PROTEIN EXPRESSION AND PURIFICATION 7, 253–261 (1996)
ARTICLE NO. 0036

Production of Human Pancreatic Ribonuclease in *Saccharomyces cerevisiae* and *Escherichia coli*

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Received August 8, 1995, and in revised form October 29, 1995

Human pancreatic ribonuclease (HP-RNase) has considerable promise as a therapeutic agent. Structure–function analyses of HP-RNase have been impeded by the difficulty of obtaining the enzyme from its host. Here, a gene encoding HP-RNase was designed, synthesized, and inserted into two expression vectors that then direct the production of HP-RNase in *Saccharomyces cerevisiae* (fused to either an unmodified or a modified **α**-factor pre–pro segment) or *Escherichia coli* (fused to the pelB signal sequence). HP-RNase produced in *S. cerevisiae* was secreted into the medium as an active enzyme, isolable at 0.1–0.2 mg/liter of culture. This isolate was heterogeneous due to extensive glycosylation and incomplete maturation of the pre–pro segment. HP-RNase produced in *E. coli* with the pET expression system was purified from the insoluble fraction of the cell lysate. Renaturation of the reduced and denatured protein produced active, homogeneous enzyme recoverable at 1 mg/liter of culture. The N terminus of the HP-RNase produced from the bacterial expression system was processed fully *in vivo*. The yeast system, combined with techniques that allow detection of picograms of ribonuclease activity, offers a sensitive probe for studies of post-translational modification and secretory targeting in eukaryotic cells. The bacterial system enables studies both to reveal new structure–function relationships in ribonucleases and to evaluate the use of HP-RNase as a cytotoxin that is tolerated by the human immune system.

Human pancreatic ribonuclease (HP-RNase[3],RNase 1, or secretory-type RNase; EC 3.27.1.5) catalyzes the cleavage of RNA specifically on the 3′-side of pyrimidine bases. HP-RNase is a member of the family of secretory ribonucleases, some of which affect the growth and differentiation of mammalian cells (1,2). For example, angiogenin promotes blood vessel formation (3,4). Bovine seminal ribonuclease (5,6) and onconase from the frog *Rana pipiens* (7,8) are potent antitumor agents.[4] Indeed, the cytotoxicity of an injected secretory-type ribonuclease can exceed those of the renown toxins ricin and α-sarcin (9). These activities of the secretory ribonucleases give them great potential as therapeutic agents and justify rigorous study of the relationship between their structure and function (10). Among the secretory ribonucleases, HP-RNase is of special interest because it is of human origin and is therefore likely to be well-tolerated by the human immune system.

Structure–function analyses of HP-RNase have been hindered by the difficulty of obtaining sufficient amounts of pure enzyme. HP-RNase can only be obtained directly from human pancreas, an organ that contains only minute amounts of the enzyme (11). We have shown that the HP-RNase isolated from humans

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[3] Abbreviations used: C > p, cytidine 2′,3′-cyclic phosphate; DTT, dithiothreitol; GSH, reduced glutathione; GSSG, oxidized glutathione; HPLC, high-performance liquid chromatography; HP-RNase, human pancreatic ribonuclease; IPTG, isopropyl-β-D-thiogalactopyranoside; M_r, relative molecular mass; PAGE, polyacrylamide gel electrophoresis; poly(C), poly(cytidylic acid); reHPR.B, recombinant human pancreatic ribonuclease from bacteria; reHPR.Y, recombinant human pancreatic ribonuclease from yeast; RNase A, bovine pancreatic ribonuclease A; SDS, sodium dodecyl sulfate.

[4] Onconase is now in Phase III human clinical trials against pancreatic cancer.

1046-5928/96 $18.00

is glycosylated extensively (12). Still, the amino acid sequence of HP-RNase has been determined (13). The cDNA that codes for HP-RNase has been cloned and shown to consist of 1620 base pairs, including an open reading frame encoding the 128-residue mature protein following a 28-residue signal sequence (14). Although no other structural information is available about HP-RNase, its amino acid sequence is 70% identical to that of bovine pancreatic ribonuclease A (RNase A), which has been the object of much seminal work in biological chemistry (15–20). This homology makes it possible to speculate on the roles of particular amino acid residues in the structure and function of HP-RNase. Still, testing such speculations requires the use of a heterologous expression system for HP-RNase. Recently, D'Alessio and co-workers reported the expression of a gene that codes for HP-RNase in Chinese hamster ovary cells (21). The resulting HP-RNase was a complex mixture of unmodified and glycosylated forms.

To enable structure–function analyses of HP-RNase, we have developed two microbial expression systems. A synthetic gene that codes for HP-RNase was designed and inserted into plasmids so as to direct the production of HP-RNase in *Saccharomyces cerevisiae* or *Escherichia coli*. The yeast system uses the pWL vector, which we had used previously to produce RNase A (22). The bacterial system uses a pET vector (23). Recombinant HP-RNase isolated from yeast (reHPR.Y) was a heterogeneous population of unmodified and modified (that is, glycosylated or not fully processed) enzyme that was recoverable at 0.1–0.2 mg/liter of culture. Recombinant HP-RNase isolated from bacteria (reHPR.B) was a homogeneous enzyme that was recoverable at 1 mg/liter of culture. The steady-state kinetic parameters exhibited by reHPR.B were similar to those of the unmodified enzyme isolated from human pancreas. Although the yeast system provides no advantage over the existing mammalian system (21) other than experimental ease, the bacterial system is the first that allows for the rapid isolation of unmodified HP-RNase in yields appropriate for structure–function analyses.

EXPERIMENTAL PROCEDURES

Materials

Unmodified HP-RNase was purified from human pancreas as described (12). RNase A was from Biozyme (Blaenovon, England).

S. cerevisiae strain BJ2168 (*MAT a prc1-407 prb1-1122 pep4-3 leu2 trp1 ura3-52*) was from the Yeast Genetic Stock Center (Berkeley, CA). Yeast expression vector pWL is a shuttle vector designed to facilitate the genetic manipulations required in protein engineering (22,24,25). *E. coli* strain BL21(DE3) [F^-, ompT, r_B^-, m_B^-] (23) and pET22b(+) were from Novagen (Madison, WI).

All enzymes for molecular biology were from Boehringer Mannheim (Mannheim, Germany), except *Acc*65I (Promega; Madison, WI) and *Msc*I (New England Biolabs; Beverly, MA). Reagents for DNA synthesis were from Applied Biosystems (Foster City, CA) except for acetonitrile, which was from Baxter Healthcare (McGaw Park, IL). Agarose was from Life Technologies (Gaithersburg, MD) and Spectra Por dialysis membrane (3500 M_r cut off) was from Spectrum Medical Industries (Los Angeles, CA). CM Sepharose CL6B cation-exchange resin and Mono-S HR 5/5 column were from Pharmacia LKB Biotechnology (Uppsala, Sweden). Protein-Pak CM 8 HR A-1 cation-exchange column and the two 510-pump HPLC system with a 996 Photodiode Array detector were from Millipore-Waters (Milford, MA).

Yeast minimal medium (SD) was prepared as described (26). Bacterial terrific broth was prepared as described (27). The different variations on standard media were as we described previously (22). Bacto yeast extract, Bacto tryptone, Bacto peptone, Bacto agar, and Bacto Yeast nitrogen base without amino acids were from Difco (Detroit, MI).

Isopropyl-β-D-thiogalactopyranoside (IPTG), dithiotreitol (DTT), oxidized glutathione (GSSG), and reduced glutathione (GSH) were from Boehringer Mannheim. Poly(cytidylic acid) [poly(C)] used for zymogram electrophoresis was from Pharmacia. Poly(C) and cytidine 2′,3′-cyclic phosphate (C > p) used in kinetic assays were from Sigma Chemical (St. Louis, MO). Reagents for polyacrylamide gels electrophoresis in the presence of sodium dodecyl sulfate (SDS–PAGE) were from Bio-Rad (Richmond, CA). All other chemicals were of reagent grade or better and were used without further purification.

Methods

Reactions involving restriction enzymes, T4 DNA ligase or T4 polynucleotide kinase were performed in the buffers provided by their suppliers. DNA restriction fragments were purified from bands in agarose (0.75%, w/v) gels with a GeneClean II kit from BIO-101 (La Jolla, CA).

Oligonucleotides were synthesized on an Applied Biosystems 392 DNA/RNA synthesizer by using the β-cyanoethyl phosphoramidite method (28) and purified by using both oligo purification cartridges (Applied Biosystems) and PAGE as described (26). DNA sequences were determined with the T7 sequencing kit from Pharmacia. Oligonucleotide-mediated site-directed mutagenesis was performed by the method of Nakamaye and Eckstein (29) using a kit from Amersham Life Science (Arlington Heights, IL). Other manipulations of DNA were performed as described (26,27).

SDS–PAGE was performed at room temperature in

gels (0.8 × 8 × 10 cm) containing polyacrylamide (15%, w/v) and SDS (0.1%, w/v) as described (30), except using a Bio-Rad Protean II dual slab cell model apparatus. Gels were fixed and stained by washing with aqueous methanol (40%, v/v) containing acetic acid (10%, v/v) and Coomassie blue (0.1%, w/v). Prestained molecular mass standards were from Bio-Rad: phosphorylase B (106 kDa), bovine serum albumin (80 kDa), ovalbumin (49.5 kDa), carbonic anhydrase (32.5 kDa), trypsin inhibitor (27.5 kDa), and lysozyme (18.5 kDa).

Protein concentrations were measured with a protein assay kit from Bio-Rad based on the method of Bradford (31). Ultraviolet and visible absorbance measurements were made with a Cary Model 3 spectrophotometer (Varian, Palo Alto, CA) equipped with a Cary temperature controller. Amino acid sequences were determined by using a Beckman LF-3000 sequencer.

Design of a synthetic gene for HP-RNase. Eight oligonucleotides were designed such that their annealing and ligation would result in a gene (Fig. 1) that codes for HP-RNase (13).[5] The design had three additional objectives: (i) to allow for the expression of the gene from yeast expression plasmid pWL, (ii) to distribute unique restriction endonuclease recognition sites throughout the gene, and (iii) to use codons that maximize protein production in *S. cerevisiae.* These objectives were accomplished by (i) adding a *Kpn*I and a *Sal*I site to the 5′ and 3′ termini of the gene to enable the ligation of the gene into plasmid pWL, (ii) identifying five unique restriction sites that could be incorporated into the HP-RNase coding region using the SILENT program from the Genetics Computer Group (Madison, WI), and (iii) using those codons that are most prevalent in *S. cerevisiae* (32).

Synthesis of the gene coding for HP-RNase. A gene that codes for HP-RNase was constructed by a method similar to that described previously (33,34). Briefly, eight overlapping oligonucleotides coding for both strands of the HP-RNase gene were synthesized chemically. The oligonucleotides, except those that constitute the two 5′ termini of the gene, were phosphorylated with the enzyme T4 polynucleotide kinase. Each pair of complementary oligonucleotides (that is, 1 and 1c, 2 and 2c, 3 and 3c, and 4 and 4c) were annealed to generate four fragments, which were then ligated using T4 DNA ligase. The assembled ligation product was gel purified and amplified by the polymerase chain reaction using oligonucleotides 1d (5′-CGACTGGGTACCTTTGGATAAAAGAAA-3′) and 4d (5′-CGACTGCTCGACTTAAGAATCTTCAAC-3′) as primers. The resulting PCR product was digested with *Acc*65I (which is an isoschizomer of *Kpn*I) and *Sal*I, and the gene was cloned into M13mp19 that had been digested with *Acc*65I and *Sal*I, to yield plasmid M13mp19.HPR.

Construction of plasmids YEpHPR.1 and YEpHPR.2. The DNA fragment containing the HP-RNase gene was isolated from M13mp19.HPR by digestion with *Acc*65I and *Sal*I and inserted into plasmid pWL to yield plasmid YEpHPR.1. This plasmid carries a region sufficient for replication (ori) and packaging as a single-stranded phagemid (f1) in *E. coli* and for replication in [cir+] *S. cerevisiae* (2 μm). *E. coli* transformed with pWL can be selected by ampicillin resistance (Amp^r). *S. cerevisiae* transformed with pWL can be selected by recovery of tryptophan (TRP1) or leucine (LEU2-d) prototrophy. In YEpHPR.1, the production of HP-RNase fused to the α-factor pre−pro segment is under the transcriptional control of the hybrid ADH2−GAPDH promoter, which is derepressed by depletion of fermentable carbon sources (35), and of the GAPDH terminator sequence. To create plasmid YEpHPR.2, oligonucleotide MR5 (5′-TACCTTTGGATAAAAGAGAGGCTGAAGCTGAAGCTGAAGCTAAGGAATCTCGAGCT-3′) was used to insert an 18-bp sequence encoding a $(Glu-Ala)_3$ nonapeptide at the C terminus of the α-factor pre−pro segment (36). This nonapeptide is recognized by the protease product of the STE13 gene product. This protease acts with the proteases encoded by genes KEX1 and KEX2 to process the α-factor pre−pro segment.

Construction of plasmid pET22.HPR. An *Msc*I site was generated at the 5′ termini of the HP-RNase gene of M13mp19.HPR by mutagenesis using oligonucleotide MR4 (5′-CGGTACCTTTGGATATGGCCAATTAATCTCGAGCT-3′). The resulting *Msc*I/*Sal*I fragment was band purified, and inserted into *E. coli* expression plasmid pET22b(+) that had been digested with *Msc*I and *Sal*I, to yield plasmid pET22.HPR.

Plate assay for ribonuclease activity. We and others have used plate assays to measure the ability of a microbe to secrete an active ribonuclease (22,25,37,38). A plate assay was used to test the ability of pET22.HPR, YEpHPR.1, and YEpHPR.2 to direct the secretion of active HP-RNase.

For *S. cerevisiae,* an aliquot (1 μl) of a culture of BJ2168 carrying pWL, YEpWL.RNase A (22), YEpHPR.1, or YEpHPR.2 and grown overnight in S4%D−leu medium was placed on a plate of YEP1%D containing Bacto agar (2%, w/v) and yeast RNA (2 mg/ml). The plates were incubated at 30°C for 3 days to allow for ribonuclease to be produced and for its enzymatic activity to be manifested.

For bacteria, an aliquot (1 μl) of a culture of BL21(DE3) carrying pET22b(+), pBXR (22), or pET22.HPR and grown overnight in LB medium containing ampicillin (400 μg/ml) was placed on a plate of the same medium containing Bacto agar (1.5%, w/v),

[5] The amino acid sequence in Ref. 13 and Fig. 1 lacks the C-terminal threonine residue that was deduced from the cDNA sequence (14) after our work had begun.

yeast RNA (2 mg/ml) and IPTG (1 mM). The plate was incubated for 8 h at 37°C.

To assess ribonuclease activity, the plates were developed by washing with aqueous perchloric acid (10%, v/v), which precipitates high molecular mass RNA. Colonies that secrete active ribonuclease produced a clearing in an otherwise foggy background.

Zymogram electrophoresis. Zymogram electrophoresis was used to assess the ability of ribonucleases to cleave poly(C) (39). Briefly, zymogram electrophoresis was SDS–PAGE in a nonreducing gel containing poly(C) (0.3 mg/ml). After electrophoresis, the SDS was extracted with aqueous isopropanol, and the gel was incubated for 30 min at pH 8.0 to allow for the enzymatic cleavage of poly(C). The gel was then stained with toluidine blue (0.2%, w/v) and destained with water until regions in the gel containing ribonuclease activity appeared as clear bands in a blue background.

Zymogram spot assay. Zymogram spot assays were used to assess rapidly the ribonuclease content of fractions produced during ribonuclease purification and to estimate the amount of RNase produced by the yeast expression system. The spot assay was performed as described (22), except that torula yeast RNA type VI (Sigma Chemical) was used instead of poly(C).

Production and purification of recombinant HP-RNase from S. cerevisiae (reHPR.Y). reHPR.Y was purified from *S. cerevisiae* strain BJ2168 harboring the plasmid YEpHPR.2. Transformed cells were stored as −70°C freezer stocks in glycerol (30%, w/v). Frozen cells were rejuvenated by plating onto 2 × S4%D–Trp containing Bacto agar (2%, w/v) and incubating the resulting plates at 30°C for 2 days. A liquid culture of 2 × S4%D–Trp was inoculated with cells from the plate and shaken at 30°C until turbid. A liquid culture of 2 × S4%D–Leu was then inoculated with the turbid culture and grown until it was turbid. The resulting culture was added to 2 × S4%D–Leu medium (1 liter), which was then shaken at 30°C for 96 h.

Cells were removed by centrifugation at 3000*g* for 10 min, and the supernatant was concentrated to 70 ml with a Minitan ultrafiltration system (Millipore, MA) using a 5000 M_r cutoff polysulfone membrane. Acetone (to 60%, v/v) was added to the concentrate, and the resulting precipitate was collected by centrifugation at 27,000*g* for 30 min. The precipitate was resuspended in a minimal volume of water and lyophilized. The lyophilysate was resuspended in and dialyzed exhaustively against 25 mM sodium acetate buffer, pH 5.5. The dialysate was loaded onto a Mono-S cation-exchange column that had been equilibrated with the same buffer. RNase was eluted with a linear gradient of NaCl (0.00–0.35 M) in 25 mM sodium acetate buffer, pH 5.5. Fractions were collected and assayed for ribonuclease activity with the zymogram spot assay. The purity of active fractions was assessed by SDS–PAGE and zymogram electrophoresis.

Production and purification of recombinant HP-RNase from E. coli (reHPR.B). A mid-log phase culture of *E. coli* strain BL21(DE3) harboring plasmid pET22.HPR in TB medium containing ampicillin (400 μg/ml) was used to inoculate a larger culture (1 liter) of the same medium containing ampicillin (50 μg/ml). The inoculated culture was shaken at 25°C until it reached late log phase (A_{550} = 1–2 O.D.) and was then induced to express the synthetic gene that codes for HP-RNase by the addition of IPTG to a final concentration of 1 mM. Shaking at 25°C was continued overnight. The cells were harvested by centrifugation for 20 min at 10,000*g* at 4°C.

The cell pellet was resuspended in solubilization buffer (200 ml), which was 20 mM Tris–HCl buffer, pH 7.8, containing urea (6 M), EDTA (1 mM), PMSF (0.2 mM), and DTT (20 mM). This suspension was shaken for 1 h at 37°C and then centrifuged for 20 min at 10,000*g* and 4°C. The supernatant was retained and the pellet was extracted again with 200 ml of solubilization buffer.

The two supernatants were pooled together and loaded onto a column (100 ml) of CM-Sepharose CL 6B that had been equilibrated with 20 mM Tris–HCl, pH 7.8, containing urea (6 M) and DTT (2 mM). The loaded column was washed with the same buffer (200 ml). HP-RNase was eluted with a linear gradient of NaCl (0.00–0.50 M) in the same buffer. Fractions (5 ml) were collected and assayed for ribonuclease activity using zymogram electrophoresis. The fractions containing RNase activity were pooled and dialyzed for 24 h at room temperature against 20 mM Tris–HCl buffer, pH 7.8, containing NaCl (0.1 M), GSH (1 mM), and GSSG (2 mM) to allow for the refolding and reoxidation of the sample. Additional dialysis against 20 mM Tris–HCl buffer, pH 7.8, was performed to remove the remaining salt and urea. The RNase was purified further by cation-exchange chromatography on a Protein-pak CM-8HR A-1 HPLC column. The elution was carried out with a linear gradient (30 + 30 ml) of NaCl (0.00–0.20 M) in 20 mM Tris–HCl buffer, pH 8.0.

Determination of steady-state kinetic parameters. Spectrophotometric assays (40) were used to determine the kinetic parameters for the cleavage of poly(C) and the hydrolysis of C > p by the various ribonucleases. For C > p, the concentration of enzyme was 0.1–0.25 μM, the initial concentration of C > p was 0.1–3 mM, and the activity was measured by recording the increase in absorbance at 296 nm [$\Delta\epsilon_{296}$ = 516.4 M^{-1} cm^{-1} (40)]. During assays of poly(C) cleavage, the concentration of enzyme was 5 nM, the initial concentration of poly(C) was 0.10–2.5 mg/ml, and the decrease in absorbance at 294 nm was monitored. All assays were

FIG. 1. Nucleotide sequence of the synthetic gene coding for HP-RNase. The gene was constructed from eight synthetic oligonucleotides. White and black regions indicate the four fragments resulting from annealing complementary oligonucleotides. Five restriction endonuclease recognition sites that are not present in yeast expression plasmid pWL are indicated.

carried out at 25°C in 0.2 M sodium acetate buffer, pH 5.5, using 1-cm path length cells for C > p and 0.2-cm path length cells for poly(C). Steady-state kinetic parameters were obtained by nonlinear regression analysis using the program Enzfitter (41).

RESULTS

Design and Construction of Synthetic Gene for HP-RNase

Eight overlapping oligonucleotides have been designed and synthesized such that their annealing and ligation result in a gene that codes for HP-RNase protein (13). Although the ligation product was designed with cohesive ends, we opted to amplify it to increase the efficiency of subcloning. PCR amplification of the ligation product generated a fragment of appropriate size (400–450 bp). This fragment was digested with *Acc*65I and *Sal*I to generate cohesive ends and then subcloned into M13mp19, yielding plasmid M13mp19.HPR. The nucleotide sequence of the synthetic HP-RNase gene was found to be as expected (Fig. 1).

Expression and Purification of HP-RNase from S. cerevisiae

The DNA fragment corresponding to the gene coding for HP-RNase was isolated from M13mp19.HPR plasmid and cloned into pWL to yield plasmid YEpHPR.1. Plasmid YEpHPR.2 differs from plasmid YEpHPR.1 by the presence of a region coding for the $(Glu-Ala)_3$ nonapeptide spacer in frame between the C-terminal Lys–Arg of the α-factor pre–pro segment and the N-terminal sequence of the HP-RNase gene. The resulting plasmids were shown to be functional as follows. Together with pWL and YEpWL.RNase A (which served as negative and positive controls for the expression of a ribonuclease), YEpHPR.1 and YEpHPR.2 were propagated in *E. coli* and conferred ampicillin resistance to transformed cells. All four plasmids were also propagated in *S. cerevisiae* and allowed transformed cells to recover tryptophan and leucine prototrophies. BJ2168 *S. cerevisiae* cells carrying YEpWL.RNase A, YEpHPR.1, or YEpHPR.2, but not pWL, tested positive in the ribonuclease plate assay under low glucose conditions, indicating the capability of these constructions to express active reHPR.Y.

The amounts of ribonuclease secreted by *S. cerevisiae* BJ2168 harboring plasmid YEpHPR.1, YEpHPR.2, and YEpWL.RNase A were difficult to compare because each sample was glycosylated extensively. Instead, we compared the production of ribonuclease activity by using a RNA zymogram spot assay as described (42) (Table 1). The ribonuclease activity emitted by yeast cells transformed with YEpHPR.2 was slightly greater than that from cells transformed with YEpHPR.1. *S. cerevisiae* cells harboring either YEpHPR.1 or YEpHPR.2 secreted 20- to 50-fold less ribonuclease activity than did cells transformed with YEpWL.RNase A.

Recombinant HP-RNase from yeast (reHPR.Y) was

TABLE 1

Analysis of the Production of Ribonucleases in *S. cerevisiae* Medium

Plasmid	Total protein (mg/ml)[a]	Ribonuclease activity (mg/liter)[b]	Ribonuclease purity (%)[c]
pWL	0.22	0.0	—
YEpWL.RNaseA	0.18	2.5	1.4
YEpHPR.1	0.22	0.042	0.019
YEpHPR.2	0.19	0.16	0.080

[a] Determined by the method of Bradford (31).
[b] Determined by the ribonuclease spot assay and reported relative to commercial RNase A.
[c] Assuming that all ribonucleases have the same catalytic activity as commercial RNase A.

isolated from the growth medium of BJ2168 *S. cerevisiae* cells harboring plasmid YEpHPR.2. SDS–PAGE and zymogram electrophoresis demonstrated that reHPR.Y was secreted into the medium in a variety of forms having diverse electrophoretic mobilities (data not shown). Previously, we had observed that *S. cerevisiae* cells carrying YEpWL.RNase A secreted the bovine enzyme into the medium in a variety of forms due to inefficient removal of the leader sequence, extensive N-glycosylation, and mild O-linked glycosylation (22). The heterogeneity observed in HP-RNase is likely due to similar occurrences. The isolated yield of reHPR.Y, as estimated from measurements of protein concentration, was 0.1–0.2 mg/liter of culture.

Expression and Purification of Recombinant HP-RNase from E. coli

Sequence analysis confirmed that pET22.HPR had been constructed as designed. *E. coli* cells carrying pET22.HPR tested positive in the ribonuclease plate assay, indicating that this construct had the ability to direct the expression of recombinant HP-RNase from bacteria (reHPR.B).

The majority of reHPR.B produced by *E. coli* BL21(DE3) cells harboring pET22.HPR was not completely released from the periplasm upon cold osmotic shock. Rather, it was distributed among the soluble and the insoluble fraction of the cell lysate. To extract all of the reHPR.B at once, the periplasmic contents were released by cell lysis using solubilization buffer.

An isolation strategy based on refolding and reoxidation prior to purification, useful in the purification of RNase A produced by *E. coli* (22), was not useful in the isolation of reHPR.B. Removal of urea from the solubilized fraction by dialysis against an acidic buffer and the oxidation of the soluble dialysate with a glutathione redox buffer caused the precipitation of reHPR.B along with other proteins. Hence, a purification step prior to an attempt to refold and reoxidize the solubilized reHPR.B was essential to isolate the recombinant enzyme. Reduced and unfolded reHPR.B did bind to a cation-exchange column equilibrated with a buffer containing urea and DTT, and the bound protein eluted with a linear salt gradient. The resulting sample was suitable for refolding and reoxidation. Folded and reoxidized reHPR.B was purified further by HPLC on a Protein-pak 8HR cation-exchange column. This procedure yielded a single protein eluting at 0.09 M NaCl. Analysis of this protein by SDS–PAGE and zymogram electrophoresis showed a single, active band with a mobility similar to that of commercial RNase A (Fig. 2, lanes 3 and 5). The isolated yield of reHPR.B, as estimated from measurements of protein concentration, was approximately 1 mg/liter of culture. The amino acid sequence of the five N-terminal residues of the protein purified by HPLC was identical to that of enzyme isolated from human pancreas.

Steady-State Kinetic Parameters

Table 2 lists the steady-state kinetic parameters for the cleavage of poly(C) and the hydrolysis of C > p by recombinant HP-RNase produced in *E. coli* (reHPR.B), HP-RNase from human pancreas, and RNase A. HP-RNase from *E. coli* displayed the same kinetic parameters for each substrate tested as did HP-RNase from human pancreas and RNase A.

DISCUSSION

The study of HP-RNase has been impeded by the difficulty of isolating adequate amounts from human pancreas and by the extensive glycosylation of the human isolates. To reveal the atomic basis for the activities of HP-RNase, we found it necessary to develop a heterologous system for its production. We have accomplished this goal by synthesizing a gene that codes for HP-RNase and producing the enzyme in both a yeast and a bacterial expression system.

In *S. cerevisiae,* HP-RNase is produced as a fusion protein with the α-factor pre–pro segment. Although for some expressed proteins the presence of $(Glu–Ala)_3$ sequence at the C-terminal of the leader peptide had

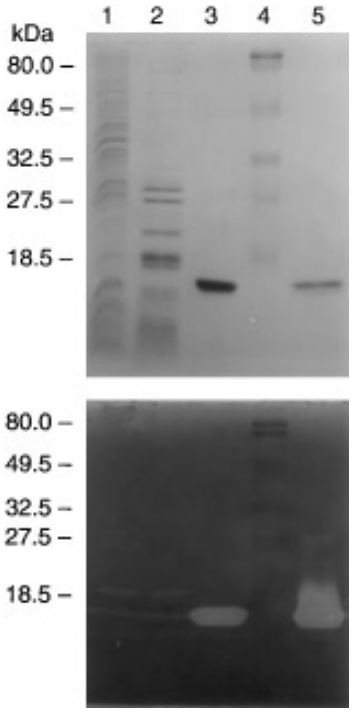

FIG. 2. Gels from SDS–PAGE (top) and zymogram electrophoresis (bottom) showing purification of HP-RNase from *E. coli*. Lane 1, protein extracted with solubilization buffer; lane 2, pooled fractions after cation exchange chromatography; lane 3, RNase A; lane 4, prestained molecular mass standards; lane 5, protein after cation-exchange HPLC.

proved to be unnecessary for efficient secretion or processing (36,43), for other proteins the presence of this nonapeptide has been demonstrated to be crucial (44). We tried both approaches.

HP-RNase isolated from the culture medium of *S. cerevisiae* cells harboring either YEpHPR.1 [which does not code for $(Glu–Ala)_3$] or YEpHPR.2 [which codes for the $(Glu–Ala)_3$] is a mixture of RNase species that differ in the extent of post-translational modification. Although the $(Glu–Ala)_3$ peptide had no apparent effect on the post-translational processing, a fourfold improvement in the overall yield was observed when the peptide was present. This increase in production when using YEpHPR.2 could be explained by synergism in processing by the protease product of the gene STE13, which removes the $(Glu–Ala)_3$ nonapeptide, and the protease products of the KEX1 and KEX2 genes, which remove the C-terminal Lys–Arg sequence of the pre–pro sequence peptide and produce mature protein (43).

Recombinant HP-RNase produced in yeast cells transformed with YEpHPR.2 is recoverable at 0.1–0.2 mg/liter of culture. The active forms appeared as multiple bands in a zymogram. This heterogeneity, together with the existence of two additional N-glycosylation sites and the N-terminal basicity in the human enzyme, suggests, when these data are compared to those obtained for heterologous RNase A expressed in yeast (22), that reHPR.Y is processed inefficiently at its leader sequence and glycosylated extensively with N-linked carbohydrates. In addition, reHPR.Y could be mildly glycosylated with O-linked carbohydrates. Such extensive glycosylation is not unusual in proteins produced in *S. cerevisiae* (45). Further, simply replacing the Asn–Leu–Thr sequence of RNase A with an Asn–Met–Thr sequence in HP-RNase may increase N-glycosylation (46).

Chromatographic procedures failed to separate unmodified reHPR.Y from forms with lower electrophoretic mobility. Thus, reHPR.Y produced using the pWL expression system is not the homogeneous enzyme required for rigorous structural or functional analysis. Nevertheless, studies on protein glycosylation or subcellular localization by *S. cerevisiae* cells may benefit from this system.

Plasmid pET22.HPR, derived from plasmid pET-22b(+) (23), directs the production of HP-RNase in *E. coli* (Fig. 2). BL21(DE3) cells harboring pET22.HPR produced the heterologous enzyme in both a soluble and insoluble form recoverable at 1 mg/liter of culture. This isolate was shown to be homogeneous by electro-

TABLE 2

Steady-State Kinetic Parameters for the Cleavage of Poly(C) and the Hydrolysis of C > p by HP-RNase from *E. coli* (reHPR.B), HP-RNase from Human Pancreas, and RNase A[a]

	Poly(C)			C > p		
Enzyme	$(V_{max}/[E_o])_{rel}$[b]	K_m (mg/ml)	$(V_{max}/[E_o]/K_m)_{rel}$	k_{cat} (min^{-1})	K_m (mM)	k_{cat}/K_m (10^3 $M^{-1}s^{-1}$)
reHPR.B	0.97	0.35 ± 0.10	1.08 ± 0.28	165 ± 20	0.83 ± 0.05	3.3 ± 0.1
HP-RNase	0.99	0.45 ± 0.06	0.86 ± 0.13	175 ± 20	0.80 ± 0.09	3.6 ± 0.2
RNase A	1.00	0.38 ± 0.05	1.00 ± 0.13	184 ± 15	0.84 ± 0.06	3.7 ± 0.1

[a] Assays were performed at 25°C in 0.20 M sodium acetate buffer, pH 5.5.
[b] $[E_o]$ is the concentration of enzyme in the assay.

phoretic and chromatographic criteria. Like recombinant RNase A produced using a pET expression system (22), reHPR.B is insoluble but mature (in that it did not contain the pelB sequence) and not recoverable from the periplasm by cold osmotic shock. This result can be explained if partially folded reHPR.B aggregated in the periplasm (47) or if the translocation of the enzyme was arrested after signal sequence cleavage due to the basicity of the protein. Such inefficient translocation has been observed for lysozyme (48) and (possibly) RNase A (22). The observed lower level of expression of HP-RNase, when compared to the expression levels observed for other enzymes, makes the second hypothesis more plausible. This lower level of expression also made necessary a purification step prior to refolding and reoxidation. Additional purification was accomplished by cation exchange chromatography under denaturing and reducing conditions. Finally, HPLC led to the desired homogeneous isolate. Unfortunately, the reason for the observed differences in production yields between the human and bovine enzyme is mysterious, especially considering that the two proteins are 70% identical in amino acid sequence. A possible explanation for this discrepancy is that the seven proline residues in HP-RNase (in contrast to only four in the bovine enzyme) vastly complicate the folding pathway of this protein.

Recently, D'Alessio and co-workers reported the synthesis and expression in Chinese hamster ovary cells of a gene that codes for HP-RNase (21). The HP-RNase produced by this mammalian system was secreted to the medium as a mixture of unmodified and glycosylated forms recoverable with an overall yield of 3 mg/liter of culture. Here, we have shown that reHPR.B isolated from *E. coli* BL21(DE3) cells harboring plasmid pET22.HPR is homogeneous. Although the mammalian system results in a threefold higher overall yield, the homogeneous enzyme from our bacterial system is a better object for rigorous structure–function analyses and for cytotoxicity assays.

ACKNOWLEDGMENTS

We thank Dr. E. Querol and Dr. J. Arino (Universitat Autònoma de Barcelona) for their advice and Dr. M. Vilanova (Universitat de Girona) for her support and review of this manuscript. We are indebted to the Fundació M.F. de Roviralta, Spain, for generous support. This work was funded by grants PM88/109 (CICYT, Spanish Ministerio de Educacion y Ciencia), AR88-5758 (CIRIT, Generalitat de Catalunya), and GM44783 (NIH). M.R. was supported by a predoctoral fellowship (PFPI) from the Ministerio de Educacion y Ciencia. S.B.delC. was supported by Cellular and Molecular Biology training grant GM07215 (NIH). R.T.R. is a Presidential Young Investigator (NSF), Searle Scholar (Chicago Community Trust), and Shaw Scientist (Milwaukee Foundation).

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