Rebuttal Letter for JoVE59391 "Nitropeptide profiling and identification illustrated by Angiotensin II"

Editorial comments:

Changes to be made by the author(s) regarding the manuscript:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We thank the editor's kind reminder. We have tried our best to correct any spelling and grammar errors.

2. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below.

3. 1.5: Please specify how to use HPLC for the purification of nitro-Angiotensin II. Alternatively, add relevant references.

Since step 1.5 is the alternative step, we have deleted this part in revised manuscript.

4. 4.3: Please describe how this step is actually done.

Following the editor's suggestion, we have added the detailed information for this step.

5. 4.5: Please specify centrifugation parameters for spinning down the beads.

Following the editor's suggestion, we have specified centrifugation parameters.

6. 4.6: Please provide the parameters for LC-MS/MS analysis.

The detailed information for LC-MS/MS has been provided in the revised manuscript.

7. Please expand to explain the Representative Results in the context of the technique you have described, e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc.

We have expanded the Representative Results to include content along the comments given by the editor in the revised manuscript. More details are described in the figure legends.

8. JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:

a) Critical steps within the protocol

b) Any modifications and troubleshooting of the technique

c) Any limitations of the technique

d) The significance with respect to existing methods

e) Any future applications of the technique

We have added a few more sentences in Discussion and ensured that all the five points are now explicitly covered. In particular, we added sentences in paragraph 1 and 2 to highlight critical steps. The limitation of the technique is described in the last paragraph.

9. Figures 2-7: Please include a title in the figure legend for each figure.

We revised all the figure legends of Figure 2-7.

10. Please upload each Figure individually to your Editorial Manager account as a .png, .tiff, .pdf, .svg, .eps, .psd, or .ai file.

We have uploaded as instructed.

11. Table of Equipment and Materials: Please sort the items in alphabetical order according to the name of material/equipment.
We have reordered the sequence of the items.

12. References: If there are six or more authors, list the first author and then “et al.”.
According the editor’s suggestion, we have corrected the format for all reference.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
Due to the challenges of detecting low abundance nitroproteins, enrichment may be necessary. The goal of this manuscript is to establish a novel method of nitropeptide enrichment by using angiotensin II as a model. This technique could be expanded to in vitro and in vivo models.

Major Concerns:
I have no major concerns regarding this work.

Minor Concerns:
The authors should also include the Zhan and Desiderio Int. J Mass Spectrometry citation from 2009 as it is applicable. The in vivo application is solely based on the authors work. It would be nice to see broader array, but this may not be possible due to the novelty of this work.
We appreciate the positive comments from the reviewer, and agree that the *in vivo* application in broader contexts will require further investigations in follow-up studies. We have now added Zhan & Desiderio as Reference#3 in Introduction.

Reviewer #2:

Manuscript Summary:

The proposal is useful and will help the scientific community dealing with this challenging PTM. It has some details to be adjusted:

We appreciate the reviewer’s positive comments on our work.

Under 1.

The composition of 150 mM PBS solution should be described in detail.
We have described the composition of PBS solution in the revised manuscript.

The concentration of peroxynitrite should be measured.Since peroxynitrite is highly unstable in neutral to acidic solution (t½ about 10 ms) the mixture with the protein should be done fast (addition in the whirl generated by a vortex). Because of that the incubation for 30 min after pH adjustment is unnecessary.
We thank the reviewer’s great suggestions. We agree with the reviewer that the nitration reaction is fast, and we confirmed by experiments that there were no significant differences in the productivity between 5

and 60 min of the reaction time. Therefore, we have shortened the reaction time to 5 min in the revised manuscript. Regarding the concentration of peroxynitrite, we have tested the amounts of peroxynitrite used in this reaction from 50 to 500 folds excess of the substrate peptide and observed no significant differences in the products. According to the product information of peroxynitrite (Millipore – Calbiochem, 516620), freshly received product will decrease its activity by ~2% /day at -20°C. Since we follow the instruction to aliquot and store at -80°C, and only use it within 4 weeks after receiving it, we think the level of drop in concentration and activity of peroxynitrite would not affect the reaction products. Therefore, measuring concentration may not be particularly useful from the user's point of view.

It would be advisable to evaluate the level of nitration obtained (Absorbance at 420/350 nm (Crow and Beckman, 1995).

Regarding evaluating the level of nitropeptide, we agree that the mentioned method of absorbance measurement is feasible. In fact, in the current protocol, we use MS to detect the nitration product of Angiotensin II, therefore we could use mass spectrum to evaluate the nitration reaction. Based on our preliminary result, over 50% of Angiotensin II was converted to nitro-Angiotensin II, and some side products formed by oxidation rather than nitration. Because our protocol is focused on derivation of the nitrated peptide and its MS characterization, which is not affected by the efficiency of nitration in the first step, we hope the reviewer could allow the performance of nitration measurement in a separate study.

Under 5.1.

Is the sentence "Prepare 3 sets of nitro-Angiotesin II solutions, each set contains 2 concentrations of the solvent: 1st set, 20 and 20 nmol; 2nd set, 10 and 20 nmol; 3rd set, 40 and 10 nmol" correct? Please revise.
We have revised it to "Prepare 3 sets of nitro-Angiotensin II solutions, each set contains 2 concentrations of nitro-Angiotensin II: Set #1, 20 nmol in tube A and 20 nmol in tube B, each in 100 ul TEAB solution; Set #2, 10 nmol in tube C and 20 nmol in tube D, each in 100 ul TEAB solution; Set #3, 40 nmol in tube E and 10 nmol in tube F, each in 100 ul TEAB solution."