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Engineering S-protein fragments of bovine ribonuclease A for targeted drug delivery

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Abstract

High affinity interaction between S-protein and S-peptide fragments of bovine pancreatic RNase A has been recently used for construction of molecular vehicles for targeted drug delivery. The vehicle is assembled as a complex of drug carrier conjugated S-protein with S-peptide-tagged targeting protein. To avoid random chemical crosslinking of drug carriers to S-protein, we constructed a mutant 16–124aa fragment of RNase A in which ^{122}ala is replaced with a cysteine residue. The mutant and the corresponding wild type fragments expressed in *Escherichia coli* are refolded into functional conformations only in the presence of S-peptide. After the removal of S-peptide, both fragments retain the ability to bind S-peptide and S-peptide-tagged proteins. The ^{122}cys residue in the mutant fragment is available for site-specific conjugation.

Limited digestion of bovine pancreatic RNase A with subtilisin results in two enzymatically inactive fragments: 1–20aa (S-peptide) and 21–124aa (S-protein). These two fragments form a stable complex ($K_d \sim 10^{-9}$ M), known as RNase S, with complete restoration of ribonuclease activity [1]. It was found that only fifteen N-terminal amino acids of RNase A (1–15aa fragment) are necessary for reconstitution of fully active RNase S [2]. Currently, this fragment is used as a fusion "S-tag" for detection and affinity purification of recombinant proteins [3].

We have reported recently that high affinity of S-protein/S-peptide interaction can be used for assembly of modular molecular vehicles for targeted drug delivery [4,5]. We proposed to create "payload" modules by conjugating drug carriers to S-protein and then to "dock" them non-covalently to S-tagged targeting proteins (Fig. 1). This approach avoids direct crosslinking of drug carriers to targeting proteins and therefore preserves their ability to interact with corresponding receptors or cell surface antigens. However, random chemical conjugation of drug carriers to S-protein still creates heterogeneous payload modules with variable affinities to S-tagged targeting proteins. We reasoned that construction of a mutant S-protein with an unpaired cysteine available for site-specific chemical modifications might overcome this problem.

To the best of our knowledge, expression of the wild type or mutant recombinant S-proteins has never been reported. In this study we describe construction, expression, and purification of two functionally active 16–124aa fragments of bovine pancreatic RNase A: the wild type and the A122C mutant with ^{122}cys available for site-specific modification.

Materials and methods

Construction of expression plasmids

The pT7-7/RNase A plasmid encoding 1–124 amino acids of the mature bovine pancreatic RNase A was provided by Dr. G. D'Alessio (Napoli Federico II University, Naples, Italy). Six different S-protein fragments were amplified by PCR from the pT7-7/RNase A plasmid with sense primers introducing ATG codon immediately upstream of the coding sequences (Table 1). An antisense primer 5′-**CTA**CACTGAAGCATCAAA

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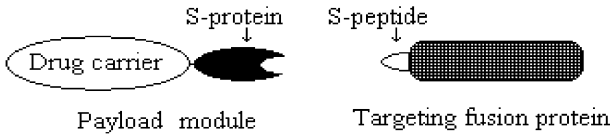

Fig. 1. Modular assembly of molecular drug delivery vehicle is based on S-protein/S-peptide interaction.

GTGGACTGGC introduced a stop-codon (bolded) immediately downstream of the ^{124}val codon. A cysteine residue in –3C-terminal position was engineered by using an antisense primer 5′-**CTA**CACTGAACAATC AAAGTGGACTGGC introducing A122C amino acid substitution. Gel purified PCR fragments were ligated separately into pETBlue-1 vector (Blunt-end ligation kit, Novagen, Madison, WI) according to manufacturer's instructions. To construct a 16–124aa RNase fragment with C-terminal ^{125}cys, PCR was done with a sense primer 5′-AACATGCATATGAGCACTTCCGC TGCCAGCAGC containing a *Nde*I restriction site and an antisense primer 5′-TACCTCGAG**CTA***GCA*CAC TGAAGCATCAAAGTGGACTGGC containing a *Xho*I restriction site, a stop-codon (bolded), and a codon for ^{125}cys (italicized). The PCR fragment was cut with *Nde*I and *Xho*I, gel purified, and cloned into pET32a(+) expression vector (Novagen) cut with *Nde*I and *Xho*I and gel purified to remove Trx-, His-, and S-tags. The resulting plasmids were confirmed by sequencing (CSU-Macromolecular Resources, Ft. Collins, CO).

Expression of BoS and BoS-cys

The wild type and A122C mutant 16–124aa fragments of RNase A designated BoS and BoS-cys, respectively, were expressed in *Escherichia coli* strain Tuner(DE3)pLacI (Novagen) grown in CircleGrow medium (Q-Biogen, Carlsbad, CA). The expression was induced by 1 mM IPTG (Life Technologies, Rockville, MD) at optical density of 0.4–0.5 U, at 600 nm. After induction, the cultures were grown for 4 h at 37 °C with shaking at 250–280 rpm; then harvested by centrifugation for 15 min at 5000*g*. BugBuster reagent (Novagen) was used to open bacteria and to wash inclusion bodies according to manufacturer's instructions. Then, inclusion bodies were also washed with CelLytic B reagent (Sigma, St. Louis, MO) diluted 1:10 with water and one more time with 1× HisBind buffer (Novagen). Purified inclusion bodies were solubilized by sonication in a buffer containing 8 M urea, 20 mM Tris–HCl, and 100 mM NaCl, pH 8.0. At this stage, the protein concentrations were determined by SDS–PAGE on 17.5% gels with the native bovine S-protein (Sigma) serving as a standard. Synthetic peptide, KETAAAKFERQH MDS, corresponding to bovine 15aa S-peptide (custom synthesized by GeneMed, San Francisco, CA) was added to solubilized inclusion bodies at the molar peptide to BoS (or BoS-cys) ratio of 1:1. The mixtures were then dialyzed as follows: first, 20 h against 20 V of 100 mM Tris–acetate, 100 mM NaCl, 0.5 M arginine, 1 mM glutathione (GSH, Novagen), and 0.4 mM oxidized glutathione (GSSG, Novagen), pH 8.6; then 24 h against 100 V of 100 mM NaPi and 150 mM NaCl, pH 7.2. To retain S-peptide in protein solutions during the dialysis step dialysis bags with MWCO of 1000 Da (Spectrum Laboratories, Rancho Dominguez, CA) were used.

Purification of BoS and BoS-cys

After the two-step dialysis, protein solutions were centrifuged at 25,000*g* for 15 min, mixed with 1/10 V of ice-cold 10% TFA, incubated on ice for 5 min, and re-centrifuged as above. The supernatants were passed through HiTrap Sepharose SP Fast Flow (1-ml prepacked columns, Amersham, Piscataway, NJ). Non-specific bacterial proteins were washed out with 20 mM NaOAc, pH 6.5; then BoS (or BoS-cys) was eluted with 1M NaCl in the same buffer. Fractions containing recombinant proteins were combined and loaded on a C8 RP HPLC Vydac 208TP510 300 μm column (250 × 10 mm), eluted at 2 ml/min, with 0.1% TFA (v/v) and a linear gradient of acetonitrile (0–65% over 30 min), and collected in tubes containing one volume of ice-cold 40 mM NaOAc, pH 6.5. Excess acetonitrile was removed from the protein preparations by passing them through HiTrap Sepharose SP column, as described above. Fractions containing BoS (or BoS-cys) were

Table 1
Sense primers used for amplification of S-protein ORFs with varying N-termini

S-protein ORF	Bovine RNase A (partial amino-acid sequence) 16	17	18	19	20	21	22	23	24	25	26	27	28	Protein expression
	Ser	Thr	Ser	Ala	Ala	Ser	Ser	Ser	Asp	Tyr	Cys	Asp	Glu	
	Sense primer													
16-124aa	AGC	ACT	TCC	GCT	GCC	AGC	AGC							+
17-124aa		ACT	TCC	GCT	GCC	AGC	AGC	TCC						–
18-124aa			TCC	GCT	GCC	AGC	AGC	TCC	AAC					+
19-124aa				GCT	GCC	AGC	AGC	TCC	AAC	TAC	TG			–
20-124aa					GCC	AGC	AGC	TCC	AAC	TAC	TGT	AAC	C	–
21-124aa						AGC	AGC	TCC	AAC	TAC	TGT	AAC	CAG	–

combined and dialyzed for 24 h against 100 V 20 mM NaOAc, 150 mM NaCl, pH 6.5, then centrifuged at 25,000*g* for 15 min, and stored at −70 °C in small aliquots. Endotoxin in purified proteins was determined by chromogenic LAL assay (BioWhittaker, Walkersville, MD) according to manufacturer's instructions.

The concentrations of proteins were determined by RP HPLC on C18 Waters Nova-Pack 5 μm column (150 × 3.9 mm) with elution at 0.75 ml/min with 0.1% TFA (v/v) and a linear gradient of acetonitrile (5–50% over 20 min). BoS and BoS-cys concentrations were calculated using 216-nm integral absorption in HPLC profiles with the native bovine S-protein serving as a standard. HP 1090 Series II instrument was used for all RP HPLC.

Ribonuclease activity assay

Ribonuclease activity assay, based on Novagen's S-tag Rapid Assay, was performed in a buffer containing 20 mM Tris–HCl, 100 mM NaCl, pH 7.5, and 0.1 mg/ml polyC. Chemically synthesized bovine 15aa S-peptide or S-tagVEGF, a recombinant human S-tagged $VEGF_{121}$ fusion protein expressed and purified as described in [6], was used for reconstitution of active ribonuclease. To determine specific activities of reconstituted ribonucleases, S-peptide (or S-tagVEGF) was added to the reaction mixtures to a final concentration of 85 nM and the reactions were initiated by addition of varying amounts of BoS, BoS-cys, or native bovine S-protein. After 5-min incubations at room temperature, the reactions were stopped by addition of 1/4 volume of ice-cold 10% TCA, incubated on ice for 5 min, and centrifuged for 10 min at 14,000*g*. Activity of the reconstituted ribonuclease was measured by absorbance of the supernatants at 280 nm, reflecting the amount of acid-soluble material released upon hydrolysis of polyC. In separate experiments, it was established that under these conditions the rate of polyC hydrolysis remains constant for at least 30 min. To estimate K_d values for complexes of S-tagVEGF with BoS, BoS-cys, or native bovine S-protein, ribonuclease activity was measured at constant concentrations of these proteins and variable concentration of S-tagVEGF. Kinetic data were used for calculation of the K_d values under the assumption that the initial rate of the hydrolysis is proportional to the concentration of reconstituted ribonuclease. DYNAFIT software was used for the global fitting with numeric iteration and calculation of the K_d values [7].

Modification with N-(1-pyrene)-maleimide

BoS-cys was incubated with 2-fold molar excess DTT in 20 mM NaOAc, 150 mM NaCl, and 1 M guanidine HCl, pH 6.5 for 4 h at room temperature. DTT was removed on a desalting column (D-Salt 5 ml, Pierce, Rockford, IL), equilibrated with 0.15 M NaCl, 0.5 M guanidine HCl, and 0.1 M NaPi, pH 7.2. *N*-(1-pyrene)-Maleimide (Molecular Probes, Eugene, OR) dissolved in DMSO was added to BoS-cys at the molar ratio of 4:5 and incubated for 40 min at room temperature. The reaction mixture was analyzed by RP HPLC on a C4 Alltech MACROSPHERE 300 5 μm column (150 × 4.6 mm) with elution at 0.75 ml/min with 0.1% TFA (v/v) and a linear gradient of acetonitrile (5–50% over 15 min) with detection at 216 and 340 nm. BoS protein was treated and analyzed under the same conditions.

Results

Construction of bovine S-proteins

We have first constructed the wild type and A122C mutant 21–124aa fragments of bovine RNase A and tried to express them in *E. coli*. Unexpectedly, both proteins were not detectable under the standard conditions of inducible protein expression (Materials and methods). In systematic search for readily expressable S-protein, we then constructed five S-protein fragments (SPf) with N-termini starting at ^{16}ser, ^{17}thr, ^{18}ser, ^{19}ala, or ^{20}ala. These five amino acids are dispensable in S-peptide for binding to S-protein and reconstitution of ribonuclease activity [2]. We therefore reasoned that the extension of S-protein N-termini by these amino acids would not affect binding of S-protein to S-peptide. We found that only two proteins were readily expressed in *E. coli*: 16–124aa SPf(A122C) and 18–124aa SPf(A122C) (Table 1). 16–124aa SPf(A122C) has been chosen for further analysis and designated BoS-cys. The corresponding wild type protein was designated BoS. It should be noted that BoS or BoS-cys in combination with 15aa S-peptide restores the complete 1–124aa sequence of RNase A with a single break between ^{15}ser and ^{16}ser. Similarly, subtilisin-generated fragments of RNase A restore its full-length amino-acid sequence with the break between ^{20}ala and ^{21}ser. We also constructed and tried to express 16–124aa SPf with an additional C-terminal ^{125}cys, but this protein was not detectable in induced bacterial cultures (data not shown).

Refolding of BoS and Bos-cys

Bos and BoS-cys were expressed in *E. coli* strain Tuner(DE3)pLacI and found in inclusion body fractions (Fig. 2A). Proteins were solubilized, refolded in the presence of equimolar amounts of S-peptide through a two-step dialysis, and purified via combination of cation-exchange chromatography and RP HPLC, as described under Materials and methods (see, Fig. 2A).

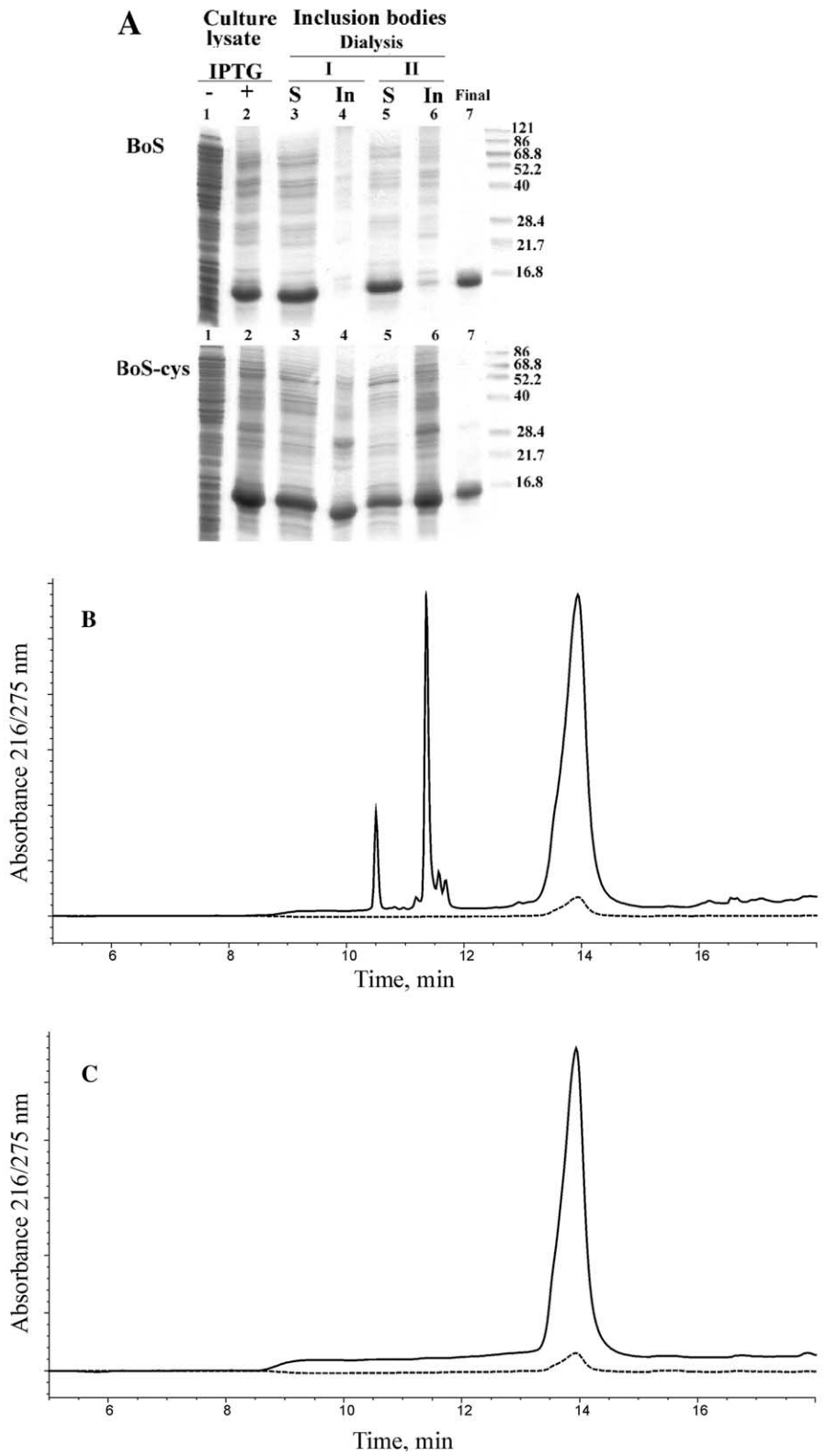

Fig. 2. BoS and BoS-cys are purified from inclusion bodies. The proteins were expressed in *E. coli* strain Tuner(DE3)pLacI and purified, as described under Materials and methods. (A) Total uninduced and induced bacterial lysates (−/+ IPTG), solubilized inclusion bodies after a two-step refolding dialysis (S, soluble, In, insoluble), and purified proteins (Final) were analyzed by SDS–PAGE on 17.5% gels, followed by SafeBlue staining. Molecular weights of protein markers are indicated. (B) RP HPLC analysis of 1/1000 part of the supernatant after two-step refolding dialysis of BoS (C18 Waters Nova-Pack 5 μm column, 150 × 3.9 mm). Retention times: 11.4 min, peptide; 14 min, BoS protein. (C) RP-HPLC analysis of purified BoS. Solid line: absorbance at 216 nm; dotted line: absorbance at 275 nm.

During purification, S-peptide was removed by RP HPLC (Fig. 2B for BoS). The proteins were ⩾99% pure, as judged by Safe-stained SDS–PAGE gels (Fig. 2A, lane 7) and RP HPLC (Fig. 2C for BoS). Endotoxin content in preparations of both proteins was 20 EU/mg, as determined by chromogenic LAL assay. The yields of the purified proteins were 10 mg/L for BoS and 8 mg/L for BoS-cys.

Purified BoS and BoS-cys were functionally active, as judged by the ability to bind S-peptide and reconstitute ribonuclease activity (see below). In contrast, refolding of BoS and BoS-cys without S-peptide yielded functionally inactive proteins (data not shown). To test how the structure of S-peptide might affect refolding of S-protein, we have performed refolding in the presence of a mutant S-peptide with the M13A amino acid substitution. It was reported that the M13A substitution does not significantly affect thermodynamic parameters associated with binding of S-peptide to native S-protein [8]. However, refolding of BoS and Bos-cys in the presence of the M13A mutant S-peptide resulted in significantly decreased functional activities of purified proteins (data not shown).

BoS and BoS-cys are functionally active

High affinity of BoS and BoS-cys to S-tagged proteins is crucial for using these proteins in molecular vehicles for targeted drug delivery. To characterize affinity of BoS and BoS-cys to S-tagged recombinant human $VEGF_{121}$ fusion protein (S-tagVEGF), we have measured ribonuclease activity under conditions of equilibrium between free and S-protein bound S-tagVEGF. To derive K_d values for complexes BoS/S-tagVEGF and BoS-cys/S-tagVEGF, the experimental data were analyzed with DYNAFIT software [7] under the assumption that the initial rate of the hydrolysis is proportional to the concentration of reconstructed ribonuclease. Native bovine S-protein was used as a standard in these experiments. We found that affinity of S-tagVEGF to BoS was ~5-fold higher and affinity to BoS-cys was similar to that of native bovine S-protein (Fig. 3).

Native bovine S-protein is used for quantitation of recombinant S-tagged proteins with Novagen's S-tag Rapid Assay Kit [3]. This assay is based on experimental observation that free and protein-fused S-peptides form equally active RNase S. To establish whether BoS

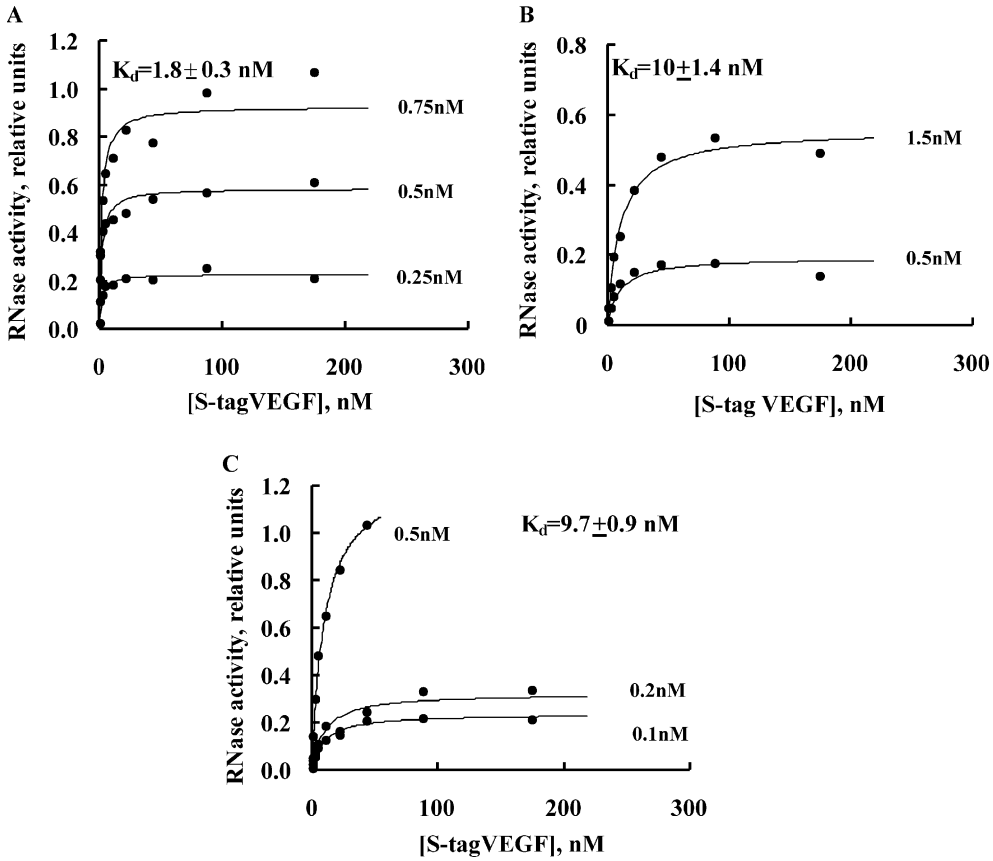

Fig. 3. Affinities of BoS and BoS-cys to S-tagVEGF are higher or similar to that of native bovine S-protein. Varying amounts of S-tagVEGF were mixed with recombinant or native bovine S-proteins and reconstituted ribonuclease activity was measured as described under Materials and methods. To estimate K_d, data were fitted (solid curves) with DYNAFIT software [7]. (A) BoS at concentrations of 0.25, 0.5, and 0.75 nM. (B) BoS-cys at concentrations of 0.5 and 1.5 nM. (C) Native bovine S-protein at concentrations of 0.1, 0.2, and 0.5 nM.

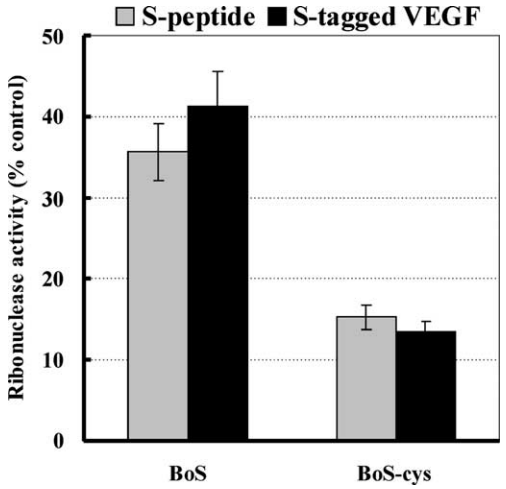

Fig. 4. Recombinant BoS and BoS-cys form similarly active RNase S with S-peptide and S-tagVEGF. Varying amounts of recombinant proteins and native bovine S-protein were mixed with 85 nM S-peptide or S-tagVEGF and restored ribonuclease activity was measured as described under Materials and methods. Specific activities of corresponding RNase S were calculated as optical density of acid-soluble material released by 1 nM of corresponding S-protein per minute. Specific activity of native bovine S-protein based RNase S was taken as 100%.

(BoS-cys) can be used for quantitation of recombinant S-tagged proteins, we have compared enzymatic activities of RNase S reconstituted by BoS (BoS-cys) mixed with chemically synthesized 15aa S-peptide or S-tagVEGF.Specific activities of BoS and BoS-cys based RNase S were, respectively, 2.8- and 6.7-fold lower than that of bovine RNase S (Fig. 4). However, the specific activities of RNase S complexes formed with S-peptide were similar to those formed with S-tagVEGF (Fig. 4). The latter results indicate that recombinant BoS can be used for quantitation of S-tagged proteins as reliably as native bovine S-protein [3].

^{122}*cys in BoS-cys can be selectively modified*

Oxidative refolding of BoS and BoS-cys has been performed in a red-ox buffer containing GSH and GSSG at the ratio of 3:1. Refolding of RNase A in red-ox buffers proceeds via multiple intramolecular and mixed disulfide intermediates until the four native disulfide bonds are finally formed [9,10]. It is likely that refolding of BoS and BoS-cys in the presence of S-peptide proceeds via the same routes, while these proteins evolve toward similar functionally active conformations. Since ^{122}cys in BoS-cys does not have a "natural" partner, it might remain in the mixed disulfide with GSH, as it was reported for other recombinant proteins with the odd numbers of cysteines [11]. We found that completely refolded BoS-cys was not modified with a thiol reagent, *N*-(1-pyrene)-maleimide, indicating that there is no free cysteine in the protein. Since the four native disulfide bonds in RNase are stable in a highly reducing environment [10], we reasoned that GS-^{122}cys would be the only mixed disulfide bond that might be disrupted by a mild DTT treatment. Equal amounts of BoS-cys and BoS were treated with DTT, purified on desalting columns, and then modified with *N*-(1-pyrene)-maleimide (see, Materials and methods). This treatment resulted in modification of cysteine in BoS-cys, as judged by increased RP HPLC elution time for ~75% of treated protein, and co-elution of pyrene moiety (measured at 340 nm) with the modified protein (Fig. 5A). In contrast, the same treatment did not affect RP HPLC elution time of either BoS or *N*-(1-pyrene)-maleimide, indicating that under the selected conditions the native disulfide bonds were unaffected (Fig. 5B).

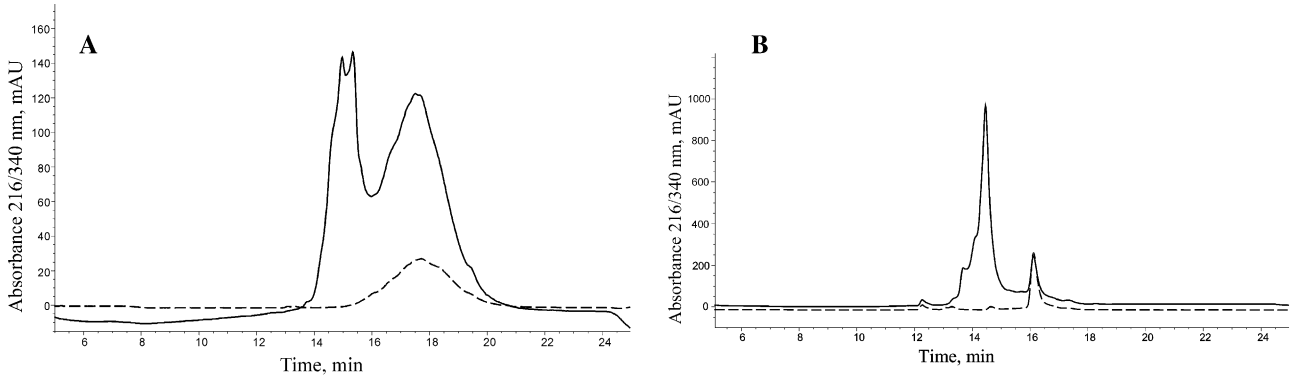

Fig. 5. BoS-cys is selectively modified with *N*-(1-pyrene)-maleimide. Purified BoS-cys and BoS were treated sequentially with DTT and *N*-(1-pyrene)-maleimide (see, Materials and methods for details). The products were analyzed by RP-HPLC (Alltech MACROSPHERE 300 5 μm column, 150 × 4.6 mm). Solid line: absorbance at 216 nm; dotted line: pyrene moiety absorbance at 340 nm. (A) BoS-cys analysis, retention times: 15.0 min, BoS-cys unaffected by treatment; 15.2 min, BoS-cys after DTT treatment; 17.6 min, pyrene modified BoS-cys. Note co-elution of pyrene (absorbance at 340 nM) and protein (absorbance at 216 nM). (B) BoS analysis, retention times: 14.5 min, BoS unaffected by treatment; 16.1 min, free *N*-(1-pyrene)-maleimide.

Discussion

We have engineered, expressed, and purified the wild type and A122C mutant 16–124aa fragments of bovine pancreatic RNase A named BoS and BoS-cys, respectively. To the best of our knowledge, this is the first successful expression of S-protein fragments capable of high affinity binding to S-peptide or S-tagged proteins. Interestingly, only two out of six possible S-protein fragments were readily expressed in a bacterial system (Table 1). The crucial role of N-terminal amino acid residues for efficiency of bacterial protein expression is well established and original findings are summarized in the "N-end rule" [12]. Surprisingly, failed S-protein variants start with amino acid residues permissible under the "N-end rule", suggesting more complex regulatory mechanisms.

BoS and BoS-cys were recovered from inclusion bodies and their refolding into functional conformation capable of binding S-peptide presented unexpected challenge. Although bovine RNase A is a classic system for protein folding studies [9,10], we did not find any reports on successful refolding of reduced S-protein fragment. Our experiments indicate that the presence of S-peptide in a red-ox refolding buffer is necessary for obtaining functional BoS and BoS-cys. This results suggest that binding of S-peptide during refolding is necessary for proper positioning of eight cysteine residues to form four disulfide bonds that stabilize the structure of RNase A. After the refolding is complete, S-peptide can be removed by RP-HPLC, but recombinant BoS and BoS-cys retain the ability to bind S-peptide and S-tagged proteins.

The affinities of BoS and BoS-cys to S-tagVEGF are, respectively, higher and similar to that of the native bovine S-protein. On the other hand, the ribonuclease activities reconstituted with recombinant proteins are somewhat lower than that obtained with the native bovine S-protein. It remains to be established whether these effects are due to differences in folding or to some post-translational modifications of the native protein. Importantly, the specific activities of complexes of recombinant S-proteins with S-peptide and S-tagVEGF were similar. If this finding holds for other S-tagged proteins, it would be possible to use BoS for quantitation of S-tagged proteins and avoid animal-derived bovine S-protein.

BoS-cys was engineered for constructing a novel type of molecular vehicle for targeted drug delivery [4,5] The vehicles are assembled via non-covalent interactions between an S-tagged targeting protein and a "payload" module made of S-protein conjugated to a drug carrier. Currently, only animal-derived bovine S-protein is available for random chemical conjugation to drug carriers. BoS-cys provides a cysteine residue for site-specific conjugation of drug carriers to the S-protein. We found that refolding in a red-ox buffer yields BoS-cys with protected ^{122}cys. However, after a mild DTT treatment ^{122}cys becomes available for a chemical modification. We expect that the option of constructing payload modules via site-specific modification of BoS-cys provides more flexibility in designing and testing modular vehicles for targeted drug delivery.

Acknowledgments

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