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Studies on Peptides. CIV.[1,2] Synthesis of Ribonuclease S'

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Ribonuclease (RNase) S' was prepared by the combination of synthetic S-peptide and synthetic S-protein. The S-peptide was synthesized by the azide condensation of three fragments, (1—8), (9—13) and (14—20), followed by deprotection with 1 M trifluoromethanesulfonic acid–thioanisole in trifluoroacetic acid. The same reagent was employed to remove all protecting groups from the protected S-protein. In order to establish the disulfide bonds of the deprotected S-protein, glutathione-mediated air oxidation was performed in the presence of the S-peptide. The product was purified by affinity chromatography followed by ion-exchange chromatography on sulfopropyl-Sephadex to afford RNase S' in 0.5% yield from the protected S-protein.

Keywords——synthetic S-peptide; synthetic S-protein; deprotection with 1 M trifluoromethanesulfonic acid in TFA; thioanisole-mediated deprotection; glutathione-mediated disulfide formation; affinity purification; RNase S' activity

After our total synthesis of a crystalline protein with the full enzymatic activity of bovine pancreatic ribonuclease (RNase) A,[4] we wish to report that we have now succeeded in generating RNase activity by the combination of synthetic S-peptide and synthetic S-protein (Fig. 1).

In 1958, Richards[5] demonstrated that combination of two inactive components, S-peptide (RNase A 1—20) and S-protein (RNase A 21—124) derived from the limited proteolysis of RNase A by subtilisin, resulted in the full regeneration of enzymatic activity by forming a complex, named RNase S. The first semi-synthesis of an enzyme (RNase S') was reported by Hofmann *et al.*[6] in 1964, from synthetic S-peptide and the natural S-protein. In 1969, when the solid phase synthesis of RNase A with partial enzymatic activity was reported by Gutte and Merrifield,[7] an alternative semi-synthesis of RNase S' was simultaneously reported by the Merck research group,[8] from synthetic S-protein and the natural S-peptide. However, in this synthesis, only 2γ of RNase S' activity in solution was recorded and no chemical characterization

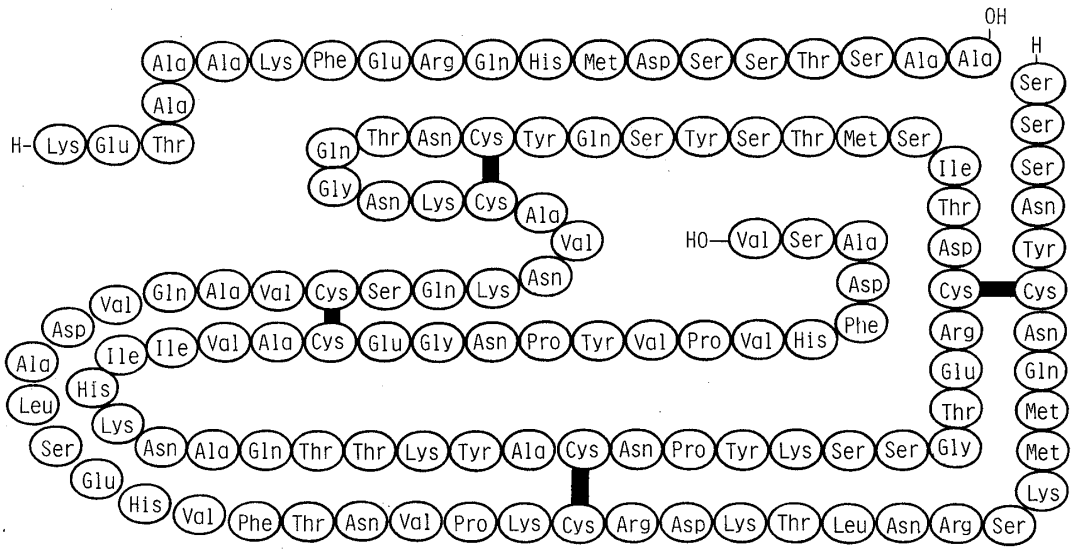
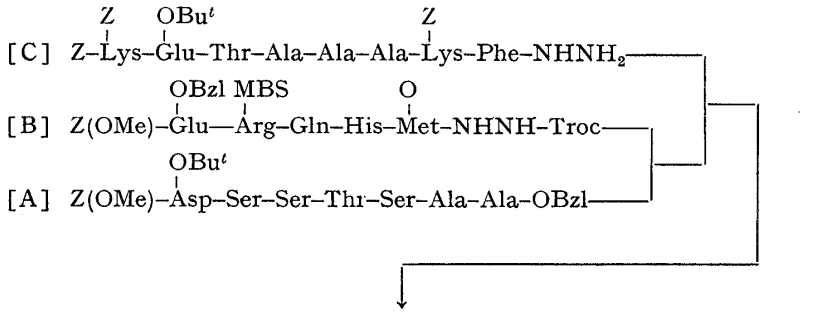


Fig. 1. Structure of RNase S'

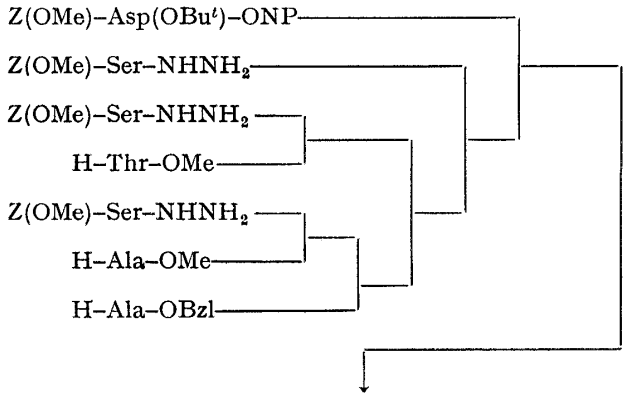
was carried out. Thus, synthetic RNase S′, including reduced-reoxidized S-protein,[9] has never been isolated for chemical characterization.

At present, a considerable amount of information about the synthesis of S-peptide and its analogs is available.[10] Alternatively, we synthesized the protected S-peptide, Z–Lys(Z)–Glu(OBu^t)–Thr–Ala–Ala–Ala–Lys(Z)–Phe–Glu(OBzl)–Arg(MBS)–Gln–His–Met(O)–Asp–Ser–Ser–Thr–Ser–Ala–Ala–OBzl, by successive azide condensation[11] of three fragments; Z–(RNase 1—8)–$NHNH_2$ (C), Z(OMe)–(RNase 9—13)–NHNH–Troc (B) and Z(OMe)–(RNase 14—20)–OBzl (A), as shown in Fig. 2. Of these, two fragments, (B) and (C), were those used for our previous synthesis of RNase A. The fragment (A) was newly synthesized starting with H–Ala–OBzl by a method similar to that employed previously, as shown in Fig. 3. The Bu^t ester of the Asp residue (position 14, the N-terminal in A) was removed by TFA, together with the Z(OMe) group, prior to the next condensation, in order to suppress base-catalyzed aminosuccinimide formation,[12] as discussed previously. The TFA-treated peptide thus obtained was subsequently condensed with the pentapeptide, Z(OMe)–Glu(OBzl)–Arg(MBS)–Gln–His–Met(O)–$NHNH_2$, derived from the fragment (B) by treatment with Zn in acetic acid for removal of the Troc group.[13] The chain elongation of the resulting protected dodecapeptide ester by condensation with the fragment (C) was performed essentially in the same manner as described previously. The protected eicosapeptide ester (protected S-peptide) was obtained in a homogeneous form, after precipitation from DMSO with MeOH.



H–Lys–Glu–Thr–Ala–Ala–Ala–Lys–Phe–Glu–Arg–Gln–His–Met–
Asp–Ser–Ser–Thr–Ser–Ala–Ala–OH

Fig. 2. Synthetic Scheme for S–Peptide



Z(OMe)–Asp(OBu^t)–Ser–Ser–Thr–Ser–Ala–Ala–OBzl

Fig. 3. Synthetic Scheme for the Protected Heptapeptide Ester, Z(OMe)–(S–peptide 14—20)–OBzl [A]

For deprotection, the TFMSA-thioanisole in TFA system[14] was employed. The protected S-peptide was treated with 1 M TFMSA–thioanisole in TFA in the presence of *m*-cresol in an ice-bath for 60 min. This treatment was repeated three times to ensure complete deprotection. It is interesting to note that a model compound, H–Met(O)–OH,[15] was reduced back to methionine in nearly 80% yield within 60 min by this thioanisole–acid system in an ice-bath. The deprotected peptide was converted to the corresponding acetate by treatment with Amberlite CG-4B and then treated with dilute ammonia at pH 10 for 30 min to reverse the possible N→O shift at the Thr and Ser residues.[16] To ensure the complete reduction of the Met(O) residue, the product was incubated with dithiothreitol[17] and the reduced product was then purified by gel-filtration on Sephadex G-25, followed by ion-exchange chromatography on CM-cellulose. The homogeneous product thus obtained was combined with the synthetic S-protein, as shown in Fig. 4.

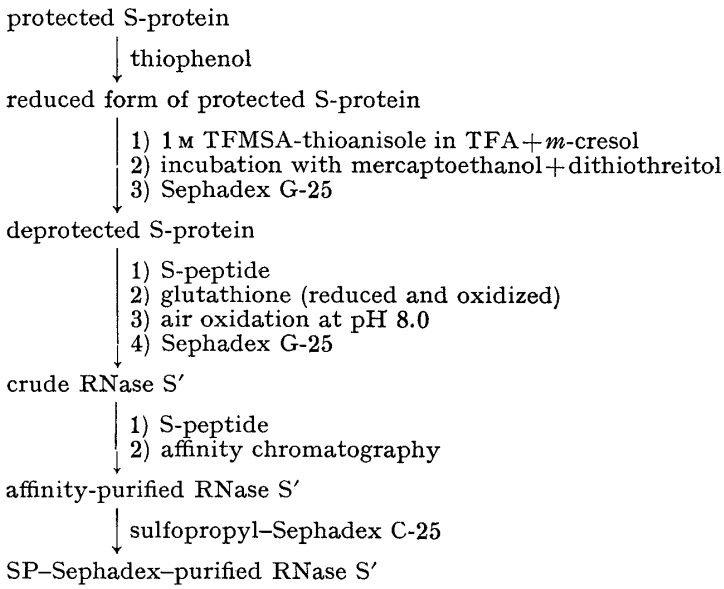


Fig. 4. Scheme for the Synthesis of RNase S′

As reported in the previous synthesis of RNase A, the protected S-protein was treated with thiophenol for reduction of the sulfoxide of Cys(MBzl) residues[18] which formed partially during the synthesis. The Met(O) residues were also reduced under this treatment. The treated sample was then deprotected with 1 M TFMSA–thioanisole in TFA in the presence of *m*-cresol. This acid treatment was repeated three times to ensure complete deprotection of all 28 protecting groups; Z(OMe) from the N-terminal, Z from Lys, Bzl from Glu, Asp and the C-terminal, MBzl from Cys and MBS[19] from Arg. The deprotected protein was reduced with thiols,[20] as performed for the synthesis of RNase A, in 0.2 M Tris–HCl buffer containing 4 M guanidine HCl at pH 8.6 and then gel-filtered on Sephadex G-25 with 0.1 N AcOH as an eluant. The synthetic S-peptide obtained above (1 eq) was mixed with the appropriate eluate fraction, though the requirement of the S-peptide for the correct disulfide bond formation of reduced S-protein has not been established.[9] Next, according to Chavez and Scheraga,[9a] the reduced and oxidized forms of glutathione were further added and the solution was diluted with 0.2 M Tris–HCl buffer at pH 8.0 to a protein concentration of 0.2 mg/ml, a ten times higher concentration than that employed in the former RNase A synthesis, as recommended by Veber *et al.*[21] After air oxidation for 5 days, the activity detected was 2 to 2.5% in the solution and 2.5% in the isolated product, after dialysis followed by gel-filtration, when assayed using yeast RNA as a substrate.[22] When the air oxidation was performed either in 2-fold diluted or concentrated solution, we could not obtain any improvement. The activity we detected

was negligible in the former experiment and only 1.6% in the latter experiment. As will be discussed later, the yield we obtained in this air oxidation step for disulfide formation was considerably lower than that in the former RNase A synthesis.

Next, we decided to purify the above air-oxidized product by means of affinity chromatography, referring to the purification of RNase A.[23] Though the activity of the crude RNase S′ obtained above did not alter, even when assayed with addition of the synthetic S-peptide, this crude product was mixed with the synthetic S-peptide and applied to a column of Sepharose-(4B)-5′-(4-aminophenylphosphoryl)-uridine-2′(3′)-phosphate. In this purification step, the activity was brought up to 62%, but the yield was only 2%. Without the aid of affinity chromatography, isolation of the highly active product in such a low yield would be an extremely difficult task. Next, referring to Chavez and Scheraga's procedure[9a] for the separation of RNase S from RNase A, the affinity purified product was subjected to further purification by ion–exchange chromatography on sulfopropyl (SP)-Sephadex C-25. CM-cellulose purification was judged to have great possibility to dissociate the S-protein and S-peptide complex once formed. No significant increase of the activity could be obtained in this purification step. The activity of the product against yeast RNA and 2′,3′-cyclic cytidine phosphate[24] was 64% and 65% of that of natural RNase A, respectively. Starting with the reduced form of protected S-protein, the total yield we obtained here was only 0.5%. Because of this low yield, we did not carry out further purification. Though the yield was low and the activity which we attained here was not fully satisfactory, the amino acid ratios in a 6 N HCl hydrolysate of the product thus purified matched those of natural RNase A fairly well. This result seems to justify the conclusion that the product we isolated as described above is the desired complex, RNase S′, formed by combination of the synthetic S-protein and the synthetic S-peptide in a molar ratio of one to one.

When the results of parallel experiments for the synthesis of RNase A (total yield 6.6%) and RNase S′ (total yield 0.5%) are compared, it can be seen that folding of a protein with a partial sequence was more difficult than that of a complete protein. From the synthetic viewpoint, our results provide additional information relating to the previous conclusion that the entire amino acid sequence of a native protein is essential for the formation of native three-dimensional structure.[9,20,25] It seems very likely that a reduced protein, such as reduced natural RNase A, still retains a significant amount of native structure, as regards the contents of α-helix and β-turn structures,[25] but this is not the case for a synthetic protein, which was exposed to acid deprotection. Unless suitable methodologies are explored in the case of deprotected synthetic proteins to establish favorable (presumably necessary) three-dimensional structures for folding, prior to air oxidation, it will be extremely difficult to duplicate exactly refolding studies on native proteins by using synthetic proteins.

Despite these difficulties, we were able to duplicate Richads' earlier observation on the interaction of native S-protein and native S-peptide with synthetic S-protein and synthetic S-peptide.

Experimental

General experimental procedures employed here were essentially the same as described in Part 88 of this series.[4] Rotations were determined with a Union PM-101 digital polarimeter. The amino acid compositions of acid hydrolysates were determined with a Hitachi model KLA-5 amino acid analyser, and values are uncorrected. TLC was performed on silica gel (Kieselgel G, Merck). *Rf* values refer to the following solvent systems: Rf_1 $CHCl_3$–MeOH–H_2O (8:3:1), Rf_2 *n*-BuOH–AcOH–AcOEt–H_2O (1:1:1:1), Rf_3 *n*-BuOH–AcOH–pyridine–H_2O (30:20:6:24). For dialysis, cellulose tubing VT-351 (Lot. No. V9H-1894) was purchased from Nakarai Chemical Co. A Hitachi wavelength–tunable effluent monitor (034-0029) was used to determine the enzymatic activity against 2′,3′-cyclic cytidine phosphate. For affinity chromatography, Sepharose (4B)-5′-(4-aminophenylphosphoryl)-uridine-2′(3′)-phosphate was prepared according to Wilchek and Gorecki.[23] The following reagents and enzymes were purchased from Sigma Chemical Co.; 2′,3′-cyclic cytidine monophosphoric acid sodium salt (Lot. No. 76C-7510), yeast RNA (Type XI, Lot. No. 124C-8150),

leucine aminopeptidase (LAP, Lot. No. 79C-8110), natural bovine pancreatic RNase A (Type XII-A, Lot. No. 49C-8049, 76 U/mg). The unit of activity was confirmed according to Kunitz.[22]

1. Synthesis of the S-Peptide——Z(OMe)–Ser–Ala–Ala–OBzl: The azide [prepared from 8.91 g (25 mmol) of Z(OMe)–Ser–Ala–$NHNH_2$[26]] in DMF (90 ml) and Et_3N (3.5 ml, 25 mmol) were added to an ice-chilled solution of H–Ala–OBzl [prepared from 17.6 g (50 mmol) of the tosylate with 7.62 ml (55 mmol) of Et_3N] in DMF (150 ml) and the solution, after stirring at 4°C for 24 h, was concentrated. The residue was triturated with ether and 5% citric acid. The resulting powder was washed with 5% citric acid, 5% $NaHCO_3$ and H_2O and recrystallized from MeOH and ether; yield 8.11 g (64%), mp 178—183°C, $[\alpha]_D^{20}$ −6.8° (c=1.0, DMF). Rf_1 0.69. *Anal.* Calcd for $C_{25}H_{31}N_3O_8$: C, 59.87; H, 6.23; N, 8.38. Found: C, 59.72; H, 6.22; N, 8.46.

Z(OMe)–Ser–Thr–Ser–Ala–Ala–OBzl: Z(OMe)–Ser–Ala–Ala–OBzl (5.51 g, 11 mmol) was treated with TFA–anisole (20 ml–5 ml) in an ice-bath for 60 min, then dry ether was added. The resulting powder was collected by filtration, dried over KOH pellets *in vacuo* for 3 h and dissolved in DMF (50 ml) containing Et_3N (1.67 ml, 12.1 mmol). The azide [prepared from 5.07 g (13.2 mmol) of Z(OMe)–Ser–Thr–$NHNH_2$[4]] in DMF (50 ml) and Et_3N (2.0 ml, 14.5 mmol) were added to the above ice-chilled solution and the mixture was stirred at 4°C for 48 h. The solvent was removed by evaporation and the residue was treated with ether and 5% citric acid. The resulting powder was washed as stated above and precipitated from DMF with MeOH; yield 6.61 g (87%), mp 230—231°C, $[\alpha]_D^{20}$ −1.0° (c=1.0, DMF). Rf_1 0.67. *Anal.* Calcd for $C_{32}H_{43}N_5O_{12}$: C, 55.72; H, 6.28; N, 10.16. Found: C, 55.46; H, 6.38; N, 10.20.

Z(OMe)–Ser–Ser–Thr–Ser–Ala–Ala–OBzl: Z(OMe)–Ser–Thr–Ser–Ala–Ala–OBzl (6.61 g, 9.6 mmol) was treated with TFA–anisole (24 ml–6 ml) and the N^{α}-deprotected peptide isolated as described above was dissolved in DMF (60 ml) containing Et_3N (1.45 ml, 10.5 mmol). The azide [prepared from 5.43 g (19.2 mmol) of Z(OMe)–Ser–$NHNH_2$] in DMF (40 ml) and Et_3N (2.6 ml, 19.2 mmol) were added to the above ice-chilled solution. After stirring for 48 h, the solution was concentrated and the residue was triturated with ether and 5% citric acid. The resulting powder was washed as described above and precipitated twice from DMF with MeOH; yield 5.39 g (72%), mp 246—251°C, $[\alpha]_D^{20}$ −1.2° (c=0.9, DMF). Rf_1 0.57. *Anal.* Calcd for $C_{35}H_{48}N_6O_{14}$: C, 54.11; H, 6.23; N, 10.82. Found: C, 53.85; H, 6.29; N, 10.74.

Z(OMe)–Asp(OBu^t)–Ser–Ser–Thr–Ser–Ala–Ala–OBzl [A]: Z(OMe)–Ser–Ser–Thr–Ser–Ala–Ala–OBzl (1.75 g, 2.25 mmol) was treated with TFA–anisole (10 ml–2 ml) and the N^{α}-deprotected peptide isolated as mentioned above was dissolved in DMF–DMSO (1: 1, 20 ml) together with Et_3N (0.68 ml, 4.96 mmol) and Z-(OMe)–Asp(OBu^t)–ONP (1.28 g, 2.70 mmol). After stirring at room temperature for 24 h, the solution was concentrated and the residue was triturated with ether and 5% citric acid. The resulting powder was washed as described above and precipitated from DMSO with MeOH; yield 1.58 g (74%), mp 229—231°C, $[\alpha]_D^{18}$ −0.9° (c=1.2, DMF). Rf_1 0.66. Amino acid ratios in a 6 N HCl hydrolysate: Asp 1.05, Ser 2.79; Thr 0.98, Ala 2.00 (recovery of Ala, 85%). *Anal.* Calcd for $C_{43}H_{61}N_7O_{17}$: C, 54.47; H, 6.49; N, 10.34. Found: C, 54.23; H, 6.50; N, 10.06.

Z(OMe)–Glu(OBzl)–Arg(MBS)–Gln–His–Met(O)–Asp–Ser–Ser–Thr–Ser–Ala–Ala–OBzl [Z(OMe)–(S-peptide 9—20)–OBzl]: The above protected heptapeptide ester [A] (952 mg, 1.0 mmol) was treated with TFA–anisole (5 ml–1 ml) in an ice-bath for 3 h and at room temperature for 30 min to ensure the complete removal of the Bu^t ester from the Asp residue. Dry ether was added and the resulting powder isolated as described above was dissolved in DMSO–DMF (1: 1, 10 ml) containing Et_3N (0.30 ml, 2.20 mmol). The azide [prepared from 1.154 g (1.0 mmol) of Z(OMe)–Glu(OBzl)–Arg(MBS)–Gln–His–Met(O)–$NHNH_2$] in DMF (10 ml) and Et_3N (0.15 ml, 1.10 mmol) were added and the mixture was stirred at 4°C for 24 h, then concentrated. The residue was triturated with ether and H_2O and the resulting powder was washed with H_2O. Precipitation from DMF with MeOH afforded the protected dodecapeptide ester; yield 1.191 g (64%), mp 216—219°C, $[\alpha]_D^{18}$ −7.9° (c=1.2, DMSO). Rf_2 0.46. Amino acid ratios in a 6 N HCl hydrolysate: Asp 1.00, Thr 0.84, Ser 2.36, Glu 2.16, Ala 1.93, Met 0.81, His 1.05, Arg 1.00 (recovery of Asp, 88%). *Anal.* Calcd for $C_{80}H_{108}N_{18}O_{29}S_2 \cdot 2H_2O$: C, 50.94; H, 5.99; N, 13.37. Found: C, 51.02; H, 6.04; N, 13.44.

Z–Lys(Z)–Glu(OBu^t)–Thr–Ala–Ala–Ala–Lys(Z)–Phe–Glu–(OBzl)–Arg(MBS)–Gln–His–Met(O)–Asp–Ser–Ser–Thr–Ser–Ala–Ala–OBzl [Protected S-Peptide]: The above protected dodecapeptide ester (724 mg, 0.39 mmol) was treated with TFA–anisole (3 ml–0.7 ml) in an ice-bath for 60 min and the N^{α}-deprotected peptide isolated as described above was dissolved in DMSO–DMF (1: 1, 10 ml containing Et_3N (118 μl, 0.86 mmol) and N-methylmorpholine (47 μl, 0.43 mmol). The azide [prepared from 523 mg (0.39 mmol) of Z–Lys(Z)–Glu(OBu^t)–Thr–Ala–Ala–Ala–Lys(Z)–Phe–$NHNH_2$] in DMF–DMSO (1: 1, 5 ml) and N-methylmorpholine (47 μl, 0.43 mmol) were added to the above ice-chilled solution. After stirring at 4°C for 48 h, the mixture was concentrated and the residue was triturated with ether and H_2O. The resulting powder was purified by washing with H_2O followed by precipitation from DMSO with MeOH; yield 808 mg (69%), mp 226°C (dec.), $[\alpha]_D^{20}$ −14.9° (c=1.2, DMSO). Rf_2 0.76. Amino acid ratios in a 6 N HCl hydrolysate: Asp 0.92, Thr 1.84, Ser 2.39, Glu 2.24, Ala 4.82, Met+Met(O) 0.83, Phe 1.00, Lys 2.26, His 0.87, Arg 0.93 (recovery of Phe, 95%). *Anal.* Calcd for $C_{138}H_{188}N_{28}O_{43}S_2 \cdot 3H_2O$: C, 54.42; H, 6.42; N, 12.88. Found: C, 54.73; H, 6.40; N, 12.54.

H–Lys–Glu–Thr–Ala–Ala–Ala–Lys–Phe–Glu–Arg–Gln–His–Met–Asp–Ser–Ser–Thr–Ser–Ala–Ala–OH [S-Peptide]: The protected S-peptide (200 mg, 66.8 μmol) was treated with 1 M TFMSA–thioanisole in TFA

(4.7 ml) in the presence of *m*-cresol (0.26 ml, 2.34 mmol) in an ice-bath for 60 min, then dry ether was added and the resulting powder was dried over KOH pellets *in vacuo* for 30 min. This treatment was repeated twice more. The deprotected peptide thus obtained was dissolved in a small amount of H_2O and treated with Amberlite CG-4B (acetate form, approximately 2 g) for 30 min. After filtration, the filtrate was adjusted to pH 10 with 5% NH_4OH and stirred in an ice-bath for 30 min. The pH of the solution was adjusted to 6 with a few drops of AcOH and the solution was lyophilized. The residue was dissolved in H_2O (2 ml) and incubated with dithiothreitol (206 mg, 20 eq) under an N_2 atmosphere at 60°C for 24 h. The solution was applied to a column of Sephadex G-25 (1.8×122 cm), which was eluted with 0.2 N AcOH. The ultraviolet (UV) absorption at 260 nm was determined in each fraction (3 ml each). The fractions corresponding to the main peak (tube Nos. 50—60) were combined and the solvent was removed by lyophilization to give a fluffy powder; yield 87.5 mg (60%). The product was dissolved in H_2O (3 ml), then applied to a column of CM-cellulose (2×3.5 cm), which was eluted first with H_2O (100 ml) and then by gradient elution with 0.03 M $AcONH_4$ (260 ml) through a mixing flask containing H_2O (150 ml). Monitoring the UV absorption at 260 nm for each fraction (4 ml) revealed the presence of the main peak with a small front shoulder in the gradient eluates (Fig. 5). The fractions corresponding to the main peak (tube Nos. 48—58) were combined and the solvent and the ammonium salt were removed by repeated lyophilization to give a fluffy white powder; yield 64.6 mg (44%), $[\alpha]_D^{20}$ −66.6° (c=0.8, 10% AcOH). Rf_3 0.12. Amino acid ratios in a 6 N HCl hydrolysate and an LAP digest (numbers in parentheses): Asp 0.98 (0.94), Thr 1.75 (Gln+Thr 2.40 Calcd as Thr), Ser 2.52 (2.71), Glu 3.05 (2.11), Ala 4.81 (4.87), Met 0.84 (0.88), Phe 1.00 (1.00), Lys 2.03 (2.11), His 0.93 (0.98), Arg 0.97 (0.94), recovery of Phe 90% (84%). *Anal.* Calcd for $C_{89}H_{144}N_{28}O_{33}S \cdot 3CH_3COOH \cdot 5H_2O$: C, 46.83; H, 6.87; N, 16.10. Found: C, 46.79; H, 6.62; N, 16.39.

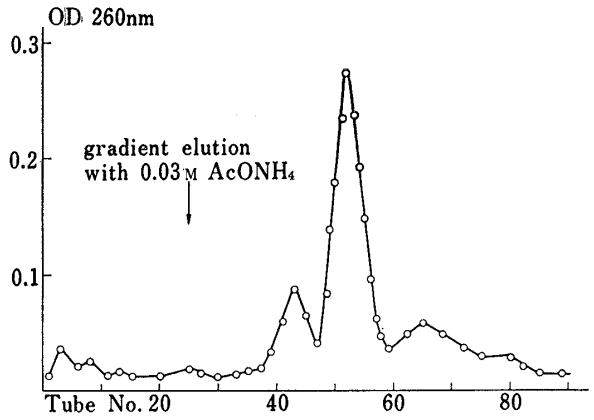


Fig. 5. Purification of Synthetic S-Peptide by Ion-exchange Chromatography on CM-cellulose

2. Synthesis of RNase S′——(i) Deprotection of the Protected S-Protein and Air Oxidation: The protected S-protein (500 mg) in HMPA–DMF (4: 1, 8 ml) was treated with thiophenol (3.8 ml) as performed for the reduction of protected RNase A; yield 397 mg (80%). The reduced form of the protected S-protein (100 mg, 6.67 μmol) was treated with 1 M TFMSA–thioanisole in TFA (1.9 ml) in the presence of *m*-cresol (196 μl, 280 eq) in an ice-bath for 60 min, then dry ether was added and the resulting powder was dried over KOH pellets *in vacuo* for 30 min. The resulting powder was treated twice more with the same reagents under identical conditions. The deprotected peptide was dissolved in 4 M guanidine HCl in 0.2 M Tris–HCl buffer at pH 8.6 (3 ml) and the solution, after incubation with mercaptoethanol (0.42 ml, 5.34 mmol) and dithiothreitol (154 mg, 1.0 mmol) at 40°C for 3 h and at room temperature for 24 h, was applied to a column of Sephadex G-25 (1.8×122 cm), which was eluted with 0.1 N AcOH. The UV absorption at 275 nm was determined in each fraction (3 ml) and the fractions corresponding to the front peak (tube Nos. 41—70) were combined. After addition of the synthetic S-peptide (16.7 mg, 1 eq), the solution was diluted with H_2O to 300 ml. The reduced and oxidized forms of glutathione (30.6 mg and 61.2 mg, 14.9 eq each) were added at this stage and the solution was further diluted with 0.2 M Tris–HCl buffer (pH 8.0) to a total volume of 400 ml (protein concentration, 1.67×10^{-2} mM). After standing at 23°C for 5 days, the solution generated RNase activity of 2.5%, when measured using yeast RNA as a substrate. After adjustment of the pH to 5 with 1 N HCl, the entire solution was lyophilized and the residue was dialyzed against H_2O using cellulose tubing VT 351. The content of the tubing was then desalted by gel-filtration on Sephadex G-25, using 0.05 M NH_4HCO_3 (pH 8.4) as an eluant. The UV absorption at 275 nm in individual fractions (3 ml each) was measured as stated above. The solvent and the ammonium salt in the main fractions (tube Nos. 24—36) were removed by repeated lyophilization to give a powder; yield 46.4 mg (50%), activity 2.5%.

(ii) Affinity Purification of Crude Air-oxidized RNase S′: The crude air-oxidized sample of RNase S′ obtained above (45.14 mg) was dissolved in 0.02 M AcONa (2 ml) at pH 5.2 and the synthetic S-peptide (7.0 mg, 1 eq) was added. This solution was applied to a column of Sepharose (4B)-5′-(4-aminophenylphosphoryl)-uridine-2′(3′)-phosphate (0.8×12.2 cm) and the column was eluted first with 0.02 M AcONa and then with 0.2 N AcOH as reported for the purification of synthetic RNase A (Fig. 6). The desired fractions (tube Nos. 34—43, 2.5 ml each) of the 0.2 N AcOH eluates were then desalted by gel-filtration on Sephadex G-25, using 0.05 M NH_4HCO_3 (pH 8.4) as an eluant. Repeated lyophilization of the desired fraction gave a fluffy powder; yield 0.91 mg (2.0%), activity 62%.

(iii) SP-Sephadex Purification of the Affinity-purified Product: The affinity-purified synthetic RNase S′ (2.18 mg, obtained by repeated experiments) was further purified by ion-exchange chromatography

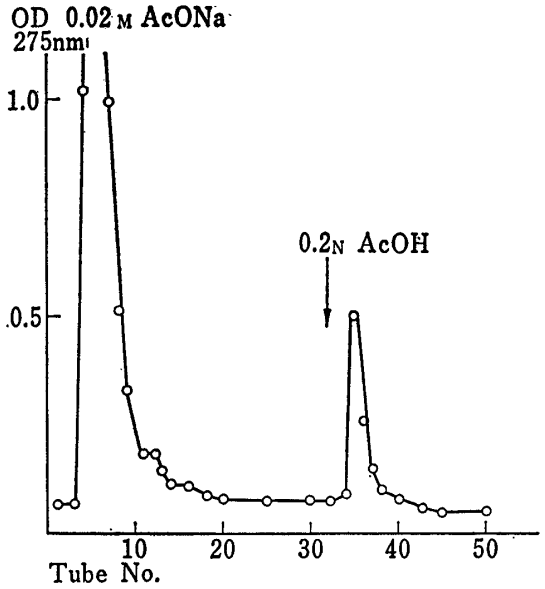


Fig. 6. Purification of Synthetic RNase S′ by Affinity Chromatography

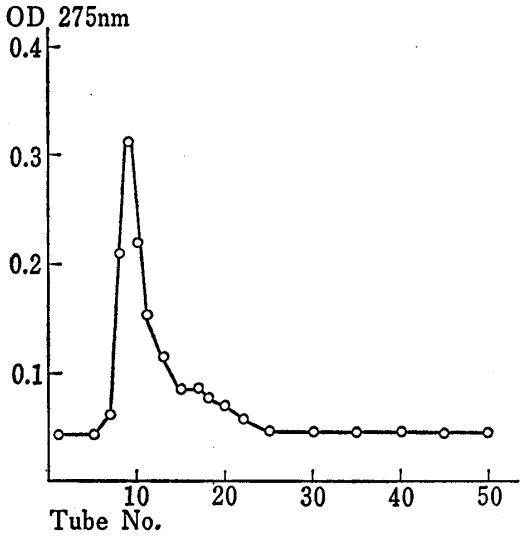


Fig. 7. Purification of the Affinity-purified Product by Ion-exchange Chromatography on SP–Sephadex

on SP-Sephadex C-25 (1.0×20.7 cm) using 0.13 M phosphate buffer (pH 6.55) as an eluant (Fig. 7). The desired fractions (tube Nos. 7—11, 2.2 ml each) were then desalted by gel-filtration on Sephadex G-25 with 0.05 M NH_4HCO_3 as an eluant. After repeated lyophilization, the product was obtained as a white fluffy powder; yield 1.12 mg (52%). The activity against yeast RNA and 2′,3′-cyclic cytidine phosphate was 64% and 65% of that of natural RNase A, respectively. In terms of the Kunitz unit,[22] the activity against yeast RNA was 48 U/mg. The Michaelis constant for yeast RNA was 1.18 mg/ml (lit.[27] RNase A, 1.25 mg/ml). Amino acid ratios in a 6 N HCl (48 h) hydrolysate (numbers in parentheses are those for natural RNase A and numbers in brackets indicate the theoretical values): Asp 15.14 (15.42) [15], Thr 9.77 (9.80) [10], Ser 13.76 (13.80) [15], Glu 12.82 (12.53) [12], Pro 4.48 (4.07) [4], Gly 3.55 (3.30) [3], Ala 12.04 (12.10) [12], Cys 3.94 (4.03) [4], Val 8.72 (8.84) [9], Met 3.64 (3.85) [4], Ile 2.29 (2.18) [3], Leu 2.00 (2.00) [2], Tyr 5.78 (5.97) [6], Phe 3.13 (2.99) [3], Lys 10.80 (10.67) [10], His 3.30 (3.23) [4], Arg 3.54 (3.70) [4]. Recovery of Leu 82.2% (72.1%).

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References and Notes

1) Peptides and their derivatives mentioned in this communication are of the L-configuration. The following abbreviations are used: Z=benzyloxycarbonyl, Z(OMe)=*p*-methoxybenzyloxycarbonyl, Bzl=benzyl, MBzl=*p*-methoxybenzyl, MBS=*p*-methoxybenzenesulfonyl, Bu^t=*tert*-butyl, DCC=dicyclohexylcarbodiimide, NP=*p*-nitrophenyl, Troc=*β*,*β*,*β*-trichloroethyloxycarbonyl, TFA=trifluoroacetic acid, DMF=dimethylformamide, HMPA=hexamethylphosphoramide, DMSO=dimethylsulfoxide, TFMSA=trifluoromethanesulfonic acid.
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3) Location: *Sakyo-ku, Kyoto, 606, Japan.*
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