DIRECT MEASUREMENT OF FATTY ACID BINDING PROTEIN FROM RAT LIVER CYTOSOL

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DIRECT MEASURMENT OF FATTY ACID BINDING PROTEIN FROM RAT LIVER CYTOSOL

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ABSTRACT

DIRECT MEASUREMENT OF FATTY ACID BINDING PROTEIN FROM RAT LIVER CYTOSOL

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Rat liver fatty acid-binding protein (LFABP) is a 14-16 kDa cytosol protein which binds long chain free fatty acids and is believed to participate in intercellular movement and distribution of free fatty acids. My studies on LFABP are expected to lead to a direct assay to quantitate rat LFABP in cytosol. This new direct assay method is designed to replace our present In-House Method (IHM) of FABP measurement which uses Sephadex G-75 column chromatography and rose Bengal assay. This direct assay method will change the IHM from a day-long method to measure LFABP to a procedure that can be done in ~2 hours. The direct assay method is based upon a fluorescence enhancement process where LFABP and rose Bengal interactions are exposed to emission of a 543 nm laser to produce a fluorescence spectrum. The procedure was found specific for homogeneous LFABP (hLFABP), while potentially interfering components of cytosol, hemoglobin, myoglobin, and serum albumin, do not fluoresce like hLFABP.

My experimental results indicate that cytosol can be directly used in the direct assay method to quantitate FABP, and the presence of other potential contaminants in

cytosol, along with FABP, do not interfere/alter the process to measure FABP. My research shows future promise in replacing the IHM and in the fields of medicine that are attempting to understand and elucidate cures for lipid- related diseases. Since LFABP takes part in dietary fatty acid transport from intestine to blood to liver, for bioenergy production and biosynthesis, any dysfunction of LFABP may cause or be associated with altered lipid uptake/metabolism. These studies can be extended in future to quantitate/study other FABPs like intestinal FABP (IFABP) and perhaps be helpful in making a biosensor for detecting FABP.

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LIST OF SYMBOLS/ABBREVATIONS

FABP = Fatty Acid Binding Protein

LFABP = Liver Fatty Acid Binding Protein

IFABP = Intestine Fatty Acid Binding Protein

IHM = In-House Method

BSA = Bovine Serum Albumin

LCFA = Long chain fatty acids

TSM = Tris Buffer

mM = millimolar

mg = milligrams

mL = milliliters

g = gram

μL = micro liters

kDa = kilo Daltons

ΔH^{o} = Standard enthalpy change of reaction

nm = nanometer

CHAPTER 1

INTRODUCTION

1.1 Proteins and Chromatography

Proteins are macromolecules which are constructed from one or more unbranched chains of amino acids. A typical protein contains 200-300 amino acids[1]. A very common technique that is used to separate biological molecules is called chromatography. Different chromatography procedures can be used to separate proteins into individual components[2].

Some common chromatography methods used to separate proteins are partition chromatography, ion exchange chromatography, and molecular sieve or gel filtration chromatography[2]. In partition chromatography, a mixture of molecules is separated based upon their relative affinity for two immiscible phases (the mobile phase and the stationary phase) [2]. Mobile phase is a buffer in which proteins are soluble and stationary phase is a column of beads containing pores.

Gel filtration chromatography which is used in our "In House Method" (IHM)[3, 4] uses size differences to separates proteins. In gel filtration chromatography a protein solution is passed through a column that is packed with semi- permeable porous resin. The resin has a range of pore sizes that determine the size of protein that can be separated with the column[2]. This is called the fractionation range or exclusion range of the resin.

In our IHM a Sephadex G-75[3,4] porous matrix made of polysaccharide beads through which the proteins can diffuse is used. It has pores that exclude molecules larger than 80 kDa[2]. The size of the pores in the matrix determines the rate at which proteins of various sizes diffuse and pass through the beads. Molecules under 80 kDa can enter the pores of the beads, which impede the movement of such molecules. The excluded, larger molecules simply go around the beads. Because these larger proteins are unable to enter the pores they pass quickly through the column in the spaces between the resin beads and elute first. Very small proteins and other low molecular weight substances that are below the exclusion range of the resin enter all the pores in the resin, and move through the column very slowly because they must pass through the entire volume of the column. These molecules pass through the end of the chromatogram in a "totally included peak"[2].

To separate a protein sample by gel filtration chromatography, the column must first be equilibrated with the desired buffer. This is accomplished by passing several column volumes of the buffer through it. Equilibration is an important step because the equilibration buffer is the buffer in which the protein sample will elute in a bioactive form. Next, the sample is loaded onto the column and allowed to enter the resin. Then more of the equilibration buffer is passed through the column to separate the sample and elute it from the column. Fractions (measured aliquots) are collected as the sample elutes from the column. Larger proteins elute in the early fractions and smaller proteins elute in subsequent fractions[2].

FABPs, Their Roles and Significance

The proteins of interest here are fatty acid binding proteins (**FABPs**). FABPs are cytosolic proteins that non-covalently bind long-chain fatty acids (LCFA)[5]. Despite many years of research, the precise biological function(s) of FABPs remain unclear[5,6]. The proposed functions can be summarized as follows: FABPs are involved in facilitating the uptake and intercellular transport of fatty acids, limiting ligand association with alternative binding sites within the cell, and modulating cell growth and differentiation[5,6].

FABPs were originally classified as Z proteins[7], a pre-existing class originally found to be a binding protein for organic anions, fatty acids and aminoazodyes. Because of the anion-binding activity of rat Z protein, it was initially thought that a single protein was responsible for all FABP activity[7]. An early hypothesis was that intracellular FABPs bind fatty acids in the cytoplasm and thereby prevent their nonspecific detergent effects on disrupting membrane function. Intracellular FABP levels are in the 0.2 -0.4 mM range in the tissues active in fatty acid metabolism[8].

Several different FABPs have been identified since then, each named after the tissue in which it was first discovered[5,6]. These include the heart, epidermis, adipose tissue, liver, and small intestine[1]. The amino acid sequences in FABPs exhibit considerable tissue specific variation[9]. FABPs are clam shell-shaped proteins consisting of two antiparallel β-pleated sheets of 5 strands apiece and 2 short α-helices forming the shell halves[10]. Rat liver FABPs are most extensively studied. They have a molecular mass of 14-16 kDa[6].

The initial binding of the LCFA to a FABP occurs between the ε-amino group on a FABP lysine residue and the carboxylate of the LCFA[10,11]. When lipids are digested,

they are broken down into glycerol and fatty acids, and these fatty acids bind to FABP. The term fatty acid identifies saturated and unsaturated monobasic carboxylic acids, and several series of substituted acids having carbon skeletons identical with normal saturated acids exist[12]. Fatty acids are important source of cellular energy and form the building blocks from which various cellular components are synthesized[13]. At least 60% of the long chain fatty acids found in the liver cytosol are bound to liver FABP[12].

My research primarily concerns liver FABP (**LFABP**) in rats. LFABP is structurally and functionally different from the other FABP types[6]. LFABP binds two fatty acids per molecule, whereas the other FABP types have a single fatty acid binding site[2]. LFABP may play a role as an intercellular acceptor of LCFA, by enhancing LCFA uptake and intercellular transport[14]. Several enzyme activities, especially enzymes involved in fatty acid metabolism, are stimulated by LFABP[14]. In contrast, intestinal fatty acid binding protein (IFABP) functions in the transfer of fatty acids from the intestine to the bloodstream [15]. LFABP binds these free circulating fatty acids as they pass through the hepatic capillary bed and helps transfer them into liver cells for metabolism[15]. Thus, IFABP contributes to plasma fatty acid content while LFABP removes fatty acids from the bloodstream[15,16].

Comparing the primary structure of the liver and intestinal FABPs indicates significant similarity as well as difference. In the amino-terminal domains there is a high degree of sequence homology, wherein 11 of the first 22 residues are identical[12]. The amino acid sequences exhibit considerable tissue specific variation[9]. Different FABP types in one species show amino acid sequence identities ranging from 20 to 70%[17]. Affinities, kinetics, and thermodynamics of FA interactions with FABP from different

tissues are quite different, but these characteristics are quite similar for FABPs from the same tissue of different species[9]. For example, while ΔH^o for adipocyte, heart, intestine, and liver from oleate binding range from -7 to -12 kcal/mol, single Alanine substitution in intestine generates proteins with ΔH^o ranging from -4 to -17 kcal/mol[9].

LFABP is an abundant protein constituting 5% of the cytosolic mass in the liver, whereas IFABP constitutes 2.5% of the cytosolic mass in the intestine[12]. LFABP has the broadest substance specificity among FABPs studied and has an aqueous diffusion mediated lipid transfer mechanism which differs from other FABPs[17,18]. LFABP is a tumour cell marker in hepatic cell malignancies, colorectal and hepatic carcinomas. LFABP is useful for detection of liver damage during and after transplantation[19, 20]. The primary function of soluble binding proteins such as FABP may be to enhance the diffusive fluxes of their ligands by reducing membrane binding[6].

LFABP takes part in dietary fatty acid transport from intestine to blood to liver, for bioenergy production and biosynthesis. Hence, dysfunction of either FABP or variation of the ratio of LFABP to IFABP[3, 4] may cause or be associated with altered lipid uptake/metabolism and lipid related diseases[21,22] such as diabetes, atherogenesis, and body fat increases often seen in old age. LFABP undergoes a conformational change upon fatty acid binding, changing its structure slightly as the fatty acid is integrated into the protein[6]. LFABP levels are known to be gender dependent, with females showing higher levels than males[10]. A low amount of LFABP relative to the amount of IFABP may correspond to a higher level of circulating fatty acids in the bloodstream, which could in turn contribute to lipid deposition in the blood vessels[3,4]. Atherosclerosis and hypertension may develop as a result[3, 4]. A significant difference of LFABP and IFABP

level/ratio was seen in different rats of both genders in the previous research in our laboratory[3, 4]. LFABP has been studied extensively, particularly in rats. Previous research shows that primary amino acid sequence of rat liver FABP is 82 % homologous to human liver FABP[5] and that rat and human Z protein exhibited similar binding affinities for various bile acids. It was also shown that administration of different drugs like clofiberate to rats increases LFABP levels [3,4]. It is thought that the increase in levels of FABP is due to retention of export proteins. LFABP is thought to covalently bond to the drugs, causing increase in levels of LFABP[3,4]. The specific mechanism for this process is not known.

1.2 In-House Method

The present in-house method (IHM) to measure fatty acid binding protein in rat liver at our laboratory was designed over ten years[3,4] with several different steps. It is complex, labor-intensive and takes a whole day's work (8 hours) to process liver and intestine samples from one rat. The most recent form of the IHM uses molecular sieve chromatography to separate a very complex mixture of proteins into simpler mixtures that allow assessment of the presence and content of LFABP in a test sample of liver cytosol. The IHM uses Sephadex G-75 polymer matrix in the chromatography column to separate FABP. The IHM requires 4 grams of tissue to be processed through a column chromatogram followed by 3 different assays to measure/quantitate LFABP (see section 1.2). The IHM has been used to identify and compare to controls FABPs present and their levels during life cycle change and as consequences of drug administration[3,4, 21]. The new method I am seeking to develop to quantitate LFABP by direct measurement of rose Bengal (RB) absorption in cytosol takes two hours to process and quantitate, using less

then one gram of tissue. The method is based upon a fluorescence enhancement process in which the FABP-rose Bengal complex in a sample is exposed to emissions of a 543.5 nm laser and produces a fluorescence spectrum. The new method uses the spectrochemical perturbation caused by binding of LFABP to rose Bengal dye for direct measurement of LFABP.

This direct assay method, when perfected, will change the IHM from a complex, labor-intensive, day long method to one that can be done in approximately two hours. I found the procedure to be specific for homogenous LFABP (hLFABP). The main potential interfering proteins in liver, hemoglobin, myoglobin and serum albumin, do not fluoresce or alter data taken with hLFABP. In addition, examination of LFABP-containing fractions from Sephadex G-75 shows that they contain about 90% of fluorescing material and the remainder of the fractions give about 10% as much fluorescence as seen with LFABP containing fractions. Several samples of cytosol that contain LFABP in known quantities were used in our new direct assay method and compared with Sephadex G-75 pool assay. It was seen that the enhancement of the emission peak was directly proportional to the amount of FABP present in the sample. The new assay method, when perfected, will facilitate uncovering FABP roles in health and disease, improving treatment of lipid related human diseases. This process will add to our ability to study FABPs and can further be used to study IFABP. This approach could possibly be extended to making a biosensor for detecting liver fatty acid binding proteins.

CHAPTER 2

OBJECTIVES

The objective is to develop a new method to directly measure LFABP from cytosol.

My work seeks to:

- Show that rose Bengal absorption enhancement by new method is proportional to LFABP concentration, with homogenous LFABP.
- Prove specificity of the LFABP effect by comparing it to hemoglobin, serum albumin and myoglobin.
- Demonstrate that cytosol proteins other than FABP do not contribute to the FABP effect.
- Show that direct FABP binding in cytosol gives results comparable to the data obtained with the IHM.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

The following instruments and chemicals were used in preparation of cytosol from liver, separation of FABP, assays, and direct measurement of FABP.

Instruments

Beckman L5-65B ultracentrifuge (Beckman Coulter, Inc., Palo Alto, CA), homogenizer apparatus (Arthur H. Thomas, Philadelphia, PA), IEC Centra-B Plus low speed centrifuge (International Equipment Company, Needham Heights, MA), Mettler Toledo electronic balance Model # AG104 (Mettler Toledo, Inc., Columbus, OH), Milton Roy Spectronic Genesys 5 (Milton Roy USA, Ivyland, PA) and Genesys 8 (Spectronic Instruments, Madison, WI) spectrophotometers, YSI-2700 Select Biochemistry Analyzer (YSI Life Sciences, Yellow Springs, OH), Ocean Optics spectrometer model # CHEM2000-UV-VIS (Ocean Optics, Inc., Dunedin, FL), and JDS Uniphase Neon Laser model # 1674MP (JDS Uniphase Corporation, San Jose, CA).

Solutions and Chemicals

TSM buffer, YSI-2357 buffer (YSI Life Sciences, Yellow Springs, OH), Sephadex G-75 resin, rose Bengal dye, and dextran-charcoal. The chemicals were purchased from Sigma-Aldrich Chemical Company (Sigma-Aldrich Corp., St. Louis, MO) unless otherwise indicated. All solutions are made in deionized water unless noted otherwise.

TSM buffer contains 0.050M trishydroxymethylaminomethane (tris base), 0.25 M sucrose, and 3.00 mM 2-mercaptoethanol (pH 7.5). It was the buffer used during all experiments unless otherwise indicated. It was made in 1 L deionized water as follows: 6.05 g Tris base, 85.5g sucrose and 0.30 ml mercaptoethanol is weighed and added to 750 ml cold deionized water (di-water) and are stirred until they dissolve. The pH of the solution is measured and is neutralized to pH 7.6, and then 250 ml of di-water is added. The solution is stirred in a cold box until it is cold, and the pH is checked again and restandardized if necessary[11, 12]. Sephadex G-75 polymer (Amersham Biosciences, Piscataway, NJ) was used to separate FABP from other proteins in cytosol by column chromatography[3,4].

40.0 mM rose Bengal solution was prepared by mixing 41 milligrams of rose Bengal in one liter of deionized water for the regular assay used in the In House Method (IHM[3,4]). The IHM uses 0.30 mL of 40 mM (~11 mM) rose Bengal solution for the assay. 10 mM rose Bengal per liter was prepared in deionized water for the direct assay method. Rose Bengal for the direct assay is made in YSI-2357 buffer. 13 milligrams YSI-2357 buffer per liter dissolved in deionized water. The use of the dye in all assay methods will be described in the second section of this chapter.

Dextran-coated charcoal was prepared by diluting 0.828 g of Na_2HPO_4 (Fisher Scientific Co., Pittsburgh, PA) with 200 mL of cold (4°C) deionized water and standardized to a pH of 7.4. Then, 1.0 g of Dextran T-170 (Fisher Scientific Co., Pittsburgh, PA) and 10.0 grams of Norit A decolorizing carbon (Fisher Scientific Co., Pittsburgh, PA) were added to the solution. Dextran-coated charcoal was used to remove rose Bengal dye which did not bind the protein.

All rats used in these studies were either purchased from Harlan-Sprague Dawley (Indianapolis, IN), or bred from animals purchased from Harlan-Sprague Dawley. The strains used were spontaneously hypertensive rats (SHRs) and Holtzman rats. The breeding and housing of all rats was in the University of Dayton animal facility. Strain differences, diets, and drugs including clofibrate (Ayerst Laboratories, Collegeville, PA), and fenofibrate (Abbott Laboratories, North Chicago, IL) were used as described elsewhere[11,12] to cause the variations of FABP levels denoted in the results section of this thesis.

3.2 Routine In-House Method

Sacrificing the Animals and Preparing Cytosol

In this method, devised and refined over ten years in our laboratory[3,4], all animals used were killed by decapitation after their weights were recorded. Then, their livers were removed, and placed on ice. After weighing each liver, about 4 grams representative samples were collected. Each sample was homogenized in a glass tube (Potter-Elvhjem homogenizer Bellco Glass, Inc., Vineland, NJ) with 1 mL of TSM buffer per gram of tissue[3,4]. Each homogenate was next poured into a centrifuge tube, placed in a Beckman fixed-angle 40-centrifuge rotor (Beckman Coulter, Inc., Palo Alto, CA) and centrifuged (105,000 x g) for 60 minutes at 2^{o} C. After each centrifugation, the supernatant (cytosol) was separated from the sediment, and its volume was measured and recorded.

Sephadex G-75 Chromatography

A 1.25 x 70 cm column was prepared for use with each cytosol sample prior to each experiment[11, 12]. The cytosol from one liver sample was pipetted into the top of the column; the column was run until the sample (cytosol) entered the polymer matrix. 6 ml

of TSM was added to the column, and a TSM drip was applied to elute the column. A fraction collector collected sixty aliquots of 1.5 mL (fractions) over 2 to3 hours. All the proteins are eluted at different times and into different fractions. The tubes were then assayed to check protein content and LFABP.

FABP Measurement

The fractions collected were subjected to three assays for quantitation. These were the A-280 column assay for protein content, the A-560 column assay for the LFABP content and the pool assay which quanitated tissue LFABP content. The A-280 and the A-560 assays which utilized the fractions collected from G-75 columns will be called **Reference Assays** to differentiate them from the pool assay which uses pooled column fractions shown to contain FABP by the A-560 column assay.

Assay Methods

A-280 Reference Assay: 0.1 mL of sample was removed from chosen fractions from the G-75 column. They were each mixed with 0.9 mL of ice cold TSM buffer in separate test tubes and read in the Milton-Roy spectrophotometer at 280 nm. Absorbance readings were graphed against fraction numbers as shown in Figure 1. The resultant large peak on the graph represents the majority of non-FABP proteins. The FABPs, which elute near the end of the chromatogram due to their small size, are found along the downward slope of the curve.

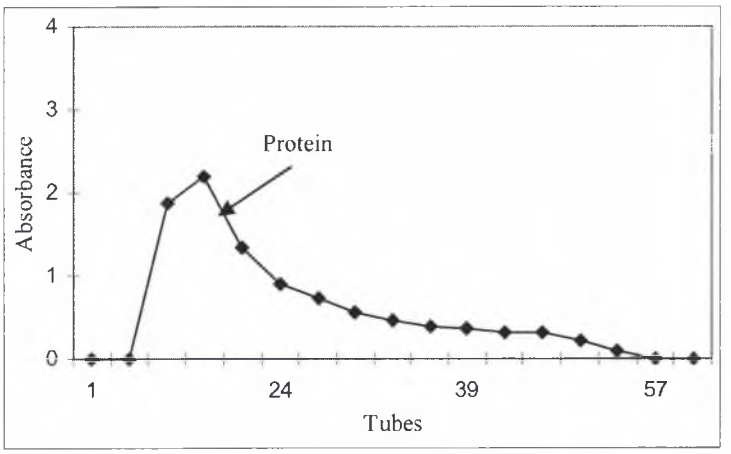


Figure 1: 280 nm absorbance from A-280 assay

A-560 Reference Assay: This assay uses the same samples from the column fractions as the 280 assay. First, 0.1 mL of each sample is mixed with 0.75 mL TSM and 0.3 mL of 40 mM rose Bengal. The mixture was allowed to sit on ice for five minutes. Then, 0.15 mL of Dextran-charcoal was added and the mixture was kept on ice for eight minutes. Next, the samples were centrifuged in Centra-B Plus centrifuge for eight minutes, and finally the absorbance was measured and graphed as shown in Figure 2. After graphing the absorbance of 280 nm and 560 nm assays, the results were analyzed. The 280 nm peak confirmed that there are proteins present. The first 560 nm assay peak identifies most cell proteins. The second 560 nm peak shows which fractions contain FABP. The graph is plotted for two different Y-axis because they are read at different wavelengths and show different absorbance.

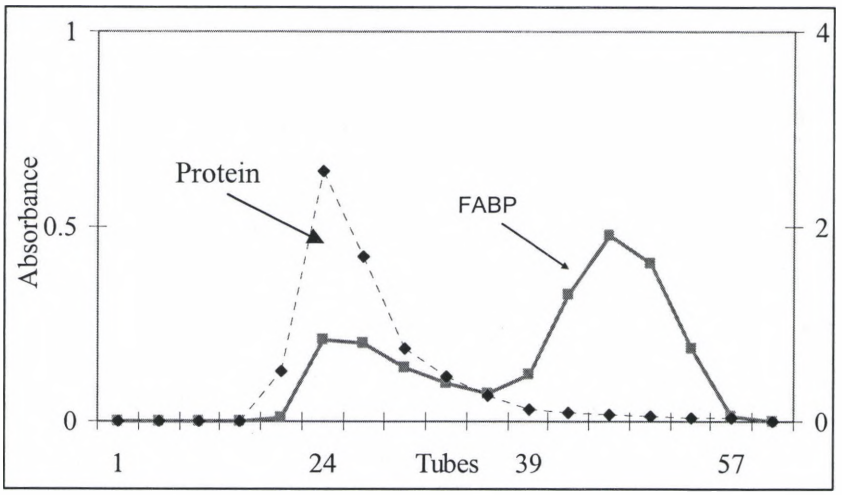


Figure 2: A-560 assay indicating the fractions containing FABP

Pool Assay (Reference): The fractions that contained FABP were pooled. Five samples of the pool were then diluted with TSM for assay as shown in Table 1 [3,4].

Table 1: Pool Assay to quantitate FABP by IHM

Tube Number	0	1	2	3	4	5
FABP pool (ml)	0.000	0.050	0.100	0.200	0.400	0.600
TSM (ml)	0.750	0.700	0.650	0.550	0.450	0.350

Six tubes were filled as shown. Following TSM addition, rose Bengal was added to each tube, the tubes were vortexed and allowed to stand on ice for 5 minutes, and Dextran charcoal was added. The mixtures were kept on ice for eight more minutes and centrifuged in the Centra-B Plus centrifuge for eight minutes. The supernatants were drawn off and read at 560 nm[3,4].

3.3 Calculations

A series of calculations were used to obtain the amount of FABP in milligrams per gram liver. First, the absorbance read for the pool assay was divided by the volume of the pool to obtain absorbance per milliliter.

$$\text{Absorbance / mL pool} = \frac{\text{Absorbance 560}}{\text{Pool volume in sample}}$$

Then the readings were averaged, and the total absorbance in the pool (in red binding units, RBU) was calculated by multiplying the average by 0.67, a factor devised to correct for changes in assay volumes in 1997 in our laboratory[3,4].

$$\text{Total RBU} = \frac{\text{(average RBU/mL) X (Pool Volume)}}{0.67}$$

The milligrams of FABP per gram tissue was calculated by dividing the total red binding protein units by 3.69 (the product of assay correction factor 2.86 and 1.29, the absorption of 1 mg pure LFABP in the assay[3,4]) and the weight of the tissue chromatographed.

$$\text{mg FABP/g liver} = \frac{\text{Total RBU}}{\text{(3.69) X(corrected weight)}}$$

CHAPTER 4

DIRECT ASSAY METHOD

4.1 Experimental Setup for Direct Assay

The assay developed here uses cytosol directly. I am developing this assay in an effort to replace the much slower, more complicated IHM. This assay uses the YSI-2700 Select Biochemistry Analyzer which is added to an Ocean Optics Spectrometer (model # CHEM2000-UV-VIS). A JDS Uniphase Neon Laser (model # 1674MP) of wavelength 543.5 nm is used as a light source to produce the emission as shown in Figure 3.

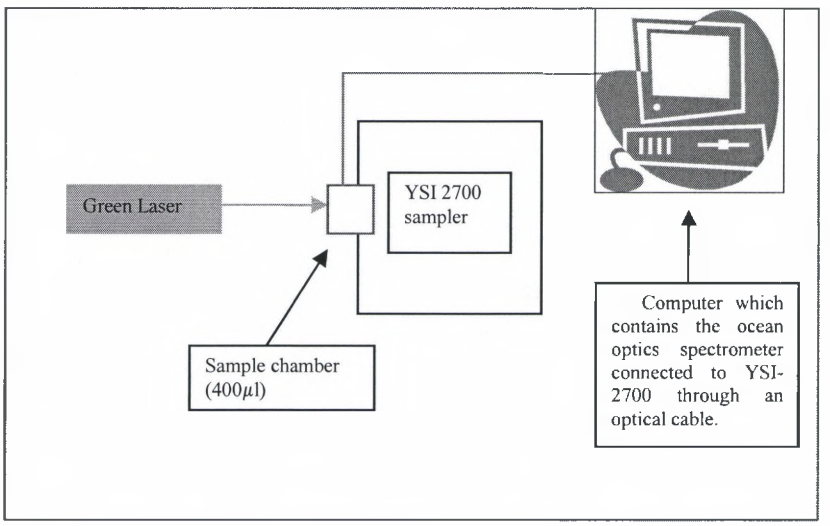


Figure 3: Experimental setup for the direct assay

The YSI-2700 Select Biochemistry Analyzer consists of a sampling chamber (400 μL volume) and a sipper which injects the test solution into the chamber. The sipper injects 25 μL of sample per injection. An optical cable is attached to the sampling chamber at right angles to the laser beam which is the light source and a quartz mirror is placed

opposite to the optical cable so any light reflected away from the optical cable is redirected back to it to provide a strong signal. The optical cable is connected to the spectrophotometer. Initially the sampling chamber is flushed with 10 mM rose Bengal dye and the emission peak of the dye is recorded on the computer via the Ocean Optics software supplied with the spectrophotometer. The analyzer is designed to flush the chamber three times, every time the flush button is used. Next, cytosol is injected into the sampling chamber which contains rose Bengal dye and mixed quickly by the instrument. Liver FABP binds rose Bengal and the change in the emission peak is recorded by the software. The binding of LFABP and rose Bengal dye enhances the emission peak height. This process is repeated with several different samples of diluted cytosol (1:1, 1:3, 1:5, 1:8 and 1:10 dilutions), and the emission peak is recorded for each sample. All the samples are diluted in TSM buffer. The sampling chamber is flushed with rose Bengal dye before and after injecting the cytosol because the rose Bengal emission peak is used as a reference to record the change in emission for different dilutions of cytosol. The recorded emission peak is plotted on an Excel spreadsheet and the data are related and compared with the results obtained using the IHM.

4.2 Data Plotting and Calculations

The emission spectrum is recorded and stored on the computer using the Ocean Optics software. The recoded data can then be opened in an Excel spreadsheet. The data gives the wavelength from 178 nm to 879 nm and the respective intensity of the emission peak next to it as shown in the sample data below. The data are plotted using the XY (scatter) chart type. The emission peak of rose Bengal and the enhanced emission peak

due to FABP binding are plotted. Then the highest point of both the emission peaks are recorded and the highest point of the enhanced emission peak is subtracted from the highest point of the rose Bengal emission peak which gives the actual effect, or effective peak height, due to the presence of FABP.

The same procedure is repeated for each concentration of FABP or cytosol used and then the concentration of FABP in the chamber is plotted against the effective peak height. It was seen that the concentration of FABP was proportional to the enhancement of the emission peak. The data are shown below in Table 3 and plotted in Figure 4.

Table 2: Sample emission peak data

Wavelength (nm)	Intensity (counts)
178.4	0
228.7	0.90
278.5	-0.90
328.8	-1.4
378.0	0.10
428.7	1.3
478.8	2.9
528.9	7.4
578.8	331
628.8	123
678.5	16

Table 3: FABP concentration against effective emission peak height

Dilution ratio	[FABP]	Enhanced Emission Peak	Rose Bengal Emission Peak	Effective peak Height
1:1	12.5	602	231	369
1:3	8.3	474	222	251
1:5	5.0	389	232	156
1:10	2.5	318	244	73

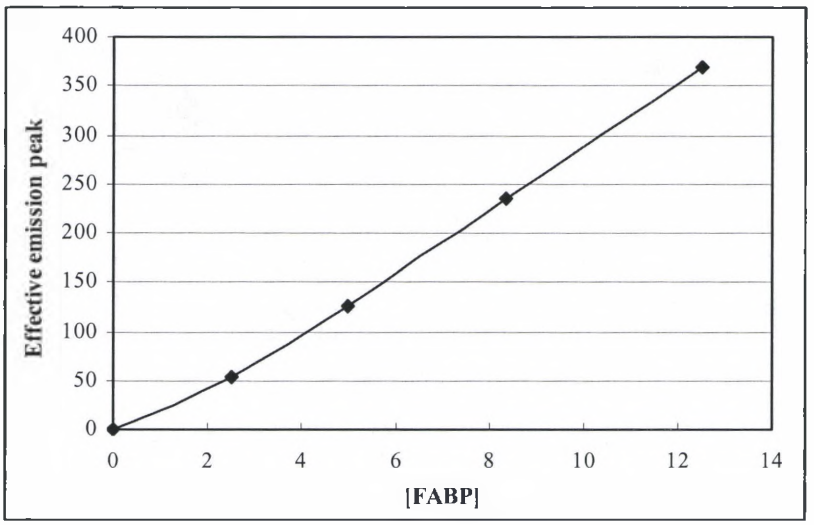


Figure 4: Effective emission peak plotted against the FABP concentration

CHAPTER 5

Results

5.1 Rose Bengal

10 mM rose Bengal was made in YSI-2357 buffer, as explained in Material and Methods discussion. The rose Bengal solution was connected to a pump which allows the YSI-2700 Select Bioanalyzer to use it to flush the sampling chamber through a sipper. The sampling chamber is flushed three times in between uses. The laser and the equipment are set as explained in Section 2.1. The emission peak of the rose Bengal solution in the sampling chamber is then recorded on the computer using Ocean Optics software. The recorded spectral data are then plotted in an Excel spread sheet. The intensity of the emission is then plotted against the wavelength (e.g. Figure 5).

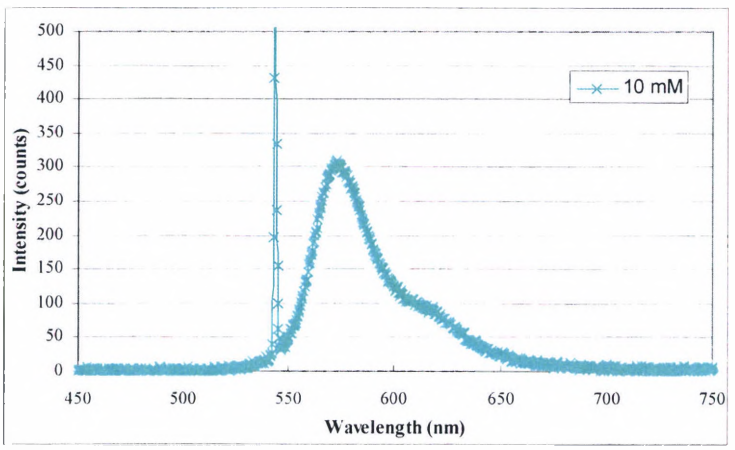


Figure 5: Emission peak of 10 mM rose Bengal solution

Different concentrations (10 mM, 20 mM, 50 mM and 100 mM) rose Bengal solutions were made in YSI-2357 buffer. These solutions were used to flush the sampling chamber as already explained. The solutions are used one after the other and the emission peak of each solution is recorded. The data are then stored and plotted in Excel. As shown in Figure 6, higher concentrations of rose Bengal gave smaller emission peaks since the solutions were hazy allowing less light to penetrate through to the optical cable. The results show that 10 mM rose Bengal solution is the optimum concentration to be used in our direct assay method.

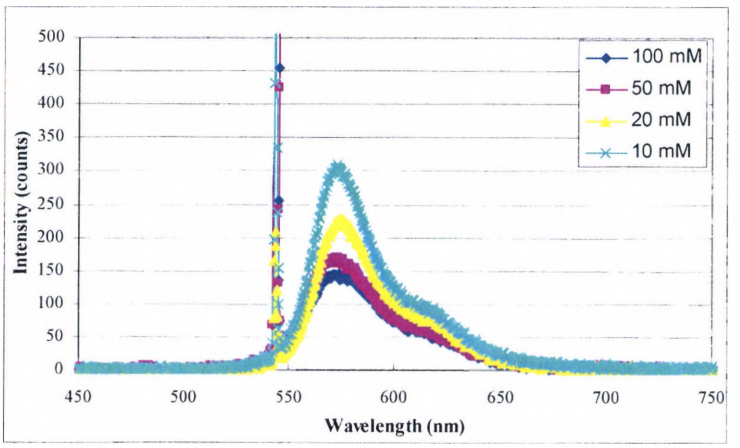


Figure 6: Different concentration of rose Bengal used to check the concentration which is optimum to use

Initially partial purified LFABP (9.5mg/ml) was used. The LFABP was purified at Dr. Singer's laboratory using different chromatography columns [G-75, A-50 and DEAE-Sephadex A-50[11, 12]]. The LFABP was stored in 500 µl aliquots to minimize FABP breakdown on repeated thawing and freezing. 10 mM rose Bengal solution is used to flush the sampling chamber and the emission spectrum is recorded. The LFABP (9.5mg/ml) is placed in the sample holder from which the sipper injects 25 µl samples into the sampling chamber. The injected LFABP is mixed with the rose Bengal solution

present in the sampling chamber by a magnetic stirrer. The LFABP binds with rose Bengal causing an enhancement in the rose Bengal emission peak. The enhanced emission peak is then recorded and plotted (e.g. Figure 7).

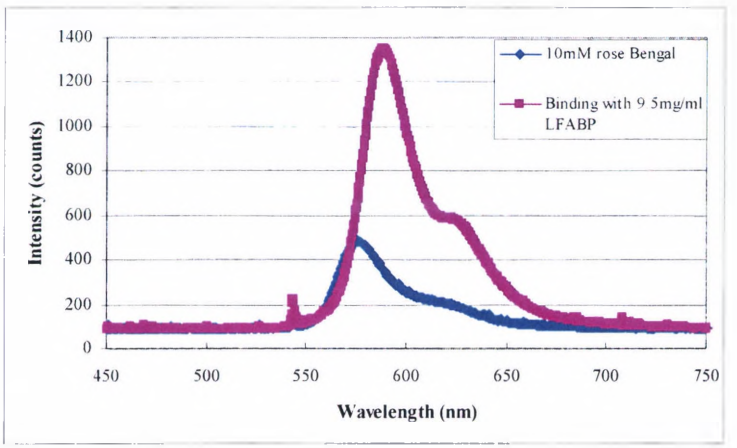


Figure 7: Enhanced emission peak due to binding of 9.5 mg/mL LFABP with 10 mM rose Bengal solution

Several different concentrations of LFABP (9.5mg/mL, 4.75mg/mL, 2.4mg/mL, 0.95mg/mL, and 0.095mg/mL) were used, consecutively, in the direct assay. Initially 10 mM rose Bengal solution was used to flush the chamber and then 9.5mg/mL LFABP was injected into the sampling chamber. The emission peak of rose Bengal and the enhanced emission peak due to LFABP binding with rose Bengal were recorded. The sampling chamber is always flushed with rose Bengal solution before and after injecting LFABP. The LFABP concentration cannot be diluted below 0.095mg/mL because at this level no change in the emission peak occurs. Figure 8 shows that the emission peak ratio, which is the difference between the emission peak height due to LFABP and rose Bengal divided by the emission peak ratio of rose Bengal alone shows a linear profile for hLFABP.

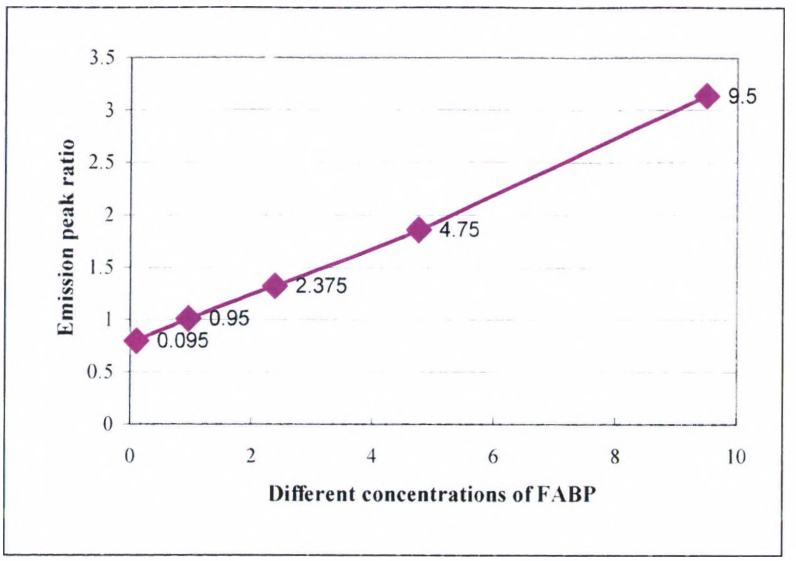


Figure 8: Effect of different concentrations of LFABP on the emission peak of 10 mM rose Bengal solution

$$\Delta E, \text{ Emission peak ratio} = \frac{\text{Enhanced emission peak height due to LFABP - RB binding}}{\text{Emission peak height of Rose Bengal alone}}$$

To check the specificity of the direct assay method, bovine serum albumin (BSA), myoglobin and hemoglobin were used in the processes described earlier for FABP. The serum albumin was used first. 10mM rose Bengal solution is used to flush the sampling chamber and the emission spectrum was recorded (Figure 9). Then BSA was placed in the sample holder from which the sipper injects 25 μL samples into the chamber. The BSA binds rose Bengal causing the emission peak to quench slightly, as shown in Figure 9. The quenched emission peak is then recorded and plotted. Three different concentrations (0.1 mg/mL, 1 mg/mL and 10 mg/mL) of BSA were used as explained earlier with LFABP. As shown in Figure 9, 0.1 mg/mL BSA quenches the emission peak and 10 mg/mL BSA causes both peak shifting and quenching.

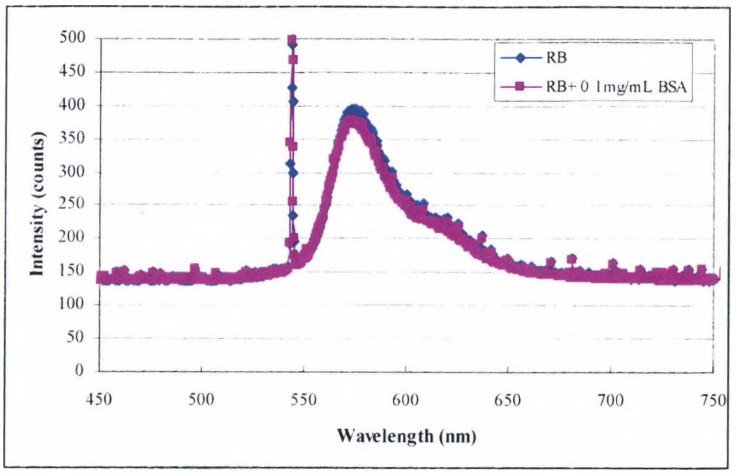


Figure 9: Effect of 0.1mg/mL BSA on the emission peak 10 mM rose Bengal

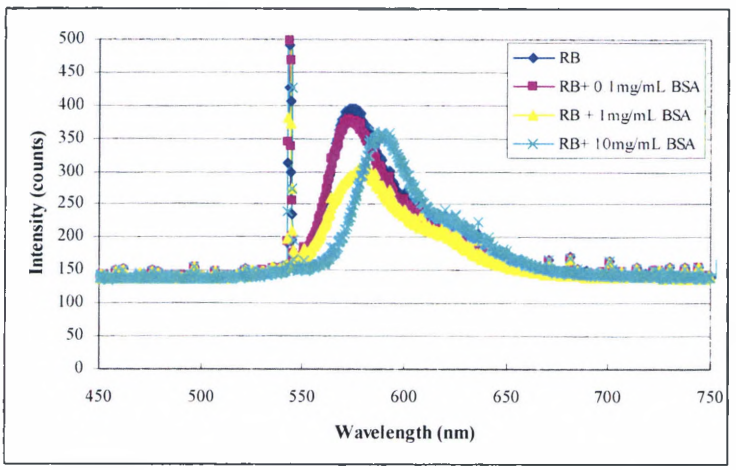
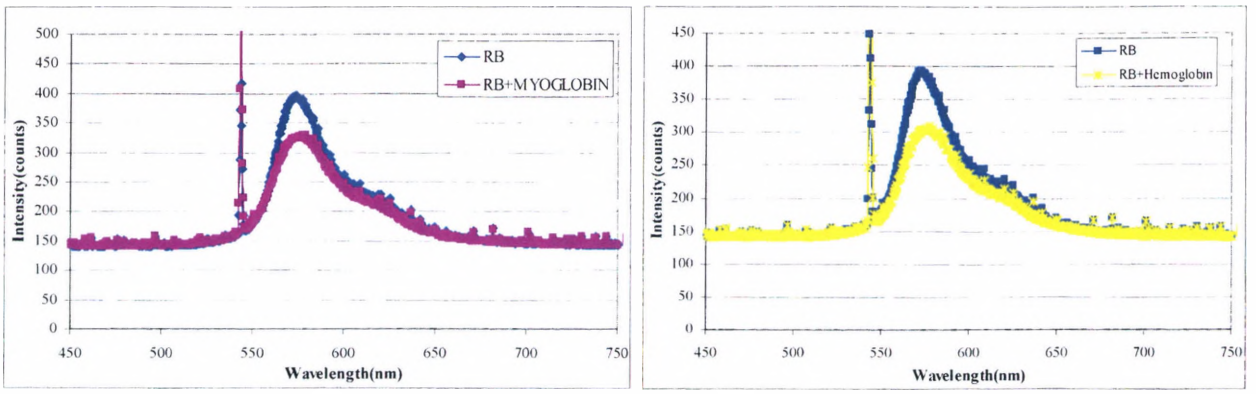


Figure 10: Effects of different concentrations of BSA on the emission peak of 10 mM rose Bengal solution

The same process was repeated for myoglobin and hemoglobin. When 10 mg/ml myoglobin and hemoglobin was used it was seen that both quench the rose Bengal emission peak (Figures 11a and 11b). The same results were seen with lower concentrations of myoglobin and hemoglobin. So, all three proteins quenched the emission peak whereas LFABP enhanced the emission peak.



(a) (b)

Figure 11: Effect of 10 mg/ml Myoglobin (a) and Hemoglobin (b) on the emission peak of 10 mM rose Bengal

Next, effects of BSA, myoglobin, and hemoglobin on the enhanced emission due to presence of LFABP were tested. Initially, 9.5 mg/mL LFABP was injected into the sampling chamber with help of the sipper and the enhanced emission peak was recorded. Then we compared this data to those with mixtures of the LFABP and BSA (0.1 mg/ml, 1 mg/ml, and 10 mg/ml). BSA had very little effect on the enhanced emission peak of LFABP (Figure 12).

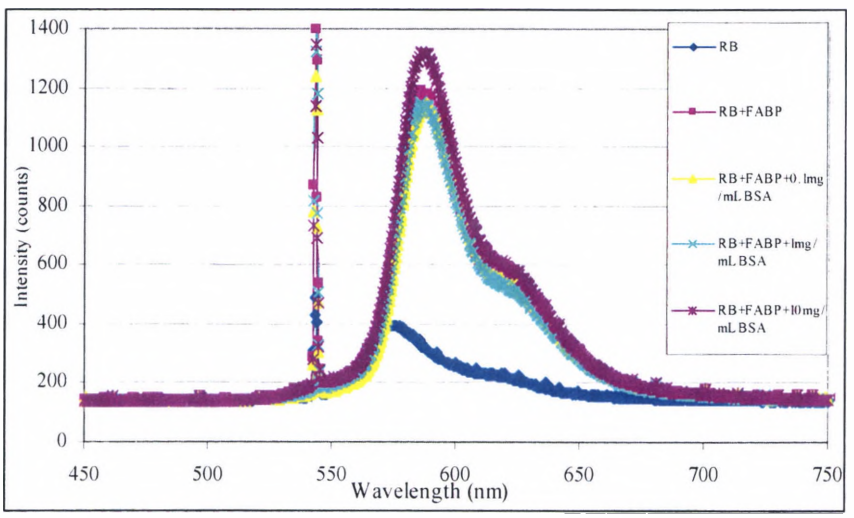


Figure 12: Effect of different concentrations of BSA on the enhanced emission peak of 10 mM rose Bengal

The same process was repeated to see the effect of different concentrations of LFABP on the quenched emission peak due to binding of 0.1 mg/mL and 10 mg/mL BSA with rose Bengal. The results were recorded and plotted as shown in Figure 13. The figure shows that the presence of 0.1 mg/mL BSA does not cause any effect of the enhancement of emission peak due to the binding of LFABP with the dye.

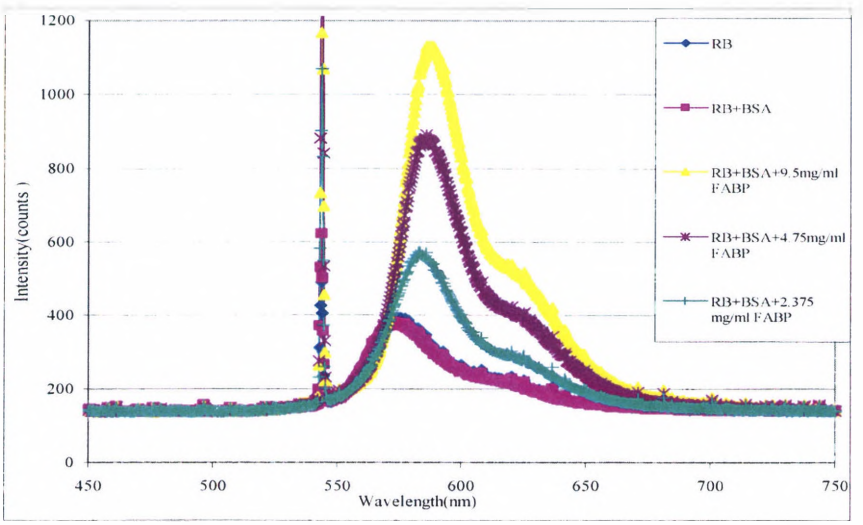


Figure 13: Effect of different concentrations of FABP on the quenched emission peak using 0.1 mg/mL BSA

The process was repeated for 1mg/mL and 10 mg/mL BSA with different concentrations of LFABP. The quenched emission peak due to binding of rose Bengal dye with 1 mg/mL BSA is used as a control between the different concentrations of LFABP and similarly for 10 mg/mL BSA. The control is the reference peak. The presence of 1 mg/mL and 10 mg/mL BSA did not affect the enhancement of the emission peak as shown for 10 mg/ml BSA in Figure 14.

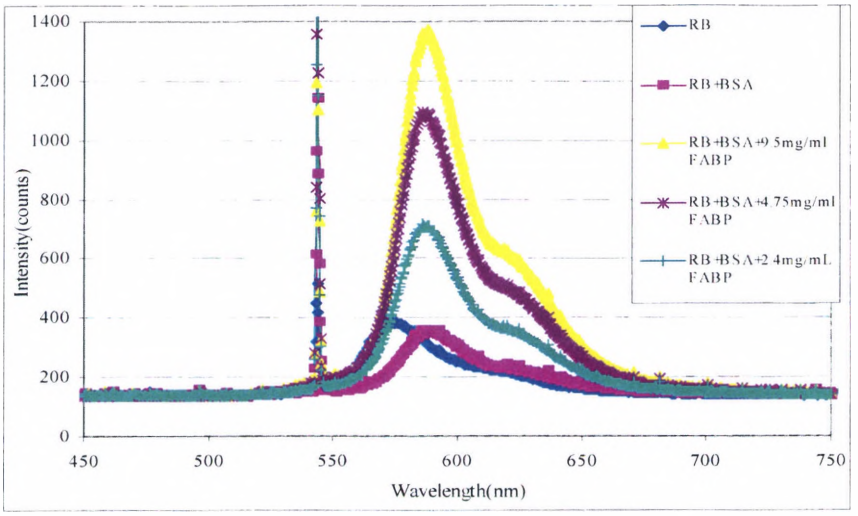


Figure 14: Effect of different concentrations of FABP on the quenched emission peak due to 10 mg/mL BSA

The process was also repeated with myoglobin and hemoglobin to see their effects on the emission peak of rose Bengal. Once again 0.1 mg/ml, 1 mg/ml, and 10 mg/ml samples of myoglobin and hemoglobin were compared. As shown in Figures 15 and 16, myoglobin had no effect on LFABP binding to rose Bengal; LFABP enhanced the emission peak normally in the presence of myoglobin.

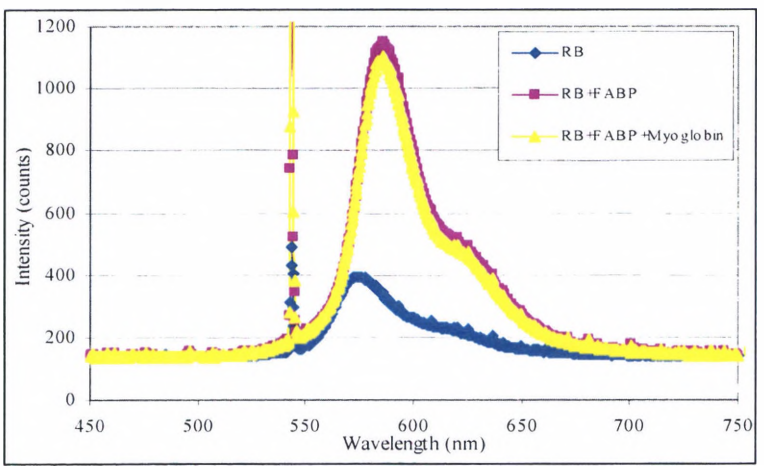


Figure 15: Effect of 10 mg/ml myoglobin on the enhanced emission peak by 9.5 mg/ml LFABP

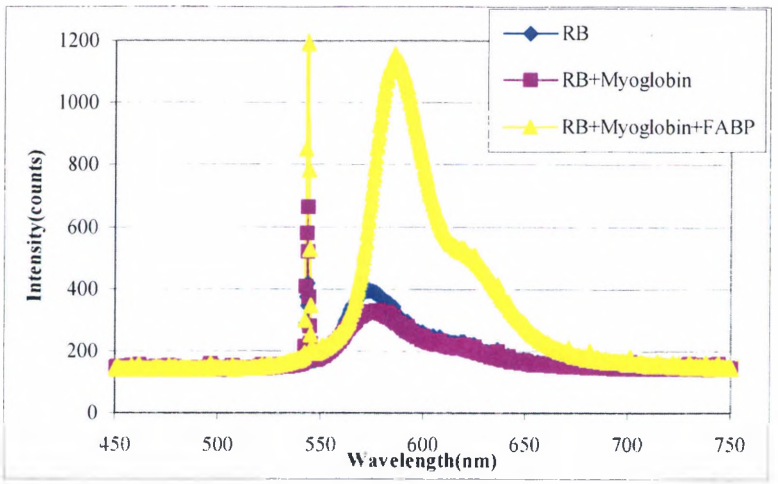


Figure 16: Effect of 9.5 mg/ml LFABP on the quenched emission peak by 10 mg/mL myoglobin

The experiments shown in Figure 15 and 16 are done one after the other in a short period of time. The LFABP preparation used in both experiments was the same and the emissions can be compared. The heights of the enhanced emission peak caused by LFABP were found to be similar in both cases. The same result was seen in Figure 17 when the process was repeated for hemoglobin.

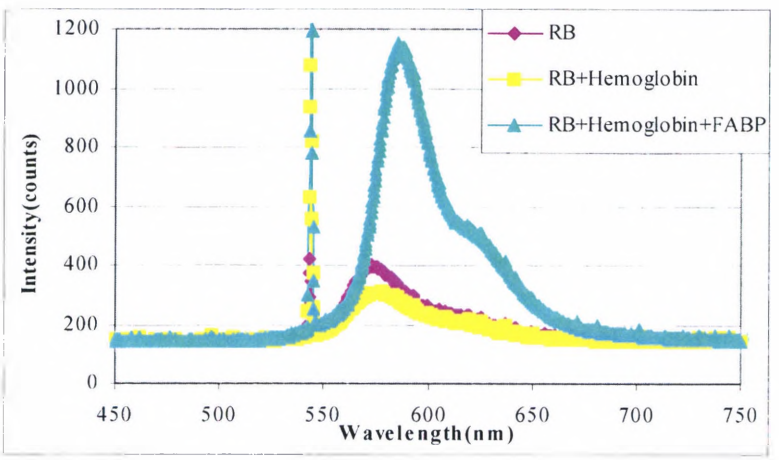


Figure 17: Effect of 9.5 mg/ml LFABP on the quenched emission peak due to 10 mg/mL hemoglobin

Table 4: Sample calculation of [FABP] from the graph for myoglobin

FABP concentration used	Emission peak ratio for Myoglobin	FABP concentration calculated from the graph
9.5 mg/ml	3.08	9.2 mg/ml
4.75 mg/ml	1.87	4.42 mg/ml
2.375 mg/ml	1.33	2.31 mg/ml

The data of Figures 9 through 17 qualitatively illustrate that the direct assay method is specific to LFABP, and is not strongly influenced by the presence of other similar potentially interfering species. The data of these figures can also be investigated quantitatively using the relation between emission peak ratio and LFABP of Figure 8. Table 4 compares the known LFABP concentrations with those calculated using measured emission peak ratios in the presence of myoglobin and the relation between this parameter and LFABP concentration shown in Figure 8. As this comparison indicates, the direct assay method provides reasonable estimate of the LFABP concentration.

Next, the samples collected from the column chromatography in our IHM were used in our direct assay method. As explained previously IHM produces 60 samples of cytosol eluted from the column. The A-560 assay indicated the tubes which contain the FABP and tubes that lack FABP as shown in Figure 18. The tubes which contain FABP and which lack FABP are pooled separately and are used in the direct assay method one after the other. The YSI-2700 Select Biochemistry Analyzer was then used to study the emission peak produced by interaction of rose Bengal with LFABP.

It was seen that the FABP in "FABP pool" detected by IHM caused enhanced emission peak, while the non-FABP containing pool did not enhance the peak (Figure

19). Since the pool collected from the Sephadex G-75 column tubes was dilute, we concentrated it by 8 fold using an Amicon stirred cell model # 8400 before using it in the direct assay. The pool lacking FABP contained most other proteins (e.g. serum albumin and hemoglobin). Myoglobin was present in the pooled FABP-containing fractions.

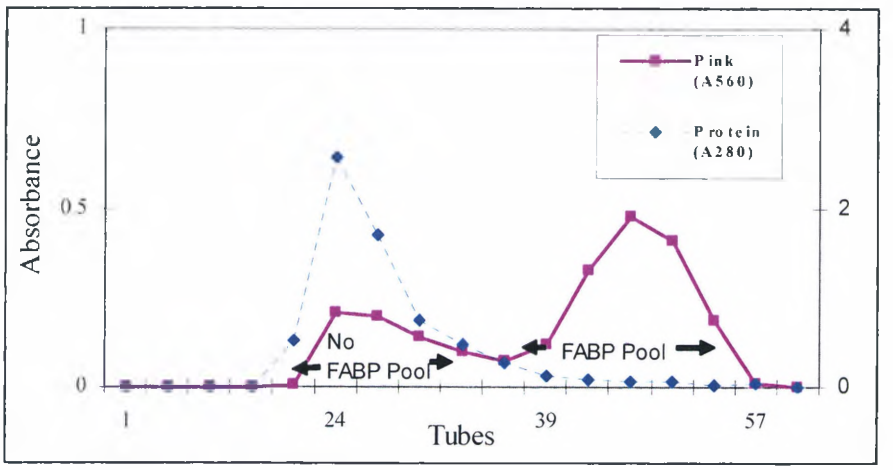


Figure 18: Sample Sephadex G-75 Chromatogram from the IHM

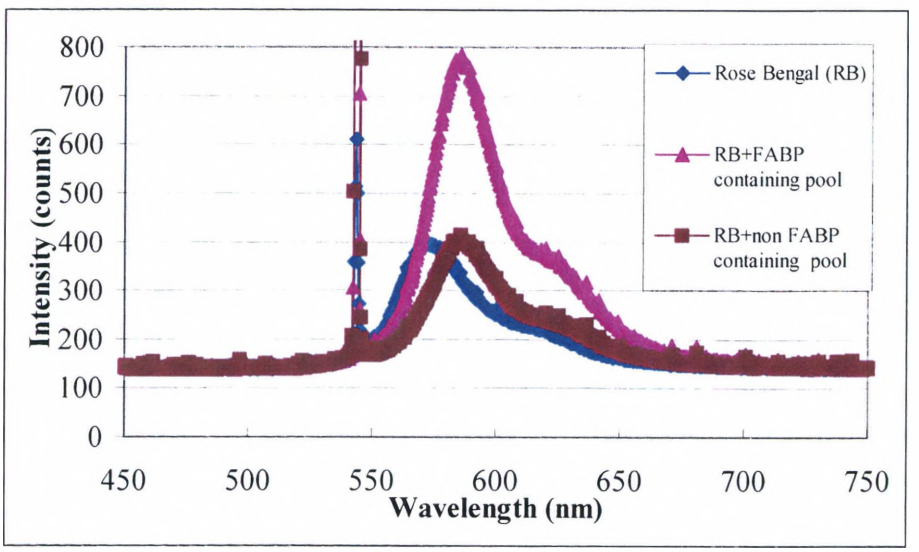


Figure 19: Effect of FABP and non-FABP containing pool on the emission peak of rose Bengal

In the final step to quantitate LFABP by the direct assay method, cytosol was used directly. The previous experiments had shown that the direct assay method was specific for FABP and once the specificity of the assay was clear cytosol was tested in the direct assay. Cytosol was prepared as explained in Section 1.2. When injected into the analyzer the LFABP present binds with rose Bengal present in the sampling chamber causing enhancement in its emission peak (Figure 20). The magnitude of enhancement depends upon the amount of FABP present in the tissue. Previous research conducted in our laboratory[3, 4] and by others[21] showed that animals on special diets like clofiberate and fenofibrate will have higher amounts of FABP in there tissue hence causing larger enhancement (Figure 21).

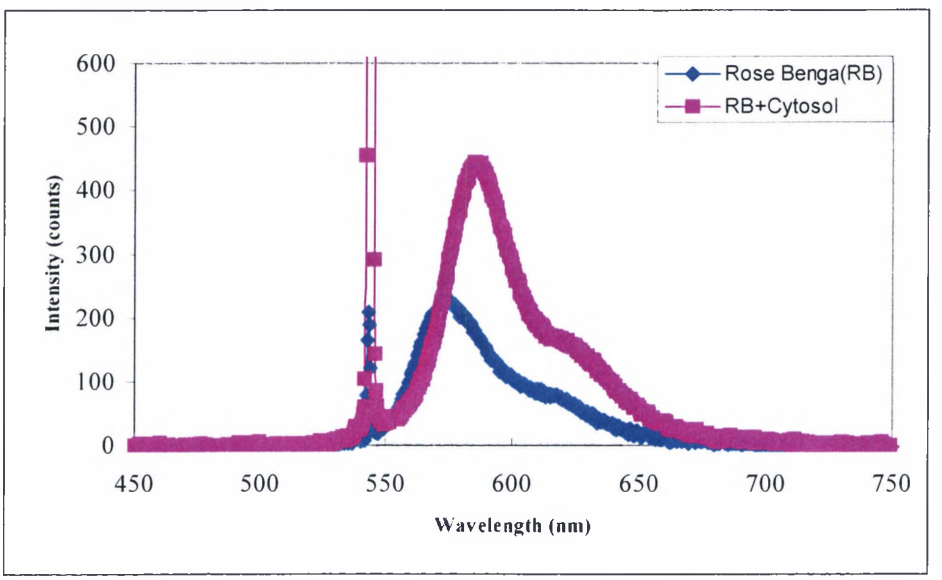


Figure 20: Effect of cytosol on the emission peak of 10 mM rose Bengal

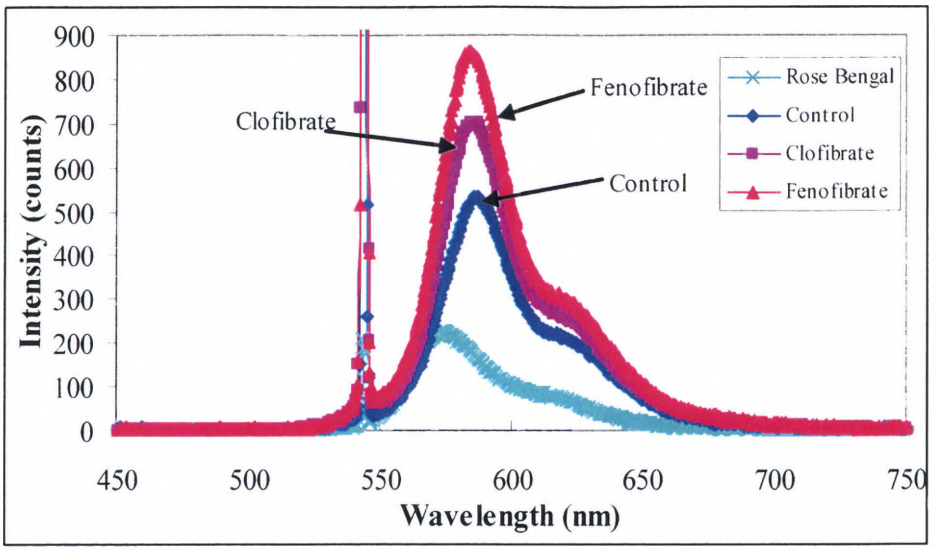


Figure 21: Enhanced emission peak of cytosol from animals on different diets

All the animals which were on special diets and the control animals were plotted against the data take by the IHM. The IHM gives the amount of FABP present per gram tissue. Figure 21 shows that animals on special diets which have higher amounts of FABP showed higher effective peak heights and they grouped together while the control animals which had lower amounts of FABP formed a second group at lower levels.

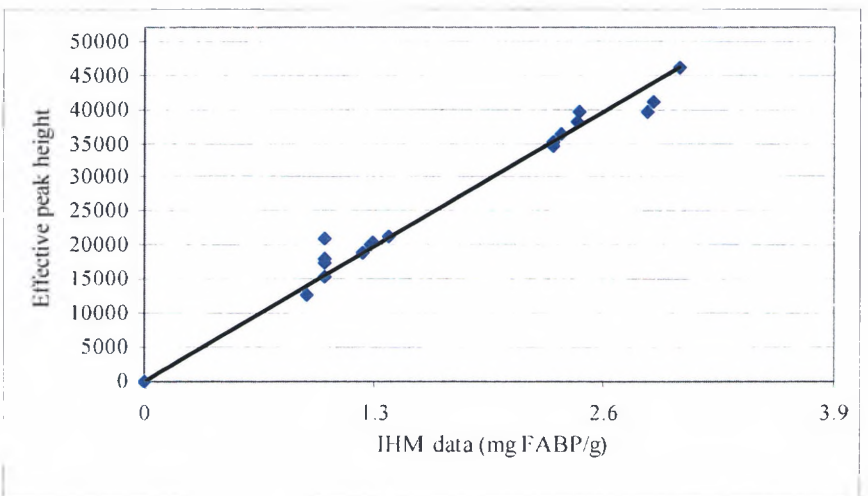


Figure 22: IHM data (mg FABP/g tissue) plotted against the effective emission peak height of the direct assay method

5.2 Calculations

The effective peak height was calculated as explained in Chapter 2 where the enhanced emission peak data was separated from the emission peak of rose Bengal to get the effective emission peak due to the presence of LFABP. The effective emission peak of LFABP is then multiplied with the dilution ratio and calculated for 1mL solution by multiplying it with 40 since only 25μL of sample was injected into the sampling chamber.

Table 5: Effective emission peak height calculated for 1mL sample

Dilution ratio	Effective peak height	Effective peak height for 1mL sample
1:2	396.94	396.64 x 2 x 40 =31755
1:3	251.89	251.89 x 3 x 40 = 30226
1:5	156.64	156.64 x 5 x 40 = 31328
1:10	73.94	73.94 x10 x 40 = 29576

As shown in Table 5 effective peak heights are calculated for different animals. The effective peak height is then plotted against the IHM data. Table 6 shows the comparison between the IHM (mg FABP/g tissue) data and the effective peak height calculated. The data in Table 6 is plotted in Figure 22.

Table 6: Comparison of IHM with direct assay method

IHM (mg FABP/g) data	Effective peak height for 1 mL sample
0.92	12745
1.02	15240
1.02	17423
1.02	17884
1.02	20867
1.24	19000
1.28	19977
1.29	20307
1.38	21267
2.31	34971
2.31	34537
2.36	35170
2.45	36235
2.46	38176
2.85	39481
2.88	41200

CHAPTER 6

DISCUSSION

The present study focuses on developing a new assay for measuring fatty acid binding proteins directly from cytosol. The new method uses the spectrochemical perturbation that is caused by the binding of LFABP to rose Bengal dye for direct measurement of LFABP. The previous research conducted by others[6] and our own IHM[3,4] used spectroscopic and binding properties of FABP for identifying and measuring fatty acid binding proteins in cytosol. In the previous research conducted by others and our own IHM, the FABP is subjected to chromatography on a Sephadex G-75 equilibrated with buffer. Elution fractions containing FABP are assayed for radioactivity[6] and absorbance at 280 nm[3, 6] and 560 nm[3, 4]. Two major peaks are obtained, the second corresponding to FABP. These methods are complex and labor intensive.

The results of the present study in developing the new assay method aimed to make the process simple, while obtaining similar results. The new direct assay method has been tested for specificity for homogenous LFABP[3, 4] (hLFABP) and LFABP in presence of other proteins (non-homogenous). Homogenous LFABP was obtained by Thomas Burnett in our laboratory[3, 4] by applying cytosol to different chromatography columns (G-75, A-50, and DEAE-Sephadex A-50[3, 4]).

The non-homogenous LFABP is found in cytosol which contains other potential contaminants[3] along with FABP. As shown Figure 7, homogenous LFABP interacts with rose Bengal dye causing an enhancement in its emission peak. When different concentrations of hLFABP were used, it was seen (Figure 8) that the response curve was linear and the new method was able to detect hLFABP within a concentration range from 9.5mg/mL to 0.095mg/mL as evidenced by the change in the enhancement of the emission peak of rose Bengal. This figure shows that the direct assay method is sensitive enough to measure FABPs at low concentrations typical of those in the physiological range.

The direct assay method also shows (Figures 10, 11) that different proteins such as bovine serum albumin, myoglobin, and hemoglobin do not fluoresce and bind to rose Bengal, quenching its emission peak. A number of proteins which bind fatty acids and other organic anions are present in the cytosol out of which serum albumin, myoglobin and hemoglobin have nucleotide sequence homologies with FABP and have similar binding properties. Serum albumin is the most abundant protein in the circulatory system and makes up a large percentage of protein in cytosol. These proteins which are components of cytosol and their presence may interfere with the assay to quantitate LFABP if the assay is not specific, as they can interfere with the IHM unless the G-75 chromatogram is carried out. This result shows that these proteins which are components of cytosol do not cause the same effect as FABP on the emission peak of rose Bengal by the direct assay method.

The specificity of the assay was determined from results in Figures 14 through 17 which show that the presence of other potential contaminants[3] of cytosol like myoglobin,

serum albumin, and hemoglobin do not interfere or alter the data taken with hLFABP. Initially these proteins were tested against the enhanced emission peak of rose Bengal due to the presence of LFABP, and it was seen that the other proteins had very little effect on the enhanced emission peak (Figures 12, 15, and 17). Next, LFABP effect on the quenched emission peak due the presence of proteins like serum albumin, myoglobin, and hemoglobin was tested. It was seen that the LFABP enhanced the quenched emission peak normally. These results show that our assay is specific for LFABP.

In the IHM[3, 4] the chromatogram collected from the Sephadex G-75 column shows (Figure 18) two major emission peaks for the A-560 reference assay. As explained in Section 1.2 the Sephadex G-75 polymer matrix separates proteins on the basis of molecular size. The proteins which are of higher molecular size such as serum albumin and hemoglobin pass around the matrix covering lesser column volume, eluting earlier than the smaller molecular size proteins such as myoglobin and FABP which elute later[2]. The A-560 reference assay uses the relationship between molecular size and elution behavior of proteins to identify most cell proteins. The first emission peak of the A-560 reference assay corresponds to a molecular mass greater then 60 kDa[6] and may reflect the presence of serum albumin[6] and other proteins with fatty acid-binding activity[6]. The second peak contains LFABP (Figure 18).

Both the FABP and non-FABP containing samples, as indicated from the emission peaks of A-560 assay, were pooled and used in the direct assay method. It was seen (Figure 19) that the pool from the second peak enhanced the emission peak of rose Bengal indicating the presence of LFABP, while the pool from the first peak did not enhance the emission peak but shifted it. This indicated the presence of proteins such as

serum albumin and other potential contaminants of cytosol.

In the final step to quantitate LFABP from cytosol, this cell fraction was directly used in the direct assay method as shown in Figure 20. It was seen that the cytosol enhanced the emission peak of rose Bengal; and on the basis of the previous results (Figures 14 through 17), it was certain that the enhancement caused by the cytosol was due the presence of FABP. Previous results also show (Figure 19) that the enhancement of the rose Bengal emission peak is only due to the presence of FABP, while the other proteins do not enhance the emission peak.

Research conducted previously in our laboratory[3, 4] and by others[21] showed that rats on special diets such as clofibrate and fenofibrate show high levels of LFABP compared with the animals on the normal diet. The cytosol from the animals on special diets and the normal diet was used in the direct assay method. It was seen (Figure 21) that the animals on the special diet had larger enhancement of emission peak indicating the presence of high levels of LFABP, while animals on normal diet had relatively smaller enhancement of emission peak indicating lower levels of LFABP. This result agrees with the previous research conducted in our laboratory[3, 4] and by others[21].

In the final stage of my effort to develop this new assay the results from the IHM were compared to and plotted against the effective peak height due to the presence of LFABP. The pool assay in the IHM gives the amount of LFABP present per gram of tissue (Liver) which was plotted against the effective peak height, showing (Figure 22) that both are directly proportional to each other. It was seen that the data from the IHM which has higher amount of LFABP present per gram of tissue had higher effective peak heights while the animals which had lower amount of FABP showed lower effective peak

heights. All animals on special diets formed a group higher than the control animals which formed a second lower group. The results plotted in Figure 22 demonstrate the relation between the direct assay method and the IHM. If we get similar results in both the methods then the data from IHM (i.e. mg FABP/g tissue) should be directly proportional to the effective emission peak in the direct assay method, which means linear data. Some points in the study show variation from linearity, which means that similar results were not always obtained for the direct assay method and the IHM.

CHAPTER 7

CONCLUSION AND RECOMMENDATIONS

The direct assay method, when perfected, will facilitate uncovering FABP roles in health and disease, improving treatment of lipid related human diseases. This process will add to our ability to study FABPs and can be used to study IFABP further. These studies, in the future may extended to make biosensors to detect fatty acid binding proteins. The direct assay method is 10 times faster than the IHM and uses less tissue. The new direct assay method can be applied to beef liver FABP to see if similar results are obtained.

While the direct assay method is promising, it will need future work to perfect it. It is recommended that direct assay method can be improved by using a spectrophotometer to replace our present experimental setup. It is also suggested that larger sample size of cytosol or LFABP may decrease the error see in the result where the data for the direct assay method is compared with the IHM. The direct assay method can be future be improved by understanding the stoichiometry of the reaction taking place between the FABP and rose Bengal dye. The use of sol-gel slides will help in understanding the interactions between the rose Bengal and FABP better if the solution can be kept cold for long periods of time.

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