Ribonuclease S redux†‡

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The S-peptide and S-protein components of bovine pancreatic ribonuclease form a noncovalent complex with restored ribonucleolytic activity. Although this archetypal protein-fragment complementation system has been the object of historic work in protein chemistry, intrinsic limitations compromise its utility. Modern methods are shown to overcome those limitations and enable new applications.

In the late 1950's, Fred Richards discovered protein-fragment complementation—the restoration of protein function by the noncovalent interaction of component polypeptides. Working at the renowned Carlsberg Laboratory, he found that the protease subtilisin catalyzes the cleavage of bovine pancreatic ribonuclease (RNase A[1,2]; EC 3.1.27.5) between residues 20 and 21.[3] The resulting complex, RNase S (wherein "S" refers to subtilisin), is composed of two fragments: S-peptide (residues 1–20) and S-protein (residues 21–124). Separation of these two components abolished ribonucleolytic activity, which was restored by their mixing.[4–6] This work, which served to launch the field of molecular recognition, was done before the three-dimensional structure of any protein was known. Later, the first 15 residues of S-peptide (S15) were found to yield a fully active complex.[7] In the last fifty years, many other proteins have ceded to fragment complementation.[8]

The RNase S system has had a singular role in protein chemistry. Prior to the advent of recombinant DNA technology, chemists synthesized S-peptide analogues and studied their complexes with S-protein.[9,10] A harbinger of current work on proteins containing nonnatural residues, these studies revealed important principles of protein folding,[11] protein–protein interactions,[12,13] and enzymology.[14] Since the 1990's, the affinity of S-peptide for S-protein has served as the basis for the fusion protein system known as "S·Tag".[15–17] In the last decade, RNase S has enabled the synthesis of protein dendrimers,[18] evaluation of peptide-bond isosteres,[19] and development of targeted drug delivery systems.[20–22]

The traditional RNase S system is, however, compromised in its utility. First, the isolation of the individual RNase S components is problematic. Subtilisin[23] is a non-specific protease that cleaves RNase A not only between residues 20 and 21, but also at other peptide bonds.[24,25] Hence, digestion is commonly quenched prior to complete cleavage at the S-peptide/S-protein boundary. The result is an inefficient conversion to RNase S,[26] and a tedious isolation from intact RNase A (which contaminates commercial preparations). Moreover, subtilisin is not suitable for selective cleavage at the analogous S-protein/S-peptide boundary of RNase A homologues from humans and other vertebrates.[26] Likewise, amino-acid substitutions near residues 20 and 21 of RNase A can hinder digestion by subtilisin.[27]

A second major problem relates to the stability of the S-protein·S-peptide complex, which has a K_d value in the micromolar–nanomolar range.[12,29] At lower concentrations, the complex is nearly fully dissociated, abolishing catalytic activity. Moreover, RNase S is less thermostable than RNase A,[30] and is more susceptible to chemical denaturation[4] and proteolytic degradation.[3,31,32]

Here, we revisit RNase S. We use modern methods of molecular biology and protein chemistry to facilitate the production and purification of S-protein and to install a covalent bond between the components, thereby generating "RNase–S" (Fig. 1). These changes overcome the major limitations of the traditional system and provide new opportunities for protein chemists.

In our hands, digestion of RNase A with subtilisin resulted in a complex mixture of products and a daunting separation (Fig. 2). We reasoned that increased specificity for proteolytic cleavage between the S-peptide and S-protein regions of RNase A would simplify the isolation procedure.

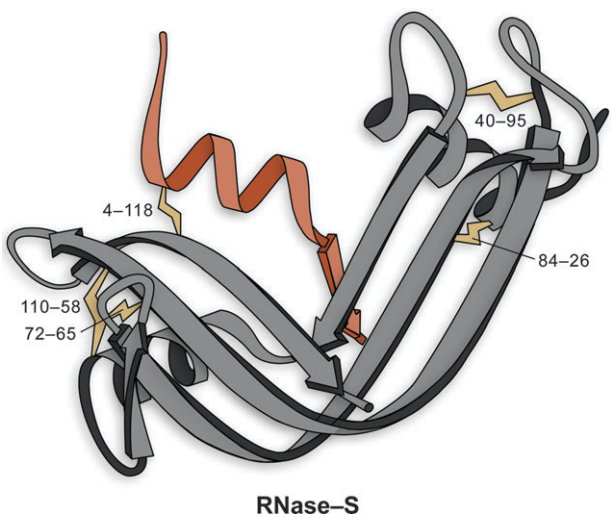

Fig. 1 Notional structure of "RNase–S", the mixed disulfide of A4C S15 (red) and V118C S-protein (gray). Disulfide bonds (yellow) form between the indicated cysteine residues. The image is based on the known structure of the noncovalent S-protein·S15 complex.[28]

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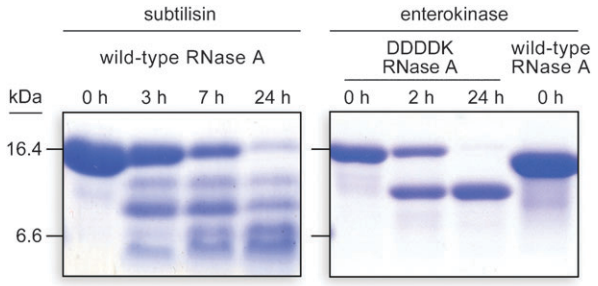

Fig. 2 Analysis of the proteolytic digestion of ribonucleases with SDS–PAGE. Left, products of the cleavage of wild-type RNase A by subtilisin. Right, products of the cleavage of DDDDK RNase A and wild-type RNase A by enterokinase.

Enterokinase is a digestive protease that catalyzes cleavage on the C-terminal side of the amino-acid sequence AspAspAspAspLys.[33] To avail ourselves of the higher substrate specificity of enterokinase relative to subtilisin, we inserted an enterokinase cleavage site between residues 20 and 21 of RNase A variants.

Enterokinase digestion of wild-type RNase A with an inserted enterokinase cleavage site (DDDDK RNase A) resulted in essentially complete conversion to the desired RNase S product (Fig. 2). S-protein and S-peptide (containing the C-terminal DDDDK sequence) were separated easily by high-performance liquid chromatography (HPLC) (Fig. 3) to yield purified components.

Next, we took advantage of previous work demonstrating that the introduction of cysteine residues at positions 4 and 118 of RNase A results in the spontaneous formation of a stabilizing disulfide bond with little effect on catalytic activity.[34–36] Analogous disulfides also stabilize homologous ribonucleases.[22,37,38] We reasoned that introducing this disulfide bond in the RNase S complex would eliminate drawbacks of the noncovalent system. Accordingly, we replaced Val118 with a cysteine residue in DDDDK RNase A.

To enhance our system still further, we added another substitution to DDDDK/V118C RNase A. His12 is the catalytic base in the active site of RNase A.[1,2] Its replacement with an alanine residue decreases ribonucleolytic activity by $>10^4$-fold without perturbing the three-dimensional structure.[39,40] As residue 12 will be discarded after digestion with enterokinase, the H12A substitution serves as a safeguard, diminishing the catalytic activity from any trace contaminant of intact enzyme in an S-protein sample. Finally, to prevent the adventitious air oxidation of Cys118, we protected the

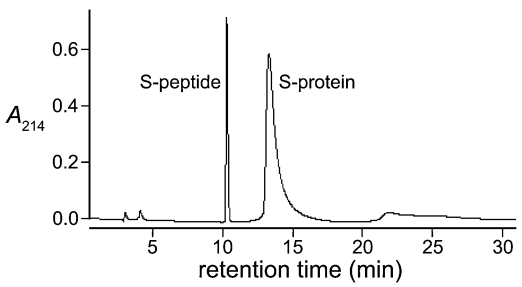

Fig. 3 Separation of S-peptide and S-protein components from DDDDK RNase A by reversed-phase HPLC.

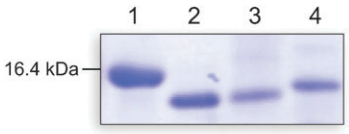

Fig. 4 Analysis of RNase–S semisynthesis with SDS–PAGE. Lane 1, RNase A; lane 2, RNase S; lane 3, S-protein derived from H12A/DDDDK/V118C RNase A; lane 4, A4C S-peptide + V118C S-protein of lane 3.

purified V118C S-protein by reaction with 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB), thereby forming a mixed disulfide.

To effect the semisynthesis of RNase–S, we added deprotected V118C S-protein to the synthetic A4C variant of S15, which had been activated as a mixed disulfide with 2-nitro-5-thiobenzoic acid (NTB). After removal of the NTB byproduct, the presence of the disulfide linkage between A4C S15 and V118C S-protein was apparent by both SDS–PAGE (Fig. 4, lane 4) and MALDI–TOF mass spectrometry (m/z 13324; expected: 13317). The semisynthesis of RNase–S in the opposite manner (that is, by reacting unactivated A4C S15 with NTB-activated S-protein) was less effective.

We analyzed RNase A and the components of RNase S with non-reducing zymogram electrophoresis, an extremely sensitive technique for detecting ribonucleolytic activity (clear bands on a dark blue background).[41] Neither V118C S-protein (Fig. 5, lane 2) nor V118C S-protein mixed with cysteine-free S-peptide (Fig. 5, lane 3) displayed detectable activity. In marked contrast, RNase–S had robust activity (Fig. 5, lane 4). The activity due to RNase A contamination of commercial RNase S is apparent (Fig. 5, lane 6).

Finally, we assessed the activities of RNase S and RNase–S as catalysts of RNA cleavage at high and low enzymic concentrations. RNase–S retains the ribonucleolytic activity of RNase A across a concentration-range of 10^4-fold (Fig. 6). In contrast, RNase S loses detectable activity over that range.

Despite many advances since the 1950's, the isolation of RNase S components has remained a challenge. In their initial isolation of S-protein and S-peptide, Richards and Vithayathil digested a whopping 730 mg of RNase A with subtilisin.[4] S-protein was separated from S-peptide by acid precipitation. In our hands, a subtilisin-digest of RNase A resulted in a complex mixture of products (Fig. 2). S-protein can be isolated from such mixtures by using S-peptide-affinity chromatography.[26] This method requires an expensive custom-made column, and still suffers from the inefficient and imprecise conversion of RNase A to RNase S. Furthermore, subtilisin is an ineffective means to generate RNase S from some RNase A variants[27] and homologues.[26] Of course, S-protein can be produced as a

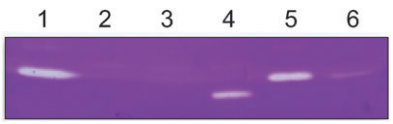

Fig. 5 Analysis of RNase–S semisynthesis with zymogram electrophoresis. Lane 1, RNase A; lane 2, S-protein derived from H12A/DDDDK/V118C RNase A; lane 3, S15 + V118C S-protein of lane 2; lane 4, A4C S15 + V118C S-protein of lane 2; lane 5, RNase A; lane 6, commercial RNase S.

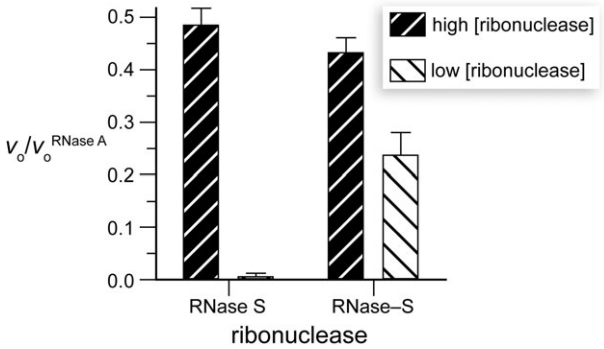

Fig. 6 Initial velocities of RNA cleavage at high (0.15 μM) and low (25 pM) ribonuclease concentrations, relative to RNase A. "RNase S" refers to the noncovalent complex of S15 and the S-protein derived from H12A/DDDDK/V118 RNase A.

polypeptide by recombinant DNA technology. But because S-protein does not fold properly in the absence of S-peptide,[11] synthetic S-peptide must be added during the folding process,[21] which is wasteful and inefficient.

Our strategy for producing S-protein has many advantages. Insertion of an enterokinase recognition site enables the complete, regioselective cleavage of RNase A (Fig. 2). The absence of side-products facilitates the separation of S-peptide and S-protein (Fig. 3). Moreover, our strategy makes S-protein accessible from RNase A homologues, including human pancreatic ribonuclease. As only bovine S-protein has been explored in detail to date, our strategy enables new applications and structure–function analyses.

The noncovalent interaction of S-protein and S-peptide limits the use and complicates the characterization of RNase S complexes. RNase S is not active at low concentrations because this noncovalent complex has modest stability that is dependent on solution conditions.[12,29] Further, the traditional RNase S system has less thermostability[30] and less resistance to denaturants[4] and proteases[3,31,32] than does RNase A.

The covalent attachment of S-peptide to S-protein endows RNase–S with high catalytic activity at low concentrations (Fig. 6). We propose that tethering single-stranded DNA to the S-peptide fragment of RNase–S could lead to sequence-specific ribonucleases that are useful *in cellulo*.[42] The 4–118 disulfide bond also enables transient tethering strategies[43] that could yield S-peptide variants with higher affinity for S-protein. Finally, the ability to assess catalytic activity at low concentrations could facilitate the discovery of new structure–function relationships.

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