

Is Porcine Pancreatic Elastase a Suitable Model for Human Leukocyte Elastase?

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Human Leukocyte Elastase (HLE) has recently been implicated as a poor prognostic marker in certain breast cancers and as a key component involved in Chronic Obstructive Pulmonary Diseases. Because HLE is so expensive, Porcine Pancreatic Elastase (PPE) has been used to model elastase activity and inhibition. This paper elucidates the differences and similarities between HLE and PPE through enzyme activity assays, protein sequence alignment, and specificity comparison. In addition, PPE is a nonglycosylated protein which is secreted as a zymogen, where HLE is glycosylated at two positions and is released as a series of isoenzymes. The enzyme activity assays help to prove that HLE and PPE have different substrate specificities. HLE prefers valine while PPE prefers alanine. The two proteins, when aligned using uniprot, show only a 47% similarity.

Enzymatic activity is essential for the activity of most, if not all, biological systems. Enzymes allow for the most specific and efficient use of nutrients and energy required for living systems to maintain their peak activity while other stresses in the environment threaten them.¹ The enzyme this study focuses on is elastase in human leukocytes. Elastase, which gets its name from its specificity to break down elastin, is a proteolytic enzyme that is naturally inhibited by the binding of alpha-1 Protease Inhibitor (α -1PI). When α -1PI is oxidized it no longer binds elastase which causes elastase to become activated. During tissue remodeling (wound healing) H_2O_2 is produced and it provides the mechanism for α -1PI oxidation.¹ Elastin is a key component in keeping parts of the body flexible, such as the elasticity in the skin and in the lungs.

The loss of this elasticity can have substantial effects on the body. In the lungs, elastin maintains the ability of the connective tissues connecting the air sacs to expand and contract. If the α -1PI is oxidized outside of normal remodeling, the lungs could lose their

ability to perform their function of breathing; this results in a family of diseases called Chronic Obstructive Pulmonary Diseases (COPDs). Cigarette smoke and other environmental pollutants oxidize α -1PI and thus activate elastase, which causes undamaged elastin to be broken down faster than it is resynthesized.

Neutrophil elastase has also been found in high concentrations in human breast cancer cells. One such breast cancer is the MDA-MB-231 strain.² In this context, elastase plays a different role. Elastase cleaves 50-kDa cyclin E into a more active 35-kDa cyclin E. The presence of 35-kDa cyclin E has been associated with a poor prognosis in patients with the MDA-MB-231 breast cancer³

Cyclin E is a cell cycle protein which is required to send the cell into the S-phase of division. The lower molecular weight cyclin E (LMW) is more active than the higher molecular weight cyclin E (HMW). As a result, cells with a predominance of LMW will progress through the cell cycle more rapidly inducing a tumorous cell. Based on the cleavage locations of the LMW it was

¹ Lazari et al. (2009)

1 Lazari et al.

2 Nguyen et al.

3 Nguyen et al.

determined that the enzyme responsible is a protease of the elastase family. To confirm this, wild type Cyclin E was partially digested using porcine pancreatic elastase. The digested cyclin E was analyzed using Western blot and compared to the *in vivo* cyclin E from tumor cells. The band patterns for the two digests were similar and showed nearly the same LMW products.⁴

Indole-3-Carbinol is a natural product found in cruciferous vegetables. Studies performed by Firestone et al. have indicated that I3C arrests G₁ cell-cycle progression in estrogen-responsive and unresponsive human breast cancer. I3C acts by fostering the accumulation of HMW as opposed to LMW. Western blot analysis indicates that MDA-MB-231 cancer cells treated with I3C show a significant decrease in the concentration of LMW. With the knowledge that elastase cleaves cyclin E, Western blot and chromogenic assays were performed to determine if I3C inhibits elastase and, if so, how it inhibits. Both assays reveal that the I3C arrests G₁ cell-cycle progression by binding elastase in a noncompetitive fashion.⁵

Studying Indole-3-Carbinol, and its binding to elastase was the important next step. Crystallographic studies appeared to be the best suited to analyze the inhibitory binding site of elastase to I3C. Approximately 20ug of HLE from Sigma Aldrich costs \$112, and since milligram amounts of protein are needed to grow a proper crystal the use of human leukocyte elastase (HLE) is too expensive. Due to the cost associated with using HLE, porcine pancreatic elastase (PPE) was considered for use in crystallography binding studies because of its cheaper price, 10mg of PPE from Elastin Products Company costs \$57.

Before the PPE could be used, it had to be verified that PPE models HLE. An assay was created using PPE to study the inhibition of PPE with I3C. The assay was adapted from

Gestin et al.⁶ The substrate used for the PPE was n-succinyl-ala-ala-ala-p-nitroanilide (SANA), a chromogenic substrate which releases a yellow color when cleaved by the elastase. Thus the kinetics, or rate of reaction, can be measured and the mode of inhibition can be determined.

Methods

PPE and SANA were found to be moderately soluble in water so Trizma-base buffer was used in the assay. The trizma buffer was titrated to a final pH of 8.0 using concentrated hydrochloric acid. The PPE was kept at a concentration of 0.508 units/mL and the concentration of the SANA varied and was comprised of four values which varied between 0.1 and 0.5 μmol/mL. The inhibitors were used at concentrations of 0 and three others ranging between 0.1 and 0.5 μmol/mL. Using a 1 mL quartz cuvette and a Varian Cary 100 Bio UV-Vis Spectrometer, the kinetics of the cleavage of SANA with PPE was monitored at 380nm. The graph of absorbance versus time was displayed. Absorbance is proportional to the concentration of the cleaved SANA. As the absorbance increases, the concentration increases and as the change in absorbance increases the rate of the cleavage of SANA increases. The slope of the initial straight portion of the graph is the value of the initial rate. Each concentration of substrate gave a different rate, and these were plotted as a Lineweaver-Burk graph, inverse of the initial rate versus the inverse of the concentration of the SANA. This is the common method for testing the mode of inhibitors for a specific enzyme.

Results

This experiment was performed on 28 separate occasions using a series of slight modifications, including concentration and temperature adjustments. Each time the experiment was performed, the results were erratic but followed a similar pattern (Figures

4 Porter et al.

5 Nguyen et al.

6 Gestin et al.

1 and 2). There was no trend distinguishable in the difference of the different trials, nor was there a trend found on the Lineweaver-Burk plot. Of the 28 experiments performed none showed any inhibition of the PPE with the I3C. After a great deal of experimentation no advancements were made on the project.

Through a search of literature a potential answer was found. In 1987 a paper was published in the Proceedings of the National Academy of Sciences (PNAS) which posed the potential answer. The PPE is secreted as a zymogen, it cleaves N-side alanine residues, it is sensitive to increased ionic strength, and synthesized as a nonglycosylated protein. HLE is a lysosomal enzyme which is electrostatically bound to an insoluble polysaccharide matrix, it cleaves at bulkier side chains such as valine, and it degrades elastin more slowly than PPE. In addition to this, the HLE shows increased activity at higher ionic strength, is synthesized as a series of isoenzymes, and it is inactivated by fatty acids and activated by fatty alcohols.⁷

This indicates that it may not be possible to use PPE as a model for HLE; therefore, an experiment was designed to test this comparison. Using a method for HLE assays which was adapted from Firestone et al.³, PPE was compared to HLE. Rather than using the SANA, a different substrate, n-methoxy succinyl-ala-ala-pro-val-p-nitroanilide, was used because it is the standard substrate for HLE activity assays. The final concentration of cleaved substrate was monitored and compared.

New Method

For both human and porcine elastases, the concentration within the well was 5×10^{-5} units. The substrate concentrations were 0.5, 3.0 and 5.0mM. Three concentrations of the inhibitor were also used at values of 0, 25, and 50 μM . The substrate and the inhibitor were dissolved in dimethyl sulfoxide (DMSO)

for solubility purposes. 100mM Trizma-HCL buffer system was used with 250 mM sodium chloride (NaCl) at a pH of 7.5, pH adjustments made using concentrated hydrochloric acid.

New Results

After allowing the solutions to incubate for 10 minutes at 37°C, they were allowed to return to room temperature and the absorbance at 410 was measured. The HLE shows an interesting trend which may be attributed to the progression of the elastase cleaving the substrate(Figure 3). When the PPE was analyzed, there was no cleavage detectedThus PPE does not cleave at the valine sites that HLE does.

The amino acid sequences of HLE and PPE were compared to one another using EMBOSS (Figure 5). The results of this analysis indicate that there is only a 47.1% similarity in amino acid sequence between the two proteins. There are identical matches of only 99 of the 295 amino acids (33.6%). These two proteins do, however, share the same active site amino acids but the surrounding amino acids differ enough to cause their substrate specificities to be different.

Using a similar method to how the HLE results were analyzed, previous data for PPE cleavage of SANA with and without I3C was revisited (Figure 4). Using the time 0.50 minutes after the start of the reaction, the absorbance values for the different substrate concentrations were plotted against inhibitor concentration. This graph should show a negative slope as inhibitor concentration increases indicating that the inhibitor does in fact bind to and inhibit elastase activity. However, the data does not show this, it shows little to no change in absorbance based on addition of inhibitor. This is the same result as seen for HLE, no I3C inhibition. The results for the HLE might be attributed to previously mentioned time lapse between first sample analyzed and last sample analyzed., however the PPE did not have a time lapse.

7 Sinha et al.

Conclusion

Based on this experimentation, it can be safely concluded that these two proteins are different. They differ in substrate specificity, size, amino acid sequence, rate of reaction, and how they are synthesized. Based on these differences it is likely that the proteins bind to inhibitors differently. For this reason, strong caution should be taken when using porcine pancreatic elastase to model human neutrophil elastase. Further studies should be conducted in order to compare relative amounts of a substrate cleaved by both HLE and PPE in order to compare reaction rate. Kinetics of inhibition of both HLE and PPE to similar substrates should also be considered for comparison.

Figures

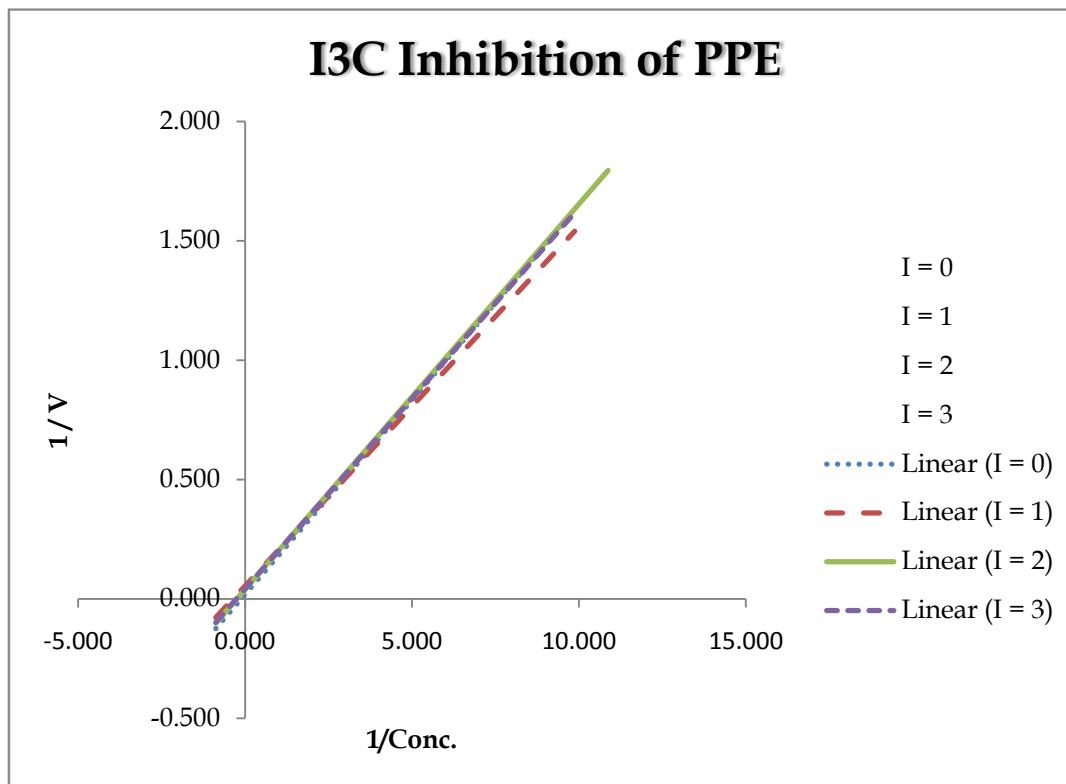


Figure 1: The graph is the Lineweaver-Burk plot which was made from the data of PPE with SANA as the substrate being inhibited by I3C. The trendlines do not behave as expected. There should be one of three graphs formed either the lines should all be parallel or they should intersect at one point. The values of I ; 0, 1.2.3; refer to the increasing concentrations of the inhibitors not the actual value of the concentrations.

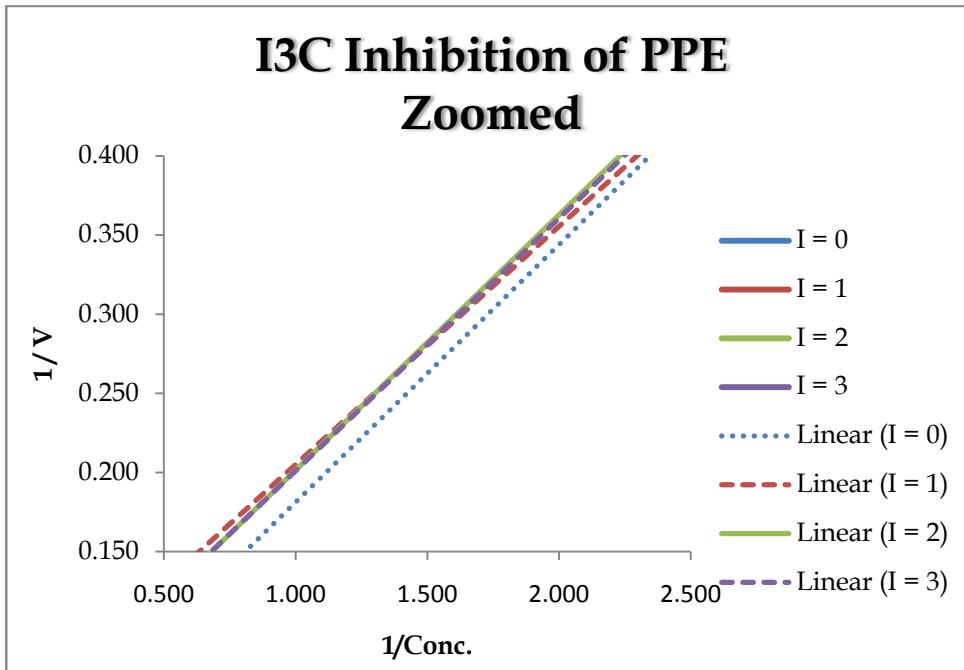


Figure 2: This is a zoomed in version of the Lineweaver-Burk from figure 1 which shows the lack of convergence.

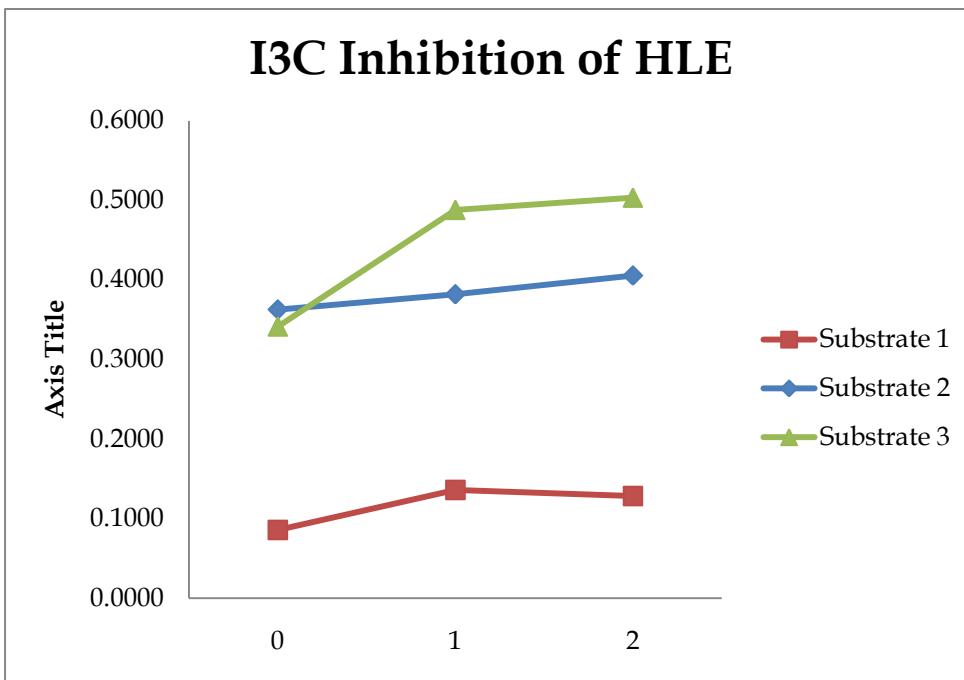


Figure 3: A plot of absorbance versus inhibitor concentration at three concentrations of substrate with HLE as the enzyme. The expected trend is a decrease in absorbance as inhibitor concentration increases. However, this is not seen. This may be attributed to the time delay between analysis at the lowest inhibitor concentration and at the highest inhibitor concentration. Data acquisition required this time lapse. Values of substrates refer to the increasing concentrations, not the actual value of the concentrations.

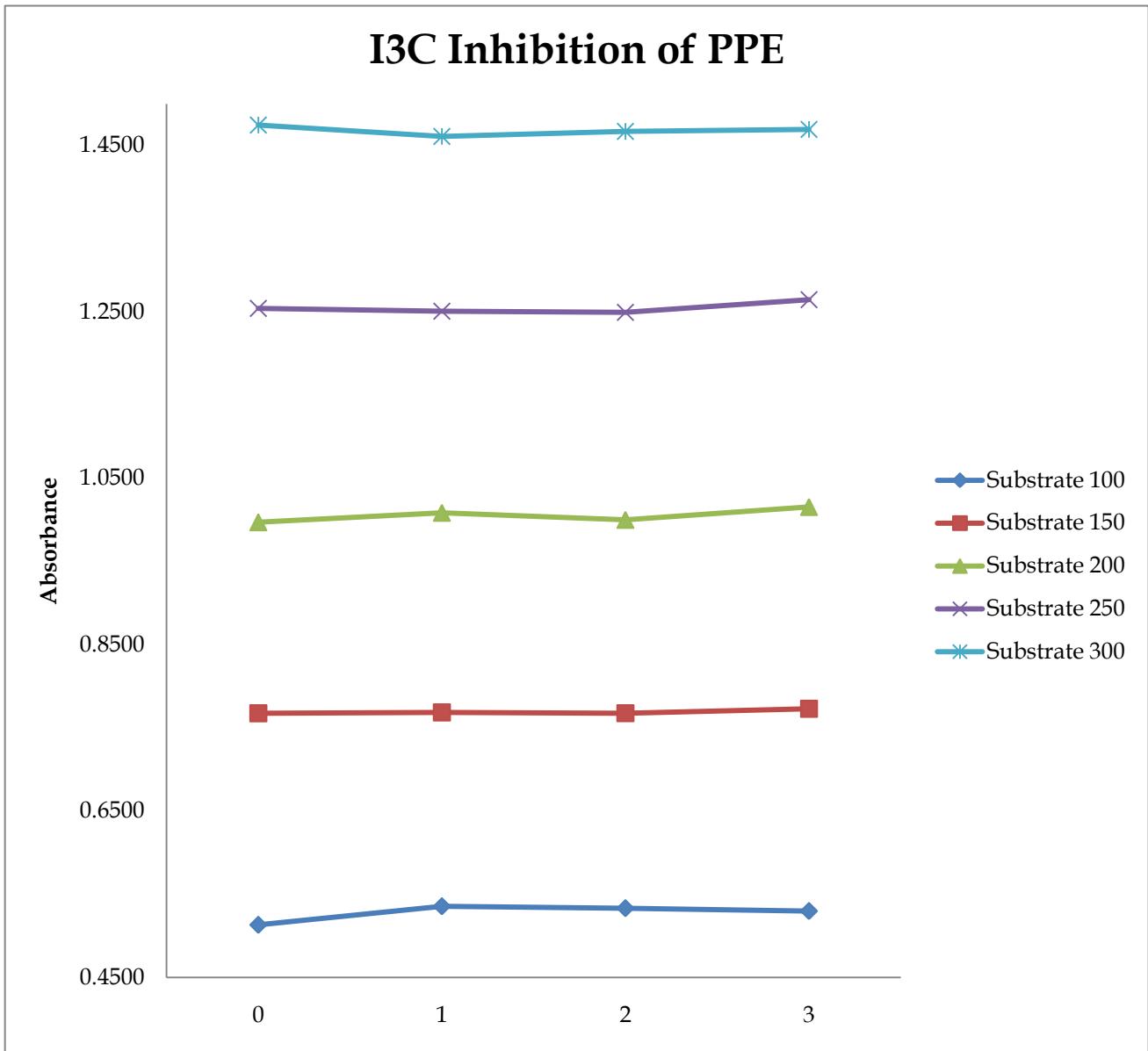


Figure 4: The data from PPE analysis in figures 1 and 2 was analyzed in a similar fashion to the HLE from figure 3. This data shows a nearly flat line for each substrate concentration which may indicate no inhibition.

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#
# Aligned_sequences: 2
# 1: ELNE_HUMAN
# 2: CELA1_PIG
# Matrix: EBLOSUM62
# Gap_penalty: 10.0
# Extend_penalty: 0.5
#
# Length: 295
# Identity:      99/295 (33.6%)
# Similarity:    139/295 (47.1%)
# Gaps:          57/295 (19.3%)
# Score:        383.0
#
#
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ELNE_HUMAN      1 MTLGRRLLACFLACVLPALLGG-TA-L--A-SEIVGGRRARPHAWPFMV
45           .|.|...|...:|.||. | . . . :|||..|.:|:|..|.
CEL A1_PIG      1 -----MLRLILVVVASLVLYGHSTQDFPETNARVVGGEAQQRNSWPSQI
42

ELNE_HUMAN      46 SLQLR-GG---HFCGATLIAPNFVMSAAHCVANVNRAVRVVLGAHNLSR
91           ||||| | |. |.|||.|.||:||||| : . . .|||:|.|||||:
CEL A1_PIG      43 SLQYRSGSSWAHTCGGTLLIRQNWWMTAAHCV-D-RELTFRVVVGEHNLNQ
90

ELNE_HUMAN      92 REPTRQVFVQRIFENGY---DPVNLLNDIVILQLNGSATINANVQAQL
138          . . .|.|...||:|...| | .|...|||.:|||..|.:|:|...|.
CEL A1_PIG      91 NDGTEQYVGVQKIVVHPYWNTDDVAAGYDIALLRLAQSVTLNSYVQLGVL
140

ELNE_HUMAN      139 PAQGRRLGNGVQCLAMGWGLLGRNRGIASVLQE-----LNVTVVTSCLC-
181          | . . .|.|...|...| .|||...|...|.:|...|: | . . .|...
CEL A1_PIG      141 PRACTILANNNSPCYITGWGLTRNGQLAQTLQQAYLPTVDYAIICSSSSYW
190

ELNE_HUMAN      182 ----RRSNVCTLVRGRQAGVCFGDSGPLVC--NG--LIHGIASFV-RGG
222          : .|.|...|...|.:| |.|||..|..| | .| .:|||..| | .|.
CEL A1_PIG      191 GSTVKNSMVCAGGDGVRSRSG-CQGDSGGPLHCLVNGQYAVHGVTSFVSR LG
239

ELNE_HUMAN      223 CASGLYPDAFAPVAQFVNWIDSIIQRSEDNPCPHPRDPDPASRTH      267
CEL A1_PIG      240 CNVTRKPTVFTRVSAYISWINNVIASN-----                  266

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Figure 5: Needle output of the alignment of HNE and PPE. ELNE_HUMAN is the Human Neutrophil Elastase, whereas the CELA1_PIG is porcine pancreatic elastase. The program analyzed the similarity between the two amino acid sequences by matching key pieces of the PPE to the HNE sequence. Identity is the exact amino acid to amino acid matching between the two elastases. The similarity is the percentage of matches between the two sequences. Vertical lines between the two sequences show identical matches.

References

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