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ISOLATION AND IDENTIFICATION OF RNASE A FROM TESTIS THROUGH HPLC AND CHROMATOFOCUSSING

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ABSTRACT

From the previous studies it was clear that RNase A is present in pancreas and our new Finding includes that RNase that is similar to RNase A was also present in testis along with other RNases in the tissue. In chromatofocussing the peak was observed in fraction 5 of PI 9.6 and in HPLC it was present in all fractions of the column. The aim of present work is to isolate and identify RNase A through HPLC and chromatofocussing. From the results we conclude that RNase A is present in testis in addition to its presence in epidydymis .Future perspectives include finding of other RNases present in the testis and also further evaluation by other methods.

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INTRODUCTION

Chromatofocussing is a powerful technique that uses ion exchange principles in Combination with PH gradient to separate protein molecules based on their isoelectric Point. The column uses two buffers one is starting buffer which has PH different to that of the Elution buffer and has different PH gradients to that of starting buffer (1). Before going through Chromatofocussing in detail we discuss about PI of the proteins and Their behaviour at different PH. PI is the isoelectric point under which the proteins Has no net negative charge. Below to the PI the proteins has positive charge and above to it Contains net negative charge. It is the one of important property that is used in chromatofocussing to separate the proteins (5). Buffer with acetic acid and sodium acetate with counter ion Na+ is used in cation exchange Chromatography in this case carboxymethyl cellulose is the choose one which is an weak Cation exchanger and the technique depends on the nature of the exchanger, amount of the Sample and the operating conditions. The column has anions bound with the counter ions. When we do it with sample Applied to the column leads to displacement of counter ions with that of proteins that have Cationic charge. After that elution buffer that has different PH to that of starting buffer was Used, depending on the PH the proteins that has PI at that PH is eluted. In this report we are focused on non porous reverse phase HPLC that was coupled with Chromatofocussing technique. The present objective is which RNase is present in testis because from previous studies of mine have shown that inhibition of RNaseA by drug metosartan . So, it is also important to address the problem that whether it is RNase A or not. So, I have used chromatofocussing and HPLC which is a fast and most reliable technique for identification of Proteins based on their retention times of the proteins. Sample can be applied directly to the Column as the impurities are removed in the guard column. The detector used is 214nm in This article. The main scope of this article was identification of RNase A in testis sample and isolation in pure manner. Up to now there is no report about the RNase that was present in the testis and from the results of chromatofocussing it is clear that there are 2 RNases in the testis in which 1 is similar to RNase A that is present in pancreas with a PI of 9.6.

In HPLC also the fractions resulted in similar peak with retention time of 9.2 as that of Standard.

MATERIALS AND METHODS

Isolation of enzyme from source: (2):

All the procedure is carried about room temperature and testis was collected and washed in distilled water after anesthising the rat, dissecting it and grinded in 30ml of buffer A (0.05 M tris- HCL (PH 8.0), 0.01M magnesium chloride, 0.01 M β-mercaptoethanol, 0.10mM EGTA, 10% (V/V) glycerol + 0.05M KCL in pestle and mortar and centrifuged at 8000g for 10 min. The supernatant was collected discarding the pellet. The supernatant was Recentrifuged at 12,000rpm and Supernatant from that was collected and recentrifuged at 12,000rpm for 3hrs and resulting fraction was applied to the DEAE cellulose column.

DEAE cellulose chromatography: (2) with minor modifications

After isolation the fraction above was applied to DEAE cellulose column which was Equilibrated with buffer A+ 0.05 M Kcl with a flow rate of 130ml/hr and washed with the Buffer A+0.05 MKcl and all the fractions are pooled and saturated with 70% with Ammoniumsulfate and the solution resulting from it was centrifuged at 12000 rpm for 20 min And dialysis was performed with buffer A+ 0.05 KCL of 1 liter for 5hrs and the final volume Was about 7.5ml.

DEAE cellulose gradient chromatography: (2)

The resulted Solution was applied to the DEAE cellulose column which was previously Equilibrated with buffer A + 0.05 M KCL and the sample was eluted with a linear gradient Of 0.1-0.30 M Kcl and the fractions was collected for every 10 min and dialysis was performed with the above eluted solution and dialysed against buffer A for 30hrs and the Buffer was replaced thrice with the fresh one. The resulting fraction was used for
Chromatofocusing.

Chromatofocussing technique: (6):

Carboxy methyl cellulose column was prepared by preparing slurry by mixing with buffer Of PH 8.0 (50mM acetic acid + sodium acetate) and equilibrated with the same. The sample Was applied to the column and allowed to separate. Elution is performed with buffer B of DEAE cellulose with gradients of PH 8.8, 9.0, 9.2, 9.4, 9.6, 9.8. The fractions of different PH gradients was analysed at 280nm to characterise RNase A which has peak maxima at 278nm by U.V –Visible absorption spectrophotometry.

Non porous reverse phase chromatography: (3):

Preparation of protein standard:

Ovalbumin (10mg/ml), Bovine serum albumin (10mg/ml), RNase A (10mg/ml) and Glutathione (10mg/ml) was prepared individually and mixture was made by mixing all the Samples in a test tube.

Procedure:

Sample eluted from the column was analysed by HPLC on c18 nonporous column detector at 214nm.The separation was done by using gradiently eluting two solvents water / TFA 0.1% (A) And ACN / TFA 0.08% (B) with a continuous flow of 0.75ml/min and detecting the protein Sample at 214nm.

RESULTS

Chromatofocussing of proteins:

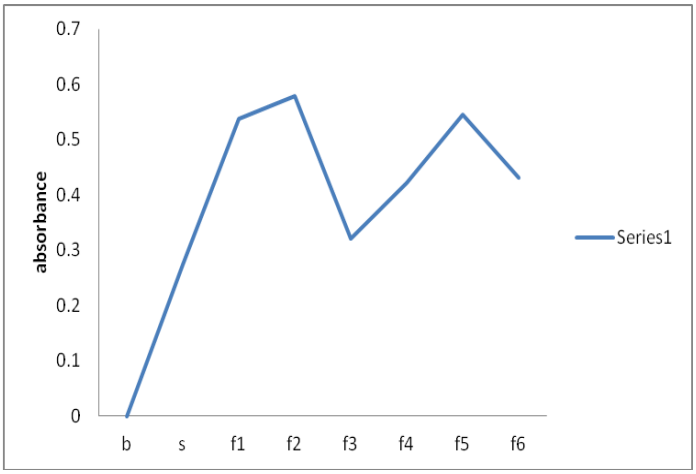

Figure:1.

Chromatofocussing of proteins at PH gradients 8.8, 9.0, 9.2, 9.4, 9.6 and 9.8. B- Blank, f1- f6 fractions of respective PH. From the figure1 it is clear that the sample from testis consists of two proteins having maxima In fraction2 and other with maxima in f5. As we know that PI of RNase A is 9.6, so it is clear That the fraction f5 consists of RNase A as it is eluted with PH of 9.6. The protein or RNase That present in fraction 2 has to find out.

Analysis of proteins using HPLC:

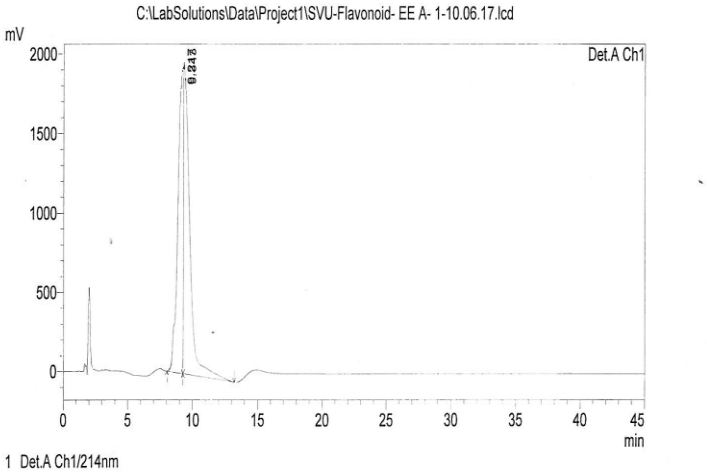

Figure: 2.

Analysis of standard sample mixture consisting of ovalbumin, bovine serum Albumin, RNase A and glutathione by HPLC

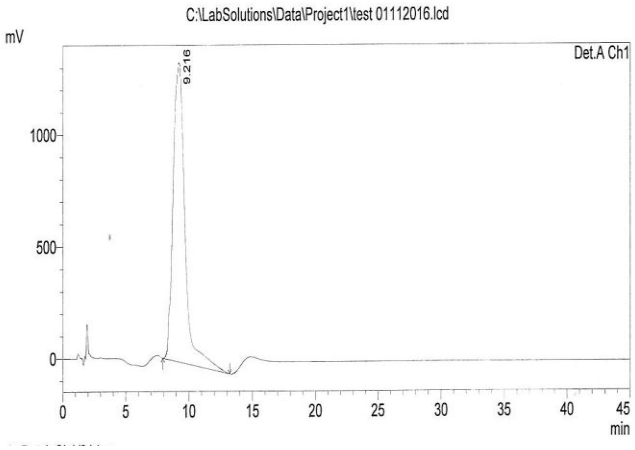

Figure: 3.

Analysis of fraction 1 By HPLC

In protein sample especially standard consists of two peaks with retention times of 9.217, 9.343 which was proved to be RNase A and Bovine serum albumin from the previous Literature (5).

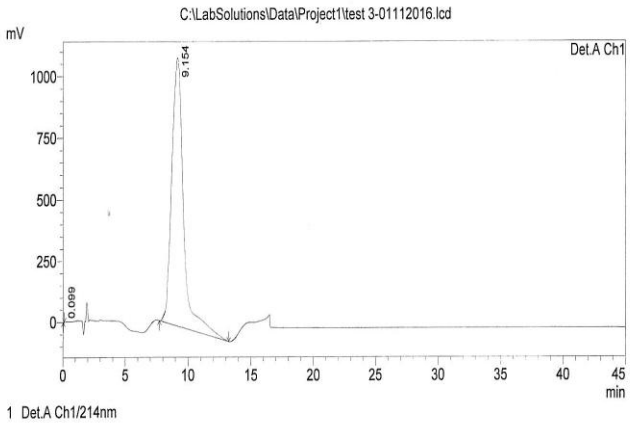

Figure: 4.

Analysis of fraction 2 by HPLC

In fraction 1 there is only one peak which corresponds to RNase A and fraction 2 .There is two peaks which has retention times 0.099 and 9.154 which corresponds to RNase A and the peak with retention time 0.099 was unknown.

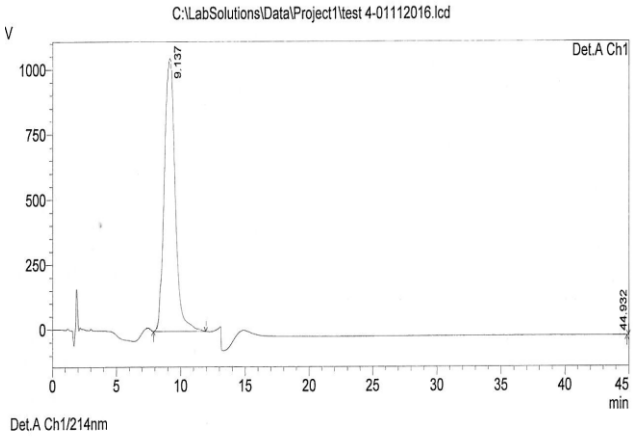

Figure5:

Analysis of fraction 3 by HPLC

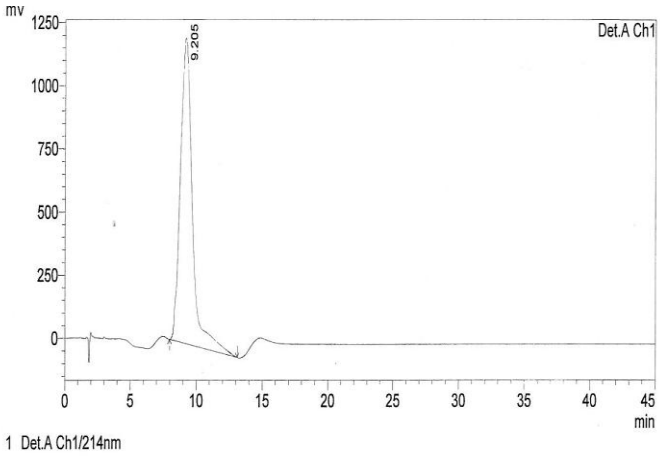

Figure: 6.

Analysis of Fraction 4 by HPLC

Fraction 2 and 3 also represents the RNase A with retention times of 9.317, 9.206.

DISCUSSION

From the previous studies and also data from now shows that HPLC and chromatofocussing Has been the most important techniques for separation of proteins. As identification of which RNase is present in testis is important to know about balance in synthesis of RNA and Degradation in order to regulate gene expression in the tissue. From the previous studies so many people studied RNase A by HPLC but nobody separated it from testis and there was no Reports of which RNase present in testis. So, chromatofocussing confirmed by this that RNase A is present in testis as PI of 9.6 is that of RNase A. Future aspects include the finding Of the RNase that has given peak at fraction 2 in chromatofocussing and f2 in HPLC. Future perspectives include which is the rapid and reliable method for isolation of RNase A and characterization of it.

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