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Expression of bovine pancreatic ribonuclease A in *Escherichia coli*

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A synthetic gene for bovine pancreatic ribonuclease A (RNase A) has been expressed in *Escherichia coli* as a fusion protein with β-galactosidase linked by the tetrapeptide Ile-Glu-Gly-Arg. RNase A was cleaved from the fusion using factor Xa, and the resulting product prufied and reconstituted. The isolated RNase A was chromatographically, catalytically, and immunologically indentical with authentic RNase A. This work argues that the method suggested by Nagai and Thogersen [Nagai, K. & Thogersen, H. C. (1984) *Nature (Lond.) 309*, 810 – 812] for releasing fusion proteins is quite general, even when applied to a particularly complicated expression problem. The procedure here makes RNase A available for the first time as a model for studying structure-function relationships in proteins using site-directed mutagenesis.

Recombinant DNA techniques are widely recognized as being powerful tools for studying problems of protein structure [1, 2]. Combined with chemical synthesis of oligonucleotides [3], these techniques can be used to prepare proteins having systematically varied structures. These mutant proteins are then useful for testing hypothesis relating structure and function.

However, much of the experimental effort required to obtain variants of proteins must be devoted to recovery, purification, and reconstitution of the proteins after they are expressed [4], especially if they have been cysteines [5, 6], are sensitive to degradation within *Escherichia coli* [7 – 11] or must reform multiple disulfide bonds to refold correctly [12]. In especially difficult cases, eukaryotic expression systems generally must be used [13, 14].

These limitations are especially constraining in view of the tradional background of enzymology. Some of the best-studied proteins, where the information is available to ask the most sophisticated questions about protein structure and catalysis, are precisely those least accessible via prokaryotic expression systems.

We recently described the synthesis and cloning of a gene coding for bovine pancreatic ribonuclease (RNase) S protein [15]. Again, because of the large literature on this protein, RNase offers many possibilities for studying catalysis, folding, and evolution in proteins. However, the expression of RNase A is complicated by three factors. First, RNase A proved to be unstable when expressed by itself in *E. coli.* Second, to reconstitute RNase to active protein, four disulfide bonds must be correctly made. Third, expression within a cell is potentially cytotoxic. Simple expedients that might solve these problems, such as construction of fusion proteins, were problematic a priori. Methods commonly used for cleavage of fusion proteins (e.g. using cyanogen bromide to release a fusion protein joined at methionine) were obviously inapplicable, as RNase A has several methionines within the polypeptide chain.

We report here a procedure for the expression of the gene for bovine pancreatic RNase in *E. coli* and a procedure for purifying and reconstituting the expressed RNase to give a homogeneous product having full specific catalytic activity.

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Abbreviations. RNase, bovine pancreatic ribonuclease A; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

Enzyme. Ribonuclease A (EC 3.1.27.5).

EXPERIMENTAL METHODS

Genetic material for RNase used in these studies originated as chemically synthesized DNA fragments. These oligonucleotides were ligated and cloned as previously described [15]. DNA was synthesized using phosphoramidite chemistry on solid supports [3]. Synthetic DNA fragments were designed to incorporate unique sites for digestion by restriction endonucleases [15]. Unique sites were also incorporated at each end of the gene to facilitate removal and replacement of the gene into cloning and expression vectors.

Construction of vector

The synthetic gene, shown in Fig. 1, codes for a protein that includes RNase fused at its amino terminus to the tetrapeptide Ile-Glu-Gly-Arg [16]. The gene was synthesized to have an *Eco*RI end at the amino terminus and a *Bam*HI end at the carboxyl terminus. The construction of the expression vector is shown in Fig. 2. The plasmid p1 (a generous gift from Prof. J. Wang, Department of Biochemistry and Molecular Biology, Harvard University), containing β-galactosidase gene, was linearized with *Nco*I, the sticky ends were filled in, and ligated with the *Bam*HI linker CGGATCCG to obtain plasmid pN1. Plasmid pN1 was cut at the unique *Eco*RI and *Bam*HI sites and the synthetic double-stranded gene (Fig. 1) was incorporated into the vector by ligation using T4 DNA ligase. The product of the construction is a gene coding for a fusion polypeptide corresponding to most of β-galactosidase, a four-amino-acid linker Ile-Glu-Gly-Arg, and residues corresponding to ribonuclease A, all behind the β-galactosidase promoter-operator sequence. Transformation was effected by

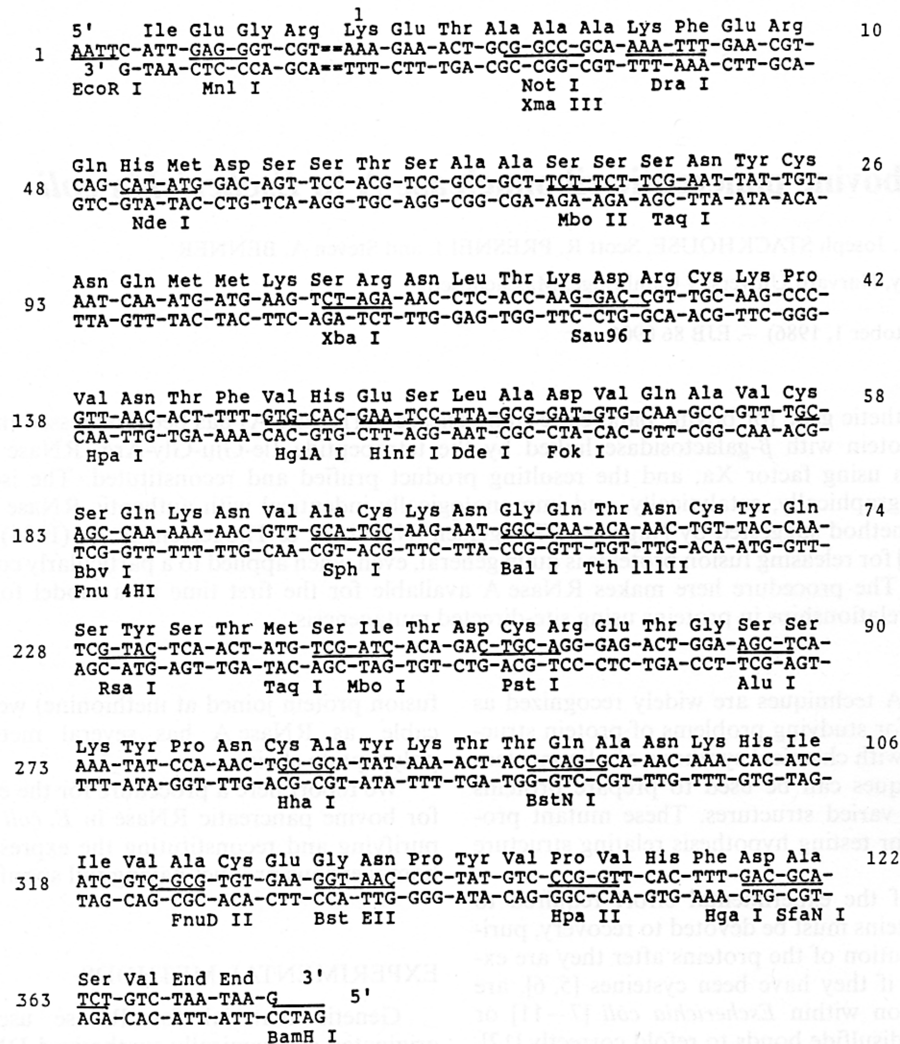

Fig. 1. *Sequence of a synthetic gene coding for RNase A fused at the amino terminus to the tetrapeptide Ile-Glu-Gly-Arg, and having sticky ends for* Eco*RI and* Bam*HI*. The numbering of nucleotides in the sequence is on the left; numbering of the amio acids is on the right, and corresponds to the numbering in native bovine pancreatic ribonuclease A. Restriction sites are underlined and labeled. Some restriction sites are not underlined, as their location adjacent to other sites makes them redundant for the purpose of cutting small segments from the gene

subjecting *E. coli* (DH1) cells to calcium shock in the presence of sulfolane [15] and transformants were selected for ampicillin resistance. The structure of the cloned gene, including the segment coding for the tetrapeptide linker, was verified by DNA squencing using the Maxam-Gilbert method [18].

Antibodies to RNase A were prepared by injecting New Zealand White rabbits with either denatured or native RNase A and Freunds complete adjuvenant [19, 20]. The rabbits were boosted at three-week intervals until a satisfactory level of antibody production was obtained. The rabbits were bled and the antibodies were assayed by radioimmunoabsorption. Antibody was immobilized on Sepharose 4B using the procedure of Porath et al. [21].

The catalytic activity of RNase A was assayed by coupling the hydrolysis of uridylyl(3′-5′)adenosine monophosphate to the deamination of adenosine catalyzed by adenosine deaminase [22]. The change in absorbance at 265 nm was monitored.

E. coli cells were grown in LB medium at 37°C to an absorbance at 550 nm of unity. Isopropyl β-D-thiogalactopyranoside (1 mM) was added to the medium to induce expression of the gene, and the cells were incubated for 4 h after induction. The cells were isolated by centrifugation and stored frozen.

All subsequent operations were performed at room temperature, unless otherwise noted [12]. The cells were suspended in Tris/sarcosine buffer (50 mM Tris, 100 mM NaCl, 100 mM sarcosine, 1 mM EDTA, pH 8.0, 3 ml buffer to 1 g wet-weight cells) and lysed in a French press (27.6 MPa). The mixture was centrifuged (10000 rpm, 5 min, SS-34 rotor) and the supernatant was discarded. The pellet was shown by gel electrophoresis to contain most of the fusion protein. The fusion protein was identified by Western blotting using antibodies against both RNase A and β-galactosidase. The pellet was then twice washed by dispersing in Triton X-100 (5%) 10 mM EDTA, and Tris/sarcosine buffer, and recovered by centrifugation.

A solution of 8 M urea in Tris/sarcosine buffer was prepared from a 9.5 M solution of urea deionized by shaking with ion-exchange resin (Bio-Rad AG-501 X-8 mixed-bed resin) [12]. The pellet was dissolved in this denaturing buffer (9 ml/g original weight of bacteria) by vigorous vortexing followed by sonication at 20 – 25°C. The turbid mixture was

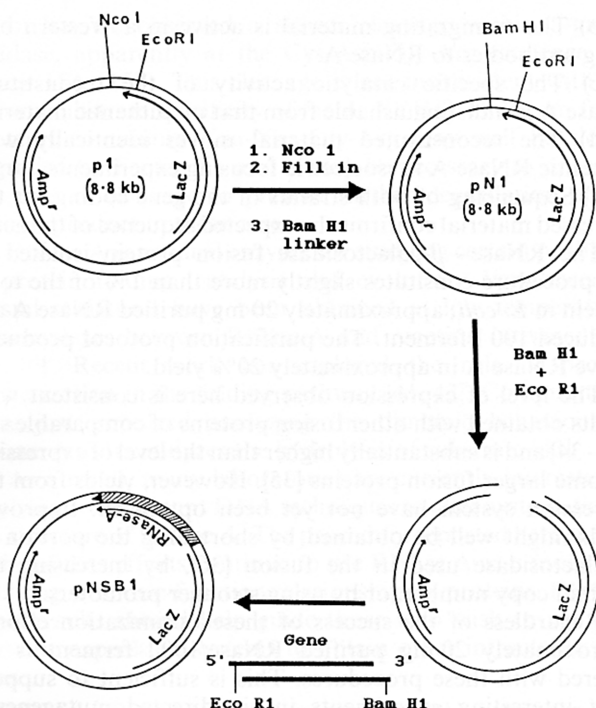

Fig. 2. *Construction of plasmid pNSB1*. Plasmid pN1 was prepared from plasmid p1 by cutting with *Nco*I, filling in and ligating with *Bam*HI linker CGGATCCG. Plasmid pN1 was cut at the unique *Bam*HI and *Eco*RI sites and the synthetic double-stranded gene (Fig. 1) was incorporated by ligation using T4 DNA ligase

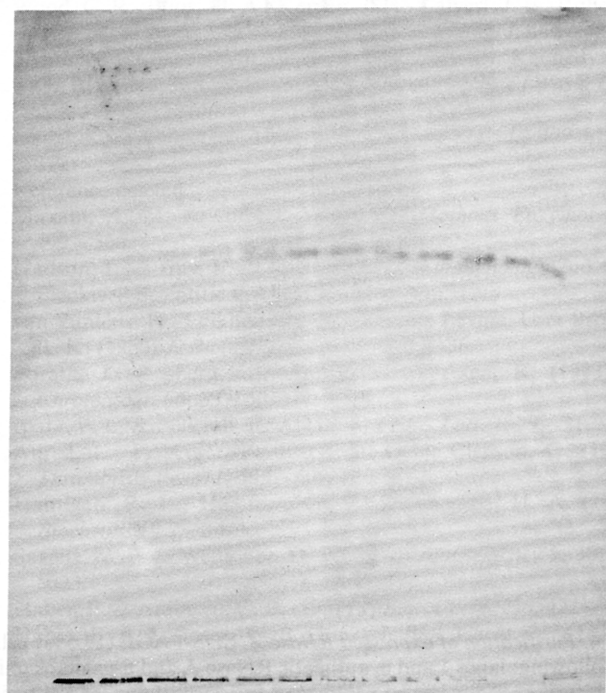

Fig. 3. *Western blot of an SDS/polyacrylamide (12.5%) gel monitoring cleavage of the fusion protein with factor Xa as a function of time*. Lane 1 contains authentic commercial RNase A (Sigma) mixed with expressed cleaved RNase A; the two bands comigrate. Lane 2 contains authentic RNase A alone. Lanes 3–11 show aliquots removed as a function of time, proceeding from right (initiation of cleavage) to left. To be able to show the progress of cleavage reaction with greater clarity, the data shown here come from an experiment where the concentration of factor Xa was a tenth of that used in standard cleavage mixtures (Experimental Methods), the reaction was run at 4°C instead of 37°C, and the time points were taken at 1, 2, 3, 7, 12, 14, 17, 21, and 24 days. With more enzyme and higher incubation temperatures as routinely used in the standard cleavage mixtures (see Methods), the reaction is complete in less than 60 h. Lane 12 shows fusion protein without added factor Xa

centrifuged (15000 rpm, 10 min, SS-34, at room temperature) and the pellet was frozen and saved for recycling. Recycling of the pellet typically yielded an additional 10–15% more fusion protein. An hour after the dissolution of the pellet, the supernatant was mixed vigorously stirred buffer (9 vol., 50 mM potassium dihydrogen phosphate, 100 mM NaCl, 1 mM EDTA, pH 10.7). The pH was then readjusted to 10.7 with 2 M potassium hydroxide. The mixture was allowed to stand for 30 min. The pH of the rapidly stirred solution was then adjusted to 8.0 with 1.2 M HCl. To the mixture was added Tris buffer (1 M, to make a final concentration of 50 mM), mercaptoethanol (0.5% final concentration) and dithiothreitol (1 mM final concentration). The mixture was allowed to stand for another hour.

The mixture was then concentrated in an Amicon hollow-fiber filter (100-kDa cut off) to about a tenth of its volume. Further concentration resulted in the undesirable precipitation of significant amounts of material. The solution was then dialyzed (Spectrapor I tubing, 50-kDa cut off) against two changes of Tris buffer (50 mM Tris, pH 8.0, 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 0.5% 2-mercaptoethanol), followed by three changes against the same buffer lacking dithiothreitol and 2-mercaptoethanol. The total absorbance at 280 nm of the final solution was 12–15. This solution could be stored at 4°C indefinitely; freezing was found occasionally to induce precipitation.

At this point, the solution of fusion protein was essentially free of urea and was ready for cleavage by factor Xa [16]. The factor Xa obtained from Boehringer-Mannheim proved to be an excellent preparation; certain preparations from other sources produced inadequate results. Factor Xa was assayed using a chromogenic substrate [23].

The fusion protein (0.3–1 mg/ml) was cleaved with factor Xa (1.5 unit/mg substrate) [23] in buffer (50 mM Tris, pH 8.0, 100 mM NaCl, 3 mM $CaCl_2$, 5 mM oxidized glutathione, 5 mM reduced glutathione, 0.1% NaN_3). Incubations lasted 72 h at 37°C. The progress of the reaction was followed by gel electrophoresis run on aliquots (Fig. 3); a single new band, comigrating with authentic RNase, appeared as the digestion progressed. Factor Xa was found to remain active under these conditions.

When the cleavage reaction was complete, the mixture was added directly to solid guanidine · HCl (final concentration 6 M guanidine). Dithiothreitol (5 mM final concentration) and 2-mercaptoethanol (0.5% final concentration) were then added. The mixture was incubated at 40°C for 30 min. The solution was then ultrafiltered (Amicon ultrafilter pressure cell, YM-100 membrane, 100-kDa cut off). The residue was mixed with 0.25 vol. 6 M guanidine · HCl and ultrafiltered again. The filtrates were combined and concentrated by ultrafiltration (YC-05 membrane, 500-kDa cut off) in an Amicon pressure cell.

When the solution was near dryness, distilled water (5–10 ml) was added directly to the pressure cell. The mixture was concentrated again, and distilled water (5–10 ml) was again added. Most of the protein precipitated and was re-

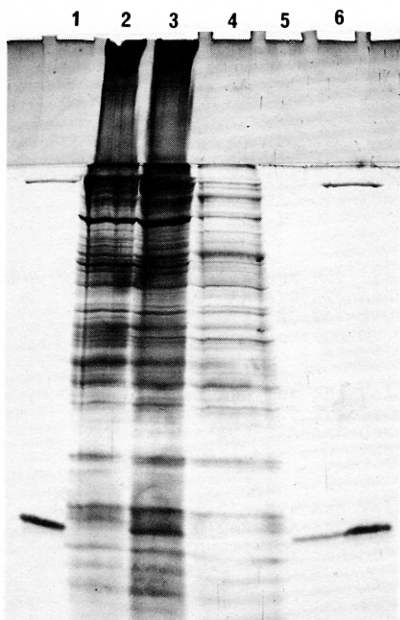

Fig. 4. *Purification of expressed RNase A*. SDS-PAGE (13.5%) gel of the following: lanes 1 and 6, authentic RNase A and β-galactosamine (Sigma); lane 2, uncleaved solubilized fusion protein; lane 3, fusion protein cleaved with factor Xa; lane 4, material from lane 3 after passage through antibody affinity column, with RNase A removed; lane 5, product RNase A obtained by eluting affinity column with glycine · HCl buffer

covered. The precipitate constained most of the RNase A. It can be stored in this form.

The solid material was then dissolved in deionized urea (10 M) in Tris/sarcosine buffer (50 mM Tris, 100 mM sarcosine, 100 mM NaCl, pH 8.0). Lower concentrations of urea did not completely dissolve the solid material. The solution was then diluted with rapid mixing into 40 vol. of phosphate buffered saline (25 mM Na_2HPO_4, 150 mM NaCl, 0.01% NaN_3, pH 7.4). Any slight precipitate formed was removed by centrifugation (10000 rpm, 5 min, SS-34 rotor).

The solution was then directly applied to an affinity column bearing immobilized antibodies to RNase A (10–40 µg binding capacity/ml, at least twofold excess over RNase A in solution [21]. The column was washed with 10 vol. phosphate-buffered saline. The RNase A was eluted from the column using 100 mM glycine · HCl (pH 2.55). The pH of the eluate was immediately adjusted to 7.0 with Tris base (2 M).

The material eluting from the affinity column was a single band by SDS gel electrophoresis (Fig. 4). The band migrated with authentic RNase A, and was detected by staining of Western blotting. The material can be stored frozen.

This material was renatured by adjusting the pH to 8.0, and adding 5 mM oxidizing and reduced glutathione [24–28]. The mixture was incubated at 25°C for 16 h. This mixture was assayed directly for RNase activity.

RESULTS AND DISCUSSION

The following evidence showed that RNase A prepared, isolated, and renatured by these procedures is identical with authentic RNase A.

a) The material comigrates with authentic RNase A during SDS gel electrophoresis.

b) The comigrating material is active in a Western blot using antibodies to RNase A.

c) The specific catalytic activity of the reconstitued RNase A is indistinguishable from that of authentic material.

d) The reconstituted material moves identically with authentic RNase A in isoelectric focusing experiments.

e) Sequencing of both strands of the gene coding for the expressed material confirms the expected sequence of the gene.

The RNase – β-galactosidase fusion protein isolated in this procedure consitutes slightly more than 1% of the total protein in *E. coli*; approximately 20 mg purified RNase A are produced/100 l ferment. The purification protocol produced native RNase A in approximately 20% yield.

The level of expression observed here is consistent with results obtained with other fusion proteins of comparable size [29–34] and is substantially higher than the level of expression of some larger fusion proteins [35]. However, yields from the expression system have not yet been optimized. Improved yields might well be obtained by shortening the portion of β-galactosidase used in the fusion [36], by increasing the plasmid copy number, or by using stronger promoters.

Regardless of the success of these optimization efforts, approximately 20 mg purified RNase/100 l ferment is recovered with these procedures. This is sufficient to support most interesting experiments in site-directed mutagenesis. Further, our experience so far in expressing variants of RNase A suggests that the procedures reported here will be satisfactory for preparing substantial quantities of variants of RNase A that might have intrinsically poorer stabilities than native RNase A in *E. coli*.

To obtain the release of RNase A from the fusion protein, we have adopted an interesting expedient suggested by Nagai and Thogersen [16]. Factor Xa recognizes and cleaves polypeptides at the carboxyl terminus of the tetrapeptide Ile-Glu-Gly-Arg. Therefore, a fusion constructed to include this tetrapeptide at the amino terminus of the desired product can be digested with factor Xa to release the desired product. The method works well for the expression of hemoglobin in *E. coli* [16], although its generality has not yet been explored.

There were questions as to whether factor Xa would be generally applicable in the release of proteins from fusions. For example, a similar fusion protein was constructed for the expression of myoglobin by Boxer and coworkers [37]. However, trypsin rather than factor Xa was used to release the fusion protein. While trypsin would not generally be useful in releasing fusion proteins, tryptic cleavage was satisfactory in the case of myoglobin, perhaps because the myoglobin portion of the fusion protein to protect other susceptible sites [37].

We have found that RNase A was not degraded by factor Xa, consistent with the fact that RNase A does not contain the sequence Gly-Arg. More critically, we have found that strongly denaturing protocols for solubilizing the fusion protein are not incompatible with subsequent treatment with factor Xa. Finally, extension of the method of Nagai and Thogersen to a second protein argues strongly that their method is general.

However, it is important to note that different commercial preparations of factor Xa have quite different catalytic activities. These differences are more apparent with a colorimetric assay than a clotting assay [38] and may be responsible for some failures obtain release of fusion proteins using factor Xa.

Further, our work does suggest one pontential limitation in the use of factor Xa as a general method for release of fusion proteins. During the digestion of the fusion protein by

factor Xa, we observed a slower cleavage within β-galactosidase, apparently at the Cys-Asn-Gly-Arg sequence (residues 154–157). This cleavage is not inconsistent with the known substrate specificity of factor Xa [16]. However, fused proteins containing Xaa-Xaa-Gly-Arg may well be slowly degraded by Factor Xa and it may be necessary to optimize the time of cleavage to prevent further degradation of the desired product in these case.

RNase A is an ideal enzyme for studying certain problems in protein folding, catalysis and evolution. Very recently, crystal structures have been obtained at low temperatures of complexes between RNase A and reaction intermediates [39, 40]. Recent work has explored the role of proline isomerization in the folding pathway [41, 42]. Computations have just appeared analyzing the dynamic behaviour of RNase A as it catalyzes the hydrolysis of substrate [43].

As a result, many laboratories are currently interested in applying recombinant DNA techniques to alter systematically the structure of RNase A. The procedure reported here should be helpful as efforts continue to use RNase A as a model for studying structure-function relationships in proteins. Further, these results should help guide those who wish to use prokaryotic expression systems to obtain other intractable proteins.

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