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Ribonuclease S-peptide as a carrier in fusion proteins

JIN-SOO KIM AND RONALD T. RAINES

Department of Biochemistry, University of Wisconsin at Madison, Madison, Wisconsin 53706-1569

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Abstract

S-peptide (residues 1-20) and S-protein (residues 21-124) are the enzymatically inactive products of the limited digestion of ribonuclease A by subtilisin. S-peptide binds S-protein with high affinity to form ribonuclease S, which has full enzymatic activity. Recombinant DNA technology was used to produce a fusion protein having three parts: carrier, spacer, and target. The two carriers used were the first 15 residues of S-peptide (S15) and a mutant S15 in which Asp 14 had been changed to Asn (D14N S15). The spacer consisted of three proline residues and a four-residue sequence recognized by factor X_a protease. The target was β-galactosidase. The interaction between the S-peptide portion of the fusion protein and immobilized S-protein allowed for affinity purification of the fusion protein under denaturing (S15 as carrier) or nondenaturing (D14N S15 as carrier) conditions. A sensitive method was developed to detect the fusion protein after sodium dodecyl sulfate-polyacrylamide gel electrophoresis by its ribonuclease activity following activation with S-protein. S-peptide has distinct advantages over existing carriers in fusion proteins in that it combines a small size (≥15 residues), a tunable affinity for ligand ($K_d \geq 10^{-9}$ M), and a high sensitivity of detection ($\geq 10^{-16}$ mol in a gel).

Keywords: activity stain; biotechnology; enzyme assay; noncovalent interaction; protein immobilization; protein purification; site-directed mutagenesis; zymogram

Ribonuclease A (RNase A; EC 3.1.27.5) catalyzes the hydrolytic cleavage of RNA (Richards & Wyckoff, 1971; Blackburn & Moore, 1982; Beintema, 1987; Eftink & Biltonen, 1987). Almost 40 years ago, Richards and coworkers demonstrated that the protease subtilisin prefers to cleave a single peptide bond in native RNase A (Richards, 1955, 1992; Richards & Vithayathil, 1959). The product of this cleavage, ribonuclease S (RNase S), consists of two tightly associated fragments: S-peptide, which derives from residues 1-20 of RNase A, and S-protein, which derives from residues 21-124. Although neither fragment alone has any ribonuclease activity, RNase S has enzymatic activity similar to that of intact RNase A. Twenty-five years ago, Wyckoff and Richards determined the structure of crystalline RNase S by X-ray diffraction analysis (Wyckoff et al., 1967a,b).[1]

The S-peptide fragment of RNase A has had a singular role in the development of protein science. Before molecular biologists were able to use recombinant DNA technology to explore protein structure-function relationships, chemists synthesized analogs of S-peptide and studied their complexes with S-protein. These studies were successful in illuminating molecular aspects of enzymatic catalysis and protein-protein interaction and were the harbinger of current work on proteins containing unnatural or otherwise mutant amino acid residues.

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 (Uhlén & Moks, 1990; Ford et al., 1991; Nilsson et al., 1992). The affinity of the carrier for a specific ligand can enable the facile detection, immobilization, and purification, of a fusion protein. The wealth of information that has been accumulated on the properties of RNase S suggests that this noncovalent complex may provide a useful carrier and ligand in a fusion protein system. We have drawn on this information to test a fusion protein system in which S-peptide is the carrier and S-protein is the ligand.

Results

Design of fusion system

A fusion protein has three parts: carrier, spacer, and target. The carriers used in this work were derived from

Address reprint requests to: Ronald T. Raines, Department of Biochemistry, University of Wisconsin at Madison, Madison, Wisconsin 53706-1569.

[1] A detail from a painting of this structure adorns the cover of *Protein Science 1*(1).

ribonuclease S-peptide. These carriers met two other design criteria.

First, a carrier should be as small as possible because small carriers are less likely than large carriers to alter the properties of a fused protein or to be immunogenic. The truncated RNase S formed from the first 15 residues of S-peptide (S15), and S-protein has the same enzymatic activity and dissociation constant as does RNase S (Potts et al., 1963). The structure of this truncated RNase S has been determined by X-ray diffraction analysis (Fig. 1; Kinemage 1). This analysis indicates that the C-terminus of the S15 fragment is accessible to solvent. Thus, the attachment of a target protein to the C-terminus of S15 should not prevent its interaction with S-protein to form a functional ribonuclease. This hypothesis is supported by the structure of bovine seminal ribonuclease, a dimeric homolog of RNase A in which the S-peptide fragments of two monomers are exchanged (D'Alessio et al., 1991). A fusion protein in which a target protein is fused to the C-terminus of S15 should therefore be of minimal size but retain its ability to bind properly to S-protein.

Secondly, a fusion system should allow for tight binding to ligand (for protein immobilization) or weak binding to ligand (for protein purification). The dissociation constant of RNase S is about 10^{-9} M. This value is small enough to immobilize a fusion protein having an S-peptide carrier to an S-protein affinity resin, but it is too small to allow for the purification of the fusion protein under nondenaturing conditions. All of the 40 known amino acid sequences for pancreatic ribonucleases have Asp as residue 14 (Beintema, 1987). In RNase A, the carboxylate group of Asp 14 forms a hydrogen bond with the phenolic hydroxyl group of Tyr 25. This interaction increases the acidity of the carboxyl group of Asp 14, which has $pK_a = 3.8$ in S15 but $pK_a = 2.4$ in the complex of S15 with S-protein (Niu et al., 1979; Cohen et al., 1980). The mutant RNase S in which this interaction is weakened by changing Asp 14 to Asn has the same enzymatic activity as does RNase S but a dissociation constant that is 20-fold higher (Filippi et al., 1975). A protein fused to D14N S15 should therefore have a dissociation constant more appropriate for affinity purification under native conditions.

Thus, the two carriers used in this work were S15, which is a truncated but otherwise wild-type S-peptide, and D14N S15, which is a truncated and mutated S-peptide. The sequences of these two carriers are

S15:

Lys-Glu-Thr-Ala-Ala-Ala-Lys-Phe-Glu-Arg-Gln-His-Met-Asp-Ser

D14N S15:

Lys-Glu-Thr-Ala-Ala-Ala-Lys-Phe-Glu-Arg-Gln-His-Met-Asn-Ser.

Recombinant DNA was used to produce proteins in which a target protein was fused to the C-terminus of these carriers (Fig. 2).

The spacer used in this work consisted of three proline residues followed by the sequence of four residues recognized by blood coagulation factor X_a, a commercial protease. This spacer was designed (1) to minimize interaction between the carrier and target because polyproline tends to adopt a rigid, extended structure (Katchalski et al., 1963), and (2) to allow the intact target to be liberated from the remainder of the fusion protein because factor X_a catalyzes the cleavage of the peptide bond after the sequence Ile-Glu-Gly-Arg (Nagai & Thøgersen, 1987).

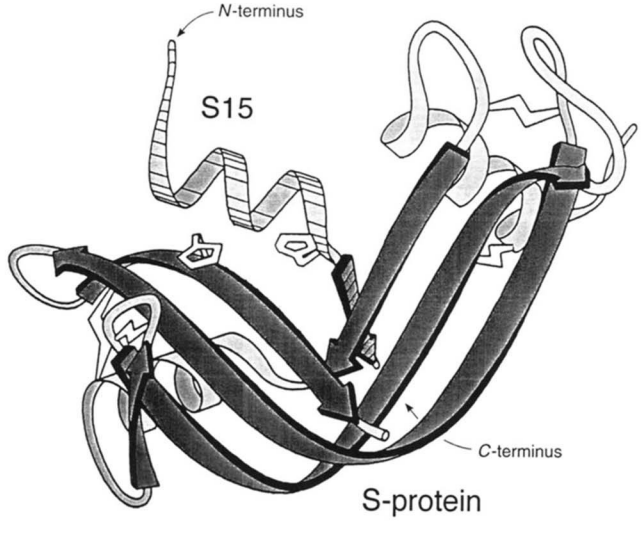

Fig. 1. Structure of crystalline complex formed by S15 and S-protein (Taylor et al., 1981). This complex is a truncated but fully active RNase S. The side chains of the two active-site histidine residues (His 12 and His 119) are shown.

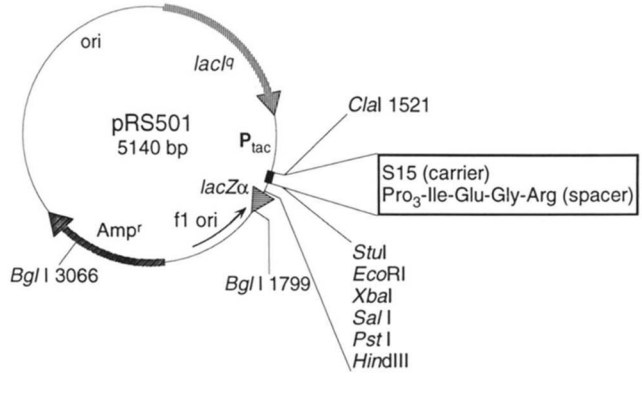

Fig. 2. Map of plasmid pRS501, which directs the production of proteins fused to the C-terminus of an S15 carrier. In this work, the gene that codes for the target protein, β-galactosidase, was inserted into the *Stu*I and *Pst*I sites of pRS501 to yield plasmid pSG601, which directs the production of S15–Pro_3-Ile-Glu-Gly-Arg-β-galactosidase. In addition, the codon for Asp 14 of S15 pSG601 was mutated to that for Asn to yield plasmid pSG919, which directs the production of D14N S15–Pro_3-Ile-Glu-Gly-Arg-β-galactosidase.

The target used in this work was β-galactosidase, an enzyme with a catalytic activity that is easy to assay and that has been either the carrier or the target in many other fusion proteins.

Production of fusion protein

Plasmids pSG601 and pSG919 directed the synthesis of a fusion protein with an apparent molecular weight of 118 kDa in *Escherichia coli* strain CSH27 (Fig. 3). The amount of fusion protein produced from an isopropyl-β-D-thiogalactopyranoside (IPTG)-induced culture of CSH27(pSG919) was twofold to threefold lower than that from an IPTG-induced culture of CSH27(pSG601).

In crude extracts prepared from uninduced and IPTG-induced cultures of CSH27(pSG601), β-galactosidase activity was measured, and, after the addition of various amounts of S-protein, ribonuclease activity was measured. Only the crude extract from the induced culture showed significant β-galactosidase or ribonuclease activity (Fig. 4). This ribonuclease activity appeared only after the addition of S-protein.

Purification of fusion protein

An affinity resin for S-peptide fusion proteins was produced in which S-protein was attached covalently to CNBr-activated Sepharose 4B. Batch adsorption to this resin of fusion protein having S15 (data not shown) or D14N S15 (Fig. 5) as carrier was complete at 4 °C in 20 mM sodium phosphate buffer, pH 7.0. Resin produced by direct coupling of S-protein had a 10-fold higher binding capacity for fusion protein than did resin produced in situ by treating RNase A–Sepharose with subtilisin. Sepharose alone did not absorb the fusion proteins.

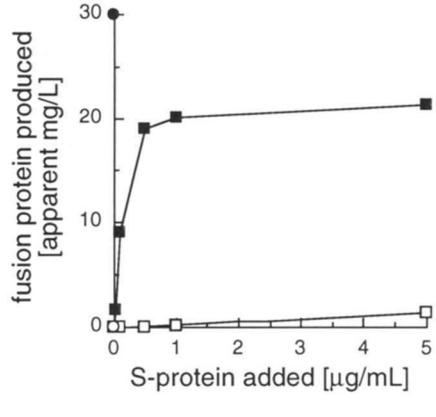

Fig. 3. Production of fusion protein with S15 as carrier. Apparent concentrations were determined by measuring β-galactosidase activity (○, ●) or ribonuclease activity after activation with different amounts of S-protein (□, ■). Cells were uninduced (open symbols) or IPTG induced (closed symbols).

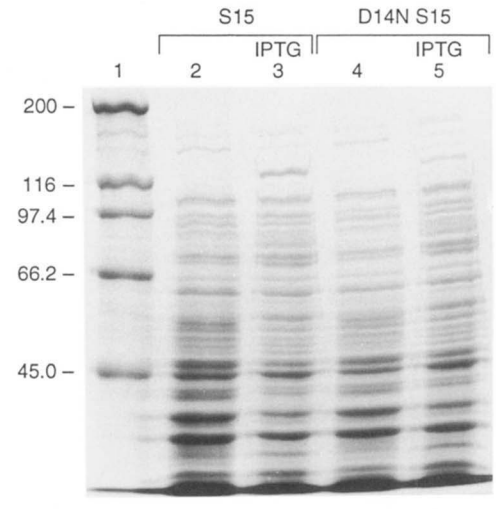

Fig. 4. Production of fusion proteins with S15 or D14N S15 as carrier. Fusion protein with S15 as carrier was produced from CSH27(pSG601) (lanes 2, 3). Fusion protein with D14N S15 as carrier was produced from CSH27(pSG919) (lanes 4, 5). Results were analyzed by staining with Coomassie brilliant blue after SDS-PAGE. Lane 1, molecular weight markers (kDa); lanes 2, 4, total protein from uninduced cells CSH27(pSG601); lanes 3, 5, total protein from IPTG-induced cells.

Nondenaturing conditions effected the elution from the affinity resin of fusion protein having D14N S15 as carrier. Raising the temperature to 37 °C of the loaded resin suspended in 20 mM Tris-HCl buffer, pH 7.4, produced fusion protein that was a single band after sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 5). The N-terminal amino acid sequence of this protein was as expected. A summary of the purification of the fusion protein having D14N S15 as carrier is given in Table 1.

Denaturing conditions (specifically, NaSCN [3 M]) were necessary to effect the elution of the fusion protein having S15 as carrier. Because target proteins vary in the efficiency with which they can be renatured, the fusion protein having S15 as carrier is more useful for immobilization than for purification of fusion proteins.

Detection of fusion protein

Fusion protein was detected by electrophoresis in a zymogram, which was an SDS–polyacrylamide gel impregnated with polyC, a ribonuclease substrate. Ribonuclease activity was produced in the gel after SDS was extracted with aqueous isopropanol, and the fusion protein was activated with S-protein. The presence of the fusion protein was revealed after intact polyC was stained with toluidine blue.

Zymogram electrophoresis of protein fused to D14N S15 produced a clear band on a dark background (Figs. 5, 6). As few as 10 pg (10^{-16} mol) of fusion protein was detectable after zymogram electrophoresis (Fig. 6, lane 4).

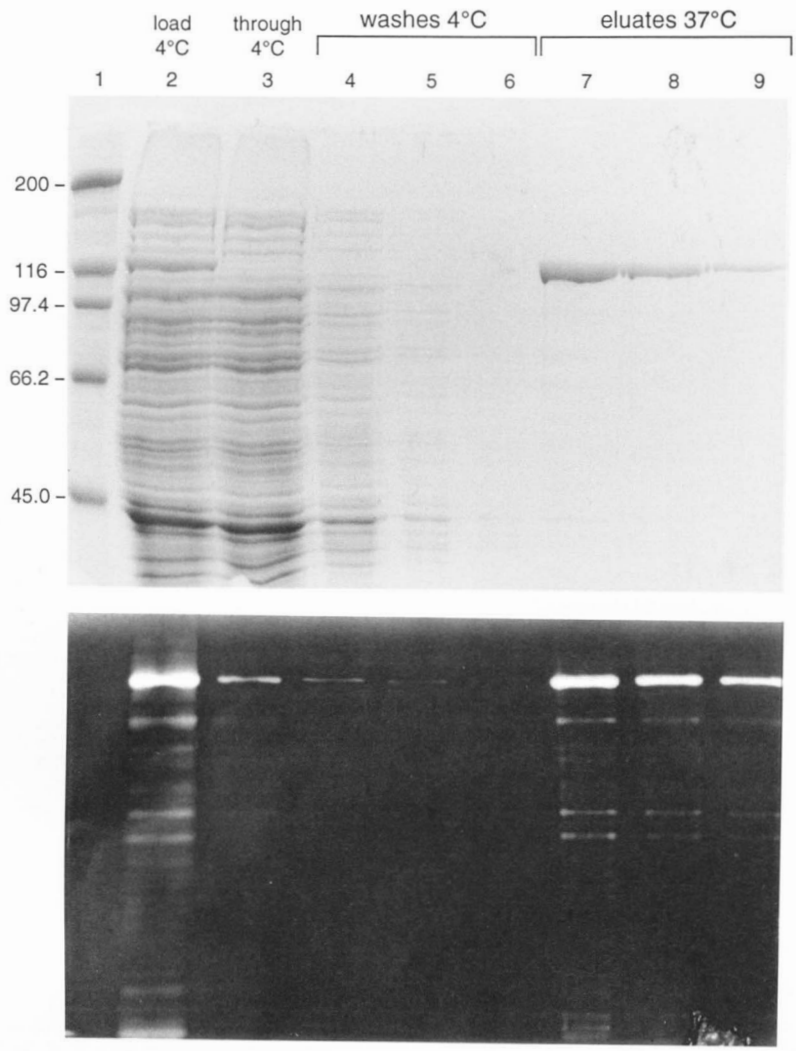

Fig. 5. Affinity purification of fusion protein under nondenaturing conditions. Fusion protein with D14N S15 as carrier was produced from CSH27(pSG919). Results were analyzed by staining with Coomassie brilliant blue after SDS-PAGE (top) or by zymogram electrophoresis (bottom). The bottom gel contains 10-fold less of each sample than the top gel. Lane 1, molecular weight markers (kDa); lane 2, soluble protein from IPTG-induced cells; lane 3, flow-through at 4 °C; lanes 4–6, buffer washes at 4 °C; lanes 7–9, buffer eluates at 37 °C.

This sensitivity decreased if activation with S-protein was performed above 4 °C or if bovine serum albumin was omitted from the activation solution.

Overloading fusion protein in a zymogram revealed the presence of several fragments (Fig. 6, lane 1) in a sample that appeared to be homogeneous by detection with protein stains (Fig. 5, lane 7). These fragments were still present in fusion protein prepared from the protease-deficient *Escherichia coli* strains: CAG 597 and CAG 629 (data not shown).

Liberation of target protein

Purified target protein was liberated from denatured, but not native, fusion protein by treatment with factor X_a.

Table 1. *Purification of D14N S15 fusion protein from a 0.1-L culture of E. coli strain CSH27 (pSG919)*

	Total catalytic activity (units)[a]	Yield (%)	Specific catalytic activity (units/mg)[a]	Purification factor	Total protein (mg)
Crude lysate	3.8×10^5	100	5.9×10^3	1	64
S-protein affinity chromatography	2.8×10^5	74	1.9×10^5	31	1.5

[a] Units refer to β-galactosidase units (Miller, 1972).

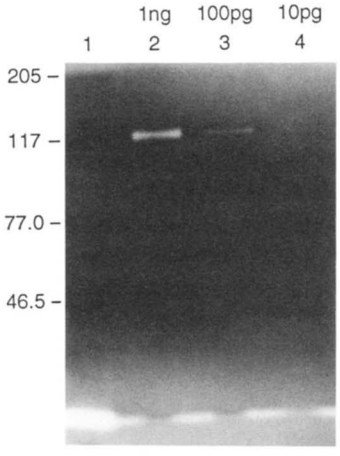

Fig. 6. Sensitivity of detection of fusion protein after zymogram electrophoresis. The sample was identical to that in Figure **5**, lane **7**. Lane **1**, molecular weight markers (kDa); lane **2, 1** ng (10^{-14} mol); lane **3,** 100 pg (10^{-15} mol); lane 4, 10 pg (10^{-16} mol).

SDS-PAGE indicated that this protease cleaved the fusion protein at several sites (data not shown).

Target protein was liberated from native fusion protein by treatment with trypsin, which cleaves proteins after Lys and Arg residues (Fig. 7). The presence of S-peptide carrier in the fusion protein was monitored by zymogram. This amount decreased with time (0–17 h). During treatment with trypsin, a peptide that co-migrated with authentic S15 appeared (0–1 h) and then disappeared (4–17 h). The quantity and migration of the protein with an apparent molecular weight >100 kDa appeared to be approximately constant. The N-terminal sequence of this protein indicated that cleavage had occurred primarily after Arg 10 of the **S15** carrier.

Discussion

Target proteins fused to a carrier polypeptide can be detected, immobilized, or purified based on the interaction of the carrier with a specific ligand. For example, the interaction of a carrier with an appropriate antibody can be used to detect as few as 10^{-13} mol of a fusion protein (Sambrook et al., 1989). Detection methods that rely on the catalytic activity of an enzymic carrier can be even more sensitive. Unfortunately, enzymes are relatively large and are therefore more likely than simple peptides to perturb a target protein or to be immunogenic. S-peptide is an unusual carrier because it combines a small size with a high sensitivity of detection.

Sensitive methods exist for assaying ribonuclease activity in solution or in a gel matrix. We have demonstrated that these methods are also applicable to fusion proteins having an S-peptide carrier. For example, as few as 10^{-16} mol of intact RNase A can be detected after its electrophoresis in a zymogram, which is a polyacrylamide gel containing a polymeric substrate for RNase A (Thomas & Hodes, 1981; Blank et al., 1982; Ribó et al., 1991; S.B. delCardayré & R.T. Raines, unpubl.). Similarly, 10^{-16} mol of a fusion protein having an S-peptide carrier can be detected after zymogram electrophoresis and activation with S-protein. This assay is most sensitive at low temperature, which is known to minimize the dissociation constant of RNase S (Schreier & Baldwin, 1977). The sensitivity is also enhanced by the presence of bovine serum albumin, which probably serves to limit the nonspecific interaction of S-protein with the gel matrix. Zymogram electrophoresis is useful not only because of its high sensitivity, but also because it reports the molecular weight of the fusion protein and reveals the presence of truncated forms that co-purify with the intact fusion protein. Finally, it may be possible to increase the sensitivity of this

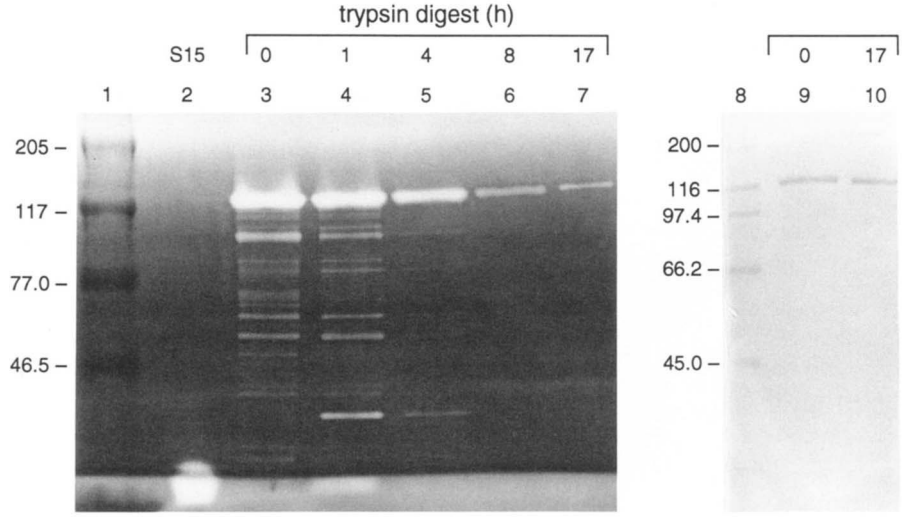

Fig. 7. Liberation of target protein by digestion of fusion protein with trypsin. Fusion protein was produced from CSH27(pSG601), purified, and digested with trypsin **for** various times. The results were analyzed by zymogram electrophoresis (left) or by staining with silver after SDS-PAGE (right). Lanes **1**, 8, molecular weight markers; lane **2,** authentic S15; lanes **3**, 9, before digestion with trypsin; lanes **4–6,** after digestion for **1** h, **4** h, or 8 h, respectively; lanes **7**, 10, after digestion for **17** h.

method even further by using cascades that amplify the signal generated by RNA cleavage (Cecchini et al., 1986; Rabin et al., 1988).

The spacer in our fusion protein was designed to allow the intact target to be liberated from the remainder of the fusion protein. Yet the native fusion protein, which contains in its spacer the Ile-Glu-Gly-Arg sequence recognized by blood coagulation factor X_a protease, is not cleaved by factor X_a. This absence of cleavage may result from an inability of factor X_a to access the spacer sequence. Similar results have been observed with another fusion protein that also contains an Ile-Glu-Gly-Arg spacer sequence preceding a β-galactosidase target protein (Maina et al., 1988). There as here, denatured fusion protein is digested by factor X_a, but several products are produced (Maina et al., 1988). This nonspecific digestion is likely due to the ability of factor X_a to catalyze cleavage at sequences other than Ile-Glu-Gly-Arg (Nagai & Thøgersen, 1987).

The native fusion protein is cleaved by trypsin. The predominant product of trypsin digestion (Fig. 7, lane 10) has an apparent molecular weight of 116 kDa and an N-terminal sequence that indicates that cleavage occurred primarily after Arg 10 of the S15 carrier. No cleavage is detected after the Arg residue of the spacer. Thus trypsin, like factor X_a, may be unable to access the Arg residue of the spacer in the native fusion protein. Unfortunately, no method currently exists for the precise liberation of an intact target protein from its carrier (Ford et al., 1991; Nilsson et al., 1992).

In addition to those described above, two other properties of S-peptide increase its versatility as a carrier in fusion proteins. First, the mature domains of exported proteins bear information necessary for the effective secretion of a protein (Wickner et al., 1991). RNase A is secreted effectively from exocrine cells of the cow pancreas and is readily translocated in both bacteria and yeast (S.B. delCardayré & R.T. Raines, unpubl.). An S-peptide is therefore unlikely to inhibit the efficient translocation of a fusion protein.

Secondly, S-peptide associates with S-protein such that both the N- and C-termini of S-peptide are accessible to solvent (Fig. 1). This topology suggests that binding of S-peptide to S-protein would not suffer if a protein were fused to either termini of S-peptide. Indeed, hybrid biopolymers in which DNA is attached to the N-terminus of S-peptide have been described, and these constructs allow for the formation of a hybrid RNase S that is an active ribonuclease (Rabin et al., 1988; Zuckermann & Schultz, 1988). The accessibility of both termini suggests that a protein fused to either (or both) termini of S-peptide would not prevent binding to S-protein.

Much is known about the biochemistry and biophysics of the intermolecular interaction that produces RNase S. Detailed studies on the binding of S-peptide to S-protein have indicated that three residues (Phe 8, His 12, and Met 13) seem to be essential for the formation of a catalytically active RNase S, and four others (Glu 2, Lys 7, Arg 10, Gln 11, and Asp 14) contribute to the stability and activity of the complex (Beintema, 1987). Variation in these seven residues creates a range of binding energies, some suitable for immobilization and others for purification of a fusion protein having an S-peptide carrier. Conversely, the residues in S-protein that interact with these seven residues in S-peptide can be varied by applying recombinant DNA technology to RNase A (S.B. delCardayré & R.T. Raines, unpubl.). Generating affinity resins from mutant S-proteins would enable a fusion protein with a single S-peptide carrier to be either immobilized or purified. This and other desirable properties make S-peptide a tag useful for detecting, immobilizing, or purifying proteins in vitro as well as for exploring the behavior of proteins in vivo.

Materials and methods

Materials

Escherichia coli strain TB1 (ara Δ[lac proAB] rpsL [φ80 lacZ DM15] hsdR) was from New England Biolabs (Beverly, Massachusetts). *Escherichia coli* strains CJ236 (dut ung thi relA; pCJ105[Cm^r]) and MV1190 (Δ[lac proAB] thi supE Δ[srl recA]306::Tn10 [tet^r] {F′: traD36, proAB, lacIqZDM15}) were from Bio-Rad (Richmond, California). *Escherichia coli* strains CAG598 (*htpR165*-Tn*10*) and CAG629 (*lon*$^-$ *htpR165*-Tn*10*) were gifts from C.A. Gross. *Escherichia coli* strain CSH27 (F^- ara Δ[lac proAB] thi tet^s recA56 srl^-) was a gift of W.S. Reznikoff. Plasmid pMAL-c was from New England Biolabs. Phagemid pBluescript SKII(−) was from Stratagene (La Jolla, California). Plasmid pMC1871 was from Pharmacia (Piscataway, New Jersey).

Reagents for DNA synthesis were from Applied Biosystems (Foster City, California) except for acetonitrile, which was from Baxter Healthcare (McGaw Park, Illinois). Oligonucleotides for DNA sequencing and mutagenesis were synthesized on an Applied Biosystems 392 DNA/RNA synthesizer by using the phosphoramidite method (Sinha et al., 1984). The restriction endonucleases *Pst*I, *Sma*I, *Stu*I, *Eco*RI, *Bgl*I, and *Cla*I were from New England Biolabs, as were DNA ligase and T4 DNA polymerase.

Bacto tryptone and Bacto yeast extract were from Difco Laboratories (Detroit, Michigan). IPTG was from Sigma. 5-Bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) was from United States Biochemical (Cleveland, Ohio). Ampicillin (sodium salt) was from International Biotechnologies (New Haven, Connecticut). CNBr-activated Sepharose 4B was from Pharmacia.

RNase A (type III-A), S-protein, adenosine deaminase, and uridylyl(3′ → 5′)adenosine were from Sigma Chemical (St. Louis, Missouri). Authentic S15 was synthesized

by Operon Technologies (Alameda, California). PolyC was from Midland Certified Reagent (Midland, Texas). [α-^{35}S]deoxyadenosine 5′-triphosphate was from Amersham (Arlington Heights, Illinois). All other chemicals and reagents were of commercial reagent grade or better and were used without further purification.

Methods

The pH was measured with a Beckman pH meter fitted with a Corning electrode, calibrated at room temperature with standard buffers from Fischer (Chicago, Illinois). Ultraviolet and visible absorbance measurements were made on a Cary 3 spectrophotometer equipped with a Cary temperature controller. DNA sequencing was performed with a Sequenase 2.0 kit from United States Biochemical. DNA fragments were isolated from agarose gels with a GENECLEAN II kit from Bio101 (La Jolla, California). Site-directed mutagenesis was performed on single-stranded DNA isolated from *E. coli* strain CJ236 according to Kunkel et al. (1987). Other manipulations of DNA were performed as described by Ausubel et al. (1989). Protein sequences were determined at the University of Wisconsin Biotechnology Center by using the method of Wadsworth et al. (1990).

Rich medium contained (in 1 L) Bacto tryptone (10 g), Bacto yeast extract (5 g), NaCl (5 g), and dextrose (2 g). All media were prepared in distilled, deionized water. Lysis buffer was 10 mM sodium phosphate buffer, pH 7.0, containing NaCl (0.5 M), Tween 20 (0.25% [w/v]), β-mercaptoethanol (10 mM), and EDTA (10 mM).

Polyacrylamide gel electrophoresis was performed in the presence of SDS (0.1% [w/v]) according to Ausubel et al. (1989). Gels were fixed and stained either by washing with aqueous methanol (40% [v/v]), containing acetic acid (10% [v/v]) and Coomassie brilliant blue (0.1% [w/v]), or by using a silver staining kit from Bio-Rad. The molecular weight standards from Bio-Rad were either unstained: myosin (200 kDa), β-galactosidase (116), phosphorylase B (97.4), serum albumin (66.2), and ovalbumin (45.0); or prestained: myosin (205 kDa), β-galactosidase (116), bovine serum albumin (80.0), and ovalbumin (49.5).

Construction of plasmids

An f1 origin was inserted into plasmid pMAL-c as follows. Plasmid pMAL-c was digested with *Bgl*I, and the 5.0-kb fragment was isolated. Plasmid pBluescript SKII(−) was also digested with *Bgl*I and the 1.3-kb fragment was isolated. These two fragments were ligated to yield phagemid pML. A unique *Cla*I site was created in phagemid pML by site-directed mutagenesis with oligonucleotide JS21 (CAAGGACCATCGATTATGAAA) to yield phagemid pML305.

The sequence coding for S15-Pro$_3$ was inserted into phagemid pML305 as follows. Phagemid pML305 was digested with *Cla*I and *Eco*RI, and the 5.1-kb fragment was isolated. Oligonucleotide RS92 (CCAACAAGGAC CATCGATT ATG AAG GAA ACC GCT GCC GCG AAA TTC GAA CGT CAG CAC ATG GAC TCC CCG CCG CCC ATC GAG GGT AGG CCT G) was annealed to oligonucleotide SR94 (AA TTC AGG CCT ACC CTC GAT GGG CGG CGG GGA GTC CAT GTG CTG ACG TTC GAA TTT CGC GGC AGC GGT TTC CTT CAT AATCGATGGTCCTTGTT). The resulting cassette was digested with *Cla*I and then ligated to the 5.1-kb fragment to yield phagemid pRS501.

The sequence coding for β-galactosidase was inserted into phagemid pRS501 as follows. Phagemid pRS501 was digested with *Stu*I and *Pst*I, and the 5.1-kb fragment was isolated. pMC1871 was digested with *Sma*I and *Pst*I, and the 3.1-kb fragment was isolated. These two fragments were ligated to yield phagemid pSG601 (Fig. 1). Phagemid pSG601 directs the production of S15-Pro$_3$-Ile-Glu-Gly-Arg fused to β-galactosidase under the control of P_{tac}, a promoter induced by IPTG.

Site-directed mutagenesis

The GAC codon for aspartate 14 of the S-peptide portion of pSG601 was converted to the AAT codon for asparagine by site-directed mutagenesis with oligonucleotide NE21 (T CAG CAC ATG AAT TCC CCG CC) to yield phagemid pSG919. This phagemid directs the production of D14N S15-Pro$_3$-Ile-Glu-Gly-Arg fused to β-galactosidase under the control of promoter P_{tac}.

Production of fusion protein

Escherichia coli strain CSH27(pSG601) or CSH27 (pSG919) was grown at 37 °C in rich media containing ampicillin (100 μg/mL) until the absorbance at 600 nm was 0.5 O.D. IPTG was then added to a final concentration of 1 mM. The culture was further incubated for 2 h at 37 °C. The cells were harvested by centrifugation, resuspended in 1/10 volume of lysis buffer, and lysed by ultrasonication. Insoluble debris was removed by centrifugation to yield crude extract.

Detection of fusion protein

β-Galactosidase activity in crude extract was measured as described (Miller, 1972). Ribonuclease activity in crude extract was measured as described (Ipata & Felicioli, 1968) after adding various amounts of S-protein and incubating the mixture for 30 min on ice. The apparent amount of fusion protein present was deduced from activity assays by using a standard curve generated with authentic β-galactosidase or RNase A.

The fusion protein was also detected in the crude extract by using a gel that allows for assay of ribonuclease activity. This gel was a Laemmli gel containing polyC (0.3 mg/mL), which is a substrate for RNase S. After electrophoresis, the activity gel was washed with a solution of isopropanol (20% [v/v]) to remove the SDS. The fusion protein was then activated for cleavage of the polyC by incubating the gel at 4 °C in 10 mM Tris-HCl buffer, pH 7.5, containing S-protein (1 μg/mL) and bovine serum albumin (1 mg/mL). After 10 min, the gel was washed with 10 mM Tris-HCl buffer, pH 7.5, to remove excess S-protein. The gel was then incubated at 4 °C with 10 mM Tris-HCl buffer, pH 7.5. After 30 min, the gel was washed with a solution of toluidine blue, which stains only polymeric nucleic acid.

Preparation of S-protein–Sepharose

S-protein was coupled to CNBr-activated Sepharose 4B as described (Kato & Anfinsen, 1969). In the coupling reaction, 2 mg of S-protein was used per 1 g of freeze-dried resin. Alternatively, RNase A was coupled to CNBr-activated Sepharose 4B according to the manufacturer's instructions. In the coupling reaction, 2–5 mg of RNase A was used per 1 g of freeze-dried resin. The resulting resin was treated with subtilisin and washed extensively with acetic acid (50% [v/v]) to elute S-peptide from the resin.

Purification of fusion protein

Crude extract containing the fusion protein was mixed with $\frac{1}{10}$ volume of swollen S-protein–Sepharose gel. After incubation for 30 min on ice, the solution was removed by filtration. The beads were washed three times, each with five volumes of lysis buffer. Elution was performed at 37 °C with 20 mM Tris-HCl buffer, pH 7.4, containing sodium chloride (0.5 M) or sodium thiocyanate (3 M).

Liberation of target protein

β-Galactosidase was liberated from the fusion protein by treating the purified fusion protein (native or denatured) with factor X_a according to the manufacturer's instructions, or by treating the purified fusion protein (native) with trypsin (0.02% [w/w]) on ice.

Note added in proof

The S-peptide fusion system has also been used to detect the heterologous production of the first tandem repeat region of link protein from the cartilage proteoglycan aggregate (P.A. Hajduk, J.L. Wood, J.-S. Kim, & L.E. Lerner, unpubl.).

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