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In-Capillary Reduction and Digestion of Proteins at Nano-Liter Volume Using Capillary Electrophoresis

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A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Chemistry

In-Capillary Reduction and Digestion of Proteins at Nano-Liter Volume Using Capillary Electrophoresis

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By

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THE UNIVERSITY OF WESTERN ONTARIO
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Abstract

To analyze the proteins present in biological samples several major steps are involved. One of the crucial steps in proteomics has been sample preparation. Prior to mass spectral analysis proteins containing disulfide bonds must be reduced which is followed by digestion. In this work, a novel way of performing protein reduction and digestion at nano-liter volume using capillary electrophoresis is presented. In this work, zone passing CE technique was utilized to analyze the reduction products in CE. After the successful in-capillary reduction, digestion of protein was also performed. A pH mediated stacking was used to bring the reduced protein and trypsin together for digestion. For performing in-capillary reactions, very low amount of sample is needed resulting in the miniaturization of the reaction. MALDI MS was used for detection considering the ease of coupling to the instrument and only nano-liter sample volume was needed for MS analysis.

KEYWORDS: capillary electrophoresis, in-capillary digestion, reduction, protein analysis, pH junction, MALDI TOF MS, sample preparation.

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I would like to thank my dear parents and my sister for their endless encouragement and support during my study.

Most of all, I would like to express my heartfelt thanks to my dearest husband, for his immense love and encouragement throughout my research life.

Dedication

To my parents and my dearest husband, without them nothing would have been possible!

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List of Symbols and Abbreviations

2D	Two-dimensional
η	Viscosity
μ_a	Apparent mobility
μ_e	Effective mobility
μ_{EOF}	Electroosmotic mobility
BSA	Bovine serum albumin
BGE	Background electrolyte
CE	Capillary electrophoresis
CHCA	α-cyano-4-hydroxycinnamic acid
cLOD	Concentration limit of detection
CPA	Cross-linked Polyacrylamide
CZE	Capillary Zone Electrophoresis
DLPC	1,2-dilauroyl-sn-phosphatidylcholine
DTT	Dithiothreitol
EMMA	Electrophoretically mediated microreaction

EOF	Electroosmotic flow
ESI-TOF MS	Electrospray time of flight mass spectrometry
FASS	Field amplified sample stacking
IAA	Iodoacetic acid
I.D	Internal diameter
L_d	Length of the capillary from the sample injection end to the detector
l_{inj}	Length of injection
L_t	Total length of capillary
MALDI	Matrix assisted laser desorption/ionization
mAU	Milli-absorbance unit
MS	Mass Spectrometry
m/z	Mass-to-charge ratio
nL	Nano liter
O.D.	Outer diameter
pH	Puissance de Hydrogen

pI	Isoelectric point
pKa	-Log of the acid dissociation constant
PMF	Peptide mass fingerprinting
PTM	Post–translational modification
TBP	Tributyl phosphine
TCEP	Tris(2-carboxyethyl)phosphine
t_{inj}	Time of injection
Tris	Tris(hydroxymethyl)aminomethane
TOF	Time of flight

Chapter 1 Introduction to Protein Analysis and Proteomics

1.1 Protein Analysis and Challenges

Proteins are the building blocks of life as they control almost every function within the cell. They are the molecular machines that are responsible for all vital functions in our body; they function as enzymes, in cellular signaling, molecular transport and many more. In order to understand the development and physiology of organisms, a thorough knowledge about protein modification, protein-protein interactions and their function during different stages of cell development are essential. Protein identification has always been a crucial task, which is necessary to determine their roles in tissues or cells as well as their normal or abnormal physiological stress for early detection and diagnosis of diseases [1, 2]. Hence, protein analysis has been a rapidly growing field of research in the recent years. Proteins are dynamic [3] and their expression varies from cell to cell with time, stress, post-translational modification and other factors [4]. An organism can have multiple protein expression in different parts of its body and at different stages of its life cycle. Protein analysis is quite challenging because of the complexity in protein structure and functions.

Proteins are complex molecules made up of amino acids, which are the building blocks of proteins. They are produced from a set of only 20 amino acids, sharing the

same basic structure; an amino group, a carboxylic group and a hydrogen atom, with only difference lying in the side chain. The presence of different side chains is responsible for the different properties of the protein. The amino acid sequence in a protein defines its primary structure, which is a polypeptide chain. Each polypeptide chain of any length has a N-terminal amino acid containing a free amino group and a C-terminal amino acid containing a free carboxyl group. The secondary structure of proteins is derived from the primary structure by hydrogen bonding between amino acids. The two motifs generated by this process are the alpha helix, and the beta pleated sheet. Tertiary structure refers to the three-dimensional structure of a single protein molecule. As a result of solvation and ionization of the amino acid groups, polar residues will be exposed to the aqueous environment and the non-polar residues are grouped inside the protein by hydrophobic forces. Tertiary structure of protein is more complex than the secondary structure. The association of two or more polypeptide chains forms quaternary structure. They are of two types depending on the nature of the polypeptide chains: homogeneous quaternary structure and heterogeneous quaternary structure.

Many proteins like enzymes and hormones most of the time has to function outside the cells. They have to protect themselves from the extracellular environments and also have to resist any denaturation and degradation. Disulfide bonds that are formed between

the cysteine residues of some proteins plays an important role in maintaining the folded conformation and the stability of the proteins. They also help in improving their thermodynamic stability [5, 6], which in turn gives the proteins a better resistance to environmental extremes.

Disulfide bonds are covalent bonds formed between cysteine residues. It is formed by the oxidation of sulfhydryl (-SH) groups of two cysteine molecules, as illustrated in figure 1-1.

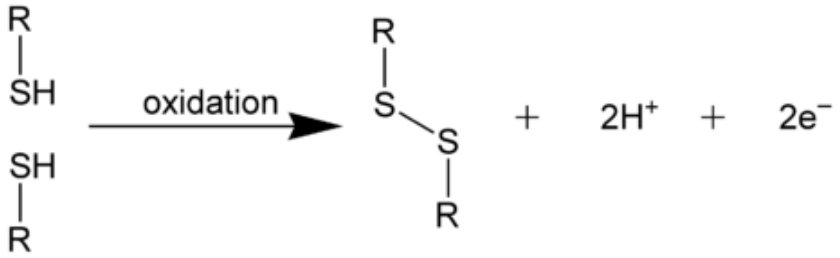


Figure 1-1 Formation of disulfide bonds.

However, many proteins can be functional only when they are folded into its three dimensional structure. In other words, unfolded proteins have to fold into their native structure in order to be biochemically active. Protein folding is a physical process by which a polypeptide folds into its characteristic and functional three-dimensional structure. Protein folding in a cell is a very complex process. When translated from a

sequence of mRNA to a linear chain of amino acids, proteins exist as unfolded polypeptides. Amino acids interact with each other to form the three-dimensional structure, which is the folded protein known as the native state [7]. If the protein fails to fold, it will result in the formation of inactive proteins, which can be toxic. However, under certain conditions like high temperature, high concentration, extreme pH and the presence of denaturants will result in the unfolding of the proteins [8].

In addition to the complexity of protein structure and functions, yet another factor that increases the level of complexity in protein identification are protein post-translational modifications. It is the chemical modification that happens after protein translation. There are over 300 different PTM's which include the diverse processes as phosphorylation [9, 10], lipidation, proteolysis, nitration, oxidation, glycosylation [11, 12], methylation [13], acylation, ubiquitination [14] and many more. Among these, phosphorylation is the most common PTM in proteins. Often, apart from the single modifications, they are even modified through a combination of posttranslational cleavage and by the addition of functional groups. These modifications can result in changing the size, charge and structure of proteins [15].

1.2 Current Approaches to Protein Analysis

As a result of all the above-mentioned complexity, protein analysis has been a challenging task. There are several steps involved in the analysis of proteins in a biological sample. The major steps include protein extraction, preparation of the sample, separation of the protein of interest, detection using MS and interpretation of the data for the identification of proteins. There are many separation techniques developed like chromatography, capillary electrophoresis and mass spectrometry, which are excellent for separating and detecting proteins. However, the key factor in determining the success of protein analysis relies on the quality of the sample. Without a properly pretreated sample, even by using the most sophisticated technique would result in the complexity of analysis.

To obtain meaningful and reliable results in proteomic analysis, protein of interest should be pretreated, purified and fractionated prior to the analysis. Sample preparation means extraction followed by the cleaning up of the sample to be analyzed, which can include the isolation of protein of interest, removal of unwanted salts and ions, chemical modification of the analyte or even concentrating the analyte of interest [16]. No universal sample preparation is available for all the analytes. It depends on the nature of the analyte and the analytical techniques further employed for the separation

and detection of it. Ideally, the number of steps required for sample preparation should be minimized and utmost care should be taken to avoid any sample loss. With the rapid development of analytical techniques for protein separation and detection, demand for quality samples have also increased. As a result, researches on improving the sample preparations of proteins have increased immensely over the past few decades.

Depending on the degree of purity to be achieved, different types of purification procedures can be utilized for the fractionation of protein mixtures. After protein fractionation and purification, proteins have to be subjected to digestion in order to efficiently sequence the amino acids. If the proteins have disulfide bonds, it will prevent denaturation and thus hinders digestion. In order to achieve effective proteolysis for MS peptide fingerprinting, these disulfide bonds must be reduced. There are many reducing agents commercially available, which are being used for the reduction of disulfide bonds. Common reducing agents include dithiothreitol (DTT), tributylphosphine (TBP), beta-mercaptoethanol and tris (2-caroxyethyl) phosphine (TCEP). Most widely used reducing agent by researchers is DTT [17]. DTT is a strong reducing agent and only a low concentration of DTT is sufficient to induce reduction.

The reduction by DTT usually involves two sequential thiol-disulfide exchange reactions (figure 1-2). The intermediate mixed-disulfide state is highly unstable as the second thiol of DTT has a tendency to form a six membered-closed ring, which is stable.

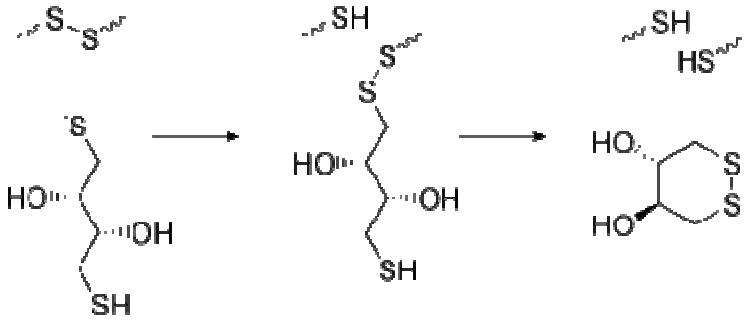


Figure 1-2 Schematic of the reduction of disulfide bonds by DTT.

Reduction reactions are usually followed by alkylation and digestion. Alkylation is done to prevent the reformation of disulfide bonds by air oxidation. Iodoacetamide (IAA) is the standard alkylating agent that has been used by most of the researches to ensure alkylation. Digestion is done by the introduction of digestive enzyme mostly trypsin and the sample is allowed to react at 37° C for a few hours [18]. Trypsin cleaves peptide bonds after arginine and lysine. Other commonly used digestive enzymes are pepsin and chymotrypsin. Pepsin is efficient in cleaving peptide bonds between hydrophobic and aromatic amino acids such as phenylalanine, tryptophan and tyrosine. While chymotrypsin cleaves peptide bonds where the carboxyl side of the amide bond is tyrosine, phenylalanine or tryptophan. Upon digestion, the protein of interest is cleaved into smaller peptide sequence and further analyzed by MS.

1.3 Protein Identification

Prior to the introduction of Mass Spectormetry, Edman degradation was the only method of choice for protein identification. This method involves the cyclic degradation of peptides based on the reaction of phenylisothiocyanate with the free amino group of the N-terminal residue such that amino acids are moved one at a time and identified as their phenylthiohydantoin derivatives. This method is very tedious and time-consuming. Besides these, one of the major limitations is that the peptides with more than 50 residues cannot be sequenced by this method. Also, this method cannot be applied if N-terminal amino acid has been chemically modified or if it is buried within the protein [19].

In 1993, protein mass fingerprinting (PMF), an analytical technique was developed for protein identification. In this method, protein of interest is first cleaved into smaller peptides and the peptide fragments are then identified by peptide mass fingerprinting, a technique in which MS is used to measure the masses of the peptide fragments [20]. This approach is also known as bottom-up protein characterization. The masses of the peptide fragments are then compared to a database containing the known protein sequence [21].

By this way a fingerprint unique to the unknown protein can be obtained. However, identification of protein by this technique faces some challenges. The protein sequence must be present in the database and the presence of post-translational modifications would further complicate the analysis. While identifying larger proteins, there is a high possibility of false positive matches.

These challenges have been overcome with the advent of MS/MS analysis. During the past two decades, mass spectrometry has become the method of choice for protein detection and identification. By combining MALDI-TOF or ESI-TOF MS, further selection and fragmentation of individual peptides from the fingerprint can be achieved [22]. This results in more accurate determination of the amino acid sequence and hence the identification of the unknown proteins. MS can be used to analyze samples ranging in attomole through nanomole quantities.

1.4 Limitations of Current Digestion Techniques

For effective protein analysis, protein digestion should be carried out efficiently and should give high reproducibility. The standard digestion methods have several drawbacks. While using 2D-gel electrophoresis for protein analysis, each spot on the gel

that corresponds to a specific protein are manually excised from the gel, and digested overnight with trypsin, peptides formed are then identified by mass spectrometry. Even though this method has been an essential step in protein analysis, it has a lot of limitations. Manual excision of the protein spots is time consuming and the various steps performed cannot be automated which eventually affects the resolution and sensitivity of the analysis. While performing in-gel digestion, some of the protein substrates are trapped in the gel and the proteolytic enzyme fail to digest them. Also, some peptides that are formed by digestion cannot diffuse out of the gel [23].

On the other hand, in-solution digestions, which are performed in a small sample vial usually suffer from diffusion-limited mass transfer. This would slow down the time needed for digestion, especially for proteins present at sub-micro molar concentration. Moreover, the several reactions that needs to be performed before digestion and the longer reaction time favors many side-reactions to occur at undesired amino acids. These in turn generate complexity in the mass fingerprint. Also, while performing digestion in the vials, the large surface area of the vials result in sample loss due to adsorption. As an alternative, bioreactors were used to digest proteins. But the time required for obtaining a homogenous solution is usually very long as the enzyme to substrate ratio is kept low to avoid any auto digestion of trypsin. In order to overcome the above-mentioned

challenges, methods and techniques which are much faster and which utilizes minimum samples were required.

1.5 Miniaturization of Protein Analysis

Currently, there has been a growing trend towards the miniaturization of the analytical methods. Miniaturization has led to the development of microfluidic and lab on a chip devices (LOC), figure 1-3 [24]. The aim of these devices is the integration of several laboratory processes on a single chip format, which further results in the down sizing of the samples used. Some of the attractive advantages of miniaturization of analytical procedures are the consumption of fewer samples, low analysis time, generation of less waste, integration and automation of various methods etc. The remarkable surface-to-volume ratio of the microchannels (of the order of 80,000 m-1) lowers the sample consumption. These devices also allow integrating the sample preparation and separation steps in one single device so that cross contamination can be avoided. This way sample handling is reduced to a considerable amount resulting in increased reproducibility of the results. In microfluidic devices, manipulation of low volumes of sample (nL to μL) using voltage and pressure is possible. Microfluidic channels are available in various widths, depths and lengths; usually the dimension ranges from millimeter to micrometer.

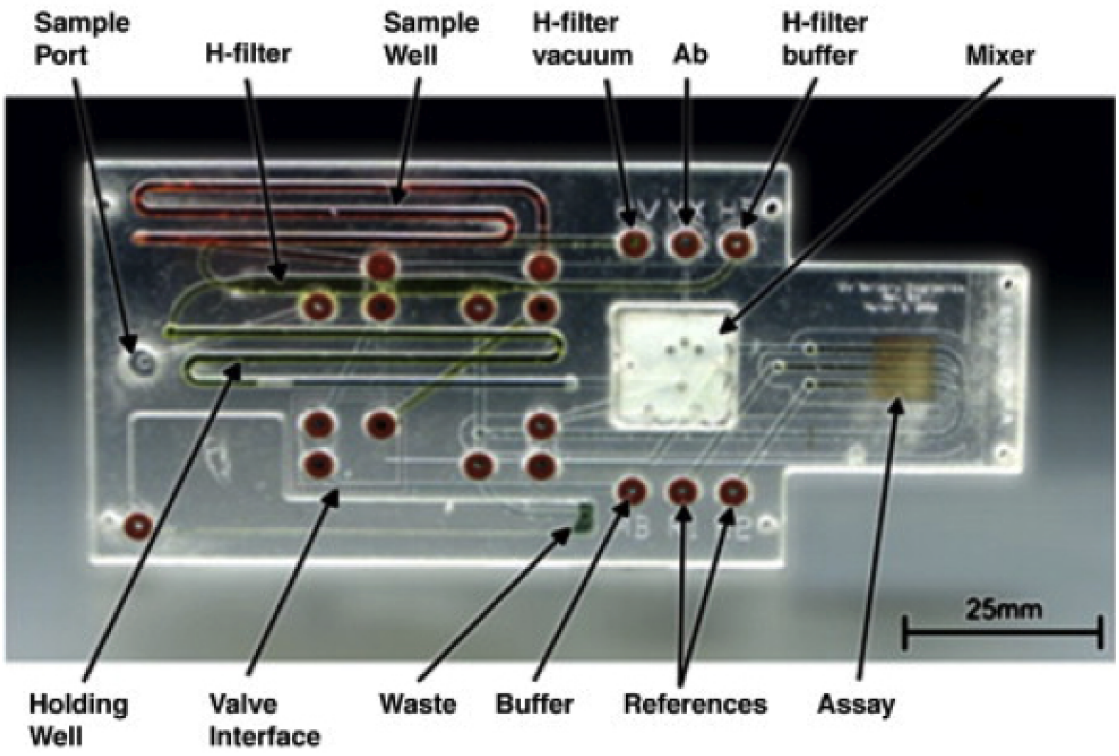


Figure 1-3 Picture of a typical Lab on a chip device [25].

To resolve the limitations of 2D-gel electrophoresis mentioned in section 1.4, and for obtaining fast and efficient separations; performing the sample preparation steps and separation on microfluidic devices have been investigated by several research groups. Gottschlich [26, 27] group successfully performed two-dimensional separation system on a micro fabricated device that utilizes open-channel electrochromatography as the first dimension and capillary electrophoresis as the second dimension. However, the two

major issues that have to overcome in order to develop a fully functional 2D chip for proteomics are sensitivity and resolution.

Microfluidic devices have been also developed for performing digestion of proteins [28, 29]. Such devices not only speeds up the reaction by consuming low sample volume (nL to μL) but also increased the enzyme to substrate ratio. One of the most common trends has been the use of enzyme immobilization on solid supports for solid-phase bioreactors. Immobilization was achieved either by chemical or physical adsorption of the enzyme to the solid support. This way a larger enzyme to substrate ratio, high efficiency and the reduction of enzyme autolysis was possible. Wang *et* al. [30] developed a microfluidic chip packed with trypsin-loaded beads in a fluidic channel. This method was found to be more advantageous than the conventional methods as the digestion was performed faster and more efficiently.

Lab on a chip and microfluidics devices are not only used for performing sample preparation steps but also for the separation of proteins and for chip/MS interface. Remarkable developments were made on interface design between the chip and the mass spectrometer.

Even though miniaturization of protein analysis using these devices sounds promising, the cost of manufacturing such devices is very high. Moreover, majority of the research in this field focuses on the development of single-function chips, which is usually separation of proteins coupled to MS. Hence, only a very few miniature alternatives to sample preparation of proteins exists. Therefore an analytical technique, which can be used to develop and optimize the processes that are performed on LOC was needed. Capillary electrophoresis (CE), which employs capillaries of internal diameter in the micrometer range, allows nanoliter volume sample consumption resulting in miniaturization of the reaction. Therefore, it is highly suitable for the integration on a chip format [31].

Capillary electrophoresis or high-performance CE, an alternative to HPLC meets most of the microfluidics requirements and it can be coupled with MS. CE with its attractive features like low sample volume inside the narrow bore capillary, voltage and pressure application to manipulate the samples and the coupling of CE with MS analysis offers an effective analytical technique towards miniaturization of protein analysis [32]. CE can be used for performing both sample preparation and separation of complex protein mixture, which makes it a superior and versatile analytical technique.

In my work CE was the analytical technique used to perform the sample preparation steps like reduction and digestion of proteins, which resulted in the miniaturization of the reactions.

1.6 Fundamentals of Capillary Electrophoresis

Electrophoresis is defined as the migration of the ions under the influence of an electric field. Tiselius first introduced electrophoresis as a separation technique in 1937. Traditional electrophoresis was performed in anti-convective media such as polyacrylamide or agarose gels. Though gel electrophoresis has been widely used for the separation of macromolecules such as proteins and nucleic acids, it did not prove to be an efficient technique. Longer analysis time, low efficiency and poor dissipation of joule heat were the major disadvantages of gel electrophoresis.

In order to overcome the limitations of gel electrophoresis, electrophoresis was performed in narrow-bore capillaries. The high surface area-to-volume ratio of the capillaries allows quick dissipation of heat, which makes it anti-convective. In 1967, Hjerten conducted open tube electrophoresis using tubes of only a few millimeter

diameters [33]. After that, advancement in this technique was made when they decreased the inner-diameter (I.D) of the capillaries. CE was soon recognized for its high resolution and high separation efficiency, which made it a superior analytical technique [34].

1.6.1 Theory and Instrumentation of Capillary Electrophoresis

In CE, electrophoresis is performed in narrow-bore capillaries, usually of inner diameter 25 to 75 μm. The capillary is mostly filled with the buffer solution or background electrolyte (BGE). The use of narrow bore capillaries has several advantages. The high electrical resistance of the capillary enables the application of very high electrical fields with the generation of less heat. Also, the large surface area-to-volume ratio of the capillary helps to dissipate the heat that is produced. As a result of using high electric fields, analysis time is shortened and results in high efficiency and resolution. In addition to these, while performing sample preparation steps inside the capillary, only a very small amount of sample is needed at low concentration. This results in the miniaturization of the reaction. Another excellent feature of CE instrument is that it can be coupled with MS for further identification of the analytes. All these features have made CE an effective separation technique compared to other chromatographic techniques [35].

Capillary Zone Electrophoresis (CZE) is the simplest and the most widely used mode in CE due to its simplicity of operation. In this mode, the capillary is only filled with the buffer and separation occurs because the analytes migrate in different zones at different velocities. Figure 1-4 represents the basic setup of a CE instrument. The basic instrumental set-up includes a high voltage power supply, a fused silica capillary, two buffer reservoirs and an online UV detector. The ends of the capillary are placed into the buffer reservoirs, which also contain the electrodes that are connected to the power supply.

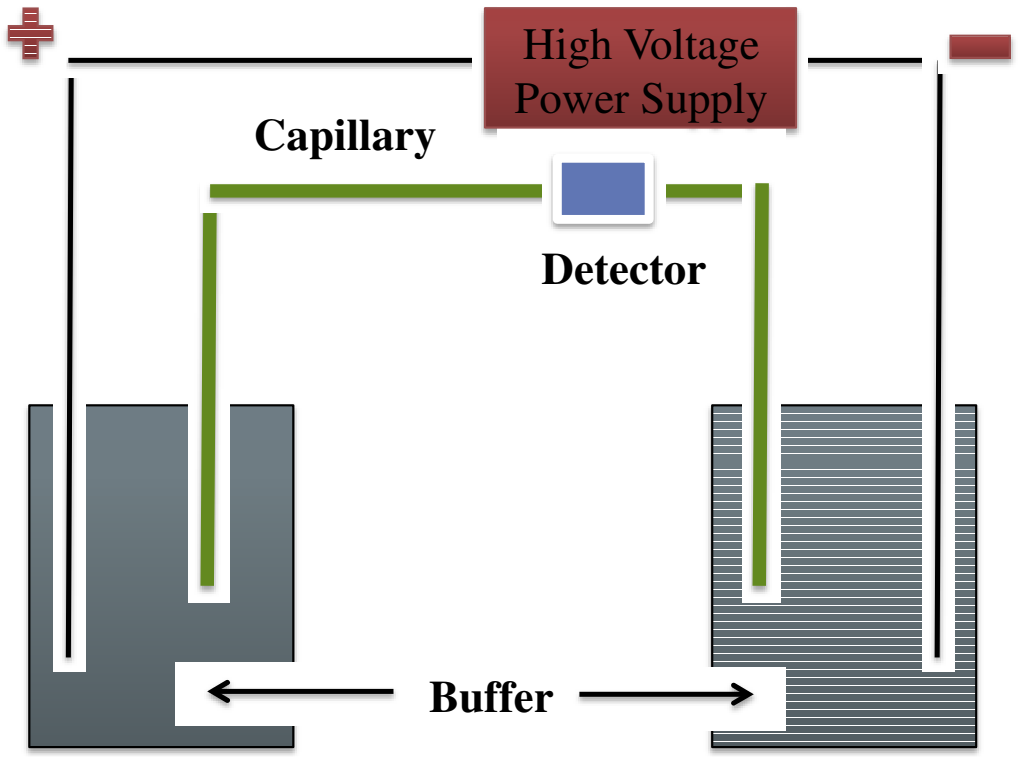


Figure 1-4 Basic setup of a CE instrument, showing each significant part.

In CZE mode, analytes are separated based on their charge-to-size ratio as they have different electrophoretic mobility. In addition to their electrophoretic mobility, analytes are also mobilized by electroosmotic flow (EOF), which sweeps the analytes towards the cathode.

1.6.2 Electroosmotic Flow (EOF)

A fundamental constituent of CE is electroosmotic flow (EOF). EOF is the bulk flow of liquid inside the capillary; it is formed by the presence of charge on the inner surface of the capillary wall. The amount of time an analyte remains in the capillary is controlled by the EOF. In fused silica capillaries EOF is strongly controlled by the silanol groups (SiOH), which can also exist in anionic form (SiO^-). The cations from the buffer will build up near the negatively charged surface in order to maintain a charge balance. This results in the formation of a double layer and a potential difference is created near to the wall. This is known as zeta potential [36]. During voltage application, the cations forming the diffuse double layer are attracted to the cathode. Because the cations are solvated, their movement results in the dragging of bulk solution inside the capillary towards the cathode. As a result, EOF causes the movement of all analytes, irrespective of charge in the same direction, (figure 1-5).

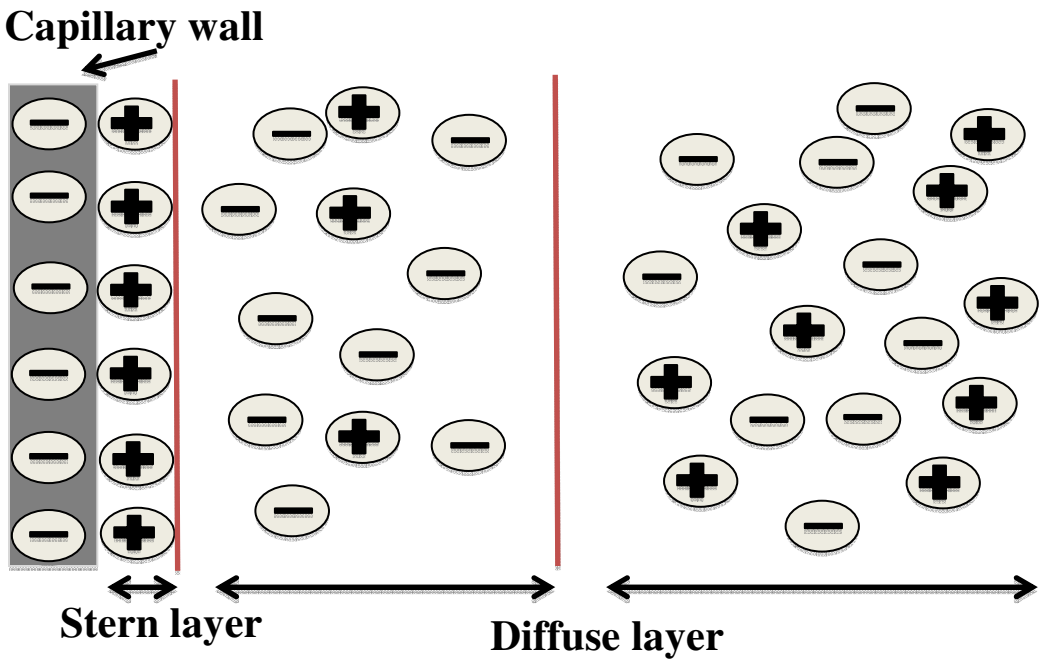


Figure 1-5 Illustration of the various layers of electric potential at the capillary surface.

In the presence of EOF, the mobility of an analyte is called the apparent mobility (μ_a). Apparent mobility of an analyte can be calculated using the equation 1.1.

$$\mu_a = \frac{L_d L_t}{tV} \qquad \text{(Eq. 1-1)}$$

where L_d is the distance from the point of injection to the detector, t is the migration time of the analyte from the point of injection to the detector, E is the applied electric field and L_t is the total length of the capillary and V is the applied voltage.

Apparent mobility depends on the electrophoretic mobility (μ_e) and electroosmotic mobility (μ_{EOF}). Therefore, apparent mobility can be expressed as the sum of electrophoretic and electroosmotic mobilities,

$$\mu_a = \mu_e + \mu_{EOF} \qquad \text{(Eq. 1-2)}$$

The mobility of the EOF [37] can be found out by measuring the apparent mobility of a neutral molecule (EOF marker) that will migrate at a velocity same as that of EOF [38]. Electrophoretic mobility (μ_e) of the analyte can be expressed as,

$$\mu_e = \frac{q}{6\pi\eta r} \qquad \text{(Eq. 1-3)}$$

where q is the charge of the ion, η is the viscosity of the buffer, and r is the radius of the ion. Since μ_e is proportional to the charge-to-size ratio of the analyte, it is evident that small highly charged analytes will have higher mobilities and vice versa.

Another unique feature of EOF inside the capillary is the flat profile of the flow, this is quite different from the laminar flow profile generated in pressure driven systems. This is because the driving force of the EOF is uniformly distributed along the capillary. This flat flow profile is advantageous by reducing the dispersion of the sample zone and results in higher separation efficiencies, (figure 1-6).

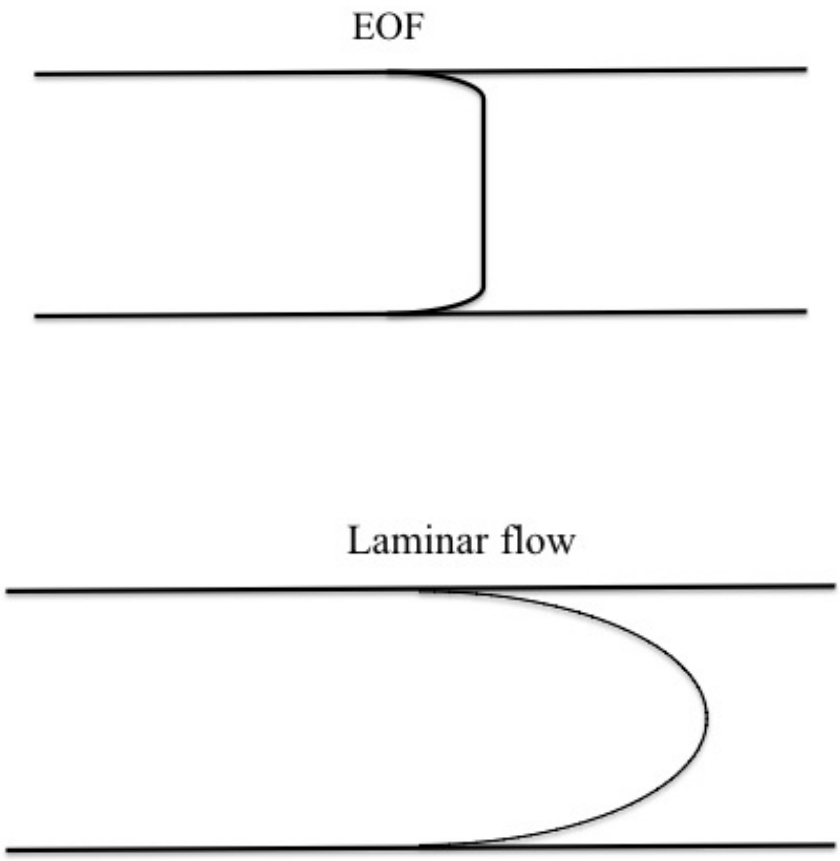


Figure 1-6 EOF flow profile and Laminar flow profile.

1.6.3 Capillary Electrophoresis for performing Sample Preparation of Proteins

Capillary electrophoresis has been used in proteomic research more as a separation tool, to separate the components in a complex mixture prior to mass spectral analysis. Recently, there has been a growing demand towards the development of analytical techniques that can integrate sample preparation and separation under a single format so as to achieve high-throughput results. As a result, significant progress has been made in the area of performing sample preparation and chemical derivatization steps on CE. Even though, the use of narrow bore capillaries reduces the consumption of sample to nanoliters, it causes a great deal of problems in sensitivity and in terms of concentration limit of detection (cLOD) while using on-capillary optical detection [39]. To overcome this, several research in CE focuses on in-capillary analyte enrichment.

Sample preconcentration in CE can be achieved by several enrichment methods. Preconcentration happens when a large volume of sample loaded onto the capillary is stacked or compressed into a narrow band. In electrophoretic sample stacking [40], the electrophoretic mobility of the sample is decreased when the molecules cross the interface from the sample zone to the background buffer, thus resulting in a narrow sample band. This is possible only if the sample zone and background electrolyte are of different composition; it could be pH, conductivity, concentration or additives. As

a result of stacking, the peak width decreases and the peak height increases, thereby enhancing the signal-to-noise ratio and improving the limit of detection. Electrophoretic sample stacking techniques include field amplified sample stacking (FASS) [41] and pH-mediated stacking.

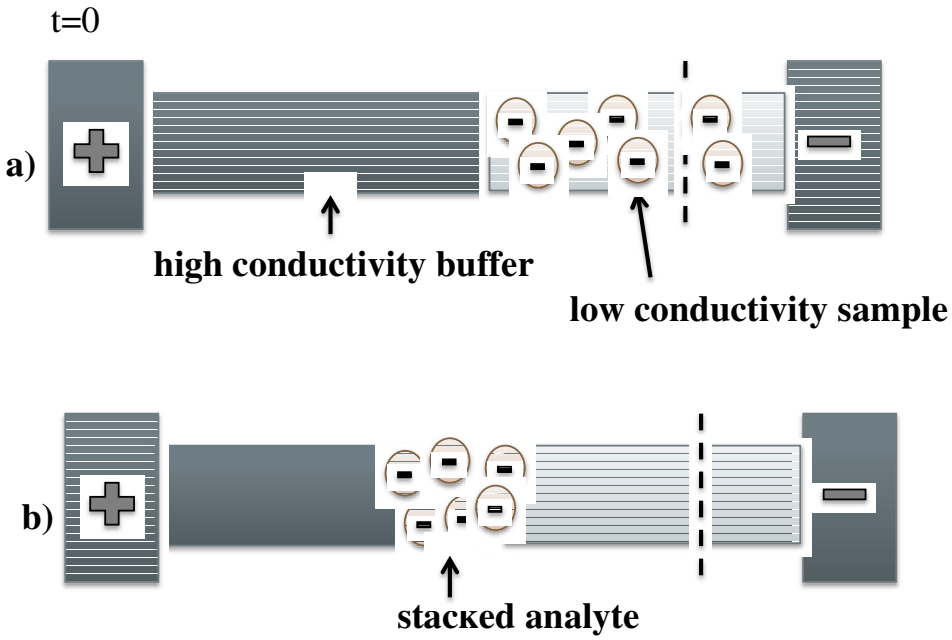


Figure 1-7 Schematic of FASS

a) Schematic of FASS of anionic species in the absence of EOF. A gradient in the background electrolyte ion concentration is established with the sample in the low conductivity zone. Upon application of an electric field, the axial gradient in conductivity results in an electric field gradient. Since area-averaged current density is uniform along the axis of the channel, the low conductivity section is a region of high electric field, and the region of high conductivity contains relatively low electric field. As sample ions exit the high field/high electrophoretic velocity region and enter the low velocity region, they locally accumulate and increase in concentration. b) stacking of analyte.

FASS is one of the most commonly used stacking techniques [42]. In this technique, the analyte is prepared in a solution with conductivity lower than that of the background buffer. When voltage is applied, due to the lower conductivity in the sample plug the local electric field will be higher than the BGE. This reduces the mobility of the analyte molecule as they cross the interface of the sample zone and the BGE, and results in stacking. Figure 1-7 represents the schematic of FASS.

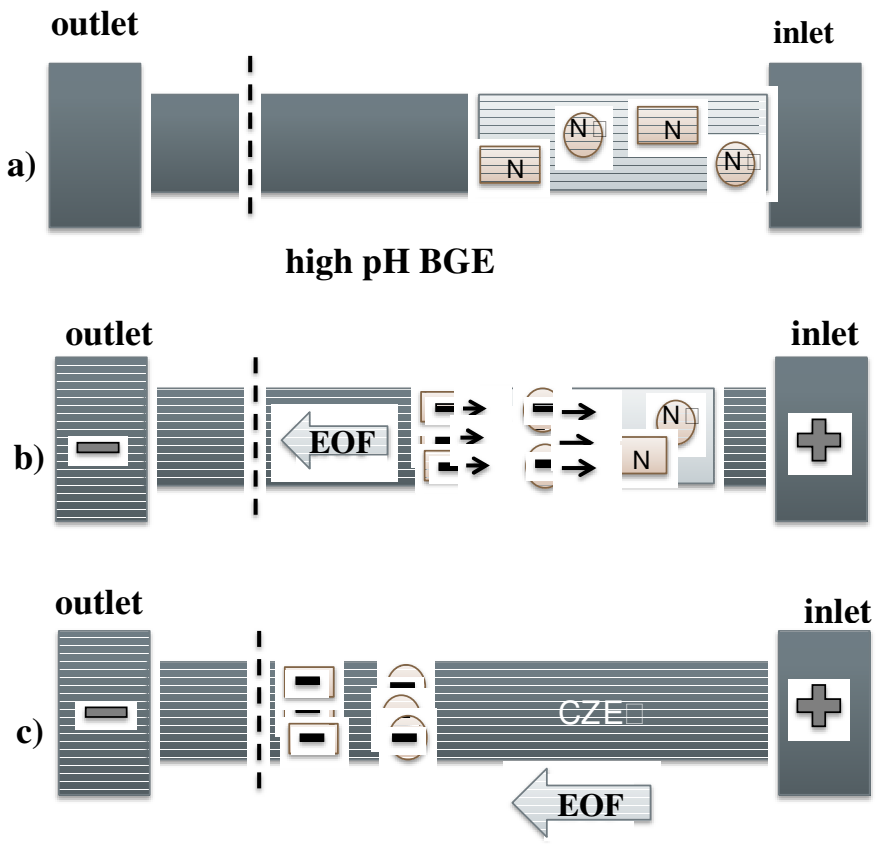


Figure 1-8 A schematic diagram of the dynamic pH junction model

a) A large sample plug at a low pH is injected into the capillary filled with a higher pH buffer; b) a steep pH boundary develops at the front end of the sample zone and sweeps throughout the sample zone during electrophoresis, converting neutral analytes into anionic analytes and hence slows down its migration velocity; c) the sample zone is fully titrated and the stacked analyte undergo separation by CZE.

In pH mediated sample stacking [43], as the name suggests the pH of the sample and/or the BGE is changed which results in stacking. In acid stacking, a plug of strong acid is introduced after the injection of the sample zone. By voltage application, the strong acid titrates the sample zone resulting a neutral and low-conductivity region through which cationic analytes move faster and gets stacked at the interface. Britz-McKibbin *et* al [44] introduced a dynamic pH junction (figure 1-8), where the analyte's ionization state is altered, hence changes the velocities across the sample zone and the BGE. Weakly acidic analytes were injected as neutral molecules at sample pH below their pKa and the pH of BGE's was kept high. When voltage was applied, the hydroxide ions from BGE titrated and raised the pH at the cathodic end of the sample zone. As the hydroxide front sweeps across the sample zone, the analytes were ionized and migrated with it resulting in stacking. By this method, 50-fold enrichment factor was reported for nucleotides. Following this work, other groups used the dynamic pH junction technique and were successful enough in achieving a 100-fold increase in UV absorbance detector response [45]. However, this technique was not applicable for high molecular weight molecules like proteins because of their slow electrophoretic migration. To overcome this, Nesbitt *et* al [46] introduced a pH-mediated stacking technique for proteins, which allowed the stacking of proteins for longer period. In their work, a pH junction was formed by placing a base (pH 9.25 ammonium) at the cathodic end of the capillary and an acid (pH 4.25 acetate) at the anode. This set-up helped to maintain the pH throughout the entire run. This technique resulted in successful enrichment of protein as well as protein mixtures and peptides.

Apart from preconcentration, CE has been employed to perform other sample preparation steps like fractionation, purification of the sample from unwanted matrices and several in-capillary reactions like reduction, digestion or derivatization of the samples. Low-volume sample preparation was possible by integrating membrane-based fractionation techniques with CE. This includes dialysis, electrodialysis and dialysis on liquid artificial membranes. Salt from enriched analytes were removed by the technique of pH junction. Microreactions with immobilized reagents were also performed in CE. Kuhr *et* al. [47] were the first group to develop enzymatic capillary reactors for online protein-digestion.

Yet another remarkable way of performing reactions inside the capillary in CE is by zone mixing of different reagents or analytes without the use of immobilization on solid supports. Besides the conventional procedures involving pre or post capillary derivatization [48], recently a lot of research has been focused on in-capillary derivatization as well [49]. In in-capillary derivatization, reaction is performed inside the capillary by promoting the mixing of analytes with the reagent. An excellent feature of in-capillary derivatization is that the capillary acts as a micro reactor, as a result the sample consumption and dilution are minimized [50]. There are different ways of performing in-capillary derivatization. If the derivatization is performed in the middle of

the capillary by passing either the analyte or reagent plug through the other by the application of an electric field, it is referred to as zone-passing. Figure 1-9 shows the schematic of in-capillary derivatization by zone passing technique. If the derivatization is performed by introducing the sample solution at the inlet of the capillary previously filled with a running buffer, which has the reagent, it is referred to as throughout capillary.

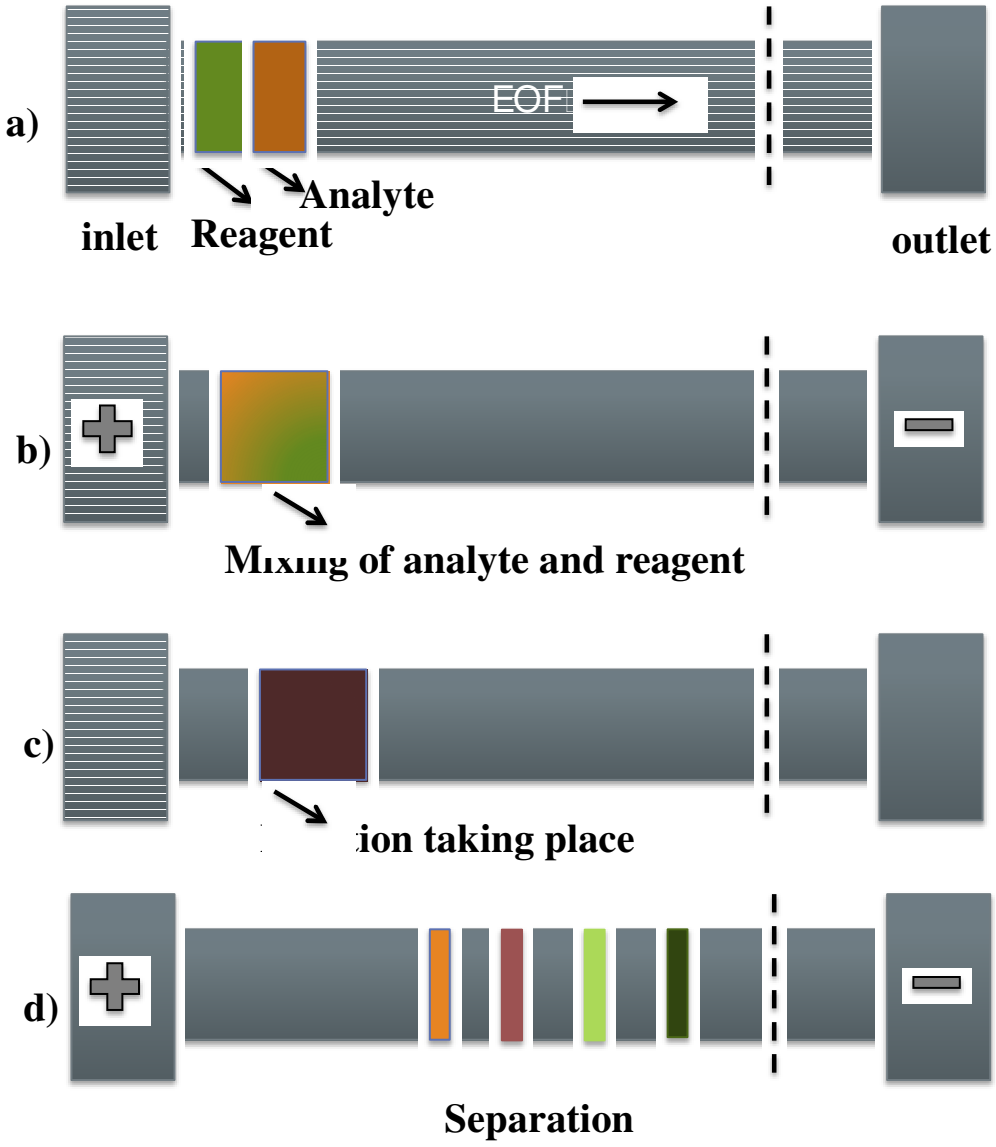


Figure 1-9 Schematic illustration for in-capillary derivatization by zone passing technique.

(a) hydrodynamic injection of the analyte and reagent plugs; (b) electrokinetic mixing of the plugs; (c) stand-by time for derivatization; (d) separation of the analytes and reagents.

Bao and Regnier [51] were the first to perform in-capillary reactions and they called it as electrophoretically mediated microanalysis (EMMA). Nesbitt *et* al. [52] successfully performed in-capillary protein enrichment, digestion, and separation using CE, followed by MALDI-MS. In this method, a discontinuous pH buffer system was utilized to enrich the proteins and the digestive enzyme, trypsin. After the digestion of proteins, voltage was applied to re-establish the pH junction and to separate the peptides according to their pI. This was followed by spotting the sample from the capillary onto the MALDI plate mixed with the matrix and peptides identified by MS. This method was found to be superior to traditional in-solution digestion.

Hence, CE has been recognized as a superior analytical technique not only for separation of biological macromolecules but also for performing sample preparation of biological samples. Though there had been a lot of developments in performing the digestion of proteins inside the capillary using EMMA and by using enzymes immobilized on solid-phases, not much work has been reported in performing the reduction of proteins in CE. Hence, my research focuses on performing the sample preparation steps like reduction and digestion of proteins using the zone passing CE technique.

1.7 Overview of this Thesis

The work presented here is an effort towards the miniaturization of reduction and digestion (using nano-liter sample volume) of disulfide containing proteins by performing the reaction in-capillary using CE, followed by MS analysis. First step was the optimization of reduction reaction, which was performed using zone passing CE technique at a weakly basic condition and at low concentration. The goal of my work was to improve the completeness of the reduction, by optimizing the reaction time and the completeness of the reaction. After successful reduction, digestion was performed in-capillary as well. A pH mediated stacking was utilized for effective mixing of the digestive enzyme trypsin and the protein. Protein was incubated for a few hours inside the capillary and MALDI MS was performed to identify the peptides.

Chapter 2 Experimental for In-Capillary Reduction and Digestion of Proteins

2.1 Materials and Apparatus

Apparatus: An Agilent Capillary Electrophoresis (Palo Alto, CA, USA) instrument with a direct UV-visible absorbance detector was used for all the experiments. Uncoated fused silica capillaries with dimensions 50 μm i.d and 364 μm o.d were purchased from Polymicro Technologies (Phoenix, AZ, USA). The total length of the capillaries used for all the experiments is 48.5 cm. A small window was burned through the polyimide coating on the silica capillary, 8.5 cm from the detector for UV-Visible absorbance detection resulting a length of 40.0 cm to the detector. Data acquisition was performed using the ChemStation software by Agilent. pH adjustments were performed using a caliberated Accumet AB15 pH meter from Fisher Scientific (Nepean, ON, Canada). Mass Spectrometry data were obtained using a 4700 Proteomics Analyzer, MALDI TOF TOF (Applied Biosystems, Foster City, CA, USA). Data acquisition and data processing were respectively done using 4000 Series Explorer and Data Explorer (both from Applied Biosystems). The estimated isoelectric points of the peptides were computed using the online pI calculator provided by ExPASy.

2.2 Chemicals and Reagents

Disulfide containing proteins like insulin from bovine pancreas and Bovine Serum Albumin were purchased from Sigma Aldrich (St. Louis, MO, USA). DTT was purchased from Sigma Aldrich. Anhydrous methanol was purchased from Sigma Aldrich (St. Louis, MO, USA), which was used in preconditioning the capillary. Anhydrous sodium phosphate dibasic was purchased from MERCK (Darmstadt, Germany); it was used to prepare the phosphate buffer that is used in all the reduction experiments. The pH of the buffer was adjusted to 7.5 using phosphoric acid, which was purchased from MERCK (Darmstadt, Germany). Reagent grade ammonium hydroxide was purchased from EM Science (Gibbstown, NJ, USA). Promega sequencing grade trypsin was obtained from Fisher Scientific and used in all digestions. Solutions were prepared using fresh deionized 18.2 MΩ water, provided by a Millipore Water Purification System (Bedford, MA, USA). α-Cyano-4-hydroxycinnamic acid (CHCA) was obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.3 Capillary Preconditioning

Fused silica capillary was prepared prior to use by first flushing with anhydrous methanol for 10 min, followed by 1 M HCl for 10 min and 1 M NaOH for 10 minutes.

The capillary was also flushed with de-ionized water for 2 minutes after flushing with methanol, HCl and NaOH. The final flushing was with buffer solution for 10 minutes. In between every run the capillary was again rinsed with 1 M NaOH for 2 minutes, de-ionized water for 1 minute and buffer solution for 2 minutes by the application of positive pressure at the inlet.

2.4 Optimizing the Conditions

Phosphate buffer at different concentration and pH was tested to find the compatibility of it with BSA, insulin and DTT. The final optimized buffer solution chosen for reduction was 80 mM phosphate buffer with pH 7.5, which was used for both in-vial and in-capillary reduction reactions. Different concentrations of DTT were also tested and finally chose 5 mM as the optimized DTT concentration for all reduction reactions. For in-capillary digestion, 80 mM ammonium phosphate of pH 8.5 and pH 2.0 were used.

2.5 Reduction Tests

2.5.1 In-Vial Reduction

At first, traditional in-vial reductions were performed by mixing 1 mg/ml of insulin (10 μL) and freshly prepared 10 mM DTT (10 μL), which resulted in the final concentration of BSA (0.5 mg/mL) and DTT (5 mM). All in-vial experiments were performed in low retention microtubes from Axygen Scientific Inc. (Union City, CA, USA). BSA and DTT mixed in the vial were allowed to sit at room temperature for different time periods. The reduced mixture was then analyzed by CE. The reduction mixture (2 μL) was mixed with the CHCA matrix (2 μL) and 0.73 μL of it was spotted on a MALDI plate for MS analysis. In-vial reductions were also performed by heating the insulin and DTT mixture at 50°C at different time periods, which was followed by CE run and MS analysis.

2.5.2 Premixed In-Vial and Allowed to React In-Capillary

Before performing the in-capillary reduction reaction, insulin and DTT were premixed in the vial and a small plug (1 mm) of the mixture was injected into the capillary from the inlet by using low positive pressure (20 mbar). It was followed by the injection of a

small plug of buffer, that too by pressure application. The mixture was allowed to stand inside the capillary for different time periods and voltage was applied to analyze the reduction products in CE. The reduction mixture was also spotted on MALDI plate from the capillary for MS analysis. In order to speed up the reduction reaction, after injecting the premixed mixture from in-vial into the capillary, the vial at the inlet was replaced by heated buffer and changed it every 5 minutes with heated ones till the reduction was complete. This was followed by CE run and MS analysis.

2.5.3 In-Capillary Reduction

In-capillary reduction reaction was performed by first injecting a small plug of insulin dissolved in basic buffer followed by another small plug of DTT also dissolved in buffer and a third plug of buffer. All were injected from the inlet by the application of positive pressure (20 mbar). Positive polarity voltage was then applied to ensure the mixing of the reagents. As soon as the mixing was completed, the mixture was incubated inside the capillary for different time periods. After reduction was complete, the reduction products were analyzed by CE and also spotted onto MALDI plate from the capillary for MS analysis. In order to speed up the reduction reaction, after injecting the reagents into the capillary from the inlet, the vial at the inlet was replaced by heated buffer and changed it every 5 minutes with heated ones and allowed the reaction to take place. After reduction was complete, the reduction products were analyzed by both CE and MS.

2.6 Digestion Tests

2.6.1 In-Vial Digestions

In-vial digestion was performed with BSA of initial concentration 1 mg/mL. 5 mM DTT was added to it and was incubated at 50 °C for 15 minutes. Trypsin of initial concentration 0.1 mg/mL was added to the reduced BSA for a w/w ratio of 20:1 molecules of substrate per trypsin molecule. The mixture was allowed to react at room temperature for 3 hours. It was then spotted on MALDI target plate combined with CHCA and analyzed by MS.

2.6.2 In-Capillary Digestions

For in-capillary digestion, first the reduction was performed in-vial. After 15 minutes of reduction at 50 °C, reduced BSA along with DTT was injected into the capillary by applying 50 mbar pressure for 24 seconds (around 40 nL volume). Positive polarity voltage (15 KV) was applied to separate reduced BSA and DTT. After DTT exits the capillary and when BSA reached near to the outlet, a small plug of trypsin (10 nL) in basic buffer was injected from the outlet. pH junction was established by replacing the outlet with acidic buffer of pH 2.0 and inlet with basic buffer of pH 8.5.

BSA and trypsin was mobilized to the junction using -15 KV for 30 seconds. After BSA and trypsin was mixed well, they were allowed to react inside the capillary for 3 hours. Digested BSA was then spotted onto a MALDI plate by flushing the contents in the capillary using high pressure for 30 seconds, followed by mixing the spot with 1 μL CHCA.

2.7 MALDI MS Analysis

MALDI MS analysis was done to further confirm the reduction reaction in both in-vial and in-capillary [53]. The instrument is equipped with a 355 nm Nd:YAG laser; the laser rate is 200 Hz. Linear positive ion modes were used. Each mass spectrum was collected as a sum of 1000 shots. MALDI matrix, α–cyano–4–hydroxycinnamic acid (CHCA), was prepared as 5 mg/mL in 6mM ammonium phosphate monobasic, 50% acetonitrile, 0.1 % trifluoroacetic acid and mixed with the sample at 1:1 ratio (v/v). There are different ways of mixing the analyte and the matrix. The dried droplet method is one of the standard ways of spotting on MALDI target [54]. In this work, this method was used for spotting the analyte mixed with the matrix, CHCA [55]. For the analysis of in-vial reduction mixture, 2 μL of reduction mixture was first combined with 2 μL of the matrix in a separate vial and around 0.73 μL of the mixture was spotted onto a polished steel MALDI plate. It was allowed to dry and the MALDI MS detection was performed. In-capillary MS analysis was performed by direct spotting of the contents of the capillary

onto a MALDI plate using 50mbar pressure application to yield 20 nL fractions, followed by immediate spotting of 0.1 μL of CHCA matrix over the reduction mixture. It was allowed to dry and the MS analysis was performed.

Chapter 3 Results and Discussion

3.1 Reduction of Disulfide containing Proteins

Proteins with disulfide bonds have to be first reduced prior to digestion to achieve effective proteolysis for MS peptide fingerprinting. Conventional approach performs these reactions in-solution in small vials [56]. Such homogenous reactions suffer from diffusion-limited mass transfer and also result in sample loss. Also, the handling of sub-microliter volumes with pipettors and vials are very difficult. Hence, my work investigates the reduction and digestion of proteins using the analytical technique of capillary electrophoresis. By using CE, sample volume is minimized to nano-liter range, which will allow for easy miniaturization of the reactions and hence mimics a lab on a chip version.

Prior to performing in-capillary reduction, the conventional in-vial reduction was done. It was followed by the optimization of the reduction time and the temperature at which reduction was performed. After the successful separation of in-vial reduced protein in CE, the reaction was investigated inside the capillary. The CE results obtained from the in-capillary reduction confirmed that the reduction was successfully done; which was further confirmed by MS. Following this, digestion of proteins was also performed inside the capillary.

Previously, Abel from our group had attempted the reduction and digestion of disulfide containing proteins in CE [57]. His work was based on an in-capillary protein enrichment technique developed in our group by Nesbitt. In his method, the capillary was filled with two buffers, an acid at the anodic end of capillary, and a base at the cathodic end. As a result of voltage application, the enrichment of BSA took place at the interface of the discontinuous buffers. Following the enrichment, DTT and trypsin were brought in sequentially to reduce and digest BSA.

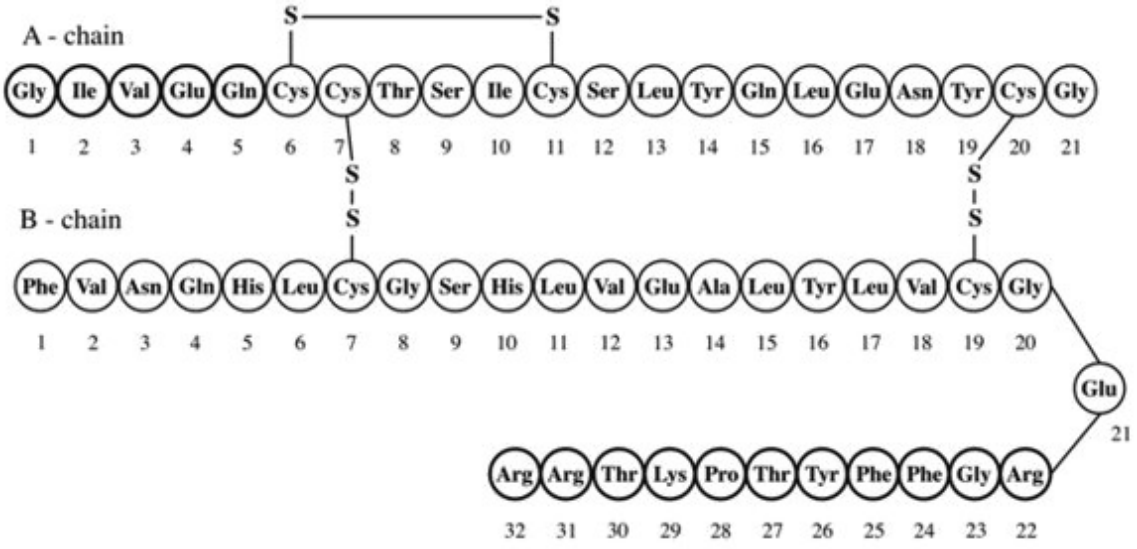
However, there were a number of limitations in the method mentioned above. Owing to the relatively slow enrichment, EOF had to be slowed down otherwise it would push the capillary content out prior to completion of enrichment. To suppress the EOF, CPA coating was selected. But the coating procedure was long, and the reproducibility was low. Precipitation of BSA at the discontinuous buffer interface was another problem faced by this method. Also, the discontinuous buffer used was good for enrichment, but not for subsequent separation. Hence, DTT could not be removed, and the separation of digested peptides by CZE was not easily achievable.

In order to overcome the limitations of Abel's work, my work investigates the reduction and digestion of disulfide proteins using zone passing CE technique. Specifically, my approach is different from the previous work in the following way:

- Conventional single buffer system was used instead of discontinuous buffers.
- Proteins were not enriched.
- Under voltage application, separation of reagents occurred based on differential electrophoretic mobilities.
- Introduction of DTT, and later trypsin, was by zone passing CE technique (discussed in Chapter 1); likewise their subsequent removal due to differential electrophoretic mobilities.
- Capillary was not coated as EOF suppression was not necessary. Alternative buffers reported in literature were explored for suppression of protein (BSA, insulin) adsorption on capillary wall.

First step illustrated in my work was the reduction of proteins. For this, proteins with disulfide bonds that can be detected in CE were required. A large number of disulfide proteins are available commercially. However, a protein that can be easily detected in CE after reducing it was necessary. In our search for such a disulfide protein, insulin presented to be ideal for performing reduction in CE. Insulin (figure 3-1) is a relatively small protein with a mass of 5733 Da. It has two sub chains referred to as insulin chain A and insulin chain B and has 3 disulfide bonds. Among the three disulfide bonds, two are inter-chain disulfide bonds which connect the two sub chains and the third one is intra

chain found in chain A [58]. Due to the presence of two inter chain disulfide bonds, reduction would yield two products of different masses. Owing to the difference in size and charge of the two chains, they can be easily detected in CE. These remarkable features encouraged us to select insulin as a model protein to perform reduction reactions. Figure 3-2 represents the flowchart for the reduction reactions performed.



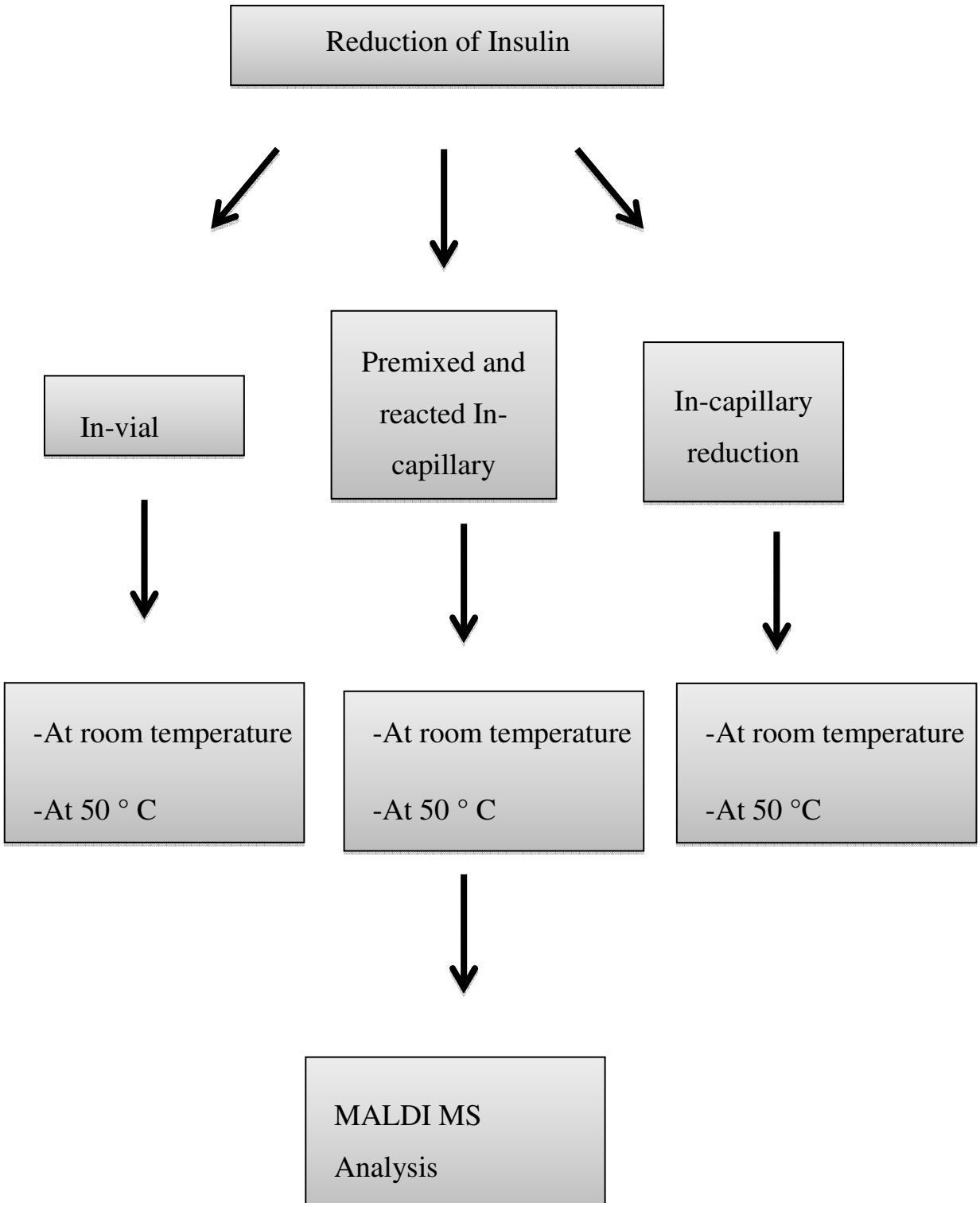


Figure 3-2 A flowchart that describes the general sequence of reduction experiments that were performed in this work.

3.1.1 Selection of Buffer for Reduction Reaction

Optimization of the conditions used in CE was the first step done prior to performing the reduction reaction. Clearly, insulin and the reduced insulin have to be detected in CE. In CE, pH and concentration of the running buffer plays a significant role in separation. Ideally, the buffer selected must be able to separate the reduction products, should result in improved peak shape, avoid the adsorption of protein and most importantly the protein should remain stable.

At first, CE run for insulin was done with the buffers like tris and acetate. Insulin peak was not detected using these buffers. This can be explained either due to the adsorption of insulin onto the capillary wall or due to the inefficient separation. In order to prevent the adsorption of protein to the silica capillary, experiments were also repeated with coated capillaries [59]. There is a wide range of permanent and semi-permanent coatings being developed for CE. Permanent capillary coatings result in covalent bonding of polymers to the capillary surface. Though they have demonstrated good stability and reproducibility in migration time, preparation of these coatings are laborious, time consuming and they are stable only at certain pH range [60]. While, semi-permanent coatings include a wide variety of neutral and ionic polymers that can be prepared fast by rinsing the capillary with the coating reagent. These coatings adsorb quite strongly

onto the capillary wall and don't have to be present in the running buffer. Among the semi-permanent coatings, one of the most commonly used is a phospholipid bilayer coating known as dilauroyl phophatidylcholine (DLPC) [61]. Lucy and co-workers were among the first who investigated phospholipids as coatings for CE. DLPC consists of a zwitterionic head group of phosphate and choline, and two C_{12} fatty acid chains as hydrophobic tails. Due to the zwitterionic nature of the bilayer surface, a highly suppressed near-zero EOF is obtained and results in separation of acidic and basic proteins.

CE run for insulin was performed with DLPC coated capillaries, however the insulin peak was not detected. After that, phosphate buffer was chosen referring to a work done by Hsin-Hua Yeh *et* al, in their work they used phosphate buffer for the simultaneous determination of regular insulin and insulin aspart by CZE [62]. Phosphate buffer has many significant features compared to tris and acetate such as providing better separation selectivity, peak shape, resolution and sensitivity of the analytes. Based on these, phosphate buffer was chosen for performing reduction reaction. 80 mM phosphate buffer at pH 7.5 was chosen as the optimized buffer condition for reduction.

Using phosphate buffer as the running buffer, CE run for insulin was done and insulin peak was detected at 200 nm wavelength. Figure 3-3 shows the electropherogram obtained from CE run for unreduced insulin.

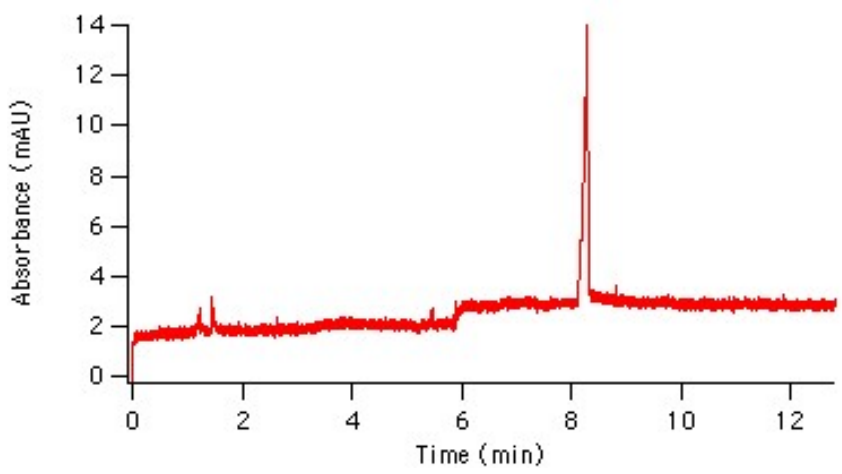


Figure 3-3 CE electropherogram of unreduced insulin at 200 nm wavelength.

Following the successful analysis of insulin in CE using phosphate buffer, further reduction reactions were done. For reducing insulin, the most commonly used reducing agent DTT was used. DTT at different concentrations were tested. No significant difference was observed in the results by using 1 mM or 50 mM DTT. Hence, 5 mM DTT was chosen as the optimal concentration for reduction.

3.1.2 Identification of the Reduced Insulin Peaks from CE Data

Before attempting in-capillary reduction, conventional in-vial reduction was done to confirm that reduction products could be separated by CE. The mixing of insulin and DTT as well as the reaction took place inside the vial. Reduction was also allowed to proceed at different time periods including overnight at room temperature. CE analysis of the reduced insulin at different time periods was done and the datas were compared. As expected the CE electropherogram showed 3 peaks, but it wasn't easy to assign the peaks just by looking at it. Figure 3-4 shows the CE electropherogram of reduced insulin after 1 hour (without assigning the peaks).

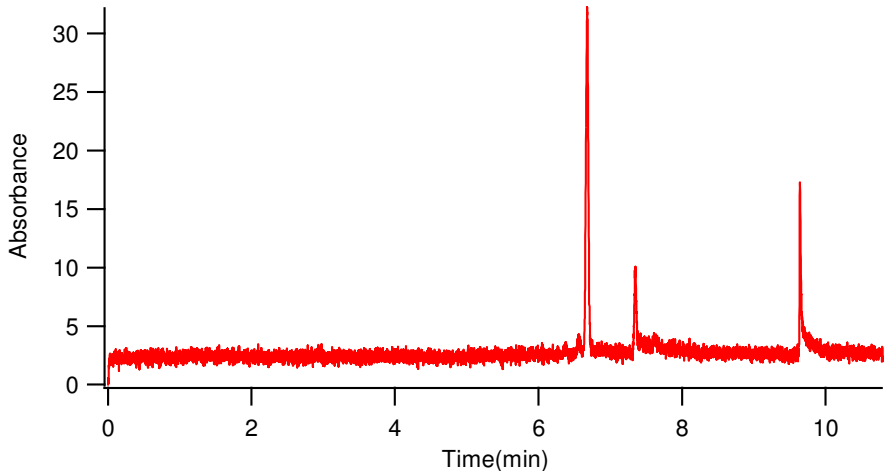


Figure 3-4 CE electropherogram of reduced insulin after 1 hour.

Since standards were not used in these experiments; to identify the peaks obtained from CE experiments a thorough understanding of how insulin, DTT and the reduction products behaved inside the capillary under the optimized conditions was necessary. Inside the capillary; insulin, DTT and reduced insulin migrated at different velocities under voltage application. The migration time depends on the charge and size of the analyte [63]. The charge of the protein depends on the pH of the buffer used for the run. Insulin is an acidic protein with a p*I* of 5.5. The pH at which the protein bears zero net charge is referred to as the p*I* of that protein. In phosphate buffer at pH 7.5, insulin carries a negative charge. While, insulin chain A with a p*I* of 3.79 is highly negative than insulin and insulin chain B. Insulin chain B with a p*I* of 6.90 is less negative than the insulin. Based on the charge and the size of the insulin and the reduction products, it can be predicted that insulin chain B will migrate faster than intact insulin and chain A. Thus, the order of migration should be insulin chain B, insulin and insulin chain A. DTT with two pKa values of 9.2 and 10.1 also bears a negative charge at pH 7.5 buffer. Considering the small size of DTT, it should migrate faster than insulin and the reduction products. Figure 3-5 shows the CE electropherogram after assigning the peaks based on the charge and the size of the reduced insulin. The pI, molecular mass, charge, pKa, of protein and DTT are listed in table 3-1 and 3-2. The electrophoretic mobility and electroosmotic mobility of DTT and insulin are listed in table 3-3.

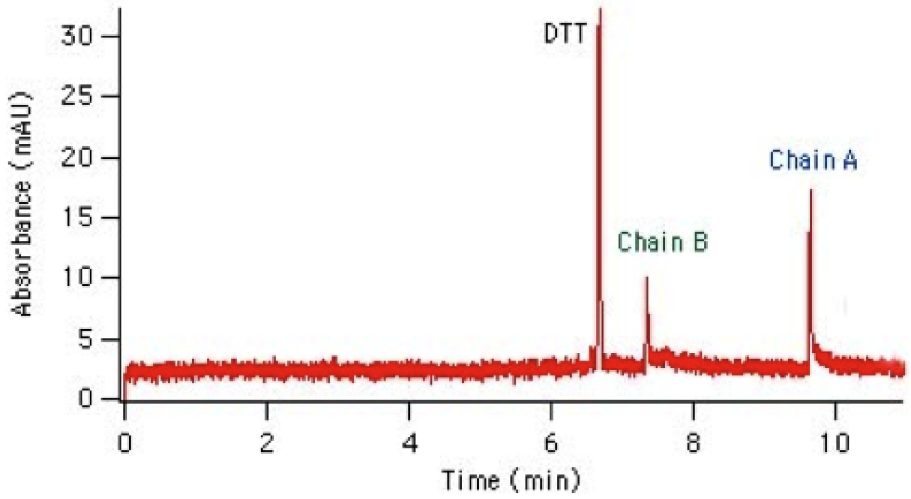


Figure 3-5 CE electropherogram after assigning the peaks based on the charge and the size of the reduced insulin.

Table 3-1 p*I*, molecular mass and charge at pH 7.5 of insulin and chains calculated.

Protein	**p*I***	**Molecular Mass**	**Charge at pH 7.5**
Insulin	5.5	5733.49	-1.8
Insulin Chain A	3.79	2339.65	-2.2
Insulin Chain B	6.90	3399.93	-0.3

Table 3-2 pKa and molecular mass of DTT.

Analyte	pKa	Molecular Mass
DTT	9.2 and 10.1	154.25gmol^{-1}

Table 3-3 Electrophoretic mobility and electroosmotic mobility of DTT and insulin calculated.

Analyte	Electrophoretic mobility (μ_e)	Electroosmotic mobility (μ_{EOF})
DTT	-0.1×10^{-4}	3.16×10^{-4}
Insulin	-0.96×10^{-4}	3.16×10^{-4}
Chain A	-0.90×10^{-4}	3.16×10^{-4}
Chain B	-0.20×10^{-4}	3.16×10^{-4}

While comparing the CE results for reduction performed at different time periods, it was clear that the reduction was completed in 1 hour at room temperature. For

comparison, Figure 3-6 shows the CE electropherogram for insulin reduced for 15 minutes at room temperature. From the graph, it can be found that insulin was not fully reduced as the insulin peak was detected after 15 minutes of reduction at room temperature.

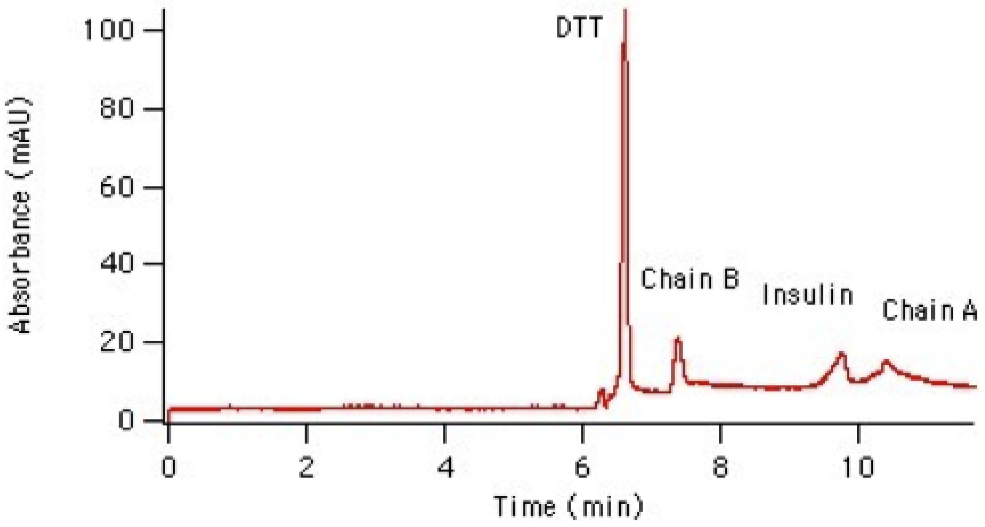


Figure 3-6 CE electropherogram for in-vial insulin reduction performed at room temperature for 15 minutes.

However, the CE electropherogram for the overnight reduction reaction was exactly the same as that of reduction performed for 1 hour (figure 3-5). In order to further confirm the reduction products, off-line CE-MALDI MS [64] analysis was performed by

spotting 0.73 µL volume of reduced insulin mixed with matrix on a MALDI plate. The spot was allowed to dry and MS analysis was performed for insulin reduced at different time periods.

MALDI mass spectrum for the unreduced insulin was also recorded; figure 3-7 shows the mass spectrum for the unreduced insulin sample. Peak at 5733 represents the unreduced insulin. MS analysis (figure 3-8) further confirmed that the reduction of insulin at room temperature took 1 hour for the reaction to go to completion. In mass spectrometry the analytes are identified based on their masses. Insulin chain A has a mass of 2339.65 and chain B has a mass of 3399.93.

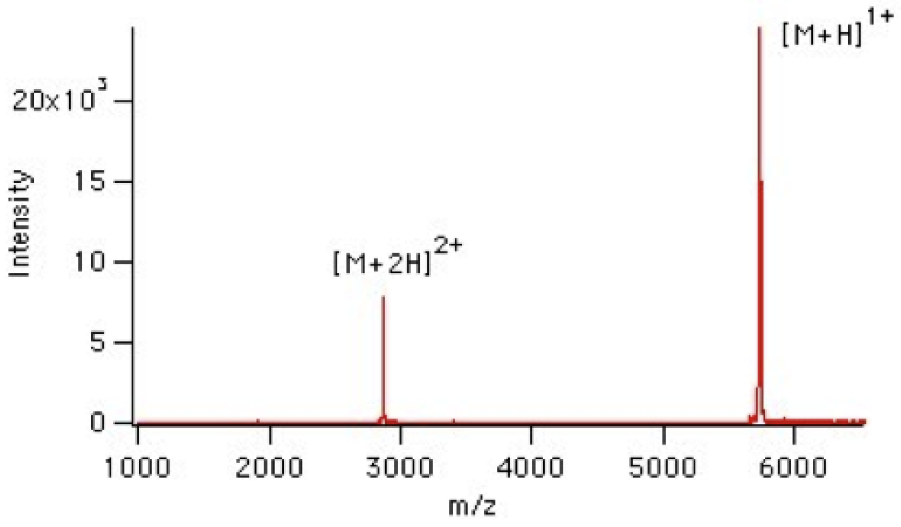


Figure 3-7 MALDI mass spectrum for unreduced insulin.

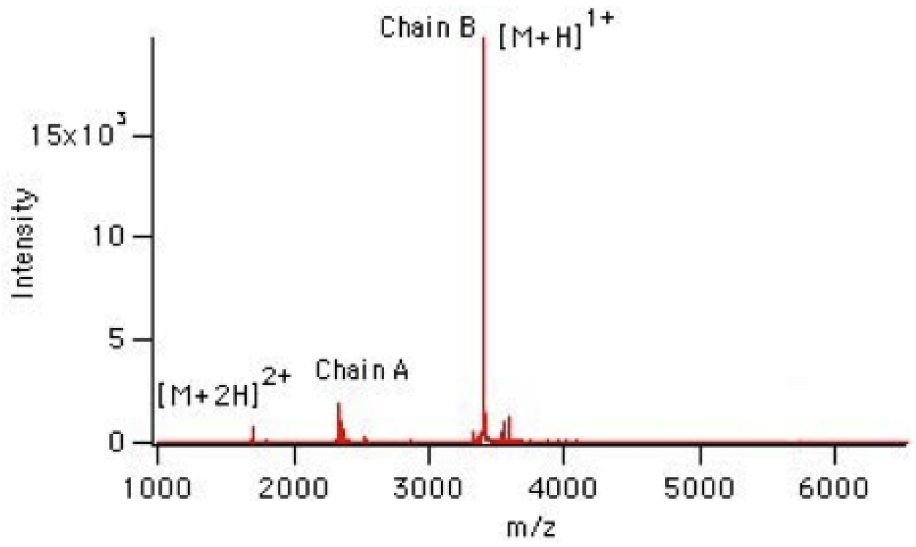


Figure 3-8 MALDI Mass spectrum of insulin sample after in-vial reduction performed at room temperature for 1 hour.

Hence by performing in-vial reduction and by analyzing the reduction products in CE confirmed that reduced insulin peaks can be detected in CE. Peaks detected were assigned based on their charge and size. Optimization of the time needed for the reduction reaction was also done, CE and MS data confirmed that it took 1 hour for the reduction to go to completion at room temperature.

3.2 Optimization of Temperature for Reduction reaction

Reduction of insulin when performed at room temperature took one hour to complete. Further investigation was needed in order to speed up the reduction reaction. A lot of research had been done regarding the optimal conditions required for the reduction of disulfide bonds. One paper reported that heating the proteins which can tolerate high temperature caused the reduction reaction to happen faster [65]. Considering this, both in-vial and in-capillary reduction reactions were carried out at 50 °C.

3.2.1 In-Vial Reduction Reaction at elevated temperature

After mixing insulin and DTT in-vial, the reagents were incubated at 50°C and CE run was performed at different time periods. The results from the runs performed at different time periods showed that when heated, it only took 15 minutes for the reduction reaction to go to completion. Figure 3-9 shows the result from CE run for in-vial reduction when heated for 15 minutes.

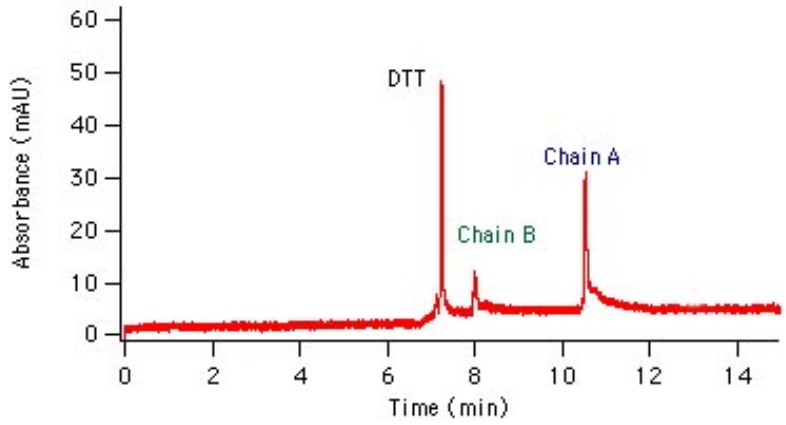


Figure 3-9 CE electropherogram of insulin sample after in-vial reduction reaction when heated for 15 minutes at 50°C.

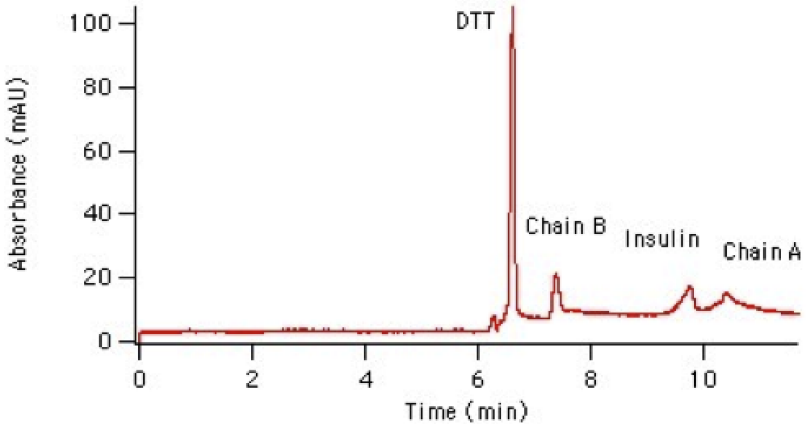


Figure 3-10 CE electropherogram for in-vial insulin reduction performed at room temperature for 15 minutes.

For comparison, figure 3-10 shows the electropherogram when the reduction reaction was performed in vial at room temperature for 15 minutes. From the electropherogram, it was clear that reduction was not completed after15 minutes when kept at room temperature as the insulin peak was still visible. While, when the sample was heated at 50°C, the reduction went to completion in 15 minutes. MS analysis further confirmed that the reduction was completed in 15 minutes when the reagents were heated in-vial. MALDI Mass spectrum for this was same as that of figure 3-8.

3.3 Premixed and Allowed to React In-Capillary at Room Temperature

Prior to performing in-capillary reduction, next approach was to premix the insulin and DTT in a vial and allow it to react inside the capillary. Since insulin and DTT was premixed in the vial, only the reaction happens inside the capillary. This step was done to monitor if the reduction reaction will work inside the capillary.

After mixing insulin and DTT in a vial, a small plug (1 mm, calculated using the equation 3.2) of the mixture was injected into the capillary from the inlet by applying

20 mbar pressure. It was followed by the injection of a plug of buffer. The reagents were allowed to be in the capillary for 1 hour at room temperature. After the incubation, CE run was performed by the application of voltage. As expected the peaks for reduced insulin was detected in CE and results were comparable to the in-vial reduction. Figure 3-11 shows the CE data obtained after 1 hour. Followed by this, MS analysis was done to further confirm the reduction products. This confirms that the reduction reaction was successfully done inside the capillary.

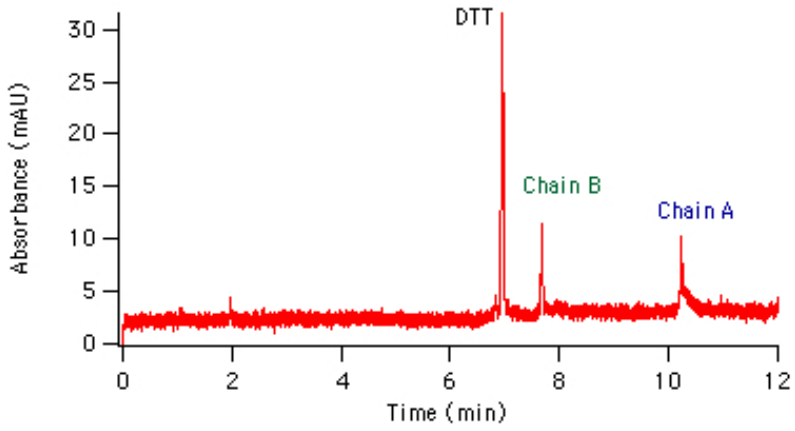


Figure 3-11 CE electropherogram for in-vial premixed to in-capillary at room temperature run performed after 1 hour.

3.3.1 Premixed and Allowed to React In-Capillary using Heated Buffers

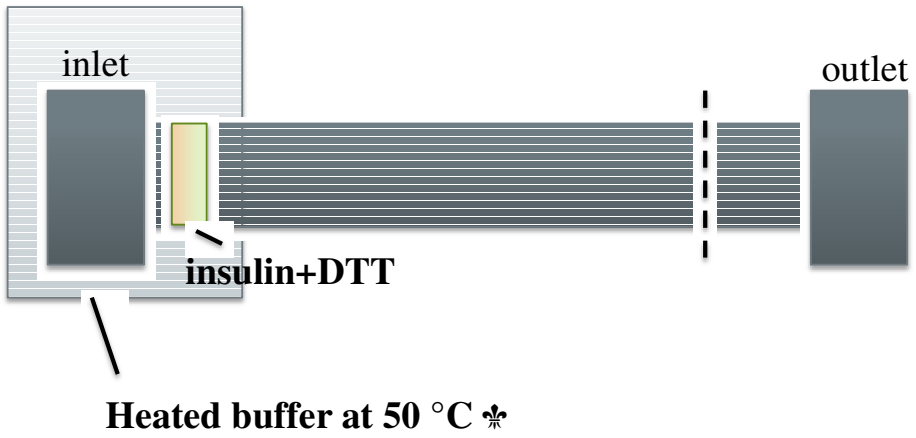


Figure 3-12 The set up for insulin and DTT reacting inside the capillary using heated (50° C) buffer at the inlet.

As the reduction was successfully performed inside the capillary at room temperature, next attempt was to perform the reaction at elevated temperature. Since, the reduction mixture was injected inside the capillary and was positioned near to the inlet, the only way to heat it was by using heated buffers. It had been already confirmed from the in-vial reduction reaction that when the reaction was done at 50 °C, the reduction was completed in 15 minutes. Based on that, buffers taken in a vial was heated in a water

bath till it reached 50° C. Every 5 minutes the buffer had to be replaced with heated ones to keep the temperature constant. Figure 3-12 illustrates the set up for this experiment. Insulin and DTT were premixed in a vial and injected into the capillary from the inlet followed by a small plug of buffer. The capillary inlet containing the insulin and DTT plug was then placed in a heated buffer at 50° C. A positive polarity voltage was then applied after 15 minutes. Figure 3-13 shows the CE data for the reduction reaction performed using heated buffer for 15 minutes.

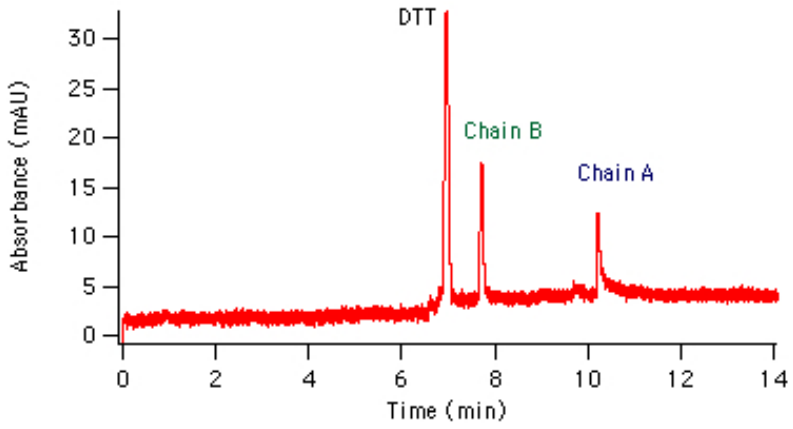


Figure 3-13 CE electropherogram for in-vial to in-capillary reduction reaction using heated buffer after 15 minutes.

So far, two experiments were conducted: insulin and DTT was mixed and reacted in-vial, premixed in-vial and reacted in-capillary. Comparing the CE and MS results of both the experiments confirmed that the reduction reaction was successfully performed inside the capillary at room temperature and at 50°. This encouraged us to perform both the mixing and reaction inside the capillary.

3.4 Mixing and Reduction inside the Capillary

In this experiment, the mixing of insulin and DTT as well as the reduction reaction was performed inside the capillary. For performing in-capillary reduction reaction, insulin and DTT were injected separately from the inlet followed by a small plug of buffer (figure 3-12). The mixing of insulin and DTT was achieved by the application of voltage. After the mixing, the reduction reaction was allowed to proceed in the capillary for 1 hour at room temperature. The reagents were injected into the capillary based on their electrophoretic mobilities and charge. Due to the presence of relative fast EOF, all the analytes migrated with the EOF and towards the cathode. The electrophoretic mobility (μ_e) of insulin and DTT were calculated using the equation 1.2. The charge of the analyte inside the capillary depends on the pH of the buffer. Since phosphate buffer at pH 7.5 was used for all the CE runs, at this pH insulin with a p*I* of 5.5 carries a negative charge. While, DTT was also negatively charged at this pH, considering the

small size of the DTT, it will migrate faster than insulin during voltage application. Prior to injecting insulin and DTT in the capillary, the length of injection of insulin and DTT and the time needed to apply voltage to result in effective mixing were calculated. At first, velocity of insulin and DTT was calculated using the equation 3.1.

$$\text{Velocity} = \frac{\text{effective length}}{\text{time}} \quad \text{(Eq. 3-1)}$$

Difference in velocity of insulin and DTT was then calculated. This was followed by calculating the length of injection by using the equation 3.2 [66].

$$l_{inj} = \frac{t_{inj} \times \Delta P \times r^2}{8 \times \eta \times L} \quad \text{(Eq. 3-2)}$$

where, t_{inj} is the time of injection, ΔP is the pressure difference across the capillary, r is the radius of the capillary, η is the buffer viscosity, L is the total capillary length. For calculating the time required for voltage application for effective mixing, the distance between the centers of the insulin and DTT plugs were calculated. Dividing this with the velocity difference of insulin and DTT gave the time required for voltage injection.

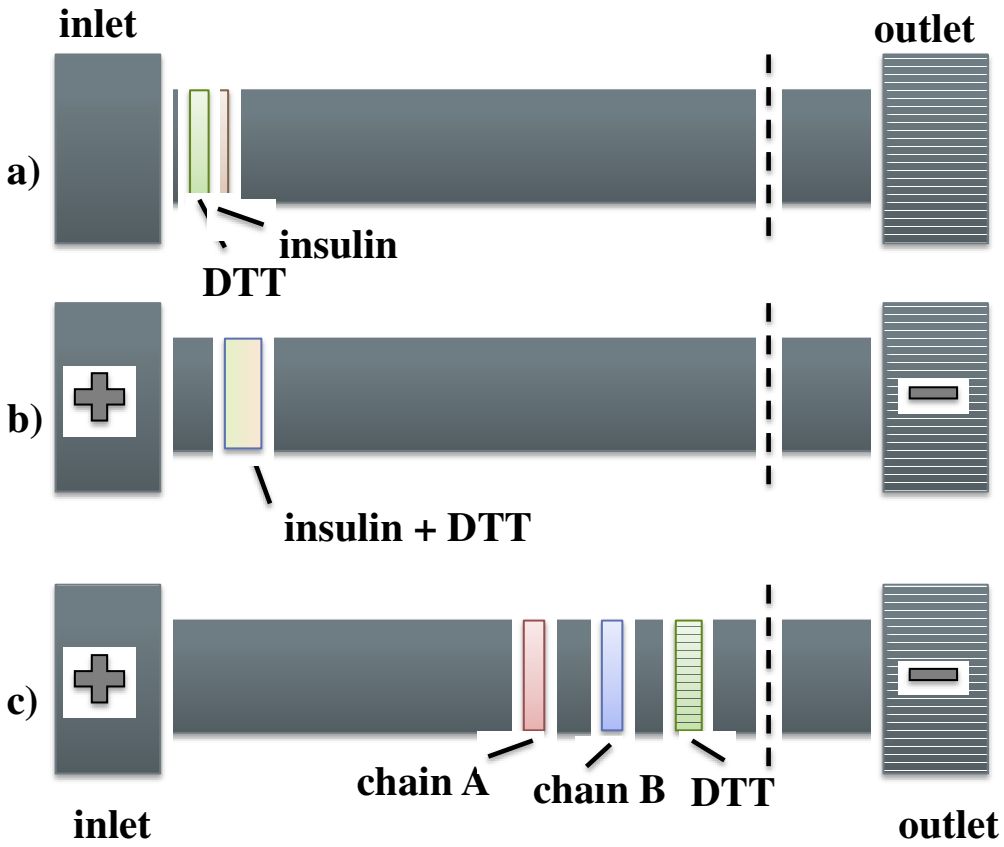


Figure 3-14 Schematic of in-capillary reduction reaction in CE.

a) Injection of insulin and DTT using pressure b) mixing of insulin and DTT by voltage application and allowed to react c) after reduction, separation of insulin chain A, chain B and DTT using voltage.

Based on the above calculations, 1 mm plug of insulin was injected first which was followed by 2 mm plug of DTT. After that a small plug of buffer was also injected. All these were injected from the inlet using a positive pressure of 20 mbar. Then a positive polarity voltage was applied for 7 sec for effective mixing of DTT and insulin. The plug of DTT moved towards the plug of insulin and the mixing will take place. Figure 3-14 shows the schematic of how in-capillary reduction reaction was performed. The reduction reaction was then allowed to proceed at room temperature for 1 hour. Figure 3-15 shows the result obtained from the CE run when reduction reaction was performed in-capillary at room temperature for 1 hour.

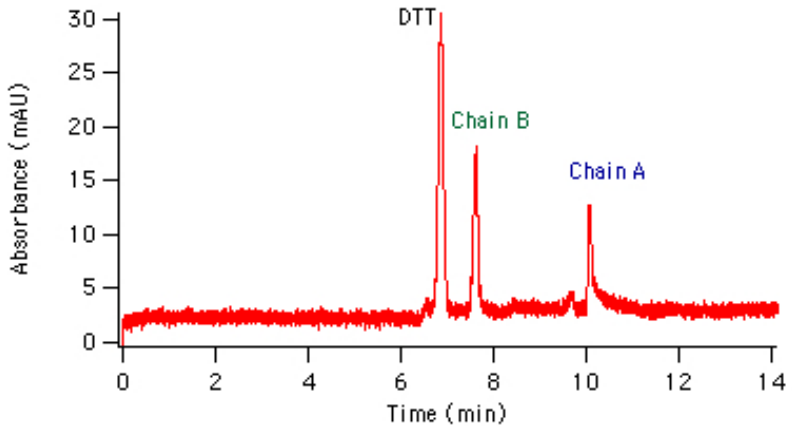


Figure 3-15 CE electropherogram for in-capillary reduction reaction performed at room temperature for 1 hour.

From CE data, it was clear that reduction worked when it was performed in-capillary. No further optimization of the voltage to mix insulin and DTT was done as the voltage calculated using the equation has resulted in efficient mixing of insulin and DTT.

In order to speed up the reaction and decrease the long reduction time, in-capillary reaction was also performed by replacing the buffer at the inlet with heated buffer at 50°C while the reaction was going on. The set up was quite similar to figure 3-14. By performing this, the reaction time was lowered to 15 minutes and the reduction went to completion. Figure 3-16 shows the CE data for in-capillary reaction performed using heated buffers.

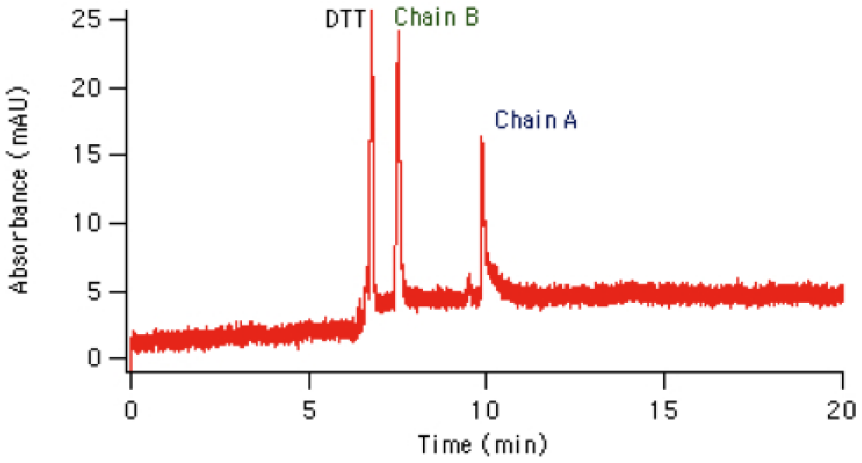


Figure 3-16 CE electropherogram for in-capillary reduction reaction performed using heated buffer after 15 minutes.

A way of miniaturizing the reduction of disulfide containing proteins by performing the reaction inside the capillary in CE was presented. Three main experiments were performed, in-vial reduction, premixed in-vial and reacted in-capillary and mixing and reaction inside the capillary. Optimization of the temperature needed for reduction was also done, 50 °C was chosen as the optimal temperature. CE data of all these experiments were compared, which led to the conclusion that reduction was successfully performed inside the capillary. MS analysis further confirmed the CE results. Migration time of each run and the relative standard deviation of all the analytes were calculated. As is shown in table 3-4, the reduction reaction is reproducible with respect to the migration time when performed inside the capillary.

Table 3-4 Migration time and Relative standard deviation % for all the analytes.

Experiment	Analyte	Mean Migration Time (min)	Relative Standard deviation %
In-vial not heated	DTT	6.76	1.1
	Chain A	9.87	2.2
	Chain B	7.49	1.7
In-vial heated	DTT	7.25	2.0
	Chain A	10.78	3.6
	Chain B	8.08	1.5
In-capillary not heated	DTT	6.90	0.7
	Chain A	10.12	1.1
	Chain B	7.68	0.8
In-capillary heated	DTT	6.78	0.7
	Chain A	9.96	1.1
	Chain B	7.51	0.8

3.5 Digestion of BSA

Since a protocol for reducing the protein inside the capillary was successfully established. Our next approach was so to perform the digestion of the reduced proteins inside the capillary. Insulin was chosen for reduction reaction as it was a small protein and the presence of two inter-chain disulfide bonds resulted in the formation of two separate chains due to reduction. As a result reduced insulin was easily detected in CE. However, it is not a good choice for digestion as it contained only a few cleavage sites to perform the digestion and is a less suitable standard for MS. Instead, Bovine serum albumin (BSA) with 17 internal disulfide bonds and many cleavage sites for digestion presented an excellent choice for performing digestion reaction. Table 3-5 shows the mass, pI and composition of BSA. Trypsin was chosen as the digestive enzyme, which is the most widely used enzyme. Trypsin cleaves at the carboxyl side of the amino acids lysine and arginine. Figure 3-17 shows the sequence of BSA, all cysteines are shown in red color. Figure 3-18 shows the sequence of BSA with lysine and arginine shown in red color.

Table 3-5 Composition, Mass and pI of BSA

Elemental composition	C2932 H4614 N780 O898 S39
Monoistopic Mass	66389.86 (25-607)
Average Mass	66432.96 (25-607)
pI	5.60

P02769|ALBU_BOVIN Serum albumin - Bos taurus (Bovine)

MKWVTFISLLLLFSSAYSRGVFRRDTHKSEIAHRFKDLGEEHFKGLVLIAFSQYL
QQCPFDEHVKLVNELTEFAKTCVADESHAGCEKSLHTLFGDELCKVASLRETYG
DMADCCEKQEPERNECFLSHKDDSPDLPKLKPDPNTLCDEFKADEKKFWGKYL
YEIARRHPYFYAPELLYYANKYNGVFQECCQAEDKGACLLPKIETMREKVLASS
ARQRLRCASIQKFGERALKAWSVARLSQKFPKAEFVEVTKLVTDLTKVHKECCH
GDLLECADDRADLAKYICDNQDTISSKLKECCDKPLLEKSHCIAEVEKDAIPENLP
PLTADFAEDKDVCKNYQEAKDAFLGSFLYEYSRRHPEYAVSVLLRLAKEYEATL
EECCAKDDPHACYSTVFDKLKHLVDEPQNLIKQNCDQFEKLGEYGFQNALIVRY
TRKVPQVSTPTLVEVSRSLGKVGTRCCTKPESERMPCTEDYLSLILNRLCVLHEK
TPVSEKVTKCCTESLVNRRPCFSALTPDETYVPKAFDEKLFTFHADICTLPDTEKQ
IKKQTALVELLKHKPKATEEQLKTVMENFVAFVDKCCAADDKEACFAVEGPKL
VVSTQTALA

Figure 3-17 Sequence of BSA with cysteines shown in red color.

MKWVTFISLLLLFSSAYSRGVFRRDTHKSEIAHRFKDLGEEHFKGLVLIAFSQYL
QQCPFDEHVKLVNELTEFAKTCVADESHAGCEKSLHTLFGDELCKVASLRETYG
DMADCCEKQEPERNECFLSHKDDSPDLPKLKPDPNTLCDEFKADEKKFWGKYL
YEIARRHPYFYAPELLYYANKYNGVFQECCQAEDKGACLLPKIETMREKVLASS
ARQRLRCASIQKFGERALKAWSVARLSQKFPKAEFVEVTKLVTDLTKVHKECCH
GDLLECADDRADLAKYICDNQDTISSKLKECCDKPLLEKSHCIAEVEKDAIPENLP
PLTADFAEDKDVCKNYQEAKDAFLGSFLYEYSRRHPEYAVSVLLRLAKEYEATL
EECCAKDDPHACYSTVFDKLKHLVDEPQNLIKQNCDQFEKLGEYGFQNALIVRY
TRKVPQVSTPTLVEVSRSLGKVGTRCCTKPESERMPCTEDYLSLILNRLCVLHEK
TPVSEKVTKCCTESLVNRRPCFSALTPDETYVPKAFDEKLFTFHADICTLPDTEKQ
IKKQTALVELLKHKPKATEEQLKTVMENFVAFVDKCCAADDKEACFAVEGPKL
VVSTQTALA

Figure 3-18 BSA sequence with lysine and arginine shown in red color. Trypsin cleaves at the carboxyl side of the amino acids lysine and arginine.

The flow chart shown below figure 3-19 shows the various steps performed for in-capillary reduction and digestion of BSA.

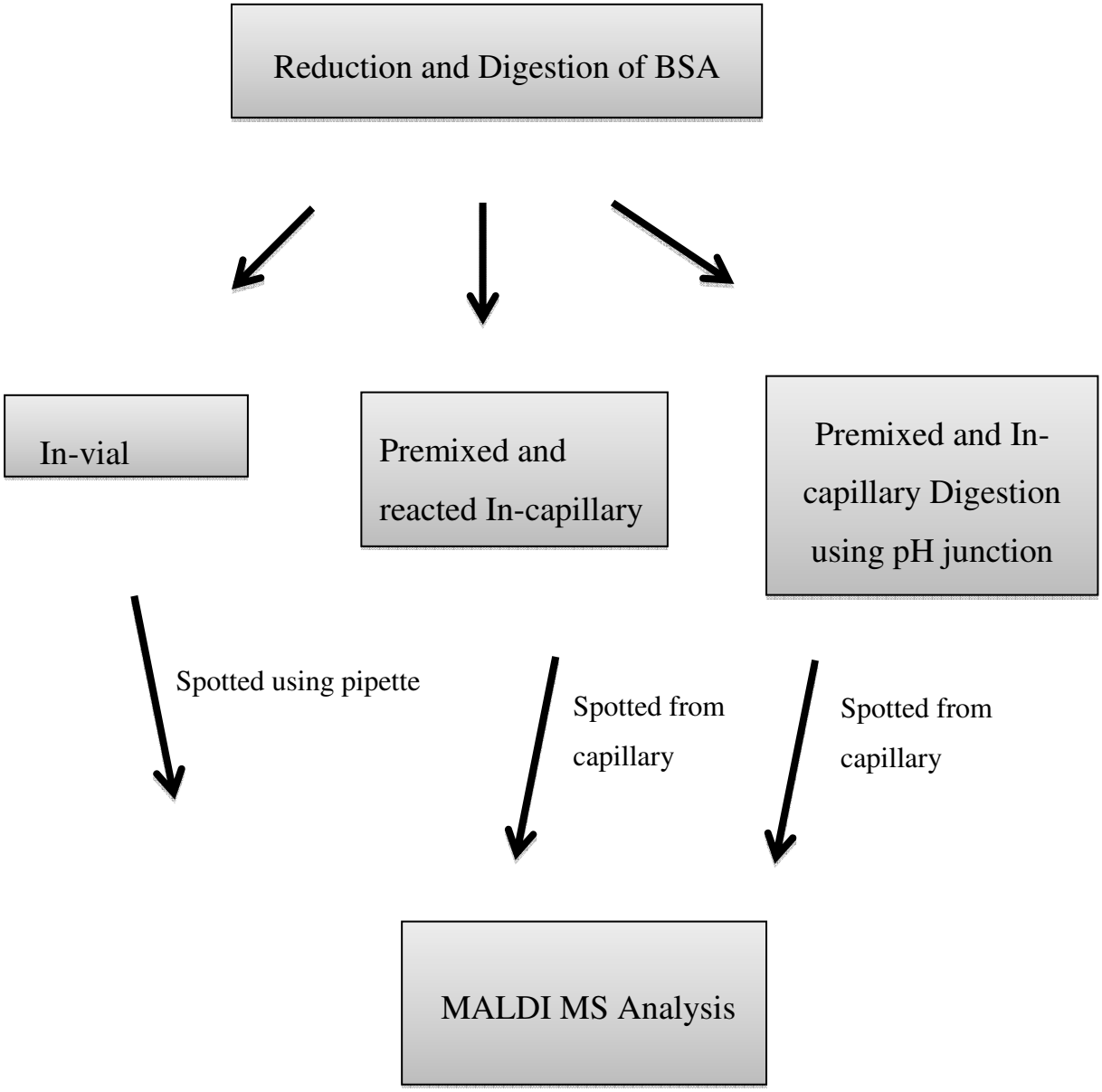


Figure 3-19 A flowchart that describes the general sequence of in-capillary digestion experiments that were performed in this work.

3.5.1 In-Vial Digestion

Since reduction of insulin was successfully performed inside the capillary, next attempt was to design a method to perform both reduction and digestion of BSA inside the capillary. Before attempting in-capillary reaction, digestion was first performed in-vial. Since CE cannot be employed for separating and detecting the peptides, a system to validate the extent of digestion was necessary. Mass Spectrometry that can detect the peptides based on their mass to charge ratio was utilized to identify the peptides [67].

First, the conventional in-vial digestion was performed in which the mixing and the reaction took place in the vial. The digested BSA was then analyzed by MS where the peptides can be detected based on their mass to charge ratio. The mass spectrum provided the information about the extent of digestion and the peptides that are formed.

For in-vial digestion, BSA with initial concentration 1 mg/mL was combined with DTT (5 mM) and incubated at 50 °C for 15 minutes. After reduction, trypsin of concentration 0.1 mg/mL was added to the vial keeping the substrate per trypsin molecule ratio as 20: 1 [68, 69]. It was then allowed to react for 3 hours at room temperature.

Digested BSA was combined with CHCA matrix and 0.73 μL was spotted on the MALDI plate and analyzed by MS.

The peptides detected from in-vial digestion are listed in table 3-6. A total of 25 peptides were detected which resulted in a sequence coverage of 80 %. Sequence coverage was obtained using Mascot search engine.

Table 3-6 Peptides detected by MALDI MS from in-vial digestion of BSA.

Theoretical MM $[M+H]^+$	Observed MM $[M+H]^+$	Peptide Sequence	Calculated pI
544.33	545.35	K.VASLR.E	9.72
648.33	649.34	R.CASIQK.F	8.22
688.37	689.38	K.AWSVAR.L	9.79
711.36	712.38	K.SEIAHR.F	6.47
840.45	841.47	R.LCVLHEK.T	6.74
926.48	927.49	K.YLYEIAR.R	6.00
1023.44	1024.46	K.CCTESLVNR.R	5.99
1051.44	1052.45	R.CCTKPESER.M	6.13
1141.70	1142.71	K.KQTALVELLK.H	8.59
1162.62	1163.63	K.LVNELTEFAK.T	4.53
1192.59	1193.61	R.DTHKSEIAHR.F	6.92
1248.61	1249.62	R.FKDLGEEHFK.G	5.45
1282.70	1283.71	R.HPEYAVSVLLR.L	6.75
1304.70	1305.72	K.HLVDEPQNLIK.Q	5.32
1348.53	1349.55	K.TCVADESHAGCEK.S	4.65
1361.66	1362.67	K.SLHTLFGDELCK.V	5.30
1478.78	1479.80	K.LGEYGFQNALIVR.Y	6.00
1566.73	1567.74	K.DAFLGSFLYEYSR.R	4.37
1638.93	1639.94	R.KVPQVSTPTLVEVSR.S	8.75
1666.80	1667.82	R.MPCTEDYLSLILNR.L	4.37
1822.89	1823.91	R.RPCFSALTPDETYVPK.A	6.06
1849.91	1850.92	R.SLGKVGTRCCTKPESER.M	8.88
1961.94	1962.97	K.LKPDPNTLCDEFKADEK.K	4.44
2075.87	2076.89	K.ECCHGDLLECADDRADLAK.Y	4.23
2315.03	2316.07	K.YNGVFQECCQAEDKGACLLPK.I	4.68

A set of 3 experiments were done, the sequence coverage was in the 75 to 80 % range, which was found to be reasonable. Following this, another experiment was attempted in

which the mixing was performed in the vial and reaction was allowed to happen inside the capillary.

3.5.2 Premixed In-Vial and reacted In-Capillary

Prior to performing in-capillary digestion (where mixing and the reaction took place inside the capillary), it was important to confirm that reaction actually worked inside the capillary. For this, the mixing of the analyte and reagents were done in a vial and it was then injected into the capillary for the reaction to happen. In this method, mixing of the reagents was done in-vial as mentioned in section 3.4.1 and a 10 cm plug was injected into the capillary from the outlet by applying -50 mbar pressure for 120 seconds followed by basic buffer plug. BSA mixed with trypsin was incubated inside the capillary for 3 hours. After 3 hours, digested BSA was then spotted from the capillary. In order to deposit the sample on the MALDI plate, the capillary was re-positioned and the capillary outlet was exposed outside of the instrument (figure 3-20). To elute the sample, constant pressure of 50 mbar was applied at the capillary inlet for 120 seconds. 5 spots each of 200 nL sample was spotted on the MALDI plate and analyzed by MS.

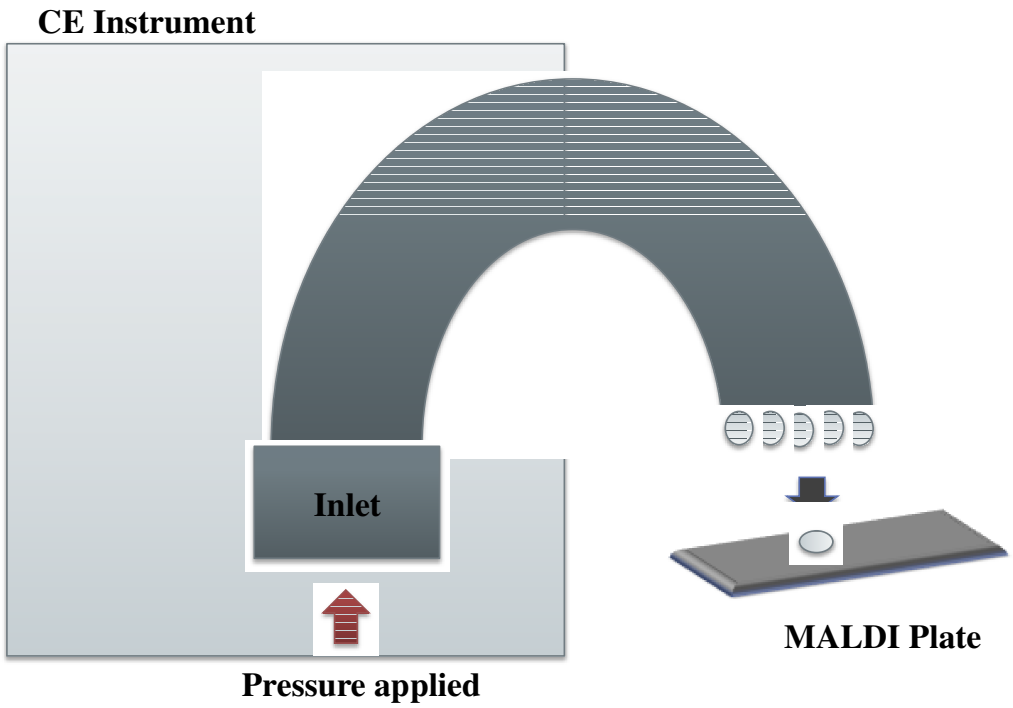


Figure 3-20 Schematic diagram of sample spotting from the capillary onto the MALDI plate using pressure.

The peptides detected are listed on the Table 3-7. Total 19 peptides were detected and resulted in a sequence coverage of 71 %. The lower sequence coverage for this method compared to in-vial digest may be attributed either due to the adsorption of some peptides onto the capillary wall or due to miss cleavage while digestion. However, the results proved that digestion worked inside the capillary; and this encouraged us to perform the digestion inside the capillary.

Table 3-7 Peptides detected by MALDI MS from premixed and reacted in-capillary digestion of BSA.

Theoretical MM $[M+H]^+$	Observed MM $[M+H]^+$	Peptide Sequence	Calculated pI
544.34	545.35	K.VASLR.E	9.72
688.37	689.38	K.AWSVAR.L	9.79
711.36	712.38	K.SEIAHR.F	6.47
840.45	841.47	R.LCVLHEK.T	6.74
926.48	927.49	K.YLYEIAR.R	6.00
1023.44	1024.46	K.CCTESLVNR.R	5.99
1162.62	1163.63	K.LVNELTEFAK.T	4.53
1192.59	1193.61	R.DTHKSEIAHR.F	6.92
1248.61	1249.62	R.FKDLGEEHFK.G	5.45
1304.70	1305.72	K.HLVDEPQNLIK.Q	5.32
1361.66	1362.67	K.SLHTLFGDELCK.V	5.30
1438.82	1439.83	R.RHPEYAVSVLLR.L	8.75
1478.78	1479.80	K.LGEYGFQNALIVR.Y	6.00
1566.73	1567.74	K.DAFLGSFLYEYSR.R	4.37
1638.93	1639.94	R.KVPQVSTPTLVEVSR.S	8.75
1666.80	1667.82	R.MPCTEDYLSLILNR.L	4.37
1822.89	1823.91	R.RPCFSALTPDETYVPK.A	6.06
2075.87	2076.89	K.ECCHGDLLECADDRADLAK.Y	4.23
2315.03	2316.07	K.YNGVFQECCQAEDKGACLLPK.I	4.68

3.5.3 *In-Capillary digestion*

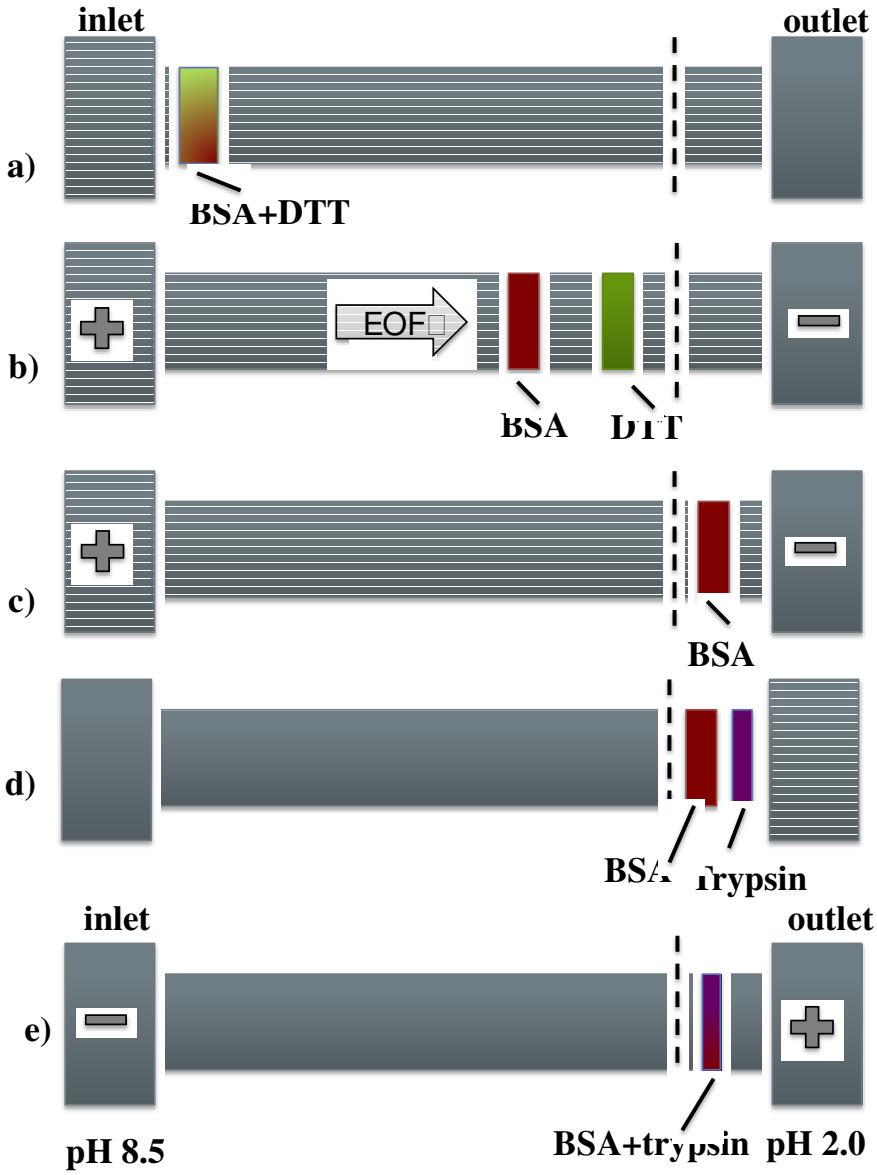


Figure 3-21 Schematic of in-capillary reduction and digestion of BSA.

a) Injection of pre-reduced BSA along with DTT into the capillary b) separation of BSA and DTT by voltage application c) DTT exits the capillary and BSA reaches near to the outlet d) trypsin injected from the outlet e) pH mediated stacking of BSA and trypsin.

Since digestion was successfully performed when the reaction was allowed to happen inside the capillary, our next attempt was to do the mixing of BSA and trypsin as well as the reaction inside the capillary. Unlike in-capillary reduction, performing digestion inside the capillary was quite challenging. In reduction reaction (mentioned in section 3.3) the position of insulin and DTT inside the capillary was accurately known, hence the voltage needed to mix them was easy to calculate.

While performing digestion, the reduced BSA was injected from the capillary inlet, followed by the separation of BSA and DTT by voltage. As DTT exits the capillary, BSA was expected to be closer to the outlet but the exact position of it couldn't be calculated. Hence to mix the reduced BSA with trypsin, a pH mediated stacking had to be employed. Optimization of the voltage needed for stacking was also done. Once, BSA and trypsin was mixed, it was allowed to react for 3 hours inside the capillary.

In this approach (figure 3-21), BSA and DTT was premixed in a vial and incubated at 50°C for 15 minutes. The reduced BSA along with DTT was injected into the capillary from the inlet and a positive polarity was applied to separate the reduced BSA from DTT. The electrophoretic mobility of DTT is much faster than BSA, hence resulted in effective separation. For this separation, 80mM ammonium phosphate of pH 8.5 was used. After

DTT exits the capillary and when BSA reached near to the outlet, the voltage was suspended. A small plug of trypsin was then injected from the outlet.

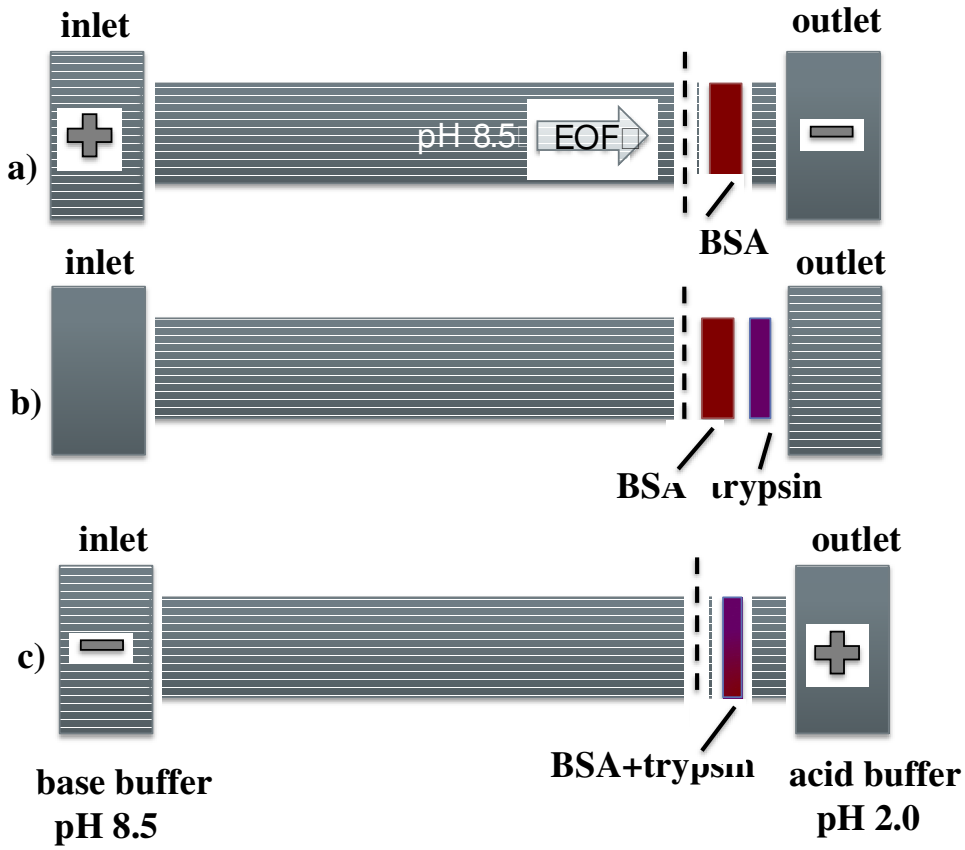


Figure 3-22 Schematic representation of pH mediated stacking of BSA and trypsin.

a) After DTT exits, BSA near to the outlet b) trypsin injected from the outlet c) outlet buffer was replaced with acid buffer (pH 2.0) and inlet buffer was basic (pH 8.5), application of voltage resulted in the pH mediated stacking of BSA and trypsin.

In order to promote effective mixing of reduced BSA and trypsin, a pH mediated stacking has to be utilized, figure 3-22 illustrates the schematic of pH mediated stacking of BSA and trypsin. Reduced BSA and trypsin was preconcentrated at the pH junction using discontinuous buffers (mentioned in chapter 1). The pH of the buffers used was higher than BSA's pI on one end and lower than the pI on the other end. To create a pH junction, outlet was placed in the acidic buffer and the inlet was placed in the basic buffer. A negative potential voltage was applied which caused the electromigration of reduced BSA and trypsin towards the pH junction from the outlet. This will result in effective mixing of reduced BSA and the trypsin, hence they stacked together as one plug. Voltage was suspended; preconcentrated BSA and trypsin were incubated for 3 hours inside the capillary. After 3 hours, the digested BSA was spotted on to the MALDI target plate by flushing the contents in the capillary using high pressure (figure 3-20).

3.5.4 Optimization of Time needed for Voltage application for pH mediated stacking

Prior to performing in-capillary digestion optimization of the time needed for voltage application, which resulted in the mixing of BSA and trypsin, were essential. The pH profile of the discontinuous buffer determines the effectiveness of the preconcentration. To achieve pH junction, the two buffers selected should only provide significant buffering capacities at the acidic and basic regions but little or no buffering capacities at the junction. For this work, ammonium phosphate buffers with pH 8.5 and 2.0 were selected as the discontinuous buffers. After injecting trypsin, voltage was applied for different time periods (0, 15, 20, 25 and 30 seconds) and the preconcentrated BSA and trypsin was mobilized using low pressure (50 mbar) from the outlet towards the detector. Figure 3.23 shows the electropherogram of BSA and trypsin stacking at pH junction by voltage application at different time period.

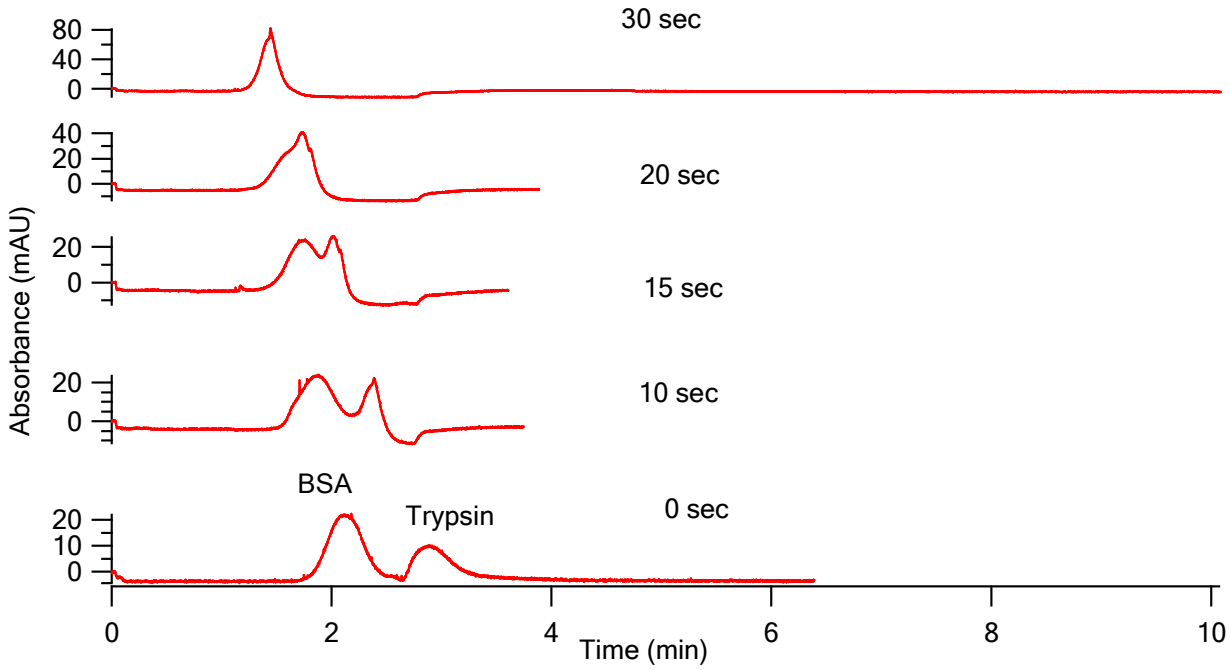


Figure 3-23 BSA and trypsin preconcentration

a) BSA and Trypsin pushed with pressure, no voltage application b) voltage applied for 10 seconds c) 15 seconds voltage application d) 20 seconds voltage application e) 30 seconds voltage application.

When voltage was not applied, the broad peak of BSA and trypsin was detected, the absorbance intensity was very low around 30 mAU for BSA and 10 mAU for trypsin. After 15 seconds of voltage application, the peaks of BSA and trypsin were found to overlap. When the voltage was increased to 25 to 30 seconds, an intense peak of very high absorbance was detected, suggesting the completion of preconcentration. Based on

these data, voltage was applied for 30 seconds to promote the mixing of BSA and trypsin inside the capillary.

After preconcentration of BSA and trypsin, they were allowed to react inside the capillary for 3 hours. The digested BSA was then spotted onto a MALDI plate by flushing the contents in the capillary by the application of high pressure for 30 seconds. The spot was then combined with CHCA matrix, allowed to dry and analyzed by MS. Total 11 peptides were detected which resulted in 21% sequence coverage. Peptides detected are listed on Table 3-8.

Table 3-8 Peptides detected by MALDI MS from in-capillary digestion of BSA.

Theoretical MM $[M+H]^+$	Observed MM $[M+H]^+$	Peptide Sequence	Calculated pI
544.34	545.35	K.VASLR.E	9.72
688.37	689.38	K.AWSVAR.L	9.79
711.36	712.38	K.SEIAHR.F	6.47
926.48	927.49	K.YLYEIAR.R	6.00
1162.62	1163.63	K.LVNELTEFAK.T	4.53
1192.59	1193.61	R.DTHKSEIAHR.F	6.92
1248.61	1249.62	R.FKDLGEEHFK.G	5.45
1478.78	1479.80	K.LGEYGFQNALIVR.Y	6.00
1566.73	1567.74	K.DAFLGSFLYEYSR.R	4.37
1638.93	1639.94	R.KVPQVSTPTLVEVSR.S	8.75
1822.89	1823.91	R.RPCFSALTPDETYVPK.A	6.06

This low sequence coverage suggests that BSA has not undergone a complete digestion. It could be either due to sample loss, as BSA has to migrate from the inlet to the outlet or due to insufficient mixing of the BSA and trypsin. In order to confirm the reasons for incomplete digestion, another experiment was performed. In this method, reduced BSA was injected into the capillary from the outlet followed by trypsin injection. pH junction was introduced to mix the BSA and trypsin by voltage application and allowed to react for 3 hours. After 3 hours, the capillary content was

flushed using high pressure for 30 seconds and spotted onto the MALDI target plate and analyzed by MS. The sequence coverage was obtained using Mascot search and a total sequence coverage of around 20% was obtained which was quite comparable to table 3-8.

From this, it was clear that the incomplete digestion was not due to sample loss (confirmed by the second experiment where BSA was injected from the outlet) but could be due to the insufficient mixing of BSA and trypsin. This proves that further optimization of the mixing of reduced BSA and trypsin was needed.

A novel way of performing in-capillary digestion using CE has been investigated. The experimental set-up was extremely simple using simple buffers and the sample consumption was reduced to nano-liter volume range. Reduced BSA was separated from DTT by CZE. A pH mediated stacking was employed which resulted in the mixing of BSA and trypsin together inside the capillary and the digestion was allowed to proceed for 3 hours. MALDI MS analysis was used to identify the peptides. The sequence coverage for in-vial digestion, premixed and reacted in-capillary and in-capillary digested was calculated. The results proved that digestion did happen inside the capillary,

however the sequence coverage was low compared to in-vial digestion. This could be explained as a result of insufficient mixing of BSA and trypsin.

Chapter 4 Conclusion and future work

4.1 Conclusions

Sample preparation is a crucial step in protein analysis and its adequacy is a key factor in determining the success of the analysis. Ideally, the steps involved in sample preparation must be kept minimal and it should be carried out without the loss of analytes. CE with its remarkable features like low analysis time, high resolution power and consumption of sub-micro liter sample volume have made it a superior analytical technique [70]. Recently, CE has been recognized as an efficient technique to integrate sample preparation and separation under a single format resulting in the miniaturization of the analysis.

This work was an effort towards miniaturization of the sample preparation steps like reduction and digestion of disulfide containing proteins using CE analytical technique. In-capillary reduction was performed at room temperature and at 50°C followed by MS analysis. From the CE and MS data it was clear that when the reduction reaction was performed at elevated temperature (50°C), it only took 15 minutes for the reaction to go to completion, which is much faster than at room temperature. Hence, a successful in-capillary reduction of disulfide containing protein was achieved using CE. Advantages of performing in-capillary reduction are nano-liter volume sample consumption and the reaction mixture can be spotted directly from the capillary onto the MALDI target.

After the successful in-capillary reduction of insulin, digestion was also done inside the capillary. In order to obtain effective mixing of the reduced BSA and trypsin inside the capillary, a pH mediated stacking was utilized. Total sequence coverage of 80 % was obtained when digestion was performed in-vial. While only 21 % sequence coverage was obtained for in-capillary digestion. This low sequence coverage was assumed due to the insufficient mixing of the BSA and trypsin which has to be resolved.

Hence, CE has been recognized as a powerful tool not only for separation but also for performing sample preparation of biological samples. CE represents a promising technique for future development in an integrated proteomics lab on a chip version.

4.2 Future Work

One hypothesis for the low sequence coverage for in-capillary BSA digest could be the insufficient mixing of BSA and trypsin by voltage application. Further optimization of mixing of BSA and trypsin has to be done. Since in-vial digestions are done in the presence of DTT and it did not complicate the MS data. Yet another proposal is to investigate the three components mixing of BSA, DTT and trypsin inside the capillary by zone passing technique. BSA, DTT and trypsin have to be injected into the capillary based on their electrophoretic mobility, mixing has to be done by voltage application and

reacted inside the capillary. After digestion, the content in the capillary can be spotted on the MALDI target plate and the peptides can be detected by MS analysis. Once a working protocol for in-capillary digestion has achieved, this technique can be applied to real biological samples.

References

1. Bakry, R.H., Huck, C.W.; Najam-ul-Haq, M.; Bonn, G.K. Journal of Separation Science. 2007, 30, 192-201.

2. Guo, T. L., Lee, C.S.; Wang, W.; DeVoe, D.L.; Balgley, B.M. Electrophoresis. 2006(27), 3523-3532.

3. Desiderio, C., Rossetti, D.V.; Iavarone, F.; Messana, I.; Castagnola, M. Journal of Pharmaceutical and Biomedical analysis. 2010, 53(5), 1161-9.

4. Huck, C.W., Bakry, R.H; Najam-ul-Haq, M.; Rainer, M.; Bonn, G.K. Journal of Separation Science. 2007, 30(2), 192-201.

5. Bulaj, G. Biotechnology Advances. 2005, 23(1), 87-92.

6. Nesbitt, C.A.L., Yeung, K.K.C. Journal of Chromatography A. 2005, 1073, 175-180.

7. Thornton, J.M. Journal of Molecular Biology. 2002, 293(2), 333-342.

8. Zweifel, M.E.; Barrick, D. Biophysical chemistry. 2002, 101-102, 221-37.

9. Ficarro, S.B.; McCleland, M.L.; Stukenberg, P.T.; Burke, D.J.; Ross, M.M; Shabanowitz, J.; Hunt, D.F.; White, F M. Nature Biotechnology. 2002, 20(3), 301-305.

10. Xu, C.F.L., Lu, Y.; Ma, J.H.; Mohammadi, M.; Neubert, T.A. Molecular & Cellular Proteomics. 2005, 4(6), 809-818.

11. Zaia, J. Mass Spectrometry Reviews. 2004, 23(3), 161-227.

12. Marino, K.B., Bones, J.; Kattla, J.J.; Rudd, P.M. Nature Chemical Biology. 2010, 6, 713-723.

13. Ong, S.E.M., Mittler, G.; Mann, M. Nature Methods. 2004, 1(2), 119-126.

14. Denison, C.K., Kirkpatrick, D.S.; Gygi, S.P. Current Opinion in Chemical Biology. 2005, 9(1), 69-75.

15. Clark, R.S.B., Bayir, H.; Jenkins, L.W. Critical Care Medicine. 2005, 33(12), S407-S409.

16. Canas, B., Pineiro, C.; Calvo, E.; Lopez-Ferrer, D.; Gallardo, J.M. Journal of Chromatography. A. 2007, 1153(1-2), 235-58.

17. Thomas, A., Bewley.; Li, C.H. Journal of Proteome Research. 1968, 117-124.

18. Walker, J.M. The Protein Protocols Handbook. 2002(2nd ed).

19. Graves, P.R.H., Haystead, T.A.J. Microbiology and Molecular Biology Reviews. 2002, 66, 39-63.

20. Thiede, B.; Hohenwarter, W.; Krah, A.; Mattow, J.; Schmid, M.; Schmidt, F.; Jungblut, P. R. Journal of Methods. 2005, 35(3), 237-47.

21. James, P. Quarterly reviews of biophysics. 1997, 30(4), 279-331.

22. Chapman, J.R. Journal of Mass Spectrometry. 2000, 538

23. Gilar, M., Bouvier, E.S.P; Compton, B.J. Journal of Chromatography. A. 2001, 909(2), 111-135.

24. Mouradian, S. Current opinion in Chemical Biology. 2002, 6(1), 51-6.

25. Yager *et* al. Nature. 2006, 442, 412-418

26. Gottschlich, N., Jacobson, S. C.; Culbertson, C. T.; Ramsey, J. M. Analytical Chemistry. 2001, 73(11), 2669-2674.

27. Rocklin, R.D., Ramsey R.S., Ramsey, J.M. Analytical Chemistry. 2000, 72(21), 5244-9.

28. Lazar, I.M., Ramsey, R.S.; Ramsey, J.M. Analytical Chemistry. 2001, 73(8), 1733-1739.

29. Ekstrom, S.; Onnerfjord, P.; Nilsson, J.; Bengtsson, M.; Laurell, T.; Marko-Varga, G. Analytical Chemistry. 2000, 72(2), 286-293.

30. Wang, C., Oleschuk, R.; Ouchen, F.; Li, J.J.; Thibault, P.; Harrison, D.J. Rapid Communications in Mass Spectrometry. 2000, 14(15), 1377-1383.

31. Campana, A.M.G., Baeyens, W.R.G.; Aboul-Enein, H.Y.; Zhang, X.R. Journal of Microcolumn Separations. 1998, 10(4), 339-355.

32. Deyl, Z., Miksik, I.; Tagliaro, F. Forensic science international. 1998, 92, 89-124.

33. Pritchet, T.L., Pritchet, J.P. Handbook of Capillary Electrophoresis, CRC Press, Inc., Boca Raton, FL, 1997.

34. Heiger, D. Electrophoresis. 2000, 135.

35. Monton, M.R.N.; Terabe, S. Journal of chromatography. B. 2006 (841), 88-95.

36. Shaw, D.J. Electrophoresis. 1969, 144.

37. Wang, W., Zhou, F.; Zhao, L.; Zhang, J.R.; Zhu, J. J. Journal of Chromatography. A. 2007, 1170, 1-8.

38. Camilleri, P. Capillary Electrophoresis. 1998. 2nd ed, 551.

39. Osbourn, D.M.; Weiss, D. J.; Lunte, C. E. Electrophoresis. 2000, 21, 2768-2779.

40. Shihabi, Z.K. Journal of Chromatography. A. 2000, 902(1), 107-117.

41. Chien, R.L.; Burgi, D.S. Analytical Chemistry. 1992, 64(8), A489-A496.

42. Mikkers, F.E.P.; Everaerts, F.M.; Verheggen, T.P.E.M. Journal of Chromatography. 1979, 169, 1-10.

43. Britz-McKibbin, P.; Terabe, S. J. Journal of Chromatography. A. 2003, 1000, 917.

44. Britz-McKibbin, P., Bebault, G. M.; Chen, D.D.Y. Analytical Chemistry. 2000, 72, 1729-1735.

45. Monton, M.R.N., Imami, K.; Nakanishi, M.; Kim, J.-B.; Terabe, S. Journal of Chromatography. A. 2005, 1079, 266-273.

46. Nesbitt, C.A., Lo, J.T., Yeung, K.K.C. Journal of chromatography. A. 2005, 1073(1-2), 175-80.

47. Licklider, L.; Kuhr, W.G. Journal of the American Chemical Society. 1994, 207.

48. Han, Y.L.; Chen, Y. Electrophoresis. 2007, 28(15), 2765-2770.

49. Ptolemy, A.S.; Britz-McKibbin,P. Journal of Chromatography. A. 2008, 133(12), 1643-8.

50. Le Potier, I.; Franck, G.; Smadja, C.; Varlet, S.; Taverna, M. Journal of Chromatography. A. 2004, 1046(1-2), 271-6.

51. Bao, J. M.; Regnier, F.E.J. Journal of Chromatography. 1992, 608, 217-224.

52. Nesbitt, C.A.J., Yeung, K. K.C. Electrophoresis. 2008, 29, 466.

53. Russell, D. H.; Williams, B. J.; Russell, W. K. Analytical Chemistry. 2007, 79(10), 3850-3855.

54. Kussmann, M.; Nordhoff, E.; RahbekNielsen, H.; Haebel, S.; RosselLarsen, M.; Jakobsen, L.; Gobom, J.; Mirgorodskaya, E.; KrollKristensen, A.; Palm, L.; Roepstorff, P. Journal of Mass Spectrometry. 1997, (32(6)), 593-601.

55. Whittal, R.M.; Keller, B.O.; Li, L. Analytical Chemistry. 1998, 70, 5344.

56. Ethier, M.H., W.; Duewel, H.S.; Figeys, D. J. Journal of Proteome Research. 2006, 5, 2754-2759.

57. Abel, A.J. In-capillary Reduction and Enzymatic Digestion of Low Nanogram Quantities of Disulfide Containing Protein Using Capillary Electrophoresis. 2009, The University of Western Ontario.

58. Tang, J.G., Yuan, Y.; Wang, Z,H. Journal of Biochemistry. 1999, 343, 139-144.

59. Jurcic, K.; Yeung, K.K.C. Electrophoresis. 2009, 30(10), 1817-1827.

60. Graf, M., Galera Garcia, R.; Watzig, H. Electrophoresis. 2005, 26(12), 2409-17.

61. Cunliffe, J.M.; Baryla, N.E.; Lucy, C.A. Analytical Chemistry. 2002(74), 776-783.

62. Yeh, H.H.; Wu, H.L.; Lu, C.Y. Journal of Pharmaceutical and Biomedical analysis. 53(2), 145-50.

63. Winzor, D.J.; Jones, S.; Harding, S.E. Analytical Biochemistry. 2004, 333(2), 225-9.

64. Chakel, J.A.; Pungor, E.; Hancock, W.S.; Swedberg, S.A.J. Journal of Chromatography. B. 1997(689), 215-220.

65. Scigelova, M. Journal of Mass Spectrometry. 2001, 7, 29-34.

66. Desiderio, C.; Iavarone, F.; Rossetti, D.V.; Messana, I.; Castagnola, M. Journal of Separation Science. 2010, 33(16), 2385-93.

67. Steen, H.; Mann, M. Molecular Cell Biology. 2004, 5, 699-711.

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

69. Craft, D.; Li, L. Analytical Chemistry. 2005(77), 2649-2655.

70. Wu, X.Z. Analytical Chemistry. 2003, 22(1), 48-58.

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