University of Alberta

Bioanalytical methods to study low activity enzymes and low abundance proteins

by

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Abstract

Low abundance enzymes and proteins were studied in this thesis. Glycosyltransferase A in HT29 colon cancer cells were used for single cell analysis in microreaction chambers. Single cell reactions were sampled over time using a nanopipettor. While enzyme activity was low, it was detectable by capillary electrophoresis-laser induced fluorescence (CE-LIF). CE-LIF was also used in this thesis to detect enzyme activity in mutant *Helicobacter pylori* fucosyltransferases. Analyses showed that by changing a critical amino acid from phenylalanine to tyrosine, α-1,4 transfer activity was increased. Low abundance proteins from the outer membrane of *Escherichia coli* were also studied. Using different surfactants, a protocol was tested for its ability to remove high abundance proteins from the membrane pellet. Other methods were also tested to evaluate the best strategy to solubilize membrane proteins.

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Dedication

This work is dedicated to my family for which I am very grateful. I also dedicate this work to my grandfather, Simon Chan, and friend, Nigel Scott who passed away during the writing of this thesis. You are an inspiration in my life and I will always remember you.

Lastly, thank you to God for allowing me this opportunity to complete this thesis. He has helped me through many hard times before and during this work. I thank Him for allowing me to share my skills with the world.

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List of Abbreviations

2DE	Two-dimensional gel electrophoresis
2D-LC	Two-dimensional liquid chromatography
ALT	Alanine transaminase
BSA	Bovine serum albumin
CAI	Codon adaptation index
CERB	Capillary electrophoresis running buffer
CGE	Capillary gel electrophoresis
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
CIEF	Capillary isoelectric focusing
CITP	Capillary isotachophoresis
CZE	Capillary zone electrophoresis
DMEM	Dulbecco Modified Eagles' Media
DTT	Dithiothreitol
EOF	Electroosmotic flow
EPF	Electrophoretic flow
FucT	Fucosyltransferase
GalNAc	N-acetylgalactosamine
GDP-Fuc	Guanosine diphosphate β-L-fucose
GlcNAc	N-acetylglucosamine
LacNAc	N-acetyl-β-lactosamine
LAP	Low abundance protein
LC-MS	Liquid chromatography-mass spectrometry

LDH	Lactate dehydrogenase
MBS	MOPS buffered saline
MEKC	Micellar electrokinetic capillary chromatography
MES	2-(N-Morpholino)ethanesulfonic acid hemisodium
MOPS	4-Morpholinepropanesulfonic acid
MRC	Microreaction chamber
MS	Mass sepectrometry
MS/MS	Tandem mass spectrometry
NAD^+	Oxidized nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide
NDA	Naphthalene-2,3-dicarboxaldehyde
PHEMA	Poly(2-hydroxyethyl methacrylate)
RCF	Relative centrifugal force
SDS	Sodium dodecyl sulfate
SPE	Solid phase extraction
TFA	Trifluoroacetic acid
UDP-Gal	Uridine 5′-diphospho-galactose
UDP-GalNAc	Uridine 5′-diphospho-N-acetylgalactosamine
αFuc(1,2)Gal	α-L-fucopyranolsyl-(1,2)-D-galactopyranose
αGal(1,3)[αFuc (1,2)]Gal	α-D-galactopyranosyl-(1,3)-[α-L-fucopyranolsyl-(1,2)]-D-galactopyranose
αGalNAc(1,3) [αFuc(1,2)]Gal	2-Acetamido-α-D-galactopyranosyl-(1,3)-[α-L-fucopyranolsyl-(1,2)]-D-galactopyranose

Bioanalytical methods to study low activity enzymes and low abundance proteins

Chapter 1: Introduction to low abundance enzymes and proteins

Low activity enzymes and low abundance proteins represent an often overlooked class of biological compounds in part due to the lack of analytical methods to characterize them. Most molecular biology studies deal with techniques that amplify the gene or protein of interest (e.g. polymerase chain reaction, overexpression of proteins, *etc.*) in order to increase the amount of the molecule of interest. However, this requires previous extensive knowledge of the gene or protein and may not be possible for novel genes and proteins. Even though they are difficult to study, low activity enzymes and low abundance proteins are undoubtedly physiologically important. This thesis explores different bioanalytical methods to study these often ignored compounds.

1.1 Single cell analysis

Enzymes are proteins that catalyze biological reactions[1]. Typically, the gene encoding the enzyme of interest is cloned, overexpressed, and purified for analysis. This requires some prior knowledge of the gene and the enzyme and requires weeks, months, or even years of work. Also, some proteins are toxic to cells and cannot be overexpressed[2, 3]. Single cell analysis offers a faster way to study these proteins and enzymes and with smaller amounts. However, the amount of enzyme present within a single cell is often quite low. While some enzymes are present in large amounts, others may be present in smaller amounts or have slow activity. Unfortunately, one cannot

compensate for the slow activity of low activity enzymes by using more enzyme as it runs counter to the objective of single cell analysis.

While there are drawbacks in performing single cell analysis, there are advantages in studying individual cells. Information unique to individual cells is not lost in the background with other cells[4, 5]. This is similar to flying over a forest. At high altitudes, one cannot distinguish between the different species of trees as they all appear as a blur with different shades of green. At lower altitudes, one can distinguish individual trees and can tell if they are birch, popular, pine, spruce, *etc.* with individual colors. Likewise, single cell analysis offers the benefits of observing the individual characteristic of each cell. Furthermore, low volume single cell assays can be used where sample amount is limited, such as, tumor biopsies or developing tissues harboring deleterious gene mutations[6].

Single cell analyses have been performed using several techniques, namely flow cytometry[7-9], confocal microscopy[10, 11], microfluidics[12-15] and capillary electrophoresis (CE)[16-18]. New techniques for single cell analysis include antibody based assays[19] and *in vivo* studies using two-photon tissue imaging[7]. Clearly, this field is rapidly developing and this thesis deals with single cell analysis by CE.

The first single cell study by CE analyzed the amino acid content from large cells (e.g. oocytes, neurons)[20]. The introduction of laser induced fluorescence (LIF) techniques allowed smaller cells to be analyzed for their proteins[21], nucleic acids[22, 23], peptides[24], and metabolites[25]. More recent single cell studies have dealt with subcellular particles such as mitochondria[26] and nuclei[27]. An application of single cell analysis of

proteins is the use of LIF techniques to study changes in the proteome during the cell cycle[28].

This thesis only deals with glycosyltransferases in single cells. Glycosyltransferases catalyze the addition of monosaccharides onto proteins and other carbohydrates to form various glycoproteins such as antigens and enzymes for proper cellular function[29]. As part of the biosynthetic pathway to form human blood antigens, glycosyltransferase A (GTA) was studied in this thesis using HT29 human colorectal cancer cells.

1.2 Low abundance proteins

Low abundance proteins (LAPs) are present in small amounts in a cell. Their low concentration makes them difficult to detect, whether by conventional proteomic methods, such as gel electrophoresis, or by more recent chromatography techniques[30]. Nevertheless, they hold important biological information and their detection and characterization are desired.

Several research groups have studied LAPs in several organisms[31-34]. There are several strategies to study LAPs: removal of high abundance proteins (HAP) by antibodies[35], enrichment of LAPs[36, 37], prefractionation of the protein samples[38] and a combination of several of the above techniques[39]. Each of these techniques varies in the approach to detect LAPs and they all have their advantages and disadvantages. For example, removal of HAPs can be done with commercial kits but may also remove LAPs[40]. Protein prefractionation minimizes sample loss but increases the analysis time exponentially by adding another separation dimension[41].

In human serum, a handful of proteins (e.g. albumin, fibrinogen, transferrin, haptoglobin, IgG, IgA, IgM) represent 95% of the proteins present by weight while LAPs are present at much lower levels[42]. Detection and characterization of the LAPs would be much improved if the HAPs were removed [43, 44]. This is due to the phenomena of ion suppression or abundance suppression[45, 46]. This effect was first observed in gel electrophoresis based experiments but affects liquid chromatography-mass spectrometry (LC-MS) based methods equally if not more so. Due to the sheer numbers, HAPs are preferentially detected. In gel based systems, their large protein spots cover nearby proteins. In LC-MS methods, high abundance peptides and proteins co-elute with other proteins. Unless the mass-to-charge ratios of these high abundance peptides are known and manually excluded from analysis, the mass spectrometer will preferentially detect these peptides while neglecting the low abundance ions.

As mentioned above, protein fractionation has been used to study LAPs. However, if HAPs could be selectively left behind during sampling, then protein fractionation could be avoided to reduce sample handling and analysis time. While selecting proteins to include in a sample is difficult to do in a homogenous mixture, it is easier to accomplish in a heterogeneous sample. The carbonate extraction method used in this thesis work provides a heterogeneous mixture of solid membrane pellet and soluble proteins. Thus, this thesis work analyzed methods to selectively include LAPs in the sample while leaving behind HAPs from the outer membrane of *Escherichi coli*.

1.3 Summary of thesis

This thesis highlights work done in several areas of biology using sensitive analytical chemistry techniques. The first example is the study of the blood group enzyme glycosyltransferase A in single HT29 human colon cancer cells. This study was done at low volumes and the analytes separated and detected by CE-LIF.

The second example is the analysis of mutant fucosyltransferases from *Helicobacter pylori*. These enzymes have a very low activity and could not be studied by radiochemical assay. Thus, CE-LIF was used to determine its substrate specificities and reaction characteristics.

Lastly, different solubilization methods were tested in a study of the *E. coli* outer membrane proteome. Different methods were analyzed for their ability to remove HAPs from the membrane pellet. Methods were also tested to maximize the number of proteins solubilized from the outer membrane pellet in order to get a complete characterization of the *E. coli* outer membrane proteome.

1.4 References

(1) Barman, T. E. *Enzyme Handbook*; Springer-Verlag: New York, 1969.

(2) Minakhin, L.; Severinov, K. *Protein Expression and Purification* **2005**, *41*, 1-8.

(3) Quesada-Moraga, E.; Vey, A. *Mycological Research* **2004**, *108*, 441-452.

(4) Woods, L. A.; Roddy, T. P.; Ewing, A. G. *Electrophoresis* **2004**, *25*, 1181-1187.

(5) Krylov, S. N.; Arriaga, E.; Zhang, Z. R.; Chan, N. W. C.; Palcic, M. M.; Dovichi, N. J. *Journal of Chromatography B* **2000**, *741*, 31-35.

(6) Gillmor, C. S.; Poindexter, P.; Lorieau, J.; Palcic, M. M.; Somerville, C. *Journal of Cell Biology* **2002**, *156*, 1003-1013.

(7) Katsuragi, T.; Tani, Y. *Journal of Bioscience and Bioengineering* **2000**, *89*, 217-222.

(8) Mizuarai, S.; Takahashi, K.; Kobayashi, T.; Kotani, H. *Histology and Histopathology* **2005**, *20*, 139-146.

(9) Danna, E. A.; Nolan, G. P. *Current Opinion in Chemical Biology* **2006**, *10*, 20-27.

(10) Lemasters, J. J.; Qian, T.; He, L. H.; Kim, J. S.; Elmore, S. P.; Cascio, W. E.; Brenner, D. A. *Antioxidants & Redox Signaling* **2002**, *4*, 769-781.

(11) Dobrucki, J.; Darzynkiewicz, Z. *Micron* **2001**, *32*, 645-652.

(12) Yi, C. Q.; Li, C. W.; Ji, S. L.; Yang, M. S. *Analytica Chimica Acta* **2006**, *560*, 1-23.

(13) Liu, R. H.; Dill, K.; Fuji, H. S.; McShea, A. *Expert Review of Molecular Diagnostics* **2006**, *6*, 253-261.

(14) Lagally, E. T.; Medintz, I.; Mathies, R. A. *Analytical Chemistry* **2001**, *73*, 565-570.

(15) Yamada, M.; Seki, M. *Analytical Chemistry* **2004**, *76*, 895-899.

(16) Ahmadzadeh, H.; Krylov, S. In *Methods in Molecular Biology*; M.A., S., Lagu, A. L., Eds.; Humana Press: Totowa NJ, 2004; Vol. 276, pp 29-38.

(17) Wang, Z. Q.; Yeung, E. S. *Journal of Chromatography B* **1997**, *695*, 59-65.

(18) Lillard, S. J.; Yeung, E. S. *Journal of Chromatography B* **1996**, *687*, 363-369.

(19) Cox, J. H.; Ferrari, G.; Janetzki, S. *Methods* **2006**, *38*, 274-282.

(20) Kennedy, R. T.; Oates, M. D.; Cooper, B. R.; Nickerson, B.; Jorgenson, J. W. *Science* **1989**, *246*, 57-63.

(21) Hu, S.; Lee, R.; Zhang, Z.; Krylov, S. N.; Dovichi, N. J. *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences* **2001**, *752*, 307-310.

(22) Matsunaga, H.; Anazawa, T.; Yeung, E. S. *Electrophoresis* **2003**, *24*, 458-465.

(23) Zabzdyr, J. L.; Lillard, S. J. *Analytical Chemistry* **2001**, *73*, 5771-5775.

(24) Tong, W.; Yeung, E. S. *Journal of Chromatography B-Biomedical Applications* **1996**, *685*, 35-40.

(25) Anderson, A. B.; Ciriacks, C. M.; Fuller, K. M.; Arriaga, E. A. *Analytical Chemistry* **2003**, *75*, 8-15.

(26) Anderson, A. B.; Xiong, G. H.; Arriaga, E. A. *Journal of the American Chemical Society* **2004**, *126*, 9168-9169.

(27) Gunasekera, N.; Olson, K. J.; Musier-Forsyth, K.; Arriaga, E. A. *Analytical Chemistry* **2004**, *76*, 655-662.

(28) Hu, S.; Zhang, L.; Krylov, S.; Dovichi, N. J. *Analytical Chemistry* **2003**, *75*, 3495-3501.

(29) Beeley *Glycoprotein and proteoglycan techniques*; Elsevier Science: New York, 1985.

(30) von Horsten, H. H. *Current Analytical Chemistry* **2006**, *2*, 129-138.

(31) Zhang, H. T.; Cheng, X.; Richter, M.; Greene, M. I. *Nature Medicine* **2006**, *12*, 473-477.

(32) Wienkoop, S.; Weckwerth, W. *Journal of Experimental Botany* **2006**, *57*, 1529-1535.

(33) Palmer, D. J.; Kelly, V. C.; Smit, A. M.; Kuy, S.; Knight, C. G.; Cooper, G. J. *Proteomics* **2006**, *6*, 2208-2216.

(34) Ahmed, N.; Rice, G. E. *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences* **2005**, *815*, 39-50.

(35) Greenough, C.; Jenkins, R. E.; Kitteringham, N. R.; Pirmohamed, M.; Park, B. K.; Pennington, S. R. *Proteomics* **2004**, *4*, 3107-3111.

(36) Pedersen, S. K.; Harry, J. L.; Sebastian, L.; Baker, J.; Traini, M. D.; McCarthy, J. T.; Manoharan, A.; Wilkins, M. R.; Gooley, A. A.; Righetti, P. G.; Packer, N. H.; Williams, K. L.; Herbert, B. R. *Journal of Proteome Research* **2003**, *2*, 303-311.

(37) Fountoulakis, M.; Juranville, J. F. *Analytical Biochemistry* **2003**, *313*, 267-282.

(38) Righetti, P. G.; Castagna, A.; Herbert, B.; Reymond, F.; Rossier, J. S. *Proteomics* **2003**, *3*, 1397-1407.

(39) Anderson, N. L.; Polanski, M.; Pieper, R.; Gatlin, T.; Tirumalai, R. S.; Conrads, T. P.; Veenstra, T. D.; Adkins, J. N.; Pounds, J. G.; Fagan, R.; Lobley, A. *Molecular & Cellular Proteomics* **2004**, *3*, 311-326.

(40) Granger, J.; Siddiqui, J.; Copeland, S.; Remick, D. *Proteomics* **2005**, *5*, 4713-4718.

(41) Tang, H. Y.; Ali-Khan, N.; Echan, L. A.; Levenkova, N.; Rux, J. J.; Speicher, D. W. *Proteomics* **2005**, *5*, 3329-3342.

(42) Anderson, N. L.; Anderson, N. G. *Molecular & Cellular Proteomics* **2002**, *1*, 845-867.

(43) Bjorhall, K.; Miliotis, T.; Davidsson, P. *Proteomics* **2005**, *5*, 307-317.

(44) Raida, M.; Schulz-Knappe, P.; Heine, G.; Forssmann, W. G. *Journal of the American Society for Mass Spectrometry* **1999**, *10*, 45-54.

(45) Sun, W.; Wu, S. Z.; Wang, X. R.; Zheng, D. X.; Gao, Y. H. *European Journal of Mass Spectrometry* **2005**, *11*, 575-580.

(46) Wienkoop, S.; Glinski, M.; Tanaka, N.; Tolstikov, V.; Fiehn, O.; Weckwerth, W. *Rapid Communications in Mass Spectrometry* **2004**, *18*, 643-650.

Chapter 2: Multiple sampling in a single cell enzyme assay[1]

2.1 Introduction

Low volume assays are an extension of the drive to miniaturize *in vitro* analysis of chemical and biochemical reactions. Low volume assays can be used to detect the activity of a single cell or a small group of cells. This is beneficial over traditional bulk cell assays as it avoids the averaging bias inherent in bulk assays[1, 2]. As well, cells can be chosen for analysis after initial screening based on visual morphology or staining characteristics. Furthermore, low volume single cell assays can be used where sample amount is limited, such as tumor biopsies or developing tissues harboring deleterious gene mutations[3]. Single cell assays can also be used to detect changes in the intracellular level of cell cycle dependent enzymes and proteins[4, 5]. Understanding these enzymes and proteins may lead to a more complete understanding of cell cycle regulation.

Flow cytometry has been used extensively to study cells individually[6]. This established method uses antibodies to detect the presence of proteins and antigens present in a cell or on its surface. While this method is fast and can study many parameters at once, it is mainly limited to antigens to which antibodies have been raised and cannot readily study parameters that have not been initially characterized. To study the contents of a cell to which antibodies have not or could not be raised, single cell assays have been done by capillary electrophoresis (CE). The first single cell study by CE analyzed the amino acid content of a snail neuron cell[7]. The introduction of fluorescently labeled substrates lowered the detection limit to attomolar levels[8] and ushered in new areas of

[1] Portions of this chapter have been published in *Analytical Chemistry* **2005**, *77*, 3132-3137 by Shoemaker *et al.* Other portions are being considered for submission to Analytical and Bioanalytical Chemistry.

analyses, such as proteins[9], nucleic acids[10], and peptide hormones[11]. Fluorescently labeled substrates also allowed the study of cells smaller than neurons and oocytes. With the introduction of new sample handling techniques, subcellular organelles, such as mitochondria[12] and nuclei[13], have been studied. In general, the chemical analysis of single cells has been termed chemical cytometry[14]. Metabolic cytometry is a subset of chemical cytometry and refers to the products of enzymatic pathways within a cell.

2.1.1 Human blood groups and glycosyltransferase A

Human blood groups are defined as the different surface antigens present on the human blood cell surface[15]. Of the twenty-six identified blood groups, four groups (ABO(H), P, Lewis, and Hh) are based on the carbohydrate structure on the cell surface and many more are based on the cell surface glycoprotein. Thus, carbohydrate modifying enzymes are critical in proper blood antigen formation. Being on the surface of blood cells, they are known to function as membrane transporters, receptor and adhesion molecules, regulatory signals, enzymes, and structural proteins, and to form part of the glycocalyx. The ABO(H) blood group is the most characterized blood group and is a major factor taken into consideration when determining blood donor/recipient compatibility. Other blood groups also play an important role in maintaining homeostasis, and are reviewed elsewhere[16].

Glycosyltransferase A (GTA) is a member of the human ABO(H) blood group enzymes. As such, it is one of two enzymes responsible for determining an individual's ABO(H) blood type[17]. Individuals with type A blood express this enzyme in their hematopoietic stem cells[18]. GTA catalyzes the addition of N-acetylgalactosamine

(GalNAc) from uridine 5'-diphospho-N-acetylgalactosamine (UDP-GalNAc) to the H-antigen, α-L-fucopyranosyl -(1,2)-D-galactopyranose (αFuc(1,2)Gal), to form the A antigen, 2-acetamido-α-D-galactopyranosyl-(1,3)-[α-L- fucopyranosyl -(1,2)]-D-galactopyranose (αGalNAc(1,3)[αFuc(1,2)]Gal), (Figure 2.1). People with type B blood express the enzyme glycosyltransferase B (GTB)[19] which transfers galactose from uridine 5'-diphospho-galactose (UDP-Gal) and adds it to the H antigen to form the B antigen, α-D-galactopyranosyl-(1,3)-[α-L- fucopyranosyl -(1,2)]-D-galactopyranose, (αGal(1,3)[αFuc(1,2)]Gal). People with type AB blood express both GTA and GTB while people with type O blood express neither functional GTA nor GTB. GTA and GTB are highly homologous and differ in only 4 out of 268 amino acids[20]. The products of these enzymes are also similar in that they only differ in the substitution on the terminal saccharide of an acetamido or hydroxyl group. Despite their similarities, giving blood transfusion patients the wrong type of blood can have fatal consequences.

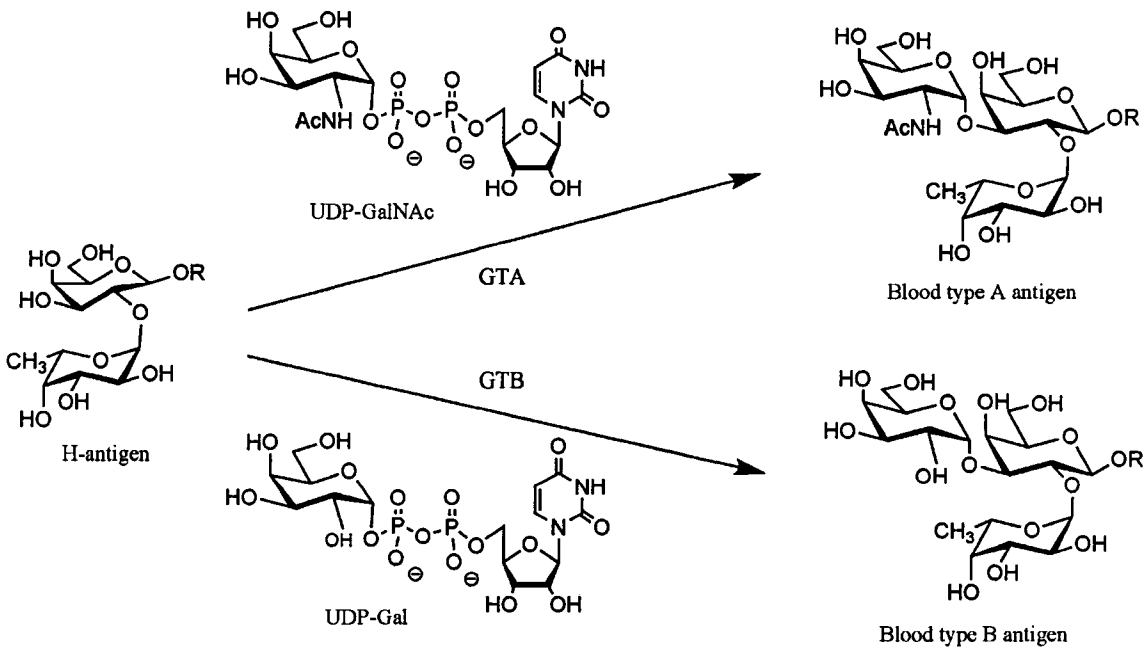

Figure 2.1: Schematic representation of the reactions catalyzed by GTA and GTB.

GTA is a relatively slow enzyme when compared to other enzymes (Table 2.1). The k_{cat} of an enzyme measures the number of reactions that enzyme can perform in one second. Because the k_{cat} for GTA is 5 s^{-1}, either more enzymes must be used or the reaction must be allowed to proceed for longer time periods in order to achieve the same amount of product as other faster enzymes. For single cell assays of GTA, using more enzymes is not applicable, so increasing incubation time and decreasing the total volume (to increase enzyme concentration) must be used to compensate the low activity of the enzyme.

Table 2.1: k_{cat} values for a few selected enzymes [21-23b].

Enzyme	k_{cat} (s^{-1})
Carbonic anhydrase	1×10^{6}
Urease	1×10^{5}
Fumerase	8×10^{2}
Chorismate mutase	5×10^{1}
Glucoamylase	2×10^{1}
β-glucosidase	8
GTA	5
Trypsin	7×10^{-1}
Chicken egg white lysozyme	2×10^{-1}

2.1.2 HT29 human colorectal cancer cell line

The cell line HT29 was first isolated from a 44 year old female Caucasian[24]. It was isolated from colorectal adenocarcinoma tissue and is generally used as a

transfection host. These adherent secondary culture cells have been used for single-cell enzyme and protein studies[25, 26] and are relatively easy to maintain and are of average size. These adherent epithelial cells are known to express the blood group A antigen, rhesus factor, and several HLA antigens. Since they express the blood group A antigen, they must possess GTA and can be used as a model for single cell GTA assays. The expression of GTA in HT29 has been found to be correlated to the level of malignancy in primary cells[27, 28]. Thus, they serve as a good model to demonstrate the ability of single cell assays to detect cell-to-cell heterogeneity.

Flow cytometry can be used to confirm the validity of the single cell GTA experiments. Although the flow cytometry protocol used here measures the presence of surface antigens and the single cell experiment done here measures intracellular enzyme activity, they are linked due to the nature of the enzymatic pathway[18]. The A antigens are synthesized in the Golgi bodies of the cell and are eventually transported to the surface of the cell. Flow cytometry has the advantage of high throughput screening but is generally limited to proteins or antigens at a single time point; it cannot readily detect enzyme activity over time. While the single cell experiment described here cannot be used for high throughput applications, it can however detect and monitor enzymatic reaction over time.

2.1.3 Capillary electrophoresis and laser induced fluorescence

Capillary electrophoresis (CE) is a powerful separation technique capable of providing excellent resolution[29]. CE is amenable to low volume assays as it only requires a small amount of analyte to be used in each analysis. The principles and practice of CE

can be found elsewhere[30, 31]. In brief, there are two types of movement in CE that when combined, provide for the separation of analytes. The first movement is electroosmotic flow (EOF). This is caused by the interaction of the negatively charged silanol groups on the wall of the capillary with the positive ions in the buffer. When a voltage is applied across the length of the capillary, the cations in the diffuse part of the double layer move towards the cathode. The momentum of the cations is imparted on the buffer and the contents of the capillary moves in bulk towards the cathode. The second movement is electrophoretic flow (EPF). This is the movement of positively or negatively charged analytes towards the cathode or the anode of the capillary, respectively. EPF is the main separation force as it separates the analytes based on charge. While EPF can either be "forward" (towards the detector) or "backwards" (away from the detector) in terms of the capillary, the EOF is usually greater than the EPF and sweeps all analytes towards the detector.

There are five main separation modes of CE: capillary zone electrophoresis (CZE), micellar electrokinetic capillary chromatography (MEKC), capillary isoelectric focusing (CIEF), capillary gel electrophoresis (CGE), and capillary isotachophoresis (CITP). The high resolution of CE analysis is especially useful in the separation of carbohydrates as they are a class of similar polyhydroxylated compounds with numerous isomers[32].

Of the different kinds of CE, MEKC offers the most separation power to mixtures of oligosaccharides. In MEKC, micelles are formed by including surfactant molecules, usually sodium dodecyl sulfate (SDS), in the running buffer at a concentration higher than its critical micellar concentration. Since the micelles are negatively charged, they

have an EPF against the EOF and will have a longer migration time than neutral or positively charged molecules. As the different neutral analytes partition between the aqueous phase and the micellar phase, they are gradually separated from each other based on the amount of time each analyte spends in the two different phases. An analyte that spends no time in the micellar phase will move with the EOF while an analyte that spends all of its time in a micelle will move at a slower rate. For a more comprehensive review of MEKC, the reader is referred to the work of Mazzeo[33].

The capillary electrophoresis running buffer (CERB) used in this work contained SDS for the formation of micelles, borate and phenylborate for complexation with the oligosaccharides, and phosphate for pH control. For most neutral oligosaccharides to be separated by CE, they must be first complexed with borate ions to form charged species[34]. In basic solutions, borate exists as tetrahydroxyborate, $B(OH)_4^-$, and reacts with vicinal diols in carbohydrates to form negatively charged complexes. Phenylboric acid also forms these complexes and has been found to improve the resolution of oligosaccharide isomers[35] and was used here. It is postulated that the phenyl group increases the complexes' hydrophobicity and introduces steric constraints within the complex. By using both borate and phenylborate, the differences in the oligosaccharides' partition coefficients in the micellar phase are enhanced and resolution is improved.

To detect small amount of analytes inherent in low volume assays, a sensitive detection method must be used. Furthermore, oligosaccharides have very low UV absorption and are not amenable to UV-visible spectrometry. There are several detection methods available for CE, such as UV absorption, electrochemical detection, and mass spectrometry. However, derivatization with fluorophores is usually used for

oligosaccharide analysis. Laser induced fluorescence (LIF) of the attached fluorophore is the most sensitive method[36, 37]. The limit of detection is usually in the range of tens to hundreds of molecules[38] but the detection of two molecules has been reported[39].

In the present system, fluorescence of the analyte is obtained by attaching the succinimidyl esters of carboxy-tetramethylrhodamine (TMR) to the oligosaccharide. This fluorophore is commonly used to label proteins and nucleic acid (Figure 2.2). The excitation wavelength maxima of TMR at 555 nm closely matches the 543 nm line of He-Ne lasers and is used in many bioanalytical applications[31]. The power of LIF as a detection system is also enhanced by the construction and geometry of the detector. Background noise in the fluorescent emission at 580 nm is reduced by collecting the emitted light at a right angle to the incident light and using a bandpass filter. Furthermore, the use of a sheath flow cuvette allows for post-column detection and minimizes scattering due to the capillary wall and changes in the refractive index of the buffer.

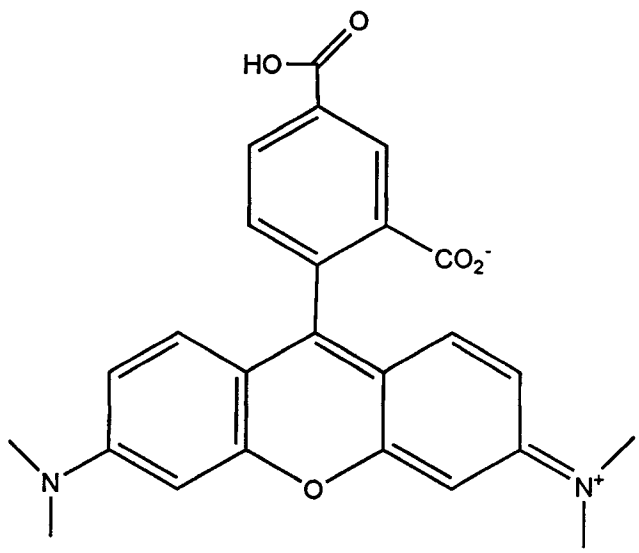

Figure 2.2: Structure of tetramethyrhodamine.

2.1.4 Nanopipettor

As the name implies, low volume assays deal with very small volumes, usually in the nanoliter range. Most micropipets can deliver 0.2 to 0.5 μL with good precision and accuracy and common molecular biology polypropylene tubes can also accommodate this volume. However, this volume is too large for single cell reactions where the enzyme being studied has low activity, such as GTA with a k_{cat} of 5 s^{-1}. Thus, many single cell reactions have taken place within the CE capillary[40, 41]. While this approach minimizes the dilution of enzymes and analytes, the entire reaction is analyzed at once and cannot be repeated. Thus, replicate analysis and time course studies cannot be done. To sample a low volume assay multiple times, the enzyme reaction must occur independently from the analysis. This requires a way to manipulate liquids at the nanoliter level in order to setup the enzyme reaction and sample a low volume assay.

To transfer minute amounts of liquids, a previously described nanopipettor was used[42]. Briefly, this locally designed tool is built using a 1 mL Hamilton syringe fitted with a fused silica capillary *via* a PEEK zero-dead-volume union. It can reproducibly deliver a small volume of liquid by changing the pressure within the Hamilton syringe for a given amount of time. In summary, the volume aspirated into the capillary is described by the equation

$$V_{aspirated} = \frac{p_0\left(1-\frac{V_0}{V_f}\right)\pi d^4 t}{128\eta L} \qquad \text{Equation 2.1}$$

where $V_{aspirated}$ is the volume aspirated into the capillary, P_0 is the initial pressure inside the syringe (101.3 kPa), V_0 is the initial volume of the syringe, V_f is the final volume of the syringe, d is the inner diameter of the capillary, t is the amount of time that the

capillary is immersed in the solution of interest, η is the solution's viscosity (0.001 kg m^{-1} s^{-1} for aqueous solutions), and L is the length of the capillary.

To aspirate a liquid, the capillary is first immersed in the solution of interest. The plunger is then quickly withdrawn to a previously determined volume. After the desired amount of time has elapsed, the liquid is immediately removed and the capillary is transferred to the receiving vessel. The plunger is then depressed beyond its original position to expel the liquid. To avoid changing the temperature of the syringe cylinder, and hence its pressure, the apparatus is mounted on a ring stand with clamps and only the plunger is in contact with the experimentalist. Several factors can be easily altered by the experimentalist to change the volume delivered: the size and length of the capillary, the time that the capillary is immersed in the liquid, and the initial and final volume of the syringe. While the parameters in the original work showed that the device can reproducibly deliver aliquots of liquids at the single nanoliter range, it can be changed so that hundreds of nanoliters can be delivered by using a 12 cm long capillary of 10 μm inner diameter.

2.2 Experimental conditions

2.2.1 Enzyme assay conditions for GTA analysis

While the H antigen constitutes the minimal acceptor for GTA, the enzyme can also transfer to other oligosaccharide acceptors as long as the terminal saccharides resemble the H antigen. In this experiment, H-type II antigen was used as the acceptor. H-type II antigen differs from H antigen in that the former has an additional N-acetyl-

glucosamine (GlcNAc) moiety attached to the galactose in a 1,4 manner (Figure 2.3). H-type II antigens were labeled with TMR to allow for fluorescent detection and were available from previous work in the group[36, 43].

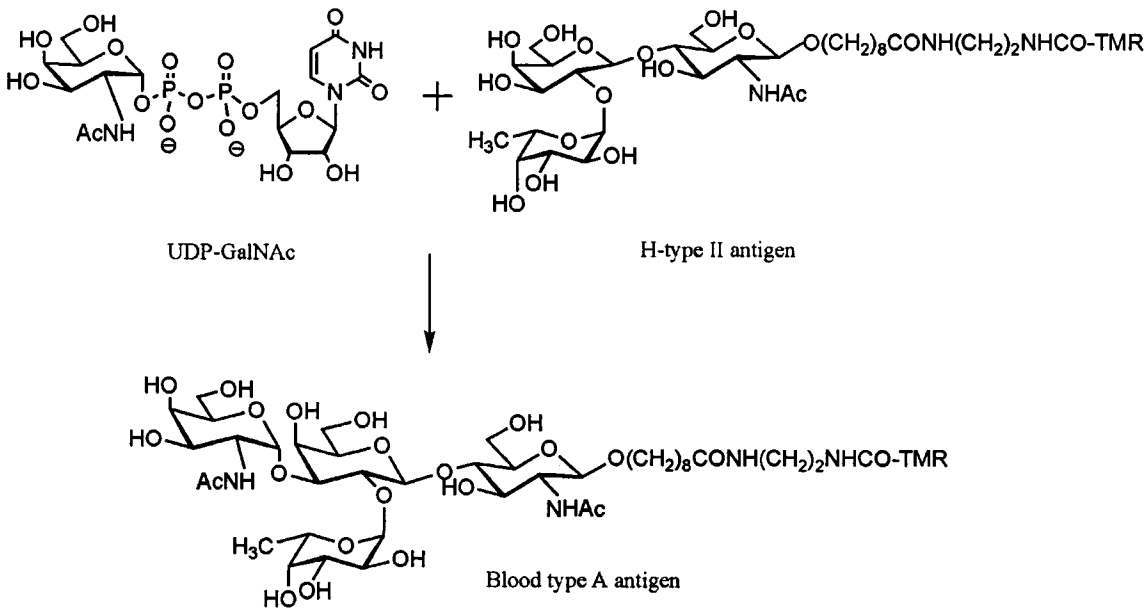

Figure 2.3 Schematic representation of the GTA reaction under assay in this experiment.

HT29 cells were obtained from American Type Culture Collection (Manassas VA). They were grown in 25 cm^2 cell culture flasks with Dulbecco Modified Eagles' Media (DMEM) with 4 mM L-glutamine and supplemented with 10% fetal calf serum and 1 mM sodium pyruvate (Invitrogen, Burlington ON). The flasks were incubated at 37 °C with 5% atmospheric CO_2 and grown to *ca.* 75% confluence. The cells were then passaged by trypsinizing the cells, diluted with fresh media and used to reseed the flask. Cells for low volume assays were collected at this time by centrifugation at 1500 RPM and resuspended in MOPS buffered saline (MBS). After the thirtieth passage, the cells were frozen at -80 °C and another vial of stock HT29 cells were thawed and maintained as above.

For GTA assays, the cell suspension was diluted with MBS instead of media and then spotted onto a glass microscope slide. Cells were then selected for single cell and ten cell analysis by using a locally constructed micromanipulator described previously[44]. This micromanipulator uses a series of stepping motors attached to translational stages to adjust the three-dimensional position of a capillary. A 50 μm (i.d.) fused silica capillary (Polymicro Technologies, Phoenix AZ) is attached to a 50 μL Hamilton syringe. The plunger of the syringe is controlled by another stepping motor to control the rate of withdrawal and expulsion. The capillary and microscope slide are coated before use with poly(2-hydroxyethyl methacrylate), (PHEMA) (Sigma Aldrich, St. Louis MO) to reduce the adhesion of HT29 cells to bare silica[45].

The capillary filled with buffer was placed adjacent to a single cell with normal morphology. Suction was then applied *via* the Hamilton syringe at a rate of 5 nL s^{-1} for 10-15 s (50-75 nL). The cell suspension on the microscope slide was monitored visually to confirm entry of the cell into the capillary. The capillary was then moved to a fresh spot of MBS for 2 minutes to allow the pressure inside the capillary to equilibrate. The capillary was then placed into a microreaction chamber (MRC) containing 100 nL of the reaction buffer. The contents of the capillary were then expelled by moving the Hamilton syringe at 15 nL s^{-1} for 5-7 s (75-105 nL) and allowed to equilibrate for 2 minutes. After expelling the cell, the capillary was returned to the microscope stage and the remaining buffer was expelled to visually confirm the absence of any remaining cells. Blank reactions were done as negative controls. In the blank reactions, MBS surrounding the cells were drawn into the micromanipulator at the same rate, allowed to equilibrate, and

expelled into MRCs containing 100 nL of the reaction buffer at the same rate and allowed to equilibrate and incubated.

The MRC was made by heat-sealing the end of a gel-loading tip to form a narrow, water-tight tube. After all manipulations were done, the MRC was then spun briefly in a microcentrifuge to collect the cell and reaction buffer at the bottom. The MRC was then sealed with a silicone-plugged 10 μL micropipette tip, wrapped with paraffin wax and incubated at 37 °C and 100% relative humidity.

Low volume GTA assay conditions were based on bulk enzyme assays conditions[46]. The reaction buffer consisted of 100 μM H-type II-TMR, 200 μM UDP-GalNAc, 0.5% Triton X-100, 6X complete EDTA-free protease inhibitor, 10 mU mL^{-1} alkaline phosphatase, 1 mg mL^{-1} BSA, 50 mM MOPS, 20 mM $MnCl_2$ and buffered at pH 7.2. The protease inhibitor and alkaline phosphatase were obtained from Roche Diagnostics (Laval QC)) and the other reagents were obtained from Sigma Aldrich (St. Louis MO).

After the appropriate incubation time, 26 nL aliquots were withdrawn for analysis using the nanopipettor. This was accomplished by using a 15 cm by 20 μm capillary and changing the syringe volume from 300 μL to 600 μL while the capillary was immersed in the MRC for 20 s. The samples were then expelled into a vessel containing 3 μL of CERB for CE-LIF analysis by changing the syringe volume to 100 μL until air was seen bubbling out of the capillary.

2.2.2 CE-LIF conditions

The CE-LIF instrument has been previously described[38]. In brief, one end of a 40 cm by 10 μm fused silica capillary (Polymicro Technologies, Phoenix AZ) was placed in

CERB and in contact with the anode of a CZEI000R high voltage power supply (Spellman, Plainview, NY, USA). The other end of the capillary was placed into a sheath flow cuvette and grounded electronically. A 1.0 mW helium-neon laser beam, λ=543 nm (Melles Griot, Carlsbad CA), was focused at the end of the capillary. Post column fluorescence was collected at a right angle with a high numerical aperture microscope objective (0.7 NA, 60×), spectrally filtered by a 580DF40 band-pass filter (Omega Optical, Vermont NE), and detected with a Hamamatsu R1477 photomultiplier tube (Bridgewater, NJ). The data were then digitized by a National Instruments NB-MIO-16X data acquisition board (Austin TX) and processed on a Macintosh computer.

Samples were electrokinetically injected into the anode end of the separation capillary by applying a 4000 V potential for 4 s. An electric field of 450 V cm^{-1} was then applied across the capillary to effect separation at room temperature.

The CERB consisted of 50 mM SDS, 2.5 mM sodium borate, 10 mM phenylboronic acid, and 10 mM sodium dihydrogen phosphate, pH 9.3. The solution was filtered through a 0.22 μm filter before use. The same buffer was also used as sheath fluid in the sheath flow cuvette.

Peak identities were confirmed by co-injecting small amounts of A-TMR along with the reaction mixture. Co-migration of the product and the standard showed that the reaction mixture contained the standard compound.

Data analysis was performed using Igor Pro (WaveMetrics, Lake Oswego OR). The CE traces were overlaid and shifted for visual comparison to show the increase in the A-TMR peak over time. Peak integration was performed and the peak area was used to

determine the ratio of A-TMR to H-type II-TMR. This ratio is given the term "percent conversion."

2.2.3 Flow cytometry conditions

Procedures for flow cytometry were adapted from Jackson and Warner[47] and Hakomori *et al*[27]. In brief, 0.5 μg of the primary antibody (anti-A antigen or anti-B antigen raised in mice [DakoCytomation, Mississauga ON]) were added to cells suspended in fresh DMEM with 0.1% sodium azide. The mixture was incubated on ice for 30 minutes and then the cells were gently spun down, washed in PBS containing 0.1% azide and resuspended. Then, 1 μg of anti-mouse fluorescein-linked secondary antibody (Jackson ImmunoResearch Laboratories, West Grove PA) was added and incubated for 30 minutes on ice. The cells were centrifuged, washed, and resuspended with PBS containing 0.1% azide (w/v) and 1% paraformaldehyde (w/v). Control incubations with no primary antibody and anti-B-antigen antibodies were also done to account for non-specific adsorption of the secondary antibody. The cells were then analyzed by a BD Bioscience FACScan™ cytometer (San Jose CA). Cells were initially plotted according to their forward and side light scatter, which measured their size and granularity, respectively. Cells of the appropriate size and granularity were then selected, or "gated," for analysis of their fluorescence signal. Higher fluorescence signal indicated a greater concentration of the antigen on the cell's surface.

2.2.4 Mycoplasma detection procedures

The frozen cells were subsequently tested for mycoplasma contamination using a MycoSensor™ PCR Assay Kit (Stratagene, La Jolla CA) and the accompanying instructions. HotMaster™ Taq DNA polymerase from Eppendorf (Hamburg, Germany) was used for the mycoplasma assay. The PCR products were separated on a 2% agarose gel and stained with ethidium bromide.

2.3 Results and Discussions

2.3.1 One hundred cell assay

GTA assays of HT29 cells were first done at the 100 cell level to determine if the reaction conditions were appropriate and to measure an approximate rate of reaction. A 0.2 μL drop of 5×10^5 cells mL^{-1} cell suspension was added to 0.8 μL of the reaction buffer. Aliquots of 0.5 μL were then placed into two MRCs for incubation so that each MRC had 1 cell per 10 nL. Samples were taken after 90, 136, 185, 235, and 281 h and analyzed by CE-LIF as described above.

Figure 2.4 is a trace of the TMR labeled oligosaccharide peaks observed during the CE run. The peak at 10.2 minutes is the A-TMR product peak and the peak at 10.5 minutes is the H-type II-TMR substrate peak. Samples from each timepoint were analyzed by CE-LIF and the peak areas integrated as described above. Visual estimation suggests that only a small amount of A-TMR is formed after several days of incubation. As shown in Figure 2.5, the percent converted is approximately 5% after approximately 4 days and continues to slowly increase. This shows that both the GTA enzyme and the A-antigen are stable over long periods in the reaction conditions used.

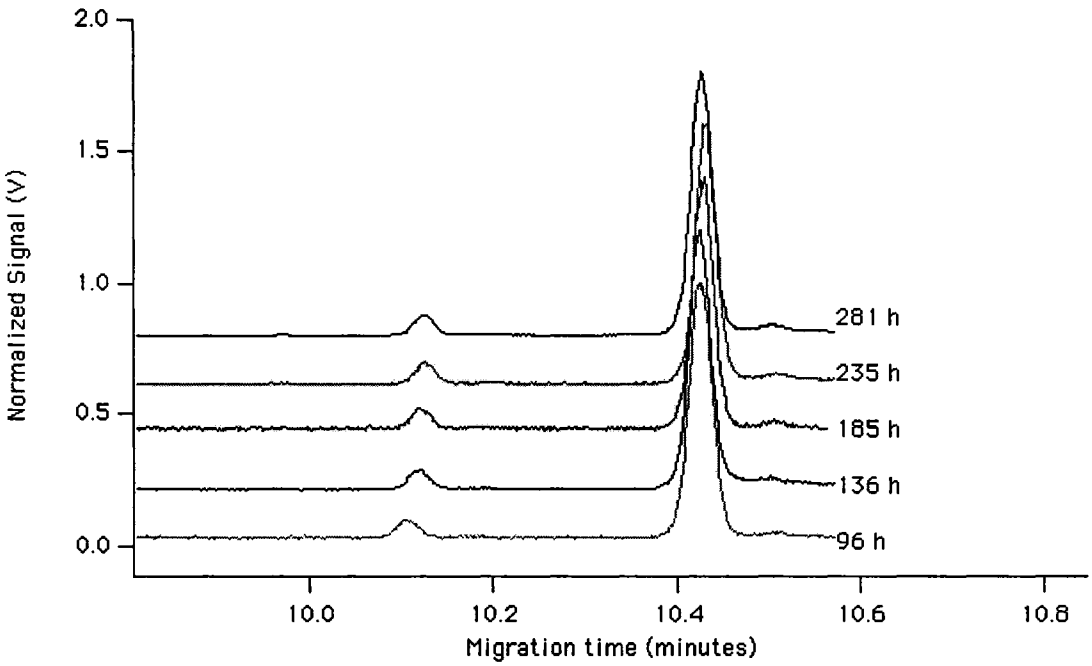

Figure 2.4: CE-LIF electropherograms of samples taken from 100 cell assays. Samples from one of the MRCs discussed in the text was taken after the indicated time and analyzed by CE-LIF. The peaks are normalized so that the intensity of the H-type II-TMR peak has an intensity of 1.0V.

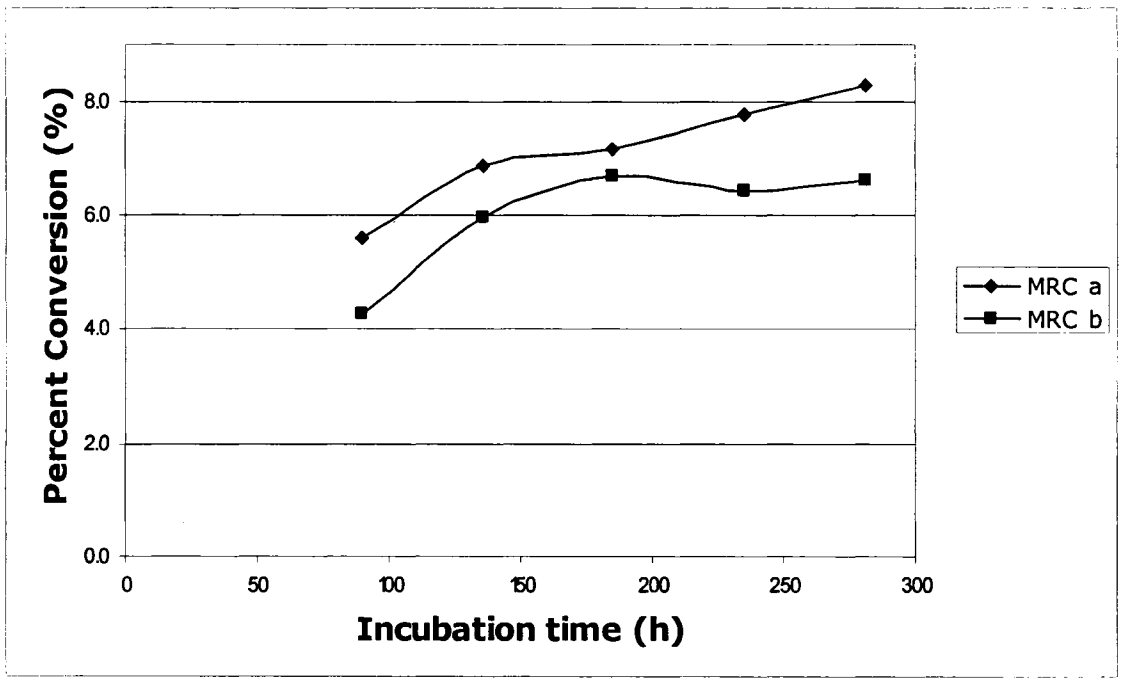

Figure 2.5: Graph of percentage of A antigen formed over the incubation period in two MRCs using 100 HT 29 cells.

Using approximately 100 cells in 1 μL (1 cell per 10 nL), a 5% conversion after 4 days (96 h) is relatively slow compared to α-glucosidase II from *Spodoptera frugiperda* which is also studied in our research group. The latter has approximately 1700 times more activity than GTA and 20% conversion to product could be achieved in 4.5 h using a single cell in 200 nL of reaction buffer. To increase the rate of the GTA reaction, it is imperative that the reaction volumes be kept low as low as possible to increase the enzyme concentration. Unfortunately, this is restricted by the limits of the MRC. The minimum volume in a MRC assay is approximately 100 nL. Volumes less than 100 nL are difficult to confirm visually and are prone to evaporation. To achieve volumes less than 100 nL, microfluidic applications may be required.

2.3.2 Ten cell assay

After the 100 cell assays, ten cell assays were attempted. The reaction buffer was the same as above with the exception that the HT29 cells were added using the micromanipulator. Ten cells were counted visually to have entered the capillary. The total volume of buffer aspirated was kept to a minimum (100 nL) to avoid excessive dilution. The cells were expelled into an MRC containing 200 nL of the reaction buffer and incubation was done as described above. Due to the limits of the MRC and micromanipulator, the volume of reaction buffer per cell had increased from 1 cell per 10 nL in the 100 cell assay to 1 cell per 30 nL in the 10 cell assay. Together, four sets of experiments totaling fifteen MRCs were done over the course of several weeks. For the purpose of discussion, only the fourth set is shown. For this set, duplicate samples were removed at 66, 138, and 210 h for CE-LIF analysis.

Figure 2.6 shows the electropherogram of the TMR labeled oligosaccharide peaks in samples taken from a representative MRC within the fourth set. The A-TMR peak can be clearly seen at 9.4 minutes along with the H-type II-TMR peak at 9.7 minutes. Other peaks from impurities and degradative products can be seen as well. The degradative product at 10.0 minutes is most likely due to the actions of endogenous glycosidases to form N-acetyl-β-lactosamine (LacNAc)-TMR, GlcNAc-TMR and/or octamethylethylenediamine-TMR. These peaks are not considered during calculations of A-TMR formation as the substrate H-type II antigen is present in large excess. Small changes in the level of H-type II antigen over the course of the incubation are unlikely to affect the rate of GTA activity.

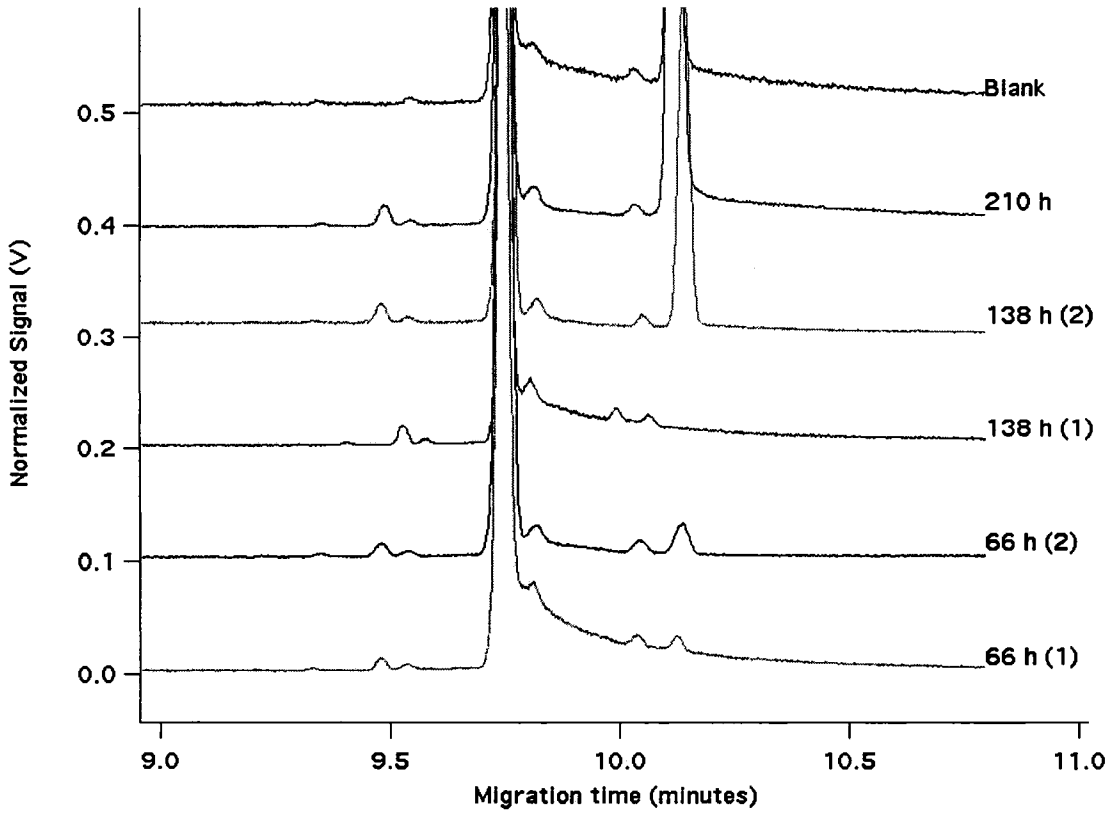

Figure 2.6: CE-LIF electropherogram of samples from a representative 10 cell GTA assay exhibiting GTA activity after incubation. Samples in this series were drawn and analyzed in duplicate. The peaks are normalized so that the intensity of the H-type II-TMR peak has an intensity of 1.0V.

Figure 2.7 shows the percent of H-type II antigen converted to A antigen over the course of incubation for the six MRCs within the fourth set. Similar to the 100 cell assay, there is an increasing amount of A antigen formed over time. After 210 h, 0.6%; 1.1%; 1.6%; 2.3%; 2.4%; and 2.9% of the H-type II antigen had been converted to A antigen in each of the six MRCs. Table 2.2 summarizes the percent conversion seen in other MRCs at the 10 cell level. After 334 h of incubation, an average of 0.5% was converted in the first set. The second set showed 1.1% conversion after 406 h and the third and fourth sets

showed 1.6% and 1.8% after 238 h and 210 h, respectively. Within each set, there is a large degree of variation in the amount converted. The relative standard deviation is between 23 and 49%. The variability may represent the heterogeneous nature of GTA expression in HT29 cells. Human error and/or adhesion of HT29 cells to the capillary wall may also be a factor. Allowing for the lower number of cells as well as the lower total volume, the rate of A antigen formation in the 10 cell assays was approximately the same as the 100 cell assays. Section 2.3.1 showed that using 1 cell per 10 nL, 5% conversion could be achieved in about 100 h. Thus, by extrapolation, an assay using 1 cell per 30 nL (the assay condition for 10 cell assays) should achieve 3.3% conversion after 200 h. The best 10 cell assay experiment (MRC 15) showed that a 2.9% conversion was achieved after 210 h. This is consistent with the results from 100 cell assays.

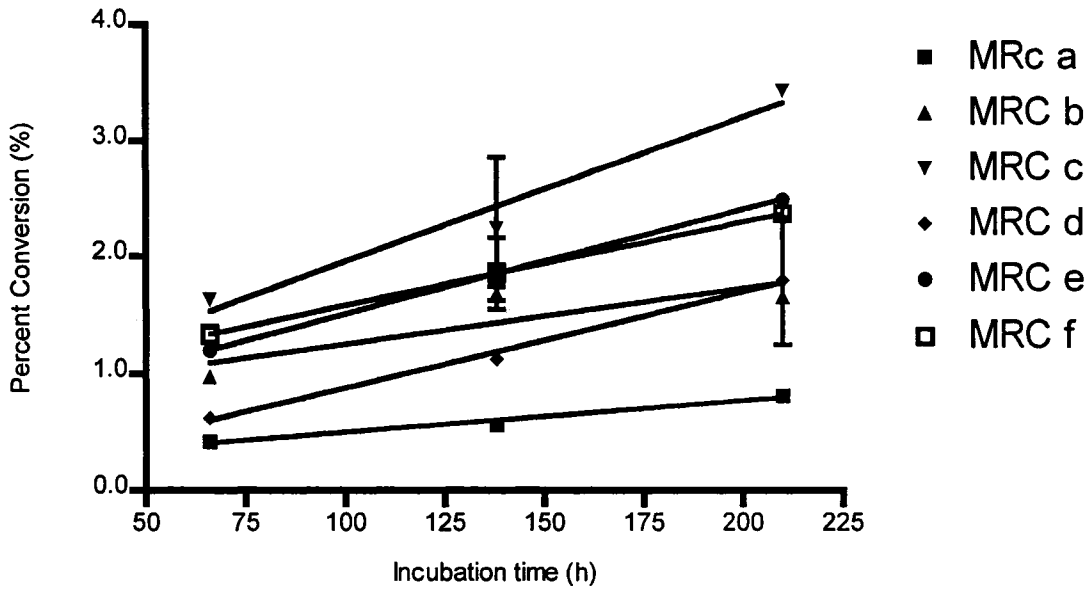

Figure 2.7: Graph of percentage of A antigen formed over the incubation period in individual MRC containing 10 HT 29 cells (1 cell 30 nL). Samples were taken and analyzed in duplicate.

Table 2.2: Summary of different 10 cell assays done in different MRC within different sets of experiment.

Set	Incubation Time (h)	MRC	Percent Converted (%)	Average (%)	Standard Deviation (%)	Relative Standard Deviation (%)
1	334	1	0.7	0.5	0.2	49
		2	0.2			
		3	0.7			
		4	0.4			
2	406	5	0.7	1.1	0.5	44
		6	0.9			
		7	1.6			
3	238	8	1.3	1.6	0.4	23
		9	1.8			
4	210	10	0.6	1.8	0.9	48
		11	1.1			
		12	1.6			
		13	2.3			
		14	2.4			
		15	2.9			

2.3.3 Single cell assay

Lastly, single cell assays were done. The reaction buffer and protocols were the same as the ten cell reaction except that only 100 nL of the reaction buffer was added to

each MRC using the nanopipettor and only one cell was added to each MRC. The volume of cell suspension added was approximately 75 nL. Unfortunately, the volume per cell had increased yet again to 1 cell per 175 nL. Thus, the reaction rate was expected to further decrease. The incubation was carried out as above and sampled at various times. A total of forty-two single cell studies were done in five sets of experiments over several weeks. For the purpose of discussion, only the fourth set is shown. Samples of 26 nL were withdrawn after 22, 48, and 80 h of incubation.

Figure 2.8 shows the trace of the TMR labeled oligosaccharide peaks in samples taken from the fourth set of single cell studies. This set contained nine reaction MRCs and one blank run. The A-TMR peak is found at 9.6 minutes and the H-type II-TMR peak at 9.9 minutes. It can be seen in this figure that only five of the nine MRCs assayed contained GTA activity. This is representative of the overall results discussed below. Figure 2.9 shows the overlaid traces of the seventh reaction of this set (trace g in Figure 2.8), A-TMR standard, and the co-injection of the seventh reaction with the A-TMR standard. The co-migration of the standard compound with the product in the reaction sample shows that the two are identical.

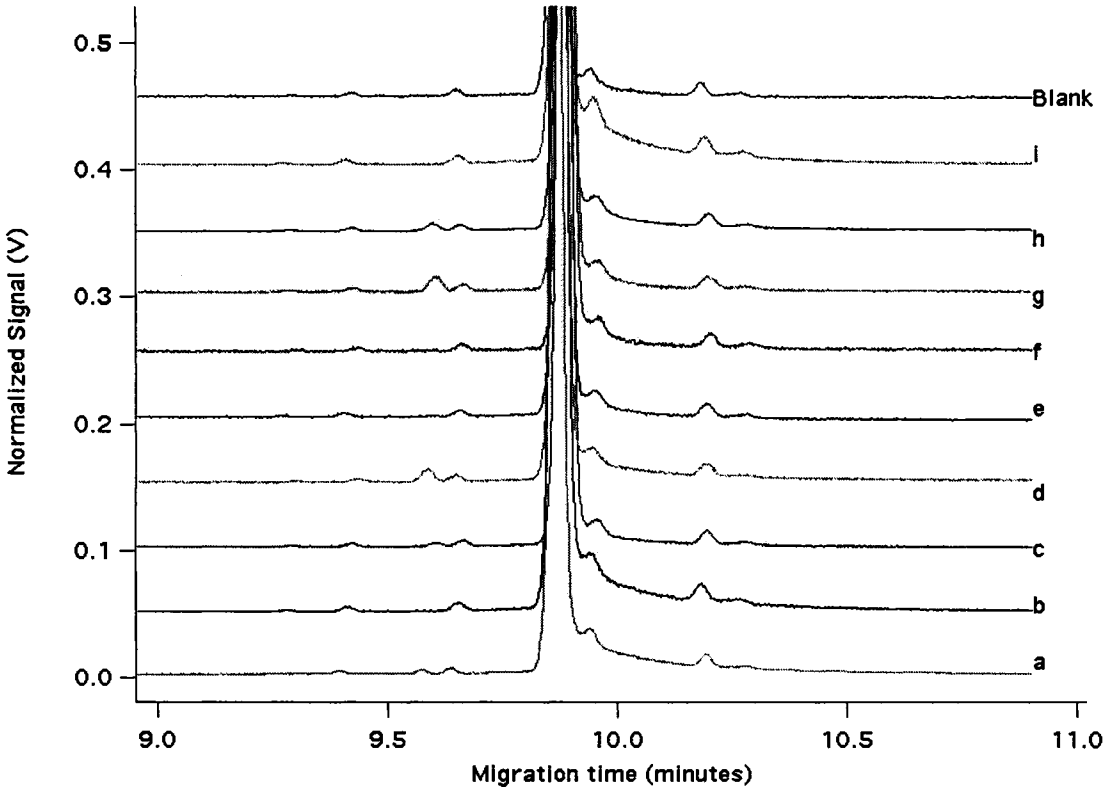

Figure 2.8: CE-LIF traces of samples from individual MRCs containing a single cell after 80 h of incubation. The peaks are normalized so that the intensity of the H-type II-TMR peak has an intensity of 1.0V.

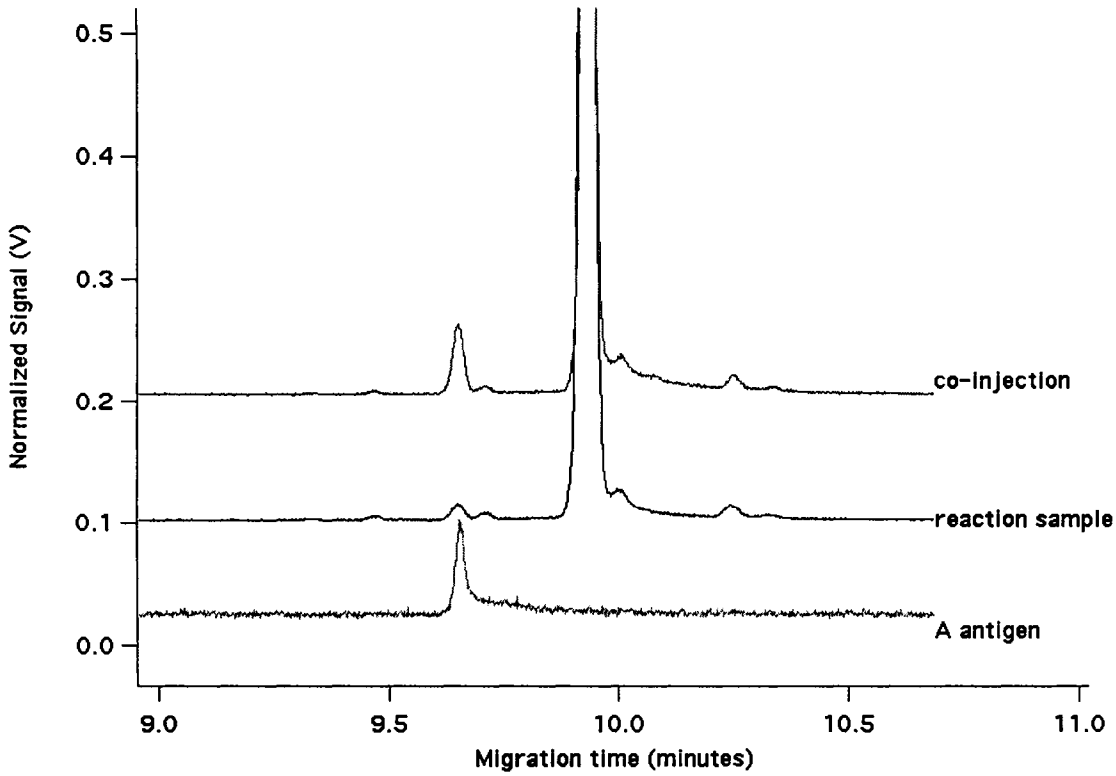

Figure 2.9: Individual traces of one MRC sample taken after 80 h of incubation, the A antigen alone, and co-injection of both A antigen and the sample. Co-migration of the A antigen with the peak in the sample shows that the two compounds are identical. With the exception of the A antigen trace, the peaks are normalized so that the intensity of the H-type II-TMR peak has an intensity of 1.0V.

Figure 2.10 shows the percent of H-type II antigen converted to A antigen over the course of incubation for those MRCs from the fourth set of single cell studies that contain a detectable amount of A antigen. Similar to the reaction profiles shown for 100 and 10 cell assays, the amount of H-type II antigen converted to A antigen increases over time. However, the amount converted is smaller, ranging in this set from 0.3% to 1.1%. Of all the sets of MRCs done, this set has the highest average amount of conversion. Using the average value of 0.6% conversion after 80 h by a single cell in 175 nL for

comparison against 100 cell assays, it can be seen that the single cell reactions have 2.5 times more activity than the 100 cell assays. Such discrepancy may be attributable to biases introduced during selection for single cells during single cell assay. Ichikawa *et al.* noted that the rate of GTA expression increased as HT29 cells become more malignant[27]. Since malignant cells are less adherent, it is possible that these cells are favorably sampled by the micromanipulator as they are less likely to stick to the microscope slide.

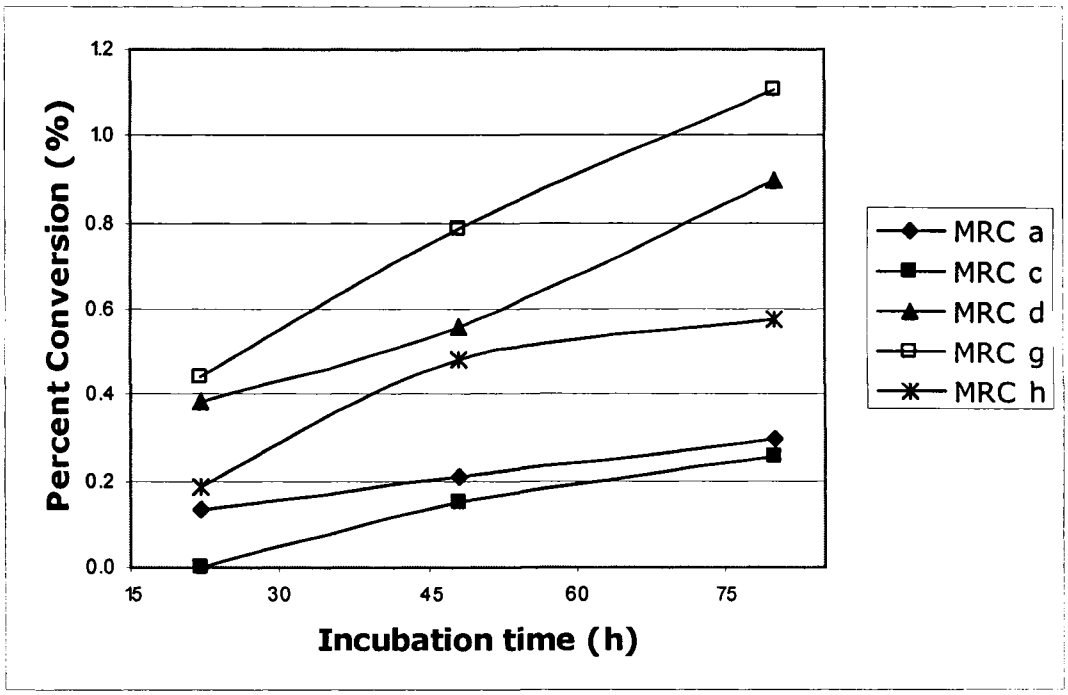

Figure 2.10: Graph of percentage of A antigen formed over the incubation period in MRCs containing a single cell showing detectable amounts of A antigen.

The low amounts of A antigen formed over the incubation period are most likely attributable to the low activity of GTA. In addition, GTA is most likely to be of low abundance in HT29 cells. In order to determine the amount of GTA present within HT29 cells, ELISA assays may be done if anti-GTA antibodies are available. Furthermore,

different types of human epithelial cells can be screened to determine which tissue contains the most GTA. With this information, those cells could be used to perform single cell GTA analysis in a shorter time period and/or higher reaction volumes.

Table 2.3 shows the percent converted in other single cell MRCs analyzed in all of the experiments. Of the forty-two MRCs tested, only twenty-four showed detectable GTA activity. This represents 57% of all of the single cells tested. The power of single cell analysis is shown here as the cells were individually tested for GTA activity. Had this been a bulk cell assay, the cells that did not express GTA would be mixed with GTA expressing cells and one would not have been able to distinguish the difference. In this experiment, the cells were chosen at random. With additional detailed observations and inference, it may be possible to determine a correlation between cell morphology and GTA expression.

Table 2.3: Summary of different single cell assays done in different MRCs within different sets of experiment.

Set	Incubation Time (h)	MRC	Percent Converted (%)	Average (%)	Standard Deviation (%)	Relative Standard Deviation (%)
1	52	1	0.1	0.4	0.3	89
		2	0.3			
		3	0.8			
		4	0.2			
2	46	5	0.1	0.3	0.2	85
		6	0.4			

3	50	7	0.1	0.1	0.05	40
		8	0.1			
		9	0.2			
		10	0.1			
4	80	11	0.3	0.6	0.4	59
		12	0.3			
		13	0.9			
		14	1.1			
		15	0.6			
5	120	16	0.1	0.2	0.1	55
		17	0.3			
		18	0.1			
		19	0.2			
6	75	20	0.1	0.2	0.1	58
		21	0.2			
		22	0.3			
		23	0.5			
		24	0.1			

To investigate the precision of the percent conversion data, samples taken from the first set of MRCs after 52 h of incubation were analyzed in triplicate by CE and integrated separately. As shown in Figure 2.11, there is a negative correlation between the percent converted and the relative standard deviation. This is most likely explained by the poor signal to noise ratio. Despite manual input of the peak limits, integration of

very low peaks is inaccurate and not reproducible, hence leading to the high relative standard deviation. To improve the precision of the results, assay conditions must be changed or modified so that a greater percent conversion is obtained.

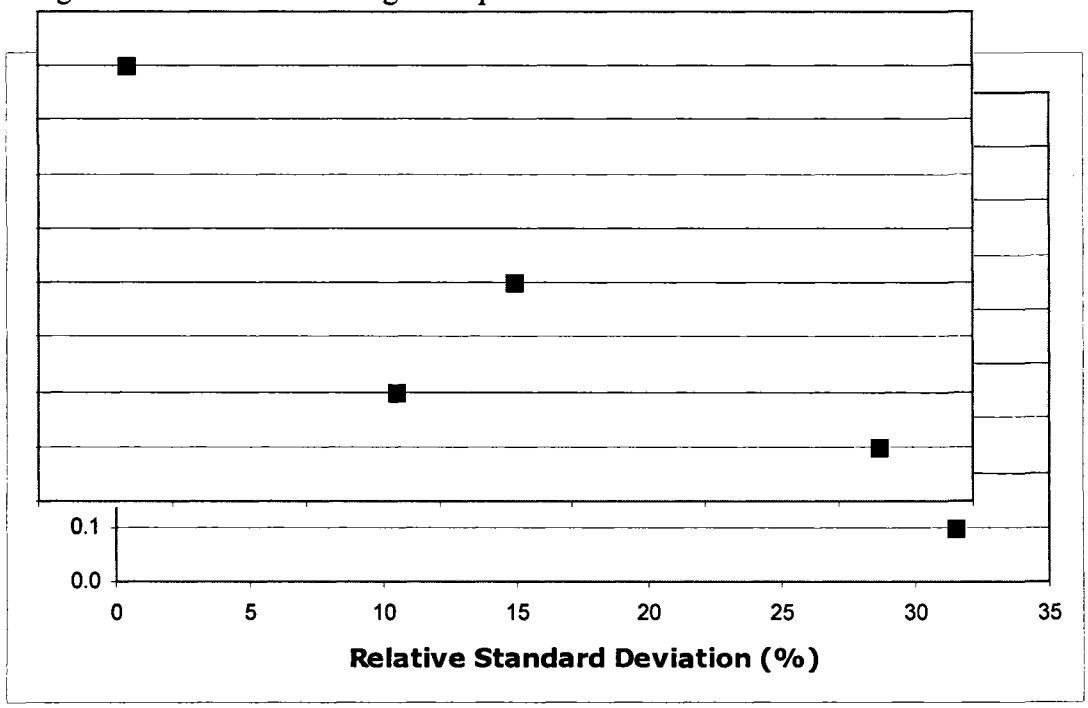

Figure 2.11: Scatter plot of the relationship between the percent of H-type II antigen converted to A antigen and the standard deviation of the value.

2.4 Comparison between single cell assay and flow cytometry

In section 2.5.3, it was determined that 56% of the cells tested showed detectable GTA activity. This is comparable to flow cytometry studies carried out by Hakamori *et al*[27]. They reported that approximately 50% of HT29 cells expressed the A antigen. To confirm their findings, flow cytometry was also performed here. The experiment done here only measured the level of A antigens and not GTA activity. Because the antigen will persist on the surface of the cell after synthesis by GTA, the level of antigen on the surface is not a direct measure of GTA activity. No correlation between antigen

abundance and enzyme activity can be determined using the protocol used here. It can only be used to determine the presence or absence of GTA within the cells.

Control incubations were done without secondary and primary antibodies to determine the background fluorescent signal (Figure 2.12). Figure 2.12.a shows the fluorescent signal from cells incubated without the fluorescent secondary antibody and shows the signal due to scattering. Figure 2.12.b shows the signal from cells incubated without the primary anti-A-antigen antibody and represents the non-specific adherence of the antibody to the cells in addition to scattering. Thresholds were set (M1 and M2) so that only signals above that intensity were analyzed in subsequent analysis.

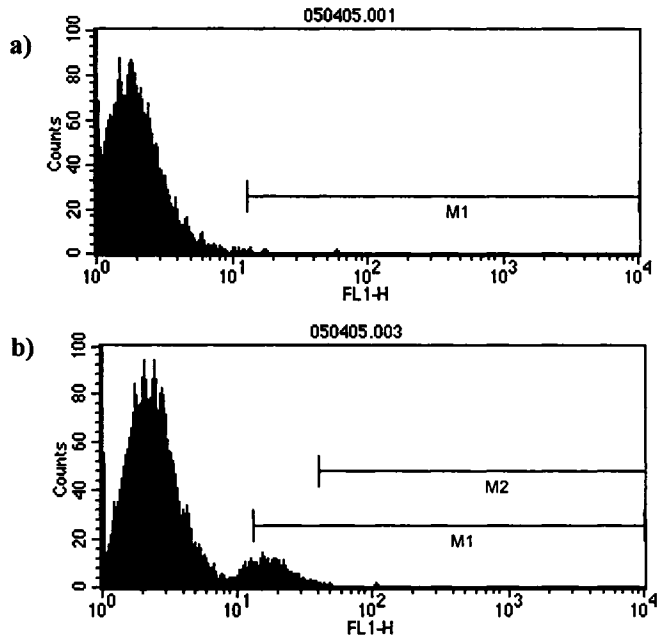

Figure 2.12: Fluorescence signal received of control incubations as analyzed by flow cytometry. Incubation in (a) was done without the fluorescent secondary antibody while incubation in (b) was done without the primary anti-A-antigen antibody.

As shown in Figure 2.13, 84% of the cells within the normally gated region (as specified in the protocol used) have a fluorescence signal above the background signal. In other words, 84% of the cells express the A antigen on their surface. However, if the gates are adjusted so that only cells smaller than the median cell size are analyzed, only 74% of the cells express the A antigen on the cell surface. Furthermore, if only the smallest 15% of the cells are analyzed, only 60% of the cells express the A antigen. This finding is significant because the bigger cells tend to sink faster on the microscope slide while on the micromanipulator. Even with a PHEMA coating on the slides, these cells are strongly adhered to the slide and are very difficult to retrieve for single cell analysis. Thus, smaller cells are more likely selected for single cell analysis. Thus, it is not surprising that only 56% of the cells tested in section 2.5.3 show any GTA activity.

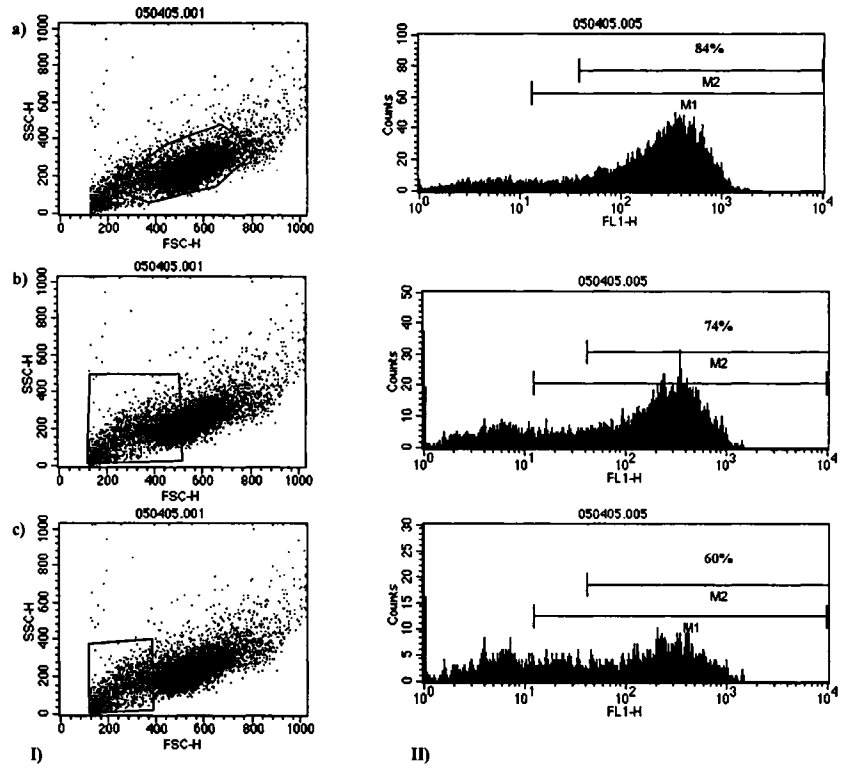

Figure 2.13: Flow cytogram of HT 29 cells stained with fluorescein linked secondary antibody to determine rate of A antigen expression. (I) scatter plots of side light scatter (SSC-H) and forward light scatter (FSC-H). Cells within the boxed region were selected for further analysis. Cells were selected based on: (a) established protocols, (b) smallest 50% of the cells, and (c) smallest 15% of the cells. (II) The cells selected from (I) were analyzed for their fluorescence intensity (FL1-H). The percentage of cells analyzed with fluorescence intensity above the threshold is shown.

The size of the cell and its fluorescence intensity was examined to ensure that the increased fluorescence was not caused by a larger cell with more A antigen on the surface. Figure 2.14 shows that there is a minimal positive correlation between the forward light scatter and the fluorescent intensity. However, the correlation is not strong

enough to explain the relationship between cell size and the proportion of cells with GTA expression.

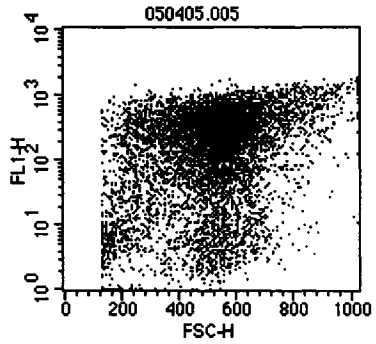

Figure 2.14: Scatterplot of the fluorescence signal (FL1-H) versus the cell size (measure in terms of forward light scatter, FSC-H).

2.5 Mycoplasma detection

According to the instructions provided with the mycoplasma detection kit, the presence of a 315 bp band in the PCR product indicates the presence of mycoplasma DNA and by inference, mycoplasma contamination. This band is clearly seen in the positive control lane of the agarose gel and not in the other lanes (Figure 2.15). The 500 bp band in each lane serves as an internal control to ensure that the PCR reaction occurred without error in each sample. These assays show that the cell cultures used were not contaminated with mycoplasma and the results from the GTA assays were not affected by mycoplasma.

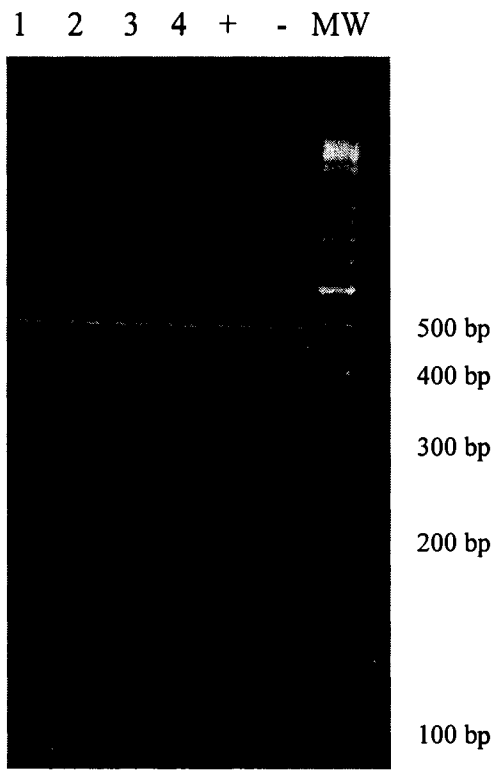

Figure 2.15: Products of the mycoplasma detection kit PCR products separated on a 2% agarose gel and stained in ethidium bromide. The lack of a 315 bp band in the samples indicate that the cell culture was not contaminated with mycoplasma. Lane 1 to 4 are from HT29 cell lines used in GTA assays; Lanes 5 and 6 are positive and negative controls, respectively.

2.6 Conclusions

In this chapter, the effective use of a micromanipulator and nanopipette to perform single cell assays is described. The power of single cell assays in analyzing GTA activity within single HT29 cells has been demonstrated. The nanopipette allows multiple samples to be taken from a single low-volume enzyme assay, which cannot be done using traditional single cell assays methods. The techniques may be refined further

to study enzyme kinetics. It is of note that these methods can be extended to any enzyme reaction that involves a fluorogenic substance.

The results obtained from the single cell assays agree with the results from flow cytometry. Approximately 50-60% of the smaller HT29 cells express GTA. To study larger cells, the microscope slide may need to have a coating different from PHEMA to reduce cell adhesion. Another alternative may be to modify the viscosity of the cell suspension so that the larger cells do not sink as quickly.

A drawback of this experimental protocol is the inability to confirm if a cell has been deposited into the MRC. While one can visually confirm the entry of a cell into the capillary and the absence of any cells in the capillary after deposition into the MRC, there are no direct methods to confirm the entry of a cell into the MRC. Attempts have been made by our research group to label amino acids from the cell with the fluorogenic tag naphthalene-2,3-dicarboxaldehyde (NDA) but they have been unsuccessful. Another alternative is to perform polymerase chain reaction on the genome of the cell but a suitable protocol to amplify a target present in high copy number has yet to be identified.

Lastly, modern flow cytometers are capable of sorting single cells into 384 and/or 1536 well plates. Thus, future experiments could use the flow cytometer to determine the amount of blood group A antigen on the surface of a cell. If the cell possesses an extraordinary amount of antigen, it could be selected for single cell analysis. In this fashion, the time-consuming task of selecting for single cells by the micromanipulator would be avoided. However, other concerns such as evaporation and sampling with the nanopipettor must be addressed.

2.7 References

(1) Woods, L. A.; Roddy, T. P.; Ewing, A. G. *Electrophoresis* **2004**, *25*, 1181-1187.

(2) Krylov, S. N.; Arriaga, E.; Zhang, Z. R.; Chan, N. W. C.; Palcic, M. M.; Dovichi, N. J. *Journal of Chromatography B* **2000**, *741*, 31-35.

(3) Gillmor, C. S.; Poindexter, P.; Lorieau, J.; Palcic, M. M.; Somerville, C. *Journal of Cell Biology* **2002**, *156*, 1003-1013.

(4) Li, H.; Sims, C. E.; Kaluzova, M.; Stanbridge, E. J.; Allbritton, N. L. *Biochemistry* **2004**, *43*, 1599-1608.

(5) Hu, S.; Zhang, L.; Krylov, S.; Dovichi, N. J. *Analytical Chemistry* **2003**, *75*, 3495-3501.

(6) Muirhead, K. A.; Horan, P. K.; Poste, G. *Bio-Technology* **1985**, *3*, 337-356.

(7) Kennedy, R. T.; Oates, M. D.; Cooper, B. R.; Nickerson, B.; Jorgenson, J. W. *Science* **1989**, *246*, 57-63.

(8) Hogan, B. L.; Yeung, E. S. *Analytical Chemistry* **1992**, *64*, 2841-2845.

(9) Hu, S.; Lee, R.; Zhang, Z.; Krylov, S. N.; Dovichi, N. J. *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences* **2001**, *752*, 307-310.

(10) Han, F. T.; Lillard, S. J. *Analytical Biochemistry* **2002**, *302*, 136-143.

(11) Tong, W.; Yeung, E. S. *Journal of Chromatography B* **1997**, *689*, 321-325.

(12) Anderson, A. B.; Xiong, G. H.; Arriaga, E. A. *Journal of the American Chemical Society* **2004**, *126*, 9168-9169.

(13) Gunasekera, N.; Olson, K. J.; Musier-Forsyth, K.; Arriaga, E. A. *Analytical Chemistry* **2004**, *76*, 655-662.

(14) Krylov, S. N.; Zhang, Z. R.; Chan, N. W. C.; Arriaga, E.; Palcic, M. M.; Dovichi, N. J. *Cytometry* **1999**, *37*, 14-20.

(15) Daniels, G. *Human Blood Groups*, Second ed.; Blackwell Science: Berlin, 2002.

(16) Daniels, G. *Blood Reviews* **1999**, *13*, 14-35.

(17) Watkins, W. M.; Morgan, W. T. J. *Nature* **1957**, *180*, 1038-1040.

(18) Kobata, A.; Grollman, E. F.; Ginsburg, V. *Archives of Biochemistry and Biophysics* **1968**, *124*, 609-&.

(19) Kobata, A.; Grollman, E. F.; Ginsburg, V. *Biochemical and Biophysical Research Communications* **1968**, *32*, 272-&.

(20) Yamamoto, F.; Clausen, H.; White, T.; Marken, J.; Hakomori, S. I. *Nature* **1990**, *345*, 229-233.

(21) Fersht, A. *Enzyme Structure and Mechanism*; W.H. Freeman and Co.: New York, 1985.

(22) Radzicka, A.; Wolfenden, R. *Science* **1995**, *267*, 90-93.

(23) Banerjee, S. K.; Rupley, J. A. *Journal of Biological Chemistry* **1975**, *250*, 8267-8274.

(23a) Thorsen, T. S.; Johnsen, A. H.; Josefsen, K.; Jensen, B. *Biochim Biophys Acta* **2006**, *1764*, 671-676.

(23b) Christakopoulos, P.; Goodenough, P. W.; Kekos, D.; Macris, B. J.; Claeyssens, M.; Bhat, M. K.; Thorsen, T. S.; Johnsen, A. H.; Josefsen, K.; Jensen, B. *Eur J Biochem* **1994**, *224*, 379-385.

(24) Fogh, J.; Trempe, G. In *Human Tumor Cells*; Fogh, H., Ed.; Plenum Press: New York, 1975.

(25) Laferte, S.; Chan, N. W. C.; Sujino, K.; Lowary, T. L.; Palcic, M. M. *European Journal of Biochemistry* **2000**, *267*, 4840-4849.

(26) Ye, M. L.; Hu, S.; Quigley, W. W. C.; Dovichi, N. J. *Journal of Chromatography A* **2004**, *1022*, 201-206.

(27) Ichikawa, D.; Handa, K.; Hakomori, S. *International Journal of Cancer* **1998**, *76*, 284-289.

(28) Iwamoto, S.; Withers, D. A.; Handa, K.; Hakomori, S. *Glycoconjugate Journal* **1999**, *16*, 659-666.

(29) Harris, D. C. *Quantitative Chemical Analysis*, Fifth ed.; Freeman and Co: New York, 1999.

(30) Li, S. F. Y. *Capillary electrophoresis : principles, practice, and applications*; Elsevier, New York, 1992.

(31) Beckman Instruments. *Introduction to Capillary Electrophoresis*; Beckman Instruments: Fullerton CA, 1991.

(32) Suzuki, S.; Honda, S. *Electrophoresis* **1998**, *19*, 2539-2560.

(33) Mazzzeo, J. R. In *Handbook of capillary electrophoresis*; Landers, J. P., Ed.; CRC Press, 1997.

(34) Scherz, H. *Electrophoresis* **1990**, *11*, 18-22.

(35) Le, X. C.; Zhang, Y.; Dovichi, N. J.; Compston, C. A.; Palcic, M. M.; Beever, R. J.; Hindsgaul, O. *Journal of Chromatography A* **1997**, *781*, 515-522.

(36) Zhang, Y. N.; Le, X. C.; Dovichi, N. J.; Compston, C. A.; Palcic, M. M.; Diedrich, P.; Hindsgaul, O. *Analytical Biochemistry* **1995**, *227*, 368-376.

(37) Zhao, J. Y.; Dovichi, N. J.; Hindsgaul, O.; Gosselin, S.; Palcic, M. M. *Glycobiology* **1994**, *4*, 239-242.

(38) Le, X. C.; Scaman, C.; Zhang, Y. N.; Zhang, J. H.; Dovichi, N. J.; Hindsgaul, O.; Palcic, M. M. *Journal of Chromatography A* **1995**, *716*, 215-220.

(39) Craig, D.; Arriaga, E. A.; Banks, P.; Zhang, Y.; Renborg, A.; Palcic, M. M.; Dovichi, N. J. *Analytical Biochemistry* **1995**, *226*, 147-153.

(40) Krylov, S. N.; Arriaga, E. A.; Chan, N. W. C.; Dovichi, N. J.; Palcic, M. M. *Analytical Biochemistry* **2000**, *283*, 133-135.

(41) Zarrine-Afsar, A.; Krylov, S. N. *Analytical Chemistry* **2003**, *75*, 3720-3724.

(42) Lorieau, J.; Shoemaker, G. K.; Palcic, M. M. *Analytical Chemistry* **2003**, *75*, 6351-6354.

(43) Khare, D. P.; Hindsgaul, O.; Lemieux, R. U. *Carbohydrate Research* **1985**, *136*, 285-308.

(44) Shoemaker, G. K.; Lorieau, J.; Lau, L. H.; Gillmor, C. S.; Palcic, M. M. *Analytical Chemistry* **2005**, *77*, 3132-3137.

(45) Krylov, S. N.; Dovichi, N. J. *Electrophoresis* **2000**, *21*, 767-773.

(46) Seto, N. O. L.; Compston, C. A.; Evans, S. V.; Bundle, D. R.; Narang, S. A.; Palcic, M. M. *European Journal of Biochemistry* **1999**, *259*, 770-775.

(47) Jackson, A. L.; Warner, N. L. In *Manual of Clinical Laboratory Pathology*; Rose, A. L., Ed.; American Society of Microbiology: Washington DC, 1986, pp 226-235.

Chapter 3: Sensitive detection of activity in a low activity mutant enzyme[2]

3.1 Introduction

Traditional enzyme assays methods have usually employed detection methods such as absorbance spectroscopy, scintillation counting, or changes in pH or dissolved oxygen[1]. These techniques rely on the enzyme to modify the substituents of the reaction buffer so that a change in absorbance, attachment of the radioisotope, or the detection of a byproduct can be monitored.

In general, enzyme assays can be classified by their detection methods: direct or indirect[2]. One type of direct detection is spectroscopy. This is a fast, continuous, and simple method that monitors the presence or absence of a compound that absorbs UV or visible light. The change in the concentration of reduced nicotinamide adenine dinucleotide (NADH) during an enzyme assay is often monitored by spectroscopy since it has a strong absorbance at 340 nm. An example where this detection method is used is the assay for alcohol dehydrogenase. This enzyme is assayed by the detection of NADH as the enzyme oxidizes methanol to formaldehyde with the concomitant reduction of NAD^+ to NADH.

Indirect detection methods are methods in which the product of the enzyme of interest is used as substrate for another enzyme[3]. This is required as the product of the first enzyme is difficult to detect. However, the introduction of a second enzyme adds to the complexity of the assay conditions. The conditions must be adjusted so that there is minimal lag time in the response of the second enzyme and must be optimal for both

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[2] This chapter has been published as sections within *The Journal of Biological Chemistry* **2005**, *280*, 36848-36856 by Ma *et al.*

enzyme reactions. An example of this enzyme detection method is the assay for alanine transaminase (ALT) (Figure 3.1). ALT catalyzes the formation of pyruvate from alanine. Pyruvate is then used by lactate dehydrogenase (LDH) to form lactate with the concomitant oxidation of NADH to NAD^+. The decrease in NADH concentration is then monitored by UV-visible spectroscopy.

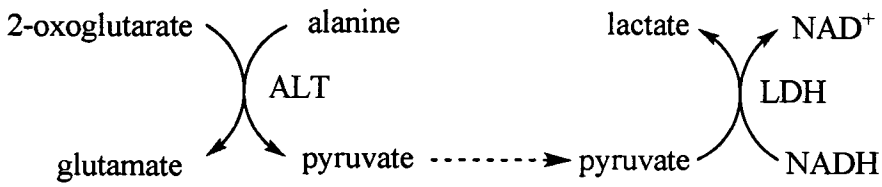

Figure 3.1: Schematic representation of ALT enzyme assay coupled to LDH to detect the loss of NADH.

A common indirect detection method used in the assay of glycosyltransferases is the use of radioisotopes and scintillation counting[4, 5]. A radioisotope, usually ^3H, is incorporated into one of the enzyme substrates, the monosaccharide of the nucleotide donor. During the incubation period, the monosaccharide containing ^3H is transferred from the donor to the acceptor molecule. The unreacted donor and acceptor are then separated on reversed-phase cartridges and the amount of ^3H incorporated into the acceptor is determined by scintillation counting. This assay method can provide accurate enzyme kinetic values but may give ambiguous results for low activity enzymes if less than 1 picomole (10^{-12} moles) of the product is formed[6].

In contrast to the relatively high detection limit of scintillation counting, CE-LIF can provide sensitive detection at tens of zeptomole (10^{-20}) levels[7]. This is done by attaching fluorophores to the substrate so that changes in the reaction mixtures can be

monitored by fluorescence spectroscopy. Fluorescence detection has been used to assay enzymes such as kinases[8], phosphatases[9], glycosidases[7], and glycosyltransferases[10].

In the previous chapter, the use of the separation power of CE and the sensitivity of LIF to assay for enzyme activity in a single cell were demonstrated. In this chapter, the use of CE-LIF to determine the activity of a low activity mutant *Helicobacter pylori* α-1,4 fucosyltransferase is described.

3.1.1 Fucosyltransferase in *H. pylori*

Fucosyltransferase (FucT) are enzymes that catalyze the addition of fucose from guanosine diphosphate β-L-fucose (GDP-Fuc) to the OH-3 and/or OH-4 of N-acetylglucosamine (GlcNAc) in N-acetyllactosamine (LacNAc). α-1,3 FucT transfers the fucose moiety onto the OH-3 of GlcNAc in Type II (LacNAc) acceptors while α-1,4 FucT transfers the fucose onto the OH-4 of GlcNAc in Type I acceptors (Figures 3.2 and 3.3). In *H. pylori*, they are involved in the biosynthesis of Lewis antigens[11]. These Lewis antigens are thought to play a role in the adhesion of the bacteria to the gastric lining[12], internalization by the gastric cells[13], and evasion of the host immune system by molecular mimicry[14]. If left to persist in the gastrointestinal tract, *H. pylori* can lead to peptic ulcers or gastritis. A more thorough understanding of the Lewis antigen biosynthetic pathway is sought in order to develop new therapies against *H. pylori* adhesion and internalization.

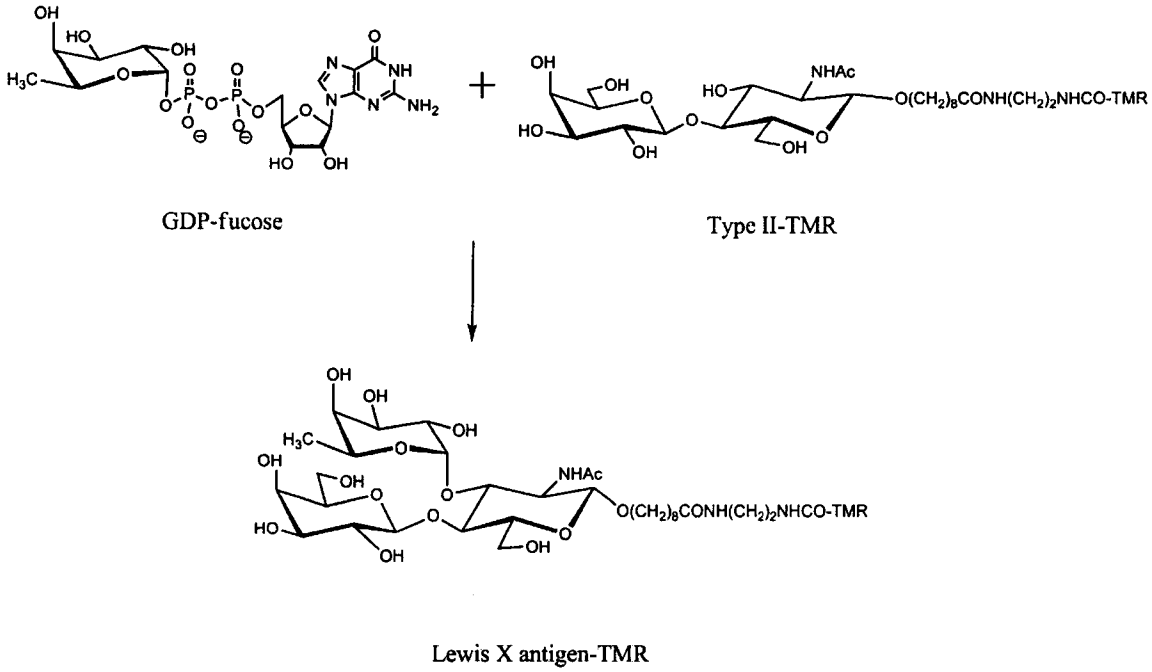

Figure 3.2: Schematic representation of the reaction catalyzed by α-1,3 FucTs. While not necessary for the enzyme to work, the TMR tag allows for LIF detection of the oligosaccharide.

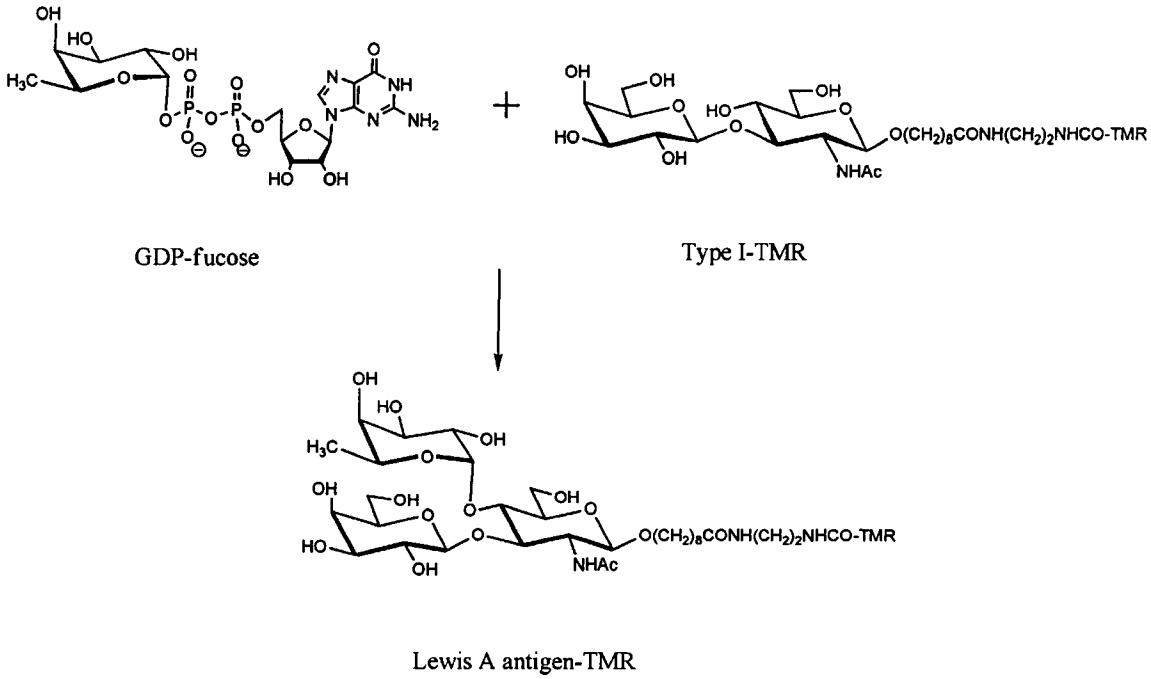

Figure 3.3: Schematic representation of the reaction catalyzed by α-1,4 FucTs. While not necessary for the enzyme to work, the TMR tag allows for LIF detection of the oligosaccharide.

Lewis X antigens are formed by α-1,3 FucTs while Lewis A antigens are formed by α-1,4 FucTs. Of the two strains of *H. pylori* studied in this work, NCTC11639 and UA948, the FucT expressed in 11639 is known to be a strict α-1,3 FucT while the enzyme expressed in UA948 is an α-1,3/4 FucT. This means that UA948FucT has both α-1,3 and α-1,4 activity and can form both Lewis X and Lewis A antigens. In both of these strains, the FucT is encoded by the *futA* gene and they are similar in amino acid sequence. Previous studies have shown that the α-1,4 activity in UA948FucT is conferred by a short hypervariable sequence in the C-terminal region of the enzyme's amino acid sequence[15]. While the sequence of most *H. pylori* FucTs is highly conserved, this hypervariable sequence is different between each enzyme (Figure 3.4). Replacement

of this sequence with the corresponding sequence from 11639FucT abolishes α-1,4 activity in the hybrid UA948 FucT. To understand better which amino acids within this sequence are responsible for α-1,4 activity, point mutations were made in this sequence for both 11639 and UA948 FucTs, and the mutant enzymes were assayed for α-1,4 activity. The hypothesis was that the tyrosine residue at position 350 in UA948 is critical for α-1,4 activity. Thus, this residue was mutated to phenylalanine, tryptophan, alanine, and glycine. A corollary was that by changing the phenylalanine in 11639FucT to tyrosine, α-1,4 activity can be conferred to this enzyme. Since there are two phenylalanine residues in the hypervariable region of 11639, both were mutated independently. The two phenylalanines were also changed to alanine to determine the effect of a non-aromatic amino acid at that position. Radioisotopes and scintillation counting were used to determine its specificity and activity but the results were ambiguous as the level of activity was too low.

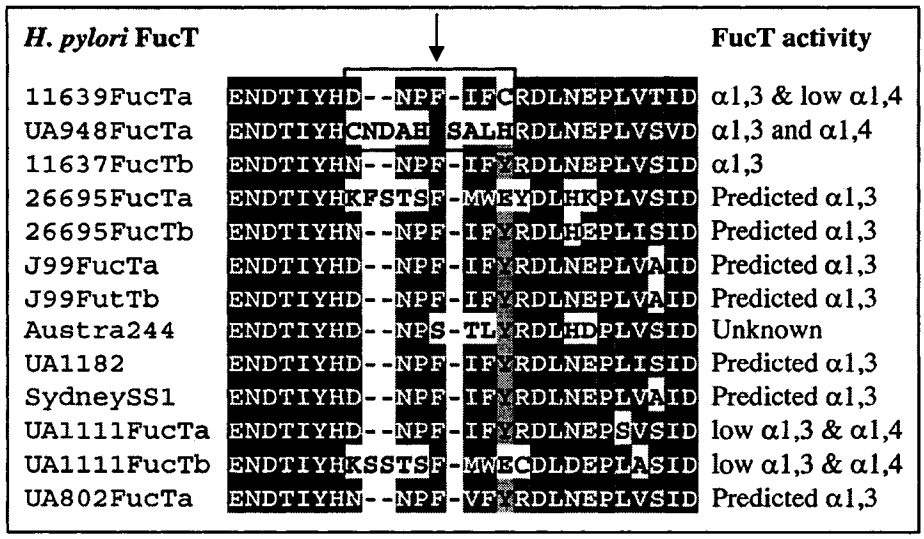

Figure 3.4: Alignment of the C-terminal hypervariable loop region of thirteen *H. pylori* α-1,3/4 FucT. The dark boxes represent highly conserved residues and grey boxes represent partially conserved residues. The boxed region highlights the residues identified as being responsible for determining α-1,3 or α-1,4 activity in 11639 FucT and UA948FucT. The arrow denotes the position of amino acid 350 in the sequence of UA948FucT. Modified with permission from Ma *et al*[16].

Identification of peaks in CE-LIF is based on comparing the migration time with a known standard. However, if another species has the same migration characteristic as the analyte of interest, then another method must be used for confirmation. To confirm that the putative Lewis antigen is a product of a FucT, fucosidase can be added to the final reaction mixture. Whereas FucTs add a fucose moiety to the carbohydrate acceptor molecule, fucosidases remove it. By monitoring the decreasing amount of the Lewis antigen present in a fucosidase reaction, one can confirm that the original FucT product is formed by the addition of a fucose molecule and that the detection of the CE-LIF peak is

not due to a co-migrating compound. Thus, fucosidase was used here to confirm the formation of Lewis A by the α-1,4 FucTs assayed.

3.2 Experimental conditions

3.2.1 Construction and expression of mutant enzymes

This part of the experiment was performed by Dr. Bing Ma and is documented in detail within her Ph.D. thesis[17].

3.2.2 Enzyme assay conditions for FucT

The FucT enzyme conditions were modified from the standard FucT assay conditions described previously[18]. The incubation mixture included 6 μL of the crude *E. coli* extract, 10 mM Type I-TMR, 200 mM GDP-Fuc in a final volume of 10 μL. The solution was buffered with 20 mM HEPES (pH 7.0), 100 mM NaCl, 35 mM $MgCl_2$, 1 mM adenosine triphosphate, 20 mM $MnCl_2$, and 5 mg mL^{-1} bovine albumin serum. The reaction was carried out in 0.6 mL PCR tubes and incubated at 37 °C. Samples were taken at 0, 0.5, 1, 2, and 5 h and diluted twenty-five fold with CERB to quench the reaction. Negative control reactions consisted of time zero samples as well as a reaction where the *E. coli* vector pGEM was expressed without the *futA* gene.

Fucosidase treatment was done by heating 4 μL of the 5 h reaction mixture to 90 °C for 10 minutes. Then, 20 mU of α-1,3/4 fucosidase was added along with 50 mM sodium phosphate (pH 5.0) to a final volume of 40 μL. Incubation was then carried out at 37 °C for 6 h. Samples were taken and quenched with CERB as above.

3.2.3 CE-LIF conditions for FucT

The CE-LIF conditions were similar to those described in Chapter 2 with the exception of the CERB. Based on the work of Chan *et al.*[19] on the separation of Lewis antigens by CE, the CERB was modified to contain 10 mM SDS, 10 mM sodium borate, 10 mM phenylboronic acid, and 10 mM sodium dihydrogen phosphate, pH 9.3. The solution was filtered through a 0.22 μm filter before use. Sample injection, electrophoresis, detection, and integration remained the same as in Chapter 2.

The percent conversion of mutant Type I-TMR to Lewis A-TMR was normalized against the wildtype enzyme using total protein expression and FucT expression. Methods used for protein and FucT assays were carried out by Dr. Bing Ma and are described in the published manuscript[16].

3.3 Results and discussion

3.3.1 NCTC11639 mutant α-1,3 FucT

Initial experiments were done with GDP-[^{3}H]Fuc. In this assay method, the [^{3}H]Fuc was transferred to the type II acceptor molecule. The acceptor molecule was then separated from the unreacted [^{3}H]Fuc by reversed-phase separation and the amount of [^{3}H]Lewis X antigen was detected by scintillation counting.. This assay showed that the wildtype 11639FucT had only trace levels of α-1,4 activity. Upon concentrating the crude cell extract, its activity was determined to be 0.05 mU mg^{-1} (*i.e.* 0.05 nmol of product was generated per minute per mg of protein as one enzyme unit (U) is equivalent

to the formation of 1 μmol of product per minute). This translates to less than 1 pmol of product formed during the course of the incubation. This is close to the detection limit of a radiochemical assay with scintillation counting so CE-LIF was used to determine the activity more accurately. In addition to the wildtype enzyme, four mutants were also tested: F350Y, F352Y, F350A, and F352A.

CE-LIF analysis showed that after an incubation of 2 h, 6% of the Type I-TMR substrate had been converted to Lewis A-TMR by the wildtype enzyme (Figure 3.5). This is in contrast to radiochemical assays that suggested that the enzyme was minimally active. As well, the two tyrosine mutants had *ca.* 50% more conversion than the wildtype, while the two alanine mutants had an activity that was the same as or less than the wildtype. These results show that the hydroxyl group in tyrosine had conferred increased α-1,4 activity to 11639FucT. Also, the aromatic nature of the residues at 350 and 352 are required for α-1,4 activity. Curiously, another aromatic amino acid, tryptophan, has been implicated in conferring α-1,4 activity in a human FucT[20]. After 5 h of incubation, the total amount of Lewis A made by each of the enzymes increased but the relative amounts remained approximately the same.

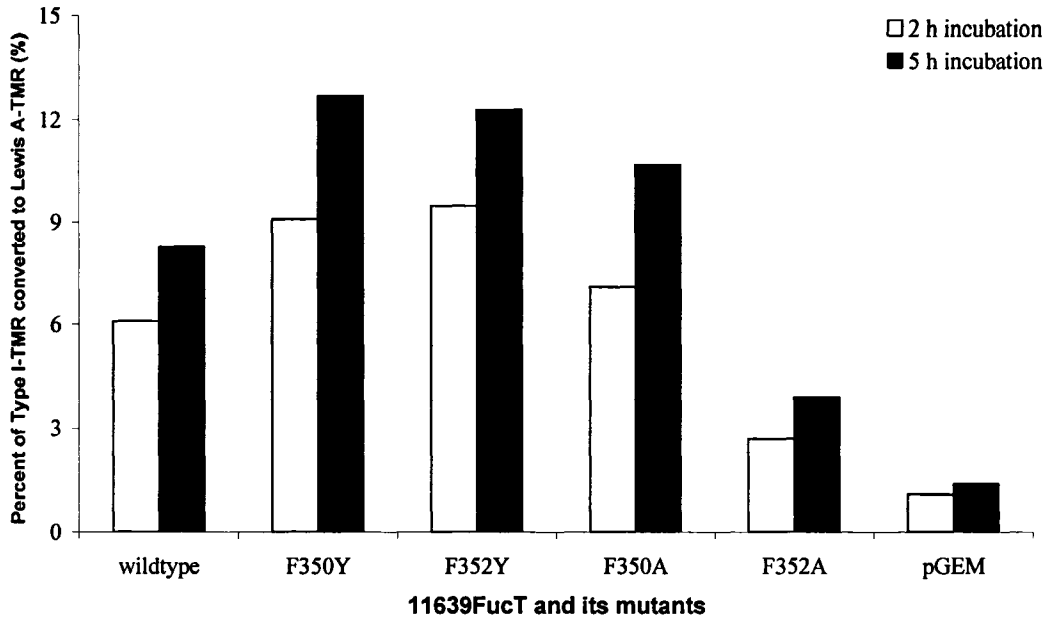

Figure 3.5: Amount of Lewis A-TMR formed after 2 and 5 h incubations of NCTC11639 wildtype and mutant FucTs. The pGEM reaction is a negative control consisting of the expression vector only.

Figure 3.6 shows the CE traces of all the mutants along with the wildtype enzyme. The traces have been offset for clarification. It is of note that the pGEM negative control also contained a small peak co-migrating with the Lewis A-TMR peak. The area of this peak was subtracted from all other results before analysis. While the amount of Lewis A-TMR formed is small, it is detectable by LIF.

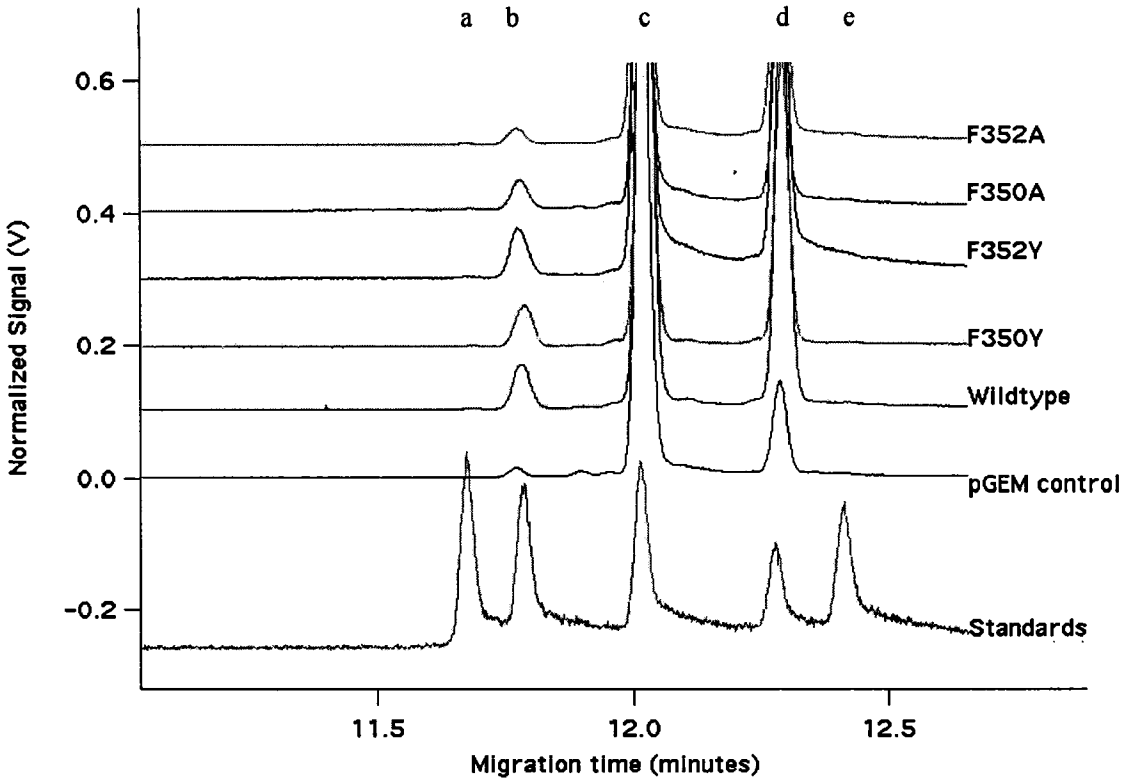

Figure 3.6: CE-LIF trace of the NCTC11639 series of reactions after 5 h of incubation. The standards run consisted of Lewis B-TMR (peak a), Lewis A-TMR (peak b), Type I -TMR (peak c), GlcNAc-TMR (peak d), and octamethyl-ethyldiamine-TMR (peak e). The identical migration pattern between the samples and standards suggests that the product peak is Lewis A-TMR. The peaks are normalized so that the intensity of the Type I-TMR peak has an intensity of 1.0V.

An injection of a mixture of oligosaccharide-TMR standards was also made. This standard included Lewis B-, Lewis A-, Type I-, GlcNAc-, and octamethyl-ethyldiamine - TMR. The migration characteristic of the Lewis A-TMR standard is identical to the peaks in the reaction samples so the reaction product peak can be identified as Lewis A-TMR. Also, the degradative product seen in the reaction traces suggests that endogenous *E. coli* β-galactosidases had converted some Type I-TMR to GlcNAc-TMR. However,

Type I-TMR is present in large excess and the minute decrease in its concentration should not affect the reaction kinetics of the FucT being assayed.

3.3.2 UA948 mutant α-1,3 /4 FucTs

Radiochemical assays of the UA948 alanine mutants had showed that the alanine mutants contained no detectable activity. CE-LIF was used to confirm these findings. After 1 h of incubation, approximately 6% of the Type I-TMR was converted to Lewis A-TMR by the two alanine mutant enzymes (Figure 3.7). Compared to the wildtype enzyme, this is a 12-fold decrease in activity but the alanine mutants are not totally inactive as determined by radioisotopes and scintillation counting.

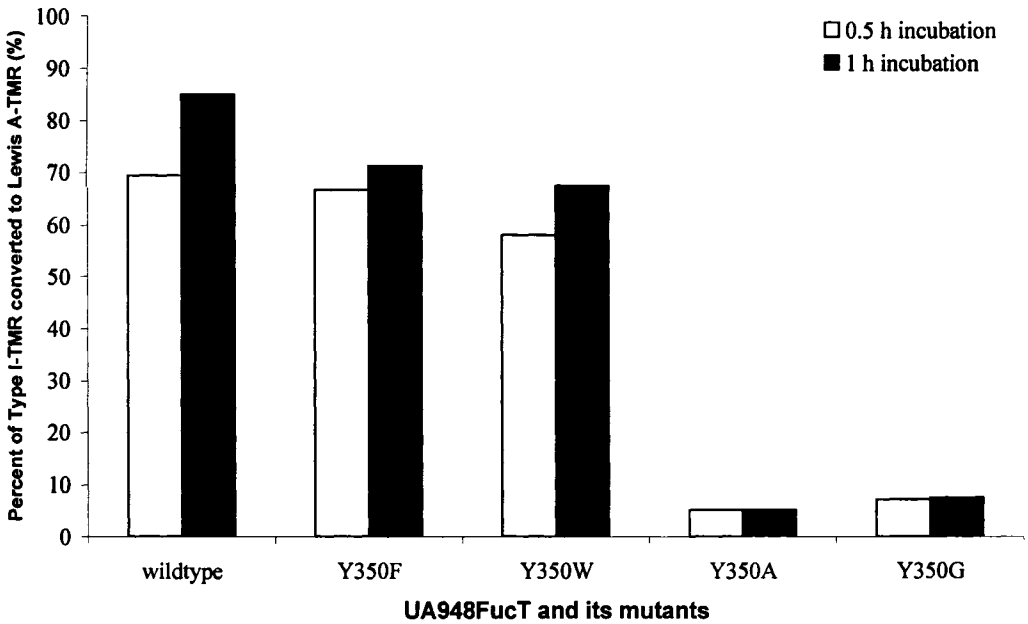

Figure 3.7: Amount of Lewis A-TMR formed after 0.5 and 1 h incubations of UA948 wildtype and mutant FucTs.

Figure 3.8 shows the electropherogram of the UA948 wildtype enzyme along with the mutants created. The Lewis A-TMR peak in the wildtype, Y350F, and Y350W traces towers over the Lewis A-TMR peak in the Y350A and Y350G traces. This shows that CE-LIF is good for high activity enzymes in addition to low activity enzymes analyzed in the previous section. As long as the percent of substrate converted is between 0 and 100%, then accurate integration of the substrate and product peaks can be done.

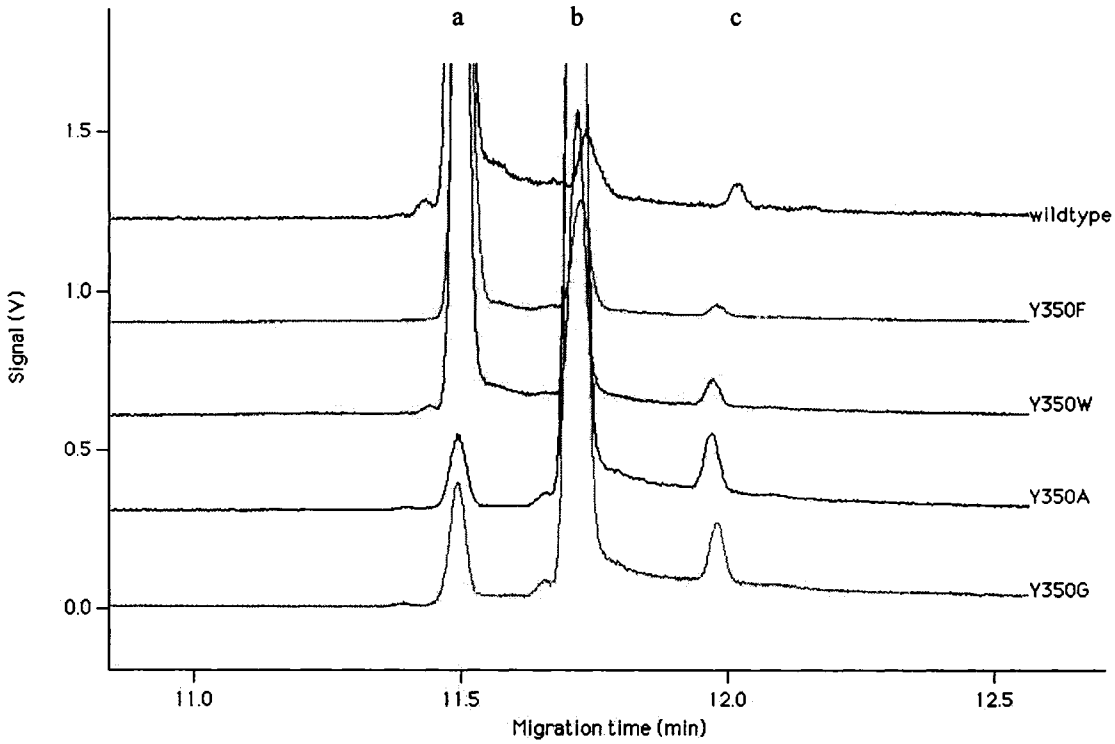

Figure 3.8: CE-LIF trace of the UA948 series of reactions after 1 h of incubation. The peaks for Lewis A-TMR (a), Type I-TMR (b), and GlcNAc-TMR (c) can be clearly seen. The peaks in this electropherogram were not normalized as the Type I-TMR peaks are small in the traces for the wildtype, Y350F, and Y350W enzymes.

The phenylalanine and tryptophan mutants had similar levels of activity to that of the wildtype. This suggests that the aromatic nature of the residue is responsible for α-

1,4 activity. While the hydroxyl group in tyrosine contributes to optimal α-1,4 activity, it is not essential. The CE-LIF provides complementary data to the radioassay findings. At the present level of data analysis, CE-LIF offers a qualitative measure of FucT activity in that it can accurately distinguish between enzymes with no activity or enzymes with low activity. It is also a quantitative method as it allows the researcher to determine the percentage of substrate that has converted to product. Radioassay is another quantitative method but it has the advantage of being able to determine enzyme kinetic values. While it would be a time consuming assay method, CE-LIF assay conditions can be adjusted so that it can also provide kinetic values. This would then be useful in determining the reaction kinetics of very low activity enzymes.

3.3.3 Fucosidase treatment of reaction products

As noted previously, the pGEM negative control incubation also produced a peak with migration characteristics similar to Lewis A-TMR. To ensure that this peak is not a true Lewis A-TMR peak, fucosidase was added to the original enzyme incubations. Fucosidases remove fucose moieties from their substrates. If the peak in question is a true product of FucT, incubation with fucosidase should reduce the amount of Lewis A-TMR over time.

After 6 h of incubation with fucosidase, the ratio of Lewis A-TMR to Type I-TMR decreased in all of the FucT enzyme incubations but not the pGEM control incubation (Figures 3.9 and 3.10). Furthermore, the control reaction did not show a decrease in the ratio measured and shows that the decrease in Lewis A-TMR is due to fucosidase and not a non-specific degradation. Taken together, this confirms that the

product peak in the FucT incubations is mostly due to the formation of Lewis A-TMR. The minor peak formed in the pGEM samples is most likely due to another glycosyltransferase other than FucT present in the *E. coli* crude extract.

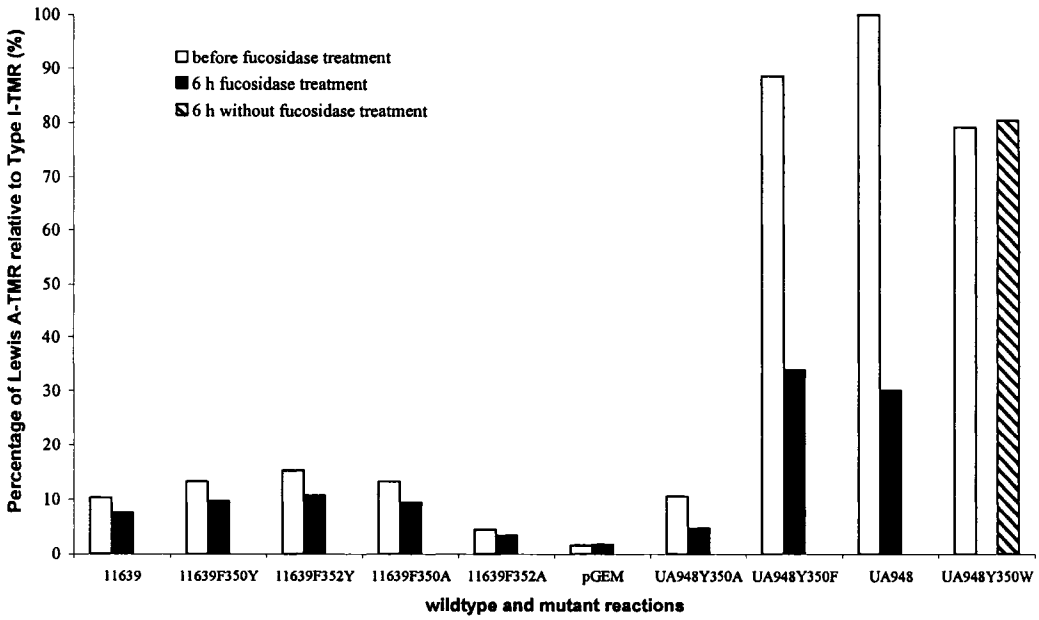

Figure 3.9: Amount of Lewis A-TMR formed after 0.5 and 1 h incubations of UA948 wildtype and mutant FucTs.

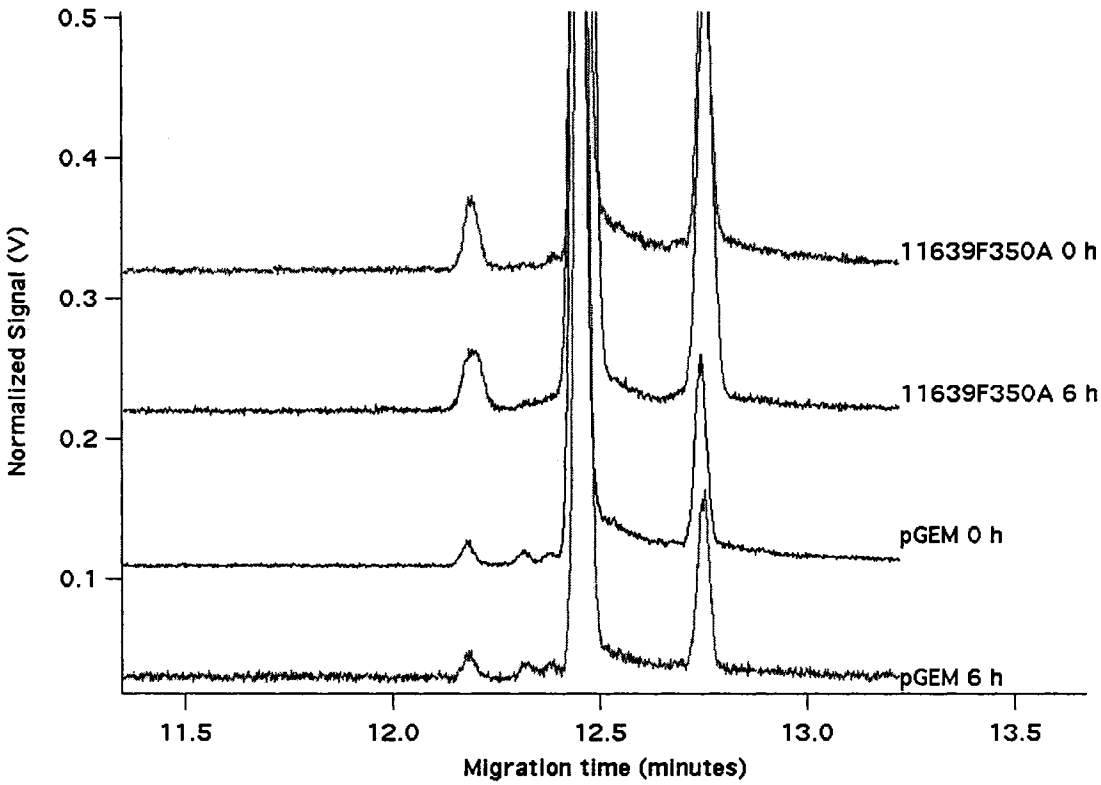

Figure 3.10: CE-LIF trace of the fucosidase reactions after 0 and 6 h of incubation. The decrease in the relative peak area of Lewis A-TMR compared to Type I-TMR shows that the peak was indeed Lewis A-TMR. The peaks are normalized so that the intensity of the Type I-TMR peak has an intensity of 1.0V.

3.4 Conclusion

This chapter has shown that in addition to low volume single cell assays, CE-LIF can be used to give qualitative and quantitative data in the assays of very low activity enzymes. Using CE-LIF, it has been shown that the a FucT in *H. pylori* NCTC11639 previously know to be a strict α-1,3 FucT does in fact have a low level of α-1,4 activity. These experiments also show that an aromatic amino acid in the hypervariable region of

FucT is required for α-1,4 activity. The presence of a hydroxyl group in tyrosine further increases the rate of reaction. This conclusion is similar to conclusions drawn from human FucT studies. Taken together, the results here add to the knowledge of *H. pylori* FucTs in understanding its evolutionary history and potential targets for antibiotic therapy.

3.5 References

(1) Cheetham, R. S. J. In *Handbook of Enzyme Biotechnology*; Wiseman, A., Ed.; John Wiley and Sons: New York, 1986, pp 54-142.

(2) Hawcroft, D. *Diagnostic Enzymology*; Wiley and Sons: New York, 1987.

(3) Barman, T. E. *Enzyme Handbook*; Springer-Verlag: New York, 1969.

(4) Seto, N. O. L.; Compston, C. A.; Evans, S. V.; Bundle, D. R.; Narang, S. A.; Palcic, M. M. *European Journal of Biochemistry* **1999**, *259*, 770-775.

(5) Polakowski, R.; Yazer, M.; Palcic, M. M. *Transfusion Medicine and Hemotherapy* **2005**, *32*, 5-10.

(6) Zhang, Y. N.; Le, X. C.; Dovichi, N. J.; Compston, C. A.; Palcic, M. M.; Diedrich, P.; Hindsgaul, O. *Analytical Biochemistry* **1995**, *227*, 368-376.

(7) Le, X. C.; Scaman, C.; Zhang, Y. N.; Zhang, J. H.; Dovichi, N. J.; Hindsgaul, O.; Palcic, M. M. *Journal of Chromatography A* **1995**, *716*, 215-220.

(8) Li, H.; Sims, C. E.; Kaluzova, M.; Stanbridge, E. J.; Allbritton, N. L. *Biochemistry* **2004**, *43*, 1599-1608.

(9) Meredith, G. D.; Sims, C. E.; Soughayer, J. S.; Allbritton, N. L. *Nature Biotechnology* **2000**, *18*, 309-312.

(10) Le, X. C.; Zhang, Y.; Dovichi, N. J.; Compston, C. A.; Palcic, M. M.; Beever, R. J.; Hindsgaul, O. *Journal of Chromatography A* **1997**, *781*, 515-522.

(11) Wang, G.; Ge, Z. M.; Rasko, A.; Taylor, D. E. *Molecular Microbiology* **2000**, *36*, 1187-1196.

(12) Edwards, N. J.; Monteiro, M. A.; Faller, G.; Walsh, E. J.; Moran, A. P.; Roberts, I. S.; High, N. J. *Molecular Microbiology* **2000**, *35*, 1530-1539.

(13) Lozniewski, A.; Haristoy, X.; Rasko, D. A.; Hatier, R.; Plenat, F.; Taylor, D. E.; Angioi-Duprez, K. *Infection and Immunity* **2003**, *71*, 2902-2906.

(14) Moran, A. P.; Knirel, Y. A.; Senchenkova, S. N.; Widmalm, G.; Hynes, S. O.; Jansson, P. E. *Journal of Biological Chemistry* **2002**, *277*, 5785-5795.

(15) Ma, B.; Wang, G.; Palcic, M. M.; Hazes, B.; Taylor, D. E. *Journal of Biological Chemistry* **2003**, *278*, 21893-21900.

(16) Ma, B.; Lau, L. H.; Palcic, M. M.; Hazes, B.; Taylor, D. E. *Journal of Biological Chemistry* **2005**, *280*, 36848-36856.

(17) Ma, B., University of Alberta, Edmonton, 2006.

(18) Palcic, M. M.; Heerze, L. D.; Pierce, M.; Hindsgaul, O. *Glycoconjugate Journal* **1988**, *5*, 49-63.

(19) Chan, N. W. C.; Stangier, K.; Sherburne, R.; Taylor, D. E.; Zhang, Y. N.; Dovichi, N. J.; Palcic, M. M. *Glycobiology* **1995**, *5*, 683-688.

(20) Dupuy, F.; Germot, A.; Julien, R.; Maftah, A. *Glycobiology* **2004**, *14*, 347-356.

Chapter 4: Optimal solubilization techniques for *E. coli* outer membrane pellet[3]

4.1 Introduction

This chapter deals with the field of proteomics using mass spectrometry. This is a large field and several general reviews have been published[1, 2]. The scope of this thesis is limited to the analysis of the *E. coli* outer membrane proteome.

4.1.1 Bacteria outer membrane proteome

In general, bacteria grow quickly and are adaptable to many different types of environments. As such, they require a large repertoire of proteins to deal with their changing environments, such as nutrient availability, presence of toxic compounds, other bacteria of the same species, and other friendly or unfriendly microorganisms. As its environment changes, the bacterium will respond with changes in its proteome[3] and metabolome[4]. To understand these changes and to develop new anti-bacterial drugs and treatments, mass spectrometry and proteomics offers a way to link the fully characterized genome with the partially described proteome[5].

As the outermost barrier between a bacterium and its environment, the outer membrane serves as a gatekeeper for molecules entering and leaving the cell[6, 7]. The movement of these molecules is mostly controlled by various porin proteins and they constitute the majority of the outer membrane proteome. Just as the proteome of the bacterium changes with its environment, the subproteome of the outer membrane will change as well. The role of efflux pumps in the outer membrane is especially interesting as they have been implicated in antibiotic resistance[8, 9]. Thus, it would be useful to

[3] The author is editing a version of this chapter for submission to a yet-to-be determined journal.

identify which efflux pumps are being induced or repressed as antibiotics are added to the culture. Because these efflux pumps are usually present in low numbers, mass spectrometry offers a sensitive method to detect these changes.

Membrane proteins in general are notoriously difficult to work with[10, 11]. As they are either partially or completely embedded in the lipid bilayer of the membrane, the exterior of the protein is usually hydrophobic. Thus, it is often difficult to solubilize them in aqueous buffers commonly used for molecular biology applications. To overcome this problem, several methods such as differential centrifugation[12], free-flow electrophoresis[13], surfactants and inorganic salts[14], amphipathic polymers[15] and carbonate extraction[16] have been developed. However, all of these methods have their own disadvantages, such as contamination with cellular debris, limited application range, or incompatibility with downstream analytical methods. For example, solubilization with surfactants requires dilution or dialysis to remove them before trypsin digestion. Some possible solutions are to solubilize the protein in organic solvents, which has the bonus effect of facilitating trypsin digestion[17, 18], or to perform the process at a higher temperature or with agitation. The research taken for this thesis used a combination of these approaches to determine the best way to solubilize *E. coli* outer membrane proteins.

The membrane proteome of some Gram negative and positive bacteria, such as *Pseudomonas*[19], *Pasteurella*[20], *Mycobacteria*[21], and *Staphylococcus*[22] have been investigated by several groups. These studies have used a variety of methods to isolate the membrane proteins, such as carbonate extraction of Molloy *et al.*[23], sacrosine precipitation of Trigo *et al.*[24], and the ProteoPrep® Membrane Extraction Kit from Sigma-Aldrich (St. Louis MO). The proteins are then solubilized using a variety of

methods, usually involving denaturing agents, such as SDS and urea. The proteins are then analyzed by a variety of two-dimensional separation methods, both gel-based and gel-free methods, before final analysis by mass spectrometry. In addition to the above bacteria, the outer membrane proteomes of enteric bacteria have also received some attention[25, 26]. In this thesis work, outer membrane from *E. coli* was isolated, its proteins solubilized and digested with trypsin, and subjected to two-dimensional separation before analysis by mass spectrometry.

4.1.2 High and low abundance proteins

As the name suggests, high abundance proteins (HAPs) are proteins that are present in high amounts compared to other proteins. In a manner analogous to single cell assays described in Chapter 2, HAPs hamper the search for more interesting, lower abundance proteins. Due to their high concentration, HAPs are preferentially detected and obscure the detection of proteins of lower abundance.

In contrast to HAPs, low abundance proteins (LAPs) are present at low to very low numbers per cell. In *Saccharomyces cerevisiae*, the definition of a low abundance protein is less than 10,000 copies per cell[27]. The number of proteins per cell was determined by the codon adaptation index (CAI) of the corresponding gene. The CAI is a value that predicts the frequency of gene expression by comparing its DNA sequence with the organism's codon usage[28]. It has been used to determine the number of low abundance proteins found in *S. cerevisiae*[29, 30]. Using two dimension-liquid chromatography techniques, Washburn *et al.* found that the ratio of LAPs to HAPs identified was less than the predicted ratio, suggesting many LAPs were not detected.

However, the results in bacteria are less conclusive. In *E. coli,* Goetz and Fuglsang found that there was a positive correlation between the CAI and the number of mRNA transcripts[31] while dos Reis *et al.* found that there was a negative correlation between the CAI and the level of protein expression[32]. In *Steptomyces coelicolor* and *Streptomyces avermitilis*, Wu *et al.* found a positive correlation between the CAI and the level of protein expression [33]. In light of these conflicting results in the use of CAI in prokaryotes, the definition of a low abundance protein has not been determined in bacteria.

In human serum, the 22 most abundant proteins represent 99% of the proteins present by weight[34]. The remaining proteins are present in amounts ten orders of magnitude or more less than the highest 22 proteins. If these HAPs are removed, then the detection of low abundance proteins can be greatly improved[35-37]. Commercially available antibodies have been commonly used to achieve this goal[38] and are commonly bound to chromatographic resin to facilitate sample processing[39].

However, such antibodies are not yet commercially available for bacterial HAPs. An obvious solution would be to raise antibodies against the HAPs found in the bacteria of interest and then affix them to chromatography resins. However, this is time- and labor- intensive and is not within the scope of feasibility in most laboratories. Thus, a way was sought to remove these HAPs in the outer membrane of *E. coli* through sequential solubilization with increasing concentrations of surfactants. It was hoped that the high abundance, loosely associated membrane proteins could be removed with low concentrations of surfactants. The low abundance, integral membrane proteins could then be solubilized with a higher concentration of surfactants.

4.1.3 Orthogonal separation in proteomics

Traditional proteomics studies have been done by two-dimensional gel electrophoresis (2DE)[40]. Of these two dimensions, the first is separation by the protein's isoelectric point and the second is by the size of the protein. These two methods of separation can be considered orthogonal as the separation mechanisms are different. Orthogonal separations are desired in order to reduce the complexity of the protein or peptide sample[41]. Ideally, the total separation process would be able to isolate all species within a mixture so that they can be detected and identified with ease. Multidimensional orthogonal separations also provide a way to combat the dynamic range challenge inherent in many proteomic samples[42].

In modern-day proteomics, 2DE has been replaced by the gel-free technique of two-dimension liquid chromatography (2D-LC). Chromatographic techniques are preferred so that sample handling can be automated and has minimal sample loss compared to 2DE[43]. Currently, there are many chromatographic techniques used in proteomics, such as reversed-phase, ion-exchange, size exclusion, affinity, and hydrophilic interaction chromatography, just to name a few. In order to determine a suitable pair of separation methods, one must consider the peak capacity in each dimension as well as the separation orthogonality.

One of the first orthogonal separations of peptides was the MuDPIT (*mu*lti*d*imensional *p*rotein *i*dentification technology) technique developed by Yates *et al.*[29, 44]. This technique combines strong cation exchange in the first dimension with reversed-phase chromatography in the second dimension. This combination has become

popular among different research groups[45] and other good combinations include size-exclusion chromatography with reversed-phase chromatography, hydrophilic interaction chromatography with reversed-phase chromatography, and alkaline reversed-phase chromatography with acidic reversed-phase chromatography[46].

Various systems have been designed to couple the two chromatographic steps. They can generally be classified as "online"[47] or "offline"[48]. Online methods allow for automation and less sample handling while offline methods allow for operator intervention in between the two steps. Furthermore, offline methods allow fractions to be collected from the first dimension separation to be stored or analyzed repeatedly. Given the benefits of offline coupling, this method was used in this thesis work.

4.2 Experimental Conditions

4.2.1 *E. coli* outer membrane preparation

E. coli K12 (ATCC Strain 470760) was grown to mid-stationary phase in 1 liter of MES minimal media. MES minimal media is a defined minimal media commonly used for bacteria growth. Per liter, it contained 9.7 g 2-(N-Morpholino)ethanesulfonic acid hemisodium (MES), 1.1 g KOH, 6 g glycerol, 1.2 g NH_4Cl, 0.35 g K_2HPO_4. This solution was adjusted to pH 6.6 and autoclaved. After cooling, sterile stock supplements were added so that the final concentration of essential supplements were 0.5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 7 μM $Fe(NH_4)_2(SO_4)_2$, 5 μg mL^{-1} thiamine, and 1× trace element solution. One liter of the 1000× trace element solution contained 2.86 g H_3BO_3, 1.81 g $MnCl_2 \bullet 4H_2O$, 0.22 g $ZnSO_4 \bullet 7H_2O$, 0.39 g $Na_2MoO_4 \bullet 2H_2O$, 0.079 g $CuSO_4 \bullet 5H_2O$, and

0.0494 g $Co(NO_3)_2 \bullet 6H_2O$ and was sterilized by passing the solution through a sterile 0.22 μm filter. All chemicals used were obtained from Sigma Aldrich (St. Louis MO).

The *E. coli* culture was started from an isolated colony streaked from a frozen working stock. An isolated colony was incubated in 1 mL of MES minimal media at 37 °C with shaking at 250 RPM. After 6 h, the solution was transferred to 50 mL of the same media in a 250 mL baffled Erlenmeyer flask and incubated at the same conditions. After 24 h of incubation, the culture was used to inoculate 1 L of pre-warmed MES minimal media so that the initial OD_{600} was 0.02. The culture was allowed to incubate at the above conditions in a 4 L baffled Erlenmeyer flask.

After 20 h of incubation, the OD_{600} of the culture had reached 3.5 and the culture was harvested and all subsequent manipulations were done at 4 °C. The cells were centrifuged for 10 minutes at 2800 RCF, washed with 500 mL of 50 mM MOPS (pH 7.3) and centrifuged again. The cells were finally suspended in 50 mL of 50 mM MOPS and divided equally into 5 tubes. The tubes were centrifuged for 10 minutes at 3 000 RCF. The supernatant was discarded and the cell pellets (*ca.* 1.5 g wet cells) were frozen at -80 °C until needed.

To isolate the outer membrane of *E. coli*, the modified methods of Molloy *et al.*[23] were used. One aliquot of *E. coli* was thawed and diluted with 15 mL of 50 mM MOPS (pH 7.3) and DNase I (Invitrogen) was added to a final concentration of 1 mg mL^{-1}. The solution was passed through a French pressure cell twice at a pressure of 14 000 psi. The lysate was centrifuged for 10 minutes at 2 300 RCF and the pellet was discarded. 2 mL of the lysate (*ca.* 60 mg total protein) was diluted to 10 mL with 50 mM MOPS. To this solution, 110 mL of 0.1 M Na_2CO_3 (pH 11.0) was added slowly and stirred for 1 h at 0

°C. The solution was then ultracentrifuged for 65 minutes at 115 000 RCF. The supernatant was discarded while the pellet was resusupended in 50 mM MOPS and divided into three tubes. The tubes were ultracentrifuged for 25 minutes at 115 000 RCF. The supernatant was discarded while the pellet was kept at -80 °C until needed for solubilization as described below.

4.2.2 Solubilization of outer membrane proteins by different surfactants

Solubilization of the outer membrane pellet was attempted using several different surfactants: SDS, Tween 20, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), deoxycholic acid, and Triton X-100 (All chemicals were obtained from Sigma Aldrich St. Louis MO). In SDS solubilization, an increasing concentration of SDS (0, 0.1%, 0.15%, 0.5%, 1.0%, 2.5%, and 5.0% [w/v]) in a 10% (v/v) methanol solution buffered with 100 mM NH_4HCO_3 was used. 250 μL of the appropriate solution was added to a 1.6 mL Eppendorf tube containing the outer membrane pellet. The tube was vortexed to disperse the pellet and then incubated at 37 °C with shaking at 250 RPM to facilitate solubilization. After 4 h, the tube was centrifuged for 10 minutes at 16 000 RCF and the supernatant was saved for analysis by SDS-PAGE. To the pellet, 250 μL of the next higher concentration of SDS was added. The pellet was then dispersed and incubated with shaking. This process was repeated until the SDS concentration reached 5%. It was noticed that even after 5% SDS, a pellet remained in the tube. To achieve complete solubilization, 100 μL of stock SDS-PAGE loading buffer (Bio-Rad Laboratories, Hercules CA) was added and heated to 95 °C for 30 minutes. After this step, the pellet was completely solubilized.

In solubilization with Tween 20, CHAPS, and deoxycholic acid, 0.05%, 0.10%, and 0.25% (w/v) of the surfactant was dissolved in an aqueous solution with 1% dithiothreitol (DTT) and 50 mM Tris (pH 6.8). For Triton X-100, the same concentrations were used in addition to 0.50% and 1.0% (w/v). For each different concentration and type of surfactant, solubilization was carried out by adding 200 μL of the solution to a 1.6 mL Eppendorf tube containing the outer membrane pellet. After dispersing the pellet and 20 minutes of incubation at 50 °C, the tube was centrifuged for 10 minutes at 16 000 RCF and the supernatant was saved for analysis by SDS-PAGE. To the pellet, 200 μL of the same concentration of surfactant was added. Dispersion, incubation, and centrifugation was carried out one more time. After the supernatant was removed, the next higher concentration of surfactant was used as above to solubilize the pellet.

All of the supernatants were analyzed by SDS-PAGE to evaluate how well the proteins were solubilized in each step. 10 μL of the supernatant was added to a stock solution of SDS-PAGE loading buffer so that the final concentration was 62.5 mM Tris (pH 6.8), 2% SDS (w/v), 5% β-mercaptoethanol (v/v), 25% glycerol (v/v), and 0.01% Bromophenol Blue (Bio-Rad Laboratories, Hercules CA). The solution was heated for 10 minutes at 95 °C and loaded onto a 12% (w/v) bis-acrylamide gel with a 4% stacking gel using Laemmli's methods[49]. Once the dye front had reached the bottom of the gel, the gel was fixed with 50% methanol and 10% acetic acid and stained with Biosafe Coomassie Blue (Bio-Rad Laboratories, Hercules CA) and recorded into computer memory by a flatbed scanner.

4.2.3 Solubilization of outer membrane proteins by methanol and SDS

Three aliquots of outer membrane preparation were solubilized by sequentially using 60% (v/v) methanol and SDS under the conditions stated in Table 4.1. For methanol solubilization, 150 μL of 60% methanol was added to the tubes. The pellet was dispersed by vortexing and incubated at the appropriate temperature for 10 minutes. After 10 minutes of centrifugation at 16 000 RCF, the supernatant was collected and the procedure repeated two more times. After methanol treatment, the pellets were solubilized with SDS at the corresponding concentration and temperature. 125 μL of SDS solution was added to the tube. After vortexing and incubating the tubes for 10 minutes, the tubes were centrifuged for 10 minutes at 16 000 RCF and the supernatant collected and the procedure repeated two more times.

Table 4.1: Conditions used for outer membrane solubilization with methanol and SDS.

Method name	Methanol	SDS	
Cold/cold	25 °C	25 °C	2.0%
Cold/hot	25 °C	60 °C	0.2%
Hot/hot	60 °C	60 °C	0.2%

Protein concentration in the supernatants was estimated using a Bradford Protein Assay Kit (Bio-Rad Laboratories, Hercules CA). The proteins' disulfide bonds were reduced with 3 mM DTT for 30 minutes at 37 °C and the resulting cysteine residues alkylated with 5 mM iodoacetamide for 15 minutes at 25 °C in the dark. The solutions containing SDS were diluted with 100 mM NH_4HCO_3 so that the final concentration of SDS was 0.1%. The alkylated proteins were digested with trypsin at a ratio of 1:50 (w/w)

in a buffered solution of 100 mM NH_4HCO_3 and incubated overnight at 37 °C. The peptides in the separate supernatants were pooled according to Table 4.1 and concentrated by centrifugation under vacuum.

A solid phase extraction (SPE) procedure was used to remove excess inorganic salts that might interfere in subsequent analysis. SPE cartridges were obtained from Waters (Milford MA) and prepared for use according to the manufacturer's directions. Samples were washed with an aqueous solution of 0.1% (v/v) trifluoroacetic acid (TFA) before elution with 50% (v/v) acetonitrile and 0.1% TFA and concentration by centrifugation under vacuum.

Strong cation exchange separation of the peptides from each method listed in Table 4.1 was done on a SP1500 (150×4.6) mm column from Biochrom Labs (Terre Haute, IN). The peptides were eluted using an increasing gradient of sodium chloride as specified in Table 4.2. Elution of the peptides was monitored using UV absorption at 210 nm. The eluate was collected in ten one-minute fractions starting at 18 minutes and covered the entire elution profile of the peptides. Shoulder fractions were pooled to save subsequent analysis time.

Table 4.2: Elution program used during strong cation exchange of solubilized *E.coli* outer membrane peptides. The A solvent is 20% (v/v) acetonitrile and 0.1% TFA aqueous solution and the B solvent is 20% acetonitrile (v/v), 0.1% TFA, 1 M NaCl aqueous solution.

Time (minutes)	**%A**	**% B**
0	100	0
15	100	0
22	70	30
24	50	50
30	50	50

For optimal performance during reversed-phase chromatography, the inorganic salts were removed from the strong cation exchange eluates[50]. This was done by using C_{18} ZipTip™ from Millipore (Billerica MA). The manufacturer's directions were followed and the eluted peptides were once again concentrated by centrifugation under vacuum to *ca.* 3 μL.

Reversed-phase separation was done just prior to analysis by mass spectrometry. 1 μL of salt-free cation exchange eluate was injected onto a monolithic polystyrene-divinylbenzene (PS-DVB) column (200 mm×5 cm; Dionex, Sunnyvale CA) and eluted using a gradient of acetonitrile as specified in Table 4.3. A monolithic PS-DVB column was chosen instead of the more common C_{18} packed columns due to its superior

chromatographic properties in the separation of peptides[51-53]. The eluate was then directed into a LCQ-Deca mass spectrometer (ThermoFinnigan, Waltham MA) using electrospray ionization in the positive mode. For each survey MS scan, the two most intense ions were selected for MS/MS analysis with fragmentation by collision induced dissociation. Each strong cation exchange eluate fraction was analyzed three times by reversed-phase chromatography MS and MS/MS.

Table 4.3: Elution program used during reversed phase separation of solubilized *E.coli* outer membrane peptides. The A solvent was 5% (v/v) acetonitrile and 0.1% TFA aqueous solution and the B solvent was 0.1% TFA in acetonitrile.

Time (minutes)	**%A**	**% B**
0	95	5
40	75	25
45	5	95
48	5	95
54	95	5
60	95	5

The data produced from the mass spectrometer were searched against Swissprot's *E. coli* sequence database using the search program Mascot[54]. Only peptides that were confidently identified to an *E. coli* protein ($P<0.05$, where P is the probability of a random match) were kept. The number of proteins identified in each of the triplicate analyses was combined and compared to determine the reproducibility of the reversed-

phase separation and MS detection. Similarly, the data obtained from each method (cold/cold, cold/hot, and hot/hot) were compared to determine the best solubilization method.

4.3 Results and Discussions

4.3.1 Solubilization of outer membrane proteins by different surfactants

SDS-PAGE was used as a quick way to evaluate the effectiveness of protein solubilization using different surfactants. The amount and type (high or low abundance) of protein solubilized by each method can be quickly visualized instead of running a full proteomic protocol involving digestion, two-dimensional separation, and mass analysis. It was hoped that the majority of the membrane associated HAPs could be solubilized with low concentrations of surfactants while leaving behind the low abundance integral membrane proteins. The outer membrane pellet was first solubilized by SDS at various concentrations. Figure 4.1a shows an image of the gel containing the different supernatants. It could be seen that even without SDS, HAPs could be found (Lane 2). While two proteins (35 kDa and 33 kDa) could be found in significant amounts in the supernatant with 0.1% SDS, they were also found ubiquitously throughout the supernatants of higher SDS concentration. This suggests that these proteins are present in very high amounts and that they cannot be easily solubilized at a low concentration of SDS. Medium to low abundance membrane proteins were solubilized with 0.1% to 1.0% SDS. However, these fractions all contain HAPs and did not achieve the initial objective of selectively removing HAPs.

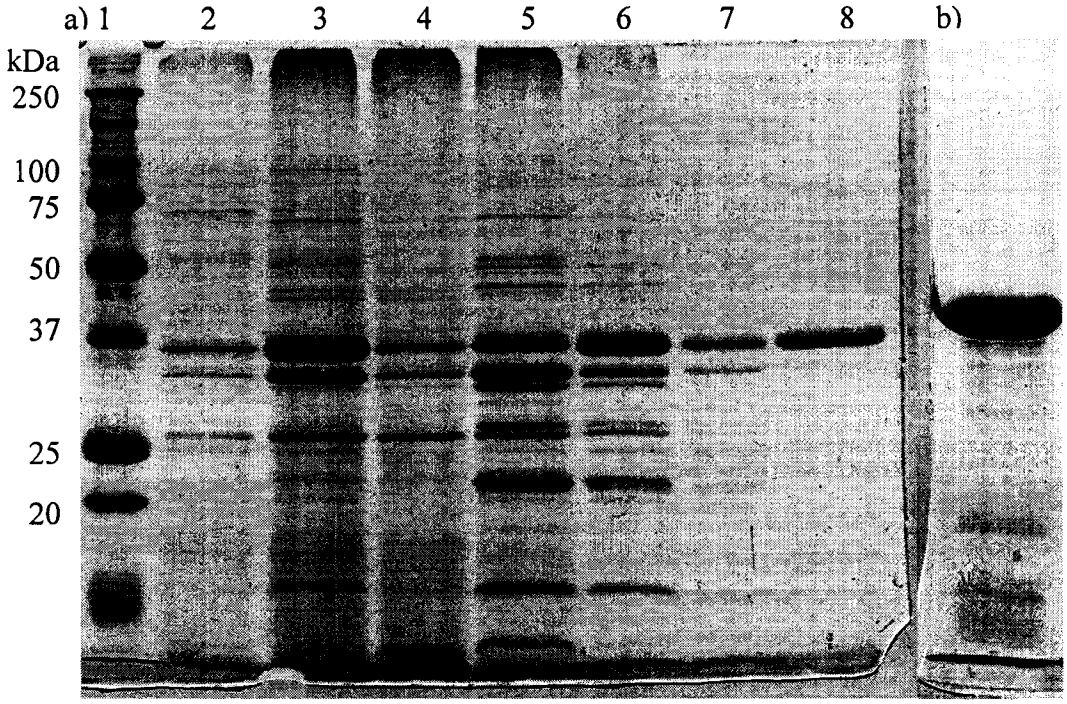

Figure 4.1a): Solubilization of protein pellet using SDS in 10% methanol and 100 mM NH_4HCO_3. Molecular weight markers, Lane 1; 0% SDS, Lane 2; 0.1% SDS, Lane 3; 0.15% SDS, Lane 4, 0.5% SDS, Lane 5, 1.0% SDS, Lane 6; 2.5% SDS, Lane 7; and 5.0% SDS, Lane 8. 1b): SDS-PAGE analysis of the pellet solubilized by SDS-PAGE loading buffer and heated to 95°C.

It is of note that the 35 kDa HAP could still be found in the supernatant of 5% SDS. It is also interesting that even after an overnight solubilization with 5% SDS, a small pellet remained in the tube. This pellet was eventually solubilized with the stock solution of SDS-PAGE loading buffer and heated at 95 °C for 30 minutes. SDS-PAGE analysis of this solution showed that the 35 kDa protein was still found within the pellet. (Figure 4.1b)

Observing that the hot SDS-PAGE loading buffer was able to completely solubilize the pellet, it was hypothesized that the higher temperature and the presence of reducing agents may help dissolve a pellet. As well, since SDS is a strong surfactant and affects downstream processing, different surfactants were sought to replace SDS. Thus, Tween-20, CHAPS, deoxycholic acid, and Triton X-100 were used in an attempt to dissolve the pellet at a higher temperature. Solubilization at each concentration of surfactant was done twice in order to remove traces of proteins solubilized during the first addition.

Figures 4.2a and 4.2b show the SDS-PAGE analysis of the supernatants from Tween-20 and CHAPS solubilization, respectively. As in the SDS solubilization experiment, the 35 kDa protein predominates throughout the gel. Other HAPs could be seen in supernatants of higher surfactant concentration as well.

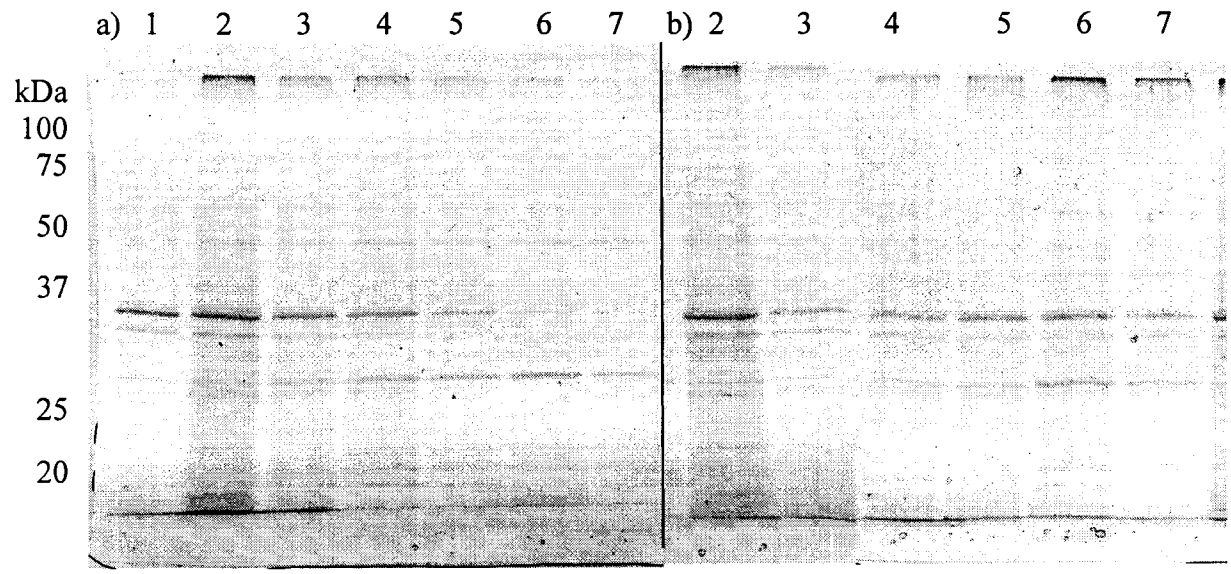

Figure 4.2: Solubilization of protein pellet using different concentrations of Tween-20 (a) and CHAPS (b) in 1% DTT and 50 mM Tris. Aqueous supernatant, Lane 1; 0.05%, Lanes 2 and 3; 0.10%, Lanes 4 and 5; 0.25%, Lanes 6 and 7.

Deoxycholic acid is a bile salt used by the human body to emulsify lipids for absorption in the digestive tract. *In vitro*, it can be used to solubilize membranes and membrane proteins. It was used in increasing concentrations here to solubilize *E. coli* outer membrane proteins. While the 35 kDa HAP was effectively removed by 0.05% and 0.10% deoxycholic acid, the 33 kDa HAP remained at 0.25% (Figure 4.3).

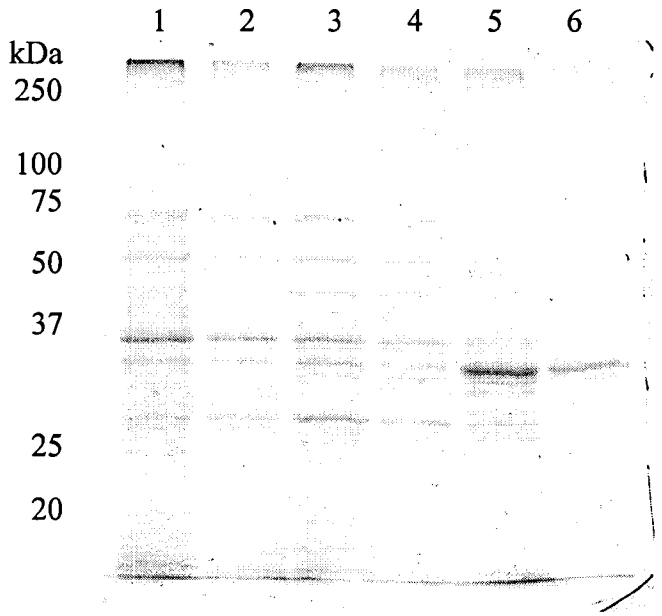

Figure 4.3: Solubilization of protein pellet using different concentrations of deoxycholic acid in 1% DTT and 50 mM Tris. 0.05%, Lanes 1 and 2; 0.10%, Lanes 3 and 4; 0.25%, Lanes 5 and 6.

Lastly, Triton X-100 was used to solubilize the outer membrane pellet. Once again, different HAPs could be found at different concentrations (Figure 4.4). Membrane associated HAPs were solubilized at low concentrations of Triton X-100 while integral membrane HAPs were solubilized at higher concentrations. No one concentration could effectively remove all HAPs while leaving behind medium to low abundance proteins.

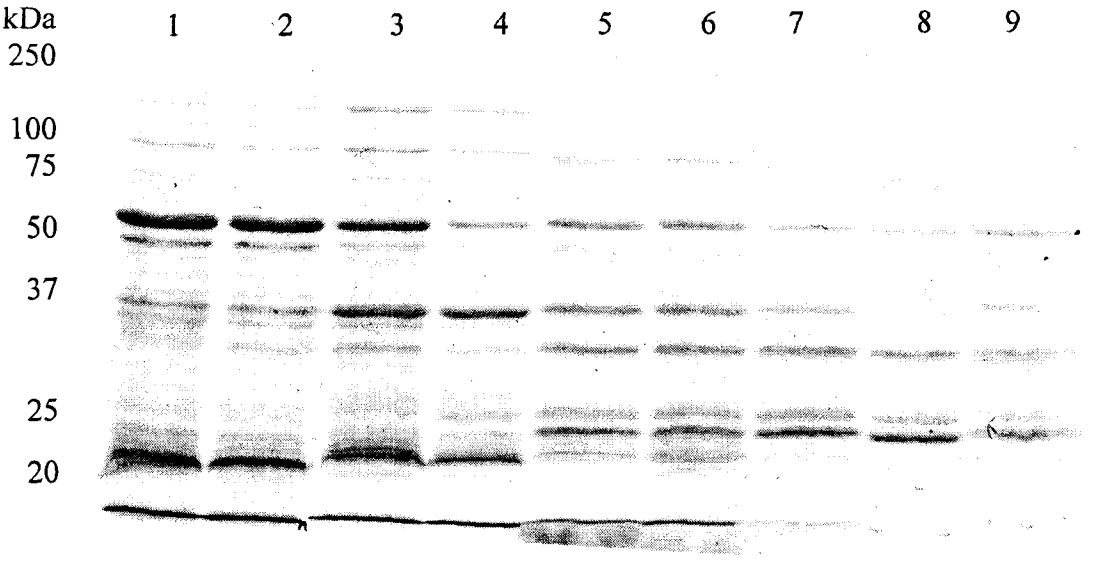

Figure 4.4: Solubilization of protein pellet using Triton X-100 in 1% DTT and 50 mM Tris. 0.05%, Lanes 1 and 2; 0.10%, Lanes 3 and 4; 0.25%, Lanes 5 and 6; 0.50%, Lanes 7 and 8; 1.0%, Lane 9.

Unfortunately, the different surfactants were unable to achieve the desired effect of removing HAPs. While some HAPs were removed at lower concentration of surfactants, others remained and were solubilized only with higher concentrations. Overall, this work did not find one concentration of any one surfactant that could effectively remove all HAPs.

4.3.2 Solubilization of outer membrane proteins by methanol and SDS

The number of proteins positively identified as an *E. coli* protein from each solubilization method was totaled after triplicate analysis (Figure 4.5). While the majority of the proteins identified could be found in all three methods, the hot methanol/hot SDS method was able to solubilize the most proteins. This is most likely

attributed to the increase in the incubation temperature. The higher kinetic energy of the molecules in both the outer membrane pellet and solvent may have allowed the solvent molecules to better remove the membrane lipids and solubilize the proteins. As well, solubility generally increases as temperature increase. In addition to solubilizing a greater number of proteins, the hot SDS method used a lower concentration of SDS. This is advantageous as less extensive dilution and/or dialysis is required before trypsin digestion.

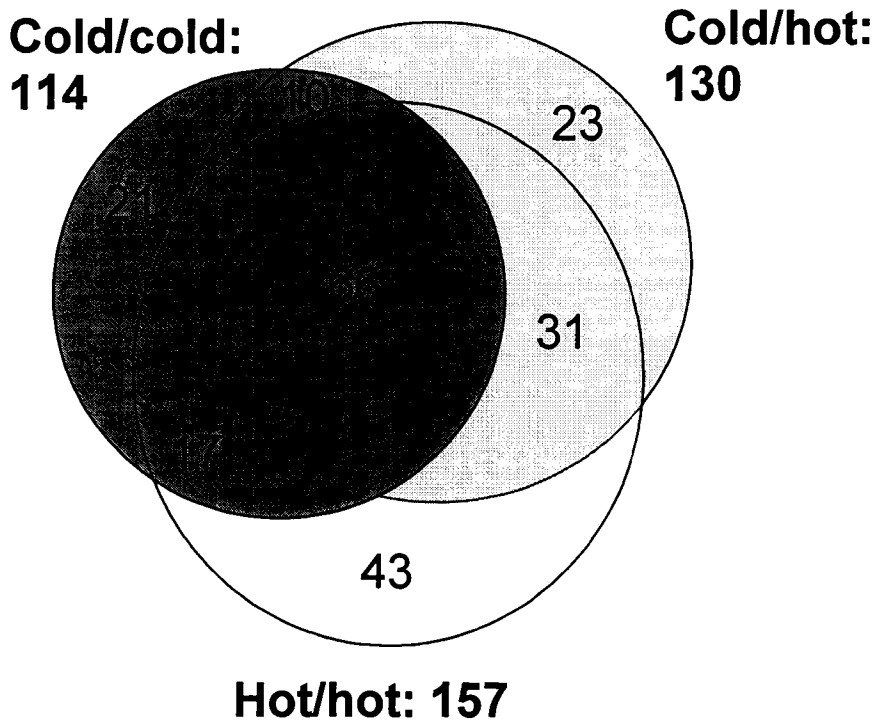

Figure 4.5: Schematic representation of the number of proteins identified from each of the solubilization method. The terms "cold/cold, cold/hot, and hot/hot" are defined in Table 4.1.

The difference in protein numbers shown in Figure 4.5 may also be due to the degree of reproducibility associated with LC-MS/MS experiments. To examine this

issue, triplicate analysis of the hot/hot solubilization method was carried out in a separate experiment and the results are shown in Figure 4.6. One can see that the majority of the proteins could be found in two or more analysis (Figure 4.6). Seventy-two percent of the proteins identified were found in two or more analyses while 61% were found in all three analyses. While the proteins identified from one run to another may be somewhat different, the total number of proteins identified was almost the same (128, 127 and 126). This reproducibility behavior of LC-MS/MS experiments is in agreement with previous studies by Toll *et al.*[53]. Thus, the difference in the number of proteins observed in Figure 4.5 is mainly due to the different sample composition as a result of the three different methods used.

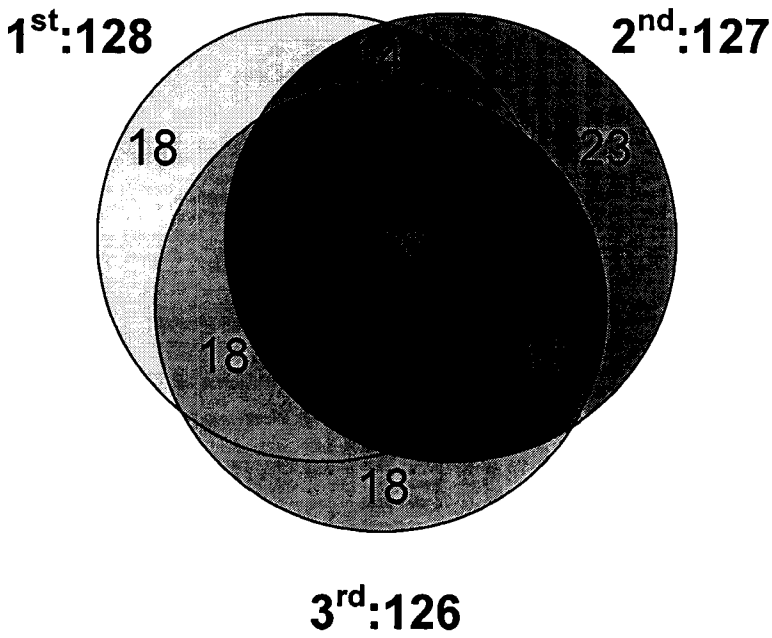

Figure 4.6: Schematic representation of the number of proteins identified during triplicate analysis of the hot/hot solubilization method. Three LC-MS and MS/MS runs were independently processed and the number of proteins found in one, two, or all three analyses is represented.

Lastly, the subcellular locations of the identified proteins were determined according to Swissprot classification. Of the 127 proteins identified using the hot/hot solubilization method in the second analysis (Figure 4.6), ninety-six proteins (76%) were classified as belonging to the peripheral membrane (*i.e.* outer membrane, periplasm, and inner membrane). Of the ninety-six, twelve proteins were identified as being from the outer membrane. Nine proteins (7%) were classified as belonging to the cytoplasm and twenty-two (17%) were not classified. Proteins identified as part of the ribosome were grouped with membrane proteins as they were most likely isolated while they were attached to the membrane during co-translational insertion of membrane proteins. Many

of the unclassified proteins were theoretical proteins that have not been previously identified experimentally. Other researchers have used the *E. coli* genome and computational software to predict that there are 1084 proteins present in the peripheral membrane[55, 56]. Of these, 91 are localized to the outer membrane. Compared to the results obtained here, we have only identified *ca.* 10% of the *E. coli* membrane proteome. Future work will be focus on the use of these digestion methods, in combination with improved mass spectrometric instruments (*e.g.* quadrupole time-of-flight mass spectrometer), to generate a comprehensive profile of the outer membrane proteome.

4.4 Conclusions

This chapter documents attempts to obtain a better view of the low abundance proteins in the membrane of *E. coli*. Different surfactants and incubation conditions were used in an attempt to remove HAPs so that low abundance proteins could be more easily identified. However, surfactants alone cannot remove all HAPs as the HAPs vary in their degree of hydrophobicity and they cannot all be removed at one critical or "magic" concentration of surfactant.

However, it was found that solubilization with a lower concentration of SDS at an elevated temperature could solubilize more proteins than using a higher concentration of SDS at room temperature incubation. The reproducibility of reversed-phase chromatography and MS and MS/MS analysis was demonstrated to be comparable to that obtained by other authors.

In order to fully understand the function of low abundance proteins in the membrane of *E. coli*, it may be necessary to completely solubilize the pellet in SDS at an

elevated temperature and then perform protein level fractionation to remove HAPs. These fractionation methods may include hydrophilic interaction chromatography[57], free-flow electrophoresis[13], and/or isoelectric focusing[58]. By separating the HAPs from the LAPs, LAP can be carefully analyzed without the overwhelming background signal from HAPs.

4.5 References

(1) Lane, C. S. *Cmls-Cellular and Molecular Life Sciences* **2005**, *62*, 848-869.

(2) Guerrera, I. C.; Kleiner, O. *Bioscience Reports* **2005**, *25*, 71-93.

(3) Han, M. J.; Lee, S. Y. *Microbiology and Molecular Biology Reviews* **2006**, *70*, 362-+.

(4) Koek, M. M.; Muilwijk, B.; van der Werf, M. J.; Hankemeier, T. *Analytical Chemistry* **2006**, *78*, 1272-1281.

(5) Corbin, R. W.; Paliy, O.; Yang, F.; Shabanowitz, J.; Platt, M.; Lyons, C. E.; Root, K.; McAuliffe, J.; Jordan, M. I.; Kustu, S.; Soupene, E.; Hunt, D. F. *Proceedings of the National Academy of Sciences of the United States of America* **2003**, *100*, 9232-9237.

(6) Koebnik, R.; Locher, K. P.; Van Gelder, P. *Molecular Microbiology* **2000**, *37*, 239-253.

(7) Achouak, W.; Heulin, T.; Pages, J. M. *Fems Microbiology Letters* **2001**, *199*, 1-7.

(8) Nikaido, H. *Seminars in Cell & Developmental Biology* **2001**, *12*, 215-223.

(9) Pages, J. M. *M S-Medecine Sciences* **2004**, *20*, 346-+.

(10) Ahram, M.; Springer, D. L. *Expert Review of Proteomics* **2004**, *1*, 293-302.

(11) Kalipatnapu, S.; Chattopadhyay, A. *Iubmb Life* **2005**, *57*, 505-512.

(12) Simpson, R. J.; Connolly, L. M.; Eddes, J. S.; Pereira, J. J.; Moritz, R. L.; Reid, G. E. *Electrophoresis* **2000**, *21*, 1707-1732.

(13) Cutillas, P. R.; Biber, J.; Marks, J.; Jacob, R.; Stieger, B.; Cramer, R.; Waterfield, M.; Burlingame, A. L.; Unwin, R. J. *Proteomics* **2005**, *5*, 101-112.

(14) Ruth, M. C.; Old, W. M.; Emrick, M. A.; Meyer-Arendt, K.; Aveline-Wolf, L. D.; Pierce, K. G.; Mendoza, A. M.; Sevinsky, J. R.; Hamady, M.; Knight, R. D.; Resing, K. A.; Ahn, N. G. *Journal of Proteome Research* **2006**, *5*, 709-719.

(15) Picard, M.; Duval-Terrie, C.; De, E.; Champeil, P. *Protein Science* **2004**, *13*, 3056-3058.

(16) Blonder, J.; Terunuma, A.; Conrads, T. R.; Chan, K. C.; Yee, C.; Lucas, D. A.; Schaefer, C. F.; Yu, L. R.; Issaq, H. J.; Veenstra, T. D.; Vogel, J. C. *Journal of Investigative Dermatology* **2004**, *123*, 691-699.

(17) Russell, W. K.; Park, Z. Y.; Russell, D. H. *Analytical Chemistry* **2001**, *73*, 2682-2685.

(18) Strader, M. B.; Tabb, D. L.; Hervey, W. J.; Pan, C. L.; Hurst, G. B. *Analytical Chemistry* **2006**, *78*, 125-134.

(19) Blonder, J.; Goshe, M. B.; Xiao, W. Z.; Camp, D. G.; Wingerd, M.; Davis, R. W.; Smith, R. D. *Journal of Proteome Research* **2004**, *3*, 434-444.

(20) Boyce, J. D.; Cullen, P. A.; Nguyen, V.; Wilkie, I.; Adler, B. *Proteomics* **2006**, *6*, 870-880.

(21) Hughes, M. A.; Silva, J. C.; Geromanos, S. J.; Townsend, C. A. *Journal of Proteome Research* **2006**, *5*, 54-63.

(22) Nandakumar, R.; Nandakumar, M. P.; Marten, M. R.; Ross, J. M. *Journal of Proteome Research* **2005**, *4*, 250-257.

(23) Molloy, M. P.; Herbert, B. R.; Walsh, B. J.; Tyler, M. I.; Traini, M.; Sanchez, J. C.; Hochstrasser, D. F.; Williams, K. L.; Gooley, A. A. *Electrophoresis* **1998**, *19*, 837-844.

(24) Zhao, G. S.; Pijoan, C.; Choi, K. W.; Maheswaran, S. K.; Trigo, E. *Canadian Journal of Veterinary Research-Revue Canadienne De Recherche Veterinaire* **1995**, *59*, 46-50.

(25) Nandakumar, M. P.; Cheung, A.; Marten, M. R. *Journal of Proteome Research* **2006**, *5*, 1155-1161.

(26) Molloy, M. P.; Phadke, N. D.; Maddock, J. R.; Andrews, P. C. *Electrophoresis* **2001**, *22*, 1686-1696.

(27) Gygi, S. P.; Corthals, G. L.; Zhang, Y.; Rochon, Y.; Aebersold, R. *Proceedings of the National Academy of Sciences of the United States of America* **2000**, *97*, 9390-9395.

(28) Sharp, P. M.; Li, W. H. *Nucleic Acids Research* **1987**, *15*, 1281-1295.

(29) Washburn, M. P.; Wolters, D.; Yates, J. R. *Nature Biotechnology* **2001**, *19*, 242-247.

(30) Pedersen, S. K.; Harry, J. L.; Sebastian, L.; Baker, J.; Traini, M. D.; McCarthy, J. T.; Manoharan, A.; Wilkins, M. R.; Gooley, A. A.; Righetti, P. G.; Packer, N. H.; Williams, K. L.; Herbert, B. R. *Journal of Proteome Research* **2003**, *2*, 303-311.

(31) Goetz, R. M.; Fuglsang, A. *Biochemical and Biophysical Research Communications* **2005**, *327*, 4-7.

(32) dos Reis, M.; Wernisch, L.; Savva, R. *Nucleic Acids Research* **2003**, *31*, 6976-6985.

(33) Wu, G.; Culley, D. E.; Zhang, W. W. *Microbiology-Sgm* **2005**, *151*, 2175-2187.

(34) Anderson, N. L.; Anderson, N. G. *Molecular & Cellular Proteomics* **2002**, *1*, 845-867.

(35) Raida, M.; Schulz-Knappe, P.; Heine, G.; Forssmann, W. G. *Journal of the American Society for Mass Spectrometry* **1999**, *10*, 45-54.

(36) Govorukhina, N. I.; Keizer-Gunnink, A.; van der Zee, A. G. J.; de Jong, S.; de Bruijn, H. W. A.; Bischoff, R. *Journal of Chromatography A* **2003**, *1009*, 171-178.

(37) Bjorhall, K.; Miliotis, T.; Davidsson, P. *Proteomics* **2005**, *5*, 307-317.

(38) Greenough, C.; Jenkins, R. E.; Kitteringham, N. R.; Pirmohamed, M.; Park, B. K.; Pennington, S. R. *Proteomics* **2004**, *4*, 3107-3111.

(39) Gong, Y.; Li, X.; Yang, B.; Ying, W. T.; Li, D.; Zhang, Y. J.; Dai, S. J.; Cai, Y.; Wang, J. L.; He, F. C.; Qian, X. H. *Journal of Proteome Research* **2006**, *5*, 1379-1387.

(40) Lambert, J. P.; Ethier, M.; Smith, J. C.; Figeys, D. *Analytical Chemistry* **2005**, *77*, 3771-3787.

(41) Corthals, G. L.; Wasinger, V. C.; Hochstrasser, D. F.; Sanchez, J. C. *Electrophoresis* **2000**, *21*, 1104-1115.

(42) Issaq, H. J.; Chan, K. C.; Janini, G. M.; Conrads, T. P.; Veenstra, T. D. *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences* **2005**, *817*, 35-47.

(43) Millea, K. M.; Krull, I. S. *Journal of Liquid Chromatography & Related Technologies* **2003**, *26*, 2195-2224.

(44) Washburn, M. P.; Ulaszek, R.; Deciu, C.; Schieltz, D. M.; Yates, J. R. *Analytical Chemistry* **2002**, *74*, 1650-1657.

(45) Nagele, E.; Vollmer, M.; Horth, P.; Vad, C. *Expert Review of Proteomics* **2004**, *1*, 37-46.

(46) Gilar, M.; Olivova, P.; Daly, A. E.; Gebler, J. C. *Analytical Chemistry* **2005**, *77*, 6426-6434.

(47) Fujii, K.; Nakano, T.; Hike, H.; Usui, F.; Bando, Y.; Tojo, H.; Nishimura, T. *Journal of Chromatography A* **2004**, *1057*, 107-113.

(48) Fujii, K.; Nakano, T.; Kawamura, T.; Usui, F.; Bando, Y.; Wang, R.; Nishimura, T. *Journal of Proteome Research* **2004**, *3*, 712-718.

(49) Laemmli, U. K. *Nature* **1970**, *227*, 680-685.

(50) Le Bihan, T.; Duewel, H. S.; Figeys, D. *Journal of the American Society for Mass Spectrometry* **2003**, *14*, 719-727.

(51) Ivanov, A. R.; Zang, L.; Karger, B. L. *Analytical Chemistry* **2003**, *75*, 5306-5316.

(52) Oberacher, H.; Premstaller, A.; Huber, C. G. *Journal of Chromatography A* **2004**, *1030*, 201-208.

(53) Toll, H.; Wintringer, R.; Schweiger-Hufnagel, U.; Huber, C. G. *Journal of Separation Science* **2005**, *28*, 1666-1674.

(54) Perkins, D.; Pappin, D.; Creasy, D.; Cottrell, J. *Electrophoresis* **1999**, *20*, 3551-3567.

(55) Rey, S.; Acab, M.; Gardy, J. L.; Laird, M. R.; DeFays, K.; Lambert, C.; Brinkman, F. S. L. *Nucleic Acids Research* **2005**, *33*, D164-D168.

(56) Gardy, J. L.; Laird, M. R.; Chen, F.; Rey, S.; Walsh, C. J.; Ester, M.; Brinkman, F. S. L. *Bioinformatics* **2005**, *21*, 617-623.

(57) Alpert, A. J. *Journal of Chromatography* **1990**, *499*, 177-196.

(58) Gan, C. S.; Reardon, K. F.; Wright, P. C. *Proteomics* **2005**, *5*, 2468-2478.

Chapter 5: Conclusions and Future Work

This thesis has shown several applications of bioanalytical chemistry. Chapter 2 demonstrated the power of CE-LIF in the analysis of a low-activity enzyme in a single cell reaction. The sensitivity of CE-LIF was once again demonstrated in Chapter 3 in the analysis of a low-activity mutant enzyme. While not related to CE-LIF, Chapter 4 also dealt with low-abundance biological information in the area of proteomics.

In Chapter 2, a micromanipulator was used to isolate and transport single cells into a small reaction vessel to assay for GTA activity. A nanopipettor was used to withdraw samples from the vessel at different times. CE-LIF was used to separate and detect the product blood group A antigen and the substrate H-Type II antigen. Quantitation by integration determined the amount of substrate converted to product. While the amount converted was small (at times less than 1% conversion), it was detectable by CE-LIF. Of the many single cell reactions analyzed, about 60% contained signs of GTA activity. Flow cytometry confirmed that a similar proportion of small HT29 cells contained GTA activity.

To further develop this single cell assay method, further reduction in volume is necessary. However, it is difficult to physically manipulate volumes less than 100 nL. Thus, it may be necessary to use microfluidic technologies. One must keep in mind that a method that allows for multiple sampling is preferable as it can perform replicate analysis or time-course studies. While the micromanipulator used here can be used for visual screening before selecting cells for analysis, it is a bottleneck as it is time and labor intensive. Furthermore, it is difficult to ascertain if one has expelled a cell from the capillary into the receiving vessel.

Chapter 3 showed another application of CE-LIF in a crude enzyme assay. A mutant enzyme previously thought to be inactive was shown to contain a low level of activity. By showing that the mutant enzyme had a low level of activity, CE-LIF was able to make a contribution to the elucidation of FucT enzymatic mechanism. Further improvements for this application could include adjustments to the CE-LIF parameters to achieve a lower detection limit and better separation.

In contrast to the low activity enzymes studied in Chapters 2 and 3, Chapter 4 dealt with low abundance proteins in the field of proteomics. A method was sought to detect low abundance proteins in the outer membrane of *E. coli*. The first approach tried was solubilization with increasing concentrations of various surfactants. The hypothesis was that high abundance peripheral membrane proteins could be solubilized at low concentrations and less abundant integral membrane proteins solubilized at high concentrations. However, the HAPs were distributed ubiquitously among the different concentration of surfactants. During this procedure, a higher temperature was found to better solubilize the membrane pellet. Thus, the use of a higher temperature was incorporated into the method tested for complete solubilization of the outer membrane pellet. It was found that solubilization in 60% methanol followed by 0.2% SDS at 60 °C was able to solubilize more proteins than other methods at lower temperatures and higher concentration of SDS. Triplicate analysis was also performed to determine the reproducibility of LC-ESI-MS and MS/MS analysis. The results were comparable to other studies.

During one of several analyses, 127 proteins were confidently identified, with more than three-quarters of them known to be localized to the peripheral membrane of *E.*

coli. Of these proteins, nine were localized to the outer membrane. In all, this represented *ca.* 10% of the proteins that are theoretically localized to the peripheral and outer membrane. To increase the number of proteins identified, more sensitive instruments can be used in conjunction with protein level fractionation to remove the HAPs from lower abundance proteins.

This thesis has dealt two different areas of bioanalytical chemistry. In both of these areas, the goal was to characterize enzymes or proteins that were present in low activity or amounts. While it is easy to detect the high activity or abundance species, it is more difficult to observe the less active or abundant ones. However, with new advances in various fields of bioanalytical chemistry such as microfluidics and protein level fractionation, it may become easier to study these low activity or abundant proteins.