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Expression and purification of soluble E-Syt2: Low protein stability impedes tag removal

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ABSTRACT

Affinity tags are valuable tools for high-throughput protein isolation in automated screenings or downstream processing approaches and are also widely used in laboratory applications for quick and easy access to many proteins. Here, we describe the preparative purification of soluble extended synaptotagmin 2 (rE-Syt2) at bench scale for basic structural and functional studies. Due to the low protein stability, a classical purification procedure without affinity tag was more powerful than isolation of $His_{(6)}$-tagged rE-Syt2 and subsequent proteolytic tag-removal. Furthermore, expression analysis of truncated rE-Syt2 variants suggested a concept of interdependent-domain organization in proteins containing multiple C2 domains.

1. Introduction

After genome sequencing, a tremendous number of genes encoding for proteins with yet undefined structure and/or function await characterization in industrial and academic science. In high-throughput approaches several structural genomics centers as well as pharmaceutical institutions aim to identify proteins, which are easily accessible using recombinant expression systems and standardized protocols [1]. For comprehensive protein isolation strategies, the use of affinity tags has proven very beneficial in saving time, costs and effort [2]. In this context, the hexahistidine-tag ($His_{(6)}$-tag) emerged as a popular tool to facilitate purification of different proteins independent of their individual properties [3,4]. In automated settings using 96-well formats, expression and solubility of numerous tagged target proteins can be tested at the same time [5] and large numbers of individual proteins or libraries of mutated candidates can be isolated for functional screenings [6,7].

Since plenty of affinity tags and systems for tag removal are commercially available [8,9], tagged strategies have also become generic procedures for protein preparation at laboratory scale. Although the use of tags is well established to simplify the preparation of recombinant proteins, removal of tags is desirable for most clinical and structural applications to prevent artificial influence of the engineered region on protein activity. Even the relatively short $His_{(6)}$-tag was reported to influence binding properties or the structure of some proteins [10,11]. However, limitations in proteolytic tag removal are frequently encountered including inefficient proteolysis and non-specific secondary cleavage sites [3,4]. Furthermore, the scissile bond may be buried in the target protein making it inaccessible for the protease applied. Unfortunately, constraints of the methodology are difficult to predict in advance for a given protein of interest and both optimization of cleavage conditions and substitution of the protease utilized can be very time-consuming. Nevertheless, the "old-fashioned" alternative to isolate a protein solely by its biochemical and physical properties is mostly abandoned in favour of affinity-tagged approaches promising high yields using standardized protocols with moderate requirements of optimization [2,9].

Here, we compare a tag-based versus a tag-free strategy for the purification of recombinant extended synaptotagmin-like protein 2 (rE-Syt2). The new protein family of E-Syts was recently introduced [12,13] with E-Syt2 containing at least one transmembrane domain and three C2 domains. Detailed knowledge on structure and function of human and murine C2 motifs was acquired from studies of isolated recombinant C2 domains expressed in *E. coli* [14–16]. The intracellular localization of proteins containing C2 domains (C2 proteins) can be modulated by virtue of the specialized lipid binding properties of their C2 motifs directing these proteins to particular membranes [17,18]. For many C2 domains, phospholipid binding is calcium dependent [19,20] rendering these C2 proteins sensitive to one of the most important second messengers in eukaryotic

Abbreviations: CD, circular dichroism; E-Syts, extended synaptotagmin-like proteins; GdmCl, guanidinium chloride; MCTPs, multiple C2 domain and transmembrane region proteins; PKC, protein kinase C; SEC, size exclusion chromatography; TM, transmembrane domain.

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cells. Most C2 proteins contribute to processes of cellular signal transduction such as protein kinase C [21], phospholipase A and C isoforms [22–24] or play a role in membrane trafficking as described for synaptotagmins (Syts, [25]). With the exception of synaptotagmins proteins containing more than one C2 domains (multi-C2 proteins) are only sparsely characterized and the functional context, in which E-Syts act also remains to be elucidated [12,26]. Furthermore, most biochemical studies on multi-C2 proteins focus on single C2 domains rather than taking into account the whole C2 content of the respective proteins. Among the few examples describing tag-free multi-C2 proteins with all inherent C2 domains are reports on proteins comprising merely two C2 domains, such as Syt 1 [27–29], Syt 3 [16] or Copine [30,31]. In contrast, our work on the structural characterization of E-Syt2 [13] is to our knowledge the first and only description of a purified protein without affinity tag exhibiting three C2 domains.

In the present paper, we describe the isolation of tag-free rE-Syt2 in detail and provide to our knowledge the first protocol for the purification of a multi-C2 protein containing three C2 domains. Homogenous preparation of a $His_{(6)}$-tagged version not only involved nickel affinity chromatography, but also required two ion exchange chromatography steps. Subsequent tag removal was unrewarding due to unexpected limitations of a well established protein purification system. In contrast, the successful preparation of untagged rE-Syt2 using standard chromatography proved to be equally time-consuming and highlighted the efficiency of conventional strategies. Additionally, for the first time we observed a rather low thermal and chemical stability for rE-Syt2 which might further impede the tag-cleavage steps. Furthermore, to consider the possible interdependence of tandemly repeated C2 domains *in vitro*, expression studies of truncation mutants were carried out, from which a concept of the mutual relationship between individual C2 domains of E-Syt2 was developed.

2. Experimental

2.1. Cloning

The E-Syt2 fragments for recombinant protein production were amplified by PCR using oligonucleotides as follows with restriction sites underlined. The forward primers were GCCTCGAGAAGGGTGTCCTAAGGA (His-rE-Syt2), GCTCATGAAGGGTGTCCTAAGGATTCACT (rE-Syt2), GCCTCGAGGCACTGCTGATCTTGT (His-rE-Syt2ΔC2A) and GCGATATCTGATTAATGCACTGCTGATCTTGTATTTG (His-rE-Syt2ΔC2AΔC2C). The reverse primers were AAACTCGAGCTACGTTATCACCTGTGGCCT (His-rE-Syt2, rE-Syt2, His-rE-Syt2ΔC2A) and ATCTCGAGCTATTGGGAGCTGTGCCGGAT (His-rE-Syt2ΔC2AΔC2C). Compatible cohesive ends were utilized where appropriate to insert purified DNA into the pET-15b expression vector (Novagen, Darmstadt, Germany) into NcoI/XhoI sites for untagged or NdeI/XhoI sites for $His_{(6)}$-tagged proteins. All constructs were confirmed by sequencing.

2.2. Recombinant protein expression

For production of recombinant E-Syt2 proteins, *E. coli* BL21(DE3) transformed with one of the cloned expression plasmids was cultivated at 25 °C using a preculture for inoculation, which was grown over night in the presence of 1% (w/v) glucose. At an OD_{600} of 0.8, bacteria were induced with 1 mM IPTG and harvested after 14 h in culture by centrifugation at 3000 × *g* and 4 °C for 15 min. Pellets were resuspended in lysis buffer (25 mM phosphate, 5 mM β-mercaptoethanol, 0.1% (w/v) CHAPS, pH 8.0) supplemented with protease inhibitors (complete EDTA-free, Roche, Mannheim, Germany) and disruption of bacteria was carried out using a French pressure cell (SLM instruments, Urbana, USA) in three repeat cycles at 33,000 psi. The obtained lysates were pulse-sonicated (Sonifier B-12, Branson, Danbury, USA) three times for 10 s and cleared by centrifugation at 20,000 × *g* and 4 °C for 30 min. Supernatants were applied to column chromatography as described in the results section.

2.3. Chromatography

All chromatographic separations were performed using an ÄKTA basic-system (GE Healthcare, Uppsala, Sweden) at flow rates of 1 ml/min for ion exchange and affinity matrices and 0.75 ml/min for gel filtration. Absorption at 280 nm was monitored to detect proteins in the flow. The column types (all from GE Healthcare, Uppsala, Sweden) used for protein purification are listed below with the respective buffer conditions specified in parentheses. His-rE-Syt2: HisTrap SP, 5 ml (lysis buffer); Resource Q, Resource S, 1 ml (25 mM phosphate, 2 mM DTT, 0.1% (w/v) CHAPS, pH 8.0). rE-Syt2: HiTrap SP FF, 5 ml (lysis buffer); Resource Q, Resource S, 1 ml (25 mM phosphate, 4 mM DTT, 0.1% (w/v) CHAPS, pH 6.5); Superdex 75 HiLoad 16/60 pg, 120 ml (20 mM Tris, 4 mM DTT, 300 mM NaCl, pH 8.0). Details on gradients applied are given in the respective elution profiles.

2.4. Circular dichroism spectroscopy

The thermal unfolding transition of structural elements was determined by Far-UV-CD spectroscopy, using a Jasco J-715 spectropolarimeter (Jasco, Großumstadt, Germany) equipped with a PTC 343 peltier unit. The experiments were carried out in quartz cuvettes with 0.02 cm pathlength at a protein concentration of 0.3 mg/ml. Thermal melting was recorded following the decrease in CD signal at 218 nm in 20 mM Tris, 4 mM DTT, pH 8.0 with a constant heating rate of 30 °C/h. Far UV-spectra were recorded from 200 to 260 nm in 5 °C steps. To test the influence of calcium on the thermal stability of rE-Syt2, 10 mM Ca^{2+} was added to the protein samples during thermal unfolding. For better comparability the fractional change of the structural content was plotted, normalizing the native samples to 100% folded.

2.5. Fluorescence spectroscopy

GdmCl-induced unfolding of rE-Syt2 was monitored by following its fluorescence at 340 nm. Samples of rE-syt2 (end concentration 0.05 mg/ml) were incubated at 16 °C in 20 mM Tris, 4 mM DTT, pH 8.0 and varying concentrations of GdmCl for 16 h. Fluorescence spectra of the respective protein samples were recorded in 10 mm cells in a Spex III (Jobin Yvon, Grabrun, Germany) fluorimeter at an excitation wavelength of 280 nm and scanning the emission from 300 to 450 nm. Buffer samples without protein were used for correction of all individual spectra. The change of the fluorescence signal at 340 nm was used to determine the loss in structure in dependence of the GdmCl concentration with the signal of native rE-Syt2 set to 100% folded. For measurements of emission spectra, fluorescence intensities were normalized to the maximum signal of native rE-Syt2.

2.6. Electrophoresis

Protein samples were analyzed by SDS-PAGE according to [32] using 12% slab gels in a SE 260 apparatus (Hoefer, San Francisco, USA) run at a constant current of 30 mA per gel. For Coomassie staining, gels were incubated in 50% (v/v) ethanol, 7% (v/v) acetic acid, 0.0625% Coomassie brilliant blue R-250 (Sigma, Schnelldorf, Germany) for 30 min and background was destained over night in 7% (v/v) acetic acid. For quantification of protein purity the ImageJ

software package was utilized [33] to determine the signal intensity of rE-Syt2 bands as a percentage of the total signal detected per lane.

2.7. Western blotting

Proteins were transferred from SDS-gels to a PVDF membrane (Schleicher & Schuell, Dassel, Germany) by electroblotting in 50 mM Tris, 40 mM glycin, 20% (v/v) methanol, 1% (w/v) SDS, pH 9.2 at 1.5 mA/cm^2 for 18 Vh using a semi-dry apparatus (Cti, Idstein, Germany). Blots were treated with blocking buffer (5% milk powder in 10 mM Tris, 100 mM NaCl, 0.05% (v/v) Tween-20, pH 7.4) for 1 h and then incubated over night with an anti-His-tag antibody (His·Tag Monoclonal Antibody, Novagen, Darmstadt, Germany) diluted 1:1000 in blocking buffer. Then, the membranes were washed three times for 10 min with wash buffer (10 mM Tris, 100 mM NaCl, 0.05% (v/v) Tween-20, pH 7.4) and afterwards incubated for 2 h with HRP-conjugated secondary antibody (Dianova, Hamburg, Germany) in blocking buffer. Subsequently, the membranes were washed as described above and bands were visualized using the ECL Plus detection system (GE Healthcare, Munich, Germany) and a ChemiLux Imager (Intas, Göttingen, Germany) according to the manufacturer's instructions.

2.8. Dialysis and ultrafiltration

Buffers were exchanged by dialysis using ZelluTrans membranes (Roth, Karlsruhe, Germany, MWCO 10,000) according to the manufacturer's instructions. Precipitates were removed by centrifugation at 2800 × *g* and 4 °C for 10 min. Pooled fractions of pure His-rE-Syt2 or rE-Syt2 were dialyzed against 20 mM Tris, 4 mM DTT, pH 8.0 and concentrated in a Centricon YM-30 device (MWCO 30,000, Millipore, Eschborn, Germany) according to the manufacturer's recommendations.

2.9. Thrombin cleavage

For analytical thrombin cleavage, typically 10 μg $His_{(6)}$-rE-Syt2 were digested with 0.1 U of protease from two different sources (GE Healthcare, Munich, Germany and Novagen, Darmstadt, Germany). The reactions were carried out for different periods of time as indicated at 22 °C in PBS or in buffer supplied with the enzyme. To examine the kinetics of proteolysis, 500 μg $His_{(6)}$-rE-Syt2 in PBS, 2 mM DTT were incubated with 10 U of thrombin (GE Healthcare, Munich, Germany) at room temperature removing aliquots for SDS-PAGE at the time points specified.

2.10. Preparation of inclusion bodies

For isolation of recombinant protein deposited in inclusion bodies from IPTG-induced bacteria, bacterial protein extraction reagent (B-Per, Pierce, Rockford, USA) was utilized following the manufacturer's instructions.

3. Results

3.1. Expression and purification of His-rE-Syt2

The recombinant $His_{(6)}$-tagged construct His-rE-Syt2 covering all three C2 domains (C2A, C2B and C2C) of murine E-Syt 2 (Fig. 1A) was expressed in *E. coli*. Homogenous His-rE-Syt2 was prepared by column chromatography in three steps as summarized in Fig. 1. The progress of purification was monitored by SDS-PAGE starting from total lysates of induced bacteria (Fig. 1B), in which an additional band near the 66 kDa marker indicated the expression of the recombinant 63 kDa target protein compared to the non-induced culture. After mechanical disruption of the bacteria, the soluble fraction was obtained by centrifugation and loaded onto a nickel affinity column to capture His-rE-Syt2. Non-specifically bound proteins were eliminated in a step-gradient of 75 and 150 mM imidazole (Fig. 1C). His-rE-Syt2 was strongly bound to the matrix and eluted at an imidazole concentration of 300 mM. As shown by SDS-PAGE of the eluted fractions (Fig. 1D), the recombinant protein was efficiently separated from most contaminating proteins, but was not entirely free of impurities. After buffer exchange, the protein was applied to an anion exchange column, which removed several further contaminating proteins (Fig. 1E), but did not bind His-rE-Syt2. The flow-through was subsequently applied to cation exchange chromatography, where His-rE-Syt2 was bound to the resin and sharply eluted in a linear salt gradient (Fig. 1F) yielding highly pure His-rE-Syt2 as shown by SDS-PAGE (Fig. 1G). Pooled fractions were concentrated by ultrafiltration and the resulting His-rE-Syt2 preparation was 96% pure as determined by SDS-gel analysis (Fig. 1H). Typically, 5 mg His-rE-Syt2 were obtained per 12.5 g wet weight of bacterial cells.

3.2. Tag removal by thrombin cleavage

To prevent possible interference of the flexible fusion-tag with activity assays or structural studies of recombinant E-Syt2, the $His_{(6)}$-part of His-rE-Syt2 was intended to be removed proteolytically producing tag-free ΔHis-rE-Syt2. For this purpose, His-rE-Syt2 contained a thrombin cleavage site, which was unique in the protein sequence according to ExPASy's PeptideCutter software [34]. In initial screening experiments, thrombin from two different suppliers was used to digest purified His-rE-Syt2 analytically to test buffer conditions and incubation times. However, all enzyme treatments produced at least two fragments as deduced from SDS-PAGE analysis of the cleavage reactions (Fig. 2A). After protease treatment none of the obtained protein bands corresponded to the expected molecular weight of 61 kDa for ΔHis-rE-Syt2 indicating one or more unrecognized thrombin cleavage sites in His-rE-Syt2 or non-specific protease activity. To avoid proteolytic degradation at secondary sites by minimizing the temporal exposure of His-rE-Syt2 to thrombin, the progress of substrate cleavage was analyzed at various time points by SDS-PAGE (Fig. 2B). However, fragmentation of His-rE-Syt2 already occurred after 1 h incubation, whereas a control sample without thrombin showed no evidence for intrinsic proteolytic activity of rE-Syt2 indicating that the fragmentation of rE-Syt2 observed in the presence of thrombin is caused by the protease applied. Thrombin processing over night yielded two unexpected cleavage products whose molecular weights differed by more than 20 kDa from the sizes of ΔHis-rE-Syt2 and the cleaved $His_{(6)}$-tag. Altogether, proteolytic removal of the $His_{(6)}$-tag from His-rE-Syt2 using thrombin was not compatible with intrinsic properties of the recombinant protein requiring an alternative strategy for purification of tag-free rE-Syt2.

3.3. Preparation of tag-free rE-Syt2

For isolation of untagged rE-Syt2, a four-step chromatography procedure was established (Fig. 3) on the basis of the physical and biochemical properties of the recombinant protein at different pH levels without taking advantage of specific high-affinity tags. Expression of the tag-free construct in *E. coli* was carried out as described for the $His_{(6)}$-tagged version. Production of rE-Syt2 (60 kDa) in induced cultures was confirmed by SDS-PAGE of whole bacterial lysates (Fig. 3A), in which expression of the recombinant protein gave an additional band that was absent in the non-induced culture below the 66 kDa marker. Bacteria were disrupted by French press treatment and the cleared lysate was loaded onto a cation exchange column, from which bound material was eluted with a gradient up to 1 M NaCl in lysis buffer (Fig. 3B). Suitable fractions

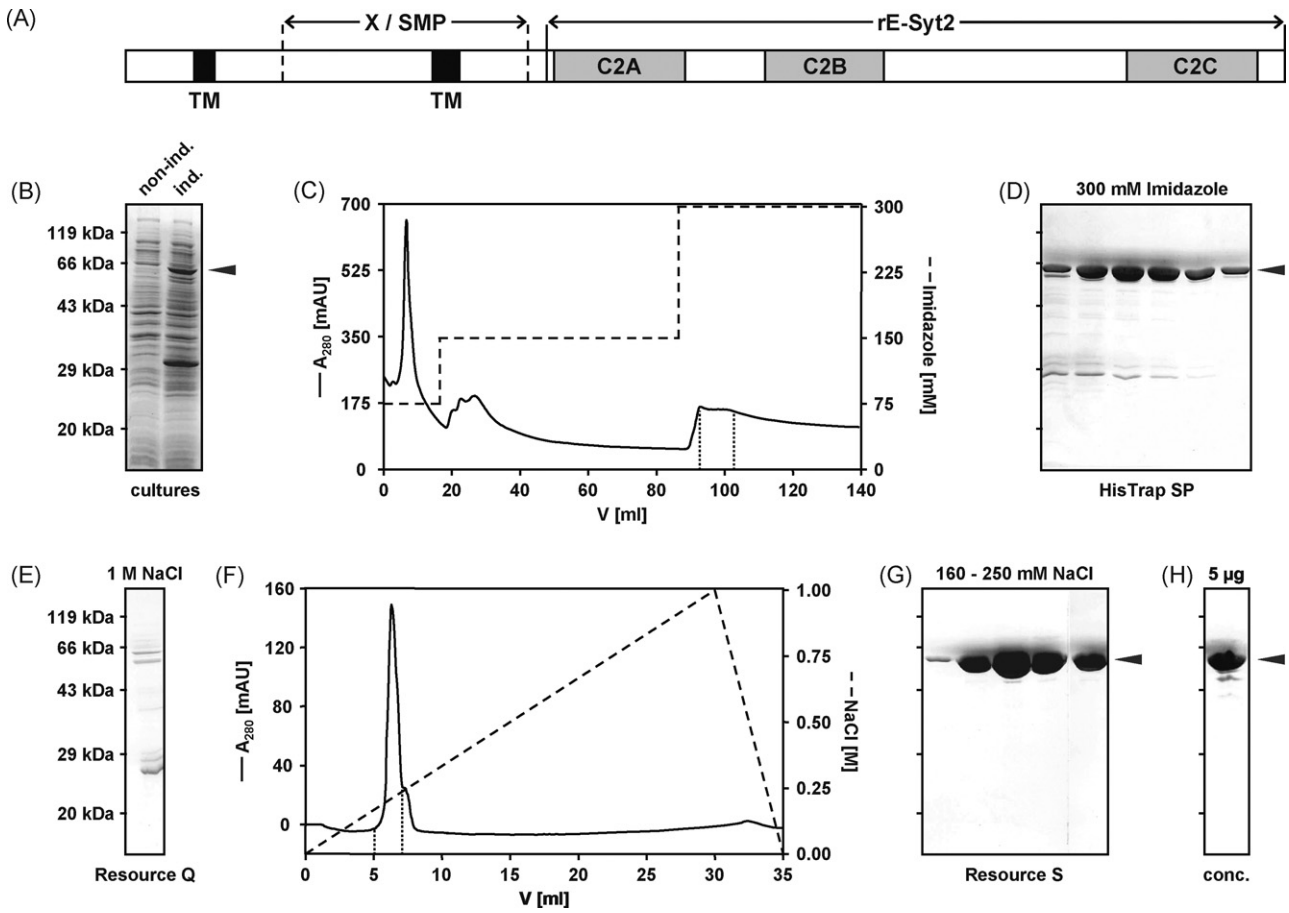

Fig. 1. Purification of His-rE-Syt2.
Murine E-Syt2 (A) is a transmembrane protein containing three C2 domains and includes a putative X or SMP domain [12,49]. A $His_{(6)}$-tagged recombinant version of soluble E-Syt2 (His-rE-Syt2) comprising the complete C2 region of the protein was isolated from induced *E. coli* cultures (B). His-rE-Syt2 was captured from bacterial lysates using a nickel affinity matrix (C, solid line: absorption at 280 nm, dashed line: imidazole gradient) and collected fractions (C, dotted lines) were subjected to SDS-PAGE (D) showing efficient enrichment of His-rE-Syt2 (63 kDa, marked with an arrow). An anion exchanger removed several contaminating proteins (E) and His-rE-Syt2 found in the flow-through was applied to a cation exchange column (F, dashed line: NaCl gradient). Fractions of the major peak (F, dotted lines) contained His-rE-Syt2 (G) and pooled concentrated fractions yielded a 96% pure His-rE-Syt2 solution, as shown by SDS-PAGE (H).

(Fig. 3C) were pooled, desalted by dialysis and applied to an anion exchanger. Similar to His-E-Syt2, several contaminating proteins bound to this matrix (Fig. 3D), whereas rE-Syt2 was present in the flow-through, which was loaded onto a second cation exchange column. Bound protein was eluted using a salt gradient (Fig. 3E) and pooled fractions (Fig. 3F) were buffer-exchanged. The dialysate was concentrated and further applied to a size exclusion chromatography column for final separation of impurities (Fig. 3G). Pooled fractions containing isolated rE-Syt2 (Fig. 3H) were dialyzed and a diluted sample of this protein solution was analyzed by SDS-PAGE

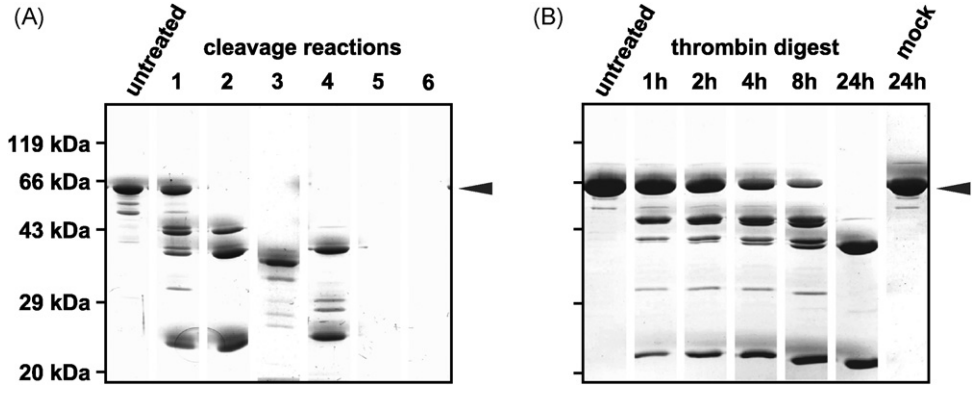

Fig. 2. Proteolytic removal of affinity tag from His-rE-Syt2.
Isolated His-rE-Syt2 was digested with thrombin from two different sources under various conditions to remove the $His_{(6)}$-tag from the recombinant protein. In most cleavage reactions multiple fragments were detected by SDS-PAGE (A) however, the expected 61 kDa product ΔHis-rE-Syt2 was not generated (arrow). Lanes: 1 = suppl. G, PBS, 16 h; 2 = suppl. N, PBS, 16 h; 3 = suppl. N, provided cleavage buffer, 16 h; 4 = suppl. G, PBS, 3 d; 5 = suppl. N, PBS, 3 d; 6 = suppl. N, provided cleavage buffer, 3 d. Kinetic analysis (B) demonstrated, that His-rE-Syt2 was already fragmented after one hour of thrombin treatment (suppl. G, PBS, 2 mM DTT) and prolonged digestion produced two bands of approximately 38 and 22 kDa.

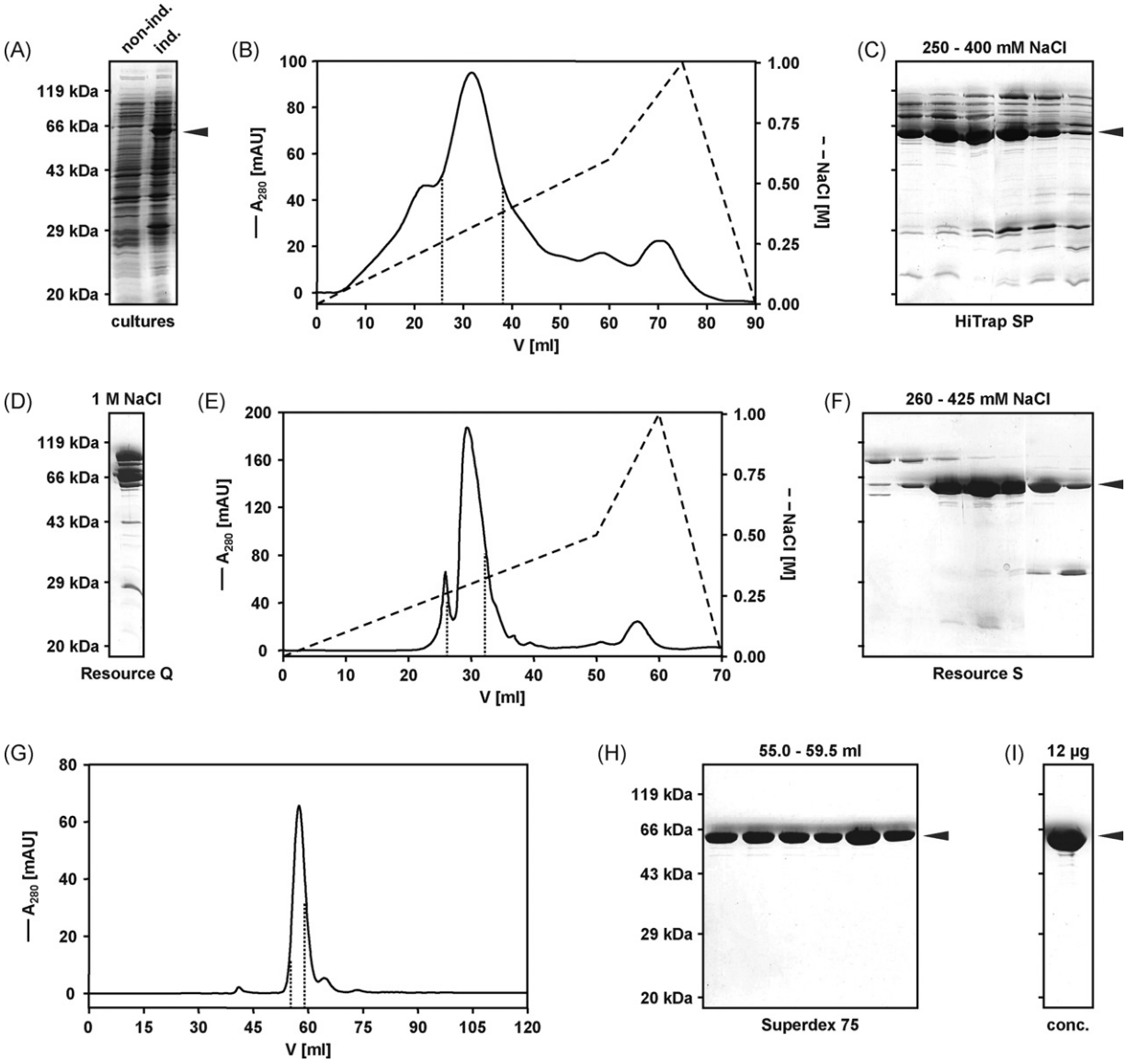

Fig. 3. Isolation of tag-free rE-Syt2.
Tag-free rE-Syt2 was prepared from lysates of induced *E. coli* cultures (A) using a cation exchanger to capture the recombinant protein (B, solid line: absorption at 280 nm, dashed line: NaCl gradient). The fractions from the main peak (B, dotted lines) were analyzed by SDS-PAGE (C) showing enrichment of protein at the molecular size of rE-Syt2 (arrow). Pooled fractions were buffer-exchanged and loaded onto an anion exchanger removing contaminants (D) without binding rE-Syt2. In subsequent cation exchange chromatography of the flow-through (E, solid line: absorption at 280 nm, dashed line: NaCl gradient), the target protein was further purified as illustrated by SDS-PAGE (F) of collected fractions (E, dotted lines). After pooling and buffer exchange, the remaining contaminants were separated in a final gel filtration step (G). SDS-PAGE of fractions (G, dotted lines) confirmed that the rE-Syt2 preparation was homogenous (H) and analysis of a diluted sample of concentrated protein solution (I) demonstrated a purity of 99%.

(Fig. 3I) demonstrating 99% purity of the preparation. Altogether, processing of 11.0 g of wet bacterial pellet yielded 4 mg of purified rE-Syt2 protein.

3.4. Thermal and chemical stability of rE-Syt2

A prominent parameter possibly promoting the degradation of rE-Syt2 during tag removal by thrombin is the intrinsic stability of the protein. The chances for a protease to recognize non-specific or alternative cleavage sites in a rather unstable protein are usually higher than in stable proteins thus increasing the risk of non-specific protein degradation instead of tag removal. Additionally, the stability of E-Syt2 is another biochemical parameter that has not yet been characterized. Therefore, we measured the thermal as well as the chemical stability of rE-Syt2 (Fig. 4). The temperature-induced loss of structure was monitored by CD spectroscopy at 218 nm giving a prominent thermal unfolding in the range from 37 to 53 °C with a midpoint at 43 °C (Fig. 4A). This process was completely irreversible as further highlighted by visible aggregation of the protein at temperatures above 50 °C. Recently, we were able to demonstrate that rE-Syt2 is a calcium binding protein forming higher oligomers in the presence of calcium [13]. To investigate the influence of calcium on the thermal unfolding of rE-Syt2, we analyzed its thermal transition in the presence of 10 mM calcium (Fig. 4A). Interestingly, the transition shifted to lower temperatures (midpoint at 37 °C), indicating a destabilizing effect of calcium. To characterize rE-Syt2 stability not only against elevated temperatures, but also against denaturing agents, the intrinsic tryptophane fluorescence of the protein was followed in dependence of rising concentrations of GdmCl. As demonstrated in Fig. 4B, rE-Syt2 proofed to be a rather labile protein with an unfolding transition between 0.2 and 2.0 M GdmCl (midpoint at 1.0 M GdmCl). Again, the unfolding process was irreversible and strong, visible aggregation was observed in the denatured samples. The presence of

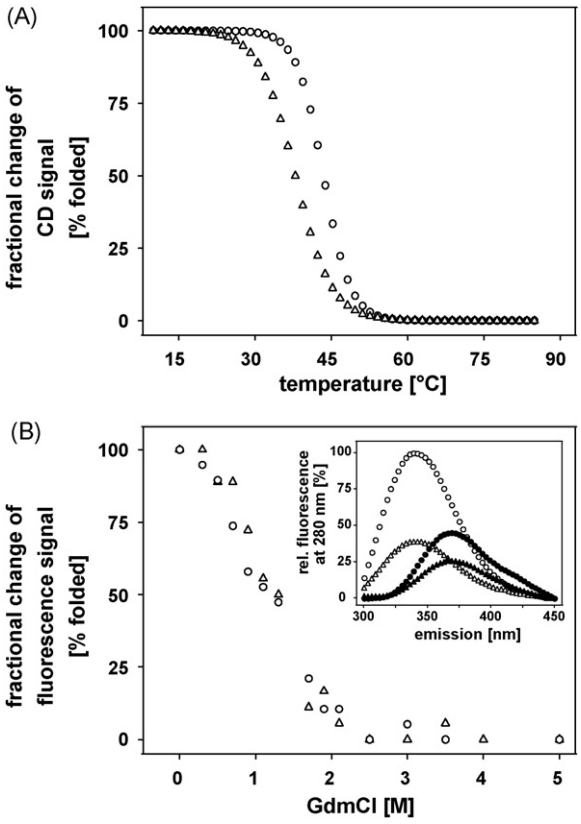

Fig. 4. Stability of rE-Syt2.
Temperature-induced unfolding of E-Syt2 was measured by CD spectroscopy of rE-Syt2 (A) in presence (△) and absence (○) of 10 mM calcium. Midpoints of transition from folded to denatured protein were detected at 37 °C and 43 °C, respectively. Chemical unfolding of rE-Syt2 was induced by GdmCl (B) in presence (△) and absence (○) of 10 mM calcium and monitored by fluorescence spectroscopy. The midpoint of transition was observed at 1.0 M GdmCl. *Inset*: comparison of fluorescence emission spectra of native (○) and denatured (●) rE-Syt2 as well as native (△) and denatured (▲) E-Syt2 in presence of 10 mM calcium.

calcium did not further destabilize the protein in a significant manner (Fig. 4B). Additionally, the intensity of the intrinsic fluorescence of rE-Syt2 was quenched in the presence of calcium (Fig. 4B, inset). Taken together, rE-Syt2 appears to be a rather unstable protein which is most likely the basis for its vulnerability to proteolytic degradation by thrombin at secondary sites during tag removal.

3.5. Interdependence of C2 domains of E-Syt2

To analyze the contribution of individual C2 domains to calcium and lipid binding properties of E-Syt2, which will be further explored in functional studies, recombinant fragments lacking one or two C2 domains were cloned and tested for soluble expression in *E. coli*. As depicted in Fig. 5A, His-rE-Syt2ΔC2A was generated by deletion of the N-terminal C2 domain (C2A) and further removal of the C-terminal C2 domain (C2C) gave His-rE-Syt2ΔC2AΔC2C. All constructs contained a $His_{(6)}$-tag allowing to assess and compare feasibility of purification of the respective proteins by affinity chromatography using a nickel matrix. The protocol for expression of His-rE-Syt2ΔC2A (45 kDa) and His-rE-Syt2ΔC2AΔC2C (32 kDa) in *E. coli* was adopted from the production of His-rE-Syt2 and rE-Syt2. For both truncated constructs additional bands of the expected molecular weight were observed in Coomassie-stained SDS-gels (Fig. 5B) of induced cultures compared to non-induced controls. Corresponding $His_{(6)}$-tag signals were detected by Western blot analysis (Fig. 5C) confirming the presence of recombinant proteins in bacteria. Lysates were prepared as specified for His-rE-Syt2 and rE-Syt2 and the soluble fraction was loaded onto a nickel affinity column. Elution of bound proteins was carried out as described for His-rE-Syt2 utilizing a step gradient of imidazole, however, neither His-rE-Syt2ΔC2A nor His-rE-Syt2ΔC2AΔC2C could be detected in the collected fractions by SDS-PAGE (data not shown). Instead, both deletion mutant proteins were exclusively found in inclusion bodies, which were prepared from induced cultures and analyzed by SDS-PAGE (Fig. 5B). In contrast, only His-rE-Syt2 was partially contained in cleared bacterial lysates (Fig. 5C) suggesting that solubility of E-Syt2 variants is severely hampered in the absence of at least one C2 domain.

From these results, a model was constructed describing the interdependence of the C2 domains of E-Syt2. As illustrated in Fig. 5D, the three C2 domains could be arranged in a way that hydrophobic surfaces of each domain are directed towards the interior of rE-Syt2 interconnecting the domains and maintaining their relative orientation. Thereby, a compact structure is likely to be formed by the C2 domains of E-Syt2 rather than separate motifs connected in series and removal of domains could expose interdomain contact areas leading to non-specific aggregation and insolubility.

4. Discussion

The majority of recombinant proteins prepared for functional or structural studies contain at least one affinity tag for ease of purification. Numerous different systems were established to provide fusion partners for efficient enrichment of protein constructs from complex mixtures [8,9,35]. For purification of recombinant E-Syt2 variants, one of the most commonly used strategies was employed. A $His_{(6)}$-tag was attached to the N-termini of target proteins allowing isolation of His-rE-Syt2 by immobilized metal affinity chromatography (IMAC). The tagged strategy demonstrated the high performance of IMAC in the extraction of His-rE-Syt2 from induced bacterial lysates. Nevertheless, two further separation steps were necessary to isolate the $His_{(6)}$-tagged version of rE-Syt2. Untagged rE-Syt2, however, was purified to homogeneity on the basis of its biochemical and physical properties without an affinity matrix in just one more chromatography step. This approach gained advantage over the tagged strategy, which additionally requires further processing of the isolated protein such as removal of the protease utilized, separation of cleaved $His_{(6)}$-tag and residual fusion protein assuming successful proteolysis. However, the tag-assisted approach already failed because of difficulties experienced during thrombin digestion making all subsequent steps inapplicable. Aiming to produce tag-free ΔHis-rE-Syt2, protease treatment caused fragmentation of His-rE-Syt2 under various conditions presumably by scission at unpredicted recognition sites that were accessible due to the low stability of soluble E-Syt2. Secondary cleavage may be enhanced in exposed regions of E-Syt2 susceptible to thrombin treatment, as enzyme activity is critically influenced by the chemical properties of the microenvironment of arginine or lysine positions [36] rather than depending on a defined recognition sequence. The problem of non-specific thrombin cleavage during removal of affinity tags has been widely discussed [2,37–39] and several previous studies report on the inappropriate fragmentation or even degradation of different target proteins [40–45]. Therefore, automated identification of thrombin recognition sites in primary sequences is limited and may also account for other proteases frequently used for processing of fusion proteins such as enterokinase or factor Xa. The problem of non-specific cleavage is further highlighted by the inconsistent pattern of bands observed in thrombin cleavage reactions even for the same protease under different buffer conditions, which impaired the identification of the respective non-specific thrombin recognition sites. As a conse-

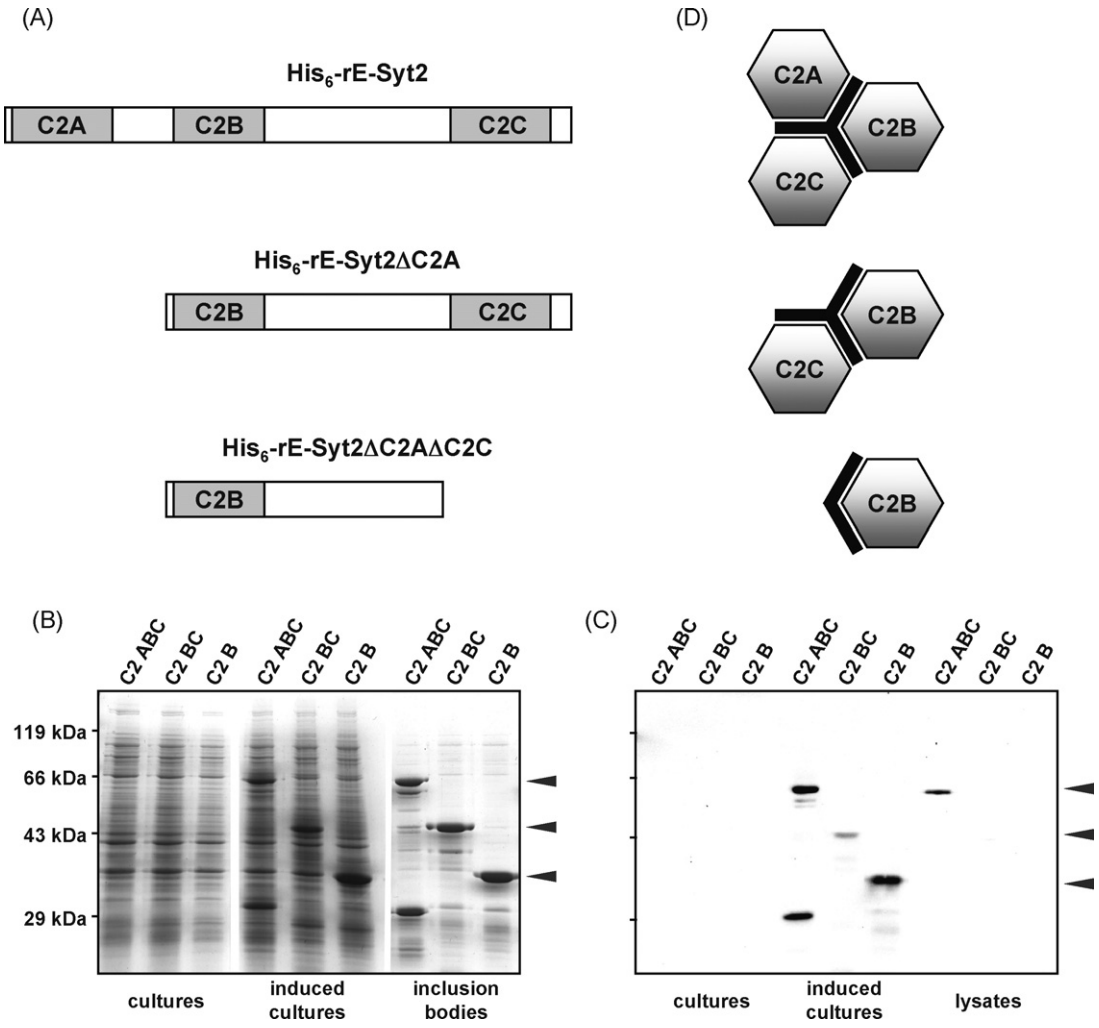

Fig. 5. Interdependence of C2 domains of E-Syt2.
Truncated versions of His-rE-Syt2 (A) were generated by deletion of one (His-rE-Syt2ΔC2A) or two (His-rE-Syt2ΔC2AΔC2C) C2 domains. SDS-PAGE of induced bacterial lysates (B) showed expression of all constructs (marked with arrows), that were also found in inclusion bodies. For simplicity, labelling of lanes refers to the C2 domains present in individual variants. The presence of recombinant proteins in induced cultures was confirmed by Western blot analysis (C, anti-$His_{(6)}$-tag), however, in soluble lysates only His-rE-Syt2 containing the entire C2 portion of E-Syt2 was detected. In a model representation of rE-Syt2 (D), the relative orientation of C2A, C2B and C2C domains (hexagons) is stabilized by interdomain contact regions (black areas). Deletion of single domains exposes internal, presumably hydrophobic regions of the protein leading to low solubility, non-specific intermolecular binding and accumulation in inclusion bodies during overexpression in *E. coli*.

quence, internal protease cleavage of recombinant constructs can only be detected reliably after proteolytic digest of purified protein, however, homogenous isolation often requires more than just one affinity step and is thus very time-consuming. For removal of the $His_{(6)}$-tag by an alternative protease, substitution of the protease cleavage site involves further cloning associated with additional loss of time. Taken together, purification of rE-Syt2 using a combination of three ion exchangers and gel filtration has proven to be more efficient than a procedure based on a specific affinity step.

Analysis of thermal and chemical stability of rE-Syt2 identified the protein to be rather labile, aggregation-prone and to exhibit a low melting temperature compared to isolated C2 domains from synaptotagmins [46,47]. As demonstrated previously, rE-Syt2 assembles into higher oligomers upon binding of calcium ions [13]. Interestingly, the presence of calcium ions further destabilized the protein possibly by inducing its aggregation, although previous reports show, that calcium binding enhances thermal stability of single C2 domains [46,47]. Thus, especially after metal-affinity chromatography the presence of bivalent metal atoms might promote rE-Syt2 instability and in summary, the low stability of the protein seems to be the crucial determinant for the strong non-specific degradation of the protein by thrombin observed during the tag removal step.

Following a common strategy to dissect the contributions of individual C2 domains to the functionality of multi-C2 proteins, we aimed to produce and isolate truncated rE-Syt2 variants containing only one or two of the three C2 domains. Availability of such constructs enables to analyze the involvement of particular C2 domains in the interaction with binding partners such as calcium, lipids or other proteins in future characterization studies. However, His-rE-Syt2ΔC2A and His-rE-Syt2ΔC2AΔC2C were insoluble and deposited in bacterial inclusion bodies. In recent studies on E-Syts and MCTPs, similar purified mutants lacking at least one C2 domain were employed to investigate interaction with calcium and lipids *in vitro* [12,48]. Stability of these constructs may be explained by the use of a large GST-tag that was not removed for measurements after one-step purification presumably increasing the solubility of the respective fusion partners. Utilization of a small $His_{(6)}$-tag may have emphasized intrinsic properties of the recombinant proteins and differences in solubility of the expressed constructs may indicate the mutual influence of C2 domains on each other.

The tendency towards low solubility of rE-Syt2 mutants shortened by one or two C2 domains under conditions for growth and

induction, that produced soluble rE-Syt2 comprising all three C2 domains, highlights the structural dependence of individual C2 domains in E-Syt2. Moreover, enhanced lability in the absence of at least one C2 domain suggests interconnected domains, whose contacts may also be a basic requirement for E-Syt2 stability *in vivo*.

5. Conclusion

Fusion tag technology offers a vast repertoire of optimized methods for protein purification with the potential to save costs and effort. However, for recombinant E-Syt2 a tag-free approach has proven beneficial and even more effective with less effort than the $His_{(6)}$-tagged procedure. The homogenous preparation of His-rE-Syt2 required further steps in addition to affinity chromatography and tag cleavage was impractical. The observed fragmentation of rE-Syt2 after protease treatment may be associated with the moderate stability of the protein exhibiting a low melting temperature and weak resistance against denaturing agents. In line with these findings, all C2 domains are required for soluble expression indicating a concept of three interdependent C2 domains.

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