STABILITY OF C-REACTIVE PROTEIN IN SALIVA

USING AN ELISA TEST

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Undergraduate Honors Thesis Approved:

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CHAPTER I

ACKOWELEDGMENTS

I would like to thank Dr. John McDevitt for supporting me in doing research. I would also like to thank Dr. Nick Christodoulides for guiding me through this last year in doing different experiments and helping me understand research experiments. I would also like to thank Archana Raamanathan, Shelley Acosta, and Mitra Mohanty for helping me in the experiments that were done this past year. I would also like to thank the Dean's Scholars Honors Program for helping me become a scientific leader rather than a follower. I would also like to thank my parents, Seetharamulu Peddaiahgari and Umamangala Peddaiahgari for being there for me and helping me through all the hard times and the good times. I would also like to thank my friends for being good friends for me.

CHAPTER II

ABSTRACT

This project was done in order to measure the stability of proteins in general; in particular C-Reactive Protein (CRP) which is very important in cardiovascular studies. Current methods of handling CRP is to freeze the sample at -80°C for long term use, or -20°C for medium term use, or 4°C for short term use. This experiment was done in order to measure how much CRP will degrade after a certain time with different matrices. From this project, no conclusive data was gathered to give a certain approximation on the stability of CRP. The data that was gathered did tell us that CRP is most stable with PBS with 0.5% Albumin (PBSA) than saliva, which is more stable than in nanopure water. Furthermore, the data showed that samples kept at -20°C were more stable than those kept at 4°C.

CHAPTER III

INTRODUCTION

BACKGROUND

Cardiovascular disease is one of the most prominent cause of death in the United States. Finding an early detection method would prove to be a useful way to diagnose a patient and to improve the patient's health. As such, C-Reactive Protein (CRP) have been seen to increase when there is tissue damage. Concentration of serum proteins, such as CRP, increase before a major heart attack. This increase in CRP has been caused from the atherosclerosis (major clotting of the heart). As such, diagnosis of increased levels of CRP can help prevent major onset of cardiovascular disease.

C-REACTIVE PROTEIN (CRP)

C-Reactive Protein (CRP) has been discovered by Tillett and Francis[1], and Abernethy and Avery[1] in 1903[2]. It was found that this protein bound to C-polysaccharide on the cell wall of *Streptococcus pneumonia*[1] As such, it has been called C-Reactive Protein due to this reaction where upon binding to *Streptococcus pneumonia*, the complement cascade is activated[2]. CRP has been showed to bind to phosphocholine[2] which are lipids found within the cell membrane. The following is a figure that represents the structure of CRP:

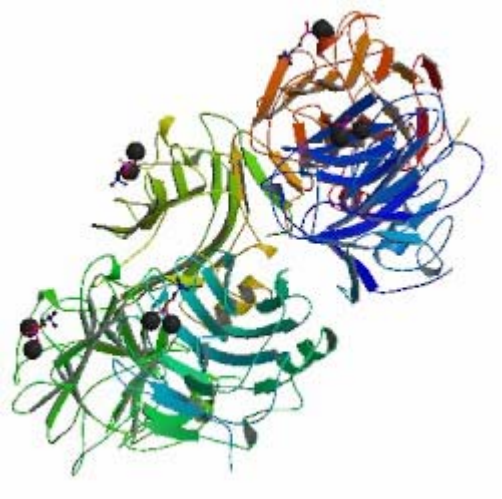

Figure 1: Structure of C-Reactive Protein (Protein Data Bank, structure ID 1b09)

CRP has been characterized as being cyclic and composed of five identical noncovalently subunits that are bound to make up the protein[1] with a molecular weight of 118,000 Daltons[1].

CRP is synthesized within liver cells (hepatocytes), where it is secreted in the presence of interleukin-6[1]. Within humans, it has been shown that there is an increase in the levels of serum CRP within 1 to 2 days after major tissue damage[1]. CRP studies have found that increased levels of CRP leads to atherogenesis[3,5]. It has been reported by Fiedel and Gewurz that platelet aggregation occurs due to CRP concentration increase in serum[1].

C-Reactive Protein has been shown to increase when there is a site of inflammation, up regulated during myocardial infarction, diabetes, and acute and chronic hypertension[1]. There is a direct relationship between the high incidence LDL cholesterol and the increase of serum CRP levels. This can be seen in the following figure:

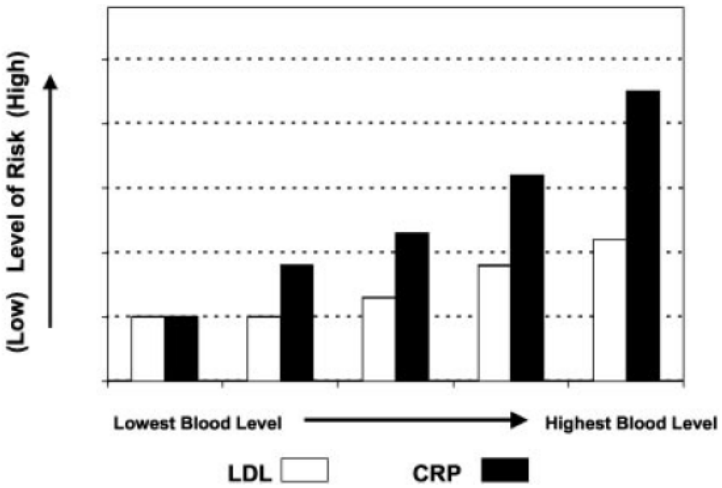

Figure 2: Increase level of myocardial infarction is strongly directly related to serum CRP proteins and weakly directly related to LDL[8]

Individuals with increased CRP levels have been seen to have a higher risk of deaths related to cardiovascular disease[8]. Furthermore, CRP has been shown to be a better indicator than LDL for cardiovascular disease. This can be seen in the following figure:

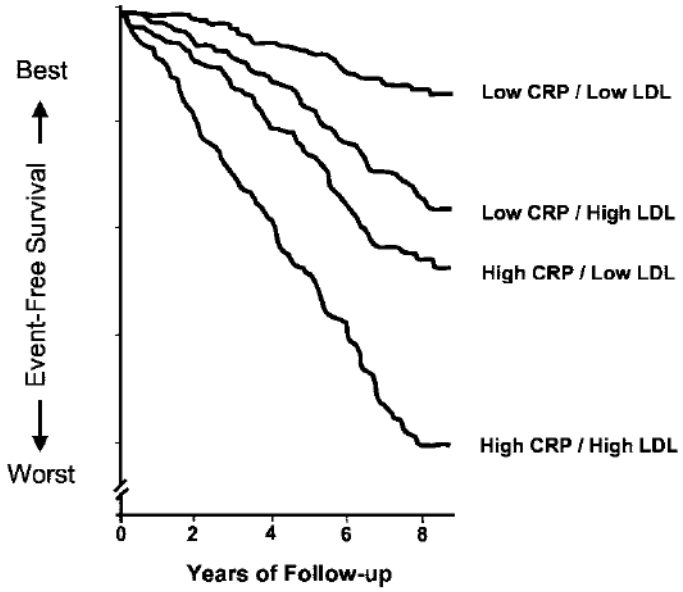

Figure 3: Serum CRP as a better indicator of mortality rate related to cardiovascular disease than LDL concentration[8]

Average serum concentration of healthy humans has been seen to be less than 2 mg/L[1]; where elevated levels are found to be greater than 3 mg/L[1]. Stability tests have been done

on CRP and have been found that CRP has been stable at 4° and 21°C for 5 days and stable at 30°C for 2 days[4] and have a half life of 19 hours in serum.

ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

This is a type of technique that is developed in order to measure proteins using antibodies that can fluorescence. An antibody is attached to the polystyrene of the 96 plate well that binds well to CRP. The sample is added so that the CRP, and only CRP binds, to the antibody that is attached to the polystyrene.

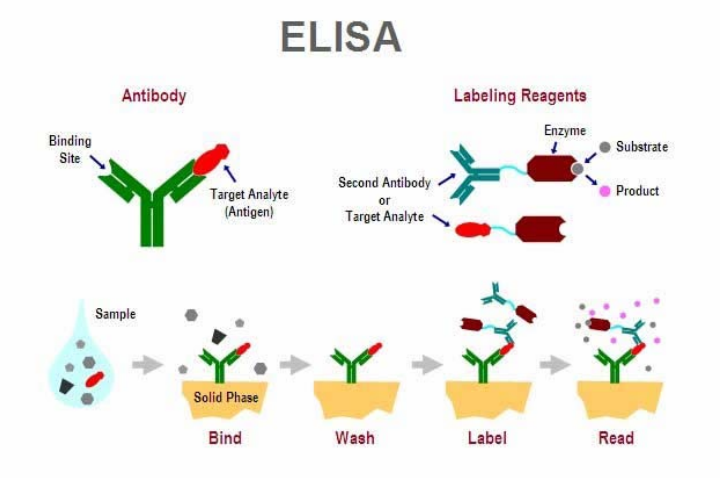

Figure 4: Summary of ELISA (http://64.202.120.86/upload/image/articles/2006/biopen/biopen-elisa-schematic.jpg)

After several washes to remove the excess sample, a secondary antibody is added. This secondary antibody has an enzyme that will catalytically convert a substrate so that the product can fluoresce. As such, when the secondary antibody is added to the well, it will bind to the CRP that is already bound to the primary antibody on the polystyrene. A substrate is added into each well so that the enzyme that is covalently linked to the secondary antibody will convert the substrate into a product that can fluoresce. This product can then be used to detect the concentration of the protein in question.

CHAPTER IV

EXPERIMENTAL

MATERIALS

- ALPCO EIA Kits for C-Reactive Protein (hsCRP)
- Human Saliva
- Refrigerator
    - 4°C
    - -20°C

METHODOLOGY

Samples for Time Decay of CRP:

- Un-stimulated saliva was taken and used as a matrix
- 1 mL of Saliva with 100 ng/mL of CRP was added to each sample
- Variations from using 0.5% PBS with Albumin (PBSA) as a diluent and water were done when diluting CRP
- 3 day and 2 day storage of the samples at both 4°C and -20°C

Samples for Freeze Thaw of CRP:

- 100 ng/mL of CRP in 1 mL of matrix
    - Matrix consisted of either water, PBSA, or saliva
- Flash freeze thaw cycles of 5, 4, 3, 2, 1 times were done in which each cycle consisting of 10 minutes at -80°C and 10 minutes at 25°C

ELISA:

- Using ALPCO EIA Kits for C-Reactive Protein (hsCRP) CRP was measured at 405 nm

CHAPTER V

RESULTS/CONCLUSION

RESULTS/CONCLUSION

From the ELISA plate, the following table is the CRP concentration reading in ng/mL:

[CRP] (ng/mL)	***PBSA diluent with Saliva***	***H_2O diluent with Saliva***	***Saliva (Control)***
3 day			
4°C	13.80	-3.14	-8.49
-20°C	28.83	23.60	-8.07
2 day			
4°C	12.30	-0.64	x
-20°C	8.28	21.69	x

[CRP] (ng/mL)	***PBSA***	***H_2O***	***Saliva***
Freeze Thaw 1	126.85	-8.05	55.01
Freeze Thaw 2	53.89	0.18	31.60
Freeze Thaw 3	106.85	-4.88	33.80
Freeze Thaw 4	117.89	-3.11	39.39
Freeze Thaw 5	141.11	-6.20	27.37

This ELISA plate is giving readings that are not supposed to be there. This is because the highest concentration should be no more than 100 ng/mL (the initial spike) and no less than 0 ng/mL. Even from these major deviations, one can see that PBSA (0.5% PBS with Albumin) is a better choice as a matrix to use to store samples when compared to water as the matrix. Furthermore, pure saliva was better than water, but not better than PBSA. This would suggest that the concentration of proteins found within the saliva is lower than that found within 0.5% PBS with Albumin (PBSA). Furthermore, using -20°C would degrade the samples less than if kept at 4°C.

FUTURE STUDIES

This experiment has to be redone. This experiment was supposed to be done in sync with the Luminex instrument, but other factors inhibited that study to be done. As such, in the future, Luminex should be taken into account for better results. Futhermore, the time frame should be increased in order to measure stability of CRP.

CHAPTER VI

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CHAPTER VII

APPENDIX

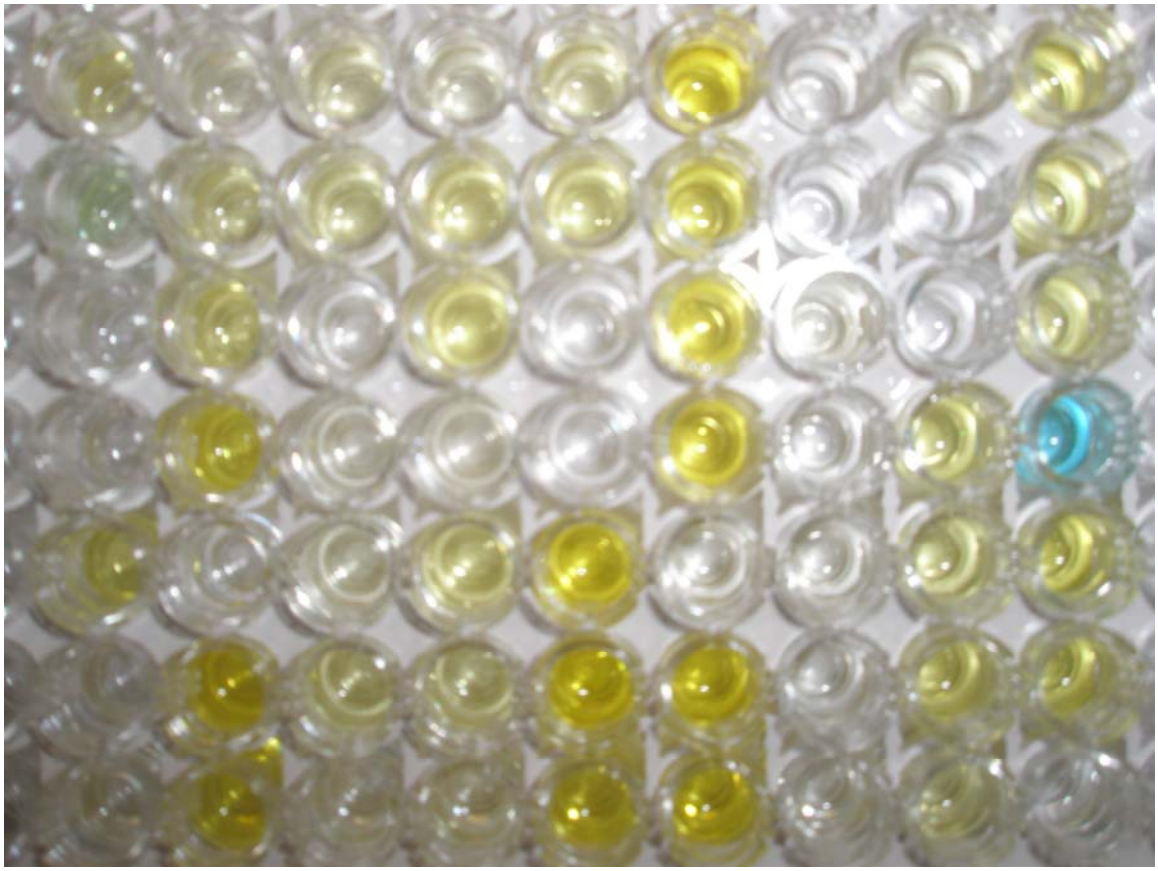


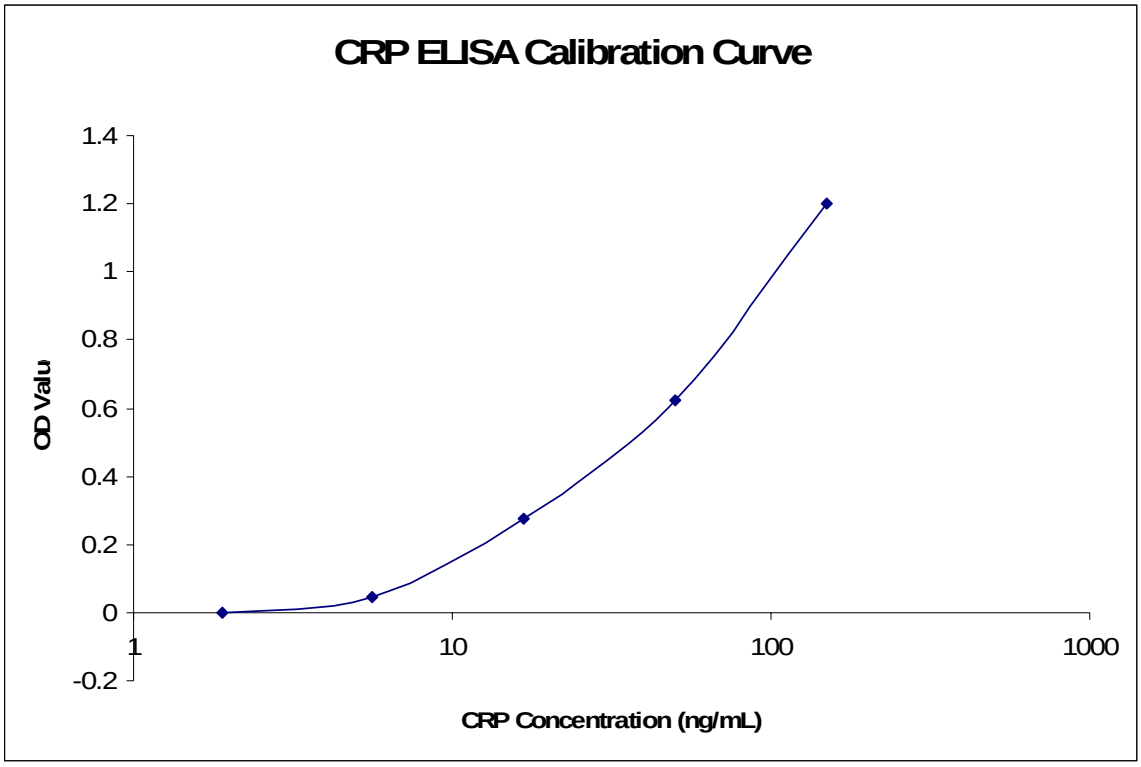
Figure 5: Picture of CRP ELISA Plate

Location

Control	5.6 ng/mL	PBSA 3 day 4°C	H_2O 2 day 4°C	H_2O 2 day -20°C	PBSA Freeze Thaw 4	H_2O Freeze Thaw 5	H_2O Freeze Thaw 2	Saliva Freeze Thaw 3
Control	16.7 ng/mL	PBSA 3 day 4°C	PBSA 3 day -20°C	H_2O 2 day -20°C	PBSA Freeze Thaw 3	H_2O Freeze Thaw 5	H_2O Freeze Thaw 1	Saliva Freeze Thaw 3
0 ng/mL	16.7 ng/mL	H_2O 3 day 4°C	PBSA 3 day -20°C	Saliva 4°C	PBSA Freeze Thaw 3	H_2O Freeze Thaw 4	H_2O Freeze Thaw 1	Saliva Freeze Thaw 2
0 ng/mL	50 ng/mL	H_2O 3 day 4°C	H_2O 3 day -20°C	Saliva -20°C	PBSA Freeze Thaw 2	H_2O Freeze Thaw 4	Saliva Freeze Thaw 5	Saliva Freeze Thaw 2
50 ng/mL	1.9 ng/mL	PBSA 2 day 4°C	H_2O 3 day -20°C	PBSA Freeze Thaw 5	PBSA Freeze Thaw 2	H_2O Freeze Thaw 3	Saliva Freeze Thaw 5	Saliva Freeze Thaw 1
1.9 ng/mL	150 ng/mL	PBSA 2 day 4°C	PBSA 2 day -20°C	PBSA Freeze Thaw 5	PBSA Freeze Thaw 1	H_2O Freeze Thaw 3	Saliva Freeze Thaw 4	Saliva Freeze Thaw 1
5.6 ng/mL	150 ng/mL	H_2O 2 day 4°C	PBSA 2 day -20°C	PBSA Freeze Thaw 4	PBSA Freeze Thaw 1	H_2O Freeze Thaw 2	Saliva Freeze Thaw 4	

OD Values

0.2274	0.0644	0.1374	0.0801	0.1914	1.2002	0.0217	0.0981	0.4587
0.1825	0.2221	0.1978	0.2564	0.2706	0.9268	-0.008	-0.0083	0.3284
-0.0096	0.3326	0.0202	0.3205	-0.0116	0.9043	0.0505	-0.0077	0.3107
0.0096	0.8452	0.0426	0.0484	-0.0082	0.948	0.0129	0.2981	0.023
0.3962	0.01	0.1012	0.2464	1.1047	0.0317	0.0271	0.2552	0.5288
-0.0133	1.452	0.2099	0.1709	1.2772	1.2355	0.0078	0.3733	0.469
0.0236	0.9517	0.0229	0.0756	0.8084	0.9172	0.0181	0.179	



OD value = slope * CRP Concentration + intercept

y = 0.0080383 x + 0.056677

R^2 = 0.9568277

[CRP] ng/mL

21.24	0.96	10.04	2.91	16.76	142.26	-4.35	5.15	50.01
15.65	20.58	17.56	24.85	26.61	108.25	-8.05	-8.08	33.80
-8.25	34.33	-4.54	32.82	-8.49	105.45	-0.77	-8.01	31.60
-5.86	98.10	-1.75	-1.03	-8.07	110.88	-5.45	30.03	-4.19
42.24	-5.81	5.54	23.60	130.38	-3.11	-3.68	24.70	58.73
-8.71	173.58	19.06	14.21	151.84	146.65	-6.08	39.39	51.29
-4.11	111.34	-4.20	2.35	93.52	107.05	-4.80	15.22	

[CRP] (ng/mL)	***PBSA diluent with Saliva***	***H_2O diluent with Saliva***	***Saliva (Control)***
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4°C	13.80	-3.14	-8.49
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CHAPTER VII

VITA

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