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Capture-and-release of a sulfoquinovose-binding protein on sulfoquinovose-modified agarose

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Abstract

The solute-binding protein (SBP) components of periplasmic binding protein-dependent ATP-binding cassette (ABC)-type transporters often possess exquisite selectivity for their cognate ligands. Maltose binding protein (MBP), the best studied of these SBPs, has been extensively used as a fusion partner to enable the affinity purification of recombinant proteins. However, other SBPs and SBP-ligand based affinity systems remain underexplored. The sulfoquinovose-binding protein SmoF, is a substrate-binding protein component of the ABC transporter cassette in *Agrobacterium tumefaciens* involved in importing sulfoquinovose (SQ) and its derivatives for SQ catabolism. Here, we show that SmoF binds with high affinity to the octyl glycoside of SQ (octyl-SQ), demonstrating remarkable tolerance to extension of the anomeric substituent. The 3D X-ray structure of the SmoF•octyl-SQ complex reveals accommodation of the octyl chain, which projects to the protein surface, providing impetus for the synthesis of a linker-equipped SQ-amine using a thiol-ene reaction as a key step, and its conjugation to cyanogen bromide modified agarose. We demonstrate the successful capture and release of SmoF from SQ-agarose resin using SQ as competitive eluant, and selectivity for release versus other organosulfonates. We demonstrate that SmoF can be captured and purified from a cell lysate, demonstrating the utility of SQ-agarose in capturing SQ binding proteins from complex mixtures. The present work provides a pathway for development of 'capture-and-release' affinity resins for the discovery and study of SBPs.

Introduction

Periplasmic binding protein-dependent transport systems employ substrate-binding proteins (SBPs) that specifically capture small molecules and deliver them to their cognate ATP-binding cassette (ABC)-type transporter.[1] These integral membrane protein complexes couple ATP hydrolysis to the transport of solutes across the periplasmic membrane.[2] SBPs have evolved to recognise a diverse array of molecules, such as sugars, inorganic ions, amino acids, peptides and vitamins.[3, 4]

SBPs typically consist of two domains linked by a hinge region. Upon binding their cognate ligands, SBPs undergo a substantial conformational change, using a 'venus flytrap'-like mechanism to encapsulate their cargo. The closure of the SBP results in creation of a new interface, allowing the complex to dock to the corresponding integral membrane proteins of the ABC cassette, and allowing delivery of the cargo to its designated transporter.[5]

One of the most extensively studied SBPs is maltose binding protein (MBP), an *Escherichia coli* protein with binding affinity for amylose and oligomaltosides.[6] MBP is commonly used as a fusion partner in the production of recombinant proteins. It serves dual purposes as a solubilization tag and facilitates the purification of fusion proteins through affinity chromatography on crosslinked amylose resin.[7, 8] Widely employed in the biotechnology sector, MBP comes with readily available expression plasmids and affinity purification resin.[7, 9, 10] However, a limitation arises from residual amylase activity in cell lysates, which degrades the crosslinked amylose resin during protein purification, limiting the number of times that the resin can be regenerated and reused. Exploring other alternative SBPs and potential SBP-specific affinity could provide a solution to this issue, yet this concept remains underexplored.

In a previous work, we reported the discovery of SmoF, a sulfoquinovose binding protein, which binds sulfoquinovose (SQ, 6-deoxy-6-sulfo-D-glucose), simple SQ glycosides, and sulfoquinovosyl diacylglycerides (SQDGs). It forms part of an SQ-specific ABC transporter cassette found in *Agrobacterium tumefaciens*.[11, 12] This transporter facilitates the import of SQ and SQ and its glycosides, supporting the catabolism of these sulfosugars through a sulfolysis pathway in *A. tumefaciens*. Similar ABC transporter cassettes, associated with other SQ sulfolysis and sulfoglycolysis pathways, have been identified in other bacteria.[12, 13] The 3D X-ray structures of SmoF bound to different SQ glycosides revealed its capacity to accommodate a variety of anomeric substituents.[12] This malleability likely evolved to enable the import of SQ and its various naturally occurring glycosides, maximise the utility of this bacterial SQ catabolic pathway.

In this study, we showcase the affinity purification of the SQ binding protein SmoF from *A. tumefaciens* using SQ-modified agarose resin. The crystal structure of SmoF bound to a

lipidic glycoside of SQ is presented, which demonstrates the tolerance of the protein towards a linker-like aglycone. Our findings reveal that such a linker can access the bulk solvent even after the SBP has 'closed' around it. Leveraging this structural information, we designed and synthesized a linker-equipped SQ derivative, and covalently conjugated it to cyanogen bromide-modified agarose to generate SQ-modified agarose (**Figure 1**). This modified agarose effectively captured SmoF and, using SQ as eluent, the SmoF protein could be released and recovered under mild conditions. Expanding our work, we demonstrate the successful capture of SmoF directly from cell lysate. This study underscores the utility of SQ-agarose in capturing SQ-binding proteins from complex mixtures and establishes a foundation for the further development of orthogonal and complementary SBP-based affinity purification systems.

Results

To determine whether SmoF can bind an SQ glycoside bearing an extended alkyl chain, we synthesized octyl-SQ **3** in two steps from octyl α-D-glucopyranoside **1**. Exposure of **1** to NBS/Ph_3P in DMF[14] gave 6-bromo-6-deoxy-α-D-glucopyranoside **2**. Subsequent treatment with sodium sulfite in water at reflux afforded octyl-SQ **3** (**Figure 2a**). Binding of octyl-SQ **3** to recombinant SmoF was studied using nano-differential scanning fluorimetry (nano-DSF), a technique that measures protein folding state by monitoring changes in intrinsic tryptophan fluorescence as a function of temperature. An increase of 15 °C in the melting temperature (Tm) of SmoF was observed in the presence of 2 mM SQ-octyl, which is indicative of significant fold stabilisation and involves substantial conformational change (**Figure S1**). This compares with ∆Tm = 10.3 ºC for SQ and ∆Tm = 15.3 ºC for sulfoquinovosyl glycerol (SQGro).[12] The thermodynamic parameters of binding were quantified by isothermal titration calorimetry. Octyl-SQ **3** binds to SmoF with K_d = 1.5 ± 0.6 μM, ΔH = -3.2 ± 0.2 kcal/mol, and ΔS = 15.6 cal/mol/deg (**Figure S2**). By comparison, SQ binds with K_d = 2.4 μM, showing that the octyl group does not materially affect binding to SQBP.[12]

To determine how octyl-SQ **3** is accommodated within the binding cleft of SmoF, we determined the 3D structure of SmoF•**3** using X-ray crystallography. Co-crystallization yielded a complex in $P2_12_12_1$ space group that diffracted to a resolution of 1.8 Å (**Table S1**). Clear density was observed for **3**, including all eight carbons of the octyl chain, with the ligand bound between the two α/β globular domains in the 'closed' state (**Figure 3a**, **Figure S3**). Dyndom analysis[15] of these dynamic domains revealed a hinge rotation of about 32° to achieve a closed state when compared to ligand-free SmoF in an 'open' state (7NBZ.pdb), which is similar to that seen with SQ and a range of other SQ-derived ligands[12] (**Figure S4**). The sugar and

sulfonate group make numerous hydrogen bonding interactions with the residues in the binding cleft. C1-O of the glycosidic bond H-bonds Trp276 (3.1 Å), C2-OH of the sugar is hydrogen-bonded to Asp113, and C3-OH and C4-OH both H-bond to Arg345 and Asp67. The characteristic anionic sulfonate binds Thr220 (2.5 Å), Ser43 (2.8 Å) and the backbone amide of Gly166 (2.6 Å) (**Figure 3b,c**). These interactions are similar to those observed previously for the SmoF•SQ complex (7YZS.pdb),[12] but with complete closure the two α/β globular domains prevented by the octyl chain, which extends through the cleft causing disorder in the local region and no observable density of amino acid residues 11-16. The octyl chain of the ligand is accommodated in hydrophobic region of the cleft flanked by Phe111, Leu312, Tyr240 and Trp18. The ligand binding pocket volume was computed using the CastP server.[16] The pocket volume of the SmoF•octyl-SQ complex is 360 Å^3, which is expanded compared to 297 Å^3 for the SmoF•SQ complex (**Figure S5**).[12] The plasticity of the binding pocket to accommodate a long linker chain at the α-anomeric position highlights SmoF as a promising binding protein for exploring affinity-based purification approaches.

Based on the evidence that the long alkyl chain of octyl-SQ **3** can be accommodated within the SmoF binding cleft, we set about the synthesis of an extended linker suitable for attachment of SQ to a solid support (**Figure 2b**). Treatment of PEG-diamine **4** with ethyl trifluoroacetate at -65 ºC, as described by Carrié *et al*.[17] gave trifluoroacetamide **5** in 41% yield. EDC-mediated coupling of **5** with 4,4'-dithiobutyric acid **6** gave disulfide **7** in 64% yield. Reduction of the disulfide with TCEP in pH 4 acetate buffer, followed by work-up using deoxygenated EtOAc afforded crude thiol **8**. Without purification, thiol **8** and allyl-SQ **9**[18] were coupled in a thiol-ene reaction using Vazo-44[19] at 50 ºC to give sulfide **10**. Under these conditions some loss of trifluoroacetamide group occurred, and the resulting amine impurity could be easily removed by passage through cation exchange resin (H^+ form). This linker was susceptible to autooxidation, and to provide a chemically stable linker, the thioether of **10** was oxidized to the sulfone with mCPBA to give the sulfone **11** in 90% yield. Finally, cleavage of the trifluoroacetamide of **11** was achieved using NaOMe in MeOH, affording linker equipped SQ-amine **12**. To provide a control to establish whether the linker binds protein, we also synthesized a butyramide-capped amine **13** in one step from reaction of butyric anhydride and PEG-diamine **4**.

SQ-amine **11** and butyramide-capped amine **12** were conjugated to cyanogen bromide-activated Sepharose 4B at a loading of 5 μmole/ml resin, then unreacted groups blocked with glycine, to give SQ-agarose and control-agarose, respectively. Initially, we examined whether SQ-agarose could capture and release purified recombinant SmoF (**Figure 4a**). 50 μl of swollen SQ-agarose resin treated with 50 μg of SmoF (lane 1) showed binding of SmoF through lower intensity of protein in the flowthrough (lane 2). After washing with 20

volumes of binding buffer, residual protein bound to the resin was released under denaturing conditions to reveal that substantial SmoF (40% by densitometry) was retained by the resin (lane 3). Additional washing steps led to low level release of SmoF (lanes 4 and 5), consistent with binding to the resin being an equilibrium process. SmoF was almost completely eluted from the resin using 1 mM SQ (lane 6; 36% of input by densitometry), with only traces of SmoF retained as shown upon denaturation of the eluted resin (lane 7). Equivalent experiments performed on the butyramide-capped control-agarose revealed no binding of SmoF in the absence of the SQ moiety, and thus that SmoF does not bind to the linker or agarose. Based on the reduction in intensity of the loading sample versus the flowthrough in Figure 4a, we estimate that the SQ-agarose has a binding capacity of 0.4 mg of SmoF per millilitre.

We next explored the optimal concentration of SQ to elute SmoF, and whether it can be eluted with alternative sulfonates (**Figure 4b**). SmoF was loaded onto the SQ-resin and washed with loading buffer, then eluted with increasing concentrations of SQ. Two column volumes of 0.2 mM SQ eluted the majority of the SmoF, while increasing the concentration eluted additional SmoF. However, even after elution with 5 mM SQ, a trace amount of SmoF was retained on the resin. 2,3-Dihydroxypropanesulfonate (DHPS) and sulfolactate (SL) are 3-carbon, polar organosulfonates with some structural similarities to SQ. Attempted elution of SmoF with increasing concentrations of each of these compounds resulted in low levels of release that was concentration independent, suggesting that SmoF was slowly leaching in a sulfonate-independent but equilibrium-based manner from the resin. A similar result was obtained with the sulfonated Good's buffer, HEPES at 250 mM. Collectively these data show that elution of SmoF from the SQ-resin is specific for SQ, relative to other structurally distinct organosulfonates.

Finally, we explored whether SQ-resin could be used to affinity purify SmoF from a cell lysate (**Figure 5**). A cell lysate containing SmoF was obtained by IPTG induction of *E. coli* transformed with a pET vector carrying the *smoF* gene (lane 1). Treatment of the cell lysate with SQ-resin led to depletion of SmoF in the lysate (lane 2), which was retained after washing (lane 3) and could be eluted with 6 column volumes of 5 mM SQ (lanes 4-6), with no residual SmoF detected on the resin (lane 7).

Discussion and Conclusions

Solute binding proteins are widely produced by bacteria and interact with diverse ligands. Previous efforts to develop SBPs as tags for affinity purification have mainly focused on MBP, which has also benefitted from the property of MBP as a solubility enhancing expression tag. SBPs have additional applications as biosensors,[20] and when immobilized, as stringent capture reagents for their specific ligands.[21]

Creation of a solid-supported ligand for capturing the cognate SBP involves identifying an appropriate site on the ligand for covalent modification and assessing the tolerance of the SBP for an extended linker. We previously showed that SmoF could bind SQ and various anomeric derivatives, with a constellation of interactions between protein and ligand at the sulfonate group and other non-anomeric sites that are presumably required for SQ specificity. Here, we show that SmoF accommodates SQ modified with an 8-carbon octyl linker attached at the anomeric position, with little perturbation to binding as assessed by thermal stability and isothermal titration calorimetry. The 3D crystal structure of octyl-SQ bound to SmoF confirmed the octyl linker is well ordered and projects towards the protein surface. Utilizing this information, we designed and synthesis of a polyethylene glycol (PEG) linker-equipped SQ-amine, which was then conjugated to cyanogen bromide-modified agarose to create an SQ-agarose resin. This proof-of-concept could potentially be applied to other glycoside-binding SBPs, involving attachment of a similar linker to the anomeric position and immobilisation to resin to give orthogonal solubilisation/affinity purification systems.

Few examples have been published describing the attachment of a small molecule SBP ligand through a linker to a solid support for capturing the cognate SBP. One instance studying thiamine binding protein used a synthetic linker connecting thiamine and biotin, allowing the display of the thiamine ligand indirectly through binding of the biotin to streptavidin-coated polystyrene.[22] That work showed an extended linker comprised of PEG11 was effective, while a shorter PEG3 linker was not. The extended length of the required linker is likely because of the need to span deep binding clefts in both streptavidin and thiamine binding protein. The present study demonstrated the effectiveness of a shorter linker for the direct attachment of SQ to the agarose support.

The SQ-agarose developed here exhibited a binding capacity estimated at 0.4 mg per ml. This compares with commercially available cross-linked amylose resins (eg amylose resin high flow, New England Biolab #E8022S), which has a capacity of >4 mg per ml for MBP5-paramyosin δSal fusion (equivalent to >2.5 mg per ml for MBP). Notable differences between SmoF and commercial MBP proteins are that variants of the latter (eg MBP5) have been engineered with enhanced affinity for amylose resin, and the addition of residues encoding a spacer for linking to fusion partners. Another important difference is that in this work we grafted the ligand to crosslinked agarose resin, while for MBP crosslinked agarose is the resin and ligand, which likely provides higher binding capacity. Possibly, higher loading could be achieved by introduction of branched linkers bearing additional copies of SQ. We note that low levels of SmoF elute upon washing in the absence of SQ. This may reflect sub-optimal affinity of SmoF for SQ and the equilibrium nature of binding.

Affinity based capture methods are valuable for discovering and annotatiing new proteins. This approach could allow the capture of novel SQBPs produced by microorganisms from environmental samples or in monoculture experiments, followed by identification through proteomics. Broadly speaking, this work outlines a pathway for developing capture and release methods for an SQ binding protein that could be extended to other members of this protein family with diverse ligand preferences. This method holds promise for discovering new SBPs through affinity capture.

Author contributions

Conceptualization: S.J.W., E.D.G.-B., G.J.D. Formal Analysis: M.L., M.S., E.D.G.-B., G.J.D., S.J.W. Funding acquisition: S.J.W., E.D.G.-B., G.J.D. Experimental/Methodology: T.A., A.J.D.S., M.L., M.S., Y.Z., J.P.L. Supervision: S.J.W., E.D.G.-B., G.J.D. Writing – original draft: S.J.W. Writing – review & editing: M.L., S.J.W., E.D.G.-B., M.S.

Conflicts of interest

There are no conflicts of interest to declare.

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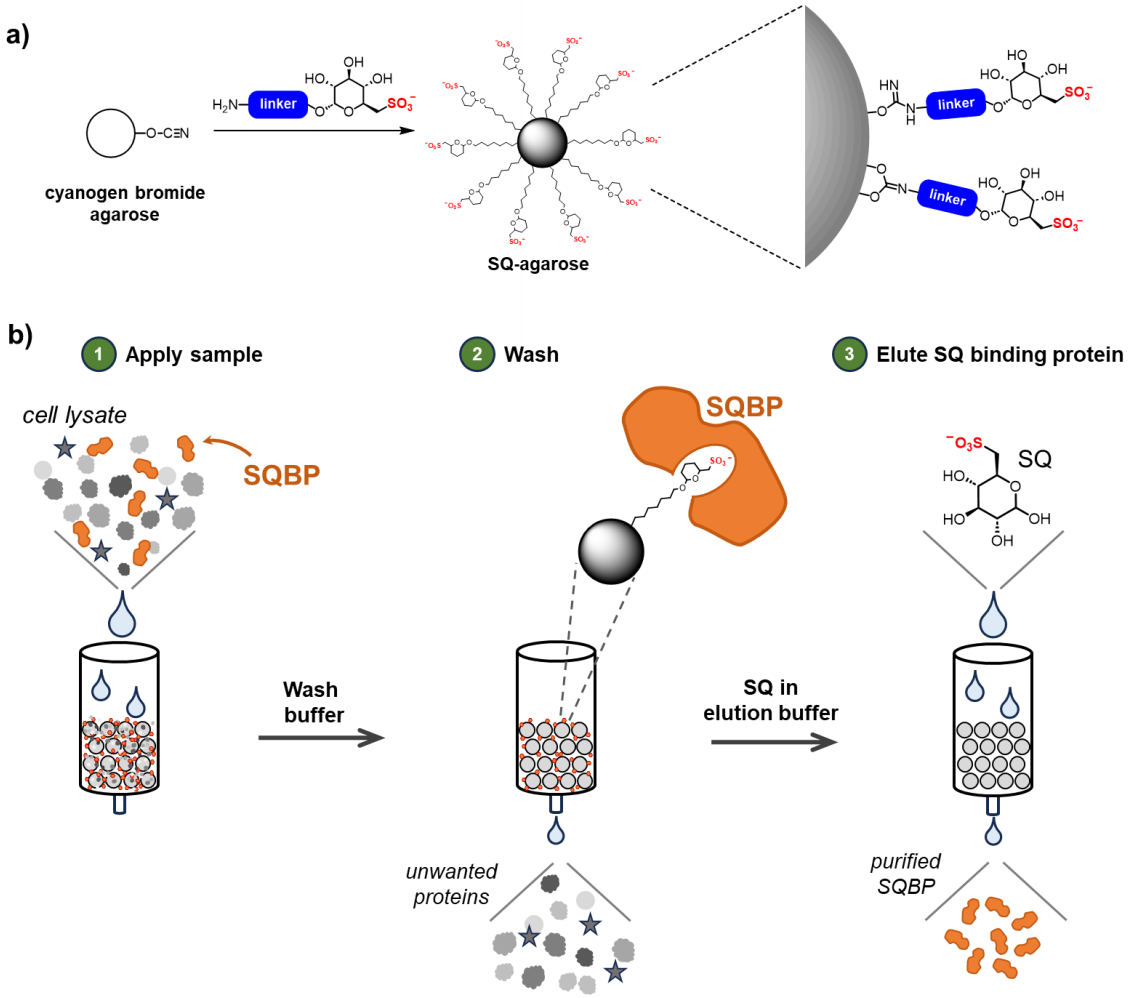

Figure 1. Strategy for affinity capture-and-release of sulfoquinovose-binding proteins (SQBPs) on SQ-agarose. **(a)** Conjugation of a linker-equipped SQ-amine with cyanogen bromide modified agarose. **(b)** Use of SQ-agarose for affinity purification of SQBPs, with elution using sulfoquinovose (SQ).

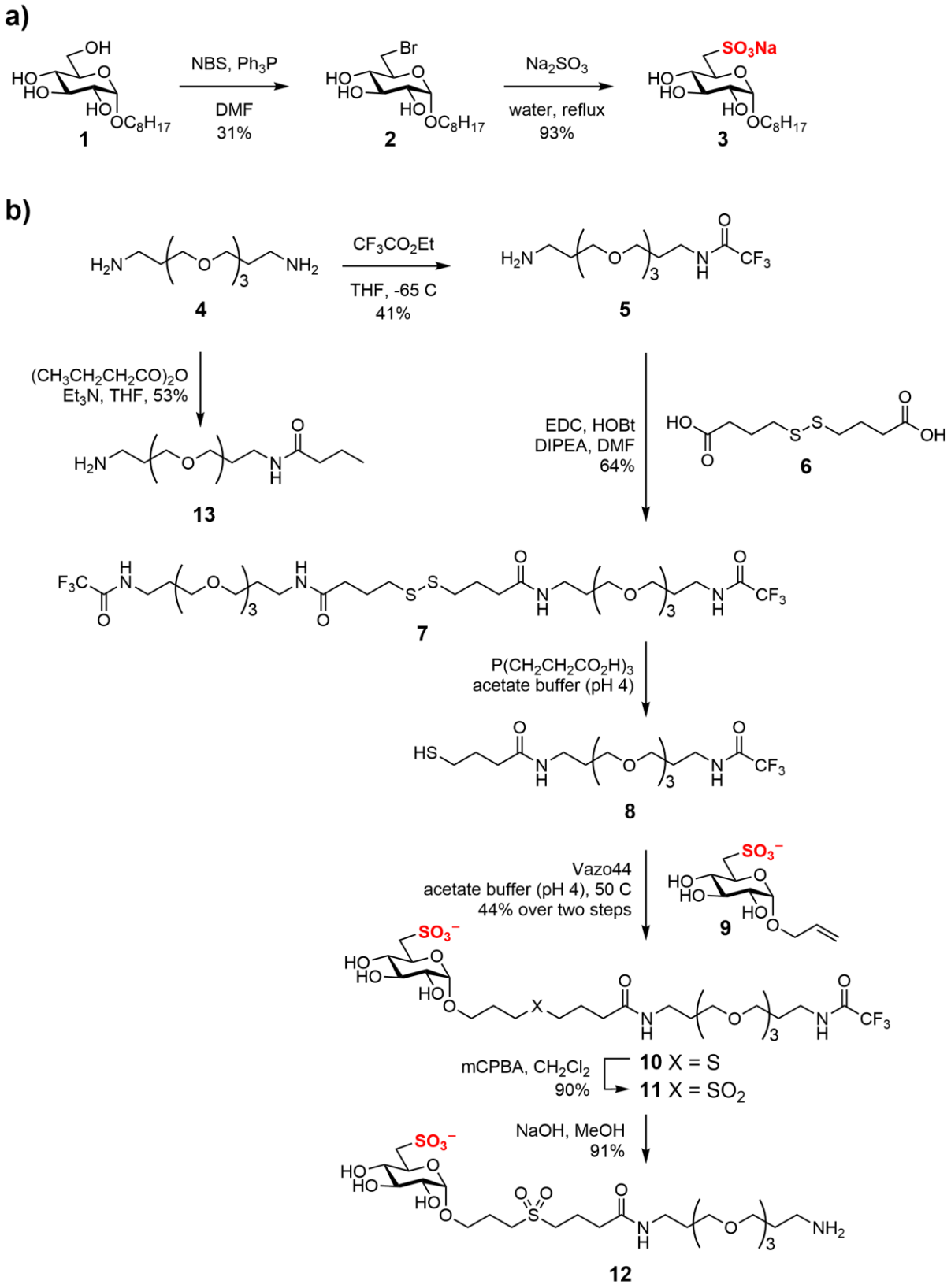

Figure 2. Synthesis of **(a)** octyl α-D-sulfoquinovoside (octyl-SQ, **3**) and **(b)** linker-equipped SQ (SQ-amine, **12**) and butyramide-capped linker (control-amine **13**).

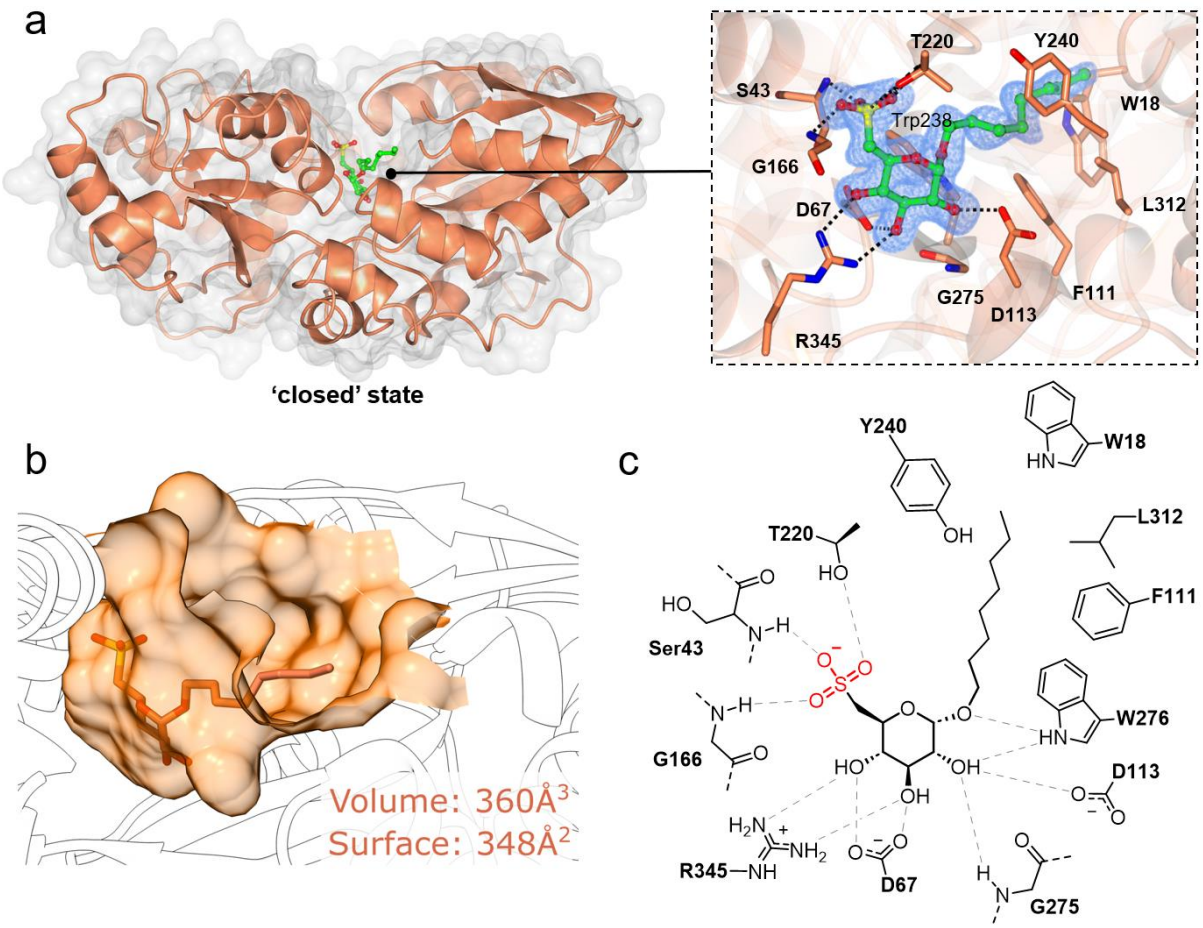

Figure 3. 3D X-ray crystal structure of SQ binding protein SmoF from *A. tumefaciens* with octyl-SQ. **(a)** Overall fold of SmoF•octyl-SQ in a closed conformation (left) and close-up view of showing active site interactions with the ligand. Side-chain interactions with amino acid residues in the binding cleft are shown within 4 Å distance. Electron density in blue mesh corresponds to $2F_o - F_c$ map contoured at 0.8 σ (0.302 electrons/Å3). (**b**) Volume and surface of SmoF ligand binding pocket visualized using CASTp server;[16] surface of the binding pocket octyl-SQ **3** is depicted at a transparency of 0.5 with ligand **3** shown as cylinders. The pocket occupies a 360 Å^3 volume. (**c**) Cartoon of binding site interactions with octyl-SQ **3**.

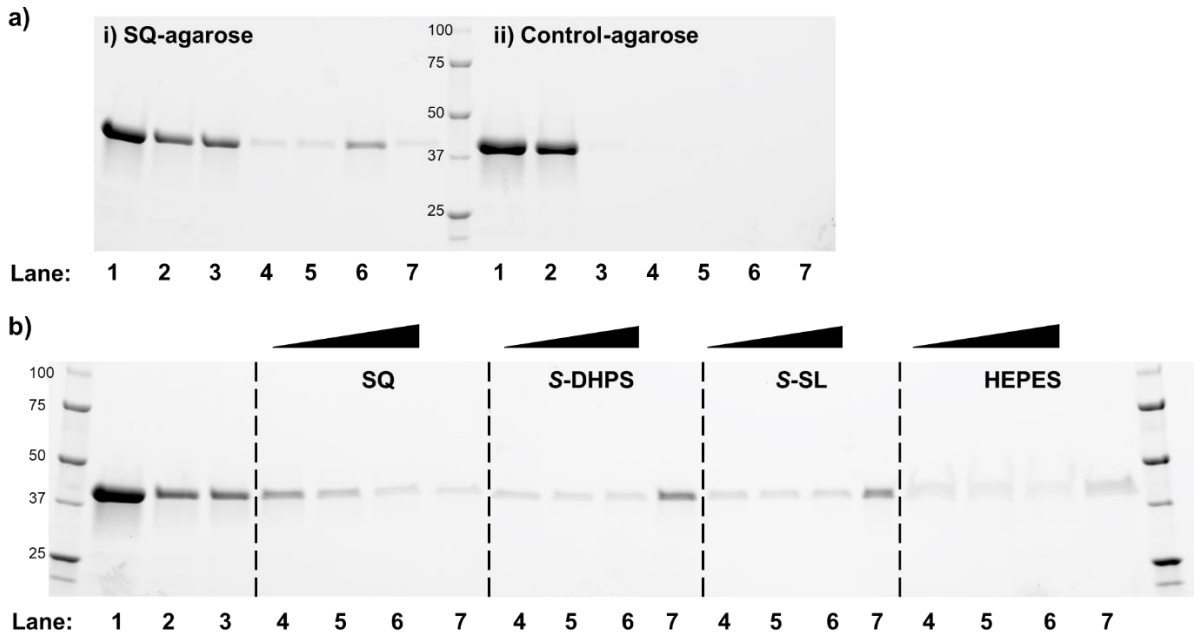

Figure 4. Binding and elution of SQ binding protein SmoF. **(a)** Comparison of i) SQ-agarose and ii) Control-agarose (butyramide-capped). 50 μg of SmoF applied to 50 μl resin. Lane 1: SmoF protein input (5 μg), lane 2: flow through of unbound protein (10 μl of 100 μl eluate), lane 3: resin washed with 20 vol binding buffer, then bound SmoF released by boiling a portion of washed resin with loading dye including SDS (5 μl of 50 μl), lane 4: wash with 1 column volume (CV) binding buffer (5 μl of 50 μl eluate) after 20 CV wash, lane 5: wash with 1 CV binding buffer (5 μl of 50 μl eluate) after 21 CV wash, lane 6: elution with 1 mM SQ (5 μl of 100 μl eluate), lane 7: bound SmoF released after denaturing portion of eluted resin (5 μl of 45 μl). **(b)** Release of SmoF is be achieved specifically by SQ. 50 μg of SmoF applied to 50 μl resin. Lane 1: SmoF protein input (5 μg), lane 2: flow through of unbound protein (10 μl of 100 μl eluate), lane 3: resin washed with 20 vol binding buffer, then bound SmoF released by denaturing of portion of washed resin (5 μl of 50 μl), lanes 4, 5, 6: elution with 2 CV each SQ (0.2, 1, 5 mM), *S*-DHPS (0.2, 1, 10 mM), *S*-SL (0.2, 1, 10 mM) or HEPES (0.25 M, lanes 4-6), lane 7: bound SmoF released after denaturing portion of eluted resin (5 μl of 45 μl). Binding buffer is 20 mM Tris.HCl (pH 7.5), 0.2 M NaCl.

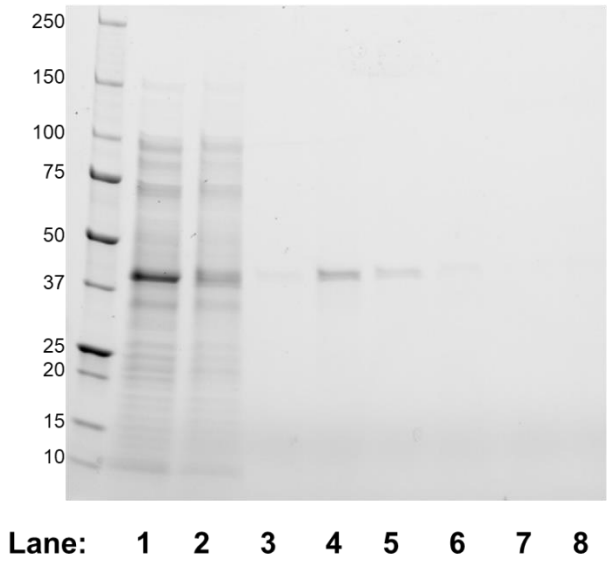

Figure 5. Capture-and-release of SQ binding protein: affinity purification of SmoF from *E. coli* cell lysate. Input is 100 μl of soluble fraction of *E. coli* lysate applied to 50 μl SQ-agarose. Lane 1: Lysate input (10 μl), lane 2: flow through of unbound proteins (10 μl of 100 μl eluate), lane 3: resin washed with 21 CV binding buffer (5 μl of 50 μl of final wash), lane 4: bound protein released by denaturing portion of washed resin (5 μl of 50 μl); lane 5, 6, 7: sequential elution with 2 CV 5 mM SQ (5 μl of 100 μl eluate, each), lane 8: bound protein released by denaturing portion of residual resin (5 μl of 45 μl).