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An improved system for expressing pancreatic ribonuclease in *Escherichia coli*

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An improved method for expressing and purifying bovine pancreatic ribonuclease from a synthetic gene using the λ promoter controlled by a temperature-sensitive repressor is described. The procedure involves isolation in the presence of a refolding buffer containing oxidized and reduced glutathione, under conditions where RNase can refold, but where proteases presumably do not. Yields are approx. 2 mg purified protein per l ferment.

RNase; Purification; Gene expression

1. INTRODUCTION

During work applying modular mutagenesis methods on a synthetic gene for bovine pancreatic ribonuclease A (RNase) [1–3], it became clear that certain physical studies would require more protein than could be conveniently prepared as a fusion with β-galactosidase [4,5]. Therefore, we have developed a method for expression of bovine pancreatic RNase in *E. coli*, directly behind a λ promoter. In view of the many laboratories interested in expressing and studying RNase, it now seems appropriate to report the details of this method.

2. MATERIALS AND METHODS

Oligonucleotides were synthesized on an Applied Biosystems model 380A synthesizer using phosphoramidite chemistry and purified by silica gel chromatography using a mixture of butanol, ammonia, and water (55:35:10) as eluant. Complementary oligonucleotides (Mut1a/b) were annealed, kinased with [γ-^{32}P]ATP and ligated according to Maniatis et al. [6]. The gene for RNase was obtained from a synthetic gene [1,4] cloned in vector pN1, and transferred into plasmid pAL181 (a generous gift from the Genetics Institute, Cambridge, MA), together with a linker containing the methionine start codon. In this plasmid, the RNase gene begins just after the Shine-Dalgarno sequence in pAL181. A strain of *E. coli* (N4830) was transformed with the plasmid containing the RNase gene by the procedure of Hanahan [7], and plasmids from positive transformants were isolated and sequenced. Quantitative protein determination was by UV (for RNase ϵ_{278} = 9700) and amino acid analysis. Rabbit anti-RNase serum was prepared as described [4].

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2.1. *Expression and purification of RNase*

LB medium [6] (50 ml) containing ampicillin (125 μg/ml) was inoculated with a fresh colony of N4830/p_L RNase and then grown to high density overnight. This culture was used to inoculate four 5-l Erlenmeyer flasks each containing 1 l of LB/amp (100 mg/ml) medium. These were grown at 30°C to an A_{550} value of 1.2 in a shaker. The flasks were then transferred to a shaker warmed to 42°C, and LB medium (400 ml) preheated to 72°C was added to each flask. This brought the temperature of each quickly to 42°C. Shaking was continued for 25 min, and the cells were recovered by centrifugation (10 min at 4000 × *g*). The bacterial paste was resuspended in 10 × (v/w) denaturing buffer (8 M urea, 40 mM sarcosine, 20 mM Tris, 30 mM mercaptoethanol, 1 mM EDTA, pH 7.8). The suspension was passed through a French press (4000 lb/inch^2) and the insoluble materials removed by centrifugation for 10 min at 15000 × *g*. The clarified material was dialyzed (M_r > 3500 cutoff) vs two changes of refolding buffer (100 mM NaCl, 50 mM Tris, 4 mM oxidized glutathione, 4 mM reduced glutathione, 1 mM EDTA, 0.4 mM phenylmethylsulfonyl fluoride (PMSF), 0.02% NaN_3, pH 7.6). The dialysate was loaded onto a Sephadex G-50 sizing column and eluted at a linear flow rate of 2.5–3 ml·h^{-1}·cm^{-2} using

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elution buffer (100 mM NaCl, 50 mM Tris, 0.4 mM PMSF, 0.02% NaN_3). Fractions containing RNase were identified by catalytic activity against UpA [9]. These were pooled and concentrated 4-fold using an Amicon ultrafilter device with a YM5 filter ($M_r > 5000$ cutoff). This solution was dialyzed into a new buffer (100 mM NH_4OAc, 0.02% NaN_3, pH 6.1) and then applied to a column of affinity resin (4 ml, pUp Sepharose, Pharmacia) by gravity. The eluate under these conditions was essentially free of RNase activity. The column was washed with 10–20 vols buffer, and then RNase was eluted using a new buffer (200 mM NH_4OAc, 300 mM cytidylic acid, pH 6.1). Small fractions (1.5 ml) were collected. Fractions containing RNase activity were pooled and dialyzed vs two changes of buffer (100 mM NH_4OAc, pH 6.1) to remove the cytidylic acid. The solution was placed in a sterile tube and stored at 4°C.

3. RESULTS

RNase was isolated in essentially pure form in yields of ~2 mg/l ferment. Optimal levels of RNase were observed when cells were harvested 30 min after induction. After longer periods, some degradation of the expressed protein could be seen by Western blotting.

Renatured RNase was recovered from the Sephadex G-50 column as a rather broad peak eluting about one-third of the way through the elution. The specific catalytic activity of the preparation against UpA is high at this point, indicating both that the rather low M_r of RNase allows it to be separated from > 80% of other cell debris in one step, and that refolding was essentially complete. Affinity chromatography with a pUp Sepharose resin yields essentially homogeneous product as judged by gel electrophoresis with SDS and by immunoblotting.

4. DISCUSSION

The salient features of the system are: (i) use of the strong P_L promoter of phage I; (ii) reconstitution of RNase activity from an unfolded state under conditions where proteases and other nucleases are unlikely to refold; (iii) purification of the enzyme to homogeneity in two chromatographic steps. We believe that the relative success of this system is due to the controlled nature of the λ promoter system, the optimization of harvesting time following induction, and the special reconstitution and purification method. Many of these details are applicable only to small, easily refolded proteins such as RNase. However, for the many laboratories interested in such proteins, even this rather specialized protocol should be useful.

The RNase obtained in this system has formylmethionine group at the amino-terminus of the protein. Whether or not this additional residue is undesirable depends on the intended application of the system. However, it should be noted that additional amino acids at the amino-terminus are tolerated in the folded structure of RNase. Indeed, RNase from rat contains a 3 amino acid extension at this position [8].

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