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PCR mutagenesis, cloning, expression, fast protein purification protocols and crystallization of the wild type and mutant forms of tryptophan synthase

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TITLE:

PCR mutagenesis, cloning, expression, fast protein purification protocols and crystallization of the wild type and mutant forms of tryptophan synthase

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Tryptophan synthase, recombinant protein, mutant protein, protein expression, protein purification, ammonium sulfate precipitation, size exclusion chromatography, protein crystallization

SUMMARY

This article presents a series of consecutive methods for the expression and purification of *Salmonella typhimurium* tryptophan synthase comp this protocol a rapid system to purify the protein complex in a day. Covered methods are site-directed mutagenesis, protein expression in *Escherichia coli*, affinity chromatography, gel filtration chromatography, and crystallization.

ABSTRACT

Structural studies with tryptophan synthase (TS) bienzyme complex ($\alpha_2\beta_2$ TS) from *Salmonella typhimurium* have been performed to better understand its catalytic mechanism, allosteric behavior, and details of the enzymatic transformation of substrate to product in PLP-dependent enzymes. In this work, a novel expression system to produce the isolated α- and isolated β-subunit allowed the purification of high amounts of pure subunits and $\alpha_2\beta_2$ *St*TS complex from the isolated subunits within 2 days. Purification was carried out by affinity chromatography followed by cleavage of the affinity tag, ammonium sulfate precipitation, and size exclusion chromatography (SEC). To better understand the role of key residues at the enzyme β-site, site-direct mutagenesis was performed in prior structural studies. Another protocol was created to purify the wild type and mutant $\alpha_2\beta_2$ *St*TS complexes. A simple, fast and efficient protocol using ammonium sulfate fractionation and SEC allowed purification of $\alpha_2\beta_2$ *St*TS complex in a single day. Both purification protocols described in this work have considerable advantages when compared with previous protocols to purify the same complex using PEG 8000 and spermine to crystalize the $\alpha_2\beta_2$ *St*TS complex along the purification protocol. Crystallization of wild type and

some mutant forms occurs under slightly different conditions, impairing the purification of some mutants using PEG 8000 and spermine. To prepare crystals suitable for x-ray crystallographic studies several efforts were made to optimize crystallization, crystal quality and cryoprotection. The methods presented here should be generally applicable for purification of tryptophan synthase subunits and wild type and mutant $\alpha_2\beta_2$ *St*TS complexes.

INTRODUCTION

The tryptophan synthase (TS) bienzyme complex ($\alpha_2\beta_2$) is an allosteric enzyme, catalyzing the last two steps in the biosynthesis of the amino acid L-Tryptophan in bacteria, plants, and fungi[1-3]. Bacterium *Salmonella enterica* serovar *typhimurium* (*St*) causes a severe gastrointestinal infection in humans and other animals. Since humans and higher animals do not have TS (EC 4.2.1.20), the inhibition of *S. typhimurium* $\alpha_2\beta_2$ TS complex ($\alpha_2\beta_2$ *St*TS) has been explored as a potential drug target for the treatment of cryptosporidiosis and tuberculosis[4], genital and ocular infections[5], and for potential herbicide utilization in agriculture[6]. The α-subunit catalyzes the aldolytic cleavage of indole-3-glycerol-phosphate (IGP) to glyceraldehyde-3-phosphate (GAP) and indole, through the formation of an indolenine tautomer intermediate and subsequently carbon-carbon bond cleavage to produce GAP and indole[3,6]. The β-catalytic site contains a pyridoxal 5'-phosphate (PLP) cofactor molecule bound to β-Lys87 via a Schiff base, which functions as an electron sink in the course of the reactions at the enzyme β-subunit[3,7]. The β-site catalyzes the replacement of the L-Serine side-chain hydroxyl by indole to give L-Tryptophan and a water molecule in a PLP-dependent reaction. *St*TS serves as a longstanding model for the investigation of substrate channeling and allosteric communication within multi-enzyme complexes[2,3]. Bidirectional allosteric communication between the α- and β-subunits of TS is necessary to synchronize the catalytic steps and prevent indole release during L-Tryptophan synthesis[3]. To extend this effort, we have prepared several mutants (β-Gln114Ala, β-Lys167Thr, and β-Ser377Ala) by single point mutation to be used in further explorations of the relationship between enzyme structure, mechanism and function at the catalytic site of the *St*TS β-subunit.

Detailed research on the catalytic mechanism of $\alpha_2\beta_2$*St*TS was initiated by the research group of Edith W. Miles. Early studies with native *Escherichia coli* $\alpha_2\beta_2$ TS complex have focused on the purification and characterization of the isolated α-subunit[8,9], isolated β-subunit[10,11] and the reconstitution of the $\alpha_2\beta_2$ TS complex from the isolated subunits[12]. Purification was carried out by ammonium sulfate precipitation, sample dialysis, DEAE-Sephadex chromatography, dialysis, and a second chromatographic round on a DEAE-Sephadex column[12]. In another protocol, the purification of the same complex was improved by loading the clarified cell lysate on a DEAE-Sephadex column followed by a chromatographic step on a Sepharose 4B column, ammonium sulfate precipitation and dialysis[13]. Both purification protocols last for 4-5 days. *Escherichia coli* $\alpha_2\beta_2$ TS complex crystallized but crystals were not suitable for X-ray diffraction at that time.

In a novel study, recombinant and wild type forms of *S. typhimurium* $\alpha_2\beta_2$ TS complex were purified and crystalized[14, 15]. The recombinant $\alpha_2\beta_2$ *St*TS complex was overexpressed in *E. coli* strain CB149 carrying the pEBA-10 expression vector. Initial crystallization and X-ray diffraction data collection and analysis of the $\alpha_2\beta_2$ *St*TS complex were reported[14]. However, long and thin needle like $\alpha_2\beta_2$ *St*TS crystals impaired structural studies. In an attempt to collect better X-ray

diffraction data, another purification protocol was described to purify the wild type and mutant forms of the $\alpha_2\beta_2$ *St*TS complex[15]. Purification was carried out with an initial precipitation using spermine and PEG 8,000 into the clarified cell lysate and a large bulky precipitate was removed by centrifugation. The supernatant fraction containing high amounts of $\alpha_2\beta_2$ *St*TS complex was stored for 16-48 h at 4 °C until yellow crystals precipitated. Crystals were washed and extensively dialyzed against different buffers. Protein complex was recrystallized in buffer containing ammonium sulfate and dialyzed[15]. Although, protein crystallization depends on protein and precipitant concentrations in solution, it is difficult to monitor, predict, and reproduce purification for other mutant forms of $\alpha_2\beta_2$ *St*TS complex in solution. This protocol has the advantage that it does not use any chromatographic methods; however, the disadvantages are the long purification time necessary to crystallize, dialyze, and recrystallize, typically requiring 5-7 days. To obtain crystals suitable for X-ray data collection, more than 600 crystallization conditions were evaluated using a combination and variation of protein concentration, temperature, precipitants (PEG 4,000, 6,000, and 8,000), and additives ($CaCl_2$, $MnCl_2$, $ZnCl_2$, cadaverine, putrescine, spermine, or spermidine)[15]. Crystals had a better crystalline form and grew faster in conditions containing 12% PEG 8,000 and 2 mM spermine. Crystallization was more favorable at 25 °C rather than at 4, 30, or 42 °C and grew to maximum dimensions within 3 days[15]. Several $\alpha_2\beta_2$ *St*TS crystal structures were reported at that time (1996-1999)[16-21] and many other structures have been published to date.

Here, the main purpose is to present alternative protocols to purify tryptophan synthase and optimize protein crystallization. The present work shows significant improvements to purify the wild type isolated α-subunit (α*St*TS), isolated β-subunit (β*St*TS), reconstituted $\alpha_2\beta_2$ *St*TS complex from the isolated subunits, and wild type and mutant forms of the $\alpha_2\beta_2$ *St*TS complex. The advantages over past protocols are considerable since purification time was reduced significantly and crystallization and cryoprotection were optimized. Mutant forms of $\alpha_2\beta_2$ *St*TS complex engineered in this work have crystallized near the same condition used for the wild type form. However, fine crystallization optimization was necessary to obtain large single crystals of sufficient quality for structure determination at near atomic resolution. To date, there are 134 tryptophan synthase crystal structures deposited in the Protein Data Bank (PDB), accounting 101, 31 and 2 crystal structures, respectively, for bacteria, archaea and eukaryote. Nicely, 73 structures belong to *S. enterica* serovar *typhimurium* and 5 crystal structures of the $\alpha_2\beta_2$ *St*TS complex have resolution limits higher than 1.50 Angstroms. Not surprisingly, 4 out 5 were prepared in our research group (PDB IDs:5CGQ at 1.18 Å, 4HT3 at 1.30 Å, 4HPJ at 1.45 Å, 6DZ4 at 1.45 Å resolution). The refined crystal structures of mutant form of $\alpha_2\beta_2$ *St*TS complex are anticipated to provide new insights into the mechanism and roles played by essential amino acid residues involved in L-Tryptophan synthesis.

PROTOCOL

1. Fast protocol to purify the α- and β-subunit and the recombined $\alpha_2\beta_2$ *St*TS complex

1.1. DNA subcloning into pETSUMO expression vector

1.1.1. Obtain the translationally coupling gene (trpA and trpB) encoding the α- and β-subunits of the tryptophan synthase from bacterium *Salmonella enterica* serovar *typhimurium* cloned in the pEBA-10 expression vector[22]. Use pEBA-10 vector as a DNA template.

NOTE: Alternatively, the listed primers below can be used to amplify both genes from the *Salmonella enterica* serovar *typhimurium* genome. All molecular biology steps were followed as described in Molecular Cloning: A Laboratory Manual[23].

1.1.2. Use polymerase chain reaction (PCR) to amplify individually the full-length polynucleotide sequence of the α-subunit (α*St*TS) with primers α*St*TS-FW-Bam and α*St*TS-Rev-Eco and the full-length sequence of β-subunit (β*St*TS) with primers β*St*TS-FW-Bam and β*St*TS-Rev-Hind. Use a melting temperature of approximately 55 °C and a polymerase extension time of 2 min.

1.1.2.1. Use high-fidelity DNA polymerase (e.g., Phusion) and the manufacturer's protocol to amplify the DNA sequences. For a 50 µL PCR reaction add 34 µL of nuclease-free water, 10 µL of 5x reaction buffer, 1 µL of 10 mM dNTPs, 1 µL of 10 µM forward primer, 1 µL of 10 µM reverse primer, 1 µL of Template DNA (200 ng), 1.5 µL of DMSO, 0.5 µL of Phusion DNA polymerase.

1.1.2.2. For the PCR program, use a hot start (180 seconds at 98 °C) followed by 30 amplification cycles (30 seconds at 98 °C, 30 seconds at 55 °C and 120 seconds at 72 °C), and a final extension (300 seconds at 72 °C).

NOTE: The italicized sequences correspond to the *Bam*HI, *Eco*RI, *Bam*HI and *Hind*III restriction sites, respectively. Enzyme cleavage efficiency close to the termini of PCR fragments were enhanced by adding extra bases (lowercase sequences).
α*St*TS-FW-Bam: 5'-cgc*GGATCC*ATGGAACGCTACGAAAA-3'
α*St*TS-Rev-Eco: 5'-ccg*GAATTC*TTATGCGCGGCTGGC-3'
β*St*TS-FW-Bam: 5'-cgc*GGATCC*ATGACAACACTTCTCAAC-3'
β*St*TS-Rev-Hind: 5'-ccc*AAGCTT*TCAGATTTCCCCTC-3'

1.1.3. Load PCR product on 0.8% agarose gel in 1x TAE buffer (40 mM Tris, 20 mM acetic acid and 1 mM EDTA) at 6 V/cm, gel extract the DNA band of interest, and gel purify the PCR fragment using a silica bead kit following the instructions from the manufacturer.

1.1.3.1. Digest at least 200 ng of each DNA fragment with appropriate restriction enzymes and conditions recommended by the manufacturer for 2 hours at 37 °C.

1.1.3.2. To set up a 50 µL restriction digestion reaction add 34 µL of nuclease-free water, 10 µL of DNA (200 ng), 5 µL of 10x reaction buffer, 0.5 µL of restriction enzyme 1, and 0.5 µL of restriction enzyme 2.

1.1.3.3. Load the digestion product on 0.8% agarose gel on 1x TAE at 6 V/cm, gel extract, and gel purify the digested fragment using a commercial kit.

1.1.4. Subclone individually each fragment into the *E. coli* expression modified vector pET SUMO, previously digested with appropriate enzymes and gel purified.

NOTE: This vector is a modified version of the commercial pET SUMO. This vector has been optimized for restriction enzyme cloning. The multi cloning site (MCS) of pET28b vector (*Bam*HI, *Eco*RI, *Sac*I, *Sal*I, *Hind*III, *Not*I, and *Xho*I) was inserted in the pET SUMO cloning site. This vector contains an N-terminal 6x-Histidine tag in frame with the Small Ubiquitin-like Modifier protein (SUMO) and multiple cloning sites.

1.1.5. Ligate 100 ng of modified pET SUMO and 50 ng of PCR fragment with T4 DNA ligase for 2 hours at 25 °C.

1.1.6. Transform the constructed plasmid into competent cells of *E. coli* strain DH10Bα cells. Plate cells on LB agar plates containing 35 µg/mL kanamycin. Incubate the plate inverted overnight at 37 °C.

1.1.7. Select a single colony from each transformation, prepare ultra-pure plasmid DNA, and perform DNA sequencing to verify that α*St*TS or β*St*TS were cloned in frame with the N-terminal His6-SUMO tag.

NOTE: Prepare glycerol stocks of cell culture (turn cell suspension in 16% final concentration of sterile glycerol) and store them long-term at -80 °C.

1.1.8. Transform the expression plasmid SUMO-α*St*TS or SUMO-β*St*TS individually into competent cells of *E. coli* expression strain Rosetta (DE3) *pLysS* with a T7 promoter-based system. Plate the recombinant cells on Luria Bertani (LB) agar plates containing 35 µg/mL kanamycin and chloramphenicol. Incubate the plate inverted overnight at 37 °C.

1.1.9. After successful colony formation, pick one single colony (without any satellite colonies) and disperse it in 5 mL of LB medium with both antibiotics. Culture cells overnight with shaking at 200 rpm at 37 °C.

NOTE: Prepare glycerol stocks of cell culture, store long-term at -80 °C or use immediately for recombinant protein expression.

1.2. Expression of the SUMO-α*St*TS and SUMO-β*St*TS subunits

1.2.1. Inoculate fresh *E. coli* strain Rosetta (DE3) *pLysS* cells harboring SUMO-α*St*TS or SUMO-β*St*TS constructs or scrape some of the frozen glycerol stock into a 50 mL culture of LB containing 35 µg/mL kanamycin and chloramphenicol. Grow cells overnight with shaking at 200 rpm at 37 °C.

1.2.2. Next morning, inoculate 5 mL of the overnight cell culture in a fresh and sterile 2x 1000 mL of LB containing 2% glycerol plus kanamycin and chloramphenicol (2.8 L Fernbach flask). Grow cell culture with shaking at 200 rpm at 37 °C.

NOTE: The expression of SUMO-α*St*TS or SUMO-β*St*TS in LB broth yields 125-150 mg of tagged protein per liter. Consider scaling protocol up or down to fulfil specific demands.

1.2.3. Induce recombinant protein expression when the OD_{600} reaches 0.6-0.8 by addition of isopropyl β-D-1 thiogalactopyranoside (IPTG) at a final concentration of 0.4 mM followed by incubation at 30 °C overnight with shaking at 200 rpm.

1.2.4. Harvest the cells by centrifuging at 4,000 x *g* at 4 °C for 20 min. Remove the supernatant and re-suspend the cell pellets with cold lysis buffer 1 (50 mM Tris-Cl, pH 8.0, containing 500 mM NaCl, 5% glycerol, 10 mM 2-mercaptoethanol, and 40 mM imidazole-Cl) to a final volume of 60 mL.

NOTE: Cells can be stored long-term at -80 °C or used immediately for protein purification. To store cells, split the cell suspension into 2 x 50 mL disposable centrifuge conical tubes and keep cell pellets at -80 °C until the protein purification step.

1.3. Purification of the α- and β-subunits of tryptophan synthase

NOTE: All procedures are to be conducted at 4 °C unless otherwise stated. To reduce purification time, equilibrate Ni-NTA agarose nickel-charged affinity columns and size exclusion column in buffer prior to protein purification or during recombinant protein expression.

1.3.1. Disrupt cell pellet by sonication using a 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.

1.3.2. Centrifuge the cell lysate at 30,000 x *g* for 30 min. Aspirate supernatant, ensuring the pellet does not dislodge from tube. Filter the supernatant with a 0.45 µm filter unit on ice and flow it through a 15 mL Ni-NTA agarose nickel-charged affinity column pre-equilibrated in lysis buffer 1.

NOTE: Each Ni-NTA agarose column will be used to purify either SUMO-α*St*TS or SUMO-β*St*TS recombinant protein. Purification can be performed in a 2x 5 mL Ni-NiTA column attached to a fast protein liquid chromatography system. Purify one protein at a time.

1.3.3. Wash Ni-NTA agarose column in 100 mL of lysis buffer 1.

1.3.4. Proceed with an 80 mL one-step elution with buffer E1 (25 mM Tris-Cl buffer, pH 7.8, containing 200 mM NaCl, 5% glycerol, and 400 mM imidazole-Cl).

NOTE: SUMO-protease tolerates up to 300 mM imidazole and the membrane of centrifugal filter devices tolerates up to 100 mM imidazole. We recommend performing an ammonium sulfate precipitation to remove high amounts of imidazole and decrease the purification time instead dilute the protein sample and waste time with protein concentration.

1.3.5. Assess the initial volume of the supernatant fraction. Slowly add small amounts of ammonium sulfate at a time, until a 60% saturation (39.48 g/ 100 mL) at 25 °C is reached. Gently stir the solution for 30 min and avoid bubbles. Centrifuge at 10,000 x *g* for 15 min.

1.3.6. Aspirate and discard the supernatant fraction carefully. Resuspend the pellet fraction in 20 mL of sample buffer (20 mM Tris-Cl, pH 8.0, 300 mM NaCl and 5% glycerol).

1.3.7. For His-SUMO-tag cleavage with SUMO-protease, add the recombinant fragment of Ubl-specific protease 1 from *Saccharomyces cerevisia*e in a 1:1000 ratio and incubate the mixture for 2 hours at 4 °C.

1.3.8. Centrifuge the digestion product 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.

1.3.9. Remove traces of His6-SUMO tag passing the 20 mL resuspended sample on a 15 mL Ni-NTA agarose column, previously equilibrated in lysis buffer 1.

1.3.10. Collect the pass-through sample containing the non-tagged α*St*TS or β*St*TS.

1.3.11. Wash the Ni-NTA column in 20 mL of buffer 1 to collect leftovers of non-tagged α*St*TS or β*St*TS.

1.3.12. Concentrate each subunit separately with a 15 mL 10 kDa cutoff centrifugal filter unit by spinning at 3,000 x *g* at 4 °C. Transfer the concentrated protein to a fresh 2.0 mL tube and microcentrifuge (10,000 x g, 10 min, 4 °C) to remove aggregates. Determine protein concentration[24], prepare 1 mL aliquots at 20-25 mg mL^{-1}, label, flash-freeze in liquid nitrogen, and store them at -80 °C.

NOTE: Pre-purified protein samples can be stored long-term at -80 °C or used immediately for protein purification on a size exclusion chromatography column (SEC).

1.3.13. Take a protein aliquot (20 mg) and microcentrifuge at 10,000 x *g* for 10 min to remove aggregates prior SEC.

1.3.14. Load sample on a size exclusion chromatography column (e.g., HiPrep 16/60 Sephacryl S-200 HR) attached to a fast protein liquid chromatography at a flow rate of 0.5 mL min^{-1}, previously equilibrated in SEC buffer (10 mM Tris-Cl, pH 7.8, 100 mM NaCl, 5% glycerol, 0.1 mM pyridoxal phosphate).

NOTE: To purify higher amounts of isolated α*St*TS or β*St*TS subunit and decrease the number of SEC rounds, load a 5 mL sample at 20-25 mg mL^{-1} on a size exclusion chromatography column at a flow rate of 1.5 mL min^{-1}.

1.3.15. Assess the quality of α*St*TS or β*St*TS in the peak fraction using a 12% or a 15% sodium dodecyl sulfate-gel electrophoresis (SDS-PAGE), respectively, stained with Coomassie brilliant blue stain[25].

1.3.16. Concentrate protein with fresh 15 mL 10 kDa cutoff centrifugal filter units, determine protein concentration[24], prepare 0.5 mL aliquots at 20-25 mg mL^{-1}, label, flash-freeze in liquid nitrogen, and store them at -80 °C.

1.4. Purification of the $\alpha_2\beta_2$ *St*TS complex from the α- and β-subunits

NOTE: Equilibrate the size exclusion chromatography column (Sephadex S-200 HR or Superdex 200 pg) in SEC buffer (10 mM Tris-Cl, pH 7.8, 100 mM NaCl, 5% glycerol, 0.1 mM pyridoxal phosphate).

1.4.1. To purify the wild-type $\alpha_2\beta_2$ *St*TS complex from the α- and β-subunits, thaw concentrate samples of α*St*TS and β*St*TS subunits at 4 °C.

1.4.2. Combine aliquots (1.2 α*St*TS: 1.0 β*St*TS molar ratio) and incubated at 4 °C for 1 h.

NOTE: Excess of αStTS is necessary to ensure that most of β*St*TS will be incorporated into the $\alpha_2\beta_2$ *St*TS complex.

1.4.3. Remove protein aggregates by microcentrifugation (10,000 x g, 10 min, 4 °C).

1.4.4. Load the clarified supernatant onto the size exclusion column.

1.4.5. Assess the quality of α*St*TS or β*St*TS in the peak fraction using a 12% or a 15% sodium dodecyl sulfate-gel electrophoresis (SDS-PAGE), respectively, stained with Coomassie brilliant blue stain[25].

1.4.6. Determine protein concentration[24], prepare 250-1000 µL aliquots at 15-20 mg mL^{-1}, flash-freeze in liquid nitrogen, and store at -80 °C.

2. Purification of the wild type or mutant form of the $\alpha_2\beta_2$ *St*TS complex

2.1. Site-directed mutagenesis to prepare mutant β*St*TS

2.1.1. Use construct pEBA-10 expression vector[22] as the DNA template during two-steps of polymerase chain reaction to introduce specific point-mutations in the β-chain TS polynucleotide sequence.

NOTE: This protocol can be used to introduce a single point mutation in either α-or β-subunit from *Salmonella typhimurium* tryptophan synthase. For additional, new oligonucleotide primers containing the desirable mutation must be appropriately designed. In this work, mutations βQ114A, βK167T, βS377A are listed.

2.1.2. Perform PCR reactions using pairs of nucleotide primers TS-FW-NcoI/Q114A-Rev, TS-FW-NcoI/K167T-Rev, and TS-FW-NcoI/S377A-Rev to generate fragments A1, B1, and C1, respectively.

NOTE: Oligonucleotide TS-NcoI-FW and TS-SacI-Rev, respectively, anneals upstream and downstream of the αβ *St*TS polynucleotide sequence cloned in the pEBA-10 vector.

2.1.2.1. Use high-fidelity DNA polymerase (e.g., Phusion) and the manufacturer's protocol to amplify the DNA sequences. For a 50 µL PCR reaction, add 34 µL of nuclease-free water, 10 µL of 5x reaction buffer, 1 µL of 10 mM dNTPs, 1 µL of 10 µM forward primer, 1 µL of 10 µM reverse primer, 1 µL of Template DNA (200 ng), 1.5 µL of DMSO, 0.5 µL of DNA polymerase.

2.1.2.2. For the PCR program, use a hot start (180 seconds at 98 °C) followed by 30 amplification cycles (30 seconds at 98 °C, 30 seconds at 55 °C and 120 seconds at 72 °C), and a final extension (300 seconds at 72°C).

NOTE: All molecular biology steps were followed as described in Molecular Cloning: A Laboratory Manual[23]. While a lowercase sequence corresponds to a restriction site, a bold and italic sequence corresponds to a mutation.
TS-FW-NcoI: 5'-CAA TTT CAC ACA GGA AAC AGA *cca tgg*-3'
Q114A-Rev: 5'-C AGA GGC GAC GCC GTG C***GC*** ACC GGC GCC GGT TTC-3'
K167T-Rev: 5'-CTC GTT ACA GGC ATC ***TGT*** TAG CGT AGC GGA GCC-3'
S377A-Rev: 5'-C TTT ATC TCC GCG GCC AG***C*** GAG ATT GAC CAC CAG-3'

2.1.3. Perform PCR reactions using pairs of nucleotide primers TS-Rev-SacI/Q114A-FF, TS-Rev-SacI/K167T-FF, and TS-Rev-SacI/S377A-FF to generate fragments A2, B2, and C2, respectively.
TS-Rev-SacI (5'-TTA *tgc gcg* GCT GGC GGC TTT CAT GGC TGA G-3'
Q114A-FF 5'-GAA ACC GGC GCC GGT ***GC***G CAC GGC GTC GCC TCT G-3'
K167T-FF 5'-GGC TCC GCT ACG CTA ***ACA*** GAT GCC TGT AAC GAG-3'
S377A-FF 5'-CTG GTG GTC AAT CTC ***G***CT GGC CGC GGA GAT AAA G-3'

2.1.4. Use polymerase chain reaction to individually amplify the partial fragments. Use a melting temperature of 55 °C and a polymerase extension time of 2 min.

2.1.5. To perform the second round of PCR reactions, load the PCR product above on 0.8% agarose gel on 1x TAE at 6 V/cm and gel extract and gel purify the fragments of interest.

2.1.5.1. Mix fragments A1/A2, B1/B2, and C1/C2 separately in equimolar amounts, heat denature the mixture for 10 min at 96 °C, and anneal at 25 °C. Extend the recombinant strands

with a high-fidelity polymerase and deoxyribonucleotides according to the manufacturer's protocol.

2.1.6. To generate a high copy number of the full-length mutant DNA fragment, add oligo primers TS-FW-NcoI and TS-Rev-SacI to perform a second PCR.

2.1.7. Load PCR product on 0.8% agarose in 1x TAE buffer at 6 V/cm, gel extract the DNA band of interest, gel purify the PCR fragment, and proceed with appropriate restriction digestion for 2 hours at 37 °C following the appropriate buffer and conditions recommended by the manufacturer.

2.1.7.1. Digest at least 200 ng of each DNA fragment with appropriate restriction enzymes and conditions recommended by the manufacturer for 2 hours at 37 °C. To set up a 50 µL restriction digestion reaction add 34 µL of nuclease-free water, 10 µL of DNA (200 ng), 5 µL of 10x reaction buffer, 0.5 µL of restriction enzyme 1, and 0.5 µL of restriction enzyme 2 . Load digestion product on 0.8% agarose in 1x TAE buffer at 6 V/cm, and purify the digested PCR fragment.

2.1.8. Digest 200 ng of pEBA-10 construct with restriction enzymes *Nco*I and *Sac*I following the manufacturer's recommendation. Load the digestion product on 0.8% agarose gel in 1x TAE buffer at 6 V/cm, excise the band correspondent to the vector, and gel purify the digested vector.

2.1.9. Ligate 100 ng of vector with 50 ng of PCR fragment, previously digested and purified, with T4 DNA ligase for 2 hours at 25 °C. Transform the constructed plasmid into competent cells *E. coli* strain DH10B cells. Plate cells on LB agar plates containing 50 µg/mL ampicillin. Incubate the plate inverted overnight at 37 °C.

2.1.10. Pick a single colony (without any satellite colonies) from each cell transformation and disperse it in 5 mL of LB medium containing ampicillin. Grow cells overnight with shaking at 200 rpm at 37 °C. Prepare 10 µg of ultra-pure plasmid DNA from each engineered construct. Prepare glycerol stocks of cell culture (turn cell suspension in 16% final concentration of sterile glycerol) and store long-term at -80 °C.

2.1.11. Perform DNA sequencing to confirm the full-length sequence encoding the α- and β-subunits of tryptophan synthase. Confirm each individual single mutation and discard any plasmid construct containing undesirable random mutation. The oligonucleotide primers used in this study are listed below.

TS-1F: 5'-ATGACAACACTTCTCAAC-3'
TS-1R: 5'-GAAATGCCAGAACATTAC-3'
TS-2F: 5'-CAGTCGCCGAACGTC-3'
TS-3F: 5'-GATGATGCAAACAGC-3'
TS-4F: 5'-CTGGCATTGAACAGTC-3'
TS-5F: 5'-CGTTGCATCATCTCATTG-3'

NOTE: Oligonucleotide primers TS-1F, TS-1R, TS-2F, and TS-3F anneal on the polynucleotide sequence of the β-subunit (GenBank accession code: CP051286.1). Primers TS-4F and TS-5F anneal on the polynucleotide sequence of the α-subunit (GenBank accession code: CP053865.1).

2.1.12. Use 200 ng of each plasmid construct (pEBA-10-βQ114A, pEBA-10-βK167T, and pEBA-10-βS377A) to transform competent cells of *E. coli* expression strain CB149, lacking the *trp* operon[26]. After successful colony formation, pick one single colony and grow the cells in 5 mL of LB medium containing 50 µg/mL ampicillin. Culture in the bacterial incubator at 37 °C overnight. Prepare glycerol stocks of cell culture, store long-term at -80 °C or use immediately for recombinant protein expression.

2.2. Expression of wild type and mutant form of $\alpha_2\beta_2$ *St*TS complex in *E. coli* strain CB149

2.2.1. Grow *E. coli* strain CB149 harboring the desirable construct in a fresh and sterile 50 mL of LB medium containing 50 µg/mL ampicillin. Grow cells overnight with shaking at 200 rpm at 37 °C.

2.2.2. Add 5 mL of the overnight cell culture in a fresh and sterile 2x 1000 mL of LB containing 2% glycerol plus ampicillin (2.8 L Fernbach flask). Grow cell culture with shaking at 200 rpm at 37 °C.

2.2.3. Induce recombinant protein expression when the OD_{600} reaches 0.6-0.8 by addition of IPTG at a final concentration of 0.4 mM followed by incubation at 30 °C overnight with shaking at 200 rpm.

2.2.4. Harvest the cells by centrifuging at 4,000 x *g* in 4 °C for 20 min. Remove the supernatant and re-suspend the cell pellets with cold lysis buffer 2 (50 mM Tris-Cl, pH 7.80, containing 100 mM NaCl, 5 mM dithiothreitol, 1 mM EDTA, and 1 mM PMSF) to a final volume of 50 mL buffer.

NOTE: Cells can be stored long-term at -80 °C or used immediately for protein purification. To store cells, split the cell suspension into 2 x 50 mL disposable centrifuge conical tubes and keep cell pellets at -80 °C until the protein purification step. The expression of mutant and wild type form of $\alpha_2\beta_2$ *St*TS complex in LB broth yields 125-150 mg of tagged protein per liter. Consider scaling protocol up or down to fulfill specific demands.

2.3. Purification of wild type or mutant form of $\alpha_2\beta_2$ *St*TS complex

NOTE: The following protocol is intended to purify the non-tagged recombinant wild type or mutant form of the recombinant $\alpha_2\beta_2$ *St*TS complex within 1 day, depending on skill set and efforts. To reduce purification time, equilibrate size exclusion column in buffer prior protein purification/expression. Purification of wild type or mutant $\alpha_2\beta_2$ *St*TS complex is carried out by a two-step purification comprising ammonium sulfate fractionation and a size exclusion chromatography. This protocol yields 60-100 mg of pure complex from 1 L of LB medium.

2.3.1. Disrupt cell pellet by sonication using a 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.

2.3.2. Centrifuge the cell lysate at 30,000 x *g* for 30 min at 25 °C. Aspirate supernatant, ensuring the pellet does not dislodge from tube. Filter the clarified supernatant fraction with a 0.45 µm filter at room temperature.

2.3.3. Assess the initial volume of the clarified supernatant fraction. Slowly add small amounts of ammonium sulfate at a time, until a 20% saturation is reached (11.51 g / 100 mL). Carry out ammonium sulfate fractionation out at 25 °C. Gently stir the solution for 10 min and avoid bubbles.

2.3.4. Centrifuge at 30,000 x *g* for 10 min at 25 °C. Transfer the 20% supernatant fraction into a clean flask. Gently resuspend 20% pellet fraction in 20 mL of sample buffer 2 (50 mM Tris-Cl, pH 7.80, containing 100 mM NaCl, 1 mM EDTA, and 2 mM dithiothreitol) and prepare a sample to run SDS-PAGE gel. Discard the 20% pellet fraction.

2.3.5. Assess the initial volume of the 20% supernatant fraction. Add ammonium sulfate to 30% saturation is reached (5.94 g / 100 mL), stir solution, avoid bubbles, and centrifuge as before. Transfer the 30% supernatant fraction into a clean flask. Gently resuspend 30% pellet fraction in 20 mL of sample buffer 2 and prepare a sample to run SDS-PAGE gel. Discard the 30% pellet fraction.

2.3.6. Assess the initial volume of the 30% supernatant fraction. Add ammonium sulfate at a 40% saturation is reached (6.14 g / 100 mL), stir solution, avoid bubbles, and centrifuge as before. Transfer the 40% supernatant fraction into a clean flask. Gently resuspend 40% pellet fraction in 10 mL of sample buffer 2 and prepare a sample to run SDS-PAGE gel. Discard the 40% supernatant fraction.

NOTE: Pre-purified protein aliquots of the pre-purified $\alpha_2\beta_2$ *St*TS complex can be stored long-term at -80 °C or used immediately for protein purification on a size exclusion chromatography column (SEC).

2.3.7. Microcentrifuge the protein sample at 10,000 x *g*, 20 min, 4 °C and load the supernatant fraction on a HiPrep 16/60 Sephacryl S-200 HR column attached to a fast protein liquid chromatography at a flow rate of 0.5 mL min^{-1}, previously equilibrated in SEC buffer (10 mM Tris-Cl, pH 7.8, 100 mM NaCl, 5% glycerol, 0.1 mM pyridoxal phosphate).

NOTE: To purify large amounts of complex, load a 5 mL sample at 20-25 mg mL^{-1} on a HiLoad 26/600 Superdex 200 pg column at a flow rate of 1.5 mL min^{-1}.

2.3.8. Assess samples collected along the ammonium sulfate fractionation and peak fractions from the SEC column on a 12% SDS-PAGE gel stained with Coomassie brilliant blue stain[25].

2.3.9. Concentrate the wild type or mutant $\alpha_2\beta_2$ *St*TS complex with a 15 mL 100 kDa cutoff centrifugal filter unit by spinning at 3,000 x *g* at 4 °C. Transfer the concentrated protein to a fresh 2.0 mL tube and microcentrifuge (10,000 x g, 10 min, 4 °C) to remove aggregates.

2.3.10. Determine protein concentration[24], prepare 0.5 mL aliquots at 20-25 mg mL^{-1}, label, flash-freeze in liquid nitrogen, and store them at -80 °C.

3. Optimized crystallization for wild type and mutant form of the $\alpha_2\beta_2$ *St*TS complex

NOTE: The 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].

3.1. Prepare stock solutions prior to the crystallization assays to achieve a better crystallization reproducibility. To perform structural studies with TS, crystallize protein with Na^+ or Cs^+ ion at the metal coordination site of the bienzyme complex.

3.1.1. Prepare 5 mL stock solution of 200 mM spermine in water and keep 500 µL aliquots at -20 °C.

3.1.2. Prepare 50 mL stock solution of 30% (w/v) PEG 8000 in water and keep 25 mL aliquots in a 50 mL disposable centrifuge conical flasks at 25 °C.

3.1.3. Prepare a 25 mL stock solution of 1 M CsCl in water and keep at 25 °C.

3.1.4. Prepare 25 mL stock solution of 1 M NaCl in water and keep at 25 °C.

3.1.5. Prepare 3x 50 mL stock solution of 1 M bicine and titrate with CsOH or NaOH to obtain buffered solutions at pH 7.6, 7.8, and 8.0. Keep 25 mL aliquots at 4 °C.

3.2. Thaw a sample of $\alpha_2\beta_2$ *St*TS complex (20-25 mg mL^{-1}) on an ice bath.

3.3. Microcentrifuge sample at 10,000 x *g* for 10 min at 25 °C to remove protein aggregates.

3.4. Transfer the cleared supernatant fraction into a clean microcentrifuge tube.

3.5. Estimated protein concentration[24] and dilute protein aliquots (150-200 µL) at 15 mg mL^{-1} with 50 mM bicine-CsOH or -NaOH, pH 7.8 containing 50 mM CsCl or NaCl. Keep protein sample at 25 °C.

3.6. Prepare the 500 µL reservoir solutions for 3x 24-well sitting drop plates in sterile 1.5 mL labeled microcentrifuge tubes. The reservoir solution contains 50 mM bicine-CsOH or -NaOH, 50 mM CsCl or NaCl, and PEG 8000. Vary the concentration of PEG 8000 (6-11%) on the plate columns and the concentration of spermine (2-8 mM) on the plate rows. Change buffer pH (pH

7.6, 7.8 and 8.0) for each set.

3.7. Cap the tubes and vortex vigorously at least 10 sec. Centrifuge tubes at 10,000 x *g* for 10 min at 25 °C to remove bubbles. Dispense 500 µL buffered solution in each labeled reservoir.

3.8. Use a P-10 micropipette and dispense 5 µL of protein solution at 15 mg mL^{-1} per each sitting drop well. Avoid bubbles. Add 5 µL of each correspondent reservoir solution to the protein drop. Avoid bubbles during mixing and do not pipette up and down to homogenize the mixture.

3.9. Tape the plate with a transparent adhesive tape and store the plate at 25 °C. Crystals appear in 2-5 days and grow to their full dimensions within two weeks.

4. X-ray diffraction data collection and $\alpha_2\beta_2$ *St*TS complex structure solution

NOTE: Prior to X-ray diffraction data collection, prepare cryoprotectant solution for each crystal in advance. Use the specific reservoir solution to prepare 3 aliquots containing increasing concentrations of dimethyl sulfoxide in solution (10, 20, and 30% v/v) and specific ligand (s). Dimethyl sulfoxide was found to be a better cryoprotectant than glycerol, ethylene glycol, and PEG 200-300.

4.1. Harvest a large single crystal using a cryoloop under the stereoscopic microscope.

4.2. Pipette 2 µL of each cryoprotectant solution containing the precipitation solution plus higher concentrations of dimethyl sulfoxide and ligand (s) onto a new cover slide.

4.3. Sequentially, soak crystal in each drop and let the crystal equilibrate for 30 s in the cryoprotectant solution.

4.4. Flash-cooling the cryoprotected crystal using a gaseous nitrogen stream at -173 °C (100 K) or immerse in liquid nitrogen for long-term storage in pucks.

NOTE: Fill a foam Dewar with liquid nitrogen, pre-cool a crystal puck, store crystals in the cryogenic storage Dewar, and ship crystals to the synchrotron using a dry shipper.

4.5. Proceed with X-ray diffraction data collection at -173 °C. Record X-ray diffraction data using a 0.5-4.0 s exposure time and 0.5° oscillations. Rotate crystal 180-360°.

4.6. Process the X-ray diffraction images with iMosflm[27] to generate reflection file in the appropriate space group. Generally, $\alpha_2\beta_2$ *St*TS crystals belong to the space group C 121 (C2).

4.7. Scale together multiple observations of reflections with Scala[28], implemented in the CCP4 package[29].

4.8. Solve the structure of the high-resolution $\alpha_2\beta_2$ *St*TS complex by molecular replacement using MolRep[30] and an appropriate search model. Inspect the model and the electron density map in Coot[31] after a successful structure solution.

NOTE: There are many TS crystals structures deposited in the Protein Data Bank with different ligand (s). For a better molecular replacement step and decreased time fitting the newer crystal structure, use the best search model containing ligand (s) of interest. Proceed with crystal structure refinement in CCP4[29,30] or Phenix[31].

4.9. Make manual adjustments to the model using Coot[32], followed by automatically refinement with Refmac[29,33] or phenix.refine[31,34]. Build and refine the final model by running iterative rounds of model building in Coot[32] and automated refinement.

4.10. Proceed with coordinate and structure factors deposition in the Protein Data Bank web site.

REPRESENTATIVE RESULTS

Purification of the α- and β-subunits of the tryptophan synthase

The α-subunit (α*St*TS) and the β-subunit (β*St*TS) of the *Salmonella typhimurium* tryptophan synthase were subcloned in the modified pET SUMO vector. **Figure 1A** shows representative SDS-PAGE results of two strong bands corresponding to the His6-SUMO-α*St*TS (lane αON) and His6-SUMO-β*St*TS (lane βON) fusion protein. The purification protocol described in this work allowed purification of both subunits individually within 2 days. 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. **Figure 1B** and **1C** show representative SDS-PAGE results of the α-subunit and the β-subunit purification, respectively. On the second day, the concentrate α-subunit, β-subunit, and the $\alpha_2\beta_2$ *St*TS complex from the α- and β-subunits were loaded on a size exclusion chromatography column. **Figure 1D** shows a typical elution profile of α*St*TS, β*St*TS, and $\alpha_2\beta_2$*St*TS complexes on a S-200 HR size exclusion column. **Figure 1E** shows a representative SDS-PAGE result of the collected peak fractions. The purest peak fractions were pooled, concentrated, and the $\alpha_2\beta_2$ *St*TS complex was used for protein crystallization studies.

Purification of the wild type and mutant $\alpha_2\beta_2$ *St*TS complex

Another rapid and efficient protocol to purify the wild type and mutant form of the $\alpha_2\beta_2$ *S. typhimurium* tryptophan synthase complex is described in this work. **Figure 2** shows a representation of the pEBA-10 construct containing the wild type translationally coupling gene (trpA and trpB) encoding for the α- and β-subunits[22]. The two-step PCR mutagenesis protocol to generate mutant forms of the $\alpha_2\beta_2$ *St*TS complex is depicted in **Figure 3**.

The coding regions of mutant $\alpha_2\beta_2$ *St*TS complex in pEBA10 were confirmed by DNA sequencing and used to transform *E. coli* strain CB149 cells[26]. The wild type and mutant form of the $\alpha_2\beta_2$ *St*TS complex were overexpressed and the recombinant proteins were purified successfully within 1-2 days. Ammonium sulfate fractionation at room temperature readily removed most of the

contaminant proteins from the heterologous expression system (**Figure 4A, lanes 20P, 30P and 40S**). A representative elution profile with relative elution position of $\alpha_2\beta_2$ *St*TS (143.06 kDa) complex on a HiPrep 16/60 Sephacryl S-200 HR size exclusion chromatography column is shown in **Figure 4B**. The purity of the excluded peak fractions was SDS-PAGE analyzed before pooling (**Figure 4C**).

Optimization of wild type and mutant $\alpha_2\beta_2$ tryptophan synthase complex crystallization

Aliquots of wild type and mutant $\alpha_2\beta_2$ *St*TScomplex at 15 mg ml^{-1} were used to set up 24-well sitting drop plates. Typically, droplets consisting of 5 µL protein solution and the equivalent volume of reservoir solution were equilibrated against 500 µL of reservoir solution (**Figure 5**). Spermine is required to crystallize the wild type and mutant $\alpha_2\beta_2$ *St*TS complex[22]. While the final concentration of spermine to crystallize the wild type is 2 mM, the concentration of spermine to crystalize the mutant complex in this work showed to be slightly higher (4-8 mM).

Large single crystals were obtained through a fine crystallization optimization, varying PEG 8000 (6-11%) and bicine buffer pH. Crystals with different morphologies appeared in 2-5 days and crystals grew to full size within two weeks (**Figure 6**). Prior to X-ray diffraction data collection, crystals were soaked in cryoprotectant solution (reservoir buffer containing up 30% dimethyl sulfoxide). The optimized process resulted in quality crystals suitable for X-ray diffraction measurements at near atomic resolution.

X-ray diffraction data analysis

A crystal structure of the wild type $\alpha_2\beta_2$ *St*TS complex was prepared with methods described in this article and X-ray diffraction data was collected at near atomic resolution. The crystal was soaked in cryoprotective solution containing F9 inhibitor (2- ({[4-(Trifluoromethoxy)Phenyl]Sulfonyl}Amino)Ethyl Dihydrogen Phosphate) and L-Tryptophan.

A complete X-ray diffraction data set was collected on the SIBYLS synchrotron beamline 12.3.1 at the Advanced Light Source (Berkeley-CA) by rotating the crystal 360° in increments of 0.5°. X-ray diffraction intensities were processed, and data-collection statistics are summarized in **Table 1**. Symmetry analysis indicates that the crystal belonged to the monoclinic space group C2. The unit-cell parameters are *a* = 182.55, *b* = 59.30, *c* = 67.37Å, α = 90.00, β = 94.82, γ = 90.00°. The calculated value of the Matthews coefficient (Vm = 2.57 $Å^3$ Da^{-1}) suggests the presence of one TS heterodimer molecule (αβ *St*TS) in the asymmetric unit of the crystal with a solvent content of 52.08% [35,36]. All X-ray data were collected at low temperatures (100 K) to improve the diffraction quality and decrease the radiation decay. The $\alpha_2\beta_2$ *St*TS crystal structure in complex was solved by the molecular replacement method using the wild type αβ *St*TS model in complex with the inhibitor F9 at the α-site and cesium ion at the metal coordination site (PDB ID code: 4HT3). The final coordinate file and the structure factors were deposited in the PDB with accession code 5CGQ (**Figure 7A**). The crystal structure of the wild type $\alpha_2\beta_2$ *St*TS complex with inhibitor F9 at the enzyme α-site (**Figure 7B**), cesium ion at the metal coordination site (**Figure 7C**), the cofactor pyridoxal 5′-phosphate covalently bonded to βLys87 (**Figure 7D**), and the product L-tryptophan at the enzyme β-site (**Figure 7E**) was solved at 1.18 Angstrom resolution. Model 5CGQ is the highest resolution $\alpha_2\beta_2$ *St*TS crystal structure deposited in the PDB to date.

FIGURE AND TABLE LEGENDS:

Figure 1. Purification of the α- and β-subunits and the $\alpha_2\beta_2$*St*TS complex. (A) Recombinant protein expression. 12% SDS-PAGE gel of the overnight expression profile of SUMO-α*St*TS (αON) and SUMO-β*St*TS (βON) after IPTG induction at 30 °C (α/β0 prior IPTG induction). (**B**, **C**) Ni-NTA affinity chromatography followed by ammonium sulfate precipitation (60% saturation), (S) clarified crude extract (FT1) Ni-NTA column pass through sample (W) column wash sample (E) eluate sample (60S) and (60P) supernatant and precipitate fractions after high-speed centrifugation, respectively (D) SUMO-protease digestion product (FT2) Ni-NTA column pass through sample containing the tag-less α*St*TS or β*St*TS subunit. (**D**) elution profile of α*St*TS subunit (28.67 kDa), β*St*TS subunit (42.86 kDa), and $\alpha_2\beta_2$ *St*TS complex (143.06 kDa) with a HiPrep 16/60 Sephacryl S-200 HR size exclusion chromatography column. Each run was performed separately. (**E**) SDS-PAGE gels of the excluded peak fractions from each individual chromatography. While 15% SDS-PAGE gels were prepared to analyze $\alpha_2\beta_2$ *St*TS complex and α*St*TS subunit, a 12% SDS-PAGE gel was prepared to analyze the β*St*TS subunit. Lane MW, molecular-weight markers in kDa.

Figure 2. **Representation of the construct pEBA-10.** (**A**) Representation of the wild type translationally coupling gene (trpA and trpB) encoding the α- and β-subunits of the tryptophan synthase from bacterium *Salmonella enterica* serovar *typhimurium* (Yang, Ahmed et al. 1996). (**B**)The vector contains an ampicillin resistance (amp) gene, a replication origin (ori), a $lacI^q$ gene to better shutdown a lac promoter in absence of IPTG inducer, and the LacI-repressed promoter.

Figure 3. Overall representation of the two-step PCR mutagenesis protocol. The pEBA-10 vector was used as a DNA template. The first round of PCR was prepared with primers TS-FW-NcoI and MUT-REV (a reverse primer containing a mutation) to generate the first fragment and primers TS-Rev-SacI and MUT-FW (a forward primer containing a mutation) to generate the second fragment). Fragments were gel purified and equimolarly combined, heat denatured, and annealed. The recombinant strands were extended with polymerase and deoxyribonucleotides. The second round of PCR was prepared with primers TS-FW-NcoI and TS-Rev-SacI.

Figure 4. **Purification of wild type and mutant form of $\alpha_2\beta_2$ *St*TS complex**. (**A**) 12% SDS-PAGE gel of samples collected along the ammonium sulfate precipitation using 20, 30, 40 and 50% ammonium sulfate saturation at room temperature: (CE) crude extract (S) and (P) supernatant and precipitate fractions after high-speed centrifugation. (**B**) Elution profile of $\alpha_2\beta_2$ *St*TS (143.06 kDa) complex with a HiPrep 16/60 Sephacryl S-200 HR size exclusion chromatography column. (**C**) 12% SDS-PAGE gel picture of the excluded peak fractions. Lane MW, molecular-weight markers in kDa (Precision Plus Protein Unstained Standards, Bio-Rad).

Figure 5. **Crystallization optimization for wild type and mutant form of $\alpha_2\beta_2$ *St*TS complex.** Crystals were grown in 50 mM Bicine-CsOH buffer containing 50 mM $CsCl_2$. The concentration of polyethylene glycol 8000 (6-11%) and spermine (2-8 mM) were varied to obtain single large crystals forms to perform structural studies by X-ray protein crystallography. (**A**) Bicine-CsOH, pH 7.6. (**B**) Bicine-CsOH, pH 7.8. (**C**) Bicine-CsOH, pH 8.0.

Figure 6. **Photomicrograph of crystals of wild type and mutant form of $\alpha_2\beta_2$ *St*TS complex**. Crystals differ in morphology, but they belong to the space group C2. The crystals grew to their full dimensions in the final conditions after two weeks. Crystals of approximately 0.20 x 0.15 x 0.10 mm in size. (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).

Figure 7. Overall visualization of crystal structure and validation of electron density maps obtained after crystal structure refinement. (**A**) crystal structure of the wild type $\alpha_2\beta_2$ *St*TS complex with inhibitor F9 at the enzyme α-site (yellow colored), cesium ion at the metal coordination site (blue colored), the cofactor pyridoxal 5′-phosphate covalent bonded to βLys87 (green colored), and the product L-tryptophan at the enzyme β-site (cyan colored) at 1.18 Angstrom resolution. While the α-subunit is colored in light blue, the β-subunit is colored in salmon. (**B-E**) Electron density maps contoured at 1.0 r.m.s. level around (**B**) inhibitor F9 (**C**) cesium ion (**D**) pyridoxal-5′-phosphate, and (**E**) L-Tryptophan.

Table 1. Data collection and processing. Values in parentheses are for the outer shell.

DISCUSSION

We have successfully engineered mutant form $\alpha_2\beta_2$ βQ114A, $\alpha_2\beta_2$ βK167T, and $\alpha_2\beta_2$ βS377A *St*TS complexes for structure-function correlation studies. Initially, we have tried to purify the mutants using a previous purification protocol[22], which requires $\alpha_2\beta_2$ *St*TS complex crystallization with PEG 8000 and spermine during purification. 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. Crystallization could be achieved either after long periods (48-96 h) or being necessary the addition of extra amounts of PEG 8000 after crystallization initiation[15].

Unfortunately, mutant forms of $\alpha_2\beta_2$ *St*TS complex presented in this work were not successfully purified using this protocol since they failed to crystallize during the initial steps of the protocol, impairing crystallographic studies. Therefore, we have created a simple and efficient purification protocol comprising ammonium sulfate fractionation and size exclusion chromatography, which give high yields of wild type and mutant form of $\alpha_2\beta_2$ *St*TS complex. This protocol is faster (1-2 days) and reproducible when compared with the previous protocol (5-7 days)[15,22], since there is no crystallization requirements and protocol troubleshooting along purification. In addition, we have created new expression constructs and protocol to purify high amounts of the α-subunit, β-subunit and the reconstitute $\alpha_2\beta_2$ *St*TS complex from the isolates.

Future application includes the recombination of wild type and mutant sub-units to perform functional and structural studies. Mutant $\alpha_2\beta_2$ βQ114A, $\alpha_2\beta_2$ βK167T, and $\alpha_2\beta_2$ βS377A complex crystallized in conditions containing higher concentrations of spermine (4-8 mM) when compared with the wild type form (2 mM). Therefore, it is worthwhile spending time on improving the quality of protein crystals by varying the concentration of precipitants and buffer pH. Single large

crystal forms randomly grew in bicine buffered solution (pH 7.6-8.0) containing 6-11% PEG 8,000. The methods described in this work will be used to prepare crystal structures of the wild type and mutant forms of the $\alpha_2\beta_2$ complex with different ligands within the α- and β-active sites, mimicking different intermediates and transition states involved in the conversion of indole and serine to tryptophan. The crystal structures of these mutants are anticipated to provide new insights into the mechanism and roles played by key residues in L-Tryptophan synthesis.

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DISCLOSURES

The authors have nothing to disclose and declare no competing financial interests.

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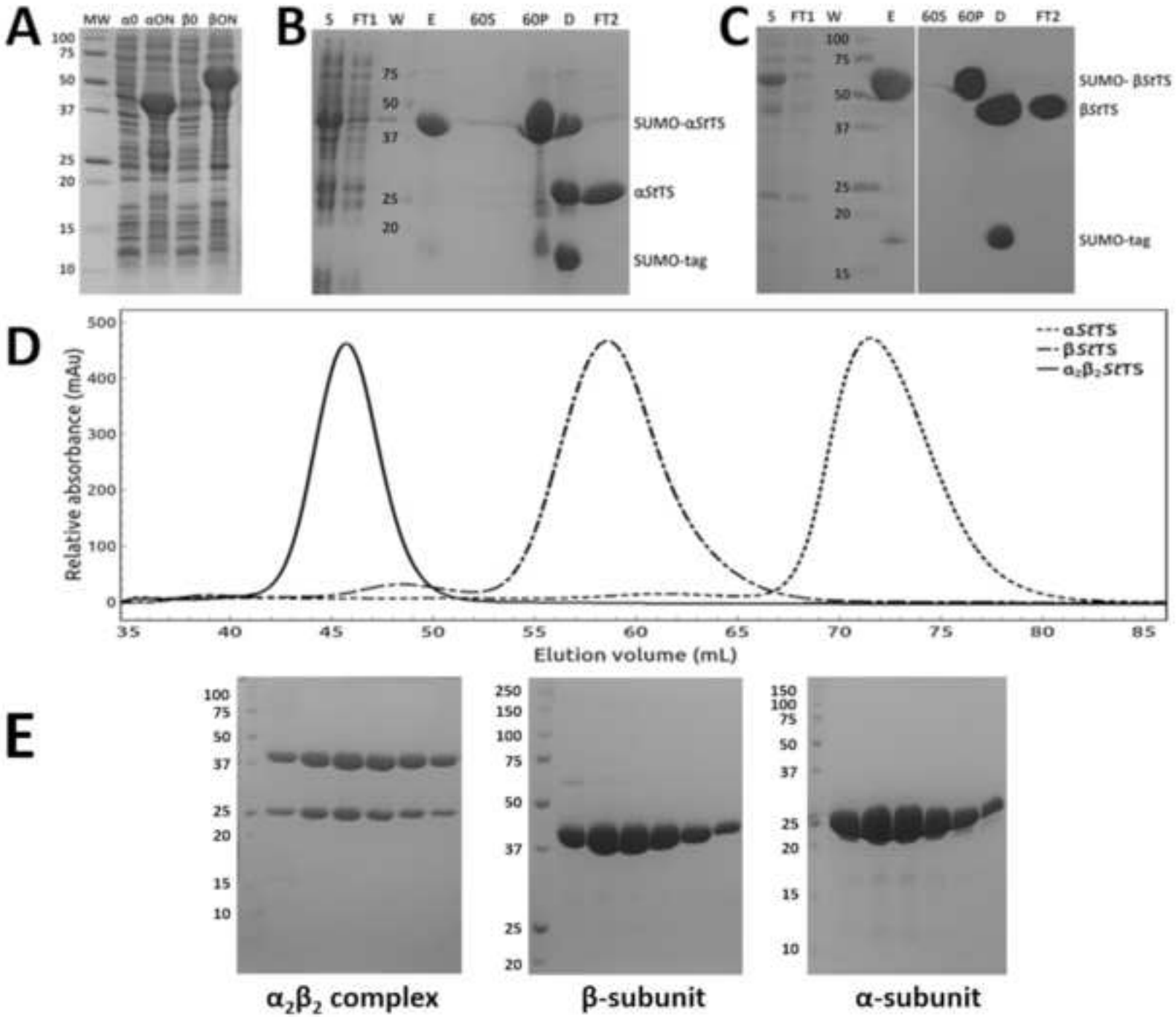

Figure 2

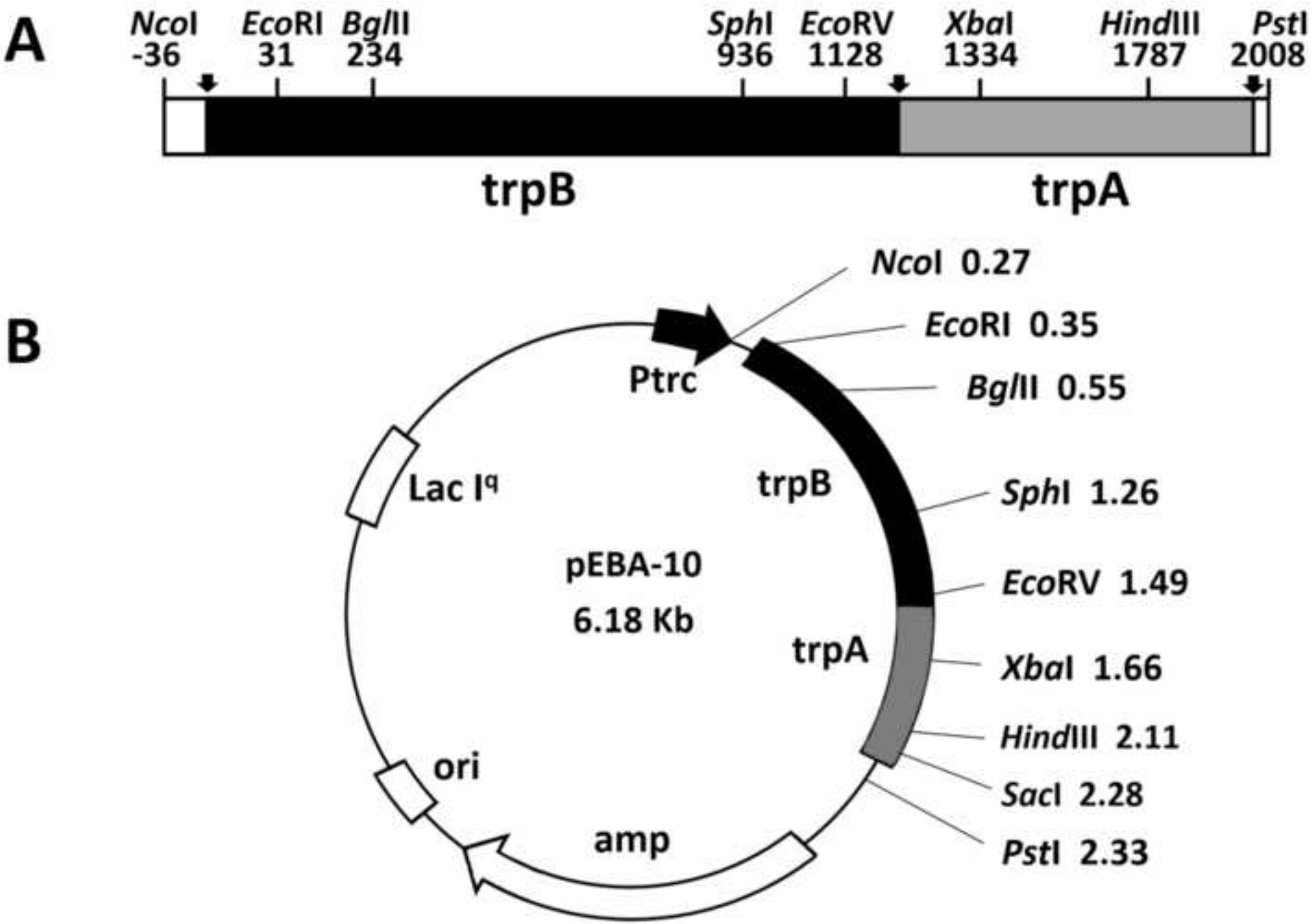

Figure 3

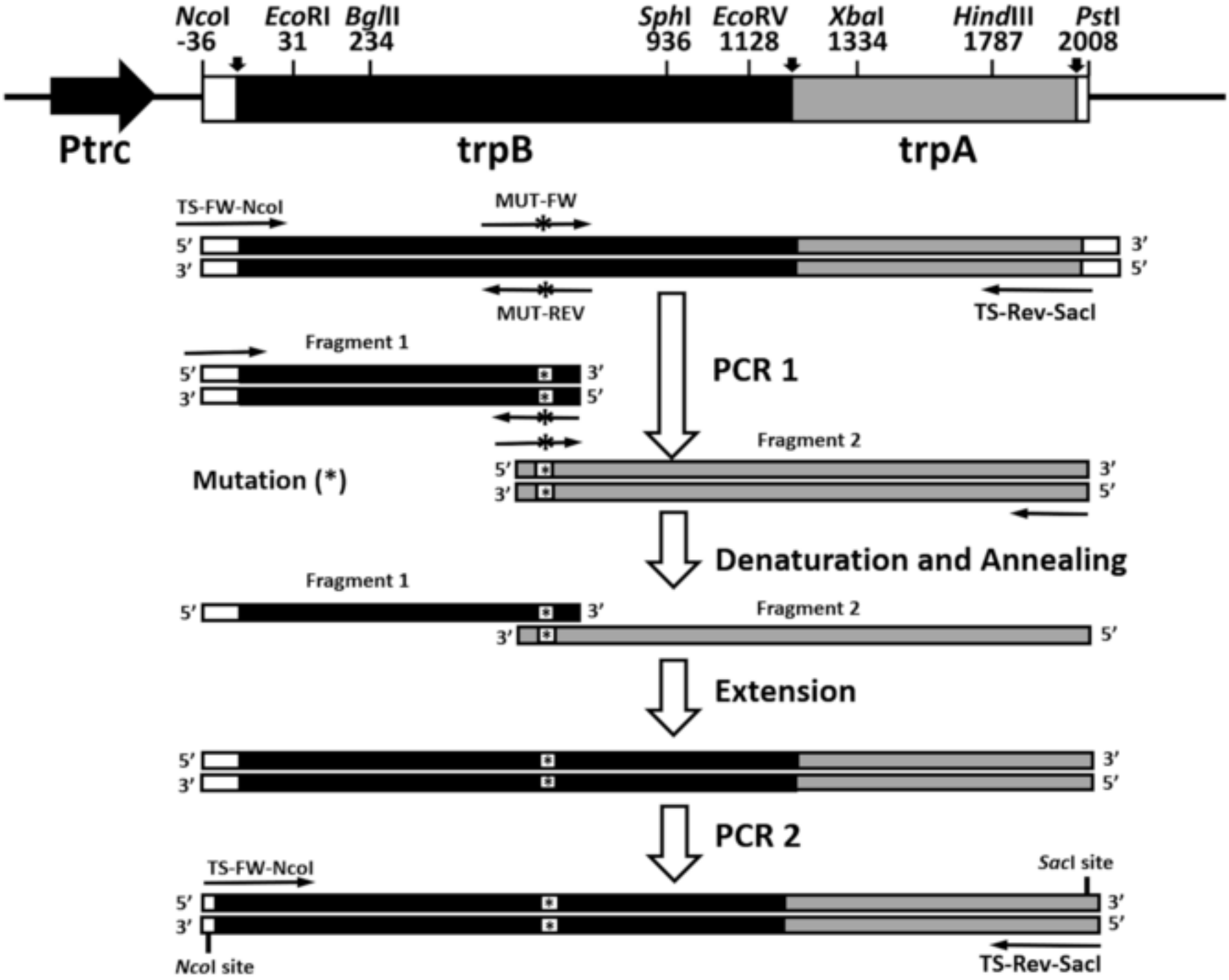

Figure 4

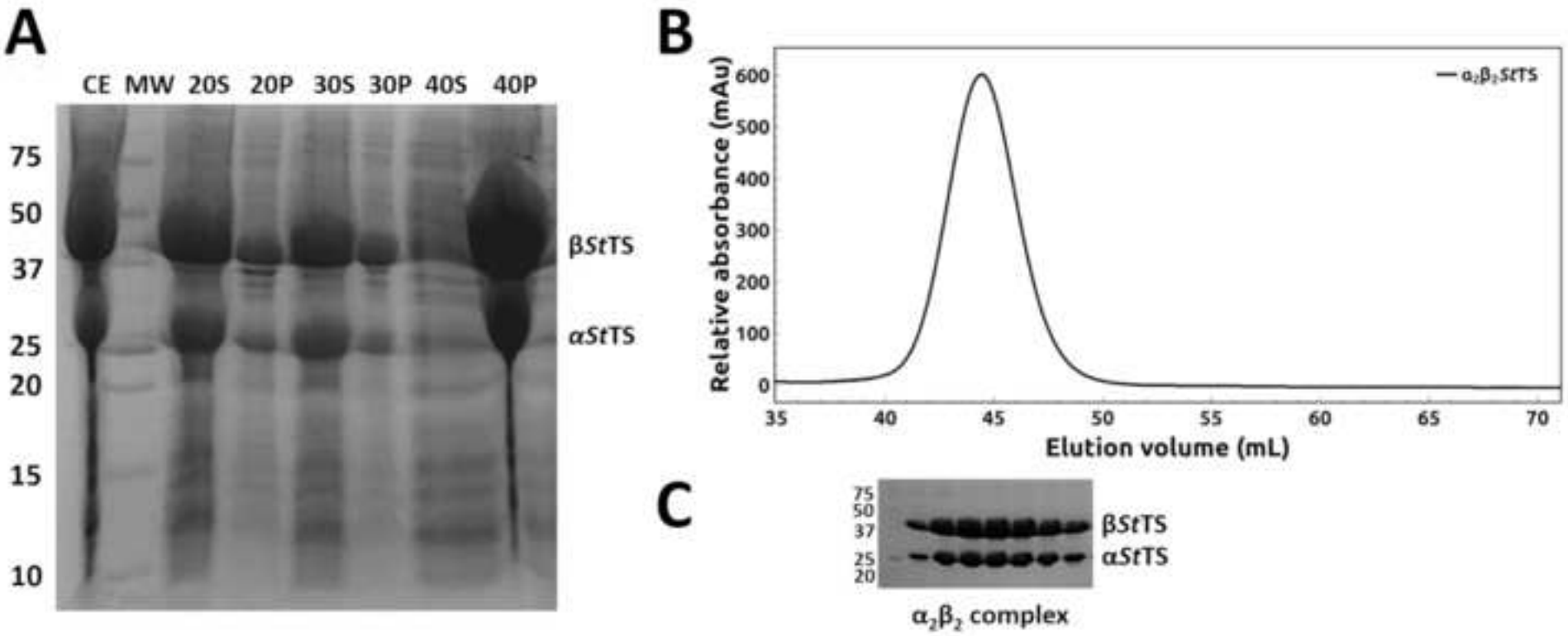

Figure 5

Click here to access/download;Figure;Figure5.tif

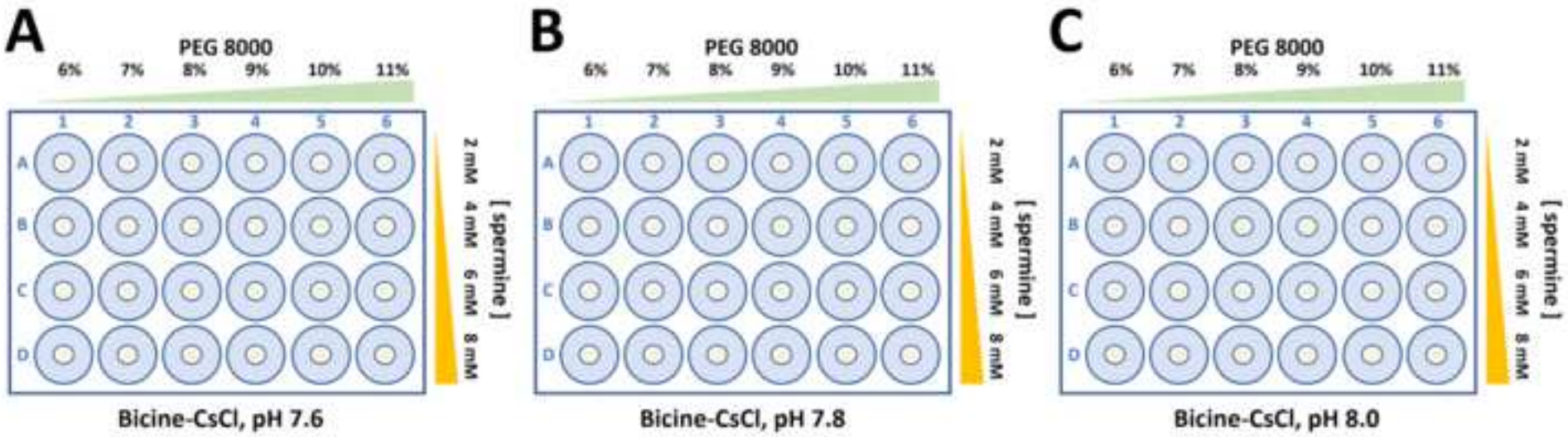

Figure 6

Click here to access/download;Figure;Figure6_new.tif

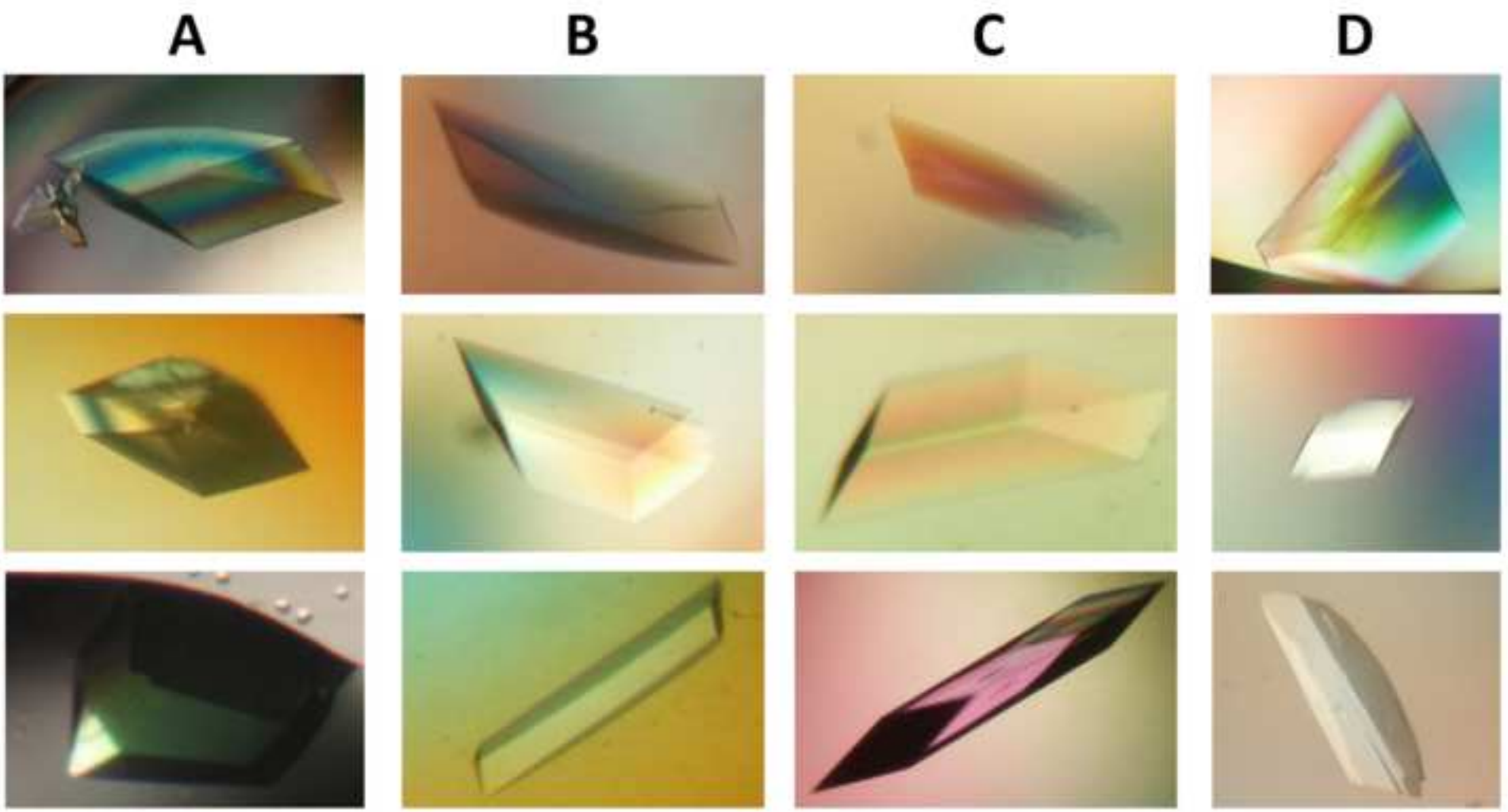

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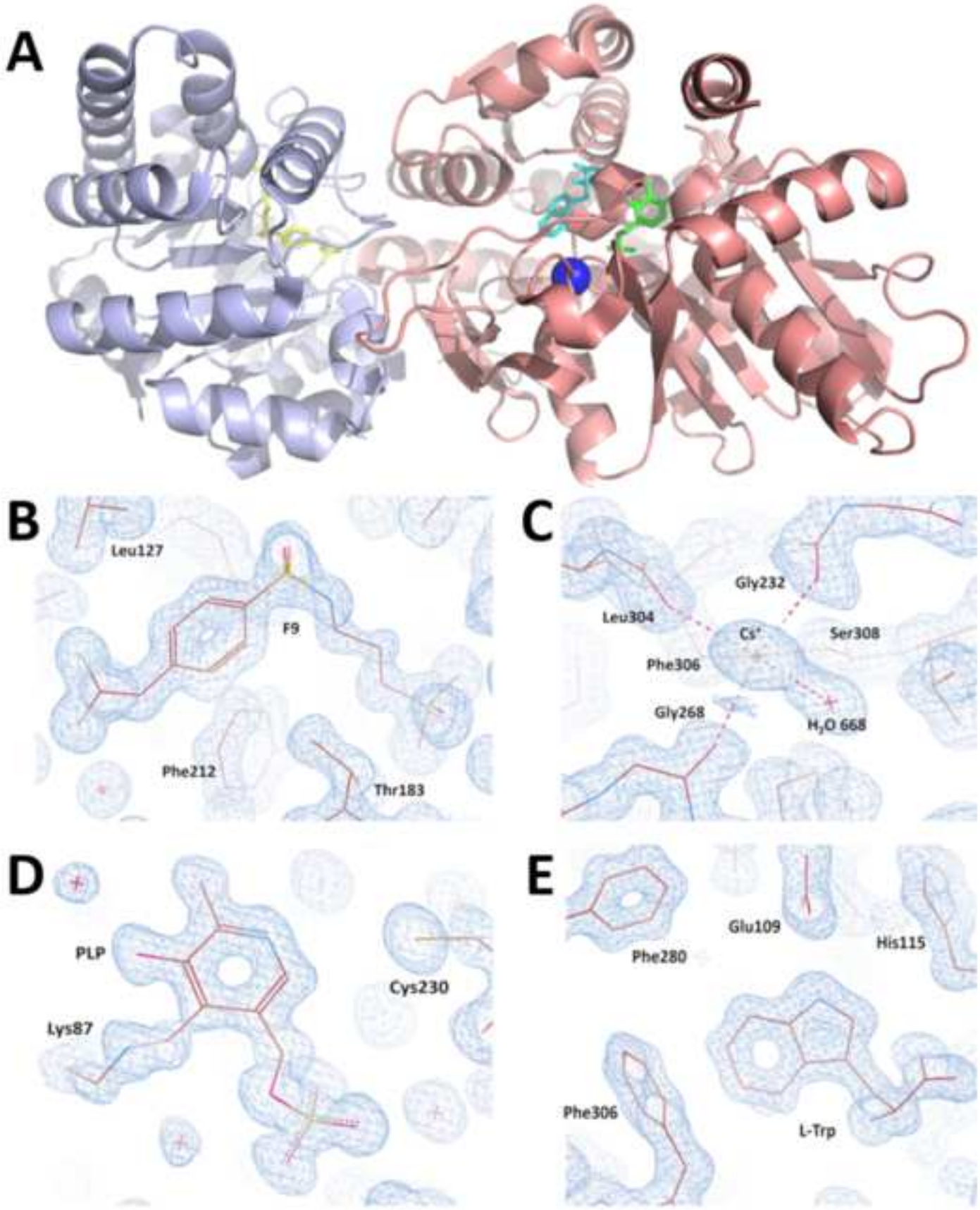
Figure 7



Data collection and processing	
X-ray source / Beam line	ALS Beamline 12.3.1
Wavelength (Å)	10,000
Resolution (Å)	40.00 - 1.18 (1.24 - 1.18)
Total number of reflections	2151280 (252941)
Total number unique reflections	231646 (32187)
Space group for indexing, scaling and merging	C 1 2 1
Cell dimensions	
a, b, c (Å)	182.55, 59.30, 67.37
α, β, γ (°)	90.00, 94.82, 90.00
Mosaicity	0.61
Matthews volume VM ($Å^3$ Da^{-1})	2.57
Rmeas (%)	8.6 (93.0)
<I/σ(I)>	14.7 (3.0)
CC1/2 (%)	0.999 (0.778)
Completeness (%)	98.6 (94.2)
Multiplicity	9.3 (7.9)
Refinement statistics	
Rwork/Rfree (%)	14.04 / 16.05
RMSD bond length (Å)	0.0120
RMSD bond angle (°)	14,059
Ramachandran favored	515 (96.44%)
Ramachandran allowed	16 (3.00%)
Ramachandran disallowed	3 (0.56%)

Table of Materials

Click here to access/download;Table of Materials;JoVE_Materials (1).xls

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
15 mL 10 kDa filter	MilliporeSigma	UFC901024	centrifugal filter unit
15 mL 100 kDa filter	MilliporeSigma	UFC910024	centrifugal filter unit
2 mL cryogenic vials	Corning	CLS430489	Cryogenic vials
2 mL microcentrifuge tubes	Fisher Scientific	05-408-141	microcentrifuge tubes
24-well Cryschem Plate	Hampton Research	HR3-158	24-well sitting drop plates
2-mercaptoethanol	Fisher Scientific	O3446I-100	Chemical
50 mL centrifuge conical tubes	Thermo Scientific	12-565-270	centrifuge conical tubes
AB15 ACCUMET Basic	Fisher Scientific	13-636-AB15	pH meter
Agarose	Fisher Scientific	BP1356-100	Agarose gel
ammonium sulfate	Fisher Scientific	A702-500	Chemical
Ampicillin	Fisher Scientific	BP1760-5	Antibiotic
Bacterial incubator	Fisher Scientific	S35836	incubator.
BamHI	New England Biolabs	R0136S	Restriction enzyme
bicine	Fisher Scientific	BP2646100	Chemical
Branson 450 Digital Sonifier	Brason	B450	Cell disruptor
Cesium chloride	Fisher Scientific	BP210-100	Chemical
Cesium hydroxide	Acros Organics	AC213601000	Chemical
Chloramphenicol	Fisher Scientific	BP904-100	Antibiotic
dimethyl sulfoxide	Fisher Scientific	D1391	Chemical
dithiothreitol	Fisher Scientific	BP172-5	Chemical
DNA Polymerase	Thermo Scientific	F530S	HF polymerase
dNTP Set	Invitrogen	10-297-018	dNTPs set
EcoRI	New England Biolabs	R0101S	Restriction enzyme
Ethylenediaminetetraacetic acid	Fisher Scientific	S311-100	Chemical
Excella E25R Orbital Shaker	Eppendorf New Brunswick	M1353-0004	Orbital incubator
GE AKTA Prime Plus	GE Healthcare	8149-30-0004	FPLC
Gel Extraction Kit	Invitrogen	K210012	DNA purification kit
Glycerol	Fisher Scientific	G33-500	Chemical
HindIII	New England Biolabs	R0104S	Restriction enzyme
His-Trap columns	GE Healthcare	GE17-5255-01	5 mL Histrap column
imidazole	Fisher Scientific	O3196-500	Chemical

IPTG	Thermo Fisher Scientific	R0392	Inducer
Kanamycin	Fisher Scientific	BP906-5	Antibiotic
Kelvinator Series-100	Kelvinator	discontinued	Ultra low freezer
LB broth	Fisher Scientific	BP1426-500	Liquid broth
Luria Bertani agar	Fisher Scientific	BP1425-2	Solid broth
NaCl	Fisher Scientific	S271-500	Chemical
NcoI	New England Biolabs	R0193S	Restriction enzyme
Ni-NTA affinity beads	Thermo Fisher Scientific	R90115	Ni-NTA agarose beads
PEG 8000	Fisher Scientific	BP233-100	Chemical
phenylmethylsulfonyl fluoride	Fisher Scientific	44-865-0	Chemical
pyridoxal phosphate	Acros Organics	AC228170010	Chemical
S-200 HR	Cytiva	45-000-196	Size exclusion column
SacI	New England Biolabs	R0156S	Restriction enzyme
Sodium hydroxide	Fisher Scientific	S318-100	Chemical
Sorvall RC-5B centrifuge	Sorvall	8327-30-1004	Floor cetrifuge
Spermine	Acros Organics	AC132750010	Chemical
Superdex 200 prep grade	Cytiva	45-002-491	Size exclusion column
T4 DNA ligase	New England Biolabs	M0202S	DNA liagse
Tris	Fisher Scientific	BP152-500	Chemical
Ubl-specific protease 1	Thermo Scientific	12588018	SUMO Protease

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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.