

## Effect of Onconase on Double-stranded RNA *In Vitro*

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**Abstract.** *Onconase is a cytotoxic ribonuclease which targets tumor cells in vivo and in vitro. To date, cellular tRNA appeared to be the major target for Onconase mediated cytotoxic activity. Most recently we demonstrated that Onconase can also cleave double-stranded RNA (dsRNA). Incubation of Onconase at 37°C with GAPDH gene-dsRNA (~440 bp long) and dsRNA ladder showed degradation of dsRNA into a spectrum of smaller dsRNA fragments. Moreover, incubation of dsRNA substrates at 40°C under similar conditions markedly potentiated further cleavage of dsRNAs. The recently discovered double-stranded RNase activity of Onconase suggests another mechanism for inducing cell death/apoptosis in malignant phenotypes via the RNA interference mechanism involving siRNA and miRNA.*

Onconase, also known as ranpirnase, is a novel ribonuclease isolated from the *Rana pipiens* frog eggs and early embryos (1-3). It is currently in a confirmatory Phase IIIb clinical trial for malignant mesothelioma (4, 5) and has been granted orphan-drug and fast-track status by the US Food and Drug Administration. Onconase is the smallest known ribonuclease of the RNase A super-family and shares 30% amino acid sequence identity with its homolog of bovine pancreatic ribonuclease (RNase A) protein. It is a basic protein consisting of a single polypeptide chain of 104 amino acids with a molecular weight of 11,820 and an iso-electric point (pI) of 9.7. Its unique features include: (a) N-terminal pyroglutamic acid residue is an integral part of the active site and is essential for Onconase enzymatic and biological activities, (b) Compact tertiary structure contributing to its high conformational stability with high denaturation temperature ( $T_m$ ) of 90°C and resistance to proteolysis and (c) Its resistance to mammalian cytosolic ribonuclease inhibitor protein (RI). The exceptional high conformational stability coupled with RI-evading ability contributes towards

its cytotoxic activity. Onconase has also been shown to possess anti-viral activity against HIV-1 virus (6, 7).

Cellular tRNA appears to be the major substrate for Onconase action. Degradation of tRNA by Onconase once internalized into cells leads to inhibition of protein synthesis and is considered to be one of the mechanism(s) of its cytotoxicity (8, 9). Among synthetic substrates, Onconase preferentially cleaves UpG and CpG dinucleotides. Studies involving *E. coli* tRNAs as substrate, showed Onconase has an unusual specificity for G-G sites (located either in D-arm of tRNA<sup>fMet</sup> and tRNA<sup>Val</sup> or in variable loop of tRNA<sup>Lys</sup>) in addition to U-G and C-G dinucleotide bonds (10, 11). The degradation of tRNA alone may not be the only mechanism for its cytotoxic activity and suggests the existence of additional or alternative mechanism of Onconase mediated pathway(s).

Onconase suppresses proliferation and is cytotoxic to several tumor cell lines. It inhibits growth of certain tumors in mice (12) and enhances the activity of several chemotherapeutic agents when used in combination (13-17). The induction of p16<sup>INK4A</sup>, p21<sup>WAF1/CIP1</sup> and p27<sup>KIP</sup> and decreased pRb phosphorylation was observed in U-937 cells after the cells were treated with Onconase (18). Onconase-induced enhancement of radiation response in A549 human lung cancer *in vivo* has also been reported (19).

Onconase is known to induce apoptosis in a number of tumor cell lines (15, 20). Induction of notable apoptosis was reported in human lymphoblastoid TK6 cells by Onconase under mild hyperthermic conditions (21). The present study was aimed to evaluate the Onconase activity on double stranded RNA as a substrate and the effect of mild-hyperthermia conditions on Onconase mediated dsRNase activity. Hyperthermia alone or in combination with chemotherapy and or radiotherapy is an accepted modality in cancer therapy (22).

### Materials and Methods

Gene silencing kit "Silencer™ siRNA Cocktail Kit (RNase III)" (Cat. #1625) was purchased from Ambion. Double stranded RNA (dsRNA) was transcribed using Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) template DNA according to instructions provided with the kit. Double stranded RNA ladder (Cat. #N0363S) was purchased from New England Biolabs. The dsRNA prepared using the ambion kit and the dsRNA ladder were used for digestion

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with Onconase for evaluating its activity. The reaction buffer used was 20 mM MES buffer of pH 6.0. Incubation of the dsRNA was carried out for 60 min. at 37°C as well as at 40°C. Experiments were conducted using RNase free water and sterile pipette tips and tubes.

**Ribonucleolytic activity assay.** Double stranded ribonucleolytic activity of Onconase was determined by assaying the cleavage of dsRNA using gel electrophoresis. The dsRNA transcribed using ambion kit as well as dsRNA ladder were incubated for 60 min. at 37°C in 10 µl of reaction volume containing 20 mM MES buffer pH 6.0 (reaction buffer), and Onconase at various concentrations. After the incubation, whole reaction mixture volume was analyzed by 2% agarose-TBE mini gel electrophoresis using 1X TBE buffer; pH 8.3 as running buffer. Gel was run for 40 min. at 100V. The dsRNA was visualized after staining with ethidium bromide.

Reaction mixture of GAPDH-dsRNA digested products was also analysed on 12% non denaturing polyacrylamide gel using 1X TBE as running buffer and visualized by ethidium bromide staining.

## Results and Discussion

Onconase cleaved the GAPDH-dsRNA substrate, when the reaction was done at  $1 \times 10^{-6}M$ ,  $1 \times 10^{-7}M$   $1 \times 10^{-8}M$  concentrations of Onconase. The digestion was more pronounced at higher concentrations of  $1 \times 10^{-6}M$  in agarose gel (Figure 1A) and in acrylamide gel (Figure 1B). Onconase showed a significant cleavage of the dsRNA, when the reaction was carried out in 20 mM MES buffer at pH 6.0. At pH 6.5 and 7.0 the effect was less pronounced. (results not shown). Earlier studies have also revealed the pH 6.0 for optimum activity of Onconase using purified yeast ribosomal RNA as a substrate (2).

Onconase cleaved the dsRNA into small fragments of various sizes as represented by smear on gel analysis (Figure 1A and 1B). The newly discovered dsRNase activity of Onconase was also confirmed by using dsRNA ladder as substrate (Figure 2). Cleavage of GAPDH-dsRNA substrate by Onconase generated dsRNA cocktail of 20-400 bp fragments, large dsRNA fragments were more pronounced than smaller ones.

The question arises as to what is the significance and role of these large and small dsRNA fragments in the cell? Small RNAs are widely known to silence the genes whereas, a number of studies have reported that long endogenous double stranded RNAs (800 bp) also induce complete gene silencing in mammalian cells and primary cultures (23), efficient specific silencing of gene expression has also been reported by the use of large exogenous dsRNAs (24-26).

While dsRNA may occur in cells as a result of viral infections, there appears to be an abundance of it expressed from within (27). These dsRNA present inside the cell might also act as a substrate for Onconase, generating dsRNAs of different sizes. These dsRNAs could trigger their cellular response, utilizing one or more signaling pathways dependant on their length and concentration, as reviewed by Wang *et al.* (28).

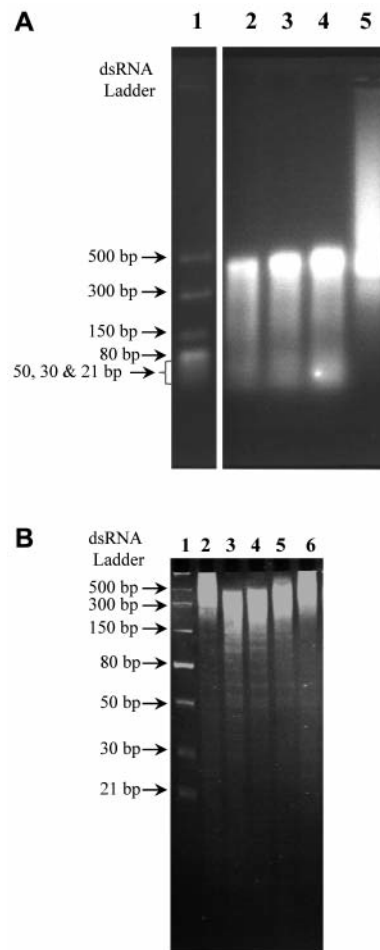


Figure 1. (A) 2% agarose-TBE gel electrophoreses analysis showing cleavage of GAPDH-dsRNA (440 bp) by Onconase. The sample details are as follows: 0.5 µg dsRNA ladder (lane 1), 1 µg GAPDH-dsRNA treated with onconase  $1 \times 10^{-6} M$  (lane 2),  $1 \times 10^{-7} M$  (lane 3),  $1 \times 10^{-8} M$  (lane 4), untreated control GAPDH-dsRNA in reaction buffer (lane 5). (B) 12% Non denaturing acrylamide gel electrophoreses analysis showing cleavage of GAPDH-dsRNA (440 bp) by Onconase. The sample details are as follows: 0.5 µg dsRNA ladder (lane 1), 1 µg GAPDH dsRNA untreated control in reaction buffer (lane 2 and 6), 1 µg GAPDH dsRNA treated with Onconase  $1 \times 10^{-6} M$  (lane 3),  $1 \times 10^{-7} M$  (lane 4),  $1 \times 10^{-8} M$  (lane 5).

Double-stranded RNA longer than 30bp induce the activation of protein kinase R (PKR), which phosphorylates and inactivates the translation factor, eukaryotic initiation factor 2α (eIF-2α), leading to a generalized shutdown of protein biosynthesis (29, 30). PKR could activate apoptotic gene expression and induce apoptosis by activation of the Fas-associated death domain/caspase 8 pathway (31) or of caspase 9 (32). Double stranded RNA activated PKR could also mediate signal transduction by phosphorylating transcription factor inhibitor IκB protein resulting in its release from the transcription factor NF-κB followed by its

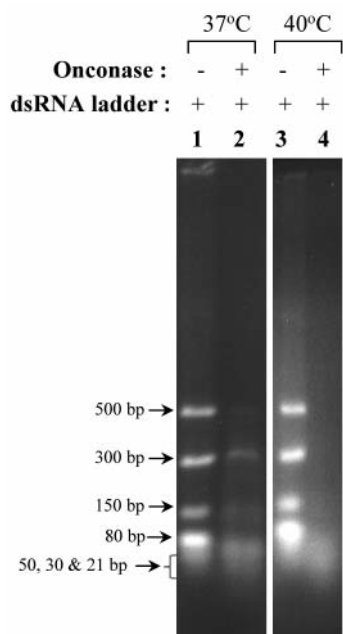


Figure 2. 2% agarose-TBE gel electrophoreses analysis showing significantly higher dsRNase activity of Onconase at 40°C. The reaction was performed using 1  $\mu$ g dsRNA ladder and Onconase at conc. of  $1 \times 10^{-6}$  M in 20 mM MES buffer at pH 6.0. The sample are as follows: dsRNA ladder control at 37°C (lane 1), dsRNA ladder + Onconase at 37°C (lane 2), dsRNA ladder control at 40°C (lane 3), dsRNA ladder + Onconase at 40°C (lane 4).

translocation to the nucleus where it activates the expression of apoptotic genes having NF- $\kappa$ B binding sites; for example, beta interferon (33), p53 (34), Bax (35).

Potential of tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) induced apoptosis by Onconase has also been reported (15). These results could be explained on the basis of the observed dsRNase activity of Onconase, because TNF- $\alpha$  has been reported to up-regulate PKR activating protein and phosphorylate PKR (36, 37). The same dsRNase activity of Onconase may be responsible for the demonstrated interferon induced enhanced activity of Onconase as reported by Vasandani *et al.* (20) since interferon induces apoptosis via dsRNA activated protein kinase (38).

Double-stranded RNA shorter than 30bp produced could enter the sequence specific RNA interference (RNAi) pathway, where they mediate the destruction of targeted mRNAs (39) and silence the specific genes. Using its dsRNase activity Onconase may also act through RNAi mechanism by silencing the genes of some growth factors for example VEGF, because high VEGF levels have been correlated with poor prognosis in patients with lung cancer (40).

Additionally, Onconase may mimic the activity of Dicer, a member of RNase III enzyme family, by cleaving the large dsRNA transcripts and generating microRNAs (miRNAs) with gene silencing properties (41). The deregulation of small

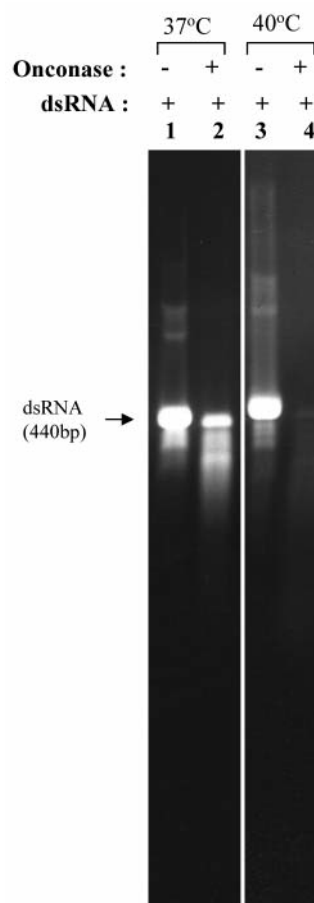


Figure 3. 2% agarose-TBE gel electrophoreses analysis showing significantly higher dsRNase activity of Onconase at 40°C. The reaction was carried out using 1  $\mu$ g GAPDH dsRNA and Onconase at conc. of  $7.2 \times 10^{-6}$  M in 20 mM MES buffer at pH 6.0. The samples are: GAPDH dsRNA in reaction buffer at 37°C (lane 1), dsRNA+ Onconase at 37°C (lane 2), GAPDH-dsRNA in reaction buffer at 40°C (lane 3), dsRNA+ Onconase at 40°C (lane 4).

noncoding miRNAs has been reported to contribute to the development and progression of several types of solid tumors by Croce *et al.* (42, 43). Relative expression of miR-155 and miR- 21 microRNA was decreased after Onconase treatment in mesothelioma cell line H2452 (unpublished data).

Mild hyperthermia increases the cytotoxic activity of Onconase *in vitro*. Treatment of human lymphoblastoid TK6 cells with Onconase at 40°C for 24 or 48 hours led to 64-200% enhancement in the incidence of apoptosis (21). This enhanced apoptosis could be attributed to the marked degradation of dsRNA by Onconase at 40°C, as reflected by the almost complete digestion of dsRNA ladder (Figure 2) and 400 bp GAPDH-dsRNA substrate (Figure 3). Thus the abundant availability of large and small dsRNA fragments may contribute to the enhanced apoptosis/or cytotoxic activity of Onconase.

While Onconase and RNase A share structural similarity, Onconase is a much less efficient enzyme than RNase A (2). RNase A is known to degrade single-stranded RNA (ssRNA) only. Onconase in addition to targeting the ssRNA is also capable of degrading dsRNA like the RNase III enzymes. The intrinsic basic nature of Onconase protein (pI 9.7) may contribute to the required destabilization of the secondary structure of dsRNA for its cleavage. It has been reported that the high basicity appears to be essential for dsRNA cleavage (44).

In summary, this is the first report which demonstrates that Onconase targets dsRNA also for its cytotoxic effects. The action of Onconase on 440bp dsRNA produced a spectrum of smaller dsRNA species. These large and small dsRNAs fragments generated by Onconase may inhibit target gene expression through diverse cellular signaling pathways.

In the cytoplasm, long dsRNAs could activate the potent interferon and protein kinase R (PKR) antiviral pathways, resulting in non-sequence-specific effects leading to apoptosis while short dsRNAs enter the sequence-specific RNA interference (RNAi) pathway targeting specific mRNAs for gene silencing. Onconase plays a key role during embryonic development in *Rana pipiens* (cell growth, differentiation and cell death) and therefore, this newly identified dsRNase activity may account for the gene regulation during early development and its role in host defenses. Currently, work is in progress to evaluate the effect of Onconase generated dsRNAs fragments on target gene expression.

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