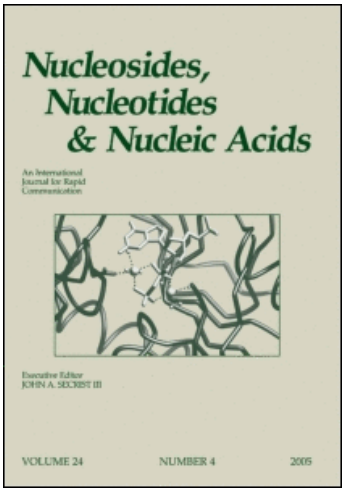

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Expression of Ribonuclease T1 in Escherichia Coli and Rapid Purification of the Enzyme

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EXPRESSION OF RIBONUCLEASE T1 IN *ESCHERICHIA COLI* AND RAPID PURIFICATION OF THE ENZYME

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Abstract: An *Escherichia coli* clone secreting native ribonuclease T1 into the periplasm has been constructed. In a two step purification procedure 20 mg of pure enzyme can be isolated starting from 1 litre of liquid culture.

Ribonuclease T1 (RNase T1; EC 3.1.27.3) from the fungus *Aspergillus oryzae* consists of 104 amino acids of known sequence[1] and hydrolyses single stranded ribonucleic acid specifically after guanosine residues producing 3'-phosphorylated mono- or oligoribonucleotides. The enzyme has been extensively studied by biochemical and biophysical methods. The tertiary structures of RNase T1 complexed with two inhibitors, 2'GMP and 2',5'GpG, and one of its possible products, 3'GMP, have been reported (for review see 2,3,4).

As a prerequisite for protein design with RNase T1 we have chemically synthesized a corresponding gene and constructed an overproducer e.g. an *E. coli* clone secreting the enzyme into the periplasm. The RNase T1 gene was cloned downstream to the region coding for the ompA protein (a major outer membrane protein of *E. coli*)[5].

Due to the synthesis strategy and to the secretion vector used (pIN-III-ompA2[6]), there was a four codon linker between the genes for the ompA signal peptide and native RNase T1 (pA2T1-1 in Fig. 1A upper part). This led to the production of a ribonuclease with an N-terminal extension of four amino acids. The protein showed the same behaviour as RNase T1 from *A. oryzae* with respect to catalysis and specificity[5] (Rao, personal communication).

In this paper we describe the construction of an *E. coli* clone producing native RNase T1 via deletion of the twelve base pair linker between the region

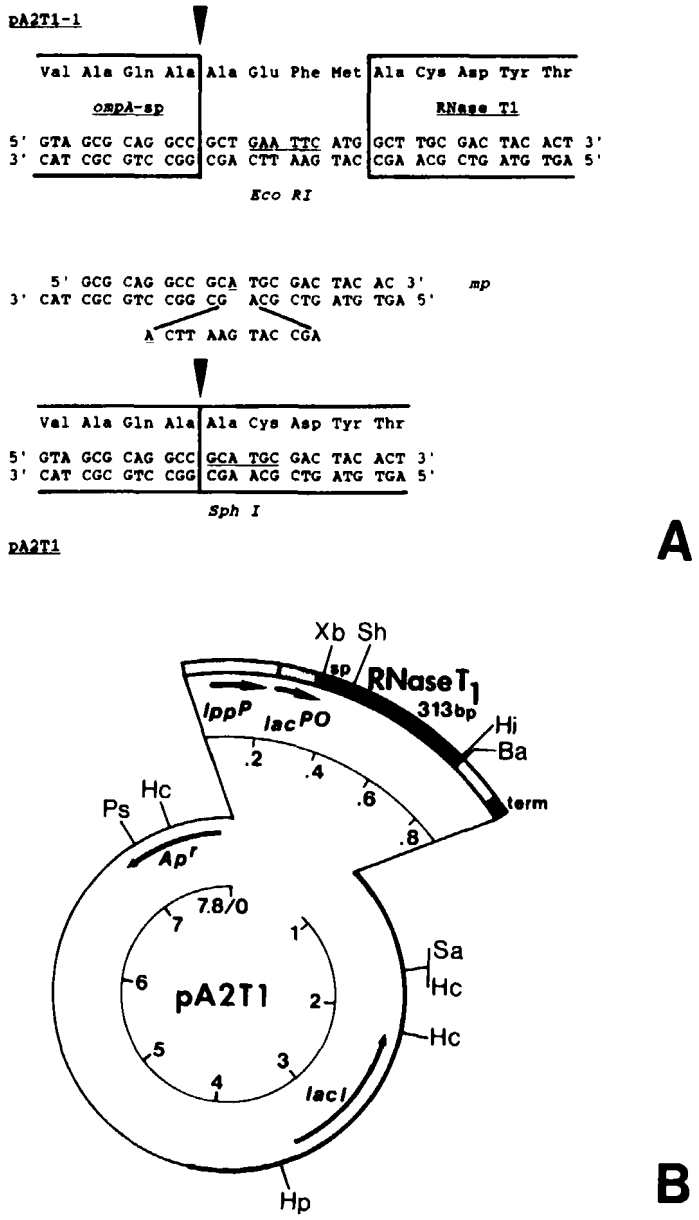

FIG. 1: Signal peptide- RNase T1 junction and map of the expression/secretion plasmid pA2T1. (A) Mutation from pA2T1-1(upper part)[5] to pA2T1 (lower part) was accomplished with the mutagenesis primer (mp). (B) Map of pA2T1. Abbreviations are: Ba, Bam HI; Hc, Hinc II; Hi, Hind III; Hp, Hpa I; Ps, Pst I; Sa, Sal I; Sh, Sph I; Xb, Xba I; lppP, lpp promoter; lacPO, lac promoter-operator; sp, signal peptide; term, transcription terminator (for further details see ref. 5).

coding for the signal peptide and RNase T1, and the purification in two steps of the enzyme from the host's periplasm.

RESULTS AND DISCUSSION

Mutagenesis

Standard cloning techniques were applied as described[7]. Oligonucleotide-directed mutagenesis was performed using the pMa/c system (Stanssens et al., in preparation). The mutagenesis primer 5′ GCG CAG GCC GCA TGC GAC TAC AC 3′ (mp in Fig. 1A) was synthesized on an Applied Biosystems 380A DNA synthesizer and purified as reported[5]. Fig. 1A shows in detail the junction between the genes for the signal peptide and RNase T1 as constructed by Quaas et al.[5], and schematically the mutagenesis experiment, the result of which is the plasmid pA2T1 (Fig. 1B). Here (i) the twelve base pair linker between the signal peptide and the RNase T1 gene has been deleted and (ii) to compensate for the loss of the Eco RI-site, an Sph I-site has been created by a T to A exchange in the wobble position of an Ala codon. This maintains a unique restriction endonuclease cleavage site at this junction.

Purification of RNase T1 from E. coli

Growth of *E. coli*(pA2T1) and preparation of the periplasmic fraction was as described elsewhere[5]. The periplasmic fraction (600 ml starting with a 6 l liquid culture) was applied to a DEAE-Sepharose CL-6B column (Pharmacia; 200 ml column volume) equilibrated with TE-100 (50 mM Tris, 10 mM EDTA, 100 mM NaCl, pH 7.5). After washing with the same buffer and gradient elution (see Fig. 2A) active fractions were pooled, lyophilized, dissolved in 10 ml running buffer (50 mM ammonium acetate) and passed through a Bio-Gel P-30 column (Bio Rad; 1.5 x 70 cm). The course of purification is demonstrated in Fig. 2C.

Finally, 20 mg of pure RNase T1 could be isolated per 1 litre liquid culture. This enzyme and Lys25-RNase T1 (see ref. e) from *A. oryzae* share an identical N-terminus and have been indistinguishable in preliminary kinetic experiments (data not shown).

The described overproducer is the starting point for a variety of mutants which will be studied by biochemical and biophysical methods to lead to a deeper insight into the structure and function of RNase T1.

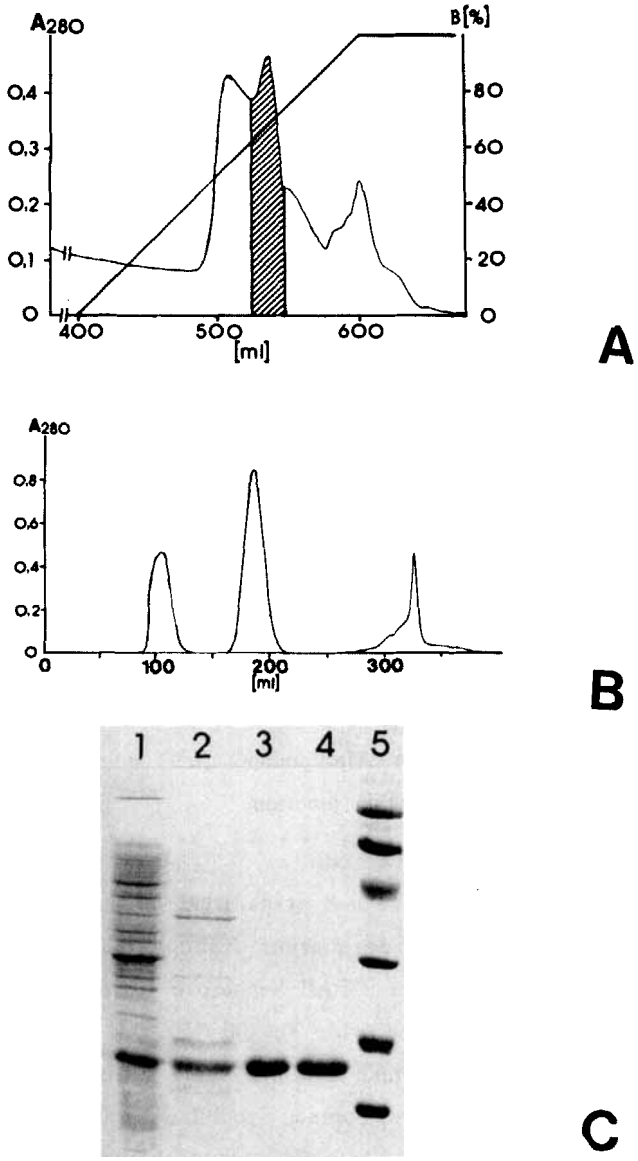

FIG. 2: Purification of RNase T1 from *E. coli*(pA2T1). Panel A: Elution profile after anion exchange chromatography. The hatched area indicates the pooled fractions containing ribonuclease activity determined as described[5]. Buffer A: TE-100 (see above); buffer B: TE-700 (TE-100 containing 700 mM instead of 100 mM NaCl). Panel B: Elution profiles after gel filtration. RNase T1 activity was recovered in the peak eluted between about 170 and 210 ml. Panel C: 15 % SDS-PAGE showing the course of purification of RNase T1 from the periplasmic fraction (lane 1) of the overproducer, pooled fractions containing RNase T1 activity after anion exchange chromatography (lane 2) and after gel filtration (lane 3). Lane 4 shows RNase T1 with a four amino acid N-terminal extension. On SDS-PAGEs the apparent molecular weight of RNase T1 is about 17 000 instead of about 11 000 as has been determined from the amino acid composition[1]. Lane 5: molecular weight standard containing a) phosphorylase b (92,500), b) bovine serum albumin (66,200), c) ovalbumin (45,000), d) carbonic anhydrase (31,000), e) soybean trypsin inhibitor (21,500) and f) lysozyme (14,400). For further details see text and ref. 5.

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