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August 30, 2020

Dear Editor,

We would like to thank you and the reviewers for all constructive comments and your precious time reviewing our manuscript. We notice that none of the reviewers flagged a major concern regarding this manuscript and we were happy to receive a positive feed-back from all.
Based on the reviewing comments, we made revision accordingly and made small trackable changes along the manuscript. We believe the manuscript is much better now since we addressed all the concerns from the reviewers and JOVE editorial comments.
Here, I response to the comments of the editorial and reviewers' one by one in the following:

Editorial comments:
Changes to be made by the Author(s):
1) Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
Response: We have thoroughly proofread the manuscript and fixed several issues flagged by different reviewers.

2) Please add more details to your protocol steps. 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.
Response: We complemented several points from the protocol section adding additional information about PCR protocol and reaction setup, restriction digestion reaction set up, gel concentration and composition to make each point better and more reproducible.

3) 1.1.2: What is the PCR program (temperature and time) used?
Response: we added the program details and recommendations in points 1.1.2 and 2.1.2

4) 1.1.3: What is the gel percentage? V/cm? How was the gel purification done? What is the RE digestion protocol?
Response: We added the gel concentration, composition, and Voltage/cm along the manuscript. The gel extracted DNA slices were purified using QIAEX II Gel Extraction Kit (Qiagen) and we inserted the name of the kit along the cloning protocols. We added more details for restriction enzyme digestion as well.

5) Are the PCR programs and gel purification protocols the same throughout the protocol? They are done again in step 2.1.
DNA purification protocol was the same for all cloning protocols and performed with QIAEX II Gel Extraction Kit (Qiagen). We have inserted kit/manufacturer along the cloning section. For

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PCR, we strongly recommend researchers to follow the PCR protocols and conditions described by the DNA polymerase manufacture, but we included a basic protocol in points 1.1.2 and 2.1.2

6) 1.3.7: Incubate at what temperature for the cleavage?
Response: We added a note above point 1.3.1 to conduct all steps at 4 °C unless otherwise stated along the specific protocol but we added a redundant information in point 1.3.7 to perform SUMO-protease cleavage at 4 °C as well.

7) Please specify all volumes and concentrations throughout.
Response: We believe that we specified most of volumes and chemical concentration along the protocol section of this manuscript.

Reviewer #1:
This reviewer concluded that "The manuscript presents a methodology for purification of the isolated α- and β- subunits of the recombinant enzyme tryptophan synthase from Salmonella typhimurium (StTS), which allows obtaining the α2β2 StTS complex from, in a fast way. The manuscript also presents protocols for protein crystallization in native or mutant forms, as well as in the presence of the F9 ligand."

Major Concerns:
None

Minor Concerns:
1) The proposed protocol is very detailed, which is undoubtedly a contribution for those who need to obtain pure protein in its biological unit, for both kinetic and/or crystallographic studies. However, the title of the manuscript does not reflect all the work presented. The reference of the protocol for crystallization and solving the protein structure was omitted. I recommend reviewing the title for a broad one.
Response: we changed the original title from "Fast purification protocols for wild type and mutant form of *Salmonella typhimurium* tryptophan synthase" to a broad title "PCR mutagenesis, cloning, expression, fast protein purification protocols and crystallization of the wild type and mutant forms of tryptophan synthase".
The reference for crystallization (reference #22) was previously inserted in item 3 from the representative results. We included the same reference as a NOTE in item 3 from the protocols as well. "NOTE: Initial crystallization condition for the $\alpha_2\beta_2$ *St*TS complex was previously reported in conditions containing 12% PEG 8,000 and 2 mM spermine[22]".
The references for the x-ray data processing, molecular replacement and crystal structural refinement were previously inserted along item 4 from protocols, references 27-34 listed along the subitems 6-10.

2) The legend in figure 6 does not specify which crystal presented refers to the native structure, nor which refers to the mutant structure. The legend also does not specify which mutation in

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protein is crystalized. I recommend correlate crystals with structures and conditions (apo, holo...) using the letters (a), (b), (c)...

Response: we have modified figure 6 and correlated the crystal pictures with their protein form (wild type or mutant form). We added "(A-D) PLP holo-crystals in complex with cesium ion at the metal coordination site of the wild type form (column A), mutant form $\alpha_2\beta_2$ βQ114A (column B), $\alpha_2\beta_2$ βK167T (column C), and $\alpha_2\beta_2$ βS377A (column D)." in the end of the figure 6's legend.

Reviewer #2:

This reviewer concluded that "The manuscript by Hilario at al. presents improved protocols for purification of tagged and untagged versions of the Salmonella typhimurium L-tryptophan synthase complex as well as its individual subunits α and β. Moreover, it also describes construction of plasmids encoding several enzyme variants and purification of the respective protein complexes. Lastly, it presents optimization of protein crystallization conditions for all L-tryptophan synthase complex variants described and results of X-ray diffraction data analysis for the wild-type enzyme form with F9 inhibitor, cesium ion, pyridoxal-5'-phosphate and L-tryptophan in the active site. The protocols proposed by the authors, in comparison to previously published ones, decreased the amount of time required for purification of the enzyme and its components. It also reduced complexity of the whole procedure. All procedures were clearly described and can be valuable for researchers working on L-tryptophan synthases or perhaps other similar enzymes."

Major Concerns:
None

Minor Concerns:
1) Page 7, line 219-225 (points 5-7 of the protocol): Could the authors explain in the manuscript why is ammonium precipitation required prior to SUMO-tag cleavage? The protein seems rather pure already after Ni-NTA agarose purification step. Was decreasing the volume beneficial for sumo digestion? The protein is further concentrated after removal of the cleaved His-tag using filters, could ammonium sulfate precipitation step (point 5) be substituted by centrifugal concentrators as well? Also, in point 8 of the same protocol it is mentioned that " digestion product was centrifuged….prior to ammonium sulfate precipitation" but no precipitation seems to take place after the tag has been removed. Also - the order of events is reversed in the Results section (Page 13, line 482-483), first SUMO cleavage is mentioned and then ammonium sulfate precipitation. Please clarify that.
Response: SUMO-protease is tolerant to imidazole up to 300 mM but we have used 400 mM imidazole (point 4), since 300 mM imidazole is not enough to elute all tagged protein from the beads. High amounts of imidazole (>60 mM) from initial purification of the SUMO fusion protein (point 4) will interfere with binding of the SUMO-protease and the cleaved SUMO-tag in

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Ni-NTA agarose column (point 9). In addition, It is not recommended to use protein concentrators such as popular Amicon Ultra centrifugal filter devices (Millipore) since the membrane of the filter is sensitive to high amounts of imidazole. Manufacturer advises to concentrate solutions containing up to 100 mM imidazole. This explains why we precipitated the protein with ammonium sulfate prior SUMO-digestion instead wasting time with protein solution dilution, which will be increasing the solution volume and time to concentrate the SUMO-*αSt*TS or SUMO-β*St*TS protein prior SUMO-protease digestion. We added a note between points 4-5 addressing this matter "NOTE: SUMO-protease is tolerant up to 300 mM imidazole and the membrane of centrifugal filter devices tolerate up to 100 mM imidazole. We recommend performing an ammonium sulfate precipitation to remove high amounts of imidazole and decrease purification time."
We fixed as "8. The digestion product was centrifuged at 10,000 x g in 25 °C for 20 min to remove protein aggregates prior to load the sample through an affinity chromatography column."
We fixed the order of the events in the representative results section "The first day was used to purify each protein by Ni-NTA affinity chromatography, ammonium sulfate precipitation followed by His-SUMO-tag cleavage, removal of His-SUMO-tag traces, and protein concentration."

2) Page 4, line 101: "the long purification time necessary to crystallize, extensively dialysis, and recrystallization" - please change the names of subsequent steps to all nouns or all verbs in.e. dialyze, recrystallize
Response: we fixed as "… are the long purification time necessary to crystallize, dialyze, and recrystallize, typically requiring 5-7 days."

3) Page 5, line 132: please change translational coupling gene to translationally coupled genes. It's actually a pair of genes. It repeats also further in the manuscript.
Response: we fixed 3 occurrences found in point 1.1 from protocol, point 2 from representative results, and figure 2 legend)

4) Page 5, line 134: the PCR product bearing trpB or trpA sequences is subcloned using pEBA-10 vector as a template for amplification. Do the listed primers enable also amplification of trpBA from the Salmonella genome? Perhaps it's worth mentioning for readers who might not have an easy access to pEBA-10 vector?
Response: The listed primers can be used to amplify both genes from the *Salmonella enterica* serovar *typhimurium* genome. We have added this comment in the note between points 1-2 "Alternatively, the listed primers below can be used to amplify both genes from the *Salmonella enterica* serovar *typhimurium* genome as well. All molecular …"

5) Page 6, line 175: "Culture in the bacterial incubator at 37 °C overnight" - with aeration?
Response: we replaced the sentence in point 9 from "Culture in the bacterial incubator at 37 °C overnight." to "Culture cells overnight with shaking at 200 rpm at 37 °C."

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6) Page 7, line 216: "step gradient elution" - one step elution? Only one concentration of imidazole seems to have been used.
Response: we fixed from "step gradient elution" to "one step elution"

7) Page 7, line 225 (point 7) - please add incubation temperature
Response: there is an initial note in protocol 1.3 to conduct all procedures at 4 °C unless otherwise stated but we added "at 4 °C" in point 7 as well.

8) Page 7, line 244-245: does pyridoxal phosphate has to be present in the buffer for purification of both subunits? I am not sure how expensive this compound is, but since it's the β subunit that needs this cofactor perhaps it can omitted for purification of α?
Response: pyridoxal phosphate (PLP) is not required to purify the alpha-subunit but PLP it is not expensive when used at a concentration of 0.1 mM. Since the costs to run a protein purifier equipment, buffer preparation, extensive time to equilibrate a size exclusion chromatography column, and labor wages are much more expensive than few milligrams of PLP it is worth to keep columns in buffer containing PLP. This is a common procedure for those people working with PLP-dependent enzymes.

9) Page 9, lines 302-304, Point 6 seems to repeat point 5
Response: we remove point 5 and renumbered the other points

10) Page 9, lines 308-309, please specify PCR conditions
Response: we have added the PCR conditions and volumes.

11). Page 10, line 347: please change "amplify" to grow or culture
Response: we have changed "amplify" to "grow" in protocol item 2.2 subitem 1 as well as in protocol item 2.1 subitem 12.

12). Page 16, line 604-605: "Although crystallization rate depends on the mutant form and on the concentration of the complex in solution." The sentence seems unfinished or its unclear what the authors mean.
Response: We changed sentence from "Although crystallization rate depends on the mutant form and on the concentration of the complex in solution, being difficult to predict when crystals appear in large solution volumes." to "Although crystallization rate depends on the mutant form and on the concentration of the complex in solution, being difficult to predict when crystals appear in a large solution volume."

Additional comment: I think it is more correct to speak of protein variants and mutations in a gene, not in a protein sequence.
Response: we have seen the term "mutant protein" in many articles published in top journals such as Nature, Science, Cell, JBC, Biochemistry and the utilization of the term could reflect in personal preferences and written style.

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Reviewer #3:

This reviewer concluded that "The procedure presented by Hilario et al was generally easy to follow and concisely written. The development of new crystallization conditions for the St TS complex is exciting. The manuscript could benefit from some attention to grammar and spelling, particularly in the introduction & discussion. The only missing element from our read was on the potential generalizability of these procedures. The authors do a good job describing how a point mutation can alter conditions needed for purification, what about 3 or 4 mutations? How robust would they expect the protocol to be? Since the goal of the journal is to help teach such protocols, commenting on limitations is a strength, not a weakness of the manuscript itself. We recommend publication after addressing the minor points below."

Major Concerns:
None

Minor Concerns:
1) 1.1.4 - SUMO stands for 'small ubiquitin-like Modifier', not 'ubiquitin small modifier protein'
Response: we corrected to "Small Ubiquitin-like Modifier"

2) 1.1.6 - Why list two strains for sub-cloning? Are there any meaningful differences?
Response: we removed the strain DH5α from points 1.1.6 and 2.1.10 as well.

3) 1.2.1 - Why use a 50 mL starter culture when you only need 10 mL to inoculate the expression culture? Feels like something might be out of order here.
Response: We usually inoculate 5 mL of the overnight cell culture in each 1 L fresh LB broth as described in our protocol. LB media is a very cheap media. If you inoculate exactly 10 mL LB media and grow overnight, next morning you will end up with less than 8 mL culture due to the evaporation. In attempt to decrease the effect of evaporation, cellular stress, cell growth delay and weak protein expression we prefer to inoculate a 250 mL Erlenmeyer flask containing 50 mL fresh broth with appropriate antibiotics.

4) 1.2.4 - The lysis buffer here does not contain PMSF, but later (2.2.4) this inhibitor is added. Confirm protocols are complete. If the difference is trivial, leave as is. If it is meaningful, please explain.
Response: Due our extensive work with recombinant tryptophan synthase (TS) protein expression and purification, we noticed that TS is not attacked by proteases from the host *E. coli*. Since PMSF is a little expensive and toxic we are trying to decrease initial hazardous waste along initial purification steps. The effective concentration of PMSF is between 0.1 - 1 mM. The half-life of PMSF is short in aqueous solutions (110 min at pH 7.0; 55 min at pH 7.5; and 35 min at pH 8.0).

5) 1.3.1 - Sonication settings vary a lot by manufacturer. Can you give further details that might make the protocol translatable? Also, you say 20x 10s pulses of sonication. How long is the rest period between pulses?

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Response: Indeed, the sonication step makes a very significant difference at the beginning of the protein purification step. We wrote in the manuscript point 1.3.1 and 2.3.1 “Disrupt cell pellets by sonication (20 cycles of 10 s pulse at 80% amplitude duty cycle) with an interval of 20 s or until complete disruption…”
We meant 20 cycles of 10 s pulse and 20 s rest, but we modified the sentence as “Disrupt cell pellet by sonication using a Branson 450 Digital Sonifier with 1/2" Horn probe (or a similar equipment). Perform 20 cycles at 80% amplitude duty cycle on ice water bath using 10 s pulse and 20 s rest or until complete cell disruption.”

6) 1.3.4 - Here and throughout the rest of the protocols, please indicate where in the gradient the desired proteins eluted.
Response: the recombinant SUMO-α*St*TS and SUMO-β*St*TS elutes in buffer containing 400 mM imidazole. We state to perform a one-step elution with 400 mM imidazole in point 1.3.1, a unique occurrence along the manuscript.

7) 1.3.15 - a 12-15% gel indicates a gradient. For example, 4-20% is a common gradient to use. I2-15% is unusual; do the authors mean either/or?
Response: we did not used gradient gels and we meant 12% SDS-PAGE or 15% SDS-PAGE gels. We have used 12% or a 15% SDS-PAGE gel respectively for β*St*TS (42.8 kDa) or α*St*TS (28.7 kDa). We fixed the information in points 1.3.15 and 1.4.5

8) 2.1.4 - the authors indicate a 'melting temperature of 55 °C'. Would 'annealing temperature' be more accurate if the process happening in solution requires the two strands to meet one another?
Figure 1E - Would prefer for y-axis beside gels to be the same for all 3 images, as this is an indicator of purity as well as identity.
Response: The melting temperature of 55 °C was adequate to amplify all fragments described in this work without any difficulties using the listed primers from the protocol section.
We believe the original figure 1E is more aesthetical, but we prepared another version of figure 1 to show recombinant protein purity and identity.

We would like to thank all three reviewers and the Editor for the valuable comments and suggestions to improve our manuscript. We look forward to hearing from you soon.

Sincerely,

Eduardo Hilario, Ph.D.