Studies on Peptides. 103.[1] Chemical Synthesis of a Crystalline Protein with the Full Enzymatic Activity of Ribonuclease A

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Abstract: Improved chemical synthesis of bovine pancreatic ribonuclease (RNase) A was achieved by applying a new deprotecting procedure with trifluoromethanesulfonic acid–thioanisole in combination with a modified air oxidation procedure in the presence of glutathione for the disulfide formation. After purification by affinity chromatography on Sepharose 4B-5′-(4-aminophenylphosphoryl)-uridine-2′(3′)-phosphate followed by ion-exchange chromatography on CM-cellulose, a protein with the full enzymatic activity was obtained. According to Kunitz, the synthetic protein was subsequently crystallized from aqueous ethanol. A totally synthetic protein with full RNase A activity was thus obtained in a crystalline form for the first time.

In 1969, two groups of investigators reported the synthesis of proteins with partial enzyme activity of ribonuclease (RNase) A. Gutte and Merrifield[2] performed the automated solid phase synthesis and reported that they obtained a supernatant solution with a specific activity of 78%, after ammonium sulfate fractionation of the trypsin-resistant material. The Merck group[3] undertook the synthesis of S-protein[4] and obtained a solution containing ca. 2γ of RNase S′ activity, upon combination with the S-peptide[4] derived from the natural source. The final product in both syntheses was not chemically characterized.

We wish to report the first chemical synthesis of a crystalline protein with the full enzymatic activity of RNase A (Figure 1). Recently, in a preliminary communication[5] followed by a series of six papers,[6] we reported the total synthesis of a protein with the full RNase A activity. We have now succeeded in crystallizing the synthetic enzyme, according to Kunitz.[7] Improvement in the yield of the enzyme was attained at the final step of the synthesis by using a new deprotecting procedure with TFMSA–thioanisole[8] and subsequent air oxidation in the presence of glutathione for the disulfide bridge formation.[9]

In the final step of our previous synthesis of RNase A, MSA[10] was used, together with *m*-cresol as a cation scavenger, for removal of various protecting groups employed; Z from Lys and the N-terminal, Bzl from Glu, Asp, and the C-terminal, *t*-Bu from Glu (position 2), MBS[11] from Arg, and MBzl[12] from Cys. The HF deprotection[13] afforded the comparable results. The yield of the fully active product obtained after disulfide formation[14] and subsequent purifications by affinity chromatography[15] and ion-exchange chromatography on CM-cellulose[14b,16] was less than 5% in both experiments. The incorrect disulfide formation and various unsuppressed side reactions during the deprotection were most likely responsible for this low yield. Among possible side reactions which may take place under acidolytic deprotecting conditions, the acid-catalized aminosuccinimide formation of the Asp residue[17] seems to be a major unsolved problem. Recently, Schön and Kisfaludy[18] reported that not only the Asp(OBzl) residue in peptides, but also the Asp residue with the free carboxyl group has the tendency to undergo the aminosuccinimide formation, depending on the acid employed and the sequence involved. We also noticed such tendency of the Asp residue during the synthesis of vasoactive intestinal polypeptide.[19] These findings indicated that when the Bzl ester group was removed acidolytically from the Asp(OBzl) residue, the resulting Asp residue still has the tendency to form the aminosuccinimide derivative to a certain degree. This hitherto unknown side reaction, which seems to proceed via the carbonium cation, has now been investigated with use of two model peptides, H-Asp-Ser-OH (position 14-15) and

Table I. Formation of the Aminosuccinimide Derivatives (%) from Two Asp-Peptides[a]

sample	MSA[b]	MSA–thioanisole[b] (60 equiv)	1 M TFMSA–thioanisole in TFA[c]	2 M TFMSA–thioanisole in TFA[c]
Z(OMe)-Asp(OBzl)-Ala-OBzl	14.6	14.2	≒0	6.8
H-Asp-Ser-OH	20.2	21.6	≒0	11.5

[a] Every reaction was performed in the presence of *m*-cresol (30 equiv). [b] 24 °C for 60 min. [c] 0 °C for 60 min.

(1) A preliminary communication of this paper has appeared in *Chem. Pharm. Bull.* **1981**, *29*, 600. Amino acids, peptides, and their derivatives mentioned in this paper are of the L configuration. Abbreviations used are those recommended by IUBAC-IUB Commission of Biochemical Nomenclature: *Biochemistry* **1966**, *5*, 2485; **1967**, *6*, 362; **1972**, *11*, 1726. Z, benzyloxycarbonyl; Bzl, benzyl; *t*-Bu, *tert*-butyl; MBS, *p*-methoxybenzenesulfonyl; MBzl, *p*-methoxybenzyl; Z(OMe), *p*-methoxybenzyloxycarbonyl; DCC, dicyclohexylcarbodiimide; MSA, methanesulfonic acid; TFMSA, trifluoromethanesulfonic acid; DMF, dimethylformamide.
(2) Gutte, B.; Merrified, R. B. *J. Am. Chem. Soc.* **1969**, *91*, 501; *J. Biol. Chem.* **1971**, *246*, 1922.
(3) Hirschmann, R.; Nutt, R. F.; Veber, D. F.; Vitali, R. A.; Varga, S. L.; Jacob, T. A.; Holly, F. W.; Denkewalter, R. G. *J. Am. Chem. Soc.* **1969**, *91*, 507.
(4) Richards, F. M. *Proc. Ntal. Acad. Sci. U.S.A.* **1958**, *44*, 162. Richards, F. M.; Vithayathil, P. J. *J. Biol. Chem.* **1959**, *234*, 1459.
(5) Yajima, H.; Fujii, N. *J. Chem. Soc., Chem. Commun.* **1980**, 115.
(6) Fujii, N.; Yajima, H. *J. Chem. Soc., Perkin Trans. 1* **1981**, 789, 797, 804, 811, 819, 831.
(7) Kunitz, M. *J. Gen. Physiol.* **1940**, *24*, 15.
(8) (a) Yajima, H.; Fujii, N.; Ogawa, H.; Kawatani, H. *J. Chem. Soc., Chem. Commun.* **1974**, 107. (b) Irie, H.; Fujii, N.; Ogawa, H.; Yajima, H. *Ibid.* **1976**, 922. (c) Fujii, N.; Funakoshi, S.; Sasaki, T.; Yajima, H. *Chem. Pharm. Bull.* **1977**, *25*, 3096. (d) Kiso, Y.; Ito, K.; Nakamura, S.; Kitagawa, K.; Akita, T.; Moritoki, H. *Ibid.* **1979**, *27*, 1472. (e) Kiso, Y.; Nakamura, S.; Ito, K.; Ukawa, K.; Kitagawa, K.; Akita, T.; Moritoki, H. *J. Chem. Soc., Chem. Commun.* **1979**, 971.
(9) (a) Chavez, L. G., Jr.; Scheraga, H. A. *Biochemistry* **1980**, *19*, 996. (b) Konishi, Y.; Scheraga, H. A. *Ibid.* **1980**, *19*, 1308. (c) Szajewski, R. P.; Whitesides, G. M. *J. Am. Chem. Soc.* **1980**, *102*, 2011.
(10) Yajima, H.; Kiso, Y.; Ogawa, H.; Fujii, N.; Irie, H. *Chem. Pharm. Bull.* **1975**, *23*, 1164.
(11) Nishimura, O.; Fujino, M. *Chem. Pharm. Bull.* **1976**, *24*, 1568.
(12) Akabori, S.; Sakakibara, S.; Shimonishi, Y.; Nobuhara, Y. *Bull. Chem. Soc. Jpn* **1964**, *37*, 433.
(13) Sakakibara, S.; Shimonishi, Y.; Kishida, Y.; Okada, M.; Sugihara, H. *Bull. Chem. Soc. Jpn* **1967**, *40*, 2164.
(14) (a) Anfinsen, C. B.; Haber, E. *J. Biol. Chem.* **1961**, *236*, 1361. (b) White, F. H. Jr. *Ibid.* **1961**, *236*, 1353.
(15) Wilchek, M.; Gorecki, M. *Eur. J. Biochem.* **1969**, *11*, 491.
(16) Taborsky, G. *J. Biol. Chem.* **1959**, *234*, 2652.
(17) (a) Bodanszky, M.; Natarajan, S. *J. Org. Chem.* **1975**, *40*, 2495. (b) Natarajan, S.; Bodanszky, M. *Ibid.* **1976**, *41*, 1269. (c) Bodanszky, M.; Martinez, J. *Ibid.* **1978**, *43*, 3071. (d) Bodanszky, M.; Kwei, J. Z. *Int. J. Pept. Protein Res.* **1978**, *12*, 69. (e) Martinez, J.; Bodanszky, M. *Ibid.* **1978**, 12, 277. (f) Yang, C. C.; Merrifield, R. B. *J. Org. Chem.* **1976**, *41*, 1032.
(18) Schön, I.; Kisfaludy, L. *Int. J. Pept. Protein Res.* **1979**, *14*, 485.
(19) Yajima, H.; Takeyama, M.; Koyama, K.; Tobe, T.; Inoue, K.; Kawano, T.; Adachi, H. *Int. J. Pept. Protein Res.* **1980**, *16*, 33. Takeyama, M.; Koyama, K.; Inoue, K.; Kawano, T.; Adachi, H.; Tobe, T.; Yajima, H. *Chem. Pharm. Bull.* **1980**, *28*, 1873.

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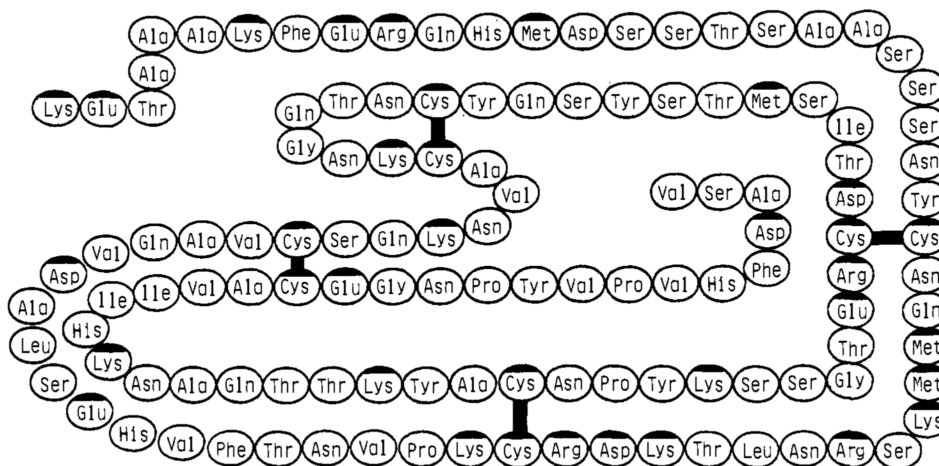

Figure 1. Structure of bovine pancreatic ribonuclease A. Partial shading indicates protecting groups removed at the final step of the synthesis: Asp(OBzl), Glu(OBzl), Lys(Z), Cys(MBzl), Arg(MBS), Met(O), Glu(OBu) (position 2).

Z(OMe)-Asp(OBzl)-Ala-OBzl (position 121-122). In our synthesis of protected RNase A, the β-carboxyl protecting group of Asp (position 14) was previously removed in order to avoid the base-catalized aminosuccinimide formation.[17]

The deprotection of the Bzl type protecting groups by TFMSA in TFA was previously reported[8a] and the role of thioanisole as an effective alkyl cation scavenger was pointed out.[8b,c] Recently, Kiso et al.[20] observed that thioanisole accelerated the TFA deprotection of the Z group by forming the *S*-benzylsulfonium compound and they proposed the "Push-Pull" mechanism to explain the phenomenon. The deprotection by TFA in the presence of thioanisole, instead of anisole or *m*-cresol, seems to be a sort of S_N2 type reaction, instead of the simple acidolysis. However, this new deprotecting procedure still required 3 and 8 h for complete removal of the Z and Bzl groups and a much longer time for the MBS and *S*-MBzl groups. We have found that 1 M TFMSA–thioanisole in TFA can cleave all of the aforementioned protecting groups within 60 min and gave a much more homogeneous product than MSA–thioanisole or MSA–*m*-cresol with the two model peptides, H-Asp-Ser-OH and Z(OMe)-Asp(OBzl)-Ala-OBzl. The amount of the byproduct, the aminosuccinimide derivatives, increased according to higher concentration of TFMSA in TFA in both cases (Table I).

Considering these experimental results, together with a superior property of *m*-cresol[21] to anisole as a scavenger for trapping alkyl cations, which are derived directly from the protecting groups by acid and in addition presumably through the thioanisole–*S*-sulfonium compound[22] formed according to the "Push-Pull" mechanism, we decided to apply 1 M TFMSA–thioanisole in TFA plus *m*-cresol as a deprotecting reagent for the improved synthesis of RNase A (Figure 2).

The protected RNase A was treated with thiophenol as described previously to reduce the sulfoxide of the Cys(MBzl) residues,[23] which formed partially during the synthesis. The Met(O) residues[24] were also reduced under this treatment. As reported,[6] this reduction was conducted until two model sulfoxides, Z(OMe)-Cys(MBzl) (O)-OH and Z(OMe)-Met(O)-OH, were completely reduced under identical conditions. The treated sample was then exposed to 1 M TFMSA–thioanisole in TFA in the presence of *m*-cresol (10 equiv for each protecting group) in an

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Protected RNase A
      ↓ Thiophenol
Reduced form of protected RNase A
      | 1) 1M TFMSA-thioanisole in TFA + m-cresol
      | 2) Mercaptoethanol + dithiothreitol
      ↓ 3) Sephadex G-25
Deprotected protein
      | 1) Air oxidation with glutathione
      ↓ 2) Sephadex G-75
Crude synthetic RNase A
      ↓ Affinity chromatography
Affinity purified product
      ↓ CM-cellulose chromatography
CM-purified product
      ↓ 95% Ethanol
Crystalline synthetic RNase A
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Figure 2. Scheme for the improved synthesis of RNase A.

ice bath for 60 min. This treatment was repeated three times to ensure the complete removal of all of the protecting groups (a total of 33 groups). It seems worthwhile to note that this reagent has an ability to reduce Me(O) to Met in nearly 80% within 60 min at 0 °C.

The deprotected peptide was then incubated with mercaptoethanol and dithiothreitol at pH 8.6 as described previously according to Anfinsen and Haber.[14a] This treatment was judged effective for reduction of any remaining Met(O) residues, if present, and for removal of the S-alkyl groups from the Met residues, if such products were formed during the deprotection. After removal of reducing reagents by gel filtration on Sephadex G-25, the desired eluates were diluted with water. Next, according to the procedure published recently by Chavez and Scheraga,[9a] equal amounts of the reduced and oxidized forms of glutathione were added and the solution was further diluted with 1 M Tris–HCl buffer to a protein concentration of 0.08 mg/mL. The solution, after adjusting the pH to 8.0, was kept left standing at 23 °C for the disulfide bond formation. As discussed by Chavez and Scheraga[9a] and Szajewski and Whitesides,[9c] the reaction seems to proceed via thiol–disulfide interchange reactions. The solution generated RNase A activity and after 3 days it reached the constant value of 13.8%, using yeast RNA as a substrate.[25]

After lyophilization and desalting by gel filtration on Sephadex G-75, the activity of the crude product we obtained was 18.9%

(20) Kiso, Y.; Ukawa, K.; Akita, T. *J. Chem. Soc., Chem. Commun.* **1980**, 101.

(21) Yajima, H.; Takeyama, M.; Kanaki, J.; Mitani, K. *J. Chem. Soc., Chem. Commun.* **1978**, 482. Yajima, H.; Takeyama, M.; Kanaki, J.; Nishimura, O.; Fujino, M. *Chem. Pharm. Bull.* **1978**, *26*, 3752.

(22) Lundt, B. F.; Johansen, N. L.; Vølund, A.; Markussen, J. *Int. J. Pept. Protein Res.* **1978**, *12*, 258.

(23) Yajima, H.; Funakoshi, S.; Fujii, N.; Akaji, K.; Irie, H. *Chem. Pharm. Bull.* **1979**, *27*, 1060. Funakoshi, S.; Fujii, N.; Akaji, K.; Irie, H.; Yajima, H. *Ibid.* **1979**, *27*, 2151.

(24) (a) Iselin, B. *Helv. Chim. Acta* **1961**, *44*, 61. (b) Fujii, N.; Sasaki, T.; Funakoshi, S.; Irie, H.; Yajima, H. *Chem. Pharm. Bull.* **1978**, *26*, 650.

(25) (a) Kunitz, M. *J. Biol. Chem.* **1946**, *164*, 563. (b) Anfinsen, C. B.; Redfield, R. R.; Choate, W. L.; Page, J.; Carroll, W. R. *Ibid.* **1954**, *207*, 201. (c) Egami, F.; Takahashi, K.; Uchida, T. *Prog. Nuclecic Acid Res. Mol. Biol.* **1964**, *3*, 59.

Table II. Summary of the Improved Synthesis of RNase A[a]

	product, mg	yield, %	activity, %	Kunitz, U/mg
protected RNase A	100.0			
air oxidized	51.0	65	18.9	14
affinity purified	8.2	16	81.3	62
CM purified	4.6	63	113.1	86

[a] Total yield, 6.6%.

and the yield was 65% from the reduced form of protected RNase A (total units 733U). In a parallel experiment, when the air oxidation was performed without glutathione, the activity of the product was 16.7% and the yield was 60% (total units, 595 U). By comparing these total units with those obtained in the former experiment by MSA–*m*-cresol deprotecting procedure (389 U), it can be seen that a definite improvement has been achieved in the new deprotecting step and the air oxidation step as well.

The above air-oxidized product was then purified by affinity chromatography on Sepharose 4B–5'-(4-aminophenylphosphoryl)-uridine-2'(3')-phosphate[15] followed by ion-exchange chromatography on CM-cellulose[14b,16] as performed previously. The purified product migrated in the field of isoelectric focusing on Ampholine (pH 3.5–10) as a single component with the identical mobility of natural RNase and the amino acid ratios in 6 N HCl hydrolyzate were also in excellent agreement with those of natural RNase A. Its physicochemical constants, specific rotation,[14b] Michaelis constant,[26] and extinction coefficiency in UV absorption[14b] matched fairly well with those of literature values and measured values on native RNase A. The activity of the finally purified product was 86 U/mg (113% of natural RNase A) against yeast RNA and the overall yield in the deprotection followed by purification steps was 6.6%. As summarized in Table II, a higher yield was attained than those previously obtained by MSA or HF deprotection.

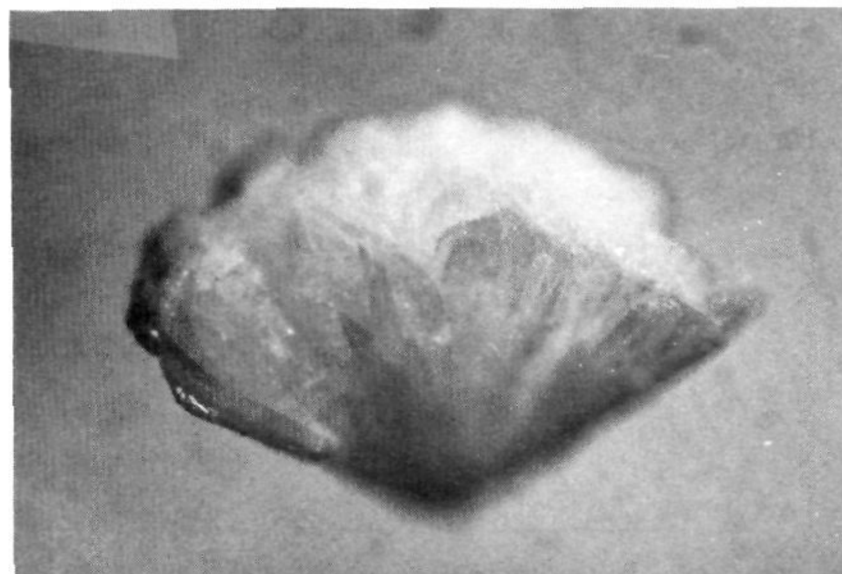
Crystallization of the synthetic RNase A was next performed according to Kunitz[7] with use of the salt-free procedure. A turbid solution formed by addition of 95% ethanol to an aqueous solution of synthetic RNase A obtained above was kept in a refrigerator for 3 months. During this period, small transparent, plate-like single crystals grew into multioriented polycrystals with rosettes (Figure 3a) or stalagmitic shape (Figure 3b). However, attempts to pick up a suitable single crystal for X-ray analysis[27] have been unsuccessful. We confirmed that the enzymatic activity of a crystalline form of synthetic RNase A toward yeast RNA and 2',3'-cylic cytidine phosphate[28] bears a very favorable comparison with that of natural RNase A.

As described above, we were thus able to synthesize a crystalline protein with the full enzymatic activity of bovine pancreatic RNase A, the structure of which was firmly established by Smyth, Stein, and Moore[29] in 1963.

Experimental Section

Melting points are uncorrected. Rotations were determined with a Union digital polarimeter PM-101. Amino acid compositions of acid hydrolyzates were determined with a Hitachi amino acid analyzer, Model KLA-5, and values are uncorrected for amino acid destruction. TLC was performed on silica gel (Kieselgel G, Merck) and *Rf* values refer to the following solvent systems: R_f^{I} $CHCl_3$/MeOH/AcOH (9:1:0.5), R_f^{II} *n*-BuOH/AcOH/pyridine/H_2O (4:1:1:2). A Shimadzu dual wavelength TLC scanner CS-900 was used for quantitative determination of aminosuccinimide derivatives from Asp-peptides. UV absorption was measured by a Hitachi model 200-20 spectrophotometer. 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 the procedure given by Wilchek and Gorecki.[15] The following substrates and enzyme were

(a)



(b)

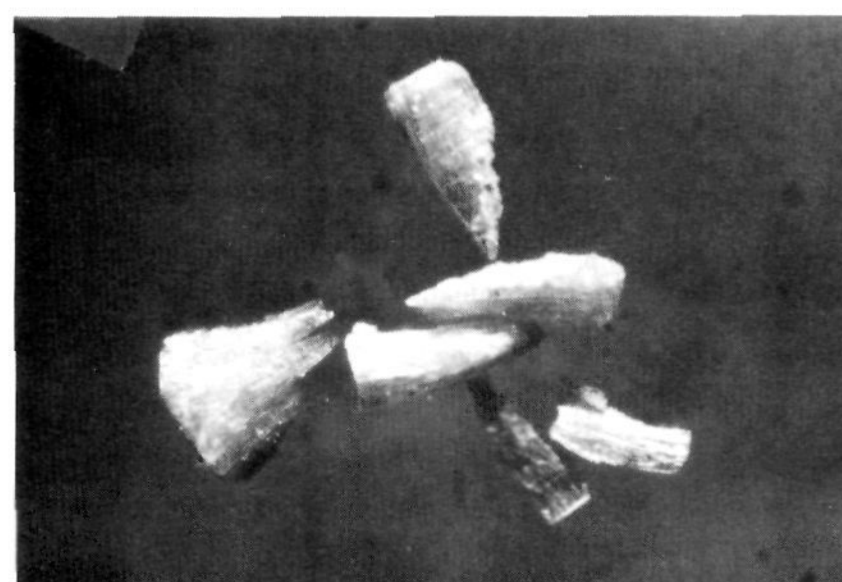


Figure 3. Crystals of synthetic RNase A: (a) × 50; (b) × 26.

purchased from Sigma Chemical Co.: 2',3'-cyclic cytidine monophosphoric acid sodium salt (Lot. No. 76C-7510), yeast RNA (Type XI, Lot. No. 124C-8510), and natural bovine pancreatic RNase A (Type XII-A, Lot. No. 49C-8049, 76 Kunitz U/mg). The activity of this commerical RNase A was reexamined according to the procedure given by Kunitz.[25] The values we obtained in three different concentrations were 78.2 U/mg in 1 μg/mL, 75.3 U/mg in 1.25 μg/mL, and 75.1 U/mg in 1.5 μg/mL. The average value we obtained, 76.2 U/mg, was consistent with the recorded value.

Z(OMe)-Asp(OBzl)-Ala-OBzl. In the usual manner, Z(OMe)-Asp-(OBzl)-OH (3.51 g, 10 mmol) and H-Ala-OBzl [prepared from 3.51 g (10 mmol) of the tosylate as usual] in DMF (30 ml) were condensed by DCC (2.27 g, 11 mmol). After being stirred at room temperature for 48 h, the solution was filtered, the filtrate was concentrated, and the residue was dissolved in AcOEt. The organic phase was washed with 5% citric acid and H_2O-NaCl, dried over Na_2SO_4, and concentrated. Trituration of the residue with ether afforded a powder, which was recrystallized from AcOEt and ether: 2.25 g (41%); mp 117–120 °C; $[\alpha]_D^{24}$ −15.8° (*c* 0.8, MeOH); R_f^{I} 0.71. Anal. Calcd for $C_{30}H_{32}N_2O_8$: C, 65.68; H, 5.88; N, 5.11. Found: C, 65.48; H, 5.84; N, 5.36.

Z(OMe)-Asp(OBzl)-Ser-OBzl. The title compound was similarly prepared by the DCC procedure and purified by recrystallization from AcOEt and *n*-hexane: yield 41%, mp 126–129 °C; $[\alpha]_D^{24}$ −7.3° (*c* 0.6, MeOH); R_f^{I} 0.69. Anal. Calcd for $C_{30}H_{32}N_2O_9$: C, 63.82; H, 5.71; N, 4.96. Found: C, 63.77; H, 5.84; N, 4.92.

H-Asp-Ser-OH. Z(OMe)-Asp(OBzl)-Ser-OBzl (2.21 g, 3.91 mmol) in 80% aqueous MeOH (20 mL) containing a few drops of AcOH was hydrogenated over a Pd catalyst in the usual manner and the product was recrystallized from EtOH and ether: 522 mg (61%); $[\alpha]_D^{20}$ +13.7° (*c* 1.1, 0.1 N AcOH); R_f^{II} 0.13 (lit.[30a] $[\alpha]_D^{27}$ +24.0° in 1 N HCl; lit.[30b] $[\alpha]_D^{25}$ +17.8° in H_2O; lit.[30c] mp 179–180 °C; $[\alpha]_D^{24}$ +17.1° in H_2O).

(26) Edelhoch, H.; Coleman, J. *J. Biol. Chem.* **1956**, *219*, 351.
(27) King, M. V.; Magdoff, B. S.; Adelman, M. B.; Harker, D. *Acta Crystallogr.* **1956**, *9*, 460. King, M. V. *Biochim. Biophys. Acta* **1964**, *79*, 388.
(28) Fruchter, R. G.; Crestfield, A. M. *J. Biol. Chem.* **1965**, *240*, 3868.
(29) Smyth, D. G.; Stein, W. H.; Moore, S. *J. Biol. Chem.* **1963**, *238*, 227.
(30) (a) Folsch, G. *Acta Chem. Scand.* **1966**, *20*, 459. (b) Buchanan, D. L.; Haley, F. E.; Dorer, F. E.; Corcoran, B. *J. Biochem.* **1966**, *5*, 3240. (c) Hofmann, K.; Finn, F. M.; Limmetti, M.; Montibeller, J.; Zanetti, G. *J. Am. Chem. Soc.* **1966**, *88*, 3633.

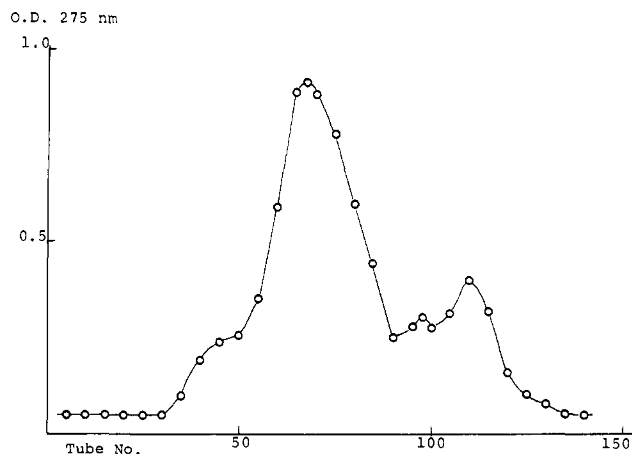


Figure 4. Purification of the air-oxidized product by gel filtration on Sephadex G-75.

Anal. Calcd for $C_7H_{12}N_2O_6$: C, 38.18; H, 5.49; N, 12.72. Found: C, 37.47; H, 5.45; N, 11.91.

Acid Catalized Aminosuccinimide (Asc) Formation of Asp-Peptides. In the presence of *m*-cresol (30 equiv), Z(OMe)-Asp(OBzl)-Ala-OBzl or H-Ap-Ser-OH (0.1 mmol each) was treated for 60 min with the following reagents (3 mL each): (1) MSA at 24 °C; (2) MSA-thioanisole (60 equiv) at 24 °C; (3) 1 M TFMSA-thioanisole in TFA at 0 °C; and (4) 2 M TFMSA-thioanisole in TFA at 0 °C. Ether was added and the resulting oily precipitate was washed with ether. The Asc derivative in each product was qualitatively identified by measurement of the typical carbonyl band in IR (ν_{max} (Nujol) cm^{-1} 1775, besides 1680). The residue of the sample was dissolved in H_2O (1 mL) and a part of the solution was examined by TLC. The ninhydrin color intensity of the respective R_f^1 spots (H-Asp-Ala-OH 0.21; H-Asc-Ala-OH 0.32; H-Asp-Ser-OH 0.13; H-Asc-Ser-OH 0.28) was quantitatively determined by a dual wavelength chromatographic scanner and the results are listed in Table I.

Improved Synthesis of RNase A. Deprotection with 1 M TFMSA-Thioanisole in TFA. The thiophenol-reduced form of protected RNase A (100 mg, 5.63 μmol) was treated with 1 M TFMSA-thioanisole (330 equiv each) in TFA (1.86 mL) in the presence of *m*-cresol (0.2 mL, 330 equiv) in an ice bath for 60 min and then dry ether was added. The resulting powder was collected by filtration and dried over KOH pellets in vacuo for 3 h. This treatment was further repeated two times and the resulting deprotected protein was dissolved in a solution of 4 M guanidine-HCl in 0.2 M Tris-HCl buffer (1.5 mL) at pH 8.6. After adjusting the pH to 8.6 with 5% $MeNH_2$, the solution was incubated with β-mercaptoethanol (0.35 mL, 100 equiv each for 8-Cys) and dithiothreitol (174 mg, 50 equiv each for 4-Met) under a nitrogen atmosphere at 40 °C for 5 h. β-Mercaptoethanol (0.18 mL, 50 equiv each for 8-Cys) was further added and incubation was continued at room temperature for 24 h as reported previously.

Air Oxidation in the Presence of Glutathione. The above solution was adjusted to pH 4 with 3 N HCl and applied to a column of Sephadex G-25 (1.6 × 107 cm), which was eluted with 0.1 N AcOH at a flow rate of 30 mL/h. The UV absorption at 275 nm was measured in each fraction (3 mL). The fractions corresponding to the front peak (tube Nos. 26–43) were combined and the combined solution was diluted with ice-chilled H_2O to 800 mL. After addition of reduced glutathione (23.6 mg, 13.6 equiv) and oxidized glutathione (47.1 mg, 13.6 equiv), the solution was brought up to 1000 mL with 1 M Tris-HCl buffer, pH 8.0 (200 mL), under gentle stirring in an ice bath (final protein concentration, 0.08 mg/mL). After adjusting the pH to 8 with 5% Tris, the solution was left standing at 23 °C for 5 days. The activity generated in the solution was measured periodically, using yeast RNA as a substrate. The activities we detected were 10.4% after 18 h, 12.8% after 36 h, 13.8% after 72 h, and 13.6% after 120 h.

After 5 days, the pH of the solution was adjusted to 4 with 2 N HCl and the entire solution was lyophilized. The residue was dialyzed three times against H_2O in a cellulose tubing (VT-351) and the content of the tube was lyophilized. The resulting hygroscopic powder was next desalted by gel filtration on Sephadex G-75 (2.8 × 84 cm) with 0.05 M NH_4HCO_3 as an eluant and the UV absorption at 275 nm was measured in each fraction (5.1 mL) (Figure 4). The fractions corresponding to the main peak (tube Nos. 54–88) were combined and the solvent and ammonium salt were removed by repeated lyophilization to give a fluffy white powder; yield 51.04 mg (65%); activity against yeast RNA was 18.9% (total units 733). When this air-oxidation was performed without addition of

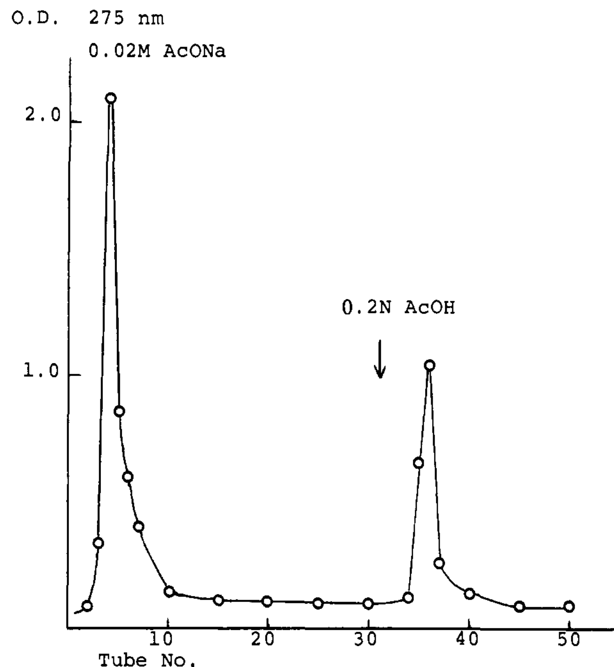


Figure 5. Purification of the G-75-purified product by affinity chromatography on Sepharose 4B-5′-(4-aminophenylphosphoryl)-uridine-2′-(3′)-phosphate.

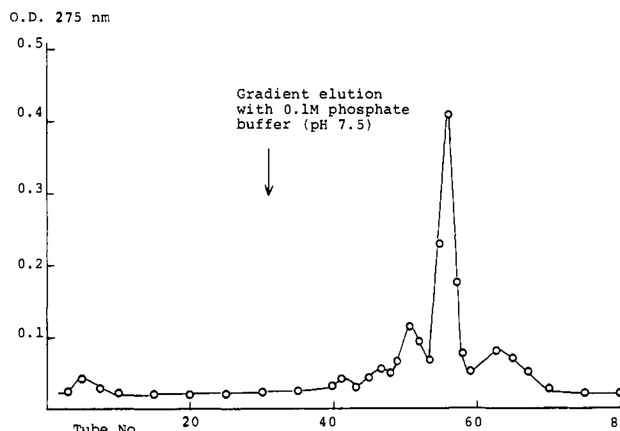


Figure 6. Purification of the affinity-purified product by ion-exchange chromatography on CM-cellulose.

glutathione, the yield was 46.89 mg (59.9%) and the activity was 16.7% (total units 595).

Purification of the G-75-Purified Product by Affinity Chromatography. Essentially in the same manner as reported previously, the G-75-purified product (50.05 mg) was purified by affinity chromatography on Sepharose 4B-5′-(4-aminophenylphosphoryl)-uridine-2′(3′)-phosphate (0.8 × 14.4 cm), using first 0.02 M sodium acetate (pH 5.2) and then 0.2 N AcOH as eluants (Figure 5). The UV absorption at 275 nm was measured in each fraction (2.5 mL). Peak I (the fractions, tube Nos. 3–15, in sodium acetate eluates were desalted on Sephadex G-25): yield 28.28 mg, inactive. Peak II (the fractions, tube Nos. 34–43, in 0.2 N AcOH eluates were desalted on Sephadex G-25): yield 8.17 mg (16.3%), activity 81.3% (total units 505).

Purification of the Affinity-Purified Product by Ion-Exchange Chromatography on CM-Cellulose. As described previously, subsequent purification of the above affinity-purified product (7.37 mg) was performed by ion-exchange chromatography on CM-cellulose (1 × 13.2 cm), using gradient elution with pH 7.5, 0.1 M phosphate buffer (200 mL) through a mixing flask containing pH 6.0, 0.01 M phosphate buffer (120 mL) (Figure 6). The fractions corresponding to the main peak (tube Nos. 54–58) were desalted by gel filtration on Sephadex G-25. After lyophilization, the desired product was obtained as a fluffy white powder; yield 4.62 mg (63%), activity 113.1% against yeast RNA (compared with natural RNase A, total units 397).

The product behaved as a single component in disk isoelectrofocusing on 7.5% polyacrylamide gel with the identical mobility of natural RNase

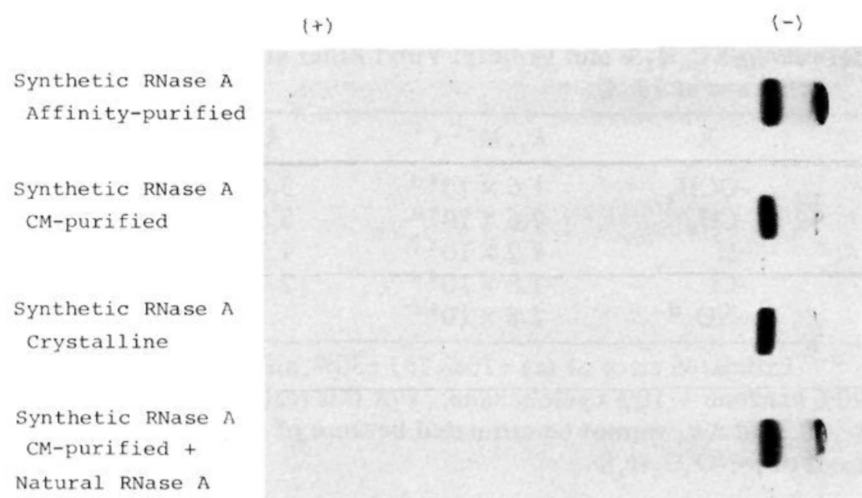


Figure 7. Disk isoelectrofocusing of synthetic RNase A on Ampholine, pH 3.5–10.0 (10 μg each).

A [Ampholine pH 3.5–10, at 200 V for 5 h, stained by Coomassie Brilliant Blue G-250 (Sigma)] (Figure 7); $[\alpha]^{20}_D$ −69.7° (c 0.2, in 0.1 M KCl) (lit.[11b] $[\alpha]^{20}_D$ −73.3° in 0.1 M KCl); UV $\epsilon_{max\ 277.5\ nm}$ 9734 in 0.1 M KCl (lit.[11b] 9800); Michaelis constant for yeast RNA, 1.21 mg/mL (lit.[23] 1.25 mg/mL). Amino acid ratios in 6 N HCl (48 h) hydrolyzate (numbers in parentheses are those of natural RNase A and numbers in brackets indicate the theory): Asp 14.94 (15.07) [15], Thr 9.79 (9.64) [10], Ser 13.80 (13.67) [15], Glu 12.53 (12.47) [12], Pro 4.27 (4.42) [4], Gly 3.38 (3.29) [3], Ala 11.96 (12.21) [12], Cys 3.82 (3.79) [4], Val 9.19 (8.92) [9], Met 4.20 (3.94) [4], Ile 2.28 (2.19) [3], Leu 2.00 (2.00) [2], Tyr 5.87 (5.94) [6], Phe 3.07 (3.11) [3], Lys 10.57 (10.44) [10], His 3.86 (3.66) [4], Arg 4.01 (4.11) [4], recovery of Leu 79% (77%).

Crystallization of the CM-Purified Product from Aqueous Ethanol. The CM-purified sample (3.4 mg) was dissolved in H_2O (50 μl). This solution was cooled to 5 °C and then 95% EtOH (60 μl) of the same temperature is added with gentle shaking until a very faint turbidity appeared. The solution was kept in a refrigerator for 3 months. During this period, a fine precipitate changed into a mass of small transparent, platelike crystals and then grew to multioriented polycrystals with rosettes (Figure 3a) or stalagmite shapes (Figure 3b). They were collected by centrifugation and washed with 95% EtOH; yield 1.92 mg. Activity against yeast RNA and 2′,3′-cyclic cytidine phosphate was 114% and 112% of natural RNase A, respectively. When measured at the protein concentration, 1.5 μg/ml, this crystalline sample exhibited the RNase A activity of 86.0 Kunitz U/mg.

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Evaluation of Addition Rates of Thiyl Radicals to Vinyl Monomers by Flash Photolysis. 3.[1] Polar Effect in Addition Reactions of Substituted Benzenethiyl Radicals

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Abstract: Rate constants for addition (k_1) of the para-substituted benzenethiyl radicals (p-$XC_6H_4S\cdot$) to vinyl monomers ($CH_2{=}CHY$) such as isobutyl vinyl ether and vinyl acetate (nonconjugated monomers) and acrylonitrile (conjugated one) have been determined by means of flash photolysis. The rate constants for the reverse reaction (k_{-1}) and the equilibrium constants ($K = k_1/k_{-1}$) have been estimated in the forms of k_{-1}/k_2 and Kk_2, respectively, where k_2 is the rate constant for the reaction between p-$XC_6H_4SCH_2C\cdot HY$ and oxygen; oxygen was used as a selective radical trap to the carbon-centered radicals. From the linear correlations obtained in the Hammett plots of log k_1, log (k_{-1}/k_2), and log Kk_2 vs. the substituent constants (σ^+), the reaction constants (ρ^+) were estimated for each vinyl monomer. The $\rho^+(Kk_2)$ values were invariant with a change in vinyl monomers ($\rho^+(Kk_2)$ = 1.37). The $\rho^+(k_1)$ values increase with an increase in the electron densities of the double bonds in vinyl monomers. This suggests that a part of the polar effects in the reactivities is determined by the polar effect in the thermodynamic stabilities of the p-$XC_6H_4S\cdot$ and that another part of the polar effects is caused by the polar resonance structures in the transition state of which contribution varies with the electron-donating or -withdrawing ability of vinyl monomers.

Since Walling and Mayo applied the Hammett equation to radical addition reactions,[2] the polar effects in the free-radical reactions have been the subject of many studies.[3-5] The polar effects in various radical reactions have been rationalized in terms of the contributions of the polar resonance structures in the transition state. In the hydrogen abstraction, Zavitsas and Pinto have recently proposed an idea that the Hammett reaction constants (ρ or ρ^+) do not reflect the polar effects in the transition state but they reflect the exo- or endothermicity of the reactions.[6] Davis and Pryor[7] have presumed that both effects are effective, and Tanner and his co-workers[8] have pointed out that the discussion should be made on the basis of the accurate relative rate constants.

For the polar effects in the addition reactions, such arguments have not been made because of lack of thermodynamic data for both reactant radicals and product radicals. For reactions of the

(1) Part 2: Ito, O.; Matsuda, M. *J. Am. Chem. Soc.* **1979**, *101*, 5732.
(2) Walling, C.; Biggs, E. R.; Wolfstirn, K. B.; Mayo, F. R. *J. Am. Chem. Soc.* **1948**, *70*, 1537.
(3) Walling, C. "Free Radicals in Solution"; Wiley: New York, 1967; Chapter 8.
(4) Russell, G. A. In "Free Radicals", Kochi, J. K., Ed.; Wiley: New York, 1973; Vol. I, pp 293–8.
(5) Imoto, M.; Kinoshita, M.; Nishigaki, M. *Makromol. Chem.* **1966**, *94*, 238.
(6) Zavitsas, A. A.; Pinto, J. A. *J. Am. Chem. Soc.* **1972**, *94*, 7390.
(7) Davis, W. H.; Pryor, W. A. *J. Am. Chem. Soc.* **1977**, *99*, 6365.
(8) Tanner, D. D.; Samal, P. W.; Ruo, T. C. R.; Henriquez, R. *J. Am. Chem. Soc.* **1979**, *101*, 1168.

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