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Characterization of Glutaraldehyde-Immobilized Chymotrypsin and an *In-Situ* Immobilized Enzyme Reactor Using Capillary Electrophoresis-Based Peptide Mapping

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Résumé

La digestion enzymatique des protéines est une méthode de base pour les études protéomiques ainsi que pour le séquençage en mode « bottom-up ». Les enzymes sont ajoutées soit en solution (phase homogène), soit directement sur le gel polyacrylamide selon la méthode déjà utilisée pour l'isolation de la protéine. Les enzymes protéolytiques immobilisées, c'est-à-dire insolubles, offrent plusieurs avantages tels que la réutilisation de l'enzyme, un rapport élevé d'enzyme-sur-substrat, et une intégration facile avec les systèmes fluidiques. Dans cette étude, la chymotrypsine (CT) a été immobilisée par réticulation avec le glutaraldehyde (GA), ce qui crée des particules insolubles. L'efficacité d'immobilisation, déterminée par spectrophotométrie d'absorbance, était de 96% de la masse totale de la CT ajouté. Plusieurs différentes conditions d'immobilisation (*i.e.,* réticulation) tels que la composition/pH du tampon et la masse de CT durant la réticulation ainsi que les différentes conditions d'entreposage tels que la température, durée et humidité pour les particules GA-CT ont été évaluées par comparaison des cartes peptidiques en électrophorèse capillaire (CE) des protéines standards digérées par les particules. Les particules de GA-CT ont été utilisés pour digérer la BSA comme exemple d'une protéine repliée large qui requit une dénaturation préalable à la digestion, et pour digérer la caséine marquée avec de l'isothiocyanate de fluorescéine (FITC) comme exemple d'un substrat dérivé afin de vérifier l'activité enzymatique du GA-CT dans la présence des groupements fluorescents liés au substrat. La cartographie peptidique des digestions par les particules GA-CT a été réalisée par CE avec la détection par absorbance ultraviolet (UV) ou fluorescence induite par laser. La caséine-FITC a été, en effet, digérée par GA-CT au même degré que par la CT libre (*i.e.,* soluble). Un microréacteur enzymatique (IMER) a été fabriqué par immobilisation de la CT dans un capillaire de silice fondu du diamètre interne de 250 μm prétraité avec du 3-aminopropyltriéthoxysilane afin de fonctionnaliser la paroi interne avec les groupements amines. Le GA a été réagit avec les groupements amine puis la CT a été immobilisée par réticulation avec le GA. Les IMERs à base de GA-CT étaient préparé à l'aide d'un système CE automatisé puis utilisé pour digérer la BSA, la myoglobine, un peptide ayant 9 résidus et un dipeptide comme exemples des substrats ayant taille large, moyenne et petite, respectivement. La comparaison des cartes peptidiques

des digestats obtenues par CE-UV ou CE-spectrométrie de masse nous permettent d'étudier les conditions d'immobilisation en fonction de la composition et le pH du tampon et le temps de réaction de la réticulation. Une étude par microscopie de fluorescence, un outil utilisé pour examiner l'étendue et les endroits d'immobilisation GA-CT dans l'IMER, ont montré que l'immobilisation a eu lieu majoritairement sur la paroi et que la réticulation ne s'est étendue pas si loin au centre du capillaire qu'anticipée.

Mots-clés: Cartographie peptidique, Réticulation, Glutaraldéhyde, Chymotrypsine, Électrophorèse capillaire, Réacteur d'enzyme immobilisé (IMER)

Abstract

Digesting proteins using proteolytic enzymes is a standard method in proteomic studies and bottom-up protein sequencing. Enzymes can be added in solution or gel phase depending on how the protein has been isolated. Immobilized, *i.e.,* insoluble, proteolytic enzymes offer several advantages such as reusability of enzyme, high enzyme-to-substrate ratio, and integration with fluidic systems. In this study, we prepared glutaraldehyde-crosslinked chymotrypsin (GA-CT), which creates insoluble particles. The immobilization efficiency was determined by absorbance spectrophotometry and found to be 96% of the total amount of chymotrypsin added. Different immobilization (*i.e.,* crosslinking) conditions such as buffer composition/pH and initial mass of CT during crosslinking as well as different storage conditions such as temperature, time and humidity for the GA-CT particles were evaluated by comparing capillary electrophoretic (CE) peptide maps of protein standards digested with the particles. The GA-CT particles were used to digest BSA as an example of a large folded protein that needs denaturation prior to digestion, and casein-fluorescein isothiocyanate (FITC) as an example of a small, labeled substrate to test enzyme activity in the presence of substrate-bound fluorescent groups. Peptide mapping of digests from GA-CT particles was achieved by CE with ultraviolet (UV) absorbance or laser induced fluorescence (LIF) detection. FITC-labeled casein was digested by GA-CT to the same extent as with free (*i.e.,* soluble) CT. An immobilized enzyme microreactor (IMER) was fabricated by immobilizing CT inside a 250 μm i.d. fused-silica capillary tube pre-treated with 3-aminopropyltriethoxysilane to functionalize the inner walls with amine groups. Glutaraldehyde was reacted with the amine groups and then CT was immobilized by crosslinking to the GA. IMERs based on GA-CT were fabricated using an automated CE system and used to digest BSA, myoglobin, a 9-residue peptide and a dipeptide as examples of large, medium and small substrates. Digests were studied by comparing peptide maps obtained by CE coupled to either UV or mass spectrometric (MS) detection in order to evaluate immobilization conditions as a function of buffer composition/pH and reaction times. A separate study, which used fluorescence microscopy to investigate the extent and location of GA-CT immobilization in the IMER, showed that immobilization

only takes place primarily near the capillary walls and that crosslinking does not extend as far into the center of the IMER as had been expected.

Keywords: Peptide mapping, Crosslinking, Glutaraldehyde, Chymotrypsin, Capillary electrophoresis, Immobilized enzyme reactor (IMER)

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List of Abbreviations and Acronyms

3-APTES	(3-aminopropyl)-triethoxysilane
API-ES	Atmospheric pressure electrospray ionization
BAEE	N-α-benzoyl-*L*-arginine ethyl ester
B-Arg	N-α-benzoyl-*L*-arginine
BGE	Background electrolyte
BSA	Bovine serum albumin
CE	Capillary electrophoresis
CGE	Capillary gel electrophoresis
CIEF	Capillary isoelectric focusing
CITP	Capillary isotachophoresis
CLCE	Crosslinked enzyme crystal
CLEA	Crosslinked enzyme aggregates
CPG-Trypsin	Controlled pore glass-Trypsin
CSLM	Confocal laser scanning microscope
CT	Chymotrypsin
CZE	Capillary zone electrophoresis
DAD	Diode array detector
DPSS	Diode-pumped solid state
DTT	Dithiothreitol
EOF	Electroosmotic flow
F	Phenylalanine (Phe)
f.s.	Fused silica capillary
FH	Phe-His-O-CH_3

FITC	Fluorescein isothiocyanate
FRQNT	Fonds de recherche du Québec – Nature et technologies
GA	Glutaraldehyde
GA-CT	Glutaraldehyde crosslinked chymotrypsin
H	Histidine (His)
HPLC	High performed liquid chromatography
ID	Inner diameter
IMER	Immobilized enzyme reactor
LC	Liquid chromatography
L_d	Total length to capillary detector
LIF	Laser induced fluorescence
L_t	Total length
MS	Mass spectrometry
NCBI	National Center for Biotechnology Information
NSERC	Natural Sciences and Engineering Research Council of Canada
OD	Outer diameter
Phe	Phenylalanine
PMT	Photomultiplier tube
RSD	Relative standard deviation
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulphate- Polyacrylamide gel electrophoresis
SIM	Single ion monitoring
SRM	Selective reaction monitoring

TPCK-Trypsin	Tosyl phenylalanyl chloromethyl ketone-Trypsin
TRIS- HCl	Trizma hydrochloride
Trp	Tryptophan
Tyr	Tyrosine
W	Tryptophan (Trp)
Y	Tyrosine (Tyr)
Z-Arg	N2-Carbobenzyloxy-L-arginine

Count what is countable, measure what is measurable, and what is not measurable, make measurable.

— Galileo Galilei

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Chapter 1. General Introduction, Literature Review and Research Objectives

1.1. Determination of Proteins

The human body is made of billions of living cells, each containing a vast number of biomolecules. Knowing the composition of cells, tissues and biological fluids plays an important role in state-of-health studies. Abnormalities in cellular functions can cause different responses, usually a disease state [1, 2]. Biochemical studies have provided much information about diseases and biochemical markers such as proteins are used as indicators for diagnosis [1-3]. While DNA is considered the blueprint of life, proteins can be considered as its tools. To study proteins' functions, it is important to understand their structure, composition and interactions. The field of research aimed to study proteins emerged in 1994 and Wilkins named it "proteomics" [4]. The word proteomics is based on the philosophy of the previously introduced terms "genomics" and "genome" describing the sets of genes in an organism, where the term "omics" indicates the overall understanding of living cells [5, 6]. Proteomics studies include protein separation, identification, quantification and sequence analysis, and provide a means for understanding and mapping protein function in cells. Additionally, proteomics studies have provided opportunities for introducing new medicins. While many earlier medicines are small non-protein molecules, several new drugs have been derived from proteins in last two decades. Proteomic studies have helped in reconstituting endogenous proteins such as insulin, growth hormones, blood clotting factors and reproductive hormones as medicines with pivotal impact in the treatment of many diseases [7]. The proteome of a cell can show large variations that depend on cellular and environmental conditions. The proteome has a dynamic nature because of many molecular interactions and post-translational modifications. The number of modified and unmodified proteins in a cell is higher than the number of genes that code for them. Additionally, many of these proteins are at low abundance, which makes proteomic analysis a challenge. Such a complex sample requires very robust separation techniques prior to sequence analysis or quantification as well as highly sensitivity detectors.

The ultimate goal of analytical methods used in proteomics is to identify and quantify all individual proteins in a cell. Finding a single method is challenging, however a combination of sequential methods can provide high resolution analysis for a complex

protein mixture. Mass spectrometry (MS) and multidimensional liquid chromatography (LC) are the two dominant analytical separation techniques used in structural proteomics studies [8]. A high quality separation is essential for protein identification as T. Rabilloud, a leader in the field of protein separation, emphasized: "Because of the complexity of biological samples, it is absolutely necessary to separate (and sometimes quantify) the analytes prior to their measurement with mass spectrometry" [9].

Proteins are made of linear chains of amino acids. These amino acids are linked together by a peptidic bond between the α-amino group of one amino acid and the α-carboxylate group of another. Proteins have four levels of structure, namely: primary, secondary, tertiary and quaternary. The specific number and sequence of amino acids in a linear polypeptide is called the primary structure. Analytical separations of polypeptides by techniques such as LC and MS are usually based on the primary amino acid sequence.

A protein's secondary structure is the local structure of neighboring amino acids. Once the primary structure is formed, the single chain can twist into an α-helix, or lie alongside itself and form a β-pleated sheet. These structural conformations are called secondary structure. All proteins are defined first by their primary structure and then secondary structure. Larger proteins such as myoglobin or bovine serium albumin (BSA) can have a tertiary structures and quaternary structures.

Tertiary structure refers to the three dimensional shape formed when the peptide chain curls and folds. Some forces such as covalent disulfide bonds between two cysteines, electrostatic (ionic) interactions mostly between acidic and basic side chains, hydrogen bonds, van der Waals forces, and hydrophobic side chains pushed away from water create and hold the tertiary structure. It is this structure that gives a protein its unique function [10]. Not all proteins have quaternary structure. Quaternary structures exist only for proteins containing more than one polypeptide chain bonded to the others in the same protein via non-covalent interactions.

Identifying and quantifying a large protein can be quite challenging. One way to study large proteins is to cleave them into smaller segments called polypeptide chains. This is frequently accomplished by enzymatic digestion. Enzymes are incredibly important

biological catalysts exerting the ability to cut proteins by specific recognition of certain amino acids in the primary structure. An enzyme catalyzes a single reaction or class of reactions with its protein substrates, a property known as enzyme specificity. For example, chymotrypsin cleaves peptide bonds primarily on the carboxy-terminal side of the amino acid residues phenylalanine, tryptophan and tyrosine in a protein. These amino acids all contain an aromatic group, which makes chymotrypsin specific for this class of molecules. Enzymes are very specific and a powerful means of cleaving proteins into smaller polypeptides to allow analysis. However, enzymes are sometimes not able to function if the protein substrate cannot reach the enzyme's active site. When a protein is large and highly folded, the enzyme has very limited access to the internal amino acid residues and therefore it will have limited cleavage sites accessible. This affects the amount of polypeptide fragments that can be created and may affect the analytical methods for protein identification. In order to increase the extent of enzymatic digestion for large or heaviliy folded proteins, the protein can be unfolded, i.e., the proteins lose their tertiary structure. This can be achived by denaturing the protein [10], generally by heat or use of chemical reactions. At high temperatures, the energy of heat can overcome the hydrophobic interactions that hold a protein together and cause the protein to partially unfold; however, this is a reversible process and the protein may refold at lower temperature. Chemical chaotropic reagents such as urea [11] can break the strong hydrogen bonds within a protein's secondary and tertiary structure and reducing agents such as dithiothreitol can break the intramolecular disulphide bonds between two cysteines allowing the protein to be unfolded.

1.1.1. Sample Preparation for Structural Proteomics Studies

Proteins are usually contained within intracelleular strucutres inside a cell. Often cell wall disruption is required before proteins can be effectively solubilized and extracted. Various chemical and physical techniques can be used to destroy the cell wall [12, 13]. The next step involves protein solubilisation and extraction. This is often aided by surfactants such as sodium dodecyl sulfate (SDS). Due to the wide range of proteins and interfering

contaminants that SDS solubilisation can produce, protein separation at this point is imperative. Protein enrichment during separation is the next step, which increases the concentration of the proteins of interest and decreases the concentration of interfering compounds. Proteins treated with SDS are usually fractionated either by two dimensional polyacrylamide gel electrophoresis (2D-SDS-PAGE), which separates proteins based on their isoelectric point (p*I*) and molecular mass, or by chromatographic methods such as LC, depending on the sample complexity (Figure 1.1) [14]. Isolation (fractionation) is usually followed by protein digestion using proteolytic enzymes. The resulting polypeptides are then separated by LC methods with higher resolution than those used for proteins, or by MS or capillary electrophoresis (CE) because analysis of peptides yields higher resolution and more accurate identification than analysing the intact proteins.

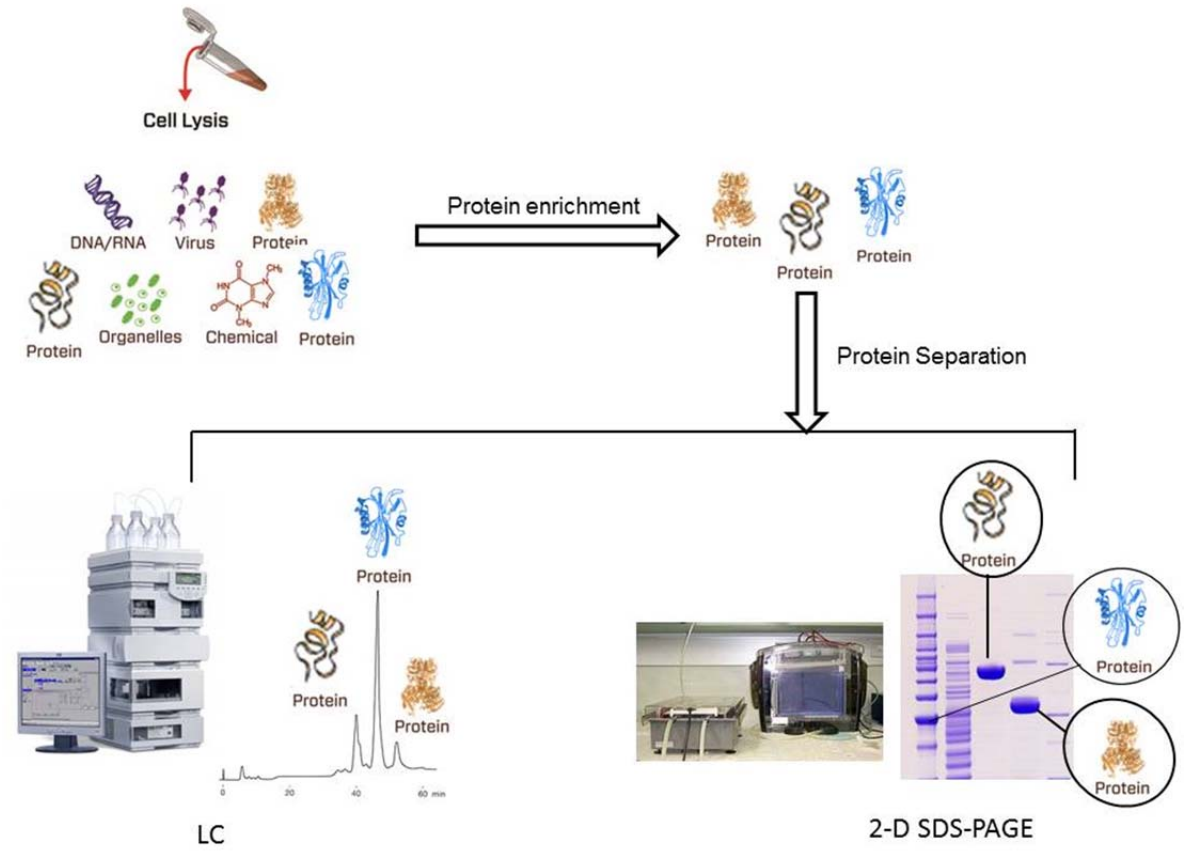

Figure 1.1. General schematic for protein extraction, enrichment and separation.

Since the early days of biotechnology, some proteins have been known to be difficult to digest due to their tight folding. As mentioned above, in order to conduct proteomic studies on large proteins, digestion by using denaturation is usually necessary. For example, Figure 1.2 shows the undenatured (folded) native tertiary structure of bovine serium albumine (BSA) with the locations of Phe (F), Tyr (Y) and Trp (W) highlighted; the amino acid residues that should be cleaved by using chymotrypsin (Figure 1.2). However, due to the folded structure of BSA these sites are not readily accessible for cleavage, which makes denaturation a critical step in protein analysis of BSA [15].

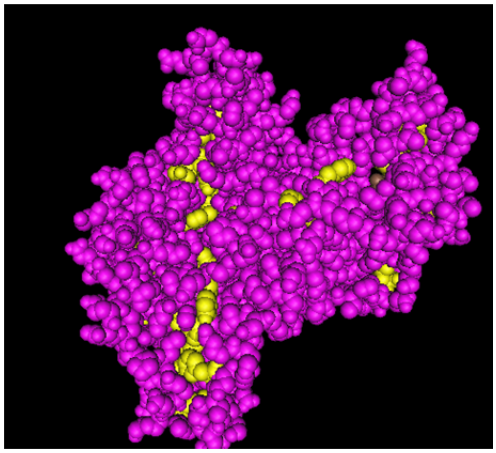

Figure 1.2. Tertiary structure of the native protein, the yellow sites indicate the presence of F, W or Y in BSA crystal (http://www.ncbi.nlm.nih.gov/Structure/MMDB/mmdb.shtml).

Although fewer sample preparation steps are needed for thermal denaturation, this method can lead to protein aggregation or renaturation upon cooling. The mechanisms of protein aggregation are not fully understood, but it is known that protein aggregation causes insolubility of proteins [16]. On the other hand, chemical denaturation is commonly used as an initial step for digesting large proteins. It involves unfolding with urea, reduction of disulfide bonds and then alkylation of the cysteine –SH groups to prevent reformation of disulfide bridges. In the present study, for most experiments, BSA was chemically denatured in this way prior to digestion by chymotrypsin. Undenatured BSA was also digested using this enzyme in a comparative way to study the effect of denaturation.

1.1.2. Enzymatic Digestion

Proteomic studies typically proceed by one of two types of digestion: “in-gel” or “in-solution”. The in-solution digestion is used for proteins fractionated by LC, with collected fractions redissolved in buffer and then enzyme added to the solution in order to digest the protein substrates. The in-gel digestion is for proteins fractionated by gel electrophoresis. The isolated protein is embedded in a gel matrix, already extracted from the whole gel, and the enzyme solution is added to the slice of the gel. In-gel digestion offers the benefit of removal of low molecular weight impurities [17]. It normally includes destaining, reduction, and alkylation of –SH groups, enzymatic cleavage of protein, peptide extraction from the gel, and finally analysis by MS or LC-MS, including sequencing by tandem MS [14].

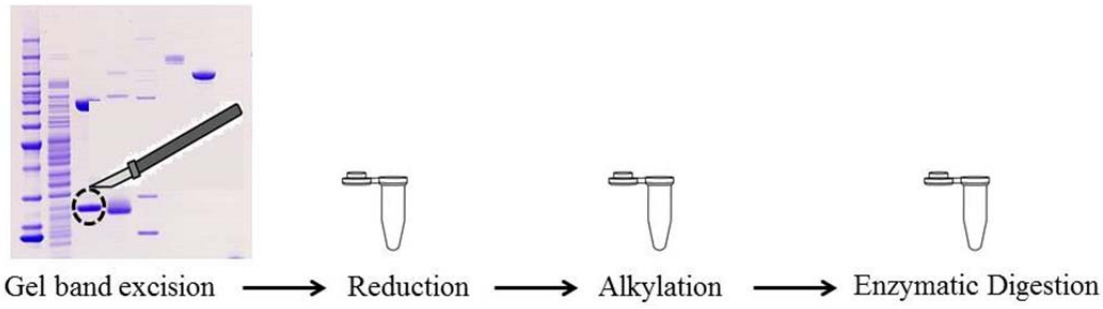

Figure 1.3. The workflow of in-gel protein digestion including gel band excision, reduction, alkylation and finally enzymatic digestion.

Proteolytic enzymes can undergo autoproteolysis, which is self-digestion. Autoproteolysis leads to interfering background peptides during identification and quantification. In both in-solution and in-gel digestions, the enzyme-to-substrate ratio must be low to avoid having autoproteolysis products detected as they are interfering background peptides. Since enzymes are catalysts and not consumed in the substrate digestion, using low amounts of enzymes typically suffice in these reactions. However, such enzyme reactions are slow, as the reaction rate correlates positively with the amount of the enzyme used. In-gel digestions are often carried out for 18-24 h (“over night”) to allow complete

digestion. An alternative approach is to use a larger amount of enzyme for faster enzymatic reaction, or turnover. This is achieved by immobilizing enzyme, which can greatly reduce autoproteolysis and thus the nuisance of interfering background peptides. There is a limitation to increasing the enzyme concentration that depends on the turnover number of the enzyme. This is further described below under Section 1.2.

Various proteolytic enzymes are used for protein digestion, including trypsin, chymotrypsin and pepsin. Trypsin is one of the most commonly used enzymes for this purpose. It cleaves peptide bonds at the C-terminal side of Lys and Arg residues with very high selectivity, producing a modest number of polypeptides of small and medium length which are easily separated by most analytical separation techniques. The separation of such peptides is called “peptide mapping” of the parent protein and is described further in the next section. Traditional enzymatic digestion in solution or in gel can be time consuming because of the low concentration of enzyme [14], a drawback that is addressed further under Section 1.2.

1.1.3. Peptide Mapping

Proteomics analysis can be performed in either “bottom-up” (also called shotgun), or “top-down” mode (Figure 1.4). In the latter mode, protein fragmentation is not achieved by using enzymes; it takes place in a mass spectrometer due to specific collisionally induced dissociation. The “bottom-up” approach involves digestion of proteins using a proteolytic enzyme that cleaves at well-defined sites to create a peptide mixture. For both modes, proteins from biological samples are extracted then fractionated by gel electrophoresis or LC (Figure 1.1) because of the complexity of the sample [18].

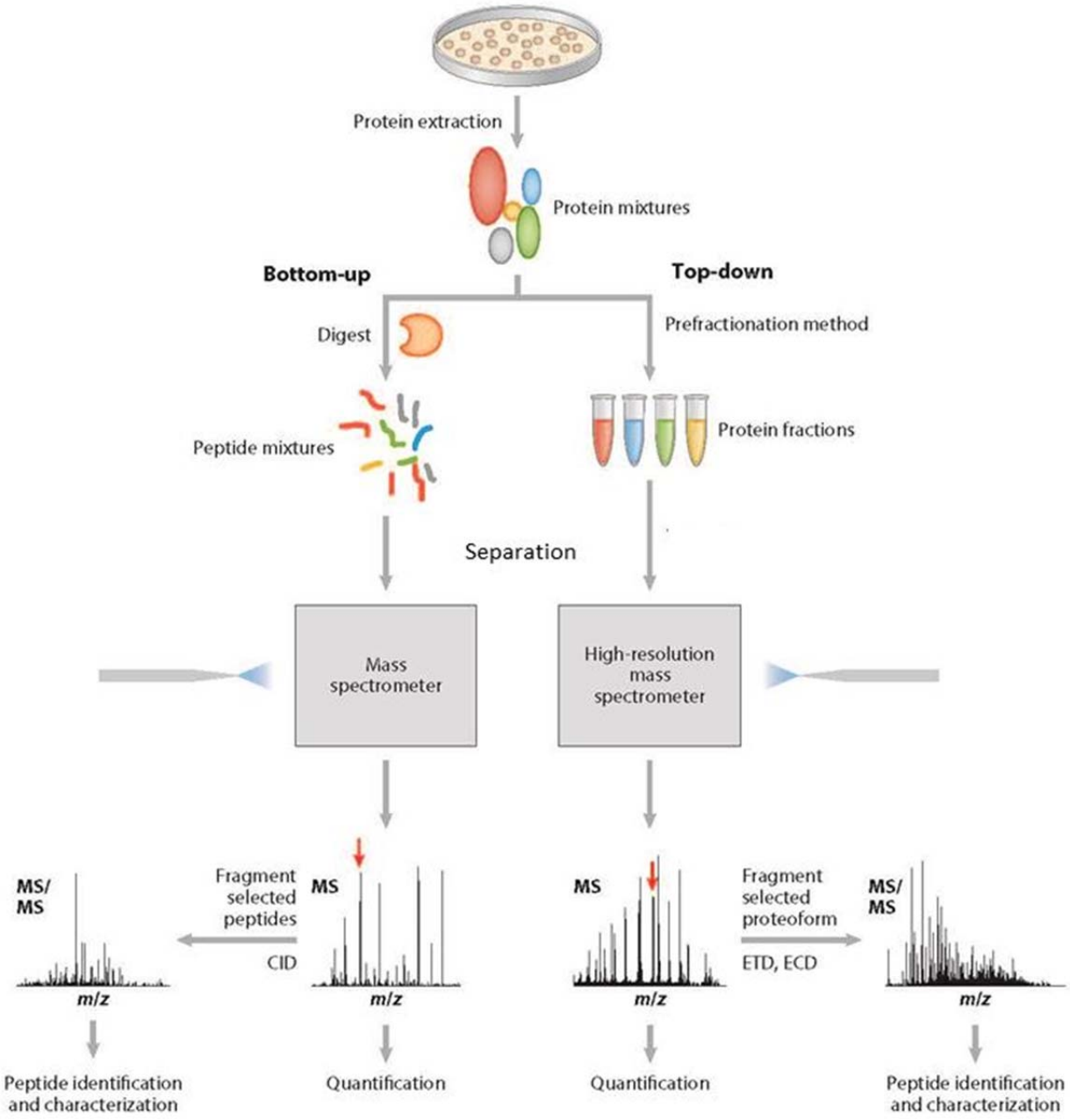

Figure 1.4. General workflow for bottom-up and top-down proteomics [19].

In the “bottom-up” approach to a proteomics study, the sample preparation usually includes proteolysis of proteins into peptides (protein digestion) by using an enzyme leading to a more effective analysis because peptides are less complex than proteins and easier to separate by analytical techniques. Every enzyme cleaves only specific peptide bonds according to the amino acid residue. Therefore, the peptides resulting from the action of a known enzyme on each given protein are easier to identify using protein databases (e.g., National Center for Biotechnology Information (NCBI) protein resources or UniProtKB/Swiss- Prot) if mass spectrometric detection is used and thus the exact masses of the peptides are known. This is referred to as peptide mass mapping and can oftern be carried out without the need of chromatography techniques, for example by matrix assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF MS). Additionally, the peptides can be sequenced if an MS/MS (tandem MS) analyser is used, which will fragment each parent peptide (precursor) separated first in the LC or CE “map” and/or the first analyser of the spectrometer. This reveals the amino acid sequence of the peptides and provides a very high confidence in protein identification using comparison with genomic databases. It also provides primary sequence information for newly discovered proteins and is used in determining post-translational modifications (PTMs) of proteins.

Peptide mapping has also been conducted by CE, a complementary technique to HPLC, either to compare the fingerprint separation of one sample to another, or as CE-MS and CE-MS/MS to identify peptides according to their mass followed by identifying the proteins via genomic database searching [18, 20]. The map or fingerprint alone (the electropherogram or the chromatogram) acquired with photometric detection can be used to compare two samples without the need to identify each peptide by its mass.

The amount of a sample available for proteomics studies is often limited, which can affect the accuracy of an analysis. CE is an ideal technique in this respect because it requires only small amounts of sample and can achieve separation efficiencies of over one million theoretical plates [20-26]. CE is a powerful tool for peptide separation such that peptide mapping was one of the first applications shown when CE was first described in the literature [27-31]. For unambiguous identification of peptides separated by CE, it is coupled

with MS. Kasicka published a very good review on different peptide separation methods using CE [32, 33].

Regardless of the technique chosen for peptide mapping, the use of enzymes is an unavoidable step to preparing the protein substrates. Due to the drawbacks of using enzymes in solution or for in-gel digestion, as described under Section 1.1.2., the enzyme immobilization approach has been used widely and has recently grown in popularity.

1.2. Enzyme Immobilization

The classical method of enzyme digestion, whether done in solution or "in-gel" following PAGE fractionation, suffers from long digestion time and interference of enzyme autoproteolysis products during the quantification and identification. To decrease the autoproteolysis, typically a low enzyme to substrate ratio is used, usually 1:20 to 1:100, which indicates that the enzymatic reactions are slow (16-24 h) [34-37]. Using enzyme immobilization can address this limitation and expedite digestion without the interference from autoproteolytic peptides.

1.2.1. Immobilized Proteases

The immobilization of enzymes for chemical analysis applications has become more popular during the last two decades. In this technique, an enzyme can be immobilized by several methods. These methods include: binding to a solid-phase carrier, [38, 39] or glass beads as a support [40-44]; encapsulating by a polymer [45]; entrapping using sol-gels [46, 47]; and crosslinking as a carrier free immobilized enzyme [48-53]. Several immobilization approaches have been demonstrated, including entrapment, adsorption, covalent binding, crosslinking or affinity-based interaction.

1.2.1.1. Covalent Immobilization

In the covalent binding method, a biocatalyst is bound to a surface or solid support through functional groups. These functional groups are those which are not essential for the catalytic activity of the biocatalyst. The binding is normally carried out by initial activation of the surface using multifunctional reagents (e.g., glutaraldehyde), and then enzyme coupling to the activated support. Although this method provides a high stability of enzyme immobilization, the reproducibility is sometimes poor and it needs high amounts of bioreagent [51, 54, 55]. It is still one of the most popular methods of immobilization since it provides very strong binding of enzyme with minimal leakage. Non-covalent bonds are normally weaker and changing the pH, temperature, or ionic strength can compromise the enzyme and thus its reaction with the substrate [56].

1.2.1.2. Entrapment

Enzymes can be immobilized in three-dimensional matrices such as an electropolymerized film, carbon paste, a photopolymer, a polysaccharide or a silica-based sol-gel. This immobilization method is easy to perform and the activity of the enzyme is preserved during the immobilization process because there is no chemical reaction between the monomers and the enzyme. The drawbacks for this method include biocomponent leaching and possible diffusion barriers [49].

1.2.1.3. Adsorption

One of the easiest methods of immobilization is enzyme adsorption onto a solid support. In this method, the enzyme is dissolved in a solution and a solid support is in contact with the solution for a fixed period of time. The adsorption is based on weak bonds such as van der Waals forces and hydrophobic interactions. The drawback for this method includes desorption of the enzyme if there are changes in temperature, pH and ionic strength [55].

1.2.1.4. Affinity

This method provides an immobilization based on enzyme orientation and molecular recognition. It creates an affinity bond between an activated support and a specific group of the enzyme sequence. This technique allows controlling the enzyme orientation in order to reduce enzyme inactivation and site blocking. The limitation of this method is the need for the presence of specific groups on the enzyme. In some cases the affinity tag needs to be attached to the protein sequence by genetic engineering [57], but the most common practice is to covalently bind biotin to the enzyme and have it react strongly with streptavidin that is attached to a solid support.

1.2.2. Immobilization by Glutaraldehyde-Mediated Crosslinking

There is an increasing interest in using carrier-free immobilized enzymes such as crosslinked enzyme crystals (CLECs). These do not lead to extra inactive mass, as a solid carrier does, which decreases the volumetric activity. One of the carrier-free immobilization methods is crosslinked enzyme aggregates or CLEAs [52, 58]. This method includes changing the hydration state of an enzyme or altering the electrostatic constant of the enzyme solution by adding appropriate aggregation agents. The soluble enzyme precipitates as insoluble aggregated enzyme with native confirmation retained. The next step is crosslinking the aggregated enzyme by adding a crosslinking agent. CLEAs need highly purified protein and thus a labour-intensive recrystallization step [48]. Using the glutaraldehyde immobilization technique described in this thesis, the enzyme is immobilized directly by adding the crosslinking agent and without need of crystallization.

In crosslinking techniques, enzymes are immobilized using a bifunctional agent such as glutaraldehyde. This technique is simple and yet provides fairly strong chemical crosslinks between enzyme molecules, depending on the pH. The main drawback however is the loss of enzyme activity due to distortion of the active enzyme's conformation during crosslinking [40, 53, 57, 59].

In the present study, enzyme immobilization was conducted by using the crosslinking technique. Chymotrypsin, the enzyme used for the study, was immobilized (i.e., insolubilized) using glutaraldehyde as a crosslinking agent. Chymotrypsin has been immobilized on several sorbents according to the literature [60-65]. Using an immobilized protease overcomes the previously discussed drawbacks. Additionally, this method improves the stability of the enzyme and offers the benefit of reusability. Since the enzyme stability is promoted in this method, a highier enzyme-to-substrate ratio can be used and it diminishes the autoproteolysis significantly [41, 53, 66-71]. One of these methods, glutaraldehyde (GA)-mediated crosslinking, has been used for the research described in the present thesis and is thus discussed further.

Glutaraldehyde is one of the most common protein crosslinking agents. It is an inexpensive and commercially available agent. GA exists in several forms (monomerique and oligomerique) in aqueous solution which can all react with lysine residues (the ε-amino group) of proteins. Most proteins contain several lysine residues that are usually located on the protein surface because of the polar nature of the amine group. The lysine residues in chymotrypsin are not involved in its catalytic site, which allows GA crosslinking to maintain the enzyme's conformation and therefore its biological activity. One of the common mechanisms proposed for crosslinking is between an aldehyde group of GA and an ε-amino group of lysine in the enzyme (Figure 1.5) [50]. In the present study, we used GA-mediated crosslinking of chymotrypsin. The exact mechanism of GA reaction with a protein's amino groups is not clearly understood and a large number of mechanisms have been reported in the literature [50]. It seems that more than one mechanism could be responsible for GA crosslinking with chymotrypsin, leading to a range of conjugates, but this aspect was not studied in the current work.

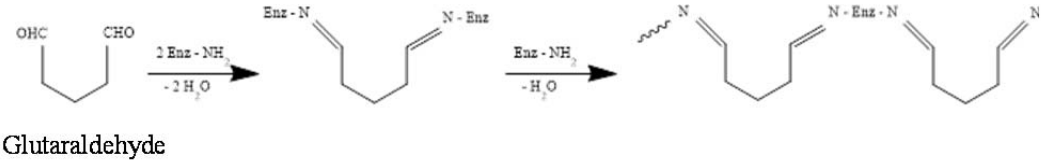

Figure 1.5. One of the common crosslinking reactions between glutaraldehyde and an enzyme, were Enz-N represents a lysine ε-amino group of the enzyme[50].

Although we expect a partial enzyme inactivation because of the crosslinking, sufficient enzyme activity was retained in most of our experiments. Additionally, our GA-crosslinked enzyme generally shows a good stability and can be recovered and reused, as despcribed in Chapters 3 to 6.

Immobilized enzyme can be used in batch form or in flow systems such as immobilized-enzyme reactors (IMERs). An immobilized enzyme reactor (IMER), usually in the format of a flow-through cartridge, can be prepared from various stationary phases and offers reduced cost of solvent and solution in the case of expensive enzymes or low amounts of substrate [72]. Since IMERs can operate in a fluidic style, they can be coupled with micro-column protein purification upstream and with high sensitivity peptide mapping methods downstream for protein characterization. Using the IMER also makes automation of digestion easier. The IMER is usually coupled with other instruments such as capillary electrophoresis [32, 73-76], HPLC [77, 78] or mass spectrometry [27, 72, 79, 80].

The on-line digestion of proteins can be automated and therefore faster protein identification made. By using this method, manual sample handling can be greatly reduced because the reactor can also be coupled on-line to separation and detection schemes, with the advantage of automation being especially important in microfluidic systems.

1.2.3. Immobilized Enzyme Reactor (IMER)

Miniaturising devices for chemical analysis has been a rapidly growing trend in recent years [81-83]. Small sized immobilized enzyme reactors (IMERs) are not exceptions. Several studies have been conducted on IMERs for proteomics studies. Since the analytical

performance of an IMER is directly affected by the immobilization process, many studies have been done to develop successful immobilization strategies to advance sensitivity and stability of IMERs. The best method for enzyme immobilization depends on whether higher sensitivity or more stability is desired for each application. Other factors such as reproducibility, cost and difficulty of the immobilization process need to be considered [56].

Several IMER preparation techniques have been reported in the literature, using most often either a packed column or by derivatizing the enzyme on the inner wall of an open tube capillary. Bonneil *et al*.[40, 84] used enzyme-bound beads which is one of the popular methods because of its simplicity. They used CPG-Trypsin packed inside the IMER and digested β-casein and insulin chain B as substrate. Although the separation efficiency was poor because of valve design for the on-line method, the system was reproducible in terms of migration time [40, 84, 85].

One of the first studies on trypin immobilization coupled to CE separation was performed by Kuhr's group [86]. They demonstrated that trypsin can be immobilized onto the inner wall of a fused-silica capillary to produce an open tubular heterogeneous enzyme reactor. Shan *et al.* [87] used δ-gluconolactone as an enzyme to prepare an IMER. Their preparation method took about 10 h and needed high temperature (180 °C) and nitrogen gas. Although they prepared the IMER, they only used it to separate peptides and DNA markers and not to digest substrate. Another IMER was described by Krenkova *et al.* [88] using TPCK-treated trypsin and pepsin A as the enzymes. They attached the enzyme on the inner wall of a capillary. The IMER preparation method they used needed more than 30 h, 100 °C and nitrogen gas where cytochrome C and β-casein as substrates were passed through the IMER using pressurized nitrogen. They only used the native protein and not denatured substrate. Another study was conducted by Wood *et al.* [89] who also used TPCK-trypsin and had a long IMER preparation time of about 35 h and needed an oven to reach 110 °C. They digested Nα-benzoyl-L-arginine ethyl ester (BAEE) and measured the ratio of Nα-benzoyl-L-arginine (B-Arg) peptide from substrate to internal standard of Nα-Z-L-arginine (Z-Arg) in order to calculate the enzyme activity. The above studies only represent a fraction of those reported for proteolytic IMERs in the past 20 years.

In the methods mentioned above, the IMER preparation took a long time and needed instruments other than the CE itself, such as an oven and nitrogen gas. In our study, we attempted to simplify these steps and used only a common CE system with the standard injection and flushing pressures. We used a temperature as high as was possible in the CE itself, and not a complementary oven for IMER preparation. The simplified technique helps to achieve automation with less complexity and fewer steps. Additionaly, we used denatured substrate as well as the native forms to study the effect of denaturing agents on the IMER digestion by GA-crosslinked chymotrypsin.

1.3. Chymotrypsin as the Protease

In principle, any enzyme can be immobilized for a vast range of applications. Since the current study is focused on application to proteomics, only the proteases trypsin and chymotrypsin are discussed below.

Several immobilized proteases have been reported for proteomics studies, ed such as pepsin [90], trypsin [42, 72, 86, 91-94], and chymotrypsin [53, 64]. Although the Waldron research group had used trypsin for previous studies, chymotrypsin has been used in the past few years for reasons described below. The highly specific cleavage of peptide bonds by trypsin at the C-terminal side of residues lysine and arginine in a substrate [72] indicates that these two amino acids must be “available” in their native state to be recognized by the enzyme’s active site. On the other hand, our group was interested in using the ultra-high sensitivity of CE-LIF-based peptide mapping as a means to characterize the efficiency of glutaraldehyde (GA)-crosslinked enzyme for low substrate concentrations. Since many fluorescently labeled proteins have been derivatized at the ε-amine group of lysine residues, trypsin will not cleave the protein here. [95-100]. Therefore, trypsin cleaves the labeled substrate only at its arginine sites,, which is a less abundant residue in many proteins. Because of this drawback for using trypsin, chymotrypsin was selected as an alternative to allow characterization of immobilized enzyme to benefit from using CE-LIF for peptide mapping.

Chymotrypsin cleaves the substrate on the C-terminus of phenylalanine (Phe), tyrosine (Tyr), and tryptophan (Trp) residues [101, 102]. An advantage of this is that all chymotryptic peptides contain one aromatic amino acid which allows their detection by UV-absorbance, although the molar absorptivity of the peptide is low. Chymotrypsin is a 25 kDa protease with a single polypeptidic chain of 245 amino acid residues. It has been used in food industry and exerts pharmaceutical properties [103, 104]. For example, protein hydrolysis using chymotrypsin enhances nutritional characteristics in food products and reduces allergic properties and bitterness. Since large amounts of this enzyme are required for industrial purposes, its recovery and reuse is documented [105]

In the present study chymotrypsin was immobilized using GA with a similar procedure developed previously in our lab for trypsin [51, 106].

1.4. Research Objectives and Thesis Structure

A key step in obtaining proteomics information includes enzymatic digestion of protein into smaller peptides, which is the “bottom-up” approach [107]. The enzyme can be used in immobilized form, which offers several benefits including its reusability, reducing its autoproteolysis and, therefore, the possibility of using higher enzyme-to-substrate ratios [36]. Immobilized enzymes can be used batch-wise or in flow systems such as IMERs [85] and various studies have been conducted for both formats [36, 49, 51, 84]. Nevertheless, there is a need for additional investigation on immobilizing enzymes by GA, especially in micro-reactors, and studying their effectiveness in digesting different sizes of substrates.

Therefore, the general hypothesis that guided the current work was to develop and characterize a simple and rapid *in situ* enzyme immobilization procedure for GA-crosslinked enzymes to make a microreactor (IMER) compatible with microcolumn separations and mass spectrometry (MS). The novelties of this approach includes using chymotrypsin, which provides cleavage sites different of those trypsin and facilitating digestion of fluorescently labeled proteins, making an *in-situ* IMER using chymotrypsin, and digesting different substrates and separating the digests. The objectives of the present study include the short term goal of making carrier-free immobilized enzyme preparations

and an IMER by using GA as a crosslinking agent. The long term goal is finally coupling the IMER with microcolumn protein purification upstream and with high sensitivity peptide mapping methods downstream for protein characterization.

Using polymers and other supports as enzyme carriers lead to “dilution of activity” of enzyme due to the presence of a large amount of non-catalytic mass. Compared with immobilizing the enzyme on a solid support, the GA-based crosslinking method has the advantage of higher volumetric activity because of the absence of extra inactive mass, e.g., the carrier [48]. It also eliminates the necessity of packing micro-columns with enzyme-bound beads. The GA crosslinking immobilization method in bulk format, its optimization, and the effect of buffers are examined in Chapter 3. The preparation of substrate is also discussed. In order to achieve a more complete digestion, denaturing the substrate is needed to expose more residues and subsequently make more cleavage sites accessible to enzyme, especially when a large folded protein substrate is used [108]. BSA was chosen as a large model substrate, which was first denatured, reduced and alkylated and then digested using GA-immobilized chymotrypsin.

In the present study, we used chymotrypsin as the enzyme instead of the most commonly used enzyme in proteomics: trypsin [40, 84, 88, 89]. When substrate concentration is low, peptide mapping by CE-UV can be substituted by CE-LIF for fluorescently labeled substrates digested with chymotrypsin, as described above in Section 1.3. Since chymotrypsin cleaves the substrate at residues Phe, Tyr and Trp, fluorescence labeling at lysine does not interfere with the digestion. Digesting fluorescently labeled substrates using immobilized chymotrypsin as well as comparing GA-immobilized to soluble chymotrypsin are discussed in Chapter 4. We also show that immobilzed chymotrypsin can be used to digest denatured BSA after being stored wet (i.e., in solution) or dried, where the digests were mapped using CE-UV. Of most significance in Chapter 4 is the first example of making our *in situ* IMER based on GA crosslinking of chymotrypsin. We describe how chymotrypsin is immobilized in a fused silica capillary using the CE system to automate reagent deliver. Then myoglobin, as a medium sized substrate, is digested in the IMER and the digests mapped using CE-MS. This latter technique is described briefly in Chapter 2.

In Chapter 5, the digestions of substrates of different sizes using the IMER are discussed. Various times and pressures were applied using the automated CE system to pass the substrates through the IMER. Peptide mapping of the digests was made using CE-UV and/or HPLC-MS. We also conducted certain experiments to study the effect of denaturation agents on the IMER digestion of BSA, a large model substrate.

In order to better understand the extent of immobilization of enzyme in the IMER capillary, a fluorescent microscopy imaging study was done, as described in Chapter 6. The IMERs were prepared in two lengths, 3 cm and 43 cm. A fluorescein isothiocyanate solution was passed through the IMER at various stages of fabrication and the confocal laser scanning microscopy (CLSM) images show its attachement to the enzyme and/or the amine-functionalised capillary wall. The CLSM system is shown in Chapter 2.

Chapter 7 presents a summary of the results obtained from chapters 3 to 6 and puts them into prespective with respect to the short term goals of the research and to other studies already published. Future experiments are proposed to improve on the results obtained and to work towards the long term goals.

Chapter 2. Analytical Techniques

Several analytical techniques were used in the present study to perform enzyme immobilization, digestion, peptide separation (mapping) and identification. These techniques are described in the present chapter. Separations were mostly performed by capillary electrophoresis (CE) using ultraviolet (UV) absorbance detection, CE with laser induced fluorescence (LIF) detection, and CE coupled to a single quadrupole mass spectrometer (MS). To investigate the crosslinked enzyme inside the immobilized enzyme reactor (IMER) capillary, a confocal laser scanning microscope (CLSM) was used to visualise fluorescence labeling of the enzyme.

2.1. Capillary Electrophoresis

CE is a high resolution low solvent consumption analytical separation technique that is powerful enough to give good peptide maps using UV detection because peptide bonds absorb UV. However, the limit of detection (LOD) is high and it is challenging using solutions with very low concentrations [40, 109]. On the other hand, CE coupled with laser induced fluorescence (LIF) can provide high sensitivity detection for fluorescent analytes [110, 111]. CE is somewhat more difficult to interface with MS due to limitations including the need of a volatile separation buffer and a low flow regime. This is because the CE flow rate is lower than the electrospray ionisation (ESI) make-up flow, which needs to be at the range of several μL/min. However, CE-MS offers the benefit of accurate peptide identification for known proteins in the many proteomic databases [20, 28, 112, 113].

The CE technique was first described in 1967 by Hjerten [114]. The technique became well known in the 1980s when Jorgenson and Lukacs showed that using CE could increase the resolution of liquid phase separations to over 100,000 theoretical plates[115]. CE has been used successfully in separation and analysis of various molecules such as vitamins, drugs, peptides, proteins, nucleotides and nucleic acids [22, 40, 42, 45, 50, 96, 116]. Some inherent characteristics of CE include advantages such as small sample size, rapid analysis, low solvent consumption, high resolution and high efficiency of analysis. These advantages have made CE an important complementary technique to HPLC for instrumental analysis in

chemistry, biochemistry and clinical laboratories. Due to these advantages, CE has been widely used in several proteomic studies [40, 88, 117].

CE is performed in a fused silica capillary normally coated by polyimide on the outside for protection and flexibility (Figure 2.1). The capillary length usually varies between 20-60 cm and the inner diameter (ID) is between 50-250 μm. The capillary is filled with an appropriate electrolyte that exerts sufficient buffer capacity. The sample injection is performed at the capillary inlet by either hydrodynamic pressure of 5-50 mbar or electrokinetically by applying a voltage of 1-5 kV, for a few seconds in both cases. The injection could also be made by applying a vacuum at the capillary outlet. After sample injection, both inlet and outlet of the capillary are immediately immersed into vials containing about 1 mL buffer known as the background electrolyte (BGE). Subsequently, a separation voltage of 10-30 kV is applied to induce electromigration of charged analytes injected at the inlet toward the outlet. In buffers of pH > 2, the silanol groups (Si-OH) on the surface of the inner wall of the capillary create a net negative charge. An adsorbed layer of cations from the BGE forms here, as well as a second diffuse layer, which make up the electric double layer composed of BGE ions. When a voltage is applied acoss the capillary, bulk flow of BGE, which moves from the positive (anode) to the negative end (cathode) and is called electroosmetic flow (EOF), occurs. The bulk flow of BGE is due to the electrical double layer's cationic nature being attracted to the cathode. The strong EOF insures that the analyte is directed toward the outlet passing the detector, which is placed near the capillary outlet where the cathode is. The analyte ion's intrinsic velocity, typically in 0.1-1 mm/s range, depends on the charge and size of the ion. The net migration velocity of the analyte depends on its electrophoretic mobility and additional factors such as the electroosmotic flow (EOF), and all of these depend on pH and viscosity of the buffer solution and the possible interaction of the analyte with capillary walls [22, 116, 118-120].

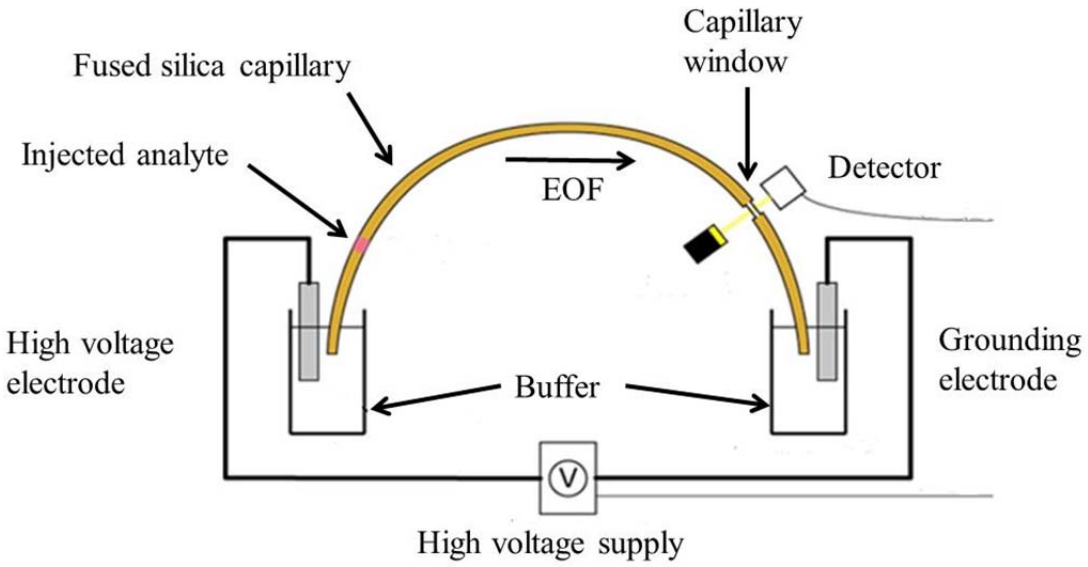

Figure 2.1. Schematic of capillary electrophoresis instrumentation.

Two CE instruments were used during the course of the research presented in the present thesis. The first was an Agilent HP^{3D}CE system (Agilent Technologies, Waldbronn, Germany) equipped with a UV/Vis diode array detector, although detection was generally monitored at 200 nm. For certain experiments this CE was coupled with MS (MSD SL System, Agilent Technologies, Santa Clara, CA, USA). More information about CE-MS is described in the next section. The second system was a Beckman P/ACE MDQ system (Beckman Coulter, Fullerton CA, USA) equipped with a 3 mW argon ion laser having an excitation wavelength of 488 nm and a 520 nm wavelength band-pass emission filter was for CE-LIF experiments.

2.1.1. Peptide Mapping by CE-UV for digests obtained by using immobilized chymotrypsin pellet

Peptide mapping of digested substrate was performed on the Agilent CE System (Agilent Technologies, Waldbronn, Germany) equipped with a UV/Vis diode array detector (DAD). The electropherograms were recorded at 200 nm. Separations were performed at

+15 kV at 25 ^{0}C in a fused silica capillary (43 cm total length, 75 µm id). The BGE consisted of 50 mM sodium phosphate, pH 2.5. All buffers were prepared in Milli Q water and were filtered through a 0.22 µm nylon syringe filter (Chromatographic Specialties, Brockville, ON, Canada) prior to use.

2.1.2. Peptide Mapping by CE-UV for digests obtained by using IMER

Peptide mapping of substrate digests from the IMER was performed on the Agilent CE System with the same capillary dimensions and applied voltage as described above in Section 2.1.1. The following buffers were prepared to study the effect of buffer on separating IMER digests: 25, 50, and 75 mM sodium phosphate, each at pH 2.5, 4.4 and 6.8; 50 mM ammonium bicarbonate at pH 4.4 and 6.9; 25, 50, and 75 mM sodium borate at pH 8.6. All buffers were prepared in Milli-Q water and filtered as above.

2.1.3. Peptide Mapping by CE-LIF

CE-LIF peptide mapping was performed on a Beckman P/ACE MDQ instrument (Beckman Coulter, Fullerton CA, USA) equipped with a 3 mW argon ion laser with an excitation wavelength of 488 nm and emission wavelength of 520 nm. Separations were performed at +20 kV, and 25 °C, in an uncoated fused silica capillary (43 cm total length, 75 µm I.D.). The BGE consisted of 50 mM sodium phosphate with pH 7.0 was chosen based on previouse study by S-M Gan [121]. All buffers were prepared in Milli Q water and filtered as above.

2.2. Capillary Electrophoresis-ElectroSpray Ionization / Mass Spectrometry (CE-ESI/MS)

Coupling CE to MS provides a sensitive, rapid and powerful technique that has been used since late 1980s. Many research and review articles have been published regarding the use of CE-MS particularly for proteomic studies [98, 112, 122-128], even though the

technique has not been nearly as popular as HPLC-MS. There are several ionization techniques reported for coupling CE with MS, including atmospheric pressure chemical ionization, thermospray ionization and MALDI. However, electrospray ionization (ESI) is by far the most popular ionization technique [113]. ESI is a primary choice for coupling CE to MS since it allows the detection of large molecules by generating ions with multiple charges. Using a commercial nano-spray interface, the analyte is transferred from CE buffer to gas phase ions by ESI [129]. More recently, improved and simplified electrospray interfaces have been reported [130-132] and CE-MS technique become more popular.

Despite the new CE-MS interfaces, most reported analyses have been conducted using commercially available interfaces with a sheath liquid, usually for nanospray HPLC-MS, and their robustness is not always ideal [53, 125]. In the present study, we used CE-ESI-MS to separate and identify peptides after protein digestion with GA-immobilized chymotrypsin. All analyses were performed on an Agilent single quadrupole MS (MSD SL System, Agilent Technologies) interfaced with an orthogonal sheath flow ESI-MS sprayer ion source (Agilent Technologies, Figure 2.2 A and B). The Agilent HP^{3D}CE system was coupled to the ESI probe using a specially designed CE cassette to allow the capillary to exit the CE instrument without losing too much of the temperature thermostatting. All separations were performed at 25 °C.

The flow splitter was made using 21 cm of blue PEEK tubing bringing solvent (sheath liquid) from the pump to a Tee connection. The Tee connector splits the flow between a 75 µm ID, 35 cm long capillary going to the nebulizer carrying the sheath liquid, and a 250 µm ID, 56 cm long blue PEEK tubing going back to the bottle to recycle the unused buffer (Figure 2.3 A and B).

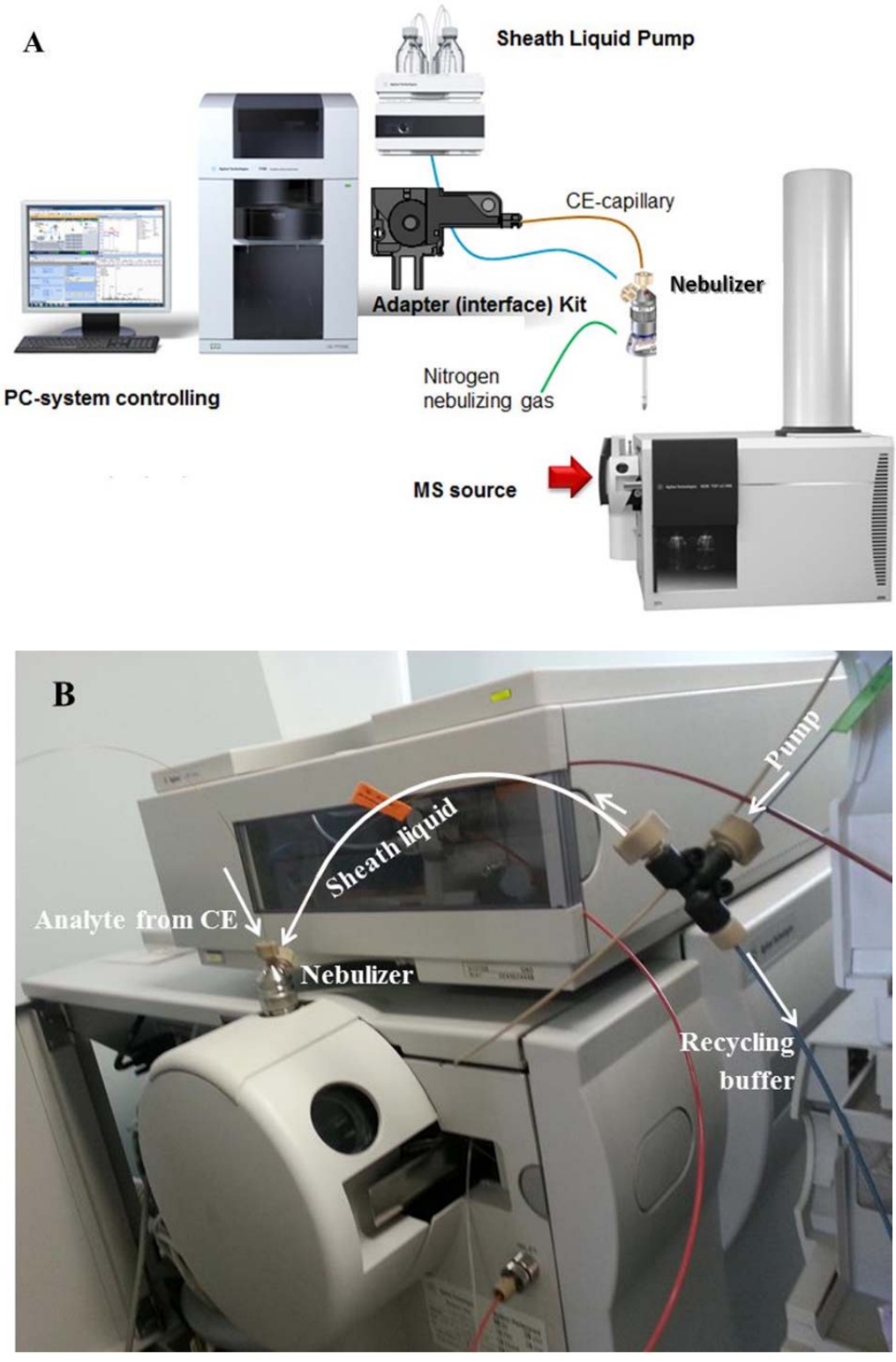

Figure 2.2. A) Schematic of CE coupled to an ESI-MS via the interface. B) A photo of the ESI-MS flow splitter used to connect the CE capillary outlet to the MSD for peptide mapping.

The BGE pH is important since it determines the degree of protonation or deprotonation of liquid phase and, therefore, affecting the charged ions in the gas phase. The BGE was composed of 10 mM ammonium acetate at pH 6.7. The sheath liquid mixes with the CE electrolyte solution at the CE outlet tip to provide the electrical contact for the CE capillary and spraying tip. It also offers sufficient flow rate for ESI and, therefore, improves the electrospray stability. Sheath liquids should be compatible with BGE and volatile, exert low surface tension to facilitate droplet break-up in the ion evaporation process, and be sufficiently conductive to permit ESI [133, 134]. However, it should not be too conductive to suppress the ionization of analytes by the competing ions in the droplet. Typically, the sheath liquid has high content of non-aqueous solvent with low surface tension such as alcohols, and a volatile electrolyte such as ammonium acetate or formate, acetic acid, or formic acid [135, 136]. The sheath liquid used in the present study was 0.1% formic acid in 50% methanol/water, with the flow rate set at 10 μL/min. In order to achieve this low flow rate, a sheath flow splitter was used to decrease the isocratic Agilent LC pump flow by a factor of 25 from 250 μL/min. The sheath flow running in blue PEEK tubing from one side and the analyte in BGE running in capillary from the other side, go to the nebulizer and are sprayed to the MS (Figure 2.2). Both BGE and sheath liquid affect the transfer of analyte ions from the liquid to the gas phase.

Nitrogen as a nebulizing (drying) gas assists the ion evaporation process in ESI. The nitrogen gas for nebulization was regulated at 130 °C and 170 kPa to provide a flow rate of 10 L/min to facilitate the desolvation.

To connect the CE to the ESI source, the outlet of the separation capillary was placed in the ion source as an emitter tip (Figure 2.3). To increase the stability of the spray, about 1 cm of the insulating polyimide coating was removed from the capillary tip and the glass was cut carefully. The tip of the capillary should extend beyond the nebulizer tip slightly. The optimum length that yielded the best results was about 2 mm (Figure 2.3).

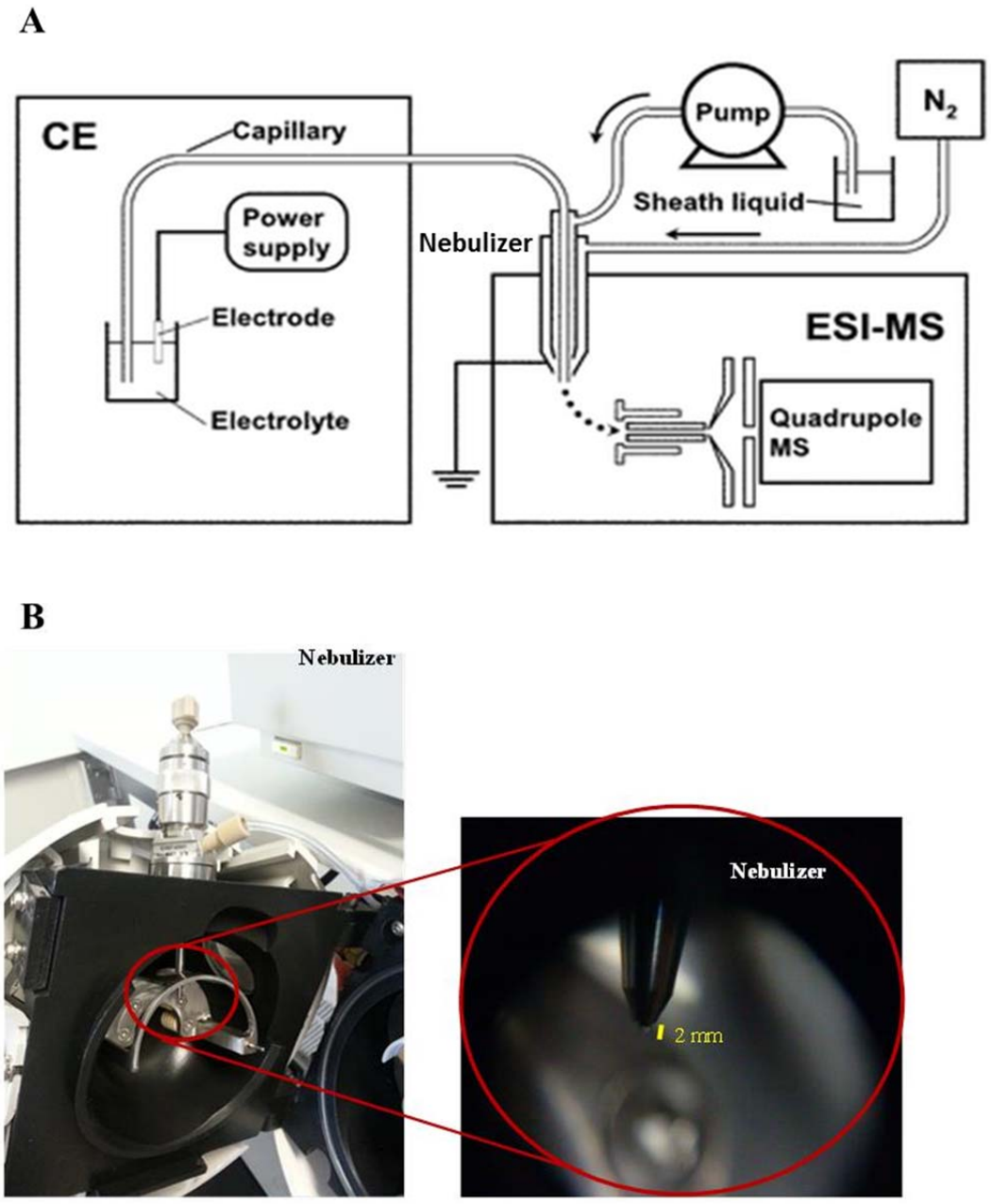

Figure 2.3. A) Schematic presentation of CE connected to ESI-MS showing analyte from CE, sheath flow, and drying gas going to nebulizer and then MS [137]. B) ESI nebulizer (left panel) showing the outlet of the CE capillary placed slightly beyond the end of the nebulizer tip (right panel).

For peptide mapping by CE, a low pH buffer is preferred to render all peptides cationic, thus a better detection sensitivity is achieved for small positively charged peptides. Therefore, the MSD was operated in positive ion detection mode. The MSD parameters were set to: 200 to 1000 m/z mass range, fragmentor was set at 65 V, detector gain at 3, cycle time of 0.97 s per cycle. Single ion monitoring (SIM) mode was used for 50% of a cycle time.

2.3. Confocal Laser Scanning Microscope (CLSM)

Confocal laser scanning microscopy is a well-known technique used for high resolution or 3D imaging [138-140]. One of the advantages of confocal microscopy over a wide-field microscope is its ability to produce imaging for thick samples at different depths. The CLSM was developed in 1957 [141] and became more popular years later, owing more popularity to the progress in laser technology and computer science power [142]. CLSM imaging is point by point and, instead of projecting through an eyepiece, the image is processed by a computer [143]. A laser or arc-discharge source is usually used for excitation due to its high output power that improves fluorescence emission intensity. The detector is usually a photomultiplier tube (PMT) which has high quantum efficiency in the near-UV, visible and near IR.

In the present study, a Leica TCS SP5 II CLSM equipped with point laser sources controlled by high speed acousto-optic tuneable filters was used (Figure 2.4). The excitation sources include a helium neon (λ=633 nm), diode-pumped solid state (DPSS: λ=561 nm), and argon ion (λ=458/488/514 nm) laser. The source used for the studies presented in Chapter 6 was the 488 nm argon ion line, since we used fluorescein isothiocyanate (FITC) as our fluorescent label. The emission was measured at 500 nm.

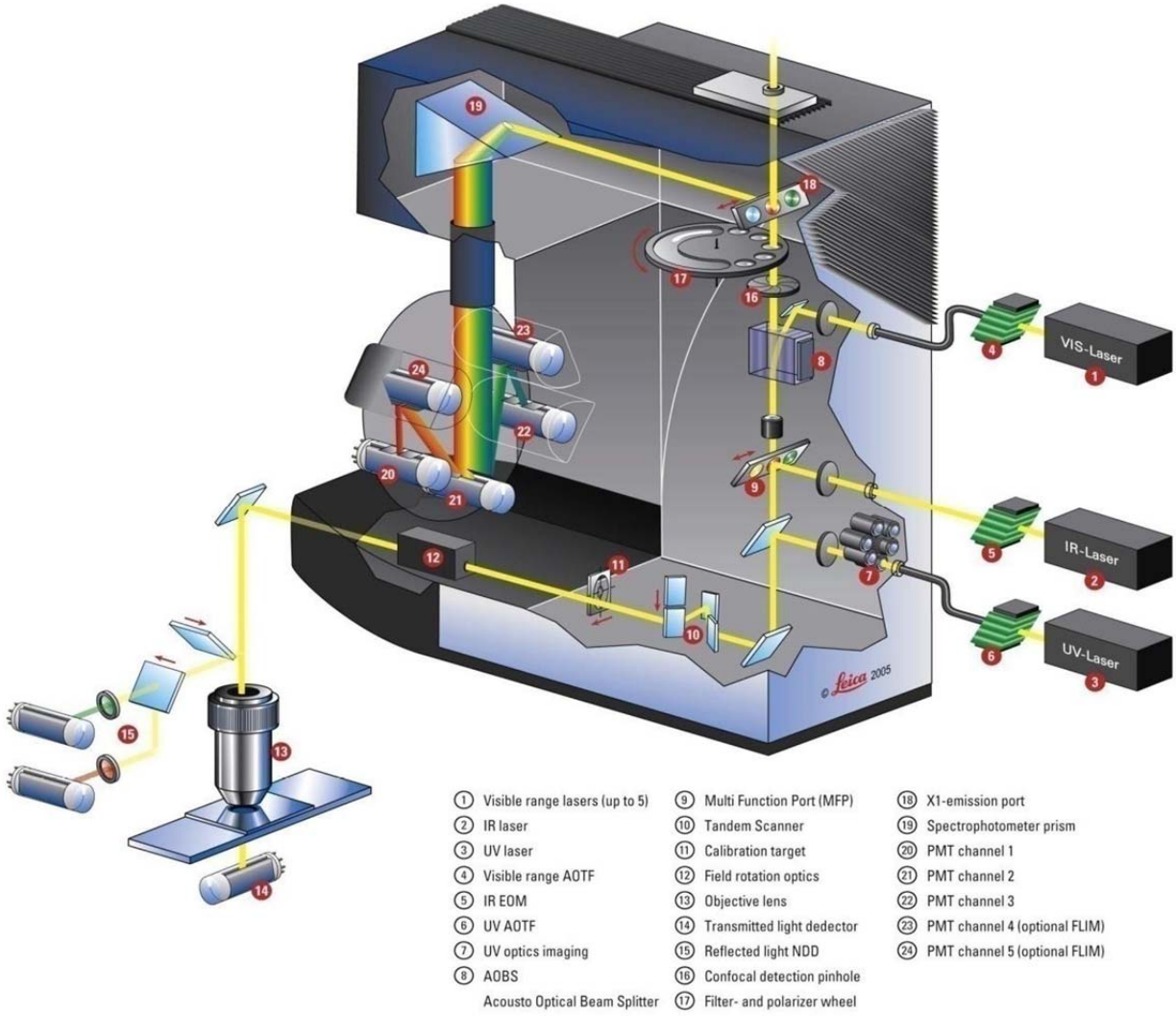

Figure 2.4. A schematic presentation of confocal laser scanning microscope (Leica TCS SP5).

Chapter 3. Capillary Electrophoretic Peptide Mapping to Probe the Immobilization/Digestion Conditions of Glutaraldehyde-crosslinked Chymotrypsin

A version of this chapter has been submitted[1] on April 29, 2015, to *Current Analytical Chemistry* with authorship by Golfam Ghafourifar and Karen C. Waldron

[1] The manuscript, accepted during thesis corrections, will be published as *Curr. Anal. Chem.*, **2016**, *12*, 65-73.

3.1. Abstract

The use of immobilized enzymes in proteomics studies has increased over the last decade due mostly to their ease of use in fluidic systems but also because autoproteolysis is suppressed allowing use of high ratios of enzyme-to-substrate for rapid digestion. Chymotrypsin immobilization by crosslinking with 2.5% aqueous glutaraldehyde has been used to produce a gel-like agglomerated pellet of high enzyme loading capable of digesting protein. The efficiency of the immobilized enzyme for protein digestion was followed by capillary electrophoretic peptide mapping with absorbance detection at 200 nm. A study comparing preparation methods showed that making a small immobilized enzyme pellet leads to better and more reproducible digestions (i.e., peptide maps) compared to using either a five-fold larger pellet or a portion of the latter having a similar mass to the small pellet. When three different buffers for the enzyme crosslinking reaction were compared, sodium phosphate at pH 6.4 showed better batch-to-batch reproducibility of the enzyme and better long-term storage (28 days at -20°C). The immobilized chymotrypsin pellet could be used for two consecutive digestions of BSA.

3.2. Introduction

Proteomic studies, both structural and functional, play an important role in the post-genomic era, with increasing importance for diagnosis and treatment of disease. Based on estimations, human serum and plasma contain more than 100,000 proteins [144]. This complexity of samples presents a major challenge for identification and quantification in proteomics studies. Peptide mapping is a tool widely used to aide in protein identification and characterization; protein is digested using a proteolytic enzyme and the digests, composed of peptides, are separated and identified using one or more analytical methods such as mass spectrometry (MS), liquid chromatography (LC) and LC-MS, capillary electrophoresis (CE) and CE-MS, and capillary electrochromatography (CEC) [35, 111, 124, 145-149].

Compared to LC, CE has been less widely used in proteomic studies; however, it can provide valuable information about the size and charge of peptides. It offers the benefit of rapid separation, high resolution, decent sensitivity, good reproducibility with optimized rinsing, and small sample and reagent consumption [147]. Whereas the exact identification of the peptides is not conclusive using CE with UV diode array detection, it can be coupled with MS and the peptide masses used to identify the protein substrate. On the other hand, peptide mapping used as a comparative tool can be highly effective in that the peptide fingerprint can have diagnostic value. In the current study, peptide mapping of bovine serum albumin (BSA) protein by CE with absorbance detection at 200 nm was used to evaluate the efficiency of the chymotrypsin immobilization method and the effects of using different conditions for the enzyme preparation and digestion.

Typically, enzymatic digestion is carried out in solution with enzyme present at 25-50 times less than the protein substrate so that autoproteolysis peptides will be undetected in peptide maps. This practice leads to long incubation times of up to 24 h and implies a single use of enzyme. Enzyme immobilization offers the benefit of facile separation from the digest for its re-use and supressed autoproteolysis, which means a high enzyme-to-substrate (E:S) ratio can be used for faster enzymatic reaction [36, 42, 150, 151]. Immobilization may also improve an enzyme's performance by increasing its stability [152, 153] and, in some cases, enzymatic activity and specificity [154, 155]. Many different enzyme immobilization methods have been reported. These can be divided into four main categories [58]: first, binding to a carrier, for example magnetic particles [38, 39] or glass beads and polymers as a support [42-44, 156]; second, encapsulation using a polymer [45]; third, entrapment using sol-gels [46, 47]; and forth, by crosslinking of enzyme molecules [48-53]. Enzyme crosslinking is a carrier-free method and, in comparison with binding to a solid support or encapsulation, can potentially contain a high proportion of active enzyme [51, 52].

One of the most common protein crosslinking agents is glutaraldehyde (GA), which is commercially available and inexpensive. Crosslinking occurs between the tertiary amine groups of the enzyme's lysine residues and the two aldehyde functional groups of GA [50]. Glutaraldehyde has been studied by our group as a crosslinking agent for trypsin and

chymotrypsin [41, 51, 53], specifically to develop simple and inexpensive immobilization methods for proteomics applications and enzyme microreactors. In this study, chymotrypsin was immobilized using GA-mediated crosslinking, which produces a soft, spongy polymeric particle resembling an agglomerated pellet, and the effects of certain enzyme preparation parameters on the immobilization and digestion procedure have been investigated. Specifically, the amount of immobilized enzyme (the pellet's relative size), the effect of three different buffers used during the immobilization reaction, the effect of digestion time, and the effect of denaturing the substrate have all been evaluated by comparing the quality of the CE-based peptide maps of BSA. The potential for re-usability of the GA-crosslinked chymotrypsin was also assessed. The current immobilization method differs significantly from that used to prepare crosslinked enzyme aggregates (CLEAs) for industrial scale catalysis [52, 58] in which the enzyme is first precipitated and then the crosslinked product is dried and crushed to a powder for use.

3.3. Experimental

3.3.1. Reagents and Chemicals

α-Chymotrypsin from bovine pancreas type II, glutaraldehyde (GA; 25% aqueous solution), bovine serum albumin (BSA), monobasic sodium phosphate, dibasic sodium phosphate, tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), 2-amino-2-(hydroxymethyl)- 1,3-propanediol (Tris base), phosphoric acid, dithiothreitol (98% purity), ammonium phosphate and ammonium bicarbonate were purchased from Sigma Aldrich (Oakville, ON, Canada). Calcium chloride, sodium hydroxide, sodium acetate, sodium chloride, glycine, urea, iodoacetamide and acetic acid were purchased from Anachemia (Montreal, QC, Canada) and used as is. Methanol was purchased from BDH (West Chester, PA, USA). Hydrochloric acid was from EMD Millipore (Gibbstown, NJ, USA). Fused silica capillary tubing for CE separations (75 μm I.D., 375 μm O.D.) was from Polymicro Technologies (Phoenix, Arizona, USA). A multi-cartridge Milli-Q filtration/deionization system (Millipore, Bedford, MA, USA) was employed to purify the distilled water used in preparation of all solutions and buffers.

3.3.2. Substrate Denaturation

The protein substrate chosen for all tests was BSA, a large protein with 17 disulfide bridges. Denaturation was carried out using common methodology as follows: 8 mg BSA was dissolved in 800 µL ammonium bicarbonate solution (0.4 M, pH 8.0) containing 8 M urea. Reduction of disulfide bonds was achieved by adding 120 µL dithiothreitol (45 mM in water) to the substrate solution and reacting at 50 °C for 15 min. The solution was then cooled to room temperature, and then 120 µL of iodoacetamide (100 mM in water) was added to prevent thiol re-oxidation and reforming of the disulfide bridges between cysteine residues in the substrate. The solution was diluted with Tris-HCl (100 mM, pH 7.8) containing 10 mM calcium chloride to a final concentration of 2 mg mL^{-1} BSA.

3.3.3. Preparation of Immobilized Chymotrypsin

The following procedure was adapted from a previous method for trypsin crosslinking and preliminary studies using chymotrypsin [51, 157]. In a 1.5 mL microcentrifuge vial, 200 µL chymotrypsin stock solution was diluted to 0.15 mM using 50 mM phosphate pH 6.4. Then 390 µL GA, diluted to 2.5% (v/v) in water, was added drop-wise to the enzyme solution, representing a large molar excess over the chymotrypsin (260 nmol). The immobilization reaction was allowed to proceed without stirring for 2 h at room temperature. The solution was then centrifuged at 3000 rpm for 2 min and the supernatant was decanted off leaving a soft, gel-like mass of agglomerated particles that settle to resemble a large pellet. The insoluble product was gently washed to remove unreacted enzyme and excess GA with 3 × 1 mL buffer, then 3 × 1 mL NaCl (500 mM in water), and finally with 1 ×1 mL buffer, each time by decanting. The decanted washings were kept for further analysis to study the washing effect on removing un-bound chymotrypsin from the GA-crosslinked enzyme product, which is referred to herein as the pellet. Finally, 1.0 mL glycine (200 mM in phosphate buffer) was added to react with any remaining free aldehyde groups for 3 h at room temperature. The final product was washed with 3 × 1 mL buffer followed by 3 × 1 mL Milli-Q water. The immobilized chymotrypsin was stored at -20 °C in 1 mL Milli-Q water.

A small-scale preparation of immobilized chymotrypsin was also made and involved the same procedure above but with all volumes reduced five-fold, including washes. The small-scale immobilized product, i.e., the enzyme pellet, was kept in 200 µL Milli-Q water at -20 °C.

3.3.4. Digestion of Substrate by Immobilized Chymotrypsin

For digestion using the small-scale preparation, BSA substrate was added directly to defrosted immobilized chymotrypsin (already in 200 µL water) to reach the intended E:S ratio as indicated in the Results and Discussion section. The digestion was carried out in the microcentrifuge tube for 4 h at 37 °C with gentle shaking at 50 min^{-1} using a model SK-10 shaker (BEA-Entprotech Corp, Hyde Park, MA). The substrate was typically added at a molar ratio equal to or less than the effective mass of enzyme to achieve sufficient digestion activity. The mixture was buffered by residual Tris-HCl, pH 7.8, added in conjunction with the denatured substrate. The digest solution was decanted off without prior centrifugation and analysed by CE (peptide mapping) without prior filtration, pre-concentration or desalting. These latter procedures may contribute to analyte loss and decreased method reproducibility, which could hamper our goal of comparing subtle differences in immobilized enzyme preparation and use. Digestion using the full-scale preparation was carried out in the same manner, but with removal of some storage solution first to achieve the desired E:S ratio. Digestion using 20% of the full-scale preparation was attempted, in order to have the same effective mass of enzyme as the small-scale preparation. However, it wasn't possible to weigh out $1/5^{th}$ of the gel-like pellet, and withdrawing 200 µL of a suspension of the particles after vigorously shaking the 1 mL preparation gave highly irreproducible peptide maps so this was not pursued further.

3.3.5. Effect of Digestion Time

BSA substrate solution was added as three aliquots to three separate batches of immobilized chymotrypsin made using the small-scale preparation such that the apparent

E:S ratio was 1:1 (mol:mol). The three solutions were then digested for 4, 8 and 24 h at 37 °C and 40-50 µL of digest solution was withdrawn immediately for peptide mapping by CE.

3.3.6. Effect of Buffer Composition During Enzyme Crosslinking Reaction

Enzyme immobilization is usually carried out in a buffered medium thus three crosslinking buffers were evaluated for the small-scale enzyme preparation method: 50 mM sodium acetate buffer at pH 5.5, 50 mM sodium phosphate buffer at pH 6.4, and 50 mM sodium phosphate buffer at pH 6.8. These buffers were used for the subsequent washing steps. Digestions (4 h) of denatured BSA and peptide mapping were carried out as indicated in the Results and Discussion section.

3.3.7. Effect of Substrate Denaturation on Digestion

To see if native BSA could undergo partial digestion by the immobilized chymotrypsin preparation, two fresh batches of immobilized enzyme were used to digest denatured and native (undenatured) BSA, both dissolved in Tris-HCl buffer (100 mM, pH 7.8, with 10 mM $CaCl_2$). The two substrates were also analysed by CE to identify their migration times.

3.3.8. Reusability of the Immobilized Chymotrypsin

An initial aliquot of denatured BSA was digested using the small-scale enzyme preparation and all supernatant decanted off then analysed by CE. The remaining enzyme pellet was washed with water then sodium phosphate buffer, each 200 µL × 2 times. A second aliquot of substrate was then added and the volume brought to 200 µL for the second digestion. Upon washing the immobilized enzyme after the second digestion and decantation, the pellet did not stay agglomerated and became too finely dispersed for a third use. To test for possible carry-over between the two digestions, two control experiments

were performed. For the first control, a blank substrate solution containing all denaturation reagents except BSA was "digested" by a fresh batch of immobilized chymotrypsin for 4 h at 37 °C. The supernatant was decanted off and mapped by CE. The enzyme pellet was then washed as above with water and buffer. Then, an aliquot of denatured BSA was added for the second digestion followed by peptide mapping by CE. For the second control, the opposite experiment was carried out: denatured BSA was digested first then the blank substrate solution after washing the enzyme pellet.

3.3.9. Peptide Mapping by Capillary Electrophoresis

Peptide mapping by CE was performed on an HP^{3D}CE instrument (Agilent Technologies, Waldbronn, Germany) equipped with a UV/Vis diode array detector monitored at 200 nm. Separations were performed at +15 kV at 25 °C in an uncoated fused silica capillary (43 cm total length, 34 cm effective length). The background electrolyte consisted of 50 mM sodium phosphate buffer, pH 2.5, prepared in Milli Q water and filtered with a 0.22 μm nylon syringe filter (Chromatographic Specialties, Brockville, ON, Canada) prior to use. The capillary column was conditioned at 950 mbar with 0.1 M HCl then buffer for 3 min each before injection, and then with 0.1 M NaOH and Milli-Q water for 1 min each and 0.1 M HCl for 2 min after each run. Samples were injected by applying 34.5 mbar × 5 s at the inlet followed by a plug of buffer (34.5 mbar × 2 s) to prevent the sample from diffusing out of the capillary before the high voltage is applied.

3.4. Results and Discussion

3.4.1. The Effect of Preparation Method on Immobilized Enzyme Performance

For the enzymatic digestion of proteins, the typical E:S ratio of 1:25 (weight-to-weight or mole-to-mole) [158] presumes that both reagents are in solution and the enzyme activity is high. Similar nomenclature can be used for immobilized enzymes, even though their specific activity is greatly reduced compared to the soluble form. For example, we reported that GA-crosslinked trypsin had a specific activity 4000 times lower than soluble trypsin

[51]. The mass of enzyme was determined from its immobilization efficiency (> 95%) in that study. As a result, immobilized trypsin digestions were made at an “apparent” E:S ratio of 2:1 (w/w) for 4 h at 37 °C, which was comparable to soluble trypsin digestions made at an E:S of 1:25 (w/w) for 24 h [41, 51]. The immobilization efficiency of GA crosslinking of chymotrypsin in terms of total mass of enzyme was recently determined as 94±2% [53], which translates to a quantity of 240 nmol chymotrypsin for the full-scale enzyme preparation.

Using the full-scale enzyme preparation at an apparent E:S ratio of 8:1 (mol/mol) showed almost no digestion after 4 h at 37 °C based on the CE peptide maps (data not shown). Increasing the E:S ratio to 26:1 showed a better digestion (Figure 3.1 A), however the electropherograms suggest incomplete digestion of BSA, as evidenced by the large protein peak at 14 min. On the other hand, an apparent E:S ratio of 8:1 (mol/mol) when using the small-scale enzyme preparation showed good digestion in 4 h (Figure 3.1 B) as evidenced by numerous peptide peaks of intensity comparable to the remaining undigested BSA. The poor digestion using the full-scale method might be caused by adsorption of substrate and/or product peptides on the large enzyme pellet, thus inhibiting substrate digestion and/or product release into the supernatant. Replicate CE injections are shown in Figure 3.1 as a measure of instrumental reproducibility; peak area precision ranged from 1.4 to 22% RSD measured across 12 peaks in the 3 runs from Figure 3.1 B. The 12 peaks marked with asterisks were chosen to span the elution window from 8-22 min and be confidently seen in all three runs. Migration time reproducibility was not evaluated because electropherograms can be realigned by normalisation with internal peptide standards to give very good presicsion when needed.

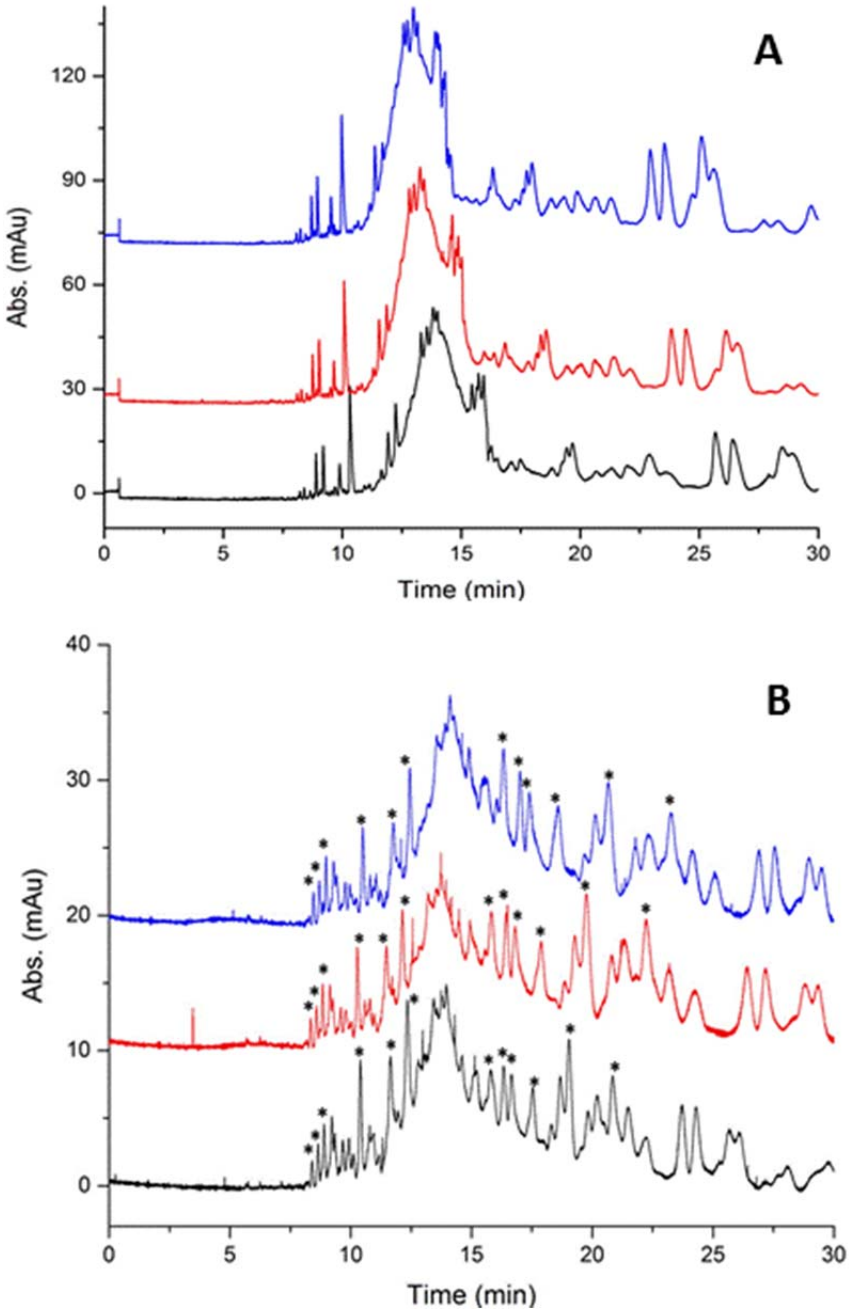

Figure 3.1. Peptide maps showing the effect of the preparation scale of immobilized enzyme on its overall performance. (A) CE-UV peptide maps of BSA (9 µM) digested by GA-crosslinked chymotrypsin prepared using the full-scale procedure and with an apparent E:S ratio of 26:1 (mol:mol); (B) CE-UV peptide maps of BSA (22.5 µM) digested by GA-crosslinked chymotrypsin prepared using the small-scale procedure and with an apparent E:S ratio of 8:1 (mol:mol). In both cases, digestion was performed for 4 h at 37 °C. Separations were performed at +15 kV in 50 mM sodium phosphate buffer, pH 2.5, with detection at 200 nm. Triplicate injections into the CE are shown for both preparation methods. Peaks with asterisks were used to calculate area reproducibility.

The wash solutions during the immobilization were reserved to monitor the efficiency of removing unreacted chymotrypsin that could induce free-solution digestion of substrate. The triplicate decanted washes for the full-scale method were pooled resulting in three samples for CE analysis: 3 mL of buffer, 3 mL of NaCl and 1 mL of buffer from the various series of washing. No chymotrypsin peak was detected in any of these. The same negative result was obtained for the pooled washes from the small-scale method. Given the modest detection limits of CE, the solutions were concentrated 10-fold by centrifugal evaporation using a Savant Speed Vac, then re-analysed. No chymotrypsin was detected in the washes from the small-scale immobilization procedure whereas a small peak for chymotrypsin was detected in the 10× concentrated washes from the full-scale preparation (Figure S3.1 in Supporting Information, Section 3.7), with decreasing amounts in successive pooled washes (Table 3.1). There was no detectable chymotrypsin in the washing steps after glycine addition, even when washes were concentrated.

Table 3.1. The pooled washes from the full-scale enzyme immobilization procedure were collected and the solutions concentrated 10 times by evaporation under centrifuge. The concentrates were injected to the CE and the chymotrypsin concentration was calculated based on a calibration curve.

	Unreacted [CT] in concentrates	**Estimated unreacted [CT] in pooled washings**
First series of washing (3 × 1 mL buffer)	0.36 μM	0.036 μM
Second series of washing (3 × 1 mL NaCl)	0.24 μM	0.024 μM
Third series of washing (1 × 1 mL buffer)	0.14 μM	0.014 μM

3.4.2. Effect of Digestion Time

Immobilized enzymes are frequently used at high apparent E:S ratios to not only accelerate the enzymatic reaction but also to compensate for the reduced activity per gram of product. Digestions on the order of a few minutes have been demonstrated with solid-phase trypsin [42, 151], which is ideal when substrates are small or when highly incomplete digestions can be tolerated, i.e., for protein identification by MS mapping. Since our previous GA crosslinking studies used 4 h digestions, this duration was retained to ensure there would be enough peptide peaks to evaluate the crosslinking conditions. With the small-scale enzyme preparation and an apparent E:S ratio of 1:1 (mol:mol), the peptide maps of BSA were essentially identical after 4 and 8 h digestion whereas 24 h of digestion resulted in around 10 more peptide peaks (Figure S3.2 in Supporting Information, Section 3.7). A 1 h digestion of BSA using the full-scale enzyme preparation at the higher E:S ratio of 26:1 was quite acceptable (Figure S3.3 in Supporting Information) but was irreproducible from batch to batch (data not shown). The more efficient digestions observed at the lower E:S ratio implies that the small-scale GA crosslinking procedure provides higher relative activity than the full-scale method.

3.4.3. Effect of Buffer Composition During the Enzyme Crosslinking Reaction

The buffer conditions, particularly pH, during enzyme immobilization influence the reaction between the enzyme and GA. Migneault [159] tested a range of buffers for the GA crosslinking procedure with trypsin, from phosphoric acid at pH 2 to sodium borate at pH 10. She showed that for pH $\leq$ 4 and pH $\geq$ 9, no insoluble product could be formed, and at pH 5 it took 150 min to make the insoluble GA-crosslinked trypsin. The fastest reactions leading to insoluble immobilized enzyme were obtained between pH 5-8, with pH 6.8 chosen for peptide mapping in those studies [159]. Accordingly, we chose to investigate the effect of crosslinking buffer on the chymotryptic peptide maps of BSA for three buffer systems: 50 mM sodium acetate at pH 5.5 and 50 mM sodium phosphate at pHs 6.4 and 6.8. Each immobilized chymotrypsin was prepared in triplicate batches for each buffer using the small-scale procedure. The washing steps were made using the respective buffers.

Digestions were carried out identically for the nine samples using nine aliquots of a single solution of denatured BSA as substrate.

By visual inspection, both phosphate buffers (pH 6.4 and 6.8) led to a more robust enzyme pellet after washing compared to using the acetate buffer at pH 5.5. In comparing the peptide maps obtained from digestions with three batches of enzyme prepared in the pH 5.5 acetate buffer (see Figure S3.4-A in Supporting Information, Section 3.7), some variability was observed in the number of peptides and their peak areas in each digest (Figure 3.2 A), which suggests that the crosslinking process and/or the washing steps were not very reproducible. The latter hypothesis is plausible and would lead to variation in the final mass of immobilized enzyme available for digestion and thus a variation in specific activity. Using the pH 6.4 phosphate buffer during crosslinking resulted in better batch-to-batch reproducibility, reflected as more reproducible peptide peak areas in the BSA digests (Figure 3.2 B) and also more peptides (Figure S3.4-B in Supporting Information) compared to using either the pH 6.8 buffer (Figure 3.2 C) or the pH 5.5 buffer during enzyme crosslinking. The quantitative differences between the three crosslinking buffers were compared by calculating the peak area RSD (n=3) per peptide per buffer system (Table S3.1 in Supporting Information) as well as the overall variation in peak area for each buffer by averaging the peak area RSDs. These results are summarized in Table 3.2, along with the number of peptides identified for each batch of enzyme used, and demonstrate that enzyme crosslinking in phosphate buffer, pH 6.4, leads to the highest and most reproducible batch-to-batch enzymatic activity as measured by BSA digestion under the reported conditions.

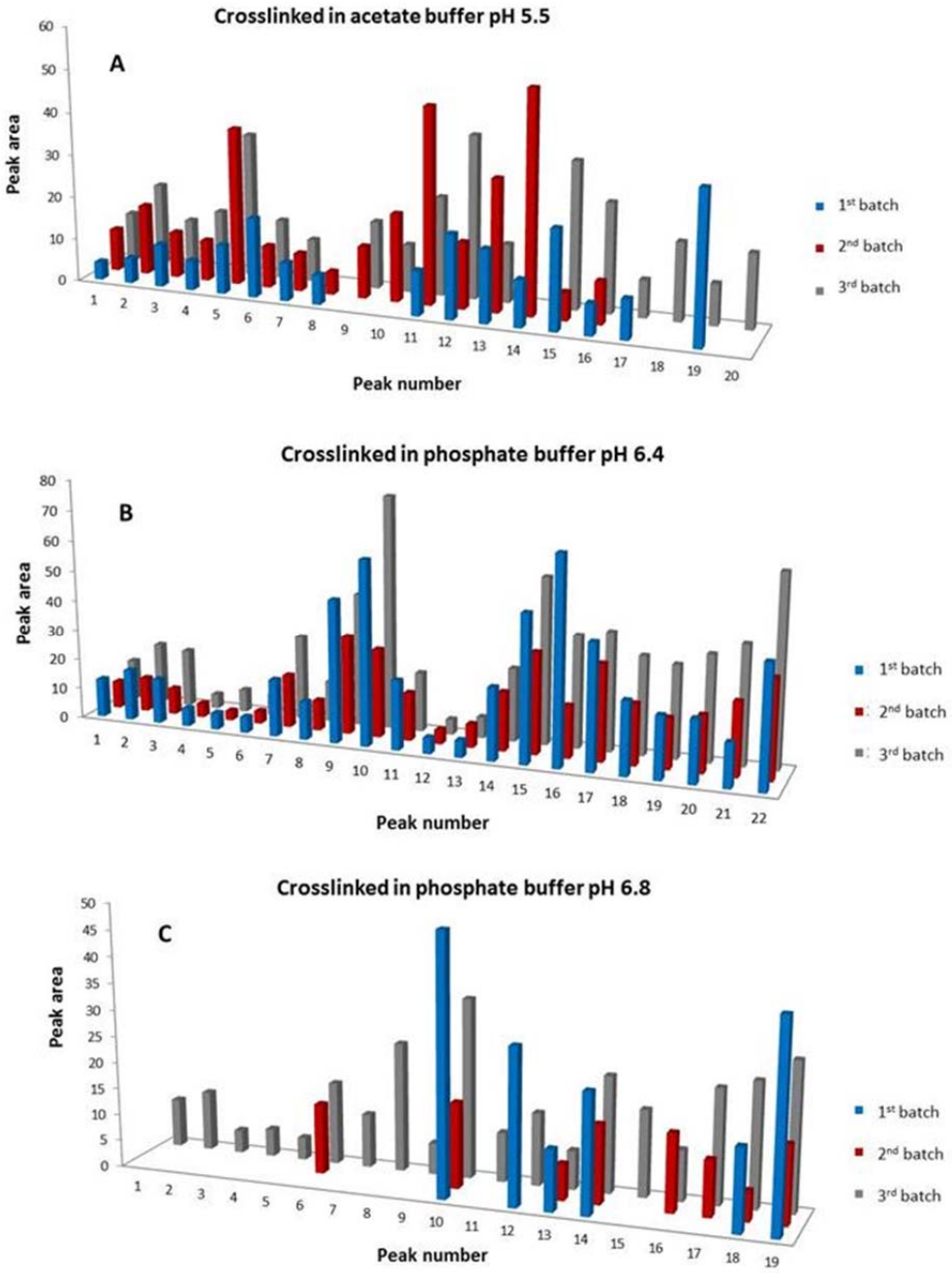

Figure 3.2. Graphs of peptide peaks in BSA digests showing batch-to-batch (n = 3) reproducibility of immobilized chymotrypsin for three different buffers tested during enzyme crosslinking: (A) acetate buffer, pH 5.5; (B) phosphate buffer, pH 6.4; (C) phosphate buffer, pH 6.8. All 9 batches of immobilized chymotrypsin were prepared by the small-scale procedure. Nine aliquots of the same substrate (BSA, 22.5 μM) were used. The E:S ratio was 8:1 (mol:mol) and digestions were performed for 4 h at 37 °C then peptideseparations were carried out as in Figure 3.1.

Table 3.2. The effect of crosslinking buffer composition on immobilized enzyme reproducibility and activity (batch-to-batch) evaluated by peptide mapping of BSA (22.5 μM) at an apparent E:S ratio of 8:1 (mol:mol). Separations were carried out as in Figure 3.1.

Crosslinking buffer	Immobilized enzyme batch	Total no. of identified peptidic peaks [a]	Overall peak area RSD [b,c]
Acetate pH 5.5	1st	16	43%
	2nd	16	
	3rd	18	
Phosphate pH 6.4	1st	22	27%
	2nd	22	
	3rd	22	
Phosphate pH 6.8	1st	6	39%
	2nd	8	
	3rd	19	

[a] Presence of a peptide molecule confirmed by diode array spectra.

[b] The raw data for each peptide peak (triplicate digestions) and calculated RSD values are given in Table S1 of Supporting data, Section 3.7.

[c] The peak area RSD arising from technical variation alone (i.e., the CE mapping method), ranges from 1.4 to 22% RSD, with an average of 11% RSD, calculated from Fig. 1 B for 12 peptide peaks (triplicate injections)

3.4.4. Reusability of Immobilized Chymotrypsin

Immobilized enzyme reusability was evaluated using the same BSA substrate solution and a single batch of enzyme prepared using the small-scale procedure. Figure 3.3 shows a decreased intensity of peptidic peaks obtained from the second digestion, likely because of loss of immobilized enzyme in the pellet during the first digestion and/or the washing steps. A third digestion was not possible as there was such extensive break-up of the enzyme pellet that the dispersed particles could not agglomerate and be separated from the wash solutions by decanting or centrifugation. To verify that the peptides observed in the second digest were the result of enzymatic digestion and not carry-over from the first digest, or the

result of chymotrypsin autoproteolysis, two control experiments were performed. In the first, a substrate-free blank solution was digested, followed by the BSA substrate with washing of the pellet in between. In the second, the BSA substrate was digested, followed the blank solution (after washing). Figure S3.5-A (Supporting Information, Section 3.7) shows some chymotrypsin autoproteolysis peaks for the blank digestion done first then the complete peptide map of BSA when the real substrate was digested in the second use of the enzyme. For the reverse experiment, Figure S3.5-B (Supporting Information) shows the expected BSA peptide map for the first use of enzyme and then only small autoproteolysis peaks for the blank digestion in the second use. These results confirm that the enzyme pellet is robust enough to withstand the washing regime after the first digestion, maintaining good activity, and also that washing the immobilized enzyme after the first digestion adequately removed peptides that may have been adsorbed on the enzyme pellet.

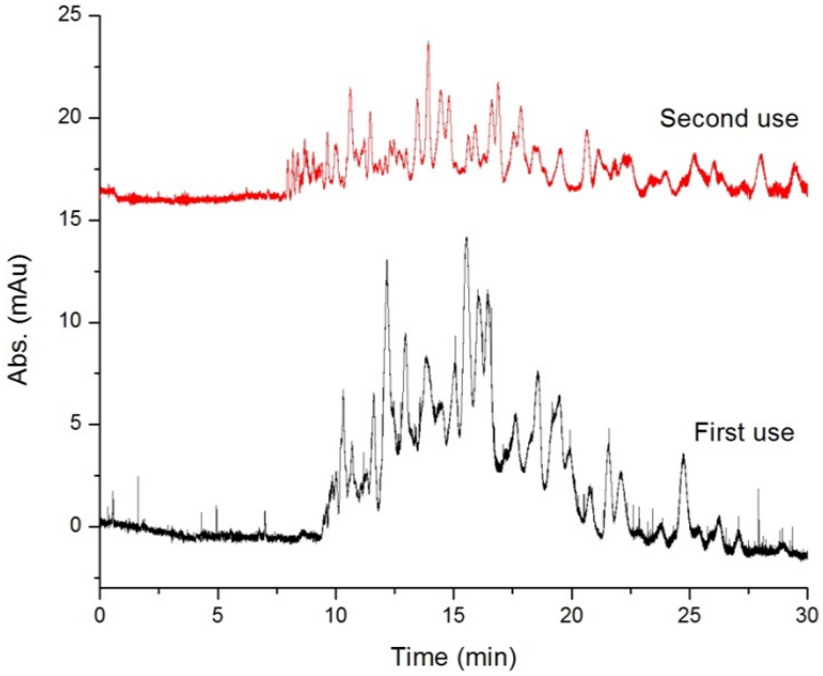

Figure 3.3. Peptide maps showing immobilized enzyme reusability for digestion of BSA (22.5 μM) by a single batch of GA-crosslinked chymotrypsin prepared using the small-scale procedure. The enzyme pellet was washed 4 times: 2 × 200 μL each with water and crosslinking buffer. The apparent E:S ratio was 1:1 (mol:mol) during both digestions carried out for 4 h at 37 °C. Separations were carried out as in Figure 3.1.

3.4.5. The Effect of Denatured Versus Native Substrate[1]

The necessity of denaturing a large substrate like BSA before digestion with the GA-crosslinked chymotrypsin was investigated because when a partial digestion is sufficient for an application, eliminating the time consuming steps of denaturation, reduction and alkylation is desirable. The BSA substrates (denatured and native) were analysed by CE before their digestion, revealing that the native protein elutes about 5.5 min faster than the denatured form in pH 2.5 separation buffer (Figure 3.4 A and C). Intuitively, the denatured protein has a larger hydrodynamic radius and thus a slower migration time caused by frictional drag during electrophoresis compared to the compact, native form of BSA. Both substrates were added to the immobilized chymotrypsin prepared using the small-scale method and digested under identical conditions then mapped by CE (Figures 3.4 B and D). The peptide map of native BSA (Figure 3.4 D) shows partial digestion as well as undigested substrate (peak at 9 min), not only because the highly folded protein protects many cleavage sites but possibly also because the enzyme pellet is not sufficiently porous to allow much of the protein backbone to reach the active sites of chymotrypsin. In contrast, Figure 3.4 B shows more peptidic peaks centered around 11 min due to more extensive digestion of denatured BSA by the immobilized chymotrypsin pellet. Nonetheless, the GA-crosslinked enzyme shows good potential for use with simplified substrate preparation for MS-based proteomics studies.

[1] The study was done with different concentrations of urea and $CaCl_2$ and the best concentration was 8M and 10 mM respectively. More details about the effect of [urea] and [$CaCl_2$] are described in Appendix A.

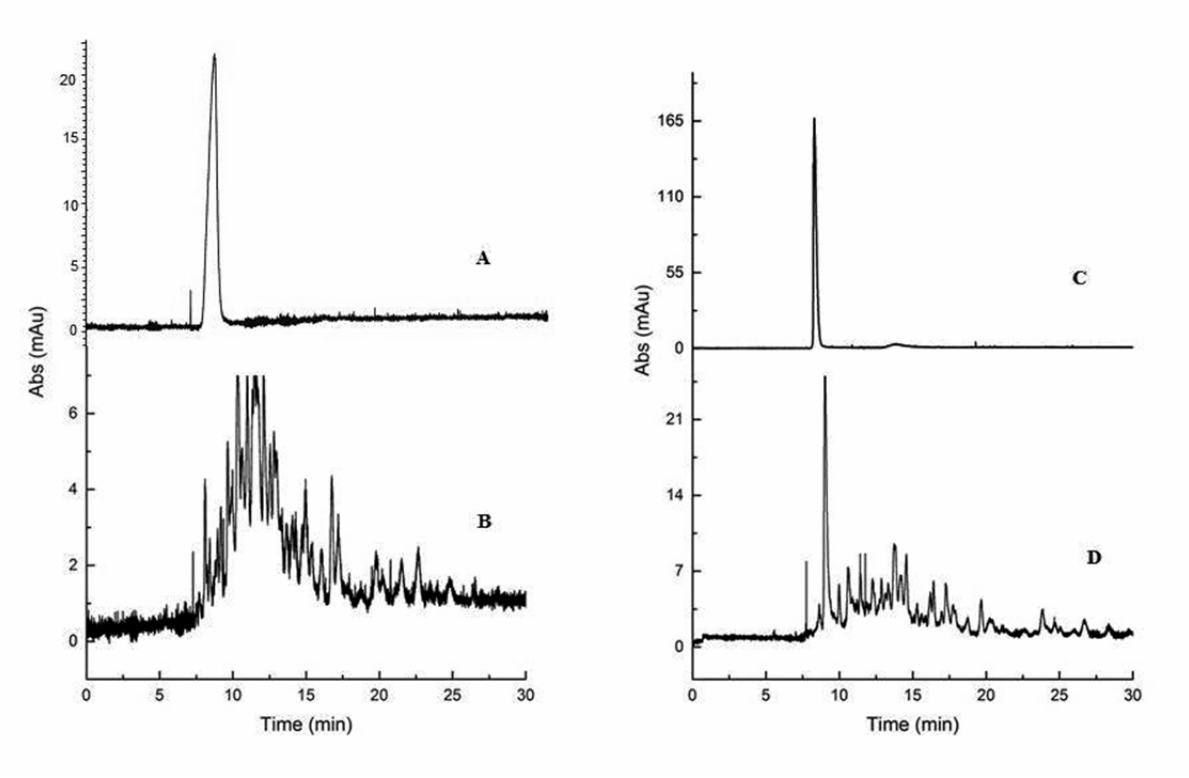

Figure 3.4. Comparison of immobilized enzyme digestion efficiency for denatured versus native substrate. (A) Electropherogram of denatured BSA; (B) peptide map of digested denatured BSA (22.5 μM); (C) electropherogram of native BSA; (D) peptide map of digested native BSA (22.5 μM). Digestions were performed for 4 h at 37 ^{0}C using the small-scale preparation of GA-crosslinked chymotrypsin at an apparent E:S ratio of 1:1 (mol:mol). Separations were carried out as in Figure 3.1.

3.5. Conclusions

Chymotrypsin immobilized by GA crosslinking was demonstrated for BSA peptide mapping studies, although further optimization is still required to improve the enzyme's reusability. Nonetheless, this rapid and inexpensive immobilization method offers the benefits of using a high enzyme-to-substrate ratio and reuse of the enzyme. A study of the conditions for immobilized enzyme preparation showed that the size of the enzyme pellet and the composition of the crosslinking buffer—most importantly its pH—influence the reproducibility of peptide maps and, by extrapolation, the activity of the immobilized enzyme. The gentle washing protocol was able to remove a significant amount of non-

reacted or adsorbed chymotrypsin from the enzyme pellet, although it was observed that there was a limitation to the washing, which eventually caused disintegration or dissolution of the immobilized enzyme. The solution to this problem is using the immobilized enzyme in a microreactor format with on-line peptide mapping and with fewer washing steps in order to conserve the GA-crosslinked chymotrypsin.

3.6. Acknowledgements

This research was supported in part by NSERC (Natural Sciences and Engineering Research Council of Canada) and FRQNT (Fonds de Recherche du Québec – Nature et Technologies). G. Ghafourifar gratefully acknowledges the U. of Montréal Chemistry Department for stipend assistance.

3.7. Supporting Information

The following section is adapted from the Supporting Information submitted with Ghafourifar and Waldron "Capillary Electrophoretic Peptide Mapping to Probe the Immobilization/Digestion Conditions of Glutaraldehyde-crosslinked Chymotrypsin" to Current Analytical Chemistry**.**

Supporting data includes: electropherograms of residual chymotrypsin in washings after enzyme immobilization; BSA peptide maps for digestion of 1, 4, 8 and 24 h; BSA maps showing batch-to-batch reproducibility of immobilized chymotrypsin prepared in three crosslinking buffers; a table quantifying the peptide peaks and areas as a function of crosslinking buffer composition and batch number; peptide maps for the carry-over study to evaluate GA-crosslinked chymotrypsin reusability using substrate blanks as controls.

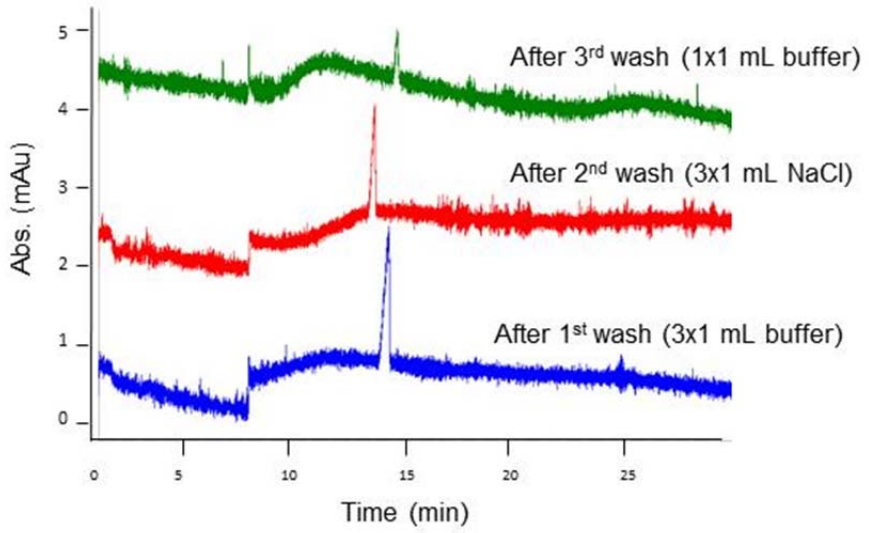

Figure S3.1. Electropherograms showing the residual, unreacted chymotrypsin removed after each stage of washing the immobilized enzyme aggregated particle that was made using the full-scale procedure. Each pooled wash solution was concentrated 10 fold using a Speed Vac before injection into the CE for separation. Separations were carried out by capillary electrophoresis at +15 kV (75 µm i.d. × 43 cm capillary) in 50 mM sodium phosphate buffer, pH 2.5, with detection at 200 nm.

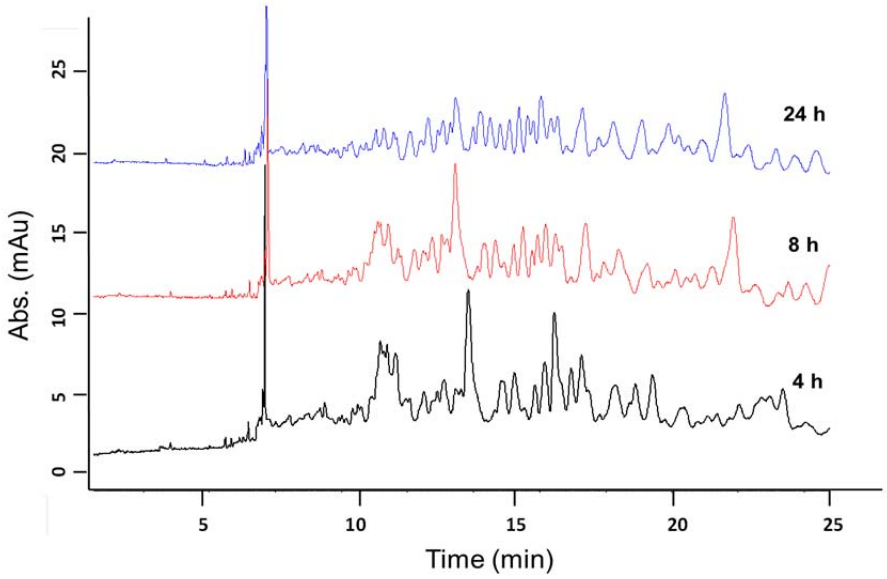

Figure S3.2. Peptide maps showing the effect of digestion time for BSA (22.5 µM) digested at 37°C using GA-crosslinked chymotrypsin prepared by the small-scale procedure and with an apparent E:S ratio of 1:1 (mol:mol). Separations by CE-UV were carried out as described in Figure S3.1.

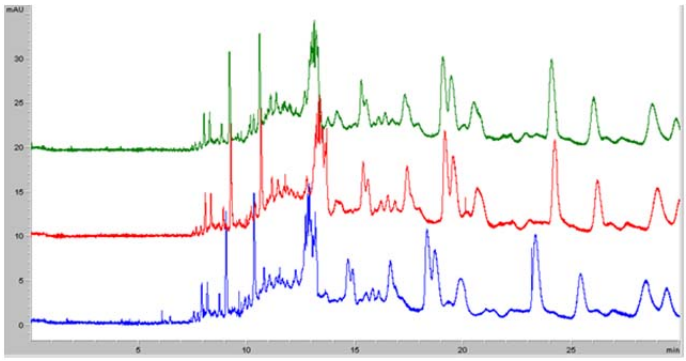

Figure S3.3. Peptide map of BSA (9 µM) digested for 1 h at 37 °C by GA-crosslinked chymotrypsin prepared using the full-scale procedure and with an apparent E:S ratio of 26:1 (mol:mol). Separations by CE-UV were carried out as described in Figure S3.1. Triplicate injections of a single digest into the CE are shown.

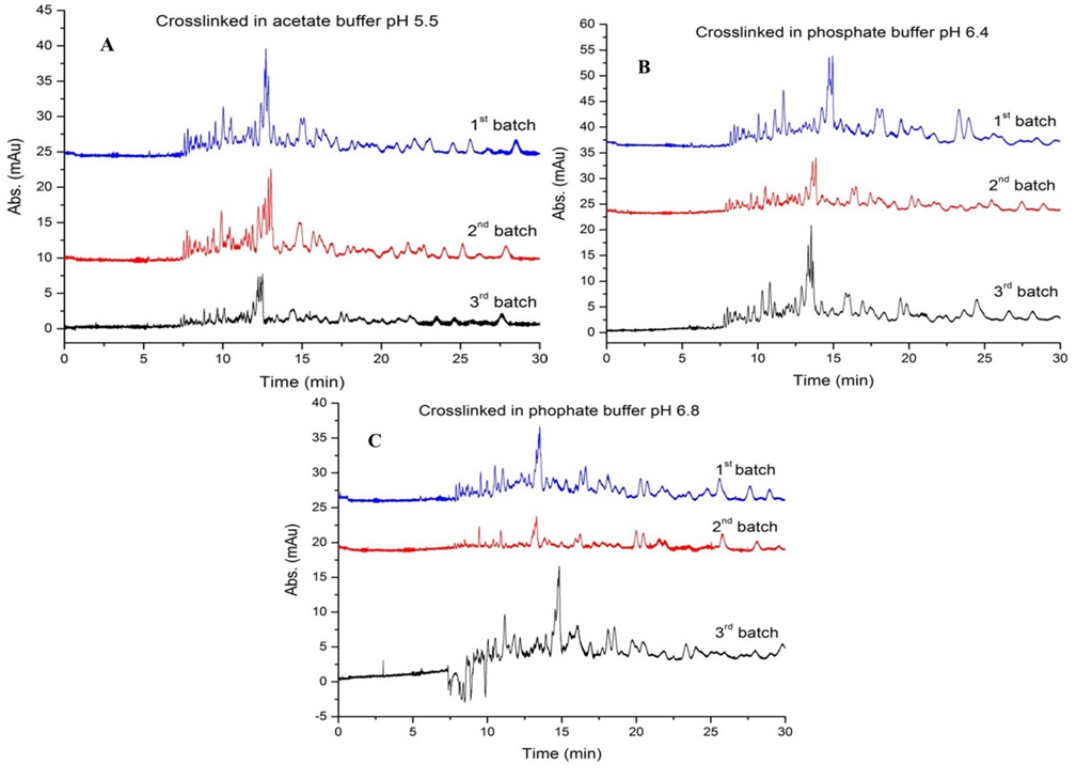

Figure S3.4. Peptide maps for BSA digestions showing batch-to-batch (n = 3) reproducibility of immobilized chymotrypsin prepared using three different buffers during enzyme crosslinking: (A) acetate buffer, pH 5.5; (B) phosphate buffer, pH 6.4; (C) phosphate buffer, pH 6.8. All 9 batches of immobilized chymotrypsin were prepared following the small-scale procedure. Nine aliquots of the same substrate (BSA, 22.5 µM) were used. The apparent E:S ratio was 8:1 (mol:mol) and digestions were performed for 4 h at 37 °C. Separations by CE-UV were carried out as in Figure. S3.1.

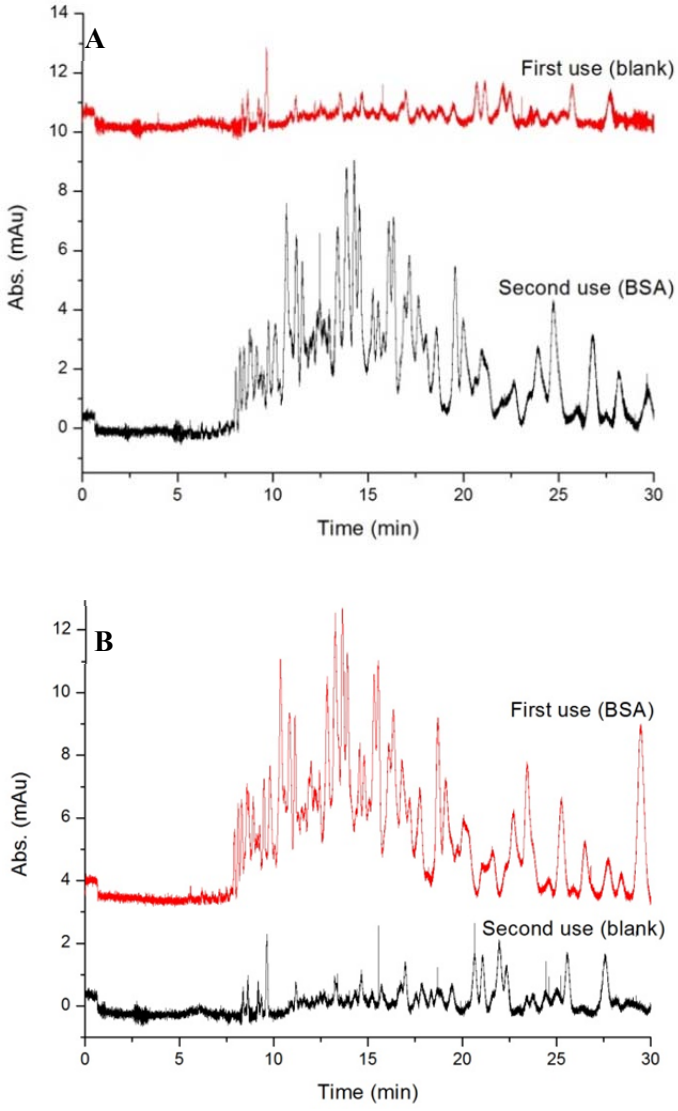

Figure S3.5. Carry-over study for assessing GA-crosslinked chymotrypsin reusability using substrate blanks as controls for either the first use or the second use of enzyme. (A) GA-crosslinked chymotrypsin was used to "digest" a blank solution containing all denaturation reagents except BSA, then the crosslinked particle was washed, then reused with denatured BSA (22.5 µM) as substrate for the second digestion. (B) GA-crosslinked chymotrypsin was used to digest denatured BSA (22.5 µM) as substrate in the first use of the enzyme particle, followed by the same washing procedure as above, then reused with the blank substrate solution "digested" in the second use of enzyme. The immobilized chymotrypsin for both batches (A and B) was prepared using the small-scale procedure. All digestions were performed at 37 ^{0}C for 4 h and the E:S ratio for BSA was 1:1 (mol:mol). Separations (peptide mapping) by CE-UV were carried out as in Figure S3.1.

Table S3.1. The effect of crosslinking buffer composition on GA-crosslinked chymotrypsin activity evaluated by comparing digests of BSA (22.5 µM) at an E:S ratio of 8:1 (mol:mol). Peptide mapping was carried out by CE at +15 kV (75 µm i.d. capillary) in 50 mM sodium phosphate buffer, pH 2.5, with detection at 200 nm. For each buffer composition, three batches of crosslinked enzyme were made and digestion was carried out with aliquots of the same BSA solution (denatured, reduced and alkylated)..

Acetate buffer pH 5.5					**Phosphate buffer pH 6.4**					**Phosphate buffer pH 6.8**				
	Peptide peak areas					*Peptide peak areas*					*Peptide peak areas*			
Peak number	**1st batch**	**2nd batch**	**3rd batch**	**% RSD**	**Peak number**	**1st batch**	**2nd batch**	**3rd batch**	**% RSD**	**Peak number**	**1st batch**	**2nd batch**	**3rd batch**	**% RSD**
1	4	10	12	45	**1**	13	9	13	21	**1**	ND	ND	9	-
2	6	16	19	51	**2**	17	11	20	28	**2**	ND	ND	11	-
3	10	11	11	7	**3**	15	9	19	37	**3**	ND	ND	4.3	-
4	7	9	14	37	**4**	6	4	5	15	**4**	ND	ND	5.2	-
5	11	37	34	51	**5**	5	3	7	40	**5**	ND	ND	4.2	-
6	18	10	14	31	**6**	5	4	7	26	**6**	ND	13	15	11
7	9	9	10	7	**7**	19	17	28	27	**7**	ND	ND	10	-
8	7	5	ND	18	**8**	12	10	13	15	**8**	ND	ND	24	-
9	ND	12	16	19	**9**	47	32	44	19	**9**	ND	ND	6	-
10	ND	20	11	42	**10**	61	29	77	44	**10**	49	16	34	50
11	10	46	23	68	**11**	23	16	19	18	**11**	ND	ND	9	-
12	20	15	38	49	**12**	5	5	5	6	**12**	29	ND	14	51
13	17	31	14	44	**13**	5	8	7	16	**13**	11	7	7	30
14	11	52	ND	92	**14**	24	19	24	12	**14**	23	15	22	22
15	23	6	34	66	**15**	48	33	55	24	**15**	ND	ND	16	-
16	7	10	26	69	**16**	68	17	37	63	**16**	ND	15	10	29
17	9	ND	9	4	**17**	41	31	39	13	**17**	ND	10	22	49
18	ND	ND	18	-	**18**	24	20	32	25	**18**	15	6	23	60
19	35	ND	10	81	**19**	20	16	30	32	**19**	39	15	28	44
20	ND	ND	17	-	**20**	20	18	35	36					
-					**21**	14	24	39	48	**-**				
-					**22**	40	32	62	35	**-**				
Average RSD				**43%**					**27%**					**39%**

Chapter 4. Development of Glutaraldehyde-Crosslinked Chymotrypsin and an *In Situ* Immobilized Enzyme Microreactor with Peptide Mapping by Capillary Electrophoresis

Golfam Ghafourifar, Antoine Fleitz, and Karen C. Waldron, *Electrophoresis,* **2013**, 34, 1804–1811.

Note on author contributions: All research in this paper was carried out by Golfam Ghafourifar. Antoine Fleitz, a summer student co-supervised by Golfam Ghafourifar with K.C. Waldron, also contributed to the CE-MS method development. The whole manuscript was written by Golfam Ghafourifar; corrections were done by supervisor Karen C. Waldron.

4.1. Abstract

Immobilized proteolytic enzymes present several advantages over their soluble form, not the least of which is suppression of autoproteolysis peaks even at high enzyme-to-substrate ratios. We have made immobilized chymotrypsin by directly crosslinking it with glutaraldehyde to produce polymeric particles. Digestion of two model substrates using the particles was followed by capillary electrophoresis (CE) peptide mapping with detection by UV absorbance or laser induced fluorescence. Results showed that autoproteolysis was highly suppressed and that different storage conditions of the particles in the short term (24 h) did not affect digestion of denatured BSA. As well, the chymotrypsin particles were indifferent to the presence of fluorescein groups on a casein substrate. Glutaraldehyde crosslinking of chymotrypsin inside a fused silica capillary column to make an immobilized enzyme reactor (IMER) was achieved in a series of reagent addition and washing steps, entirely automated using a commercial CE instrument. Digestion of myoglobin in the IMER for 30 min at 37 °C followed by peptide mapping by CE-MS of the collected digest allowed identification of 17 chymotryptic peptides of myoglobin, or 83% primary sequence coverage.

4.2. Introduction

Proteomic studies require rapid peptide mapping methods for protein identification and quantification. Drawbacks of a classical proteolytic digestion, whereby soluble enzyme is added to the protein, include long incubation times and single use of the enzyme [72, 79, 88, 92]. Advantages of immobilizing the enzyme include using a high enzyme-to-substrate ratio to accelerate digestion, better enzyme stability by reducing the enzyme's flexibility, fewer autoproteolysis products and reuse of the enzyme [72, 86, 88, 160, 161]. Many immobilization methods for proteolytic enzymes have been reported [22, 42, 85, 86, 161-164], several of which use glutaraldehyde (GA), a common crosslinking agent [50, 111, 161, 165]. GA is highly reactive towards primary amine groups making it suitable either as a linker to solid supports or as a polymerizing agent [43, 51, 166]. Immobilized enzymes in general have wide ranging use but for proteomics they facilitate automated protein

digestion [79, 88, 167] in the form of immobilized enzyme reactors (IMERs) that can be coupled to separation and detection systems enabling high throughput proteomic analysis [79, 151, 167-170]. There are several excellent reviews on immobilized protein reactors [72, 79, 164, 171-173].

The digests from proteolytic IMERs have been analyzed by different on-line and off-line mapping methods such as direct injection MS [9, 15, 45, 80, 112, 122, 124, 131, 147, 148, 150, 167-169, 172, 174-183], LC-MS [149, 166, 184-186], CE-MS [28, 33, 39, 49, 52, 75, 78, 79, 111, 113, 123, 138, 147-149, 152, 168, 187-195], CE-laser induced fluorescence (LIF) [28, 186] and CE-UV [40, 109]. Capillary electrophoresis offers certain advantages compared to other methods, specifically the use of minute amounts of sample, low solvent consumption and compatibility with a wide range of detection techniques [22, 96, 116]. Limitations due to low sample concentrations can be overcome by using in-capillary preconcentration strategies [22, 40, 171]. Within an IMER, the enzyme can be immobilized on a variety of supports; particles [196], a membrane [180, 187] or the capillary inner wall [109, 168] to name just a few.

While essentially any enzyme could be immobilized inside an IMER, trypsin is the most commonly used for proteomic studies because of its highly specific cleavage, which is limited to the residues lysine and arginine [72] and produces medium length peptides that are mostly cationic. To evaluate IMER performance (activity, reproducibility, stability, etc) at sub-nanomolar protein substrate concentrations, a sensitive detection method such as LIF is needed [95, 96]. However, fluorescent derivatization has two drawbacks for proteomics studies: post-digestion, the peptides at sub-nM concentrations react too slowly with fluorescent labeling reagents; predigestion, fluorophore-labeled ε-lysine residues are not recognized by trypsin resulting in cleavage only at arginine, the less abundant residue. Our approach is to use chymotrypsin as an alternative to trypsin so that IMER characterization can profit from using CE-LIF and fluorescently labeled protein as a test substrate. Therefore, in the present study, chymotrypsin was immobilized by crosslinking it with GA using a similar procedure developed in our lab for trypsin [51, 111]. CE coupled to UV, LIF and MS detectors was used for peptide mapping in this study as a means to evaluate

certain aspects (e.g., short-term storage conditions) of GA-crosslinked chymotrypsin particles and the ability of a fabricated IMER to digest myoglobin.

4.3. Materials and Methods

4.3.1. Reagents and Materials

α-Chymotrypsin from bovine pancreas type II, glutaraldehyde (25% aqueous solution), bovine serum albumin (BSA), horse myoglobin-α, monobasic sodium phosphate, dibasic sodium phosphate, triza hydrochloride, Trizma base, calcium chloride, sodium hydroxide, fluorescein isothiocynate (FITC) conjugated to bovine casein, (3-aminopropyl)triethoxysilane (3-ATPES, 99% purity), sodium acetate, sodium chloride, glycine, urea, formic acid, iodoacetamide and acetic acid were from Anachemia (Montreal, QC, Canada). Phosphoric acid, dithiothreitol (DTT, 98% purity), ammonium phosphate and ammonium bicarbonate were purchased from Sigma Aldrich (Oakville, ON, Canada). Methanol was purchased from BDH (West Chester, PA, USA). Hydrochloric acid was from EMD Millipore (Gibbstown, NJ, USA). Fused silica capillary tubing for the IMER (250 μm I.D., 360 μm O.D.) was obtained from Chromatographic Specialties Inc. (Brockville, ON, Canada). Fused silica capillary tubing for CE separations (50 μm or 75 μm I.D., 375 μm O.D.) was from Polymicro Technologies (Phoenix, Arizona, USA). A multi-cartridge Milli-Q filtration/deionization system (Millipore, Bedford, MA, USA) was employed to purify the distilled water used in preparation of all solutions and buffers.

4.3.2. Protein Substrate Denaturation

Eight milligram of substrate in 800 μL ammonium bicarbonate solution (0.4 M, pH 8.0) containing 8 M urea was reacted with 120 μL DTT (45 mM in water) at 50 °C for 15 min to reduce disulfide bonds. After cooling to room temperature, 120 μL of iodoacetamide (100 mM in water) was added to prevent thiol oxidation and reformation of the disulfide bridges between cysteine residues in the substrate. Tris-HCl (100 mM, pH 7.8) containing

10 mM calcium chloride was added to dilute the denatured protein substrate to a final concentration of 2 mg/mL.

4.3.3. Digestion of Substrate by Soluble Chymotrypsin

Chymotrypsin stock solution (1.3 mM) was added to substrate (either FITC-casein solution or Milli-Q water for the autoproteolysis study) and digested at 37 °C. The duration and the enzyme:substrate ratio are indicated in the results section.

4.3.4. Preparation of GA-Crosslinked Chymotrypsin Particles

The following procedure was adapted from our previous method for trypsin and from preliminary studies using chymotrypsin [41, 106]. A 50 mM sodium phosphate buffer, pH 6.4, was used for several preparation and washing steps in this section. In a 1.5 mL microcentrifuge vial, the chymotrypsin stock solution was diluted to 0.15 mM using phosphate buffer. GA, diluted to 2.5% (v/v) in water, was added dropwise (*ca.* 80 µL) to the enzyme solution. This represents a large molar excess over chymotrypsin. The immobilization reaction was allowed to proceed without stirring for 2 h at room temperature. The solution was then centrifuged at 3000 rpm for 2 min and the supernatant was decanted off. The particles were washed to remove unreacted enzyme and excess glutaraldehyde with 3 × 200 µL phosphate buffer, 3 × 200 µL NaCl (500 mM), then once again with 200 µL buffer. Finally, 200 µL glycine (200 mM, in phosphate buffer) was added to react with any excess aldehyde groups for 3 h at room temperature. The final product was washed with 3 × 200 µL phosphate buffer followed by 3 × 200 µL Milli-Q water. The immobilized crosslinked chymotrypsin particles were stored at -20 °C in 200 µL Milli-Q water. The immobilization yield was measured by absorbance spectroscopy using a Cary 100 Spectrophotometer from Varian.

4.3.5. Digestion of Substrate by Immobilized Chymotrypsin Particles

The protein substrate was added to the immobilized chymotrypsin preparation (particles in 200 μL) to reach a given enzyme:substrate ratio (mol:mol), as indicated in each figure, where the enzyme typically had a mole ratio equal to or higher than the substrate. Digestion was performed in a 1.5 mL microcentrifuge tube in batch format with gentle shaking for 4 h (or 24 h) at 37 °C. The digest solution was decanted off and then was injected into the CE (see description below) for separation without pre-concentration or filtration.

4.3.6. CE-UV and CE-LIF Separations

CE-UV peptide mapping of BSA digests was performed on an Agilent System (Agilent Technologies, Waldbronn, Germany) equipped with a UV/Vis diode array detector (DAD) but monitored at 200 nm. CE-LIF peptide mapping of FITC-labeled casein digests was performed on a Beckman MDQ instrument (Beckman Coulter, Fullerton CA, USA) equipped with a 3 mW argon ion laser having an excitation wavelength of 488 nm and a 520 nm emission wavelength band-pass filter. Separations were performed at +15 kV in the Agilent CE and at +20 kV in the Beckman CE, all at 25 °C in an uncoated fused silica capillary (43 cm total length, 75 μm I.D.). The background electrolyte (BGE) consisted of 50 mM sodium phosphate, pH 2.5, for CE-UV mapping and 50 mM sodium phosphate pH 7.0 for CE-LIF mapping. All buffers were filtered with a 0.22 μm nylon syringe filter (Chromatographic Specialties, Brockville, ON, Canada) prior to use. The capillary was flushed with 0.1 M NaOH for 1 min, Milli-Q water for 1 min, 0.1 M HCl for 2 min, then buffer for 3 min. Sample injection for the Beckman system was performed by applying 0.5 psi × 5 s at the inlet followed by 2 s of buffer using the same pressure. On the Agilent system, sample was injected at 34.5 mbar × 5 s followed by 2 s of buffer.

4.3.7. Microreactor (IMER) Construction and Characterization

4.3.7.1. In Situ IMER Fabrication

A selected length of fused silica capillary (43 cm × 250 μm I.D.) was installed in the Agilent CE system, rinsed at 50 mbar with NaOH (1 M) for 1 h, followed by Milli-Q water then methanol, each for 10 min. The capillary was dried by flushing (at 950 mbar) with air for 10 min at room temperature. The dried capillary was then activated with HCl (1 M) by rinsing it at 50 mbar for 1 h at room temperature. The capillary wall was then derivatized with (3-aminopropyl)triethoxysilane (3-APTES, 10% (v/v) in methanol) at 60 °C by rinsing it for 1 h at 50 mbar, then letting it sit for 3 h. The amino-functionalized capillary was rinsed for 3 min at 50 mbar with Milli-Q water to remove unreacted 3-APTES. After cooling to room temperature, GA (diluted to 2.5% (v/v) in water) was passed through the capillary for 1 h at 50 mbar followed by rinsing for 3 min at 50 mbar with phosphate buffer. Next, chymotrypsin (1.3 mM in water) was continuously passed at 50 mbar into the capillary for up to 60 min. The sequence of GA, buffer then chymotrypsin was repeated three times. The capillary was then left at room temperature for 3 h to complete the crosslinking reaction. Finally, the IMER was rinsed with water for 2 min at 50 mbar.

4.3.7.2. Digestion of Substrate in the Immobilized Chymotrypsin Microreactor

The protein substrate (18 mg/mL myoglobin with no denaturation or 2 mg/mL BSA after denaturation) was injected into the IMER at 50 mbar for 2 min. Even after 2 min of injection, little or no substrate was seen at the IMER outlet. Digestion was allowed to proceed for 30 min at 37°C. Then, 50 mM phosphate buffer was passed through the IMER capillary at 50 mbar for 2 min to flush the digest to the outlet. The digest and buffer rinse (total volume *ca.* 80 μL) were collected and analyzed by CE-MS without pre-concentration or filtration.

4.3.7.3. CE-MS Separation

Separation was performed on the Agilent system in a bare fused silica capillary (75 µm I.D., 360 µm O.D., 70 cm total length, 21 cm to the UV detector) at 25 °C under an applied voltage of +35 kV in a buffer composed of 10 mM ammonium acetate, pH 6.7. Detection was made with a single quadrupole mass sensitive detector (MSD SL System, Agilent Technologies, Santa Clara, CA, USA) interfaced using an orthogonal sheath flow CE- MS sprayer ion source (Agilent Technologies). The MSD was operated with an atmospheric pressure electrospray ionization (API-ES) source in positive ion detection mode. The source parameters were as follows: drying gas flow of 10 L/min at 130°C, 170 kPa; sheath liquid composed of 0.1% formic acid in 50% methanol operated at a flow rate of 250 µL/min. The MSD parameters were: 200 -1000 m/z mass range, fragmentor set at 65 V and detector gain at 3, cycle time of 0.97 s per cycle. Selected ion monitoring (SIM) mode was used for 50% of a cycle time.

4.4. Results and Discussion

4.4.1. Immobilization Efficiency

The immobilization efficiency, or yield, of the GA-chymotrypsin reaction was evaluated by UV-Vis absorbance spectroscopy. The spectra of chymotrypsin and GA overlap significantly (Figure 4.1), including the absorbance region specific to aromatic residues. Therefore, two graphical methods were used to quantify the amount of chymotrypsin remaining in the supernatant and washes after the crosslinking reaction, where there was still the presence of excess GA.

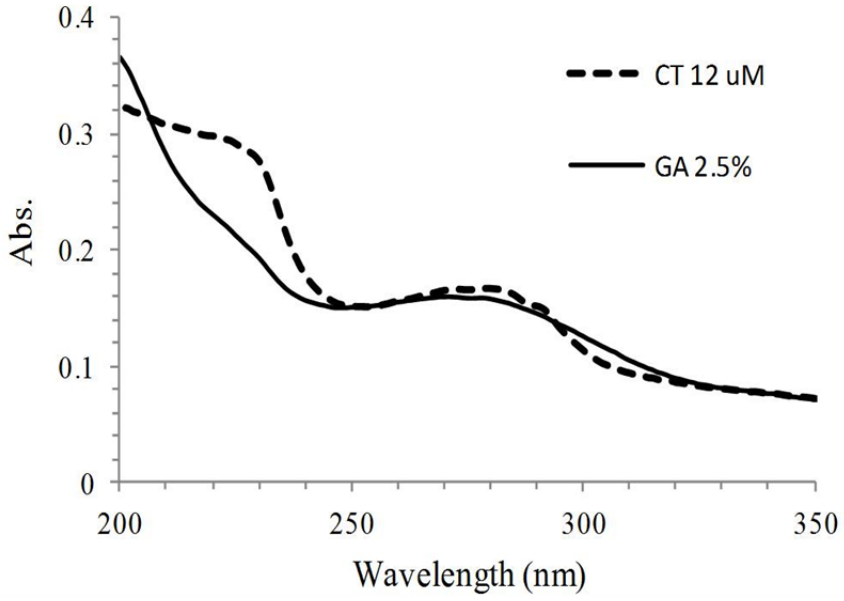

Figure 4.1. UV-Vis absorbance spectra of chymotrypsin (12 μM) and glutaraldehyde (2.5%). The reference solution was Milli-Q water.

The first method involved using the fourth derivative spectra measured at 288 nm where the absorption for glutaraldehyde is zero but that of chymotrypsin shows a strong negative band (the spectra are presented in Appendix B). A calibration curve for chymotrypsin (for concentrations from 4 to 20 μM) in the presence of excess GA was made using the fourth derivative absorptions at 288 nm of mixtures of chymotrypsin and GA. The absorption of chymotrypsin in solution (supernatant and washed) after the immobilization reaction was subtracted from the initial concentration. The second method involved the commonly used "analysis of a mixture" [197] which requires absorbance measurements, at several wavelengths, of three solutions: the mixture, a chymotrypsin standard and a GA standard. A plot of $A_{mix}/A_{CT,st}$ as a function of $A_{GA,st}/A_{CT,st}$ was made where A_{mix} is the absorption of the supernatant after crosslinking, $A_{CT,st}$ is the absorption of diluted chymotrypsin standard solution ($[CT]_{st}$ =6 μM) and $A_{GA,st}$ is the absorption of diluted GA standard solution ($[GA]_{st}$ =0.47%). The absorbances were measured at 265, 268, 271 and 275 nm. From equation (4.1), the slope and intercept, $[GA]/[GA]_{st}$ and $[CT]/[CT]_{st}$ respectively, permitted estimations of [GA] and [CT] remaining in solution after the crosslinking reaction.

$$\frac{A_{mix}}{A_{CT,st}} = \frac{[CT]}{[CT]_{st}} + \frac{A_{GA,st}}{A_{CT,st}} \cdot \frac{[GA]}{[GA]_{st}} \quad (4.1)$$

Combining the results for both spectral methods, the average immobilization efficiency was determined to be as 94 ± 2% (n=5)[1].

4.4.2. Autoproteolysis of Chymotrypsin

Autoproteolysis, which leads to interfering background peptides during separation and identification, is unavoidable unless very low enzyme concentrations are used, thereby necessitating long incubation times. Immobilization is proposed to decrease autoproteolysis by reducing the enzyme's flexibility [160]. Trypsin immobilized via the ε-amino groups of lysine residues is inherently stabilized against autoproteolysis because a large proportion of its cleavage sites are literally "tied up" [42]. Chymotrypsin, which cleaves substrate at aromatic residues, does not benefit from this so it is important to carefully evaluate the autoproteolysis of this enzyme once it is insolubilized. We prepared immobilized chymotrypsin using GA as a crosslinking agent, which results in formation of irregularly shaped, soft particles. To fairly compare the autoproteolysis of soluble versus GA-crosslinked chymotrypsin, a blank substrate sample (i.e., 0 mg of BSA) was subjected to all denaturation steps (Section 4.2.2), incubated with each form of chymotrypsin at 37 °C for 24 h, then analyzed by CE-UV. Figures 4.2A and B show that the soluble chymotrypsin underwent extensive autoproteolysis, whereas there was very limited autoproteolysis using an equivalent amount of GA-crosslinked immobilized chymotrypsin. This suggests that enzyme flexibility is indeed reduced or at least that the crosslinked chymotrypsin does not present significant cleavage sites.

[1] More details about calculating immobilization efficiency are presented in Appendix B.

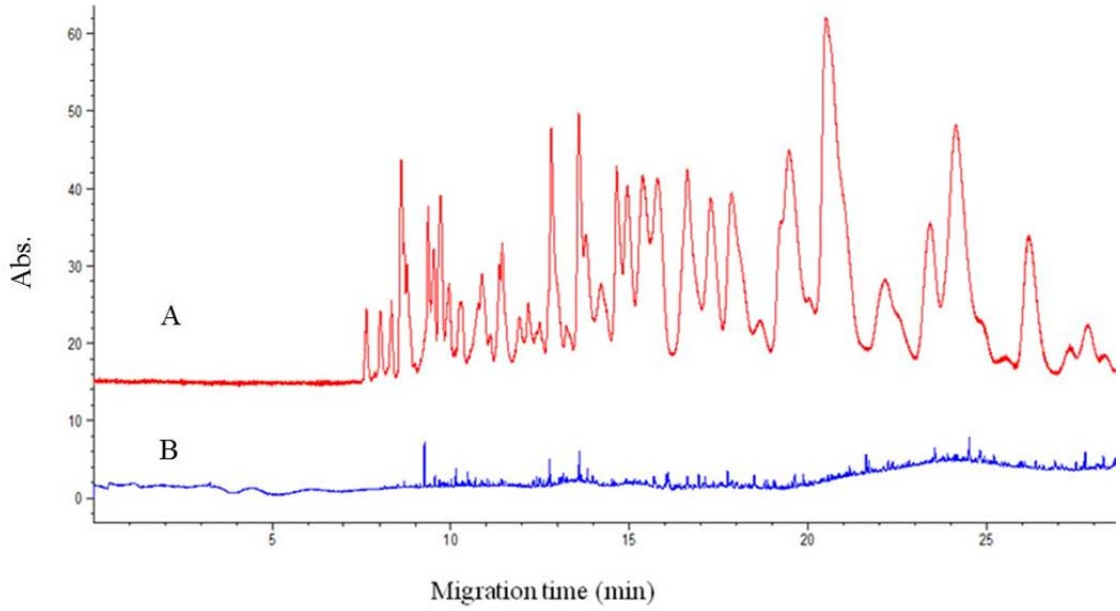

Figure 4.2. CE-UV peptide maps showing autoproteolysis of soluble chymotrypsin (A) and GA-crosslinked (immobilized) chymotrypsin (B), both incubated at 37 °C for 24 h. Milli-Q water as a substrate was subjected to the denaturation conditions described in the text. Separations were performed at +15 kV using 50 mM phosphate buffer, pH 2.5. The concentration of chymotrypsin was 0.12 mM in both experiments.

4.4.3. Storage Conditions

Immobilized enzymes are kept in either dry or wet conditions, i.e., in a known amount of water. We compared the effect of storage temperature and humidity condition of GA-crosslinked chymotrypsin on its relative activity for BSA digestion. Six batches of GA-chymotrypsin particles were prepared; three were kept wet according the procedure above and three were dried using a Savant Speed Vac, then they were stored at -20, 4 and 22°C for 24 h. The vacuum dried particles became a thin gel at the bottom of the microcentrifuge tube. Six aliquots of denatured BSA were added to the six tubes and incubated for 24 h at 37°C. Despite minor differences between the electropherograms, both wet (Figure 4.3A) and dried (Figure 4.3B) GA-chymotrypsin maintained similar activity regardless of storage temperature for the digestion of denatured BSA. In a separate experiment, we confirmed that the GA-chymotrypsin particles stored wet at -20°C for 4 months were still active

toward BSA (data not shown). These experiments suggest that although the GA-chymotrypsin particles are a soft material, they are fairly robust.

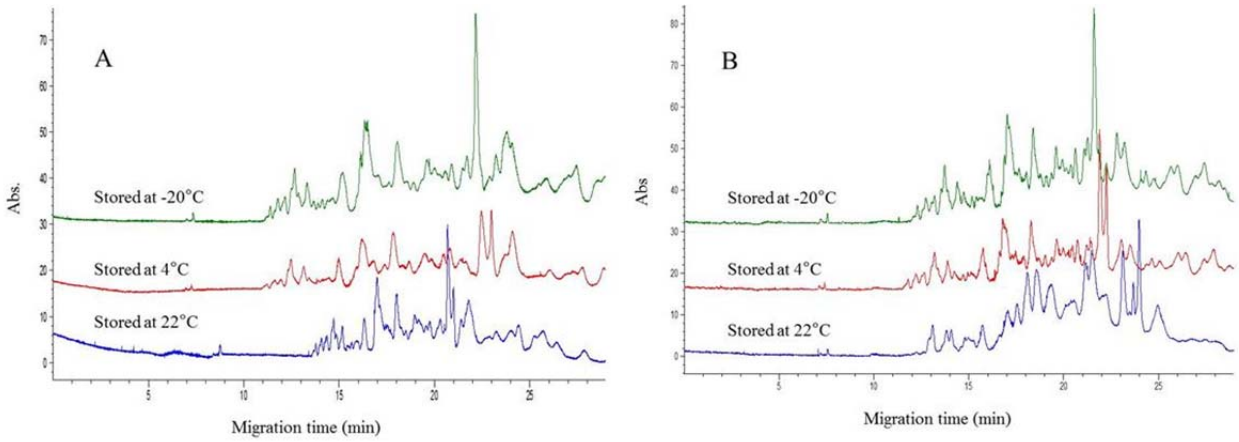

Figure 4.3. CE-UV peptide maps of BSA (2 mg/mL) showing the effect of different short-term storage conditions on immobilized enzyme activity where the GA-chymotrypsin particles were stored either wet for 24 h at the indicated temperatures (panel A), then used to digest three aliquots of denatured BSA, or stored dry for 24 h at the indicated temperatures (panel B), then used to digest three more aliquots of denatured BSA. For both wet and dry storage conditions, the enzyme:substrate ratio was 20:1 (mol:mol), and digestion was for 24 h at 37 °C. Separation conditions were the same as in Figure 5.2.

4.4.4. Digestion of Fluorescently Labeled Protein using GA-Chymotrypsin Particles

At low substrate concentrations (< 1 μM), peptides in a diluted digest cannot be easily detected by CE-UV [186]. Therefore, CE-LIF detection of peptides from a fluorescently labeled protein offers an alternative method for characterizing immobilized enzymes at low substrate concentration. The commercially available substrate FITC-casein was digested without prior denaturation by both soluble and GA-crosslinked chymotrypsin and the digests were analyzed by CE-LIF. Figure 4.4 shows that the presence of fluorescein groups on the substrate does not block the activity of the GA-crosslinked chymotrypsin. The CE-LIF separation conditions were not further optimized in this study once we confirmed that a similar number of peaks were seen in both electropherograms. On the other hand, the different peak intensities observed by CE-LIF are probably due to a difference in enzymatic

activity between the soluble and the GA-crosslinked chymotrypsin. Injection of undigested substrate shows only a single peak at 1.75 min (Figure S4.1, Supporting Information).

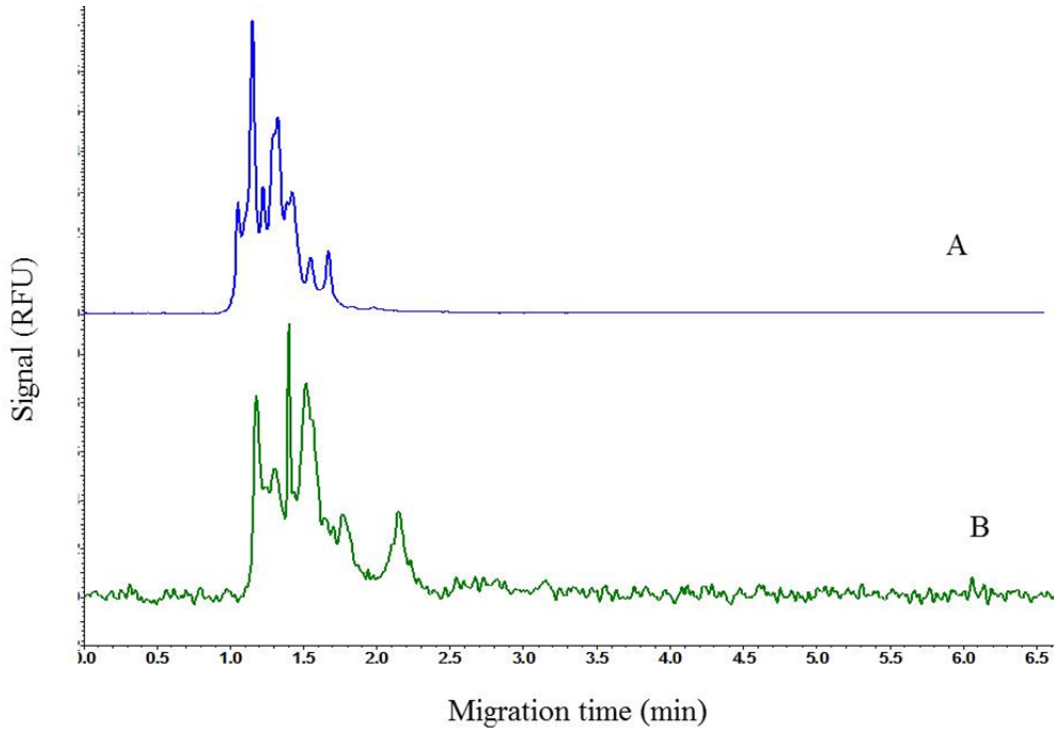

Figure 4.4. CE-LIF peptide maps of FITC-casein substrate (400 nM) digested for 4 h at 37 °C by GA-crosslinked chymotrypsin (A) and soluble chymotrypsin solution (B). The separation was carried out at +20 kV, in 50 mM phosphate buffer, pH 7.0. The E:S ratio was 30:1 (mol:mol).

4.4.5. IMER Digestion

The procedure for aminopropyl functionalization of the capillary using 3-APTES and enzyme immobilization was inspired by the methods reported by Krenkova *et al.* for a trypsin microreactor [88] and by Shan *et al.* for a δ-gluconolactone modified capillary [87]. The Krenkova method required more than 30 h and both reports required nitrogen gas and high drying temperatures. Our method required about 15 h and temperatures no higher than 60 °C. Figure 4.5A shows a simple stereomicroscope image (Motic Model BA310 microscope, Hong Kong) of the capillary after derivatizing it with 3-APTES but prior to

chymotrypsin immobilization. Liquids can still pass freely through the capillary. Figure 4.5B shows the capillary after passing through GA and chymotrypsin three times, in which a heterogeneous solid material can be seen as evidence that crosslinked chymotrypsin was made. At this point, flow through the capillary was encumbered but not totally blocked and it was not clear whether a thick layer of GA-crosslinked chymotrypsin was formed along the walls, or whether the polymeric network extended across the width of the IMER. When then same procedure was attempted in 75 μm I.D. capillaries they became blocked at the chymotrypsin addition step, which is why we moved to a larger I.D. column.

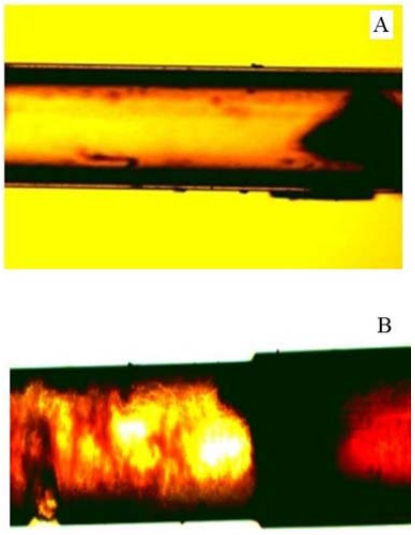

Figure 4.5. Stereomicroscope images taken from the window section of the IMER capillary after passing the 3-APTES through (image A), and then after passing the glutaraldehyde and chymotrypsin through (image B), leading to GA-chymotrypsin immobilized inside the IMER.

We were able to digest myoglobin substrate in 30 min, although the total volume injected was not calibrated. It is important to note that when we tried to digest denatured BSA in the IMER we saw some peaks in the CE-MS analysis but none of the m/z values could be attributed to BSA chymotryptic peptides. It is not clear whether the residual denaturation reagents caused a problem in the IMER or whether the BSA was too large to digest. If substrate size were the issue, then this would clearly limit the application of the

IMER. We are investigating these limitations using heat denaturation of BSA, other large protein substrates and chemical denaturation of myoglobin before IMER digestion.

4.4.6. CE-MS Characterization

Addition of mass spectral detection to CE permits peptide mass mapping, which facilitates protein identification by comparing the experimental m/z values of peptides to those from theoretical digestion of known proteins in a given genomic database. The ExPASy (http://www.expasy.org) and Protein Prospector (http://prospector.ucsf.edu) databases were used to determine the theoretical chymotryptic sites for digestion of myoglobin (Figure 4.6) as well as for autodigestion of chymotrypsin (31 cleavage sites). The only optimization of the CE-MS parameters consisted of investigating acetic acid and sodium acetate along with ammonium acetate for the BGE and the effect of a few different flow rates for the sheath liquid. CE-UV data are also obtained from the Agilent CE-MS system for a given sample but are not always meaningful because the effective separation length to the UV detector is short (21 cm) and the buffer, which must be compatible with MS detection, generally has poor resolving power for CE-UV peptide mapping.

MGL / SDGEW / QQVL / NVW / GKVEADIAGHGQEVL / IRL / F / TGHPETL / EKF / DKF / KHL /

KTEAEMKASEDL / KKHGTVVL / TAL / GGIL / KKKGHHEAEL / KPL / AQSHATKHKIPIKY / L /

EF / ISDAIIHVL / HSKHPGDF / GADAQGAMTKAL / EL / F / RNDIAAKY / KEL / GF / QG

Figure 4.6. Primary sequence of myoglobin with slashes indicating the expected (theoretical) chymotryptic cleavage sites and with CE-MS identified peptides underlined to visualize the sequence coverage and missed cleavages.

For the IMER-based myoglobin digest, the CE-UV peptide map had poor S/N ratio but in the CE-MS total ion current electropherogram, peptides (although poorly resolved) eluting between 6 and 11 min could be seen. Approximately 12 mass spectra across this elution window were examined. Scanning the mass range up to 1,000 Da was deemed

adequate because the largest peptides are highly basic and thus can be detected in their higher charge states. The measured m/z values obtained for chymotryptic digestion of myoglobin in the IMER were compared to the theoretical m/z values to identify the peptides. The experimentally identified peptides are shown in Table 4.1 along with their m/z values, charge states, theoretical m/z values and number of missed cleavages. The identified peptides are also underlined in Figure 4.6. The sequence coverage was 83.8% according to the amino acid residues in the identified peptides. It should be noted that three chymotryptic cleavage sites in myoglobin result in single amino acid residues that are below the mass range scanned.

Table 4.1. Experimentally identified residues from horse myoglobin digested by chymotrypsin IMER and identified by CE-MS.

Measured m/z [a]	Theoretical m/z (mi)	Position	MC[b]	Peptide sequence [a] detected by CE-MS
204.1	204.098	153-154	0	(F)QG(-)
274.7^{2+}	274.681^{2+}	31-34	1	(L)IRLF(T)
389.7^{2+}	389.708^{2+}	148-154	2	(Y)KELGFQG(-)
644.4	644.398	71-77	1	(L)TALGGIL(K)
950.5	950.505	140-147	0	(F)RNDIAAKY(K)
593.2	593.220	4-8	0	(L)SDGEW(Q)
407.3^{2+}	407.211^{2+}	42-47	1	(L)EKFDKF(K)
295.1	295.129	106-107	0	(L)EF(I)
418.2	418.208	13-15	0	(L)NVW(G)
487.2	487.287	9-12	0	(W)QQVL(N)
475.9^{2+}	475.756^{2+}	140-147	0	(F)RNDIAAKY(K)
778.1	778.409	148-154	2	(Y)KELGFQG(-)
924.6	924.432	117-124	0	(L)HSKHPGDF(G)
762.0^{2+}	761.897^{2+}	16-30	0	(W)GKVEADIAGHGQEVL(I)
295.1^{4+}	294.918^{4+}	78-87	0	(L)KKKGHHEAEL(K)
566.9^{2+}	567.285^{2+}	125-136	0	(F)GADAQGAMTKAL(E)
433.8^{4+}	434.506^{4+}	91-105	1	(L)AQSHATKHKIPIKYL(E)
579.2^{3+}	579.005^{3+}	91-105	1	(L)AQSHATKHKIPIKYL(E)
894.3	894.366	1-8	1	(-)MGLSDGEW(Q)
544.3^{4+}	544.300^{4+}	51-70	1	(L)KTEAEMKASEDLKKHGTVVL(T)

a. for $[M+H]^+$ unless otherwise indicated; the MSD only reports mass to ± 0.1 Da.

b. number of missed cleavages.

Table 4.1 and Figure 4.6 show there were a few more missed cleavages than what has been seen for other microreactor configurations [51, 124, 198]. This may be a result of not denaturing the myoglobin substrate before digestion or an indication that there was less contact than anticipated between the substrate and GA-chymotrypsin in the IMER. Although myoglobin has no disulfide bonds that require chemical denaturation, it is an extremely compact protein [199] so some of its aromatic residues are probably buried when in the native conformation making them less accessible to chymotryptic cleavage. It is important to note that this situation is less problematic for trypsin because lysine and arginine—the tryptic cleavage sites—should be nearer to the surface of a compact protein. Batch digestion of undenatured myoglobin with GA-chymotrypsin particles showed ca. 18 peptidic peaks by CE-UV analysis (Figure S4.2 in Supporting Information), which is similar to the number of peptides identified by CE-MS after IMER digestion. The peptides in Figure S4.2 presumably include some missed cleavages and some of the 26 expected chymotryptic peptides for myoglobin, suggesting that the IMER format provided as much enzyme-substrate "contact" as did a suspension of GA-chymotrypsin particles. Batch digestion using soluble chymotrypsin was also carried out, but unfortunately we had instrument problems with the API-ES interface to the MSD when the soluble chymotrypsin digest of myoglobin was analyzed and we are working to fix this. A further study will compare the digestion of denatured to undenatured myoglobin for soluble, crosslinked and IMER-bound chymotrypsin formats.

IMER autoproteolysis activity was examined in the CE-MS results by searching the mass spectra for peaks corresponding to m/z values of chymotryptic peptides of chymotrypsin. No such peptides were found, even when including three missed cleavages and charge states above $(M+4H)^{4+}$. Similarly, trypsin activity toward myoglobin was searched in the spectra because our chymotrypsin was not sequencing grade, but no tryptic peptides were found.

4.5. Concluding Remarks

Immobilized enzymes play an important role in proteomics. Chymotrypsin immobilization mediated by GA crosslinking was achieved to make insoluble enzyme particles as well as an IMER directly in a capillary using the CE instrument for fluid handling to deliver all reagents. The present study shows that the short-term storage temperature and humidity level of the GA-crosslinked chymotrypsin particles have little effect on BSA digestion. Furthermore, bulky fluorophore moieties on the substrate do not block digestion by the GA-chymotrypsin particles. A separate manuscript that describes optimizing the conditions for particle formation is concurrently in preparation. We succeeded in fabricating an in situ IMER and good myoglobin digestion (83% sequence coverage) was observed by CE-MS peptide mass mapping. Further optimization of the IMER preparation and digestion conditions is underway and we are investigating alternative methods to evaluate the extent of crosslinking that occurs. The goal is to couple the GA-crosslinked enzyme IMER to different separation and identification techniques to show its viability for on-line peptide mapping.

4.6. Acknowledgments

We thank NSERC (Natural Sciences and Engineering Research Council of Canada) and FRQNT (Fonds de recherche du Québec – Nature et technologies) for financial support and University of Montréal Chemistry Department for stipend assistance for G.G. We also thank M-C. Tang from the Chemistry Department's Regional MS Laboratory for help with interfacing the CE to the MSD and Prof. J-F. Masson for kindly letting us use his spectrophotometer and stereomicroscope. We are grateful to Dr. A. Fürtös of the Regional MS Laboratory for her advice in preparing this manuscript.

4.7. Supporting Information

The following section is adapted from the Supporting Information published with Ghafourifar et al. "Development of glutaraldehyde-crosslinked chymotrypsin and an *in situ* immobilized enzyme microreactor with peptide mapping by capillary electrophoresis"., in *Electrophoresis.*

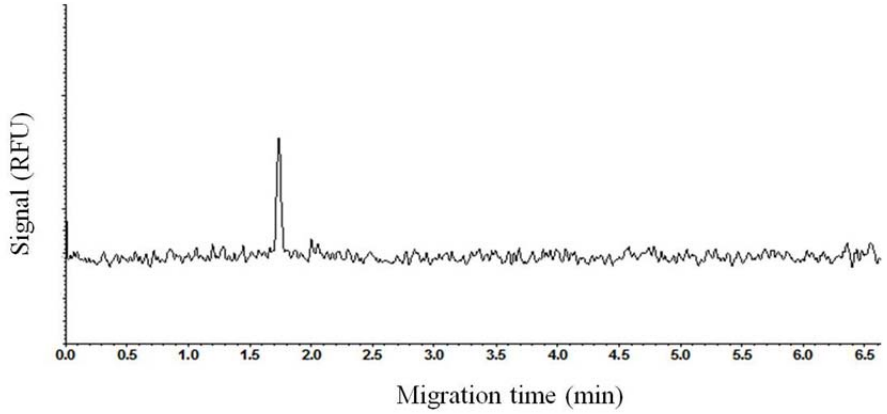

Figure S4.1. CE-LIF peptide map of FITC-casein substrate (1 μM) before digestion. The separation was carried out at +20 kV, in 50 mM phosphate buffer, pH 7.0. All other separation and detection conditions were the same as in section 4.3.6.

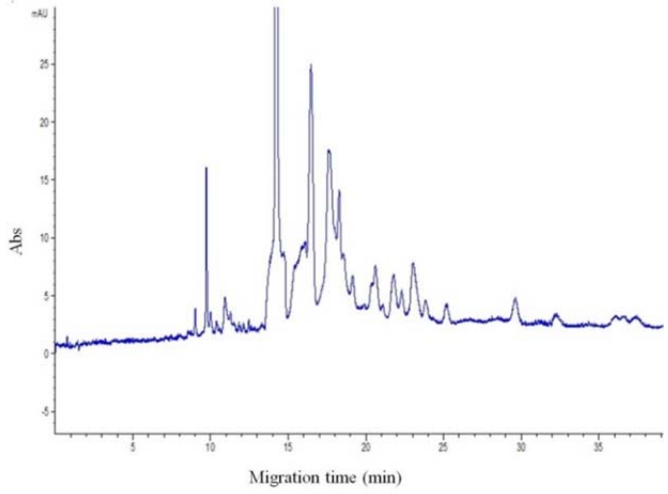

Figure S4.2. CE-UV peptide map of undenatured myoglobin (2 mg/ml) digested using GA-crosslinked chymotrypsin particles for 4 h at 37 °C. The enzyme:substrate ratio was 10:1 (mol:mol). The separation was carried out at +15 kV in 50 mM phosphate buffer, pH 6.4 (baseline noise was high at the typical separation pH of 2.5). All other separation conditions were the same as in section 4.2.6.

Chapter 5. A Study of the Chymotrypsin IMER Digestion Efficiency as a Function of Substrate Size

A version of this chapter will be submitted for publication to *Analytical Sciences* with authorship by Golfam Ghafourifar, Brian Fleury and Karen C. Waldron

Note on author contributions: All research in this paper was carried out by Golfam Ghafourifar. Brian Flury, a college-level student co-supervised by Golfam Ghafourifar during his 6-week internship, contributed to the experiments regarding separation buffer choice. The whole manuscript was written by Golfam Ghafourifar; corrections were done by supervisor Karen C. Waldron.

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5.1. Abstract

Enzymatic digestion of proteins, an essential technique used in bottom-up proteomics, can be achieved with immobilized, i.e., insoluble, enzymes. This method offers several benefits over soluble enzymes including reusability, high enzyme-to-substrate ratio for rapid digestion, limited autoproteolysis and integration with fluidic systems. We are using chymotrypsin as the proteolytic enzyme and glutaraldehyde (GA) as a crosslinking agent to immobilize chymotrypsin without the need for a secondary solid support. The resulting polymeric, spongy product of crosslinked GA-chymotrypsin prepared in batch form can be prepared inside a capillary column to fabricate an immobilized enzyme microreactor (IMER). IMERs based on GA-chymotrypsin were prepared using an automated capillary electrophoresis (CE) system for delivery of reagents and to digest BSA as an example of a medium-to-large sized protein substrate, a 9-residue peptide as an example of a small substrate, and finally a dipeptide. During digestion of these substrates, the conditions investigated were buffer composition, pH, applied pressure and reaction time in the IMER. The performance of the IMERs was followed by comparing peptide maps obtained by CE or HPLC coupled to either UV or mass spectrometric (MS) detection.

5.2. Introduction

The use of micro-scale immobilized enzyme reactors (IMERs) based in proteases has grown in recent years to address the need for fast digestions, reduced autoproteolysis and multiple usage especially for expensive enzyme catalysts [36, 40, 72, 92, 94, 151, 160, 167, 200]. Immobilization of enzymes essentially eliminates their intermolecular collisions and thus the potential for autoproteolysis. This, in turn, allows using a higher enzyme-to-substrate ratio than the typical ratio used in both soluble protease reactions and in-gel digestions and leads to a faster reaction. For example, Liang *et al.* reported that using a trypsin-IMER allowed a ten to one hundred fold increase of the enzyme concentration in comparison with a solution-phase digestion [189]. Dartiguenave *et al.* showed that efficient digestions of β-casein could be achieved in 80 s in a trypsin-IMER and that over 20 digestions could be made reproducibly [42]. On the other hand, Rivera-Burgos *et al.*

observed that the peptides generated from trypsin-IMER digested protein are not always identical to those obtained with the traditional 24 h digestion protocol using soluble trypsin, although this is less of a problem in tandem mass spectrometry (MS/MS) based peptide identification with sequence analysis[201]. Migneault *et al.* showed that autolysis peaks using immobilized trypsin were indistinguishable from background noise [41]. An advantage of the proteolytic IMER is that it can be coupled to high-resolution separation and detection instruments such as liquid chromatography-MS (HPLC-MS), capillary electrophoresis-MS (CE-MS), CE-UV and CE-laser induced fluorescence (LIF) to automate peptide mapping [72, 85, 132, 194, 202]. Although a certain number of IMER devices with enzymes immobilized on agarose-gel or silica particles are commercially available, a large number of novel IMERs have been described and may become commercialized.

Enzyme immobilization is relatively straightforward, whereas creating an efficient microIMER is not. There are several techniques to immobilize enzymes such as non-covalent adsorption, entrapment and encapsulation, ionic binding, covalent binding and enzyme crosslinking, to name a few [31, 183, 195, 198, 203]. Although immobilization through formation of a covalent bond requires several chemical steps, it boasts the highest popularity over other immobilization techniques because it is robust [156, 169]. Crosslinking an enzyme using a bifunctional agent such as glutaraldehyde (GA) is an example of covalent binding that requires the least chemical modification [204, 205]. GA is a well-known crosslinking agent used for more than 40 years [48, 50, 52, 106, 206].

Theoretically, any enzyme can be immobilized to make a microIMER; however, trypsin is the most commonly reported due to its high specificity (cleavage of peptide bonds only at the C-terminal side of lysine and arginine residues) and its rendering of medium-sized peptide fragments having a positive charge and, thus, its preference for protein digestion and proteomics studies [40, 41, 169, 207-210]. For use with amine-derivatized substrates, such as fluorescently labeled proteins, trypsin is not the ideal enzyme because it does not recognize the labeled lysine side chains as a cleavage site. The alternative to this, chymotrypsin, probably the second most commonly used proteolytic enzyme, cleaves proteins at the C-terminal side of aromatic residues so it can be used as a

viable alternative to trypsin in IMERs when the substrate is fluorescently labeled at its amino groups, allowing fluorescent detection of products to be used [53, 72]. We have shown that GA-crosslinked chymotrypsin can be used to digest fluorescein isothiocyanate (FITC) labeled casein in batch format and also that chymotrypsin can be immobilized inside a 250 μm ID, 40 cm long, fused silica capillary using GA-mediated crosslinking to produce a microIMER [53]. In the present work, GA-chymotrypsin IMERs were fabricated in a similar manner and the effects of certain reagent conditions (flow rate, pH) on digestion of small and large substrates were studied with the goal of identifying a universal or optimum digestion protocol. The digests of various substrates were analyzed by CE-UV or HPLC-MS, depending on their complexity.

CE is a high-resolution separation technique that can be sensitive to certain buffer conditions. The proper selection of buffer composition and pH is essential to obtain good peptide mapping. In most cases, a CE buffer of sodium phosphate at pH ≤ 2.5 insures all peptides are positively charged and silanol dissociation on the inner capillary wall is suppressed so that electroosmotic flow is very low and thus good resolution is achieved [42, 51, 117]. In previous studies from our group, CE-UV based peptide maps were obtained in these conditions. However, the digests in the current IMER study could not be separated by CE in phosphate buffer pH 2.5 for unknown reasons, but somehow related to the peptides coming from digestion in the GA-chymotrypsin microreactor. Therefore, we examined three additional buffer systems: ammonium bicarbonate at pHs 4.4 and 6.9 and sodium tetraborate at pH 8.6, to find the optimum CE-UV conditions for peptide mapping of the chymotryptic-IMER digests.

5.3. Materials and Methods

5.3.1. Reagents and Materials

α-Chymotrypsin from bovine pancreas type II, GA (25% aqueous solution), bovine serum albumin (BSA), monobasic sodium phosphate, dibasic sodium phosphate, acetic acid, trizma hydrochloride, Trizma base, calcium chloride, sodium hydroxide, (3-

aminopropyl)triethoxysilane (3-APTES, 99% purity), urea, and iodoacetamide were from Anachemia (Montreal, QC). The nonapeptide WAGGDASGE, sodium acetate, sodium borate, and *DL*-phenylalanine, phosphoric acid, tryptophan and dithiothreitol (DTT) (98% purity) were purchased from Sigma Aldrich (Oakville, ON). Hydrochloric acid was from EMD Millipore (Gibbstown, NJ). The dipeptide H-Phe-His-OMe·2HCl (FH-OMe) was purchased from Bachem (Torrance, CA). Sodium dodecyl sulfate (SDS) was from Fisher Scientific (Hanover Park, IL). Fused silica capillary tubing for the IMER (250 µm ID, 360 µm OD) was obtained from Chromatographic Specialties Inc. (Brockville, ON). Fused silica capillary tubing for CE separations (75 µm ID, 375 µm OD) and Inner-Lok connectors were from Polymicro Technologies (Phoenix, AZ). Syringes were purchased from BD (Franklin lakes, NJ). A multi-cartridge Milli-Q filtration/deionization system (Millipore, Bedford, MA) was employed to purify the distilled water used in preparation of all solutions and buffers.

5.3.2. BSA Substrate Denaturation

BSA was first denatured in an ammonium bicarbonate solution (0.4 M, pH 8.0) containing 8 M urea, then disulfide bonds were reduced with DTT (45 mM in water) followed by alkylation with iodoacetamide (100 mM in water), as described previously [53]. The final solution was diluted using Tris-HCl (100 mM, pH 7.8) containing 10 mM $CaCl_2$ to reach a final substrate concentration of 2 mg/mL.

5.3.3. Digestion of Substrate by Soluble Chymotrypsin

Chymotrypsin solution (1.3 mM in water) was added to substrate to reach a given enzyme:substrate ratio (mol:mol), as indicated in the following sections, and the solution was incubated at 37 °C for 4 h, then analysed by peptide mapping.

5.3.4. Preparation of Glutaraldehyde-Immobilized Chymotrypsin Particles

Chymotrypsin stock solution (1.3 mM in water) was diluted using 50 mM phosphate buffer pH 6.4 to reach 0.15 mM. A diluted solution of GA (2.5% in water) was added drop-wise to the enzyme solution and the immobilization reaction proceeded for 2 h at room temperature. The solution was then centrifuged at 3000 rpm for 2 min and the supernatant was decanted off. The insoluble product was gently washed to remove excess GA and unreacted enzyme with 3 × 200 µL buffer, then 3 × 200 µL NaCl (500 mM), and finally with 1 ×200 µL buffer. Next, 200 µL glycine (200 mM in phosphate buffer) was added to react with any remaining unreacted aldehyde groups for 3 h at room temperature. The final product was washed with 3 × 200 µL buffer followed by 3 × 200 µL water. The immobilized chymotrypsin was stored at -20 °C in 200 µL water.

5.3.5. Digestion of Substrate by Immobilized Chymotrypsin Particles

The substrate solution was added to immobilized chymotrypsin to reach the enzyme:substrate ratio (mol:mol, based on 94 ± 2% immobilization efficiency [53]) as indicated in the following sections. Digestion was performed in a 1.5 mL microcentrifuge tube by shaking gently (50 min^{-1}) using a model SK-10 shaker (BEA-Entprotech Corp, Hyde Park, MA) at 37 °C for 4h. The digest solution was then decanted off and injected directly into the CE for separation.

5.3.6. *In situ* IMER Fabrication

A series of six IMERs in total were fabricated using a procedure similar to that reported previously where the IMER internal wall was derivatized by sequentially passing 3-ATPES, glutaraldehyde and chymotrypsin through the capillary [53]. Specifically, a 43 cm length of fused silica capillary (250 µm ID, internal volume of 21.1 µL) installed in the CE system (Agilent Technologies, Waldbronn, Germany) was first rinsed with 1 M NaOH for 1 h followed by water for 10 min and then methanol for 10 min. All the solutions were passed through the capillary at 50 mbar using the Agilent CE system autosampler,

unless stated otherwise. The capillary was then dried by flushing with air at 950 mbar for 10 min. The internal wall was then activated by rinsing with 1 M HCl for 1 h. Then 3-APTES (10% (v/v) in methanol) was passed continuously through the capillary at 60 °C for 1 h, and then left at rest for an additional 3 h to functionalize the internal wall with amino groups. The capillary was cooled to room temperature and then rinsed for 3 min with water. Next, GA (2.5% v/v in water) was passed through the capillary for 1 h at room temperature followed by rinsing for 3 min with phosphate buffer, pH 6.4. Chymotrypsin (1.3 mM in water) was then continuously passed into the capillary for up to 1 h. The sequence of adding GA, buffer then chymotrypsin was repeated three times. The capillary was then left at room temperature to allow the crosslinking reaction to occur. After 3 h, the IMER was rinsed with water for 2 min.

Substrate was passed through the IMER using different protocols as described below to find the best method for digestion. The digests were collected at the IMER outlet into 20 µL water (to prevent evaporation as the digest exited the capillary) and then subjected to analysis (peptide mapping) by CE or HPLC.

5.3.7. Separation Buffers for Peptide Mapping by CE-UV

The following buffers were prepared to identify the most suitable conditions for separating IMER digests with the highest resolution of peaks: 25, 50, and 75 mM sodium phosphate, each at pH 2.5, 4.4 and 6.8; 50 mM ammonium bicarbonate at pH 4.4 and 6.9; 25, 50, and 75 mM sodium borate at pH 8.6. All buffers were prepared in Milli-Q water and filtered through a 0.22 µm nylon syringe filter (Chromatographic Specialties) prior to use.

5.3.8. Digestion Using the IMERs

Different experimental methods were applied for the six IMERs to increase substrate digestion as evidenced first by the collection of sufficient digest at the IMER outlet and then by the quality of peptide maps, *i.e.,* higher intensity and number of expected peaks. Substrates with different size and complexity were passed through the IMERs using

different parameters of applied pressure (which dictates flow rate and thus residence time in the IMER), rinsing time and loading time (*i.e.*, injection volume). The digestion methods are described in the sections below according to the substrate tested and the IMER fabricated. In all cases, the IMER was thermostated at 37°C during digestions.

5.3.8.1. IMER Digestion of the Dipeptide Phe-His-OMe (FH-OMe)

FH-OMe (1.6 mM in water) as substrate was passed through two different IMERs using the methods summarized in Table 5.1. In Method 1, 200 μL FH-OMe solution was passed through IMER #1 in four steps: first, by applying the "normal" Agilent CE injection pressure (34.5 mbar) for 0.15 min followed by a second, lower pressurization (2 mbar) for longer time (30 min) and then another 5 mbar pressure injection for even longer (190 min), which emptied the inlet vial. Since the full 200 μL had still not eluted at the capillary outlet, the IMER was flushed at 950 mbar, which is the Agilent CE "wash" pressure, for 0.10 min to push out all the digest. Almost all 200 μL of the digest were collected by the end of these 4 pressurization steps, and this solution was mapped by CE-UV.

In Method 2a, 200 μL of FH-OMe solution was passed through a new IMER (IMER #2) by applying 50 mbar for 120 min, leading to collection of only 40 μL of digest the IMER outlet whereas the inlet vial was more-or-less empty. It should be noted that applying 50 mbar × 120 min corresponds to an equivalent volume of 50 mL passing through an open tube of 43 cm × 250 μm ID.

In Method 2b, the 40 μL of digest collected from Method 2a was reinjected into the IMER #2 to increase the extent of digestion. Approximately 30 μL was collected at the outlet and mapped by CE-UV.

Table 5.1. Methods used to pass substrate FH-OMe through an IMER for digestion using the programmable CE system.

Substrate solution	Pressure applied to injection vial (mbar)	Duration (min)	Approximate volume collected
Method 1 (IMER #1)			
FH-OMe (200 µL)	34.5	0.15	0
	2.0	30	0
	5.0	190	~ 200 µL
	950	0.10	A few µL
Method 2a (IMER #2)			
FH-OMe (200 µL)	50.0	120	40 µL
Method 2b (IMER #2)			
Solution collected from Method 2a (~ 40 µL)	50.0	120	30 µL

5.3.8.2. IMER Digestion of the peptide WAGGDASGE

The nonapeptide substrate WAGGDASGE (1.3 mM in water) was passed through a new reactor (IMER #3) using two different methods, as summarized in Table 5.2. Since 50 mM acetate buffer pH 5.6 had been used previously for digesting substrate by GA-immobilized enzyme particles in batch format, this buffer was passed through IMER #3 prior to introducing substrate. The digestion in Method 3a was devised to simulate conditions of digestion using the enzyme particles. The buffer was flushed through IMER #3 by applying 950 mbar for 0.50 min prior to injecting the substrate. WAGGDASGE (200 µL) was then flushed through the IMER for 2.00 min at the same pressure.

In Method 3b, IMER #3 was reused and flushed with 50 mM acetate buffer pH 5.6 for 0.20 min to push out possible remaining digest from inside the reactor, produced during Method 3a.

Table 5.2. Methods used to pass substrate WAGGDASGE through the IMER for digestion using the programmable CE system.

Substrate solution	Pressure applied to injection vial (mbar)	Duration (min)	Approximate volume collected
Method 3a (IMER #3)			
Ammonium acetate buffer, 50 mM, pH 5.6	950	0.50	(not measured)
WAGGDASGE (200 µL)	950	2	100 µL
Method 3b (IMER #3)			
Ammonium acetate buffer, 50 mM, pH 5.6	950	0.20	200 µL

5.3.8.3. IMER Digestion of the protein BSA

The substrate BSA (0.12 mM in water, denatured) was passed through new IMERs using four methods, as summarized in Table 5.3. In Method 4, BSA substrate solution was flushed through IMER #4 for 2 min. The collected digest (150 µL) at the outlet vial was re-injected to the same reactor (IMER #4) by applying 50 mbar for 30 min to increase the level of digestion.

In Method 5a, denatured BSA solution (0.12 mM) was flushed through a new reactor (IMER #5) for 0.20 min followed by pulsed flushes for 0.40, 0.60, and 0.80 min until finally the digest was seen at the outlet. At the end, we were able to collect about 60 µL of digest, which was analysed by peptide mapping.

In Method 5b, the collected digest from Method 5a was flushed through IMER #5 again for 1.00 min at 950 mbar to increase the extent of digestion. This injection was followed by flushing water through IMER #5 for 0.20 min to push out the entire digest from IMER #5, yielding a collected volume of 80 µL.

In Method 6, denatured BSA solution (0.12 mM) as substrate was passed slowly through a newly prepared reactor (IMER #6) by applying 50 mbar pressure for 60 min

followed by flushing at 950 mbar for 1 min. The digest (200 µL) was collected at the outlet and subjected to peptide mapping.

Table 5.3. Methods used to pass substrate BSA (denatured) through the IMER for digestion using the programmable CE system.

Substrate solution	Pressure applied to injection vial (mbar)	Duration (min)	Approximate volume collected
Method 4 (IMER #4)			
BSA (200 µL)	950	2	150 µL
Solution collected from Method 4 (150 µL)	50	30	50 µL
Method 5a (IMER #5)			
BSA (200 µL)	950	0.20	0
		0.40	0
		0.60	0
		0.80	60 µL
Method 5b (IMER #5)			
Collected solution from Method 5a (~ 60 µL)	950	1.00	(not measured)
Water	950	0.20	80 µL
Method 6 (IMER #6)			
BSA (200 µL)	50	60	(not measured)
	950	1.00	~ 200 µL

5.3.9. Peptide Mapping by CE-UV

Peptide mapping of substrate digests was performed on the Agilent CE System which is equipped with a UV/Vis diode array detector (DAD). The electropherograms were recorded at 200 nm. Separations were performed at +15 kV at 25 ^{0}C in a fused silica

capillary (43 cm total length, 75 µm id). The capillary was rinsed with 0.1 M HCl, then water, each for 3 min at 950 mbar pressure prior to each injection. After each separation, the capillary was rinsed with 0.1 M NaOH, then water, for 1 min each, then with 1 M HCl for 2 min and finally with SDS for 3 min, each by applying 950 mbar pressure. The sample (digest) was injected by applying 34.5 mbar pressure for 5 s followed by buffer injection for 2 s.

5.3.10. Peptide Mapping by HPLC-MS

Digests were submitted to the Regional Mass Spectrometry Laboratory in the Chemistry Department at Univeristé de Montréal where analyses were made on an Acquity ultra-performance liquid chromatography Class I system (Waters Ltd., Milford, MA) coupled to a Waters Synapt G2-S quadrupole-time-of-flight mass spectrometer with electrospray ionization HPLC-ESI-TOFMS in positive ion mode. MassLynx 4.1 software was used to control the system and process the data.

Separations were carried out using an Acquity CSH C18 column (75 × 2.1 mm, 1.7 µm particles) from Waters Ltd., maintained at 40^{o}C. The auto-sampler temperature was set at 10^{o}C to avoid sample degradation. The eluents consisted of 0.1% of formic acid in water (eluent A) and 0.1% of formic acid in acetonitrile (eluent B), and the initial mobile phase contained 5% B. The following gradient elution was applied at a flow rate of 400 µL/min: 5 to 90% B in 7 min; hold 90% B for 1 min. Eluent B was then decreased from 90 to 5% from 8 to 8.5 min and held constant for up to 12 min to permit column equilibration. The injection volume was 3 µL.

The electrospray interface was operated in positive ion mode. The capillary voltage was 0.8 kV, the source temperature 100 ^{o}C and the desolvation temperature 250^{o}C. Mass spectra were acquired in MS^{E} resolution mode from m/z 100-3200 in both functions. A linear collision energy ramp of 20-35 was applied in function 2 in the transfer cell. Accurate mass values from function 1 were used for peptide elemental composition confirmation while manually aligned fragments from function 2 were used for peptide sequence confirmation. The mass range scanned was up to m/z 3000. The databases

ExPASy (http://www.expasy.org) and Protein Prospector (http://prospector.ucsf.edu) were used to determine the theoretical chymotryptic peptides for all three substreates: FH-OMe, WAGGDASGE and denatured BSA. The maximum number of missed cleavages was set to two and the peptide masses investigated were for the ions $[M+H]^{+}$and $[M+2H]^{2+}$only.

5.4. Results and Discussion

5.4.1. Choosing the Separation Buffer for Peptide Mapping by CE-UV

For unknown reasons, using the traditional phosphate buffer at pH 2.5 for peptide separation was problematic because the capillary wall was somehow changed after the first injection of any IMER-digested sample, and this sabbotaged additional analyses with the same capillary until very intense washes were made with 1 M NaOH and HCl. The problem usually involved a shift in the electropherogram baseline, which drifted both in the positive and negative directions dramatically. In addition, the baseline become excessively noisy and the current dropped to zero after a few minutes of applying voltage, which caused the run to be aborted.

As a result of the above problems we decided to increase the pH, which would increase the EOF, hoping to facilitate the peptides' migration through the capillary. However, the problems with current drop and drifting baseline continued, even after changing the phosphate buffer pH from 2.5 to 4.4 and 6.8 (Figure S5.1, Supplementary data), or changing the buffer concentration from 25 to 50 or even to 75 mM (data not shown), in an effort to modify the electric double layer in the capillary by modifying ionic strength. In order to get around this problem and find a suitable background electrolyte (BGE), several other buffers and pHs were studied.

Using the 50 mM ammonium bicarbonate buffer at pHs 4.4 and 6.9 provided a more stable current. However, the baseline drifted noticeably up and down throughout the electropherogram (Figure S5.2, Supplementary data), so this was abandoned. Using a 50 mM sodium borate buffer pH 8.6 gave the most stable current and baseline during the

separation, so this was investigated further to find the best conditions for separation (peptide mapping).

5.4.2. The Effect of Borate Buffer Concentration on CE-UV Mapping

To study the effect of borate buffer concentration on CE separation of digests from the IMERs, we used BSA digested using Method 5b as the sample. Sodium borate buffer pH 8.6 was investigated at three different concentrations: 25, 50 and 75 mM. Although all three concentrations tested showed stable current and baseline with digests from the IMERs, using 50 mM buffer yielded a better separation than at 25 and 75 mM. We could identify 4 significant peaks in BSA digests from Method 5b using the 50 mM borate buffer as BGE as shown by the peak resolutions given in Table 5.4, calculated between neighboring peaks.

Table 5.4. The peak resolutions calculated between two neighbouring peaks as a function of borate buffer concentration (pH 8.6), using BSA digests from IMER #5 digested by Method 5b as the sample. The separations were performed by CE-UV.

Borate buffer concentration (mM)	**$R_{1,2}$**	**$R_{2,3}$**	**$R_{3,4}$**
25	0.65	0.09	4^{th} peak was not detectable
50	1.01	1.03	0.39
75	2.78	3^{rd} peak was not detectable	

As seen in Table 5.4, changing the buffer concentration to 25 mM diminished the resolution between the first and the second peaks and the forth peak was not detectable. Using the 75 mM buffer provided a much better resolution between the first and the second peaks, however the third peak was not detectable. Therefore, the 50 mM borate buffer was used for all subsequent CE separations.

5.4.3. Blank Digestion Using an IMER

A blank experiment was performed to investigate whether any IMER fabrication reagents remained in our collected digest samples which might interfere with our separation and detection. As the blank, 200 µL pure water was passed through an IMER by applying 50 mbar pressure for 1 h. The collected solution at the outlet was mapped using CE-UV. The electropherogram did not show any peaks that could be attributed to the presence of reagents from the IMER. The sample was spiked with FH-OMe, which showed the electropherogram expected for the standard solution of 80 µM FH-OMe (Figure 5.1).

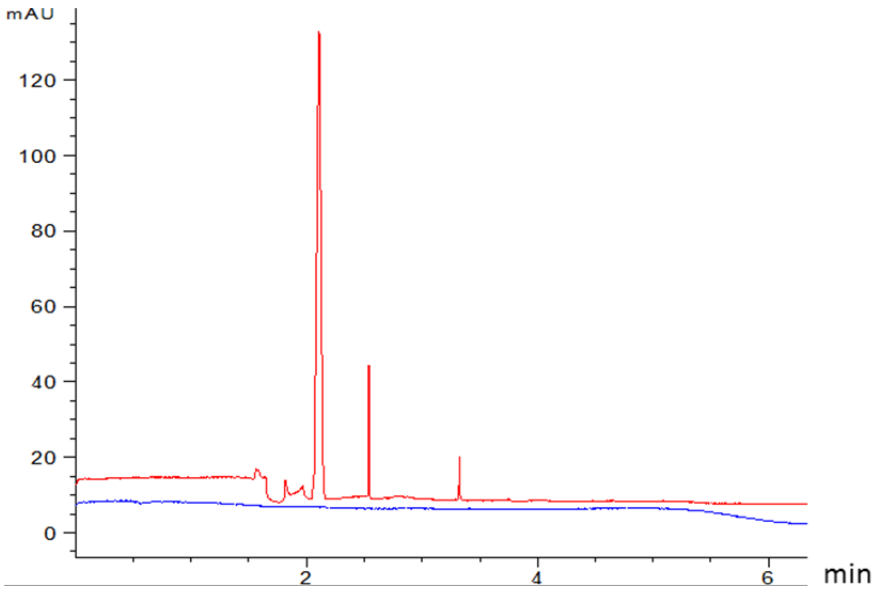

Figure 5.1. Blank digestion (blue trace) using water passed through the IMER for 1 h at 50 mbar pressure. The blank digest spiked with 80 µM FH-OMe (red trace). CE-UV separations were performed at +15 kV using 50 mM borate buffer, pH 8.6, at 25 °C in an uncoated fused silica capillary (43 cm total length, 75 µm I.D.) with detection at 200 nm.

There was no significant difference between the blank digest spiked with FH-OMe and the standard solution of FH-OMe. Thus, there appeared to be no interference caused by the IMER reagents in analysing the collected sample by CE-UV.

5.4.4. IMER Digests of Substrate FH-OMe

In the first digestion method, the single substrate sample (200 µL FH-OMe) required four attempts to flow through the reactor using the pressures and times shown in Table 5.1. Using Method 1, FH-OMe substrate was first injected into IMER #1 for 0.15 min while applying 34.5 mbar pressure, and then pushed through continuously by applying 2 mbar for 30 min, which should lead, respectively, to a 3.3 µL then a 116 µL injection plug in an open capillary of the same dimensions (43 cm × 250 µm ID). Although the second injection should have pushed the entire digest to the outlet, no liquid was collected in the outlet vial. This indicates that 2 mbar pressure was insufficient to pass the substrate through the IMER, either because there is a blockage or just a very narrow flow path. Therefore, the pressure and the time were increased to 5 mbar ×190 min. The total volume of substrate in the inlet was equal to 200 µL and it was mostly passed through the IMER; however, a few drops were left in the inlet. To insure the substrate was passed through completely and no substrate was left in the inlet or inside the IMER, we applied 950 mbar (i.e., the Agilent flushing pressure) for 0.1 min. The total time for digesting the substrate using this method was 220 min. The CE-UV peptide map is shown in Fig. 5.2, blue trace. The digest solution was then spiked with 2 mM Phe to verify the migration time of the Phe product peak (Figure 5.2, red trace). There was no standard for the other product, H-OMe, which should have a similar CE migration time to the substrate at pH 8.6.

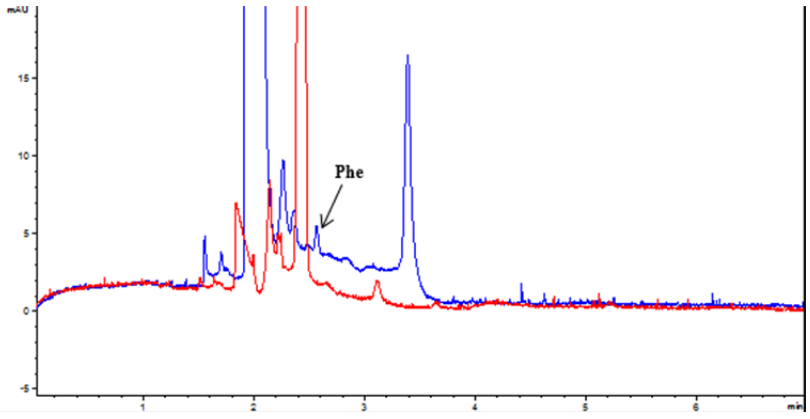

Figure 5.2. CE-UV peptide map FH-OMe (1.6 mM) digested by IMER #1 using Method 1 (blue electropherogram). The digest solution was spiked with phenylalanine solution (2 mM) in order to determine the Phe peak (red electropherogram). Separation conditions are the same as in Figure 5.1.

In order to quantify the concentration of Phe in the IMER digest, different concentrations of the standard Phe solution were injected into the CE and the areas were measured. Each standard sample was injected three times and the average area was plotted to make the calibration curve shown in Figure 5.3. The larger error bar for 0.08 mM Phe might be due to a faulty injection.

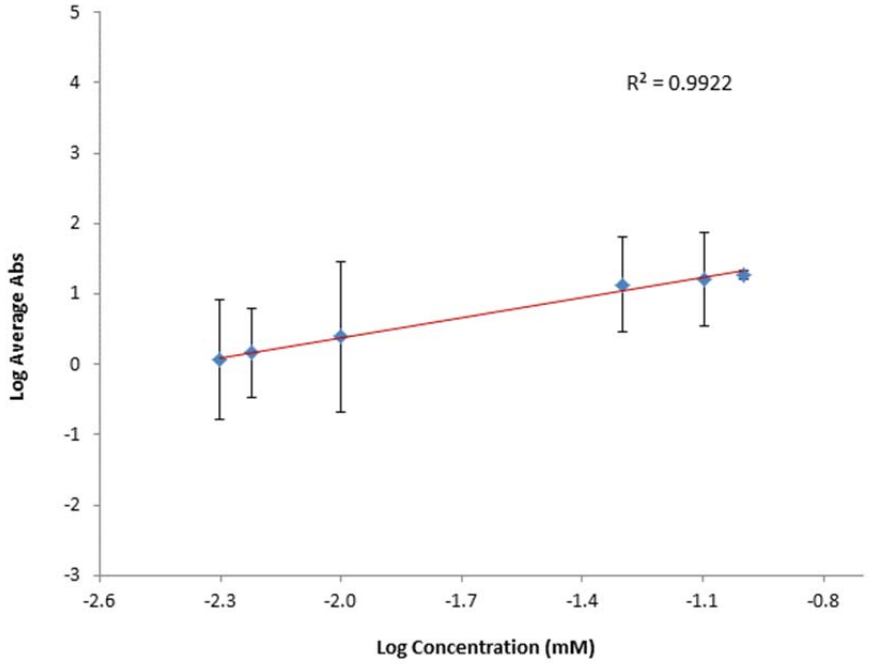

Figure 5.3. Calibration curve (log-log plot) for different concentrations of Phe standard injected the CE. The average area was measured for three injections.

The limit of detection (at 3σ) for analysis of Phe standard by CE-UV was calculated to be 0.7 μM and the limit of quantification was 2.4 μM. In order to calculate the background noise (i.e., the σ value), the average area and the standard deviation of 10 peaks corresponding to noise were measured for the electropherogram representing the lowest concentration (5.0 μM). According to the calibration curve, the concentration of Phe from the IMER #1 digested FH-OMe using Method 1 was measured as 0.05 mM, which is only about 3.1% of the initial substrate concentration prior to digestion. However, because of the possibility of having some reagents from the IMER present, and dilution of digests, we also

measured the ratio of Phe peak area to undigested FH-OMe peak area in the electropherogram. Using this method, the ratio was calculated as 31.9%, which is 57.7% of the ratio when GA-immobilized chymotrypsin was used in batch format (particles) instead of IMER format for digestion of the same substrate and concentration. It is important to point out that the enzyme-to-substrate ratio is unknown for the IMER format.

As a result of the low amount of digest collected at the outlet (30 μL compared to the expexted 200 μL), the difficulty with injecting substrate using low pressures (2 – 50 mbar), and the limited digestion of FH-OMe according to the peptide map in Figure 5.2 and calibration curve data, the overall digestion method was adjusted. Method 2a, using new IMER #2, implied increasing both pressure and time to pass substrate through the IMER. Based on Poiseuille laminar flow in an open tube calculations, 50 mbar applied for 120 min should be enough to pass all 200 μL substrate through the IMER to the outlet. However, because of the immobilized enzyme layers on the inside wall, a longer time and higher pressure should be needed to pass all 200 μL of substrate through.

Using Method 2a, FH-OMe digest collected in the outlet vial was only about 40 μL. A tiny portion of this was subjected to CE-UV analysis (Figure 5.4-A). The peak area ratio of Phe to undigested FH-OMe was again 31.9% . The rest of the collected digest (40 μL) was then re-injected to IMER #2 for further digestion (Method 2b) for another 120 min at 50 mbar. The total digestion time was now 240 min. However, because of the small volume of collected sample using Method 2b, only about 30 μL was collected at the IMER #2 outlet using Method 2b. The peak area ratio between Phe and FH-OMe decreased to 25.0%, which is 45.3% of the ratio of digestion by immobilized chymotrypsin particle in batch format. Both Phe and undigested FH-OMe peaks showed lower intensity after the second digestion, as shown in Figure 5.4-B. This suggests that IMER reagents may be leaching from the capillary into the collection vial and causing background in the CE peptide mapping.

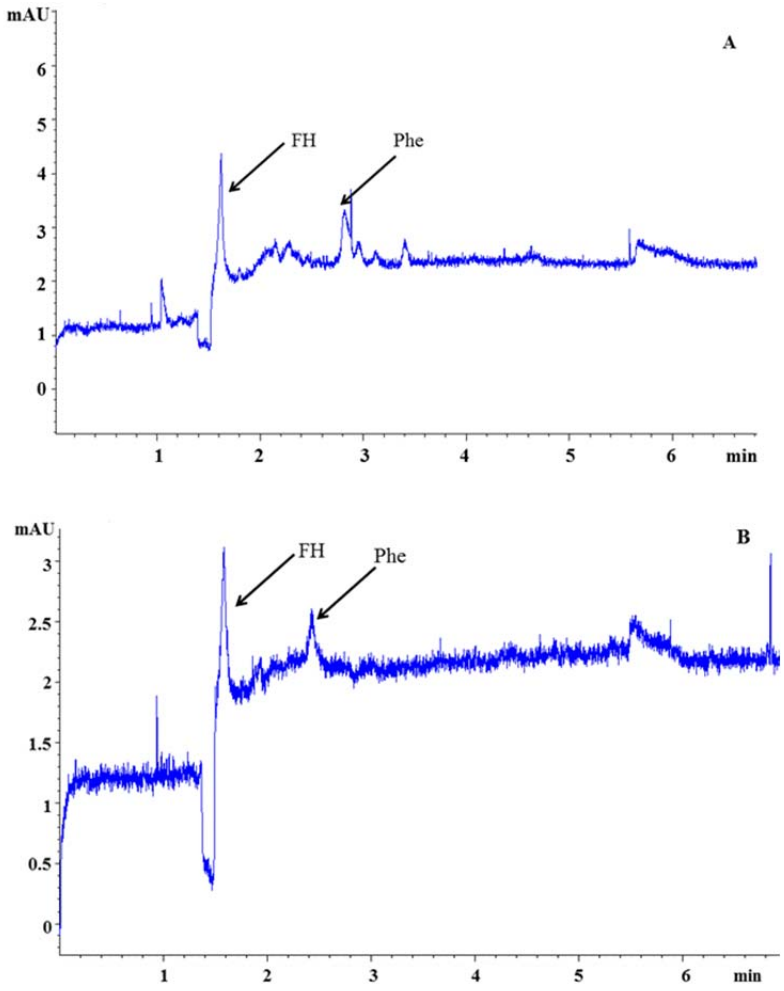

Figure 5.4. CE-UV map of the peptides collected by passing FH-Ome through IMER #2 using Method 2a (A) and Method 2b (B). The separation conditions were the same as in Figure 5.1

Both collected samples from Methods 2a and 2b were also analysed by HPLC-MS (data not shown) where we were able to identify two fragments for each digest: Phe at m/z 166 and His-OMe at m/z 317.

5.4.5. IMER Digestion of WAGGDASGE

A 50 mM ammonium acetate buffer, pH 5.6, was flushed (950 mbar) through IMER #3 for 0.5 min prior to introducing substrate to see if changing the pH inside the IMER would

have any effect on the digestion. The nonapeptide substrate at 1.3 mM (200 µL) was flushed (950 mbar) through IMER #3 for 2 min such that about 100 µL was collected in the outlet vial (Method 3a). The collected digest was mapped by CE-UV, giving the electropherogram in Figure 5.5 (blue trace), which shows several small, separated peaks and a large peak. Only two peaks are expected because only the peptide bond between the first residue Trp (W) and the second one, Ala (A), are theoretically cleaved by chymotrypsin. The digest was spiked with a standard solution of Trp (5 mM) to determine its migration time (1.6 min) in the electropherogram (Figure 5.5, red trace).

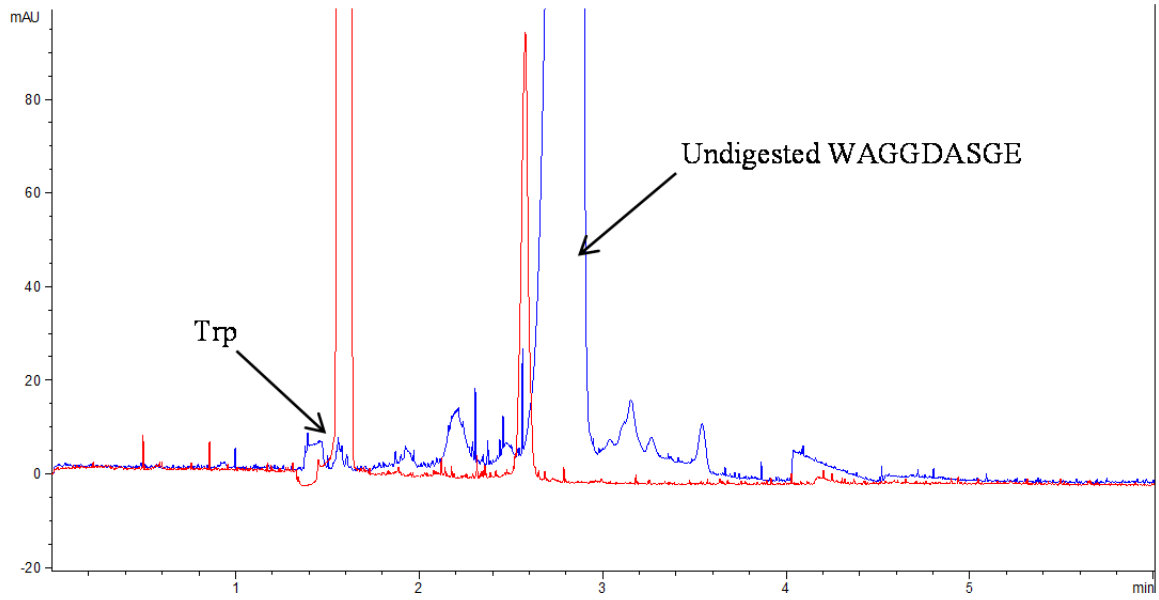

Figure 5.5. CE-UV peptide maps for the nonapeptide substrate WAGGDASGE (1.3 mM) digested by IMER #3 using Method 3a. Separation conditions for CE-UVwere the same as those given in Figure 5.1. The blue trace shows the electropherogram of collected digest and the red trace is the same sample spiked with 5 mM Trp standard.

The collected digest from IMER #3, Method 3b, was also analysed using HPLC-MS (data not shown) and a fragment with m/z 663 representing AGGDASGE was identified, as we expected. The peak for Trp was not within the scanned mass range.

Different concentrations of the Trp standard were injected into the CE-UV system in triplicate and the relative area was measured in each electropherogram, providing the

calibration curve shown in Figure 5.6. The limit of detection for measuring the concentration of Trp standard by CE-UV was calculated to be 0.37 μM and the limit of quantification as 1.2 μM.

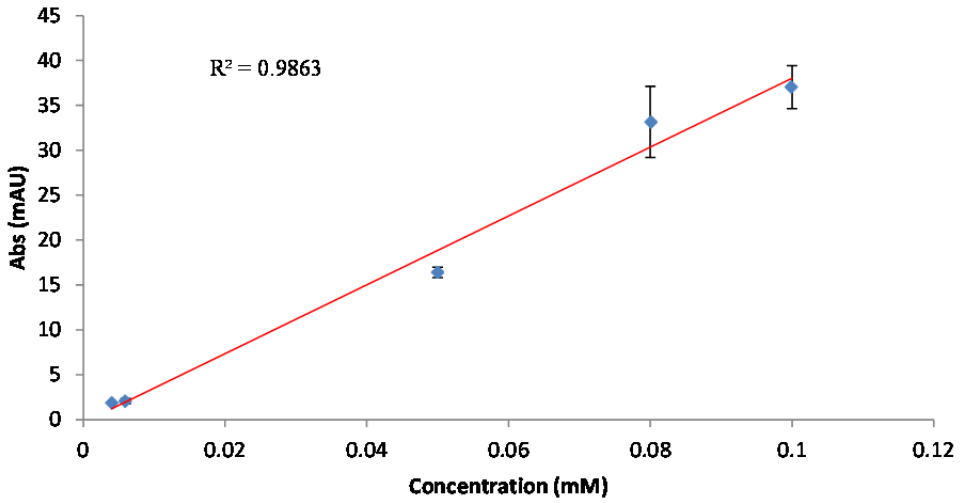

Figure 5.6. Calibration curve of standard Trp at 5 concentrations, each injected three times and the average area plotted with error bars for each concentration. Analyses were done by CE-UV using the separation conditions as in Fig. 5.1

Using the calibration curve, the concentration of Trp in the nonapeptide digest was calculated as 0.17 mM, which is 13.1% of the initial concentration of the substrate. The same method was used to calculate the concentration of Trp in a digest of the nonapeptide made using the GA-immobilized chymotrypsin in particle format for batch digestion (Figure 5.7, blue trace). The enzyme-to-substrate ratio was 1:1 for the batch format, and the ratio of Trp peak area to substrate was 71.4%, showing that it is more efficient for digestion than then IMER, either because the E:S ratio is higher, or because there is less interference with the peptide mapping by CE-UV. The Trp peak was identified in the digest by spiking the latter with a standard solution of Trp (Figure 5.7, red trace).

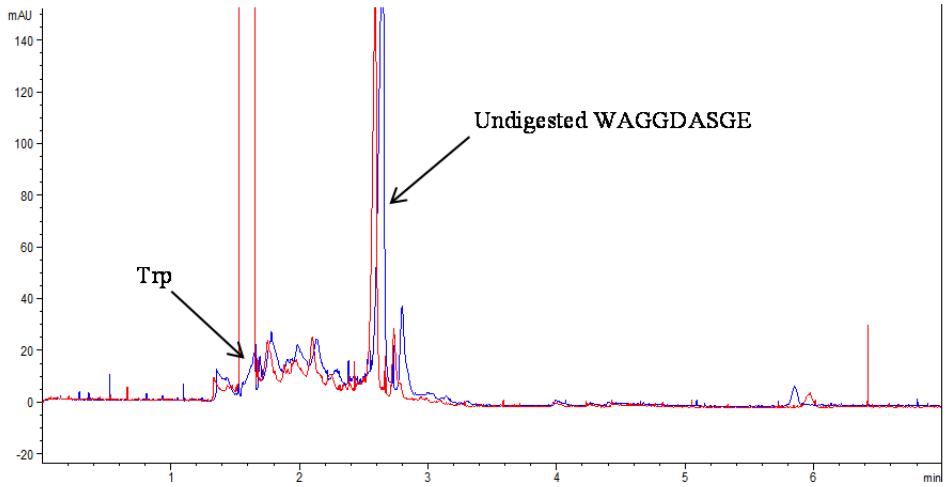

Figure 5.7. CE-UV peptide maps for substrate WAGGDASGE (0.21 mM) digested by GA-immobilized chymotrypsin particles in batch format for 4h at 37 ^{0}C, E:S of 1:1 mole ratio (blue trace). The red trace is the digest sample spiked with 5 mM Trp standard. Separations were carried as in Figure 5.1.

The IMER #3 was next rinsed by flushing ammonium acetate buffer though for 0.2 min at 950 mbar to push out any remaining digest (or undigested substrate) from the IMER (Method 3b). Although about 200 µL was collected in the outlet vial, the CE analysis did not show any detectable amount of either substrate or Trp product.

5.4.6. IMER Digestion of Denatured BSA

We chose BSA, a highly folded protein that needs to be denatured, reduced and alkylated before digestion, as an example of a large substrate in order to evaluate the IMER efficiency. However, it should be noted that according to Rivera-Burgos *et al.* [108] "limit peptides" (peptides without missed-cleavages) are not necessarily equimolar to the parent protein. These authors indicate that, although breaking disulfide bonds and adding urea makes more cleavage sites accessible, it is not enough to "globally unmask the peptide bonds required" [108]. Therefore, the complete digestion of BSA is less likely and we expect to see some missed-cleavages, which might impact the identification, which is especially true for digestion of large or complex proteins.

The protein substrate was denatured and the final concentration of 0.12 mM BSA was digested using IMERs #4, 5 and 6 by applying different digestion protocols (Table 5.3). The denatured BSA solution was flushed through IMER #4 (950 mbar pressure) for 2 min using Method 4. About 150 µL was collected at the outlet. The collected solution was re-passed through IMER #4 much more slowly (50 mbar for 30 min) to gain more digestion, without analyzing it first. The collected digest this time was only about 50 µL. The digest was analysed by HPLC-MS peptide mapping and the total ion chromatogram is shown in Figure 5.8. We were able to look at the MS spectra for all the retention times and identify 130 amino acids out of 607 in BSA (see Table S5.1 in Supplementary Data for the 29 identified peptides), which corresponds to 21% coverage of the primary sequence using digestion Method 4. The sample was also mapped by CE-UV, as shown in Figure 5.9.

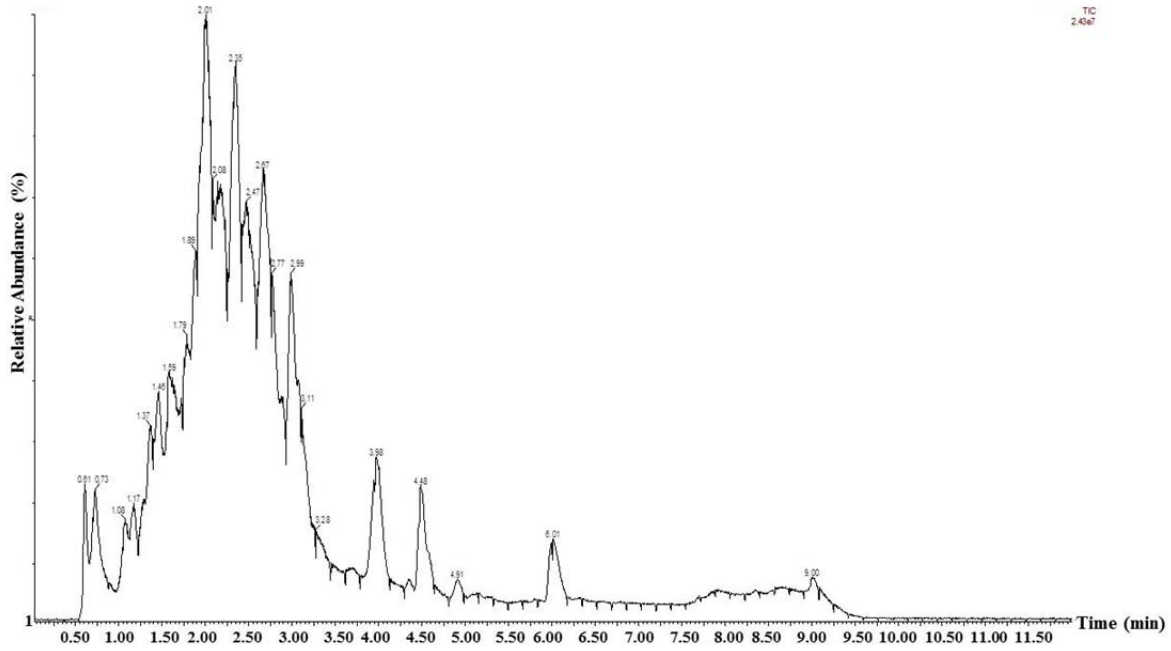

Figure 5.8. HPLC-MS peptide map (total ion chromatogram) for denatured BSA (0.12 mM) digested by the chymotrypsin IMER using Method 4 with IMER #4 (see Table 5.3) for a total substrate digestion/residence time of 32 min. Analysis conditions are given in the experimental section.

Although digestion Method 4 allowed us to identify many of the expected peptides, the HPLC-MS peptide map (Figure 5.8) and CE-UV peptide map (Figure 5.9) did not show good separations for the BSA digest. The large peak in Figure 5.9 represents the undigested BSA in the CE-UV electropherogram. Most of the peptides show low intensity signals indicating low concentrations, and the peaks mostly overlapped (Figure 5.9).

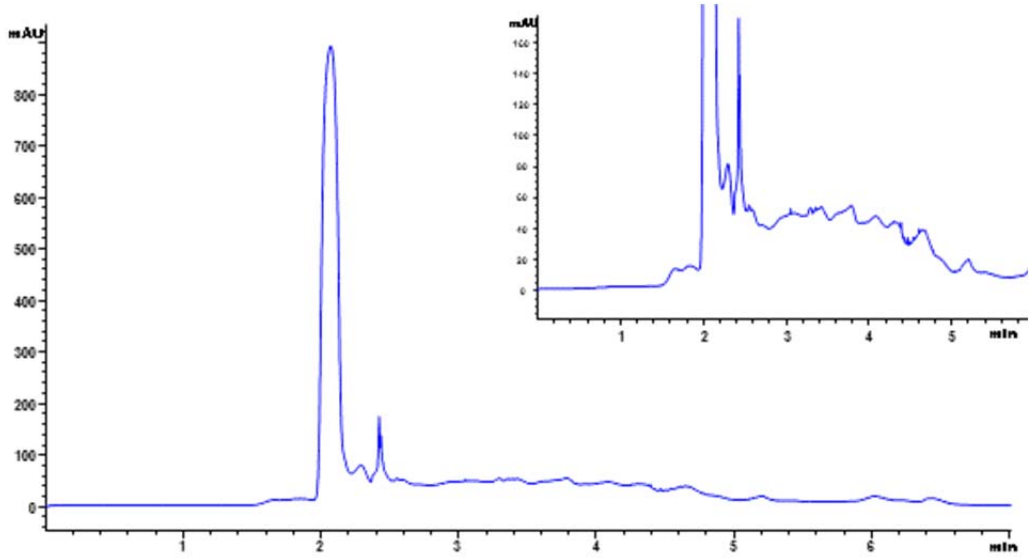

Figure 5.9. CE-UV peptide map for denatured BSA (0.12 mM) digested by chymotrypsin IMER #4 using Method 4 for a total substrate digestion/residence time of 32 min. The separation conditions were the same as in Figure 5.1. The insert represents the zoomed in electropherogram.

To decrease the digestion time for BSA in the IMER, we used only high pressure (flushing by applying 950 mbar) for both Methods 5a and 5b. A denatured BSA solution was passed through IMER #5 using Method 5a. The BSA solution was flushed through the IMER for 0.20 min. Although flowing a solution for this time and pressure would normally correspond to over 17 fold of the total volume of an empty capillary with the same dimensions, nothing eluted from the IMER, suggesting it was blocked or at least not very porous. The flushing time was then increased to 0.40 min and then 0.60 min, but with no volume change either at the outlet or inlet observed. Therefore, the flushing time was

increased yet again to 0.80 min and finally about 60 μL was collected at the outlet. It was not clear what happened to the loss of injected volume. It may have been evaporated from the inlet vial because of applying pressure here. It is also possible that a small leak through the CE pre-punch hole occurred, although it was hard to find evidence for this. The collected digest solution was analysed by CE-UV. However, the electropherogram did not show a good digestion for Method 5a (data not shown). Therefore, the digest solution was re-passed through IMER #5 according to Method 5b (see Table 5.3), with a water rinse following the sample introduction. The digest collected at the outlet was mapped by CE-UV and HPLC-MS. The CE-UV electropherogram showed more digestion than for Method 5a (data not shown). Peptide mapping by HPLC-MS allowed us to identify 34 peptides in the MS spectra corresponding to 205 amino acids, which represents a sequence coverage of 34% for BSA (Table S5.2, Supplementary Data) for a total substrate digestion/residence time of only 3.2 min. On the other hand, both CE and HPLC peptide maps showed weak separation eficiency accompanied by overlapping peaks (data not shown), which is undesirable.

IMER #6 was prepared and digestion Method 6 (Table 5.3) applied using a protocol that was almost the inverse of Method 4. A lower pressure and slower digestion of denatured BSA was done first (50 mbar × 60 min) and then flushing at 950 mbar for 1 min to investigate whether switching these orders would improve the digestion. Using Method 6, about 200 μL was collected in the outlet vial. The collected digest was mapped by CE-UV, which is shown in Figure 5.10 where there are now more peptidic peaks with a better separation efficiency and lower intensity of the undigested BSA, indicating a better digestion of the substrate compared with the other methods in Table 5.3. Similarly, the HPLC-MS peptide map was slightly better (Figure 5.11). The collected peptides were identified in the HPLC-MS spectra, resulting in 210 amino acids of a total 607 being identified across 38 peptides. Thus using Method 6 to digest the denatured BSA gives 35% primary sequence coverage (see Table S5.3, Supplementary Data).

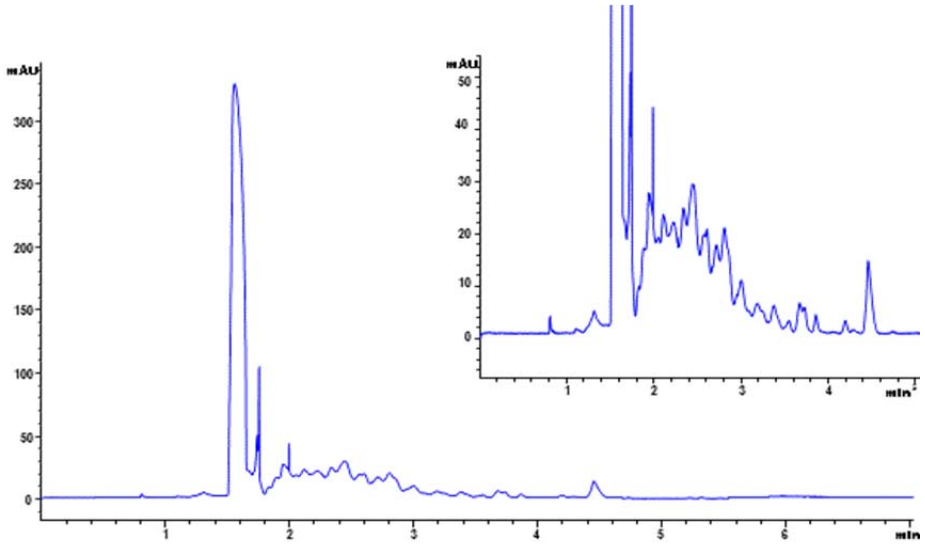

Figure 5.10. CE-UV peptide map for denatured BSA (0.12 mM) passed through IMER #6 using digestion Method 6 (see Table 5.3) for a total substrate digestion/residence time of 61 min. The separation conditions were the same as in Figure 5.1. The insert represents the zoomed in electropherogram.

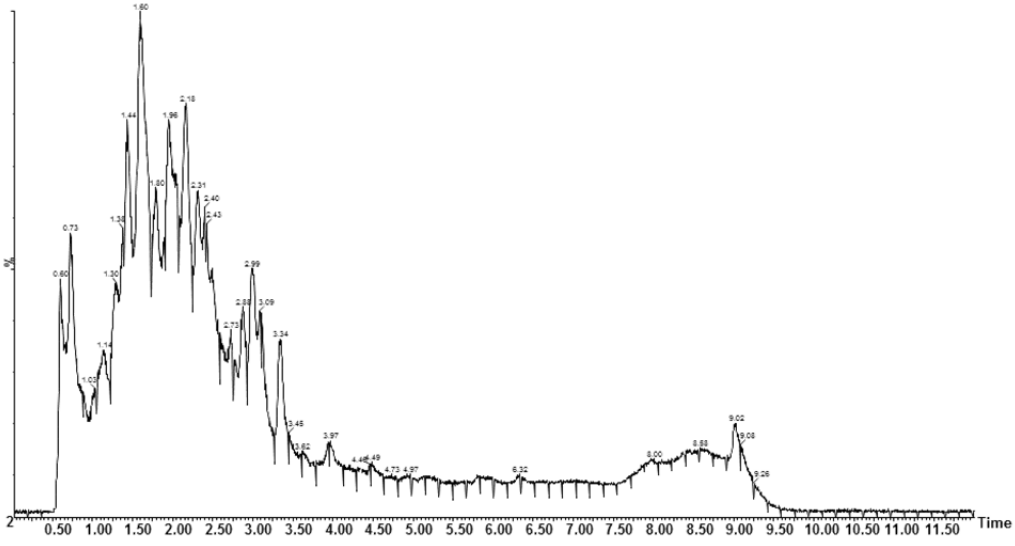

Figure 5.11. HPLC-MS peptide map (total ion chromatogram) of denatured BSA (0.12 mM) passed through IMER #6 using Method 6 (see Table 5.3) for a total substrate digestion/residence time of 61 min. The separated conditions for HPLC-MS are given in the experimental section.

5.5. Conclusions

Our study of several BGE buffers and concentrations shows 50 mM borate buffer at pH 8.6 gives us a stable current and baseline and better separation resolution for CE-UV peptide mapping. Passing water through the IMER and the blank digestion shows no significant background peaks, although actual digests showed more peaks than expected based on the theoretical cleavage by chymotrypsin. We were able to partially digest FH-OMe and WAGGDASGE solutions, identifying the peptide fragments in CE-UV maps by spiking or in HPLC-MS maps by knowing the masses of expected products. An estimation of the extent of digestion was made by quantifying amino acid fragments by CE-UV using a calibration curve. Denatured BSA was successfully digested by the IMER with 3 different digestion methods. The best digestion method (Method 6), where slower passage of substrate for 60 min then a 1-min flush at the end was done, led to the highest sequence coverage (35%) and more peptidic peaks in both CE and HPLC peptide maps with better resolution.

5.6. Supplementary Data

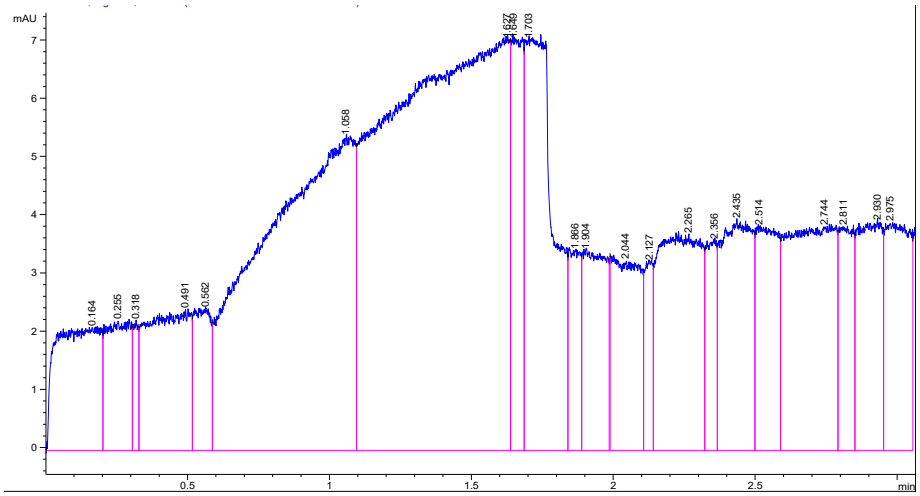

Figure S5.1. The denatured BSA (0.12 mM) passed through the IMER using Method 4. The digests were separated using CE-UV in the BGE of 50 mM sodium phosphate pH 6.8.

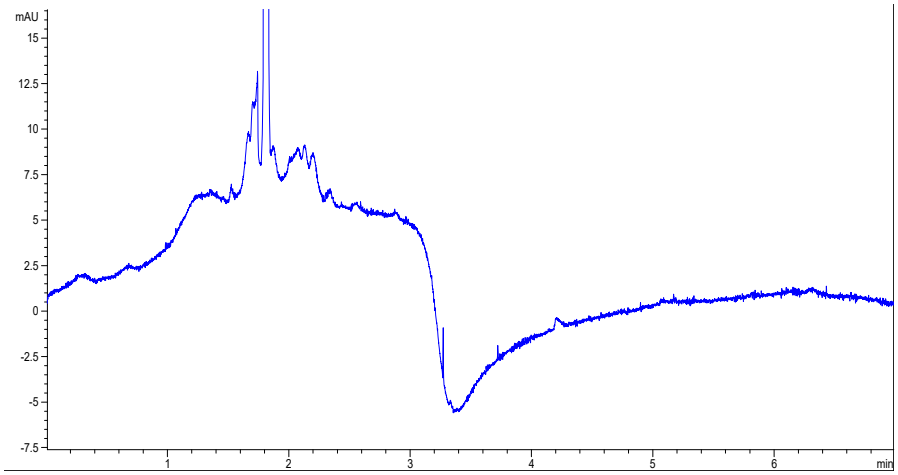

Figure S5.2. The denatured BSA (0.12 mM) passed through the IMER using Method 4. The digests were separated using CE-UV using the BGE of 50 mM ammonium bicarbonate buffer pH 4.4.

Table S5.1. Experimentally identified peptides by HPLC-MS from BSA digested by the GA-crosslinked chymotrypsin IMER #4 using Method 4.

Measured m/z [a]	Theoretical m/z	Position	MC[b]	Peptide sequence [a] detected by HPLC-MS
453.2295	453.2344	394-397	0	STVF
561.3342	561.3395	521-525	0	VPKAF
658.3187	658.3195	180-184	1	YANKY
705.4595	705.3817	174-179	0	APELLY
735.4514	735.3494	55-60	0	LQQCPF
974.4466	974.4577	36-43	0	KDLGEEHF
976.8726	976.5574	427-434	0	QNALIVRY
996.4440	996.4520	512-520	0	SALTPDETY
1051.5491	1051.5571	151-158	1	KADEKKFW
1180.6316	1180.6473	425-434	1	GFQNALIVRY
1212.5839	1212.5895	343-353	1	QEAKDAFLGSF
1488.7245	1488.7369	343-355	2	QEAKDAFLGSFLY
1538.7517	1538.7737	512-525	1	SALTPDETYVPKAF
1572.7758	1572.7904	61-73	0	DEHVKLVNELTEF
1583.8699	583.9002	592-607	0	AVEGPKLVVSTQTALA
368.1893^{2+}	368.1783^{2+}	55-60	0	LQQCPF
456.7289^{2+}	456.7323^{2+}	181-188	1	ANKYNGVF
487.7303^{2+}	487.7325^{2+}	36-43	0	KDLGEEHF
526.2755^{2+}	526.2822^{2+}	151-158	1	KADEKKFW
590.3668^{2+}	590.8273^{2+}	425-434	1	GFQNALIVRY
606.7955^{2+}	606.7984^{2+}	343-353	1	QEAKDAFLGSF
670.8265^{2+}	670.8389^{2+}	25-35	0	DTHKSEIAHRF
676.3416^{2+}	676.3489^{2+}	164-173	1	EIARRHPYFY
744.8649^{2+}	744.8721^{2+}	343-355	2	QEAKDAFLGSFLY
844.8262^{2+}	*844.8393^{2+}*		*0*	*(Cys_CAM: 581, 582, 590)*
769.8766^{2+}	769.8905	512-525	1	SALTPDETYVPKAF
786.8948^{2+}	786.8988^{2+}	61-73	0	DEHVKLVNELTEF
792.4492^{2+}	792.3903^{2+}	95-108	0	GDELCKVASLRETY
792.4492^{2+}	792.4538^{2+}	592-607	0	AVEGPKLVVSTQTALA
1145.0420^{2+}	1145.0646^{2+}	55-73	1	LQQCPFDEHVKLVNELTEF

[a] For $[M+H]^+$ ions unless otherwise indicated.

[b] Number of missed cleavages.

Table S5.2. Experimentally identified peptides by HPLC-MS from BSA digested by the GA-crosslinked chymotrypsin IMER #5 using Method 5b.

Measured m/z [a]	Theoretical m/z	Position	MC[b]	Peptide sequence [a] detected by HPLC-MS
367.1936	367.1976	159-161	0	GKY
397.1751	297.1718	52-54	0	SQY
453.2312	453.2344	394-397	0	STVF
495.2831	495.2562	181-184	0	ANKY
643.3423	643.3450	159-163	1	GKYLY
658.3190	658.3195	180-184	1	YANKY
699.3718	699.3712	350-355	1	LGSFLY
865.4722	865.4778	151-157	0	KADEKKF
930.5115	930.5155	230-237	0	GERALKAW
944.4660	944.4697	358-364	0	SRRHPEY
996.4511	996.4520	512-520	0	SALTPDETY
1193.6532	1193.6564	521-530	1	VPKAFDEKLF
1212.5888	1212.5895	343-353	1	QEAKDAFLGSF
1340.6719	1340.6705	25-35	0	DTHKSEIAHRF
1488.7397	1488.7369	343-355	2	QEAKDAFLGSFLY
322.1753^{2+}	322.1761^{2+}	159-163	1	GKYLY
326.1702^{2+}	326.1710^{2+}	526-530	0	DEKLF
456.7321^{2+}	456.7323^{2+}	181-188	1	ANKYNGVF
465.7610^{2+}	465.7614^{2+}	230-237	0	GERALKAW
526.2818^{2+}	526.2822^{2+}	151-158	1	KADEKKFW
597.3312^{2+}	597.3319^{2+}	521-530	1	VPKAFDEKLF
670.8356^{2+}	670.8389^{2+}	25-35	0	DTHKSEIAHRF
676.3439^{2+}	676.3489^{2+}	164-173	1	EIARRHPYFY
681.4107^{2+}	681.4112^{2+}	365-376	0	AVSVLLRLAKEY
744.8709^{2+}	744.8721^{2+}	343-355	2	QEAKDAFLGSFLY
792.4515^{2+}	792.4538^{2+}	592-607	0	AVEGPKLVVSTQTALA
908.5022^{2+}	908.5038^{2+}	36-51	1	KDLGEEHFKGLVLIAF
1019.5301^{2+}	1019.5309^{2+}	164-179	2	EIARRHPYFYAPELLY
1052.3928^{2+}	1052.3974^{2+}	109-126	0	GDMADCCEKQEPERNECF
1086.0456^{2+}	1086.0489^{2+}	512-530	2	SALTPDETYVPKAFDEKLF
1097.5802^{2+}	1097.5807^{2+}	36-54	2	KDLGEEHFKGLVLIAFSQY
1124.0301^{2+}	1124.0302^{2+}	74-94	0	AKTCVADESHAGCEKSLHTLF
1148.5544^{2+}	1148.5589^{2+}	25-43	1	DTHKSEIAHRFKDLGEEHF
1166.4810^{2+}	1166.4819^{2+}	377-397	1	EATLEECCAKDDPHACYSTVF

[a] For $[M+H]^+$ unless otherwise indicated.

[b] Number of missed cleavages.

Table S5.3. Experimentally identified peptides by HPLC-MS from BSA digested by the GA-crosslinked chymotrypsin IMER #6 using Method 6.

Measured m/z [a]	Theoretical m/z	Position	MC[b]	Peptide sequence [a] detected by HPLC-MS
205.0922	205.0971	158-158	0	W
295.1617	295.1652	354-355	0	LY
295.1617	295.1652	162-163	0	LY
336.1915	336.1918	575-577	0	VAF
397.1710	297.1718	52-54	0	SQY
423.2227	423.2238	350-353	0	LGSF
453.2334	453.2344	394-397	0	STVF
495.2556	495.2562	181-184	0	ANKY
553.2761	553.2769	158-161	1	WGKY
561.3387	561.3395	521-525	0	VPKAF
651.3327	651.3348	526-530	0	DEKLF
705.3815	705.3817	174-179	0	APELLY
735.3487	735.3494	55-60	0	LQQCPF
912.4567	912.4574	181-188	1	ANKYNGVF
944.4692	944.4697	358-364	0	SRRHPEY
996.4517	996.4520	512-520	0	SALTPDETY
1113.5030	1113.5033	52-60	1	SQYLQQCPF
1238.7141	1238.7143	44-54	1	KGLVLIAFSQY
1351.6904	1351.6905	164-173	1	EIARRHPYFY
1512.7236	1512.7230	354-364	2	LYEYSRRHPEY
1517.6057	1517.6069	578-591	0	VDKCCAADDKEACF
2959.4970	2959.4982	394-418	1	STVFDKLKHLVDEPQNLIKQNCDQF
277.1430^{2+}	277.1421^{2+}	158-161	1	WGKY
294.1389^{2+}	294.1392^{2+}	354-357	1	LYEY
322.1751^{2+}	322.1761^{2+}	159-163	1	GKYLY
415.2146^{2+}	415.2158^{2+}	158-163	2	WGKYLY
526.2812^{2+}	526.2822^{2+}	151-158	1	KADEKKFW
590.8263^{2+}	590.8273^{2+}	425-434	1	GFQNALIVRY
619.8602^{2+}	619.8608^{2+}	44-54	1	KGLVLIAFSQY
676.3479^{2+}	676.3489^{2+}	164-173	1	EIARRHPYFY
681.4111^{2+}	681.4112^{2+}	365-376	0	AVSVLLRLAKEY
721.3892^{2+}	712.3899^{2+}	521-532	2	VPKAFDEKLFTF
744.8716^{2+}	744.8721^{2+}	343-355	2	QEAKDAFLGSFLY
756.8633^{2+}	759.8651^{2+}	354-364	2	LYEYSRRHPEY
792.3901^{2+}	792.3903^{2+}	95-108	0	GDELCKVASLRETY
908.5020^{2+}	908.5038^{2+}	36-51	1	KDLGEEHFKGLVLIAF
987.4486^{2+}	987.4491^{2+}	333-349	1	AEDKDVCKNYQEAKDAF
2083.4978^{2+}	2083.4982^{2+}	358-393	2	SRRHPEYAVSVLLRLAKEYEATLEECC AKDDPHACY
2576.3642^{2+}	*2576.3646^{2+}*			*(Cys_CAM: 537)*
2892.5240^{2+}	*2892.5231^{2+}*			*(Cys_CAM: 537)*

[a] For $[M+H]^+$ unless otherwise indicated.

[b] Number of missed cleavages.

Chapter 6. Fluorescence microscopy imaging of an immobilized enzyme microreactor to investigate glutaraldehyde-mediated crosslinking of chymotrypsin

A version of this chapter has been submitted[1] on April 30, 2015, to *Analytical Letters* with authorship by Golfam Ghafourifar and Karen C. Waldron

[1] The manuscript, accepted during thesis corrections, is available on-line since Sept. 6, 2015 (DOI:10.1080/00032719.2015.1075128.).

6.1. Abstract

Immobilized enzyme microreactors based on proteases for proteomics studies are usually made from enzyme bound to a solid-phase support such as particles packed into a cartridge. Our group has developed a support-less immobilization strategy that uses glutaraldehyde-mediated crosslinking to render proteolytic enzymes insoluble for facile protein digestion. While this works well in batch format, in-situ crosslinking within a microcolumn-based enzyme microreactor is less straight-forward. A microreactor was fabricated by immobilizing chymotrypsin, a proteolytic enzyme, inside a 250-µm i.d. fused silica capillary that was first functionalized with amino groups before adding glutaraldehyde and enzyme. The extent and location of enzyme immobilization within the capillary tubing was probed by reacting fluorescein isothiocyanate with residual amino groups in the microreactor then imaging the capillary by confocal laser fluorescence microscopy. The images imply that chymotrypsin immobilization occurred mostly near the wall and did not extend into the center of the microreactor as a crosslinked porous network. On the other hand, this structure facilitates the passage of substrate through the reactor. Digestion of denatured bovine serum albumin by flowing it through the 43-cm long crosslinked chymotrypsin microreactor for a total of 3.2 min produced 29 peptides, corresponding to 34% primary sequence coverage..

6.2. Introduction

Enzymes have been used in analytical chemistry for a wide range of assays because of their selectivity. Immobilizing enzymes offer the advantage of their reuse because they retain most of their activity [211], leading to cost benefits. Proteolytic enzymes are no exception. As a result of immobilization, autoproteolysis decreases significantly; therefore, a proteolytic enzyme can be used at high enzyme-to-substrate ratio conditions for faster reaction [41, 42, 51]. Immobilization also increases the stability of the enzyme against denaturation induced by heat, pH extremes and organic solvents [160, 173]. Immobilized enzyme reactors, once seen only in process chemistry, are being used more and more frequently in proteomics studies for protein digestion [36, 47, 79, 164]. Commercially

available cartridges containing proteases bound to a solid-phase can be easily connected to liquid chromatography-mass spectrometry (LC-MS) systems for rapid digestion and peptide mass mapping, or sequencing, in order to identify proteins in a complex sample [79, 151, 184, 211]. For microLC and capillary electrophoresis (CE), novel small scale enzyme cartridges based on attachment to particles or magnetic beads, immobilization on the capillary inner wall, or hybridization to bound aptamers, to name just a few examples, have been reported as enzyme microreactors [39, 109, 212, 213].

An alternative immobilization strategy uses glutaraldehyde as a protein crosslinking agent, avoiding the need for a solid support [50, 51, 53]. This method results in high activity immobilized enzyme particles that are gel-like when prepared in batch form; however, the nature of glutaraldehyde-mediated crosslinking within a microcolumn-sized reactor is not necessarily the same, and is more difficult to characterize. In a previous study on myoglobin digestion in a glutaraldehyde-mediated immobilized chymotrypsin microreactor [53], we found that the order in which glutaraldehyde and chymotrypsin were passed though the capillary plays an important role in immobilizing the enzyme without clogging the reactor. Ideally, a porous network of glutaraldehyde-enzyme across the capillary would provide fast mass transfer from the reduced diffusion path length of substrate and thus increase the contact time with enzyme, as seen in monolithic trypsin microreactors [168, 177, 200, 214]. To better understand the nature of the glutaraldehyde-crosslinked enzyme in the microreactor and to visualize the extent and location of the enzyme, we devised a fluorescence imaging experiment using fluorescein isothiocyanate. At various stages, the immobilized enzyme microreactor fabrication was stopped and fluorescein derivative was passed through the capillary to react with residual amino groups. The partial (or complete) microreactor treated with the fluorescein probe was imaged with a confocal laser scanning microscope to deduce the presence or absence of immobilized enzyme. The digestion efficiency of the crosslinked chymotrypsin microreactor was assessed using bovine serum albumin, a large protein substrate.

6.3. Materials and methods

6.3.1. Reagents and materials

α-Chymotrypsin from bovine pancreas type II, glutaraldehyde (25% aqueous solution diluted in water to 2.5% for all reactions), phosphoric acid, monobasic sodium phosphate, dibasic sodium phosphate, (3-aminopropyl)-triethoxysilane (99% purity, used at 10% v/v in methanol for all reactions), fluorescein isothiocyanate, bovine serum albumin (BSA), and tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) were purchased from Sigma Aldrich (Oakville, ON, Canada). Sodium hydroxide, sodium chloride and calcium chloride were purchased from Anachemia (Montreal, QC, Canada). Methanol was purchased from BDH (West Chester, PA). Hydrochloric acid was from EMD Millipore (Gibbstown, NJ). Fused silica capillary tubing for the immobilized enzyme microreactor (250 µm i.d., 360 µm o.d.) was obtained from Chromatographic Specialties Inc. (Brockville, ON, Canada). Fused silica Inner-Lok connectors were from Polymicro Technologies (Phoenix, AZ). The Inner-Lok connector, which makes a simple compression fitting with polyimide coated capillary tubing, was cut in half and fused to a 1-mL disposable tuberculin syringe with male luer (BD, Franklin Lakes, NJ) to facilitate manual rinsing of capillaries at a slow rate (1 mL over approx. 10 s). A multi-cartridge Milli-Q filtration/deionization system (Millipore, Bedford, MA) was employed to purify the distilled water used in preparation of all solutions and buffers and for all washing steps.

6.3.2. *In situ* IMER fabrication: 3 cm × 250 µm microreactor

The following procedure was used to make batches of partial and complete immobilized chymotrypsin microreactors. A 3-cm length of capillary tubing (250 µm i.d., 1.5 µL internal volume) was washed using the homemade InnerLok-adapted syringe by pushing through 1 mL each of 1 M NaOH and methanol. It was dried by slowly pushing air through, followed by rinsing with 1 mL of 1 M HCl. Next, 1 mL of aminopropyl triethoxysilane (10% v/v in methanol) was passed through, allowing the last 1.5 µL to remain in the capillary. Both ends were sealed with Parafilm and the capillary was kept at

60 ^{0}C for 1 h. The capillary was then rinsed with 1 mL water and either imaged with the confocal laser microscope, photographed, treated with fluorescein isothiocyanate (described below) or used for the subsequent step. Next, 1 mL glutaraldehyde (2.5% v/v in water) was introduced the same way as the aminopropylating agent, resealing the ends again with Parafilm and leaving the capillary at rest for 1 h at room temperature. The capillary was then rinsed with 1 mL of 50 mM phosphate buffer, pH 6.4, and either imaged, treated with fluorescein probe or used for the subsequent steps. As with the glutaraldehyde step, 1 mL chymotrypsin (1.3 mM in water) was reacted in the sealed capillary for 1 h. The resulting enzyme microreactor was then rinsed with 1 mL water and imaged as is or treated with fluorescein probe. In each case above, treatment with fluorescein isothiocyanate (0.23 mM in water) involved introducing 1 mL and re-sealing the capillary ends with Parafilm. The fluorescein-treated enzyme microreactor was kept in the dark over night at room temperature with gentle shaking, which was achieved by taping it inside a centrifuge tube and allowing the tube to roll back and forth on the shaker operated at 50 min-1. It was rinsed with 1 mL water before microscopy imaging.

6.3.3. *In situ* IMER fabrication: 43 cm × 250 µm microreactor

The longer immobilized chymotrypsin microreactor was prepared with the same reagents and order as above for the small microreactor but using automated delivery of the solutions pushed through at 50 mbar with an Agilent CE instrument (Agilent Technologies, Waldbronn, Germany) and also repeating the sequence of adding glutaraldehyde and chymotrypsin two more times as described previously [53]. Once completed, the microreactor was rinsed continuously with 0.23 mM fluorescein isothiocyanate for 3 h at 50 mbar and finally rinsed with water for 10 min at 50 mbar before fluorescence microscope imaging. A second 43-cm long chymotrypsin microreactor was prepared without the fluorescein rinsing and left at room temperature for 3 h. It was then rinsed with water for 2 min and used for digestion of protein substrate.

6.3.4. Fluorescence microscope imaging

A Leica Microsystems TCS SP5 II (Concord, ON, Canada) confocal laser scanning microscope was used to image the immobilized enzyme microreactors. The excitation was at 488 nm for fluorescein and emission was measured at 500 nm. The transmission image was acquired simultaneously using the transmitted light detector. In all cases, microreactor capillaries were fixed on a glass microscope slide using a droplet of nail polish as glue in order to stabilize them for imaging.

6.3.5. Digestion of Bovine Serum Albumin in the Microreactor

The protein substrate BSA was denatured, reduced and alkylated as described previously [53] then diluted in Tris-HCl buffer (100 mM, pH 7.8) containing 10 mM $CaCl_2$ to reach a final concentration of 120 µM BSA. The substrate was flushed (at 950 mbar) through the 43-cm long microreactor for 2.0 min using the automated CE system set at 37°C, resulting in approx. 60 µL of digest collected at the outlet. This volume was re-introduced into the microreactor and flushed through for 1.0 min, followed by water for 0.2 min, yielding a final collected volume of 80 µL. The digest was analyzed directly by ultra-performance liquid chromatography on an Acquity UPLC Class I system (Waters Ltd., Milford, MA) coupled to a Waters Synapt G2-S quadrupole-time-of-flight mass spectrometer with electrospray ionization in positive ion mode. The separation was carried out at 40°C on an Acquity CSH C18 column (1.7 µm, 2.1 mm × 75 mm) from Waters Ltd. at a flow rate of 400 µL/min using the mobile phase constituents 0.1% formic acid in water (eluent A) and 0.1% formic acid in acetonitrile (eluent B) with the following gradient: 5 to 90% B from 0 to 7 min, hold at 90% B for 1 min, 90 to 5% B from 8 to 8.5 min, hold at 5% B for 12 min to permit column equilibration. The injection volume was 3 µL.

6.4. Results and discussion

The order in which reagents are passed through the capillary plays an important role in fabricating the crosslinked enzyme microreactor. In preliminary studies, it was found that

initial functionalization of the capillary inner wall to create amino substituents as an anchor for glutaraldehyde-immobilized chymotrypsin was necessary. We also found that pre-mixing glutaraldehyde and chymotrypsin then passing the mixture immediately through the capillary was impossible because the crosslinking reaction was too fast; chymotrypsin particles started to appear less than 1 min after mixing, which caused the capillary to clog. In an attempt to slow down the immobilization reaction, glutaraldehyde was added to the chymotrypsin in a tube placed in a dry ice bath. However, the immobilization reaction started immediately after adding reagent and a suspension of glutaraldehyde-chymotrypsin particles formed after removing the tube from the bath. Therefore, we proceeded to use sequential addition of the crosslinker and enzyme.

Fabrication of the glutaraldehyde-crosslinked chymotrypsin microreactor involved serially flushing the capillary with the reagents. During development of the 43-cm IMER [53], various sequences of reagent addition were tried: passing chymotrypsin first and then glutaraldehyde without rinsing in between; the opposite order without rinsing; passing chymotrypsin first, rinsing and then adding glutaraldehyde. In the first two sequences, there was capillary clogging and in the second, no clogging but also no digestion because no immobilization occurred. Therefore, it was deemed important to better understand the microreactor's inner surface, both in nature and spatial distribution of reagents after each fabrication step (functionalization, glutaraldehyde-activation, chymotrypsin immobilization). Confocal laser fluorescence imaging of the microreactor capillaries with concomitant acquisition of the transmitted light image was thus carried out after each reaction step.

6.4.1. Direct monitoring of enzyme immobilization

Several stereo-photographs (Figure 6.1) were taken of the 3-cm microreactor capillary after each step of fabrication using a stereomicroscope (Motic, Model BA310, Hong Kong), taking care to keep the plane of focus in the same place each time. The capillary appeared clear inside after initial washings and prior to passing through the aminopropyl functionalization reagent (Figure 6.1, left-most column). After flowing the

aminopropylation agent through and then glutaraldehyde, the capillary still appeared clear implying there was no heterogeneous material such as polymerized glutaraldehyde inside the capillary (Figure 6.1, center two columns). However, after passing chymotrypsin through, the presence of immobilized enzyme particles inside the capillary could be seen (Figure 6.1, last column). The cartoon images in Figure 6,1 imply that immobilization takes place mostly at the capillary wall but this cannot be confirmed visually.

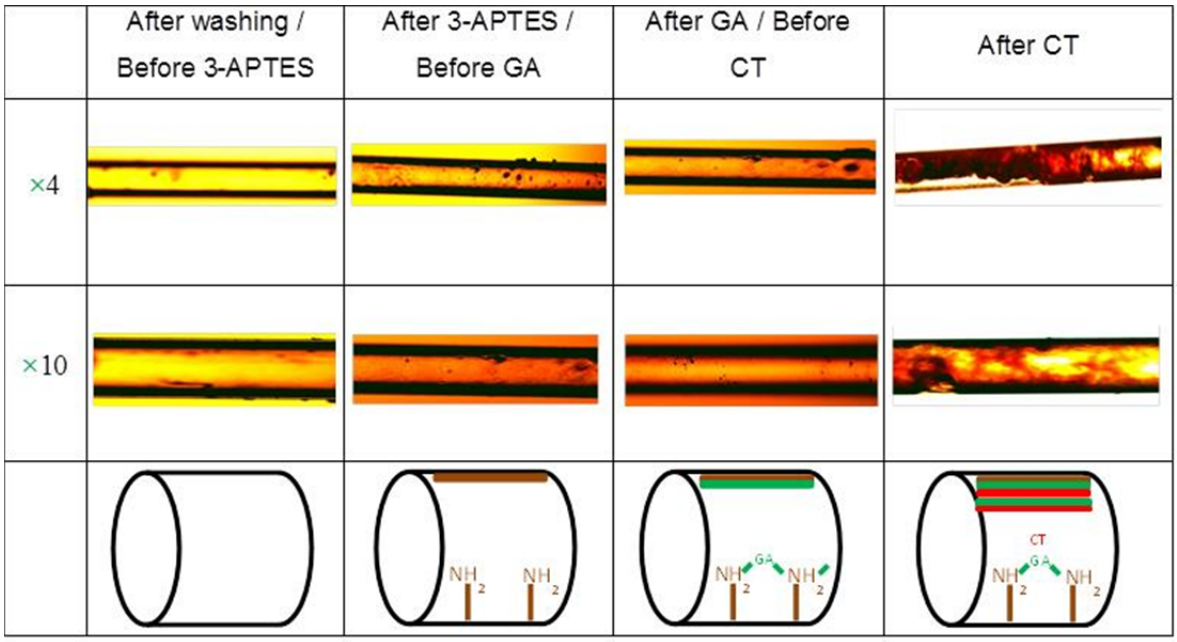

Figure 6.1. Stereomicroscope images taken from the capillary window before and after each step of enzyme microreactor fabrication. All images were taken in ×4 and ×10 magnification.

6.4.2. Indirect monitoring of enzyme immobilization by fluorescence imaging

Fluorescein isothiocyanate is reactive towards nucleophiles like primary and secondary amino groups, even at neutral pH in its monobasic form. We anticipated that by passing this fluorescent derivative through the microreactor at various stages of fabrication, it could react with the aminopropyl groups on the capillary wall and with free ε-amino groups from lysine residues of chymotrypsin that had not been crosslinked by glutaraldehyde. This

information was expected to reveal the extent of crosslinking. To image the enzyme microreactor and detect fluorescence using the confocal laser scanning microscope, a "window" was made in the capillary tubing, before adding microreactor reagents, by removing (burning off) a few millimeters length of the protective polyimide coating (Figure 6.2.). In one batch of 3-cm microreactors, the window was left intact and in another, it was cut after various stages of preparation to allow capturing an image from the cross-section. Figure 3 shows the fluorescence images and simultaneously acquired grey transmitted light images from several 3-cm microreactors. For the transmission signal, excitation was from the same 488 nm laser.

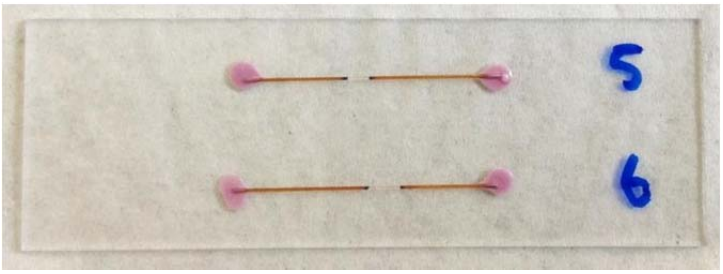

Figure 6.2. An example of two 3 cm-long microreactor capillaries (intact) glued to a microscope slide with nail polish in preparation for imaging by confocal laser fluorescence microscopy. Both microreactors were functionalized with 3-aminopropyl-triethoxysilane, activated with glutaraldehyde, crosslinked with chymotrypsin and then treated with fluorescein isothiocyanate.

Figures 6.3A-C show, respectively, capillaries treated with fluorescein probe after the first (aminopropyl functionalization), second (glutaraldehyde activation) and third (enzyme immobilization) stages of microreactor fabrication. In all three images, the window had been cut in half before imaging. The focal plane was close to the centre of the capillary tube, essentially imaging across the full diameter of each microreactor. A strong fluorescence signal was observed at the capillary walls in Figure 6.3A corresponding to fluorescein isothiocyanate having reacted with amino groups on the aminopropylated inner wall. The transmitted light signal (Figure 6.3A, right side) was quite dark because of strong absorbance by the abundant fluorescein groups. For the microreactor capillary in which

fluorescent probe treatment followed glutaraldehyde activation, the fluorescence signal was not as strong (Figure 6.3B, left) and more transmitted light was observed (Figure 6.3B, right) corresponding to less binding of the fluorescein probe added at this stage of fabrication. This results from having tied up many of the aminopropyl groups with glutaraldehyde, providing fewer reaction sites for fluorescein isothiocyanate. A further decrease in fluorescence and increase in transmitted light signal is seen in Figure 6.3C where glutaraldehyde-crosslinked chymotrypsin is present. This result was surprising, as we expected strong fluorescence to extend further into the capillary due to the fluorescein probe's reaction with free ε-amino groups from the enzyme. On the contrary, the weaker signal implies that chymotrypsin was extensively crosslinked with glutaraldehyde such that the fluorescein could react only with the residual aminopropyl groups at the capillary wall and, perhaps, a limited number of lysine side chains. An uncut microreactor containing glutaraldehyde-crosslinked chymotrypsin with no added fluorescent probe (Figure 6.3D), thus serving as a blank, shows no fluorescence as expected. The transmitted light image (Figure 6.3D, right) looked clear because there were no absorbing species at the excitation wavelength of 488 nm and thus high transmission. The cross-sectional fluorescence image (Figure 6.3E) from a cut capillary shows that fluorescein was not evenly distributed on the inner surface of the capillary and presumably the same is true for the crosslinked enzyme distribution.

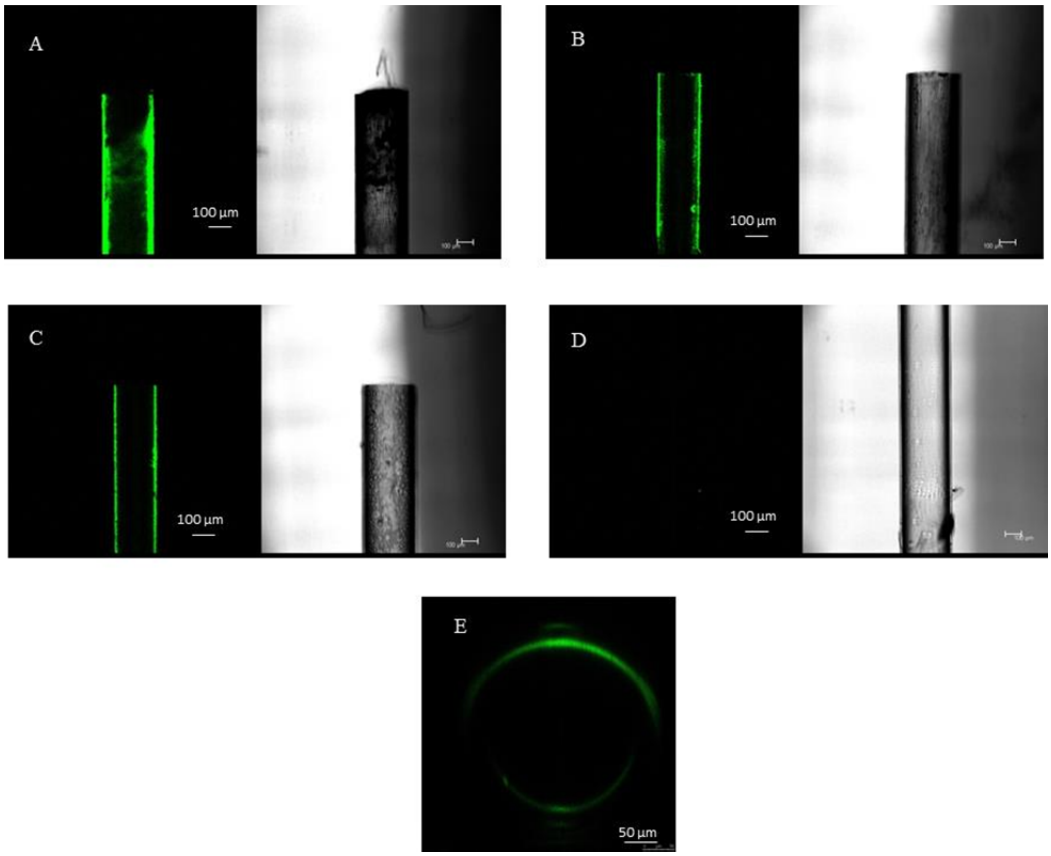

Figure 6.3. Fluorescence (left side) and transmitted light (right side) images of five small (3-cm) microreactor capillaries showing approx. 1 mm of the broken or intact window: (A) partially prepared microreactor with only aminopropyl functionalization before treatment with fluorescent probe; (B) partially prepared microreactor with aminopropyl functionalization and glutaraldehyde activation, then treatment with fluorescent probe; (C) full microreactor prepared with crosslinked chymotrypsin then treated with fluorescent probe; (D) same as (C) but no fluorescent probe passed through (i.e., the blank); (E) cross-section of a broken capillary prepared as in (C).

A second study was conducted to further investigate the immobilized enzyme location in the microreactor. After making a 3-cm microreactor and reacting it with fluorescein probe, fluorescence was recorded continuously while stepping the microscope slowly downward along the optical axis in the z direction (Figure 6.4A), from near the top of the capillary to near the middle, in cross-section. Eight screen shots from the depth-coded map are shown in Figure 6.4 where image #1 is from the top of the microreactor and image #8 is 105 µm further down the optical axis, so approaching the full width (diameter) of the

capillary microreactor, showing its cross-section. The confocal microscope's field of view corresponds to the red box around the microreactor photo shown in Figure 6.4B. Near the top of each image, fluorescence from the capillary's polyimide coating is seen and then a dark area below that corresponding to burnt polyimide. The results from images #1-8 (Figure 6.4) confirm that fluorescein is bound mostly near the wall of the microreactor and suggest it may also be within 15-25 µm of the wall because two sources of fluorescence can be seen in image #4 where the focal plane is 25 µm down (in z direction) from the top of the reactor. This may be explained by two mechanisms: the crosslinked enzyme did not completely cover the capillary wall thus exposing some aminopropyl sites for derivatization with fluorescein, and the enzyme was partially derivatized with fluorescein but is only immobilized near the wall and not throughout the entire capillary.

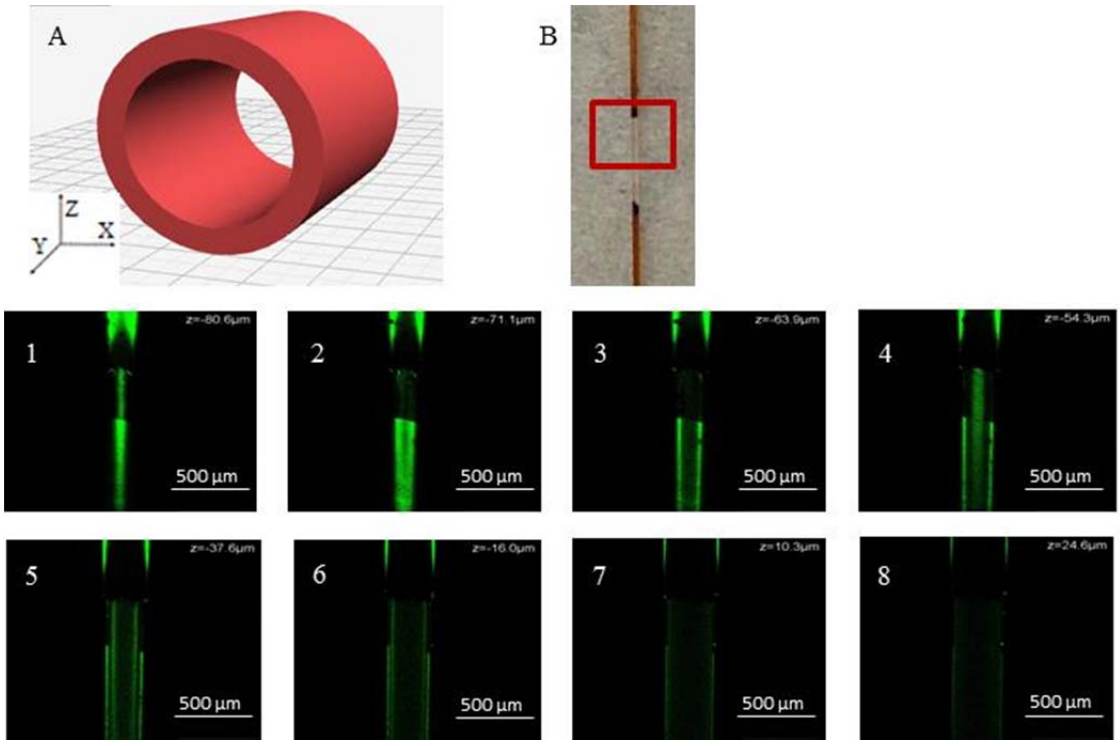

Figure 6.4. Images obtained from continuously moving the confocal laser scanning microscope from the top of the capillary to the middle along the optical axis, i.e., the z direction as in panel (A), showing its cross-section for a complete 3-cm microreactor with glutaraldehyde-crosslinked chymotrypsin, treated with fluorescein isothiocyanate. (B) Section of microreactor imaged to obtain screen shots #1 through 8 (lower panels), which span a total depth or vertical distance imaged of 103 µm.

A long (43 cm) IMER was fabricated using the method developed previously [53] and then treated with fluorescein isothiocyanate to study the consistency of chymotrypsin immobilization along the length of the microreactor by fluorescence imaging. Four windows were made at 2, 7, 16 and 26 cm from the microreactor inlet before flowing through the reagents for enzyme immobilization and finally the fluorescein probe. Fluorescence and transmission images were recorded for each window immediately following treatment with the probe. Surprisingly, no fluorescence signals were observed at any of the four locations implying that no amino groups were available for derivatization. This suggests that aminopropyl groups at the wall were completely masked by the crosslinked chymotrypsin, the latter of which had no free ε-amino groups. However, as Figure 6.5 shows, after the microreactor was left for 24 h at room temperature in the dark, weak fluorescence signals were finally observed at 2, 7 and 26 cm from the inlet and a slightly stronger signal observed at 16 cm. The transmittance signal at the 16-cm window also showed more fluorescein present than elsewhere. One hypothesis to explain the results seen both before and after the 24-h period is that the chymotrypsin is so highly crosslinked at the wall that: a) diffusion of fluorescein isothiocyanate toward the wall baring the free aminopropyl groups was hindered and thus very slow, *e.g.*, requiring 24 h, and b) there were very few uncrosslinked lysine side chains thus few ε-amino groups for reaction with the fluorescent probe. These are plausible given the triple layering of glutaraldehyde and chymotrypsin during fabrication of the 43-cm long microreactor whereas the 3-cm microreactors had only a single layer. The images in Figure 5 show also that the crosslinked enzyme was not homogeneously distributed throughout the microreacotr. Nonetheless, we know it is sufficiently abundant because microreactors made with the same procedure have been used to digest myoglobin [53], small peptides [215] and BSA as described below.

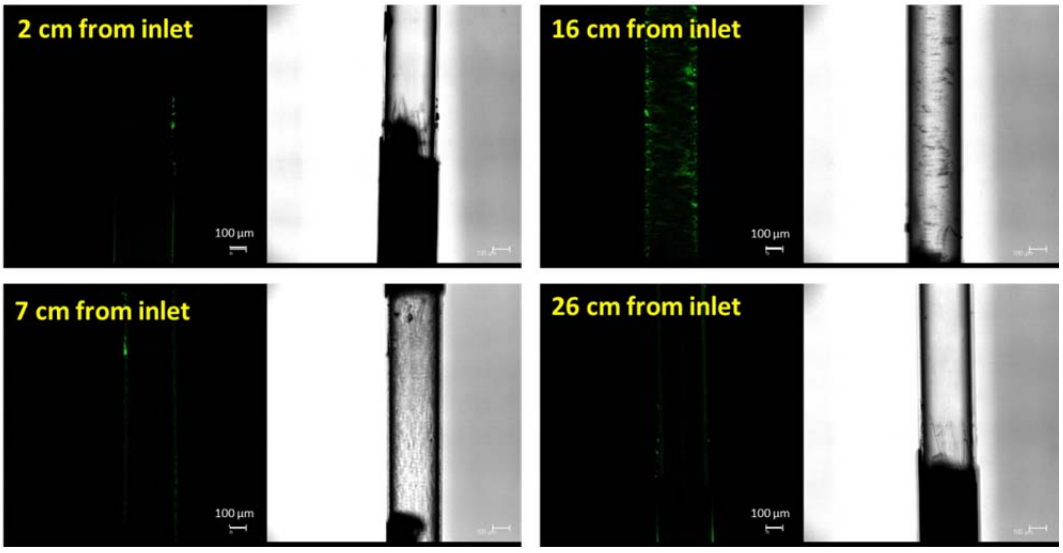

Figure 6.5. Fluorescence microscope imaging at different lengths along the capillary of a complete 43-cm microreactor with glutaraldehyde-crosslinked chymotrypsin applied in three layers and treated with fluorescein isothiocyanate then left at room temperature for 24 h. Windows were burned at 2, 7, 16 and 26 cm from the inlet before adding microreactor fabrication reagents.

6.4.3. Digestion of bovine serum albumin in the microreactor

Immobilization of a proteolytic enzyme tends to decrease its activity, so high enzyme loading is used to compensate. For example, we reported that glutaraldehyde-crosslinked trypsin particles have a specific activity 4000 times lower than soluble trypsin as determined using a small amino acid-based substrate and based on the crosslinking efficiency (95% total mass of trypsin) [51]. In that study, 4 h protein digestions made with glutaraldehyde-trypsin particles showed comparable activity to 24 h digestions made with soluble trypsin. Although the enzyme loading and specific activity of the crosslinked enzyme microreactor are expected to be lower than for the particles, there is still good digestion efficiency. BSA, a highly folded protein, was denatured and passed through the 43-cm long chymotrypsin microreactor at a high flow rate, resulting in a total residence time of 3.2 min for digestion. Peptide mass mapping by LC-MS (Figure 6.6) allowed us to identify 29 chymotryptic peptides of BSA, as shown in Table I, accounting for 205 amino acid residues of BSA, which is a primary sequence coverage of 34%. The digestion

efficiency would likely appear higher if all 80 μL of collected digest had been analyzed after preconcentration. Digestion carried out at a slower flow rate in the microreactor is also expected to improve efficiency.

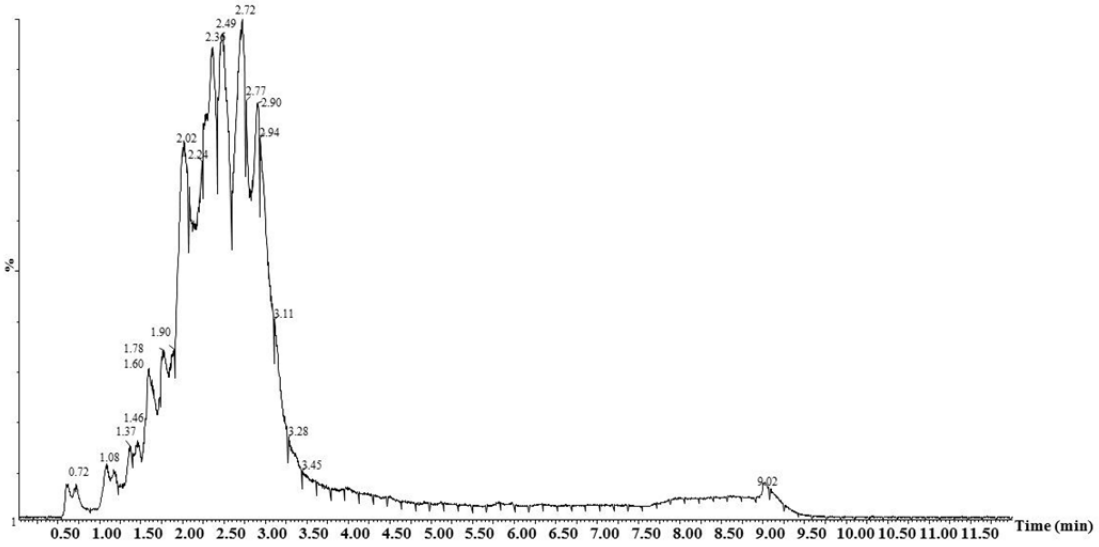

Figure 6.6. Total ion chromatogram of the digest of BSA collected after digestion for 3.2 min at 37 °C of the substrate in a 43-cm long glutaraldehyde-crosslinked chymotrypsin microreactor. The separation was performed by ultra-performance liquid chromatography coupled to a quadrupole-time-of-flight mass spectrometer and using a C18 column and gradient elution with water and acetonitrile (both with 0.1% formic acid) at a flow rate of 400 μL/min. The injection volume was 3 μL. All other details are in the experimental section.

6.5. Conclusion

Fluorescein isothiocyanate was chosen to probe the location of the glutaraldehyde-crosslinked enzyme in a microreactor due the former's known reactivity with ε-amino groups from the enzyme's lysine side chains. We expected to see a strong fluorescence signal from fluorescein derivatization in the presence of chymotrypsin. However, the fluorescence signal decreased after both activation of the reactor with glutaraldehyde and crosslinking with chymotrypsin, suggesting that the fluorescent probe reacts more efficiently with free aminopropyl groups on the functionalized capillary wall than with the

ε-amino groups of the enzyme. This explains why the fluorescein isothiocyanate reaction with amino groups took a long time when it was added after glutaraldehyde-chymotrypsin crosslinking in layers, which resulted in limiting fluorescein probe's diffusion to the microreactor wall. The fluorescence images suggest that immobilization was achieved mostly near the wall and not throughout the whole capillary as a crosslinked network. On the other hand, this structure permits passage of substrate through the immobilized enzyme microreactor because it has some open-tube character, as demonstrated by the rapid, partial digestion of BSA.

6.6. Acknowledgements

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Chapter 7. Conclusion and Perspectives on Future Work

Drawbacks for protein digestions in solution phase and in gels include long incubation times, single use of the enzyme and enzyme autolysis leading to creation of background peptides that interfere in separation and identification of peptides from the substrates of interest. By using immobilized enzymes, auto-proteolysis can be greatly reduced, allowing a high enzyme concentration be used, which helps to also achieve faster digestion of substrate. Likewise, immobilized enzymes can be reused, which offsets the extra expenses when they are used in larger quantities. Automation in fluidic and microfluidic systems is made possible by using immobilized enzyme microreactors (IMERs).

Our first objective was to immobilize chymotrypsin by using glutaraldehyde as a crosslinking agent. The crosslinking procedure was similar to the one our group has used previously [106]. This immobilization method is rapid and inexpensive and allowed us the benefit of using high enzyme-to-substrate ratios. Crosslinking chymotrypsin by glutaraldehyde is a novel approach since proteolytic enzymes are immobilized mostly by attachment to solid supports [51, 63, 64].

The general condition of chymotrypsin immobilization presented in Chapter 3 involved studying the effect of buffer and pellet size on immobilization and consequently on digestion and peptide mapping. The results showed that the immobilized enzyme pellet size and the buffer used during crosslinking can influence the reproducibility of peptide mapping. The washing steps during the immobilization process remove a significant amount of adsorbed or non-reacted chymotrypsin. However, it was observed that a portion of the enzyme is lost during the washing steps and the pellet size is reduced after several step of manipulation. In future studies, a “spacer” molecule incorporated during the crosslinking procedure might help with better robustness of the glutaraldehyde-enzyme product. Immobilized chymotrypsin was used to digest BSA in both native and denatured forms. We showed in Chapter 4 that short term storage temperature and humidity of the immobilized chymotrypsin have minimal effects on BSA digestion. The results also show that the presence of fluorescent groups on the protein substrate do not hinder the activity of immobilized chymotrypsin.

Developing an *in-situ* IMER is important for achieving an automated fluidic system. Therefore, an IMER was fabricated by adapting existing methods by Krenkova *et al.* [88] who made a trypsin reactor, and Shan *et al.* [87] who reported δ-gluconolactone modified capillary. The IMER preparation method developed in the current thesis decreases the fabrication time by half in comparison with those methods. The enzyme chymotrypsin was successfully immobilized by glutaraldehyde crosslinking inside a 250-µm i.d. capillary to fabricate an IMER. Myoglobin was digested using the IMER and CE-MS analysis showed 83% sequence coverage (Chapter 4). Digestion a larger substrate like BSA after its denaturation and reductive alkylation was problematic at first but finally achieved as shown in Chapter 5 for both fast (3 min) and slow (60 min) digestions in the chymptrypsin IMER.

To understand and measure IMER efficiency, the concentration of enzyme and substrate need to be known to estimate the E:S ratio. However, because of the heterogeneity of immobilized enzyme and the fluidic nature of introducing the substrate, the E:S ratio is different at each part of the capillary IMER and at each moment. The peptides Phe-OMe and WAGGDASGE, and the protein BSA, representing substrates of different sizes, were digested using the chymotrypsin IMER with various protocols for substrate injection by pressurisation with an automated CE system. Phe-OMe and WAGGDASGE digestion efficiencies were quantified by using calibration curves of the product peptides after their identification by spiking and their presence confirmed by HPLC-MS. Quantification of the mass of enzyme in the IMER is important for future studies and could be achieved by complete hydrolysis of the crosslinked chymotrypsin followed by amino acid analysis.

Digestion of denatured BSA, a large and complex substrate, was successfully achieved using different flow protocols. In one method, a total IMER digestion time of 30 min led to identification by HPLC-MS of 29 peptides from BSA, representing 21% sequence coverage (Method 4, Chapter 5). In another method, the total BSA digestion time in the IMER was doubled (60 min), which improved sequence coverage to 35% from 38 peptides (Method 6, Chapter 5). A rapid digestion of 3 min required re-introduction of the digest into the IMER for a second “pass”, which resulted in identification of 34 peptides and 34% sequence coverage (Method 5a and 5b, Chapter 5). The longer digestion in Method 6 produced better peptide maps by CE-UV and HPLC-MS, with better peak resolution. Zhang *et al*. reported

33% sequence coverage digesting BSA by their IMER [216] and Wu *et al.* reported 51% [217]. Yu *et al.* were able to recover 51% of BSA sequence using their IMER to digest a mixture of three substrates including myoglobin, cytochrome c, and BSA. Their study indicates digesting myoglobin itself led to 82% sequence coverage [189], which is similar to our results. In future work, it would be desirable to also quantify undigested protein more accurately using a protein assay, independent of the peptides.

The extent of immobilization inside the capillary was studied by fluorescently labeling the enzyme with FITC and imaging the IMER by confocal laser scanning microscopy. It was found that the FITC probe has a higher tendency to react with unreacted amines from 3-APTES functionalization of the capillary wall than with the unreacted lysine ε-amino groups of the chymotrypsin (Chapter 6). The fluorescence images show that immobilization was achieved mostly near the wall, but extended a little into the center channel of the IMER. This explains why a lower pressure could be used to pass the substrate through the IMER. The results show the benefit of lower back pressure and reducing diffusion paths, compared to common methods using bulk immobilization through the whole volume of the capillary reactor [156, 200, 218, 219]. This leads to a faster mass transfer and subsequently faster digestion. To visualize the location of the enzyme more selectively, without revealing derivatization of the amino groups at the wall, a second fluorescent probe could be used that is SH-reactive or COOH-reactive to show evidence of the probe-chymotrypsin reaction.

Glutaraldehyde-mediated immobilization of chymotrypsin in the capillary to make an IMER shows promise since partial digestion of small, medium and large denatured protein was achieved. Compared with the current reported methods to immobilize and reuse of trypsin, the stability of our immobilized chymotrypsin as a batchwise particle or in an IMER format needs to be improved to increase the digestion efficiency and reusability [152, 153].

As a long-term goal, the IMER could be connected to microcolumn protein fractionation/purification upstream and analytical peptide separation strategies downstream, as suggested in Figure 7.1.

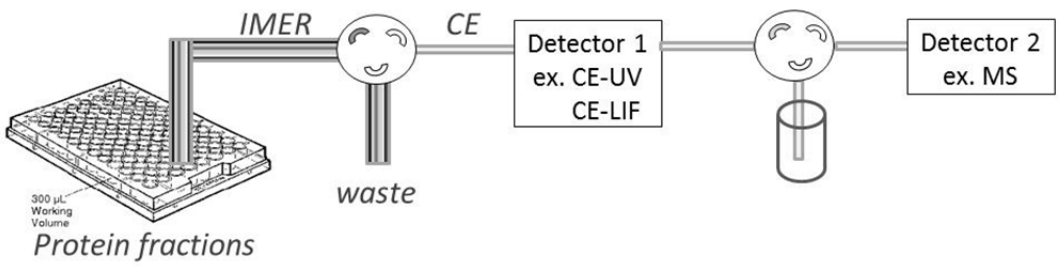

Figure 7.1. Proposed protein characterization system with coupling the IMER to CE-UV or CE-LIF to separate the peptides and finally the MS (or MS/MS) to identify and sequence them.

The format shown in Figure 7.1 allows digesting the isolated protein using immobilized enzyme (IMER) followed peptide mapping and sequencing to identify proteins in a sample. The transferability of our device to automated microfluidic platforms to address high throughput of protein analyses in the long term could be pursued.

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Appendix A. The Effect of Urea and Calcium Chloride on Denaturation of the Protein HbA

The HbA (Hemoglobin A) was denatured following the procedure stated in Chapter 3. Three different concentrations of urea (2, 5, and 8 M) were studied. We also investigated the effect of different calcium ion concentrations (0, 2, 5, and 10 mM) on denaturation.

The 2 mg/ml denatured HbA was digested using CT-GA (enzyme: substrate 1:25). The digests were separated by CE. We measured the peak areas of 6 peptides in the map and noted how they varied according to the 12 different denaturation conditions tested. Some of peptide maps showed no peak or the peak showed was overlapping with its neighbouring peak, thus could not be quantified. Table A.1 shows the overall highest peak areas present when using different concentration of urea and calcium chloride to denatured HbA.

Table A.1. Identified peptides by CE-UV from HbA digested by GA-crosslinked chymotrypsin enzyme : substrate ratio is 1:25.

[urea]	**Elution time (min)** / **[$CaCl_2$]**	**Peak area (relative units) of six different peptide peaks**					
		6.7 ± 0.3	**6.9 ± 0.3**	**7.3 ± 0.3**	**7.6 ± 0.3**	**8.4 ± 0.3**	**10.3 ± 0.3**
2 M	0 mM	31	43	10	33	40	47
	2 mM	20	25	12	16	10	61
	5 mM	9	N/D	N/D	12	10	16
	10 mM	21	28	18	21	20	12
5 M	0 mM	31	49	28	34	57	72
	2 mM	22	32	10	16	23	28
	5 mM	38	32	27	23	30	26
	10 mM	22	35	21	25	35	23
8 M	0 mM	29	40	32	26	43	47
	2 mM	48	56	41	30	60	64
	5 mM	15	22	30	19	12	34
	10 mM	**50**	**70**	**45**	**39**	**59**	**67**

Under low urea concentration a large intense peak at about 40 min was observed. This is due to the presence of insufficient urea to denature all of the protein, so the enzyme is not able to digest the protein properly either. Consequently, a large peak representing undenatured and therefore undigested protein is observed.

Using the Chem-Station software allows us to choose any area on electropherogram and look at the spectra. We were able to distinguish between peptidic peaks and noises knowing that proteins and peptides show signature absorptions between 190 to 230 nm because of their different amino acids contents. The absorption of the chromophoric amino acids is the result of side chain chromophore and carboxylate group absorption [220]. Figure A.1 shows the different amino acids spectra. Due to that carboxylic groups are used in polymerization the absorption intensity for the amino acids will be less comparing with free amino acid [220].

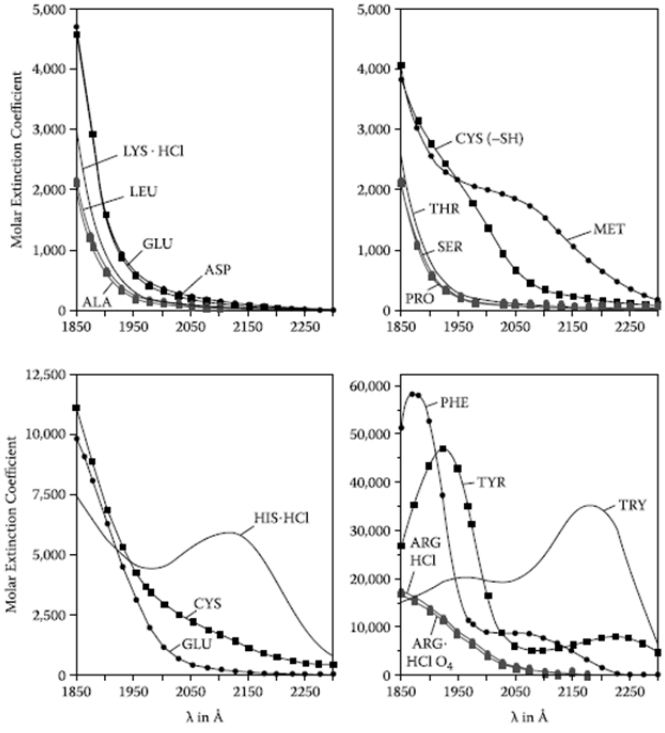

Figure A.1. UV absorption spectra for different amino acids (pH 5) from 185 to 230 nm. The cysteine spectrum was measured at pH 3 [220].

Figure A.2 represents some of the spectra obtained by choosing different peaks at HbA digested by immobilized chymotrypsin electropherogram and looking at their spectra. It allows us to classify peptides as well as non-peptidic peaks or noises.

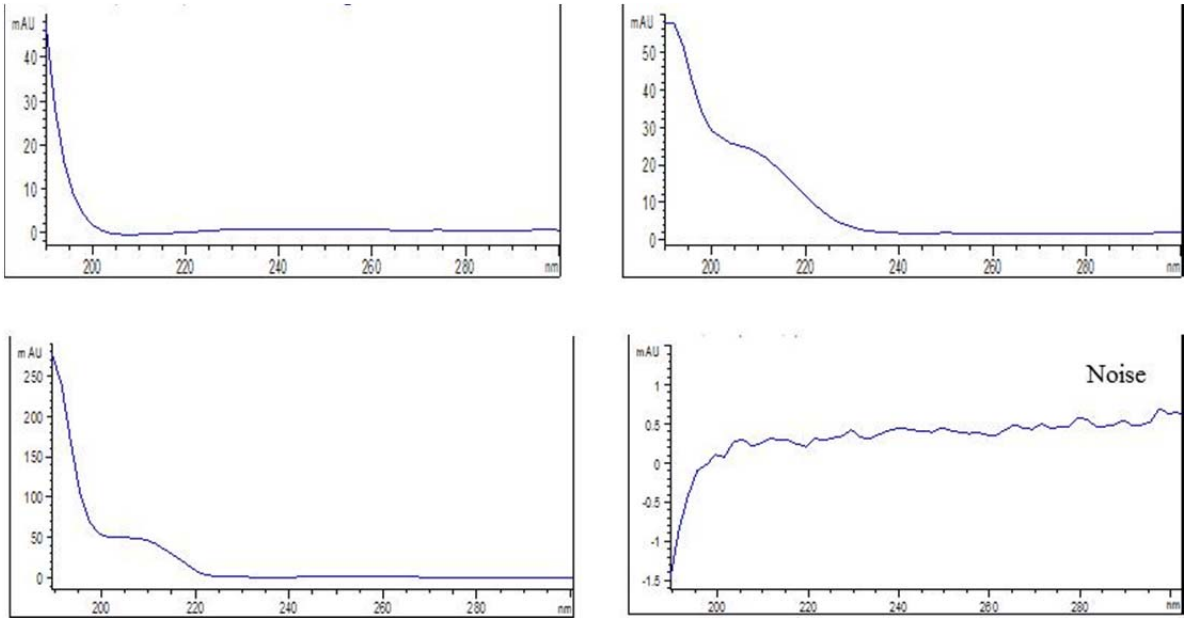

Figure A.2. Spectra obtained from choosing some peaks on denatured and digested HbA using immobilized chymotrypsin electropherogram.

Using 8 M urea and 10 mM calcium chloride shows more peptic peaks comparing with other conditions whereby lower concentration of urea or calcium chloride were used. Therefor, these concentrations were chosen as the optimum concentrations for denaturation and used thereafter throughout the present study.

In another experiment, the effect of urea on enzyme autoproteolysis was studied. The presence of urea shows a significant effect on digestion especially for large proteins. However, it also increases the enzyme autoproteolysis. The effect of urea on enzyme autoproteolysis was studied using both soluble chymotrypsin and trypsin as the enzymes. First, a blank solution of denatured protein containing all chemicals using in denaturation but protein, was added to enzyme, both chymotrypsin and trypsin separately, to reach an E:S ratio of 1:25 as if there was any denatured HbA. The solution was incubated at 37 ºC

for 4 hours. Figure A.3 shows the electropherogram after analysing the solution using CE containing numerous peptidic peaks due to the high rate of autoproteolysis.

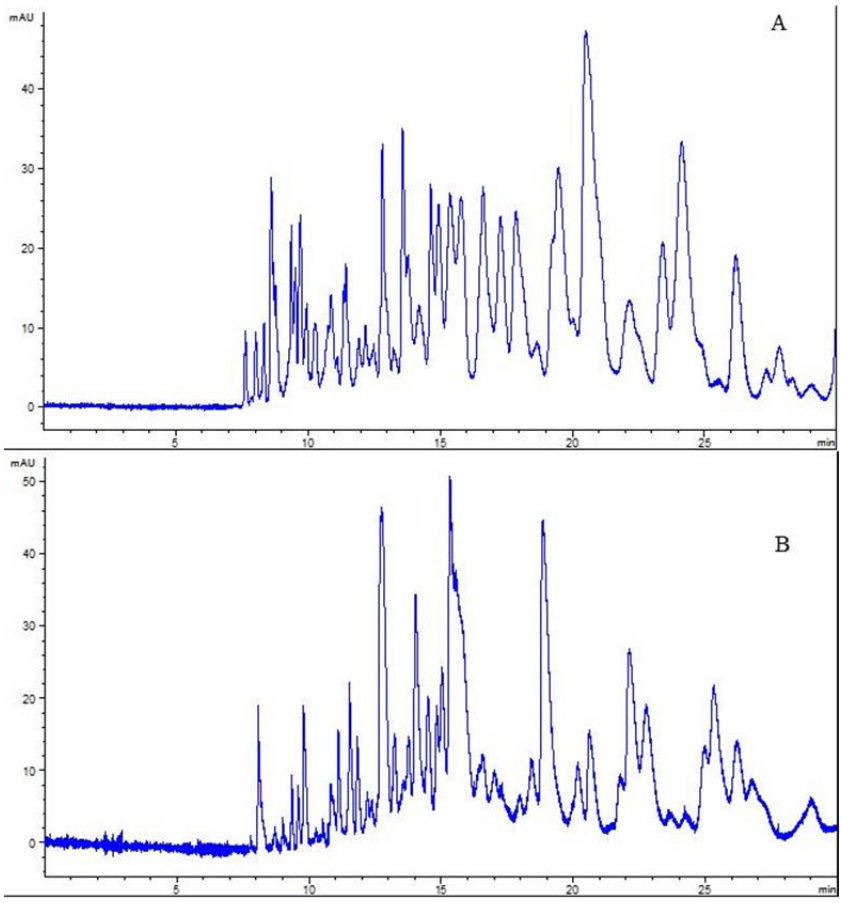

Figure A.3. A blank solution of denatured protein containing all denaturation chemicals except the protein substrate. The amount of protein was replaced with water. An enzyme, (A. free chymotrypsin and B. free trypsin) was separately added, to reach an E:S of 1:25 as if there was any protein. The digestion was carried out at 37 ^{0}C for 4 hours.

Next, a similar study was conducted in which urea was replaced with water, as well as protein, in the solution. The samples were incubated at the same digestion conditions and studied using CE. Figure A.4 shows the electropherogram of this study that represents less peptidic peaks confirming the presence of urea can increase the autoproteolysis significantly.

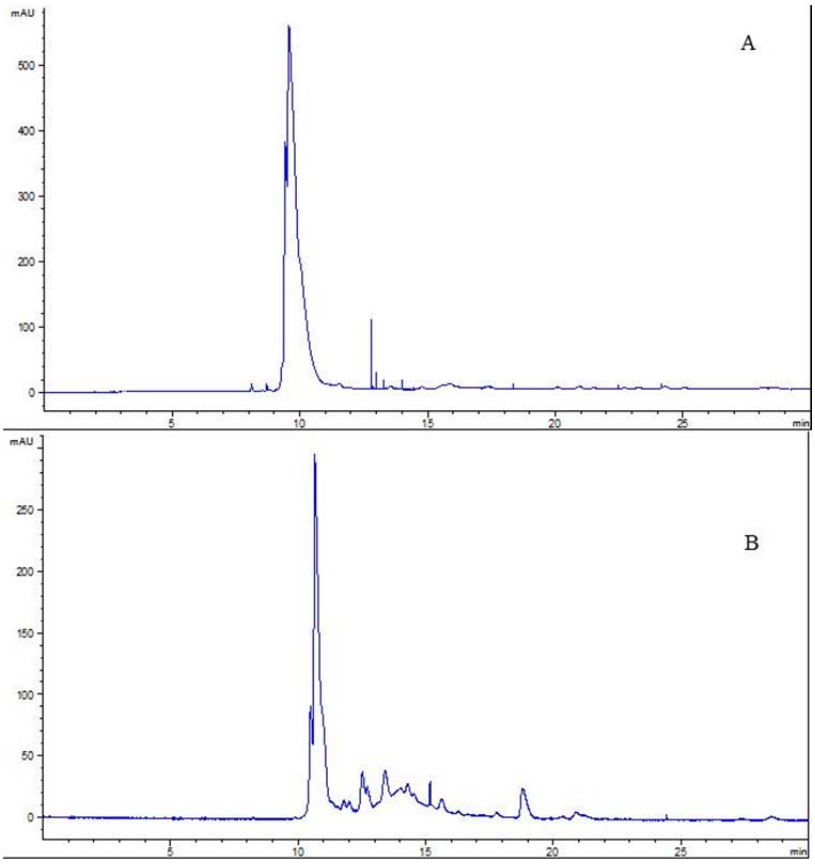

Figure A.4. A blank solution of denatured protein containing all denaturation chemicals except the protein substrate and urea. The urea and protein were replaced with water. An enzyme, (A. free chymotrypsin and B. free trypsin) was separately added, to reach an E:S of 1:25 as if there was any protein. The digestion was carried out at 37 ^{0}C for 4 hours.

Although using urea in denaturation procedure leads to higher enzyme autoproteolysis when using free (soluble) enzyme, urea is necessary to properly digest large proteins such as HbA or BSA as described under Chapter 3. Additionally, using immobilized enzyme decreases the autoproteolysis significantly even in the presence of urea.

Appendix B. Determining Immobilization Efficiency by UV-Vis Absorbance Spectroscopy

To determine the immobilization efficiency, the amount of chymotrypsin is measured in the supernatant after immobilization. We measured the absorption of chymotrypsin and glutaraldehyde (GA) prior to mixing them together, i.e., before crosslinking reaction. Figure 5-1 shows that high concentrations of glutaraldehyde and chymotrypsin exert similar spectral characteristics with the most overlap in absorption regions specific to aromatic residues.

Due to this overlap, we determined that a method is needed to carryover the amount of CT immobilized in the presence of GA. Two methods were used: The analysis of a mixture and the fourth derivative, as described below under Sections B.1 and B.2, respectively.

B.1 Analysis of a Mixture

At each wavelength, absorption of the supernatant after crosslinking reaction is the sum of the absorptions of CT and GA. Therefore:

$$\lambda: \qquad A_{mix} = \varepsilon_{CT}[CT].l + \varepsilon_{GA}[GA].l \tag{B.1}$$

For each standard solution of CT and GA:

$$A_{CT,st} = \varepsilon_{CT}[CT]_{st}.l \qquad \varepsilon_{CT} = {A_{CT,st}}/{l.[CT]_{st}} \tag{B.2}$$

$$A_{GA,st} = \varepsilon_{GA}[GA]_{st}.l \qquad \varepsilon_{GA} = {A_{GA,st}}/{l.[GA]_{st}} \tag{B.3}$$

Dividing Equations 9.2 and 9.3 then 9.1:

$$A_{mix} = \frac{A_{CT,st} \cdot [CT]}{[CT]_{st}} + \frac{A_{GA,st} \cdot [GA]}{[GA]_{st}} \tag{B.4}$$

Therefore:

$$\underbrace{\frac{A_{mix}}{A_{CT,st}}}_{y} = \underbrace{\frac{[CT]}{[CT]_{st}}}_{B} + \underbrace{\frac{A_{GA,st}}{A_{CT,st}}}_{x} \cdot \underbrace{\frac{[GA]}{[GA]_{st}}}_{m} \qquad (B.5)$$

Based on the Equation B.5 and Figure B.2 the concentrations of CT and GA can be calculated.

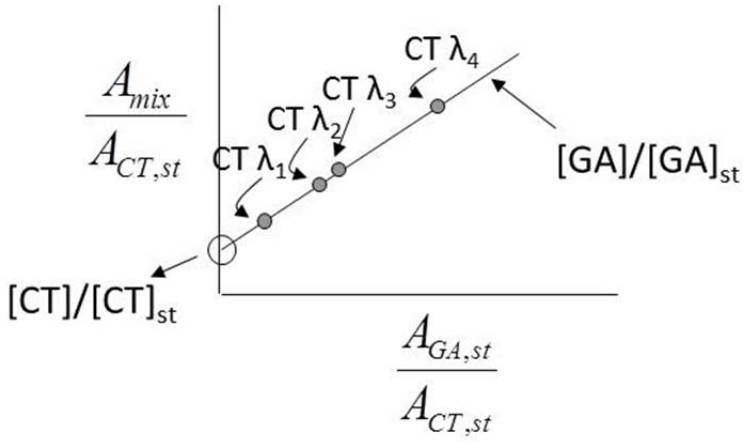

Figure B.1. Drawing the graph using Equation B.5 allows calculating the concentration of chymotrypsin based on the intersect value.

The measurements are conducted at four different wavelengths, 265, 268, 271, and 275 nm. We used chymotrypsin 6 μM and glutaraldehyde 0.45% V/V in water as the standard solutions. The supernatant mixture absorption (A_{mix}) was measured after the crosslinking reaction. By using figure B.1 the chymotrypsin concentration was measured, which shows 95% ± 2 of chymotrypsin is in the immobilized form for 5 measurements (Figure B.3).

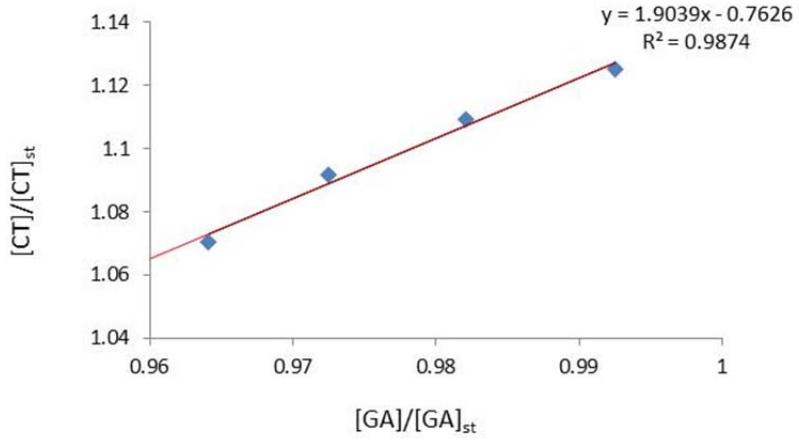

Figure B.2. Measuring absorption of supernatant and standard solution of chymotrypsin and glutaraldehyde at four different wavelengths, 265, 268, 271, and 275 nm and drawing the graph using Equation B.5.

B.2 Fourth Derivative Method

Since the spectra for chymotrypsin and glutaraldehyde are similar and show overlaps (Figure B.1), fourth derivative method was used as an alternative to carry over the amount of chymotrypsin after crosslinking reaction. In this method, fourth derivative spectra of glutaraldehyde (Figure B.3A) and chymotrypsin (Figure B.3B) were calculated mathematically.

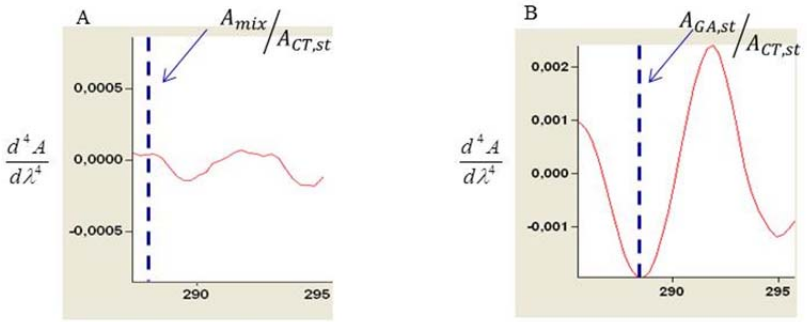

Figure B.3. (A) 4th derivative spectrum of GA, (B) 4th derivative spectrum of CT.

A Calibration curve of $d^4A/d\lambda^4$ as a function of CT concentration was constructed. The chymotrypsin immobilization efficiency was calculated as 94 ± 2% for 5 measurements.