[23] The S·Tag Fusion System for Protein Purification

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Fusion Proteins

The detection, immobilization, and purification of proteins is idiosyncratic and can be problematic. Fortunately, these processes can be generalized by using recombinant DNA technology to produce fusion proteins in which target proteins are fused to carrier polypeptides. The affinity of the carrier for a specific ligand enables the facile detection, immobilization, and purification of a fusion protein.

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Ribonuclease S

Bovine pancreatic ribonuclease A (RNase A; EC 3.1.27.5) catalyzes the cleavage of RNA.[1] The protease subtilisin prefers to cleave a single peptide bond in native RNase A. The product of this cleavage, RNase S (where "S" refers to subtilisin), consists of two tightly associated fragments: S-peptide (residues 1–20) and S-protein (residues 21–124).[2–4] Although neither fragment alone has any ribonucleolytic activity, RNase S is approximately as active as intact RNase A. The three-dimensional structure of RNase S is essentially identical to that of RNase A.[5,6]

The S-peptide fragment of RNase A has played an important role in the history of biochemistry. Before molecular biologists were able to use recombinant DNA technology to explore protein structure–function relationships, organic chemists synthesized analogs of S-peptide and studied their complexes with S-protein.[7,8] These studies provide much information on the role of individual residues in RNase S. Most significantly, only residues 1–15 of S-peptide were found to be necessary to form a fully functional complex with S-protein.[9] This shorter fragment is called "S15" or the "S·Tag" sequence.[10] (S·Tag, pBAC, and Perfect Protein are trademarks of Novagen, Inc., Madison, WI.)

In addition to structural information, extensive data have been acquired on the stability of RNase S. The value of K_d for RNase S is dependent on pH, temperature, and ionic strength.[1] Isothermal titration calorimetry has shown that the $K_d = 1.1 \times 10^{-7}$ *M* for the S-protein–S·Tag complex at 25° in 50 m*M* sodium acetate buffer, pH 6.0, containing NaCl (0.10 *M*).[11] Only a low yield of native S-protein (which contains four disulfide bonds) is isolable from the air oxidation of reduced S-protein.[12] The recovery of native S-protein is complete, however, if

[1] R. T. Raines, *Chem. Rev.* **98,** 1045 (1998).
[2] F. M. Richards, *C. R. Lab Carlsberg (Sér. Chim.)* **29,** 322 (1955).
[3] F. M. Richards and P. J. Vithayathil, *J. Biol. Chem.* **234,** 1459 (1959).
[4] F. M. Richards, *Protein Sci.* **1,** 1721 (1992).
[5] H. W. Wyckoff, K. D. Hardman, N. M. Allewell, T. Inagami, L. N. Johnson, and F. M. Richards, *J. Biol. Chem.* **242,** 3984 (1967).
[6] H. W. Wyckoff, K. D. Hardman, N. M. Allewell, T. Inagami, D. Tsernoglou, L. N. Johnson, and F. M. Richards, *J. Biol. Chem.* **242,** 3749 (1967).
[7] E. A. Barnard, *Annu. Rev. Biochem.* **38,** 677 (1969).
[8] F. M. Richards and H. W. Wyckoff, *Enzymes* **IV,** 647 (1971).
[9] J. T. Potts, Jr., D. M. Young, and C. B. Anfinsen, *J. Biol. Chem.* **238,** 2593 (1963).
[10] M. McCormick and R. Mierendorf, *Novations* **1,** 4 (1994).
[11] P. R. Connelly, R. Varadarajan, J. M. Sturtevant, and F. M. Richards, *Biochemistry* **29,** 6108 (1990).
[12] E. Haber and C. A. Anfinsen, *J. Biol. Chem.* **236,** 422 (1961).

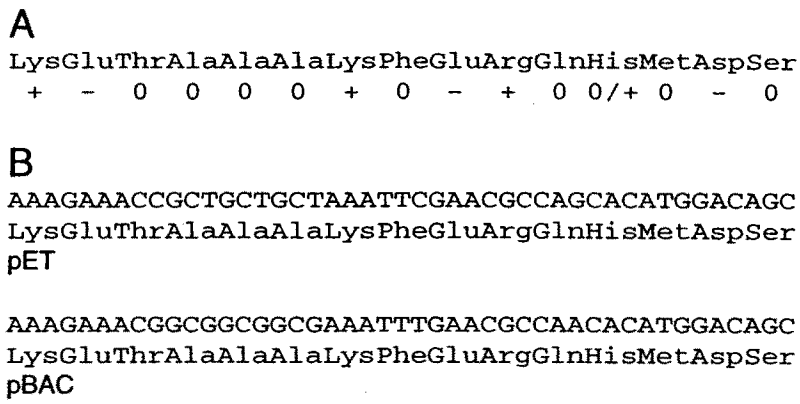

FIG. 1. (A) Amino acid sequence of S·Tag, which corresponds to the first 15 amino acid residues of ribonuclease A. The net charge on each residue at neutral pH is indicated by +, 0, or −. (B) Nucleotide sequences of S·Tag coding regions used in expression vectors. Sequences are shown that optimize codon usage in *Escherichia coli* (pET vectors) and baculovirus (pBAC transfer vectors).

oxidation is performed in the presence of S-peptide, which serves as a template for proper folding.[13]

The S·Tag Carrier

The wealth of information that has been accumulated on RNase S has enabled the development of this noncovalent complex as the basis for a fusion protein system.[14–17] In this system, S·Tag is the carrier and S-protein is the ligand. The S·Tag carrier combines a small size (15 amino acid residues) with a high sensitivity of detection (20 fmol, which is 1 ng of a 50-kDa fusion protein, in solution or on Western blots). S·Tag has several additional properties that are desirable in a carrier. For example, S·Tag is composed of four cationic, three anionic, three uncharged polar, and five nonpolar residues (Fig. 1A). This composition makes S·Tag an excessively soluble peptide with little structure and net charge near neutral pH. The S·Tag carrier is therefore unlikely to interfere with the proper folding or function of a fused target protein. The S-peptide portion of RNase A is

[13] I. Kato and C. B. Anfinsen, *J. Biol. Chem.* **244,** 1004 (1969).
[14] J.-S. Kim and R. T. Raines, *Protein Sci.* **2,** 348 (1993).
[15] V. N. Senchenko, M. V. Dianova, V. Y. Kanevskii, A. L. Bocharova, and M. Y. Karpeiskii, *Mol. Biol.* **27,** 565 (1993).
[16] M. Y. Karpeisky, V. N. Senchenko, M. V. Dianova, and V. Y. Kanevsky, *FEBS Lett.* **339,** 209 (1994).
[17] J.-S. Kim and R. T. Raines, *Anal. Biochem.* **219,** 165 (1994).

not antigenic.[18] The exacting nature of the interaction between the S·Tag peptide and the S-protein minimizes the likelihood of interference from naturally occurring molecules. Also, the topology of RNase S is such that target proteins fused to either terminus of S·Tag allow for binding to S-protein. It appears that the peptide can also reside internally within fusion proteins and remain fully accessible for S-protein binding. For example, fusion proteins in which the S·Tag sequence is located between N-terminal glutathione *S*-transferase, thioredoxin, or cellulose-binding domains, and C-terminal β-galactosidase domains are detected on Western blots with the same sensitivity as fusion proteins that carry the S·Tag peptide at either terminus (data not shown). (Although the S·Tag peptide is hydrophilic and thus expected to also be solvent accessible under native conditions, it is possible that the folding of certain target proteins could mask the peptide and prevent efficient binding of S-protein, which would compromise the S·Tag rapid assay and affinity purification procedures described herein.) Finally, the affinity between S·Tag and S-protein can be fine-tuned by rational mutagenesis.[14] Together, these properties make S·Tag an extremely useful and versatile carrier in fusion protein systems.

Expression Vector Design

A variety of vectors has been used successfully for the expression of S·Tag fusion proteins in prokaryotic, eukaryotic, and *in vitro* systems. Expression levels appear to be unaffected by the presence of the S·Tag sequence in all constructs we have used. Examples of codon-optimized nucleotide sequences for expressing the S·Tag peptide in bacterial and baculovirus vectors are shown in Fig. 1B. Transcripts of both sequences are also efficiently translated in rabbit reticulocyte lysates. If the potential for translation initiation at Met13 is a concern, it can be replaced by the isoleucine codon ATC with no effect on the interaction with S-protein. Expression vectors having various configurations of S·Tag fusion sequences, as well as S·Tag detection and purification reagents, are available commercially from Novagen.

S·Tag Rapid Assay

A unique feature of this fusion system is that the S·Tag sequence confers the ability to quantify target proteins using a simple enzymatic assay, which is based on the reconstitution of ribonucleolytic (RNase S) activity. A crude or purified sample containing the fusion protein is added to

[18] G. W. Welling and G. Groen, *Biochim. Biophys. Acta* **446,** 331 (1976).

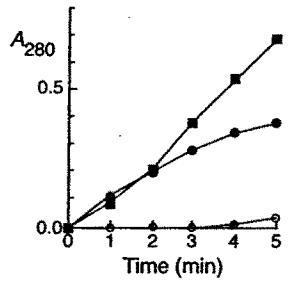

FIG. 2. Time course of an S·Tag rapid assay. Reactions with the indicated samples were performed as described in the text. (■) Rabbit reticulocyte lysate *in vitro* translation of S·Tag β-galactosidase. (●) Crude *E. coli* extract of a pET construct that directs the expression of S·Tag β-galactosidase. (○) Blank—*in vitro* translation reaction with no template.

a buffer containing excess purified S-protein and the ribonuclease substrate poly(C). After a brief incubation, the reaction is stopped with trichloroacetic acid (TCA) and the resulting precipitate is removed by centrifugation. Activity is measured by reading the absorbance of the supernatant at 280 nm, which increases as the poly(C) is broken down into acid-soluble nucleotides by the enzyme.[19] By comparing the results with a known S-peptide standard, the molar concentration of target protein in the sample can be determined. With this assay, as little as 20 fmol of target protein can be detected in a 5-min incubation.

Typical assay profiles are shown in Fig. 2. A linear signal was obtained during a 5-min incubation at 37°. In this experiment, 2 μl of crude translation mix and 2 μl of a 1/100 dilution of *Escherichia coli* lysed with 1% (w/v) SDS were used as samples. Appropriate blanks serve as controls for any endogenous ribonucleolytic activity, which appears to be minimal under the conditions of the assay.

Unlike amino acid incorporation assays (both radioactive and nonradioactive), the S·Tag rapid assay is independent of protein size, amino acid composition, and endogenous amino acid pool size. The method is also extremely versatile for measuring protein expression in cells, and it can be applied to both soluble and insoluble proteins. Multiple samples can be screened easily for expression levels by preparing crude extracts of whole cells in 1% (w/v) SDS. Because the assay detects as little as 20 fmol target protein in a 5-min incubation, even poorly expressed proteins can be measured with a high degree of accuracy.

The S·Tag rapid assay relies on ribonucleolytic activity. One might expect some level of background activity due to the presence of endogenous ribonucleases, especially in crude extracts. In practice, however, we have

[19] S. B. Zimmerman and G. Sandeen, *Anal. Biochem.* **10**, 444 (1965).

observed negligible background signals using crude *E. coli* extracts, insect cell extracts, and rabbit reticulocyte lysates. In many applications the expression levels are such that crude protein samples are diluted 10- to 500-fold prior to use in the assay, which further mitigates any background activity. Although we have less experience with mammalian cell extracts, we would expect that with few exceptions (e.g., pancreatic or liver tissue) most sources would not pose a significant background problem. The use of appropriate control samples, prepared in a manner identical to test samples but lacking expressed target protein, allow the direct measurement of any background signal. Recommended control samples for the rapid assay are uninduced crude extracts (prokaryotic), nontransfected crude extracts (eukaryotic), or *in vitro* translation reactions lacking template.

S·Tag Rapid Assay Protocol

Materials needed to perform 100 S·Tag rapid assays are as follows.

1 ml S·Tag grade S-protein 50 ng/μl (Novagen)
4 ml 10× S·Tag assay buffer [0.20 *M* Tris–HCl buffer, pH 7.5, containing NaCl (1 *M*) and poly(C) (1 mg/ml)]
50 μl S·Tag standard (20 amino acid residues; 0.05 pmol/μl) (Novagen)
40 ml sterile deionized water
10 ml 25% (w/v) TCA at 4°
Microcentrifuge at 4°
UV spectrophotometer set at 280 nm

Samples can be total, soluble, or insoluble protein fractions dissolved in denaturing buffers (see later). In general, crude cellular samples need to be diluted from 1:10 to 1:500 in water to be in the linear range of the assay. Up to 10 μl of a 1/10 dilution of 1% (w/v) SDS or 10 μl of a 1/100 dilution of 6 *M* urea or 6 *M* guanidine–HCl can be added to the assay with little effect. For each set of samples, a blank without added target protein and the S·Tag standard are run in parallel.

1. Assemble the following components in a set of sterile 1.5-ml microcentrifuge tubes. This example uses one unknown (in tube 3).

Component	Tube 1	Tube 2	Tube 3
Sterile water	348 μl	346 μl	348 μl
S·Tag standard	—	2 μl	—
Sample extract	—	—	2 μl
Blank extract	2 μl	2 μl	—
10× S·Tag assay buffer	40 μl	40 μl	40 μl
S·Tag grade S-protein	10 μl	10 μl	10 μl

2. Start the reactions by adding the S-protein at timed intervals (e.g., every 20 sec).

3. Incubate the tubes at 37° for exactly 5 min.

4. Stop the reactions by adding 100 μl ice-cold 25% (w/v) TCA, vortex, and place on ice for 5 min.

5. Centrifuge the tubes at 14,000*g* for 10 min.

6. Read the absorbance of the supernatants at 280 nm. Zero the spectrophotometer with sample 1. If the absorbance of the sample is greater than 1.5, the assay should be repeated with a more dilute sample to stay within the linear range.

7. To calculate the concentration (in pmol/μl) of S·Tag protein in the sample, use the following equation:

$$[\text{S·Tag}] = (A_{280} \text{ of tube 3}/2\ \mu\text{l}) \times (0.1 \text{ pmol S·Tag standard}/A_{280} \text{ of tube 2})$$

For example, if the A_{280} for the S·Tag standard (No. 2) is 0.5 and the A_{280} of a 1:200 dilution of the sample (No. 3) is 1.0, then [S·Tag] = (1.0 × 200/2 μl) × (0.1 pmol/0.5) = 20 pmol/μl. If the target protein has a molecular mass of 50 kDa, then its concentration is 20 pmol/μl × 0.05 μg/pmol = 1 μg/μl.

Notes

a. The blank extract for tube 1 should be prepared under conditions identical to the test sample but without expression of the S·Tag fusion protein (e.g., a blank *in vitro* translation reaction lacking target mRNA, extracts of cells lacking expression vector constructs). This preparation provides a control for the enzymatic and nonenzymatic contribution to the absorbance reading from endogenous sample components. In addition, the buffer composition of the blank should be as similar to that of the unknown as possible.

b. Best results are obtained using a special grade of S-protein ("S·Tag grade") that has been purified to remove residual RNase A and RNase S, which commonly contaminate commercial preparations of S-protein.

c. When assaying purified proteins, use a buffer containing 10 m*M* Tris–HCl buffer, pH 7.5, containing Triton X-100 (0.1%, v/v) to dilute the sample to avoid loss of material on tube surfaces.

d. The assays just described are designed for using standard 1-ml cuvettes; the assay volume can be scaled down proportionately to accommodate smaller cuvettes.

e. Keep in mind that RNase is being reconstituted in this assay and could contaminate cuvettes used for reading the results. Clean the cuvettes with 0.5 *N* NaOH or a strong quaternary amine detergent to prevent carryover.

S·Tag Western Blots

S·Tag fusion proteins produced by *in vitro* translation, eukaryotic, or prokaryotic expression are detected quickly and with high sensitivity by Western blot analysis. Blots are prepared by conventional SDS–PAGE and Western transfer protocols, blocked briefly with nonfat dry milk, and then incubated with S-protein horseradish peroxidase (HRP) or alkaline phosphatase (AP) conjugate. Target bands are visualized with colorimetric or chemiluminescent substrates. An optimized protocol allows fully developed blots to be produced in 45 min with detection of nanogram amounts of target proteins.

A time course of induction of β-galactosidase expressed from the bacterial vector pET-30b(+) is shown in Fig. 3. The S·Tag Western blot (Fig. 3, right) shows high specificity for target proteins (and their amino-terminal-containing breakdown products) with very low background staining of other *E. coli* proteins. This gel also contains a marker lane containing a set of seven S·Tag fusion proteins having defined sizes at convenient intervals, which serve as precise internal standards for S·Tag Western blots.

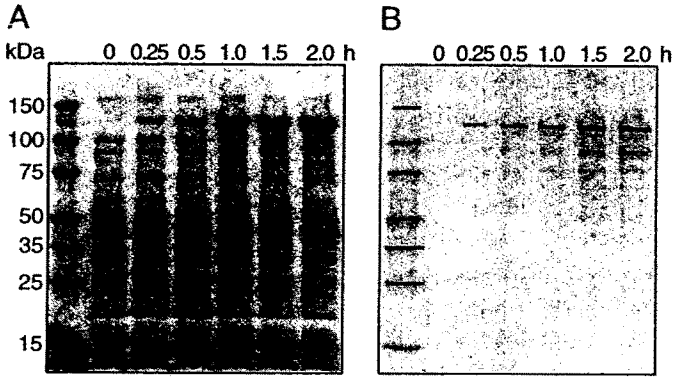

FIG. 3. SDS–PAGE and S·Tag Western blot analysis of an S·Tag fusion protein expressed in *E. coli*. An *E. coli* host carrying a pET construct encoding S·Tag β-galactosidase (118 kDa) was grown in culture. Whole cell extracts were prepared at the indicated times following the addition of IPTG to induce expression of the fusion protein. The marker lane on the left side of each panel contains a mixture of S·Tag fusion proteins having defined sizes (Perfect Protein Markers, Novagen). (A) SDS–PAGE gel stained with Coomassie blue. (B) S·Tag Western blot. A gel was loaded with a 1/100 dilution of the samples used in A. Proteins were transferred to nitrocellulose and incubated with S-protein alkaline phosphatase conjugate followed by color development with NBT/BCIP substrates.

S·Tag Western Blot Protocol

Materials needed for the development of 25 medium size (10 × 10 cm) blots are as follows.

50 μl S-protein conjugate with HRP or AP (Novagen)
25 g nonfat dry milk
200 ml 10× TBST (0.10 *M* Tris–HCl buffer, pH 8.0, containing NaCl (1.5 *M*) and Tween 20 (1% v/v)
HRP or AP buffer and development substrates (colorimetric or chemiluminescent)

The blot development procedure begins with a membrane (preferably nitrocellulose) containing proteins transferred from an SDS–polyacrylamide gel. Standard procedures for SDS–PAGE and transfer are suitable.

S·Tag Western Blot Development

1. Prepare blocking solution by dissolving 1.25 g nonfat dry milk in 25 ml of 1× TBST with stirring. This solution will block one blot.
2. Remove the nitrocellulose from the blotting apparatus and incubate in blocking solution at room temperature for 15–30 min to block excess protein-binding sites.
3. Prepare 250 ml of 1× TBST per blot by diluting the 10× stock with deionized water. Rinse the membrane for 1 min in 25 ml TBST at room temperature to remove excess blocking reagent.
4. Incubate the membrane with a 1:5000 dilution of S-protein HRP or AP conjugate in TBST for 30 min at room temperature. Use enough reagent to cover the membrane, usually about 10 ml.
5. Wash the membrane five times in 25–50 ml TBST at room temperature. This washing can be done in 1–2 min by adding the wash solution, briefly shaking, and decanting. It is important to wash thorougly the membrane at this point to achieve maximum signal:noise ratios.
6. Remove excess liquid and cover the membrane in blot development solution containing the appropriate buffer and substrates. Suitable development systems for alkaline phosphatase include a solution of NBT and BCIP for a colorimetric end point [10 m*M* Tris–HCl buffer, pH 9.4, containing NBT (0.33 mg/ml), BCIP (0.17 mg/ml), NaCl (10 m*M*), and $MgCl_2$ (1 m*M*)] and CDP-Star (Tropix) for a chemiluminescent end point. SuperSignal (Pierce) substrate is suitable for sensitive HRP-based chemiluminescent detection. Make sure that the entire surface of the membrane has been wetted with the substrate. For colorimetric substrates, incubate the blot until satisfactory signals are obtained and stop the reaction by rinsing in water. For chemiluminescent systems, incubate the blot in the substrate at room temperature for 1 min and then proceed to step 7.

7. Remove the membrane from the substrate and cover with plastic wrap. Remove any bubbles between the plastic and the membrane. Gently remove any liquid from the exterior of the plastic.

8. Place blot in a film cassette with autoradiographic film and expose for 1–10 min. Be careful not to move the film or blot after initial placement or multiple images can result. An initial exposure time of 1 min is recommended. Longer exposures can be performed, although the highest light output occurs in the first 5 min. Light output continues over several hours.

S·Tag Affinity Purification

S·Tag fusion proteins are purified rapidly by affinity chromatography using immobilized S-protein, such as S-protein covalently coupled to agarose.[1] Several purification strategies are possible, depending on the application. When fusion proteins are expressed from vectors that also encode a site-specific protease cleavage site (e.g., thrombin, LeuValProArg↓GlySer; or enterokinase, AspAspAspAspLys↓) between the S·Tag sequence and the cloning region, the target protein can be released from an S-protein matrix under native conditions simply by protease digestion. The efficiency of digestion is somewhat dependent on the target protein, as well as on the sequence context surrounding the protease cleavage site. Optimal contexts are present in relevant pET bacterial and pBAC baculovirus expression vectors available from Novagen. An example of the purification achieved under these conditions is shown in Fig. 4. Methods have been developed to allow specific, quantitative removal of the protease in the eluted fraction. For example, biotinylated thrombin is fully active and removed easily after digestion with streptavidin agarose, leaving the purified protein in solution. Under these conditions, homogeneous target proteins lacking the S·Tag peptide are recovered, and the S-protein matrix is not reused.

As an alternative, fusion proteins can be eluted from S-protein agarose under conditions that disrupt the S·Tag:S-protein interaction [e.g., 3 *M* guanidinium thiocyanate; 0.2 *M* potassium citrate buffer, pH 2; or 3 *M* $MgCl_2$]. By this method, the S·Tag peptide remains attached to the fusion protein, and the S-protein agarose can be recycled. If the target protein accumulates in cells as inclusion bodies, the insoluble fraction can be prepared and dissolved in 6 *M* urea. The sample is then diluted three fold (to 2 *M* urea) and applied to the S-protein agarose equilibrated in the same buffer. Fusion proteins are bound to the S-protein agarose under these conditions and can be eluted either with biotinylated thrombin digestion in the presence of urea or with the partially denaturing conditions listed earlier.

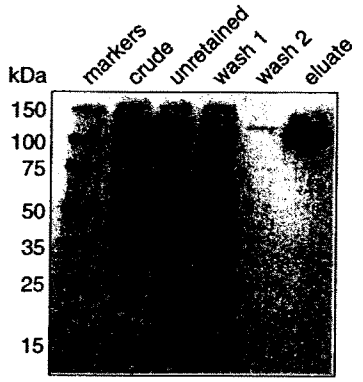

FIG. 4. S·Tag affinity purification. S·Tag β-galactosidase expressed from a pET construct was purified from a crude soluble fraction using S-protein agarose under native conditions. Elution of the target protein from the agarose was performed by digestion with thrombin, which is visible as a minor band (~33 kDa) in the eluted fraction. Use of biotinylated thrombin enables its removal with streptavidin agarose following digestion (see text). The identity of each fraction is indicated.

Binding can be performed in a column or batch mode. The capacity of S-protein agarose varies somewhat based on the size and folding characteristics of a given target protein. Under native conditions, commercial preparations of S-protein agarose exhibit a minimum binding capacity of 500 μg/ml for S·Tag β-galactosidase.

S·Tag Affinity Purification Protocol

Materials needed for the purification of 1 mg of target protein are as follows.

- 2 ml S-protein agarose [50% (v/v) slurry in 50 m*M* Tris–HCl buffer, pH 7.5, containing NaCl (0.15 *M*), EDTA (1 m*M*), and sodium azide (0.02%, w/v); Novagen]. Note that stated volumes of resins are settled bed volumes.
- 150 ml bind/wash buffer [20 m*M* Tris–HCl buffer, pH 7.5, containing NaCl (0.15 *M*) and Triton X-100 (0.1%, v/v)]
- 3 ml 10× thrombin cleavage buffer [for thrombin elution; 0.20 *M* Tris–HCl buffer, pH 8.4, containing NaCl (1.5 *M*) and $CaCl_2$ (25 m*M*)]
- 50 U biotinylated thrombin (for thrombin elution; Novagen)
- 0.8 ml streptavidin agarose [50% (v/v) slurry in sodium phosphate buffer, pH 7.5, containing sodium azide (0.02%, w/v); Novagen]
- Spin filters, 5-ml capacity (optional)

Binding to S-Protein Agarose

The purification procedure begins with a cell extract (bacterial, insect, or mammalian) or an *in vitro* translation reaction containing the S·Tag fusion protein. Note that elution with thrombin or enterokinase requires the presence of the appropriate protease cleavage site between the S·Tag peptide and the target protein sequence.

1. Gently suspend the S-protein agarose by inversion and add 2 ml of the slurry (equivalent to 1 ml settled resin) to the desired amount of soluble protein extract. If binding from partially denatured protein in 2 *M* urea, add an equal volume of 4 *M* urea in bind/wash buffer to 2 ml of resin prior to adding it to the protein extract. The resin is transferred most conveniently with a 1-ml wide-mouth pipette tip. Mix thoroughly and incubate at room temperature on an orbital shaker for 30 min. Do not shake vigorously as this will tend to denature protein.
2. Centrifuge the entire volume at 500*g* for 10 min and decant supernatant carefully.
3. Resuspend the S-protein agarose, which now contains bound S·Tag fusion protein, in 5 ml bind/wash buffer (include 2 *M* urea if using partially denatured protein). Mix by gently vortexing or by repeated inversion.
4. Repeat steps 2 and 3 twice more to wash away unbound proteins. Remove the final supernatant and elute the target protein either with biotinylated thrombin or using guanidinium thiocyanate, pH, or $MgCl_2$ as described later.

Elution with Biotinylated Thrombin

1. Prepare 15 ml thrombin cleavage buffer by diluting 1.5 ml of the 10× stock in 13.5 ml deionized water (remember to add 2 *M* urea if purifying proteins from solubilized inclusion bodies).
2. Resuspend the washed S-protein agarose containing the bound target protein in 5 ml of 1× thrombin cleavage buffer from step 1. Centrifuge at 500*g* for 10 min and remove the supernatant carefully. Resuspend and centrifuge again to fully equilibrate the resin in 1× thrombin cleavage buffer. Remove as much supernatant as possible.
3. Resuspend the washed, equilibrated agarose pellet in a final volume of 2 ml of 1× thrombin cleavage buffer (plus 2 *M* urea if necessary). Add 25 U biotinylated thrombin and incubate for up to 2 hr at room temperature on an orbital shaker. The target protein released from the agarose no longer contains the S·Tag peptide. The biotinylated thrombin is removed with streptavidin agarose. Note that biotinylated thrombin is fully active in the presence of 2 *M* urea. The recommended cleavage conditions (25 U, 2 hr)

are optimal for a variety of proteins; however, if secondary cleavage is observed with a particular protein, less enzyme can be used or the incubation duration decreased.

4. Thoroughly resuspend the streptavidin agarose by inversion. Add 800 μl of the slurry to the cleavage reaction (see note below if using urea in the buffers). Mix thoroughly and incubate for 10 min at room temperature on an orbital shaker. Note that the streptavidin agarose, in its listed buffer, can be added directly to the biotinylated thrombin/target protein/S-protein agarose mixture without the need for preequilibration. If urea has been included in the procedure, first bring the urea concentration in the streptavidin agarose slurry to 2 *M* by adding an appropriate volume from a concentrated stock solution. If desired, the streptavidin agarose can be preequilibrated in 1× thrombin cleavage buffer to avoid the addition of other components (such as inorganic phosphate or azide) in the supplied storage buffer. Proceed to step 7 if not using a spin filter to remove the agarose.

5. Transfer the entire reaction to a spin filter that has been placed in a collection tube. Centrifuge at 500*g* for 5 min.

6. Without removing the filtrate in the lower chamber, add 1.25 ml of 1× thrombin cleavage buffer (plus urea, if necessary) to the "cake" of resin in the upper chamber and centrifuge at 500*g* for 5 min. The clear filtrate contains the purified target protein, which can be used directly in many applications. See "Processing the Sample after Elution" for suggested procedures for concentration and changing the buffer.

7. Optional (if a spin filter is not used): Centrifuge at 500*g* for 5 min and transfer the supernatant, which contains the target protein, to a fresh tube. Wash the agarose pellet with an additional 1–2 ml of 1× thrombin cleavage buffer, centrifuge, and pool the second supernatant with the previous supernatant.

Elution with Guanidinium Thiocyanate, pH, or $MgCl_2$

1. Resuspend the washed resin containing the bound target protein in 1.5× settled resin volumes of one of the following solutions: bind/wash buffer containing guanidinium thiocyanate (3 *M*); 0.2 *M* potassium citrate buffer, pH 2; or 3 *M* $MgCl_2$. Incubate for 10 min at room temperature; mix gently every few minutes to keep the resin suspended. To make the sodium citrate buffer, prepare a 2 *M* stock of citric acid, adjust the pH to 2.0 with 10 *M* KOH, and dilute to 0.2 *M*.

2. Transfer the entire reaction to a spin filter that has been placed in a collection tube. Centrifuge at 500*g* for 5 min.

3. Without removing the filtrate, add 1.25 ml elution buffer to the

"cake" of resin in the upper chamber and centrifuge at 500*g* for 5 min. The clear filtrate contains the purified target protein.

4. Optional (if a spin filter is not used): Centrifuge at 500*g* for 5 min and transfer the supernatant, which contains the target protein, to a fresh tube. Wash the agarose pellet with an additional 1–2 ml of elution buffer, centrifuge, and pool the second supernatant with the previous supernatant.

5. Change the buffer in the eluted sample by one of the methods described in the next section.

6. The S-protein agarose may be recycled by washing three more times with elution buffer and then three times with bind/wash buffer. Store at 4° in bind/wash buffer containing 0.02% (w/v) sodium azide or other preservative.

Processing the Sample after Elution

The buffer of the purified sample may be changed or the sample concentrated by one of several methods. Note that, depending on the solubility characteristics of target protein, changing the buffer may result in precipitation. Three alternative procedures are as follows.

1. Dialyze into the buffer of choice. After dialysis, the sample may be concentrated by sprinkling solid polyethylene glycol (15–20 kDa) or Sephadex G-50 (Pharmacia) on the dialysis tubing. Use dialysis tubing with an exclusion limit of 6 kDa or less and leave the solid in contact with the tubing until the desired volume is reached, replacing it with fresh solid as necessary.

2. Use plastic disposable microconcentrator units (e.g., Centricon; Amicon) as directed by the manufacturer to both desalt and concentrate the sample by ultrafiltration.

3. Desalt the sample by gel filtration on Sephadex (G-10, G-25, G-50; Pharmacia) or Bio-Gel (P6DG, P-10, P-30; Bio-Rad).

Like most high-affinity chromatography methods, purification of S·Tag fusion proteins using immobilized S-protein has some disadvantages. For example, the elution of S·Tag fusion proteins from S-protein agarose requires the use of proteases or mildly chaotropic conditions to release the target protein. The use of chaotropes carries with it the risk of partial or total denaturation or inactivation of the target protein, which can be problematic when downstream applications require the isolation of a properly folded, active protein. Proteolytic elution avoids denaturing conditions, but results in the removal of the S·Tag peptide, making it unavailable for further detection or purification procedures. Proteolytic elution also carries the risk of secondary, nonspecific cleavage of the target protein. Secondary

cleavage can be reduced or eliminated in many instances with the use of lower amounts of protease, but the molecular weight and integrity of cleavage products should be confirmed by SDS–PAGE.

Prospectus

The S·Tag fusion system uniquely combines small tag size, antibody-like ligand-binding specificity, and the ability to confer an easily measured enzymatic activity to fusion proteins. The S-protein ligand is also small, relatively inexpensive, and can be used in a variety of formats to enable many applications with a single tagging system. Virtually every technique used with antibodies and their epitope tags can be applied to the S-protein:S·Tag interaction, including the blotting and purification methods described here, fusion protein immobilization, affinity capture of interacting molecules, and use of fluorophore-labeled S-protein for *in situ* affinity localization and cell sorting. The reconstitution of enzymatic activity by the simple addition of S-protein to any S·Tag fusion protein provides the platform for the development of novel assays. In particular, the discovery of a hypersensitive fluorogenic substrate for RNase A makes assays of S·Tag fusion proteins amenable to automation and thus useful in a variety of high-throughput screening applications.[20]

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The initial development of the S·Tag fusion system at the University of Wisconsin–Madison was supported by Grant GM44783 (NIH).

[20] B. R. Kelemen, T. A. Klink, M. A. Behlke, S. R. Eubanks, P. A. Leland, and R. T. Raines, *Nucleic Acids Res.* **27**, 3696 (1999).