

Original Research Article

Disulphide bond reduction of RNase A by drug metosartan a comparative study

Abstract:

RNase A is the most experimental protein in the 20th century. Disulphide bonds are necessary for enzymatic action of many proteins as it is also required for this protein. RNaseA kinetic studies is performed with the drug metosartan using RNA as the substrate Metosartan, is a drug used as blocker in excretion and found to contain inhibitory property on RNaseA. Protein degradation and thiol titration assay has found to be that the drug has reducing property on RNaseA.

Key words: RNaseA, SDS PAGE, Enzyme Kinetics, Beta blocker, Angiotensin receptor type I

1. Introduction:

Metosartan is the drug normally used as antihypertensive agent that act on heart and reduces the work load of the heart. Its components telmisartan is a angiotensin type I receptor blocker known to induce apoptosis in urinary bladder and metoprolol is the beta receptor blocker. The previous studies of eswari beeram et al., has shown that the drug inhibits RNaseA^(1,11). So, study of the enzyme kinetics at different substrate concentrations with and with out drug was proven to be challengeable in order to find the type of inhibition .

RNase A was isolated by Rene' Dubos, crystallized by Moses Kunitz, sequenced by Stanford Moore and William Stein, and synthesized in the laboratory of Bruce Merrifield, all at the

Rockefeller Institute/University⁽²⁾. It consists of four disulphides in its native position at 26–84, 40–95, 58–110, and 65–72⁽³⁾ which is necessary for three dimensional structure of RNase A. and the function of it. The 3D crystal and aqueous form of the RNaseA was previously determined.

It consists of three alpha helices, helix I (residues 3–13), helix II (residues 24–34) and helix III (50–60), and an antiparallel Beta sheet comprising Beta strand I (43–49), beta strand II (61–63), beta strand III (72–74), beta strand IV (79–87), beta strand V (96–111), and beta strand VI (116–124). There are four proline residues: Pro42, Pro93, Pro114, and Pro117. In the native state, the peptide bonds preceding Pro93 and Pro114 adopt the *cis* conformation⁽³⁾. so, the activity of the RNase A in reduced form is necessary to find out whether the disulphide bond determined 3D structure is necessary for the activity or not. But the disulphide bonds are not present in the active site.

RNaseA was known to exist in monomer, dimer, trimer and tetramer but the enzyme is highly active in the trimer state and doesn't contain tryptophan. So, oxidation with performic acid is sufficient to cleave the –s-s- bonds, but in many proteins which contain tryptophan it is more complicated as the reduction of indole group leads to undesired products⁽⁴⁾. The reduction of disulphide bonds is necessary to form intermediates between the folded and unfolded forms of the enzyme⁽⁵⁾.

Glutathione is one of the peptide that interacts with protein in most of the biological systems and the concentration of it was found to be 8mM⁽⁶⁾. So, it is necessary to study whether the pancreatic ribonuclease is active after reduction or not. Methylthioinosinedicarboxaldehyde is

one of the compound known to inhibit pancreatic RNaseA by the formation of schiffs base with ϵ - aminogroups of lysine residues that are close to the catalytic center ⁽⁷⁾ but it's inhibition by drugs is yet to be studied. Denaturing SDS PAGE was used as one of the technique used for the study and also the method of folin lowry for the estimation of RNase to find out the effect of drug on RNaseA.

2. Material and methods

2.1 Protein quantification by lowry method:

The method was followed as that of Stephen P etal⁽⁸⁾ which is explained in brief here. The RNase A along with 200 μ l of TCA was incubated in water bath at 65 °c for 15min, was centrifuged at 12,000rpm and the supernatant was discarded. Pellet retained was resuspended in 0.5ml of lowry reagent and incubated with the drug for prescribed time period and centrifuged at 12,000rpm for 20min. The supernatant was retained and estimated by lowry method or stored at -20⁰c.

2.2 SDS PAGE:

The procedure was followed as that of laemmli⁽⁹⁾ (1970).

2.3 Enzyme kinetics:

1ml of enzyme was added to 1ml of different concentrations of RNA (4.8mM,1.2mM,0.6mM and 0.3mM) and monitored with UV- Visible spectrophotometer for every minute with the and with out the drug. Michaelis menton graph was obtained from the data.

2.4 Titration of RNaseA and measurement of enzyme activity:

The protocol was followed as that of Sho takahas⁽¹⁰⁾ . Titration of thiol groups was highly sensitive at PH 8.0 in the presence of denaturant. 2g of urea, 0.2ml of 2N tris buffer (PH 8.0) and 0.1ml of 2×10^{-3} M DTNB in 0.1N tris buffer (PH 6.5) was added in the sequence. 2ml of the above solution was taken and the content of thiol groups was calculated from the absorbance at 412nm of the mixture.

3. Results: Reduction of RNaseA by drug metosartan:

Figure 1A:

1B:

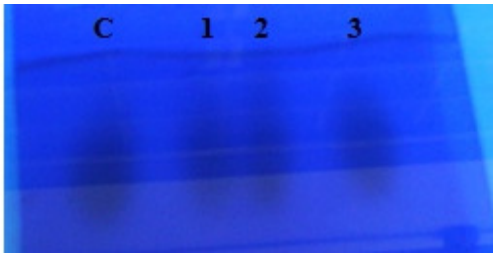
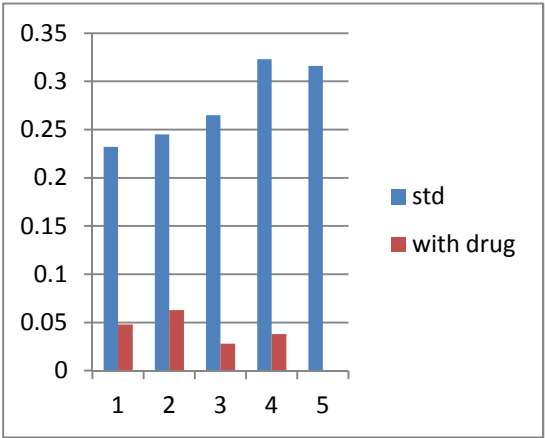


Figure 1C:

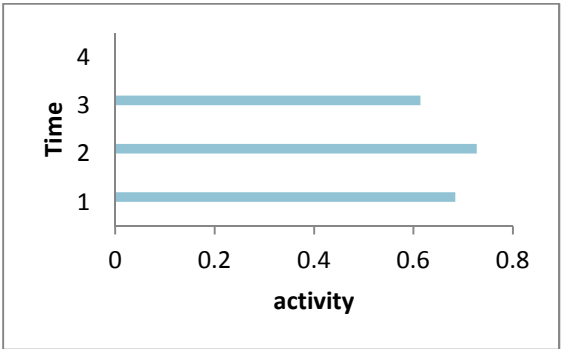


Figure:1 Reduction of RNase A by metosartan: 1A indicates the protein estimated by lowry method after extraction with TCA . It indicates that at exactly 3rd hr the protein concentration was low compared to 1st and 2nd hr even though the concentration of protein used was same . It indicates dissociation of RNaseA in to monomer . 1B indicates SDS PAGE of the control (RNase A) and drug treated RNase A. 1C indicates thiol content of RNase A. So, as a whole it indicates that the drug reduces RNaseA and recovery from it was seen again by 4th hr.

As mentioned in figure 1A cited above, exactly at 3rd hr the protein concentration was less compared to the 1st and 2nd hr and again there is increase in the concentration at 4th hr with respect to 3rd hr and less than 1st and 2nd hrs. so we may conclude from figure 1 the protein is either cleaved or lost its structure. From 1B it is clear that the 3rd hr sample was similar to that of control and from the figure 1c it is clear that the reduction of disulphide bonds present in the protein may be one of the reason for the reduced concentration .

The thiol reduction of RNaseA has shown that the activity was same or equal for all the 3 hrs so, it indicates that disulphide bonds doesn't contribute to the active site of the enzyme.

Enzyme kinetics with different concentrations of substrate with and with out drug:

Except for the highest concentration of substrate, the activity of enzyme was less or equal for the remaining substrate concentration in the presence and absence of the drug.

For the 1.2 mM concentration the enzyme activity was high with out drug and next to it is 0.3 mM concentration.

Figure 2A:

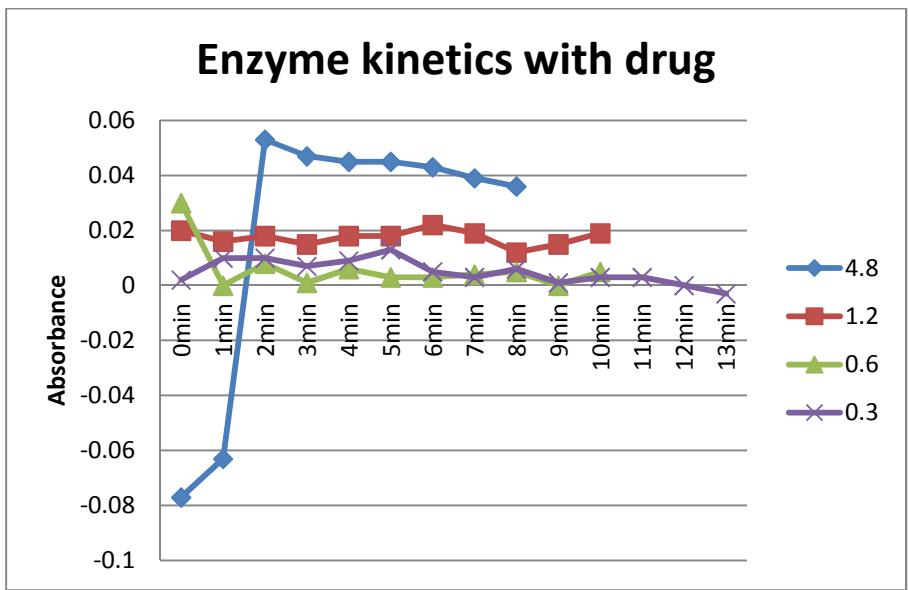


Figure 2B:

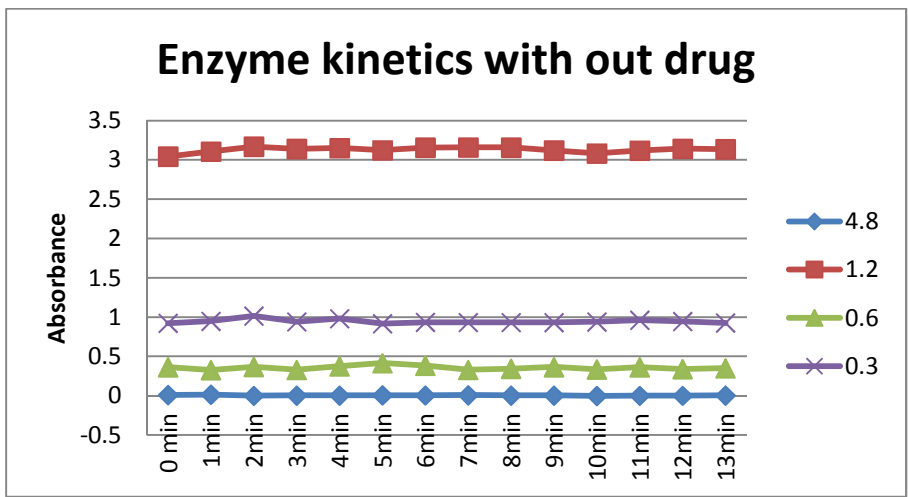
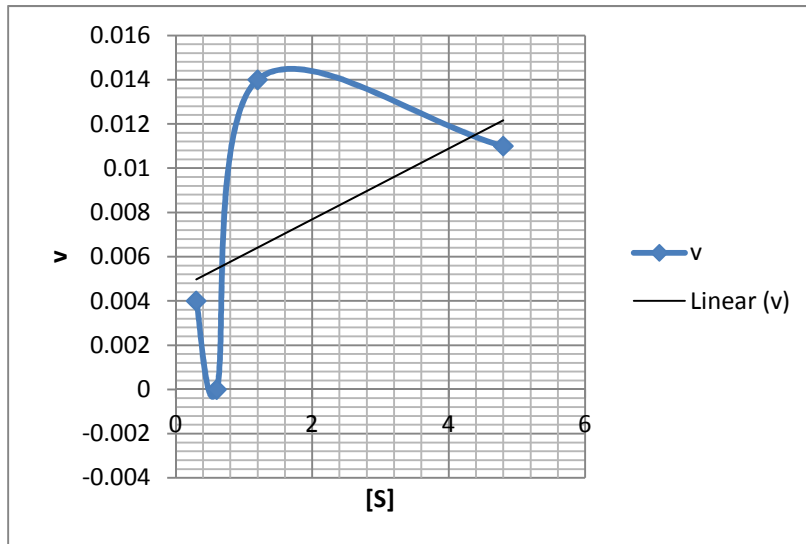


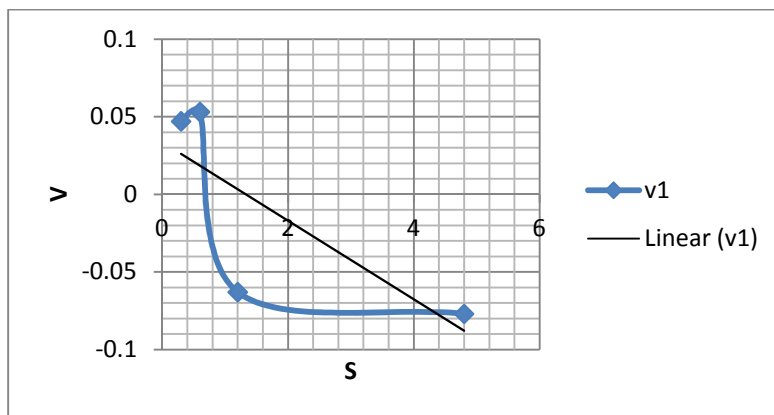
Figure :2 Enzyme kinetics of RNaseA: figure 2A indicates the graph obtained in the presence of drug and 2B indicates the graph with out any drug. From the graph it is known that the enzyme

activity was more or less same even though different substrate concentrations are used. In the graph 4.8, 1.2, 0.6, 0.3 indicates substrate concentration in mM

3.1 Michealis – Menton graph for with out drug:



3.2 Michealis – Menton graph for with drug:



From the section 3.2 we can conclude that the drug allosterically inhibit RNase A in addition to reduction of it to monomer.

4. Discussion:

From the previous data we can conclude that the disulphide bonds is required for biological activity of many proteins but here in this context for RNaseA the disulphide bonds are not required for the activity of enzyme but required for the maintainance of trimer state. Histidine is present at the active site of the enzyme. so, the imidazole groups participate in the reaction of catalytic activity. From our work we may conclude that the drug metosartan reduces the RNaseA by attacking the disulphide bonds and inhibiting the enzyme allosterically in the testis (1,11) found by titrating the enzyme with the agents that determine the thiol content and michaelis-menton graph. SDS PAGE has shown that the enzyme is dissociating in to monomer instead of dimer or trimer and tetramer as explained by smearing of the sample.

Conclusion:

We conclude that metosartin inhibits the activity of RNase A by reducing the disulphide bonds present on it. The reduction of disulphides may dissociate the enzyme to its monomer which is not highly active hence reducing the catalytic activity of RNase A.

5. References:

1. Eswari beeram.2017, Inhibition of RNase By Drug Metosartan In Testis Tissue. *Int J Recent Sci Res.* 8(6), pp. 17394-17395. DOI: <http://dx.doi.org/10.24327/ijrsr.2017.806.0345>.
2. Garland R. Marshall, Jiawen A. Feng, Daniel J. Kuster. (2007). Back to the future: RibonucleaseA Peptide Science Volume 90 / Number 3,259-277.

3. José Luis Neira and Manuel Rico (1997) Folding studies on ribonuclease A, a model protein *Current Biology*, 2:R1–R11.
4. Arthur M. Crestfield, Stanford Moore and William H. Stein. (1962). The preparation and enzymatic hydrolysis of reduced and S- carboxymethylated proteins. *The journal of Biological chemistry*. Vol. 238, No.2, 622-27.
5. Thomas E. CREIGHTON (1980) A Three-Disulphide Intermediate In Refolding Of Reduced Ribonuclease A With A Folded Conformation *FEBS LETTERS* Volume 118, number 2, 283-88.
6. B. E. DAVIDSON AND F. J. R. HIRD (1965) The Reactivity of the Disulphide Bonds of Bovine Pancreatic Ribonuclease with Glutathione *Biochem.J.* 96, 890-94.
7. T. C. Spoor, J. L. Hodnett, and A. P. Kimball (1973). The Molecular Mechanism of Action of Methyl thio inosine dicarboxaldehyde on Ribonuclease A as a Model System *CANCER RESEARCH* 33, 856-858.
8. Stephen P. Slocombe, Michael Ross, Naomi Thomas, Sharon McNeill, Michele S. Stanley (2013). A rapid and general method for measurement of protein in micro-algal biomass *Bioresource Technology* 129, 51–57.
9. Fanglian He (1970) [Bio101] Laemmli-SDS PAGE DOI: <https://doi.org/10.21769/BioProtoc.80>
10. Sho takahashin d Tatsuo OOI (1976)..Disulfide Bond Formation During Renaturation of Bovine Pancreatic Ribonuclease A. Biochemical Assignments of Location of Cystinyl Residues in the Intermediate Protein Species and Theoretical Considerations on the Folding Pathway *Bull. Inst. Chem. Res., Kyoto Univ.*, Vol. 54, No. 3, 141-155.

11. Louise Kime, Helen A. Vincent, Deena M. A. Gendoo, Stefanie S. Jourdan Colin W. G.
Fishwick, Anastasia J. Callaghan and Kenneth J (2015). McDowall The First Small-
Molecule Inhibitors of Members of the Ribonuclease E Family. Sci Rep. 2015; 5: 8028