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Recombinant Human Pancreatic Ribonuclease Produced in *E. coli*: Importance of the Amino-terminal Sequence

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SUMMARY: Human pancreatic ribonuclease 1 (hRNase 1) in the mature form has been produced in *E.coli* using T7 expression system. The recombinant hRNase 1 protein was solubilized from the inclusion bodies, refolded in glutathione redox system, and purified through chromatographic procedures by utilizing cation-exchange and reversed-phase columns. The ribonucleolytic activity of recombinant hRNase 1 was examined on yeast RNA and cytidylyl-3',5'-adenosine revealing the distinctive ribonucleolytic activity. The activity was perfectly inhibited by human placental RNase inhibitor. Truncation of 7 amino acid residues in the amino-terminal sequence resulted in much reduction in ribonucleolytic activity and in affinity to human placental RNase inhibitor with the disintegration of secondary structures of the protein observed by circular dichroism spectra. The present study has revealed the important contribution of the amino-terminal sequence of hRNase 1 to the characteristics of the protein.

Human pancreatic ribonuclease 1 (rhRNase 1), which is considered to be a counterpart of bovine pancreatic RNase A, has been isolated and characterized first by Weickmann and his associates in 1981 (1). hRNase 1, which is classified into the secretory RNase, shows its optimal enzymatic activity at alkaline pH (around 8.0) with the strong preference for poly (C) over poly (U) (2), and shows much higher activity than bovine RNase A on double stranded RNA. hRNase 1 having different glycosylation patterns have been found to be present in urine, prostate, seminal plasma, kidney, cerebrum and blood plasma (3, 4), though its biological significance and tissue distribution in detail have not yet been clarified.

The human pancreatic-type RNase family contains five different gene products at least. These RNases are widely distributed in various tissues and cells and known to have intriguing array of biological activities ranging from neurotoxic and helminthotoxic to angiogenic ones (5). Pancreatic-type RNases derived from bovine seminal and amphibian oocytes proved to have distinguished

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anti-tumor and anti-HIV activities (6-9). Such specific activities of the RNases seem to suggest that RNase may be used for the therapeutic purposes, if they have no antigenicity. However, in the RNases from human no such activities have been found yet.

To investigate the physiological functions and the pharmaceutical availability of hRNase 1, we cloned cDNA encoding hRNase 1 from human pancreatic cDNA library (10). In this paper we describe the preparation and characterization of recombinant hRNase 1 (rhRNase 1), which is active as much as that produced in human body, and the importance of the amino-terminal sequence on the activity using amino-terminally truncated rhRNase 1 (des.1-7 hRNase 1).

MATERIALS AND METHODS

Expression of hRNase 1 and des.1-7 hRNase 1 in E.coli. hRNase 1 cDNA in the plasmid pBO23 (10) was digested with *SmaI* and *BamHI*. This DNA fragment was inserted together with a pair of linker DNAs (5'-TATGAAGGAATCCC-3' and 5'-GGGATTCCTTCA-3' corresponding to $M^{-1}K^{1}E^{2}S^{3}R^{4}$) into pET3a (11) linearized with *NdeI* and *BamHI*. The resultant plasmid designated as pBO26 was designed to express mature form of hRNase 1. To design the expression of des.1-7 hRNase 1, plasmid pBO26 was cleaved with *XbaI* and *BamHI* and the hRNase 1 cDNA including ø10 promoter was cloned into pUC119. Site-directed mutatgenesis (12) was carried out to create *NcoI* site just before the codon for F^{8}. DNA fragment coding for des.1-7 hRNase 1 was cut out with *NcoI* and *BamHI*, and was cloned into pET8c (11). This resultant plasmid was designated as pBO54. The transformant expressing mature hRNase 1 was prepared by transforming *E.coli* MM294 (DE3) / pLysS (13) with plasmid pBO26 and one expressing des.1-7 hRNase 1 by transforming *E.coli* BL21 (DE3) / pLysS (11) with pBO54. These transformants were grown at 37°C in LB-broth supplemented with glucose (0.4%), ampicillin (50μg/ml) and chloramphenicol (10μg/ml) until the absorbance at 600nm reached to 0.4, isopropyl 1-thio-β-D-galactopyranoside (IPTG) was then added to a concentration of 0.4mM, and cultivation was further continued for 3 h at 37°C. Cells from 1 liter of culture medium were harvested by centrifugation and washed once with physiological salt solution and then stored at -80°C until they were used.

Purification of rhRNase 1 and des.1-7 hRNase 1. Purification of both rhRNase 1 and des.1-7 hRNase 1 was carried out through the same procedure. Bacterial cells were disrupted by osmotic shock in the presence of 20% sucrose and by freezing and thawing. The insoluble fraction containing inclusion bodies of expressed protein was collected and resuspended in 20ml of the denaturing buffer consisting of 10mM Tris-HCl (pH7.5), 8M urea, 0.1M mercaptoethanol, 10mM EDTA and 1mM phenylmethanesulphonyl fluoride (PMSF). The solution was clarified by centrifugation and passed over a column of DEAE-Toyopearl 650M (1.6 x 2.5 cm, Tosoh, Japan) equilibrated with the denaturing buffer. The flow-through fraction was further applied to a column of CM-Toyopearl 650M (2.5 x 2.1 cm, Tosoh, Japan) equilibrated with buffer A (10mM Tris-HCl (pH7.5), 10mM EDTA) supplemented with 0.25mM PMSF. After the column was washed out with buffer A containing 0.1M NaCl, the recombinant protein was eluted with buffer A containing 0.3M NaCl. Ribonucleolytic activity was generated by diluting denatured recombinant protein in the eluates to 14μM in the refolding

buffer (buffer A containing 280μM of each reduced and oxidized glutathione and 0.1M NaCl) and incubating at room temperature for 24 h. Folded recombinant RNase was purified with a cation-exchange HPLC column (CM-Toyopearl 650S, 0.4 x 25 cm, Tosoh, Japan) and a reversed-phase HPLC column (Ultron 300 C_4, 0.46 x 15 cm, Chromatopacking Center, Japan).

Circular Dichroism (CD) Spectra. CD spectra of purified recombinant RNases were determined in 10mM phosphate buffer, pH7.0 at a protein concentration of 50μg/ml at room temperature with a spectropolarimeter (Model J-500A, Jasco, Japan) using cuvettes of 0.1 cm in width.

Assay for Ribonucleolytic Activities. Ribonucleolytic activity at pH 7.5 on yeast RNA (Sigma, Type IV) purified as described by Blackburn *et al.* (14) and on cytidylyl-3',5'-adenosine (CpA) was assayed with the methods of Anfinsen *et al.* (15) and of Witzel *et al.* (16), respectively. Bovine RNase A (Sigma, Type II-A) was used as a standard of activity. Inhibitory effect of human placental RNase inhibitor (PRI) (Toyobo, Japan) on ribonucleolytic activity was assayed by the method of Yasuda *et al.* (4).

Physicochemical Analyses. Amino acid composition and carboxyl-terminal amino acid were determined by amino acid analyzer (Hitachi 835, Japan) after hydrolysis or hydrazinolysis of purified recombinant RNases. Protein sequence determinations were performed on an Applied Biosystems Model 473A protein sequencer.

RESULTS AND DISCUSSION

Expression and Purification of Recombinant RNases. As designed in Fig. 1, hRNase 1 cDNA was properly integrated into plasmid pET3a or pET8c downstream of ø10 promoter to produce rhRNase 1 and des.1-7 hRNase 1, respectively. Upon induction with IPTG, *E.coli* MM294 (DE3) / pLysS / pBO26 appeared to synthesize rhRNase 1 in about 10% of the total *E.coli* protein (Fig. 2A, *Lane 2*), while *E.coli* BL21 (DE3) / pLysS / pBO26 appeared to be inhibited from cell growth and gave extremely low yield of the target protein. Both *E.coli* BL21 (DE3) / pLysS / pBO54 and *E.coli* MM294 (DE3) / pLysS / pBO54 produced des.1-7 hRNase 1, however, the former showed higher expression level than the latter as judged

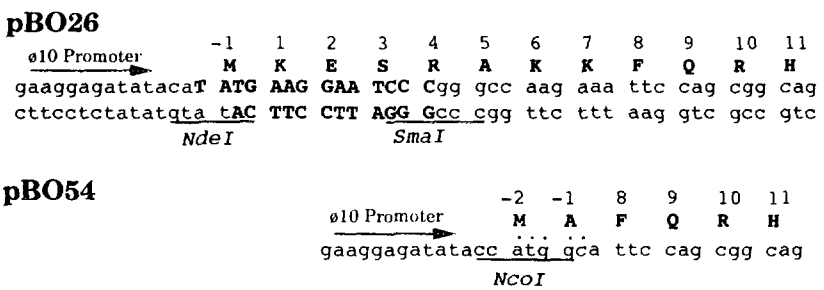

Figure 1. DNA constructs for the expression of rhRNase 1 and des.1-7 hRNase 1. (**A**) hRNase 1 cDNA was linked to downstream of ø10 promoter in pET3a with intervening synthetic linkers (upper case) for the expression of mature form of hRNase 1. (**B**) Site-directed mutagenesis was carried out on hRNase 1 cDNA to create *NcoI* site. The mutated bases are indicated with dots on the top of the bases. Utilizing the *NcoI* site, the cDNA was ligated downstream of ø10 promoter in pET8c for the expression of des.1-7 hRNase 1.

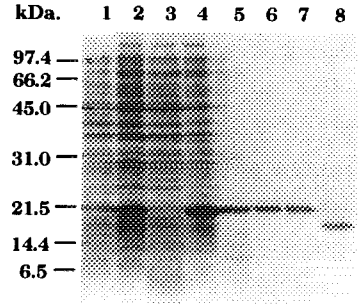

Figure 2. SDS-PAGE analyses of recombinant proteins. Samples were electrophoresed under reducing conditions. rhRNase 1 in each purification step from *E.coli*. Total cellular protein of MM294 (DE3) / pLysS / pBO26 under IPTG induced (*lane* 1) and non-induced (*lane* 2) conditions. *Lane* 3, soluble fraction of cell lysate; *lane* 4, insoluble fraction of cell lysate; *lane* 5, fraction eluted from a CM-column with 0.3M NaCl; *lane* 6, rhRNase 1 purified with CM-HPLC after refolding; *lane* 7, rhRNase 1 eluted from C4 reversed-phase HPLC; *lane* 8, bovine RNase A. The gel was stained with Coomassie Brilliant Blue R250.

by SDS-PAGE (data not shown). These results might suggest that rhRNase 1 was produced in an active form in *E.coli* BL21 that would be toxic enough to degrade endogenous RNA, therefore, in the case of des.1-7 hRNase 1, the absence of amino-terminal sequence might render itself denatured resulting in the formation of inclusion bodies even when expressed in *E.coli* BL21. Both rhRNase 1 and des.1-7 hRNase 1 appeared to be produced as an inactive form of inclusion bodies, which could be solubilized in 8M urea containing 0.1M mercaptoethanol. The recombinant proteins in inclusion bodies seemed to be protected from proteolytic degradation, because some proteolytic degradation was observed in during the following purification steps of rhRNase 1. This degradation was largely avoided by PMSF supplemented in each step.

Under the denaturing and reducing conditions, rhRNase 1 was purified with CM-column chromatography to about 90% of homogeneity as judged by SDS-PAGE (Fig. 2, *Lane 5*). Ribonucleolytic activity, which was not detected at this stage, was generated by oxidation in the folding buffer containing oxidized and reduced glutathione at pH7.5. Ribonucleolytic activity increased in a time dependent manner and reached plateau in 24 h (data not shown). Folded recombinant RNases were applied to a CM-HPLC column and eluted under a gradient of NaCl and a peak fraction detected by absorbance at 275nm was pooled (Fig. 2, *Lane 6*). This fraction was of 95.5% homogeneity as judged by C_4 reversed-phase HPLC. Through these procedures including the final reversed-phase HPLC step, 1.2 mg and 1.1 mg of active rhRNase 1 and des.1-7 hRNase 1 were obtained, respectively, from 1 liter of bacterial cell culture.

Amino-terminal sequence analysis of the recombinant RNases showed that the first 21 amino acid residues were identical to those of the construction as depicted in Fig. 1A, and that the second Ala residue in Fig. 1B was determined to

be at the amino-terminal of des.1-7 hRNase 1 indicating that the first Met residue derived from the initiation codon was processed off. The amino acid composition and amino acid residue, Thr, at the carboxyl-terminal of rhRNase 1 were coincident with those predicted from the cDNA sequence. Any free sulphydryl groups were not detected in the purified proteins with dithio-nitrobenzoic acid (17) (data not shown). Table I summarizes the ribonucleolytic activity of rhRNase 1 and des.1-7 hRNase 1 toward yeast RNA and CpA. Sorrentino and Libonati reported that the activity of hRNase 1 purified from pancreas toward yeast RNA or CpA was approximately one eighth or one third of that of bovine RNase A, respectively (2). Their results suggest that the activity of rhRNase 1 prepared here is almost equivalent to that of hRNase 1 purified from pancreas. However, the activity of des.1-7 hRNase 1 is extremely low.

Characterization of Recombinant Human RNase 1. PRI binds to mammalian pancreatic type of RNases in equimolar ratio exhibiting strong inhibition of ribonucleolytic activity (18). The activity of rhRNase 1 was also confirmed to be strictly inhibited in the presence of PRI (Fig. 3). The activity of des.1-7 hRNase 1 was also inhibited by PRI, however, three-fold amount of PRI was required to observe 50% inhibition of ribonucleolytic activity toward yeast RNA, indicating reduced binding ability of des.1-7hRNase 1 to PRI. The recent crystalographic structure of the complex between bovine RNase A and porcine RNase inhibitor showed that there was no change of conformation in RNase A with or without inhibitor (19). CD spectra of rhRNase 1 showed that the recombinant protein possessed the secondary structures characteristic to bovine RNase A, while those of des.1-7 hRNase 1 showed somewhat reduced secondary structures (Fig. 4). From these results, the amino-terminal sequence, consisting of 7 amino acid residues, of human RNase 1 was considered to contribute essentially to the tertiary structure formation of the whole protein molecule. Consisting of 20 amino acid residues, the amino-terminal sequence of bovine RNase A known as

TABLE I.

Specific Activity of Recombinant RNases

Substrate	Ribonucleolytic activity (units/mg of protein)		
	rhRNase 1	des.1-7 hRNase 1	bovine RNase A
Yeast RNA[a]	61,200	7,000	481,300
CpA[b]	2,055	85	6,505

[a] Assay of Anfinsen *et al.* (15) performed in 0.1M Mops (pH7.5) containing 0.1M NaCl. Units were defined as the amount increasing the absorbance at 260nm by 1.0 OD/min.
b Assay of Witzel *et al.* (16). Units defined in (2).

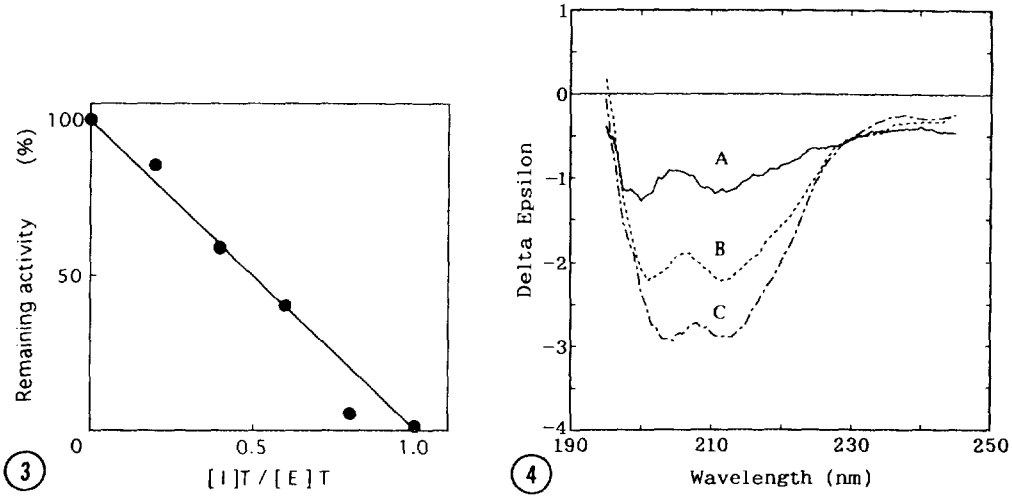

Figure 3. Stoichiometric inhibition of rhRNase 1 by PRI. rhRNase 1 (1.7 nM) was incubated with the various concentrations of PRI at 37°C for 15 min and residual ribonucleolytic activity toward yeast RNA was determined. [E]T / [I]T depicts the ratio of total concentration of rhRNase 1 to that of PRI.

Figure 4. CD spectra of rhRNase 1, des.1-7 hRNase 1 and bovine RNase A. Spectrum A, des.1-7 hRNase 1; B, rhRNase 1; C, bovine RNase A. See text in details.

S-peptide significantly contributes to maintenance of the structure of the rest of the whole molecule of S-protein, because S-protein alone would not keep its tertiary structure at all (20). However, the truncation of 7 amino acid residues seemed only to somewhat alter or destabilize the folded structure of rhRNase 1, because des.1-7 hRNase 1 retained some ribonucleolytic activity as well as PRI binding ability. These truncated amino acid residues contain 4 basic amino acid residues, K^1, R^4, K^6 and K^7. From these points of view, the reduced binding ability of des.1-7 hRNase 1 to PRI would be responsible either for the lack of direct electrostatic interaction with these basic amino acid residues as speculated by Neumann *et al.* (21), or for the altered or destabilized structure of the RNase molecule.

In the analogy of bovine RNase A, K^7 and R^{10} form P2 subsite, which is responsible for binding to phosphate, and K^1 forms B3 subsite, which is responsible for binding to base of purine or pyrimidine (22). Less catalytic activity on highly polymerized substrate such as yeast RNA observed in des.1-7 hRNase 1 might be elucidated by the absence of these subsites. The absence of these substrate binding subsites was not enough to elucidate the less catalytic activity toward dinucleotide such as CpA, which could be bound neither P2 nor B3 subsite. CD spectrum of des.1-7 hRNase 1 suggests that the decreased catalytic activity should be considered to be responsible not only for the loss of the subsites but also for the altered or destabilized structure as discussed above in the affinity to PRI.

Concluding Remarks. Russo *et al.* tried to prepare rhRNase 1 using synthetic DNA by recombinant technique, however, their design of the amino acid sequence had been mistaken in minor points (23). This may be partly because the first paper for amino acid sequence of hRNase 1 included a conflict with the cDNA sequence (24). Our report is the first preparation of rhRNase 1 expressed from the cDNA encoding complete amino acid sequence.

RNase is now considered to serve as a cytotoxic agent during host defense and physiological cell death pathways in bacteria, higher plant, and mammals (5). We are now working on the design of cytotoxic RNases using hRNase 1, potential cytotoxic RNase with selective function against malignant cells and with the lowest antigenicity. Human angiogenin (RNase 5) and eosinophil-derived neurotoxin (RNase 2) were already employed as a less antigenic toxin of selective immunotoxin (25,26). We also verified that hRNase 1 fused to ligand of cell surface molecules can be cytotoxic (unpublished data). On the other hand, cytotoxic RNases such as Onconase and bovine seminal RNase, which showed low affinity to PRI (7,27) and high affinity to cell surface are considered to internalize into cytoplasm where they degrade RNA inhibiting protein synthesis (7,28). In this analogy, the cytotoxic activity of RNases might be enhanced by reducing the affinity to PRI, therefore, modification of PRI recognition site of RNase will be effective strategy for the anti-cancer drug.

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REFERENCES

1. Weickmann, J. L., Elson, M., and Glitz, D. G., (1981) Biochemistry 20, 1272-1278.
2. Sorrentino, S. and Libonati, M. (1994) Arch. Biochem. Biophys. 312, 340-348.
3. Ribo, M., Beintema, J. J., Osset, M., Fernandez, E., Bravo, J., Llorens, R. D., and Cuchillo, C. M. (1994) Biol. Chem. Hoppe-Seyler. 375, 357-363.
4. Yasuda, T., Nadano, D., Takeshita, H., and Kishi, K. (1993) Biochem. J. 296, 617-625.
5. Youle, R. J., Newton, D., Wu, Y. N., Gadina, M., and Rybak, S. M. (1993) C.R.C. Crit. Rev. Ther. Drug Carrier Syst. 10, 1-28.
6. Vescia, S., Tramontano, D., Augusti-Tocco, G., and D'Alessio, G. (1980) Cancer Res. 40, 3740-3744.
7. Wu, Y. N., Mikulski, S. M., Ardelt, W., Rybak, S. M., and Youle, R. J. (1993) J. Biol. Chem. 268, 10686-10693.
8. Nitta, K., Ozaki, K., Ishikawa, M., Furusawa, S., Hosono, M., Kawauchi, H., Sasaki, K., Takayanagi, Y., Tsuiki, S., and Hakomori, S. (1994) Cancer Res. 54, 920-927.

9. Youle, R. J., Wu, Y. N., Mikulski, S. M., Shogen, K., Hamilton, R. S., Newton, D., D'Alessio, G., and Gravell, M. (1994) Proc. Natl. Acad. Sci. USA 91, 6012-6016.
10. Seno, M., Futami, J., Kosaka, M., Seno, S., and Yamada, H. (1994) Biochim. Biophys. Acta 1218, 466-468.
11. Studier, F. W. and Moffat, B. A. (1986) J. Mol. Biol. 189, 113-130.
12. Kunkel, T. A. (1985) Proc. Natl. Acad. Sci. USA 82, 488-492.
13. Watanabe, T., Seno, M., Sasada, R., and Igarashi, K. (1990) Mol. Endocrinol. 4, 869- 879.
14. Blackburn, P., Wilson, G., and Moore, S., (1977) J. Biol. Chem. 252, 5904-5910.
15. Anfinsen, C. B., Redfield, R. R., Choate, W. L., Page, J., and Carroll, W. R. (1954) J. Biol. Chem. 207, 201-210.
16. Witzel, H. and Barnard, E. A. (1962) Biochem. Biophys. Res. Commun. 7, 295-299.
17. Collier, H. B. (1973) Anal. Biochem. 56, 310-311.
18. Blackburn, P. and Moore, S. (1982) in The Enzymes (Boyer, P. D., Ed.), 3rd ed., Vol. XV, pp. 317-433, Academic Press, New York.
19. Kobe, B. and Delsenhofer, J. (1995) Nature 374, 183-186.
20. Doscher, M. S. and Hirs, C. H. W. (1967) Biochemistry 6, 304-312.
21. Neumann, U. and Hofsteenge, J. (1994) Protein Sci. 3, 248-256.
22. Parés, X., Nogués, M. V., de Llorens, R., and Cuchillo, C. M. (1991) Essays Biochem. 26, 89-103.
23. Russo, N., De Nigris, M., Ciardiello, A., Di Donato, A., and D'Alessio, G. (1993) FEBS Lett. 333, 233-237.
24. Beintema, J. J., Wietzes, P., Weickmann, J. L., and Glitz, D. G. (1984) Anal. Biochem. 136, 48-64.
25. Rybak, S. M., Hoogenboom, H. R., Meade, H. M., Raus, J. C. M., Schwart, D., and Youle, R. J. (1992) Proc. Natl. Acad. Sci. USA 89, 3165-3169.
26. Newton, D. L., Nicholls, P. J., Rybak, S. M., and Youle, R. J. (1994) J. Biol. Chem. 269, 26739-26745.
27. Murthy, B. S. and Sirdeshmukh, R. (1992) Biochem. J. 281, 343-348.
28. Mastronicola, M. R., Piccoli, R., and D'Alessio, G. (1995) Eur. J. Biochem. 230, 242-249.