Functional Characterization and Protein Engineering of Prolyl Endoprotease Neprosins from *Nepenthes rafflesiana*

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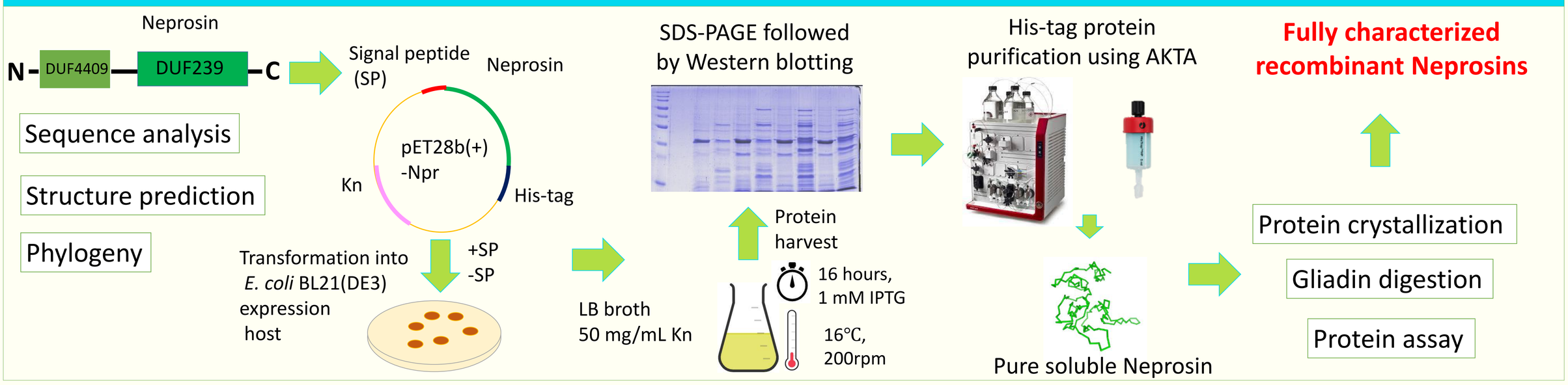
Introduction

- **Prolyl endoprotease** (PEP) belongs to a serine protease family that cleaves protein at proline residues. Previous studies(*1-3*) showed PEP cleaves gliadins, a sub-component of gluten that cannot be digested by gluten-sensitive individuals, causing intestinal inflammation in celiac disease patients.
- **Neprosin 1**(Npr1) and its homolog **Neprosin 2**(Npr2), were found to be a new PEP group discovered in *Nepenthes* which showed promising ability to cleave gliadin at low pH and concentration.

Objectives

- We aim to **functionally characterize** two novel PEPs found in the pitcher fluids of a carnivorous pitcher plant *Nepenthes rafflesiana*, Npr1 and Npr2
- To express and purify recombinant Neprosins
- To study the similarities and differences of the Neprosin homologs

Methods



Results

(a) Sequence analysis of Neprosins

Neprosins	Neprosin_propeptide domain (aa positions)	Neprosin domain
N. ampullaria 1	56-179	192-414
N. ampullaria 2	57-179	192-414
N. rafflesiana 1	37-109	151-373
N. rafflesiana 2	39-136	153-354
N. × ventrata	41-113	155-377
N. alata	41-111	155-377

(b) Percentage identity matrix

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1: Na1      100.00   85.71   36.00   40.21   40.92   42.09
2: Na2       85.71  100.00   38.00   41.02   42.28   43.70
3: Nr2       36.00   38.00  100.00   55.97   62.78   58.81
4: N.alata   40.21   41.02   55.97  100.00   84.84   89.74
5: Nr1       40.92   42.28   62.78   84.84  100.00   91.22
6: Nv        42.09   43.70   58.81   89.74   91.22  100.00
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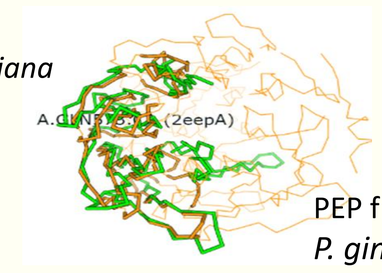
(c) Comparisons between Neprosin homologs

Proteins	No. of amino acids	MW (kDa)	pI	Instability index	Protein stability	Aliphatic index
Npr1	376	41.2	6.6	33.67	Stable	68.27
Npr2	357	39.4	7	33.6	Stable	74.68

(d) Protein structure prediction

PEP from *N. rafflesiana*



PEP from *P. gingivalis*

Figure 1. (a) Sequence analysis of Neprosins from different *Nepenthes* species. (b) Multiple sequence alignment of closely related species (*N. rafflesiana* [Nr], *N. alata, N.* x *ventrata* [Nv], *N. ampullaria* [Na]) by Clustal 2.1. (c) Comparisons between the two Neprosin homologs using ExPASy. (d) Neprosin structure (green) predicted by I-TASSER showing resemblance with a known prolyl tripeptidyl aminopeptidase from *Porphyromonas gingivalis* (yellow) through structural alignment using DALI.

Discussion

- Neprosin domains are conserved in *Nepenthes* species and has over **90%** identity with a previously reported Neprosin from *N. × ventrata* shown to degrade gliadins (*2*)
- The Neprosin structure predicted showed some similarity to the B-propeller domain of *P. gingivalis* prolyl tripeptidyl aminopeptidase. However, the length of amino acid of the aminopeptidase are almost twice the length of Neprosin with percentage similarities of only **20%** in a global alignment .
- The extraction of IPTG-induced cells grown at 16°C using glycine-HCl pH 2.5 buffer yielded **soluble** recombinant proteins at the expected sizes.

Conclusion

Recombinant Neprosins from *N. rafflesiana* has been successfully expressed by *E. coli* system. The His-tag recombinant proteins will be purified to homogeneity for further functional assays.

Gene cloning

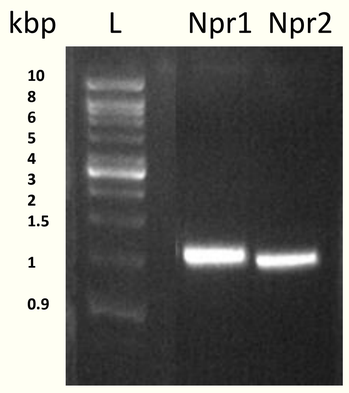


Figure 2. PCR products of Neprosin at the expected sizes without SP.

Protein expression

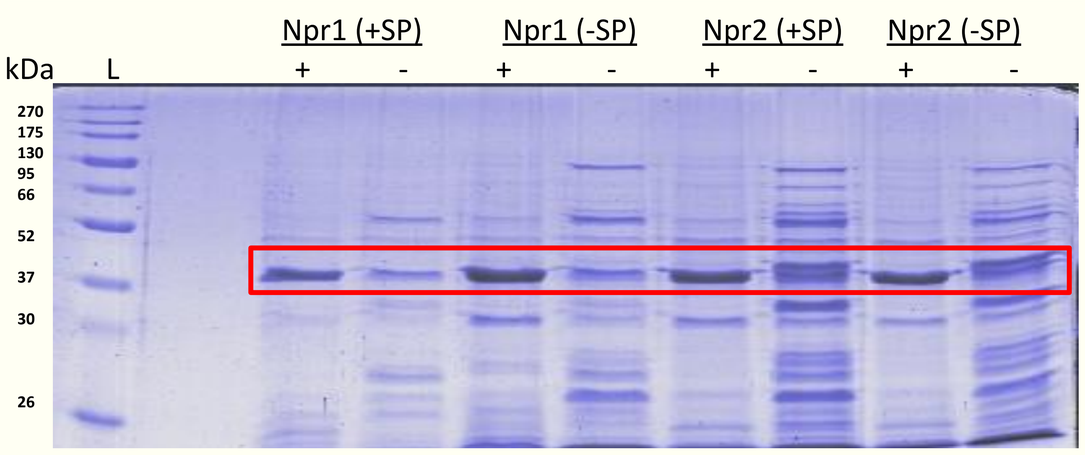


Figure 3. Expression of soluble recombinant Neprosins in the supernatant with the expected sizes after induction (+) and without induction (-) with IPTG.

References

1. Eugster *et al.*, Production and characterization of two major *Aspergillus oryzae* secreted prolyl endopeptidases able to efficiently digest proline-rich peptides of gliadin. *Microbiology* **161**, 2277-2288 (2015).
2. Rey *et al.*, Addressing proteolytic efficiency in enzymatic degradation therapy for celiac disease. *Sci Rep* **6**, 30980 (2016).
3. Moreno Amador *et al.*, A new microbial gluten-degrading prolyl endopeptidase: Potential application in celiac disease to reduce gluten immunogenic peptides. *PloS ONE* **14**, e0218346 (2019).