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Isolation, purification and characterization of high sensitive C-Reactive protein (hsCRP) from Lipemic serum samples

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Abstract

High sensitive C-reactive protein (hsCRP) from the Lipemic serum hsCRP was isolated by using calcium dependent affinity chromatography of CNBr activated Sepharose – 4B covalently coupled to p- diazonium phenyl phosphoryl choline (DPPC)and beads coated with anti hsCRP. The molecular weight of hsCRP was determined using SDS- PAGE. The purity was also determined using radial immunodiffusion technique. The elute obtained from affinity column was evaluated using nephlometer to know the hsCRP levels in serum. The differences in the concentration of elute of affinity chromatography was also studied. The whole study was done using serum samples having very high cholesterol, triglyceride and LDL cholesterol values. The purified hsCRP was homogeneous as determined by using immunodiffusion technique. The molecular weight of hsCRP was approximately 23.6 KD - SDS polyacrylamide gel electrophoresis. The results of single radial immunodiffusion technique showed a better and a larger ,clear ring both in the case of males and females than the well with direct serum. The present investigation deals with study of relationship between hsCRP and high amount of lipids in the serum .and also involves the analysis of hsCRP in predicting early cardiovascular problems. Early prediction of cardiovascular problems can help in reducing the mortality rate due to cardiac diseases which is now becoming the reason for the relatively high rate of deaths across the world.

Key words : C-reactive protein, Calcium dependent affinity chromatography, immunodiffusion technique, lipids.

INTRODUCTION

C-reactive protein (CRP) is a nonspecific marker of inflammation that, in healthy individuals, has been shown to be associated with future incidence of cardiovascular disease (CVD). . Volanakis *et al* .(1978) purified CRP by using C-polysaccharide and phosphorylcholine-coupled Sepharose -4B affinity column. Hundreds of risk factors have been associated with cardiovascular disease (CVD). Recent extensive evidence supports inflammation as a key mechanism of pathogenecity in the development and progression of atherosclerosis and in triggering clinical atherothrombotic CVD events. High sensitive C-reactive protein (hsCRP) is one possible marker of vascular inflammation, and that hsCRP plays a direct role in promoting vascular inflammation, vessel damage, and clinical CVD events. Many epidemiologic studies show positive associations between elevated hsCRP levels and incident CVD. Use of hsCRP may add to risk estimation in a limited subset of individuals who are at the intermediate predicted risk according to the Framingham risk score. Isolation of hsCRP from patient serum was first reported by MacLeod and Avery (1941), and subsequently two other methods were also reported by Volanakis and Kaplan. (1971). Characterization of hsCRP has also been done by using Single radio Immunodiffusion as (Mancini *et al*., 1968).

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MATERIALS AND METHODS

Samples

Serum Samples having high lipid content were collected from both males and females and also classified, using vacuum blood collection tubes (BD.Diagnostics) containing clot activator. The collected samples were centrifuged and individually tested for the presence of hsCRP. The reactive samples were pooled separately for males as well as for females. These reactive samples were stored at 4° C, by adding 0.1 % sodium azide.

Preparation of p-Diazonium phenylphosphoryl choline (DPPC)

DPPC was prepared using the procedure described by Umezawa *et al*.(2004)

Preparation of DPPC affinity resin:

The affinity resin was prepared by adding 1.8 mmol of glycyl-L-tyrosine to 250 ml of CNBr-activated Sepharose-4B. This immobilized peptide was then made to react for 60 h at room temperature with 0.5 mmol of p-diazonium phenylphosphorylcholine in borate buffered saline, at the pH of 8.0.

The mixture was stirred at room temperature overnight and washed with 50 mM tris-HCL, at pH 8.0, containing 0.15 M NaCl and 10 mM CaCl2

Isolation of hsCRP

hsCRP was isolated by the method described by Pepys *et al.(1977).* The elution from the column was collected

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Table:-1 Summary of the yield and the obtained concentrations

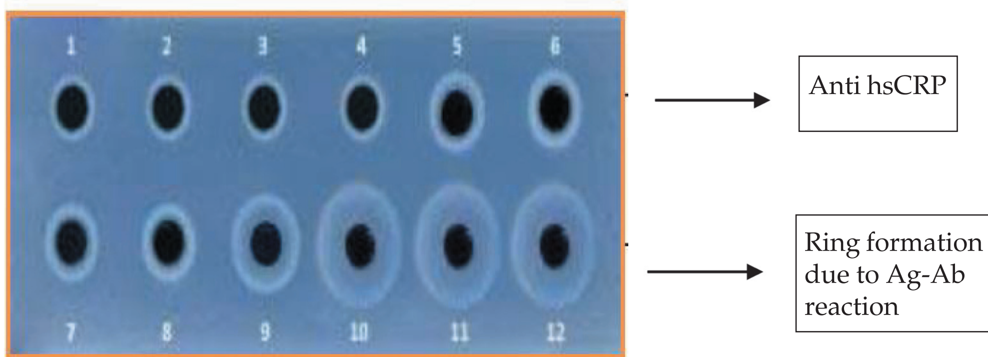
Sample No:	**hsCRP concentration in mg/L**		**Difference in Yields**	**Yield %**
	Before Affinity Chromatography	**After Affinity Chromatography**		
1	0.56	0.21	0.35	37.5
2	0.82	0.42	0.4	51.2
3	1.11	0.54	0.57	48.6
4	1.65	0.62	1.03	37.6
5	2.08	1.13	0.95	54.3
6	2.26	1.42	0.84	62.8
7	3.21	2.86	0.35	89.1
8	3.48	3.1	0.38	89.1
9	4.32	3.98	0.34	92.1
10	4.54	4.22	0.32	93.0
11	5.68	5.5	0.18	96.8
12	5.92	5.79	0.13	97.8
13	6.54	6.4	0.14	97.9
14	7.11	7	0.11	98.5
15	8.26	7.99	0.27	96.7
Std-1	0.75	0.71	0.04	94.7
Std-2	2.4	2.38	0.02	99.2
Std-3	6	5.8	0.2	96.7

Table -2 Results of single radial immunodiffusion

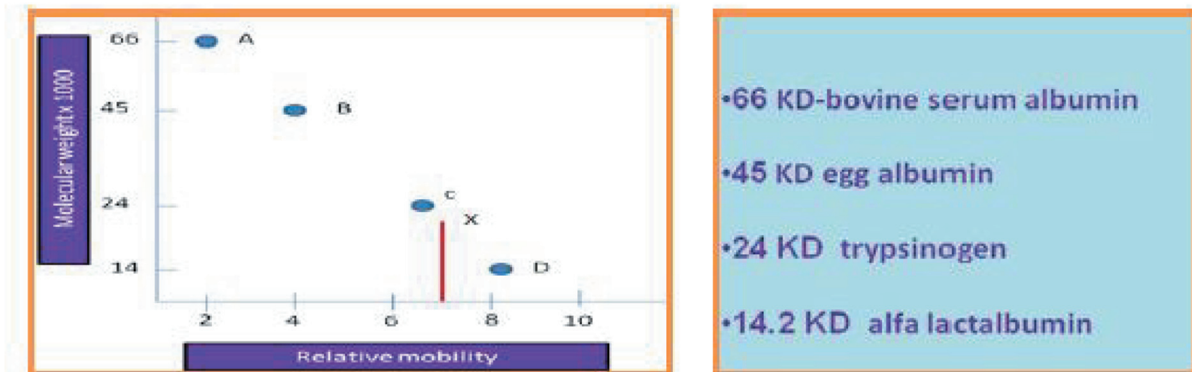
Sample No:	Diameter in mm of sample	
	Before Affinity Chroma tography	**After Affinity Chromato graphy**
1	3	2
2	4	3
3	5	4
4	5	5
5	7	6
6	7	6
7	9	8
8	10	10
9	11	11
10	12	13
11	13	14
12	13	15
13	14	16
14	15	17
15	17	17

Characterization of hsCRP by Immuniduffusion

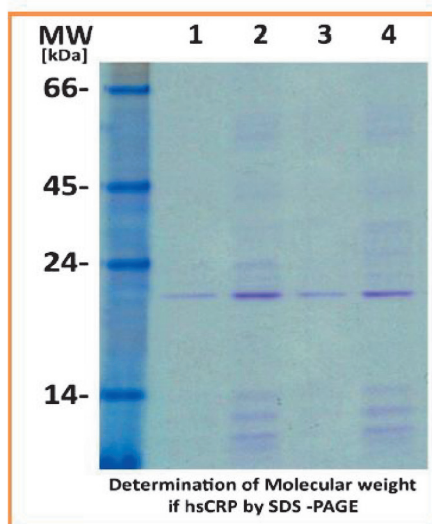
Ring formation due to Ag-Ab reaction Anti hsCRP



Analysis of Molecular Weight by SDS-PAGE



Analysis of Molecular Weight by SDS-PAGE



Determination of Molecular weight if hsCRP by SDS -PAGE

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and concentrated by ultrafilteration and dialyzed against 20 mM potassium phosphate buffer at the pH of 7.2. The elution was further purified using hydroxylapatite chromatography using a 1.5 X 7 cm column. hsCRP fractions were eluted at 15 ml/h using a linear gradient buffer of 20-500mM potassium phosphate at the pH of 7.2. Both the male and the female serum samples and a known hsCRP solution was loaded on to the columns to analyze the yield, and the total quantity of the hsCRP obtained from the elute was determined separately.

Determination of Molecular weight:

The molecular weight of hsCRP was determined using SDS-PAGE,as described by Laemmli *et al. (1970).* The Stacking gel contained 4% acrylamide and the Running gel contained a concentration of 12 % acrylamide. The samples used for the SDS-PAGE are as follows

- Elute obtained from the male serum
- Elute obtained from the female serum
- Known purified hsCRP solutions
- BSA used as marker
- Alfa-Lactalbumin used as a marker
- Trypisinogen used as a marker

Determination of Purity of hsCRP

The purity of hsCRP present in the elution of the column was determined using Single radial Immunodiffusion, as described by Mancini *et a1.(1968).* The gel was prepared using 1 % agarose in PBS and a pH of 7.2

The commercially available Anti hsCRP was incorporated into the agarose gel, and the elutes obtained from the affinity chromatography, that is

- Elute from the column where the male serum was loaded
- Elute from the column where the female serum was loaded
- Known standard of low concentration
- Known standard of High concentration

A calibration curve was prepared by running various known concentrations of hsCRP (standards). The unknown sample concentrations were plotted against the standard calibration curve.

Estimation of Purified hsCRP By Nephlometrys

The elutes obtained from the columns where the male, both high and low concentrations were loaded, and also from the columns where the female samples, both the high and low concentrations were loaded, were analyzed using Immunoturbidity kit (Futura Systems). The concentration of the known sample was also checked after passing through the DPCC affinity resin.

Results

The isolation of hsCRP using DPPC coupled sepharose-4 B chromatography, yielded good concentrations of hsCRP from the serum samples. The yield obtained from the high concentration samples of both male and female samples was around 89% and 96% respectively and that of the purified known standard was found to be 99 %. However, the yield and concentration of lower concentration sample, both in the case of Males and Females were found to be very low from the original concentration. The yield was only 47 % and 53% respectively as in case of Males and females.

The summary of the yield and the obtained concentrations are given in table:-1

The results from the SDS PAGE , showed that the bands obtained in the case of elute from Male serum, elute from the female serum and the known standard were more or less at the same level in the gel. This indicates that there is no difference in the molecular weight as in the case of males and females with respect to hsCRP. The molecular weight markers used were BSA, having a molecular weight of 66K,alfa lactalbumin having a molecular weight of 14.2 K and Trypsinogen having a molecular weight of 24 K. The bands formed by all the 15 samples and 3 standards were just above the band produced by trypsinogen, which indicates the molecular weight of hsCRP is approximately in the range of 25K.

The elutes obtained from the affinity column were subjected to check the purity using Single radial immunodiffusion technique. The results show a slight difference in the diameter of rings formed for the same concentrations in the same sample when run before the affinity column and after affinity column. The diameters obtained in the case of samples before affinity chromatography correlated with the standard calibration curve. The diameter of samples after affinity chromatography is radial Immuno diffusion was less. The minimum diameter was observed in the case of sample with concentration of 0.56 mg/L ,that is 3 mm, and the highest diameter was obtained in the case of sample having concentration of 8.26 mg/L, that is 17 mm. as in the case of samples before affinity chromatography. The minimum diameter was observed in the case of sample with concentration of 0.56 mg/L, that is 2 mm, and the highest diameter was obtained in the case of sample having concentration of 8.26 mg/L, that is 17 mm. as in the case of samples after affinity chromatography.The diameters obtained are shown in Table 2.

DISCUSSION

In SDS PAGE, the bands formed in the case of male serum, female serum and the known standard were more or less at the same level in the separating gel, which

indicate that the molecular weight is same with respect to hsCRP. The results of the Single radial immunodiffusion technique, indicate that the samples which were passed through the affinity columns showed a better and a larger clear ring both in the case of males and females than the well in which the direct serum was added as in the case of males and females. The well in which the purified standard was added also showed a clear and larger ring. This indicates that the affinity column was successful in removing the impurities and other contaminating serum proteins from that of hsCRP.

CONCLUSION

The purified hsCRP was found to be homogeneous by immunodiffusion..The molecular weight of hsCRP was approximately 23.6 KD by SDS polyacrylamide gel electrophoresis. The results of the Single radial immunodiffusion technique indicate that the samples which were passed through the affinity columns showed a better and a larger clear ring both in the case of males and females than the well in which the direct serum was present.

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