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In Vacuo Glycation of Enzymes: A Novel Approach for Increasing Enzyme Stability

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Thesis submitted to the School of Graduate Studies and Research in partial fulfillment of the degree of Master of Science in Chemistry

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Abstract

A novel approach for the thermostabilization of proteins was investigated. It is well established that proteins that are naturally highly glycosylated show an increased resistance to inactivation at high temperatures. However, non-enzymatic attachment of carbohydrate to proteins, otherwise known as protein glycation, under aqueous conditions is very difficult and has not been used as a general approach for increasing the thermostability of proteins. In the present study, advantage was taken of the *in vacuo* protein glycation procedure recently developed by Kaplan and his co-workers by which proteins can be extensively glycated in the absence of water and chemical reagents. In this procedure, reducing sugars react with the ε-amino groups of lysine side-chains to form a stable ketoamine derivative. The primary objective of the research presented in this thesis, was to determine the extent to which the thermostability of proteins can be increased by *in vacuo* glycation with reducing monosaccharides such as glucose. Another objective of this study was to investigate possible practical applications of this thermostabilization technology.

Trypsin and chymotrypsin were chosen as test proteins because their properties make them particularly suitable for development of the thermostabilization methodology and the practical objectives of the research. Both of these enzymes are among the most highly studied and characterized enzymes and their activities can be easily quantified, and they have relatively poor thermostabilities. Due to the fact that trypsin and chymotrypsin are proteolytic enzymes, these enzymes undergo significant autolysis even at 37°C. This makes them particularly sensitive to thermal inactivation as any loss of activity due to thermal denaturation will be further amplified by increased autolysis.

From a practical viewpoint, both enzymes, and especially trypsin, are widely used in proteomics and the development of highly thermostable forms of these enzymes would find useful applications.

Bovine pancreatic α-chymotrypsin that was glycated with glucose showed a greatly enhanced thermostability. For example native chymotrypsin incubated at pH 8 at a concentration of 0.100 mg/mL (4.0×10^{-6} M) at 55°C for 90 minutes lost its activity, whereas the glycated chymotrypsin retained 27% of its original activity. Even after 3 hours of incubation at 55°C the glycated sample still retained 16% of its activity. At 50°C the native chymotrypsin lost all of its activity after 3 hours of incubation however, the glycated chymotrypsin retained 42% of its original activity. At lower temperatures, such as 37°C, which is routinely used for protein digestions, the extent of thermostabilization over extended periods of time is even more striking.

The *in vacuo* glycated bovine trypsin (glycotrypsin) also showed a greatly enhanced thermostability. At 60°C for example, native trypsin incubated at pH 8 and a concentration of 0.100 mg/mL (4.3×10^{-6} M), lost 90% of its activity after incubation for 30 minutes, whereas the glycated trypsin retained 60%. After incubation at this temperature for 3 hours, the native trypsin was completely inactivated, but the glycated trypsin still retained 16% activity. In this particular case, the thermostabilization is due to two factors. One being the structural stabilization as observed with chymotrypsin, and the other results from a decrease in autolysis due to the glycation of lysine residues preventing trypsin from proteolytic cleavage at these sites. It was determined that the rate of autolysis of the glycotrypsin is approximately one third that of the native trypsin, so it appears that at least 50% of the observed effect is structural stabilization.

In order to determine if the nature of the reducing sugar had a large effect on the degree of thermostabilization that would result from *in vacuo* glycation of trypsin and chymotrypsin, a sample of trypsin was glycated with galactose and a sample of chymotrypsin was glycated with mannose. These samples did not show a significant increase or decrease in the degree of thermostabilization from the samples of trypsin and chymotrypsin glycated with glucose. Although these initial results are not definitive they indicate that the nature of the reducing monosaccharide is likely not important in the extent of thermostabilization observed.

The *in vacuo* protein chemical modification technology has also been applied to immobilize porcine trypsin to a functionalized solid support. An immobilized sample of porcine glycotrypsin was prepared by *in vacuo* cross-linking of glycated trypsin to aminated glass beads through an amide linkage. This immobilized glycated trypsin showed a remarkable resistance to thermal inactivation. When incubated at 60°C at a solution equivalent of 0.200 μg/mL, (8.5×10^{-9} M), at pH 8 for 4 hours, the immobilized glycated sample retained 100% of its original activity. On the other hand, after a 2 hour incubation at 60°C at pH 8 at a concentration of 0.200 μg/mL, the native trypsin lost 80% of its activity, and after 4 hours the native trypsin was inactivated.

Current protein digestion protocols require a time-consuming preliminary step in which the protein is denatured prior to addition of enzyme. To investigate whether this step could be eliminated, simply by digesting at elevated temperatures, the thermostable immobilized glycotrypsin was used to digest native chicken egg white lysozyme at 60°C. The results clearly demonstrated that while native trypsin did not yield any digestion

products under these conditions, the immobilized glycated trypsin did effectively digest native lysozyme.

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Chapter 2

List of Abbreviations

AANHS	acetic acid *N*-hydroxysuccinimide ester
AGE	advanced glycation endproduct
Arg	arginine
Asn	asparagine
Asp	aspartic acid
ATEE	*N*-acetyl-L-tyrosine ethyl ester
BAEE	*N*-benzoyl-L-arginine ethyl ester
BAPNA	*N*-benzoyl-L-arginine p-nitroanilide
BTEE	*N*-benzoyl-L-tyrosine ethyl ester
CM-cellulose	carboxymethyl cellulose
CNBr	cyanogen bromide
ddH_2O	distilled deionized water
DEAE	diethylaminoethyl
DEPC	diethyl pyrocarbonate
DFP	diisopropylflurophosphate
dH_2O	distilled water
DNA	deoxyribonucleic acid
ESI-MS	electrospray ionization mass spectrometry
GalNAc	*N*-acetyl-D-galactosamine
GlcNAc	*N*-acetyl-D-glucosamine
His	histidine
HTLE	Hunter thin layer electrophoresis

HVPE	high voltage paper electrophoresis
ICAT	isotope coded affinity tag
LpH	lyophilized pH
Lys	lysine
MPEG	methoxypolyethylene glycol
MS	mass spectrometry
MWCO	molecular weight cut-off
m/z	mass to charge ratio
NMR	nuclear magnetic resonance
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PEG	polyethylene glycol
pH	negative logarithm of the proton concentration
pK_a	negative logarithm of the acid ionization constant
ppm	parts per million
Q-TOF	quadrupole time of flight
RNase	ribonuclease
RP	Ruhemann's purple
SDS	sodium dodecyl sulfate
Ser	serine
T_g	glass transition temperature
Thr	threonine
TPCK	tosyl-L-phenylalanine chloromethyl ketone

Chapter 1: Main Introduction

Chapter 1: Main Introduction

1.1 Protein Chemical Modification

With the initial study of the chemistry of amino acids, scientists laid the foundation for the study of protein chemistry. Major advances in protein chemistry were made during the 1940s as scientists were beginning to analyze and investigate the amino acid composition of proteins. Included in the new techniques being developed, in order to delve into the world of proteins, was the establishment of methods to chemically modify proteins. The chemical modification of proteins was one of the first techniques used to elucidate structure-function relationships and this technique has provided much information regarding many aspects of the general nature of proteins. Although initiated in the middle of the 1940s, protein chemical modification continues to expand and develop as new methods and reagents are continually being created. Early endeavours in protein chemical modification employed the use of reagents and reactions commonly used in organic chemistry at the time. These methods included modifications such as methylation, acetylation, iodination and deamidation reactions (Means and Feeney, 1990). The expansions made to protein chemistry since these early endeavours are numerous and include the development of a wide array of reagents which serve purposes ranging from the alteration of electrostatic charge to the introduction of fluorescent or radio-labels (Lundblad, 1995).

The reactive nucleophilic groups of proteins are primarily located on the side-chains of certain amino acid residues, and include imidazole, ε-amino, sulfhydryl,

phenolic hydroxyl, and carboxyl groups (Creighton, 1993). Other reactive groups in proteins include the N-terminal amino group and the C-terminal carboxyl group.

The side-chain specific chemical modification of proteins is a powerful method for the elucidation of protein structure-function relationships and involves use of a reagent which will specifically react with one type of protein functional group and not any others. This type of modification is often difficult to achieve, as most reagents have the tendency to react with more than one reactive group in the protein. The selectivity of the side-chain specific reagents can be, to a limited extent, controlled by managing parameters such as reaction time, the pH of the solution and the concentration of the reagents. Another means targeting of side-chain specific modification is by virtue of relative nucleophilicity of the amino acid's reactive group. For example, the nucleophilicity of cysteine's sulfhydryl group far exceeds that of any other group in proteins, therefore the selective modification of such group can be achieved fairly readily (Lundblad, 1995).

As the range of reagents and methods currently used in the chemical modification of proteins is extremely vast, some of the more commonly used chemical reagents and methods will be elaborated on in this introduction. These include methods and reagents which are primarily used to provide information on the structure-function relationships of proteins, to attach special function groups to proteins, to immobilize enzymes by covalent attachment of the enzyme to a solid support, or to increase a protein's stability under denaturing conditions.

1.2 Chemical Modification to Elucidate Structure-Function Relationships

The selective modification of amino acid side-chains can be employed in order to determine the identity of groups essential for catalytic activity, or the physical structure of the protein can be modified in order to elucidate information on the protein structure (Means and Feeney, 1971). Experiments preformed by Dumas and Raushel (1990) on phosphotriesterase illustrate an example of chemical modification where the goal was to determine the essential groups for enzyme activity. In such an experiment, the retention of biological activity after the modification of functional groups provides evidence that the modified group(s) is/are not necessary for catalytic activity (Means and Feeney, 1990). The activity of phosphotriesterase was investigated by modifying the enzyme with a variety of reagents specific for modification of cysteine, lysine, histidine, glutamate and aspartate residues. It was found that the activity of the enzyme sample that was modified with diethyl pyrocarbonate (DEPC) was significantly reduced. The use of DEPC for protein modification will target modification of the histidine imidazole ring, and is associated with an increase in absorbance at 240nm. The modification is easily reversed in alkaline conditions and by the introduction of a nucleophile such as hydroxylamine (Lundblad, 1995). The results from the chemical modification experiments preformed by Dumas and Raushel indicated that there was no significant enzyme inactivation with any of the chemical modifiers used except for DEPC (Dumas and Raushel, 1990). This data provided evidence of a histidine residue necessary for catalytic activity in the active center of phosphotriesterase.

The nature of protein physical structure can also be investigated by the use of chemical modifiers. Succinic anhydride has long been used for this purpose, where protein chemical modification *via* succinic anhydride can selectively target the ε-amino groups of lysines, and results in the placement of anionic carboxylate groups in the place of cationic amonium groups (Lundblad, 1995). This creates electrostatic repulsion and frequently results in the dissociation of multimeric proteins. Kuronen and co-workers treated cytochrome oxidase by progressive succinylation of 14% to 68% of the proteins lysine residues, which resulted in an increase in the amount protein appearing the dissociated subunit form (Kuronen *et al.,* 1975). These results confirmed the fact that cytochrome oxidase exists in a dimeric form, and that strong protein-protein interactions occur between the two subunits.

1.3 Chemical Modification to Attach Special Function Groups to Proteins

Chemical modifications can also provide a means for attachment of special function groups to proteins. A good example of this is the modification of proteins with the isotope coded affinity tag (ICAT) reagent, used in proteomics research to measure levels of differential protein expression between two cell states by mass spectrometry. In 1999, Gygi and his colleagues, (Gygi *et al.,* 1999), designed a reagent that consists of a biotin derivative, used for the selective isolation of labelled peptides, an isotopically labelled linker that exists in a light and heavy form, and an iodoacetyl reactive group specific for the modification of cysteine sulfhydryls. The iodoacetyl reactive group will

selectively target sulfhydryls due to their high nucleophilicity, where the reactivity of sulfhydryls with haloacetates far exceeds that of other protein groups under almost all conditions (Lundblad, 1995). This allows for the derivitization of proteins to occur in the presence of stabilizing agents such as sodium dodecyl sulphate (SDS), urea, salts and other chemicals which do not contain a reactive thiol group.

1.4 Chemical Modification to Immobilize Enzymes

Chemical modification of proteins has been used in order to immobilize enzymes, where immobilization occurs as a result of the establishment of a covalent bond between the soluble enzyme and an insoluble solid support. The immobilized complex exhibits the physical characteristics of the solid support, while maintaining the activity of the free enzyme. Chemical enzyme immobilization generally involves the activation of a solid support with a reactive group, as most supports initially exist in an unreactive form, followed by reaction of the activated support with reactive groups of the enzyme. For example, a commonly used solid support for enzyme immobilization is carboxymethyl cellulose (CM-cellulose). As the hydrazide of CM-cellulose is commercially available, this support requires activation with nitrous acid to produce the acid azide. The enzyme can then be coupled to the support by an acylation reaction involving the ε-amino groups of lysine to produce an amide linkage between the enzyme and support (Zaborsky, 1973).

Immobilized enzymes are most commonly used in industrial processes where enzymatic catalysis of large batches is undertaken. An example of such a process is the

use of immobilized glucose oxidase to isomerize glucose to fructose in the production of high fructose corn syrup (Swaisgood, 1985). The primary benefit of using immobilized enzymes over free enzymes in such processes is the retention of the catalyst during the batch process. For example, if a free soluble enzyme is used in a batch reactor, the flow of substrate solution through the reactor will inevitably sweep the catalyst along with it. This will result in the contamination of the product and will require removal or deactivation of the catalyst. The use of an immobilized enzyme allows for the substrate solution to be passed over a column containing bound enzyme, preventing loss and contamination of the product.

1.5 Chemical Modification to Enhance Enzyme Stability

Enzymes have been chemically modified in order to increase their stability against denaturing factors. A variety of enzymes are currently used as biocatalysts in industrial processes involving the synthesis of pharmaceuticals, peptides and compounds such as alcohols, steroids and organic acids (Poulsen, 1983). Members of the protease family are routinely used in the food processing industry in such processes as meat tenderization, cheese production and flavour development (Chibata, 1978). For many of these industrial processes, highly stable proteolytic enzymes are needed, due to the extreme reaction conditions including catalysis in organic solvents, reaction at extremes of pH and temperature, and the possible presence of enzyme inhibitors (Simon *et al.*, 1997; Venkatesh and Sundaram, 1998). Therefore, much attention has focused on the

production of highly thermostable enzymes *via* various methods of chemical modification or protein engineering using recombinant deoxyribonucleic acid (DNA) technologies. Also, current techniques used in protein and proteome analysis require protein digestion by proteases such as trypsin, where highly stable autolysis resistant enzymes are desired (Twyman, 2004).

The production of enzymes with an increased stability has been accomplished *via* site directed mutagenesis or directed evolution techniques. Stabilization of enzymes is brought about by altering the enzyme structure in such a way to introduce internal or surface disulfide bridges, increase internal hydrogen bonding, increase the number of external salt bridges and improve internal hydrophobic packing (Eijsink *et al.,* 1992; Janeček, 1993; Li *et al.,* 1998; Várallyay *et al.,* 1998). This is accomplished by making a point mutation in a sequence of DNA that encodes for a particular protein. This will result in the substitution of a different amino acid in the protein primary structure of the mutant as compared to the wild type after translation (Strachan and Read, 1999). The creation of such mutant proteins often results in an alteration of protein function or stability (Li *et al.,* 1998; Várallyay *et al.,* 1998).

Protein engineering techniques such as site directed mutagenesis tend to be labour intensive, therefore much interest has been placed in the production of enzymes with enhanced stabilization *via* methods of chemical modification. It is desirable to produce enzymes with an enhanced resistance to thermal denaturation, as several advantages arise from carrying out enzymatic reactions at elevated temperatures. These advantages include the prevention of microbial contamination, the increased rate of reaction, an increase in the solubility of substrates and products, and a decrease in the viscosity of the

reaction medium (Villalonga *et al.*, 2003). It is known that the covalent attachment of carbohydrate moieties to proteins imparts a degree of enhanced stability against extreme conditions. Therefore, many of the current enzyme chemical modification techniques concerned with the improvement of enzyme stability, involve the covalent attachment of a carbohydrate moiety to the enzyme (Fernández *et al.*, 2002; Marshall, 1978; Venkatesh and Sundaram, 1998; Villalonga *et al.*, 2003). However, as will be discussed, there is a lack of a general method to covalently attach carbohydrate moieties to proteins, as the current methods tend to involve protein-specific processes.

In certain cases it has been shown that the process of enzyme stabilization *via* chemical modification has provoked a loss in catalytic activity (Darias and Villalonga, 2001; Murphy and Fágáin, 1996). Therefore, interest has been placed in the development of chemical methods aimed at creating enzymes with enhanced stabilization which also preserve catalytic properties.

There are several shortcomings associated with the chemical modification of proteins in aqueous conditions. Firstly, the modification of proteins in an aqueous environment requires careful control of conditions such as reaction time, concentration of the reactants and pH in order to achieve successful and specific derivitization. Also, all reagents, reaction intermediates and products must be miscible and stable in water. In most cases, water acts as a competing nucleophile with reactive groups in the protein, therefore reagents must be present in excess to achieve adequate derivitization. In turn, this results in the necessity to quench excess reagent and subsequent purification of the modified protein by some form of chromatography or dialysis. And, perhaps most importantly, aqueous modification techniques involve the derivitization of enzymes in

conditions where enzymes are susceptible to proteolysis, conformational dynamics, and possible denaturation.

1.6 *In Vacuo* Protein Chemical Modification

The *in vacuo* protein chemical modification technique developed by Kaplan and co-workers, (Kaplan *et al.,* 2002; Kaplan and Taralp, 1997; Taralp and Kaplan, 1997), is a novel process where protein derivitization occurs in the lyophilized state at high temperatures under vacuum. This methodology circumvents many of the problems associated with protein modification in the aqueous phase. In the absence of water, proteins are not able to exhibit conformational dynamics and in the case of enzymes, proteolysis does not occur, hence the stability of proteins in the lyophilized state far exceeds that when in solution.

Freeze-drying or lyophilization is a process where water is removed from a frozen sample by sublimation and desorption (Hayes, 2002). Despite the stability exhibited by proteins in the lyophilized state, the stresses associated with this process can often cause damage to protein structure and protein aggregation upon rehydration, which can ultimately result in the loss of biological activity (Roy and Gupta, 2004). Therefore excipients such as non-reducing sugars, polyols and polymers are usually added to the lyophilization formulation to act as stabilizers (Heller *et al,* 1999). These compounds reduce the likelihood of protein denaturation during lyophilization by stabilizing protein

structure and minimizing protein-protein interactions in the dry state by providing separation between protein molecules (Patist and Zoerb, 2005).

Despite some of the problems associated with the lyophilzation of proteins, the *in vacuo* protein modification technique provides several advantages over traditional protein modification processes in solution. Proteins are much more thermostable in the lyophilized state as compared to being dissolved in solution, therefore much higher reaction temperatures can be employed, which will ultimately enhance the rate of derivitization. Another advantage of the *in vacuo* procedure, is that only small amounts of reagents are required, and upon completion of the derivitization, any excess reagent can be removed and subsequently re-used. This is important when high cost reagents such as isotopically labelled compounds are being used.

Since current techniques used to chemically modify enzymes in the hopes of the developing a product with an enhanced stability can be laden with difficulties, and may result in the production of an enzyme with a reduced biological activity, the *in vacuo* modification approach may provide a useful alternative.

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Chapter 2: *In Vacuo* Glycation of Trypsin and Chymotrypsin

Chapter 2: *In Vacuo* Glycation of Trypsin and Chymotrypsin

2.1 Introduction

As previously mentioned, there is a vast array of chemical modification techniques that can be applied to proteins. The traditional modification techniques carried out in aqueous solution described in the main introduction can provide useful information regarding many aspects of protein structure and stability. There also exists an extensive repertoire of protein modifications that occur *in vivo*, some resulting from enzymatic catalysis, whereas others arise from non-enzymatic chemical processes. An example of an *in vivo* protein chemical modification which occurs non-enzymatically is the inevitable oxidation of proteins by oxygen and other oxidants such as peroxides and hydroxyl radicals that are generated by metabolism (Creighton, 1993). However, most proteins in the cell are modified *via* reactions which are catalyzed by enzymes. These enzymatic modification processes include proteolytic processing, alteration of C-terminal or N-terminal amino acid residues, and the covalent attachment of groups such as acetyl, phosphoryl, methyl and glycosyl to a protein's structure (Creighton, 1993).

2.1.1 Protein Glycosylation

Among the covalent modifications that occur to proteins *in vivo*, the attachment of carbohydrate moieties is among the most prevalent, and this process has been termed glycosylation. This process is strictly an enzymatic process, and the subsequent proteins which result from glycosylation are termed glycoproteins. Examples of glycoproteins are

numerous and include enzymes, protein hormones, structural proteins and cell surface proteins (Creighton, 1993). The process of glycosylation involves the formation of a glycosidic linkage between an oligosaccharide and the side-chains of specific amino acid residues, of which there are only three. The formation of a glycosidic linkage between an oligosaccharide and the nitrogen atom of the asparagine (Asn) side-chain is known as an *N*-glycosylation, and similarly, the formation of a glycosidic linkage between an oligosaccharide and the hydroxyl group of serine (Ser) or threonine (Thr) residues is known as *O*-glycosylation (Creighton, 1993). The biochemical pathways which are taken to bring about *N*-glycosylation or *O*-glycosylation are different.

The *N*-linked glycosylation pathway is the best understood route to protein glycosylation (Taylor and Drickhamer, 2003). As mentioned, this process involves the attachment of a saccharide component to the asparagine side-chain, and it can be broken down into three main steps: (1) the formation of a lipid linked precursor oligosaccharide, (2) the transfer of the pre-assembled oligosaccharide to a polypeptide chain and (3) the processing of the attached oligosaccharide. In the first step of *N*-glycosylation a lipid linked glycan is assembled from *N*-acetlyl-D-glucosamine (GlcNAc), monosaccharide units, nucleotide sugar donors and dolichol. This is accomplished by enzyme catalysis in the cytoplasmic side and lumen of the endoplasmic reticulum (Kornfeld and Kornfeld, 1985). Once synthesized, the glycan portion of the donor is transferred to a polypeptide chain *via* catalysis by an oligosaccharyltransferase. This is a co-translational event, with the energy required to form the glycan-asparagine linkage coming from the cleavage of the glycan-phosphate bond that exists between the glycan and the dolichol unit of the donor (Taylor and Drickhamer, 2003). The asparagine residues to be glycosylated must

be present on the surface of the protein and found in the specific sequences of Asn-X-Ser or Asn-X-Thr, where X can be any amino acid residue except for proline. The final stage of *N*-glycosylation is the processing of the attached oligosaccharide. This is achieved by two families of enzymes, namely glycosidases and glycosyltransferases. Various glycosidases will remove sugars from the oligosaccharide to create a core usually consisting of two GlcNAc units and three units of mannose, and glycosyltransferases will add sugars onto this core to create highly diverse *N*-linked glycans (Taylor and Drickhamer, 2003).

The process of *O*-glycosylation differs from *N*-glycosylation in several aspects. Firstly, glycosylation occurs at serine or threonine residues, with oligosaccharides attaching to the hydroxyl group of these side-chains. Secondly, sugars are added in a stepwise fashion one at a time rather than transferred as a pre-assembled core as in *N*-glycosylation. The saccharide unit which directly forms the *O*-glycosidic linkage with serine or threonine residue is *N*-acetyl-D-galactosamine (GalNAc) (figure 2.1.1). Thirdly, there are no simple target sequences within the primary structure of the protein that are analogous with the Asn-X-Ser/Thr sequences targeted in *N*-glycosylation (Taylor and Drickhamer, 2003). This is because a single oligosaccharyltransferase catalyzes the transfer of the core glycan to asparagine residues in *N*-glycosylation, and in the case of *O*-glycosylation there a numerous transferases that accomplish the transfer of saccharide units to serine or threonine side-chains. These different oligosaccharyltransferases have different substrate targets making it difficult to identify target sequences. Lastly, *N*-glycosylation is a co-translational event occurring in the endoplasmic reticulum, whereas

O-glycosylation occurs post-translationally in the Golgi apparatus (Taylor and Drickhamer, 2003).

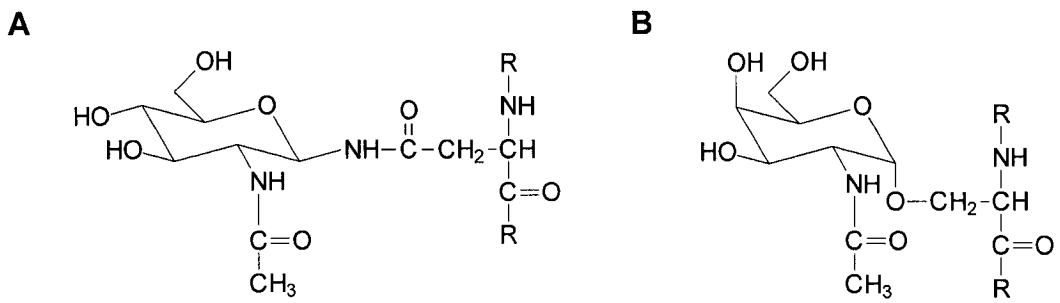


Figure 2.1.1: Nature of the *N*-glycosidic linkage between *N*-acetyl-D-glucosamine (GluNAc) and the side-chain of an asparagine residue (A), and the nature of the *O*-glycosidic linkage between *N*-acetyl-D-galactosamine (GalNAc) and the side-chain of a serine residue (B) (Bill *et al.,* 1998; Taylor and Drickhamer, 2003)

Glycoproteins represent the most diverse group of biologically occurring macromolecules known to occur (Schulz and Schirmer, 1979), and the protein bound oligosaccharides have been classified as either having a non-specific or specific function (Bill *et al.,* 1998). Non-specific functions include the maintenance of protein conformation, stability or solubility. Mucins are glycoproteins where the attached oligosaccharides can be considered to have a non-specific function. These are heavily *O*-glycosylated proteins, where carbohydrate accounts for 70-85% of their dry weight (Gallagher and Corfield, 1978). Mucins exist as networks of extended tangled macromolecules, which are able to bind large quantities of water, giving them viscous and gel-like properties (Bill *et al.,* 1998). These glycoproteins are secreted by a cell layer

which lines body cavities of the respiratory, digestive and urogenital tract, and provide lubrication and protection to the underlying tissue against mechanical or chemical degradation (Hughes, 1983). Other non-specific functions of protein bound oligosaccharides include protective effects, as influencing the stability of glycoproteins with respect to degradation, or functional effects, where the biological activity of enzymes have been altered by glycosylation (Bill *et al.,* 1998). Specific functions of the oligosaccharide components of glycoproteins primarily include biological recognition events. This is due to the fact that carbohydrates can be linked together in many different ways to form branched structures giving them the potential to carry more biological information than other similarly sized bio-molecules (Paulson, 1989). The bound carbohydrate moieties of glycoproteins play important roles in cell-cell and cell-extracellular matrix adhesion events and protein targeting events such as trafficking and catabolism. An example of such a protein targeting event is the recognition by lysozyme of a phosphorylated high mannose oligosaccharide present on certain glycoproteins which are to be degraded by this enzyme (Bill *et al.,* 1998).

2.1.2 Protein Glycation

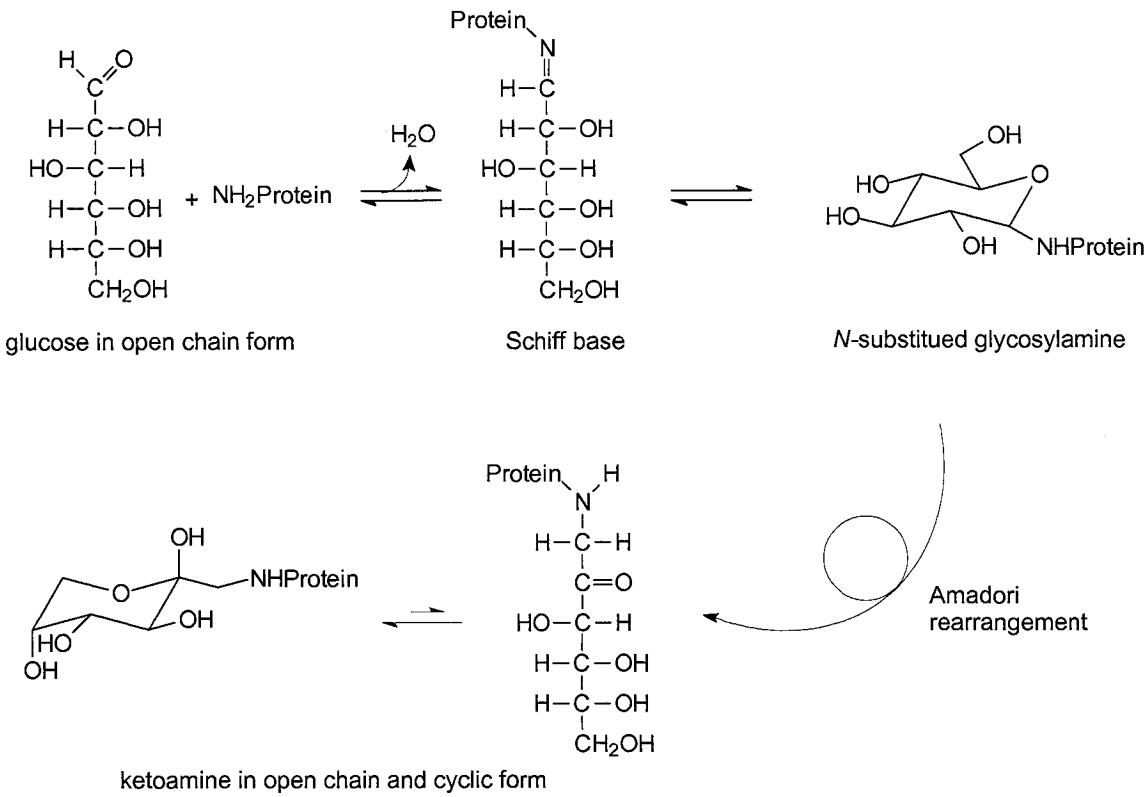
The covalent attachment of sugars to proteins can also occur non-enzymatically and to distinguish it from the enzymatic process, it is termed glycation. In 1912, Louis-Camille Maillard observed the formation of brown pigments upon heating a mixture of lysine and glucose (Mauron, 1981). The creation of the brown pigments or "melanoidins" begins with an initial condensation reaction between reducing sugars and amino groups. The breakdown of this initial condensation product *via* a cascade of

secondary and tertiary reactions results in the production of insoluble brown pigments (Baltes, 1982). This process has been termed the Maillard reaction, in honour of the French chemist who first studied it, and it includes the whole network of reactions stemming from the initial amino group and reducing sugar condensation event (Yaylayan and Huyghues-Despointes, 1994). It is well established that the Maillard reaction is responsible for the production of flavours, aromas and the browning of food during cooking. In foodstuffs the Maillard reaction primarily occurs between reducing sugars and the primary amino groups represented by the ε-amino group of lysine and to a very small extent the α-amino groups of *N*-terminal amino acids (Mauron, 1981).

As the Maillard reaction has been extensively characterized by food chemists, it has subsequently been broken down into three general stages (Hodge, 1953). The first stage is known as the early Maillard reaction and consists of the well defined initial condensation reaction that occurs between proteins and reducing sugars to yield a single product. The advanced Maillard reaction is the second stage and it consists of the degradation of the early Maillard reaction product to produce volatile or soluble substances. The last stage is known as the final Maillard reaction, and consists of further degradation of the initial product leading to the production of melanoidins (Mauron, 1981). For the purposes of this thesis, the relevant portion of the Maillard reaction includes only the early reaction, in which the process of protein glycation occurs.

Protein glycation *via* the early Maillard reaction, as illustrated in reaction scheme 2.1.1, begins with a condensation reaction between a species of reducing sugar, such as glucose and a free amino group. More specifically, a nucleophilic attack by the ε-amino of lysine or the α-amino group of the N-terminal amino acid residue occurs at the

carbonyl function of the open chain form of the reducing sugar. The condensation product rapidly loses a molecule of water and is converted to a Schiff base, which can cyclize to form an *N*-glycosylamine. The *N*-glycosylamine then undergoes the Amadori rearrangement to form a ketoamine, which is the final product of the early Maillard reaction. The Amadori rearrangement proceeds by acid/base catalysis to render an unstable enaminol intermediate which rearranges to afford the more stable ketoamine product (Baltes, 1982).



Reaction Scheme 2.1.1: Protein glycation *via* the early stages of the Maillard reaction (Baltes, 1982; Hodge 1953; Mauron, 1981).

Protein glycation *via* the Maillard reaction is not limited to foodstuffs, as this reaction has been identified to occur as a biological process in mammals (Bill *et al.,* 1998; Mester *et al.,* 1981; Monnier *et al.,* 1981; Stitt, 2001). The modification of proteins by the Maillard reaction *in vivo* provides proteins with the ability to cross-link with other proteins and over time will generate what is known as "advanced glycation endproducts" (AGEs) (Bill *et al.,* 1998). During the last 20 to 25 years the significance of this reaction and its relevance to the modification of proteins with regards to the productions of AGEs during the natural ageing process and in diseases such as diabetes and atherosclerosis has become apparent (Monnier *et al.,* 1981; Stitt, 2001). In diabetics, proteins with a slow turnover which are exposed to hyperglycemic conditions, such as haemoglobin, lens proteins, myelin and collagen tend to be modified by the Maillard reaction, and finally result as forms of AGEs. Retinopathy is a progressive complication of diabetes, where ocular arteries degenerate eventually leading to a decrease in vision quality and possibly the total loss of vision. AGEs can initiate abnormal growth responses in retinal vascular cells, which ultimately lead to the degradation of the ocular vascular system (Stitt, 2001).

A small amount of protein glycation by the Maillard reaction has been observed to occur in pharmaceutical preparations of therapeutic lyophilized protein or peptide preparations which contain reducing sugars as excipients (Li *et al.,* 1996; Quan *et al.,* 1999; Tarelli *et al.,* 1994). This dry-state glycation occurs without heating as an unwanted side reaction. The long term storage of these preparations results in the conversion of some of the therapeutic protein or peptide into compounds with a reduced stability and biological activity. Other sources report that the dry-heating of lyophilized

protein and reducing sugar mixtures leads to protein glycation through the Maillard reaction (Kańska and Boratyński, 2002; Naranjo *et al.,* 1998; Yeboah *et al.,* 1999). These sources have used this technique to investigate the pathophysiological role of early glycation products in diseases such as diabetes (Kańska and Boratyński, 2002), and to investigate the extent of lysine loss in nutritional proteins by various reducing sugars (Naranjo *et al.,* 1998). However, the production and isolation of early glycation products has proven to be difficult by way of the Maillard reaction, as this reaction often results in the production of a large number of unwanted and uncharacterized AGEs which manifest themselves as insoluble complexes (Kańska and Boratyński, 2002; Yeboah *et al.,* 1999).

2.1.3 Enzyme Glycation to Enhance Stability Properties

It is known that many glycoproteins exhibit a greater stability than carbohydrate-free proteins (Imperiali and O'Connor, 1999; Marshall, 1978; Sasvári and Asbóth, 1998; Venkatesh and Sundaram, 1998). Enzymes with an increased stability in denaturing conditions, in particular enzymes with an increased thermostability, are in high demand for various industrial applications. Therefore, many techniques have been developed to produce enzymes coupled to carbohydrates with the hopes of producing enzymes which exhibit an increased resistance to denaturation under stressing conditions. There exists a vast repertoire of chemical modification techniques used to covalently modify enzymes with carbohydrate moieties. Some specific examples of such techniques include the attachment of carboxymethyl cellulose derivatives to trypsin *via* reductive alkylation (Villalonga *et al.,* 2000), the linkage of chitosan to cellulase through covalent attachment to periodate-activated moieties of the enzyme (Darias and Villalonga, 2001), and the

reaction of trypsin with activated monomethoxypolyethylene glycol derivatives to produce a covalently modified trypsin derivatives (Gaertner and Puigserver, 1992). Other techniques include the two step process of chemoenzymatic glycosylation (Longo and Combes, 1999) and the coupling of carbohydrate moieties to enzymes by the use of bi-functional cross-linking agents (Marshall, 1978). These chemical modification techniques used to create enzyme-carbohydrate conjugates in aqueous conditions, are frequently laden with shortcomings as reagents and/or proteins may require prior chemical activation into a more reactive species, and extensive purification of the final enzyme product is often necessary as many unwanted side products may form. Also, as these modification techniques are carried out in aqueous conditions, large quantities of reagents may be required as water has the capacity to act as a competing nucleophile.

The development of an *in vacuo* protein chemical modification technique by Kaplan and co-workers (Kaplan *et al.,* 2002; Kaplan and Taralp, 1997; Taralp and Kaplan, 1997) provides an alternative method to glycated proteins, and this technique is also an advantageous method in several respects. The *in vacuo* protein glycation technique removes water and oxygen from the reaction environment preventing the formation of unwanted products. This results in the formation of a single product (Stewart, PhD Thesis, 2004). Also, the *in vacuo* glycation technology takes advantage of the modification of proteins in the lyophilized state, as proteins are more stable in this state as compared to aqueous conditions. This allows for higher reaction temperatures to be used, which subsequently increases the rate of derivitization.

The disaccharide trehalose has been selected as the excipient, since its stabilizing properties concerning the lyophilization and storage of proteins has been well established

(Allison *et al.,* 1999; Carpenter and Crowe, 1989; López-Díez and Bone, 2004; Patist and Zoerb, 2005; Roy and Gupta, 2004). The process of lyophilization imparts stresses on proteins and which can ultimately have deleterious effects on structure and the recovery of enzyme activity upon rehydration. The stabilizing properties of trehalose during the storage and lyophilization of protein preparations has been rationalized by two explanations. The first explanation concerns the ability of disaccharides to form glassy matrices with a high viscosity, restricting molecular motions, which in turn, reduces degradation of protein structure (López-Díez and Bone, 2004). The glass transition temperature (T_g) is the temperature at which such glassy matrices begin to behave as a supercooled melt when heated (Patist and Zoerb, 2005). Therefore, at temperatures below the T_g of a glassy matrix, translational and vibrational molecular motions are restricted and the substance remains in a "frozen" state. Trehalose has a high T_g (115˚C) (Patist and Zoerb, 2005), and the molecular motions exhibited by a protein interacting with this sugar will be restricted under conditions which occur at temperatures below this value. Another stabilizing property of trehalose can be explained by the fact that during the process of lyophilization, extremely dry conditions are achieved, where a protein's hydration shell is removed. Stabilizing hydrogen bonds form between essential water molecules and the polar functional groups of proteins, and the loss of these water molecules can result in binding sites engaging in hydrogen bonding with other parts of the protein structure (López-Díez and Bone, 2004). This can lead to a loss of the protein's active conformation, and subsequent irreversible inactivation upon rehydration (Carpenter and Crowe, 1989; López-Díez and Bone, 2004). Molecules of trehalose will assume the role of the essential water molecules and form hydrogen bonds with the

protein's polar groups, which helps to maintain the active conformation (Allison *et al.*, 1999; Carpenter and Crowe, 1989; López-Díez and Bone, 2004).

The *in vacuo* glycation of Ribonuclease A (RNase A) and human haemoglobin has been previously preformed and characterized (Stewart, PhD Thesis, 2004). However when considering the important roles that proteolytic enzymes play in current research techniques and industrial processes, it is of interest to investigate whether or not the *in vacuo* glycation imparts beneficial properties to such enzymes. Therefore, the present research was undertaken with the objective of characterizing the *in vacuo* glycation of the proteolytic enzymes trypsin and chymotrypsin and the subsequent analysis of the new properties that the *in vacuo* glycation has to bring to these enzymes.

2.2 Materials and Methods

2.2.1 Proteins and Reagents

Bovine pancreatic trypsin, porcine pancreatic trypsin and bovine pancreatic α-chymotrypsin were purchased from the Sigma-Aldrich Chemical Company. With regards to the purity of the proteolytic enzyme preparations used in experiments described in this thesis, it can be stated that these preparations are 99% pure. All experimental samples of either trypsin or chymotrypsin were taken from Sigma-Aldrich stock preparations, where the lot numbers of porcine pancreatic trypsin, bovine pancreatic trypsin and bovine pancreatic chymotrypsin were 034K7423, 121K7692 and 27H7007 respectively. In the case of the trypsin preparations, these samples were treated

with tosyl-L-phenylalanine chloromethyl ketone (TPCK) in order to inactivate any pseudotrypsin or chymotrypsin present in the stock preparations. The specific activities of the enzyme samples used for all experiments are as follows; bovine trypsin (Lot #: 121K7692) 14 100 Units/mg with BAEE as the substrate, porcine trypsin (Lot #: 034K7423) 15 500 Units/mg with BAEE as the substrate and bovine chymotrypsin (Lot #: 27H7007) 49 Units/mg with BTEE as the substrate. All enzyme samples were dialyzed before use to remove any traces of lower molecular weight impurities. Upon subjecting samples of the native enzyme preparations to non-denaturing polyacrylamide gel electrophoresis (PAGE) (see section 2.2.3), the presence of only one distinct band is detected , indicating the absence of other protein impurities.

D-(+)-glucose was purchased from the BDH Chemical Company and D-(+)-trehalose was purchase from the Fluka Chemical Company. D-(+)-galactose and D-(+) mannose were purchased from the Sigma-Aldrich Chemical Company. All other chemicals, reagents and solvents were high purity preparations obtained from the indicated commercial sources.

2.2.2 In Vacuo *Glycation of Enzymes*

Samples of native trypsin and chymotrypsin were reconstituted in dH_2O to create a stock solution at a concentration of 5.00 mg/mL. Accurately weighed samples of D-trehalose and D-glucose were dissolved in dH_2O and added to the protein solution so that the final weight ratio of protein to glucose to trehalose was 5:1:0.1. Also, the glycation of trypsin was preformed using galactose as the reducing sugar, and a sample of chymotrypsin was glycated using mannose as the reducing sugar. Using a calibrated

Radiometer Copenhagen type PHM26 pH meter fitted with a Beckman Futura™ refillable combination electrode, the pH of the protein and sugar solution was adjusted to 7.00 with 0.100 N NaOH (purchased from VWR) *via* micro-syringe. Solution aliquots of 1.00 mL were transferred to Pyrex® borosilicate test tubes and flash frozen by immersing in an ethanol/dry ice slurry. The samples were lyophilized using either a commercial VIRTIS-24 freeze dryer or a home-made freeze dryer consisting of an ethanol/dry ice trap attached to a 70 mtorr vacuum pump. The glass tubes were sealed under a vacuum of 50 mtorr, and subsequently placed in an oven at 85°C for 40 hours. After thermal incubation, the vacuum was released and the protein samples were reconstituted with dH_2O and placed in a Spectra/Por MWCO 6-8000 dialysis membrane. Dialysis was carried out against a dilute solution of acetic acid in order to remove trehalose and unreacted reducing sugar. After 24 hours of dialysis the protein samples were recovered and re-lyophilized. The samples were stored in screw-capped vials at 4°C until needed.

In the case of chymotrypsin, after the initial lyophilization and glycation, a sample was re-dissolved in dH_2O and the entire procedure was repeated up to four times. A sample of glycated product was removed and saved for analysis after each round of the procedure.

2.2.3 Analysis and Characterization of Glycated Enzymes

The glycated enzymes prepared by the *in vacuo* glycation procedure were analyzed by non-denaturing polyacrylamide gel electrophoresis (PAGE), following a modified protocol originally outlined by Waterborg (2002) and using the BioRad Mini-PROTEAN II electrophoresis system. Samples of the native and glycated proteins were

dissolved in dH_2O and, aliquots containing 5.00 μg of protein were loaded onto an acidic (pH 3.5), 1.5 mm thick, native 15% acrylamide gel. Electrophoresis was carried out in reverse polarity at a constant voltage for 225 VH at 5 mA. Upon the completion of electrophoresis, the gel was stained with coomassie brilliant blue R-250 (Pharmacia), in order to visualize the protein bands.

The mass spectrometric analysis of the glycated trypsin sample was carried out at the Centre for Biologics Research division of Health Canada. A Micromass™ Q-TOF mass spectrometer (Waters) was used to obtain the nanospray mass spectrum of a sample of glycated bovine trypsin. Deconvolution of the spectrum to produce data which reflected singly charged average masses was carried out by using MaxEnt1™ software (MassLynx™, Waters). Before mass spectrometric analysis, the sample of glycated trypsin was purified using the standard ZipTip® procedure (Millipore). This procedure consists of passing the analyte solution over a micro-volume bed of C_{18} reversed phase chromatography media whereby elution of the analytes was carried out by the use of 75% methanol, 25% water and 0.2% formic acid. After centrifugation of the sample, 2.00 μL of the purified enzyme sample was loaded onto a gold coated nanospray needle (New Objectives Picotip). The voltage parameters used for analysis of the sample were: capillary +950 V, cone +47 V and RF lens 1.05.

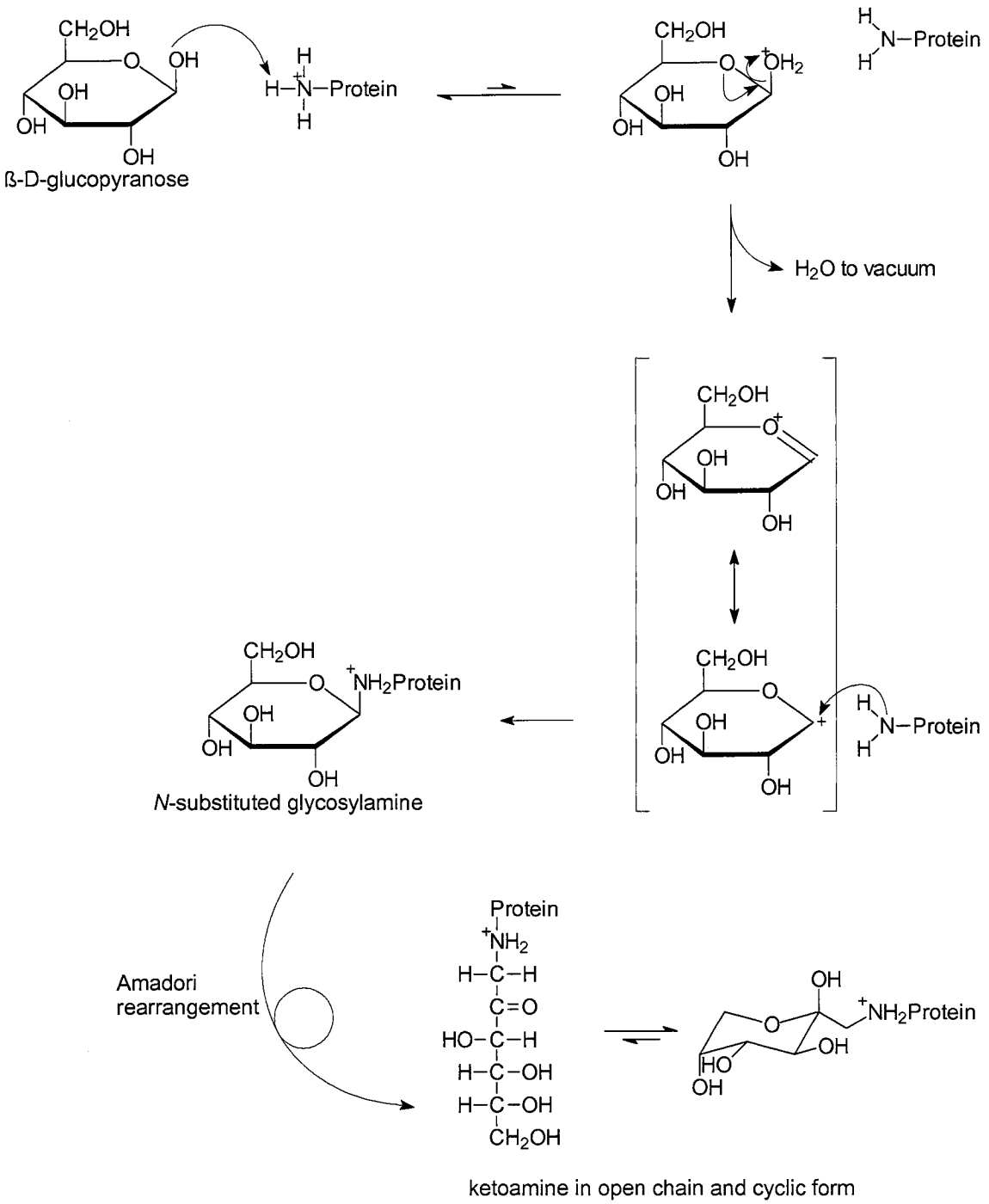
2.3 Results and Discussion

Previous work on the *in vacuo* glycation of RNase A and human haemoglobin by Stewart (PhD Thesis, 2004) has elucidated much information concerning the conditions, reaction mechanism and reaction products of this glycation procedure. It has been established that the optimal weight ratio of protein to reducing sugar to excipient is 5:1:0.1 (Stewart, PhD Thesis, 2004), and subsequently this ratio was adhered to for all glycations. This ratio of reactants has been shown to provide substantial protein glycation without collapse of the lyophilized product.

The most favourable protein to reducing sugar weight ratio has been established to be 5:1 (Stewart, PhD Thesis, 2004), where higher concentrations of sugar result in the collapse of the lyophilized product upon heating. Aldohexoses were chosen as the species of reducing sugars, since it is established that these sugars react more readily with protein amino groups than aldoketoses (Yeboah *et al.*, 1999).

The process of protein glycation in solution, involves condensation between the electrophilic aldehyde function of a reducing sugar and nucleophilic amino groups of proteins, primarily the unprotonated form of ε-amino groups of the lysine side-chain. Proteins which are lyophilized are known to exhibit a "pH memory" effect, where ionizable groups in proteins acquire the ionization state determined by the pH of the aqueous solution from which they were prepared (Zaks and Klibanov, 1988). Before lyophilization, sugar and enzyme solutions were titrated to a pH of 7.00, and based on the pK_a values of the α-amino and ε-amino groups found in proteins, it can be expected that at this pH, these groups will exist in their protonated forms. From this knowledge, it is

evident that the *in vacuo* glycation involves the reaction of protonated amino groups. Therefore, the reaction mechanism for the *in vacuo* glycation most likely differs from the mechanism found to occur in the solution glycation, where the unprotonated amino group is the reactive species. It has been suggested that the reaction mechanism for the *in vacuo* glycation, proceeds with a proton transfer from the protonated ε-amino of lysine to the cyclic form of the reducing sugar (King, PhD Thesis, 2003; Stewart, PhD Thesis, 2004). Water is lost to the vacuum, promoting the formation of the *N*-glycosylamine which undergoes rearrangement *via* the Amadori process to form a protonated ketoamine. The presence of the vacuum is essential to drive the reaction towards the formation of the glycosylamine. This is because the initial proton transfer from the protonated amino group to the sugar is a highly unfavourable process and the loss of water to the vacuum will drive the reaction towards the formation of product according to Le Châtelier's principle. The proposed reaction mechanism is illustrated in reaction scheme 2.3.1.



Reaction Scheme 2.3.1: Proposed reaction mechanism of *in vacuo* glycation of proteins with β-D-glucopyranose.

The ^{13}C-NMR of RNase A glycated *in vacuo* with $^{13}C_1$-D-glucose gives a single peak at 53.2 ppm, which corresponds to the cyclic form of the protonated ketoamine (Stewart, PhD thesis, 2004). This provides a clear indication that the only product formed from the *in vacuo* glycation is the ketoamine. This contrasts aqueous glycation *via* the Maillard reaction where the final product usually contains many different forms of AGEs.

In order to determine in a timely manner if glycation has occurred, the protein samples were subjected to non-denaturing PAGE. In the electrophoresis of native proteins, separation takes place according to the size and charge differences of the molecules. The native gel system used to identify the glycated proteins is run under acidic conditions, at a pH of 3.5, and under these conditions most proteins will have a net positive due to the presence of the basic amino acids lysine, arginine and histidine. Therefore in contrast to the usual gel electrophoresis protocols using sodium dodecyl sulfate (SDS), where the SDS-protein complexes have a net negative charge, the conditions are in reverse polarity to SDS protocols with the positively charged proteins migrating towards the negatively charged electrode.

The *in vacuo* glycation of proteins results in the attachment of sugar molecules to groups on the surface of proteins, giving modified proteins a larger size as compared to native proteins. As a protonated ketoamine is formed, the addition of the sugar moiety to the ε-amino group does not alter the charge state of this group, and hence the overall net charge of the protein remains unaltered after *in vacuo* glycation. Therefore, the native and glycated proteins will separate according to size when subjected to native acrylamide

gel electrophoresis, whereby the larger modified proteins will show a reduced electrophoretic mobility than that of their unmodified counterparts.

Upon the completion of incubation of the lyophilized trypsin and sugar mixture at 85˚C for 40 hours, a sample of the glycated trypsin was subjected to non-denaturing PAGE (figure 2.3.1). Its electrophoretic mobility was compared to that of a native sample of trypsin. A sample of control trypsin was also included in the gel. This control sample contained the same amounts of trypsin and trehalose as the glycated sample, however, inclusion of reducing sugar was omitted. Therefore, the control sample should show the same mobility as the native enzyme when subjected the electrophoresis.

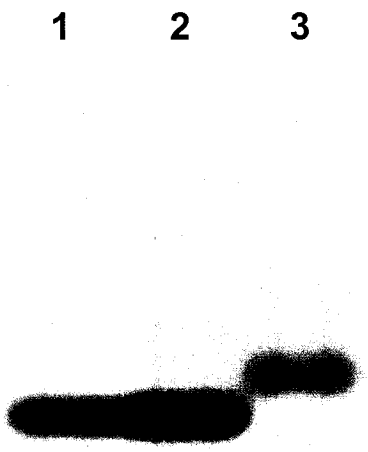


Figure 2.3.1: Non-denaturing PAGE of native and *in vacuo* glycated trypsin. A sample of trypsin was lyophilized with glucose and trehalose in a weight ratio of 5:1:0.1 and incubated under vacuum at 85°C for a period of approximately 40 hours. A 5.00 μg of each enzyme sample was subjected to non-denaturing PAGE. Lane 1: native trypsin; lane 2: control trypsin; lane 3: *in vacuo* glycated trypsin. Gel is stained with coomassie brilliant blue R-250.

From analysis of the non-denaturing gel it is clear that the glycated sample did show a significantly reduced electrophoretic mobility (band in lane 3) when compared to the native and control samples (bands in lanes 1 and 2, respectively). This indicates that the molecular size of the glycated sample exceeds that of the native and control samples. Also, as can be seen from figure 2.3.1, the band resulting from the sample of glycated trypsin (band in lane 3) is less intensely stained than native or control bands. Coomassie brilliant blue R-250 is an anionic organic dye that is electrostatically adsorbed to the positively charged groups of proteins imparting a blue colour to the protein molecules (Fazekas de St. Groth *et al.*, 1963). The reduced staining of the band displayed in lane 3 of figure 2.3.1 is consistent with the mechanism of protein glycation *via* the *in vacuo* methodology, where the ε-amino group of lysine is the site of modification. Although the modified amino group of lysine still remains positively charged, the presence of the sugar moiety would be expected to hinder the formation of intermolecular forces between this positively charged group and the anionic dye due to steric factors. The coomassie molecule will be able to interact with the positively charged groups of arginine and histidine; however the modification of the ε-amino group ultimately results in a protein that is less intensely stained as compared to the native protein. The reduced eletrophoretic mobility and the reduced stain intensity of the glycated sample, both provide evidence that the *in vacuo* glycation technique successfully enabled a reaction between glucose and the side-chains of lysine residues to afford a glycated trypsin derivative.

It is apparent that none of the original starting material remains in the glycated product as a single distinct band results on the gel (excluding the faint band located above

the glycated sample band, which will be discussed). The width of this band appears to be approximately equal to the band of the unmodified enzyme, and is therefore indicative of a relatively homogeneous product.

In order to determine whether the successive repetition of the *in vacuo* glycation procedure on the same protein sample provided a more substantial modification of the enzyme than a single round of the procedure, a sample of chymotrypsin was lyophilized with glucose and trehalose in the same ratio of 5:1:0.1, and subsequently incubated under vacuum at 85°C for 40 hours. Upon completion of the incubation, a portion of the sample was re-dissolved in water and the lyophilization and glycation procedure was repeated up to a maximum of four times. From analysis of the non-denaturing PAGE (figure 2.3.2), it appears that the sample subjected to 4 rounds of the *in vacuo* glycation (lane 5) had a lower electrophoretic mobility than that of the native chymotrypsin (lanes 1 and 6) and the sample of glycated chymotrypsin subjected to one round of the *in vacuo* procedure (lane 2). From this it can be assumed that a small number of the lysine residues after the first round of glycation remain unmodified, perhaps due to their proximity to amino acid residues with bulky side-chains, and ultimately become modified only after repetition of the procedure.

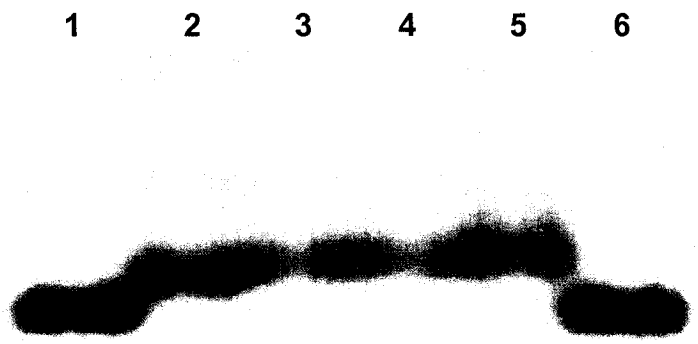


Figure 2.3.2: Non-denaturing PAGE of native and *in vacuo* glycated chymotrypsin. Samples of chymotrypsin were lyophilized with glucose and trehalose in a weight ratio of 5:1:0.1 and incubated at 85˚C for an initial period of 40 hours. After the initial glycation, a sample was re-dissolved, re-lyophilized and re-incubated at 85°C for another 40 hour period. This process was repeated up to four times, and samples which underwent the repetitive glycation appear in lanes 2 through 5. Enzyme samples of 5.00 μg were subjected to non-denaturing PAGE. Lane 1 and 6: native chymotrypsin; lane 2: glycated chymotrypsin (1 X glycation procedure); lane 3: glycated chymotrypsin (2 X glycation procedure); lane 4: glycated chymotrypsin (3 X glycation procedure); lane 5 (4 X glycation procedure). Gel is stained with coomassie brilliant blue R-250.

On both native gels (figures 2.3.1 and 2.3.2) a faint band is present above the *in vacuo* glycated samples and the control sample (figure 2.3.1). The appearance and position of this band on the native gels coupled with the knowledge that proteins will form covalent cross-links using the *in vacuo* methodology (Simons *et al.,* 2002; Simons, PhD Thesis, 2005), suggests that these faint bands present on figures 2.3.1 and 2.3.2 represent the formation *in vacuo* cross-linked of trypsin-trypsin or chymotrypsin-chymotrypsin dimers respectively. Based on the result obtained from Simons, (Simons *et al.,* 2002; Simons PhD Thesis, 2005), it can be assumed that these dimers are formed by a condensation reaction between a protonated ε-amino group of lysine and a deprotonated carboxylate group of aspartic or glutamic acid under a vacuum generating an amide linkage.

The deconvoluted mass spectrum of glycated bovine trypsin subjected to electrospray ionization mass spectrometry (ESI-MS) is shown in figure 2.3.3. Bovine trypsin contains a total of 14 lysine residues, all of which are located on the protein's outer surface, making them accessible for glycation by the *in vacuo* technology. Upon examination of the spectrum illustrated in figure 2.3.3, and with the knowledge that the native unmodified form of bovine trypsin has a mass of 23 294 Da (data not shown), it was determined that there was substantial modification of the lysine residues. Modification of a lysine residue to form a ketoamine will impart a total of 162.05 mass units to the enzyme, and the lower abundance peak located at m/z of 24 752, is indicative of a modification of a total of 9 of trypsin's lysine residues, as the difference between this mass and the native mass of the enzyme is 1458 units. Peaks representing the modification of 10, 11, 12, 13 and 14 lysines are also apparent, however, as illustrated by

the peaks with the highest abundance located at m/z of 25 078, 25 240 and 25 402, the average enzyme sample contained 11 to 13 modified lysine residues.

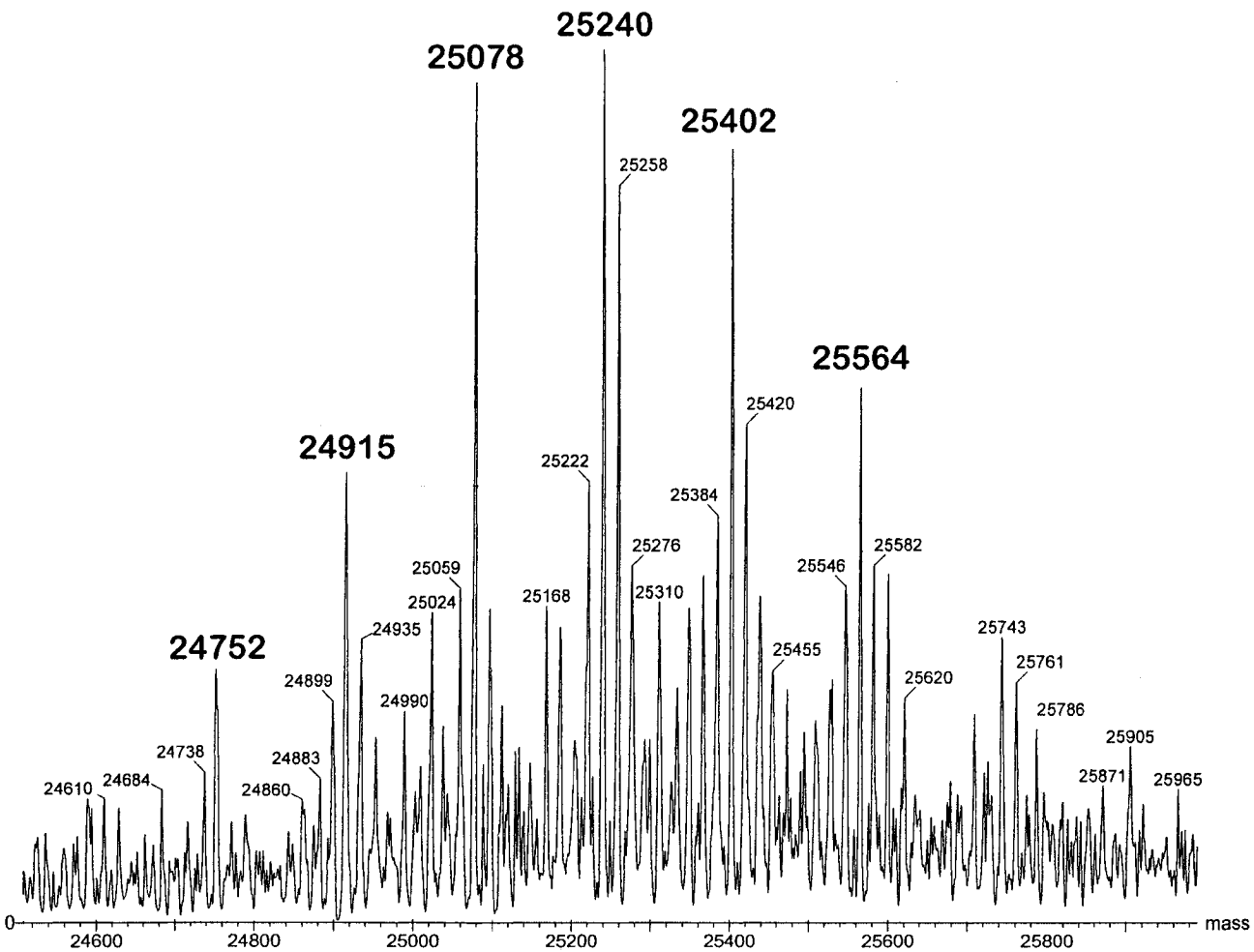


Figure 3: Electrospray ionization mass spectrum of *in vacuo* glycated bovine trypsin. Peaks generated by protein samples containing modified lysine residues are represented by m/z values of 24 752, 24 915, 25 078, 25 240, 25 402 and 25 564.

2.4 Conclusions

Although the *in vacuo* protein modification technique has previously been used to produce glycated proteins (Stewart PhD Thesis, 2004), the results presented in this thesis are the first with respect to the *in vacuo* glycation of proteolytic enzymes. From the data presented and discussed in this chapter, it is evident that the *in vacuo* chemical modification technique was applied successfully to generate glycated derivatives trypsin and chymotrypsin. Chemical and physical evidence has shown that the application of the *in vacuo* glycation procedure to trypsin and chymotrypsin has resulted in the modification of the lysine side-chains located on the surface of theses enzymes to produce a ketoamine (Stewart, PhD Thesis, 2004). The ketoamine is formed after reaction of the protonated ε-amino groups with a reducing sugar under vacuum.

The *in vacuo* protein glycation technique is a novel approach to generate glycated enzymes, and it provides several advantages to conventional protein modification techniques preformed in solution. These advantages include; (1) the derivitization of proteins with no prior chemical activation of the modifying agent or protein, (2) the removal of water and oxygen from the reaction environment (leading to the formation of fewer unwanted side products), (3) protein derivitization can be carried out with small amounts of protein modifying agent as competing nucleophiles such as water are removed, (4) the ability to use high reaction temperatures which increases the rate of derivitization, (5) the ability to re-cover and re-use any unreacted modifying agent, (6) enzymes are more stable in the dry-state reaction conditions of the *in vacuo* technology

than in the conditions of solution modification reactions, and (7) a highly derivitized protein product is produced after one round of the *in vacuo* glycation reaction.

The *in vacuo* glycation technique appears to be a simple and general procedure for the production of glycated proteins and enzymes. This technology has the potential to be a cost-effective method for the production of enzymes which exhibit a high level of stability towards denaturing factors for use in industrial processes. These aspects of protein glycation *via* the *in vacuo* glycation technique will subsequently be discussed in the following chapters.

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Chapter 3: Properties of *In Vacuo* Glycated Trypsin and Chymotrypsin

Chapter 3: Properties of *In Vacuo* Glycated Trypsin and Chymotrypsin

3.1 Introduction

3.1.1 The Serine Proteases Trypsin and Chymotrypsin

Trypsin and chymotrypsin are protein hydrolyzing enzymes synthesized in the pancreas as inactive precursors, and are activated in the small intestine by a single specific proteolytic cleavage. The primary biological function of these enzymes is protein degradation in digestion. Not only will they hydrolyze peptide bonds found in proteins, but also will act on *N*-acetylated amino acids amides and esters which fulfill the substrate specificities for the particular enzyme (Schwert *et al.,* 1948). Trypsin and chymotrypsin are categorized as serine proteases due to the presence of an essential serine residue in the active center of these enzymes.

Trypsin and chymotrypsin share a remarkable similarity in primary and tertiary structures; however, they display quite different specificities for substrates (Kurth *et al.,* 1997). Trypsin specifically catalyzes the hydrolysis of peptide bonds on the C-terminal side of lysine and arginine residues. On the other hand, chymotrypsin has a preference for peptide bonds on the C-terminal side of amino acids with aromatic side-chains such as tryptophan, phenylalanine and tyrosine. Chymotrypsin's substrate specificity is not as narrow as its close relative trypsin, as the hydrolysis at leucine, methionine, asparagine and glutamine also occurs (Gray, 1971). Differences in the substrate specificities between the two enzymes arise from the nature of their inherent substrate binding pockets. Chymotrypsin contains a pocket that is lined with two glycine residues and

contains a serine residue at the bottom, which creates a favourable environment for substrates with large hydrophobic side-chains. The binding pocket of trypsin is also lined with two glycine residues; however, an aspartic acid with a negatively charged side-chain is positioned in the pocket for optimal electrostatic interaction with the positively charged side-chains of lysine and arginine residues (Price and Stevens, 1982).

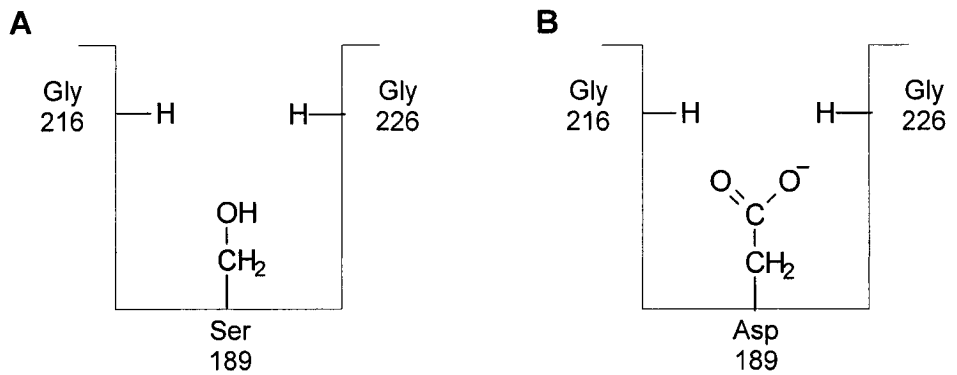


Figure 3.1.1: The nature of the substrate binding pockets in the active site of (A) chymotrypsin and (B) trypsin (Price and Stevens, 1982).

The catalytic mechanism by which chymotrypsin catalyzes the hydrolysis of its substrates has been extensively studied and is one of the best understood enzymatic mechanisms (Fersht, 1999). It was discovered that reaction of chymotrypsin and trypsin with diisopropylflurophospate (DFP) leads to a cessation in enzyme activity, and the production of a phosphate ester (Jansen *et al.*, 1949). DFP reacted with a specific serine hydroxymethyl side-chain (Ser 195), and the loss of activity was indicative of the residue's presence in the active site. This was an unusual finding due to the fact that the

hydroxymethyl group of serine is considered to be an unreactive group due to its high pK_a ($\approx$ 16) (Moran *et al.*, 1994). This residue is able to exhibit a high reactivity due to the unique interaction it shares with the side-chains of two other catalytically important residues in the active site. The active site of all serine proteases have been identified to contain what is know as a "catalytic triad" consisting of a specific arrangement of the side-chains of aspartic acid (Asp), histidine (His) and serine (Ser). The arrangement of the Asp 102...His 57...Ser 195 triad allows for what is known as a "charge relay mechanism" to occur. The proposed charge relay mechanism involves interaction of His 57 with Asp 102 by the formation of a hydrogen bond, which then allows His 57 to interact as a base with Ser 195 greatly increasing its nucleophilicity. The catalytic reaction proceeds through the formation of an acyl-enzyme intermediate which subsequently undergoes reaction with a second substrate, usually water, to complete the reaction (Price and Stevens, 1982). This catalytic mechanism is shown in detail in figure 3.1.2.

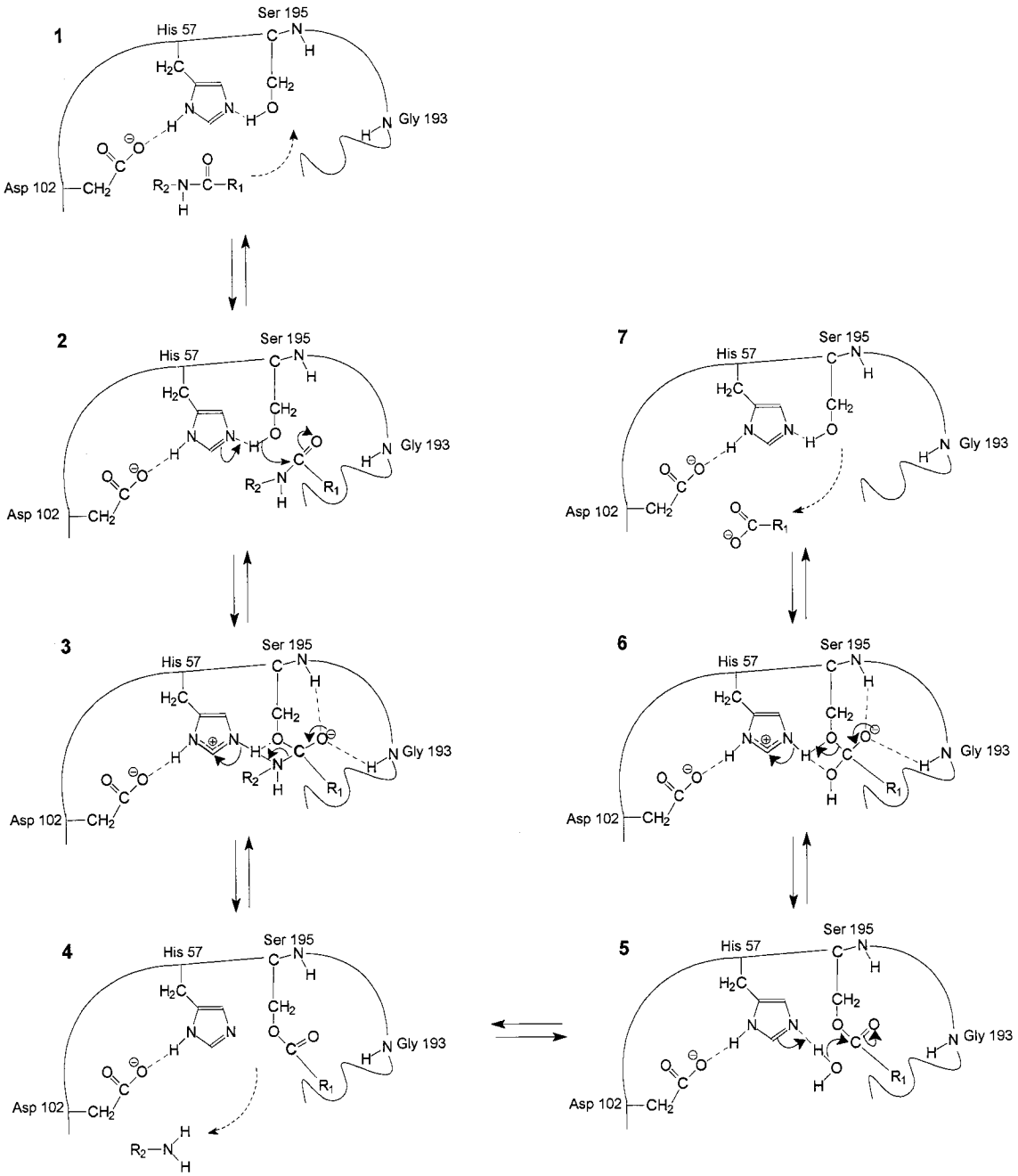


Figure 3.1.2: Catalytic mechanism of substrate hydrolysis by the serine proteases (Creighton, 1993).

3.1.2 Enzyme Stability

A protein's native conformation is stabilized by a variety of weak non-covalent forces such as hydrogen bonding, ionic, and Van der Waals interactions, and covalent interactions such as disulfide bonds, which ultimately results in a protein conformation which is often only marginally stable under stressing conditions (Creighton, 1993). As a consequence, the exposure of a protein in its native conformation to certain chemical agents or changes in environmental conditions can lead to what is known as denaturation. Denaturation has been defined as: "A conformational alteration of a biological macromolecule which entails a reversible or irreversible loss of its ability to perform a certain biological function." (Mozhaev and Martinek, 1982)

If the environmental pH is substantially raised or lowered above or below the pH at which an enzyme is most stable at, this can change the ionic state of the ionizable side-chains creating regions of charge repulsion, disrupting ion pairs or breaking hydrogen bonds, all of which can lead to the denaturation of the enzyme. Heating an enzyme can create an increase in rotational and vibrational energy which can upset the interactions stabilizing the functional folded conformation. Exposure to chemical agents such as urea, guanidinium salts or sodium dodecyl sulfate can also lead to protein denaturation as these agents affect stabilizing hydrophobic interactions (Creighton, 1993).

A loss of biological activity may be the result of exposure to harsh environmental conditions or to chemical agents; however another mechanism can cause the destruction of an enzyme's native conformation resulting in denaturation. As mentioned, trypsin begins as an inactive zymogen secreted from the pancreas. The active trypsin molecule is

created by enterokinase cleavage of a bond existing between a lysine and a valine residue near the N-terminal of the zymogen, subsequently releasing a hexapeptide and creating active trypsin (Keil, 1971). This initial cleavage is at a trypsin sensitive site and once activated trypsin is able to autocatalytically activate its own zymogen. This autocatalytic activity is not limited to the modification of the zymogen; hence once activated a molecule of trypsin has the ability to perform cleavages on other molecules of trypsin. This autodegradative process has been termed "autolysis", and it can ultimately lead to the complete destruction of enzyme's native structure and a subsequent loss in its biological activity. The nature of trypsin's substrate is a key factor in the process of autolysis. Lysine and arginine residues are primarily found on the exterior surface of proteins, since their charged side-chains are not compatible with the hydrophobic protein interior. Bovine trypsin contains 14 lysine and 2 arginine residues, all of which are located on the surface of the protein therefore, these sites are readily accessible to be targeted for hydrolysis by other molecules of trypsin. Native chymotrypsin does not exhibit autolysis in the same fashion as trypsin, since this enzyme targets the hydrolysis of bonds adjacent to residues with large hydrophobic side-chains. These residues are usually located in the hydrophobic protein core, and hence native chymotrypsin is not as susceptible to autodigestion.

Studies by Rice *et al.* have indicated that trypsin autolysis is a rapid process, where catalytic activity was reduced by approximately 50% in two hours, and by 95% in 20 hours at a temperature of 23°C and a pH of 7.8 (Rice *et al.*, 1977). Studies on rat trypsin by Várallyay and co-workers has provided much information regarding the process of trypsin autolysis and the subsequent loss of enzyme activity which results

from this process. A peptide segment stretching from Lys 61 to Arg 117 is part of the longest peptide chain not stabilized by disulfide bonds and contains a total of 5 possible sites for trypsin hydrolysis (*et al.*, 1998). Loss of activity due to autolysis could be a result of extensive degradation of this peptide segment and the consequent disruption of protein structure. Also, two important members of the catalytic triad may be affected by degradation of this peptide, as His 57 and Asp 102 lie in this portion of primary structure (Várallyay *et al.*, 1998). It has long been known that the rate of trypsin autolysis is dependant on the concentration of Ca^{2+} ions, where binding of this ion provides a stabilizing effect against autolysis (Gorini and Felix, 1953; Green and Neurath, 1953; Rice *et al.*, 1977). The exact reason for the increased resistance to autolysis exhibited by trypsin with bound Ca^{2+} remains to be fully elucidated, however, it is known that the Ca^{2+} binding loop is located in the Lys 61 to Arg 117 stretch (Várallyay *et al.*, 1998).

3.1.3 Enzyme Modification to Enhance Stability

As mentioned in the main introduction, the use of enzymes in industrial processes, where harsh environmental conditions may be used, requires the development of highly stable enzymes, and in particular, enzymes which display a high tolerance to elevated temperatures. Protein engineering techniques have been used in an attempt to create enzymes with a higher stability against denaturation by using techniques as site directed mutagenesis. Eijsink *et al.* (1992) used site directed mutagenesis to replace a lysine residue with serine or aspartic acid in the N-terminal domain of Bacillus subtilis neutral protease to improve electrostatic interactions and subsequently providing enhanced enzyme stabilization and thermostability. However, techniques such as site directed

mutagenesis tend to be labour intensive and costly, therefore chemical modification techniques appear to be more attractive for the production of enzymes which show high levels of stability.

Chemical modification was used to achieve stabilization of trypsin and chymotrypsin by artificial hydrophilization of the protein surface (Mozhaev *et al.*, 1988). It is known that many proteins contain clusters of hydrophobic amino acids on their surface, which play important roles *in vivo*; however these clusters contribute to a protein's instability *in vitro* due to unfavourable contacts with water (Mozhaev *et al.*, 1988). Therefore, it is thought that shielding of these hydrophobic clusters by the addition of a modifying agent should increase the stability of the modified enzyme. Mozhaev *et al.*, produced trypsin with a hydrophilized surface by a two step modification procedure of the surface tyrosine residues by nitration by tetranitromethane followed by reduction with sodium dithionite (Mozhaev *et al.*, 1988). The modified trypsin showed an increased resistance to thermodenaturation, where the modified enzyme retained 35% of its original activity when incubated at 56.5°C at pH 8.0 for a period of 15 minutes. The unmodified enzyme showed a complete loss of activity under these same conditions. A different approach was taken to hydrophilize the surface of chymotrypsin, where it was treated with anhydrides or chloroanhydrides of aromatic carboxylic acids allowing a number of additional carboxylic groups to be introduced onto the side-chains of lysine residues (Mozhaev *et al.*, 1988). The introduction of the bulky aromatic carboxylic groups provided shielding to some of the surface hydrophobic residues, which ultimately resulted in an enzyme with a substantially increased thermostability.

Increased stability of enzymes has also been achieved by the covalent attachment of glutaraldehyde polymers to lysine ε-amino groups (Venkatesh and Sundaram, 1998). Stabilization of enzymes is afforded by the creation of a "cage" surrounding the enzyme resulting from several intermolecular cross-links between groups of the modifier. The establishment of these intermolecular cross-links provides rigidity to the structure which restrains the unfolding of the protein molecule (Venkatesh and Sundaram, 1998). However, enzymes modified with glutaraldehyde polymers exhibited a reduced activity to protein substrates, which could be due to hindered access to the active site resulting from the establishment of the intermolecular cross-links.

Another chemical modification technique used to increase the stability of enzymes is the covalent attachment of large molecular weight polyhydroxy compounds such as polyethylene glycol (PEG) (Fágáin and O'Kennedy, 1991). Ziding Zhang and Guan modified trypsin with *p*-nitrophenyl chloroformate activated methoxypolyethylene glycol (MPEG) derivatives, which favours reaction with lysine ε-amino groups (Ziding Zhang and Guan, 1999). The results from this modification showed that the modified trypsin had a reduced rate of autolysis and thermodenaturation. Similar results were achieved by Murphy and Fágáin as they modified trypsin with acetic acid *N*-hydroxysuccinimide ester (AANHS), which also targets reaction with the ε-amino groups (Murphy and Fágáin, 1996). The reduced rate of autolysis of both the AANHS and MPEG modified trypsins is thought to be attributed to the inability of the enzyme to recognize the lysine side-chain as a substrate target due to the presence of the modifying group. The increase in thermostability can be attributed to the formation of a highly hydrogen bonded structure around the enzyme resulting from the addition of the

modifying group (Murphy and Fágáin, 1996; Ziding Zhang and Guan, 1999). However, despite the stability afforded to the AANHS and some of the MPEG trypsin derivatives, an undesirable property was also imparted to the enzyme, where the modified enzyme showed a reduced catalytic activity as compared to the native.

Villalonga *et al.* provided stabilization to trypsin by the covalent attachment of β-cyclodextrin derivatives to glutamine residues through catalysis by transglutaminase (Villalonga *et al.*, 2003). Substantial thermostability was obtained and autolysis was found to be reduced in the modified enzyme. It was assumed that the decrease in autolysis was a result of steric resistance to protease action by the bulky substituents. This steric resistance could prove to be problematic in the access of macromolecular proteinaceous substrates to the active site, and hence would result in an enzyme with increased thermostability but reduced catalytic activity.

Due to its inherent protein degradation characteristics, trypsin has proven to be an attractive protease to be employed for protein digestions for a variety of different purposes. For protein identification by tandem mass spectrometry (MS), digestion of the target protein is frequently carried out by trypsin, due to the fact that tryptic digestion is highly specific for cleavages after arginine and lysine residues. This allows for the prediction of peptides produced by digestion, simplifying the identification procedure by searching protein sequence databases which contain peptide sequences generated by trypsin digestion. This protein identification approach requires the use of autolysis-resistant trypsins, primarily because the presence of tryptic autolysis fragments in the experimental sample complicates the resulting spectra making the interpretation of results difficult. For this reason, modified trypsins which have a reduced rate of autolysis have

been produced and are available for proteomic applications using mass spectrometry. These trypsins are modified by reductive methylation, where lysine residues are converted to ε-*N, N*-dimethyllysine residues by treatment with sodium borohydride and low concentrations of formaldehyde (Means and Feeney, 1968; Rice *et al.*, 1977). Reductive methylation does not alter the total charge of the protein and there is only a modest increase in size, allowing modified proteins to retain their native physical, chemical and biological properties (Rice *et al.,* 1977). Modification of trypsin by reductive methylation produces an enzyme which is more stable to autolysis. After incubation at pH 7.8 and a temperature of 23°C, the catalytic activity of the native enzyme was reduced to half after a 2 hour incubation, whereas the reductively methylated derivative lost only 2% of its original activity. After a 20 hour incubation, under the same conditions, the native enzyme retained only 5% of its original activity, whereas the modified retained about 70% (Rice *et al.*, 1977). Although the procedure used to reductively methylate trypsin is fairly straightforward and does not require the use of any special equipment, certain shortcomings of this modification technique are apparent. The reaction proceeds most efficiently at a pH of 10, however many enzymes are unstable in these basic conditions. The reaction will proceed at slightly lower pHs, as Rice *et al.* derivitized trypsin at a pH of 9, however, the reaction proceeds extremely slowly or not at all at pH values of 7 or below, which poses a problem in the derivitization of proteins that are only stable under acidic conditions. Other shortcomings include the requirement of extensive purification of proteins which have been modified by reductive methylation, exposure to reducing conditions may affect the stability of disulfide bonds, and exposure

of proteins to carbonyl compounds such as formaldehyde can result in unwanted outcomes.

The *in vacuo* protein modification technique is a simple procedure applicable to a wide range of proteins and, it may provide an alternative to more complicated protein modification techniques carried in aqueous solution to create highly stable enzymes. This chapter deals with the investigation of stabilizing properties brought about by the modification of trypsin and chymotrypsin by the *in vacuo* methodology to produce glycated derivatives.

3.2 Materials and Methods

3.2.1 Proteins and Reagents

Bovine pancreatic trypsin (Lot #: 121K7692), porcine pancreatic trypsin (Lot #: 034K7423) and bovine pancreatic α-chymotrypsin (Lot #: 27H7007) were purchased from the Sigma-Aldrich Chemical Company. Sequence grade dimethylated trypsin was purchased from Promega. The ester substrates *N*-benzoyl-L-arginine ethyl ester (BAEE) and *N*-acetyl-L-tyrosine ethyl ester (ATEE) were purchased from the Sigma-Aldrich Chemical Company. The substrate *N*-benzoyl-L-arginine p-nitroanilide (BAPNA) was also purchased from the Sigma-Aldrich Chemical Company. All other chemicals, reagents and solvents were high purity preparations obtained from the indicated commercial sources.

3.2.2 Activity of Native Bovine Trypsin and In Vacuo *Glycated Bovine Trypsin at Elevated Temperatures*

The activity of the different trypsin samples (native trypsin, trypsin glycated with glucose and trypsin glycated with galactose) was determined by measuring the rate of BAEE hydrolysis according to the following procedure. A solution (0.100 mg/mL) of native trypsin or glycated trypsin in a 20.0 mM Tris (pH 8) buffer containing 5.00 mM $CaCl_2$ in a water bath set to an elevated experimental temperature (45°C, 50°C, 55°C or 60°C) was prepared. Aliquots of 100.0 μL were removed at timed intervals and diluted to a concentration of 0.050 mg/mL by the addition of 0.05% acetic acid and placed on ice. A 5.00 mL aliquot of substrate solution consisting of 1.00 mM BAEE, 0.100 M KCl and 1.00 mM $CaCl_2$ was transferred to the reaction vessel and subsequently titrated to a pH of 7.50 using 0.020 N NaOH (purchased from VWR) added *via* a micro-syringe. A constant stream of N_2 was blown over the reaction vessel. A 100.0 μL aliquot containing 5.00 μg of the native or glycated trypsin was added to the reaction vessel, and the reaction was allowed to proceed. The reaction was monitored by a Radiometer Copenhagen type PHM26 pH meter fitted with a Beckman Futura™ refillable combination electrode coupled to a Titration 11/Ole Dich autotitrator pH-stat assembly. An endpoint pH of 7.50 was chosen for this reaction, where a drop below this value resulted in the autotitrimetric addition of a volume of 0.020 N NaOH *via* a micro-syringe. As stated above, a sample of enzyme was removed from thermal incubation after each elapsed time period and was used to measure the initial rate of substrate hydrolysis, where the volume of base added per unit time was recorded by the pH-stat. This data (volume of base added per unit time) was used to calculate the initial rate of substrate hydrolysis. The percent relative activity was calculated by measuring the initial rate of

hydrolysis of an enzyme sample after incubation at an elevated temperature, as a fraction of the initial rate of hydrolysis of the original enzyme sample that was not incubated at an elevated temperature (i.e. enzyme at room temperature).

3.2.3 Activity of Native Bovine Chymotrypsin and In Vacuo *Glycated Bovine Chymotrypsin at Elevated Temperatures*

The method used to evaluate the activity of native chymotrypsin, chymotrypsin glycated with glucose and chymotrypsin glycated with mannose was essentially identical to the method stated in section 3.2.2 for the evaluation of trypsin activity except for a few small details. The enzyme samples were incubated in 20.0 mM Tris (pH 8) at experimental temperatures of 50°C and 55°C for various periods of time. The substrate used to measure the activity of native and glycated chymotrypsin was ATEE, present at a concentration of 1.00 mM in a solution of 0.100 M KCl. Also, a smaller amount of enzyme (1.25 μg) was used for the hydrolysis reaction. All other experimental parameters were the same as those given in section 3.2.2.

3.2.4 Activity of Native Porcine Trypsin, In Vacuo *Glycated Porcine Trypsin and Promega Sequence Grade Reductively Dimethylated Porcine Trypsin at an Elevated Temperature*

Samples of buffer consisting of 50.0 mM Tris (pH 8), 0.100 M KCl and 1.00 mM $CaCl_2$ were aliquoted to 1.5 mL eppendorf tubes and placed in a heated bath set to a temperature of 60°C. A stock solution of BAPNA was added to the buffer solutions in the bath so that the concentration of BAPNA was 0.500 mM. Solutions of native porcine trypsin, glycated porcine trypsin and Promega sequence grade trypsin were prepared and added to the buffer solution containing the BAPNA substrate so that the final enzyme

concentration was 0.010 mg/mL, and the reaction was allowed to proceed for various periods of time. Sample aliquots (130.0 μL) were removed at timed intervals and the hydrolysis reaction was halted by addition of 70 μL of 80% acetic acid and flash freezing by immersion in dry ice. Upon removal of the final sample, all samples were thawed and aliquoted into the wells of a Corning® costar 96-well polystyrene microtitre plate. The absorbance was read at 420 nm using a Molecular Devices Spectra Max plus spectrophotometer. The data was analyzed by preparing a plot of A_{420} vs. time and measuring the slope from an initial reaction period of 0.00 minutes to 5.00 minutes. The slope was also measured at various time intervals after the initial 5.00 minute reaction period. The initial slope measured from the 0.00 to 5.00 minute period was assumed as the value for 100% enzymatic activity, and the slopes taken for each time interval after the initial 5.00 minute period were measured as a fraction of this initial value.

3.2.5 The Extent of Autolysis of Native Porcine Trypsin and In Vacuo *Glycated Porcine Trypsin*

A sample of either native or glycated enzyme was accurately weighed and reconstituted in 0.100 M KCl to a concentration of 6.00 mg/mL. A 500.0 μL aliquot of the 6.00 mg/mL enzyme solution (3.00 mg of enzyme) was added to 3.00 mL of a 0.100 M KCl solution in a specially designed reaction vessel assembly equipped with an adjustable recirculating water bath set to a temperature of 37°C, which was maintained for the duration of the experiment. A constant stream of N_2 was blown over the reaction vessel. A Radiometer Copenhagen type PHM26 pH meter fitted to a Beckman Futura™ refillable combination electrode coupled to a Titration 11/Ole Dich autotitrator pH-stat assembly was used to assess the extent of autolysis by calibrating the pH meter to

measure a reaction endpoint of pH 8.50. The reaction solution containing the enzyme was titrated to this pH by adding 0.010 N NaOH (purchased from VWR) *via* a micro-syringe. The reaction was allowed to proceed, where the volume of NaOH being added per unit time was recorded by the pH-stat. In order to determine accurately the volume of base added, the micro-syringe was calibrated by measuring the mass of water delivered by the syringe per 180 mm deflection of the titrator assembly. Using the density value of 997.5415 kg/m^3 for water at 23°C (Weast, 1979) it was calculated that a volume of 1.770 μL ± 0.003 μL was delivered to the reaction solution per millimetre deflection on the autotitrator recorder.

3.3 Results and Discussion

The *in vacuo* glycation of trypsin and chymotrypsin resulted in the production of enzymes which showed an increase in stability as compared to the native enzymes at elevated temperatures. A reduced intrinsic catalytic activity as compared to the native enzymes was not observed (results not shown), suggesting that the *in vacuo* glycation did not change the active conformation of the enzyme in such a way to diminish its catalytic activity. Figure 3.3.1 shows the thermal stability profile of native bovine trypsin and glycated bovine trypsin after incubation at elevated temperatures for periods of time ranging from 20 minutes to 3 hours. The experiment outlined in section 3.2.2, where the data obtained from this experiment was used to create figure 3.3.1, was repeated at least three times, and the results displayed in figure 3.3.1 are representative of a set of

experimental data. A pH-stat was used to assess the rate of substrate hydrolysis, where by sensing a drop in pH below the reaction endpoint upon the hydrolysis of the ester bond, a volume of base would be added to the reaction medium to counter this effect. The volume of base added per unit time was recorded by the pH-stat, giving the rate of BAEE hydrolysis. This information was used to calculate the percent residual activity.

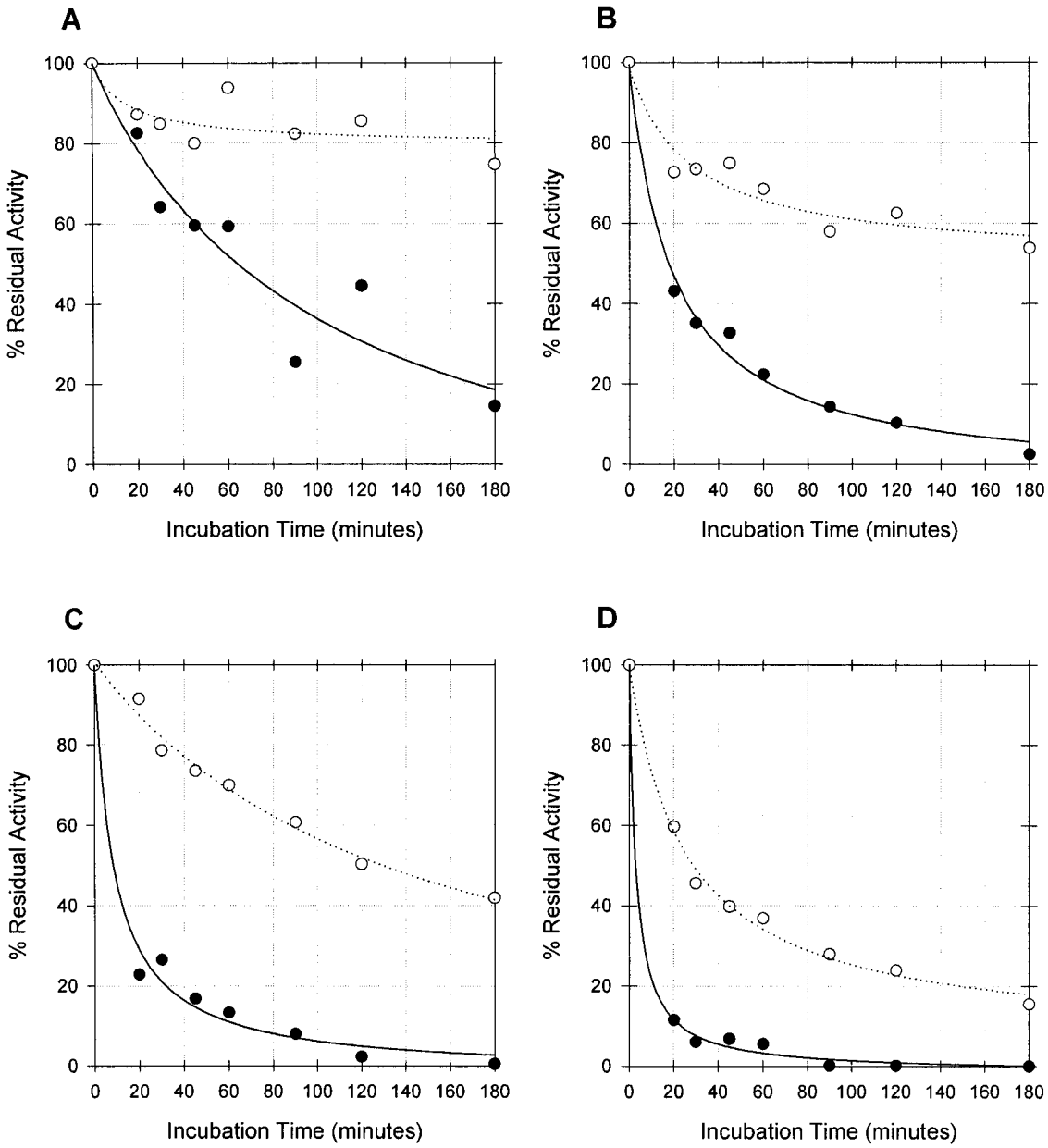


Figure 3.3.1: The effect of incubation of native and *in vacuo* glycated bovine trypsin at elevated temperatures. The rate of BAEE hydrolysis by native trypsin and glycated trypsin was measured at pH 7.50 after incubation of the enzyme sample at a concentration of 0.100 mg/mL (4.3×10^{-6} M) at pH 8 for various periods of time at different elevated temperatures; (A) 45°C, (B) 50°C, (C) 55°C and (D) 60°C. The enzyme activity of native trypsin (•) and glycated trypsin (○) is shown as % values relative to the initial activity of the samples. The % relative activity was calculated as stated in section 3.2.2.

Figure 3.3.1 illustrates that the native and glycated trypsin forms lost activity progressively with time of incubation, however it can be seen that the glycated trypsin is significantly more stable at the elevated temperatures used in this experiment. Figure 3.3.1A illustrates that incubation for 3 hours at a temperature of 45°C the native enzyme looses 86% of its original activity, whereas the glycated trypsin has lost only 25%. The results obtained from the incubation of the enzyme preparations at 50°C and 55°C (figure 3.3.1B and C) both show a sharp drop in activity of native trypsin in the first 20 minutes of incubation. After 3 hours of incubation the native preparation was left with only 2.5% of its original activity at 50°C and a residual amount of activity (0.5%) displayed after incubation at 55°C for 3 hours. On the other hand, the glycated trypsin showed a remarkable stability at 50°C as compared to the native sample, where 53% of the original activity was retained after a 3 hour incubation period. Similarly, 42% of the activity remained after incubation of the glycated sample at 55°C for 3 hours. After a 90 minute incubation at 60°C the native sample had lost all activity, whereas the stability afforded to the modified trypsin by glycation was apparent as shown by retention of 28% of its original activity (figure 3.3.1D). At the completion of the 3 hour incubation, the glycated enzyme still remained active, retaining 16% original activity.

In order to determine if modification of the lysine residues of trypsin by the *in vacuo* glycation technology provided the enzyme with resistance to autolysis, a sample of native and glycated porcine trypsin were separately incubated at a temperature of 37°C and a pH of 8.50. The extent of autolysis was measured by recording the amount of base added to the reaction per unit time resulting from a drop in pH below the endpoint value of 8.50. This drop in pH corresponds to the autolytic hydrolysis of peptide bond(s) and

the subsequent liberation of acid. Therefore the greater the extent of autolysis, the larger the volume of base added to compensate for the drop in pH. The extent of autolysis exhibited by the native and glycated samples is displayed in figure 3.3.2. The data displayed in table 3.3.1 is a numerical summary of the same results illustrated in figure 3.3.2, where at each time interval indicated in the table the relative extent of autolysis of the glycated sample was interpolated from figure 3.3.2 and compared to the extent of autolysis of the native sample.

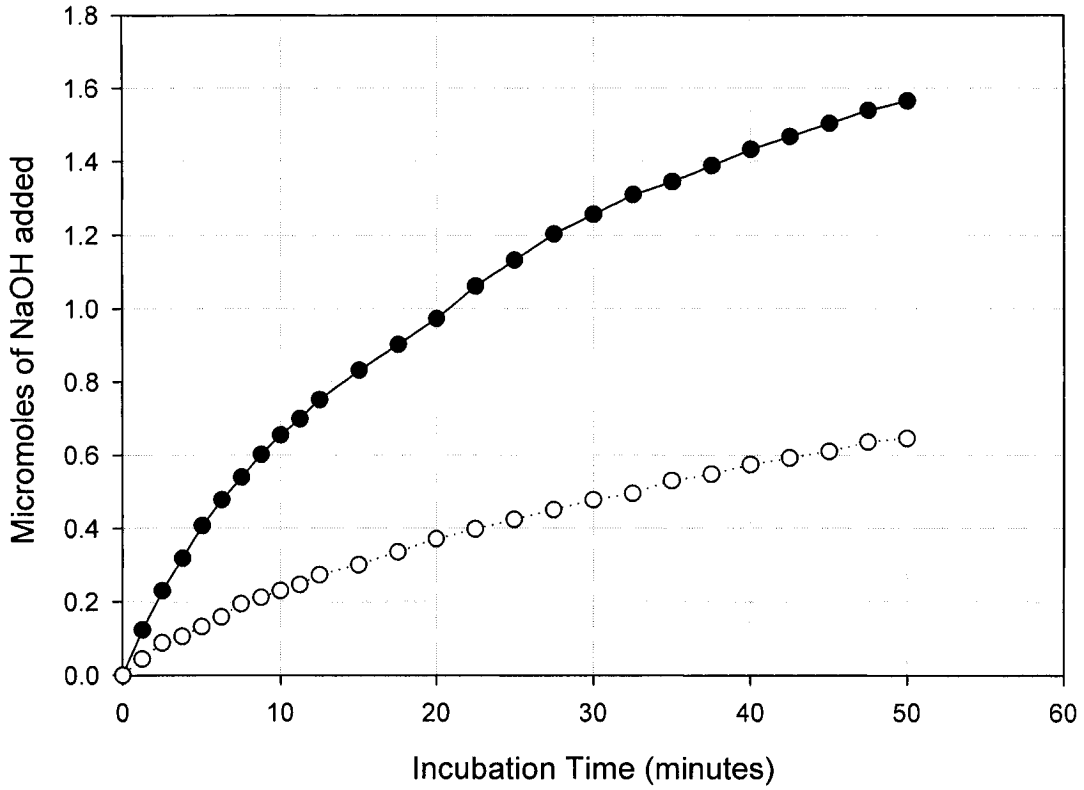


Figure 3.3.2: The extent of autolysis of native porcine trypsin (●) and *in vacuo* glycated porcine trypsin (○) at 37°C. Enzyme samples were incubated at a concentration of 1.00 mg/mL (4.3×10^{-5} M) at 37°C and the extent of autolysis was measured on a pH-stat at pH 8.50 by addition of a volume of 0.010 N NaOH to compensate for a pH drop resulting from the release of acid by cleavage of a peptide bond.

Incubation Time at pH 8.50 (minutes)	Extent of Autolysis of *In Vacuo* Glycated Trypsin Relative to the Extent of Autolysis of Native Trypsin
5	33%
10	35%
20	38%
30	38%
40	40%
50	41%

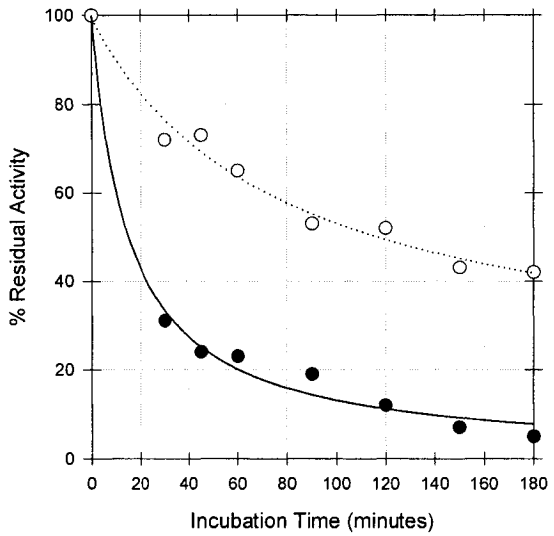
Table 3.3.1: The extent of autolysis exhibited by *in vacuo* glycated porcine trypsin measured as a fraction of the autolysis exhibited by native porcine trypsin at 37°C. Data taken from figure 3.3.2.

As illustrated in figure 3.3.2, the glycated trypsin sample showed a great reduction in the amount of autolysis occurring at pH 8.50 as compared to the native. And as displayed in table 3.3.1, after a 5 minute period of incubation, only 33% of the glycated sample had undergone autolysis as compared to the native sample, and at the end of the 50 minute incubation this value increased slightly to 41%. These results indicate that modification of the ε-amino groups of trypsin's lysine residues imparts a substantial amount of resistance to autolysis as compared to the native unmodified sample. Reduction in the extent of autolysis of the glycated sample can be explained by the fact that trypsin specifically recognizes the positively charged ammonium group at the end of a four carbon chain of lysine residues as a substrate target. The masking this group by the formation of a ketoamine resulting from *in vacuo* glycation will prevent trypsin from recognizing its substrate target and subsequently it will not be able perform hydrolysis at this particular site. Although the extent of autolysis is greatly reduced in the

glycated samples, as illustrated by figure 3.3.2 it is clear the modified trypsin sample is not completely resistant to autolysis. This can be accounted for by the fact that porcine trypsin contains four arginine residues which remain unmodified after the *in vacuo* glycation procedure and hence remain as trypsin sensitive sites for hydrolysis. Also, as illustrated by the data obtained from the mass spectrum (figure 2.3.3) of glycated bovine trypsin, the average enzyme sample did not show the derivitization of all lysine residues, indicating that some remained unmodified and sensitive to autolytic attack.

Figure 3.3.3 illustrates the thermal stability of native and glycated α-chymotrypsin after incubation at 50°C and 55°C for periods of time ranging from 30 minutes to 3 hours. The enzyme activity was measured the same way as stated for the assay of the trypsin samples stated above, however ATEE was used as the substrate. The experiment outlined in section 3.2.3, where the data obtained from this experiment was used to create figure 3.3.3, was repeated at least three times and the results displayed in figure 3.3.3 are representative of a set of experimental data.

A



B

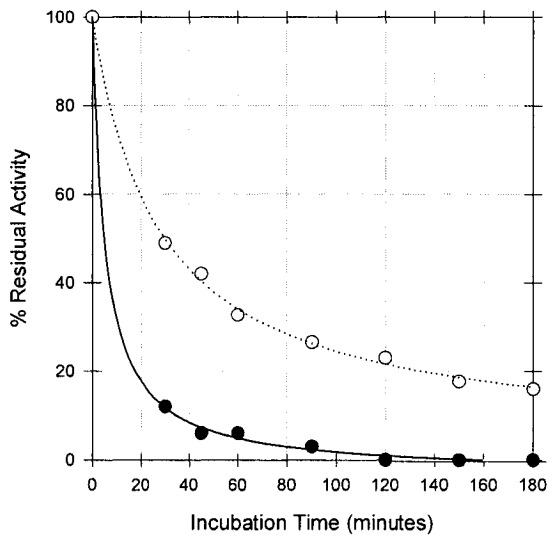


Figure 3.3.3: The effect of incubation of native and *in vacuo* glycated bovine α-chymotrypsin at elevated temperatures. The rate of ATEE hydrolysis by native and *in vacuo* glycated α-chymotrypsin was measured at pH 7.50 after incubation of the enzyme samples in pH 8 at a concentration 0.100 mg/mL (4.0×10^{-6} M) for various periods of time at a temperature of (A) 50°C and (B) 55°C. The enzyme activity of native (●) and *in vacuo* glycated (○) is shown as % values relative to the initial activity of the samples. The % relative activity was calculated as stated in section 3.2.2.

As illustrated in figure 3.3.3, activity was lost progressively with the time of incubation; however the modified form of chymotrypsin showed an impressive amount of stability as compared to the native form. Incubation at 50°C (figure 3.3.3A) showed a 69% loss in activity by the native chymotrypsin after a 30 minute incubation, and at the end of a 3 hour incubation, the native sample only retained 5% activity. The glycated sample retained an impressive 72% of its original activity after a 30 minute incubation at 50°C. The loss of activity for the duration of the 3 hour assay was gradual for the glycated sample, where 42% activity was retained and the at the 3 hour mark. This value is significant when compared to the 5% activity that remains for the native sample at the end of the 3 hour incubation. Incubation at 55°C (figure 3.3.3B) for 30 minutes results in an activity loss of 88% for the native chymotrypsin, and all activity was lost for this sample when incubated for a 2 hour period. The glycated chymotrypsin retained 50% of its original activity after a 30 minute incubation at 55°C and at the completion of a 3 hour incubation, the glycated sample still retained 16% activity.

In order to determine whether the nature of the reducing aldohexose used to glycate trypsin or chymotrypsin had an effect on the degree of thermostabilzation achieved, the activity of a sample of trypsin glycated with galactose and a sample of chymotrypsin glycated with mannose was evaluated by BAEE or ATEE hydrolysis respectively, at elevated temperatures. The results indicated that ATEE hydrolysis after incubation at 55°C for various periods of time, chymotrypsin glycated with mannose showed approximately the same activity profile as the sample glycated with glucose. Similar results were obtained when the activity measured as the rate BAEE hydrolysis at 60°C by trypsin glycated with galactose was compared to the results obtained when using

trypsin glycated with glucose as the hydrolyzing enzyme. Although not thoroughly investigated, this provides evidence that the spatial orientation of the functional groups of the aldohexoses evaluated here does not significantly affect the degree of thermostabilization observed in the derivitized enzymes.

The extra stability afforded to the glycated trypsin and chymotrypsin samples at elevated temperatures is attributed to the formation of a hydrogen bonded structure around the enzyme, resulting from the formation of a stable ketoamine derivative at the end of the lysine side-chains. The formation of these hydrogen bonds provides extra stability to the enzyme structure by making it more difficult to unfold the active enzyme conformation under heat denaturing conditions. If one was to solely examine only the results obtained from the trypsin assay, it could be inferred that the extra stability afforded to the glycated samples is an artefact of a reduced rate of autolysis, due to the masking of the lysine ε-amino group by the glycation. However, when one considers the results from the chymotrypsin assay, where the glycated samples showed a substantial resistance to heat denaturation, it can be concluded that the increased stability is not solely a result of a decreased rate of autolysis. The site of enzyme modification by *in vacuo* modification has been established to be at the ε-amino group of lysine residues. Chymotrypsin is able to undergo autolysis, however only after the native conformation has been unfolded to allow access of native molecules to perform hydrolysis of the peptide bonds on the C-terminal side of residues with aromatic side-chains. Therefore the resistance of the glycated chymotrypsin sample to heat denaturation is a result of stabilization provided by the creation of a hydrogen bonded structure around the enzyme, making it more difficult for the native conformation to unfold.

A possible application of the glycated trypsin produced by the *in vacuo* technology is for use in protein identification technologies involving peptide analysis by MS and subsequent identification by database searching. This process initially involves digestion of the target protein by trypsin into its constituent peptides, which are subsequently the target of analysis by MS. The use of native unmodified trypsin for the digestion of the target protein will inevitably result in the generation of tryptic peptides by autolysis. The presence of autotryptic fragments in the sample to be analyzed will complicate the interpretation of the resulting spectra. For this reason, there are modified forms of trypsin available, which show a reduced rate of autolysis. Promega produces a reductively methylated trypsin, where the ε-amino groups have been converted to *N, N*-dimethyl derivatives, which subsequently results in a trypsin that shows reduced autolysis (Rice *et al.,* 1977). To investigate and compare the properties of native trypsin, glycated trypsin and the Promega reductively methylated trypsin at an elevated temperature, samples of each were prepared and incubated at 60°C in buffer containing BAPNA substrate. The measurement of enzyme activity for this experiment was not carried out by monitoring BAEE hydrolysis *via* the pH-stat due to the high cost and small quantity of the Promega reductively dimethylated trypsin sample available. The measurement of enzyme activity by hydrolysis of the chromogenic substrate BAPNA requires significantly less enzyme than what is needed to measure activity by BAEE hydrolysis.

	% Residual Activity		
Incubation Time (minutes)	**Native Trypsin**	**Promega Sequence Grade Dimethylated Trypsin**	***In Vacuo* Glycated Trypsin**
Initial (0 minutes)	100	100	100
10	64	77	87
20	20	36	43
30	5	4	28
60	3	0	26

Table 3.3.2: The effect of incubation at 60°C on native porcine trypsin, Promega sequence grade dimethylated porcine trypsin, and *in vacuo* glycated porcine trypsin. Enzyme samples were incubated in pH 8 buffer at 60°C at a concentration of 0.010 mg/mL (4.3×10^{-7} M) in the presence of BAPNA substrate. Sample aliquots were removed after incubation for various periods of time, and the hydrolysis reaction was stopped by acidification and freezing on dry ice. The sample absorbance was read at 420 nm and this data was used to calculate the % residual activity as described in section 3.2.4.

As illustrated in table 3.3.2, the native and reductively methylated trypsin samples lost nearly all or all catalytic activity after a 1 hour incubation period. On the other hand, the glycated trypsin showed a substantial increase in resistance as compared to the other samples to denaturation induced by high temperature, where after a 1 hour incubation period, the enzyme sample retained 26% of its original catalytic activity. As previously explained, the glycated sample has an increased thermostabilization by modification of the lysine ε-amino groups to generate a ketoamine. A network of stabilizing hydrogen bonds will form around the enzyme by virtue of the additional functional groups

covalently attached to the enzyme resulting from the *in vacuo* glycation, and by masking of the lysine side-chain, which serves to reduce the rate of autolysis. The Promega sequence grade trypsin retained more residual activity than the native sample in the first 20 minutes of incubation, however, almost all activity was lost after a 30 minute incubation period, and no activity remained after a 1 hour incubation. Modification of lysine residues by reductive methylation to produce *N, N*-dimethyl derivatives does serve to reduce the modified sample's rate of autolysis (Rice *et al.,* 1977); however, as indicated by this experiment, trypsin modification by reductive methylation does not provide the enzyme with significant thermostabilization. The results from this assay indicate that much higher temperatures may be employed when using *in vacuo* glycated trypsin for protein digestions where analysis of the protein is carried out by MS technologies.

3.4 Conclusions

The primary objective of this study was to determine if the modification of enzymes *via* the *in vacuo* protein glycation technique resulted in the production of enzymes which exhibited an increase in thermostability. As illustrated by the results presented in this chapter, the *in vacuo* glycation of trypsin and chymotrypsin did result in the production of highly thermostable enzymes with respect to their native form. When applied to trypsin and chymotrypsin, the *in vacuo* glycation technique did not modify these enzymes in such a way that their intrinsic catalytic activity was reduced, which has

been known to occur with other protein modification techniques. *In vacuo* glycation of trypsin also resulted in a modified trypsin which showed a significant reduction in autolysis as compared to a native unmodified sample.

There are several benefits in using these *in vacuo* glycated enzymes over other chemically modified enzymes in practical applications such as protein identification technologies using MS. Reductively methylated trypsin is currently used for such applications due to the fact that it has a reduced autolysis rate as compared to native trypsin. This limits contamination of the experimental sample by trypsin autolysis fragments. The *in vacuo* glycated trypsin provides an added benefit when used for such protein identification techniques over reductively methylated preparations as this sample has proven to be stable at temperatures of 60°C, and this thermostability will permit one to carry out the digestion of native proteins. The incubation of the protein and glycated trypsin sample at 60°C will cause the protein to denature and subsequently be digested by the glycated trypsin, eliminating the need to chemically denature the protein prior to digestion. This removes a laborious step from the protein identification protocol which has potentially deleterious effects on the protein. As the glycated trypsin also shows a reduced rate of autolysis, contamination of the sample with autolysis fragments is also reduced.

With respect to the results presented in this chapter regarding the high thermostability of the glycated enzyme samples, and the reduced rate of autolysis exhibited by the glycated trypsin, it is realized that the *in vacuo* glycated trypsin and chymotrypsin samples are highly attractive products to commercialize, as there is no such

product currently available for use in proteomic applications. It follows that the *in vacuo* glycation technology is the subject of a patent application (Kaplan *et al.*, 2002).

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Chapter 4: Properties of *In Vacuo* Immobilized Glycated Trypsin

Chapter 4: Properties of *In Vacuo* Immobilized Glycated Trypsin

4.1 Introduction

4.1.1 Immobilized Enzymes

Immobilized enzymes, which are defined as "enzymes physically confined or localized in a certain defined region of space with retention of their catalytic activities, which can be used repetitively and continuously" (Chibata, 1978), were first created in the 1950s and have since been used for many industrial applications. Humans have been using enzymatic processes for various practical applications such as hide tanning, brewing and food production for thousands of years. With a greater understanding of the mechanisms of enzyme action and the expansion of industry in the 1950s, the realization of the numerous possibilities for enzymes in practical applications became apparent. However, even though enzymes are efficient and effective catalysts, it was discovered that they are not necessarily well suited to operate under the conditions used in industrial processes. Such processes require enzymatic reactions to be carried out in large batches, where enzymes operate dissolved in water in a homogeneous catalysis system. The nature of this homogeneous mixture of enzyme, substrate and product, prevents the recovery and reuse of the enzyme and contamination of the product. Hence, it is necessary to purify the product by removal of the enzyme, which is often accomplished by denaturation of the enzyme *via* pH or heat treatment (Chibata, 1978). Therefore, the use of soluble enzymes for industrial applications is highly uneconomical as large amounts of soluble enzyme would be required, quantities which may not be reasonably

supplied. Consequently, the physical confinement or localization of an enzyme which retains its catalytic properties has the potential to provide advantages over the use of soluble enzymes for industrial applications. These advantages are; (1) the easy separation of enzyme from the product and (2) the reuse of the enzyme (Tischer and Kasche, 1999).

To date, there are numerous industrial applications which currently use immobilized enzymes as catalysts in preference to solubilized enzymes, as the immobilized enzymes have proven to be more economical. Immobilized enzymes are commonly used in large scale operations in the food industry, such as the production of high fructose corn syrup, whereby immobilized glucose isomerase is used to isomerize glucose to fructose (Barker and Petch, 1985). Immobilized enzymes are also used in the manufacture of pharmaceuticals and specialty chemicals such as optically pure amino acids. For example the enantio-pure 3, 4-dihydroxy-L-phenylalanine (L-DOPA), which is used in the treatment of Parkinson's disease, is manufactured on a large scale by enzymatic catalysis *via* immobilized β-tyrosinase (Chibata *et al.,* 1985). Uses for immobilized enzymes have been realized in analytical applications, where immobilized enzymes have been integral in the automation of various clinical and chemical analysis procedures and are used in biosensors and diagnostic test strips. Immobilized enzymes have also been employed in the diagnosis and treatment of diseases in medical applications (Krajewska, 2004).

There are several different approaches which can be employed to immobilize enzymes. These approaches are generally classified either physical enzyme immobilization, immobilization by enzyme entrapment or chemical enzyme

immobilization. Physical methods involve weak non-covalent physical forces between the enzyme and solid support, entrapment involves confining enzyme(s) in a restricted area of space and chemical methods involve the formation of strong covalent interactions between a solid support and the enzyme(s), coupling the enzyme to the support (Krajewska, 2004). No single method of either physical, enzyme entrapment or chemical enzyme immobilization is best for all enzymes and their eventual application. Therefore, the optimal immobilization conditions and method for a particular enzyme and its application is often determined by a process of trial an error. This will allow one to determine which method ensures the highest level of enzymatic activity, durability and stability. Figure 4.1.1 illustrates schematic representations of commonly used approaches to immobilize enzymes.

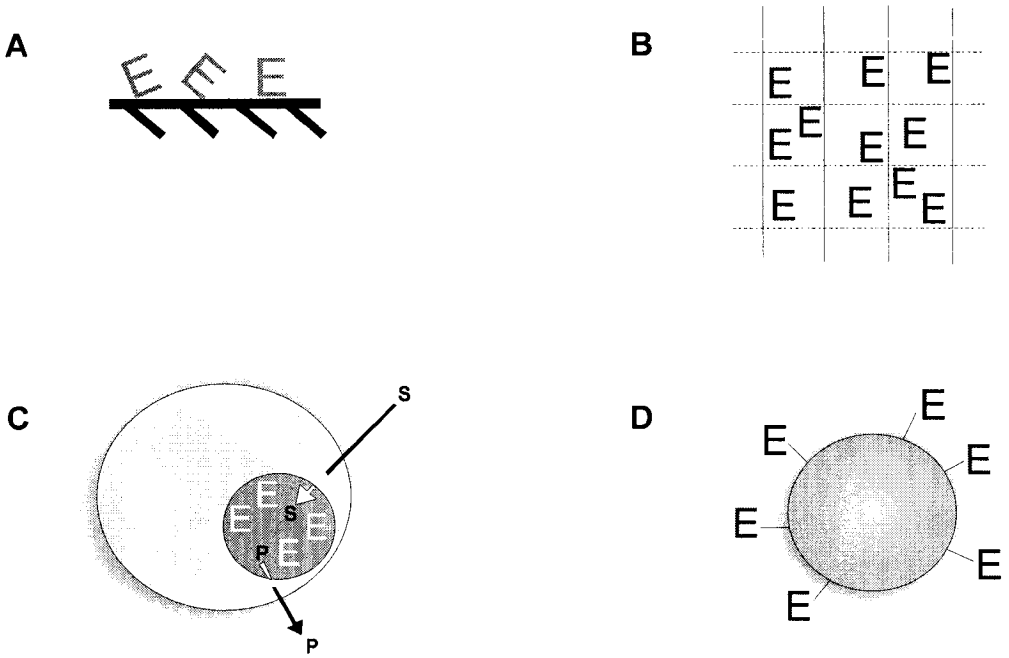

Figure 4.1.1: Schematic representations of some basic approaches to immobilize enzymes. (A) Adsorption of enzymes to the surface of a water insoluble support. (B) Entrapment of enzyme molecules within the lattice of a water insoluble cross-linked polymer, where substrate and product molecules can move freely throughout the cross-linked network. (C) Entrapment of enzyme molecules within a microcapsule with a semi-permeable membrane to allow passage of substrate and product. (D) Chemical attachment of enzyme molecules to water insoluble support through formation of a covalent bond between reactive groups on the support and the enzyme surface (Rosevear *et al.,* 1987; Zaborsky, 1973).

4.1.2 Physical Approaches to Enzyme Immobilization

Physical approaches to enzyme immobilization involve the adsorption of enzyme(s) to the surface of a solid support through formation of non-covalent forces such as hydrogen bonds, hydrophobic interactions and electrostatic forces (figure 4.1.1A) (Rosevear *et al.,* 1987; Zaborsky, 1973). The adsorption of an enzyme onto a solid support is a simple process which consists of mixing the enzyme and support under the appropriate conditions to bring about the formation of non-covalent interactions, and the subsequent separation of the soluble material from the insoluble material by centrifugation or filtration (Woodward, 1985). Solid supports commonly used for the immobilization of enzymes by adsorption are ion-exchange resins, collagen, cellulose and bentonite, and the nature of the non-covalent interactions that will arise between the enzyme and support is greatly dependant on the type of the support used (Zaborsky, 1973). Although the process of immobilization by adsorption is a very simple process, the enzyme is not firmly bound to the support. For example the adsorption of enzymes to DEAE-Sephadex occurs through multiple salt-linkages, and a subsequent change in experimental conditions such as pH, ionic strength and temperature can affect these linkages and desorb the enzyme from the support (Woodward, 1985). As a result, this method of enzyme immobilization may be not well suited to many practical applications.

4.1.3 Enzyme Entrapment Approaches to Immobilization

Immobilization may be carried out by entrapment of the enzyme molecule within a microcapsule equipped with a semipermeable membrane (Figure 4.1.1C). Although isolated from the external medium, the enzyme is able to carry out its catalytic function

due to the fact that the diameters of the membrane pores are small enough as to not allow passage of the enzyme molecule, however, are large enough to allow the entry of substrate and the subsequent exit of product (Liang *et al.*, 2000; Zaborsky. 1973). Enzymes may also be immobilized by entrapment within water insoluble polymers (Figure 4.1.1B). In this type of system, the enzyme is physically entrapped within the interstitial space of the cross-linked polymer and cannot permeate out of the lattice; however the smaller size of substrate and product molecules allows them to move freely across the cross-linked network (Zaborsky, 1973). The most commonly used polymer for this type of immobilization is polyacrylamide which is cross-linked by *N, N*-methylenebisacrylamide (Ortega *et al.*, 1998; Zaborsky, 1973). The entrapment of the enzyme is generally carried out by performing the polymerization of acrylamide and *N, N*-methylenebisacrylamide in an aqueous medium which also contains the enzyme (Chibata, 1978; Ortega *et al.*, 1998). Enzyme immobilization by entrapment is often not a suitable method when employing proteolytic enzymes, as these enzymes tend to have large substrates which may be too large to pass through membrane pores or through the interstitial space of a cross-linked polymer.

4.1.4 Chemical Approaches to Enzyme Immobilization

The chemical approach to enzyme immobilization involves the covalent attachment of water soluble enzymes to water insoluble functionalized supports through amino acid residues that are non-essential for catalytic activity (Chibata, 1978). There are a number of substances which are used as water insoluble supports in the chemical immobilization of enzymes. These supports can be organic or inorganic materials and

some commonly used examples are agarose, dextran, glass, cellulose and polyacrylamide co-polymers (Woodward, 1985; Zaborsky, 1973). In order to be effective supports, they must exhibit a large surface area to volume or mass ratio to allow for a high ratio of immobilized enzyme to support, and must exist in a form which allows easy filtration or centrifugation for retrieval of the immobilized enzyme (Zaborsky, 1973). For the most part, these supports exist in an unreactive form, and must be chemically activated before reaction with the enzyme. One of the first and most widely used methods to covalently couple an enzyme to a solid support is through the azide of carboxymethyl cellulose (CM-cellulose) (Zaborsky, 1973). The hydrazide of CM-cellulose is commercially available which must be activated by treatment with nitrous acid to produce the azide acid. The enzyme is then coupled to the support through the formation of an amide bond from the acylation of lysine's ε-amino group (figure 4.1.2A). Enzymes may be immobilized to the commercially available modified forms of the polysaccharides agarose and dextran, namely Sepharose® and Sephadex®, by cyanogen bromide activation (figure 4.1.2B). The activation involves the creation of a reactive imidocarbonate which will undergo reaction with protein amino groups hence coupling the support to the enzyme (Palomo *et al.,* 2004; Yang and Chase, 1998; Zaborsky, 1973).

Amid the array of insoluble materials used as supports for immobilization of enzymes, glass has been found to be a particularly good support. Enzyme immobilization to glass was first reported by Weetall in 1969 as trypsin and papain were covalently coupled to this support matrix (Weetall, 1969). There are several advantages of using glass as a support which includes the fact that it has a great structural stability over a wide range of environmental conditions, it is impervious to microbial attack and its shape

can be easily modified (Weetall, 1969). Weetall activated the glass surface by preparation of the aminopropyl silane derivative, which was either converted to an isothiocyanate derivative or an amino aryl derivative. The isothiocyanate glass derivative was coupled to trypsin by a sulphonamide linkage and the amino aryl derivative was coupled to papain and trypsin by reduction and establishment of an azo-linkage (Weetall, 1969). Some examples of commonly used chemical methods to immobilize enzymes are illustrated in figure 4.1.2.

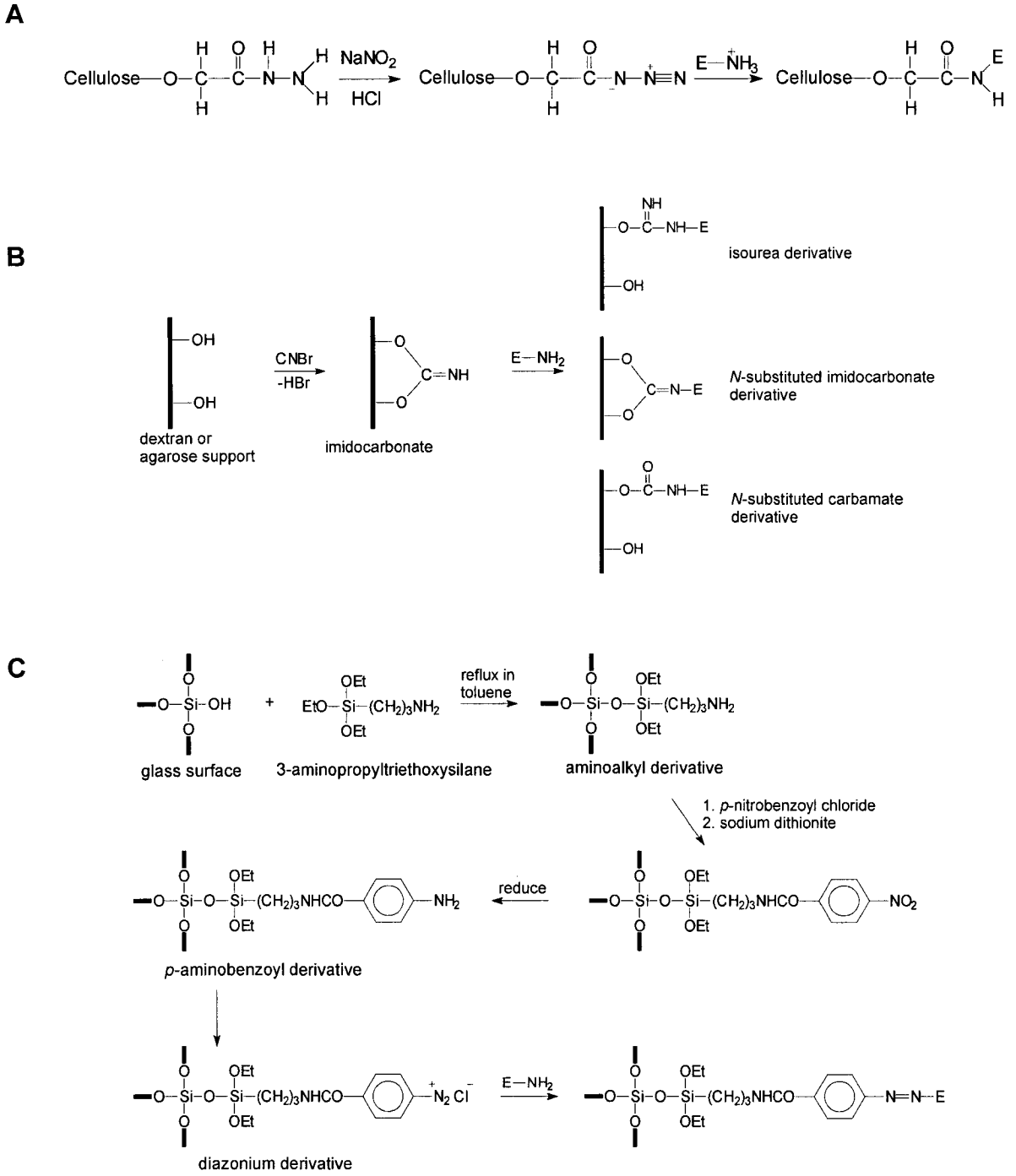

Figure 4.1.2: Some of the commonly used chemical methods to immobilize enzymes to a water insoluble solid support. (A) Enzyme immobilization to carboxymethyl cellulose by treatment with nitrous acid, (B) enzyme immobilization to dextran or agarose *via* activation by CNBr, and (C) enzyme immobilization to a porous glass surface by preparation of the aminoalkyl derivative and subsequent modification to produce the diazonium derivative (Woodward, 1985; Zaborsky, 1973).

Often the covalent coupling of an enzyme to an insoluble support affects the properties of the immobilized enzyme, where it has been observed that enzymatic activity has decreased or increased as a result of immobilization as compared to the activity of the native enzyme (Goldstein *et. al.,* 1970; Zaborsky, 1971). Goldstein *et al.,* (1970) produced an immobilized naringinase which showed a 50% decrease in activity as compared to the native free enzyme. An alteration in enzymatic activity as a result of immobilization can be due to the nature of the solid support, where the size of the support may induce steric repulsion of substrates. Also, the flexibility of the support, the degree of hydrophilicity of the support, and electrostatic interactions between the support and enzyme may have an effect on the activity of the immobilized enzyme. Changes in the properties of the immobilized enzyme may also result from alterations to the enzyme structure, namely the transformation of catalytically important residues, changes in the enzyme conformation and changes in local and net charges (Zaborsky, 1973).

4.1.5 Stability of Immobilized Enzymes

It has been observed in certain cases, that the immobilization of certain enzymes imparts a desirable property, whereby the thermostability of the enzyme has been increased (Blanco *et al.,* 1989; Martinek *et al.,* 1977; Ortega *et al.,* 1998; Weetall, 1969; Yang and Chase, 1998). This property brought about by immobilization can be viewed as an added bonus, considering that enzyme immobilization already provides two significant advantages, (1) the easy removal of enzyme from the product and (2) the reuse of the enzyme, over using native water soluble enzymes for various purposes. Weetall produced a sample of glass immobilized papain that showed no loss in activity when

incubated at 88°C for 80 minutes, whereas the soluble sample was completely thermoinactivated after a 30 minute incubation at 60°C (Weetall, 1969). Similar results were obtained with a chemically modified (acylated) form of trypsin which was immobilized by co-polymerization in an acrylamide gel matrix (Martinek *et al.,* 1977). When incubated at 60°C, the native soluble enzyme looses all activity within several minutes, whereas the immobilized derivative retained 70% activity after incubation for 3 hours (Martinek *et al.,* 1977).

4.1.6 In Vacuo *Enzyme Immobilization*

The advent of enzymes immobilized to a water insoluble support *via* the establishment of a covalent linkage between support and enzyme has proven to be advantageous for various industrial processes. However, the chemical methods used to create the enzyme-support covalent linkage are often laden with shortcomings. As illustrated in figure 4.1.2, most methods used to immobilize enzymes require activation of the support and, in some cases the activation of certain functional groups in proteins with a variety of chemical reagents. It is well established that the chemical modification of enzymes can cause an alteration in enzyme structure which can subsequently reduce its biological activity. Also, after activation of the support and coupling of the enzyme, it is likely that some of the activation chemicals remain in the preparation. Therefore, immobilized enzyme preparations may require extensive purification before use and as with aqueous protein chemical modification, a large amount of modifying, or in the case of immobilization, activating agents must be used to achieve substantial modification of the target. Therefore, the *in vacuo* protein cross-linking technique (Simons *et al.,* 2002;

Simons, PhD Thesis, 2005), which does not require the use of chemical reagents, could provide a more convenient method for immobilizing enzymes to a solid support, avoiding many of the shortcomings which are associated with aqueous modification.

Recent work by Simons (Simons *et al.,* 2002; Simons, PhD Thesis, 2005) has illustrated that proteins may be cross-linked by the *in vacuo* protein modification technique by the formation of an amide linkage under vacuum. In this protein cross-linking technology, interacting ammonium and carboxylate groups will undergo a condensation reaction to form the linkage. Therefore, the *in vacuo* methodology can be taken advantage of for enzyme immobilization as ammonium groups attached to a glass surface will subsequently interact with the carboxylate groups of glutamic or aspartic acid residues found on the enzyme surface to form the amide linkage. This technology has recently been successfully applied by Kaplan and co-workers to the immobilization of alkaline phosphatase (Taylor *et al.,* 2005), and this success provides a basis for its application to the immobilization of trypsin. Also, as it was demonstrated, the *in vacuo* glycated trypsin was significantly more resistant to thermoinactivation than the native unmodified trypsin. Therefore, it is of interest to determine whether *in vacuo* immobilization of the glycated trypsin product will provide even more stability to the enzyme structure.

4.2 Materials and Methods

4.2.1 Proteins and Reagents

Porcine pancreatic trypsin (Lot #: 034K7423) and chicken egg white lysozyme were purchased from the Sigma-Aldrich Chemical Company. The amine functionalized 3 μm glass beads were purchased from Varian Inc. All other chemicals, reagents and solvents were high purity preparations obtained from the indicated commercial sources.

4.2.2 In Vacuo *Immobilization of Glycated Porcine Trypsin to 3 μm Glass Beads*

Approximately 1 g of the glass bead sample was washed with ddH_2O and subsequently centrifuged to recover the pellet. Upon recovery, the pellet was first washed with a 1% NH_4HCO_3 solution and then washed twice with a volume of ddH_2O and re-suspended in ddH_2O. A sample of the *in vacuo* glycated porcine trypsin was re-suspended in dH_2O to a concentration of 6.00 mg/mL, and the Varian glass bead suspension was added to the enzyme solution with constant stirring. Using a calibrated Radiometer Copenhagen type PHM26 pH meter fitted with a Beckman Futura™ refillable combination electrode, the pH of the glass bead and enzyme mixture was brought up to 7.00 by addition of 0.100 N NaOH (purchased from VWR) *via* a micro-syringe. The mixture was then evenly distributed between four Pyrex® borosilicate glass test tubes and flash frozen in an ethanol/dry ice slurry. The samples were lyophilized using either a VIRTIS-24 freeze dryer or a home-made freeze dryer consisting of an ethanol/dry ice trap hooked up to a 70 mtorr vacuum pump. The sample tubes were purged with N_2 and subsequently narrowed and sealed under vacuum (50 mtorr) using an oxygen enriched

flame. The sealed tubes were placed in an oven set to a temperature of 85°C for a period of 24 hours. After thermal incubation, the sealed sample tubes were opened and 5 mL of a 0.5% acetic acid solution was added. All of the samples were then transferred to a 15 mL Corning® centrifuge tube and the sample was centrifuged to collect the pellet. Upon retrieval, the pellet was washed twice with a volume of 0.5% acetic acid and twice with phosphate buffered saline (PBS). A 3 mL volume of ddH_2O was added to the washed pellet and this was vortexed for a short period of time. The suspension was brought up to a volume of 10 mL with ddH_2O and transferred to a 20 mL screw-capped vial. The suspension was placed in an oven at 60°C for 3 hours and stirred continuously for the duration of the incubation. Upon completion of the thermal incubation, the sample was centrifuged and the pellet was collected and washed twice with PBS, twice with 0.5% acetic acid and once with ddH_2O. The pellet was once again retrieved by centrifugation and re-suspended in 3 mL of ddH_2O and lyophilized as before.

4.2.3 Activity of Immobilized Glycated Porcine Trypsin and Native Porcine Trypsin at an Elevated Temperature

A total of 40.56 mg of the immobilized glycated porcine trypsin product (glass + immobilized trypsin) was weighed out and transferred to a 50 mL Corning® centrifuge tube. A 1.00 mg/mL suspension of the immobilized glycated trypsin sample was prepared by the addition of a pH 8 buffer containing 0.100 M KCl, 1.00 mM $CaCl_2$ and 0.200 mM Tris. The sample was placed in a shaker bath set to a constant temperature of 60°C. It was necessary to allow the experimental sample to equilibrate to the experimental temperature of 60°C, therefore the first sample aliquot (t = 0.00 hrs) was removed after a five minute incubation period. Sample 5.00 mL aliquots were taken from

the bulk sample after timed periods of incubation (t = 0.00 hrs, 0.500 hrs, 1.00 hr, 2.00 hrs, 4.00 hrs and 24.0 hrs) at 60°C, and were immediately transferred to a reaction vessel equipped with a re-circulating water bath set to a temperature of 60°C. A constant stream of N_2 was blown over the reaction vessel. A 100.0 μL aliquot of a 50.0 mM BAEE solution was added to the 5.00 mL immobilized enzyme sample in the reaction vessel and the hydrolysis reaction was allowed to proceed. The reaction was monitored by a Radiometer Copenhagen type PHM26 pH meter fitted with a Beckman Futura™ refillable combination electrode coupled to a Titration 11/Ole Dich autotitrator pH-stat assembly. The reaction endpoint was set to a pH of 7.50, and a drop below this value resulted in the autotitrimetric addition of a volume of 0.020 N NaOH *via* micro-syringe. The volume of base added per unit time was recorded by the pH-stat and this data was used to calculate the rate of hydrolysis. The method used to obtain the thermostability data of the free native porcine trypsin was essentially identical to the method stated above for the immobilized sample. The native enzyme was incubated in the 0.100 M KCl, 1.00 mM $CaCl_2$ and 0.200 mM Tris pH 8 buffer at a concentration of 0.200 μg/mL, and 5.00 mL sample aliquots were taken after timed intervals of incubation in the 60°C shaker bath.

4.2.4 High Voltage Paper Electrophoresis (HVPE) of Native Lysozyme Digest by Native, Glycated and Glycated Immobilized Porcine Trypsin

Chicken egg white lysozyme was digested by either native, glycated or immobilized glycated trypsin where the digestion ratio was 1000:1 (lysozyme to trypsin). A 0.200 mg/mL solution of the native and glycated porcine trypsins in 50.0 mM NH_4HCO_3 (pH 8.0) were prepared after dilution of a 2.00 mg/mL stock solution. A 2.00 μg sample (10.00 μL aliquot of 0.200 mg/mL solution) of the native and glycated

trypsins were taken and placed in separate 20 mL screw-capped vials. It was determined that one milligram of the immobilized glycated trypsin product had the solution equivalent of 0.200 μg of soluble native porcine trypsin. Therefore, a 0.500 mL aliquot of a 20.0 mg/mL suspension of the immobilized glycotrypsin sample in NH_4HCO_3 buffer (corresponding to 10.0 mg of the immobilized product which has the equivalent activity of 2.00 μg of native soluble porcine trypsin) was taken and placed in a 20 mL screw-capped vial. All of the trypsin samples were incubated for a 5 minute period at 60°C in 50.0 mM NH_4HCO_3 buffer. After completion of the incubation, a 2.00 mg sample of chicken egg white lysozyme taken from a stock 10.0 mg/mL solution in NH_4HCO_3 buffer was added to each of the trypsin samples. Digestion of lysozyme was carried out at 60°C for 24 hours. Upon completion of the digest, a 100.0 μL aliquot of each digestion sample was transferred to a 1.5 mL eppendorf tube. The digestion was immediately stopped by the addition of 200.0 μL of pH 2.1 electrophoresis buffer (8% acetic acid and 2% formic acid) and freezing by immersion in dry ice. The samples were vacuum dried on a speed vac. The samples were reconstituted with 50.0 μL of pH 2.1 electrophoresis buffer. A 40.0 μL aliquot of each sample (80.0 μg of protein) was loaded onto a 3.3 cm wide strip of a 20 cm x 20 cm piece of Whatman chromatography paper. In order to ensure proper electrophoresis has occurred prior to staining, 5 μL aliquots of 1 mg/mL solutions of the fluorescent markers dansyl arginine and dansyl sulfonate were spread across the entire width of the paper along the origin of the sample migration. After all samples had been loaded, the chromatography paper was buffered using the pH 2.1 electrophoresis buffer. The buffered paper loaded with the digestion samples was placed in a HTLE-7002 Hunter thin layer peptide mapping electrophoresis system (C. B. S. Scientific Company

Inc.), and using the pH 2.1 electrophoresis buffer, the digested lysozyme samples were run at 20 V/cm for 2 hours (800 VH). Upon completion of electrophoresis, the paper was air-dried overnight. After drying, the paper was stained by dipping in a cadmium-ninhydrin solution (0.01g of ninhydrin/mL of ethanol, 12.5 mL of acetic acid, 1 g of $CdCl_2$/mL of ddH_2O) (Hilbert *et al.,* 1999) to visualize the peptides generated by lysozyme digestion.

4.3 Results and Discussion

The results obtained from the measurement of enzymatic activity by BAEE hydrolysis at 60°C after continuous incubation of the native trypsin and immobilized glycated trypsin samples at 60°C indicate that the immobilized product has a remarkable thermostability as compared to the native sample. As illustrated in figure 4.3.1, the immobilized glycated trypsin sample maintains 100% of its original activity after a four hour incubation period at 60°C. The native sample loses activity rapidly at this temperature, and for all practical purposes has lost its activity after 4 hours of incubation. The high level of thermostability of the immobilized sample is further demonstrated by the retention of 25% of its original activity after a continuous incubation at 60°C for a 24 hour period.

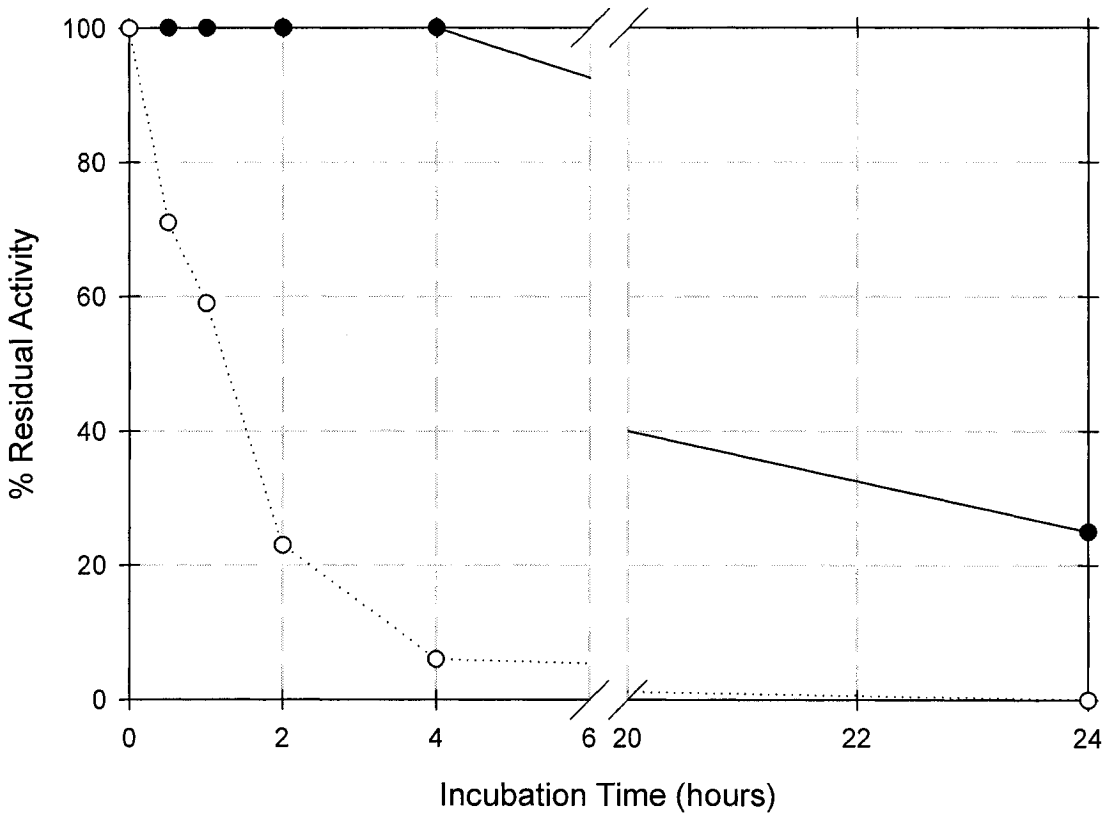

Figure 4.3.1: The effect of incubation of native porcine trypsin (○) and immobilized glycated porcine trypsin (●) at 60°C. The enzyme samples were incubated at 60°C in pH 8 buffer where the native trypsin sample was incubated at a concentration of 0.200 μg/mL (8.5×10^{-9} M) and the immobilized glycotrypsin sample was incubated at a solution equivalent of 0.200 μg/mL. The rate of BAEE hydrolysis by native and glycated immobilized porcine trypsin was measured at pH 7.50 at a temperature of 60°C. The activity is shown as % values relative to the initial activity of the samples. The % relative activity was calculated as stated in section 3.2.2.

The stability afforded to the immobilized glycated sample is the result of several factors. As discussed in the previous chapters, the *in vacuo* glycation procedure results in the modification of an average of 11 to 13 lysine residues per trypsin molecule by the creation of a stable ketoamine at the end of lysine side-chain. Creation of the ketoamine provides the enzyme structure with extra functional groups which can take place in hydrogen bonding activities, resulting in the formation of a hydrogen bonded structure around the enzyme. The creation of this hydrogen bonded structure serves to provide extra stabilization to the active conformation of the enzyme. Therefore, more energy is required to unfold the active conformation of the glycated enzyme than that required to unfold the native enzyme. In other words, the increase in translational and vibrational motions exhibited by the enzyme as a result of incubation at elevated temperatures is not as detrimental to the active enzyme conformation of the immobilized glycated trypsin as compared to the native trypsin by virtue of the stabilizing hydrogen bonds which have formed around the enzyme. Since the glycated trypsin is also covalently attached to a rigid solid support, this puts a further restriction on molecular motions which occur at elevated temperatures, resulting in an additional contribution to the preservation of the active enzyme conformation.

As was seen in chapter 3 with the soluble glycated trypsin sample, the rate of autolysis was greatly reduced as compared to a sample of native trypsin. The reduced rate of autolysis is granted by the formation of the ketoamine masking the lysine ε-amino group, making trypsin's substrate target unrecognizable to the enzyme. Since the substrate target is masked, trypsin can no longer identify the cleavage point and hydrolysis of the peptide bond will not occur, leading to a reduced autolysis rate. This

phenomenon also holds true for the immobilized sample, as the immobilized trypsin is also glycated. For the immobilized sample, a second mechanism can be used to explain the reduced autolysis rate in conjunction with the first, which ultimately contributes to the overall thermostability of the immobilized glycated preparation. Trypsin is a globular enzyme with a spherical molecular diameter of 3.8 nm (Díaz and Balkus, 1996). The glycated trypsin molecules are covalently attached to glass beads which have a diameter roughly 800 times that of the enzyme. The large diameter of the glass bead relative to the diameter of trypsin prevents the trypsin molecules from physically interacting with one another, resulting in a further reduction in the amount of autolysis which can occur.

An incubation and reaction temperature of 60°C was chosen to measure the rate of BAEE hydrolysis, as this temperature has relevance to the practical applications of the immobilized trypsin product. A primary practical application of this product would be for use in proteomic applications using trypsin as a digesting enzyme. Current protein digesting techniques usually require denaturation of the substrate protein prior to digestion by some means. Typically this is accomplished by subjecting the protein to chemical denaturants such as urea or guanidinium chloride. However, at an elevated temperature of 60°C, most proteins are not able to maintain their native conformation and will unfold. This knowledge combined with the fact the immobilized glycated trypsin product has shown to be stable at this temperature implies that this product could be used to digest proteins that have not been previously denatured. Therefore one advantage of using this product for protein digestions is that it will eliminate the need of prior denaturation by chemical methods which can be a tedious process and which can also have deleterious effects on the protein. A second advantage of performing protein

digestions at elevated temperatures is that the increased reaction temperature will increase the rate of reaction. This implies that protein digestions will be completed in a fraction of the time required to carry out digestions at room temperature. As illustrated by figure 4.3.1, the immobilized preparation maintains 100% of its original activity after 4 hours of incubation at 60°C. An incubation period of 4 hours at 60°C is more than sufficient to denature a variety of proteins and subsequently carry out digestion by the immobilized glycated trypsin. Also, due to the increased rate of reaction, a smaller quantity of enzyme will be required to complete the digestion as compared to what would be needed to complete the digestion at room temperature in a reasonable amount of time. Another advantage of using the immobilized glycated trypsin over soluble trypsin preparations is that upon completion of the digestion, the immobilized product can be removed from the reaction and re-used. The fact that small quantities of the immobilized glycated product can be used for digestions at elevated temperatures, and that this product can be easily removed after the digestion and be subsequently re-used makes the immobilized glycated trypsin preparation a highly cost effective product for practical protein digestion applications.

In light of all the possible advantages that the immobilized product possesses over soluble trypsin preparations, this sample was used to digest native chicken egg white lysozyme at 60°C in order to verify its validity as a product with benefits to offer when used for practical applications. For comparison, native soluble trypsin and glycated soluble trypsin were also used for the digestion at 60°C. After digestion the resulting lysozyme peptides were subjected to HVPE and stained with ninhydrin, to detect the peptides that are generated. These results are illustrated in figure 4.3.2.

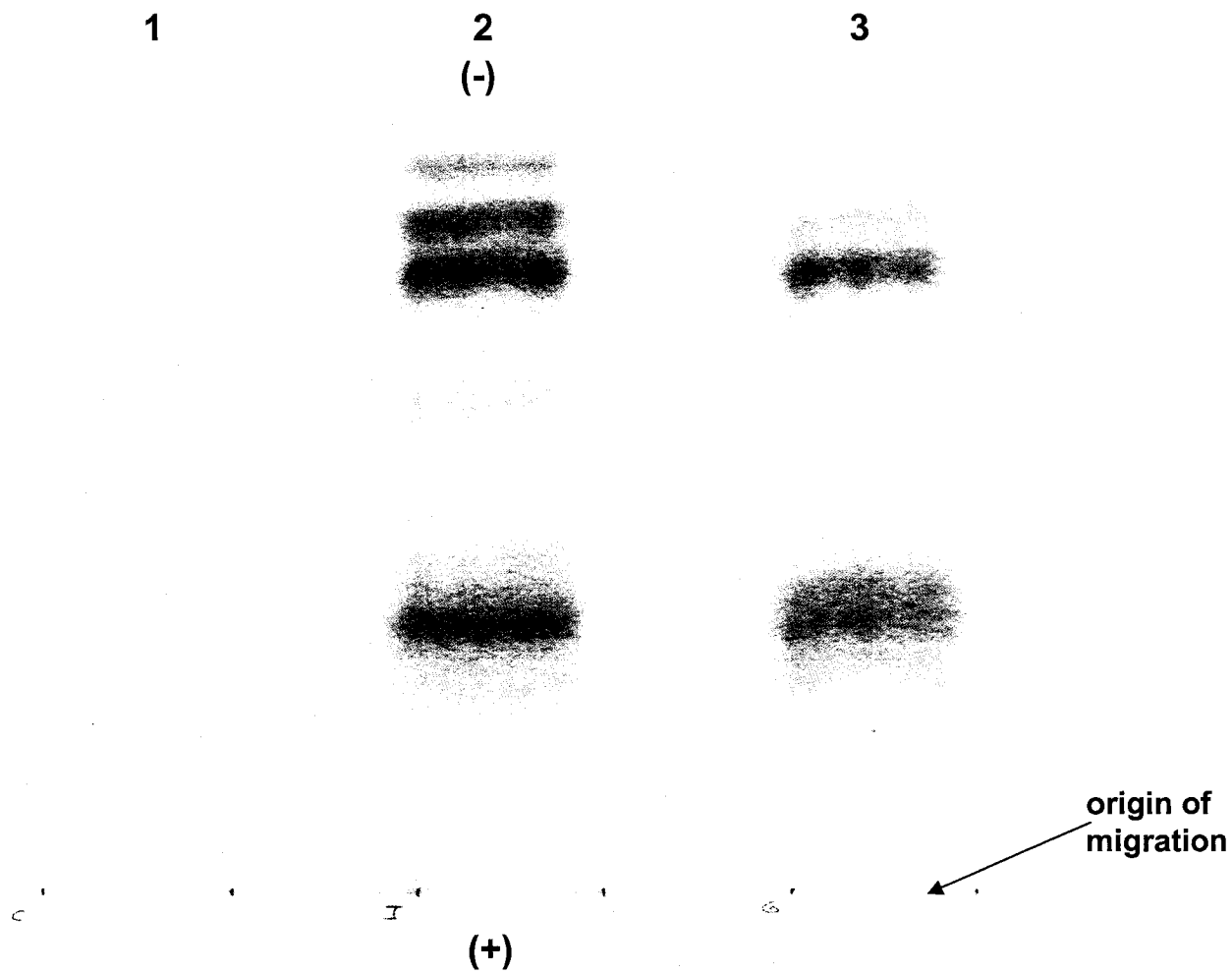

Figure 4.3.2: HVPE of peptides generated by digestion of native chicken egg white lysozyme by native porcine trypsin (lane 1), immobilized glycated porcine trypsin (lane 2) and soluble glycated porcine trypsin (lane 3). Digestion was carried out at 60°C in pH 8.0 for 24 hours at a lysozyme to trypsin ratio of 1000:1. A total of 80.0 µg of digested lysozyme sample was loaded onto each 3.3 cm lane and electrophoresis was carried out for 2 hours at 20 V/cm. After electrophoresis, the paper was dipped in a cadmium-ninhydrin solution to visualize the peptides.

Upon analysis of figure 4.3.2, it is clear that at a temperature of 60°C soluble native trypsin is unable to digest native lysozyme, as illustrated by the lack of resultant peptide bands (lane 1). This is most likely due to the fact that the native trypsin sample was denatured, whereby the active conformation of the enzyme was unable to remain folded at the high digestion temperature. However, digestion of the native protein at 60°C by the immobilized glycated and the soluble glycated trypsin samples does occur, as indicated by the peptide bands present in lanes 2 and 3 of figure 4.3.2. For both the immobilized and the soluble glycated trypsins, a total of five peptide bands can be distinguished after electrophoresis and staining of the digested native lysozyme sample. However, the ninhydrin stain intensity of the peptide bands generated by the immobilized glycated trypsin digestion of lysozyme exceeds that of the soluble glycated sample.

For many years, various analytical techniques have used ninhydrin for the detection and the quantitative estimation of α-amino acids in experimental samples (Bottom *et al.,* 1978). Ninhydrin is not limited to react only with α-amino acids, as it will undergo reaction with other molecules possessing a primary amino group, such as peptides or proteins (Friedman, 2004). The purple colour generated upon reaction of ninhydrin and a primary amino group is known as Ruhemann's purple (RP), and this reaction occurs stoichiometrically as one equivalent of RP is produced per amino group (Friedman, 2004). Therefore, by assessing the intensity of RP of the peptide bands generated in lanes 2 and 3 of figure 4.3.2, it can be determined which sample provided a more extensive digestion of the native lysozyme. From figure 4.3.2, it can be seen that the RP intensity of the peptide bands generated by digestion of lysozyme by the immobilized glycated sample is greater (lane 2) than that generated by digestion using the

soluble glycated sample (lane 3). The greater RP intensity of the peptide bands in lane 2 indicates that the immobilized glycated sample provided a more extensive digestion of the native protein than the soluble glycated sample.

Standard protein digestion protocols typically call for proteins to be digested by enzymes at ratios of 20:1 to 50:1 (substrate protein to enzyme) (Cooligan *et al.,* 2003; Kinter and Sherman, 2000; Simpson, 2003). The native lysozyme digestion by the three trypsin samples was carried out at a ratio of 1000:1. As indicated by the lack of any peptide bands present after electrophoresis of the lysozyme sample digested with native trypsin, it is clear that the native trypsin was not able to maintain its active conformation and was subsequently unable to digest the lysozyme sample to produce any resulting peptides. However, both the immobilized and soluble glycated trypsin samples were able to carry out digestion of the lysozyme, even when used in such a small quantity. This was able to occur by virtue of the extra stability afforded to these samples by glycation and immobilization. Since higher digestion temperatures can be employed, an increase in the rate of reaction will occur. The increased reaction rate will allow for the digestion to occur more efficiently, requiring less time and a reduction in the amount of enzyme that what would typically be used. A major benefit of using such a relatively small quantity of enzyme to digest a protein becomes apparent. It has been established that the amount of autolysis exhibited by the immobilized glycated trypsin and the soluble glycated trypsin is greatly reduced. This fact in combination with the fact that only relatively very small quantities of these enzyme preparations are required for digestion implies that the likelihood of the digested protein sample to be contaminated with trypsin autolysis fragments is greatly reduced. The presence of such contaminating fragments in samples

destined for analysis by MS complicates the resulting spectra and makes the interpretation of results difficult.

4.4 Conclusions

As illustrated by the results presented in this chapter, the *in vacuo* technology has successfully been applied to immobilize *in vacuo* glycated trypsin resulting in the production of a highly thermostable enzyme product. The *in vacuo* technology used to chemically immobilize the glycated trypsin provides several advantages over the conventionally used methods which occur in solution. The primary advantage is that the prior activation of the solid support or the enzyme into species which will undergo reaction to form a covalent bond is not required. Not only is this time-saving, but it eliminates the possibility of altering the enzyme in such a way as to reduce its catalytic activity, and it eliminates the possibility of denaturing the enzyme. Also, after activation and coupling of the support and enzyme in conventional solution immobilization techniques, it is likely that some of the activating chemicals remain in the preparation. This results in the necessity to purify these immobilized preparations before use. The *in vacuo* immobilization can be carried out with very small amounts of enzymes (microgram to picogram amounts). Conventional chemical immobilization techniques in solution frequently require the enzyme to be present in large quantities in order to successfully immobilize a substantial amount of enzyme.

The highly thermostable immobilized glycated trypsin is an extremely attractive product for use in practical applications, as it provides many advantages over using the conventional trypsin preparations currently available. The primary advantages occur as a result of the ability to perform protein digestions at elevated temperatures, and these advantages include; (1) the elimination of prior denaturation by chemical means of the protein to be digestion, (2) the completion of digestion in a short amount of time and (3) only a very small quantity of immobilized glycated trypsin is required to complete the digestion. Another significant advantage which arises when using the immobilized trypsin product, is that upon completion of digestion, the enzyme sample can be re-covered and re-used, making it a highly cost-effective product.

4.5 References

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Claims to Original Research

1. The demonstration of extensive glycation of bovine trypsin by the *in vacuo* glycation technology by ESI-MS.

2. The demonstration that *in vacuo* glycated trypsin is autolysis-resistant and has a greatly improved thermostability.

3. The demonstration that *in vacuo* glycated chymotrypsin has a greatly improved thermostability which greatly improves its resistance to autolysis.

4. The immobilization of *in vacuo* glycated trypsin to a functionalized glass support, and the demonstration that the *in vacuo* immobilized glycated trypsin is autolysis-free and is extremely thermostable at temperatures as high as 60°C.

5. The demonstration that the *in vacuo* immobilized native and glycated trypsin and is able to efficiently digest native proteins at elevated temperatures.