SYNTHESES OF POLYPEPTIDES CORRESPONDING TO RIBONUCLEASE T_1 AND ITS ANALOG, [59-TYROSINE]-RIBONUCLEASE T_1

Michinori Waki, Nobuo Mitsuyasu, Shigeyuki Terada, Shuji Matsuura, Tetsuo Kato and Nobuo Izumiya

Laboratory of Biochemistry, Faculty of Science, Kyushu University, Fukuoka, Japan

Received October 8,1974

SUMMARY: Two polypeptides with the sequence of ribonuclease T_1 and its analog, [59-tyrosine]-ribonuclease T_1 were prepared by the solid-phase method. After purification by gel filtration and ion exchange chromatography, the synthetic peptides afforded similar patterns on paper and disc electrophoreses to those of natural enzyme. Further purification of the peptide corresponding to ribonuclease T_1 raised the specific activity to 59% for RNA and 44% for guanosine 2',3'-cyclic phosphate. In case of [59-tyrosine]-ribonuclease T_1, the activity was raised to 61% and 57%, respectively.

Ribonuclease T_1 [ribonucleate guanine nucleotido-2'-transferase (cyclizing), EC 2.7.7.26; RNase T_1] was isolated from Aspergillus oryzae by Sato and Egami(1), and its amino acid sequence of 104 residues was determined by Takahashi(2). The enzyme is an acidic protein containing one residue of tryptophan and two disulfide bonds which can be reduced and reoxidized with essentially complete reversibility(3). We have engaged in the synthetic studies of RNase T_1 peptides with various methods: solution-phase and fragment solid-phase methods(4). Hofmann et al.(5) have also been working on the total synthesis of this protein by the solution-phase method.

We wish to report here our investigation on the stepwise solid-phase syntheses(6) of two polypeptides with the sequences of RNase T_1 and its analog, [59-tyrosine]-RNase T_1 which contains a tyrosine residue in place of tryptophan, and on some properties including the ribonuclease activity of the synthetic peptides.

EXPERIMENTAL RESULTS AND DISCUSSION

Prior to the synthesis, the stability of natural RNase T_1 to the action of hydrogen fluoride was examined and sufficient retention of the enzymatic activity was confirmed(7).

Synthesis of Protected Polypeptide-resins. Two parallel syntheses of the protected polypeptide-resins corresponding to the sequence of RNase T_1 and its analog, [Tyr^{59}]-RNase T_1 were carried out manually following the stepwise solid-phase procedure of peptide synthesis(6). Boc-Thr(Bzl)-resin was prepared from Boc-Thr(Bzl) and chloromethylated 2% cross-linked polystyrene (1.46 mmole chloride/g) derived from Bio-Beads S-X2 (200-400 mesh) in the usual manner, and treated with 1.3 N HCl in acetic acid to yield the starting Thr(Bzl)-resin hydrochloride (I) (0.21 mmole Thr/g). I(3.8 g) was used for RNase T_1 and I(1.9 g) for [Tyr^{59}]-RNase T_1. The schedule of a cycle for the incorporation of each Boc-amino acid was almost the same as described for the syntheses of pancreatic trypsin inhibitor(8) and cobrotoxin(9) with the following modifications. N^{ε}-diisopropylmethyloxycarbonyl(10) and N^{im}-Boc(11) groups were used for side-chain protection of lysine and histidine, respectively. Boc groups were removed by treatment with 1.3 N HCl in acetic acid for 30 min, except in the case of Boc-Gln, Boc-Val,^{16,33,52} Boc-Ile^{61} and Boc-His^{27} residues for which 50%(v/v) trifluoroacetic acid in methylene chloride was used for 15 min. Throughout the synthesis, the deprotection reaction was monitored by Volhard chloride titration(12). The total time required for the synthesis was 47 days. The yields of the fully protected 104-residue peptide-resins, (IIa) with the sequence of RNase T_1 and (IIb) with that of the analog were 10.3 g (54% based on weight increase and amino acid analysis) and 4.8 g (50%), respectively.

Cleavage of Polypeptides from the Resins. Cleavage of the polypeptides from the solid support together with the removal of all protecting groups was achieved by treatment with liquid hydrogen fluoride(13) in the presence of anisole (Run A, B and C; see Table 1) at 0°C. Tryptophan or tyrosine was added for Run B or Run C. The reaction time was 1 hr for Run A and 3 hr for Run B and C.

The procedure of Run B is described as follows. After evaporation of hydrogen fluoride and drying in vacuo, the residue was washed with ether.

The dried residue was extracted with 0.05 M ammonium bicarbonate (pH 7.0) under gentle stirring at 0°C. After 3 hr, the filtrate from the resin was concentrated by ultrafiltration through a Diaflo UM-2 membrane, and lyophilized. The product dissolved in 0.05 M ammonium bicarbonate (pH 7.5) was passed through a column (3.1 x 55 cm) of Sephadex G-50 with the same buffer as an eluant, and fractionated to three portions: portion 1 of polymerized aggregates, 3 mg; portion 2 with a specific activity of 0.5% and with the same elution volume as native RNase T_1, 51 mg; portion 3 of a mixture of shorter peptides, 146 mg. Thus the total amount of crude polypeptides was 200 mg (11% from the peptide-resin(IIa)).

Purification of Crude Polypeptides. The deblocked crude polypeptides were purified by gel filtration on Sephadex G-75 or 50, and by ion exchange chromatography on DEAE-cellulose. The peptides thus obtained were further purified by chymotryptic digestion (Run A) or by a column chromatography on acetylated phosphocellulose containing guanosine 2'(3')-phosphate (14)(Run B and C). The yield and activity of the peptide obtained at the various stages of purification are summarized in Table 1.

The procedure of Run B is described as follows. The crude cleaved peptide (51 mg) obtained from portion 2 was rechromatographed on the same column of Sephadex G-50 as described in the preceding section. The fractions eluted at the same volume as native RNase T_1 were reduced with 2-mercaptoethanol and reoxidized in air to form the two disulfide bridges following the procedure of Kasai(3). The reoxidized sample was subjected to repeated gel filtration on Sephadex G-50, and further chromatographed on a column (0.9 x 20 cm) of DEAE-cellulose using a linear gradient elution (0.005 M $Na_2HPO_4 \rightarrow$ 0.25 M NaH_2PO_4, 0.25 M NaCl) to afford 7.0 mg of a peptide (IIIa) with a specific activity of 5%. Paper electrophoresis (pH 8.5 borate buffer, 400 V, 1.5 hr) of IIIa showed a single Pauly-positive spot, which moved toward the anode with the same mobility (R_{Glu} 0.67) as that of native RNase T_1. Both IIIa and native enzyme afforded the same patterns on disc gel

Table 1. Yield and Activity of Synthetic Peptides obtained in Various Steps of Purification

synthetic object	RNase T_1				[Tyr^{59}]-RNase T_1	
Run	A		B		C	
	Amount[a] (mg)	SA[b] (%)	Amount[a] (mg)	SA[b] (%)	Amount[a] (mg)	SA[b] (%)
HF cleaved Crude peptide[c]	6(8[d])		200(11[d])		93(10[d])	
Gel filtration (Sephadex)	G-75		G-50		G-50	
	2.4	0.4	51	0.5	35	0.7
	0.8	1.1	(reduction and reoxidation)			
	0.5	2.2	41	1.6	13	5.9
Ion exchange chromatography (DEAE-cellulose)	0.2	23	7.0[e]	5	1.7[e]	19
			3.6	f	1.4	f
			1.5	25	0.7	46
Protease digestion (Chymotrysin-resistant peptide)	0.09	53; 41[g]				
Column chromatography (acetylated phosphocellulose containing guanosine)			0.14[e]	59; 44[g]	0.02[e]	61; 57[g]

a. Unless otherwise noted, the amount of product was determined by the absorption at 280 nm(16).
b. Specific activity; the percentage of activity relative to pure native RNase T_1 toward RNA(16).
c. IIa(0.163 g for Run A and 4.0 g for Run B) or IIb(2.0 g for Run C) was used for HF cleavage.
d. Yield of HF cleavage (%).
e. The amount of product was determined by amino acid analysis of acid-hydrolysates(17).
f. Not determined.
g. Determined with guanosine 2',3'-cyclic phosphate as a substrate(18).

electrophoresis (pH 9.5, 2 mA/tube, 2 hr). Amino acid analysis of IIIa is given in Table 2 together with that of natural RNase T_1 and synthetic [Tyr^{59}]-RNase T_1 (IIIb) at the same stage of purification.

Further twice chromatography of IIIa on DEAE-cellulose gave 1.5 mg of a peptide (IVa) with a specific activity of 25%. The peptide (IVa) was chromatographed on a column (0.8 x 20 cm) of acetylated phosphocellulose containing guanosine 2'(3')-phosphate equilibrated with 0.02 M sodium acetate

Table 2. Amino Acid Analyses[a] of Natural RNase T_1 and Synthetic Products

Amino acid	Natural RNase T_1 Expected[b]	Natural RNase T_1 Found	Synthetic RNase T_1(IIIa)	Synthetic [Tyr^{59}]-RNase T_1(IIIb)
Asp	15	15.3	14.5	14.3
Thr	6	5.7	8.2	8.3
Ser	15	13.6	11.0	10.3
Glu	9	9.0	10.3	9.2
Pro	4	4.0	3.8	3.8
Gly	12	12.0	12.0	12.0
Ala	7	7.0	7.9	7.3
Val	8	7.9	8.1	8.4
lle	2	1.6	1.3	1.8
Leu	3	2.9	2.5	2.3
Tyr	9	8.8	4.9	4.8(10[c])
Phe	4	3.9	3.8	3.7
Trp	1	0.9	0.6	0.0(0[c])
Lys	1	1.0	0.9	0.9
His	3	2.9	4.0	3.8
Arg	1	1.1	0.7	1.0

a. Samples were hydrolyzed in 6 N HCl containing 4% thioglycolic acid(17) in sealed, evacuated tube for 24 hr at 110°C and analyzed on a Hitachi amino acid analyzer. Values are expressed as molar ratios on the basis of 12.0 for Gly as standard. Cys was not determined.
b. Based on the published amino acid sequence of RNase T_1(2).
c. Expected value for an analog.

buffer (pH 5.5) followed by 60 ml of the same buffer, and then by 0.2 M Tris-HCl buffer (pH 7.5). Two peaks were obtained by measuring the optical density at 280 nm and RNA digestion activity. The contents of the two peaks were isolated through gel filtration and lyophilization with the following yields (calculated from amino acid analysis): 0.14 mg of a peptide (Va) from the first peak eluted with 0.02 M sodium acetate buffer and 0.04 mg of a peptide (VIa) from the second peak eluted with 0.2 M Tris-HCl buffer. The specific activity of Va was 59% for RNA and 44% for guanosine 2',3'-cyclic phosphate, and that of VIa was 49% and 34%, respectively. On the same column, natural RNase T_1 (1.1 mg) was also separated into two peaks: the protein (0.75 mg) from the first peak with the same nuclease activity and amino acid compositions as those of RNase T_1 and the protein (0.07 mg) from the second peak with nuclease activity but somewhat different amino acid compositions from RNase T_1.

Discussion. It is interesting to note that the fingerprints on chymotryptic digests of performic acid oxidized native enzyme and a synthetic RNase T_1 peptide with a specific activity of 3% gave practically identical patterns, and a synthetic RNase T_1 peptide with a specific activity of 19% against RNA, obtained in another run, was completely inactive toward DNA and cytidine 2',3'-cyclic phosphate. Although it is questionable that the final purified polypeptide (Va) with RNase T_1 activity contains molecules identical to natural RNase T_1, our experiment provides evidence that a polypeptide with similar properties to RNase T_1 was synthesized. The finding that the purified synthetic [Tyr^{59}]-RNase T_1 showed a fairly high nuclease activity indicates the single tryptophan residue in RNase T_1 could be replaced by another aromatic amino acid, tyrosine, without significant loss of the enzyme activity. In this respect, it is noteworthy that ribonuclease U_1 [ribonucleate guanine nucleotido-2'-transferase(cyclizing), Ustilago sphaerogena, EC 2.7.7.26] with a similar guanylic acid specificity to that of RNase T_1 has no tryptophan residue (15).

ACKNOWLEDGEMENTS

The authors are indebted to Drs. K. Takahashi, F. Sakiyama and M. Ohno for their interests and useful suggestions during this work.

REFERENCES

1. Sato, K., and Egami, F. (1957) J. Biochem. (Tokyo) 44, 753-767.
2. Takahashi, K. (1965) J. Biol. Chem. 240, PC 4117-PC 4119.
3. Kasai, K. (1965) J. Biochem. (Tokyo) 57, 372-379.
4. Terada, S., Mitsuyasu, N., Waki, M., Kato, T., and Izumiya, N. (1973) Memoirs Fac. Sci. Kyushu Univ. Chem. 8, 179-190.
5. Kawasaki, K., Camble, R., Dupuis, G., Romovacek, H., Storey, H. T., Yanaihara, C., and Hofmann, K. (1973) J. Amer. Chem. Soc. 95, 6815-6824.
6. Merrifield, R. B. (1964) Biochemistry 3, 1385-1390.
7. Izumiya, N., Waki, M., Kato, T., Ohno, M., Aoyagi, H., and Mitsuyasu, N. (1972) Chemistry and Biology of Peptides, pp. 269-279, Ann Arbor Science Publishers, Michigan.
8. Noda, K., Terada, S., Mitsuyasu, N., Waki, M., Kato, T., and Izumiya, N. (1971) Naturwissenschaften 58, 147-148.
9. Aoyagi, H., Yonezawa, H., Takahashi, N., Kato, T., Izumiya, N., and Yang, C. C. (1972) Biochim. Biophys. Acta 263, 823-826.
10. Sakakiraba, S., Fukuda, T., Kishida, Y., and Honda, I. (1970) Bull. Chem. Soc. Japan 43, 3322.
11. Li, C, H. and Yamashiro, D. (1970) J. Amer. Chem. Soc. 92, 7608-7609.

12. Stewart, J. M. and Young, J. D. (1969) Solid Phase Peptide Synthesis, pp. 55-56. W. H. Freeman and Co., San Francisco.
13. Sakakibara, S., Shimonishi, Y., Kishida, Y., Okada, M., and Sugihara, H. (1967) Bull. Chem. Soc. Japan 40, 2164-2167; Lenard, J., and Robinson, A. B. (1967) J. Amer. Chem. Soc. 89, 181-182.
14. Waki, M. and Izumiya, N. unpublished.
15. Hashimoto, J., Uchida, T., and Egami, F. (1971) J. Biochem. (Tokyo) 70, 903-911.
16. Takahashi, K. (1961) J. Biochem. (Tokyo) 49, 1-8.
17. Matsubara, H. and Sasaki, R. M. (1969) Biochem. Biophys. Res. Commun. 35, 175-181.
18. Uchida, T. and Egami, F. (1967) Methods in Enzymology, Vol. 12, pp. 228-239, Academic Press, New York.