Expression, purification and characterization of the interferon-inducible, antiviral and tumour-suppressor protein, human RNase L

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The interferon (IFN)-inducible, 2′,5′-oligoadenylate (2-5A)-dependent ribonuclease L (RNase L) plays key role in antiviral defense of mammalian cells. Induction by IFN and activation by double-stranded RNA lead to 2-5A cofactor synthesis, which activates RNase L by causing its dimerization. Active RNase L degrades single-stranded viral as well as cellular RNAs causing apoptosis of virus-infected cells. Earlier, we had reported that expression of recombinant human RNase L caused RNA-degradation and cell-growth inhibition in *E. coli* without the need for exogenous 2-5A. Expression of human RNase L in *E. coli* usually leads to problems of leaky expression, low yield and degradation of the recombinant protein, which demands number of chromatographic steps for its subsequent purification thereby, compromising its biochemical activity. Here, we report a convenient protocol for expression of full-length, soluble and biochemically active recombinant human RNase L as GST-RNase L fusion protein from *E. coli* utilizing a single-step affinity purification with an appreciable yield of the highly purified protein. Recombinant RNase L was characterized by SDS-PAGE, immunoblotting and MALDI-TOF analysis. A semi-quantitative agarose-gel-based ribonuclease assay was developed for measuring its 2-5A-dependent RNase L activity against cellular large rRNAs as substrates. The optimized expression conditions minimized degradation of the protein, making it a convenient method for purification of RNase L, which can be utilized to study effects of various agents on the RNase L activity and its protein–protein interactions.

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1. Introduction

RNase L is a uniquely regulated endoribonuclease of mammalian cells, which is a key player in the antiviral defense mechanisms induced by interferons (IFNs) (Bisbal and Silverman 2007). IFNs are a family of cytokines that are involved in the innate immunity of vertebrates against a wide range of viruses and other pathogens. IFNs induce a family of 2′,5′-oligoadenylate synthetases (OAS) (Kristiansen *et al*. 2011), which are activated by double-stranded RNAs (dsRNAs), often produced as intermediates of viral genome replication (Sadler and Williams 2008; Hovanessian and Justesen 2007). Upon activation, OAS converts ATP into a series of unique and labile cofactors, i.e. 2′,5′-linked oligoadenylates, collectively referred to as 2-5A [$5'p_X(A2'p)_n A3'$, where x=1 to 3, n=2 to ≥4], and PPi (Morin *et al*. 2010). The 2-5A binds to RNase L with high affinity, converting it from its inactive, monomeric state to an active, dimeric endoribonuclease, resulting in degradation of single-stranded viral and cellular RNAs. However, nucleoside modifications of RNA can limit OAS activation and thereby restrict RNase L-mediated RNA-degradation (Anderson *et al*. 2011). The OAS, 2-5A cofactor and RNase L together constitute the 2-5A pathway (Zhou *et al*. 1993; Liang *et al*. 2006; Silverman 2007). Certain viral mRNA can also activate RNase L and IFN-β (Luthra *et al*. 2011). Thus, RNase L is an important component of the innate immune system of vertebrates (Chakrabarti *et al*. 2011).

Keywords. 2-5A; antiviral function; cellular RNA degradation; interferons; recombinant RNase L

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Human RNase L is a 741-amino-acid-long protein (~84 kDa), with an interesting arrangement of many structural and functional domains. The N-terminal part of RNase L contains 9 ankyrin repeats, a typical domain for protein–protein and protein–nucleic acid interactions, suggesting its role in interaction with other proteins (Dong and Silverman 1997). The C-terminal part of RNase L contains the protein kinase homology (PK) domain and a RNA binding/ribonuclease domain, which is homologous to the *Ire1p* kinase/ribonuclease involved in the unfolded protein response (UPR) of yeast to humans (Dong *et al*. 2001). However, the kinase activity of RNase L has not yet been demonstrated. The N-terminal part of RNase L acts as a repressor of its C-terminal ribonuclease domain in the inactive, monomeric state. The 2-5A cofactor directly interacts with the ankyrin repeats 2 and 4 and subsequently induces a conformational change, which relieves the repression effect of the N-terminal domain on the C-terminal domain and favours dimerization of RNase L as well as catalytic activation of the C-terminal domain (Tanaka *et al*. 2004; Nakanishi *et al*. 2005).

RNase L displays a broad range of biological functions starting from antiviral, antiproliferative (Hassel *et al*. 1993), apoptotic (Castelli *et al*. 1997), antineoplastic (Liu *et al*. 2007) and immunomodulatory (Zhou *et al*. 1997) activities to its role in cellular RNA metabolism (Le Roy *et al*. 2007; Malathi *et al*. 2007), generating viral RNA signals (Malathi *et al*. 2010), translational regulation (Le Roy *et al*. 2005) and stress response (Pandey *et al*. 2004). Interestingly, RNase L is also involved in various diseases, e.g. chronic fatigue syndrome (CFS) and susceptibility to various types of cancer. Identification of RNase L gene (*RNASEL*) as a human prostate cancer (HPC1) susceptibility locus has qualified RNase L as a tumour suppressor against prostate cancer (Silverman 2003). The RNase L R462Q mutant from prostate cancer led to identification of a novel human retrovirus closely related to xenotropic murine leukaemia viruses (MuLVs), named as XMRV (Bisbal and Salehzada 2008; Chakrabarti *et al*. 2011). A recent report suggests that RNase L is not only a marker for hereditary prostate cancer but germline mutations in *RNASEL* also predict an increased risk of head and neck, uterine, cervix and breast cancer, thereby suggesting its importance in maintaining cell growth regulation in normal cells (Madsen *et al*. 2008). Dysregulation in the 2-5A pathway has been reported in immune cells from CFS patients, characterized by up-regulated 2-5A synthetase and RNase L activities, as well as by the presence of a low-molecular-weight 2-5A-binding protein of 37 kDa related to RNase L by m-Calpain and elastase-mediated proteolysis (Frémont *et al*. 2005). RNase L protein is induced by a variety of stress-inducing agents such as double-stranded RNA (poly rI:rC), chemotherapeutic anticancer drugs (e.g. cis-platin, doxorubicin, vinblastin and vincristine), hydrogen peroxide (H_2O_2), calcium chloride and tumour necrosis factor (TNF-α) in human cervical cancer cells, indicating a relatively broad range of functions for RNase L (Pandey *et al*. 2004).

RNase L mRNA expression is post-transcriptionally regulated by the conserved AU-rich elements (AREs) present in its 3′-untranslated region (3′-UTR) by binding of the HuR protein (Li *et al*. 2007). RNase L has been shown to down-modulate the expression of HuR mRNA and cell growth by binding to AREs in 3′-UTR of HuR (Al-Ahmadi *et al*. 2009). Antibacterial function of RNase L has also been reported by its role in regulation of expression of the endolysosomal protease, cathepsin E (Li *et al*. 2008). Glutamate toxicity has been shown to enhance expression of RNase L via a signal generated by *N*-methyl-D-aspartate (NMDA) receptors leading to death of cortical neurons (Sugiyama *et al*. 2008). RNase L and c-Jun NH2-terminal kinase (JNK) have been shown to function in an integrated signalling pathway, leading to elimination of virus-infected cells through apoptosis (Li *et al*. 2004). Sunitinib, an ATP-competitive inhibitor of VEGF and PDGF receptors, is a potent inhibitor of RNase L and RNA-dependent protein kinase (PKR) (Jha *et al*. 2011). Southern blot analysis of mouse *RNASEL* gene has revealed its genomic organization and upstream promoter DNA fragments (Pandey and Rath 2007), but the promoter regulation has not been reported. Thus, RNase L exhibits a broad range of cellular functions, and multiple roles of RNase L indicate its physiological significance. Here we report expression, purification and biochemical assay of recombinant GST-RNase L protein.

2. Materials and methods

2.1 *Reagents, plasmids, oligonucleotides and E. coli strains*

E. coli strains DH5-α and XL-1 blue were used as host cells for cloning and expression of recombinant human RNase L, respectively. The LB-medium and LB-agar plates were supplemented with 100 µg/mL of ampicillin. The pBluescript II SK (+) vector (Stratagene, USA) was used for cloning and the pGEX 2TK vector (Amersham, USA) was used for protein expression. Oligonucleotides were commercially synthesized (Microsynth, Switzerland) and *Pfu* DNA polymerase (Finnzymes) was used for PCR amplification of the RNase L cDNA. Glutathione–agarose beads, isopropyl thiogalactoside (IPTG), human RNase L-monoclonal antibody (cat. no. R3529), rabbit-antimouse-IgG-HRP polyclonal antibody (cat. no. A9044) and reagents used for the RNase-activity assay were purchased from Sigma-Aldrich (USA). Other biochemicals and molecular biology reagents were from Sigma-Aldrich, USA, Merck, Germany, Qualigens, India, and Spectrochem, India. The human RNase L cDNA plasmid

(pZC5) (Zhou *et al*. 1993) and 2′,5′-linked oligoadenylate (2-5A) cofactor were generous gifts from Prof RH Silverman, Cleveland Clinic Foundation, OH.

2.2 *pGEX-hRNase L construct and expression of GST-hRNase L*

The 2248 bp cDNA fragment encoding the full-length human RNase L (1–741 a.a.) was PCR-amplified using *Pfu* DNA polymerase from the pZC5 plasmid using a 5′-forward primer with a translational start codon (in bold) and a *Bam*HI site (5′-ATGGATCC**ATG**GAGAGCAGGGATCAT-3′) and a 3′-reverse primer with a complementary sequence for the translational stop codon (in bold) and a *Bam*HI site (5′-ATGGATCCAGTCCA**TCA**GCACCCA-3′). This excluded the 5′- and 3′-untranslated regions of the human RNase L cDNA. The blunt-ended PCR product was *in vitro* kinased to generate the 5′-phosphate groups by using ATP and T_4 polynucleotide kinase and ligated into pBluescript II SK (+) vector (2961 bp) at the Sma I site generating the recombinant plasmid, pBS-hRNL (clones B-4 and B-18). The 2242 bp *Bam*HI fragment was produced by restriction digestion and gel purification from the pBS-hRNL clone B-4 and was subcloned into *Bam*HI site of pGEX-2TK vector (4969 bp) generating the pGEX-hRNL clones (HR# 1-3, 5-12, 14-16, 19 and 21) in order to express the RNase L as a GST-fusion protein. The recombinant plasmids were transformed into *E. coli* DH5-α cells to prepare the recombinant hRNase L plasmid DNA and *E. coli* XL-1 blue cells to express the GST-RNase L fusion protein.

For expression, a freshly transformed colony of pGEX-hRNL construct/XL-1 blue cells (clone HR# 11), grown on LB-agar-ampicillin plate overnight at 37°C, was used to inoculate a primary culture of 10 mL LB^{Amp} and grown overnight at 37°C with shaking at 220 rpm. A secondary culture of 100 mL LB^{Amp}, 2% (w/v) glucose was inoculated with 3% (v/v) inoculum from the primary culture. The secondary culture was incubated at 37°C with shaking at 220 rpm until the O.D. at A_{600} nm of the culture reached ~0.6–0.8. The culture was then incubated at 18°C with shaking at 220 rpm for 30–40 min until the culture temperature reached approximately 18°C. Then IPTG was added up to 0.3 mM final concentration, and incubation was continued at 18°C for 18–21 h. The cells were centrifuged at 6000*g* for 10 min at 4°C in GSA rotor of SORVAL RC-5B centrifuge and the cell pellet was stored at −80°C for protein purification. A pGEX2TK vector clone was also similarly processed for IPTG induction.

2.3 *Purification of GST-hRNase L*

The cell pellet from 200 mL IPTG-induced culture was re-suspended and washed once in 10 mL Buffer A [phosphate buffered saline (PBS), 10% (v/v) glycerol, 1 mM EDTA, 0.1 mM ATP, 5 mM $MgCl_2$, 14 mM 2-mercaptoethanol, 2 μg/mL leupeptin and 2 mM PMSF]. The washed cell pellet was again re-suspended in 10 mL Buffer A supplemented with lysozyme (100 μg/mL) and incubated at 4°C for 30 min on a rocking platform. The re-suspended cells were lysed by sonication on ice at 18 microns for 15 s, five times with intermittent gap. Triton X-100 was added to a final concentration of 1% (v/v) and the cell lysate was again incubated on a rocking platform at 4°C for 30 min. The supernatant was collected after centrifugation at 20,000*g* for 15 min at 4°C in SS-34 rotor of SORVAL RC-5B centrifuge. Purification of the GST-RNase L fusion protein was performed in batch affinity as per the instructions of the manufacturer of glutathione–agarose (Sigma-Aldrich), with certain minor modifications. The glutathione-agarose [250 μL of 50% (v/v) slurry was pre-equilibrated with Buffer A] was added to the clarified cell lysate and incubated on ice on a rocking platform at 4°C for 1 h. After washing the protein–bead mixture three times with 10 mL of Buffer A and centrifugation at 1000*g* to recover the bound fraction, the fusion proteins were eluted in 0.4 mL of Buffer B [20 mM reduced glutathione, 50 mM Tris HCl (pH 8.8), 100 mM KCl, 0.1% (v/v) Triton X-100, 2 mM EDTA, 14 mM 2-mercaptoethanol, 0.2 mM ATP, 10 mM $Mgcl_2$ and 1 μg/mL leupeptin] three times with incubation at 4°C for 15 min each time. Then 20% (v/v) sterile glycerol was added to the eluted fraction and 50 μL aliquots were stored at −80°C for up to 6 months. Expression of GST-RNase L fusion protein and its purification was monitored by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie blue R-250 staining, silver staining, Western blot analysis and mass spectrometry.

2.4 *RNase L Western blot*

The purified protein (0.1 μg) was resolved in 9% SDS-PAGE, electroblotted onto nitrocellulose membrane and analysed by Western blotting. The blot was blocked with 5% (w/v) non-fat dry milk for 1 h, and a monoclonal antibody (1:5000 dilution in 1X PBST) specific for the C-terminal region of hRNase L was used to probe the protein for 1 h at room temperature with gentle shaking. The blot was washed three times with 1X PBST for 15 min each at room temperature with gentle shaking. Polyclonal rabbit-anti-mouse IgG-HRP antibody (1:5000 dilution in 1X PBST) was used to probe the blot for 1 h at room temperature with gentle shaking. The blot was washed three times with 1X PBST for 15 min each at room temperature with gentle shaking and ECL reagents (Pierce) were used to probe the signal. The blot was exposed to X-ray film.

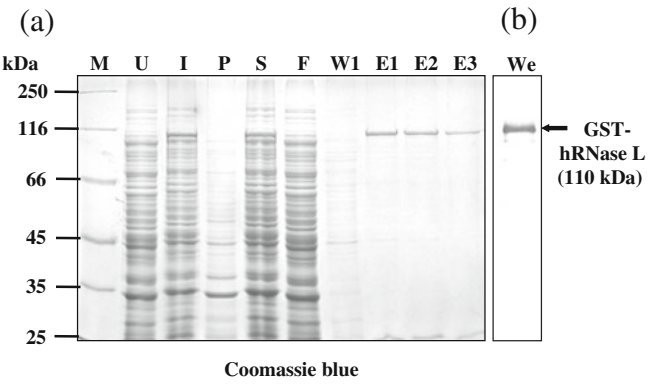

Figure 1. Purification of GST-hRNase L protein. (**a**) SDS-PAGE (9%) stained by Commassie brilliant blue R-250 showing purification of wild type GST-hRNase L using glutathione-agarose beads. (**b**) Side panel shows western blot of the purified protein against anti-human RNase L monoclonal antibody (1:5000 dilution). M – marker; U – uninduced whole cell extract; I – IPTG-induced whole cell extract; P – pellet; S – supernatant; F – flow-through; W1 – wash; E1, E2 and E3 – eluents 1, 2 and 3.

2.5 *Mass spectrometry (MALDI-TOF) of purified RNase L protein*

Characterization of the recombinant fusion protein was further carried out using MALDI-TOF analysis. The purified protein was resolved in 9% SDS-PAGE and the Coomassie brilliant blue R-250 stained band was sliced into minute pieces of approximately 1 mm^3 and de-stained by sequential washing with 40% (v/v) 100 mM ammonium bicarbonate and 60% (v/v) acetonitrile. Cysteine residues were reduced by soaking the gel pieces in 20 mM DTT and incubating at 60°C for 1 h and derivatized by treatment with 100 mM iodoacetamide at RT in dark for 20 min. After further washing with ammonium bicarbonate buffer, the gel pieces were again dehydrated with acetonitrile and dried at 37°C in a speed-vac, prior to the addition of trypsin (Sigma; 30 µL of a 10 ng μL^{-1} solution in 25 mM ammonium bicarbonate). The digestion proceeded for 16 h at 37°C, and the products were recovered by sequential extractions with 25 mM ammonium bicarbonate, 1% (v/v) trifluoroacetic acid and acetonitrile. The pooled extracts were dried in speed-vac and re-dissolved in 0.1% (v/v) trifluoroacetic acid (TFA) for mass spectrometry. The samples were then loaded onto the sample plate and subjected to laser desorption and ionization (Brüker Daltonics, Germany). Known trypsin autolysis products and keratin-derived precursor ions were automatically excluded. Protein was identified by correlation of the mass spectra to entries in MSDB using MASCOT.

2.6 *Isolation of total RNA from mouse kidney and activity of GST-hRNase L*

Mouse kidney total RNA was prepared using the LiCl-precipitation method with minor modifications (Auffray and Rougeon 1980). Freshly dissected mouse kidneys were once washed in normal saline and then homogenized in liquid nitrogen to a fine powder by a mortar and pestle. A 7.5% (w/v) homogenate was prepared in freshly prepared lysis buffer (6 M urea, 3 M LiCl, 50 mM sodium acetate, 0.1% (w/v) SDS and 200 µg/mL heparin) and sonicated at 30 microns for 30 s six times with an interval of 1 min each. The sonicated homogenate was incubated at 4°C for 16–18 h for RNA precipitation and then centrifuged at 20,000*g* for 25 min. The pellet was re-suspended in washing buffer (8 M urea and 4 M LiCl) by vigorous pipetting and incubated on ice for 15–20 min. After centrifugation at 20,000*g* for 15 min, the pellet was re-suspended in freshly prepared dissolving buffer (200 mM sodium acetate, 0.2% (w/v) SDS and 1 mM EDTA). The pellet was incubated at 4°C in the dissolving buffer for 20–25 min for complete dissolution of the ribonucleoprotein complex and then extracted once with 1:1 buffer saturated phenol and chloroform. Total kidney RNA was precipitated by ethanol at −20°C for 16 h and washed in 75% (v/v) ethanol.

The RNA degradation reaction mixture was set up in 18 µL, containing Buffer C [22.2 mM Tris-HCl (pH 7.5), 11.1 mM magnesium acetate, 8.9 mM 2-mercaptoethanol, 0.11 M KCl], ATP (0.11 mM), GST-hRNase L (50–250 ng/20 µL, 22–220 nM) and 2-5A cofactor (10 nM). The reaction mixture was pre-incubated on ice for 30–45 min for dimerization and activation of GST-hRNase L. Finally, 2 µg of mouse kidney total RNA (1 µg/ µL) was added to the reaction mixture to

Figure 2. RNA-degradation activity assay of purified GST-hRNase L. (**a**) 1.2% agarose-TBE gel showing RNA degradation profile at 30°C for 30 min. at different pH (5.0–9.0). RNA (2.0 µg/20 µL) of mouse kidney total RNA; 2-5A (10.0 nM); GST-hRNase L (300 ng), GST (purified from pGEX 2TK clone: 200 ng). (**b**) Graph depicting quantitative (IDV) data of degradation of the 28S and 18S rRNAs at different pH. (**c**) 1.2% agarose-TBE gel showing RNA degradation profile at 30°C for 10 min at different enzyme concentrations: RNA (2 µg/20 µL) of mouse kidney total RNA; 2-5A (10.0 nM); GST-hRNase L (50–500 ng in 20 µl or 22.5–225 nM), GST (purified from pGEX2TK vector clone: 200 ng). (**d**) Graph showing quantitative data of degradation of 28S and 18S rRNAs at different enzyme concentrations. (**e**) RNA degradation profile by GST-RNase L at different time points (2–12 min). (**f**) Quantitative data for degradation of 28S and 18S rRNAs. (**g**) Different RNA substrate concentrations (0.5–6.0 µg/20 µL), GST-hRNase L (50 ng/22.5 nM), GST (50 ng). (**h**) Michaelis-Menten's (left) and Lineweaver-Burk's (right) graphs for estimation of the kinetic parameters: Vmax=34.5 pmole rRNA degraded/min, Km=0.169 µM. Bars represent mean values±SD of RNase L activity of at least two independent determinations. The specific activity of GST-RNase L was 2079.6 Units mg^{-1} of protein (where 1 Unit of the enzyme activity is equivalent to 1 pmol total cellular RNA degraded per min at 37°C with10 nM 2-5A cofactor).

make the final volume to 20 µL and incubated at 30°C for 2–30 min, depending upon the experiment. The reaction was terminated by adding 4.0 µL of 6 X RNA loading dye [10 mM Tris-HCl (pH 7.5), 0.1% (w/v) bromophenol blue, 60 mM EDTA, 60% (v/v) glycerol] and 12 µL (50% (v/v) of the reaction) of each sample was loaded on 1.2% (w/v) agarose-TBE gel. RNA degradation (RNase activity) by the purified GST-hRNase L was quantified by measuring the residual intensity of the 28S and 18S rRNA bands (% 28S and 18S rRNA) in each reaction. For velocity measurement of RNase L, RNA degradation was quantified by

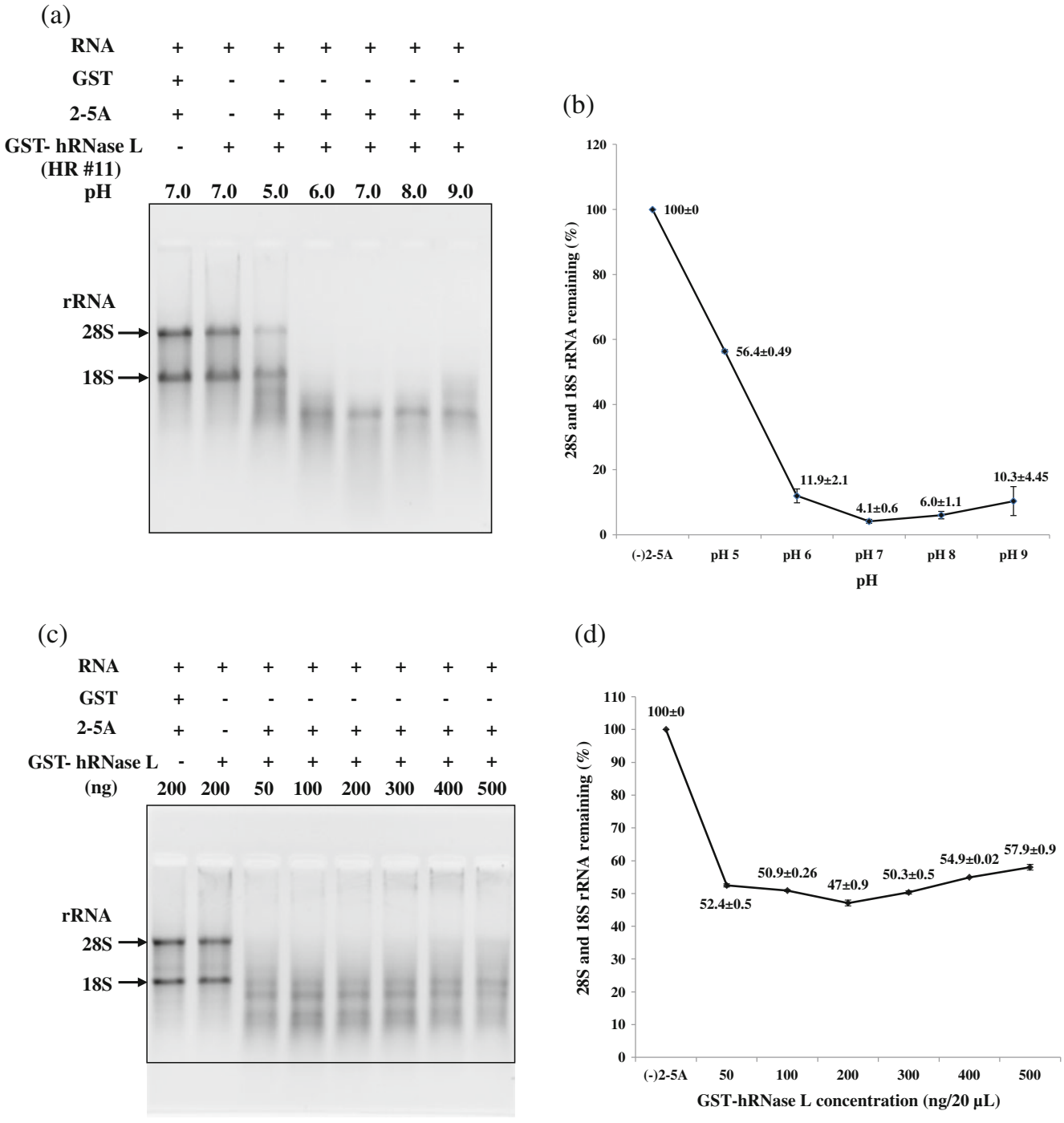

Figure 2(a–d).

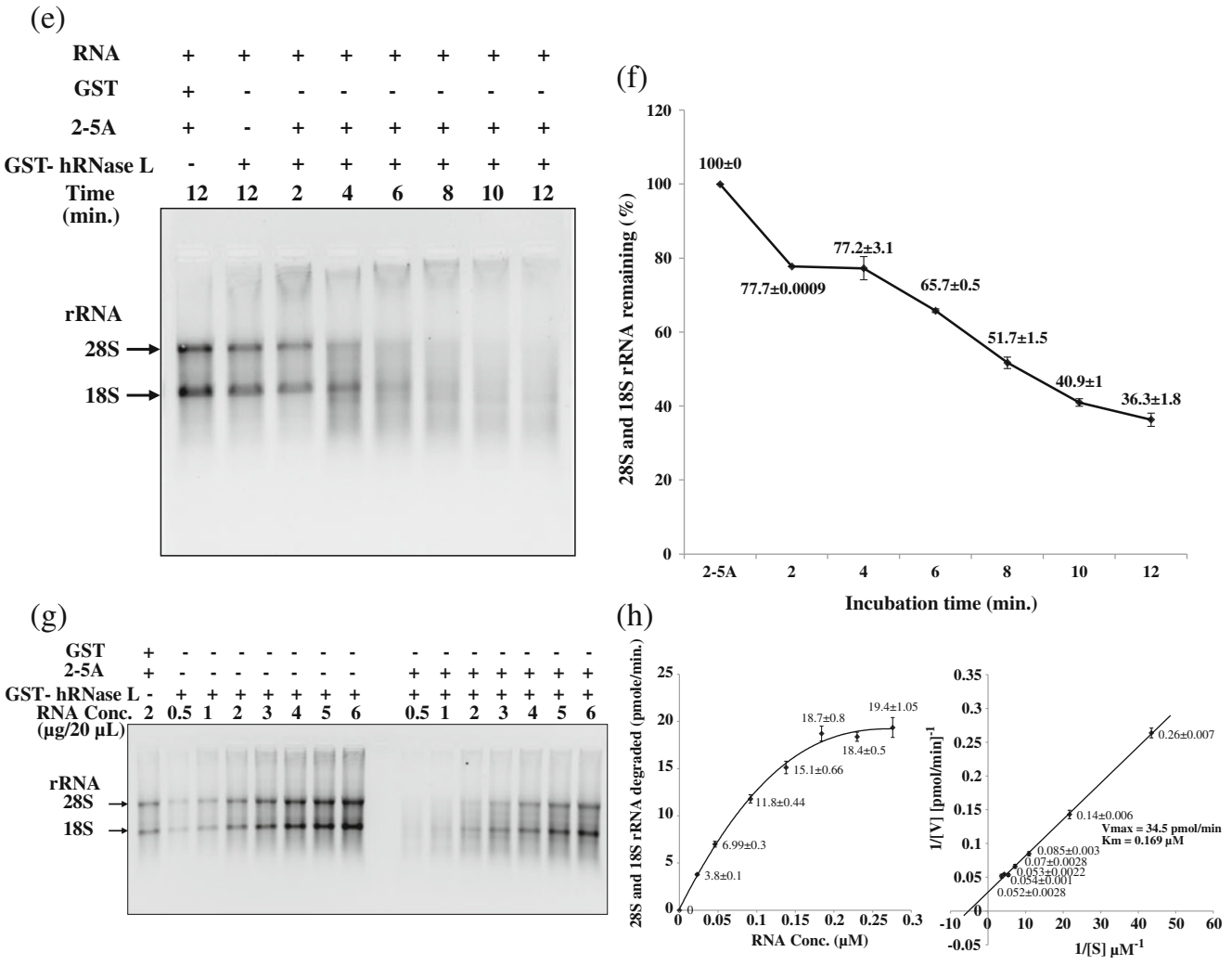

Figure 2(e–h). For caption, *see* page no. 04.

subtracting the residual rRNAs from the control reaction, which did not contain 2-5A cofactor (% 28 S and 18 S rRNA degraded). The percentage RNA degradation was converted into pmol/min of the RNA substrate degraded in 20 µL reaction volume. The intensity measurement was performed by AlphaEase FC™ software and Alpha imager 3400 (Alphainnotech corporation) comparing the total intensity of the 28S and 18S rRNAs and the degraded RNA.

3. Results and discussion

Expression of recombinant human RNase L in *E. coli* has not been an easy task as reported by many researchers. Earlier report from our laboratory showed that expression of recombinant human RNase L caused RNA degradation and inhibition of cell growth in *E. coli* in the absence of exogenous 2-5A (Pandey and Rath 2004). This suggested that recombinant human RNase L was biochemically active in *E. coli*. There was leaky expression and extensive degradation of the recombinant protein as well as inhibition of the host cell growth. Thus, human RNase L, either as a non-fusion or a fusion protein, was always expressed in limited quantity and was extensively degraded; hence, it demanded a number of chromatographic steps for its purification (Díaz-Guerra *et al*. 1997; Yoshimura *et al*. 2002; Nakanishi *et al*. 2004; Player *et al*. 1998). Partly due to these restrictions in expression of human RNase L in *E. coli*, some researchers have used alternative expression strategies like an *in vitro* transcription–translation coupled system (Zhou *et al*. 1993) or Baculovirus vector-based expression in insect cells for obtaining the recombinant protein (Dong *et al*. 1994; Dong and Silverman 1995). We have developed a convenient protocol for expression of full-length, soluble and biochemically active human

RNase L as a GST-fusion protein in *E. coli* XL-1 blue cells utilizing single step affinity purification with an appreciable yield of the purified protein. We have also developed a semi-quantitative agarose-gel-based, 2-5A-cofactor-dependent ribonuclease assay using total RNA isolated from mouse kidney as cellular RNA substrate for RNase L.

3.1 *Expression of GST-hRNase L in E. coli XL-1 blue cells*

E. coli XL-1 blue cells harbouring the pGEX-hRNL plasmid were cultured at temperatures between 18°C to 37°C. The basal level of leaky expression of RNase L retarded *E. coli* cell growth. In the pGEX-vector system, there is a *lac* promoter located upstream between the 3′-end of the *lacI*^{q} gene and the *tac* promoter. This *lac* promoter may contribute to the basal level of leaky expression of protein-coding inserts cloned in multiple cloning sites of pGEX2TK vector by *E. coli* RNA polymerase, and is subject to catabolite repression. Incorporation of 2% (w/v) glucose to the medium to reduce leaky expression of the protein significantly improved cell growth and prevented degradation of the recombinant protein. Eventually, a cell extract of *E. coli* harbouring pGEX-hRNL, grown at 18°C containing 2% (w/v) glucose in the culture medium, was found to be a suitable medium for biochemically active GST-hRNase L expression. The growth of *E. coli* cells was especially very slow in the presence of RNase L expression, irrespective of the nature of RNase L protein expressed, cell background and the medium used. Full-length his-tagged human RNase L expressed in BL21(DE3)pLysE cells caused RNA-degradation and cell-growth inhibition as reported earlier (Pandey and Rath 2004). Also, a partial his-tagged human RNase L (22-741 a.a.) expressed in BL21(DE3)pLysE cells showed decreased transformation efficiency and cell-growth inhibition in acetate-containing medium (Díaz-Guerra *et al*. 1997). Even, a non-fusion human RNase L expressed in JM105 cells also exhibited growth inhibition and formed inclusion bodies at 37°C, which could not be removed even by co-expression of thioredoxin or GroESL (Yoshimura *et al*. 2002).

Three clones of pGEX-hRNL, namely HR#11, HR#15 and HR#19, were randomly selected, which showed expression of the 110 kDa GST-hRNase L fusion protein. However, pGEX-hRNL clone HR#11 was selected for the subsequent expression and purification work. Expression of GST-hRNase L was optimized with respect to IPTG concentration (0.1 to 0.5 mM) and time period of induction from 2 to 12 h at regular intervals of 2 h and after 21 h. There was a gradual increase in the GST-RNase L protein expression (~110 kDa) from 0.1 to 0.5 mM of IPTG induction. Since higher expression of recombinant protein usually leads to formation of more inclusion bodies, while lower IPTG concentration leads to very low levels of expression, an intermediate concentration of 0.3 mM IPTG was used for the expression. There was a gradual increase in the GST-RNase L protein (~110 kDa), which peaked around 8–10 h and then decreased slightly to a lower level and was maintained till the later time point of 21 h. We observed that expression and purification of the protein at 8 h led to more degradation of the affinity purified protein as compared with the protein purified at 21 h. This may be due to stabilization and acclimatization of *E. coli* cells to the foreign protein at later time points as compared with earlier time points. After 21 h of expression of GST-hRNase L, the cells were already in the stationary phase and this might have helped the protein to escape from some proteases and other *E. coli* proteins, which were probably expressed at earlier time points (8 h). Since our earlier report (Pandey and Rath 2004) showed that the his-hRNase L protein was toxic to the *E. coli* cells, we believe that the GST-hRNase L protein might have been tolerated by the host cells at certain level of expression after some time due to metabolic adjustments and protection of the hRNase L protein from proteolytic degradation of the N-terminal GST, which is native to *E. coli* cells.

Human RNase L has been previously expressed as a GST fusion by Nakanishi *et al.* (2004), where they have expressed the fusion protein in JM109 *E. coli* strain at 30°C with 0.5 mM IPTG for 5 h, and Dong and Silverman (1997, 1999) and Dong *et al*. (2001), where they have expressed the fusion protein in *E. coli* DH5-α strain at 30°C with 0.1 mM IPTG for 3 h. The experimental design used by us differs from the above reports with respect to the optimized expression conditions (IPTG-induction at 18°C with 0.3 mM IPTG for 18–21 h) and the different *E. coli* strain (XL-1 Blue) used, which significantly improved the quality of the fusion protein by reducing degradation as well as increasing stability of the fusion protein.

In this study, the basic strategy to express the biochemically active GST-hRNase L was designed by the following modifications: (a) reducing leaky expression of the recombinant protein, (b) starting IPTG-induction with a higher cell density (A_{600}~0.8) so that the *E. coli* cells are larger in number to synthesize the protein and may also tolerate any toxic effect of the protein, (c) using relatively lower IPTG concentration (0.3 mM) to prevent inclusion body formation, (d) induction at low temperature (18°C) to obtain active protein and (e) increasing the time period of induction (18–21 h) to get enough quantity of un-degraded, soluble and biochemically active protein. For purification of GST-RNase L, fresh, cold lysis buffer, minimum freeze-thaw of the cells, sonication at lower frequency (15–18 micron) for shorter time (15 s) on ice and with intervals (1 min) for 5 times were used. For affinity purification, the sonicated supernatant was used at 4°C through the entire

process and the purified fraction was stored in buffer containing 20% (v/v) glycerol at −80°C in order to maintain its enzymatic activity.

3.2 *Purification and characterization of GST-hRNase L*

The expression conditions, mentioned above, reduced degradation of the fusion protein to a minimum and stabilized the full-length active protein. Therefore, it is one of the most convenient sources for purification of the fusion protein and facilitates functional analysis of RNase L. The single-step affinity purification method helped to retain maximum soluble and intact GST-RNase L protein (figure 1a). This protocol yielded the recombinant protein, which was approximately more than 90% soluble, 90% pure as judged by SDS-PAGE and Coomassie blue staining (figure 1a), and silver staining of the purified protein and showed 2-5A-cofactor-dependent specific RNase activity (figure 2) against mouse kidney total RNA, which is its natural cellular substrate. Approximately, 3–5 mg of the purified protein was obtained per litre of the culture by using this method of expression and purification.

The recombinant GST-hRNase L protein was characterized by Western blotting (figure 1b) using a monoclonal antibody against the C-terminal region of human RNase L protein. It detected the 110 kDa GST-hRNase L protein as a single band. The identification and characterization of the recombinant GST-hRNase L protein was also carried out by mass spectrophotometry (MALDI-TOF) analysis. The MASCOT search results of the trypsin-digested peptide ions, and the peaks matched with the original sequence from the database. These peptide ions gave a sequence coverage of 28.1% and intensity coverage of 73.4%. The highest score of 211, which is a significant score, matched with the human 2-5A-dependent RNase (accession no. A45771) in the database (table 1).

Table 1. List of trypsin-digested peptide-ions that matched with the original sequence from the database

Peptide sequence obtained	Protein identified and molecular weight (kDa)	MSDB identifier
FLYKR FYEKR GANVNLR DLEDLGR HLGLVQR HGADPVLR GANVNLRR TKEDQER LQNTEYR KHLGLVQR IYRPMIGK SFDKWTTK LQNTEYRK LFHPGEHVR EDIVELLLR RNYDHSLVK GKTPLILAVEK WAGDPQEVKR LLQPGPSEHSK AAVEDNHLLIK RAAVEDNHLLIK GNFYQNTVGDLLK DHNNPQEGPTSSSGR DHNNPQEGPTSSSGRR ENSHLVTFYGSESHR AQSNEEVVQLSPDEETK	Human 2-5A-dependent RNase (84.295)	A45771

These peptide ions gave a sequence coverage of 28.1% and an intensity coverage of 73.4%.

3.3 *Ribonuclease activity against mouse kidney total RNA*

The utility of any recombinant protein is recognized only after its enzymatic or biological activity has been established. The RNase L catalytic activity has been determined by mainly two methods, either by natural RNA substrates such as 5S, 18S and 28S rRNA degradation assay (Yoshimura *et al*. 2002; Nakanishi *et al*. 2004; Player *et al*. 1998), or synthetic poly (U) substrate degradation assay (Dong and Silverman 1995), and fluorescence resonance energy transfer (FRET) analysis (Nakanishi *et al*. 2004). Hence, RNase activity, the main functional assay for recombinant GST-hRNase L, was estimated from the degradation of 28S and 18S rRNA. Several variables of the reaction such as pH, enzyme concentration, substrate concentration and time points, *etc*., were also used to confirm its enzymatic activity.

RNA degradation using the purified enzyme and cellular total RNA substrate was tested at a pH of the reaction buffer ranging from 5.0 to 9.0 (figure 2a). Maximum RNase activity (more than 95%) was observed between pH 7.0 and 8.0, while minimum RNase activity was detected at pH 5.0 (figure 2b). Hence, pH 7.5 was used as the optimum pH of the reaction buffer for the activity. The unit of RNase L activity was defined as 2 µg mouse total kidney RNA (28S and 18S rRNAs) degraded up to more than 60% by using 50 ng (22.5 nM) of GST-hRNase L in the presence of 10 nM of 2-5A cofactor incubated at 30°C for 10 min at pH 7.5 in a reaction volume of 20 µL.

RNA degradation using different amounts of GST-hRNase L protein ranging from 50 to 500 ng (22.5–225 nM) in the presence of 2 µg mouse kidney total RNA, 10 nM 2-5A

cofactor incubated at 30°C for 10 min in a 20 µl reaction volume were also tested (figure 2c). The RNase activity of the GST-hRNase L protein within this range of enzyme and cofactor concentrations was found to be comparable (figure 2d). The basic mechanism of RNase L activity includes conformational switching leading to RNase L dimerization in the presence of 2-5A cofactor. Almost complete dimerization has been reported at a 1:1 molar ratio of RNase L and 2-5A, and highly efficient dimerization was also reported at a 2:1 ratio of RNase to $pA(2'p5'A)_3$ respectively (Dong and Silverman 1995). The molar ratio of RNase L to 2-5A cofactor in this experiment was from 2:1 to 20:1, which indicated that 2-5A cofactor concentration is limiting and maximum dimerization and hence RNase activity is already attained at 22.5 nM GST-hRNase L and 10 nM 2-5A concentration. Also, the activity slightly decreased around 400–500 ng GST-hRNase L concentration probably because of the crowding effect of the enzyme with the cofactor (figure 2d). Approximately, 4 nM 2-5A cofactor was already reported to cause 50% degradation of 1 µg of 5S rRNA by 60 nM non-fusion RNase L in 20 µL at 30°C for 30 min. (Yoshimura *et al*. 2002). Similarly, 24 nM RNase L degraded 50% of 12 nM radiolabelled poly (rU)Cp at 30°C for 30 min (Dong and Silverman 1995).

Also, since it was observed that 50 ng (22.5 nM) of GST-hRNase L protein along with 10 nM 2-5A cofactor (2:1 molar ratio) was well within the linear range of the assay for the RNA substrate used, sufficient to cause RNA-degradation, and comparable in activity as shown by other researchers, it was decided to carry out further reactions using this concentration of the protein and the cofactor. Figure 2e shows RNA degradation using 50 ng (22.5 nM) of the purified enzyme at different time points starting from 2 to 12 min in a reaction buffer of pH 7.5. It showed a gradual increase in RNA degradation and hence the RNase activity with increasing time. Approximately 60% RNA was degraded around 10 min, and RNase activity was found to be maximum at 12 min time point (figure 2f). Figure 2g shows RNA degradation using 50 ng (22.5 nM) of GST-hRNase L with increasing RNA substrate concentrations starting from 0.5 to 6 µg (0.46–5.52 pmol)/20 µL reaction. A control to each concentration of RNA substrate was also loaded without 2-5A cofactor to obtain RNase activity at that substrate concentration. The percentage IDV degraded/minute was directly converted into pmol/min of the rRNAs degraded. The Michaelis–Menten kinetics was typically represented by the recombinant GST-RNase L enzyme. The Lineweaver–Burk plot was also plotted to get the kinetic parameters of the enzyme. The enzyme showed a Vmax of 34.5 pmol/min of rRNAs degraded/minute and a Km of 0.169 µM (figure 2h). Under the conditions of the assay defined earlier and the detection method used, the calculated specific activity was 2079.6 units mg^{-1} of GST-RNase L protein (where 1 Unit of the enzyme activity is equivalent to 1 pmol RNA degraded per min) using the total mouse kidney RNA as substrate at 30°C in a total volume of 20 µL in presence of 10 nM 2-5A cofactor.

By using this method, the purified RNase L protein may be used for a variety of other experiments to study its property, e.g. 2-5A dimerization assay for the enzymatic activation, GST-pulldown assay for studying protein–protein interaction and to study effects of 2-5A analogues or other synthetic and natural modulators on RNase L activity. Since RNase L is an important antiviral, antibacterial and anticancer protein, recombinant GST-RNase L may also find application in pre-clinical research. There are several regions in the protein, e.g. the ankyrin repeats, the protein kinase homology region and the cysteine-rich domain, which are not fully and functionally dissected. The recombinant GST-hRNase L may be used to study function(s) of these regions. In addition, the tumour suppressor function of RNase L may be hidden in its different biochemical activities, which is difficult to assess in the cellular RNase L, but it can be analysed *in vitro* using the GST-hRNase L protein.

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