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Studies on Peptides C,[1,2] Chemical Synthesis of Crystalline Ribonuclease A

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 with glutathione for the disulfide formation. After purifications by affinity chromatography followed by ion-exchange chromatography, a protein with the full enzymatic activity was obtained and subsequently crystallized from 95% ethanol according to Kunitz. A totally synthetic enzyme with full RNase A activity was thus obtained in a crystalline form for the first time.

Keywords——total synthesis of RNase A; trifluoromethanesulfonic acid-thioanisole deprotection; glutathione-mediated air oxidation; affinity chromatographic purification; crystals of synthetic RNase A

Recently, in a preliminary communication,[3] followed by a series of six papers,[4] we reported the chemical synthesis of a protein with the full enzymatic activity of bovine pancreatic ribonuclease (RNase) A (Fig. 1). We wish to report that we succeded in crystallizing the fully active synthetic enzyme. Improvement in yield at the final step of the synthesis was achieved by applying a new deprotecting procedure with trifluoromethanesulfonic acid (TFMSA)-thioanisole[5] in combination with a modified oxidation procedure using glutathione for the disulfide formation.[6]

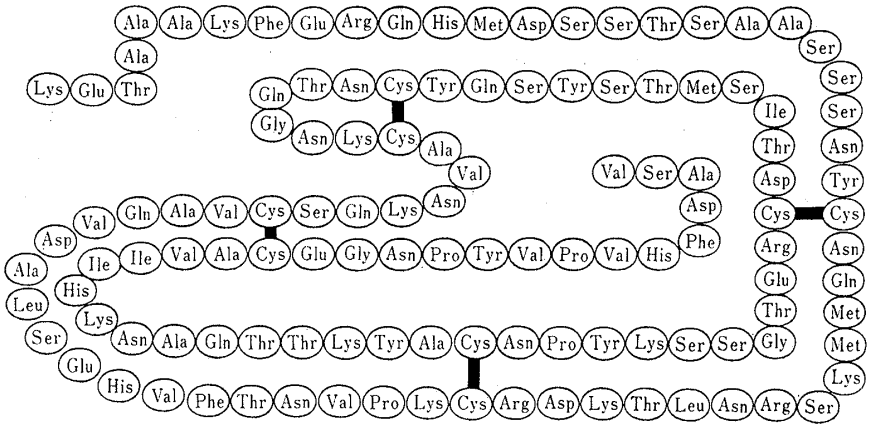

Fig. 1. Structure of Bovine Pancreatic Ribonuclease A

The protected RNase A was treated three times with 1 M TFMSA-thioanisole in TFA (0°, 60 min, each), instead of methanesulfonic acid,[7] in the presence of *m*-cresol to remove all of protecting groups employed (total of 33 groups; benzyloxycarbonyl from Lys, benzyl

from Glu and Asp, *tert*-butyl from Glu at position 2, *p*-methoxybenzyl from Cys, and *p*-methoxybenzenesulfonyl[8] from Arg) and the deprotected protein was then exposed to mercaptoethanol and dithiothreitol as described previously. Glutathione-mediated air oxidation was performed according to Chavez and Scheraga.[6] To a diluted solution of the protein (0.08 mg/ml) in pH 8.0, 0.2 M Tris-HCl buffer, reduced and oxidized glutathione (13.6 equiv. each) were added. After 5 day's of oxidation, followed by gel-filtration on Sephadex G-75, crude RNase A with an activity of 19% was obtained in 65% yield. When this oxidation was performed without addition of glutathione, the yield was 60% (activity 17%), which was still better yield than the former experiment (yield 54%, activity 12%).

Subsequent purifications by affinity[9] and ion-exchange[10] chromatographies were performed essentially in the same manner as performed previously to afford a homogeneous product, which exhibited identical mobility with that of natural RNase A in the field of isoelectrofocusing (Ampholine, pH 3.5—10, 200 V, 5 hr). Amino acid ratios in 6 N HCl hydrolysate (48 hr) were in excellent agreement with those of natural RNase A (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].

In order to obtain salt-free crystals, the method of Kunitz[11] was employed. A turbid solution formed by addition of 95% EtOH to an aqueous solution of synthetic RNase A obtained as described above was kept in a refrigerator for 3 months. During this period, small transparent, plate-like single crystals developed to multi-oriented crystals with rosette or stalagmite-shapes (Fig. 2).

Fig. 2. Crystals of Synthetic RNase A (×21)

The activity of this crystalline RNase A measured according to Kunitz[12] and Fruchter and Crestfield[13] was 114% against yeast RNA and 112% against 2′,3′-cyclic cytidine phosphate respectively. A totally synthetic enzyme with full RNase A activity was thus obtained in a crystalline form.

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References and Notes

1) Part. XCIX. H. Yajima, K. Akaji, Y. Hirota, and N. Fujii, *Chem. Pharm. Bull.*, **28**, 3140 (1980).
2) Natural bovine pancreatic RNase A (Type XII-A, Lot. No. 49C-8049, 76 Kunitz U/mg), yeast RNA (Type XI, Lot. No. 124C-8150), and 2′,3′-cyclic cytidine monophosphoric acid sodium salt (Lot. No. 76C-7510) were purchased from Sigma Chem., Co.
3) H. Yajima and N. Fujii, *J. Chem. Soc. Chem. Comm.*, **1980**, 115.
4) N. Fujii and H. Yajima, *J. Chem. Soc. Perkin I*, **1981**, in press.
5) H. Yajima, N. Fujii, H. Ogawa, and H. Kawatani, *J. Chem. Soc. Chem. Comm.*, **1974**, 107; H. Irie, N. Fujii, H. Ogawa, and H. Yajima, *ibid.*, **1976**, 922; Y. Kiso, S. Nakamura, K. Ito, K. Ukawa, K. Kitagawa, T. Akita, and H. Moritoki, *ibid.*, **1979**, 971; Y. Kiso, K. Ito, S. Nakamura, K. Kitagawa, T. Akita, and H. Moritoki, *Chem. Pharm. Bull.*, **27**, 1472 (1979).
6) L.G. Chavez, Jr. and H.A. Scheraga, *Biochem.*, **19**, 996 (1980); Y. Konishi and H.A. Scheraga, *ibid.*, **19**, 1308 (1980).
7) H. Yajima, Y. Kiso, H. Ogawa, N. Fujii, and H. Irie, *Chem. Pharm. Bull.*, **23**, 1164 (1975).

8) O. Nishimura and M. Fujino, *Chem. Pharm. Bull.*, **24**, 1568 (1976).
9) M. Wilchek and M. Gorecki, *Eur. J. Biochem.*, **11**, 491 (1969).
10) F.H. White, Jr., *J. Biol. Chem.*, **236**, 1353 (1961).
11) M. Kunitz, *J. Gen. Physiol.*, **24**, 15 (1940).
12) M. Kunitz, *J. Biol. Chem.*, **164**, 563 (1946).
13) R.G. Fruchter and A.M. Crestfield, *J. Biol. Chem.*, **240**, 3868 (1965).

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