

Original Research Article

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

Abstract:

RNase A is the most experimental protein in 20th century. Disulphide bond is necessary for enzymatic action of many proteins but in this study disulphide bonds falls outside of the enzyme activity.. The kinetic studies is performed for RNaseA with this drug on testis using RNA as the substrate. Metosartan, a drug used is blocker in excretion in kidney and found to contain inhibitory property on RNaseA. Protein degradation and thiol titration assay has found that metosartan has reducing property of disulphide bonds in 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 decrease the work load of the heart. Its components telmisartan is a angiotensin type I receptor blocker and metoprolol the beta receptor blocker. In previous studies of eswari beeram et al., indicated that the drug inhibits RNaseA^(1,11). So, study of enzyme kinetics at different concentrations with and without drug was proven to be challengeable in order to find which type of inhibition it is.

RNase A was the most studied protein in the 20th century . It 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⁾. Three

dimensional structure of proteins mainly depends on the disulfide bonds present in the protein . So, as we known the functionality of the protein RNaseA also depends on three dimensional structure of it. It consists of four disulphides in its native position at 26–84, 40–95, 58–110, and 65–72⁽³⁾ . The 3D structure of the RNaseA was determined in both crystal and aqueous form.

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 RNase A in reduced form is necessary to find out whether the disulphide bond determined 3D structure is necessary for the contribution of the active site of the protein.

RNaseA was known to exist in monomer, dimer, trimer and tetramer but the enzyme is highly active in the trimer form and it doesn't contain tryptophan so the oxidation with performic acid is sufficient to cleave the –s-s- bonds, but in proteins which contain tryptophan is going to be more complicated as the reduction of indole group leads to undesired products⁽⁴⁾ . The reduction of disulphide bonds was found to maintain 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 so it is necessary to study which of the disulphide bonds of pancreatic ribonuclease is reactive with GSH. In biological systems the concentration of GSH was found to be 8mM⁽⁶⁾ . So it is necessary

to know whether the activity of RNaseA was retained after reduction of disulphide bonds in this concentration.

Methylthioinosinedicarboxaldehyde was known to inhibit pancreatic RNaseA by the formation of schiffs base with ϵ - aminogroups of lysine residues close to the catalytic center ⁽⁷⁾but its inhibition by drugs is the required thing for the study. Denaturing SDS PAGE was used as one of the technique used here and also folin lowry estimation of protein for to determine the concentration of RNase A by the drug metosartan to find out whether the concentration matches with the thiol reduction study. Reducing property of metosartan is a new thing and the degradation of protein was seen in SDS PAGE.

2. Material and methods

2.1 Protein quantification by lowry method:

The method was followed as Stephen P etal⁽⁸⁾ which is explained in brief here. The RNase A was mixed with 200 μ l of TCA, incubated in water bath at 65 ⁰c for 15min, spun at 12,000rpm and the supernatant was discarded. The pellets were resuspended in 0.5ml of lowry reagent and with drug for prescribed time period and spun at 12,000rpm maintained at -4⁰c for 20min and the supernatant retained samples was estimated by lowry method.

2.2 SDS PAGE:

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

2.3 Enzyme kinetics:

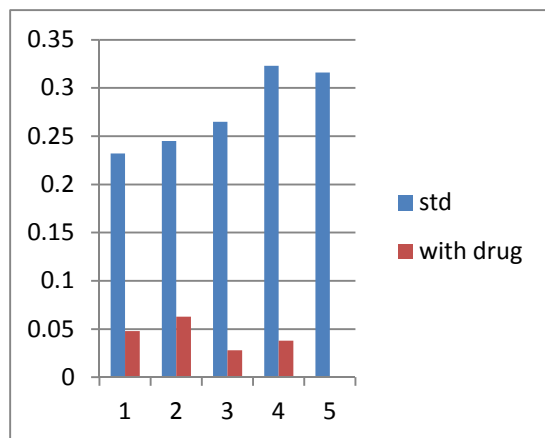
1ml of enzyme was taken with different concentrations of RNA (4.8mM,1.2mM,0.6mM and 0.3mM) as substrate and the michaelis menton graph was drawn and the same protocol was followed along with same concentration of inhibitor as that of Enzyme.

2.4 Titration of RNaseA and measurement of enzyme activity:

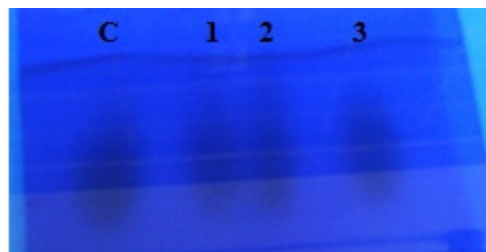
The protocol was followed as that of Sho takahas⁽¹⁰⁾. Titration of thiol groups in the presence of denaturant was highly sensitive at PH 8.0 in the presence of 2g urea, 0.2ml 2N tris buffer (PH 8.0) and 0.1ml of 2×10^{-3} M DTNB in 0.1N tris buffer (PH 6.5) in the sequence in which 2ml of a solution 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:

Figure1A:

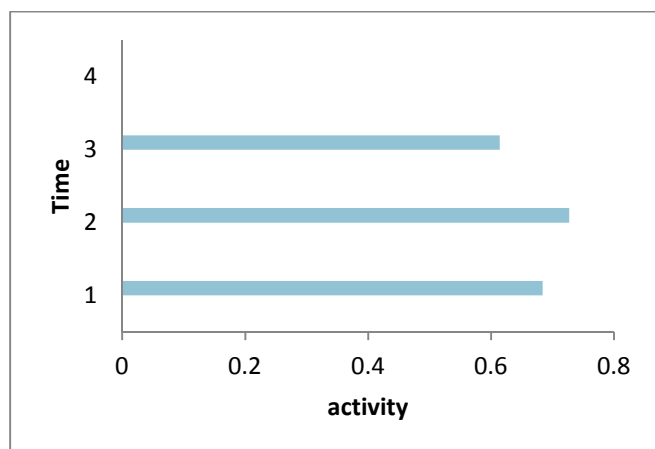


1B:



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86 Figure 1C:



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88 **Figure:1** Reduction of disulphide bonds of RNase A by metosartan: 1A indicates the protein
89 estimated by lowry method after extraction with TCA . It indicates that at exactly 3rd hr the
90 protein concentration was low compared to 1st and 2nd hr as the concentration was same. 1B
91 indicates SDS PAGE of the control (RNase A) and RNase A incubated for prescribed time
92 period with drug. 1C indicates thiol reduction of RNase A by metosartan . So, as a whole it
93 indicates that the drug reduces RNaseA and recovery from it again by 4th hr.

94 From the figure 1A it is mentioned that at exactly 3rd hr the protein concentration was less
95 compared to the 1st and 2rd hrs and again there is increase in the concentration at 4th hr so the
96 protein is either cleaved or lost its structure. From 1B it is clear that the 3rd hr sample was some
97 what similar to that of control and from the figure 1c it is clear that the reduction of disulphide
98 bonds of the protein leading to reduced concentration .

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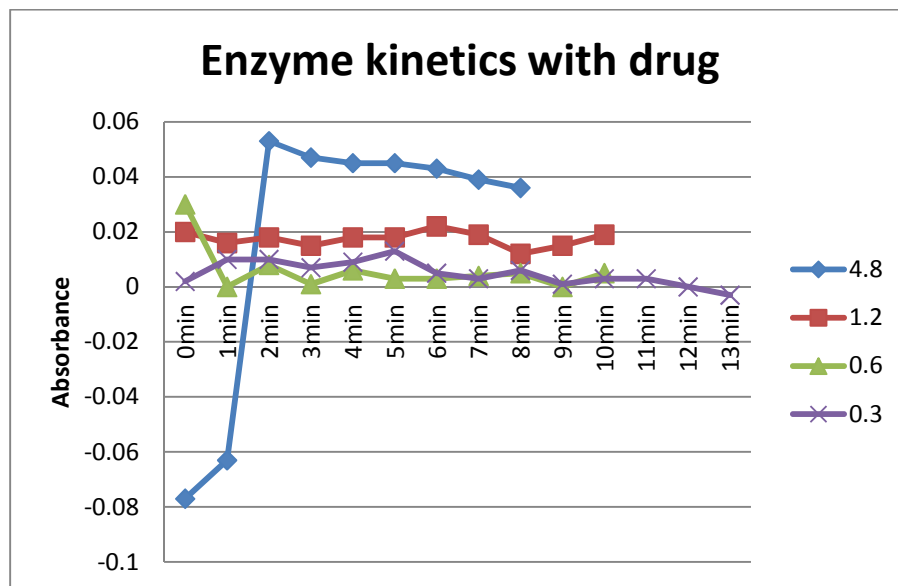
The thiol reduction of RNaseA has shown that the values of all the 3 hrs differ only by 0.2 points so, the activity was same or equal for all the 3 hrs so, it indicates that disulphide bonds doesn't contribute to the active site of the enzyme.

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

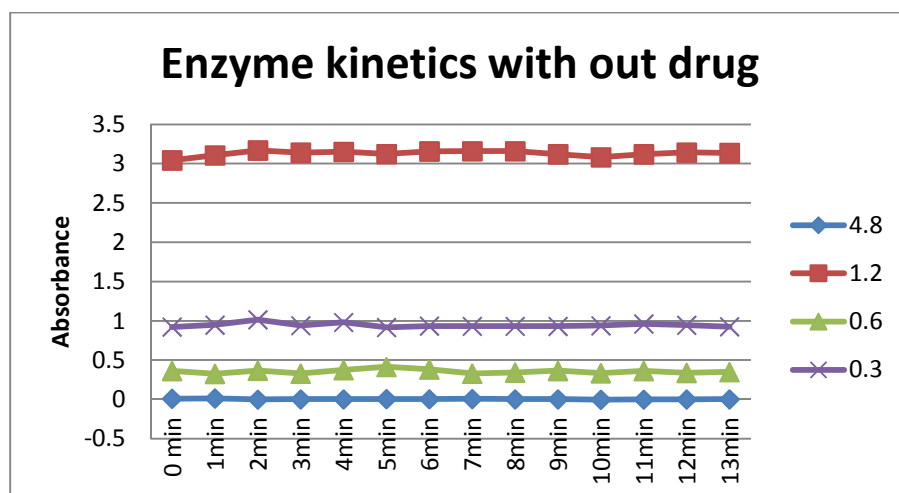
Except for the highest concentration of substrate the activity of enzyme was less or equal for the remaining substrate concentration in the presence of the drug and also in the absence of drug.

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:



114 Figure 2B:



115
116 **Figure :2** Enzyme kinetics of RNaseA: figure 2A indicates the graph obtained in the presence of
117 drug and 2B indicates the graph with out any drug. From the graph it is known that the enzyme
118 activity was same for different substrate concentrations. In the graph 4.8,1.2,0.6, 0.3 indicates
119 substrate concentration in mM

120
121 **4. Discussion:**

122 From the previous data we may conclude that the disulphide bonds is required for biological
123 activity of many proteins but here in this context for RNaseA the disulphide bonds are not
124 required for the activity as active site falls out side the disulphide region and His is present at
125 active site so, the imidazole groups participate in the reaction of catalytic activity. From our
126 work we may conclude that the drug metosartan reduces the RNaseA by attacking the disulphide
127 bonds and inhibiting the enzyme in the testis^(1,11) found by titrating the enzyme with the agents
128 that determine the thiol content. SDS PAGE has shown that the enzyme is dissociating in to
129 monomer instead of dimer or trimer and tetramer as explained by smearing of the sample.

Conclusion:

The concentration of protein at 3rd hr was found to be 28 µg where as for 1st hr it was 48µg and for 2nd hr was 63 µg. The activity of enzyme along with the incubated drug for 1st hr it was found to be 0.684 and for 2nd hr it was 0.727 and for 3rd hr it was 0.614. From this we may conclude from the study that the metosartan inhibits the RNaseA by reducing the disulphide bonds but not due to cleavage of enzyme as the enzyme dissociates in to monomer and lost the activity and as mentioned before trimer is the most active form of the enzyme.

5. References:

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