Secretion of recombinant ribonuclease T_1 into the periplasmic space of *Escherichia coli* with the aid of the signal peptide of alkaline phosphatase

Takao Fujimura, Toshiki Tanaka*, Kanako Ohara, Hiroshi Morioka, Seiichi Uesugi, Morio Ikehara* and Satoshi Nishikawa+

Faculty of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565, Japan

Received 28 March 1990; revised version received 10 April 1990

The ribonuclease T_1 (RNase T_1) gene was ligated to a synthetic gene for the signal peptide of *Escherichia coli* alkaline phosphatase. When this fusion gene was expressed in *E. coli* under the control of the *trp* promoter, active RNase T_1 having the correct N-terminal sequence was secreted into the periplasmic space, indicating that the heterologous signal peptide had been cleaved off correctly. The enzyme could be readily purified from the periplasmic fraction with a yield of 1.8 mg from 1 liter culture. Adopting the same strategy, it was possible to produce a labile mutant of RNase T_1 (Glu-58 → Ala mutant) in *E. coli*, the yield of the purified mutant enzyme being 2.0 mg from 1 liter culture.

Ribonuclease T_1; Alkaline phosphatase; Signal peptide; Secretion; Recombinant protein

1. INTRODUCTION

Ribonuclease T_1 (RNase T_1) from *Aspergillus oryzae* catalyzes specifically the hydrolysis of the phosphodiester bonds of guanosine 3′-phosphate residues in single-stranded RNA [1]. It has been used for RNA sequencing and its properties have been extensively studied [2,3].

We have chemically synthesized the RNase T_1 gene and expressed it in *E. coli* [4]. By using protein engineering techniques, we have also analyzed the functional roles of residues involved in RNase T_1 catalysis [5] and those required for recognition of guanine base [6–8]. In an attempt to reduce the potentially toxic nucleolytic activity of RNase T_1 and its mutant in *E. coli* cells, we have constructed fusion proteins in which two-thirds of human growth hormone (hGH-AB) is connected to the N-terminus via a methionine residue [9], or hGH-AB and insulin-like growth factor I are ligated to the N- and C-termini of the enzymes, respectively, again via methionine residues (sandwich method) [7]. These fusion proteins were efficiently expressed in *E. coli* and RNase T_1 and its mutants could be cut off from them by BrCN treatment. However, the drastic BrCN treatment resulted in poor yields of the enzymes, making it difficult to obtain sufficient quantities of the enzymes for physicochemical characterization. To solve this problem, we decided to employ the 'secretion methods', which has allowed Quass et al. [10] to produce nearly intact RNase T_1 in *E. coli*.

Here we report the construction of an efficient secretion vector containing the gene for the signal peptide of *E. coli* alkaline phosphatase (APase). By using this vector, it was possible to accumulate wild-type RNase T_1 and one of its mutants in the periplasmic space of *E. coli*, permitting their simple purification with high yields.

2. MATERIALS AND METHODS

2.1. *Synthesis of signal peptide gene and construction of secretion plasmids*

Based on the known primary structure of the signal peptide of *E. coli* APase [11], oligodeoxyribonucleotides (U1, U2, L1 and L2 in Fig. 1a) were synthesized by the phosphoramidite method in an Applied Biosystems DNA synthesizer. In designing these oligonucleotides, codon usage in *E. coli* [12] was taken into account. These oligonucleotides were ligated and annealed as shown in Fig. 1 to produce a synthetic gene for the signal peptide of APase. All DNA manipulations were performed as described previously [4]. pGH-L9 containing the *trp* promoter and the hGH gene [9], was digested with *Cla*I and *Sal*I (Fig. 1b). The linearized vector containing the *trp* promoter was isolated from a 1% agarose gel after electrophoresis. pPST1 (T. Tanaka and K. Ohara, unpublished results), which has been derived from pPShGH11 [13] and contains the RNase T_1 gene, was

Correspondence address: S. Uesugi, Faculty of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565, Japan

* *Present address:* Protein Engineering Research Institute, 6-2-3 Fruedai, Suita, Osaka 565, Japan

\+ *Present address:* Fermentation Research Institute, Agency of Industrial Science & Technology, Ministry of International Trade & Industry, 1-1-3 Higashi, Tsukuba, Ibaraki 305, Japan

Abbreviations: RNase, ribonuclease; APase, alkaline phosphatase; hGH, human growth hormone; IAA, 3-indoleacrylic acid; pGpC, 5′-phosphoguanylyl (3′-5′)cytidine

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/90/$3.50

digested with *Sph*I and *Sal*I (Fig. 1b). The RNase T_1 gene thus excised was isolated from a 5% polyacrylamide gel after electrophoresis. The *trp* promoter-containing vector, the RNase T_1 gene, and the synthetic gene for the APase signal peptide were mixed and incubated with T4 DNA ligase, and the desired plasma, termed pTPW1, was obtained from *E. coli* HB101 transformants by standard procedures. pW58A1 containing the gene for the (Glu-58 ⟶ Ala) mutant of RNase T_1 was constructed from pTPW1 and pT58A [5]. The nucleotide sequences of these fusion genes were confirmed by the dideoxy chain termination method [14].

2.2. *Expression in* E. coli *and purification of RNase* T_1

E. coli HB101 harboring pTPW1 (or pW58A1) was grown at 37°C in 4 liters of M9-casamino acid medium containing ampicillin (20 μg/ml). After incubation for 1.5 h (optical density of culture at 660 nm, 0.05–0.1), 3-indoleacrylic acid (IAA, 40 μg/ml) was added to the medium to induce the expression of the fusion gene. Cells were allowed to grow further for 8 h, harvested by centrifugation (3500 × *g*, 10 min, 4°C), and washed in isotonic saline. Proteins in the periplasmic space were then released by osmotic-shock treatment by a modified version of the protocol of Cornelis et al. [15]. Thus, the saline-washed cells were suspended in 1 liter of 25% sucrose containing 1 mM EDTA and 30 mM Tris-HCl (pH 8.0) and the suspension was shaken at 37°C for 30 min. The cells were then sedimented by centrifugation (14000 × *g*, 1 h, 20°C), resuspended in 1 liter of ice-cold 20 mM Tris-HCl (pH 7.5), and shaken at 0°C for 30 min. The suspension was centrifuged (10000 × *g*, 15 min, 4°C) and the resultant supernatant was used as the periplasmic fraction. The cytoplasmic fraction was prepared from the sedimented cells by disruption with lysozyme in the presence of SDS. RNase T_1 (or its mutant) was purified from the periplasmic fraction by two steps of column chromatography: first, anion-exchange chromatography on a Q-Sepharose column equilibrated with 0.15 M NaCl in 20 mM Tris-HCl (pH 7.5) and eluted with a linear NaCl gradient (0.15–0.45 M) in 20 mM Tris-HCl (pH 7.5); then gel chromatography on a Sephadex G-50 column from which the enzyme was eluted with 20 mM ammonium bicarbonate. The enzyme at each purification step was analyzed by SDS-PAGE.

2.3. *Other methods*

Protein was determined by Lowry method and in the case of the purified enzyme from the absorbance at 280 nm using an extinction coefficient of $A_{280\,nm}^{0.1\%} = 1.91$ [16].

RNase T_1 activity was assayed by measuring the hydrolysis of [5′-^{32}P]pGpC as described previously [4].

3. RESULTS AND DISCUSSION

A DNA duplex encoding the 21-residue signal peptide of *E. coli* APase was synthesized (Fig. 1a) and used for construction of the secretion plasmid pTPW1 for RNase T_1 as shown in Fig. 1b. *E. coli* HB101 was transformed with this plasmid and the APase signal peptide-RNase T_1 fusion gene was expressed by inducing the *trp* promoter with IAA. After incubation for 8 h, the culture supernatant as well as the cytoplasmic

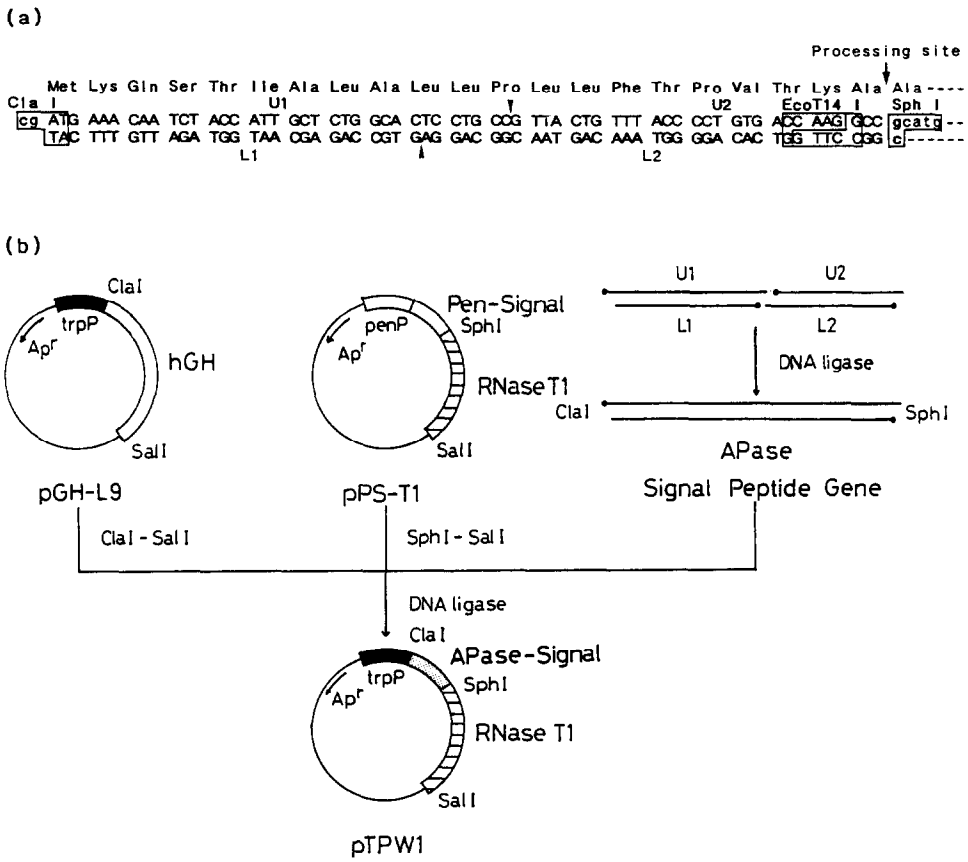

Fig. 1. Synthesis of the gene for the signal peptide of *E. coli* APase (a) and construction of the secretion plasmid pTPW1 (b). (a) Oligonucleotides termed U1, U2, L1, and L2 were chemically synthesized and ligated enzymatically (ligation sites are shown by arrowheads) to give the synthetic gene. *Cla*I and *Sph*I sites were introduced at the 5′- and 3′-ends, respectively, to facilitate subsequent DNA manipulation. The amino acid sequence of the signal peptide and the processing site are also shown. (b) The procedures are self-explanatory. trpP, *trp* promoter; penP, penicillinase promoter; Pen-Signal, penicillinase signal peptide; and APase-Signal, APase signal peptide.

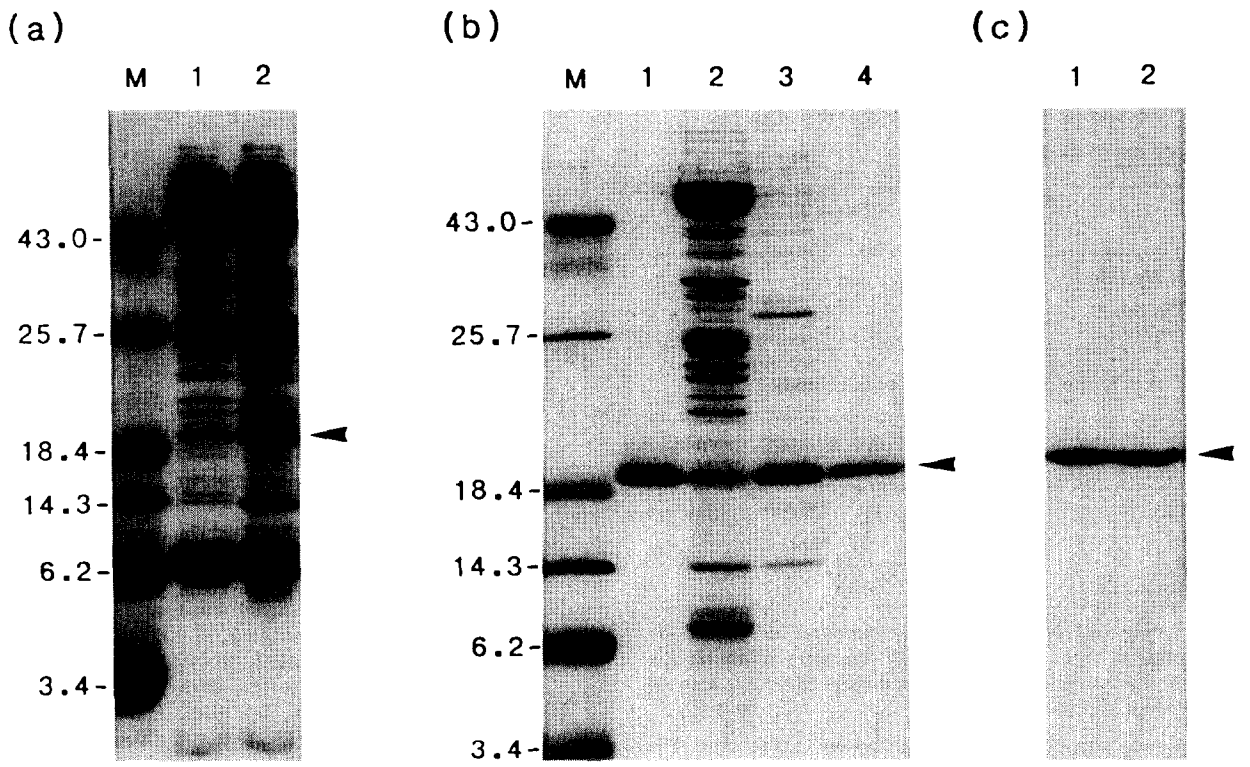

Fig. 2. SDS-PAGE (a, b) and PAGE under non-denaturing conditions (c) on 15% gels. (a) Periplasmic fractions of *E. coli* HB101/pTPW1 grown in the absence (lane 1) and presence (lane 2) of IAA (40 μg/ml). (b) Authentic RNase T_1 (lane 1). Periplasmic fraction of *E. coli* HB101/pTRW1 grown in the presence of IAA (lane 2). Fraction obtained after Q-Sepharose chromatography of the periplasmic fraction (lane 3). Fraction obtained after Sephadex G-50 gel filtration of the Q-Sepharose fraction (lane 4). (c) PAGE under non-denaturing conditions. Authentic RNase T_1 (lane 1). Purified recombinant RNase T_1 (lane 2). Lane M, molecular mass markers (values in kDa on the left). The positions of RNase T_1 is indicated by arrowheads. Gels were stained with Coomassie brilliant blue.

and periplasmic fractions (separated by osmotic shock treatment) of the cells were assayed for RNase T_1 activity. High activity was detected only in the periplasmic fraction (data not shown). Upon SDS-PAGE the periplasmic fraction of the cells harboring pTPW1 exhibited a band having the same mobility as authentic RNase T_1 and this band was induced by treatment of the cells with IAA (Fig. 2a). When the periplasmic fraction of the IAA-induced cells was subjected to Q-Sepharose column chromatography, this band was extensively purified (Fig. 2b, lane 3). Further purification of this sample by gel filtration through a Sephadex G-50 column yielded pure RNase T_1, which gave a single protein band both on SDS-PAGE (Fig. 2b, lane 4) and on PAGE under non-denaturing conditions (Fig. 1C). The yield of the pure protein was 1.8 mg from 1 liter of culture. The purified enzyme showed the same specific activity as authentic RNase T_1 (data not shown). The N-terminal sequence of the purified protein was determined to be Ala-X-Asp-Tyr-Thr-, which is identical with that of authentic RNase T_1 [2]. These results indicate that the signal peptide-RNase T_1 fusion protein synthesized in the cytoplasm was efficiently secreted into the periplasm with the aid of the APase signal peptide and the heterologous signal peptide was cleaved off at the correct site.

Although RNase T_1 is very stable, some of its mutants are labile. Especially the (Glu-58 ⟶ Ala) mutant is the most unstable among the mutants prepared in our laboratory. This mutant enzyme expressed in *E. coli* as a fusion protein with hGH-AB tends to undergo denaturation during purification procedures [5], making it difficult to obtain sufficient amounts of the pure protein. To test if this drawback can be overcome by using the secretion method, we constructed the secretion plasmid pW58A1 for the mutant in the same way as for the wild-type enzyme. Expression in *E. coli* and purification of this mutant enzyme were conducted in the same way as for wild-type RNase T_1 (data not shown). The yield of the pure mutant enzyme was 2.0 mg from 1 liter of culture. This mutant has proven to be useful for studies of the reaction mechanism of RNase T_1 [5].

In Table I the secretion method is compared with the previously used 'fusion protein method' with respect to the yield and purity of the enzyme at each step. As an example of the fusion protein method, the data for the (Tyr-45 ⟶ Trp) mutant of RNase T_1, which has a higher nucleolytic activity than the wild-type enzyme, are summarized [7]. Although a very high level of expression is observed in the crude cell extract when the fusion method is used, the yield decreases drastically

Table I

A summary of purification of wild-type RNase T_1 expressed by the secretion method (a) and that of (Tyr-45 → Trp) mutant of RNase T_1 expressed by the fusion protein method (b)

(a) Wild-type (secretion method)

Fraction	Total protein (mg)	RNase T_1 (mg)	Yield (%)
Periplasmic fraction	70	4.0	100
Q-Sepharose	7.0	2.1	53
Sephadex G-50	1.8	1.8	45

(b) (Tyr-45 → Trp) mutant (fusion protein method)

Fraction	Total protein (mg)	Mutant RNase T_1 (mg)	Yield (%)
Crude extract	243	11 (38)[a]	100
1st DE-52	75	3.6 (12)[a]	33
2nd DE-52	3.6	0.74	6.7
HPLC (DEAE-2SW)	0.13	0.13	1.2

[a] Numbers in parentheses represent the amounts of the fusion protein present in the fractions. BrCN treatment of the fusion protein should theoretically liberate the mutant enzyme in amount shown without parentheses

Purification was carried out from 1 liter of culture in both cases. The amount of enzyme in each fraction (except for the final preparation) was estimated from the catalytic activity. In (b), BrCN treatment was conducted after first DE52 chromatography. Data in (b) are from [7]

during purification, especially after BrCN treatment, which was conducted after the first DE-52 column chromatography. In contrast, in the secretion method the gene product is secreted into the periplasmic space and thus separated from numerous cytoplasmic proteins. Therefore, it can be purified by a simple two-step procedure, without substantial loss. In addition, the gene product can be processed correctly during the secretion by the host, making it possible to avoid the drastic BrCN treatment. For these two reasons the final yield of pure RNase T_1 achieved in the secretion method is much higher than in the fusion protein method, even though the level of expression is only moderate in the former method.

As mentioned earlier, Quass et al. [10] have reported the secretion of an RNase T_1 analog into the periplasm of *E. coli* using the signal peptide of OmpA protein. In their enzyme, however, Glu-25 is replaced by Lys and there are 4 extra amino acids, Ala-Glu-Phe-Met, at the N-terminus as a result of their strategy for construction of the gene. Our expression method, in which RNase T_1 is secreted in its intact form, appears to be particularly useful.

Acknowledgements: We are grateful to Dr Koki Horikoshi (RIKEN Institute) for providing the plasmid pSPShGH11 and for valuable discussions. This work was supported by a Grant-in-Aid for Specially Promoted Research from the Ministry of Education, Science and Culture of Japan and Kato Memorial Foundation (S.N.).

REFERENCES

[1] Sato, K. and Egami, F. (1957) J. Biochem. (Tokyo) 44, 753–768.
[2] Uchida, T. and Egami, F. (1971) in: The Enzymes, vol. 4, 3rd edn (Boyer, P.D. ed.) pp. 205–250, Academic Press, New York.
[3] Takahashi, K. and Moore, S. (1982) in: The Enzymes, vol. 15B, 3rd edn (Boyer, P.D. ed.) pp. 435–468, Academic Press, New York.
[4] Ikehara, M., Ohtsuka, E., Tokunaga, T., Nishikawa, S., Uesugi, S., Tanaka, T., Aoyama, Y., Kikyodani, S., Fujimoto, K., Yanase, K., Fuchimura, K. and Morioka, H. (1986) Proc. Natl. Acad. Sci. USA 83, 4695–4699.
[5] Nishikawa, S., Morioka, H., Kim, H.J., Fuchimura, K., Tanaka, T., Uesugi, S., Hakoshima, T., Tomita, K., Ohtsuka, E. and Ikehara, M. (1987) Biochemistry 26, 8620–8624.
[6] Nishikawa, S., Kimura, T., Morioka, H., Uesugi, S., Hakoshima, T., Tomita, K., Ohtsuka, E. and Ikehara, M. (1988) Biochem. Biophys. Res. Commun. 150, 68–74.
[7] Nishikawa, S., Morioka, H., Kimura, T., Ueda, Y., Tanaka, T., Uesugi, S., Hakoshima, T., Tomita, K., Ohtsuka, E. and Ikehara, M. (1988) Eur. J. Biochem. 173, 389–394.
[8] Hakoshima, T., Toda, S., Sugio, S., Tomita, K., Nishikawa, S., Morioka, H., Fuchimura, K., Kimura, T., Uesugi, S., Ohtsuka, E. and Ikehara, M. (1988) Protein Eng. 2, 55–61.
[9] Ikehara, M., Ohtsuka, E., Tokunaga, T., Taniyama, Y., Iwai, S., Kitano, K., Miyamoto, S., Ohgi, T., Sakuragawa, Y., Fujiyama K., Ikari, T., Kobayashi, M., Miyake, T., Shibahara, S., Ono, A., Ueda, T., Tanaka, T., Baba, H., Miki, T., Sakurai, A., Oishi, T., Chisaka, O. and Matsubara, K. (1984) Proc. Natl. Acad. Sci. USA 81, 5956–5960.
[10] Quass, R., McKeown, Y., Stanssens, P., Frank, R., Bloecker, H. and Hahn, U. (1988) Eur. J. Biochem. 173, 617–622.
[11] Kikuchi, Y., Yoda, K., Yamasaki, M. and Tamura, G. (1981) Nucleic Acids Res. 9, 5671–5678.
[12] Maruyama, T., Gojobori, T., Aota, S. and Ikemura, T. (1986) Nucleic Acids Res. 14, r151–r197.
[13] Kato, C., Kobayashi, T., Kudo, T., Furusato, T., Murakami, Y., Tanaka, T., Baba, H., Oishi, T., Ohtsuka, E., Ikehara, M., Yanagida, T., Kato, H., Moriyama, S. and Horikoshi, K. (1987) Gene 54, 197–202.
[14] Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.
[15] Cornelis, P., Digneffe, C. and Willemot, K. (1982) Mol. Gen. Genet. 186, 507–511.
[16] Takahashi, K. (1970) J. Biochem. (Tokyo) 67, 833–839.