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Expression and characterization of Flag-epitope- and hexahistidine-tagged derivatives of saxiphilin for use in detection and assay of saxitoxin

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Abstract

Saxiphilin is a plasma protein from the bullfrog (*Rana catesbiana*) that binds saxitoxin (STX), a causative agent of paralytic shellfish poisoning. Saxiphilin is homologous to transferrin and consists of two internally homologous domains called the N-lobe and the C-lobe. STX binds to a single site in the C-lobe of saxiphilin. In this study, cloned genes coding for recombinant saxiphilin and C-lobe saxiphilin were modified to contain two tandemly located affinity tags, Flag epitope (DYKDDDDK) and His_6 (HHHHHH), at the protein C-terminus and were expressed in cultured insect cells using baculovirus vectors. Both tagged proteins are readily detected on immunoblots by anti-Flag monoclonal antibody. Flag-His_6-tagged saxiphilin was purified to homogeneity using Ni^{2+}-chelate affinity chromatography and Heparin Sepharose chromatography. Equilibrium analysis of [^{3}H]STX binding to tagged saxiphilin and tagged C-lobe saxiphilin gave K_D values of 0.75 and 2.7 nM, respectively. Flag-His_6-tagged saxiphilin was also utilized in a microtiter well solid-phase assay with Reacti^{TM}-bind metal chelate plates to measure [^{3}H]STX binding and binding competition by unlabeled STX. Such Flag-His_6-tagged derivatives of saxiphilin have many possible applications in the assay of STX and related toxinological research.

Keywords: Insect cells; Paralytic shellfish poisoning; Protein expression; Saxiphilin; Saxitoxin

1. Introduction

Saxiphilin is a soluble protein that binds saxitoxin (STX) with high affinity and specificity (Mahar et al., 1991). STX and STX derivatives are microbial neurotoxins responsible for paralytic shellfish poisoning, which results from block of voltage-sensitive sodium channels by these toxins (Shimizu, 1996). Saxiphilin was first discovered in frogs (Doyle et al., 1982; Moczydlowski et al., 1988) but similar soluble STX binding activity has been observed in diverse species of arthropods, amphibians, reptiles, and fish (Llewellyn et al., 1997).

Cloning and sequencing of bullfrog (*Rana catesbiana*) saxiphilin cDNA revealed a protein of 91 kD that is structurally related to the transferrin family

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of proteins (Morabito and Moczydlowski, 1994a). For example, saxiphilin exhibits 51% and 42% overall amino acid sequence identity with serum transferrin from the african clawed frog (*Xenopus leavis*) and human lactoferrin, respectively. In common with many transferrin-related proteins, saxiphilin consists of two major domains called the N- and C-lobes that exhibit internal homology to each other.

Unlike transferrins, saxiphilin does not bind iron. It is also distinguished from transferrins by the presence of a unique 143-residue insertion in the N-lobe of the protein (Morabito and Moczydlowski, 1994b). This insertion consists of two tandem type-1 domains of thyroglobulin, a recognized protein module. In addition to saxiphilin, such type-1 thyroglobulin domains occur in diverse proteins that are functionally unrelated; e.g., insulin-like growth factor binding protein (Brinkman et al., 1988), invariant chain I of class II major histocompatibility complex (Koch et al., 1987), entactin or nidogen (Durkin et al., 1988), and equistatin (Lenarcic et al., 1997). A single pH-dependent STX-binding site has been localized to the C-terminal portion of saxiphilin, namely the C-lobe Morabito et al., 1995). Recombinant saxiphilin (R-Sax) and C-lobe saxiphilin (C-Sax) have been expressed in insect cells using baculovirus vectors (Morabito et al., 1995).

Paralytic shellfish poisoning is a health problem associated with toxic algal blooms and resultant shellfish contamination in coastal areas around the world (Anderson, 1989; Baden, 1988). Toxicity of shellfish extracts has been commonly determined by live mouse bioassay (Schantz et al., 1958; Sommer and Meyer, 1937). Various in vitro assays have been developed for the screening and analysis of STX and STX derivatives in shellfish extracts based on HPLC, fluorescence detection, immunochemical detection, binding to sodium channels, and binding to saxiphilin (Doucette et al., 1997; Gessner et al., 1997; Indrasena and Gill, 1998; Kralovec et al., 1996; Llewellyn et al., 1998; Negri and Llewellyn, 1998). Since bullfrog saxiphilin binds STX and related STX-derivatives with high affinity and 1:1 stoichiometry, it would be useful to have an appropriate linker coupled to this protein to facilitate biochemical manipulation and routine production of a solid-phase matrix that binds STX-related toxins. Addition of a Flag epitope (DYKDDDDK) together with hexa-histidine (HHHHHH) residues to proteins allows manipulation or detection using either anti-Flag antibody or Ni^{2+}-affinity matrices (e.g., Robeva et al., 1996). Flag-His_6 tags can be exploited for multiple applications such as purification, immunoprecipitation, and immunocytochemistry to aid in developing assays and probing functions of the tagged proteins. In this paper we describe the production and properties of Flag-His_6-tagged saxiphilin and Flag-His_6-tagged C-lobe saxiphilin that are useful for detection and assay of STX and STX derivatives.

2. Materials and methods

2.1. Materials

SF9 and High Five™ insect cells, Grace's TNM-FH insect cell media, SF900 II SFM insect cell media, penicillin-streptomycin antibiotic mixture, X-gal, and FBS (fetal bovine serum) were from Gibco (Rockville, MD). pBluebac4 vector, PCR II vector and the Bac-N-blue linear transfection kit, were from Invitrogen (Carlsbad, CA). Nickel nitrilo-triacetic acid (Ni^{2+}-NTA) super-flow resin was from Qiagen (Valencia, CA). M2 anti-Flag monoclonal antibody and mouse anti-IgG were from Sigma (St Louis, MO). Poros20 Heparin Sepharose chromatography media and ConSep LC100 Fast Performance Liquid Chromatography system were from Millipore (Bradford, MA), Reactibind™ metal chelate plates were from Pierce (Rockford, IL). Filtron Ultracassette™ tangential flow dialysis apparatus was obtained from Pall Filtron (Northborough, MA). ECL detection kit was from Pierce (Rockford, IL) and SafeScint scintillation liquid fluid was from American Bioanalytical (Natick, MA). [11−^{3}H]saxitoxin (24 Ci/mmol) was purchased from Amersham Life Science (Arlington Heights, IL).

2.2. Construction of the transfer vector

During the previous construction of C-Sax cDNA, a point mutation of Ser616Pro was inadvertently introduced by PCR (Morabito et al., 1995). This mutation was first corrected back to Ser616 by a standard site-specific mutagenesis procedure and confirmed by sequencing. A Flag-hexahistidine (FH_6) coding sequence was fused to R-Sax and C-Sax cDNA in pBluescript II KS^- as follows. An oligonucleotide primer overlapping the single *SmaI* restriction site near the C-terminal end of Sax cDNA and an antisense primer overlapping the position of the original stop codon of the Sax cDNA sequence (Fig. 1A) with the addition of the FH_6 tag extension, a new stop codon, and a new *KpnI* site were synthesized by the Yale nucleic acid facility. PCR amplification was performed using 1 μM primers and 0.1 μg R-Sax cDNA as the template, for 50 cycles (1 min at 94°C, 1 min at 42°C, 1.5 min at 72°C). The resulting 350 basepair product known as the *SmaI-KpnI* FH_6 fragment was subcloned into PCRII, a cloning vector for PCR products.

To isolate the large fragment containing the entire R-Sax or C-Sax coding sequence, R-Sax and C-Sax cDNA in pBluescript II KS^- (Morabito et al., 1995)

were first digested with *PstI*. This was followed by digestion of this fragment with *SmaI*. The resulting large *PstI-SmaI* (N-terminal) fragment was isolated and combined with the isolated *SmaI-KpnI* FH_6 (C-terminal) fragment described above. The two fragments were ligated into pBlueBac4 previously digested with *PstI* and *KpnI* to generate R-Sax-FH_6 and C-Sax-FH_6 constructs in pBluebac4 vector (Fig. 1B).

2.3. Insect cell culture and production of recombinant R-Sax-FH_6 and C-Sax-FH_6

For virus production, SF9 insect (*Spodoptera frugiperda*) ovary cells were grown as adherent cells at 28°C in Grace's TNM-FH medium supplemented with 10% FBS and penicillin and streptomycin (100 U/ml)). For protein production, High Five™ insect (*Trichoplusia ni*) cells were grown in serum free SF-900 II SFM media containing penicillin-streptomycin (100 U/ml), as suspension cultures with shaking at 150 rpm at 28°C.

pBluebac 4 vector constructs of R-Sax-FH_6 and C-Sax-FH_6 were co-transfected with Bac-N-blue™ AcMNPV DNA into SF9 cells in the presence of cationic liposomes using the Bac-N-blue linearized transfection kit (Invitrogen) and incubated for 72 h at 28°C. The supernatant from this co-transfection culture was diluted (1:10) and used in a plaque assay with agar containing X-gal to isolate pure recombinant plaques. After 7 days, blue plaques that were positive for lacZ, a marker for virus containing either R-Sax-FH_6 or C-Sax-FH_6, were picked from the plates. The recombinant virus was propagated in Grace's TNM-FH medium and purified by another round of plaque assay. Recombinant virus stocks were produced by amplification in SF9 cells, and assayed by the method of end-point dilution (Reilly et al., 1994).

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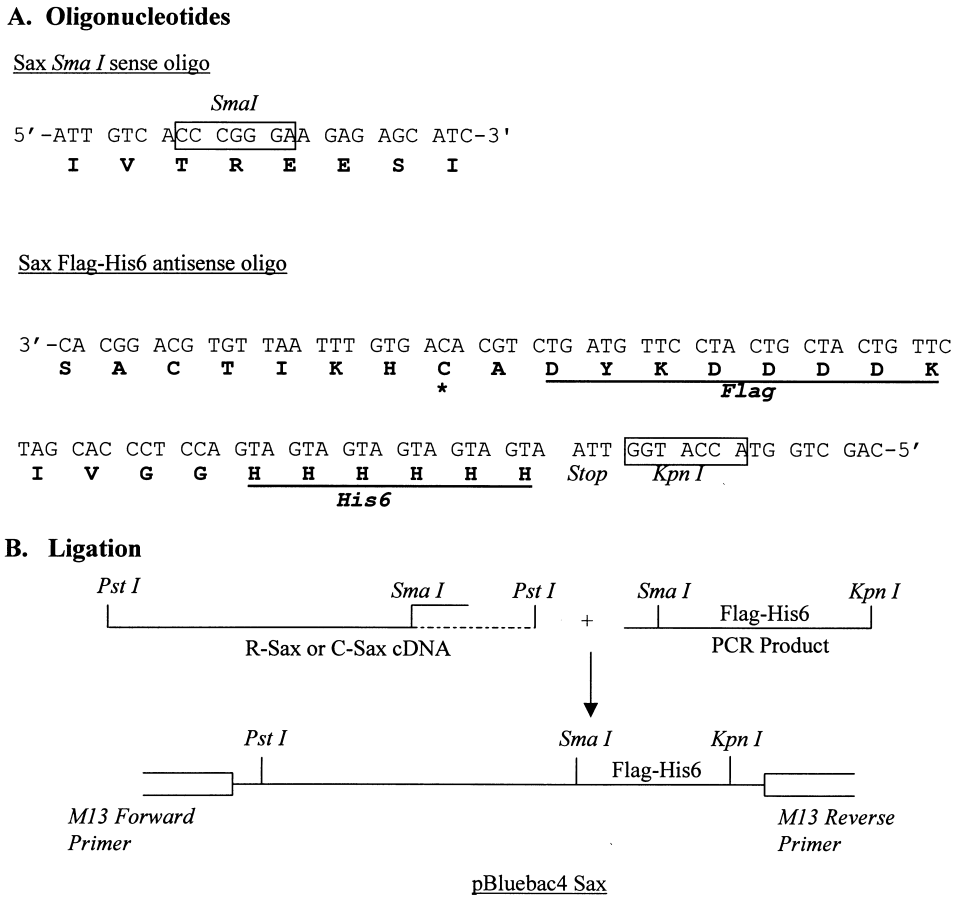

Fig. 1. Scheme for construction of Flag-His_6-tagged versions of saxiphilin and C-lobe saxiphilin. (A) Sequence of Sax *SmaI* sense oligonucleotide primers and Sax Flag-His6 antisense primer. An asterisk (*) marks the location of the original C-terminal Cys residue of saxiphilin. (B) Restriction digestion of saxiphilin R-Sax or C-Sax cDNA and the Flag-His_6-tagged PCR product followed by ligation of the selected products into the pBluebac4 vector.

cells (2×10^6 ml^{-1}) were infected at a multiplicity of 5–10 pfu (plaque forming units) of virus per cell and grown at 28°C with shaking at 150 rpm. The cell culture medium was harvested by centrifugation at 40 h post-infection, supplemented with protease inhibitors (100 μg/ml phenylmethylsulfonylfluoride, 50μg/ml leupeptin and 50 μg/ml pepstatin), and used for protein purification.

2.4. [^{3}H]STX binding assay

[^{3}H]STX binding, Scatchard plot analysis, association and dissociation kinetics of [^{3}H]STX binding to R-Sax-FH_6 and C-Sax-FH_6 were performed as described previously (Llewellyn and Moczydlowski, 1994). Binding isotherms for Scatchard analysis of R-SAX-FH_6 and C-SAX-FH_6 were obtained by varying the concentration of [^{3}H]STX from 0.05 nM to 25 nM. Samples containing saxiphilin and [^{3}H]STX were allowed to equilibrate on ice for 1–2 h. Bound [^{3}H]STX was separated from free toxin by rapid passage of 100 ml aliquots over mini columns of AG50W-X2 resin (100–200 mesh, $Tris^+$, form from Bio-Rad, Hercules, CA), pre-equilibrated with 100 mM Tris-HCl, pH 7.4, 200 mM NaCl, and 10 mg/ml of bovine serum albumin. The standard incubation buffer for [^{3}H]STX binding assay was 20 mM Mops-NaOH, pH 7.4 and 200 mM NaCl.

2.5. SDS-PAGE and immunoblot analysis

Samples were subjected to SDS-PAGE (Laemmli, 1970) using a 10% acrylamide gel. The gel was blotted onto a nitrocellulose membrane using a Bio-Rad Mini Trans-Blot Cell. The membrane was incubated with 3% BSA in 20 mM Tris-HCl, 0.15 M NaCl, pH 7.5 (TBS) for 1 h, and then incubated with 5 ml of a 10 μg/ml solution of M2 Anti-Flag antibody in TBS. After 3 washes with TBS (5 min each) the membrane was incubated with a 1:500 dilution of horseradish peroxidase-conjugated anti-mouse IgG for 1 h at 22°C. The membrane was once again washed 3 times with TBS, 5 min each, and the protein bands were visualized by chemiluminescence detection (Pierce-ECL detection kit).

2.6. Purification of R-Sax FH_6

One liter of baculovirus cell culture supernatant was concentrated to about 100 ml using a Filtron Ultracasette™ tangential flow dialysis apparatus and subsequently dialyzed against 4 l of 150 mM NaCl, 50 mM sodium phosphate, pH 8.0 at 4°C, with at least two changes of solution. NaCl was added to a final concentration of 300 mM. 8 ml of Ni-NTA superflow resin was added to the cell supernatant and gently stirred for 8 h on ice. The Ni-NTA resin containing bound R-Sax-FH_6 was packed in a column (2.0×15 cm) and washed with 10 volumes of buffer containing 50 mM sodium phosphate, 300 mM NaCl, pH 8.0, 30% glycerol, and 4 mM imidazole. R-Sax-FH_6 was eluted by addition of 50 mM sodium phosphate, 300 mM NaCl, 150 mM imidazole pH 8.0 and active fractions were pooled.

The partially purified pool of R-Sax-FH_6 was then subjected to Heparin Sepharose chromatography using a Poros 20 Heparin Sepharose column on the ConSep LC100 Millipore system as follows. Porous 20 Heparin Sepharose media was packed and equilibrated in a $1 \times$ 10 cm column according to the manufacturer's instructions. The sample of R-Sax-FH_6 was dialyzed against 4 l of 10 mM sodium acetate, 10 mM MES buffer, 5mM EDTA, pH 6.0 at 4°C and loaded on the Poros 20 Heparin Sepharose column pre-equilibrated with dialysis buffer at a flow rate of 1 ml/min. The column was washed with 30 mM sodium acetate 10 mM MES, 5 mM EDTA at pH 6.0 and R-Sax-FH_6 was eluted with an increasing NaCl gradient (30–390 mM). The flow rate was 5 ml/min, with a gradient time of 14 min. Active [^{3}H]STX-binding fractions were pooled, concentrated and analyzed by SDS-PAGE. Peak fractions were $>90\%$ pure as judged by silver or Coomassie blue protein staining. The specific activity of purified R-Sax-FH_6 was 6300 pmol [^{3}H]STX binding sites per mg protein.

2.7. Microtiter well assay of [^{3}H]STX binding and STX competition

Reacti-bind™ Ni^{2+}-chelate plates with separable wells were utilized in the following manner to assay saxiphilin-bound [^{3}H]STX. A 200 μl reaction mixture containing 25 mM Hepes-NaOH, 150 mM NaCl, pH 8.0 was incubated with 5 nM [^{3}H]STX per well. In the first type of experiment, [^{3}H]STX bound to R-Sax-FH_6 was determined by incubating 5 nM [^{3}H]STX with increasing amounts of R-Sax-FH_6. The reaction mix in the wells was incubated on ice for 3 h and removed subsequently by aspiration. The wells were then washed three times with ice-cold 100 mM Tris–HCl, pH 8.0. Individual wells were then manually separated and placed into 25 ml liquid scintillation vials. [^{3}H]STX was determined by liquid scintillation counting using 10 ml SafeScint liquid scintillation fluid. In the second type of experiment, a saturating concentration of R-Sax-FH_6 was incubated with 5 nM [^{3}H]STX and titrated with increasing amounts of unlabeled STX, as a competitor in the binding reaction. [^{3}H]STX bound in this experiment was plotted against the concentration of unlabeled STX in the reaction.

3. Results

3.1. Construction of baculovirus vectors, virus production and protein production

Expression of recombinant full-length saxiphilin (R-Sax) and C-lobe saxiphilin (C-Sax) in insect cells by use of baculovirus vectors has been described previously (Morabito et al., 1995). In the present work, we modified these latter vectors by addition of a cDNA sequence coding for 19 amino acids (ADYKDDDDKIVGGHHHHHH) at the original 3′ end. This modification was designed to introduce a Flag epitope (DYKDDDDK) followed by a hexahistidine sequence (HHHHHH) at the C-terminus of the expressed protein. Fig. 1 illustrates the construction procedure, which is described in further detail in the Methods section. Briefly, PCR was used to amplify a ~350 base pair DNA fragment that overlapped an upstream 5′ *SmaI* restriction site and replaced the original stop codon with a synthetic extension containing the Flag-His_6 coding sequence and a new stop codon followed by a 3′ *KpnI* site. A ligation reaction with a *PstI-SmaI* fragment of the original R-Sax or C-Sax DNA was then used to construct a pBluebac4 vector for production of the respective baculovirus vectors. The coding regions of the modified portions of the final R-Sax-FH_6 and C-Sax-FH_6constructions were fully sequenced to confirm that no errors were introduced.

Small scale stocks of R-Sax-FH_6 and C-Sax-FH_6 baculovirus were routinely produced by infecting SF9 cells with 0.1 pfu/cell and using this viral stock to produce the large-scale working stock for recombinant protein production. By the method of end-point dilution, the stock baculovirus titer for R-Sax-FH_6 and C-Sax-FH_6 was routinely found to be $\sim 7 \times 10^5$ pfu/ml. High Five™ cells were utilized for recombinant protein production, since they secrete larger quantities of saxiphilin than SF9 cells. Baculovirus was added at a multiplicity of infection of 5 pfu/cell to 1 l of High Five™ cells in mid-log phase. This culture was grown to a density of 2×10^6 cells/ml at 25°C with shaking at 150 rpm. After infection, aliquots of media were

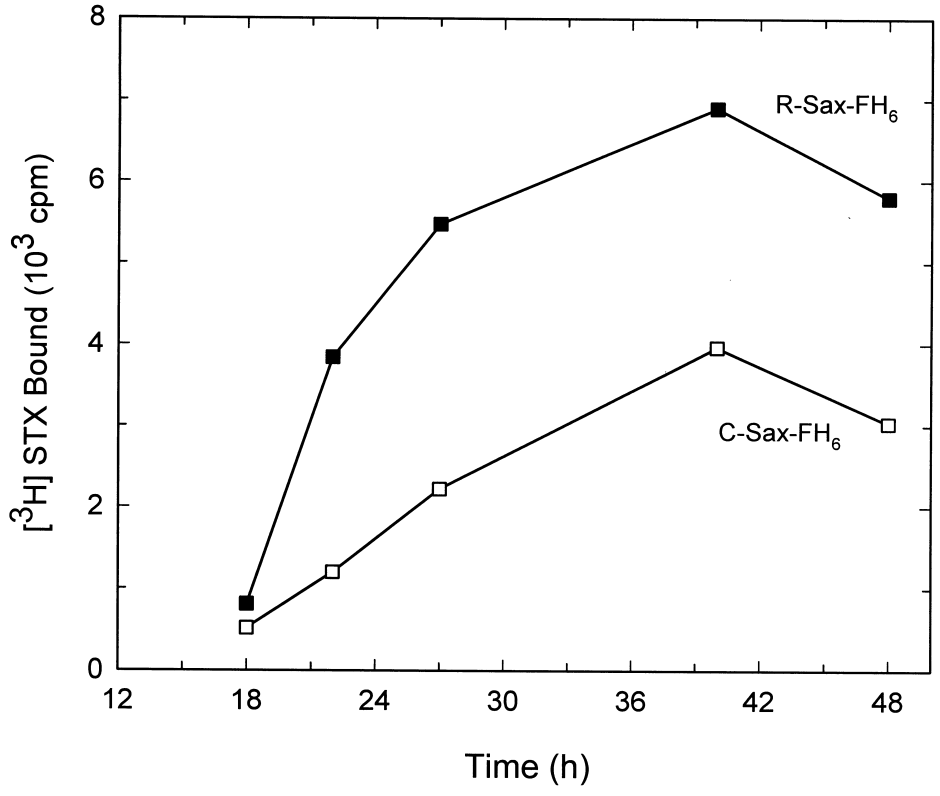

Fig. 2. Time course of expression and secretion of R-Sax-FH_6 and C-Sax-FH_6 by High Five™ cells. At various times after infection with baculovirus vector, 15 μl of the culture medium was assayed for [^{3}H]STX binding using a standard assay containing 5 nM [^{3}H]STX. Data points are corrected by subtraction of background cpm from duplicate samples measured in the presence of 10 μM unlabeled STX.

removed at various intervals and supernatant was prepared by centrifugation. [^{3}H]STX binding activity of these samples was measured to assess the time course of protein production. Fig. 2 shows the time course of increasing [^{3}H]STX-binding activity in the cell supernatant for typical cultures expressing R-Sax-FH_6 and C-Sax-FH_6. The optimal level of secreted saxiphilin activity occurred at 40 h post-transfection. Typical maximum levels of saxiphilin were 30 pmol/ml for R-Sax-FH_6 and 16.5 pmol/ml for C-Sax-FH_6 in the insect cell culture medium.

3.2. Purification of R-Sax-FH_6

In previous studies from our laboratory, native bullfrog and untagged recombinant saxiphilin were routinely purified by Heparin Sepharose chromatography followed by chromatofocusing and Sepharose gel filtration (Li and Moczydlowski, 1991; Llewellyn and Moczydlowski, 1994). The purification of Flag-His_6-tagged proteins described here is simpler and faster than this latter procedure since it eliminates the laborious chromatofocusing step and subsequent gel filtration procedure required for ampholyte removal. Fig. 3 shows a typical fractionation of R-Sax-FH_6 from concentrated baculovirus media by Ni^{2+}-NTA column chromatography. As this example illustrates, histidine-tagged proteins from crude mixtures can be readily purified by metal-chelate affinity chromatography using Ni^{2+}-NTA resin. However this procedure can also result in contamination with extraneous proteins that contain histidine rich regions or otherwise that bind to Ni^{2+}-NTA. In our case, several proteins from the insect cell media were found to co-purify with R-Sax-FH_6 on the Ni^{2+}-NTA column. Hence it was necessary to utilize an additional step of Heparin Sepharose chromatography for further purification. After this latter step, R-Sax-FH_6 was virtually homogeneous as shown in Fig. 4A, which compares purified samples of R-Sax-FH_6 and recombinant untagged saxiphilin on an SDS-PAGE gel stained with Coomassie blue. The overall yield of this purification procedure was ~20% of the original saxiphilin activity. The purified R-Sax-FH_6 preparation was also demonstrated to be homogenous by silver-staining. The molecular mass of R-Sax-FH_6 was ~2 kD larger than untagged saxiphilin due to the presence of the 19-residue Flag-His_6 tag. Purified R-Sax-FH_6 was used in subsequent [^{3}H]STX-binding experiments. C-Sax-FH_6 was partially purified using a single step of Ni^{2+}-NTA affinity chromatography. M2 anti-Flag monoclonal antibody was used to detect the Flag epitope on immunoblots. Both

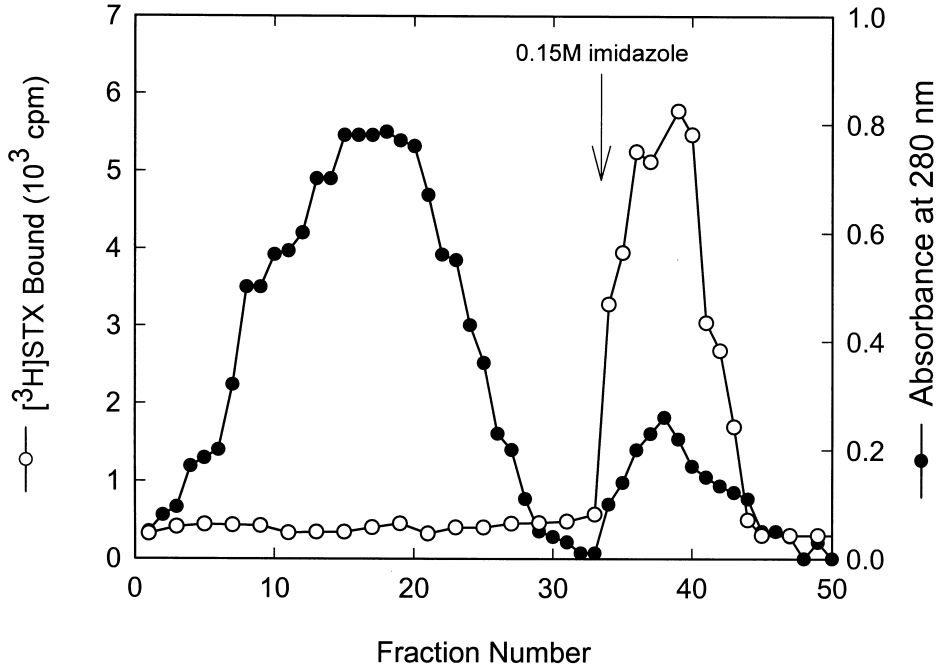

Fig. 3. Ni^{2+}-chelate affinity chromatography of R-Sax-FH_6 on Ni^{2+}-NTA resin. A sample of insect cell medium containing R-Sax-FH_6 was loaded on a column (2.0 × 15 cm) of Ni^{2+}-NTA resin. The column was washed with buffer containing 30% glycerol and 4 mM imidazole. R-SAX-FH_6 was eluted from the resin with 150 mM imidazole buffer. Fractions (4 ml) were assayed for [^{3}H]STX binding and protein absorbance at 280 nm.

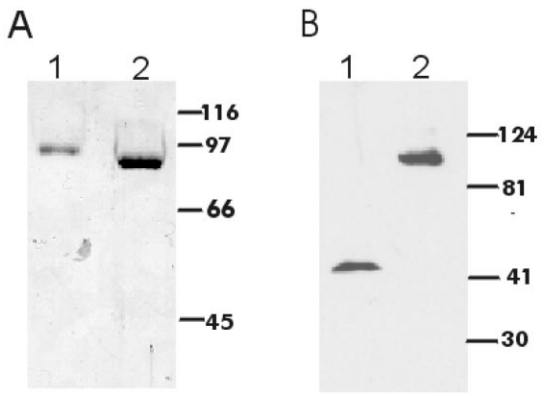

Fig. 4. Purification of R-Sax-FH_6 and immunodetection of Flag epitope. (A) SDS-PAGE of R-Sax-FH_6 purified using Ni^{2+}-NTA resin and Heparin Sepharose chromatography (lane 1) as compared with pure recombinant untagged saxiphilin (lane 2). Proteins were stained with Coomassie blue. (B) Immunoblot of partially purified C-Sax-FH_6 (lane 1) and pure R-Sax-FH_6 (lane 2). Proteins (0.5 μg) were subjected to SDS-PAGE followed by transfer to a nitrocellulose membrane. Flag-tagged proteins were detected by chemiluminescence using Anti-Flag M2 antibody. Positions of molecular weight markers (in kDa) are shown on the right.

R-Sax-FH_6 and C-Sax-FH_6 were specifically recognized by M2 antibody as 93 and 40 kD bands, respectively (Fig. 4B).

3.3. [^{3}H]STX binding to Flag-His_6 derivatives of saxiphilin

Fig. 5 shows results of equilibrium binding titrations of R-Sax-FH_6 and C-Sax-FH_6 with increasing concentrations of [^{3}H]STX. The insets are corresponding Scatchard plots for both proteins. For R-Sax-FH_6, K_D values were 1.0 ± 0.08 nM and 0.5 ± 0.03 nM from two separate experiments with a mean value of 0.75 nM. Corresponding K_D values for C-Sax-FH_6 binding were obtained as 1.58 ± 0.15nM, 2.07 ± 0.2 nM and 4.5 ± 0.45 nM from three separate experiments with a mean value of 2.7 nM. These K_D values for the Flag-His_6 tagged versions of saxiphilin and the C-lobe saxiphilin are approximately 3-fold higher than those measured for the corresponding untagged proteins (Morabito et al., 1995). The mean K_D for C-Sax-FH_6 is also approximately 3.5-fold higher than that of R-Sax-FH_6, similar to the 4-fold higher K_D previously observed for untagged C-Sax vs. R-Sax (Morabito et al., 1995). Measurements of the rates of [^{3}H]STX association and dissociation are shown in Fig. 6. Bimolecular association rate constants were 8.1×10^5 s^{-1} M^{-1} and 9.1×10^5 s^{-1} M^{-1} for R-Sax-FH_6 and C-Sax-FH_6, respectively. The rate constant for dissociation of [^{3}H]STX was measured by the rate of exchange of bound [^{3}H]STX with a large excess of unlabeled STX. The dissociation time course was fit to a single exponential decay with rate constants of 1.7×10^{-4} s^{-1} for R-Sax-FH_6 and 3.7×10^{-4} s^{-1} for C-Sax-FH_6. The dissociation rate of the tagged C-lobe is about 2.5-fold faster than tagged non-truncated version of saxiphilin. A similar difference was previously observed for untagged R-Sax and C-Sax (Morabito et al., 1995). K_D values calculated from the ratio of k_{off}/k_{on} were 0.2 and 0.4 nM for R-Sax-FH_6 and C-Sax-FH_6, respectively. The source of discrepancy between the equilibrium and kinetic measurements of K_D is unknown at present. It may be related to the difficulty of achieving true equilbrium for high-affinity ligand binding reactions. In any case, it is clear that [^{3}H]STX

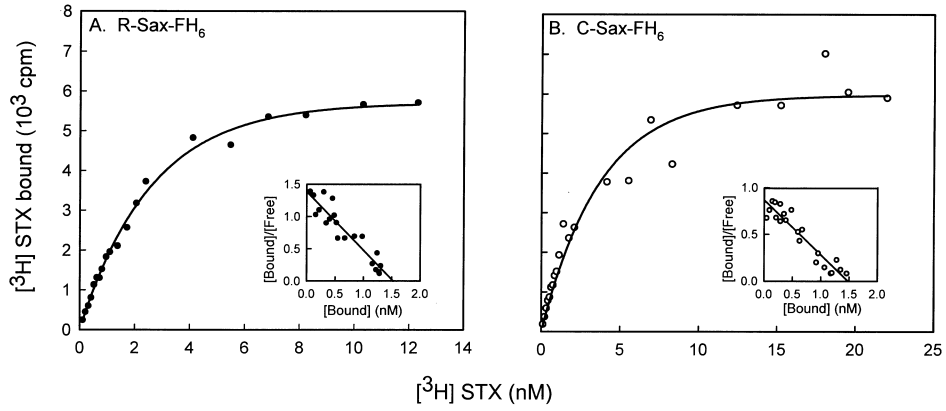

Fig. 5. Binding titration of pure R-Sax-FH_6 (A) and semi-pure C-Sax-FH_6 (B) with [^{3}H]STX. Plots show raw binding data with non-specific background subtracted. Insets represent the corresponding Scatchard plots. Data points are the mean of duplicate samples assayed in the absence and presence of 10 μM unlabeled STX for background subtraction. For the Scatchard plots the K_D was 0.75 nM for R-Sax FH_6 and 2.7 nM for C-Sax-FH_6.

K_D values for both proteins are in the low nanomolar range.

3.4. Microtitre plate assay of [^{3}H]STX binding and competition by STX

R-Sax-FH_6 binds to metal chelate affinity resins, such as the Ni^{2+}-NTA resin and Reacti-Bind™ Ni^{2+}-chelate plates, which are coated with the same functional groups. The binding of R-Sax-FH_6 to Ni^{2+}-chelate plates exhibited a sigmoidal binding curve when titrated with an increasing amount of protein as shown in Fig. 7A. The amount of R-Sax-FH_6 bound to the Ni^{2+} chelate plates can be determined from radioactivity of [^{3}H]STX bound to each well. Our calculations indicate that each well is capable of binding ~0.08 pmol of R-Sax-FH_6 at saturation. By using a constant amount of R-Sax-FH_6 and [^{3}H]STX in each well, unlabeled STX added to the reaction mixture in the range of 0–200 nM was found to compete with [^{3}H]STX binding in a concentration-dependent fashion. From titrations such as Fig. 7B, the amount of unlabeled STX in an unknown reaction mixture can be thus be estimated by assay of STX standards and unknown samples under identical conditions. The background signal of this assay method was also found to be very low, i.e., about 50 cpm/well measured in the absence of R-Sax-FH_6 or in the presence of excess unlabelled STX. This corresponds to $<2\%$ of the total cpm/well bound in the presence of a saturating amount of R-Sax-FH_6 and 5 nM [^{3}H]STX.

4. Discussion

This paper describes the construction of baculovirus vectors for expression of Flag-hexahistidine tagged saxiphilin (R-Sax-FH_6) and tagged C-lobe saxiphilin (C-Sax-FH_6) in High Five™ insect cells. Baculovirus expression vectors utilizing the original 19-residue frog secretory signal sequence at the saxiphilin N-terminus were previously found to result in efficient secretion of saxiphilin activity into the media from baculovirus-infected insect cells (Morabito et al., 1995). The Flag-hexahistidine coding sequence was therefore placed as an insertion at the C-terminus of the protein so as not to interfere with protein folding and proper secretion.

The presence of the Flag-hexahistidine tag on these recombinant STX-binding proteins greatly facilitates their initial purification from crude insect cell culture medium as shown here for R-Sax-FH_6 using Ni^{2+}-chelate affinity chromatography (Fig. 3). The Flag-epitope of the tagged proteins can also be exploited for immuno-affinity purification using commercially available anti-Flag antibodies covalently attached to a convenient chromatography matrix. As expected from the addition of a 19-residue tag, the R-Sax-FH_6 protein band migrates slightly slower than untagged recombinant saxiphilin on SDS-PAGE (Fig. 4A).

The kinetics of STX binding of R-Sax-FH_6 and C-Sax-FH_6 were compared with that of the untagged saxiphilins. The [^{3}H]STX association rates of R-Sax-FH_6 and C-Sax-FH_6 were about 2-fold lower than the corresponding untagged versions of saxiphilin, which may be due to the presence of the Flag-hexahistidine tag

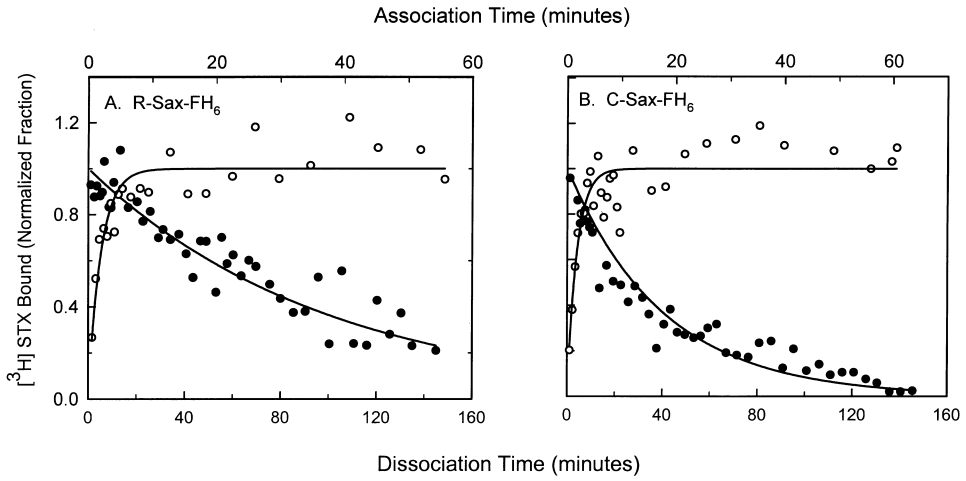

Fig. 6. Association and dissociation kinetics of [^{3}H]STX binding to pure R-Sax-FH_6 (A) and semi-pure C-Sax-FH_6 (B). Association rate data (○) measured in the presence of 10 nM [^{3}H]STX were normalized to the equilibrium level of binding and fit using a pseudo first-order rate constant of 0.0082 s^{-1} for R-SAX-FH_6 and 0.0095 s^{-1} for C-SAX-FH_6, (top horizontal axis). Dissociation rate data (●) were normalized to [^{3}H]STX bound before the addition of excess unlabeled STX and fit to a first-order rate constant of 1.68×10^{-4} s^{-1} for R-Sax-FH_6 and 3.7×10^{-4} s^{-1} for C-SAX-FH_6 (bottom horizontal axis).

sequence. However, the dissociation rate of [^{3}H]STX from R-Sax-FH$_6$ is about the same as that of untagged saxiphilin. C-Sax-FH$_6$ exhibited a faster dissociation rate (3.7×10^{-4} s^{-1}) than that of R-Sax-FH$_6$ (1.7×10^{-4} s^{-1}), but slower than that of untagged C-lobe saxiphilin (7.0×10^{-4} s^{-1}) (Morabito et al., 1995). Despite these kinetic differences, the equilibrium binding affinity of the Flag-His$_6$ derivatives of saxiphilin for STX is in the nanomolar to subnanomolar range. The STX dissociation kinetics of these proteins are slow enough to permit a wide range of manipulations such as binding and concentration of STX from crude biological extracts and isolation of STX from tagged saxiphilin attached to solid matrices. In this regard,

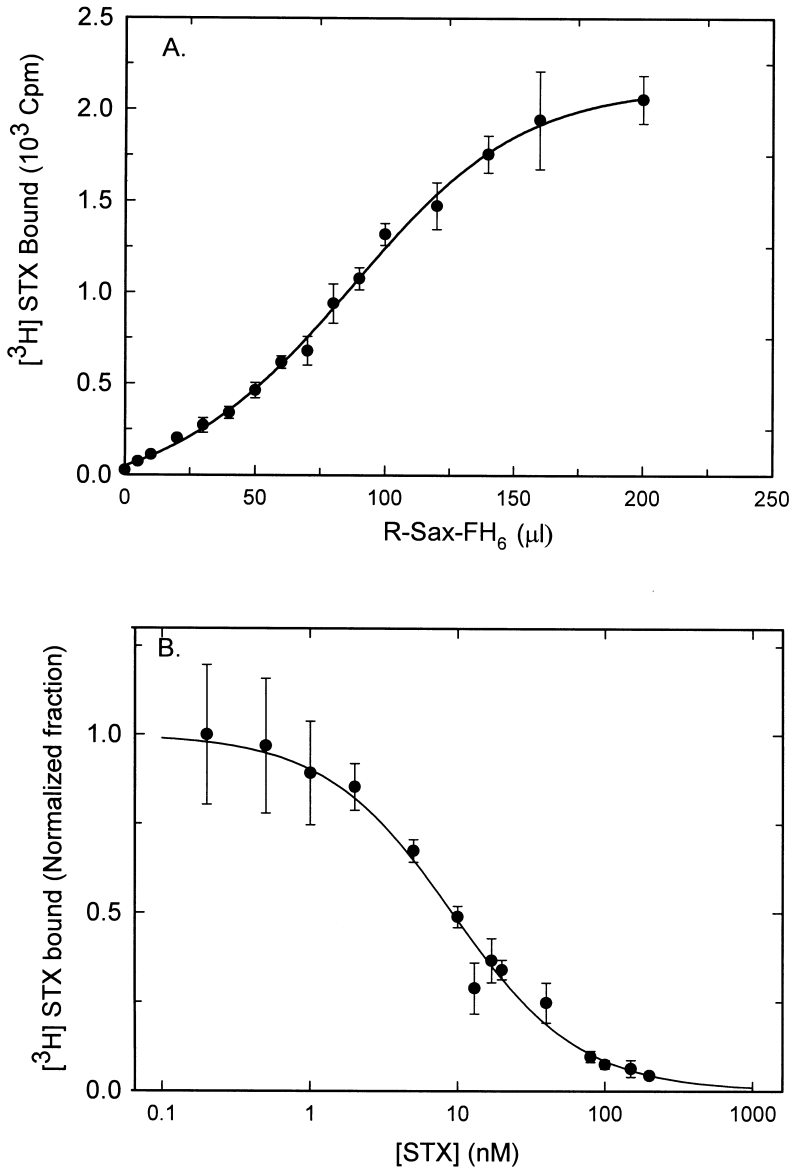

Fig. 7. Solid Phase assay of R-Sax-FH$_6$ and displacement of [^{3}H]STX binding to Reacti-bind™ metal chelate plates by unlabeled STX. (A) 5 nM [^{3}H]STX in a 200 µl reaction mixture containing 25 mM Hepes-NaOH, 150 mM NaCl, pH 8.0, was titrated with increasing amounts of R-Sax-FH$_6$ in Reacti-bind™ metal chelate wells and incubated on ice for a period of 3 h. [^{3}H]STX bound to the wells was counted after washing the wells three times with ice-cold 100 mM Tris–HCl buffer, pH 8. (B) Competition of [^{3}H]STX binding to R-Sax-FH$_6$ in Reacti-bind™ metal chelate plate wells by unlabeled STX. A fixed amount of R-Sax-FH$_6$ with 5 nM [^{3}H]STX in the standard incubation mixture was titrated with an increasing concentration of unlabeled STX.

the pH-dependence of STX dissociation from saxiphilin (Llewellyn and Moczydlowski, 1994) can be utilized to elute free STX from saxiphilin at low pH.

R-Sax-FH_6 and C-Sax-FH_6 were recognized on a protein blot, by anti-Flag M2 antibodies (Fig. 4B). We also found that M2 antibody used in conjunction with secondary fluorescein-labeled IgG can be used to identify baculovirus-infected insect cells that secrete Flag-tagged saxiphilin by fluorescence microscopy (unpublished results). In addition, the hexahistidine tag on these proteins can be effectively utilized to immobilize STX-binding sites on Reacti-bind™ Ni^{+2}-chelate microtiter plates, a procedure that could ultimately be adapted to develop a rapid quantitative assay for STX in algae or shellfish extracts. The initial application of this solid phase assay (Fig. 7) exhibited a low background of non-specific [^{3}H]STX binding ($<2\%$) and a sensitivity in the range of 1–50 nM corresponding to $\sim$20–80% displacement of 5 nM [^{3}H]STX. In previous work, native bullfrog saxiphilin was shown to bind many other natural derivatives of STX with affinities ranging from 0.1 to 170 nM (Mahar et al., 1991). Assuming that Flag-His_6-tagged versions of saxiphilin retain this broad specificity for numerous STX derivatives, the solid-phase assay introduced here may prove useful for identifying neurotoxic samples collected in connection with dinoflagellate or cyanobacteria blooms.

Saxiphilin-like STX binding activity has also been identified in a number of distantly related animal species, such as *Rana catesbiana* (North American bullfrog), *Bufo marinus* (cane toad), *Naja naja kaouthia* (Thailand cobra), *Thamnofis sirtalis* (garter snake), *Gambusia affnis* (mosquito fish), and *Ethmostigmus rubripes* (Australian centipede) (Llewellyn et al., 1997). However, only a single cDNA sequence of saxiphilin from *Rana catesbiana* has so far been determined (Morabito and Moczydlowski, 1994a). Further investigation and cloning of saxiphilin from diverse animal species may provide biochemical access to recombinant STX-binding proteins that allow enhanced expression and even more favorable properties for applications in applied and basic research.

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