Response to Reviewer 1 Comments

In the present study, the researchers have identified a kazal-type serine protease inhibitor, ShSPI from the venom of Scolopendra hainanum (centipedes). They compared ShSPI with the current pharmaceutical compound Sivelestat. They demonstrate that ShSPI showed significant inhibitory effects on porcine pancreatic elastase (Ki = 225.83 nM) and human neutrophils elastase (Ki = 12.61 nM). They propose that ShSPI may have several important physiological and clinical applications such as pharmaceutical options for the treatment of protease-related cardiopulmonary diseases.

Overall, I believe the identification of the novel kazal-type elastase inhibitor (ShSPI) from the venom of centipedes is interesting and its application in comparison to the existing SPI inhibitor compound Sivelestat will be useful from a physiological perspective for clinical researchers and enzymologists.

Comments section

-In Figure 3, it is important that the authors used the human neutrophil elastase (HNE) as a target for ShSPI inhibition in comparison with the pharmaceutical Sivelestat. However, the authors used the pancreatic version of a porcine elastase as a comparison between previous studies with Sivelestat and PPE. What is the sequence similarity between PPE and the human variant of PPE and HNE? It would be useful in this figure if the authors presented the sequences of the PPE, HNE and the human variant of PPE.

-Response: First of all, we would like to thank you for this valuable suggestion. In order to investigate the inhibit efficacy of ShSPI to diverse serine protease, PPE and HNE are used in our experiments. ShSPI exhibits different inhibitory activity to PPE and HNE from our results. However, it has been reported that sivelestat barely showed inhibitory activity to PPE (Al-Awadhi FH, Paul VJ, Luesch H. Structural Diversity and Anticancer Activity of Marine-Derived Elastase Inhibitors: Key Features and Mechanisms Mediating the Antimetastatic Effects in Invasive Breast Cancer. Chembiochem. 2018 Apr 16;19(8):815-825.). As a small molecular, sivelestat is a competitive inhibitor to elastase, and ShSPI is a noncompetitive inhibitor. After comparing the inhibitory efficiency of ShSPI and sivelestat, it was observed that ShSPI have more inhibitory activity to elastase, regardless of PPE or HNE. In terms of the sequence similarity between HNE and PPE, porcine pancreatic elastase (PPE, EC: 3.4.21.36) and human neutrophil elastase (HNE, EC: 3.4.21.37) are different groups of elastase, and they are differ in specificity on synthetic substrates and in inhibitor sensitivity.

-It would also be helpful if the authors depicted the proposed interaction site of ShSPI to HNE. A binding/interaction comparison between ShSPI and Sivelestat to the elastase would be impactful to highlight the potential clinical use of ShSPI.
**Response:** We deeply appreciate your far-sighted suggestion. As we mentioned in the previously question, sivelestat is a competitive inhibitor of HNE which bonds to the catalytic traid of elastase (Ser-195, His-57, and Asp-102), and compete with other substrate. Several important residues have been presumed from the diagram structure and alanine mutants of ShSPI, and we indicated that ShSPI could interaction with elastase as a noncompetitive inhibitor according to the results of binding kinetics. More effort should to do to identify the precise site that ShSPI interact with HNE and PPE. In order to progress the clinical use of ShSPI, a binding interaction of ShSPI to HNE and PPE will be done in the future by the X-ray crystallographic analyses.

-In Figure 1, the authors refer to Schechter and Berger for the nomenclature of the important ShSPI sites. This nomenclature is described based on the cleavage site of the substrate using an appropriate enzyme.  
Which enzyme is the reference enzyme, elastase (PPE, HNE)?

**Response:** Thank you for your comment. The reference enzyme we used in the nomenclature is HNE. We have rephrased the presentation related and another reference has been added to make this point more clearly in the revised manuscript. (Koizumi, M., Fujino, A., Fukushima, K.J., et.al. Complex of human neutrophil elastase with 1/2SLPI. J Synchrotron Radiat. 2008 May;15(Pt 3):308-11.)

-Is ShSPI being cleaved? if so, what is the proof of this? How is it acting to inhibit elastase (Kazal 1 / Kazal 2 / other mechanism?)

**Response:** We appreciate your insight. To the best of knowledge, ShSPI is not cleaved. We prefer to classify ShSPI into Kazal 2 according to the information on the website (http://pfam.xfam.org/family/PF07648), since ShSPI can act as a functional unit and showed significantly activity. The current work focuses on the discovery of elastase inhibitor from centipede’s venom, we are working on the interaction mechanism of ShSPI with elastase now and will discuss more details in the following paper.

-While the structural similarities were compared between inhibitors, Overall, more information in the manuscript (discussion) is required on how this inhibitor may be functioning as a kazal-type elastase inhibitor (atypical) with other known SPIs in its class.

**Response:** We would like to thank your suggestion. More information has been added to the discussion (line 245-257, highlighted by bright green), in which the presumed way that ShSPI could function as a kazal type elastase inhibitor and the comparison of ShSPI and other known SPIs have been discussed.

-In Table 3, the authors took an alanine mutagenesis approach to determine which sites in ShSPI were important for protease inhibition. There was a wide range of mutations all along the peptide that altered the function of the inhibitor. What information based on the structure and sequence of the inhibitor does this tell us about the protein? This has not been made clear in the paper and more rationale would be appreciated surrounding this experiment and results.
Response: Thanks for your helpful suggestion. Through the sequence and structural analysis, we found that four cysteines of ShSPI can form two pairs of disulfide bonds, which is the basis of the formation of construction (one helix and two β-sheet structures). At the same time, the folded structures of ShSPI has great spatial similarity to other inhibitors (Figure 5A-F). Accordingly, we assumed that these cysteines and sequences in α-helix/β-sheet are important for the function of ShSPI. Moreover, the paper reported by Laskowski M Jr. et.al gave us a hint that there are 10 hyper-variability sites in kazal family protein and a conserved P15’ site of Asn (N), which are important in the proteinases inhibitory activity. (Laskowski M Jr, Qasim MA, Yi Z. Additivity-based prediction of equilibrium constants for some protein-protein associations. Curr Opin Struct Biol. 2003 Feb;13(1):130-9). Taken together, those mutants were synthesized. More experiments will be done to verify the structure of other prediction mutations in the future.