



Protease column portfolio 2020

AffiPro

Perfecting your proteomics



The aim of the company is to offer qualitatively better supporting technologies for life science mass spectrometry using novel affinity approaches and smarter fine-tuned surface chemistry

The current product portfolio includes protease columns for LC-MS and protein chips for *in-situ* sample preparation in MALDI-MS

Based in Mratin near Prague, Czech Republic
www.affipro.cz

Hydrogen Deuterium Exchange

- Hydrogen deuterium exchange (HDX) mass spectrometry is a powerful tool for studying the dynamics of higher order protein structure
- The rate of hydrogen deuterium exchange on the amide hydrogen of the protein backbone provides information about solvent accessibility
- This information can be used to deduct details about protein-ligand information, protein-protein interaction, protein folding and conformational changes

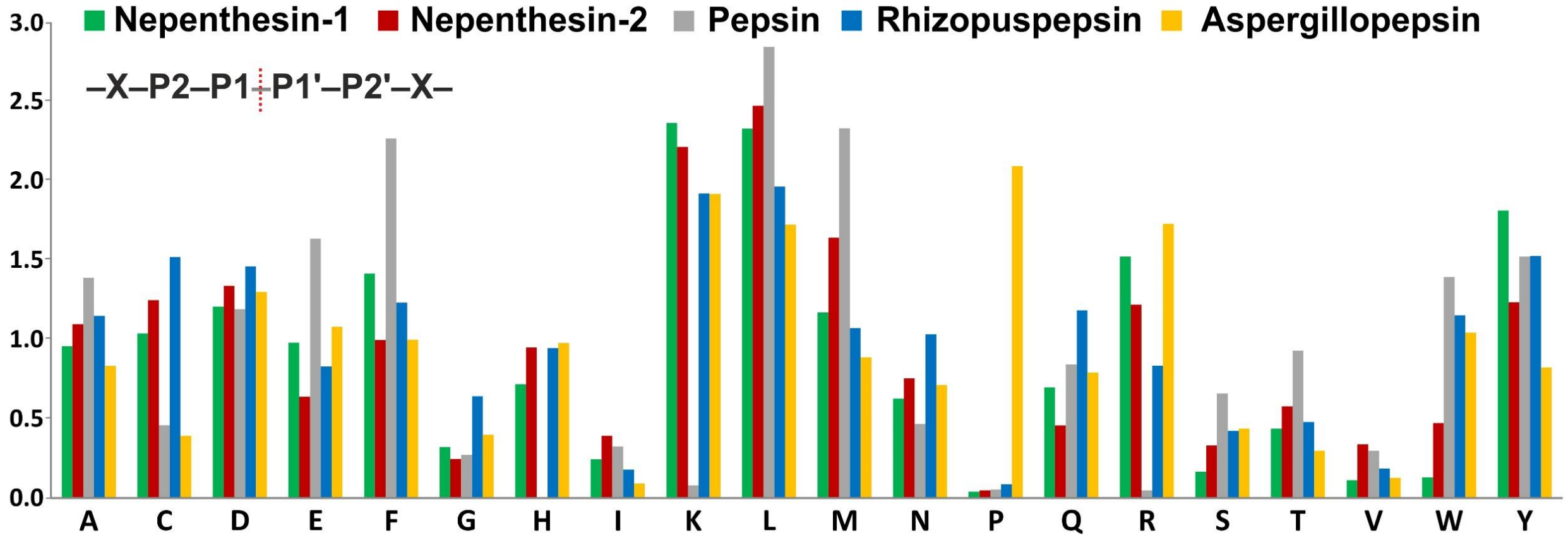
Protease digestion in HDX

- Optimization of protein digestion is a key step in the HDX experiment
- Spatial resolution and complete protein sequence coverage are the crucial factors
- Proper selection of high functioning protease column boosts digestion performance and allows to achieve full coverage of the protein sequence with many overlapping peptides
- AffiPro offers a set of five acidic protease columns with different cleavage preferences

Pepsin
Rhisopuspepsin
Nepenthesin-1
Nepenthesin-2
Aspergillopepsin



Cleavage preferences of acidic proteases



Yang M. et al., Anal Chem. 2015, Rey M. et al., Rapid Commun Mass Spectrom. 2009

Portfolio overview

PN	Protease	i.d. [mm]	Length [mm]	Volume [uL]
AP-PC-001	Pepsin	2.1	20	69.3
AP-PC-001s	Pepsin	1	20	16.2
AP-PC-002	Rhizopuspepsin	2.1	20	69.3
AP-PC-002s	Rhizopuspepsin	1	20	16.2
AP-PC-003	Nepenthesin-1	2.1	20	69.3
AP-PC-003s	Nepenthesin-1	1	20	16.2
AP-PC-004	Nepenthesin-2	2.1	20	69.3
AP-PC-004s	Nepenthesin-2	1	20	16.2
AP-PC-005	Aspergillopepsin	2.1	20	69.3
AP-PC-005s	Aspergillopepsin	1	20	16.2

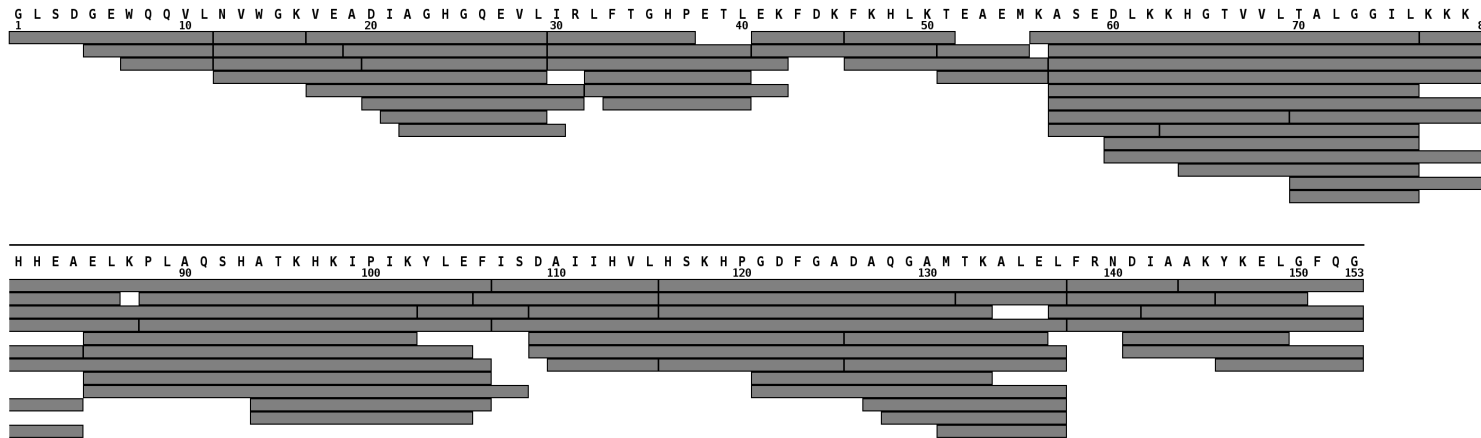


All fittings are 10-32 for 1/16" OD tubing

Nepenthesin-1

Digestion of Myoglobin, Nepenthesin-1 (AP-PC-003)

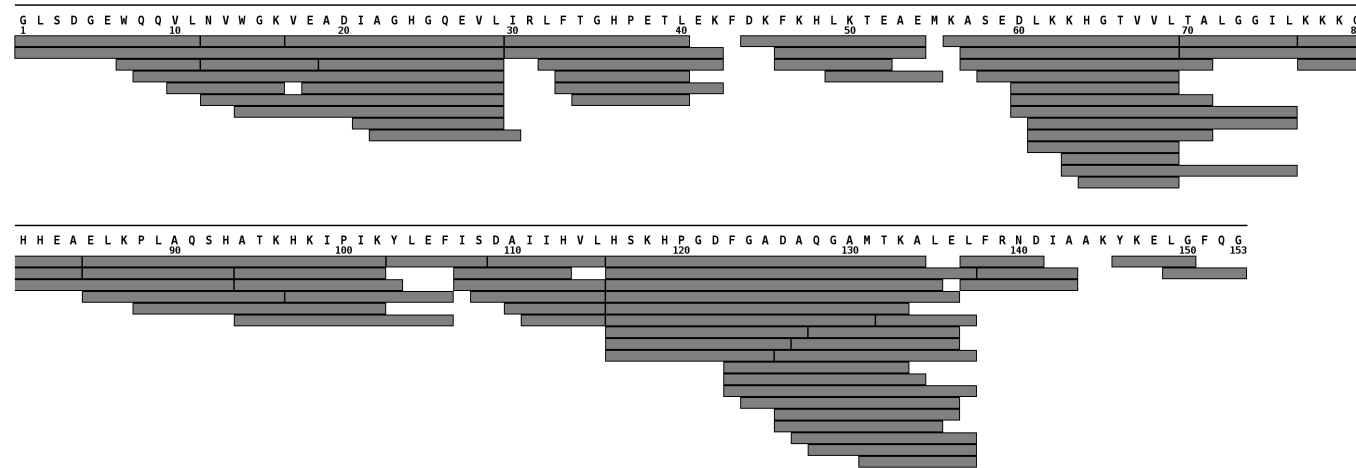
- Flow rate: **0.1mL/min**
- Temperature: 0-4°C



Nepenthesin-2

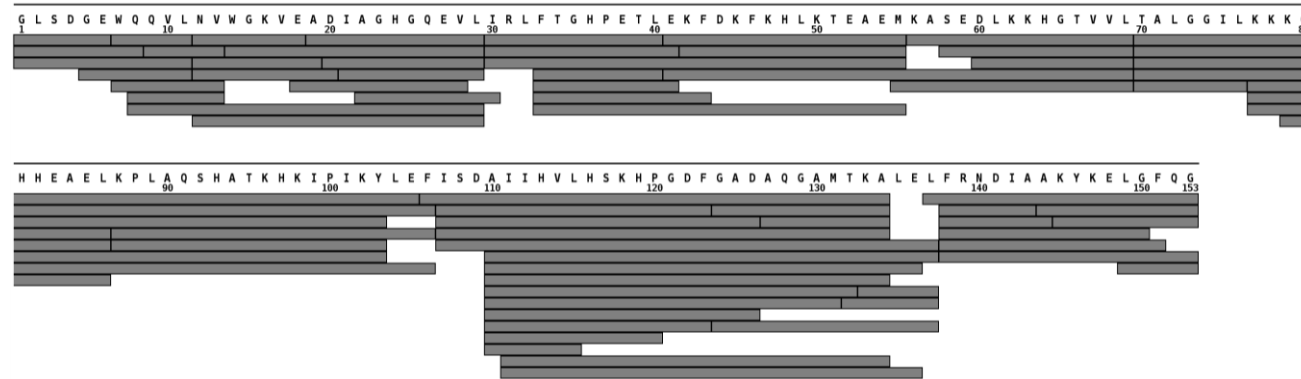
Digestion of Myoglobin, Nepenthesin-2 (AP-PC-004)

- Flow rate: **0.4mL/min**
- Temperature: 0-4°C



Digestion of Myoglobin, Pepsin (AP-PC-001)

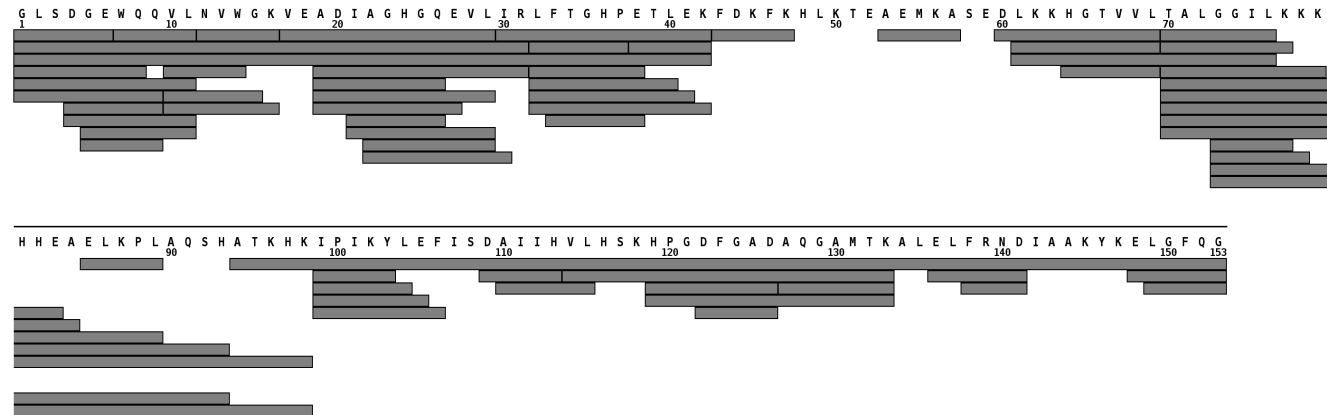
- 1pFlow rate: **0.1mL/min**
- Temperature: 0-4°C



Rhizopuspepsin

Digestion of Myoglobin, Rhizopuspepsin (AP-PC-002)

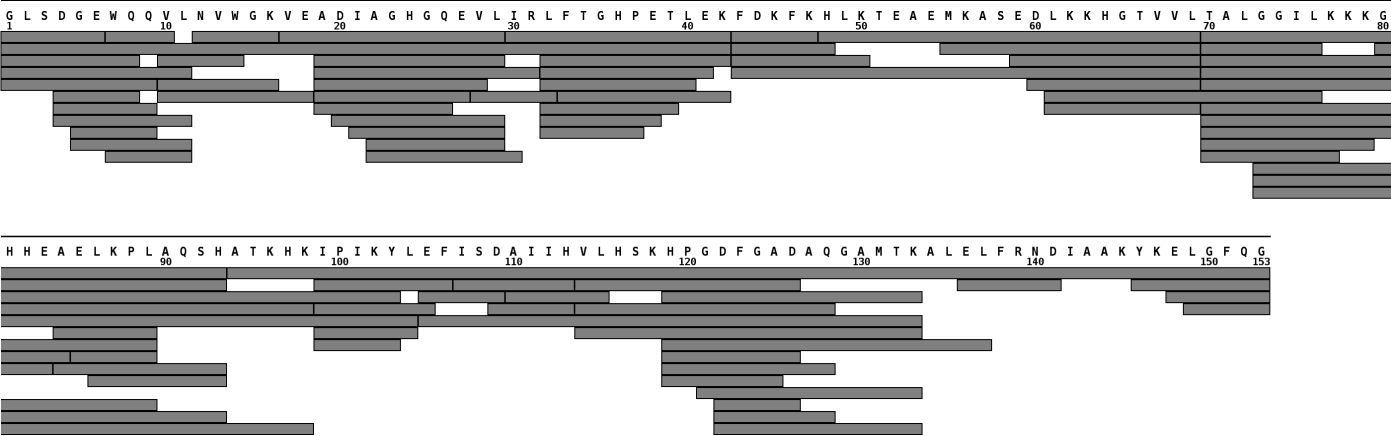
- 1pFlow rate: **0.2mL/min**
- Temperature: 0-4°C



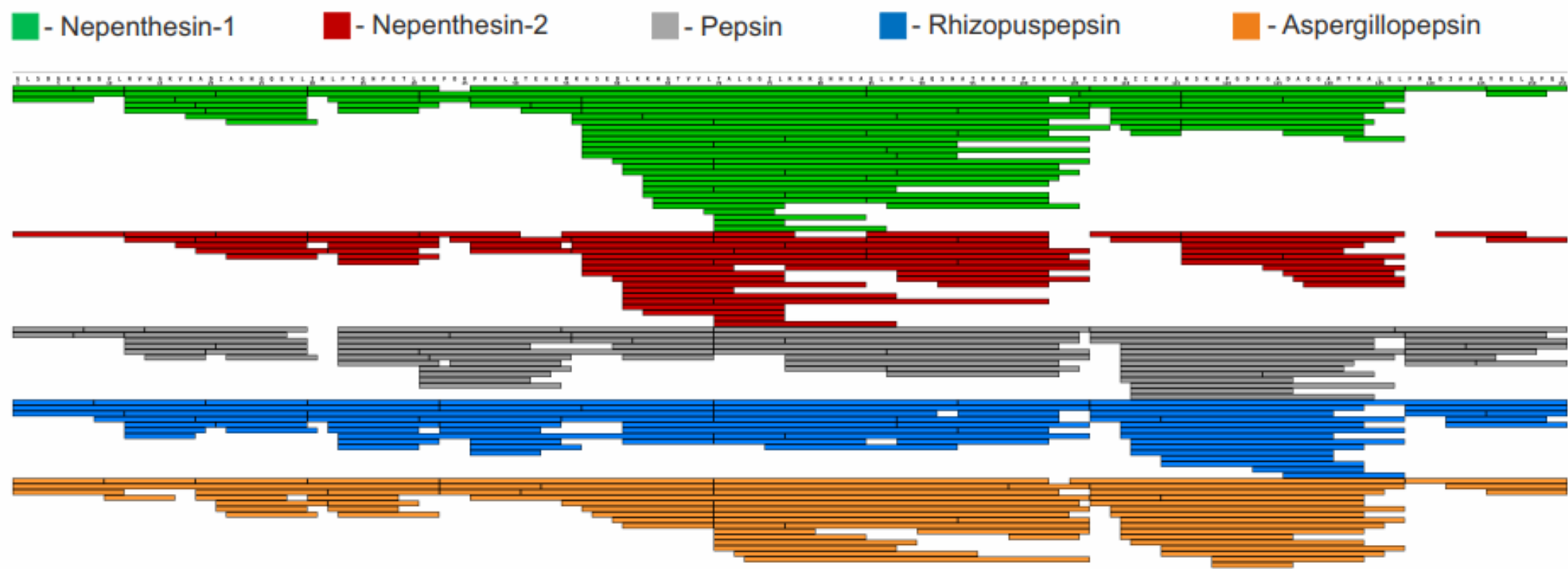
Aspergillopepsin

Digestion of Myoglobin, Aspergillopepsin (AP-PC-005)

- Flow rate: **0.4mL/min**
- Temperature: 0-4°C



Comparison of the proteolytic activity (Myoglobin)



AffiPro acidic protease columns protocol for online digestion

- The columns can be connected to any HPLC or LC/mass spectrometry system
- The degree of digestion can be controlled by changing flow rate, pressure or temperature.
- The columns are compatible with common buffers including **3 M urea, 1 M thiourea or 3 M guanidine hydrochloride**.
- The pressure limit of the columns is **2000 psi**.
- **The columns must not be exposed to solutions with pH greater than 4.5 or organic solvents.**
- The columns can be tested for enzyme activity using myoglobin.
- The columns should be stored with endcaps at 4°C in digestion buffer or in solution with pH lower than 4.5 without organic solvents.
- Before first use or after a longer storage, unplug the trap column and flush the protease column for 5min at flow rate 0.1 mL/min with digestion solvent.
- Autodigest peptides could be observed in the first run or after longer storage. Inject standard protein for several times to settle the column.

What is Nepenthesin?

New group of interesting proteolytic enzymes from carnivorous pitcher plants of the genus *Nepenthes*

Their proteolytic activity is very useful for hydrogen/deuterium exchange mass spectrometry (HX-MS)

In contrast to pepsin, it has different cleavage specificities, and despite its high inherent susceptibility to reducing and denaturing agents, it is very stable upon immobilization and withstands even high concentration of guanidine hydrochloride and reducing agents

Nepenthesin II shares many properties with Nepenthesin I, such as fast digestion at reduced temperature and pH, and broad cleavage specificity, but in addition, it cleaves C terminal to tryptophan

analytical chemistry Article
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Recombinant Nepenthesin II for Hydrogen/Deuterium Exchange Mass Spectrometry

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ABSTRACT: The pitcher secretions of the *Nepenthes* genus of carnivorous plants contain a proteolytic activity that is very useful for hydrogen/deuterium exchange mass spectrometry (HX-MS). Our efforts to reconstitute pitcher fluid activity using recombinant nepenthesin I (one of two known aspartic proteases in the fluid) revealed a partial cleavage profile and reduced enzymatic stability in certain HX-MS applications. We produced and characterized recombinant nepenthesin II to determine if a complemented nepenthesin II to nepenthesin I in HX-MS applications. Nepenthesin II shares many properties with nepenthesin I, such as fast digestion at reduced temperature and pH, and broad cleavage specificity, but in addition, it cleaves C-terminal to tryptophan. Neither enzyme reproduces the C-terminal proline cleavage that nepenthesin I, and it possesses a stability profile that is considerably more resistant to chemical denaturants and reducing agents than nepenthesin I, and it possesses a stability profile that is similar to that of pepsin. Higher stability combined with the slightly broader cleavage specificity makes nepenthesin II a useful alternative to pepsin and a more complete replacement for pitcher fluid in HX-MS applications.



Hydrogen/deuterium exchange mass spectrometry (HX-MS) provides conformational and structural data about proteins and complexes that can complement those generated by conventional structural biology methods.^{1,2} The digestion-based, or "bottom-up", approach offers a powerful way to conduct HX-MS studies. Compared to "top-down" methods, it requires low protein concentrations and low sample amounts and supports flexible buffer formulations.³ These attributes have led to new applications in the area of membrane protein analysis, complex multiprotein reconstructions, and antibody characterization.⁴⁻⁶ However, efficient digestion and antibody increases. This limitation partly arises from the suboptimal kinetics of pepsin digestion for low-temperature and short digestion work, which has prompted a search for alternative proteases.⁷

In a previous study, we demonstrated an effective alternative in an extract of the *Nepenthes* genus of carnivorous plants, otherwise known as pitcher plants or monkey cups.⁸ We showed that the proteolytic properties of the concentrated pitcher fluid are compatible with HX-MS conditions (i.e., low pH and temperature) and that the fluid possesses several advantages over pepsin, (i.e., activity was higher by >1000-fold and suppressing new cleavage sites were also observed (i.e., C-terminal to H, K, R, and P)).

Hooker observed in the 1870s that pitcher secretions contained proteolytic activity, but for a considerable period of time, the source of the activity was thought to be microbial in nature.^{9,10} Only recently has proteolytic activity been shown to

involve plant aspartic proteases called nepenthesins.^{11,12} Nepenthesins belong to MEROPS subfamily A1 of pepsin-like enzymes, which contain a typical Asp-Ser/Thr-Asp catalytic triad in the active site.¹⁴ Two isoforms of nepenthesin have been discovered and purified from *Nepenthes gracilis* plants. Nepenthesin I (NepI) and Nepenthesin II (NepII) had been partially characterized, and they demonstrate at least some classic aspartic protease features.^{13,15} They are expressed as zymogens that autoactivate in acidic pH by cleavage of a propeptide. The enzymes appear sensitive to inhibition by pepstatin A and are quite stable.

Because the fluid is an inconvenient source of enzymatic activity for HX-MS, we have begun to reconstitute fluid and characterized recombinant NepI (rNepI) from *Escherichia coli*.^{17,18} Our studies showed some differences between NepI and the native form of NepI, as well as the fluid. While the selectivity profile approached that of the fluid, there were notable exceptions. We saw little evidence of enzymatic activity and stability to W and P. We also observed that the native purified form, although the stability under reducing and denaturing conditions could be improved through immobilization.¹⁹ Native NepI is glycosylated, which likely explains its higher stability and perhaps activity, but the "missing" cleavage properties argue for other enzymes.

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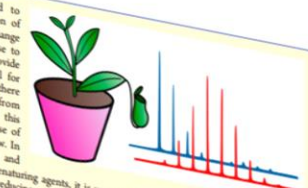
Aspartic Protease Nepenthesin-1 as a Tool for Digestion in Hydrogen/Deuterium Exchange Mass Spectrometry

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Supporting Information

ABSTRACT: Hydrogen/deuterium exchange coupled to mass spectrometry (HXMS) utilizes enzymatic digestion of proteins to localize the information about altered exchange patterns in protein structure. The ability of the protease to produce small peptides and overlapping fragments and provide sufficient coverage of the protein sequence is essential for localizing regions of interest. Recently, it was shown that there is an interesting group of proteolytic enzymes from carnivorous pitcher plants of the genus *Nepenthes*. In this report, we describe successful immobilization and the use of one of these enzymes, nepenthesin-1, in HXMS workflow. In contrast to pepsin, it has different cleavage specificities, and despite its high inherent susceptibility to reducing and denaturing agents, it is very stable upon immobilization and withstands even high concentration of guanidine hydrochloride and reducing agents. We show that denaturing agents can alter digestion by reducing protease activity and/or substrate solubility, and additionally, they influence the trapping of proteolytic peptides onto the reversed phase resin.



Protein hydrogen/deuterium exchange coupled to mass spectrometry (HXMS) is an integral part of structural biology and is routinely used in the biopharmaceutical industry.¹⁻¹⁷ It can address various questions about protein structure changes under various conditions (pH, temperature, ionic strength, etc.) or interactions with different ligands (e.g., protein, DNA, small molecule).⁸⁻¹¹ A typical workflow involves a continuous isotope exchange reaction applied to different states of a protein and sampled at select time points. The protein is then digested, and the deuterium uptake is measured by mass spectrometry for each proteolytic fragment generated. Finally, the deuteriation profiles of the individual states of the protein are compared. Despite its many advantages, HXMS suffers from low spatial resolution, which is dictated by the length of the peptides generated by the protease of choice.

Due to restrictions imposed by the quenched exchange conditions that must be maintained during the analysis (pH close to 2.5, temperature close to 0 °C), the choice of the protease is quite limited. The one most frequently used is porcine pepsin, which cleaves preferentially at the C-terminus of large hydrophobic amino acids.¹²⁻¹⁶ Pepsin is readily available at high purity and retains functionality after immobilization, which leads to increased digestion efficiency by enhancing the local protease/protein ratio and which supports higher resistance to denaturing and reducing agents. Therefore, in the immobilized form, pepsin can be used to digest proteins of highly compact structure and/or proteins stabilized by disulfide bonds.¹⁷⁻²⁰ However, there are many proteins for which pepsin does not provide sufficient spatial resolution and sequence coverage.^{11,21}

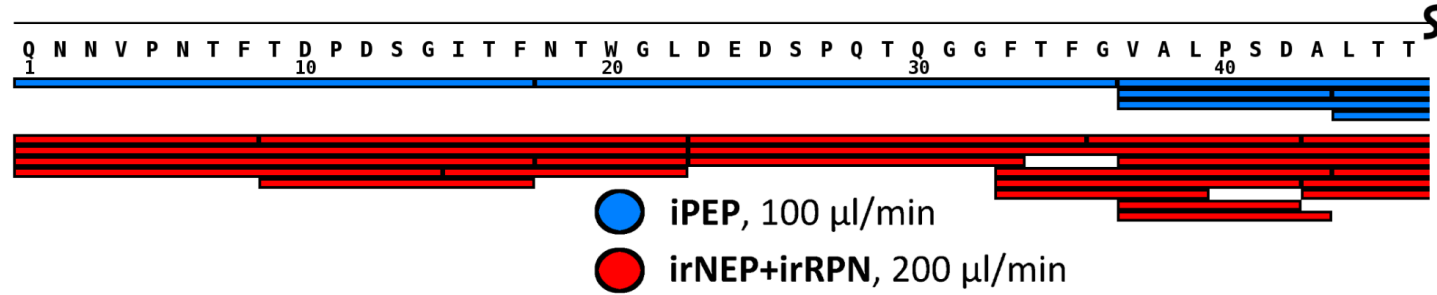
One approach to increasing the spatial resolution of HXMS involves tandem mass spectrometry. Here, techniques based on electron-induced fragmentation are promising but currently limited to small proteins or a subset of digest peptides.²²⁻²⁵ An alternative strategy is based on a computational approach requiring a large number of overlapping peptides.²⁶⁻³¹ Provided certain conditions are met,²⁹ in all cases, novel proteases with different cleavage preferences would increase the number of cleavage sites, and/or process sequence coverage and spatial resolution, and/or process proteins not easily digested by pepsin. The first report on this topic appeared ten years ago and described the use of two aspartic proteases: aspergillo- and rhizopuspepsin (known also as protease type XIII and XVIII, respectively).³² These two proteases were shown to be complementary to pepsin, based on a higher preference for cleavage after basic amino acid residues.^{33,34} Later reports described the preparation and immobilization of recombinant rhizopuspepsin and the suitability of protease type XIII for simultaneous digestion and digestion of proteins in HXMS workflow.^{33,34} Several papers also identified new proteases from various

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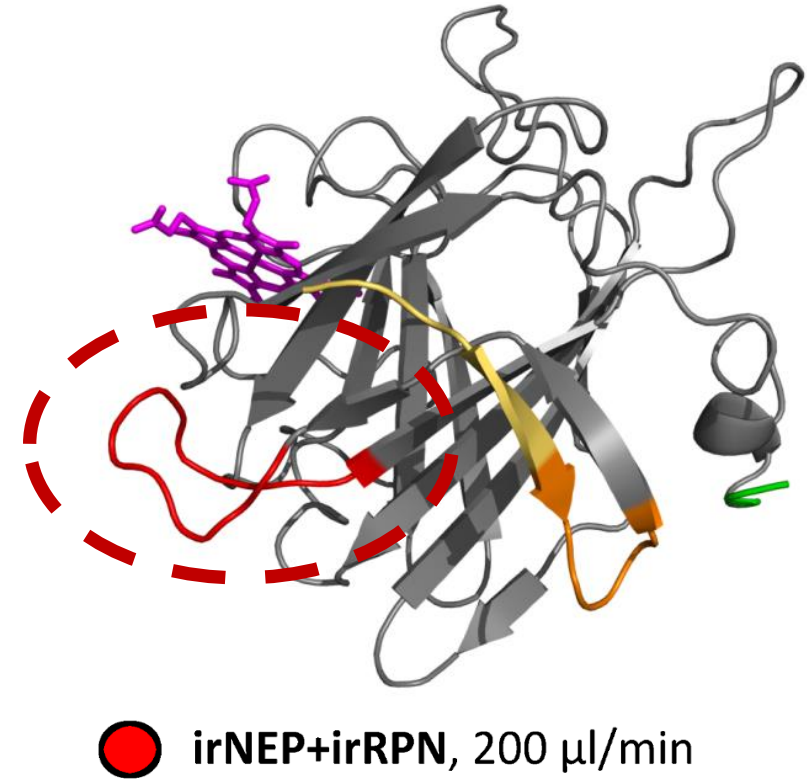
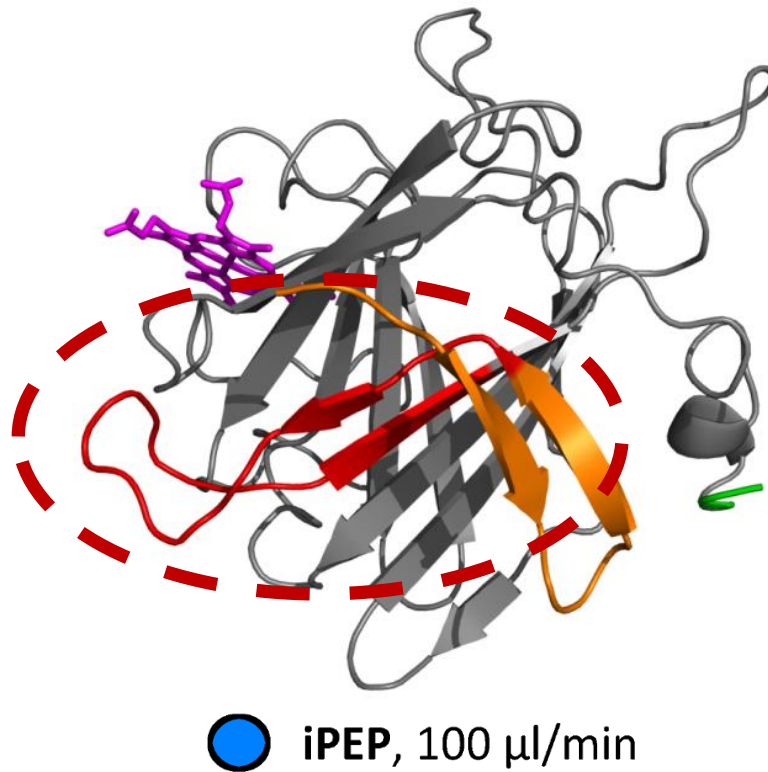
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Proteases Combination – Improved Spatial Resolution



Combining the proteases provides more information, which makes a strong scientific argument for more diverse protease column portfolio



<https://doi.org/10.1016/j.bbagen.2016.11.016> | Kadek et al., Biochim Biophys Acta (2017)

Examples of reported customer use

University of Leeds

Article | [Open Access](#) | Published: 01 May 2020

Inter-domain dynamics in the chaperone SurA and multi-site binding to its outer membrane protein clients

Antonio N. Calabrese, Bob Schiffrin, Matthew Watson, Theodoros K. Karamanos, Martin Walko, Julia R. Humes, Jim E. Horne, Paul White, Andrew J. Wilson, Antreas C. Kalli, Roman Tuma, Alison E. Ashcroft, David J. Brockwell & Sheena E. Radford [✉](#)

Nature Communications **11**, Article number: 2155 (2020) | [Cite this article](#)

2656 Accesses | **2** Citations | **93** Altmetric | [Metrics](#)

Abstract

The periplasmic chaperone SurA plays a key role in outer membrane protein (OMP) biogenesis. *E. coli* SurA comprises a core domain and two peptidylprolyl isomerase domains (P1 and P2), but its mechanisms of client binding and chaperone function have remained unclear. Here, we use chemical cross-linking, hydrogen-deuterium exchange mass spectrometry, single-molecule FRET and molecular dynamics simulations to map the client binding site(s) on SurA and interrogate the role of conformational dynamics in OMP recognition. We demonstrate that SurA samples an array of conformations in solution in which P2 primarily lies closer to the core/P1 domains than suggested in the SurA crystal structure.

<https://www.nature.com/articles/s41467-020-15702-1>

The Hebrew University of Jerusalem

Biochemistry

Defining Hsp33's Redox-regulated Chaperone Activity and Mapping Conformational Changes on Hsp33 Using Hydrogen-deuterium Exchange Mass Spectrometry

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* These authors contributed equally

Summary

One of the most challenging stress conditions that organisms encounter during their lifetime involves the accumulation of oxidants. During oxidative stress, cells heavily rely on molecular chaperones. Here, we present methods used to investigate the redox-regulated anti-aggregation activity, as well as to monitor structural changes governing the chaperone function using HDX-MS.

<https://www.jove.com/t/57806/defining-hsp33-s-redox-regulated-chaperone-activity-mapping>

Customer testimonials in the USA

Large portion of our customers in North America are in biopharma and biotech companies and do not publish nor discuss their protocols and molecules publicly. However, we can provide testimony of some leading academic KOLs in the HDX field who are using AffiPro columns.

**North Eastern University in Boston
John Engen Laboratory**

Thomas E. Wales, Ph.D.
Research professor in Engen group
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**The Engen Laboratory website:
<http://www.hxms.neu.edu/index.htm>**

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