Human Prolyl Endopeptidase: Potential in Alzheimer's Treatment

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Introduction

Human prolyl endopeptidase (hPEP) has several functions in the brain, one of which is the degradation of neuropeptides. This is significant for diseases like Alzheimer's, which is known to be caused (at least partially) by neural plaques such as amyloid-β; hPEP plays a role in generating the sticky amyloid plaques as part of its protease function. hPEP is purported to be inhibited by peptides smaller than 30 amino acids. Due to the structural similarity between hPEP and a natural glutenase, spPEP (Sunn pest prolyl endopeptidase), we hypothesize that hPEP could have a second mechanism of binding similar to that of spPEP which binds larger peptides. We are using spPEP as a control due to its 99% structural identity based on modeling and its ability to bind larger substrates, specifically gluten proteins, up to several hundred amino acids in length. This second mechanism occurs in the active site between the two lobes of the spPEP enzyme. Using peptides of varying length, we are investigating if hPEP can bind larger substrates in the same way as spPEP.

A peptide found in αS1-casein with a size of 18 amino acids is a known inhibitor of the hPEP; this inhibitor sequence is being used in αS1-casein peptides of varying length; specifically, we are using the 18-mer, 28-mer, and a 38-mer. The focus of this study is to optimize the purification process of the isolated recombinant peptides using Casein peptide 9, a 68-mer, expressed as a fusion to SUMO protein in *E. coli.* The 68-mer was chosen due to its large size making it easier to visualize upon purification. Presumably, the smaller recombinant peptides will express and purify using the same protocol developed in this study. The recombinant hPEP is also being expressed and purified during this project, which will be used with the inhibitors to investigate its kinetics to see if it does bind larger peptides than 30 amino acids in length.

Materials and Methods

- Materials used include reagents and precast gels purchased from BioRad Corp. (Hercules,CA) and cOmplete® His-Tag Purification Ni resin purchased from Sigma-Aldrich (St. Louis, MO). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).
- Methods Include:
  - **Expression and Purification of Recombinant Proteins:**
    - Recombinant *E.coli* containing the Peptide 9, a 68-mer of the N-terminus of α-S1 casein in the pCGX expression plasmid was inoculated at a 1/100 ratio of overnight culture to growth medium volume in two 1L volumes of Luria Burtani Miller broth at 37°C until the OD 600 reached 0.6. Then IPTG was added to a final concentration of 1mM. The culture continued to grow overnight at 30°C then cells were harvested by centrifugation.
    - hPEP Expression- The recombinant hPEP in the plasmid pLIC were expressed in Bl21(DE3)pLysS cells with the Lys replaced with the Trigger factor chaperone. Cells were inoculated with a 1/100 ratio of overnight culture into a 6L volume of 2X Luria Burtani Miller broth. Aeration paddles set at 400 RPM and temperature were controlled using a BioFlo110 (New Brunswick Corp. NJ). IPTG addition, temperatures of incubation and harvesting were the same as described above for the peptide.
    - Cell Lysis: After being harvested, the cells were pooled into one bottle and lysed using CelLytic Express in the 4°C cooler overnight. This process was the same for both peptide and hPEP. Cells were harvested by centrifugation.

Methods and Materials cont.

Electrophoresis:

- Pre-cast 12% SDS Polyacrylamide gels (PAGE) were used for SDS polyacrylamide electrophoresis of the hPEP and SUMOase enzyme purifications.
- SDS PAGE gels were used to monitor the purification of the hPEP and the SUMOase enzyme.
- Tris-tricine gels were used in detection of SUMOase digestion to liberate the peptide.
- The gels were stained in either Coomassie or SYPRO Ruby stain for visualization.

Assays:

- Bradford assays were made and then read with a plate reader to determine activity and amount of both peptide and enzyme. Bradford assays usually consist of two "standard" rows, the standard being bovine serum albumin (BSA). Phosphate buffer is added to the wells in amounts decreasing by 2µL, from 100 to 80 µL. The BSA is also added in amounts of increasing 2µL increments, this time increasing from 0 to 20µL, then 100µL of Bradford reagent was added to every well, totaling to 200µL. The actual samples were done in triplicate, with three wells each of 2, 5, and 10µL samples. 98, 95, and 90µL of phosphate was added to these wells, respectively. Bradford reagent was also added to the sample wells (100 µL). The Bradford assay protocols on the SoftMax Pro Plate Reader were used to read the plates at 595nm.
- **Other Methods:**
  - Other methods used include chromatography and enzyme digestion, which can be seen in more details in the results section.

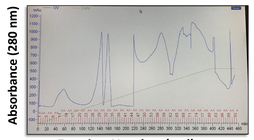

Figure 1. The chromatogram for hPEP. While the graph shows multiple peaks, the assay done on the fractions afterward indicated that only fractions 8-23 had any enzyme, indicating that the other peaks were something else, such as air bubbles.

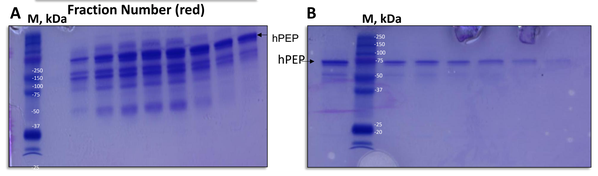

Figure 2. The gels with fractions 8-23 of hPEP. Figure A has 8-16, which shows quite a bit of contamination of other proteins. Also, despite appearing on the chromatogram, the well containing sample 8 appears to have disappeared, indicating that there wasn't any hPEP. Figure B has fractions 17-23 primarily hPEP at expected 80 KD.

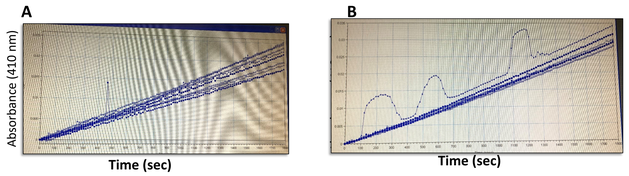

Figure 3. The graphs for activity of hPEP. Figure A contains fractions 8-16, while figure B contains fractions 17-23. Overall, hPEP has relatively low activity, which is expected. There was less activity in fractions 8-16 than 17-23 (Figure B).

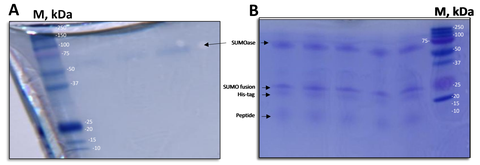

Figure 4. A) The 2 recombinant SUMO proteases compared to the commercial were all the same size, as expected. Run on SDS PAGE gels with the BioRad Dual Color marker. **B)** The peptide digestion using SUMOase run on tris-tricine gels with the BioRad Dual Xtra marker.

Results

hPEP was successfully expressed and purified using Nickel affinity chromatography. Multiple peaks were observed during the Ni purification (Figure 1). Each fraction was tested for hPEP activity. The fractions with activity were loaded onto an SDS PAGE. In the first few fractions (Figure 2A), there were many contaminating proteins. Pure hPEP was present in fractions 17-23 which were pooled. The activitiy was tested using GPpNA as substrate. All fractions containing hPEP had activity (Figure 3).

Recombinant SUMOase was successfully purified (Figure 4A). The Sumo-fusion peptide was digested using two recombinant enzymes purified in the lab, and one purchased. The recombinant Sumoase was similar in size to the commercial (Figure 4A) and all showed activity in digesting the peptide from the sumo fusion. There was some uncleaved peptide-fusion remaining under the conditions run. The liberated peptide had the expected MW of 3 KD Figure 4B).

Discussion and Conclusion

This project will continue; the next step is to optimize the SUMOase digestion of the fusion peptide to purify the peptide away from the Sumo and SUMOase. This will be done by increasing the amount of enzyme to fusion peptide and/or increasing the time of incubation. Then this method will be used to purify the different recombinant peptides. Nickel affinity chromatography will be used to remove the SUMOase and the SUMO from the peptide for purification. The peptides will then be used in inhibition kinetics to see if the peptides inhibit the hPEP. We will measure the kinetics of this process to see the rate at which hPEP is inhibited. spPEP will be used as a control. The purpose of this, specifically using sPEP as the control, is to see if hPEP has a similar mechanism of inhibition that spPEP does. These two enzymes are 99% alike structurally, yet so far hPEP has not been shown to bind larger peptides similar to spPEP, and we are not sure why that is.

This project is significant because further understanding of this enzyme and how it functions could lead to future drug development for controlling the neural plaques seen in diseases such as Alzheimer's, or even other neurological diseases like Multiple Sclerosis and Parkinson's.

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