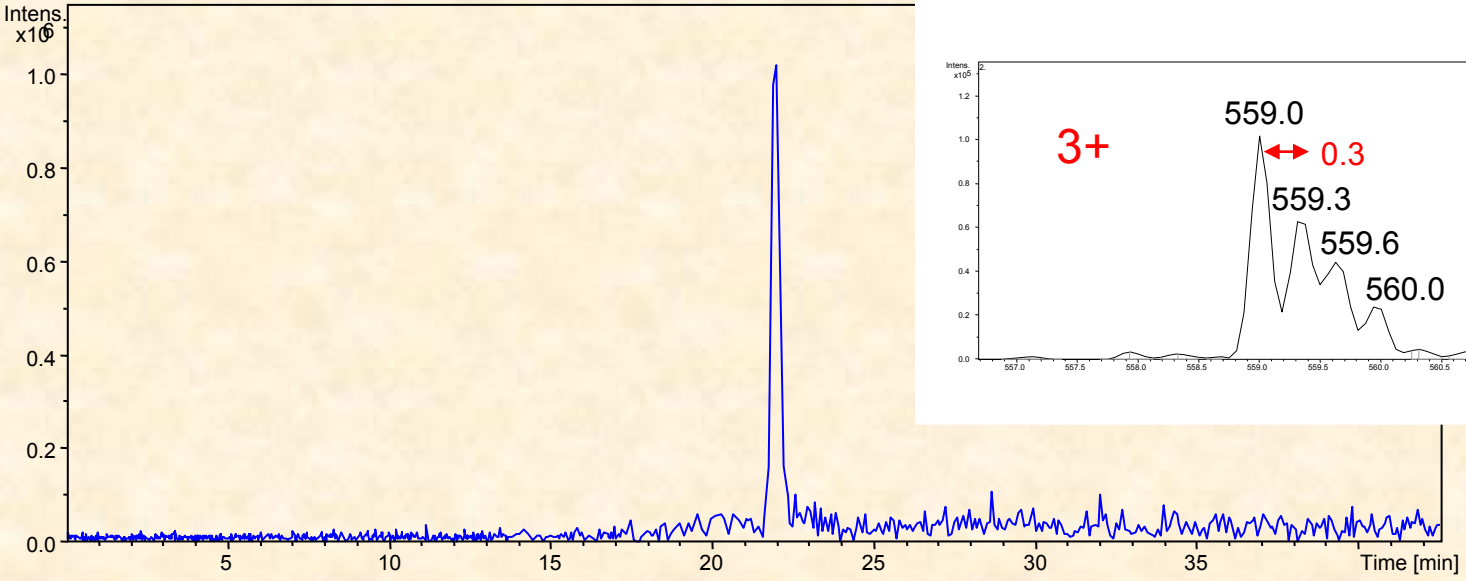
Schéma MS analýzy

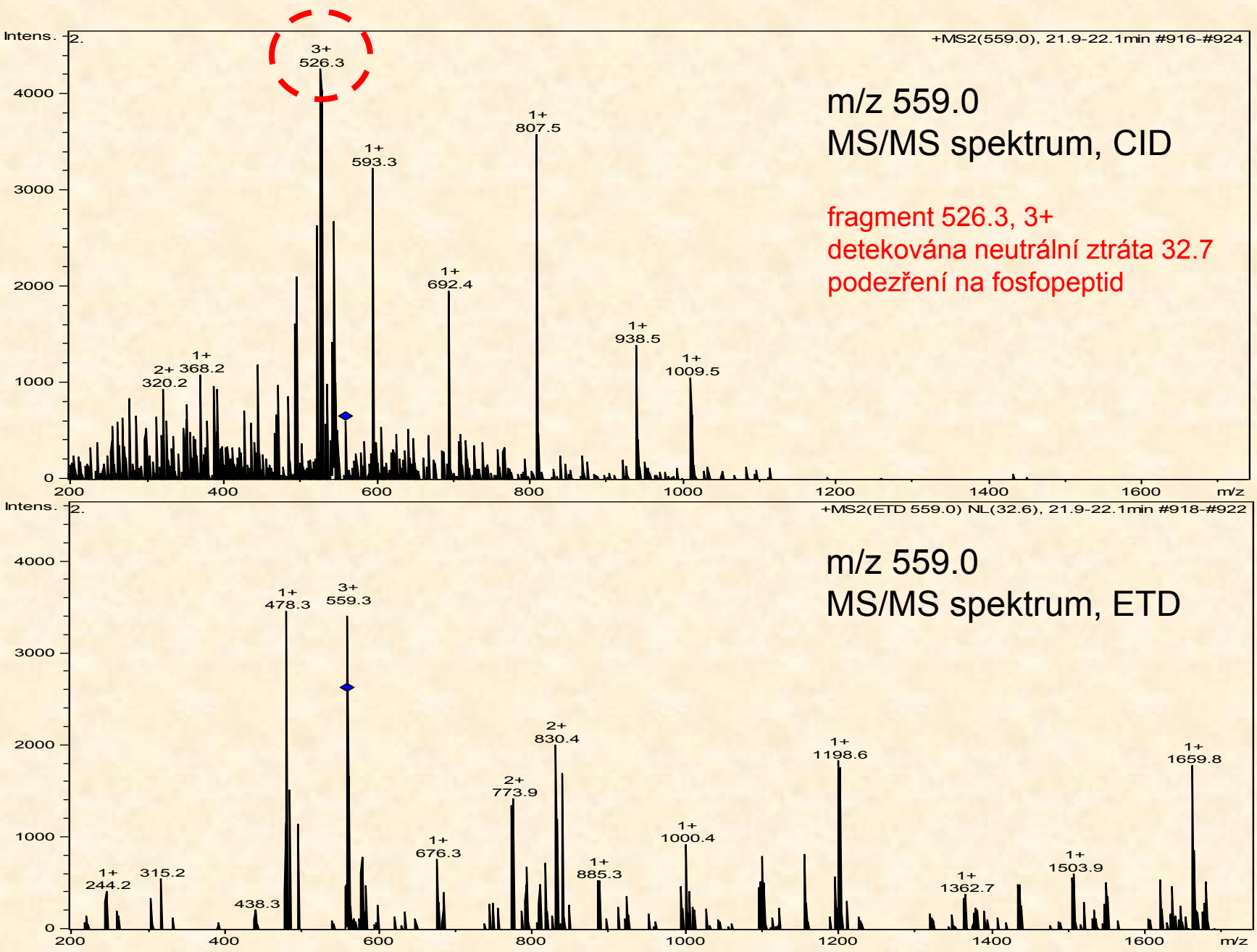


LC-MS chromatogram



MS chromatogram vybraného iontu o m/z 559.0





m/z 559.0
MS/MS spektrum, CID

fragment 526.3, 3+
detekována neutrální ztráta 32.7
podezření na fosfopeptid

m/z 559.0
MS/MS spektrum, ETD

Identifikace peptidu databázovým prohledáváním
MS/MS Ion Search (MASCOT)

m/z	Charge	RT (min)	Expect Mr
559.0	3+	22.063	1674.0

MASCOT

- gi|223582 **Mass:** 11230 **Score:** 74 **Queries matched:** 2
  histone H4

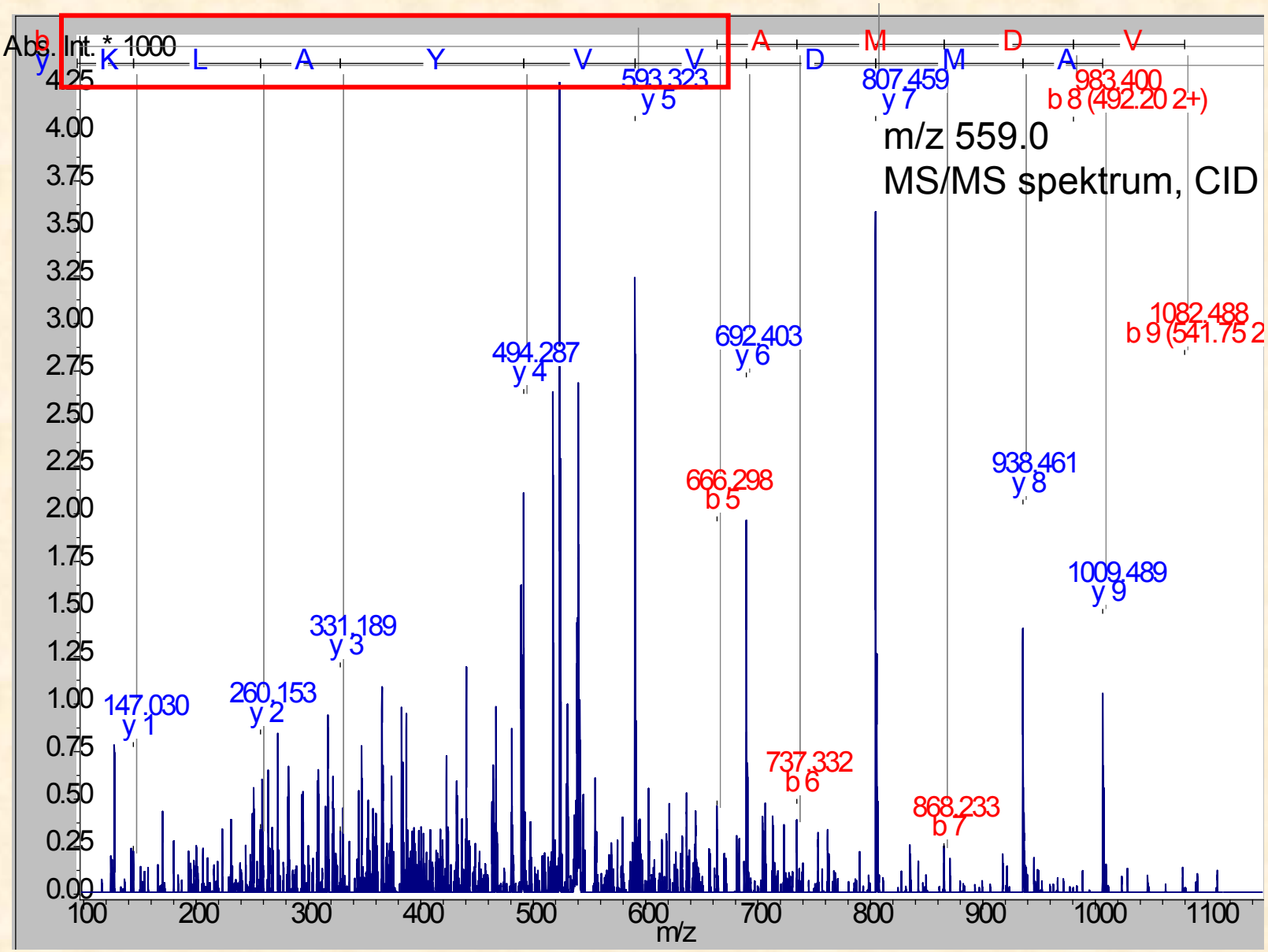
Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide	
558.98	1673.93	1673.86	0.076	2	(23)	7.8	1	K.RKTVTAMDVVYALK.R + Phospho (ST)	CID
558.98	1673.93	1673.86	0.076	2	75	5e-05	1	K.RKTVTAMDVVYALK.R + Phospho (ST)	ETD

Modifications: Optional: Phospho (ST)
Search Parameter: Charge=2+ and 3+, MS Tol.:0.500000 Da, MSMS Tol.:0.500000 Da, Trypsin
Mascot 2.2.03, NCBInr NCBInr_20081101.fasta

Biotools

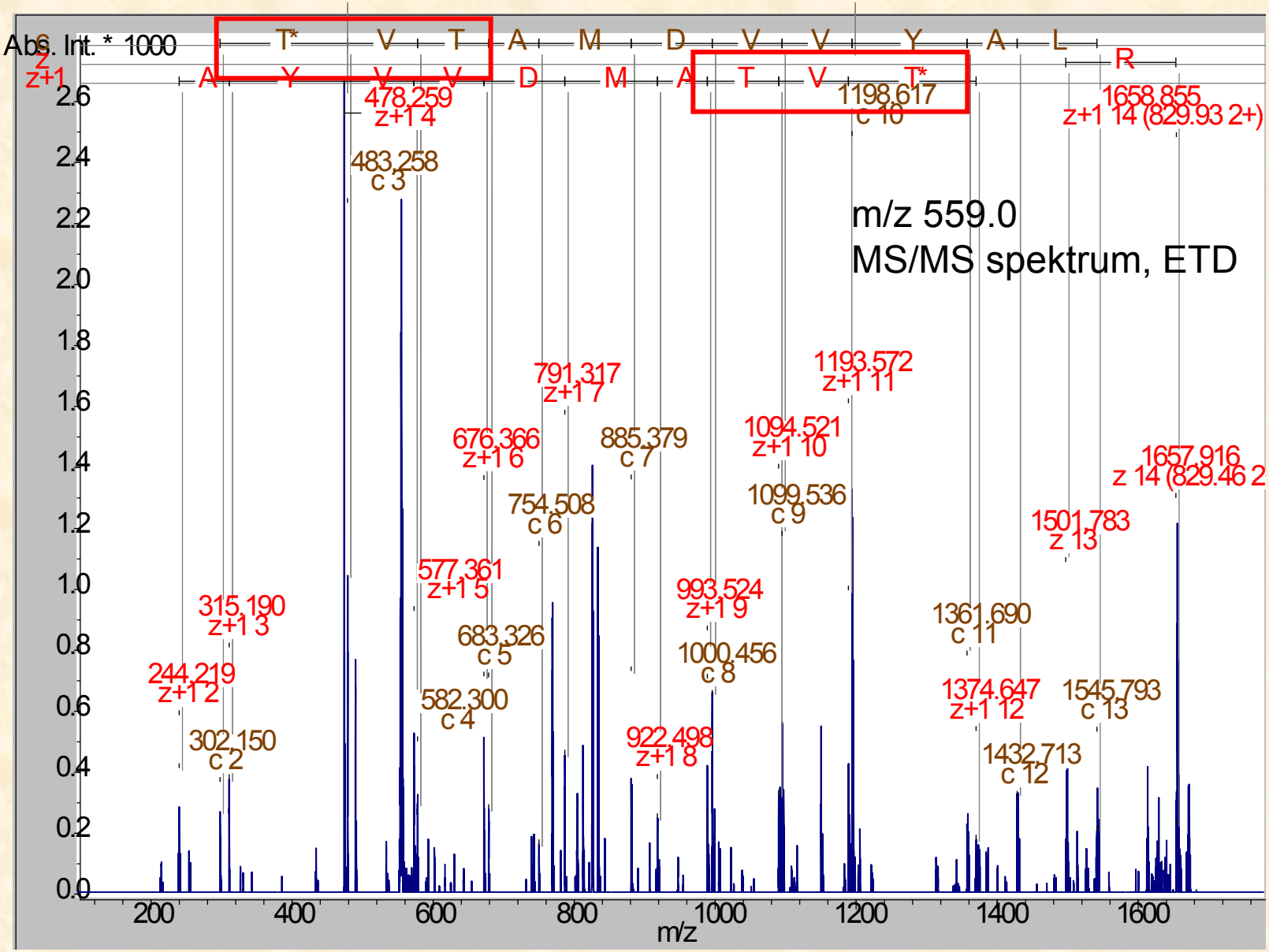
	Score (Biotools)	**Score (Mascot)**
CID		
RKTVTAMDVVYALK	520	23
RKTVTAMDVVYALK	518	22
ETD		
RKTVTAMDVVYALK	6149	**75**
RKTVTAMDVVYALK	774	47

RKTVTAMDVVYALK



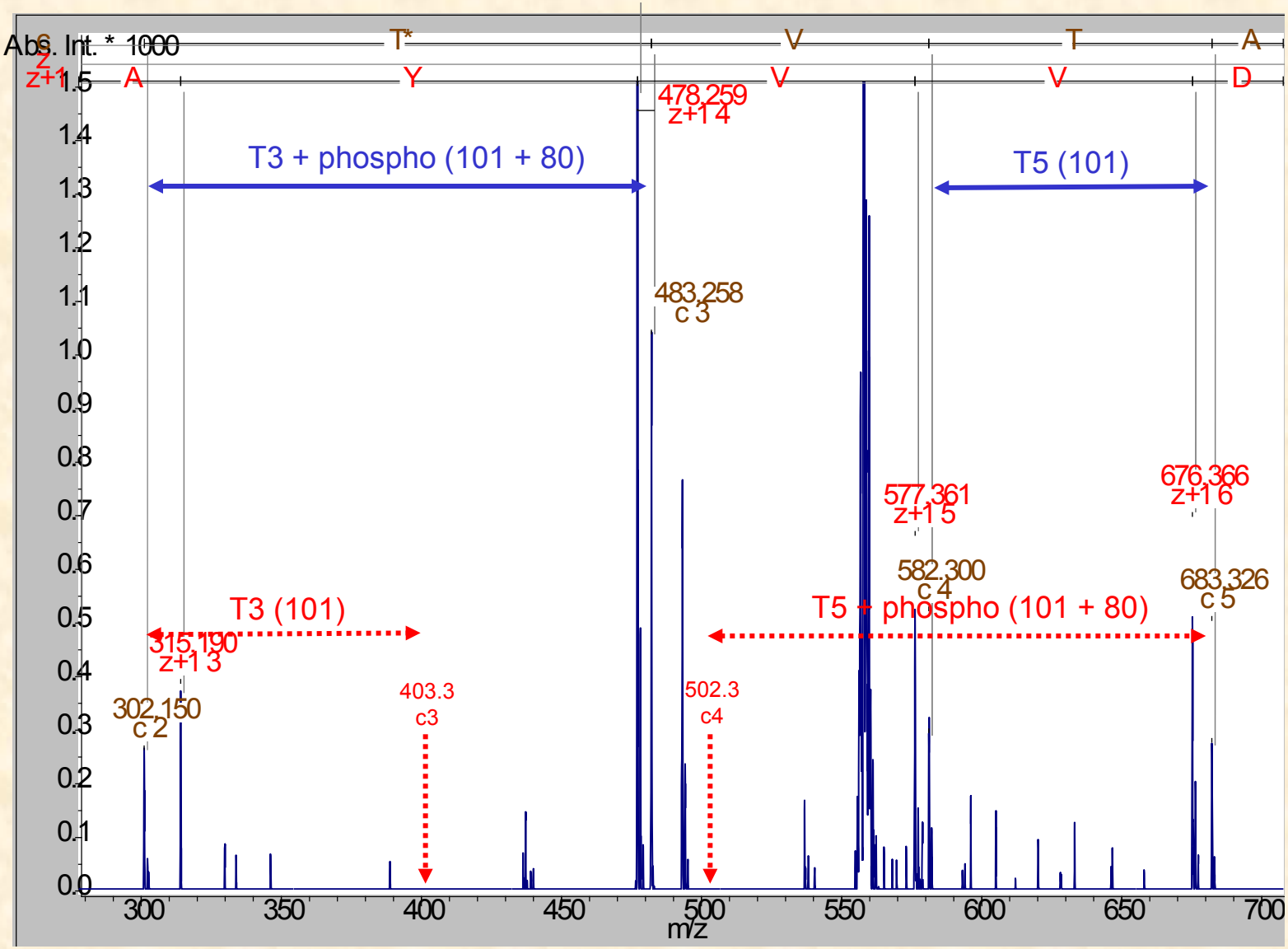
na základě CID MS/MS dat nelze fosfoskupinu lokalizovat

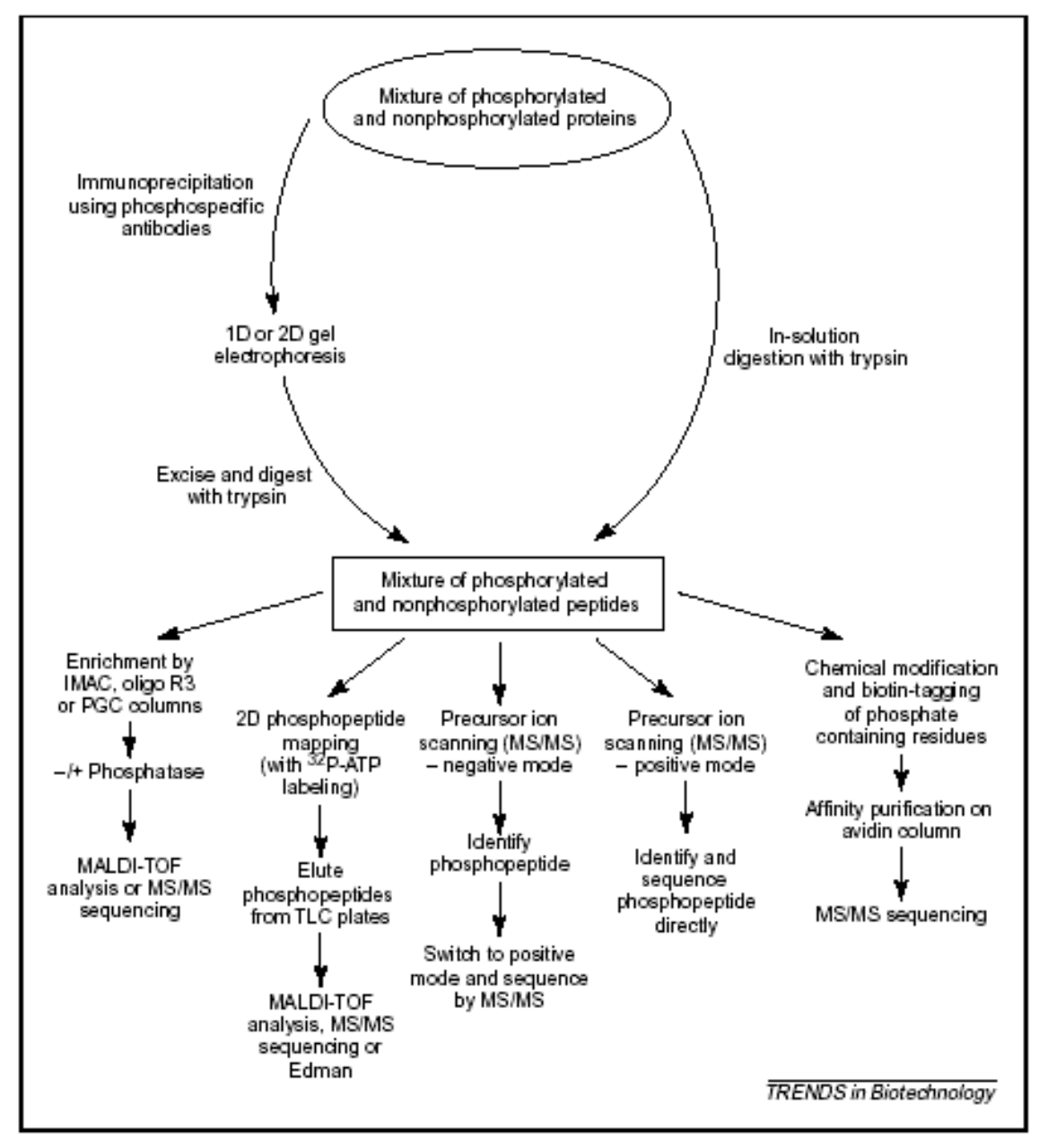
RKTVTAMDVVYALK



na základě ETD MS/MS dat lze jednoznačně lokalizovat fosfoskupinu na T(3)

T(3) x T(5)


Abs. Int. * 1000
T*
V
T
A
A
Y
V
V
D
478.259
z+1 4
T3 + phospho (101 + 80)
T5 (101)
483.258
c 3
577.361
z+1 5
676.366
z+1 6
582.300
c 4
683.326
c 5
T3 (101)
T5 + phospho (101 + 80)
315.190
z+1 3
302.150
c 2
403.3
c3
502.3
c4
m/z



M. Mann et al TRENDS in Biotechnology Vol.20 No.6 June 2002

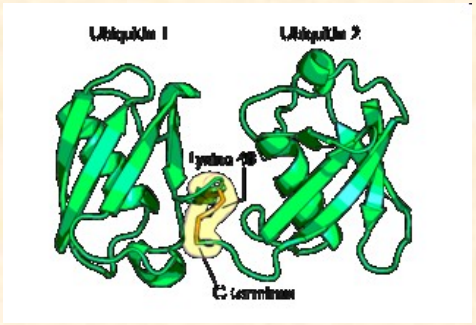

N949WP
THE SIMPSONS
FOX
Western Pacific
Photo Copyright Ralf Langer
AIRLINERS.NET

Ubikvitinace

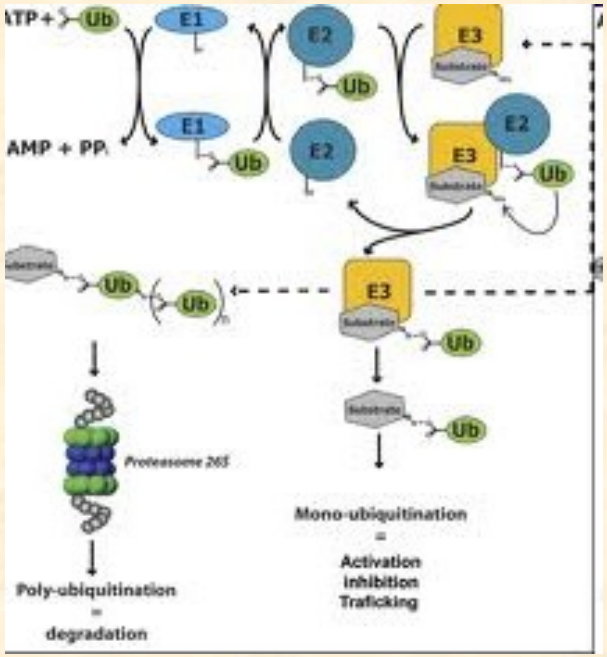
Ubiquitination is an enzymatic, protein post-translational modification (PTM) process in which the carboxylic acid of the terminal glycine from the **di-glycine motif** in the activated ubiquitin forms an amide bond to the epsilon amine of the lysine in the modified protein.

Protein ubiquitination regulates many cellular processes including transcription, endocytosis, cellcycle control, signal transduction, stress response, DNA repair as well as **proteasomal-mediated degradation**

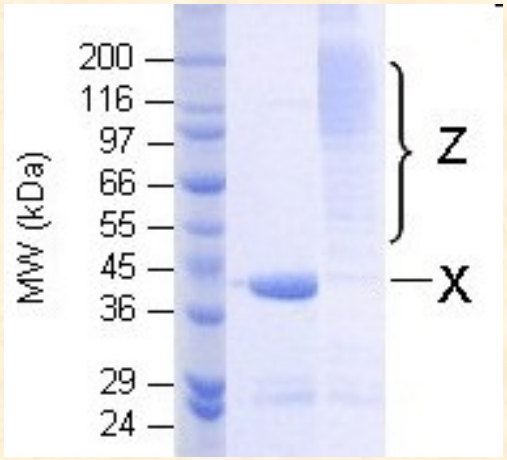
- lokalizace modifikovaných AMK
- určení vazeb v polyubikvitinu



The most studied polyubiquitin chains - lysine48-linked - target proteins for destruction



heterogenita forem

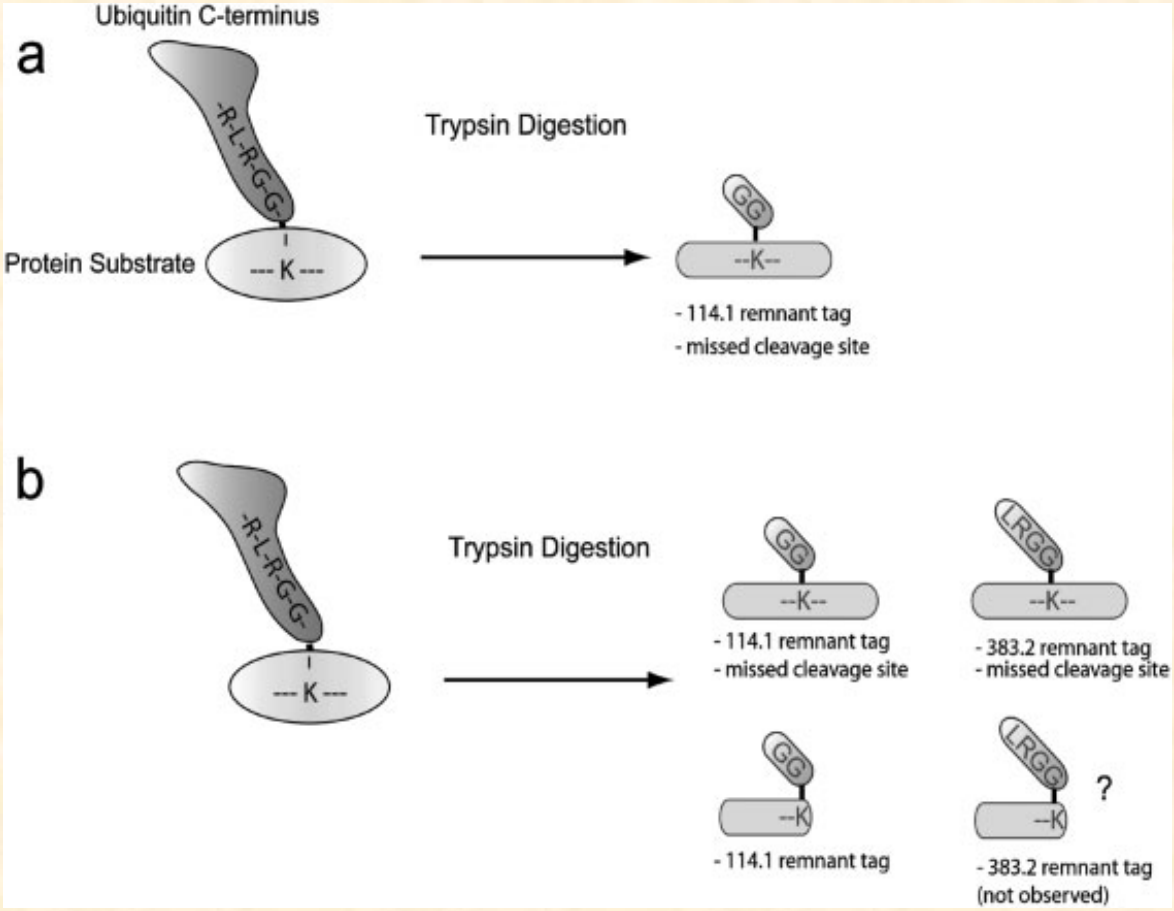


ubikvitin – protein 8.5 kDa (76 AMK)

MQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIFAGKQLEDG
RTLSDYNIQKESTLHLV LRLRGG

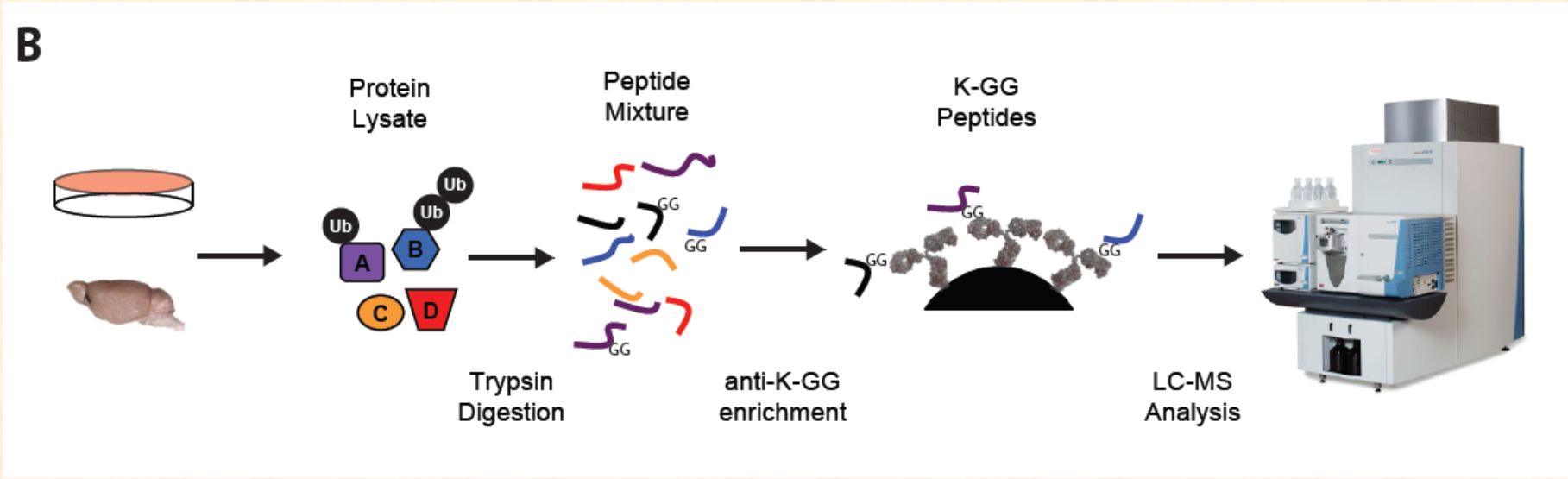
K

Strategie analýzy ubikvitinovaných míst



N.J. Denis, Proteomics 2007, *7,* 868–874

Schéma experimentu



D.Bustos, MCP, in press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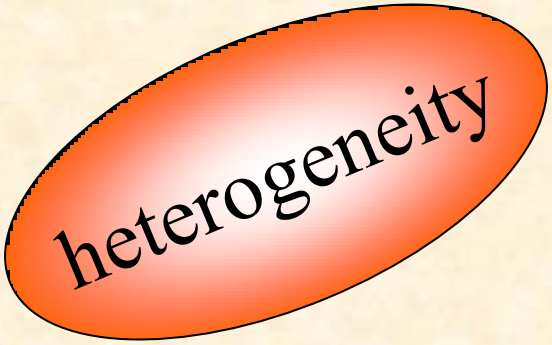
E19
Photo Copyright YOSHIAKI HOSHINA
AIRLINERS.NET

Glykosylace

one of the most common post-translational modifications of proteins in eukaryotic cells.

involved in a wide range of biological functions such as receptor binding, cell signaling, immune recognition, inflammation, and pathogenicity.

Základní typy glykanů:

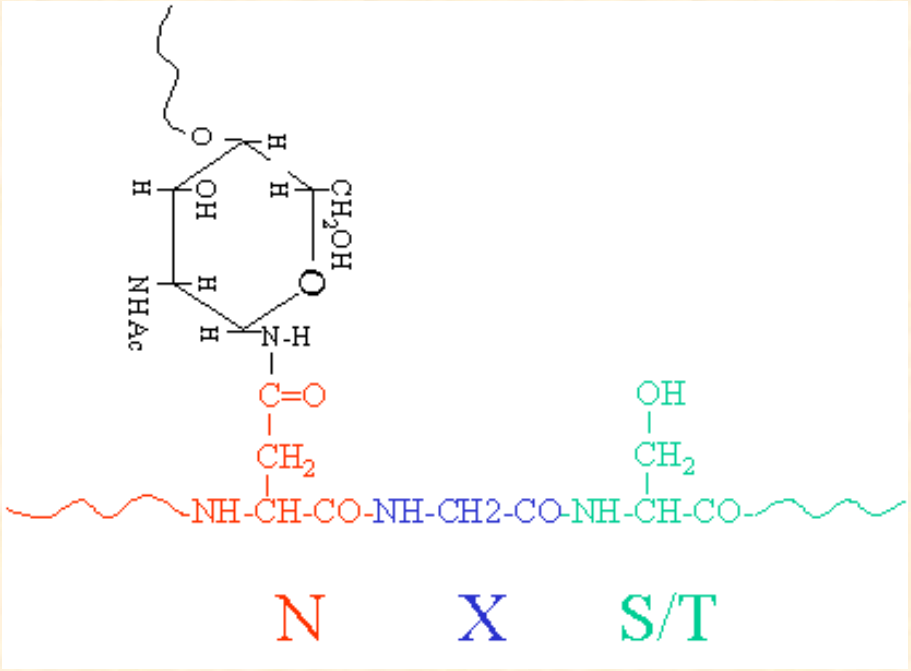


- **N-linked**
- **O-linked**
- **GPI anchors**

Variation in the degrees of saturation at available glycosylation sites results
in heterogeneity in the mass and charge of glycoproteins

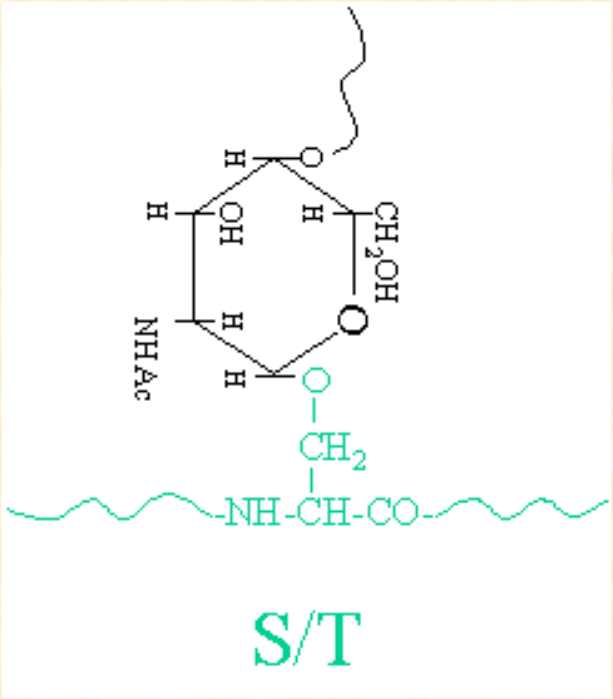
Signal Supression

http://www.expasy.ch/tools/#ptm



N - linked

O - linked



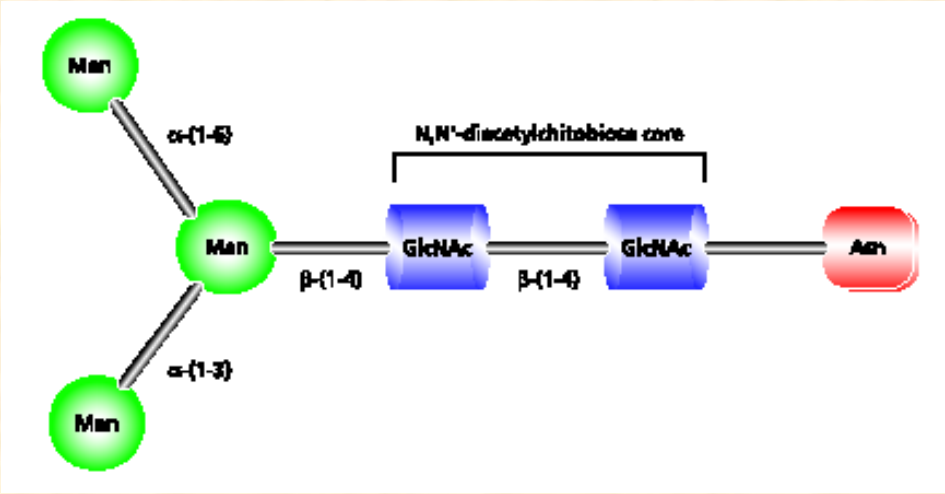
N-linked glycosylations

glycans are attached to the protein backbone via an amide bond to an asparagine *during protein synthesis*

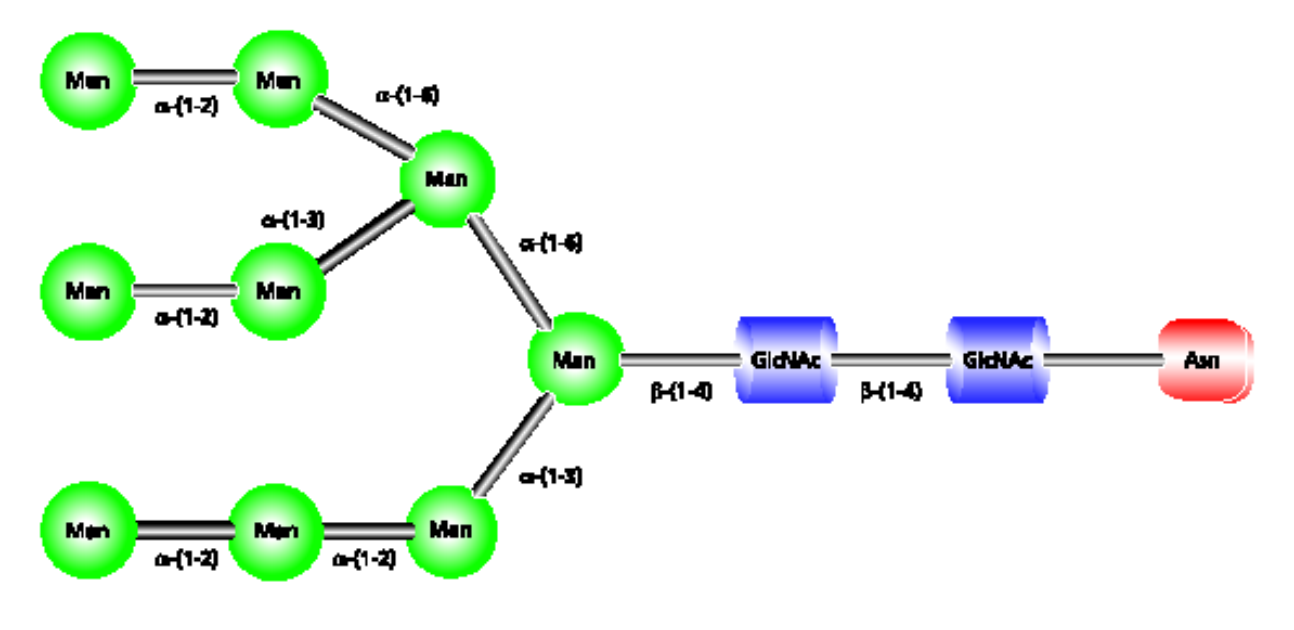
N-X-S(T) **X** nesmí být **P**

subtypes:

- **High-mannose**
- **Hybrid**
- **Complex**



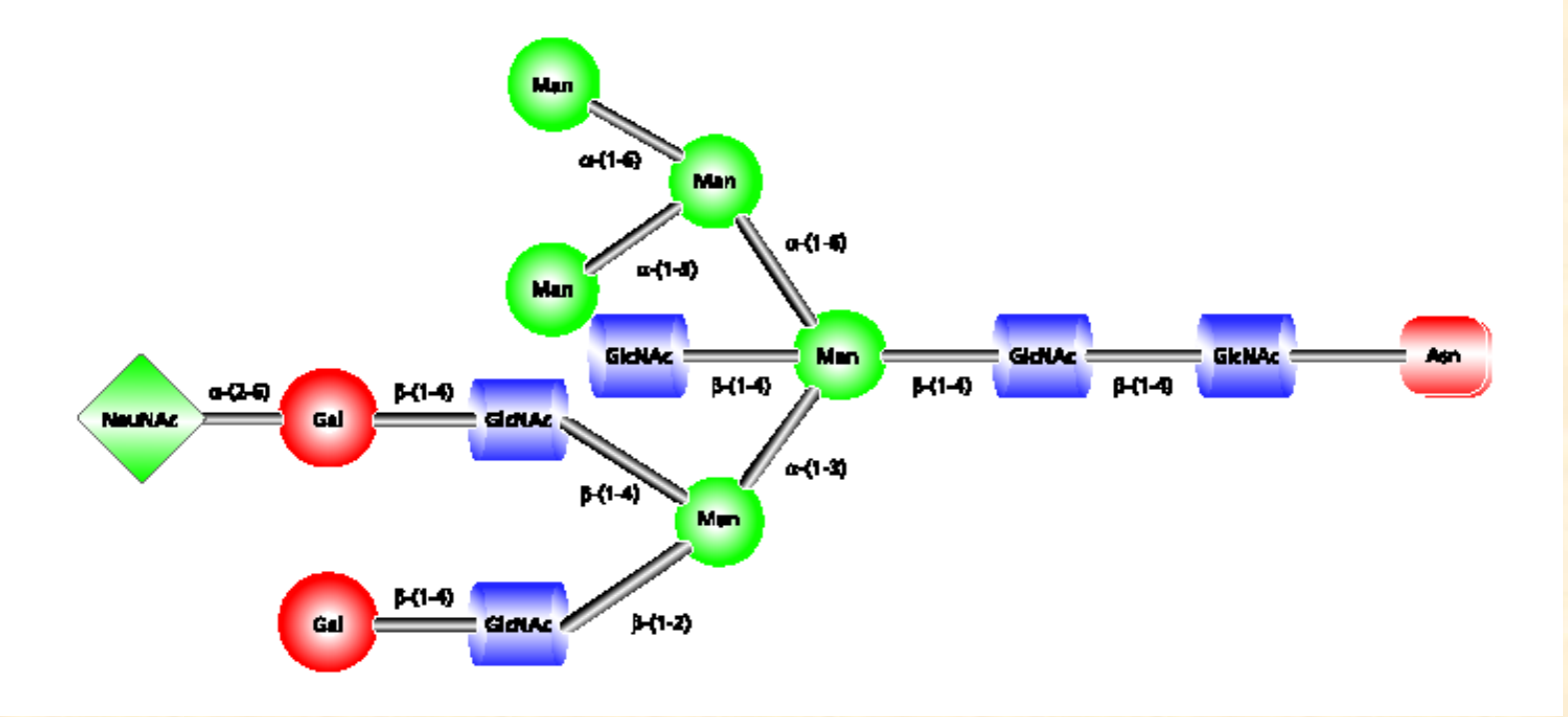
N-linked:High-mannose subtype



Man β-D-Mannose

GlcNAc β-D-*N*-Acetylglucosamine

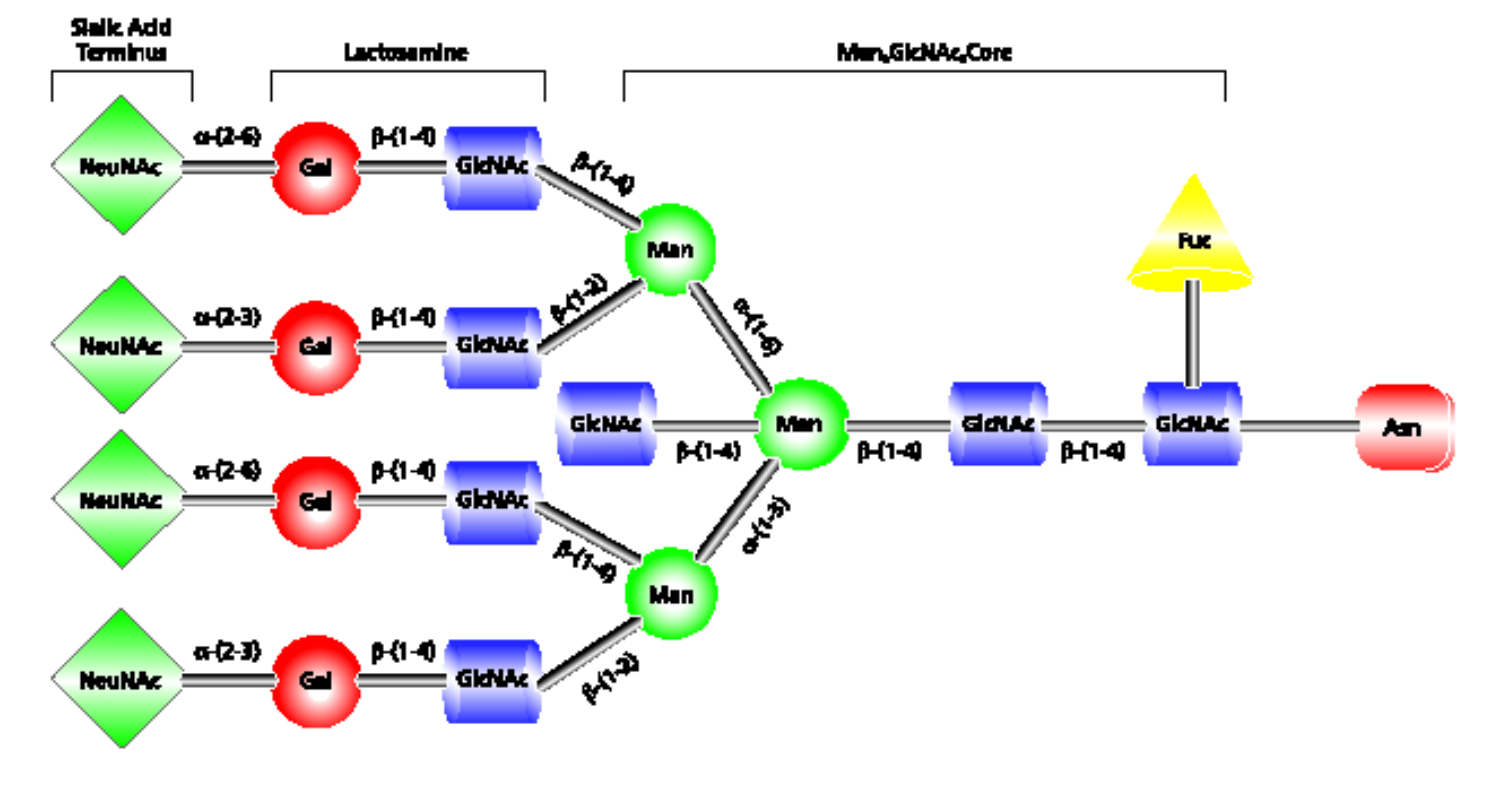
N-linked: Hybrid subtype



Gal β-D-Galactose

NeuNAc α-*N*-Acetylneuraminic acid (Sialic Acid)

N-linked: Complex subtype

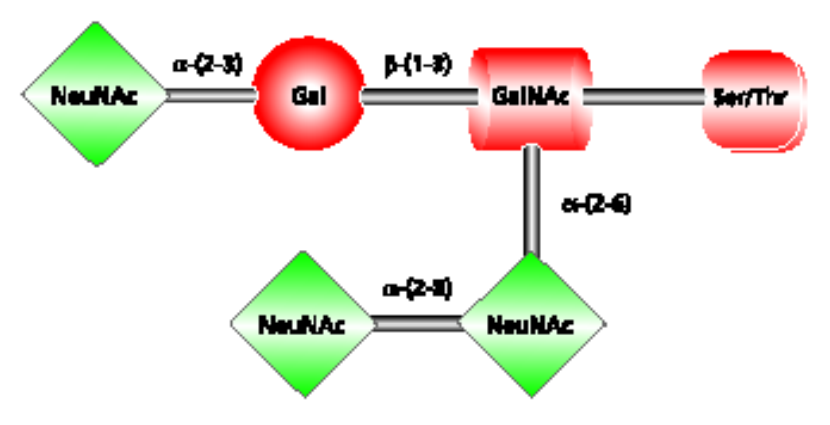


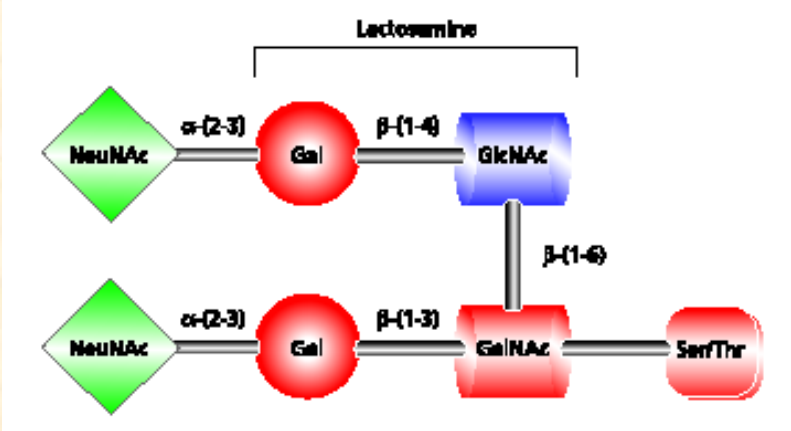
Fuc α-L-Fucose

O-linked glycosylations

glycans are linked via the hydroxyl group of serine or threonine

examples:

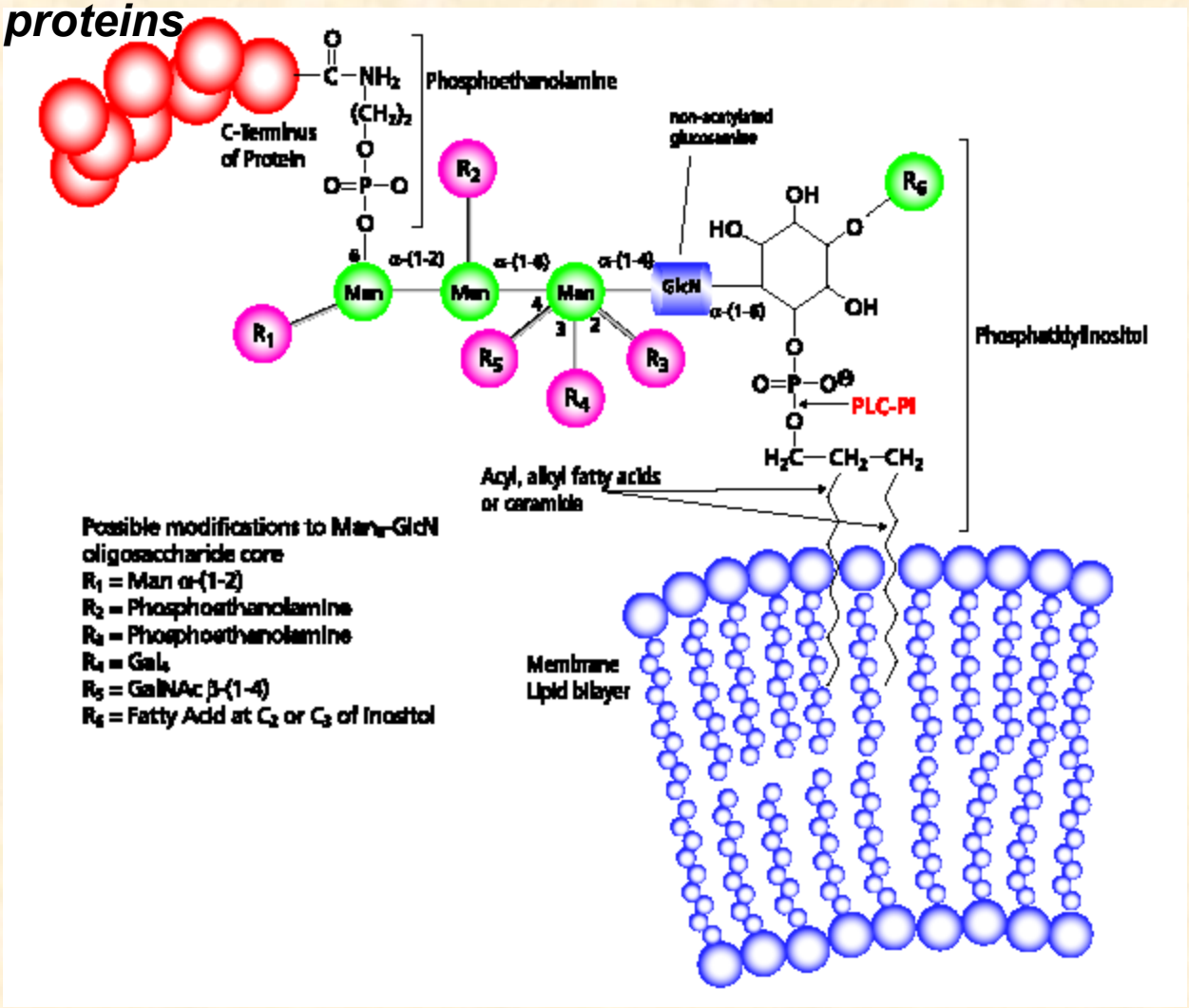




β-D-*N*-Acetylgalactosamine

GPI (glycosylphosphatidylinositol) anchors

anchors are linked via C-terminus, membrane bound proteins



Charakterizace glykoproteinů

- specifická detekce glykosylovaných proteinů
- identifikace proteinů
- určení glykosylačního místa
- určení struktury glykanu

Specifická detekce glykosylovaných proteinů

Pro-Q Emerald 300 - glyko only

Sypro Ruby - all

další techniky detekce:

kolorimetrická detekce
fluorescenční detekce

specifické obohacení:

afinitní chromatografie
(lektinové matrice.
m-Aminophenylboronic Acid)

identifikace proteinu:

Peptide mapping
MSMS Ion search

Deglykosylace

chemická:

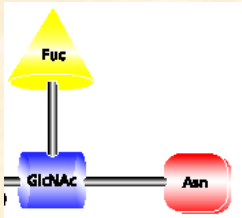
Hydrazinolysis

Hydrazine hydrolysis has been found to be effective in the complete release of unreduced O- and N-linked oligosaccharides.

Alkaline β-Elimination - jen O-linked s vyjímkami

Trifluoromethanesulfonic Acid - destrukce glykanu

enzymatická:



PNGase F N-linked, vše pryč, pokud ne

PNGase A N-linked, vše pryč, pokud ne 

Endoglycosidase H N-linked, štípe až za prvním 

Endoglycosidase F1, F2, F3 N-linked, štípou specificky ke struktuře

O-Glycosidase O-linked. vše pryč

β-galactosidase štípe před 

...

Určení místa glykosylace, resp. struktury glykanů

- **glykosylace „jen“ na S nebo T** (O-linked)
  NXS(T) (N-linked)
  lze vytipovat potencionální glyko místa
  určité strukturní typy u glykanů (high-mannose....)

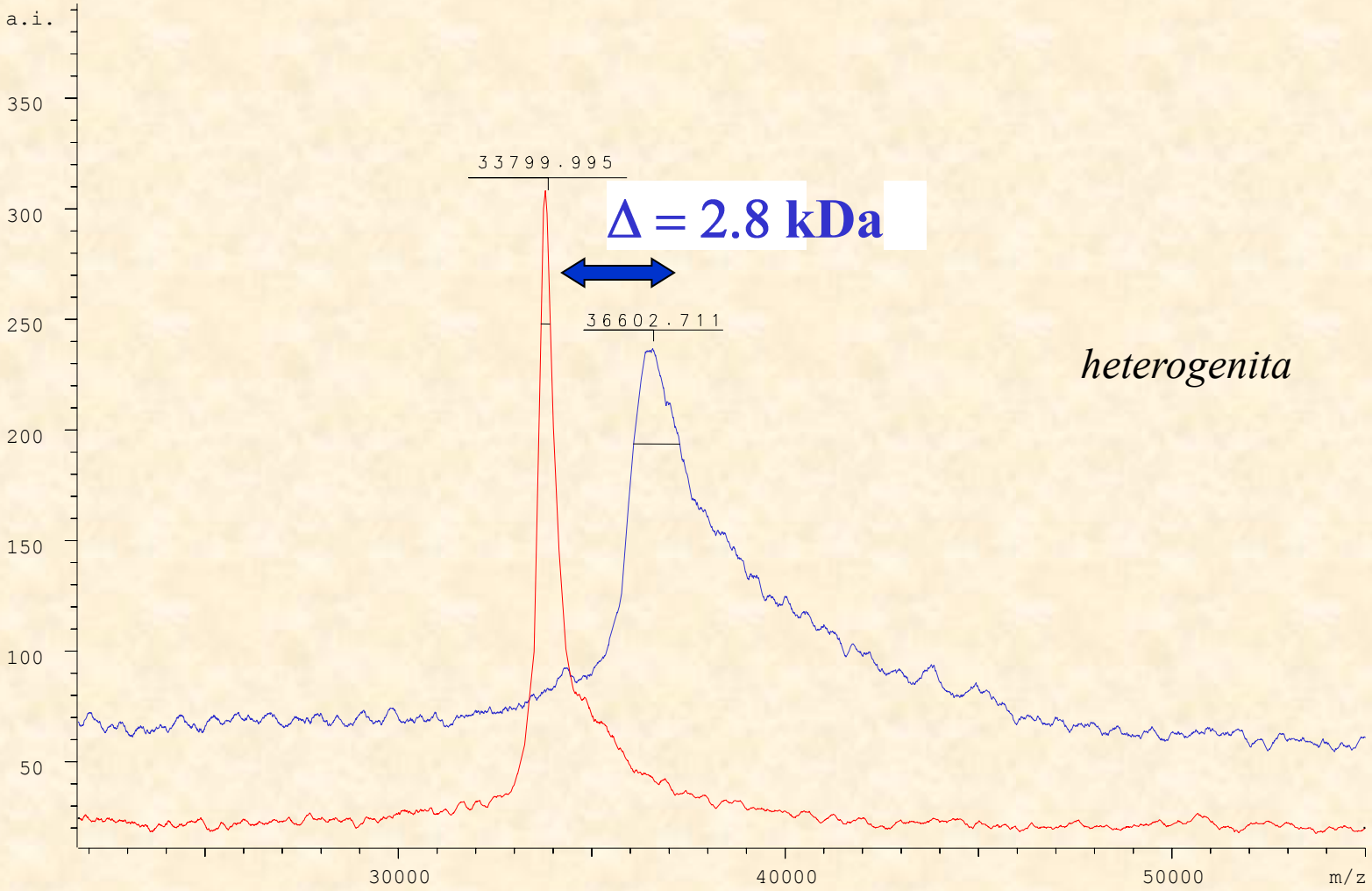
- **kombinace MS a MS/MS technik**

- **separace glykoproteinů resp. glykopeptidů**

- **vhodná deglykosylační strategie**

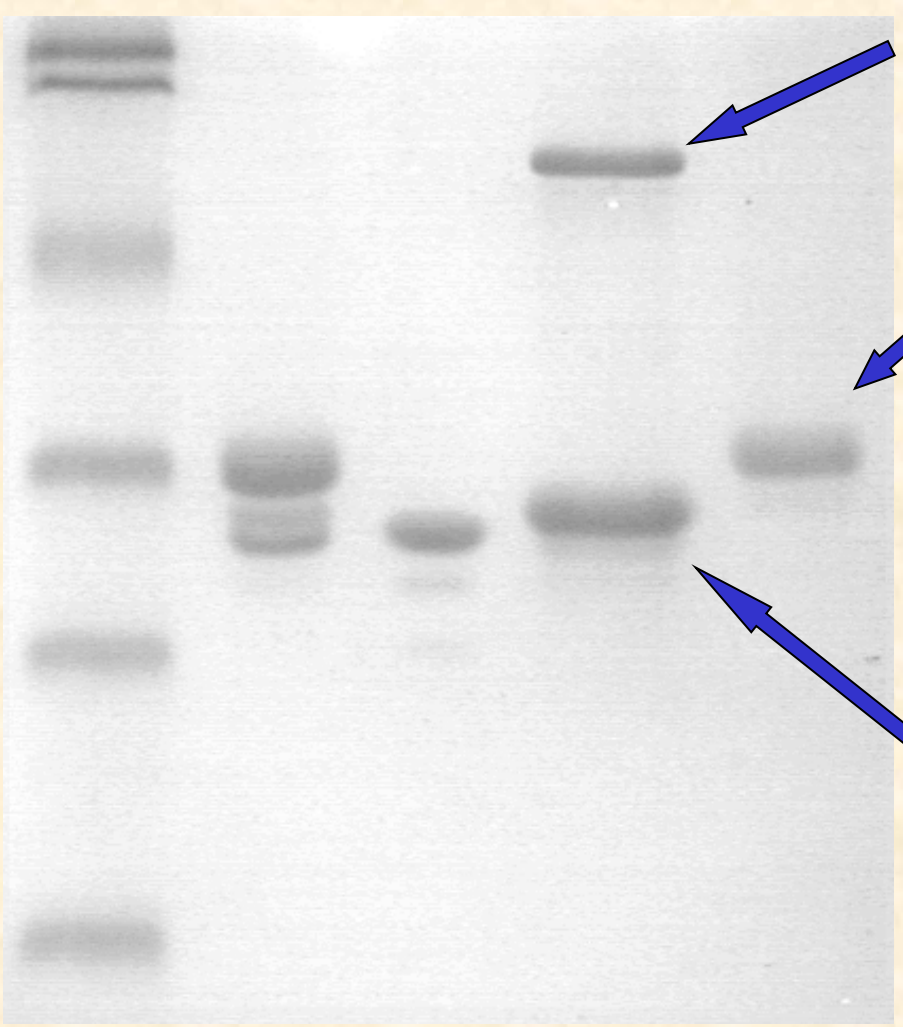
MALDI-MS spectrum of glycosylated and non-glycosylated protein

size of glycan part



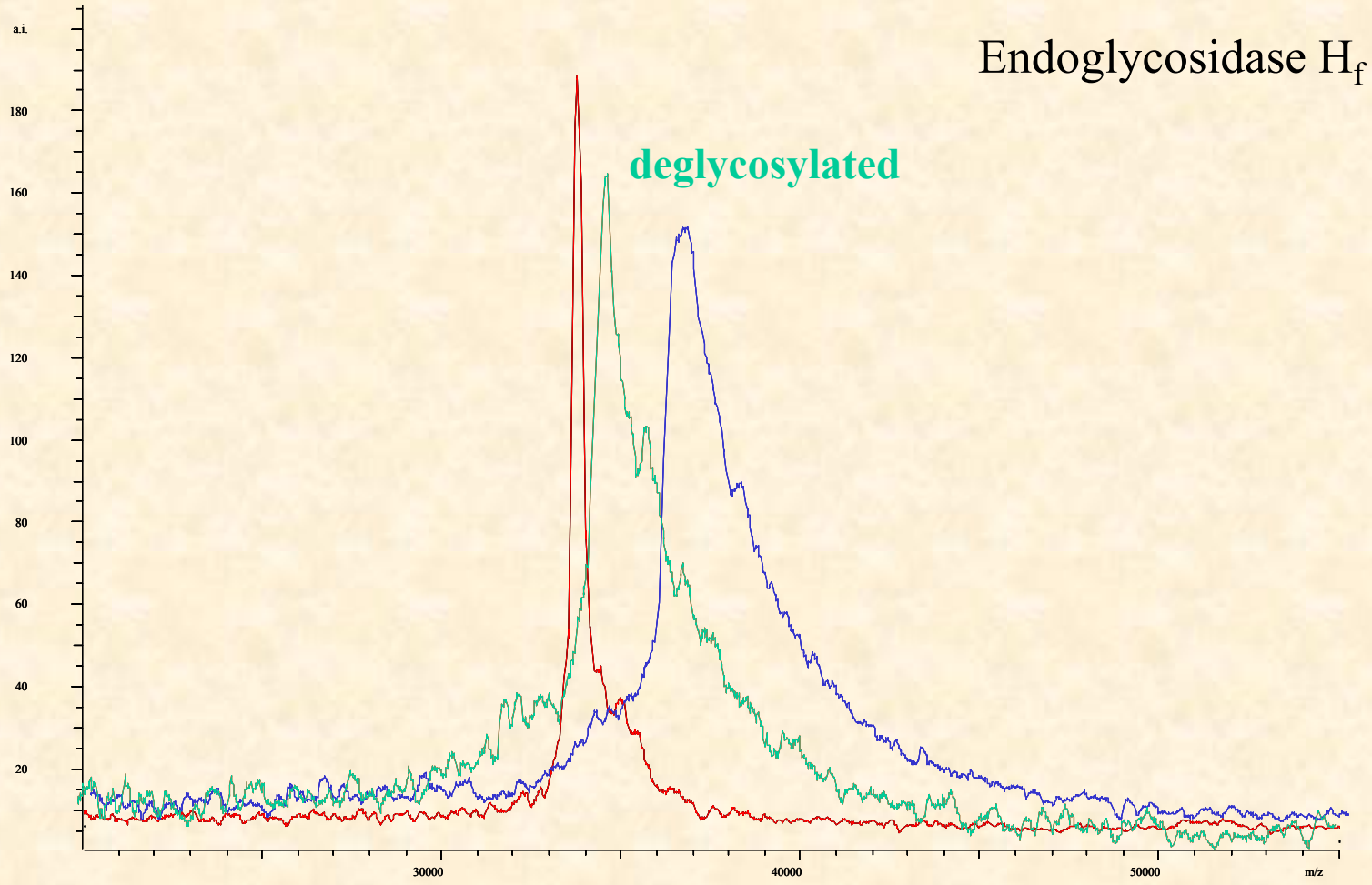
1D GE of protein before and after deglycosylation

confirmation of glycosylation

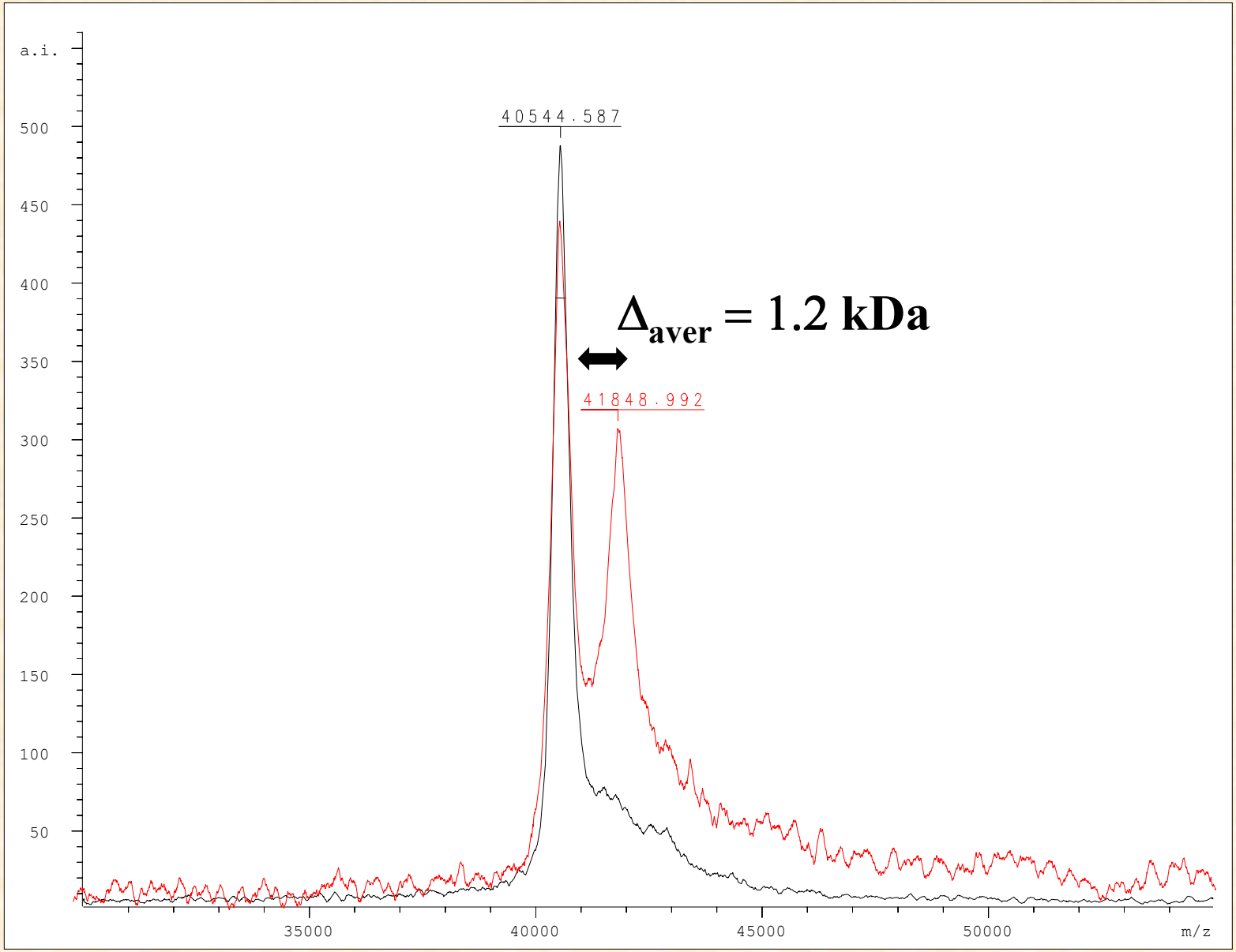


MALDI-MS spectrum of deglycosylated protein

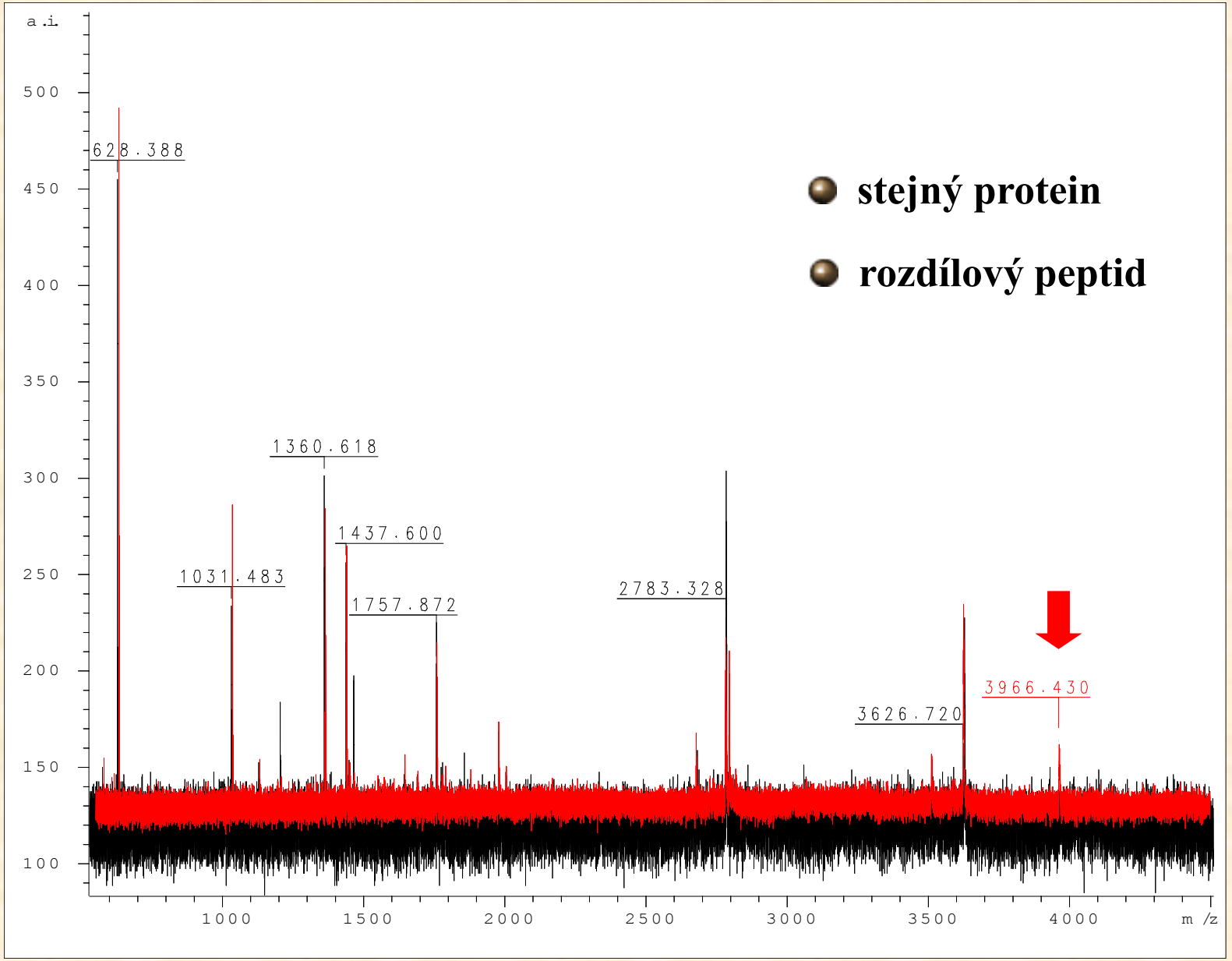
confirmation of glycosylation



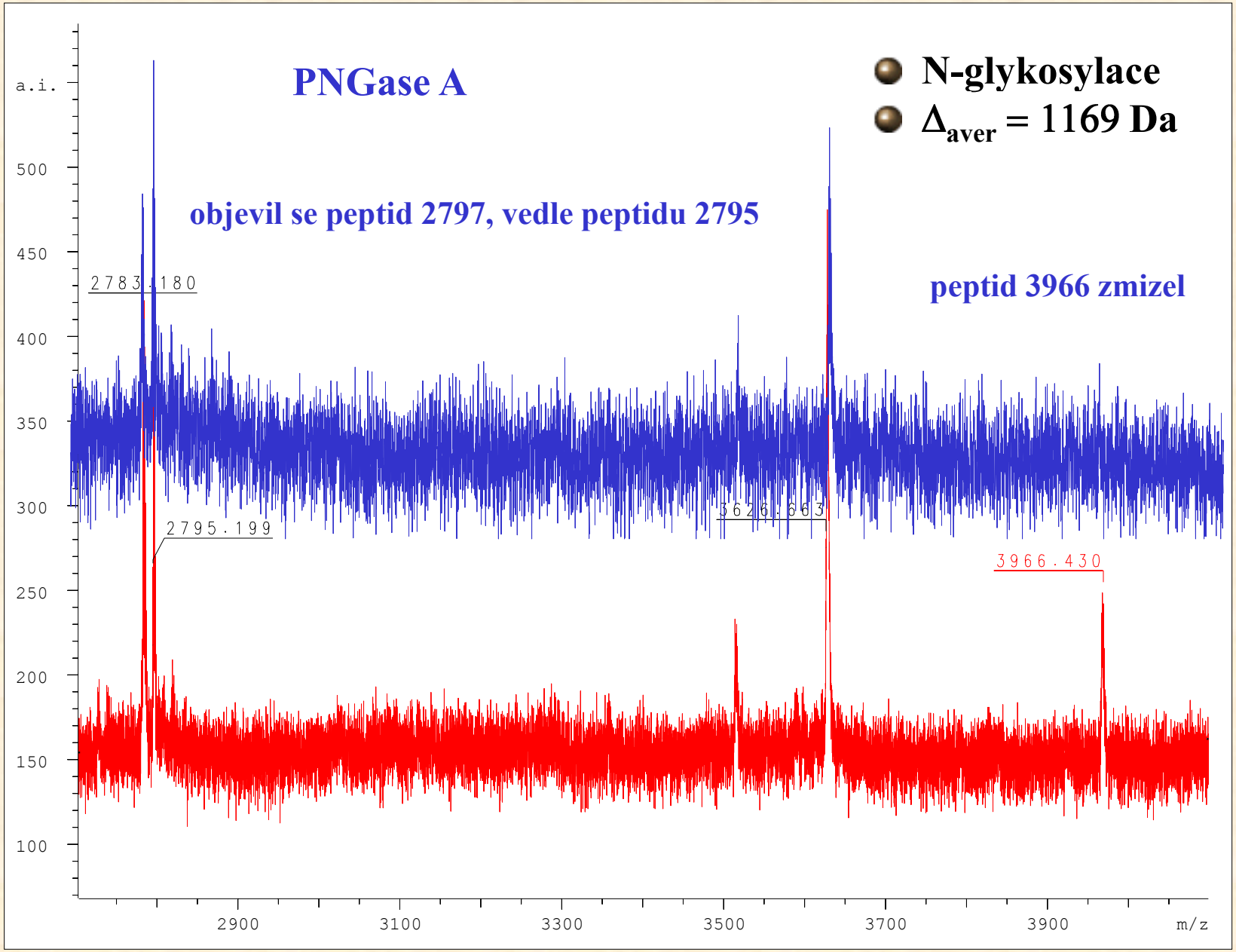
MALDI-MS celých proteinů



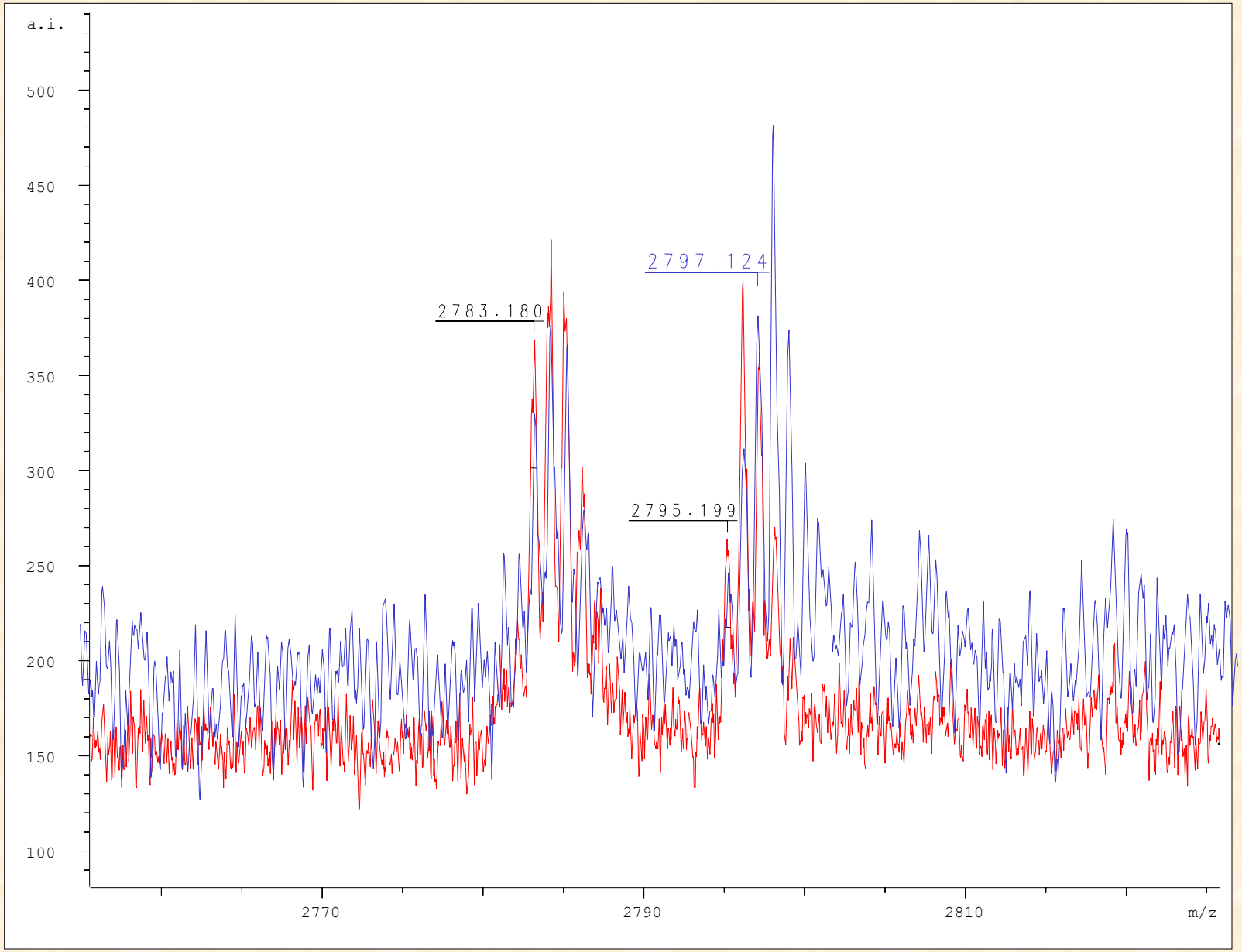
MALDI-MS tryptických digestů



Detail spekter digestů proteinu před a po deglykosylaci



Detail spekter digestů proteinu před a po deglykosylaci



Shrnutí výsledků

tryptický peptid 2796 Da …PHIF**D**YSGS… ,
kde D vzniká z N po deglykosylaci PNGasou A

původní sekvence je tedy …PHIF**N**YSGS… (hmotnost 2795 Da)

Peptid potvrzen také LCMSMS analýzou (v glykosylovaném vzorku digestu nebyl nalezen)

Hmotnost glykanu 1170 Da odpovídá
xylose+fucose+3*mannose+2*N-acetylglukosamin

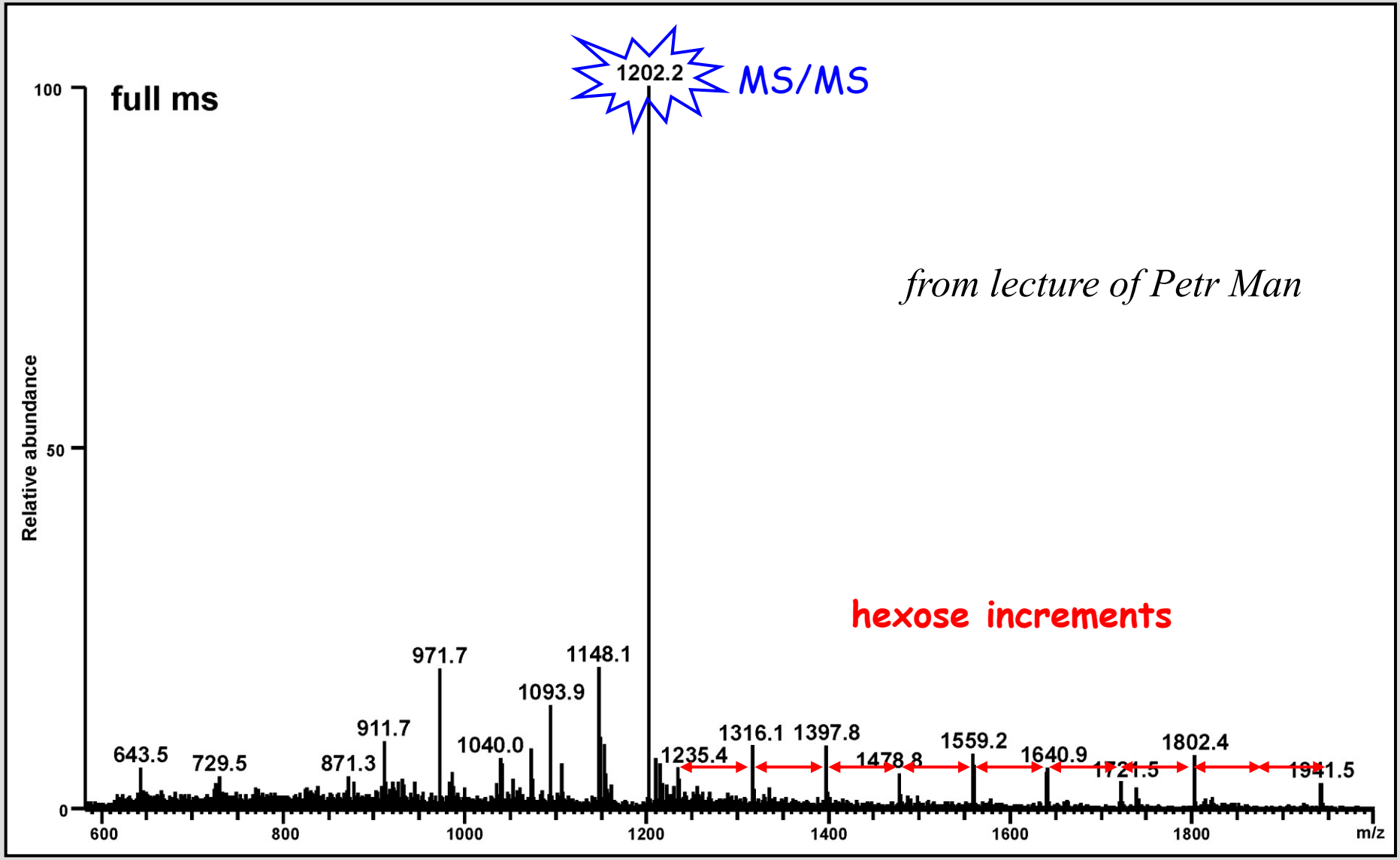
Nebyl dále potvrzen MSMS technikami

…missing parts have potential N-glycosylation sites...

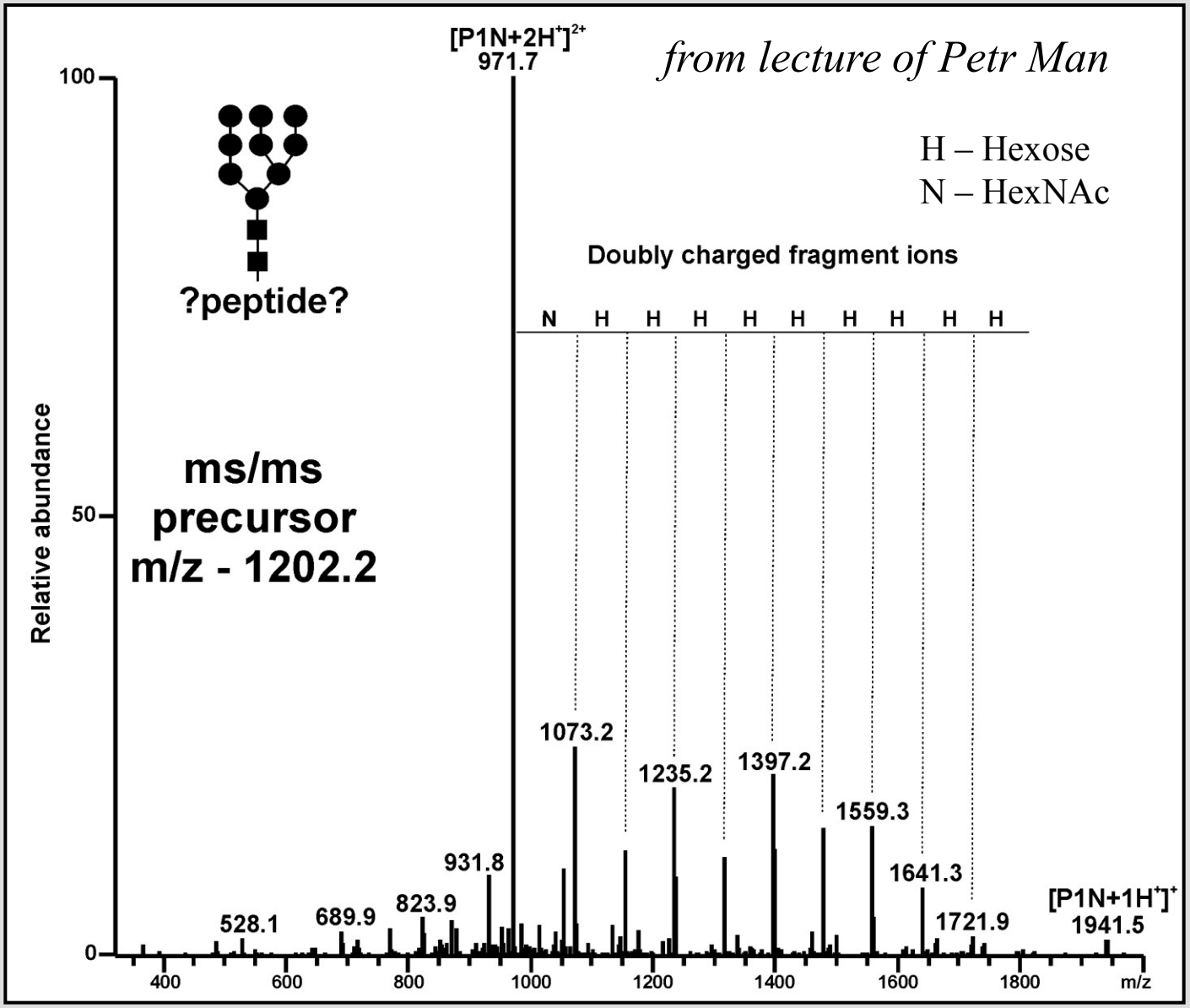
MLRNVCPVLILLIIGATAQDPTDVGEAFANVEWSVAELKRV
LVMGVPRDCGELFLSGQNHSGVYNIYPYKDSLLPVS
AYCDMETDGGGWTVFQRRGQFGNPVYYFYKKWA
DYAHGFGDPAKEYWLGNNVLHALTSDKAMSLRIE
KNHSLETLTAEYSVFKVASEEEYFKINVGGYIGSK
GSDAFSIANGSMFTASDQDHDTYTNNCAVEFKG
AWYTSCHGSNLNGLNLNGEHPSYADGIEWSAR
GGSTGLYYYSYPNVEMKVRDAHFISRVADGRAS

from lecture of Petr Man

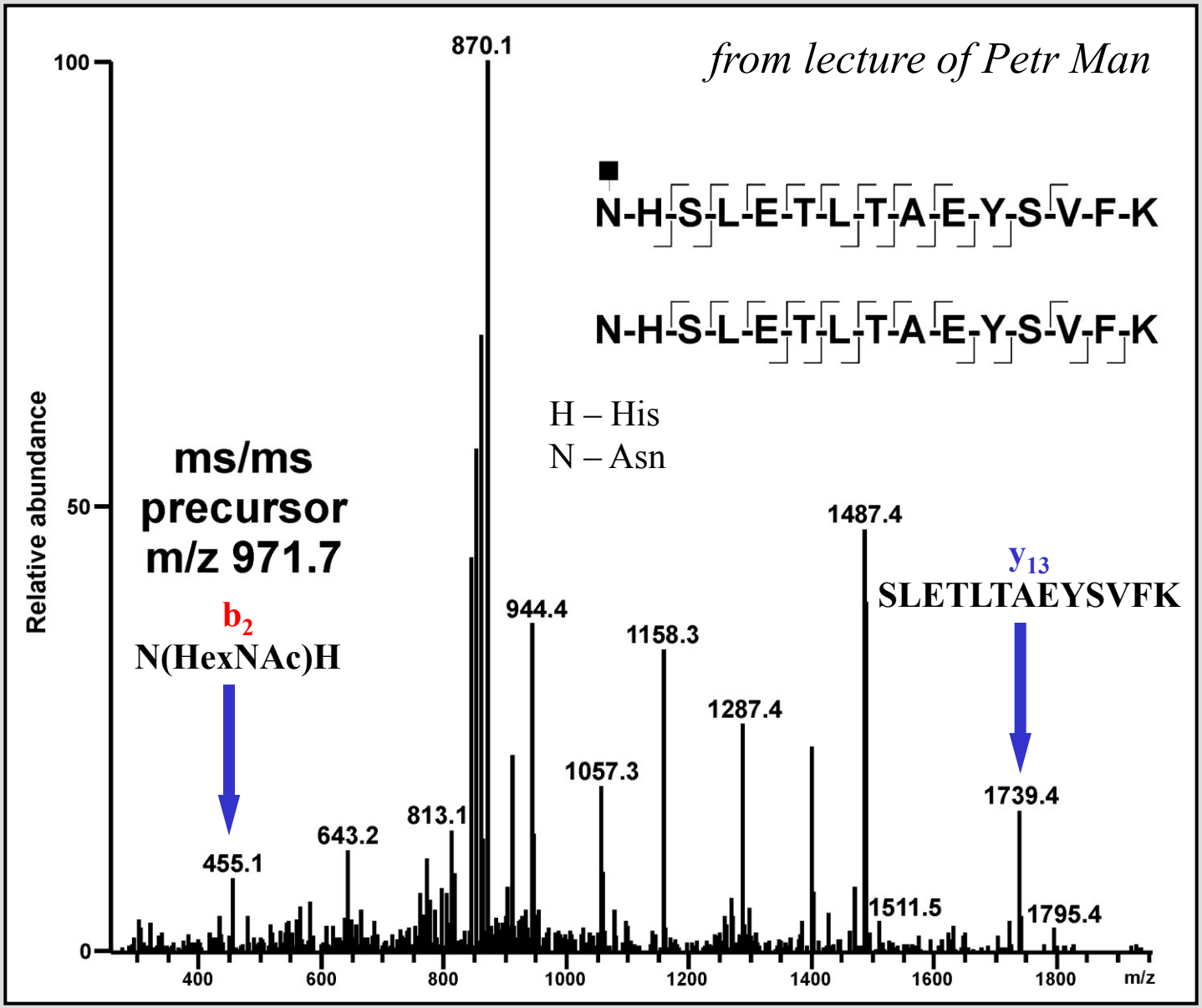
... glycopeptide...



MS/MS from 1202.2 - – glycopeptide, type of glycan identified



MS/MS from 971.7 – peptide with one HexNAc – site of glycosylation identified



MLRNVCPVLILLIIGATAQDPTDVGEAFANVEWSVAELKRV
LVMGVPRDCGELFLSGQNHSGVYNIYPYKDSLLPVS
AYCDMETDGGGWTVFQRRGQFGNPVYYFYKKWA
DYAHGFGDPAKEYWLGNNVLHALTSDKAMSLRIE
KNHSLETLTAEYSVFKVASEEEYFKINVGGYIGSK
GSDAFSIANGSMFTASDQDHDTYTNNCAVEFKG
AWYTSCHGSNLNGLNLNGEHPSYADGIEWSAR
GGSTGLYYYSYPNVEMKVRDAHFISRVADGRAS

from lecture of Petr Man

MALDI-MS spectrum of ribonuclease B

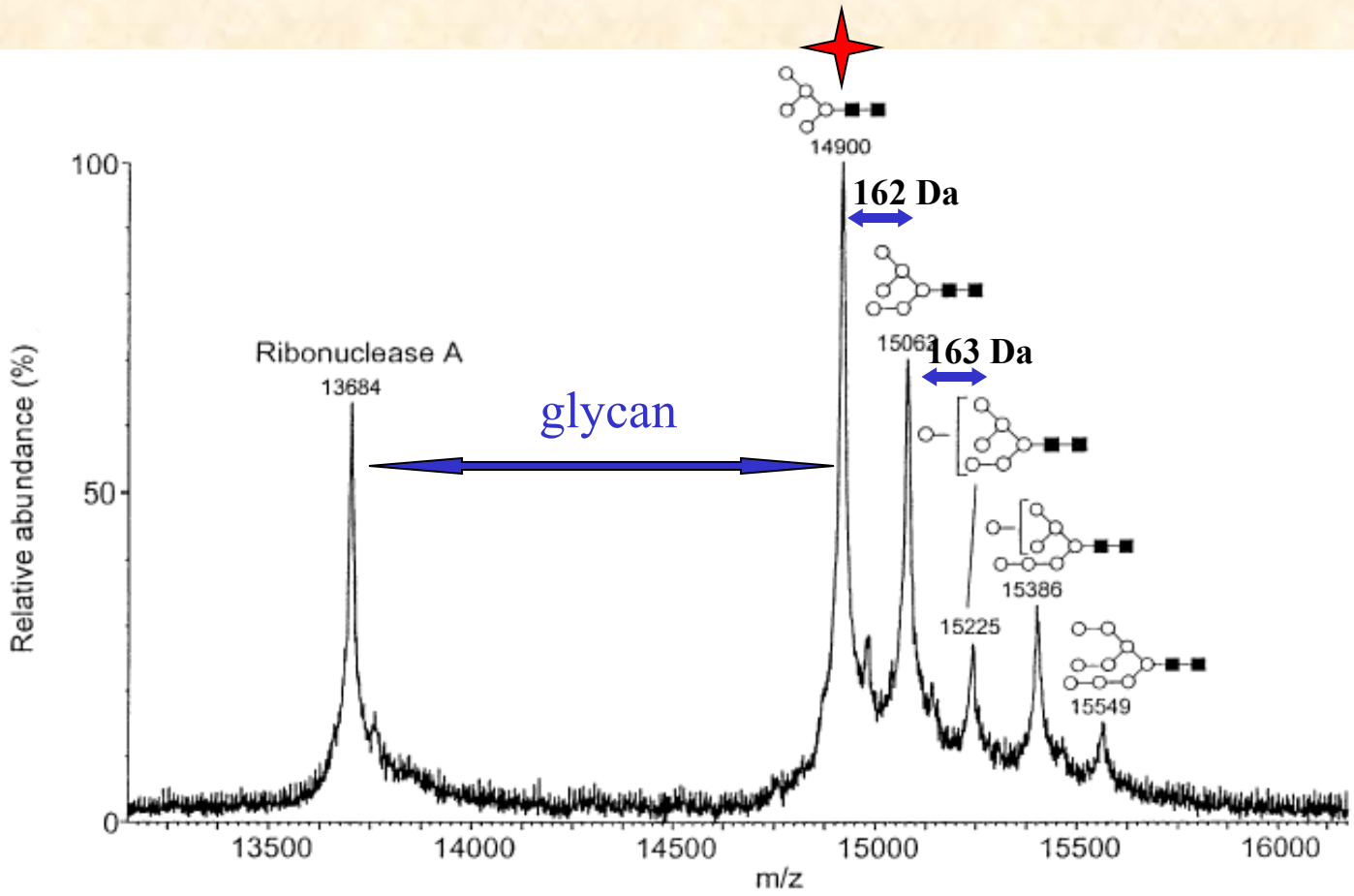


Fig. 2. Positive ion linear MALDI mass spectrum of a mixture of ribonuclease A (unglycosylated) and B (glycosylated) recorded from 4-HCCA with a Micromass TofSpec 2E mass spectrometer. The structures of the five high-mannose glycans attached to the protein to form ribonuclease B are shown. Key to symbols: (■) GlcNAc, (○) mannose.

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MSn of ribonuclease B glycans (AP-MALDI-IT MS)

Bi7050

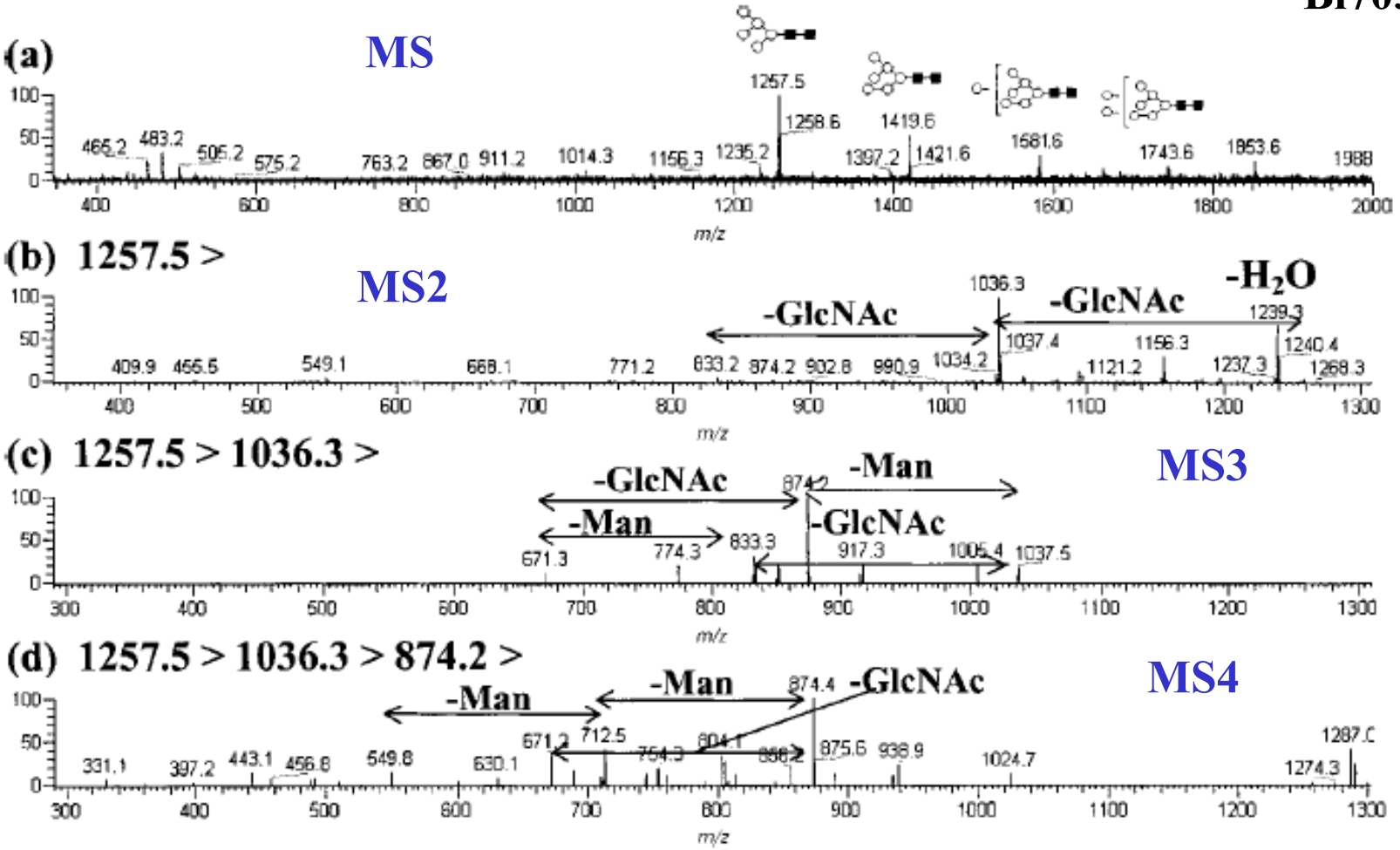
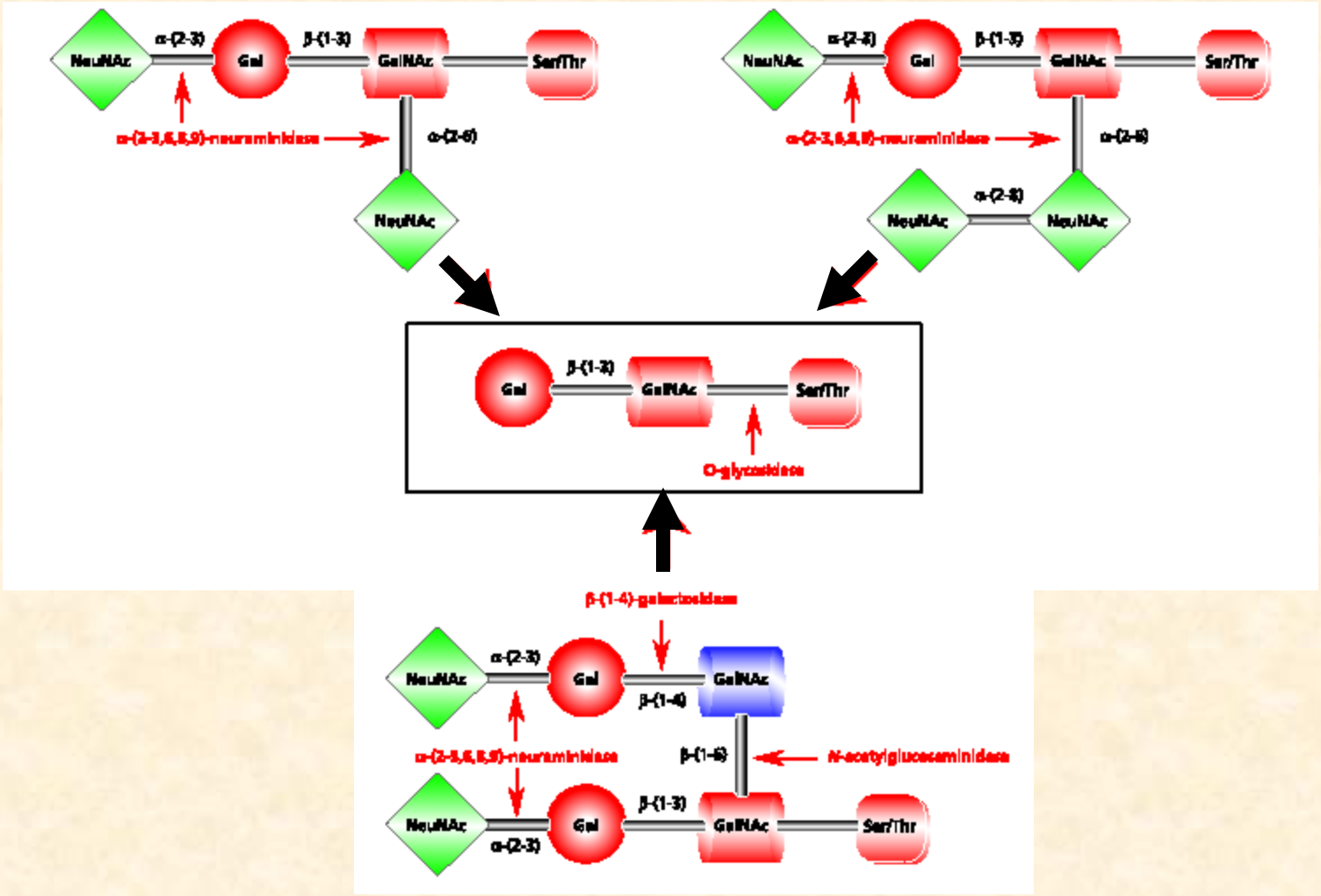


Figure 6. MS^4 analysis of ribonuclease-B glycans from a HABA matrix. (a) MS spectrum of underivatised glycan solution (500 pmol); (b) MS/MS spectrum of $(Man)_5(GlcNAc)_2$ (*m/z* 1257.5); (c) MS^3 spectrum of $(Man)_5GlcNAc$ (*m/z* 1036.3); and (d) MS^4 spectrum of $(Man)_4GlcNAc$ (*m/z* 874.2).

C. S. Creaser, J. C. Reynolds and D. J. Harvey *Rapid Commun. Mass Spectrom.* 2002; **16**: 176–184

Kombinace deglykosidačních enzymů



Postupná deglykosylace různými enzymy (MALDI-MS) **Bi7050**

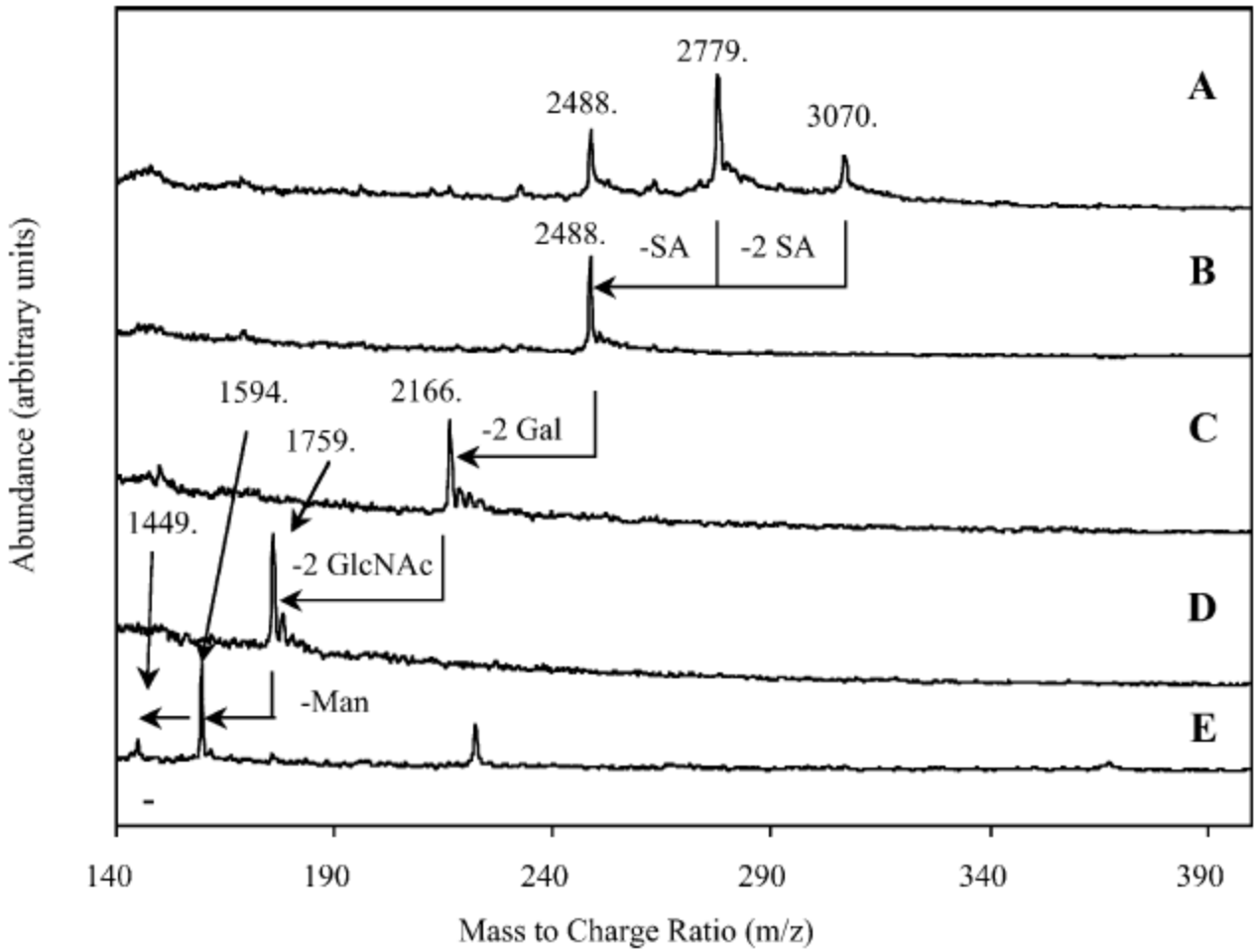


Fig. 2. MALDI-MS analysis of fraction T9 (A), after the digestion of fraction T9 with sialidase S (B), followed by β1-4 galactosidase (C) and then by β1-2-*N*-acetylglucosaminidase digestion (D). MALDI-MS analysis of fraction T9 after treatment with α1-6-fucosidase and α-mannosidase (E).

L. P. Kotra et al. / Bioorganic Chemistry 30 (2002) 356–370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