Aspergillus niger Prolyl Endoprotease from Nutritional Supplement Capsules for Use in Hydrogen-Deuterium Exchange Mass Spectrometry

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OUTLINE

Objective
Assay a commercial source for *Aspergillus niger* prolyl endoprotease (ANPEP) for potential application in hydrogen-deuterium exchange mass spectrometry (HDX MS) workflows as a complement to pepsin and nepenthesin II

Methods
SDS-PAGE, spectrophotometric enzymatic assay, LC-MS

Results
ANPEP appears to be present at usable purity and high functionality within capsules, and it cleaves at a similar efficiency to that of pepsin and nepenthesin II in a traditional HDX MS workflow

INTRODUCTION

Typical HDX MS workflows require quenching and digestive steps to stop the deuterium exchange reaction and process a target protein into measurable peptides, respectively. The low temperature and pH required for quenching severely limit the number of proteases used, leaving few industry standards such as pepsin or nepenthesin II. These enzymes often cleave poorly C-terminally to proline given its irregular structure.

Here, we investigate a novel prolyl endoprotease from *Aspergillus niger* (ANPEP), previously shown to perform well under HDX MS conditions in solution.[1] ANPEP has been studied numerous times for potential applications in celiac disease treatment through gluten degradation.[2,3] A product of these investigations is Tolerase G, nutritional supplement pills intended to aid gluten indigestion.

While previous studies of ANPEP have required expression of the enzyme in yeast or buffer-exchange purification schemes, these capsules are easily processed into a usable form.[1,4] The primary focus of this investigation is to determine whether this cheap source can be utilized in a mock-HDX MS online workflow at a similar efficacy to pepsin or nepenthesin II.

References
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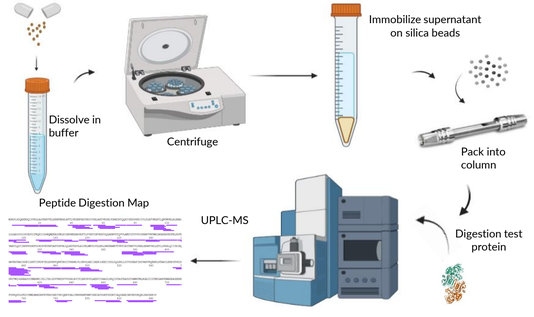
METHODS



Tolerase G capsules were processed according to a simple isolation scheme, illustrated above. ANPEP supernatant was separated by SDS-PAGE to assess molecular mass and basic digestive ability. Supernatant was additionally subjected to pH and enzyme:substrate ratio testing using Z-Gly-Pro-Ala-pNA model substrate. ANPEP column was compared against pepsin and nepenthesin II in a mock-HDX MS workflow for cleavage of six target proteins.

RESULTS AND DISCUSSION

Figure 1: Characterization of Isolated ANPEP

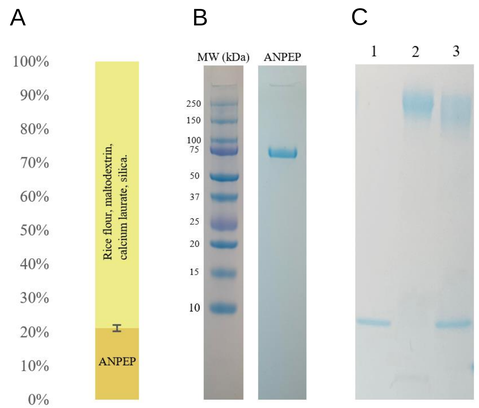


Figure 1. **A:** Percentage by mass of ANPEP in each pill as determined by Bradford. **B:** Molecular weight of ANPEP by SDS-PAGE. Expected non-glycosylated mass 58 kDa. **C:** In-solution digestions of Myoglobin (Mb, 17kDa) by ANPEP. Lane 1: Mb alone; Lane 2: Mb + ANPEP 5 min. digestion at 0°C; Lane 3: Mb + Heat-Killed ANPEP.

Figure 2: Activity Assessment

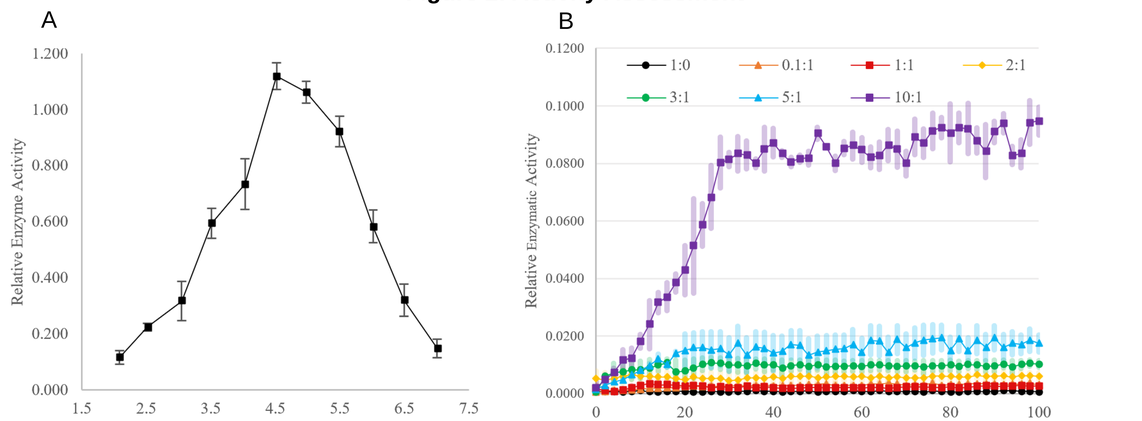
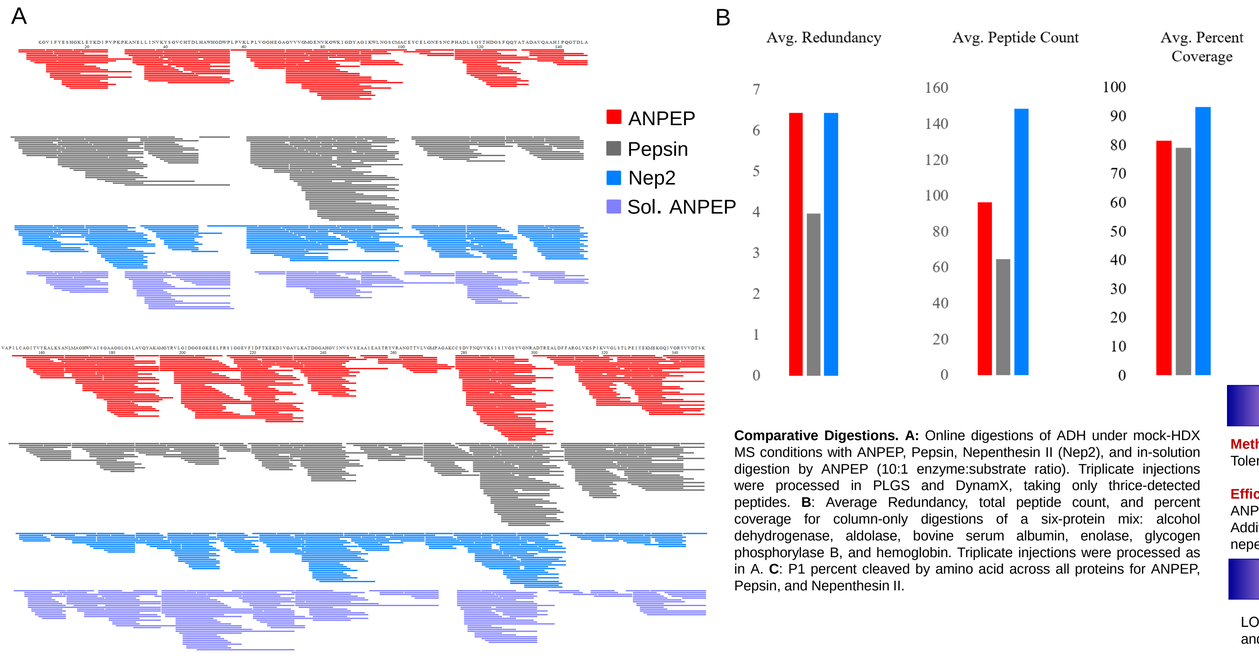


Figure 2: A: Relative enzymatic digestion by ANPEP of Z-Gly-Pro-pNA at varying pH, 10:1 enzyme:substrate ratio at 19.5 °C. Standard error of triplicate measurements is displayed.
B: Relative enzymatic digestion by ANPEP of Z-Gly-Pro-pNA over time at varying enzyme:substrate ratios. Standard error of triplicate measurements is displayed.

Figure 3: Comparative Digestions by ANPEP, Pepsin, and Nepenthesin II



Comparative Digestions. A: Online digestions of ADH under mock-HDX MS conditions with ANPEP, Pepsin, Nepenthesin II (Nep2), and in-solution digestion by ANPEP (10:1 enzyme:substrate ratio). Triplicate injections were processed in PLGS and DynamX, taking only thrice-detected peptides. **B**: Average Redundancy, total peptide count, and percent coverage for column-only digestions of a six-protein mix: alcohol dehydrogenase, aldolase, bovine serum albumin, enolase, glycogen phosphorylase B, and hemoglobin. Triplicate injections were processed as in A. **C**: P1 percent cleaved by amino acid across all proteins for ANPEP, Pepsin, and Nepenthesin II.

CONCLUSIONS

Method Feasibility
Tolerase G capsules are a cheap, quick, and relatively pure way to create an ANPEP column.

Efficacy in HDX MS Online Digestions
ANPEP performed similarly to pepsin and nepenthesin II, allowing for expected variation in digestion. Additionally, ANPEP demonstrates biased but widespread digestion different than that of pepsin or nepenthesin II, indicating its potential use as a complementary protease to current standards.

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