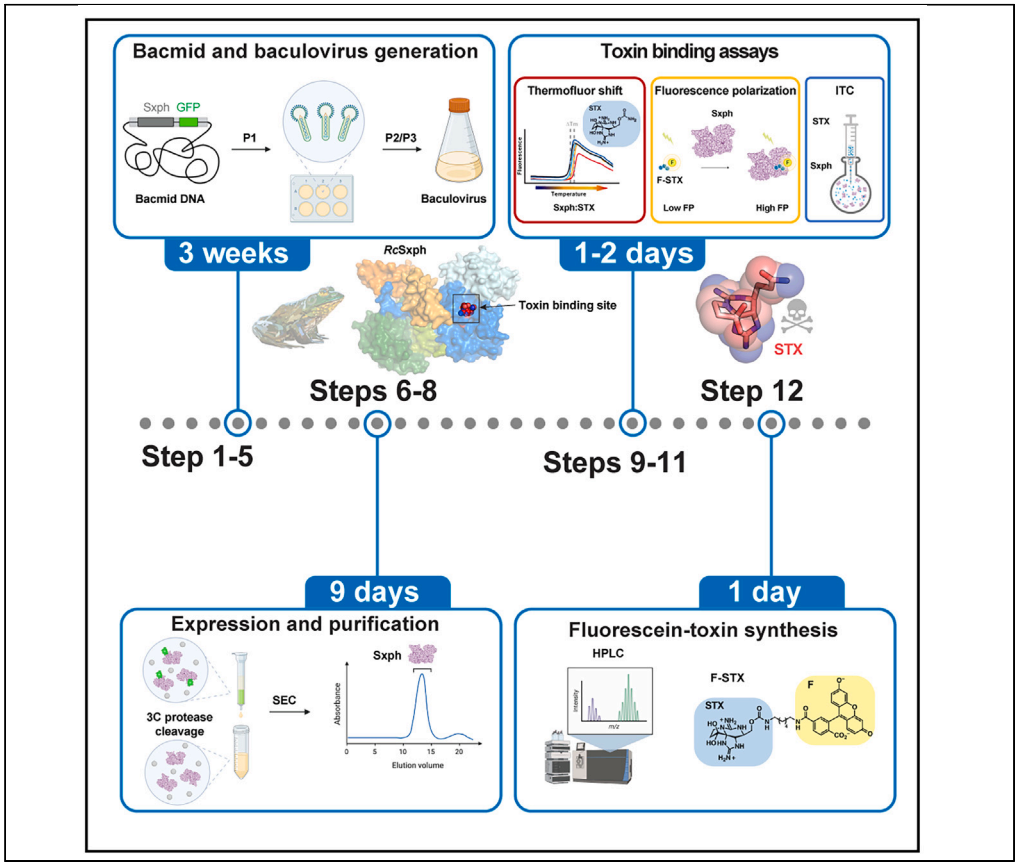
STAR Protocols

CellPress
OPEN ACCESS

Protocol

Expression, purification, and characterization of anuran saxiphilins using thermofluor, fluorescence polarization, and isothermal titration calorimetry



Anuran saxiphilins (Sxphs) are "toxin sponge" proteins thought to prevent the lethal effects of small-molecule neurotoxins through sequestration. Here, we present a protocol for the expression, purification, and characterization of Sxphs. We describe steps for using thermofluor, fluorescence polarization, and isothermal titration calorimetry assays that probe Sxph:saxitoxin interactions using a range of sample quantities. These assays are generalizable and can be used for other paralytic shellfish poisoning toxin-binding proteins.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

Zhou Chen, Sandra Zakrzewska, Holly S. Hajare, J. Du Bois, Daniel L. Minor, Jr.

jdubois@stanford.edu (J.D.B.)
daniel.minor@ucsf.edu (D.L.M.)

Highlights

Technique to produce, purify, and characterize anuran saxiphilins

Workflow for a suite of binding assays for guanidinium toxin-binding proteins

Diverse assays provide multiple ways to assess varied toxin quantities

Binding assays are adaptable to a range of saxitoxin (STX) congeners and other toxins

Chen et al., STAR Protocols 5, 102792
March 15, 2024
https://doi.org/10.1016/j.xpro.2023.102792

STAR Protocols

CellPress
OPEN ACCESS

Protocol

Expression, purification, and characterization of anuran saxiphilins using thermofluor, fluorescence polarization, and isothermal titration calorimetry

Zhou Chen,[1,7] Sandra Zakrzewska,[1,7] Holly S. Hajare,[2] J. Du Bois,[2,*] and Daniel L. Minor, Jr.[1,3,4,5,6,8,9,*]

[1]Cardiovascular Research Institute, University of California, San Francisco, San Francisco, CA 94158-2330, USA

[2]Department of Chemistry, Stanford University, Stanford, CA 94305, USA

[3]Departments of Biochemistry and Biophysics, and Cellular and Molecular Pharmacology, University of California, San Francisco, San Francisco, CA 94158-2330, USA

[4]California Institute for Quantitative Biomedical Research, University of California, San Francisco, San Francisco, CA 94158-2330, USA

[5]Kavli Institute for Fundamental Neuroscience, University of California, San Francisco, San Francisco, CA 94158-2330, USA

[6]Molecular Biophysics and Integrated Bio-imaging Division, Lawrence Berkeley National Laboratory, Berkeley, CA 94720, USA

[7]These authors contributed equally

[8]Technical contact

[9]Lead contact

*Correspondence: jdubois@stanford.edu (J.D.B.), daniel.minor@ucsf.edu (D.L.M.)
https://doi.org/10.1016/j.xpro.2023.102792

SUMMARY

Anuran saxiphilins (Sxphs) are "toxin sponge" proteins thought to prevent the lethal effects of small-molecule neurotoxins through sequestration. Here, we present a protocol for the expression, purification, and characterization of Sxphs. We describe steps for using thermofluor, fluorescence polarization, and isothermal titration calorimetry assays that probe Sxph:saxitoxin interactions using a range of sample quantities. These assays are generalizable and can be used for other paralytic shellfish poisoning toxin-binding proteins.

For complete details on the use and execution of this protocol, please refer to Chen et al. (2022).[1]

BEFORE YOU BEGIN

Saxitoxin (STX), one of the most potent paralytic neurotoxins,[2–4] is a naturally-occurring guanidinium toxin produced along with a set of equally lethal congeners by red tide-associated cyanobacteria and dinoflagellates.[5] As a result of their potent ability to inhibit select voltage-gated sodium channels (Na_Vs)[4] and inhibit bioelectrical signals in nerve and muscle, STX and its congeners cause paralytic shellfish poisoning (PSP).[2] This commercial fishing and public health hazard is a matter of increasing concern due to the effects of climate change on red tide outbreak frequency.[2,4,6] Further, due to its lethality, STX is the only marine toxin classified as a chemical weapon.[2,4] Thus, there is an interest in understanding how proteins bind and recognize STX and its congeners in order to advance new means to detect STX and its congeners in the environment and neutralize this toxin class.

Some frogs resist STX poisoning[7–10], a property attributed to high-affinity STX binding proteins called saxiphilins (Sxphs).[1,9,11] Sxph-like activity is found in arthropods,[12] amphibians,[12–14] fish,[12] and reptiles.[12] Except for frog and toad Sxphs[1] and a pufferfish protein, Pufferfish Saxitoxin and Tetrodotoxin Binding Protein, PSTBP, that binds STX and related toxin, tetrodotoxin (TTX),[15] the

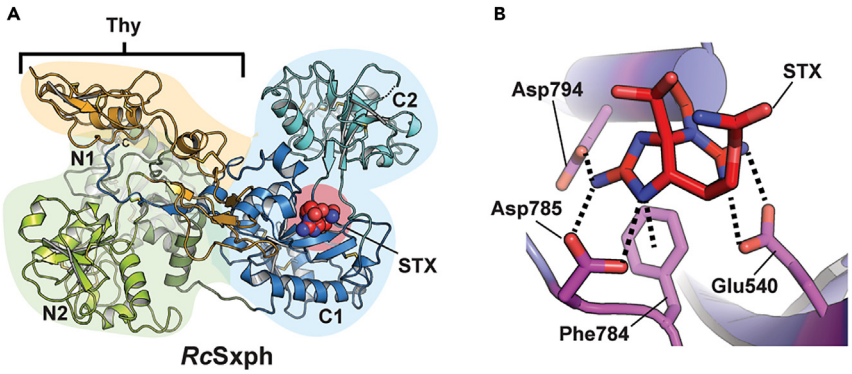


Figure 1. Saxphilin structure and STX binding site

(A) Structure of *Rc*Sxph.[11] (PDB:6O0F) N-lobe (green), C-lobe (blue), and Thy (orange) domains are indicated. STX (red) is shown in space filling representation. Red circle indicates STX binding site.

(B) Detailed view of the *Rc*Sxph STX binding site. Interactions with residues that coordinate the STX bis-guanidinium core (magenta) are shown.

molecular origins of these STX binding activities are uncharacterized. Our recent structural studies have shown that frog and toad Sxphs have a single, high affinity (Kd∼ 1 nM) STX binding site that uses an STX binding code that shares both structural and energetic binding principles with the Na_V STX binding site.[1,11] Because Sxphs are soluble proteins that are readily purified and characterized using a host of biophysical methods, this protein family provides an advantageous platform to investigate fundamental principles underlying STX recognition and a scaffold for developing STX binding assays that can be generalized to other toxin sponge proteins.

Saxiphilins (Sxphs): Soluble anuran high-affinity STX binding proteins

Sxph is a ∼91 kDa protein found in frog heart and plasma that was first characterized from the American bullfrog (*Rana catesbeiana*).[9,12,14,16,17] Recent structural studies have shown that this protein (*Rc*Sxph) is built on a modified transferrin fold scaffold that carries a single high affinity STX binding site[11](Figure 1A). The core structure comprises a butterfly-like structure formed from an N-lobe and C-lobe that each have two subdomains (N1, N2, C1, and C2). Sxphs cannot bind Fe^{3+}[17] due to the fact that nearly all of the residues in each lobe required to bind Fe^{3+} are different from those conserved in transferrins.[1,11] The single STX binding site resides on the C1 domain (Figures 1A and 1B).[11] In addition, unlike transferrins, all Sxphs carry a varied number (2–16) of thyroglobulin protease inhibitor domains that are inserted between the N1 and N2 domains of the N-lobe of the transferrin-like fold.[1,11,18] This architecture and high affinity STX binding site is found throughout the Sxph family encompassing at least 12 Sxphs from diverse types of frogs and toads whose origins are separated by ∼140 million years of evolution.[1]

Structural and mutagenesis studies show that the Sxph STX binding site is largely electronegative and uses two conserved triads of amino acids to coordinate STX.[1,11] One set engages the tricyclic STX core through a pair of carboxylate groups and cation-π interaction that has structural similarity to the Na_V STX binding site (Figure 1B).[1,11] The second triad largely interacts with the carbamate. Notably, changes to this part of the binding pocket can increase STX affinity.[1] Comparisons of apo- and STX bound structures of *Rc*Sxph,[11] *Rc*Sxph mutants,[1] and apo- and STX bound structures of Sxph from the High Himalaya frog (*Nanorana parkeri*) (*Np*Sxph)[1] show that there is very little structural rearrangement upon STX binding and indicate that this property is important for the ability of Sxphs to bind STX with high affinity.[1]

A key step enabling these studies was our establishment of baculovirus based insect cell expression system in which Sxphs bearing a C-terminal, cleavable green-fluorescent protein/His_{10} tag are secreted into the media.[1,11] To date, this system has enabled the production and characterization

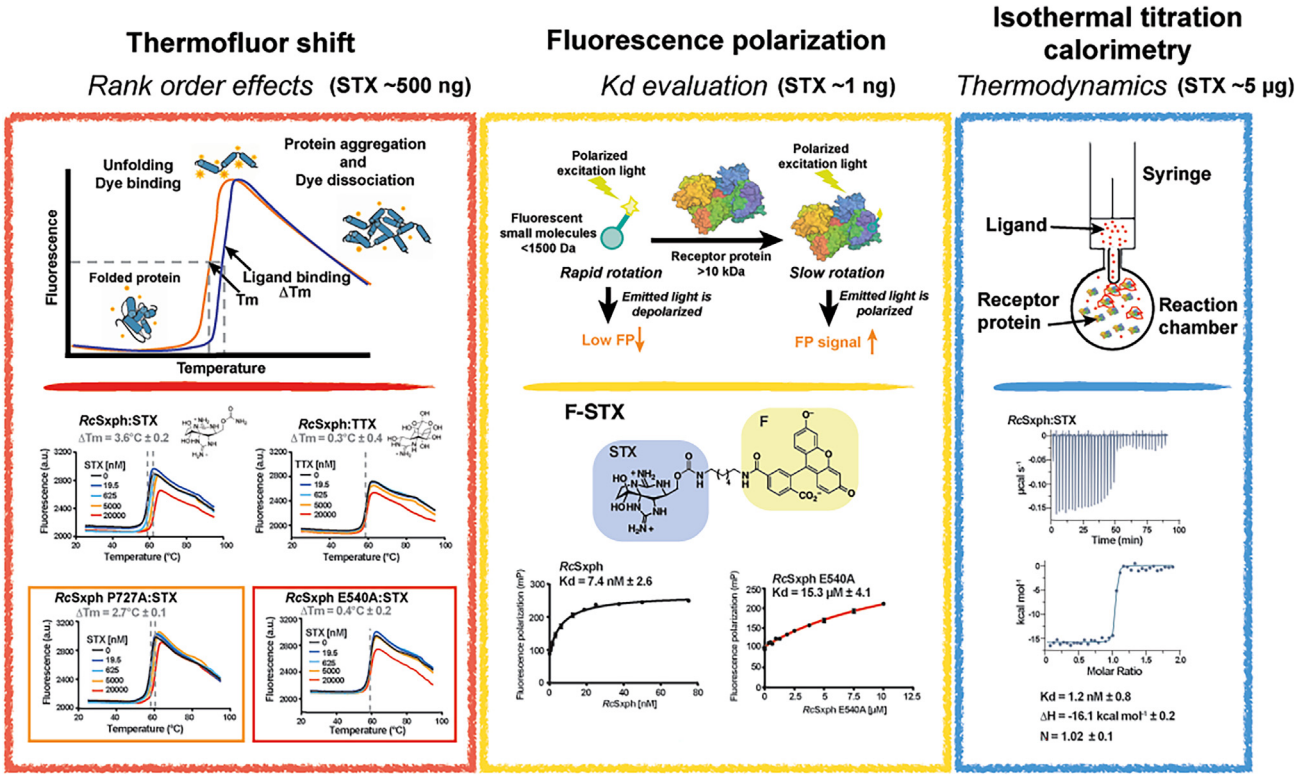


Figure 2. STX binding assays

Conceptual diagrams (top) and exemplar data (bottom) for Thermofluor shift (left), Fluorescence polarization (middle), and Isothermal titration calorimetry (right) measurements. (Left) In the TF assay,[19,20] the protein is mixed with a dye that fluoresces when it binds to hydrophobic parts of the protein. These become exposed as the protein unfolds causing a correlated increase in the fluorescence signal. At higher temperatures, the protein may aggregate and the dye also dissociates. Both events lead to a loss of fluorescent signal. Ligand binding to the folded protein stabilizes the folded state, resulting in a shift in the apparent melting temperature (ΔTm). (Center) In the FP assay,[21,22] binding of the fluorescently labeled small molecule (<1500 Da) is measured by observing a change in the amount of polarized light emitted from the fluorophore as the concentration of the receptor protein increases. Binding to a protein of >10 kDa causes dramatic change in the rotational correlation time leading to an increase in the signal. (Right) For ITC,[23] the heat of binding is measured directly for titration of the ligand into the receptor proteins and yields the thermodynamic parameters of binding. Quantities of STX required for each experiment are indicated. Error bars are SEM. Exemplar data are from ref.[1]

of >30 Sxph mutants and variants for biophysical and structural studies using the methods outlined here.

Three binding assays to measure Sxph:STX interactions

Ligand-protein interactions are at the heart of many biological processes and have been studied for decades using a wide range of methods. We developed three, complimentary assays to assess interactions between STX and Sxphs: a thermofluor assay (TF), a fluorescence polarization assay (FP), and isothermal titration calorimetry (ITC) (Figure 2). The fundamentals of these assays are described extensively elsewhere, and we point the interested user to the following general references for TF,[19,20] FP,[21,22] and ITC.[23] Here, we briefly outline the principles behind these assays and how they can be used to study Sxph:STX interactions or generalized to other guanidinium toxins and receptor proteins.

A TF assay for STX binding

TF assays are widely used to detect and compare ligand-protein interactions.[19,20] This format can be run on multiple samples at the same time using a plate reader such as the CFX Connect thermal cycler. The fundamental principle is that ligand binding increases the stability of the target receptor protein. This increase in stability can be measured by following the apparent melting curve of the protein using a dye such as SYPRO Orange that fluoresces when it interacts with hydrophobic parts of the protein of interest.

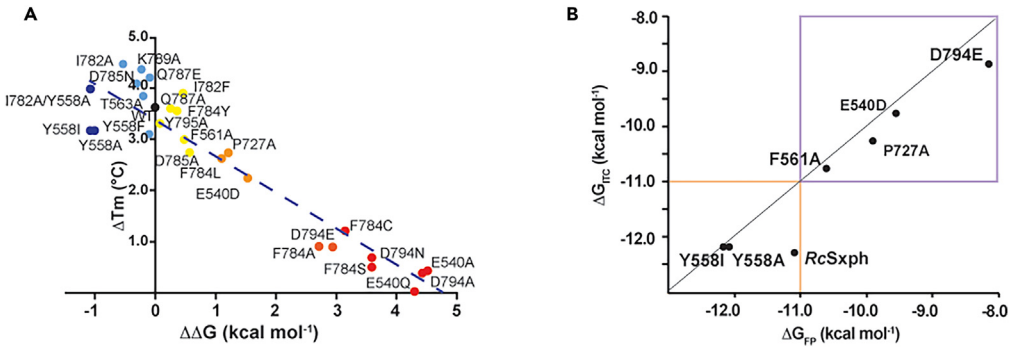


Figure 3. STX binding assay correlations

(A) Comparison of ΔTm and ΔΔG values for *Rc*Sxph and mutants (line y = 3.49–0.7523x, R^2 = 0.886).[1] Colors indicate: dark blue (ΔΔG < -1 kcal mol^{-1}), blue (-1≤ ΔΔG ≤ 0 kcal mol^{-1}), yellow (0 ≤ ΔΔG ≤ 1 kcal mol^{-1}), orange (1 ≤ ΔΔG ≤ 2 kcal mol^{-1}), red orange (2 ≤ ΔΔG ≤ 3 kcal mol^{-1}), and red (ΔΔG > 3 kcal mol^{-1}) where; ΔΔG = RT ln ($Kd_{Sxph\ mutant}$/ Kd_{Sxph}).

(B) Comparison of binding free energies for STX measured by ITC (ΔG_{ITC}) and F-STX measured by FP (ΔG_{FP}) for *Rc*Sxph and mutants.[1] Purple box highlights region of good correlation. Orange box indicates region outside of the ITC dynamic range. (line shows x = y).

Such elements generally become exposed as the protein unfolds as a result of increased temperature. As protein unfolding is a cooperative process, the observed signal should be sigmoidal, saturate, and then decrease linearly as the dye dissociates due to the increasing temperature. This decrease can also be caused by irreversible aggregation and subsequent loss of dye binding (Figure 2, left).

*Rc*Sxph is a multi-domain protein (Figure 1A) having 21 disulfides.[11] From this standpoint, it is remarkable that there is only a single unfolding transition that is observed in the TF assay, which is done in the absence of reducing agents. Some Sxphs show two thermal transitions in this assay (ex. *Ranitomeya imitator* Sxph, *Ri*Sxph).[1] For these, only one of the transitions is STX-dependent, and hence, still permits the use of the TF assay to assess binding. Importantly, for all Sxphs we have examined, a shift in melting temperature, ΔTm, is readily apparent as a function of increasing STX concentration. Tetrodotoxin (TTX), a related guanidinium toxin that is known not to bind *Rc*Sxph, has no impact on the Tm over the same concentration range (Figure 2, left), or on the Tm of other Sxphs.[1] The Tm can be easily determined by taking the first derivative of the apparent melting curve and concentration dependent changes to this value provides a clear indication of ligand binding. The relative ease of this assay allowed us to characterize >30 Sxph variants.[1]

An FP assay for STX binding

FP has distinct advantages as a binding assay as there is no requirement for separating the bound and free species,[21,22] as required for radioligand binding assays. One can observe the direct consequences of the binding event caused by the change in the emission of polarized light as the rotational correlation time of the labeled molecule changes from fast to slow upon binding to a target >10 kDa (Figure 2, middle). By connecting the STX carbamate to fluorescein via a six-carbon linker (F-STX), we created a molecule that showed a clear change in fluorescence polarization indicative of binding to the ~91 kDa *Rc*Sxph (Figure 2, middle). Similar to TF, the FP assay can be readily run on multiple samples, and enabled our measurements of >30 Sxph variants.[1] Comparison of the ΔΔG values measured by FP and the ΔTm values from TF for a series of 26 *Rc*Sxph mutants shows a strong correlation between the two assays (Figure 3A). This is indicative of the fact that the mutational changes do not cause large changes in heat capacity or entropy[24,25] and shows that in this case ΔTm provides an accurate estimate of STX binding affinity differences. Importantly, crystal structure determination of F-STX bound to *Rc*Sxph and *Np*Sxph revealed that the linker and fluorescein make no interactions with either type of Sxph[1] showing why there is such a good correlation between the TF measurements with STX and the FP measurements with F-STX.

ITC—Sxph:STX binding at the limit

ITC is the gold standard method for determining affinity and thermodynamic binding parameters.[23] This label-free method measures the heat released or absorbed when the binding partners are mixed via titration (Figure 2, right). It does have the limit of requiring the largest reagent quantity relative to the TF and FP assays (~12:8 and 100:5000 fold more protein:toxin for TF and FP, respectively), and hence, is best used to investigate the details of key mutants or samples rather than as a screening assay. Overall, the affinity constants measured directly by ITC for Sxph:STX interactions are in good agreement with those measured by FP (Figure 3B),[1] especially for those within the dynamic range for a direct titration experiment (Kds ~30–300 nM).[23] The good correlation with the FP studies (Figure 3B) provides further evidence that the linker and fluorescein do not interact with Sxph. The native Sxph:STX affinity of ~1 nM is at the limit for a direct titration experiment,[23] but nevertheless, under these conditions the method can measure enthalpy changes that provide insight into changes in the association of the toxin and receptor.

Assay correlations

The excellent correlation among TF, FP, and ITC assays provides multiple reliable ways to assess binding depending on the amount of available material (Figure 3). These assays utilize a range of sample quantities (TF: 25 μg Sxph; 600 ng STX; FP: 3 μg Sxph; 1 ng F-STX; ITC: 300 μg Sxph; 5 μg STX). This breadth of choices may be especially useful in the case of limited sample material. Further, these assays should be adaptable to other types of STX targets and STX congeners.

Comparison with other methods

Fluorescence polarization (FP) provides means of measuring the affinity constant of fluorescein-labeled STX with Sxph. Isothermal titration calorimetry (ITC) allows direct characterization of the thermodynamic parameters of the protein-toxin interactions. Both methods provide similar values that are also well correlated with the ΔTm values measured in in the TF assay.[1] Prior studies of Sxph:STX interactions used radioligand binding assays with tritiated STX.[9,12,14,16,26–30] The affinity constants and thermodynamic parameters measured by FP[1] and ITC[1] match very well with those derived from prior studies using radioactive STX.[9,16]

Applications of the method

The methods outlined here offer a robust pipeline for the production and biophysical characterization of toxin binding proteins from the Sxph family and have been used to make and characterize >30 Sxph variants.[1,10,11] These methods should be applicable to other guanidinium toxin sponge proteins such as, PSTBP,[15] or with the proteins responsible for soluble STX binding activity from various arthropods,[12] amphibians,[12–14] fish,[12] and reptiles[12] once they are identified.

Experimental design

This protocol consists of five main segments: recombinant bacmid generation and isolation (Steps 1–2), cell culture and recombinant baculovirus production (Steps 3–5); saxiphilin expression and purification (Steps 6–8); saxiphilin biochemical characterization (Steps 9–11) (Figure 4A), and F-STX synthesis (Figure 5) (Step 12).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
MAX efficiency DH10Bac chemically competent *E. coli* cells	Gibco	Cat# 10361012
Chemicals, peptides, and recombinant proteins		
LB broth (Miller)	Sigma-Aldrich	Cat# L3522
LB broth with agar (Miller)	Sigma-Aldrich	Cat# L3147

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Ampicillin sodium salt	Sigma-Aldrich	Cat# A9518
S.O.C. medium	Invitrogen	Cat# 15544034
Kanamycin sulfate	Millipore Sigma	Cat# 420311
Gentamicin reagent solution, 50 mg/mL	Gibco	Cat# 15750-060
Tetracycline hydrochloride	Sigma-Aldrich	Cat# T7660
X-gal (5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside)	Invitrogen	Cat# B1690
Isopropyl β-d-1-thiogalactopyranoside	Ambeed, Inc.	Cat# A193402
Isopropanol	EMD Millipore	Cat# PX1834-6
Ethanol	EMD Millipore	Cat# EX028503
5X Phusion HF buffer	Thermo Fisher Scientific	Cat# EX0285-3
100 mM dATP solution	Invitrogen	Cat# 10216018
100 mM dTTP solution	Invitrogen	Cat# 10219012
100 mM dCTP solution	Invitrogen	Cat# 10217016
100 mM dGTP solution	Invitrogen	Cat# 10218014
Phusion DNA polymerase	NEB	Cat# M0530S
Dimethylsulfoxide (DMSO)	NEB	Cat# B515A
Nuclease-free water	Ambion	Cat# AM9937
Agarose	Thermo Fisher Scientific	Cat# BP1356-500
Gel loading dye purple (6X)	NEB	Cat# B7024A
GeneRuler 1 kb DNA ladder	Thermo Scientific	Cat# SM0311
Ethidium bromide 1% solution	Fisher Scientific	Cat# BP130210
Acetic acid	Sigma-Aldrich	Cat# 695092
EDTA, disodium salt, dihydrate	EMD Millipore	Cat# 4050
SDS (sodium dodecyl sulfate)	Calbiochem	Cat# 7910
Glycerol	EMD Millipore	Cat# 4760
Glycine	Sigma-Aldrich	Cat# G8790
Bromophenol blue	EMD Millipore	Cat# BX1410-10
β-Mercaptoethanol	Sigma-Aldrich	Cat# M3148
Coomassie brilliant blue R-250	Bio-Rad	Cat# 1610400
ESF 921 insect cell culture medium	Expression Systems	Cat# 96-001-01
Trypan blue solution	Corning	Cat# 25900CI
Cellfectin II reagent	Thermo Fisher Scientific	Cat# 10362100
Sodium chloride	MilliporeSigma	Cat# SX0420-3
OmniPur Tris (hydroxymethyl) aminomethane	Millipore	Cat# 9230
OmniPur 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)	Millipore	Cat# 5320
Calcium chloride dihydrate	Acros	Cat# 447325000
Nickel (II) chloride	Sigma-Aldrich	Cat# 339350
CNBr-activated Sepharose 4B	Cytiva	Cat# 17043002
3C protease	Lee et al.[31]	N/A
SYPRO Orange protein gel stain	Sigma-Aldrich	Cat# S5692
Saxitoxin (STX)	National Research Council Canada; [32]	Cat# CRM-STX-g
Tetrodotoxin (TTX)	Abcam	Cat# ab120054
Fluorescein-saxitoxin (F-STX)	Chen et al.[1]	N/A
Mini-PROTEAN SDS-PAGE gels, 4–15%	Bio-Rad	Cat# 456084
Precision Plus Protein dual color standards	Bio-Rad	Cat# 1610374
Ultrafree-MC centrifugal filter, 0.22 μm pore size	Millipore	Cat# UFC30GV00
Saxitoxin-hexylamine	Andresen and Du Bois[33]	N/A
Fluorescein N-hydroxosuccinimide ester, 6-isomer (FAM-NHS)	Lumiprobe Corp.	Cat# 25120
Trifluoroacetic acid	Sigma-Aldrich	Cat# T6508
Critical commercial assays		
QIAprep Spin Miniprep Kit	QIAGEN	Cat# 27106

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental models: Cell lines		
Spodoptera frugiperda (*Sf*9) cells	Expression Systems	Cat# 94-001S
Oligonucleotides		
pUC/M13 forward primer: 5′-CCCAGTCACGACGTTGTAAAACG-3′	Invitrogen	https://tools.thermofisher.com/content/sfs/manuals/bactobac_man.pdf
pUC/M13 reverse primer: 5′-AGCGGATAACAATTTCACACAGG-3′	Invitrogen	https://tools.thermofisher.com/content/sfs/manuals/bactobac_man.pdf
Recombinant DNA		
*Rc*Sxph pFastBac1 plasmid DNA	Chen et al.[1]	Addgene:194018
Software and algorithms		
GraphPad Prism version 10.0.3	GraphPad Software	https://www.graphpad.com/features
LabSolutions CS software	Shimadzu	https://www.ssi.shimadzu.com/products/software-informatics/labsolutions-series/labsolutions-cs/index.html
MicroCal PEAQ-ITC analysis software	Malvern Panalytical	https://www.malvernpanalytical.com/en/support/product-support/microcal-range/microcal-itc-range/microcal-peaq-itc
Other		
Multiplate 96-well PCR plates, clear	Bio-Rad	Cat# MLL9601
Microseal "B" PCR plate sealing film, optically clear adhesive seal	Bio-Rad	Cat# MSB1001
Microplate, 96-well, polystyrene, flat-bottom (chimney well), black, non-binding	Greiner Bio-One	Cat# 655900
AlumaSeal II sealing film, non-sterile	Excel Scientific	Cat# AF-100
42°C heatblock	VWR	N/A
37°C New Brunswick Innova 40 shaker	Eppendorf	Cat# M1299-0090
37°C Isotemp 625D incubator	Fisher Scientific	Cat# 15-103-0513
27°C New Brunswick Innova 44 shaker	Eppendorf	Cat# M1282-0010
Corning 125 mL Erlenmeyer cell culture flask with vent cap	Corning	Cat# 431143
Corning 1 L Erlenmeyer cell culture flask with vent cap	Corning	Cat# 431147
Falcon 6-well flat bottom cell culture plate	Corning	Cat# 353046
Falcon 24-well flat bottom cell culture plate	Corning	Cat# 353047
27°C humidified incubator (model CB53)	BINDER	https://www.binder-world.com/us-en/products/growth/co2-incubators/co2-incubator-product-list?&filter[interior_volume]=53..267
Laminar flow cell tissue culture hood	Thermo Fisher Scientific	N/A
Sterile syringes (1 mL, 3 mL, and 30 mL)	Fisher Scientific	N/A
Serological pipets (5 mL, 10 mL, 25 mL, and 50 mL)	VWR	N/A
Glass Pasteur pipette	Fisher Scientific	Cat# 13-678-20C
Automated cell counter, Countess II FL	Life Technologies	https://www.thermofisher.com/us/en/home/life-science/cell-analysis/cell-analysis-instruments/automated-cell-counters/models.html
15 mL conical centrifuge tubes	MTC Bio	Cat# C2715
50 mL conical centrifuge tubes	MTC Bio	Cat# C2750
Bright-field microscope TS100	Nikon	N/A
Fluorescence microscope IX71	Olympus	https://www.olympus-lifescience.com/en/microscopes
pH meter	Mettler Toledo	N/A
Benchtop centrifuge 5424 and 5424R	Eppendorf	N/A
Centrifuge equipped with rotor for 96-well plates	N/A	N/A
Benchtop ultracentrifuge	Beckman Coulter	N/A
Ultracentrifuge tubes	Beckman Coulter	Cat# 342303
Rotor for benchtop ultracentrifuge TLA100	Beckman Coulter	https://www.beckman.com/centrifuges/rotors/fixed-angle/343840
Qsert clear glass autosampler vials (12 × 32 mm) with 9 mm screw caps	Shimadzu	Cat# 220-91521-03

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
HPLC system equipped with a fluorescent detector for the fluorescence detection size-exclusion chromatography (FSEC)	Shimadzu	https://www.shimadzu.com/an/literature/etc/jpz19004.html
Superose 6 Increase 10/300 GL column (FSEC)	Cytiva	Cat# 29091596
Superdex 200 10/300 GL column (SEC)	Cytiva	Cat# 28990944
2.8 L glass cell culture flask	Fisher Scientific	Cat# 09-552-40
Avanti centrifuge J-26 XP	Beckman Coulter	https://www.beckman.com
Centrifuge tube for Avanti rotor JLA 8.1000	Beckman Coulter	N/A
JLA-8.1000 fixed-angle rotor	Beckman Coulter	N/A
Variomag magnetic stirrer Mono Direct	Thermo Fisher Scientific	Cat# 50094713
Buchner funnel system (Buchner glass funnel and receiver flask)	VWR; Fisherbrand	Cat# 28400; Cat# FB-300-1000
Nalgene Rapid-Flow sterile 500 mL disposable 0.2 µm filter unit	Thermo Scientific	Cat# 450-0020
Syringe-driven filter units, 0.22 µm	Millipore	Cat# SLGV004SL
Amicon Ultra-15 50-kDa centrifugal filter unit	Merck Millipore	Cat# UFC905024
Econo-Pac chromatography column	Bio-Rad	Cat# 7321011
NanoDrop spectrophotometer 2000c	Thermo Scientific	Cat# ND2000CLAPTOP
Mini-PROTEAN tetra cell	Bio-Rad	Cat# 1658005EDU
Electrophoresis power supply PowerPac HC	Bio-Rad	N/A
Thermal cycler	Bio-Rad	N/A
CFX Connect real-time PCR detection system	Bio-Rad	Cat# 1855201
BioTek Synergy H1 microplate multi-mode reader	Agilent	https://www.agilent.com/en/product/microplate-instrumentation/microplate-readers/multimode-microplate-readers/biotek-synergy-h1-multimode-reader-1623193
MicroCal PEAQ-ITC calorimeter	Malvern Panalytical	https://www.malvernpanalytical.com/en/products/product-range/microcal-range/microcal-itc-range/microcal-peaq-itc
C18 HPLC column (SiliaChrom dt C18, 5 µm, 10 × 250 mm)	SiliCycle	Cat# H141805E-Q250
High-pressure liquid chromatography (HPLC) system	N/A	N/A
Bath sonicator	N/A	N/A
Lyophilizer	SP Scientific BenchTop Pro	N/A
Transilluminator	Fisher Scientific	N/A
AlphaImager	Thermo Fisher Scientific	N/A
Anti-GFP nanobody Sepharose resin	Lee et al.[31]	N/A

MATERIALS AND EQUIPMENT

- Saxitoxin (STX)-hexylamine.

Prepare STX-hexylamine as outlined in.[33]

⚠ CRITICAL: STX-hexylamine is a toxic compound. Wear proper personal protective equipment (ex. nitrile gloves) and follow institutional and governmental guidelines for waste disposal while working with toxins.

- Saxitoxin (STX).

STX can be obtained from the National Resource Council Canada (nrc.canada.ca, catalog no. CRM-STX) or synthesized, purified, and validated as described in[32]. The synthesized STX powder is directly dissolved with MilliQ water to make 1 mM STX stock. STX purchased from the National Resource Council Canada needs to be lyophilized to eliminate the solvent prior to making 1 mM

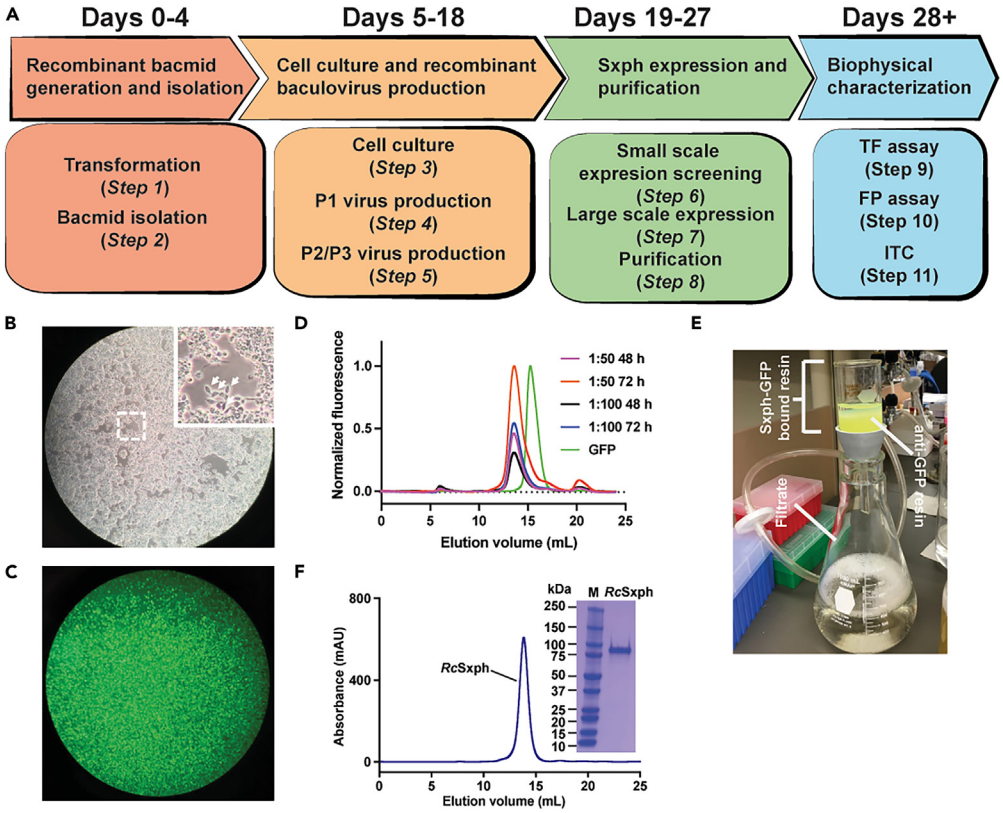


Figure 4. Sxph production flowchart

(A) Flowchart and timeline for Sxph production.

(B) *Rc*Sxph P1 baculovirus infected *Sf9* cells image, captured under a bright-field microscope, 4 days after transfection (magnification, 100x). Arrows in inset indicate cells with the signs of late-stage infection: granular appearance, cells detaching from the plate.

(C) *Rc*Sxph P2 baculovirus infected *Sf9* cells image, captured under a fluorescence microscope, 5 days after infection (magnification, 100x).

(D) FSEC small scale screening of *Rc*Sxph (Superose 6 Increase 10/300 GL).

(E) *Rc*Sxph affinity capture using anti-GFP nanobody resin.

(F) SEC of *Rc*Sxph (Superdex 200 10/300 GL). Inset shows SDS-PAGE of purified *Rc*Sxph.

STX stock using MilliQ water. STX stock can be stored at 4°C for up to 12 months. For long-term storage, STX can be stored at −20°C.

⚠ CRITICAL: STX is a toxic compound. Wear proper personal protective equipment (ex. nitrile gloves) and follow institutional and governmental guidelines for waste disposal while working with toxins.

- Fluorescein-labeled saxitoxin (F-STX) .

F-STX can be synthesized, purified, and validated as described in section 12 (ref.[1]). F-STX powder is dissolved with MilliQ water to make 1 μM F-STX stock. F-STX stock can be stored at 4°C in the dark and for up to 12 months. For long-term storage, prepare 20 μL aliquots and store in the dark at −20°C.

⚠ CRITICAL: F-STX is a toxic compound. Wear proper personal protective equipment (ex. nitrile gloves) and follow institutional and governmental guidelines for waste disposal while working with toxins.

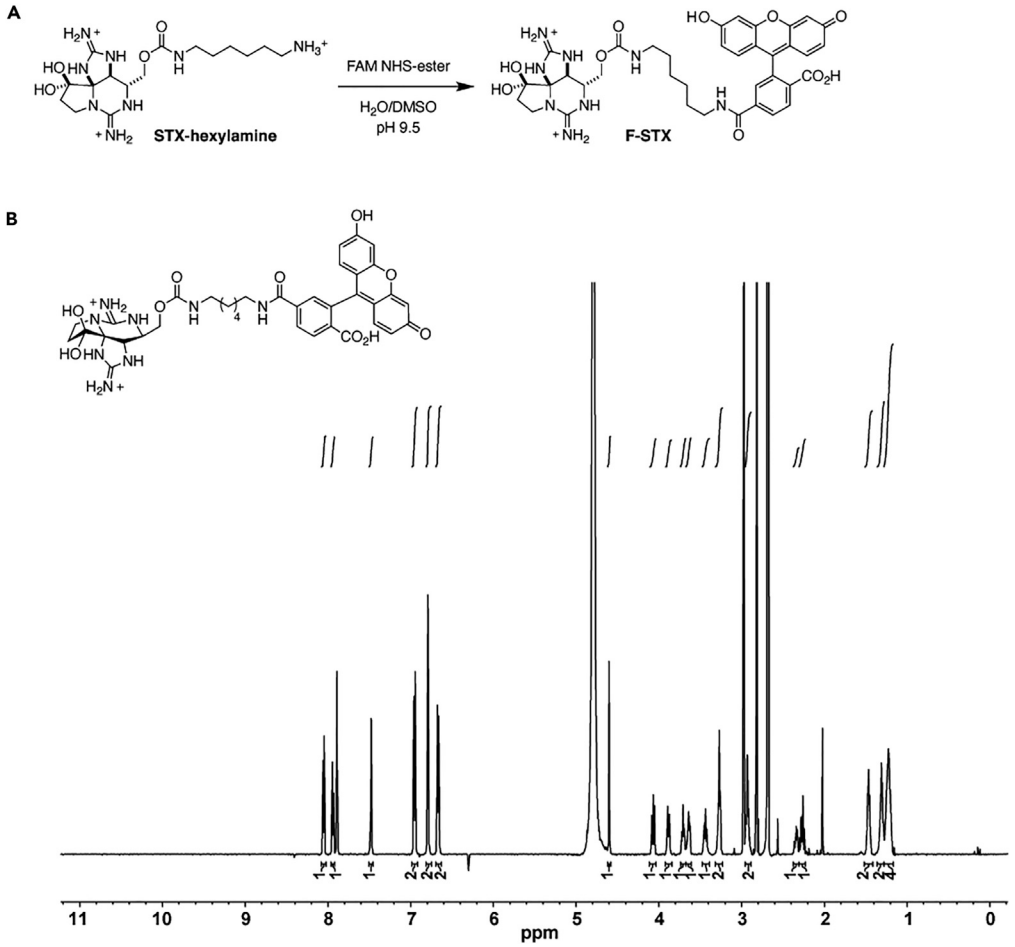


Figure 5. F-STX synthesis
(A) Reaction scheme for F-STX synthesis.
(B) Exemplar F-STX H1 NMR spectra.[1]

- 3C protease.

3C protease is produced and purified as described in.[31] Flash-freeze purified 3C protease and store at −80°C.

- Anti-GFP nanobody Sepharose resin.

Anti-GFP nanobody Sepharose resin is produced and purified as described.[31] Store at 4°C for up to 3 months.

- 20 mM dNTPs mixture.

Reagent	Final concentration	Amount
dATP (100 mM)	5 mM	5 μL
dCTP (100 mM)	5 mM	5 μL
dGTP (100 mM)	5 mM	5 μL
dTTP (100 mM)	5 mM	5 μL

(*Continued on next page*)

Continued		
Reagent	Final concentration	Amount
Nuclease-free H_2O	N/A	80 μL
Total	**20 mM**	**100 μL**

Aliquot 20 μL dNTPs into 1.5 mL tubes. Store at −20°C.

- **Tetracycline.**

Prepare 10 mg mL^{-1} in 70% EtOH, store at −20°C.

- **Kanamycin.**

Prepare 50 mg mL^{-1} in ddH_2O, store at −20°C.

- X-gal.

Prepare 40 mg mL^{-1} in 100% DMSO, store at −20°C.

- IPTG.

Prepare 1 M in ddH_2O, store at −20°C.

- Luria-Bertani (LB) agar plates for bacmid DNA generation.

Prepare 200 mL LB agar, allow it to cool and add the following reagents		
Reagent	Final concentration	Amount
Tetracycline (10 mg mL^{-1})	10 μg mL^{-1}	200 μL
Kanamycin (50 mg mL^{-1})	50 μg mL^{-1}	200 μL
X-gal (40 mg mL^{-1})	40 μg mL^{-1}	200 μL
IPTG (1 M)	1 mM	200 μL
Gentamicin reagent solution (50 mg mL^{-1})	7 μg mL^{-1}	28 μL
Total	**N/A**	**200 mL**

Pour LB agar solution into sterile Petri dishes. Store the plates at 4°C for up to 2 weeks, protected from light.

- **ESF 921 insect cell culture medium.**

No supplementation is required. Antibiotics are not recommended. Store at 4°C, protected from light. Prewarm the media up to 23 ± 2°C before the use.

- **10x TAE buffer.**

Reagent	Final concentration	Amount
Tris base	400 mM	48.5 *g*
Acetic acid (17.4 M)	200 mM	11.48 mL
EDTA, pH 8.0 (0.5 M)	10 mM	20 mL
ddH_2O	N/A	up to 1 L
Total	**N/A**	**1 L**

Dilute stock solution 10:1 to prepare a 1x TAE working solution. Store at 23 ± 2°C for up to 1 month.

- **SDS-PAGE buffer.**

Prepare 10x SDS-PAGE buffer (250 mM Tris, 1.92 M Glycine). Dilute stock solution 10:1 and add SDS to a final concentration of 0.1% to prepare a 1x working solution. Store at 23 ± 2°C for up to 1 month.

- **4x Laemmli SDS sample buffer.**

Reagent	Final concentration	Amount
Tris buffer, pH 6.8 (1 M)	240 mM	2.4 mL
SDS	8%	0.8 *g*
Glycerol (100%)	40%	4 mL
Bromophenol blue	0.04%	4 mg
β-Mercaptoethanol (99%)	20%	2 mL
ddH_2O	N/A	up to 10 mL
Total	**N/A**	**10 mL**

Aliquot 1 mL 4x Laemmli SDS sample buffer into 1.5 mL tubes. Store at −20°C.

- **FSEC buffer, anti-GFP nanobody Sepharose resin wash and elution buffer** (300 mM NaCl and 30 mM Tris-HCl, pH 7.4).

Dissolve 17.53 *g* NaCl and 4.73 *g* Tris-HCl in 900 mL ddH_2O. Adjust pH to 7.4 with HCl. Add ddH_2O to 1 L. Filter the solution through a 0.22 μm filter. Store at 4°C for up to 1 month.

- **SEC buffer** (150 mM NaCl and 10 mM HEPES, pH 7.4).

Dissolve 8.77 *g* NaCl and 2.38 *g* HEPES in 900 mL ddH_2O. Adjust pH to 7.4 with HCl. Add ddH_2O to 1 L. Filter the solution through a 0.22 μm filter. Store at 4°C for up to 1 month.

- **Coomassie Brilliant Blue staining solution.**

Reagent	Final concentration	Amount
Coomassie Brilliant Blue R-250	0.25%	2.5 *g*
Ethanol	50%	500 mL
Acetic acid (17.4 M)	10%	100 mL
ddH_2O	N/A	up to 1 L
Total	**N/A**	**1 L**

Store at 23 ± 2°C for up to 3 months.

STEP-BY-STEP METHOD DETAILS

Sxph expression and purification

Recombinant bacmid generation and isolation

Timing: 4 days

Timing: 2 days (for step 1)

Timing: 2 days (for step 2)

1. Transform *Rc*Sxph plasmid DNA (Addgene 194018) into DH10Bac *E. coli* competent cells to generate recombinant bacmid, as described in the protocol of Bac-to-Bac baculovirus expression system (Invitrogen). (https://tools.thermofisher.com/content/sfs/manuals/bactobac_man.pdf).
   a. Thaw the DH10Bac *E. coli* competent cells on ice.
   b. Add 50–100 ng plasmid DNA to 50 μL DH10Bac *E. coli* competent cells.
   c. Incubate cells on ice for 20 min.
   d. Heat-shock the cells at 42°C for 45 s.
   e. Incubate on ice for 2 min.
   f. Add 900 μL S.O.C. medium.
   g. Incubate for 5 h at 37°C with shaking.
   h. Prewarm the LB agar plates (containing 50 μg/mL kanamycin, 7 μg/mL gentamicin, 10 μg/mL tetracycline, 40 μg/mL X-gal, and 1 mM IPTG) at 37°C around 1 h before plating the cells.
   i. Spin down the cells at 2,400 *g* for 5 min.
   j. Remove 500 μL supernatant.
   k. Resuspend the cell pellet in the remaining volume.
   l. Plate 20–100 μL of the cell suspension per each LB agar plate (typically we use four plates per one construct).
   m. Incubate plates at 37°C for 2 days.

   Note: Positive colonies are picked based on the blue/white screening. Select large white colonies for the next steps.

2. Isolate recombinant bacmid using a QIAprep Spin Miniprep Kit (QIAGEN).
   a. Pick at least four single white colonies and inoculate 6 mL LB medium supplemented with antibiotics (tetracycline (10 μg mL^{-1}), kanamycin (50 μg mL^{-1}) and gentamicin (7 μg mL^{-1}). Incubate cultures at 37°C with shaking at 150 rpm for ~12–15 h.
   b. (Optional) After overnight (~12–15 h) incubation, prepare a glycerol stock for bacmid DNA isolation using 1 mL of the cells with a final glycerol concentration of 30%–40%. Store the glycerol stocks at −80°C.
   c. Harvest bacterial cell pellets by centrifugation at 3,000 *g* for 5 min.
   d. Resuspend cell pellets with 200 μL Buffer P1 and transfer the resuspension to a 1.5 mL centrifuge tube.
   e. Add 200 μL Buffer P2 to the resuspension and mix thoroughly by gently inverting the tube 4–6 times.
   f. Add 200 μL Buffer N3 and mix immediately and thoroughly by gently inverting the tube 4–6 times.
   g. Centrifuge the tube at ~16,000 *g* at 23 ± 2°C, for 10 min.
   h. Transfer supernatant to a 2 mL centrifuge tube and add 1 mL ice-cold isopropanol. Mix well by gently inverting the tube 4–6 times.
   i. Incubate the tube in a −20°C freezer for at least 10 min.
   j. Centrifuge the tube at ~21,000 *g* at 4°C for 15 min.
   k. Remove supernatant and add 1 mL 70% ice-cold ethanol. Wash the bacmid pellet by gently inverting the tube 4–6 times.
   l. Centrifuge the tube at ~21,000 *g* at 4°C for 15 min.
   m. Remove supernatant by pipetting and air-dry the pellet completely under a fume hood (~1–2 h).
   n. Resuspend the pellet with 50 μL nuclease-free water. Allow pellet to dissolve for at least 10 min on ice.

   ⚠ CRITICAL: To avoid shearing the DNA, pipette only 1–2 times slowly to resuspend.

o. Determine the concentration of bacmid DNA using a NanoDrop spectrophotometer at 260 nm. (50 ng μL^{-1} cm^{-1} at 260 nm).

Note: The following step, 2 (p), serves as a quality control step for the bacmid DNA.

p. Set up the PCR reaction mixture on ice in a 0.2 mL PCR tube for verification of the presence of the gene of interest in the recombinant bacmid DNA as described in the protocol of Bac-to-Bac baculovirus expression system (Invitrogen).
   i. Mix 4 μL 5X Phusion HF buffer, 0.4 μL dNTPs, 1 μL pUC/M13 Forward primer, 1 μL pUC/M13 Reverse primer, < 250 ng bacmid DNA, 0.6 μL DMSO, and 0.2 μL Phusion DNA polymerase. Fill up with the nuclease-free water up to 20 μL.
   ii. Transfer PCR tubes into a thermal cycler and run PCR using the following parameters:

Steps	Temperature	Time	Cycles
Initial denaturation	98°C	3 min	1
Denaturation	98°C	45 s	35
Annealing	55°C	45 s	
Extension	72°C	5 min	
Final extension	72°C	10 min	1
Hold	4°C	forever	

q. Analyze PCR products by agarose gel electrophoresis using 1% agarose gel with ethidium bromide (EtBr) at a final concentration of 0.5 μg mL^{-1}.

⚠ CRITICAL: Ethidium bromide is a toxic chemical and a mutagen. Wear proper personal protective equipment and follow institutional and governmental guidelines for waste disposal.

Note: Expected size of the bacmid transposed with pFastBac1 containing Sxph is around 5600 bp (2300 bp + size of the gene of interest including 3C protease cleavage site, GFP and His_{10}Tag).

r. Store the bacmid DNA at 4°C.

Cell culture and recombinant baculovirus production

Timing: 2 weeks

Timing: 4 days (for step 3)

Timing: 4 days (for step 4)

Timing: 6 days (for step 5)

3. Growth and maintenance of suspension *Spodoptera frugiperda* (*Sf*9) cells.

⚠ CRITICAL: Cell cultures are a potential biohazard. Ensure *Sf*9 cell work is performed in an approved laminar flow hood with aseptic technique. Wear proper personal protective equipment and follow waste disposal rules recommended by institutional and governmental guidelines while working with cell cultures.

a. Assess cell density and viability. Mix 15 μL of cell culture with 15 μL of trypan blue solution (1:1 (v/v) ratio), then check cell viability and density using a cell counting slide under a bright-field microscope. Live cells are unstained. *Sf9* cells should be in the mid-log phase (6–8 × 10^6 cells/mL) with greater than 95% viability.
b. Seed cells at 0.5–1 × 10^6 cells/mL in fresh and prewarmed (at 23 ± 2°C) ESF 921 insect cell culture medium.
c. Incubate suspension cell cultures in a 27°C non-humidified shaker, shaking at 130 rpm. Seed cells when the density reaches 6–8 × 10^6 cells/mL (normally every 3–4 d) into a new sterile Corning Erlenmeyer cell culture flask.

4. Produce recombinant P1 viral stock using *Sf9* cells.
a. Assess cell density and viability. Verify that the *Sf9* cells are in the mid-log phase (6–8 × 10^6 cells mL^{-1}) with greater than 95% viability.
b. Prepare 6 mL 9 × 10^5 *Sf9* cells using fresh and prewarmed (at 23 ± 2°C) ESF 921 medium. Aliquot each 2 mL of cells into single wells of a sterile six-well tissue culture plate.

⚠ CRITICAL: To obtain the highest transfection efficiency and produce recombinant baculovirus with higher titers, use healthy *Sf9* cells at low passage (P3-P10).

c. Incubate cells in a 27°C humidified incubator (without shaking) until more than 90% cells attach to the bottom of the plate and form a monolayer culture (~20–60 min).
d. For each well, dilute 8 μL Cellfectin II in 100 μL ESF 921 insect cell culture medium in one sterile 1.5 mL centrifuge tube. Mix well by pipetting. Incubate at 23 ± 2°C for 5 min.
e. In a new 1.5 mL centrifuge tube, dilute 8 μL recombinant bacmid (around 8 μg) (from Step 2) in 100 μL ESF 921 insect cell culture medium. Mix by pipetting gently 1–2 times. Incubate at 23 ± 2°C for 5 min.
f. Combine the diluted recombinant bacmid with diluted Cellfectin II (total volume ~220 μL). Mix gently by pipetting 1–2 times and incubate at 23 ± 2°C for 30 min.
g. Add ~220 μL bacmid/Cellfectin II transfection mixture dropwise onto the cells in each well from Step 4 (c). Shake the plate gently to ensure even distribution of the cells in each well and incubate cells in a 27°C humidified incubator (without shaking) for 4–5 h.
h. Remove media using a sterile glass Pasteur pipette and add 2 mL fresh and prewarmed (at 23 ± 2°C) ESF 921 insect cell culture medium into each well.
i. Incubate cells in a 27°C humidified incubator (without shaking) for 4–7 days.

Note: Visually check the signs of viral infection daily beginning at 72 h after transfection under a bright-field microscope. Virally-infected cells at late to very late infection stage should display the characteristics including granular appearance and detachment (Figure 4B).

j. Once the transfected cells show signs of late-stage infection (Figure 4B), collect the medium containing P1 baculovirus from each well using the sterile pipette:
i. Transfer to a sterile 15 mL falcon tube.
ii. Centrifuge at 500 *g* for 5 min.
iii. Transfer the clarified supernatant to a sterile 15 mL falcon tube and wrap the tube with aluminum foil.

Note: Use P1 baculovirus immediately to generate P2 stock or store at 4°C (protected from light) for <2 weeks to avoid possible decrease of virus titer. For long-term storage, store an aliquot of the viral stock at −80°C for later reamplification.

k. (Optional) To obtain more P1 baculovirus, gently pipette to resuspend ~50% unlysed cells from the bottom of each well. Store this P1 baculovirus stock at 4°C and protect from light.

5. Amplify P1 viral stock to generate P2/P3 stock.

a. The P1 baculovirus is a small-scale, low-titer viral stock. To generate a large-scale, high-titer P2 viral stock, seed 250 mL of 5–7.5 × 10^5 cells mL^{-1} *Sf*9 cells in a sterile 1 L Corning Erlenmeyer cell culture flask.
b. Grow cells in a 27°C non-humidified shaker, shaking at 130 rpm for 24 h. Check cell density to see if it reaches 1–1.5 × 10^6 cells/mL.
c. Add 2.5 mL P1 viral stock virus to 250 mL cells at a 1:100 (v/v) dilution ratio. Incubate cells in a 27°C non-humidified shaker, shaking at 130 rpm for 4–6 days.
d. Beginning at the 4th day of infection, check daily the signs (GFP signal) of viral infection under a fluorescence microscope.
    i. Transfer 0.5–1 mL cell culture to a sterile 24-well tissue culture plate.
    ii. Incubate cells in a 27°C humidified incubator (without shaking) for ~20 min.
    iii. Check GFP signal under a fluorescence microscope.

Note: More than 90% cells demonstrating GFP signal indicate high-titer P2 virus produced (Figure 4C).

e. Centrifuge cell culture at 3,000 *g* for 20 min. Filter the supernatant using a sterile 250 or 500 mL disposable 0.22 μm filter unit to remove cells and large debris. This is P2 viral stock.

Note: Store at 4°C and protect from light. For long-term storage, an aliquot of the P2 stock can be stored at −80°C, protected from light.

f. P2 viral stock can also be used to produce high-titer P3 stock as needed.

Saxiphilin expression screening, large-scale production, and purification

Timing: 9 days

Timing: 4 days (for step 6)

Timing: 3 days (for step 7)

Timing: 2 days (for step 8)

6. Screen conditions for optimal expression of Sxph protein using *Sf*9 cells.

Note: To maximize the yield of Sxph, expression conditions including P2/P3 virus dilution ratio (e.g. 1:50, 1:100, 1:200 (v/v) into *Sf*9 cells) used for *Sf*9 cell transduction and cell culture incubation time (e.g. 24, 48 and 72 h) should be screened at a small scale (30 mL) before proceeding to large-scale expression (1 L).

Note: See troubleshooting 1.

a. Assess cell density and viability. Verify that the *Sf*9 cells are in the mid-log phase (6–8 × 10^6 cells/mL) with greater than 95% viability.
b. For each expression condition, seed 30 mL 1 × 10^6 cells mL^{-1} *Sf*9 cells using fresh and pre-warmed (at 23 ± 2°C) ESF 921 insect cell culture medium and transfer to a new sterile 125 mL Corning Erlenmeyer cell culture flask.
c. Incubate cells in a 27°C non-humidified incubator, shaking at 130 rpm for 24 h.
d. Check cell density and viability. The viability should be > 95% and cell density should reach ~2 × 10^6 cells mL^{-1}.
e. Add P2/P3 virus into each cell culture at desired dilution ratios (e.g., 0.15 mL P2/P3 virus to 30 mL *Sf*9 cells for 1:200 v/v).

f. Incubate cells in a 27°C non-humidified incubator, shaking at 130 rpm for a desired incubation duration. Collect 1 mL cells from each flask at each time point, 24 h, 48 h and 72 h.
g. Harvest 1 mL of each culture at 3,000 *g* for 10 min. Collect supernatant and filter using a sterile disposable 0.22 μm filter unit into a 1.5 mL centrifuge tube.
h. Store the samples at 4°C until samples for all time points are collected and proceed to next step.
i. For fluorescence detection size exclusion chromatography (FSEC) analysis, collect ~150 μL supernatant from Step 6 (g) and centrifuge at ~278,000 *g* for 1 h at 4°C.
j. For each sample, transfer 120 μL supernatant into the glass vial for the FSEC autoloader.
k. FSEC is performed using a Superose 6 Increase 10/300 GL equilibrated in buffer containing 300 mM NaCl and 30 mM Tris-HCl (pH 7.4). 100 μL sample is loaded on the column and the FSEC run is performed at the flow rate of 0.3 mL min^{-1} at 4°C. (Figure 4D).
l. FSEC fluorescence signal is detected using excitation λ = 485 nm; emission λ = 512 nm and recorded using LabSolutions LC software, exported and analyzed using GraphPad Prism software.
m. The optimal condition for the large-scale Sxph protein expression should be determined based on the fluorescence signal of the GFP-fused Sxph (Figure 4D).

7. Large-scale expression of Sxph protein.
a. Seed 1 L 1 × 10^6 cells/mL *Sf*9 cells using fresh and prewarmed (at 23 ± 2°C) ESF 921 insect cell culture medium and transfer to a sterile 2.8 L glass cell culture flask.
b. Incubate cells in a 27°C non-humidified incubator, shaking at 130 rpm for 24 h.
c. Assess cell density and viability. The viability should be > 95% and cell density should reach ~2 × 10^6 cells mL^{-1}.
d. Add P2/P3 virus at an optimal dilution ratio (determined at Step 6) into *Sf*9 cells for transduction.
e. Incubate cells in a 27°C non-humidified incubator, shaking at 130 rpm for the incubation duration determined in Step 6 (typically 72 h for most Sxph variants).
f. Harvest cell cultures. Collect supernatant by centrifugation at 4,000 *g*, 25°C using an Avanti rotor JLA 8.1000 for 20 min.
g. Collect supernatant and store at 4°C for 1–2 days or proceed to next step for purification.

8. Saxiphilin protein purification.
Purification with anti-GFP Nb resin
a. Transfer the supernatant containing Sxph-GFP fusion protein from Step 7(g) to a clean 1 L beaker or glass bottle.

⚠ CRITICAL: Supernatant from Step 7(g) should be warmed up to 23 ± 2°C if stored at 4°C.

b. For each liter supernatant, the pH is adjusted to 8.0 by addition of 50 mL 1 M Tris-HCl (pH 8.0) with a final concentration of 50 mM Tris-HCl and the solution is treated with 1 mL 5 M $CaCl_2$ and 1 mL 1 M $NiCl_2$ to precipitate contaminants.[34]
c. Mix well the solution by stirring on a magnetic stirrer at 23 ± 2°C for 5–10 min.
d. Centrifuge at 23 ± 2°C, ~6,200 *g* for 20 min and transfer the supernatant to a clean 1 L beaker or glass bottle.
e. The anti-GFP nanobody Sepharose resin is prepared as previously described (Lee et al. 2021) and prewashed with ddH_2O twice and the wash buffer containing 300 mM NaCl and 30 mM Tris-HCl (pH 7.4) twice.
f. The clarified supernatant (~1 L) is incubated with 5 mL anti-GFP nanobody Sepharose resin with stirring at 23 ± 2°C for ~5 h.
g. To collect the Sxph-GFP fusion protein bound to the anti-GFP nanobody Sepharose resin, filter the supernatant through a Buchner funnel system by vacuum filtration.
h. Wash the resin with 20 column volumes (CV) (~100 mL) of the wash buffer containing 300 mM NaCl and 30 mM Tris-HCl (pH 7.4).

i. Resuspend resin with 20 mL of the wash buffer and transfer the resin by a serological pipette on an Econo-Column chromatography column (Bio-Rad).
j. On-column cleavage of the GFP-His tag from Sxph is achieved by incubating with 3C protease[31] (0.2 mg mL^{-1}, prepared in 10 mL wash buffer) at 4°C for ~12–15 h.
Purification by SEC
k. Collect the cleaved eluate into a falcon tube by a gravity flow. Wash the resin with 2 CV of wash buffer and collect the wash fraction.
l. Combine the fractions and concentrate the protein sample using an Amicon Ultra-15 50-kDa cut-off centrifugal filter unit up to 0.5–1 mL.
m. Filter the sample through the 0.22 μm filter unit before purification by size exclusion chromatography (SEC) using a Superdex 200 10/300 GL column equilibrated in 150 mM NaCl, 10 mM HEPES (pH 7.4).
n. Load protein sample into a 1 mL loop. Inject and run the SEC at 0.5 mL min^{-1} at 4°C. Collect peak fractions.
o. Collect peak fractions from SEC analysis and analyze by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie staining.

Note: This step is sufficient to separate *Rc*Sxph (91 kDa) from any residual 3C protease (22 kDa).

p. Pool the purified protein fractions.
q. Determine protein concentration using NanoDrop spectrophotometer at 280 nm and the extinction coefficient calculated using the ExPASY server (https://web.expasy.org/protparam/), 96,365 M^{-1} cm^{-1} for *Rc*Sxph.

⚠ CRITICAL: Protein concentration needs to be accurate for TF, FP, and ITC assays.

Note: See troubleshooting 2.

r. Store the protein samples at 4°C for immediate use or flash freeze in liquid nitrogen and store at −80°C for long-term storage.
s. Continue to the TF, FP and ITC assays.

Biochemical characterization of Sxph

Timing: 1–2 days

Timing: 1 day (for step 9)

Timing: 1 day (for step 10)

Timing: 1–2 days (for step 11)

Timing: 1 day (for step 12)

9. Thermofluor (TF) assay (Figure 2, left).

⚠ CRITICAL: STX and TTX are toxic compounds. Wear proper personal protective equipment (ex. nitrile gloves) and follow institutional and governmental guidelines for waste disposal while working with toxins.

a. Prepare 50 μL of 80 μM STX and TTX by mixing 4 μL of 1 mM toxin stock with 46 μL SEC buffer.
b. Prepare two-fold serial dilutions of STX or TTX.

i. Pipette 25 μL SEC buffer into each well of columns 1–11 in 96-well PCR plate.
ii. Transfer 50 μL of 80 μM STX or TTX into a well of column 12.
iii. Transfer 25 μL of toxin from well 12 into the next well 11 and pipette up and down to mix.
iv. Repeat process to serially dilute samples two-fold per series across the PCR plate until well 2.
v. Well 1 is a protein-only reference without toxin (0 nM).

c. Prepare protein sample in the SEC buffer to a final concentration of 0.15 mg mL^{-1}, and dispense 13 μL per well into a new 96-well PCR plate. For one series of toxin titration, 12 wells are used.

Note: Plan to prepare protein for at least two replicates per sample (24 wells for our study).

d. Prepare a SYPRO Orange dye by diluting the 5,000x stock to 50x (1.5 μL in 148.5 μL SEC buffer).

⚠ CRITICAL: SYPRO Orange dye is light sensitive. Store at 23 ± 2°C in light protective amber vial from supplier.

e. Add 2 μL of 50x SYPRO Orange dye into each 13 μL protein-containing well for a final concentration of 5x SYPRO Orange dye and 0.13 mg mL^{-1} protein.
f. Transfer 5 μL of each toxin dilution using a multichannel pipette to each protein sample well and pipette up and down a few times to mix (final concentration of toxin from 20 μM in well 12 to protein-only containing well 1 and a final reaction volume of 20 μL).
g. Seal the plate using the microseal 'B' PCR plate sealing film.
h. Centrifuge the plate briefly for 1 min at 230 × *g* to eliminate any air bubbles.
i. Perform fluorescence measurement using the HEX channel (excitation λ = 515–535 nm, emission λ = 560–580 nm) in CFX Connect Thermal Cycler using the program starting with the incubation at 25°C for 2 min followed by 25°C–95°C temperature gradient with a ramp rate of 0.2 °C min^{-1} and final incubation at 95°C for 1 min.
j. Fit the denaturation curves using a Boltzmann sigmoidal function in GraphPad Prism: $F=F_{min}+(F_{max}-F_{min})/(1+\exp((Tm-T)/C))$, where F is the fluorescence intensity at temperature T, F_{min} and F_{max} are the fluorescence intensities before and after the denaturation transition, respectively, Tm is the midpoint temperature of the thermal unfolding transition, and C is the slope at Tm. Alternatively, one can take the first derivative of the melting curve. Tm is the inflection point. Both methods should give similar values.

$$\Delta Tm = Tm_{Sxph+20\mu M\ toxin} - Tm_{Sxph}.$$

10. Fluorescence polarization (Figure 2, middle).

⚠ CRITICAL: F-STX is a toxic compound. Wear proper personal protective equipment (ex. nitrile gloves) and follow institutional and governmental guidelines for waste disposal while working with toxins.

a. Prepare 250 μL of 150 nM and 100 nM protein in SEC buffer.

⚠ CRITICAL: Protein concentration needs to be optimized accordingly.

Note: See troubleshooting 3.

b. Prepare twofold serial dilutions of purified protein in SEC buffer (final concentrations, 0–75 nM).
i. Transfer 120 μL SEC buffer into each well of columns 1–3 and 5–11.
ii. Transfer 250 μL of 150 nM protein into well 12 and 250 μL of 100 nM protein into well 4.

iii. Transfer 120 μL protein from well 12 to the next well 11, mix by pipetting up and down, and repeat the process until well 5.
iv. Transfer 120 μL protein from well 4 to the next well 3, mix by pipetting up and down, and repeat the process until well 2.
v. Well 1 is an F-STX-only reference.

c. Prepare 2 nM fluorescein-labeled STX (F-STX) (50 μL per well) in SEC buffer (for our study, we used 24 wells per one series per one protein sample) in 96-well black flat-bottomed polystyrene microplates. Final protein concentration in our assay: 75 nM, 50 nM, 37.5 nM, etc.
d. Seal the plate with an aluminum foil sealing film to minimize exposure to light.
e. Incubate the plate for 30 min at the desired temperature (typically 25°C) to allow reaction to attain equilibrium.

⚠ CRITICAL: Equilibrate the plate and the plate-reader at the desired temperature.

Note: See troubleshooting 4.

f. Perform fluorescence polarization measurements at the desired temperature (typically 25°C) on a Synergy H1 microplate reader using the polarization filter setting (excitation λ = 485 nm, emission λ = 528 nm).
g. The dissociation constants were calculated using GraphPad Prism by fitting fluorescence millipolarization ($mP = P \cdot 10^{-3}$, where P is polarization) as a function of Sxph concentration using the equation: $P = \{(P_{bound} - P_{free})\ [Sxph]/(K_d + [Sxph])\} + P_{free}$, where P is the polarization measured at a given Sxph concentration, P_{free} is the polarization of Sxph in the absence of F-STX, and P_{bound} is the maximum polarization of Sxph bound by F-STX.

11. Isothermal titration calorimetry (ITC) (Figure 2, right).

⚠ CRITICAL: Wear proper personal protective equipment and follow institutional and governmental guidelines for waste disposal while working with toxins.

a. Before ITC assay.

⚠ CRITICAL: The preparation of macromolecule solution and ligand solution must strictly follow ITC sample preparation guidelines.

i. The macromolecule solution must be completely dialyzed against desired buffer or completely desalted with a gel filtration/desalting column.
ii. The ligand must be prepared in the same buffer as the macromolecule.
iii. The macromolecule solution and the ligand solution should be free of air bubbles.
iv. To ensure an accurate measurement of stoichiometry (N), heat of binding (ΔH), and dissociation constant (*Kd*), a minimum concentration for the macromolecule solution is 5 μM, and the optimum ligand concentration should be 10–20 times of the macromolecule solution. For weaker binders, ligand and macromolecule concentration may need to be increased.[23]
v. The concentrations of both macromolecule solution and ligand solution should be accurately determined after final preparation.

b. ITC assay using MicroCal PEAQ-ITC calorimeter (Malvern Panalytical).

⚠ CRITICAL: Temperature difference between sample solutions and environment will cause air bubbles within the solutions. ITC measurement of Sxph/STX interaction is performed at 25°C, thus the SEC buffer should be prewarmed to 23 ± 2°C, and Sxph and STX solutions for ITC experiments should be prepared at 23 ± 2°C.

i. Before running any experiments, a water/water (MilliQ water, prewarmed at 23 ± 2°C) titration experiment must be performed to check the condition of ITC. A result of the run that must meet all the following criteria should indicate a good condition of the instrument:
ii. ITC run at set temperature and reference power.
iii. The baseline of the water/water run should be linear.
iv. The integrated peak heat produced from each 2 μL injection volume should be < 0.2 μcal.

Note: See troubleshooting 5.

v. Filter the Sxph protein purified by SEC from Step 8 using a 0.22 μm filter unit to remove any contaminants/precipitants.
vi. Determine Sxph protein concentration using a NanoDrop spectrophotometer at 280 nm, with the extinction coefficient for Sxph calculated using the ExPASY server (https://web.expasy.org/protparam/).
vii. Dilute Sxph to a final concentration of 10–30 μM with the SEC buffer containing 150 mM NaCl and 10 mM HEPES (pH 7.4).
viii. Dissolve STX powder in MilliQ water to prepare 1 mM STX stock solution.
ix. Dilute STX stock solution to a final concentration of 100–300 μM with the SEC buffer. Add MilliQ to the Sxph sample so that the final buffer composition exactly matches that of the STX sample.[1]
x. Set the following experiment settings: Syringe concentration (STX, 100–300 μM), Cell concentration (Sxph, 10–30 μM), Idle Temperature (25°C), Reference Power (5 μcal s^{-1}), Feedback (High), Stir Speed (750 rpm), Injection Spacing (150 s), Number of Injections (36 or 19), Injection Volume (0.4 μL followed by 35 injections of 1 μL for high affinity binders, 0.4 μL followed by 18 injections of 2 μL for the weak binders).
xi. Load 280 μL Sxph solution at 23 ± 2°C into the sample cell of ITC.

⚠ CRITICAL: Avoid introducing any air bubbles into the sample cell while loading Sxph solution.

xii. Load 60 μL STX solution at 23 ± 2°C into injection syringe. Check if there are any air bubbles introduced into injection syringe while loading. If the answer is 'yes', unload STX solution from injection syringe and reload it.
xiii. Run titration of STX solution into SEC buffer using the same parameter settings as for the STX titration into Sxph.
xiv. Analyze the result using MicroCal PEAQ-ITC analysis software. Subtract the heat produced in composite control experiments from each STX/Sxph experiment to correct the baseline. Choose the 'One Set of Sites' fitting model to fit the raw data points.
xv. Determine binding parameters: stoichiometry (N), heat of binding (ΔH), dissociation constant (*K*d).

Note: See troubleshooting 6.

12. F-STX synthesis.[1]

⚠ CRITICAL: STX-hexylamine and F-STX are toxic compounds. Wear proper personal protective equipment (ex. nitrile gloves) and follow institutional and governmental guidelines for waste disposal while working with toxins.

a. Add 140 μL of FAM NHS-ester, 6-isomer (2.0 mg, 4.2 μmol, 3.0 equiv, Lumiprobe Corp., Hunt Valley, MD) in 140 μL of DMSO to a 140 μL ice cold solution of 1.4 μmol STX-hexylamine[33] in pH 9.5 aqueous bicarbonate buffer (0.2 M aqueous $NaHCO_3$, adjusted to pH 9.5 with 1.0 M aqueous NaOH).
b. Stopper flask, wrap in foil to protect from exposure to light.
c. Place flask in bath sonicator for 30 s.
d. Stir mixture at 23 ± 2°C for 4 h.
e. Quench reaction by adding 0.3 mL of 1% aqueous CF_3CO_2H.
f. Dilute quenched reaction with 1.1 mL of 10 mM aqueous CF_3CO_2H and 0.3 mL of DMSO. Filter through a VWR 0.22 μm PTFE filter.
g. Purify F-STX by reversed-phase HPLC (SiliCycle SiliaChrom dt C18, 5 μm, 10 × 250 mm column) eluting with a gradient flow of 10→40% CH_3CN in 10 mM aqueous CF_3CO_2H over 40 min. Detect product using 214 nm UV detection. Expected elution time, 31 min.
h. Lyophilize purified F-STX. Purified F-STX is a dark yellow powder.
i. Validate product by ^{1}H NMR and mass spectrometry; Expected molecular mass for $C_{37}H_{40}N_8O_{10}$ 756.2867. H^1 NMR peaks as follows: (600 MHz, D_2O) δ 8.05 (d, *J* = 8.1 Hz, 1H), 7.94 (d, *J* = 8.9 Hz, 1H), 7.48 (s, 1H), 6.95 (d, *J* = 9.0 Hz, 2H), 6.79 (s, 2H), 6.67 (dt, *J* = 9.1, 2.2 Hz, 2H), 4.60 (d, *J* = 1.2 Hz, 1H), 4.09–4.05 (m, 1H), 3.89 (dd, *J* = 11.6, 5.2 Hz, 1H), 3.70 (dt, *J* = 10.1, 5.5 Hz, 1H), 3.64 (dd, *J* = 8.7, 5.4 Hz, 1H), 3.47–3.42 (m, 1H), 3.27 (t, *J* = 6.6 Hz, 2 H), 2.97–2.89 (m, 2H), 2.36–2.33 (m 1H), 2.30–2.24 (m, 1H), 1.48–1.45 (m, 2H), 1.32–1.29 (m, 2H), 1.25–1.21 (m, 4H) ppm.

EXPECTED OUTCOMES

By following this protocol, users can express, purify and characterize recombinant toxin binding proteins in around 4 weeks, or 1 week after having P2 baculovirus generated. Sxph yields are typically ~1–2 mg L^{-1} of *Sf9* cells. Protein purity is readily assessed by SDS-PAGE gels. Pure samples should yield a monodisperse peak on a size exclusion column. The biochemical evaluation of the Sxph and toxin interactions itself can be performed in 1–2 days. Toxin-binding protein and its variants are essential for understanding toxin recognition mechanism, which can be applicable for the development of the toxin sensors and antidotes. This protocol can be useful to researchers studying other secreted proteins interacting with STX or other PSP toxins.

Limitations

ITC is a label-free method for directly measuring the heat released or absorbed during the binding of an unmodified ligand to its target.[23] While this is a powerful method that can yield direct insight into thermodynamic binding parameters, it requires substantial amounts of protein (~300 μg of Sxph) and ligand (5–10 μg for STX), compared to FP and TF assays. The FP assay is very sensitive (~3 μg of Sxph and ~1 ng of ligand for a complete binding curve), and can also be run as a competition between labeled and unlabeled ligands,[21,22] but requires synthesis and purification of the fluorescent ligand. TF is the least demanding technique requiring modest amounts of material to generate a complete binding profile at using multiple toxin concentrations (~25 μg Sxph; and 600 ng STX) and is suitable for the high-throughput screening, using relatively inexpensive equipment.

TROUBLESHOOTING

Problem 1

Low Sxph expression.

Potential solution

Viral titer of P2 is low. Produce high-titer P3 viral stock.

Problem 2

Errors in TF, FP, and ITC assays resulting from poor measurement of Sxph stock concentration.

Potential solution

Inaccurate protein concentration measurement. For highest accuracy measure protein concentration using a quartz cuvette and UV spectrometer using the denaturation method as described in.[35]

Problem 3

No binding.

Potential solution

For low affinity binding variants of Sxph, the protein stock concentration has to be increased, e.g., for *Rc*Sxph E540A, the protein stock is 20 μM.

Problem 4

Reaction did not reach plateau.

Potential solution

Insufficient incubation time for the reaction equilibrium to be reached. Optimization of the incubation time, e.g., 0.5 h, 1.5 h, 4.5 h, and 24 h.

Problem 5

Reference power is offset by > 10%. Baseline is offset or noisy; or the integrated peak heat for each 2 μL injection volume is > 0.2 μcal.

Potential solution

Reference cell is drying or not clean. Rinse and refill reference cell with fresh MilliQ water weekly. Another reason may be a dirty sample cell and/or injection syringe. Wash it with 20% Contrad 70 and rinse it multiple times with MilliQ water. If the problem insists, perform more rigorous cleaning of the sample cell and injection syringe following ITC manufacturer's guidelines.

Problem 6

Stoichiometry (N) is not close to 1.

Potential solution

The concentration of Sxph and/or STX may be inaccurate. Check the concentration of Sxph or prepare new STX solution.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Daniel L. Minor, Jr. (daniel.minor@ucsf.edu).

Materials availability

Plasmids generated in this study have been deposited to Addgene:194018.

Requests for material should be sent to D.L.M.

Data and code availability

The data presented in these figures were generated as part of ref. 1. Raw files associated with Figure 4 are available from the corresponding author upon request.

ACKNOWLEDGMENTS

This work was supported by Department of Defense (DoD) grants HDTRA-1-19-1-0040 and HDTRA-1-21-1-10011 and UCSF Program for Breakthrough Biomedical Research, which is partially funded

by the Sandler Foundation, to D.L.M., NIH-NIGMS R01-GM117263-05 to J.D.B., and a DoD National Defense Science and Engineering Graduate (NDSEG) Fellowship to H.S.H. H.S.H. is a Center for Molecular Analysis and Design Fellow at Stanford University.

AUTHOR CONTRIBUTIONS

Z.C. and S.Z. contributed equally. All coauthors conceived the study. Z.C., S.Z., and D.L.M. designed and established the binding assays. H.S.H. and J.D.B. designed and synthesized F-STX. Z.C., S.Z., J.D.B., and D.L.M. wrote the protocol.

DECLARATION OF INTERESTS

J.D.B. is a cofounder and holds equity shares in SiteOne Therapeutics, Inc., a start-up company interested in developing subtype-selective modulators of sodium channels. Patent applications have been filed for this work.

REFERENCES

1. Chen, Z., Zakrzewska, S., Hajare, H.S., Alvarez-Buylla, A., Abderemane-Ali, F., Bogan, M., Ramirez, D., O'Connell, L.A., Du Bois, J., and Minor, D.L., Jr. (2022). Definition of a saxitoxin (STX) binding code enables discovery and characterization of the anuran saxiphilin family. Proc. Natl. Acad. Sci. USA *119*, e2210114119.

2. Thottumkara, A.P., Parsons, W.H., and Du Bois, J. (2014). Angew. Chem. Int. Ed. Engl. *53*, 5760–5784.

3. Hille, B. (2001). Ion Channels of Excitable Membranes, 3rd Edition (Sinauer Associates, Inc.).

4. Llewellyn, L.E. (2006). Saxitoxin, a toxic marine natural product that targets a multitude of receptors. Nat. Prod. Rep. *23*, 200–222.

5. Wiese, M., D'Agostino, P.M., Mihali, T.K., Moffitt, M.C., and Neilan, B.A. (2010). Neurotoxic alkaloids: saxitoxin and its analogs. Mar. Drugs *8*, 2185–2211.

6. Anderson, D.M., Fachon, E., Pickart, R.S., Lin, P., Fischer, A.D., Richlen, M.L., Uva, V., Brosnahan, M.L., McRaven, L., Bahr, F., et al. (2021). Evidence for massive and recurrent toxic blooms of Alexandrium catenella in the Alaskan Arctic. Proc. Natl. Acad. Sci. USA *118*, e2107387118.

7. Prinzmetal, M., Sommer, H., and Leake, C.D. (1932). The pharmacological action of "mussel poison". J Pharmacol Exp Ther *46*, 63–73.

8. Kao, C.Y., and Fuhrman, F.A. (1967). Differentiation of Actions of Tetrodotoxin and Saxitoxin. Toxicon *5*, 25–34.

9. Mahar, J., Lukács, G.L., Li, Y., Hall, S., and Moczydlowski, E. (1991). Pharmacological and biochemical properties of saxiphilin, a soluble saxitoxin-binding protein from the bullfrog (Rana catesbeiana). Toxicon *29*, 53–71.

10. Abderemane-Ali, F., Rossen, N.D., Kobiela, M.E., Craig, R.A., Garrison, C.E., Chen, Z., Colleran, C.M., O'Connell, L.A., Du Bois, J., Dumbacher, J.P., and Minor, D.L. (2021). Evidence that toxin resistance in poison birds and frogs is not rooted in sodium channel mutations and may rely on "toxin sponge" proteins. J. Gen. Physiol. *153*, e202112872.

11. Yen, T.-J., Lolicato, M., Thomas-Tran, R., Du Bois, J., and Minor, D.L., Jr. (2019). Structure of the Saxiphilin:saxitoxin (STX) complex reveals a convergent molecular recognition strategy for paralytic toxins. Sci. Adv. *5*, eaax2650.

12. Llewellyn, L.E., Bell, P.M., and Moczydlowski, E.G. (1997). Phylogenetic survey of soluble saxitoxin-binding activity in pursuit of the function and molecular evolution of saxiphilin, a relative of transferrin. Proc. Biol. Sci. *264*, 891–902.

13. Tanaka, J.C., Doyle, D.D., and Barr, L. (1984). Sodium channels in vertebrate hearts. Three types of saxitoxin binding sites in heart. Biochim. Biophys. Acta *775*, 203–214.

14. Doyle, D.D., Wong, M., Tanaka, J., and Barr, L. (1982). Saxitoxin binding sites in frog-myocardial cytosol. Science *215*, 1117–1119.

15. Yotsu-Yamashita, M., Sugimoto, A., Terakawa, T., Shoji, Y., Miyazawa, T., and Yasumoto, T. (2001). Purification, characterization, and cDNA cloning of a novel soluble saxitoxin and tetrodotoxin binding protein from plasma of the puffer fish, Fugu pardalis. Eur. J. Biochem. *268*, 5937–5946.

16. Llewellyn, L.E., and Moczydlowski, E.G. (1994). Characterization of saxitoxin binding to saxiphilin, a relative of the transferrin family that displays pH-dependent ligand binding. Biochemistry *33*, 12312–12322.

17. Morabito, M.A., and Moczydlowski, E. (1994). Molecular cloning of bullfrog saxiphilin: a unique relative of the transferrin family that binds saxitoxin. Proc. Natl. Acad. Sci. USA *91*, 2478–2482.

18. Lenarčič, B., Krishnan, G., Borukhovich, R., Ruck, B., Turk, V., and Moczydlowski, E. (2000). Saxiphilin, a saxitoxin-binding protein with two thyroglobulin type 1 domains, is an inhibitor of papain-like cysteine proteinases. J. Biol. Chem. *275*, 15572–15577.

19. Huynh, K., and Partch, C.L. (2015). Analysis of protein stability and ligand interactions by thermal shift assay. Curr. Protoc. Protein Sci. *79*, 28.9.1–28.9.14.

20. Niesen, F.H., Berglund, H., and Vedadi, M. (2007). The use of differential scanning fluorimetry to detect ligand interactions that promote protein stability. Nat. Protoc. *2*, 2212–2221.

21. Huang, X., and Aulabaugh, A. (2016). Application of Fluorescence Polarization in HTS Assays. Methods Mol. Biol. *1439*, 115–130.

22. Rossi, A.M., and Taylor, C.W. (2011). Analysis of protein-ligand interactions by fluorescence polarization. Nat. Protoc. *6*, 365–387.

23. Velazquez-Campoy, A., Ohtaka, H., Nezami, A., Muzammil, S., and Freire, E. (2004). Isothermal titration calorimetry. Curr Protoc Cell Biol Chapter *17*. Unit 17 18.

24. Becktel, W.J., and Schellman, J.A. (1987). Protein stability curves. Biopolymers *26*, 1859–1877.

25. Arrigoni, C., and Minor, D.L., Jr. (2018). Global versus local mechanisms of temperature sensing in ion channels. Pflugers Arch. *470*, 733–744.

26. Moczydlowski, E., Mahar, J., and Ravindran, A. (1988). Multiple saxitoxin-binding sites in bullfrog muscle: tetrodotoxin-sensitive sodium channels and tetrodotoxin-insensitive sites of unknown function. Mol. Pharmacol. *33*, 202–211.

27. Li, Y., Llewellyn, L., and Moczydlowski, E. (1993). Biochemical and Immunochemical Comparison of Saxiphilin and Transferrin, 2 Structurally Related Plasma-Proteins from Rana-Catesbeiana. Mol. Pharmacol. *44*, 742–748.

28. Li, Y., and Moczydlowski, E. (1991). Purification and partial sequencing of saxiphilin, a saxitoxin-binding protein from the bullfrog, reveals homology to transferrin. J. Biol. Chem. *266*, 15481–15487.

29. Krishnan, G., Morabito, M.A., and Moczydlowski, E. (2001). Expression and characterization of Flag-epitope- and hexahistidine-tagged derivatives of saxiphilin for use in detection and assay of saxitoxin. Toxicon *39*, 291–301.

30. Morabito, M.A., Llewellyn, L.E., and Moczydlowski, E.G. (1995). Expression of saxiphilin in insect cells and localization of the saxitoxin-binding site to the C-terminal domain homologous to the C-lobe of transferrins. Biochemistry *34*, 13027–13033.

31. Lee, H., Lolicato, M., Arrigoni, C., and Minor, D.L., Jr. (2021). Production of K(2P)2.1 (TREK-1) for structural studies. Methods Enzymol. *653*, 151–188.

32. Walker, J.R., Merit, J.E., Thomas-Tran, R., Tang, D.T.Y., and Du Bois, J. (2019). Divergent Synthesis of Natural Derivatives of (+)-Saxitoxin Including 11-Saxitoxinethanoic Acid. Angew. Chem. Int. Ed. Engl. *58*, 1689–1693.

33. Andresen, B.M., and Du Bois, J. (2009). De novo synthesis of modified saxitoxins for sodium ion channel study. J. Am. Chem. Soc. *131*, 12524–12525.

34. Fernandes, R.A., Li, C., Wang, G., Yang, X., Savvides, C.S., Glassman, C.R., Dong, S., Luxenberg, E., Sibener, L.V., Birnbaum, M.E., et al. (2020). Discovery of surrogate agonists for visceral fat Treg cells that modulate metabolic indices in vivo. Elife *9*, e58463.

35. Edelhoch, H. (1967). Spectroscopic determination of tryptophan and tyrosine in proteins. Biochemistry *6*, 1948–1954.