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Application of In Vacuo Chemical Modification for Protein Characterization and Enhancement of the Physico-chemical Properties of Proteins

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Application of *in vacuo* chemical modification for protein characterization and enhancement of the physico-chemical properties of proteins

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Thesis submitted to the School of Graduate Studies and Research in partial fulfillment of the degree of Doctor of Philosophy in Biochemistry

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Abstract

Chemical modification of proteins is a major experimental tool for the study of structure-function relationships in proteins and in pharmaceutical and industrial applications of proteins. Many chemical modifying reagents and strategies have been developed for a wide variety of applications. The vast majority of these procedures are designed to be carried out in aqueous solution which limits the range of chemical modifying reagents as well as the types of modifications that can be carried out. *In vacuo* chemical modification is a methodology in which proteins are chemically modified in the lyophilized state and modifications can be made which are difficult or impossible to carry out in aqueous solution. Three different *in vacuo* chemical modifications, which cannot be carried out in aqueous solution, are used to develop novel applications for structural studies on proteins, for improvement of the physical properties of proteins, and for practical applications. The first is the *in vacuo* methylation of carboxyl and amino groups of proteins with iodomethane. Novel diagonal electrophoretic procedures, which can be used in structural studies or proteomic applications, were developed for the selective isolation of peptides derived from either the carboxyl terminus or the amino terminus of proteins. The second is the *in vacuo* glycation of proteins in which a reducing sugar is covalently attached to the amino groups of a protein without the use of chemical activating reagents. Glycated trypsin, or glycotrypsin, was shown to undergo greatly reduced autolysis thereby increasing its stability and reducing contaminating autolytic products in tryptic digests of proteins. *In vacuo* glycation was also shown to improve the thermostability of trypsin and chymotrypsin, and evidence was obtained that

this phenomenon is generally applicable. The third is the *in vacuo* immobilization of proteins to solid supports without the use of activating chemicals. Immobilized trypsin and immobilized glycotrypsin were shown to have greatly improved thermostability and retained their full activity after repeated washings and multiple usages. The immobilized glycotrypsin has exceptional thermostability and it was demonstrated that it proteolyses native proteins at elevated temperatures without the requirement for prior denaturation. It was demonstrated that a complex protein structure, zona pellucida (ZP), could be immobilized *in vacuo* on a solid surface and retain its biological activity. This provides strong evidence that *in vacuo* immobilization is a benign chemical modification procedure which does not disrupt protein structure.

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List of Abbreviations

α	alpha
β	beta
μ	micro
°C	Celsius
AGE	advanced glycation endproduct
amu	atomic mass unit
Arg	arginine
Asn	asparagine
Asp	aspartic acid
ATEE	*N*-acetyl-L-tyrosine ethyl ester
BAEE	*N*-benzoyl-L-arginine ethyl ester
C-terminal	carboxyl terminal
CD	circular dichroism
CID	collision induced dissociation
CM-cellulose	carboxymethyl cellulose
CNBr	cyanogen bromide
cpm	counts per minute
d	day
ddH_2O	distilled deionized water
dH_2O	distilled water
DM	dimethylated
DNA	deoxyribonucleic acid
DNS	Dansyl (5-Dimethylamino-1-naphthalenesulfonyl chloride)
DSC	differential scanning calorimetry
ESI-MS	electrospray ionization mass spectrometry
ELISA	Enzyme-Linked ImmunoSorbent Assay
eV	electron volts
h	hour
HCl	hydrochloric acid

HI	hydriodic acid
His	histidine
HTLE	Hunter thin layer electrophoresis
HVPE	high voltage paper electrophoresis
ICAT	isotope coded affinity tag
kV	kilovolts
LpH	lyophilized pH
Lys	lysine
MALDI	matrix-assisted laser desorption/ionization
mCi	milliCurie
min	minute
mu	mass unit
MS	mass spectrometry
MS/MS	tandem MS
MWCO	molecular weight cut-off
m/z	mass to charge ratio
N-terminal	amino terminal
NaOH	sodium hydroxide
nmol	nanomole
NMR	nuclear magnetic resonance
NRS	normal rabbit serum
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
pfo	performic oxidized
pH	negative logarithm of the proton concentration
PITC	phenyl isothiocyanate
pK_a	negative logarithm of the acid ionization constant
ppm	parts per million
PSD	post source decay
Q-TOF	quadrupole time of flight
RNase	ribonuclease

TFA	trifluoroacetic acid
Thr	threonine
TOF	time of flight
T_m	melting temperature
TEMED	tetramethylethylenediamine
TPCK	tosyl-L-phenylalanine chloromethyl ketone
UV	ultraviolet
VH	volts-hour
z/m	charge to mass ratio
ZP	Zona Pellucida
ZP1	Zona pellucida protein 1
ZP2	Zona pellucida protein 2
ZP3	Zona pellucida protein 3

Chapter 1: Introduction

1-1 OVERVIEW OF CHEMICAL MODIFICATION

1-1.1 *In vivo* chemical modification

Proteins are synthesized *in vivo* as a polypeptide chain and subsequently many undergo post-translation chemical modifications. Over 200 modified amino acids have been identified; some are common, others are rare (Yan *et al.* 1989; Creighton 1993; Davis 2003). These *in vivo* modifications are carried out by specific enzymes and usually impart novel properties to the protein that are of physiological importance. Post-translational modifications of amino acids often extend the range of properties and functions of proteins by changing the chemical nature of the constituent amino acids. This is accomplished by addition of new functional groups, addition of new structural components, and alteration of the primary, secondary and tertiary structure of the protein. Some of the most common modifications are acetylation, methylation, phosphorylation, proteolytic processing, glycosylation, disulphide bridge formation and cross-linking of polypeptide chains. These modifications occur at specific sites in the cell where the relevant enzymes are located (Wold 1981).

Phosphorylation is one of the most common protein modifications that occur in animal cells (Lipmann and Levene 1932; Johnson and Lewis 2001). It is a mechanism for controlling the behaviour of a protein by regulating its activity (Nestler and Greengard 1984; Johnson and Lewis 2001). A phosphate is added and later removed to switch the enzyme or receptor "on" and "off"; thus phosphorylation is usually transient in nature. Serine side-chain is the most common site of phosphorylation followed by threonine (Deutscher and Saier 2005). Tyrosine and histidine phosphorylations also occur but are relatively rare (Heilmeyer 1993).

Following synthesis many proteins are modified at their N-terminus. Often, the initiator methionine is removed by hydrolysis and an acetyl group is added to the new N-terminal amino acid with Acetyl-CoA as the acetyl donor for these reactions (Berger 1999; Kouzarides 2000). If the donor for the modification is myristoyl-CoA, then the

myristoyl group is added to the N-terminus allowing the association of the modified protein with membranes.

Post-translational methylation typically takes place on arginine or lysine amino acid residues in proteins such as histones (Berger 1999). The activated methyl donor is S-adenosyl methionine and the methylated histones can act epigenetically to repress or activate gene expression.

Many proteins are synthesized as inactive precursors that can be turned into an active form by limited proteolysis. Inactive precursor proteins that are activated by removal of polypeptides revealing the active site are termed proproteins (Creighton 1993). Membrane bound or secreted proteins are synthesized containing an N-terminal polypeptide, termed signal sequence or signal peptide. The signal peptide is removed by signal peptidase following passage through the endoplasmic reticulum (ER) membrane (Blobel and Dobberstein 1975b; a). Proteins that contain signal peptide are called preproteins as they may be in active forms to distinguish them from proproteins. Proteins that contain both signal peptide and pro sequences are termed preproproteins. An example of a preproprotein is insulin (Clark 1999). After secretion from the pancreas the prepeptide is removed and protein folds into proinsulin. Proinsulin is further cleaved yielding active insulin which is composed of two shorter polypeptide chains linked by disulphide bridges.

1-1.2 *In vitro* chemical modification

In vitro modification of proteins using chemical reagents is a well established approach for producing proteins with novel physico-chemical properties (Means and Feeney 1971; Darbre 1986; Lundblad 1995). In the early studies on proteins, chemical modification was primarily used to elucidate protein structure, but with the development of x-ray crystallography, NMR and genetic techniques for structure determination, chemical modification is now more often used to confer new physico-chemical properties. For example, currently one of the most widely used modifications is the chemical cross-linking of enzymes to antibodies for applications in western blots and ELISAs where a new semi-synthetic protein is produced which has the binding specificity of an antibody and the catalytic activity of the enzymes (Lequin 2005). Many

such ingenious chemical modification strategies have been developed which have practical applications and the continued development of new and improved protein products by chemical modification is of academic and practical interest.

A wide array of reagents to modify the reactive groups on side-chains of amino acid residues is available. The reactive nucleophilic groups of proteins include imidazole, ε-amino, sulphydryl, phenolic hydroxyl, and carboxyl groups (Creighton 1993). The specific chemical modification of amino acid side-chains in proteins is difficult to achieve as the chosen modifying reagents are non-selective and can potentially react with all the nucleophilic functional groups in a protein. To a limited extent, by controlling the reaction conditions and types of reagents, the specificity of the side-chain modification can be improved. The relative nucleophilicity of the amino acid's reactive group available on the protein determines the selectivity of the modification. For example, the selective modification of the cysteine's sulfhydryl group can be achieved by limiting reaction time as the sulphydryl group is much more reactive than any other nucleophile in the protein (Lundblad 1995).

Most *in vitro* chemical modifications of proteins take advantage of the nucleophilic properties of the side-chains of amino acids, but there are also a few electrophilic sites that can be used for this purpose. The hydroxyl group in the aromatic side-chain of tyrosine creates positions that are reactive in electrophilic substitution reactions. This property can be used to carry out iodination or nitration of the aromatic ring of the tyrosine side-chain. Other electrophilic sites that can be used for chemical modification are the carbon atoms in the side-chains of the imidazole ring of histidine and protonated carboxyl groups of aspartic and glutamic acids (Means and Feeney 1971; Darbre 1986; Lundblad 1995). Opportunities for such electrophilic modifications are much more limited than for nucleophilic modifications due to the lower abundance and reactivity of such sites, and the number of reagents available for such modifications. However, an advantage of electrophilic modification is that it can usually be carried out with much greater specificity than the nucleophilic modifications.

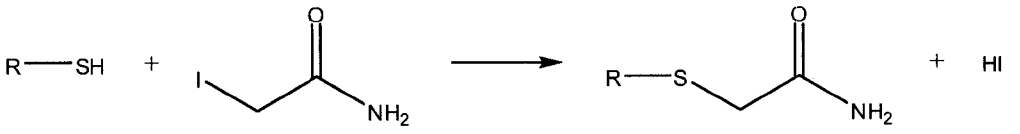
1-2 APPLICATIONS OF CHEMICAL MODIFICATIONS OF PROTEINS

1-2.1 Overview

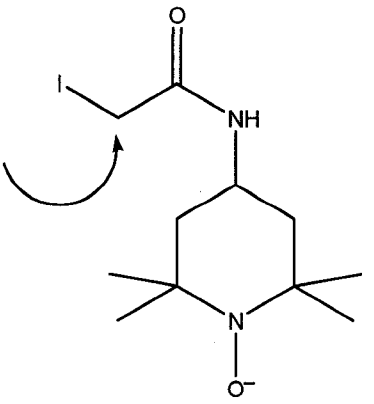
In broad terms, modification of proteins can be carried out for two purposes. The first is theoretical structure-function studies. In such research, the objective is to use chemical modification to elucidate the structural properties of proteins that confer on the protein its unique physiological activity and to determine the factors which are essential for its activity and structural integrity. The long term goal of such studies on many proteins is to obtain a better understanding of structure-activity relationships so that proteins with novel properties and activities can be designed and synthesized. The second is to use modified proteins simply as a tool in practical applications to obtain information or achieve a specific result and the protein itself is not of direct interest. An example of the latter is the use of fluorescent-labelled proteins, and in particular antibodies, in order to localize specific constituents in a cell or in a gel. Different strategies can be employed for a particular modification depending on the system and the particular objective, and many procedures that use chemical modification of proteins in highly original and ingenious ways have been developed. Some examples of the more widely-used procedures that have been developed are described below.

1-2.2 Labelling of proteins

The purpose of chemical labelling of a protein is to introduce a physical property that is not present in the naturally occurring protein. This property can be used in a variety of ways. Examples of the type of such labels are fluorescent, radioactive isotopes (^{14}C, ^{3}H, ^{32}P, ^{35}S, ^{125}I), non-radioactive isotopes (^{13}C, ^{2}H, ^{15}N, ^{18}O) and spectrophotometric (UV, NMR, electron spin). These labels can be introduced by designing reactive reagents that contain the desired label. For example, iodoacetamide is a widely used reagent for the modification of cysteine residues in proteins.



An iodoacetamide derivatized with a nitroxide ring (Esmann *et al.* 1993) can be used to introduce a spin label into a protein by reaction with cysteine residues.



The effect of the protein environment on the spin label can then be used to study the conformational dynamics (White *et al.* 2007).

The introduction of a label is the most widely applied chemical modification. Many reagents and strategies have been developed for reaction with all classes of reactive functional groups in proteins (Means and Feeney 1971; Darbre 1986; Lundblad 1995). While the nature of the group introduced and the objectives and strategies may vary greatly, they all have in common a covalent chemical modification which introduces a group with a property previously absent in the protein. Such groups are known as reporter groups. In the example above, a spin label is introduced into the protein. However, using analogous chemical modification strategies, fluorescent, spectrophotometric, NMR, radioactive isotopes and non-radioactive isotopes can be introduced (Walker 2002).

1-2.3 Immunoblotting and ELISA

Among a complex mixture of proteins, using western blotting (or immunoblotting), a single protein can be detected specifically. This technique makes use of the highly specific binding of an antibody or immunoglobulin to a target, known as an antigen. Antibodies raised against a protein antigen will bind strongly to that protein with a high specificity. In the detection procedure, a protein sample is immobilized on a solid membrane support that holds the proteins tightly and the membrane is exposed to the detection antibody to form a complex with the antigen. In the most common application, the protein mixture is first fractionated by an electrophoretic procedure and the location of the antigen of interest is located by immunoblotting, and this process is termed western blotting. At the heart of all immunoblotting procedures is the use of

labelled antibodies (radioactive or fluorescent) produced by a chemical modification. This permits detection and localisation of the bound antibodies and hence identifies the presence of the protein which the antibody recognizes.

The sensitivity of the immunoblotting procedure depends on the detection of the label attached to the antibody. Another strategy for the detection is to chemically cross-link an enzyme to the antibody and use the catalytic activity of the enzyme to produce a visible signal from a chromogenic reaction. In a broader context, such cross-linking of an enzyme to an antibody can be regarded as a labelling procedure. This can greatly increase the sensitivity of detection by allowing a longer time for the enzymatic reaction to occur. Horseradish peroxidase and alkaline phosphatase are the most common read-out enzymes covalently linked to the antibody with glutaraldehyde being the reagent used most often to effect the cross-linking (Harlow and Lane 1988; Deshpande 1996).

Enzyme-Linked ImmunoSorbent Assay, or ELISA, is a procedure that uses the high specificity of an antibody for quantifying a specific component in a complex mixture. It is similar to a western blot procedure in the use of an enzyme covalently cross-linked to an antibody. However, in the case of ELISA, conditions are more carefully controlled so that the intensity of the visible signal produced by the enzymatic reaction can be used to quantify the amount of antigen of interest.

1-2.4 Protein stabilization

Another application of chemical modifications of protein is to improve the properties of a protein so that they will function more efficiently in a specific procedure. The properties that are most often targeted are thermal stability, solubility, pH stability, and sensitivity to enzymatic degradation. An example is the use of trypsin for the digestion of proteins in proteomics applications. Natural trypsins are very susceptible to auto-digestion (autolysis) which decreases the activity of the enzyme and contaminates the peptides generated from the protein of interest (Roepstorff 2000; Hunyadi-Gulyás and Medzihradszky 2004). For this reason, trypsin in which the lysine residues are methylated by chemical modification is used in proteomics application. Methylation of the lysine residues prevents trypsin from attacking these sites and thus reduces autolysis (Rice *et al.* 1977).

1-2.5 Protein immobilization

Another widely used application of chemical modification is the immobilization of proteins by covalent attachment to a solid support. The major advantage of immobilizing proteins is that it permits control over the localization of their biological properties which is very useful in many analytical procedures and eliminates many of the complications that occur in homogeneous solution where the proteins can interfere in subsequent steps. A simple example of this is that after any process the protein can easily be removed from the other components and even recovered and possibly reused. The chemical cross-linking is usually achieved by preparing solid supports with chemically activated groups that can react with functional groups on the protein to form a covalent attachment. There are many strategies and methodologies for immobilization of proteins (Rusmini *et al.* 2007) that have been developed for a wide variety of objectives. However, regardless of the application, at the heart of all these operations is the ability to carry out a covalent chemical modification that attaches a protein to a solid support.

Such immobilized proteins are often used in purification procedures referred to as affinity chromatography (Cuatrecasas *et al.* 1968). For example, an immobilized antibody retains its highly specific binding and can be used to separate an antigen from a complex mixture by passing the mixture through such a column. The antigen binds to the antibody while all the other components pass through the column. The antigen can then be released by eluting the column under conditions that weaken the binding. In the case of immobilized enzymes, a reaction can be carried out by incubating the substrate with the enzyme immobilized on beads and simply removing the beads on completion of the reaction (Tischer and Kasche 1999). In such a case, the recovered enzyme can often be reused.

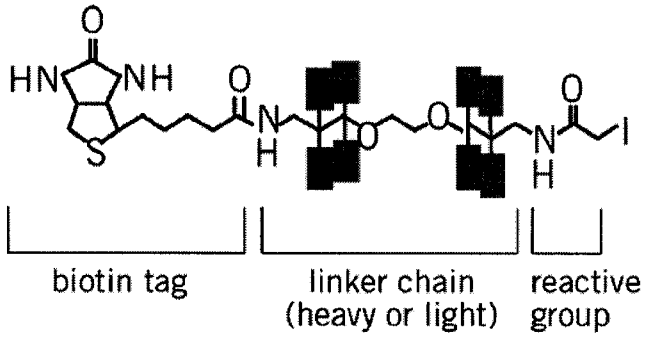
1-2.6 Isotope Coded Affinity Tagging (ICAT)

One of the major problems in proteomics is the quantification of individual proteins in a complex mixture such as a proteome. This problem has been addressed by the development of the ICAT procedure (Gygi *et al.* 1999). This procedure requires a chemical modification in which a compound containing an isotopic label and a biotin

moiety is covalently attached to the proteins in a complex mixture. An example of the chemical modifying reagent used is:

Isotope-Coded Affinity Tags

heavy reagent: D8-ICAT Reagent (X=deuterium)
light reagent: D0-ICAT Reagent (X=hydrogen)



(Gygi *et al.* 1999)

In the procedure, two separate modification reactions are carried out. One sample is modified with a D-labelled and the other with an H-labelled ICAT reagent. The two samples are mixed and digested with trypsin. The biotin tag is used to selectively isolate the labelled peptides by affinity chromatography using immobilized avidin, then the peptides are analyzed by mass spectrometry (MS) and the proteins from which they are derived identified by MS and database searches. Since two different isotopic modifying reagents are used, each peptide gives two peaks in the mass spectrum, 8 mass units apart, and the ratio of the peaks gives the relative amounts of individual peptides. One sample could be a standard, in which case the absolute amount in the other sample can be determined. Or one sample could be proteins in a normal cell and the other a diseased cell, in which case the changes in protein content in a diseased cell can be quantified.

1-2.7 Nonaqueous chemical modification

A major consideration in the chemical modification of proteins is the retention of structure and activity. In some proteomic applications, such as ICAT (Isotope Coded Affinity Tagging) for the identification and quantification of proteins in cells, this is not a consideration. However, for the production of proteins with new and/or improved properties the retention of activity is essential. Many proteins readily undergo denaturation in aqueous solution and chemical modification can increase this instability

(Hayes 2002). The challenge in carrying out such chemical modification is to select reagents and conditions that do not cause denaturation or inactivation of the protein, yet yield a modified protein with the desired properties. One of the difficulties is that each protein has its own idiosyncratic properties and often what works with one protein will not work with another. So in the production of modified active proteins it is not possible to draw up specific protocols, and hence the procedures and conditions for each new protein have to be determined. Nevertheless, one of the objectives in this field is to develop novel reagents and modification procedures that will work with a wide variety of proteins. As noted previously, there are a few potential electrophilic sites in proteins but the vast majority of reactive groups in proteins are nucleophilic in nature and as a result most chemical modification reagents are electrophiles. As is readily evident from organic synthetic procedures, water is a poor solvent for carrying out organic reactions; but as proteins are insoluble or denatured in organic solvents, conventional modification procedures described in handbooks of protein modification protocols are carried out in water, and this restricts the use of many reagents.

Taralp and Kaplan developed a methodology, *in vacuo* chemical modification, in which proteins can be modified under nonaqueous conditions (Kaplan and Taralp 1997; Taralp and Kaplan 1997). The modifications are carried out under vacuum on lyophilized proteins using gaseous reagents. One of the major advantages is that water is not present as a competing nucleophile and much smaller amounts of reagent are therefore required. In addition, when expensive isotopically labelled or radioactive reagents are used, the unreacted reagents can be recovered after the chemical modification is complete. This approach also eliminates other problems such as proteolysis and thermal denaturation often encountered under aqueous conditions. Most significantly, novel chemical modification such as trimethylation of amino groups (Kaplan and Taralp 1997; Taralp and Kaplan 1997), glycation (Kaplan *et al.* 2006), and zero-length cross-linking (Simons *et al.* 2002) of proteins can be achieved.

1-2.8 Site-directed mutagenesis

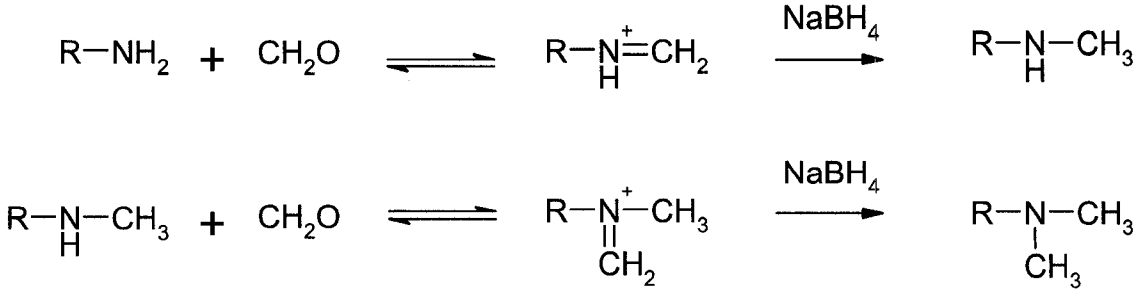
The development of site-directed mutagenesis (Hutchison *et al.* 1978), a technique pioneered by Michael Smith at University of British Columbia, provided a powerful genetic tool for the modification of proteins. This methodology makes it possible to substitute one amino acid for another at specific sites in proteins. It has proved to be invaluable in studies to determine the role of specific amino acid residues in the structure and function of proteins. For example, it was long believed that the hydroxyl of tyrosine 248 in carboxypeptidase A played an essential role in hydrogen transfer in the catalytic mechanism of this enzyme (Gardell *et al.* 1985). However, substitution of this tyrosine by a phenylalanine did not affect the catalytic mechanism. There have been many such successes with this approach but attempts to improve or introduce new properties into proteins have had only limited success (Marshall *et al.* 2003; Foley and Burkart 2007). This is due to our present poor understanding of the factors that determine the folding properties of a protein and its stability. It is possible to substitute modified amino acids but such procedures are not straightforward and substitutions can only be easily made with the 20 naturally occurring amino acids, thus limiting the chemical nature of the modifications that can be carried out. On the other hand, the number and type of potential *in vitro* chemical modifications is limited only by our ingenuity.

Chapter 2: *In vacuo* methylation: selective isolation of amino and carboxyl terminal peptides from enzymatic digests of proteins

2-1 INTRODUCTION

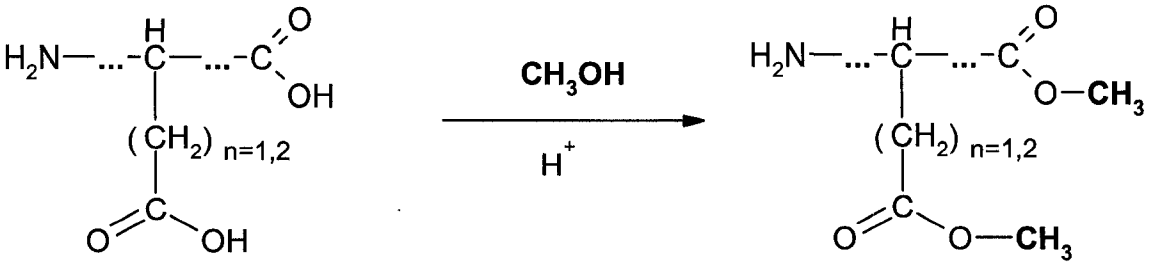
2-1.1 Overview

Reductive methylation is the most commonly used chemical modification method for protein methylation (Means and Feeney 1968; 1995). In this procedure, amino groups are dimethylated in solution using formaldehyde/borohydride as reagents.



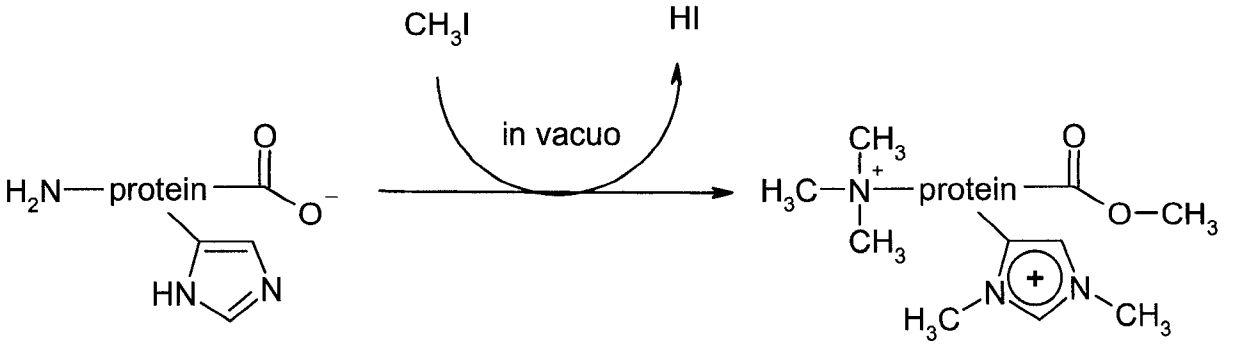
This modification reaction is relatively easy to carry out and has several desirable features. It is highly specific for amino groups and it does not alter the charge on amino groups at neutral pH values, nor in most cases does it alter the conformation and activity of the protein. On the other hand, it requires a large amount of formaldehyde and reducing reagent to drive the reaction under aqueous conditions, making the addition of labelled methyl groups inefficient. Nevertheless, this methodology has been extensively used for the radio-labelling of proteins by the introduction of ^{14}C and ^{3}H methyl groups (Heacock *et al.* 1982; Means and Feeney 1995).

Methyl groups can also be introduced into proteins by esterification of carboxyl groups with methanol (Broomfield *et al.* 1965).



The protein/methanol/H+ mixture is refluxed in a manner similar to the standard acid catalyzed esterification of small organic alcohols described in organic chemistry textbooks. These conditions inevitably result in the inactivation and some degradation of the protein. It is primarily used as a strategy when it desirable to decrease the number of acidic peptides resulting from digestion of a protein (Roepstorff 1992; Xu *et al.* 2005).

In vacuo methylation using iodomethane has been demonstrated to be a facile method for methylation of proteins (Kaplan and Taralp 1997; Taralp and Kaplan 1997).



Amino groups are converted to trimethylammonium derivatives and imidazole side chains of histidines are converted to dimethylimidazolium derivatives. These modifications result in a permanent positive charge on the derivatized amino and imidazole groups. Formation of trimethylated peptides by *in vacuo* methylation of amino groups has been demonstrated to be very useful for the identification and quantification of methylated peptides by mass spectrometry (Stewart *et al.* 2002; Poon *et al.* 2004; Laremore *et al.* 2005; Simons *et al.* 2006b). In particular, it has been shown that trimethylation of the α-amino group of a peptide increases the sensitivity of detection with MALDI-TOF-MS by at least an order of magnitude (Stewart *et al.* 2002).

Carboxyl groups in proteins can be methylated *in vacuo* in two ways, using iodomethane or methanol/HCl (Vakos *et al.* 2001). The reaction can be made relatively selective for carboxyl groups by preparing the lyophilized protein at an acid pH value (4<pH<5.5). Under these conditions, the amino and imidazole groups are protonated and should not react. In the case of methanol/HCl, the pH of lyophilisation is not as critical as the amino groups do not react. *In vacuo* reactions with both of these reagents have the advantage that very small amount of reagents are required, and in the case of labelled iodomethane or methanol, unreacted reagents can be recovered and reused.

It was noted by Vakos *et al.* (2001) that a very small but still significant amount of the two α-amino groups in insulin did react at LpH 4 (lyophilized from a pH 4 solution) and become trimethylated. This is very surprising in that the expected pK_a value of α-amino group is > 8.0 and at LpH 4 a negligible amount of deprotonated amino group should be present (< 0.01%). Similarly, a detectable amount of lysine ε-amino group with an expected pKa ~ 11 reacts at LpH 7. As only the deprotonated form of amino group can react with iodomethane, there must be some mechanism by which protons are removed from the ammonium groups to render them nucleophilic. If this explanation is correct, then it is predicted that with longer reaction time and higher temperature, protonated amino groups will become trimethylated. This can be tested by carrying out *in vacuo* methylation of protonated amino groups for a longer period of time at elevated temperature and determine the extent of trimethylation by MS analysis.

2-1.2 Protein identification

Identification of proteins in a proteome is currently one of the major research goals in proteomics. A protein is uniquely identified by its amino acid sequence and currently peptide mass fingerprints, usually of tryptic fragments, are widely used to identify proteins (Bienvenut *et al.* 2002a; Bienvenut *et al.* 2002b). The rationale behind this approach is that each protein will generate a unique peptide mass pattern (fingerprint) that can be matched to a pseudo pattern calculated from known proteins in a database and predicted proteins from a DNA-sequence database. This strategy is not straightforward because digestion is not always complete and not all fragments are detected in the mass spectrum, leading to false positives and multiple matches. Further complications arise,

because of the difficulty of obtaining pure protein samples and therefore it is not possible to be certain that all fragments arise from the same protein. This strategy can be simplified by comparing only the amino terminal (N-terminal) and carboxyl terminal (C-terminal) sequences of a protein. It has been demonstrated that knowledge of a five amino acid residue sequence at either end of the protein is sufficient to identify a protein with greater than 90% confidence (Wilkins *et al.* 1996), and using both terminal sequences it is predicted that a protein can be identified with a very high degree of confidence, greater than 99% (Wilkins *et al.* 1998). Thus, a protein can be identified by its terminal tag sequences. Moreover, the protein terminal sequences can be used to verify the correct processing of gene products and also as an indication of protein purity. This information is of particular importance to the pharmaceutical industry. In case of unknown proteins, the N and C-terminal sequences make possible the design and synthesis of oligonucleotides for selective gene isolation.

For these reasons, it would be very useful to be able to selectively isolate N and C-terminal peptides from digests of proteins. The high sensitivity of mass spectrometry could then be used to characterize and identify these peptides. N-terminal peptides have been selectively isolated from enzymatic digest of proteins (Gevaert *et al.* 2003; Stroh *et al.* 2005; Yamaguchi *et al.* 2005; Coussot *et al.* 2007); but these approaches require relatively large amounts of protein and are sequence dependent so that they are not applicable to all proteins. In addition, many proteins have blocked amino termini and any procedures requiring free amino termini will fail to detect N-terminal peptides from these proteins. Multiple-chain proteins also present difficulties for these approaches to overcome. For the selective isolation of C-terminal peptides, there is only one method that appears to be generally in its applicability and that is to amidate the carboxyl groups and subsequently to isolate the C-terminal peptides by a diagonal electrophoretic procedure (Duggleby and Kaplan 1975). However, relatively large amounts of protein (nanomole quantities) are required and as such it is not suitable for proteomic application where much smaller amounts of protein (femtomole to picomole quantities) are usually available.

The objective of the research presented in this chapter is to use *in vacuo* methylation to develop new methodology for the selective isolation of N-terminal

peptides and improved methodology for the selective isolation of C-terminal peptides from the digests of proteins. In order be useful in proteomic applications, the methodology should be applicable to all proteins including multiple-chain proteins with more than one amino terminus and proteins with blocked amino termini. It is essential that there be minimal procedural losses of peptide so only small amounts of protein are required. Another desirable feature would be that the methodology be amenable to procedures for the quantification of peptides.

2-1.3 Rationale for the selective isolation of C-terminal peptides

As noted above Duggleby and Kaplan (1975) developed a diagonal electrophoretic procedure applicable to all proteins for the isolation of C-terminal peptides from protein digests. The shortcomings of this procedure are firstly that approximately 50% of the protein is lost in the detection procedure and secondly that ninhydrin is used to detect the peptides limiting the sensitivity to nanomole quantities. However, the diagonal procedure would be applicable to much smaller quantities of proteins if the detection procedure could be improved. The scheme in **Figure 2-1** below shows how *in vacuo* methylation of carboxyl groups can be combined with the diagonal procedure to isolate selectively picomole to femtomole quantities of C-terminal peptides.

In Vacuo Methylation For Selective Isolation of C-terminal Peptides

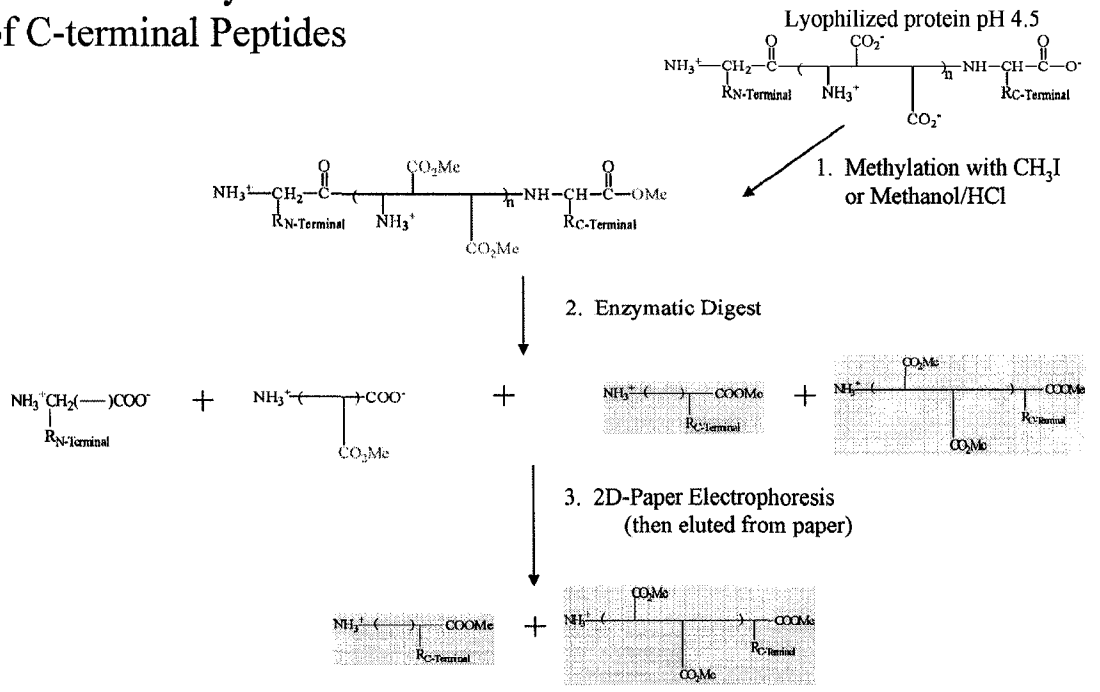
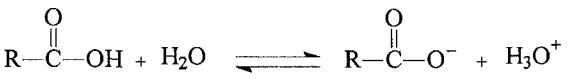


Figure 2-1. Scheme for selective isolation of C-terminal peptides.

Since proteolytic cleavage at a peptide bond will generate a free carboxyl group, all the non C-terminal peptides will have a free carboxyl group. **Figure 2-1** shows that after methylation (step 1) and enzymatic digest (step 2) only peptides derived from the C-terminus of the protein will not have a free carboxyl group. How advantage can be taken of this to isolate selectively peptides derived from the C-terminus is shown in **Figure 2-2**.

Properties of C-terminal Peptide Carboxylic Group

- At different pH (2.1 and 4.4)
  - Charge difference (gain negative charge at pH 4.4)



pK_a = 3 to 4 for carboxyl terminus

- Chemically Modified (methyl ester)
  - Structures of internal, N-terminal and C-terminal peptides
  - Methyl esters retain same charge

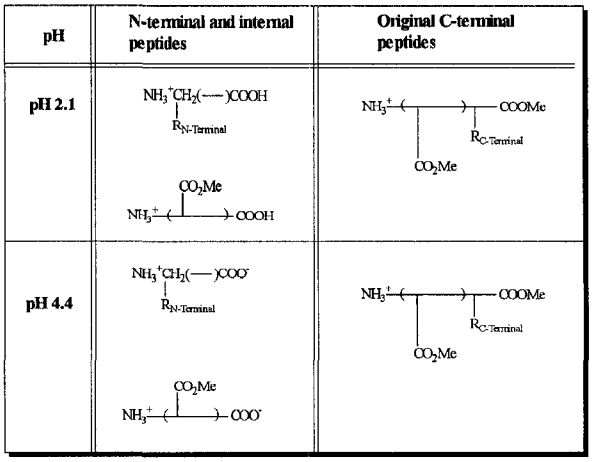
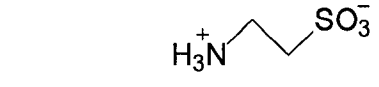


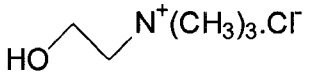
Figure 2-2. Properties of C-terminal peptide carboxylic group.

At pH 2.1, all the free carboxyl groups in the non C-terminal peptides will be protonated. If the pH is changed to 4.4, then these peptides will gain one negative charge. In contrast, peptides derived from the C-terminus will retain the same charge at pH 2.1 and 4.4. If a mixture containing C-terminal and non C-terminal peptides is subjected to electrophoresis at pH 2.1 followed by electrophoresis at pH 4.4 at a right angle to the original direction, all non C-terminal peptides with a free carboxyl group will move more slowly toward the cathode in the second dimension. In contrast, the C-terminal peptides having the same charge will have the same electrophoretic mobility at both pH values and will lie on a diagonal line of approximately 45 degrees (**Figure 2-3**). The C-terminal peptides on the diagonal can be isolated free of any non C-terminal

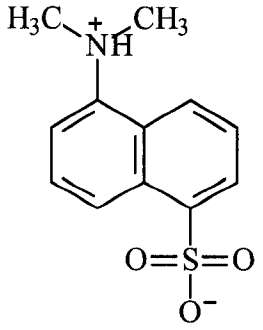
peptides. If no losses are to be incurred in locating the C-terminal peptides, the location of the diagonal needs to be delineated by non-peptide markers. This can be readily accomplished by the addition of [methyl-^{14}C]-taurine and [methyl-^{14}C]-choline chloride to the peptide digests prior to electrophoresis.



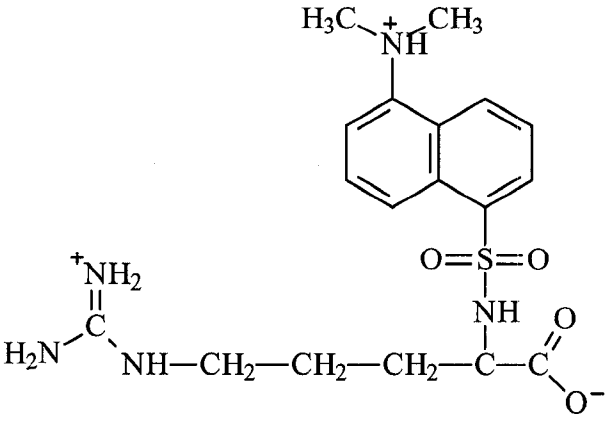
[*methyl*-^{14}C]-Taurine



[*methyl*-^{14}C]-Choline chloride



Dansyl Sulfonate



Dansyl Arginine

Figure 2-3. Radioactive and fluorescent markers used in HVPE to delineate the diagonal.

At pH 2.1 and pH 4.4 taurine will have no net charge whereas choline will have a single positive charge at both pH values. Radioautography can be used to locate them and delineate the diagonal. All C-terminal peptides will be located along the diagonal between these two markers. For routine application, radioisotopes can be avoided by using dansyl sulfonic acid (no net charge at pH 2.1 to 4.4) and dansyl arginine (+1 at pH 2.1 to 4.4) as fluorescent markers to delineate the diagonal.

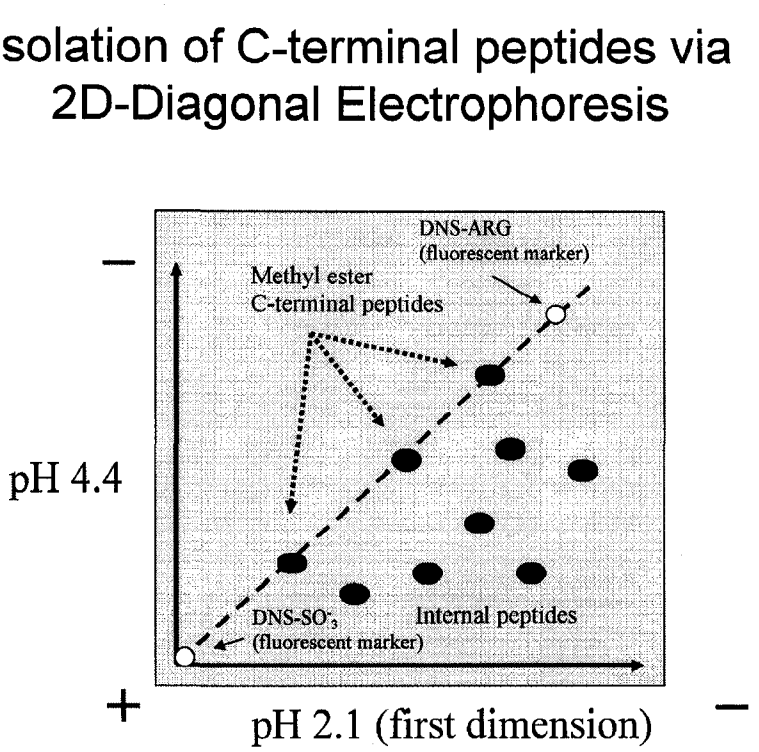


Figure 2-4. Diagonal electrophoresis for the selective isolation of C-terminal peptides from the enzymatic digest of a protein. The digest is subjected to electrophoresis at pH 2.1, then re-electrophoreses at pH 4.4 but at a right angle to the first dimension. The C-terminal peptides are located along a diagonal line delineated by fluorescent markers.

2-1.4 Rationale for the selective isolation of N-terminal peptides

Lyophilized proteins are chemically modified *in vacuo* with iodomethane resulting in the trimethylation of amino groups of the proteins (Kaplan and Taralp 1997; Taralp and Kaplan 1997). Advantage can be taken of this reaction to isolate selectively N-terminal peptides from enzymatic digest of proteins in a similar fashion adopted for the isolation of C-terminal peptides. The methylated proteins are then digested with an endoprotease to generate peptides and the peptides derived from the N-terminus are isolated as outlined by the scheme in **Figure 2-5**.

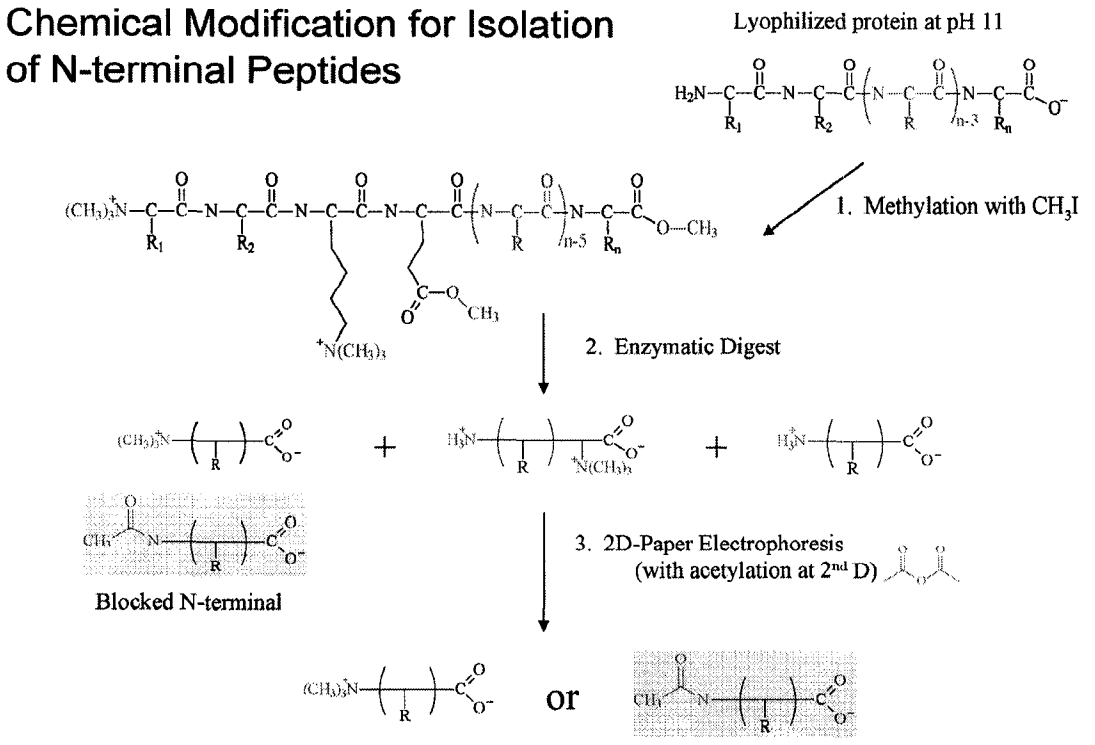


Figure 2-5. Scheme for selective isolation of N-terminal peptides.

Only peptides derived from the N-terminus will have a trimethylated α-amino group or a natural blocking group in the case of proteins with a blocked amino terminus. All other peptides generated from the interior of the protein will have a free α-amino terminus. After the first dimension, all the peptides are treated with acetic anhydride. All the non N-terminal peptides will be acetylated and lose a positive charge associated with their α-amino terminus. Peptides derived from the N-terminus will not react and retain the positive charge at their α-amino terminus. Therefore, after running a second dimension at right angles under the same electrophoretic conditions, only peptides derived from N-terminus will have the same mobility and lie on a 45 degree diagonal line (**Figure 2-6**).

Two fluororescent markers, dansyl sulfonic and dansyl arginine are used to delineate the diagonal. At pH 3.5, the charge on dansyl sulfonic will be +0 and dansyl arginine will have a charge of +1. A strip of paper can be cut out along the diagonal and eluted to obtain the N-terminal peptides. It is not necessary to visualize the peptides for isolation, since they will always be located between the two markers. However, in the

proof of principle presented here, radio-labelled [^{14}C]-iodomethane was used so that N or C-terminal peptides, as the case may be, could be visualized by radioautography. It should be noted that such radio-labelling was made possible by the *in vacuo* methylation procedure described above which permits the efficient and economical incorporation of a high degree of radio-label. Terminal peptides of various sizes may be obtained because of incomplete digestion in a non-homogenous mixture. In the case of multiple chain proteins or a mixture of several proteins, terminal peptides with different amino acid sequences with varying lengths will be obtained from the diagonal. Regardless of the situation, the diagonal methodology substantially reduces the complexity of the peptide mixtures by eliminating the non-terminal peptides, undigested proteins, enzyme, and other contaminants. Since most peptides will have a charge of +1, their mobility along the diagonal will reflect their size. Therefore, by sub-segmenting the diagonal into regions, the N-terminal peptides can be potentially simplified into peptide samples of various sizes.

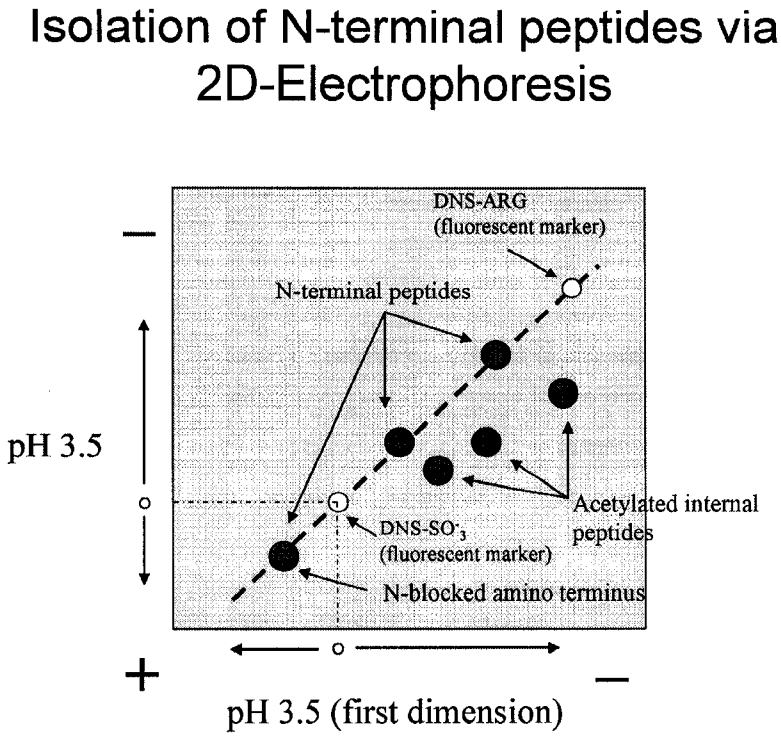


Figure 2-6. Diagonal electrophoresis for the selective isolation of N-terminal peptides from the enzymatic digest of a protein. The sample is subjected to paper electrophoresis at pH 3.5, and then the sample is acetylated with acetic anhydride before a second electrophoresis at pH 3.5 but at a right angle to the first dimension. The N-terminal peptides will be located on the diagonal delineated by the two fluorescent markers.

2-2 MATERIALS AND METHODS

2-2.1 Materials

Bovine α-chymotrypsin, bovine insulin, bovine pancreatic ribonuclease A, porcine pepsin, papain were obtained from Sigma (St. Louis, Missouri) and used without further purification. Type A allatostatin III was obtained from BACHEM (Torrance, California). Whatman 3MM paper was purchased from Sigma-Aldrich Chemical

Company (St. Louis, Missouri). Ninhydrin was obtained from Pierce (Rockford, Illinois). All other chemicals, reagents and solvents were high purity preparations obtained from commercial sources.

[^{14}C]-iodomethane (1 mCi) was purchased from Amersham Pharmacia Biotech (New England, Massachusetts). The working stock was obtained by resuspending the radiolabelled reagent in anhydrous octane to yield a specific radioactivity of 0.5 mCi/mL. The stock solution was stored at -4°C.

Three electrophoresis buffers were used for paper electrophoresis: pH 2.1 buffer (formic acid/acetic acid/water, 1:4:45, v/v), pH 3.5 buffer (pyridine/acetic acid/water, 1:10:89, v/v), pH 4.4 buffer (pyridine/acetic acid/water, 1:2:47, v/v). Amine free water was used, prepared by passing double distilled water through two column cartridges of mixed bed resin and activated carbon (Barnstead 16-D0832) purchased from VWR Canlabs (Montreal, Quebec).

2-2.2 *In vacuo* methylation of lyophilized proteins

Proteins (20 mg to 100 mg) were suspended in water (~ 10 mg/mL) and the pH of the solution was adjusted to either 4.5 or 10.0 using 1N HCl or 1N NaOH. The pH was monitored with a pH meter (Model pH-METER26; RadioMeter/Copenhagen). After the pH adjustment, additional distilled water was added to give a final protein concentration of 5 mg/mL and aliquots of 4.0 mL were transferred to Pyrex borosilicate tubes (size 13x100 mm). The samples were then flash frozen with liquid nitrogen and lyophilized; note the pH of the solution at which the samples were lyophilized is termed LpH. The tubes were shortened (13x40mm) and placed inside larger Pyrex borosilicate tubes (size 16x150 mm) for methylation under vacuum.

In vacuo methylations with [^{14}C]-iodomethane (30 μL; 15 μCi) in octane were carried out in sealed reaction vessels that were placed in an oven at 75°C for 24 h according to the procedure described by Taralp and Kaplan (1997). After the reaction, excess reagent was recovered by trapping with liquid nitrogen as previously described (Taralp and Kaplan 1997) and the reaction vessels were opened. The smaller glass inserts with the samples were then placed in new tubes (16x150 mm) and then *in vacuo*

methylation process was repeated with [^{12}C]-iodomethane (40 μL) to ensure full methylation.

2-2.3 Two-dimensional diagonal HVPE for the selective isolation of C-terminal peptides

2-2.3.1 First-dimension HVPE

The chromatography paper (Whatman 3MM, 40 cm x 57 cm) was placed on a clean glass surface and the origin line was drawn parallel but 10 cm away from the edge of the paper. The enzyme-digested α-chymotrypsin (1 mg) sample was dissolved in pH 2.1 buffer (500 μL). The solution of the dissolved sample was then applied via capillary pipette along the origin line of the paper as a band 5 cm wide. Typically, three separate samples were applied, 5 cm wide and separated by a 5 cm space between the samples. Dansyl arginine (DNS-Arg) (20 μL, 1 mg/mL) was applied at the edges and in the middle of the band as a fluorescent marker. A mixture of [methyl-^{14}C]-taurine and [methyl-^{14}C]-choline chloride (30 μL) marker was applied in the middle of the band in the same manner (to delineate the diagonal after the second dimension). The sample and markers were dried after each application with a stream of warm air. The entire paper was then wetted with pH 2.1 buffer, applied carefully at equal distance on each side of the sample band, so that the buffer front concentrates the sample evenly along the origin line; a process known as "buffering-in". Excess solvent was removed from the paper by simply blotting and the paper was subjected to HVPE.

The first-dimension electrophoresis was performed for 30 min at 2.5 kV (voltage gradient of 40 V/cm) in the tank containing pH 2.1 electrode buffer, at approximately 25°C. The paper was then removed from the tank and hung to air-dry in a well-ventilated fumehood.

2-2.3.2 Second-dimension HVPE

With the aid of the fluorescent markers, the sample band can be cut out as a strip of paper and subsequently sewn onto a second full size chromatography paper, but rotated orthogonally. The origin of the cut-out strip was aligned with the new origin. The paper was then buffered-in with pH 4.4, in the same procedure as described above.

Electrophoresis was again performed for 30 min at 2.5 kV (voltage gradient 40 V/cm), this time in the tank containing pH 4.4 electrode buffer. The paper was removed and treated in the same manner as above.

2-2.3.3 Autoradiography

The dried papers were marked with radioactive ink, prepared by the addition of waste ^{14}C isotopes to ink (~1000 cpm/μL), to aid the re-alignment of the developed film, and autoradiographed with Fuji x-ray films for 48 h. The film and chromatogram are kept in close contact in a dark room. The films were then developed and fixed with Kodak fixing reagent, with a final rinse in distilled water before drying.

2-2.4 Two-dimensional diagonal HVPE for the selective isolation of N-terminal peptides

2-2.4.1 First-dimension HVPE

The first-dimension electrophoresis of the isolation of the N-terminal peptides was carried out in the same manner as the procedure for the first-dimension run of the C-terminal peptide isolation described above. The electrophoresis buffer used was either pH 2.1 or pH 3.5.

2-2.4.2 Treatment with acetic anhydride

The sample band from the first dimension was cut out from the paper and dipped three times in acetic anhydride solution (pyridine/acetic anhydride/water/acetone, 2:8:10:80, v/v/v/v) with 15 minutes wait between dipping before drying in a fumehood for 2 h. The dipping solution cocktail can be prepared ahead of time but the acetic anhydride component was added at the time of first dipping. A control test strip with multiple spottings (1 μL) of lysine (1 mg/mL) and alanine (1 mg/mL) amino acids was also dipped in parallel with the sample band strip and then stained with cadmium/ninhydrin to verify complete reaction with acetic anhydride. The sample paper strip was then folded in half along its length and buffered-in with 2% pyridine buffer (pyridine/water, 1:50, v/v) before hanging to dry in a fumehood. This step concentrated the peptides into the center of the strip and hydrolyzed any esters formed with acetic anhydride. The sample strip was trimmed at the edge to obtain a narrower width of only

3 to 5 cm wide in situations where the sample was spotted wider than 5 cm in the first dimension, for example 20 cm wide.

2-2.4.3 Second-dimension HVPE

The strip of paper from first dimension treated with acetic anhydride was subjected to a second-dimension HVPE using the same buffer and conditions as the first dimension, but at a right angle to the first dimension.

2-2.5 Determination of digestion time required for the isolation of C-terminal peptides of α-chymotrypsin

2-2.5.1 Pepsin digest of α-chymotrypsin

The ^{14}C-labelled α-chymotrypsin sample (5.3 mg; LpH 4.5) was transferred to a 20-mL glass scintillation vial and dissolved in formic acid (88%; 600 μL) and then made up to 10% formic acid solution with distilled water (4.5 mL). Aliquots of 1.0 mL each were transferred into five 20-mL liquid scintillation glass vials. Pepsin in 10 % formic acid (20.0 μL; 1.0 mg/mL) was added to each aliquot (noting time zero) to give a protein/enzyme ratio of 50:1. The enzymatic digestion was incubated in a water bath at 37°C and allowed to proceed for time intervals of 15 min, 30 min, 1 h, 2 h and 24 h. After incubation, 5.0 mL of distilled water was added and the reaction vessel was shell frozen over liquid nitrogen and subsequently lyophilized. The samples were then subjected to HVPE as described above for the isolation of C-terminal peptides (section 2-2.3).

2-2.5.2 Papain digest of α-chymotrypsin

The ^{14}C-labelled α-chymotrypsin sample (5.4 mg) was transferred to a 20-mL glass scintillation vial and dissolved in 10% (v/v) diluted pH 4.4 buffer (5.4 mL) and 5 μL β-mercaptoethanol was added. Aliquots of 1.0 mL each were transferred into fives 20-mL liquid scintillation glass vials. Papain enzymatic solution in pH 4.4 buffer (1.0 mg papain/mL; 20.0 μL) was added to each aliquot (noting time zero) to give a protein:enzyme ratio of 50:1. The enzymatic digestion was incubated in a water bath at 37°C and allowed to proceed for time intervals of 15 min, 30 min, 1 h, 2 h and 24 h. The reactions were stopped at the specified time intervals by freezing in liquid nitrogen and

subsequently lyophilized. The samples were then subjected to HVPE as described above for the isolation of C-terminal peptides (section 2-2.3).

2-2.6 Isolation of N-blocked peptides

Bovine α-chymotrypsin was trace acetylated with [^{14}C]-acetic anhydride and followed with [^{12}C]-acetic anhydride in 8 M urea at pH 8 as described by Fraenkel-Conrat (Fraenkel-Conrat 1957) and followed with modifications (Smith 1981). The acetylated α-chymotrypsin was subjected to the N-terminal isolation procedure with *in vacuo* methylation at LpH 10 as described in section 2-2.2, digested with pepsin at a protein to enzyme ratio of 50:1 for 24 h in 5% formic (v/v), and performic oxidized (Hirs 1967) before subjecting to HVPE at pH 3.5 according to procedures outlined in section 2-2.4.

2-2.7 *In vacuo* methylation of Type A Allatostatin III at acidic pH values

Type A allatostatin III (1.20 nmol/μL) was solubilised with distilled water and a 200 μL volume of stock peptide was diluted with either 800 μL of 0.12 N HCl or TFA. Aliquots of 50 μL (10 nmol allatostatin) were pipetted into 1.5-mL microfuge tubes, briefly centrifuged before freezing with liquid nitrogen, and then lyophilized in a speed vacuum. The samples in microfuge tubes were then placed in Pyrex borosilicate tubes (size 16x150 mm) and *in vacuo* methylated with 40 μL iodomethane as described in section 2-2.2, but incubated at 75°C for various lengths of time (0 h, 1 h, 2 h, 8 h, 16 h, 24 h, 36 h, 48 h, and 72 h). A control standard sample of allatostatin III lyophilized in 1% (w/v) ammonium bicarbonate was separately *in vacuo* methylated with deuterated iodomethane (ICD_3) at 85°C for 48 h and then solubilised in 0.01% (v/v) TFA to final stock concentration of 1.00 nmol/μL. Aliquot volume of 10 μL standard (10 nmol [D]-trimethylated allatostatin III) was added to each of the test samples methylated with [H]-iodomethane and analyzed by electrospray ionisation mass spectrometry (ESI-MS). The mass spectrometric analysis of the samples was carried out by the Centre for Biologics Research division at Health Canada. A Micromass™ Q-TOF mass spectrometer (Waters) was used to obtain the nanospray mass spectrum of the peptides and

deconvolution of the spectrum to produce data which reflected singly charged average masses was carried out by using MaxEnt1™ software (MassLynx™, Waters).

2-3 RESULTS

2-3.1 Diagonal electrophoresis for C-terminal peptides

Bovine α-chymotrypsin has three polypeptide chains and therefore three C-termini. The carboxyl groups of α-chymotrypsin were methylated *in vacuo* with [^{14}C]-iodomethane, digested with pepsin (50:1), and then the digest was subjected to the diagonal electrophoresis procedure outlined in **Figure 2-4**. **Figure 2-8** shows the radioautographs of the diagonal electrophoretogram obtained for pepsin digests carried out for various lengths of time and **Figure 2-9** for papain digests. As predicted from the results reported by Duggleby and Kaplan (1975), a number of spots are visible along the diagonal between the two diagonal markers, where peptides derived from the C-terminus are expected to be located. The number and intensities of the spots (**Figure 2-7**) and hence the amounts of the various C-terminal peptides will vary with the digestion time. For this particular case, a digestion time of 30 to 60 minutes appears to be the optimum time. Interestingly, some of the non C-terminal peptides appear to form a linear pattern lying below the main diagonal. A possible explanation for this will be given the discussion section.

Insulin has two polypeptide chains and therefore two C-termini, **Figure 2-10** shows the electrophoretogram for digestion of insulin with pepsin (50:1) for one hour. Two radioactive spots are visible on the diagonal line. In the case of bovine ribonuclease which consists of a single polypeptide chain, only one major radioactive spot is visible (**Figure 2-11**) for digestion with pepsin carried out under the same conditions as for insulin.

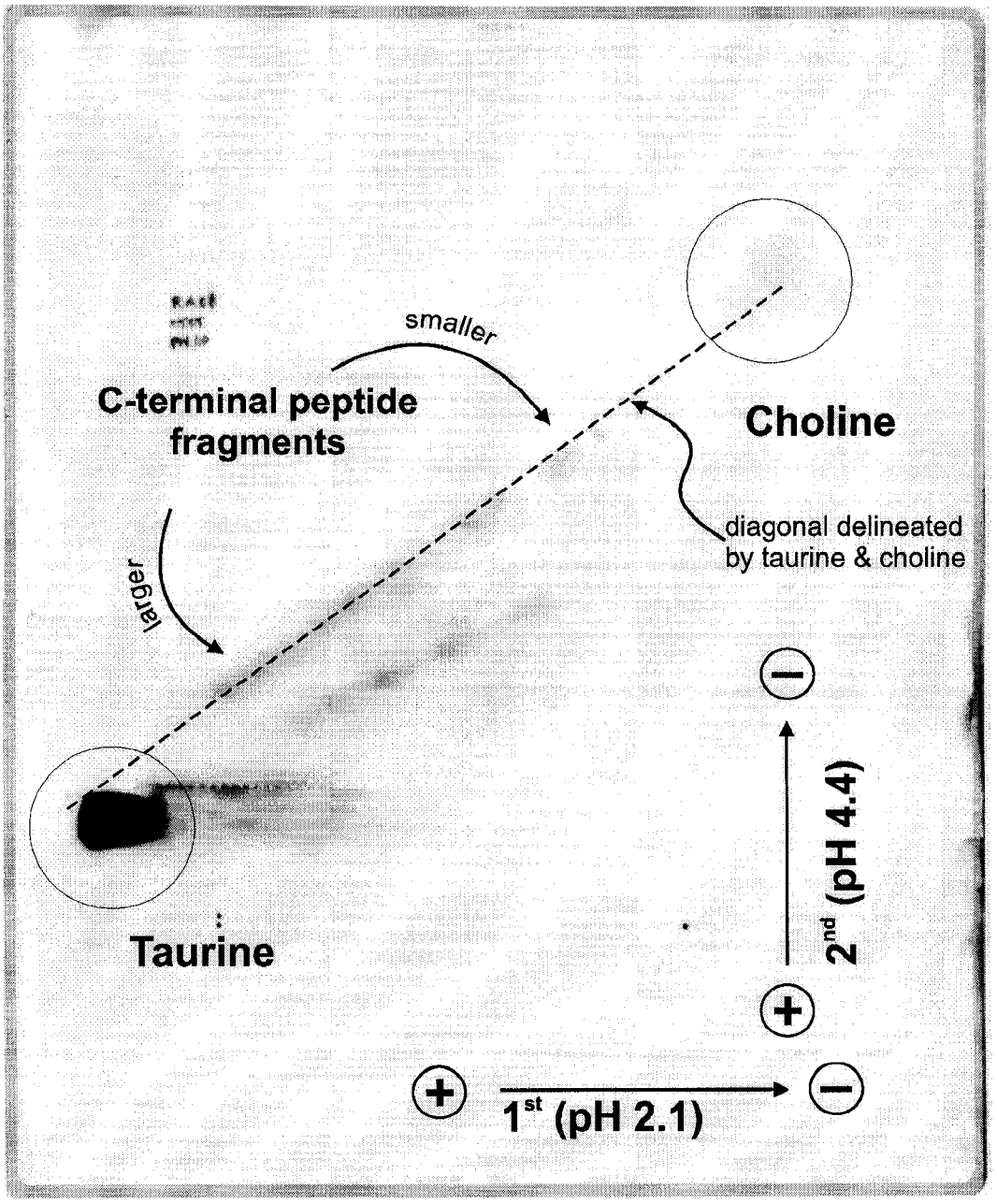


Figure 2-7. Radioautogram of a 2D-HVPE diagonal for isolation of C-terminal peptides. Pepsin digested ^{14}C-methylated α-chymotrypsin (100:1 w/w protein:enzyme ratio) for 60 min at 37°C. Radioactive markers, [methyl-^{14}C]-taurine and [methyl-^{14}C]-choline, are identified as spots at the origin and extremity of the diagonal, respectively. The large C-terminal peptide fragments have low electrophoretic mobility, and are indicated with solid arrows. Dashed line delineates the diagonal containing the C-terminal peptides.

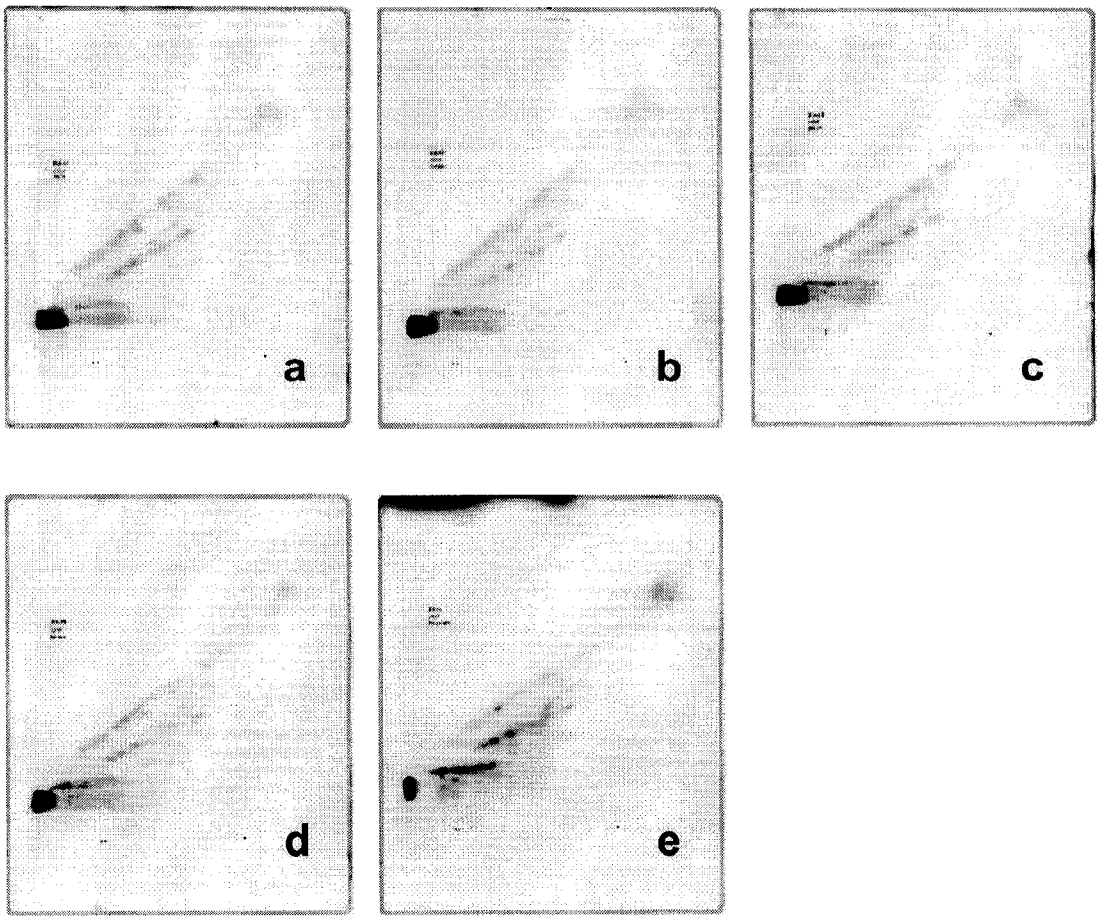


Figure 2-8. Radioautograms of C-terminal 2D-HVPE of pepsin digest ^{14}C-methylated α-chymotrypsin. Pepsin digest at 100:1 protein:enzyme ratio at 37°C. a) digestion for 15 min, b) 30 min, c) 60 min, d) 2 h, and e) 24 h.

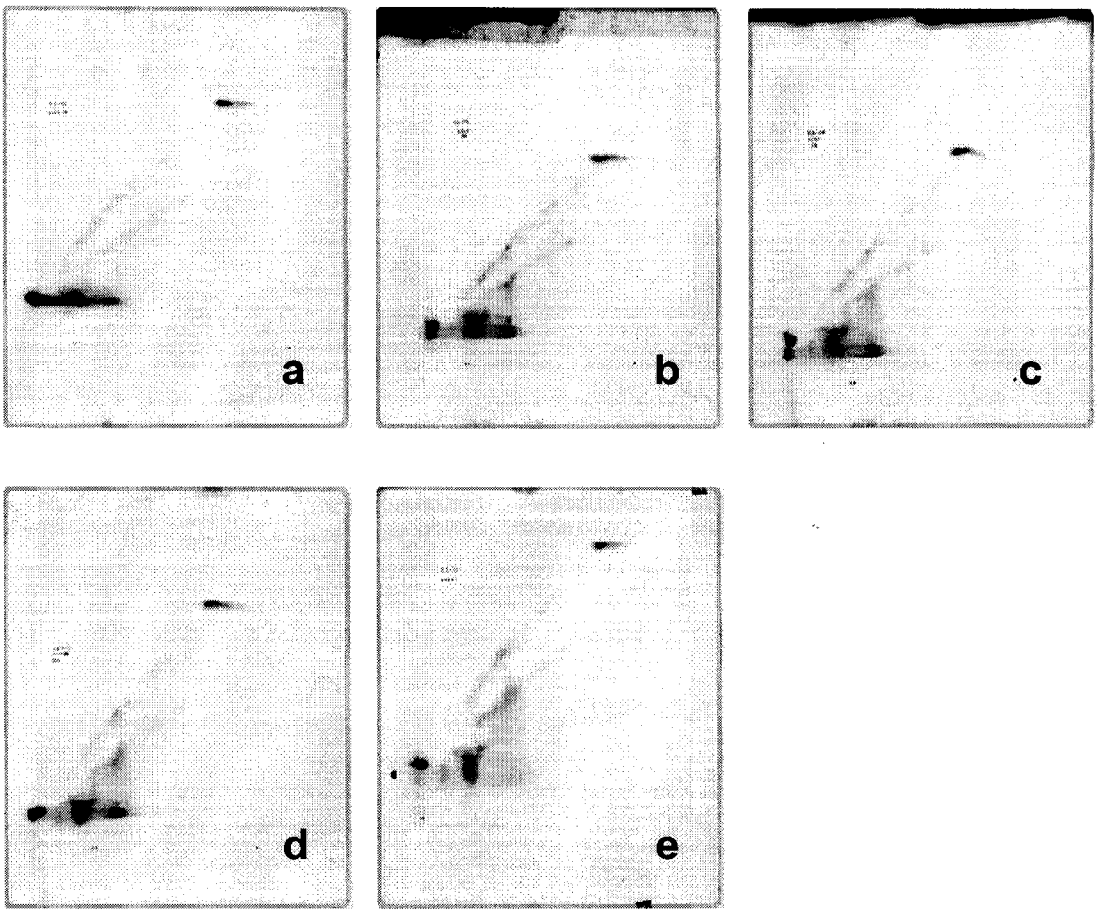


Figure 2-9. Radioautograms of C-terminal 2D-HVPE of papain digest ^{14}C-methylated α-chymotrypsin. Papain digest at 50:1 protein:enzyme ratio at 37°C. a) digestion for 15 min, b) 30 min, c) 60 min, d) 2 h, and e) 24 h.

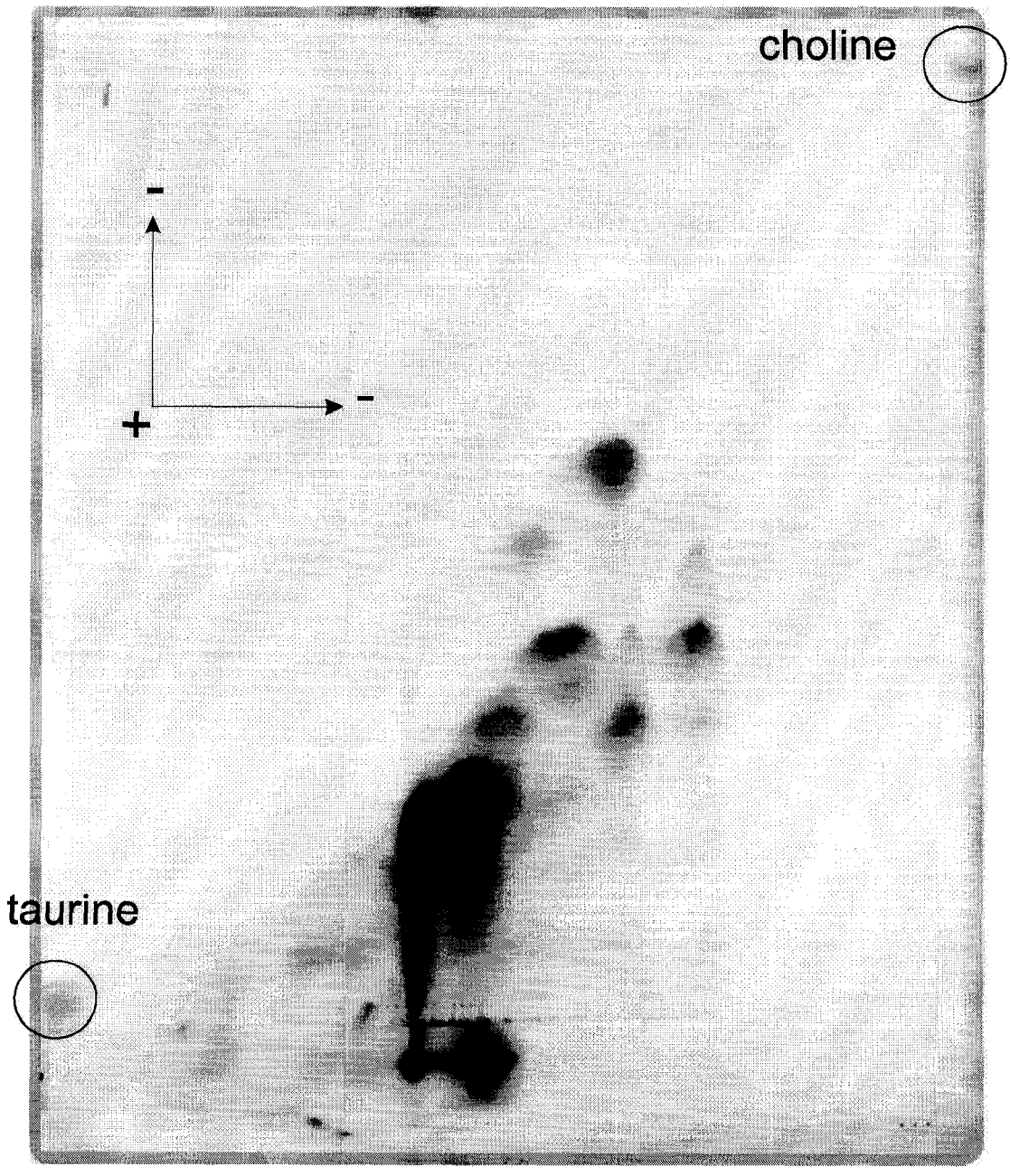


Figure 2-10. Radioautogram of C-terminal 2D-HVPE of ^{14}C-methylated bovine insulin digested with pepsin (50:1 protein:enzyme ratio) for 24 h at 37°C.

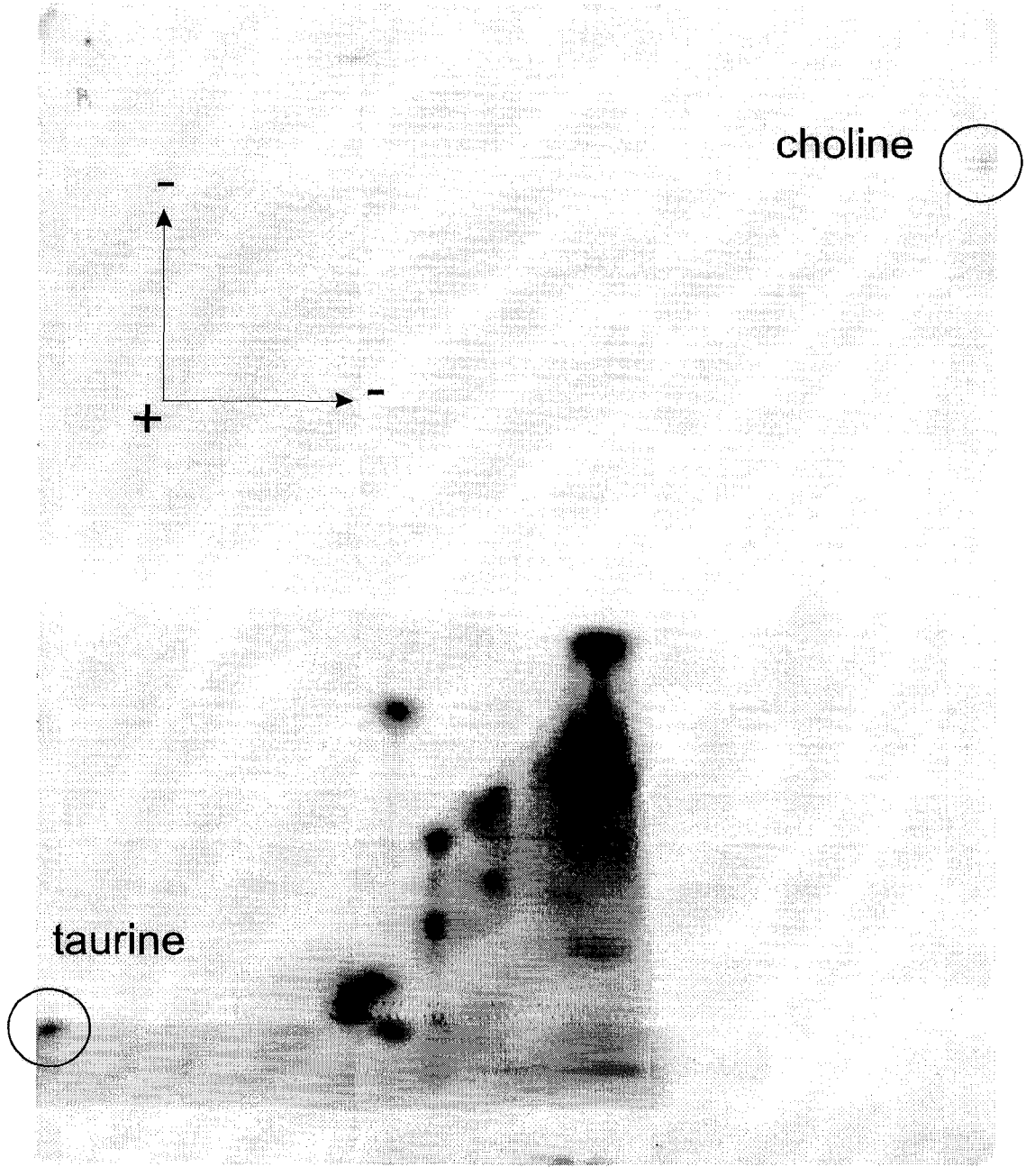


Figure 2-11. Radioautogram of C-terminal 2D-HVPE of ^{14}C-methylated bovine ribonuclease A digested with pepsin (50:1 protein:enzyme ratio) for 24 h at 37°C.

2-3.2 Diagonal electrophoresis for N-terminal peptides

The two α-amino groups of insulin were trimethylated *in vacuo* with [^{14}C]-iodomethane, digested with pepsin (50:1), and then the digest was subjected to the N-terminal diagonal electrophoresis procedure outlined in **Figure 2-6**. **Figure 2-12** shows the radioautographs of the diagonal electrophoretogram obtained for digests carried out for various lengths of time. As predicted, a number of spots are visible along the diagonal between the two diagonal markers, where peptides derived from the N-terminus are expected to be located. The number and intensities of the spots (**Figure 2-12**) and hence the amounts of the various N-terminal peptides will vary with the digestion time. Again for this particular case, a digestion time of 30 to 60 minutes appears to be the optimum time to obtain large peptides.

The radioautogram obtained for chymotrypsin (**Figure 2-13**), which has three amino termini, is shown for a one hour digest with pepsin (50:1). Similar to the C-terminal diagonal **Figure 2-7**, a large number of spots are visible between the two markers along the diagonal line where the N-terminal peptides are expected to be located.

The N-terminal peptides from proteins with blocked N-termini should also lie along the diagonal line, since they will not react with acetic anhydride and their electrophoretic mobility will not change in the second dimension. However, since these peptides will not have a free amino group, they will be negatively charged at pH 3.5, and move toward the anode. They are therefore expected to lie on the diagonal line, but on the anode side of the neutral diagonal marker. The free α-amino and ε-amino groups of α-chymotrypsin were acetylated with [^{14}C]-acetic anhydride, reacted with [^{14}C]-iodomethane, digested with pepsin (50:1), performic oxidized, and then subjected to N-terminal diagonal procedure. **Figure 2-14** shows several spots on the anode side of the diagonal where the blocked (acetylated) peptides derived from the amino terminus are expected to be located. There are also two spots on the cathode side of the diagonal and these spots can only arise from blocked N-terminal peptides containing a basic residue, either arginine or histidine.

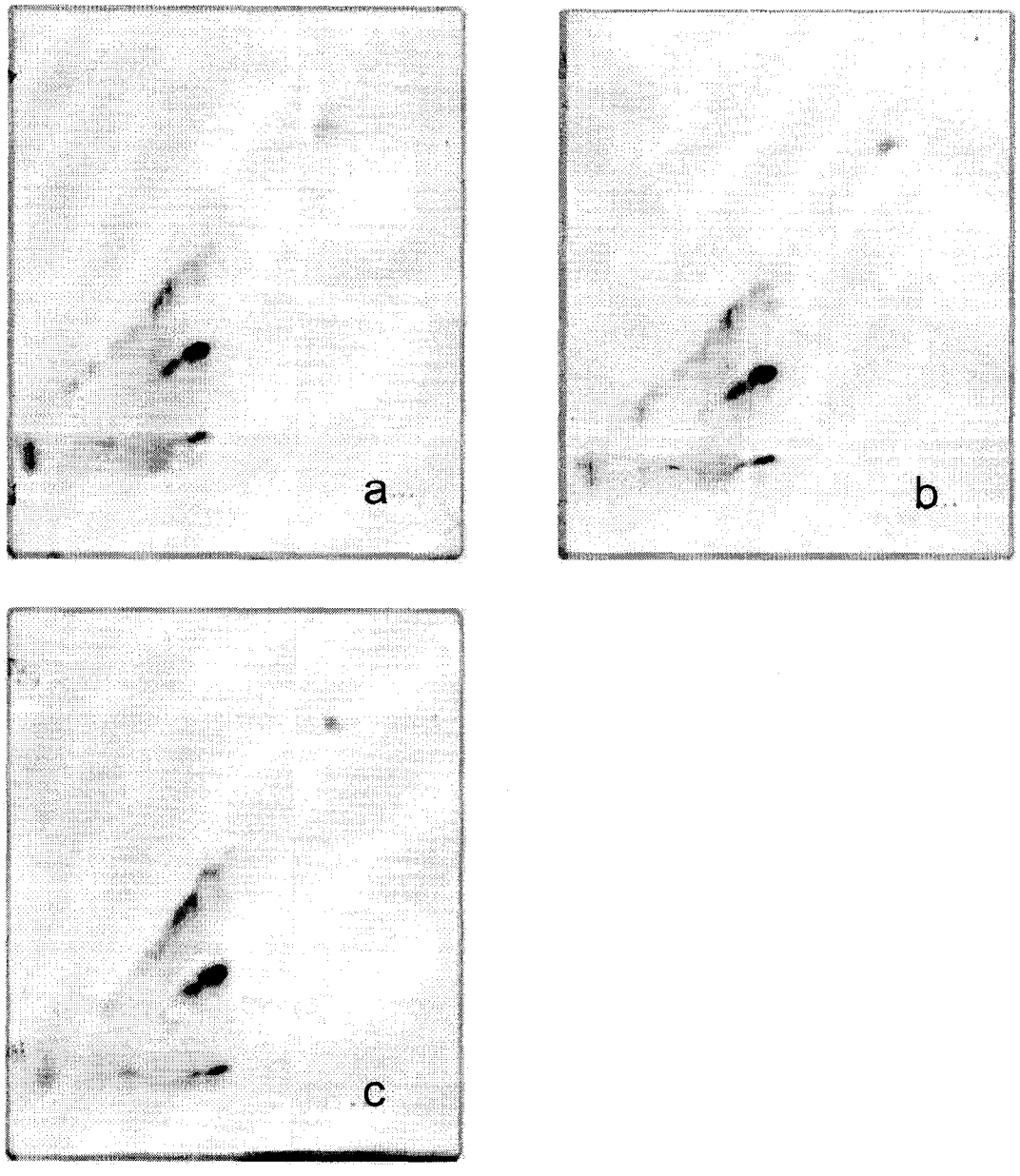


Figure 2-12. Radioautograms of N-terminal 2D-HVPE of pepsin digest ^{14}C-methylated LpH 10 insulin. Pepsin digest at 50:1 protein:enzyme ratio at 37°C. a) digestion for 1 h, b) 4 h and c) 18 h.

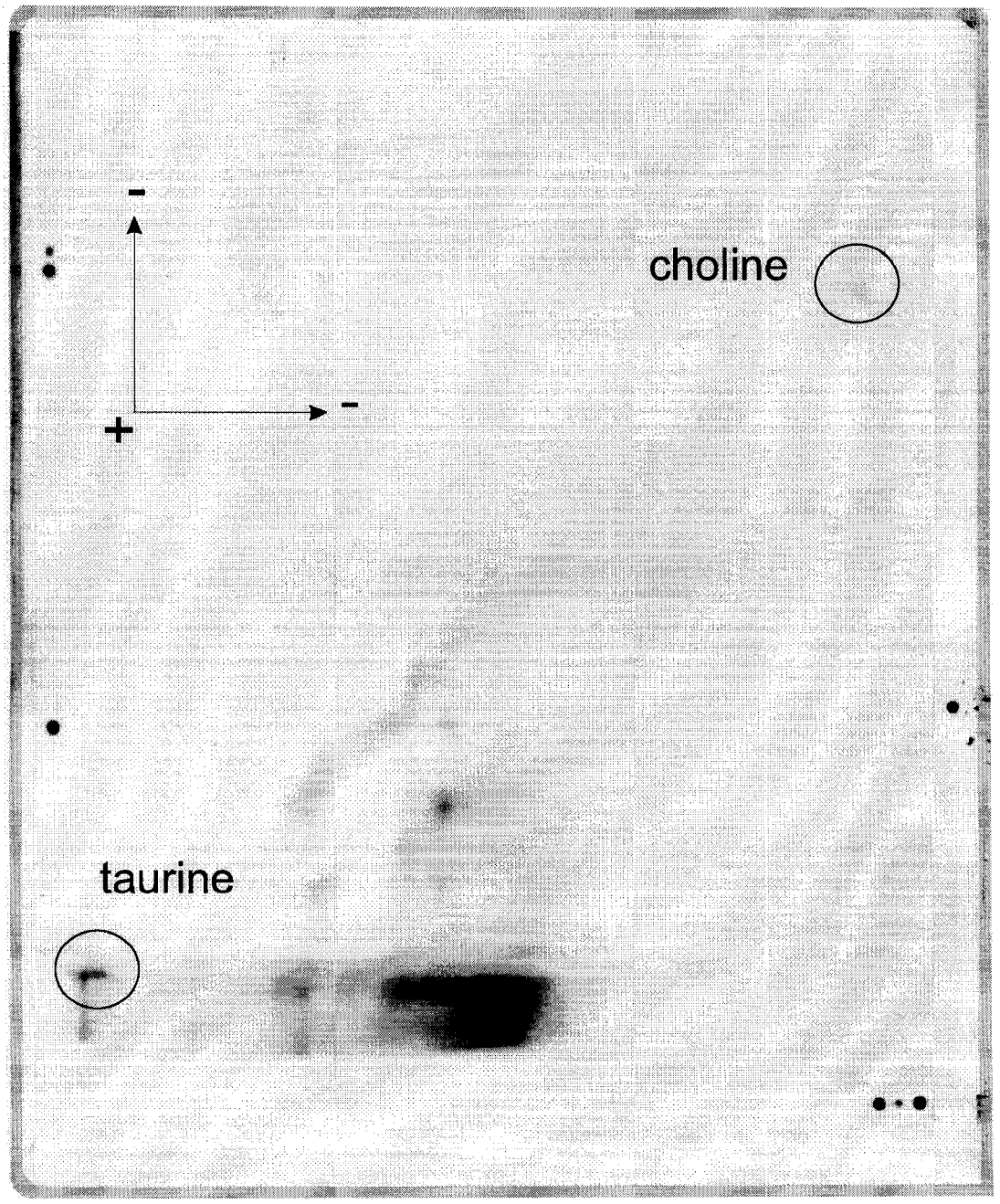


Figure 2-13. Radioautogram of N-terminal 2D-HVPE of ^{14}C-methylated α-chymotrypsin digested with pepsin (50:1 protein:enzyme ratio) for 24 h at 37°C.

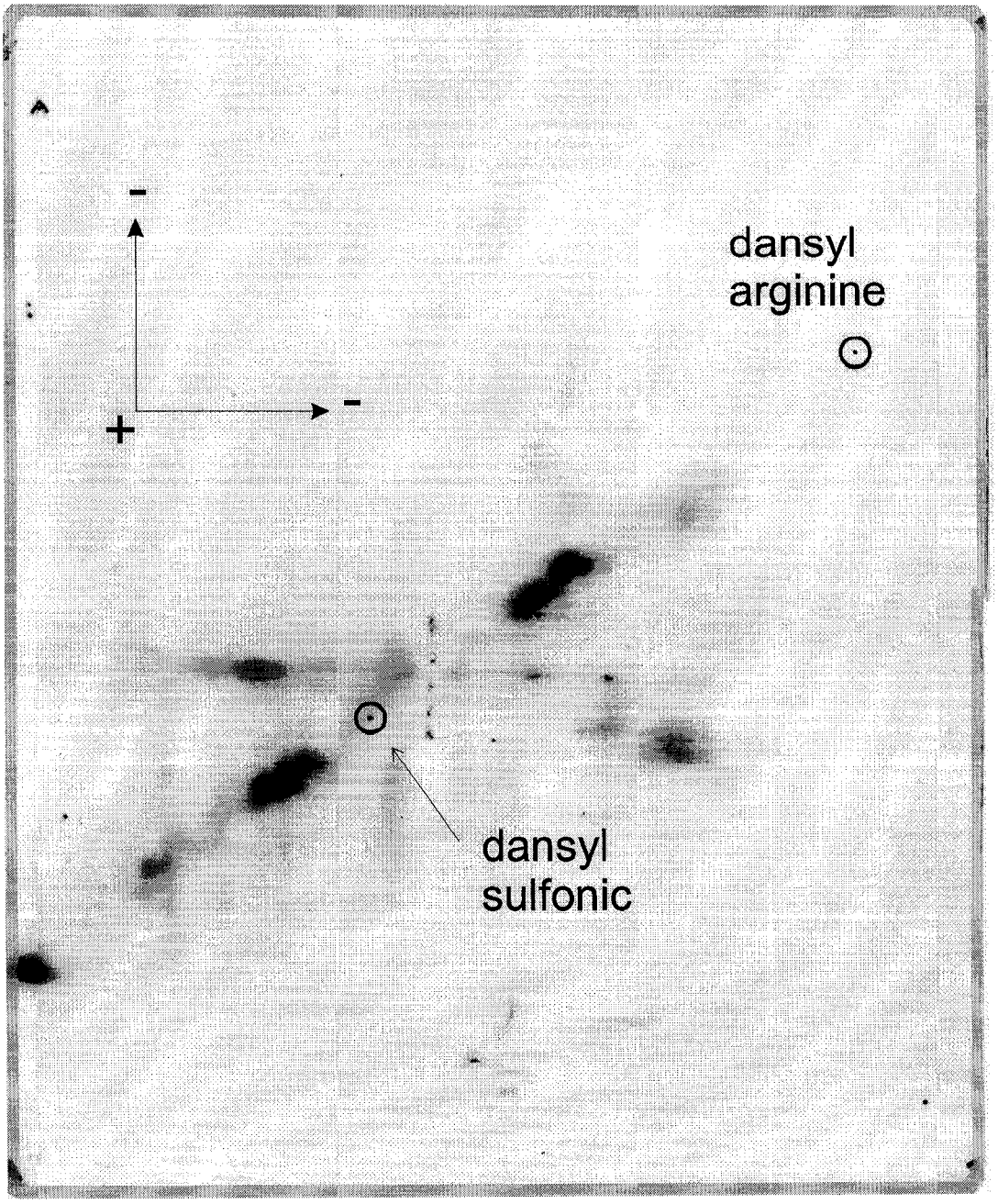


Figure 2-14. Radioautogram of N-terminal 2D-HVPE of ^{14}C-acetylated α-chymotrypsin digested with pepsin (50:1 protein:enzyme ratio) for 24 h at 37°C. Fluorescent markers dansyl sulfonic and dansyl arginine are identified as spots at the origin and extremity of the diagonal, respectively.

2-3.3 *In vacuo* methylation of amino group at acidic LpH

In vacuo reaction of type A allatostatin III with iodomethane was carried under conditions under where the amino group was protonated by lyophilisation from solution of two different acids, 0.10 N TFA and 0.10 N HCl. An amount of allatostatin III (MW 950.6) fully methylated with ICD_3 equal to the non-methylated allatostatin III (MW 899.5) was added as a standard for MS quantification (**Figure 2-15**). **Figure 2-16** and **Figure 2-17** demonstrate under these conditions where the amino group is protonated that trimethylation does occur. A greater extent of reaction does take place with the allatostatin III lyophilized from TFA than HCl (**Figure 2-18**). Assuming CD_3 (MW 950.6) and CH_3 (MW 941.5) derivatives are detected with equal sensitivity, after 72 hours approximately 50% of the amino group is trimethylated in the TFA treated sample and 10% for HCl treated samples. The result in **Figure 2-15** shows that the trimethylated peptide is detected with a much greater sensitivity than the non-methylated peptide. Based on the relative peak heights, the sensitivity is increased by a factor of 18.

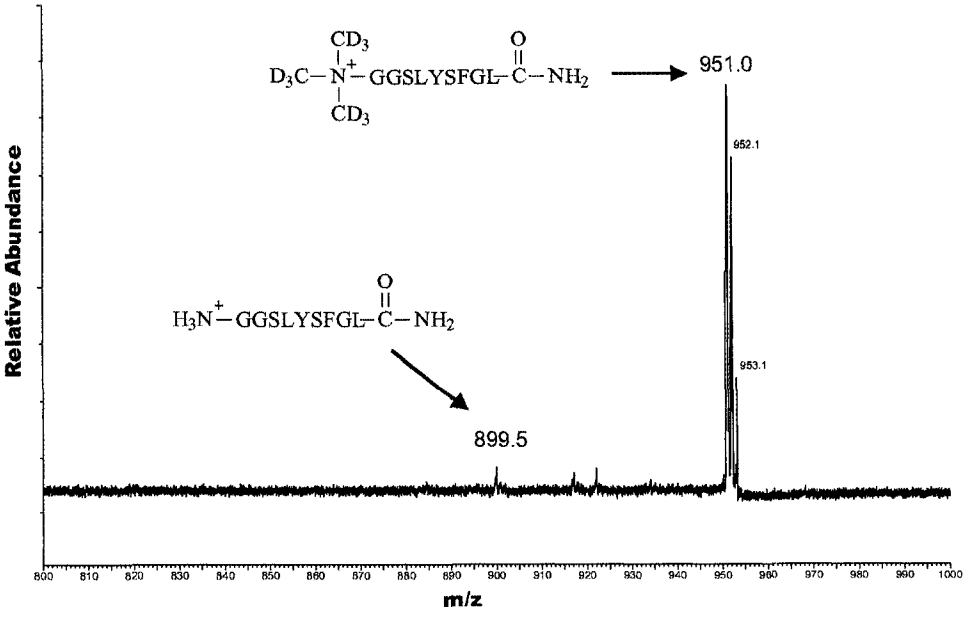


Figure 2-15. ESI-MS of equal mixture of type A allatostatin III (MW=899.5) and trimethylated type A allatostatin III (MW=951.0).

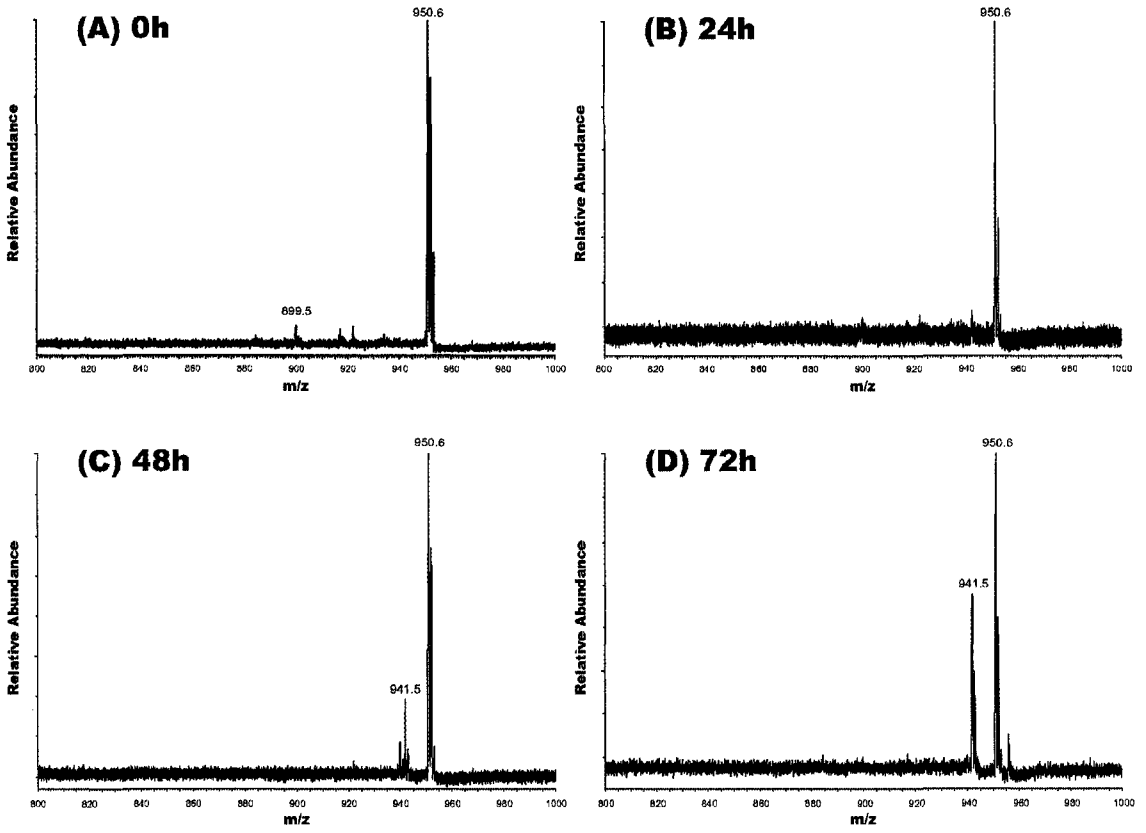


Figure 2-16. ESI-MS of type A allatostatin III lyophilized in 0.10 N TFA and *in vacuo* methylated with idomethane for various incubation time interval at 75°C, (a) 0 h, (b) 24 h, (c) 48 h and (d) 72 h. A control of equal amount of allatostatin III fully methylated with ICD_3 at LpH 8 was added to each sample.

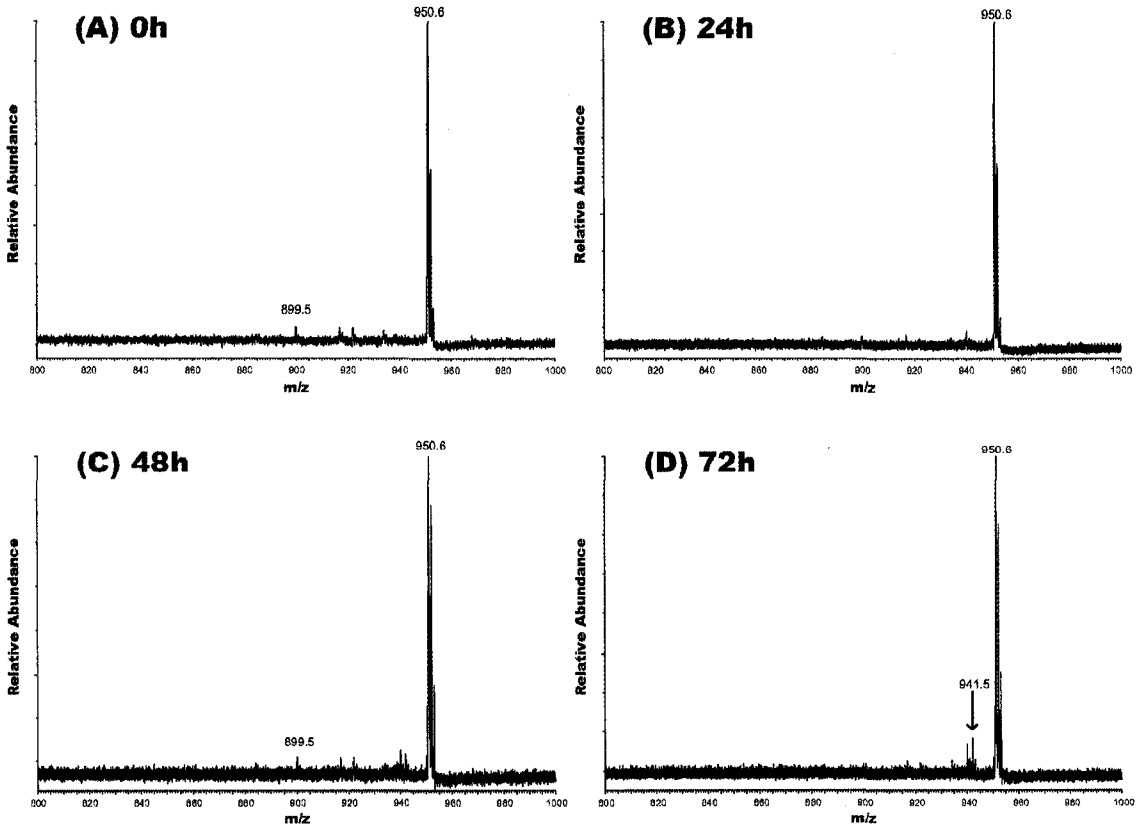


Figure 2-17. ESI-MS of type A allatostatin III lyophilized in 0.10 N HCl and *in vacuo* methylated with idomethane for various incubation time interval at 75°C, (a) 0 h, (b) 24 h, (c) 48 h and (d) 72 h. A control of equal amount of allatostatin III fully methylated with ICD_3 at LpH 8 was added to each sample.

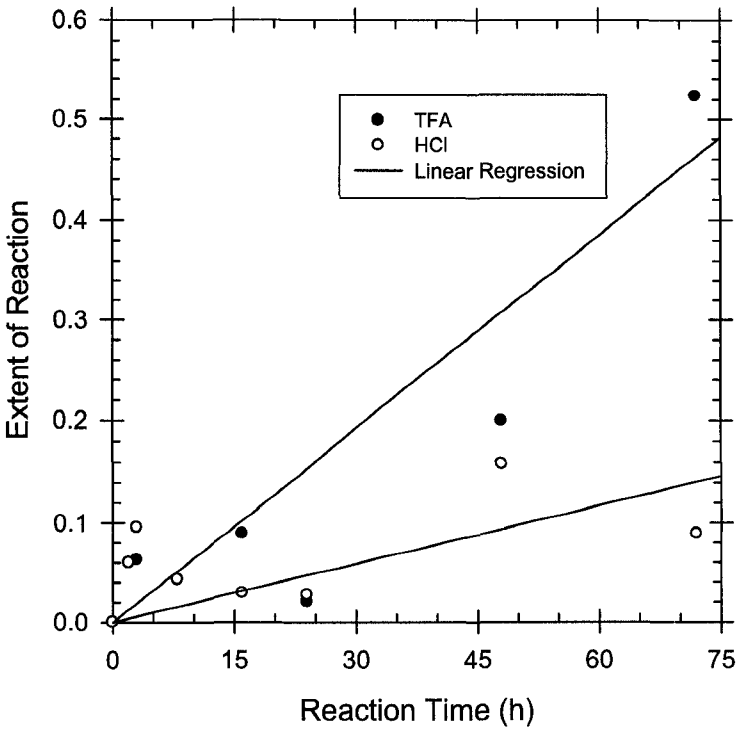


Figure 2-18. Extent of trimethylation of type A allatostatin III lyophilized with either TFA or HCl. The extent of reaction is measured by the ratio of peaks (941.5 m/z) to the fully methylated control peaks (950.6 m/z).

2-4 DISCUSSION

C-terminal diagonal procedure described in this chapter is a variation of the diagonal procedure developed by Duggleby and Kaplan (1975). The significant improvement is that much smaller of amounts of the C-terminal peptides can be isolated and therefore much smaller amounts of protein are required. In the proof of principle, the carboxyl groups of proteins were radio-labelled with [^{14}C]-iodomethane as this provided easy detection and visual proof that the C-terminal peptides do indeed lie along a diagonal line. In practice, it is not necessary to use radiolabels. It is only necessary to locate the diagonal line and this can be done using fluorescent markers, dansyl sulfonic and dansyl arginine (**Figure 2-14**). All the C-terminal peptides lie on the diagonal between these two markers and can be eluted for MS analysis.

In the case of the procedure developed by Duggleby and Kaplan (1975), each C-terminal peptide had to be isolated individually for analysis and this required the sacrifice of a significant amount the peptide (approximately 50%) for its detection and localization on the electrophoretogram. In the present procedure, all the C-terminal peptides are isolated together without any losses of peptide for detection purposes, and MS can be used to analyse each individual peptide in the mixture. Either ESI or MALDI MS can be employed to give the mass of each peptides and further tandem MS can be used to determine the sequence of the peptides. A further refinement would be to react the carboxyl groups with an equimolar mixture of ICD_3 and ICH_3. Each C-terminal peptides should give two peaks of equal intensities separated by three mass units on MS analysis. This could serve to differentiate peptide peaks from non-peptide contaminants in the MS spectrum (Stewart *et al.* 2002).

As noted previously, there is a second linear array of spots below the diagonal line in the C-terminal diagonal of α-chymotrypsin (**Figure 2-7**). They are not C-terminal peptides, but they contain radio-labelled carboxyl groups which can only arise from methylation of the carboxyl side chain of aspartic or glutamic acids. In the second dimension run (at pH 4.4), the charges on the terminal α-amino and terminal α-carboxyl groups will cancel each other out, and such peptides will be neutral and not migrate in the second dimension. As these peptides do migrate in the electric field, they must contain a basic residue (lysine, arginine, or histidine) which gives a net positive charge to the peptides. Since each of these peptides gain exactly one negative charge in the second dimension, their mobility will be proportional to their mobility in the first dimension and therefore lie on a straight line with a slope of less than one and below the main diagonal.

To our knowledge no diagonal procedures for the selective isolation of N-terminal peptides from enzymatic digest of proteins have been reported. There have been several column procedures reported (Gonzalez *et al.* 2000; Gevaert *et al.* 2003), but their applicability is limited to proteins with favourable sequences and unlike the diagonal procedure described here are not general in their applicability. Like the C-terminal diagonal procedure, the N-terminal diagonal procedure does not have any losses for detection and therefore is applicable to small amounts of protein. However, the tandem

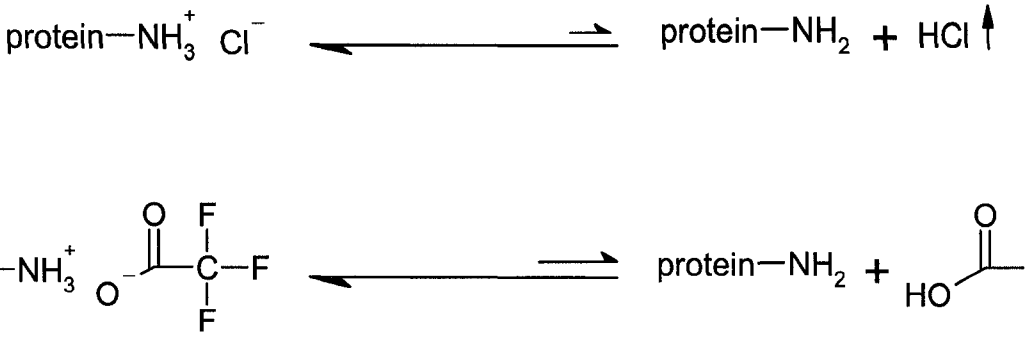
MS analysis is more complex due to the trimethylated amino terminus which does not have a mobile proton. For a permanently charge peptide, the fragmentation is governed by a mechanism called "charge-remote-fragmentation" which occurs only with high energy CID (Tomer *et al.* 1983; Burlingame *et al.* 1998; Gross 2000). Most tandem MS is a low energy CID resulting in "charge-induced-fragmentation" mechanism which requires a mobile proton on the peptide. Electrospray ionization (ESI) mass spectrometry utilizes a soft energy ionization technique, resulting in low energy CID. Indeed Poon *et al.* (2004) have shown that trimethylated amino terminal peptide does not fragment along the peptide backbone. Fragmentations using post-source decay (PSD) on a matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (MALDI-MS) results in a medium energy CID. With care and special adjustment, MALDI-PSD can obtain laboratory energy above 50 eV which can be considered as a medium high energy CID. Sufficiently high energy sources do exist but have not been applied to such peptides and such spectrometers were not available to us for analysis. A significant advantage is that trimethylated peptides are detected with at least a 10 fold increase in sensitivity by either ESI-MS or MALDI-MS (see below).

The success in demonstrating that naturally blocked N-terminal peptides can be isolated by the diagonal procedure provides the basis for a generally applicable N-terminal procedure that does not require trimethylation of amino groups. Proteins containing a free amino terminus can be acetylated with an equimolar mixture of deuterated [$(CD_3CO)_2O$] and non-deuterated [$(CH_3CO)_2O$] acetic anhydride. The acetylated N-terminal peptides can then be isolated using the diagonal procedure. These N-terminal peptides will appear in a mass spectrum as two peaks three mass units apart. As in the case of peptides isolated from the C-terminal diagonal, this has the advantage of differentiating peptides from non-peptide contaminants. However, in the case of proteins that are naturally blocked, this advantage will be lost as only one peak will be present. The ability to isolate N-terminal peptides as either their trimethylated or acetylated derivatives can be very useful in characterizing known recombinant proteins, especially those prepared for pharmaceutical applications, to verify purity and proper processing. In eukaryotes, however, approximately 80% of proteins are naturally blocked at the N-terminal (Kouzarides 2000). The proof of principle demonstrates that blocked

N-terminal peptides can also be isolated by the diagonal procedure and these peptides can be characterized by CID tandem MS.

In summary, it has been demonstrated that diagonal electrophoresis can be used to isolate selectively peptides derived either from the C-terminus or N-terminus of a protein. A potential problem is the removal of contaminants, but this is a problem regardless of the procedure used for preparing peptides for MS. It is likely that this problem can be resolved with established procedures for contaminant removal. In the development of the diagonal methodology described here, paper electrophoresis was used because it was the most convenient medium to work with. However, now that the proof of principle has been established, other media such as polyamide, cellulose or silica sheets could be used, and perhaps one of these media may prove to be superior to paper. With the impressive advances in MS, these peptides isolated from the diagonal can be characterized by established MS techniques. Such a development will be of value for characterizing pharmaceutical proteins as well as identifying unknown proteins in a proteome.

Vakos et al. (2001) in an NMR study on the *in vacuo* methylation of the carboxyl groups of insulin made an unexpected observation that a small but significant amount of trimethylation of amino groups takes place under conditions in which the amino group is protonated, and therefore should be unreactive. The present study with type A allatostatin III demonstrates that their observation was not due to some special property of the amino groups in insulin, but that protonated amino groups do indeed become trimethylated during *in vacuo* methylation with iodomethane. This result is surprising because it appears on the face of it that the protonated form of the amino group is reacting with iodomethane, but this is not possible because the protonated amino group is not nucleophilic. The only plausible explanation for such a reaction is that the protonated amino group becomes deprotonated *in vacuo* by some mechanism, and therefore can react as a nucleophile with iodomethane. Under the *in vacuo* conditions used, these amino groups exist as solid ionic salts with trifluoroacetate and chloride ions as counter ions. This suggests a possible mechanism by which an amino group can lose a proton and become nucleophilic. It is proposed that the following two equilibria exist *in vacuo*:



In each case, a small amount of deprotonated amino group is produced under *in vacuo* conditions. As the amino group becomes methylated, the equilibrium will shift to the right according to Le Chatelier's principle, thereby producing more deprotonated amino group. Such an equilibrium is consistent with the slow rate of reaction observed and also with the fact that apparently more deprotonated amino group is present with the trifluoroacetate counter ion than with the chloride ion, as HCl is a much stronger acid. There is a clear trend of an increase of trimethylation with time (**Figure 2-18**), but there is a considerable scatter in the data. This is possibly due to the presence of a small amount of water vapour in the sample which condenses with the introduction of iodomethane into the reaction vessel by liquid nitrogen trapping. Although precautions were taken to minimize this, it is likely that small amounts of water vapour that varied from sample to sample were present. It appears that the presence of water vapour facilitates the proton transfer and this would account for the scattering observed in the data. These results therefore suggest that there is an optimum amount of water vapour for *in vacuo* methylation.

It was observed in the present study that introduction of a permanent positive ion by *in vacuo* trimethylation of the α-amino group of a peptide greatly increases the sensitivity of detection by ESI-MS (**Figure 2-15**). Such an increase has previously been observed in ESI-MS for peptides containing a permanent positive charge (Chao *et al.* 2004). In these cases, the charge was introduced by chemical modification of the peptides in aqueous solution with activated reagents that were designed and prepared by chemical synthesis. In the case of the amino terminal diagonal procedure, the fact that the amino terminal peptides are isolated with their α-amino groups trimethylated is an

additional advantageous feature of the methodology as this will greatly increase the sensitivity of detection by either MALDI-MS (Stewart *et al.* 2002) or ESI-MS.

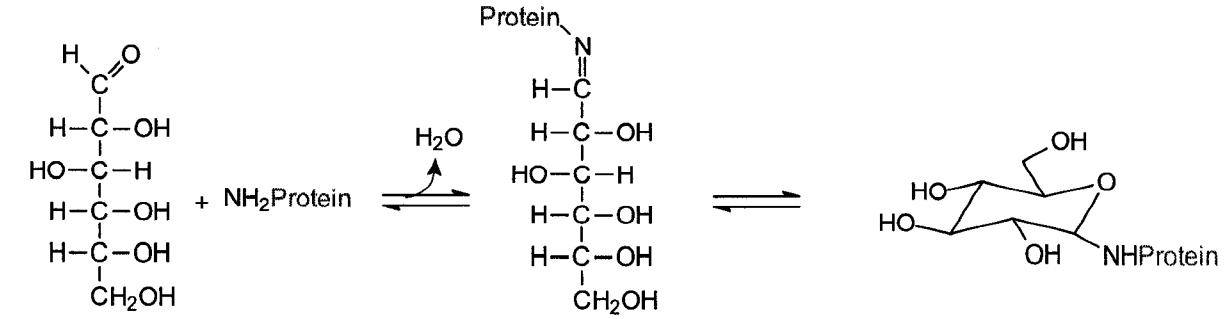
The mass spectroscopy is a major undertaking in itself and requires the dedication of substantial time on a mass spectrometer and expertise in MS of peptides, both of which were not available to us. Chao and coworkers (Chao *et al.* 2004) applied the *in vacuo* methylation and provide an example of the specialized MS facility and the expertise required for the sequencing of methylated peptides. However, the proof of principle described here has provided a sound basis and justification for the application of mass spectrometry for the identification and characterization of N and C-terminal peptides. This research is now being continued by the research group of Dr. Diethard Bohme at the Mass Spectroscopy Institute of York University who have the expertise and sufficient resources to carry out the MS analysis. Also the *in vacuo* methodology for peptide and protein methylation developed in this work is being applied by MDS Sciex Proteomics Application Group in Toronto in investigations of novel analytical applications of proteins and peptides using MS.

Chapter 3: Glycated trypsin: A novel autolysis-resistant trypsin

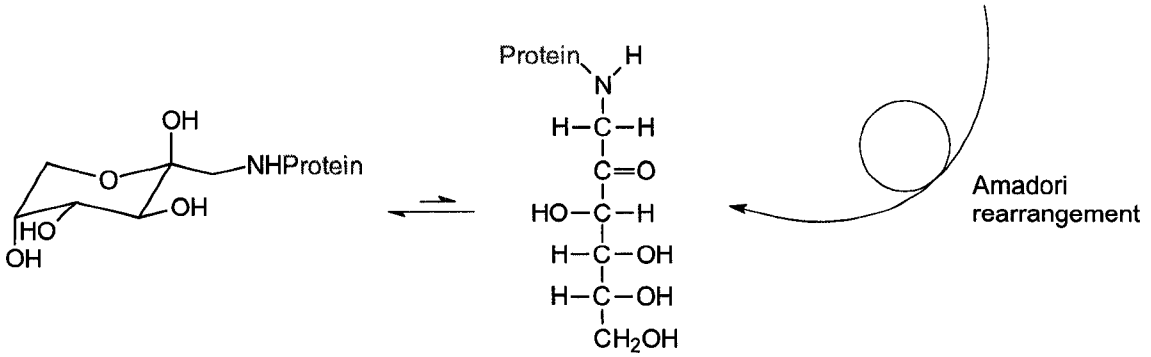
3-1 INTRODUCTION

There has been substantial effort dedicated to developing methodology for the attachment of carbohydrates to proteins (Boratynski and Roy 1998; Spiro 2002; Sinha and Surolia 2007). The interest in this stems from the observation of that there are many naturally occurring glycoproteins and that the attached carbohydrates appear to confer favourable properties such as increased thermostability, resistance to preoteolytic degradation and other special properties such as the ability to inhibit the growth of ice crystals. The attachment of carbohydrates to proteins *in vivo* is selective and mediated by enzymes called glycosyltranferases. There are many different glycans of various sizes that are covalently attached to the side-chains of asparagine, serine, and threonine in the protein (Spiro 1973; Paulson 1990; Matthews 1993). This enzyme mediated modification is referred to as glycosylation. On the other hand, glycation is a process in which carbohydrates can be covalently attached to proteins without the mediation of enzymes. Unlike glycosylation, the process is non-selective and the primary amino groups are the sites of attachments.

It has been known for almost a century that reducing sugars will react with amines when heated. This reaction was discovered by Maillard in 1912 (Cho *et al.* 2007) and gave rise to brown pigments. Initial reaction occurs by a nucleophilic attack of a free amino group with the aldehyde group of the open chain form of the sugars to give a Schiff base and a release of water (Neglia *et al.* 1983).



glucose in open chain form | Schiff base | *N*-substitued glycosylamine

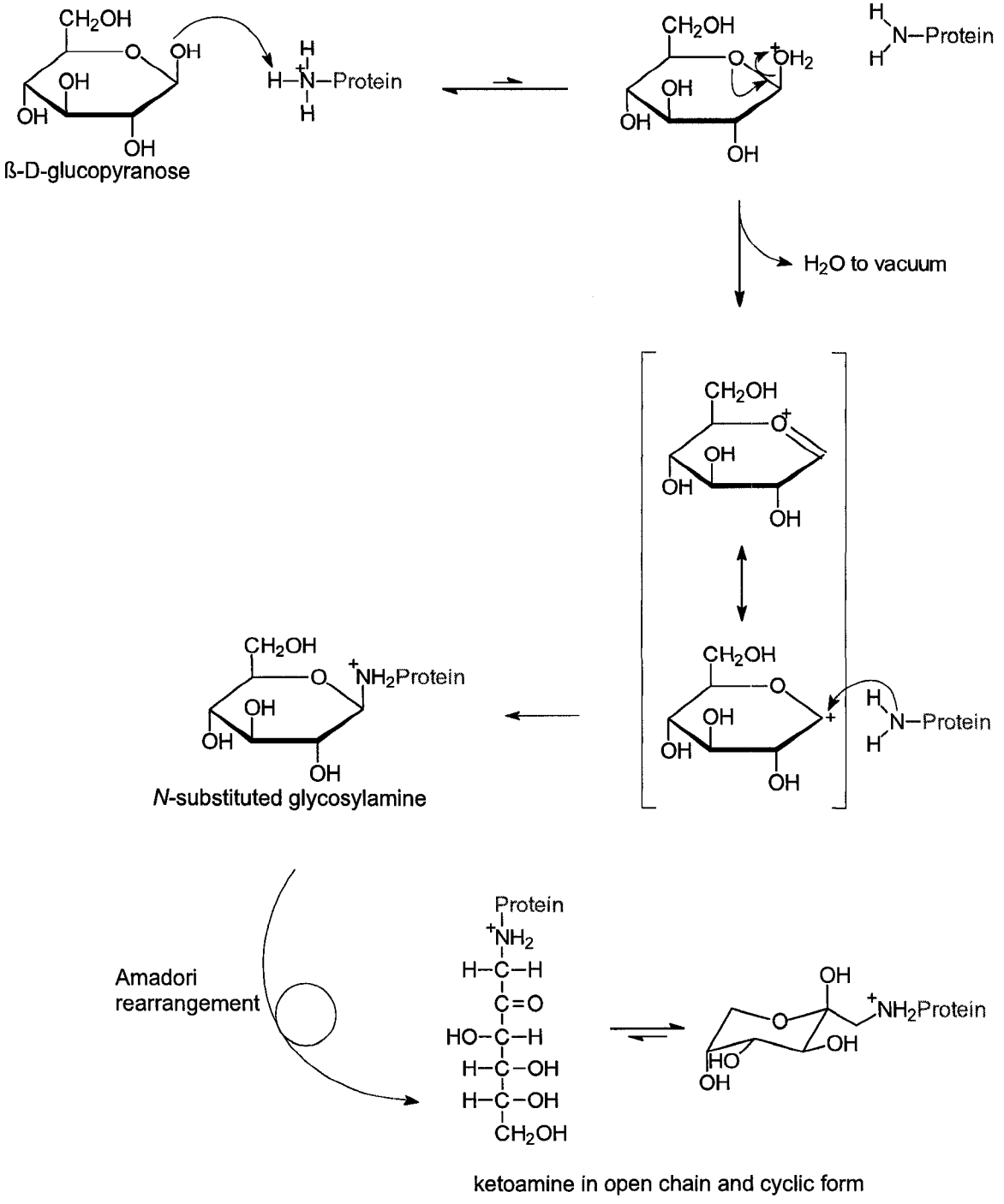


ketoamine in open chain and cyclic form

Glycation can also occur in the solid state by a complex reaction cascade, known as the Maillard reaction, giving rise to coloured products. The brownish coloured products observed on baking or cooking of foods are attributed to this reaction cascade and these products are referred to as advanced glycation end-products (AGEs). Glycation has not been widely used as a means of attaching carbohydrates to proteins because of the thermodynamically unfavourable condensation reaction and the subsequent formation of AGEs. Chemically activated sugar derivatives have been much more widely used as a means of attaching carbohydrates to proteins (Spiro 2002; Sinha and Surolia 2007).

Kaplan and coworkers have demonstrated that chemical modification of proteins can be achieved *in vacuo* on lyophilized proteins (Kaplan and Taralp 1997; Taralp and Kaplan 1997). This methodology overcomes some of the most serious difficulties encountered in solution modification of proteins, namely, protein inactivation due to pH and solution conditions, thermal induced unfolding and enzymatic degradation. Recently, it was demonstrated that proteins can by glycated *in vacuo* without the presence

of solvent or any activated chemical modifying reagents (United States Patent Application Number 20060122369; CIPO Application Number CA 2398213). The glycation is achieved simply by lyophilizing the protein with a reducing sugar and incubating at an elevated temperature (60°C to 85°C) *in vacuo* for a few hours. Under these conditions, the sugar becomes covalently attached to the amino groups on the protein forming a stable ketoamine linkage.



There are three advantageous features of this glycation methodology: 1) lyophilized proteins are much more stable than in solution, and 2) proteolytic degradation is eliminated due the absence of an aqueous environment, and 3) only the early stage of the Maillard reaction takes place and no AGEs are formed.

Trypsin has a very high specificity for proteolytic cleavage at the C-terminal side of the basic side-chains of lysine and arginine. It is therefore possible to predict the fragmentation pattern of a tryptic-digested protein that would be observed in the mass spectrum. For this reason, trypsin fragmentation is the most widely used strategy for the identification of proteins in proteomic studies. However, trypsin is known to undergo significant autolysis under mildly alkaline conditions where it is most active (Nakamura and Matsushima 1969) and its autolysis products complicate the analysis of the fragmentation pattern. To minimize autolysis, the lysine residues of trypsin have been dimethylated by reductive methylation (Rice *et al.* 1977) which reduces possible cleavage at the lysine residues. Glycation also takes place at lysine residues, and it is therefore expected that *in vacuo* glycation of trypsin would also have the effect of greatly reducing the autolysis of trypsin. It is also possible that glycation will be more effective than reductive methylation as the attached sugar will completely prevent any possibility of autolytic cleavage at lysine residues due to the fact that it is too large to fit into the active site of the enzyme. If this is the case, *in vacuo* glycation would provide a more attractive method of producing an autolysis-resistant trypsin as it is a much simpler and economical modification procedure than reductive methylation. In this chapter the test of this hypothesis is described.

3-2 MATERIALS AND METHODS

3-2.1 Materials

Bovine pancreatic trypsin and porcine pancreatic trypsin were purchased from Sigma-Aldrich Chemical Company. N-benzoyl-L-arginine ethyl ester (BAEE), N-acetyl-L-tyrosine ethyl ester (ATEE), and tosyl-L-phenylalanine chloromethyl ketone (TPCK) were from Sigma-Aldrich Chemical (St. Louis, Missouri). Standard 0.100 N NaOH was from Canada VWR (Montreal, Quebec). D-(+)-glucose was purchased from the BDH Chemical Company. All other chemicals, reagents and solvents were high purity preparations obtained from commercial sources.

3-2.2 Preparation of *in vacuo* glycated enzymes

Trypsin and chymotrypsin were glycated with glucose as previously described (Kaplan *et al.* 2002). In the case of the trypsin preparations, these samples were treated with TPCK to inactivate any pseudotrypsin or chymotrypsin present in the stock preparations. All enzyme samples were dialyzed (3.5 kDa cutoff) before use to remove any traces of lower molecular weight impurities. Enzyme (50 mg) was suspended in 10 mL in distilled water (dH_2O) and glucose (10 mg) was added. The pH was adjusted to 7.0 with 0.100 N NaOH and 2.0 mL aliquots (~10 mg protein) were lyophilized in Pyrex borosilicate tubes (size 16x100 mm). The glass tubes were then narrowed and sealed under a vacuum of 50 mTorr, and subsequently placed in an oven at 85°C for 40 h. After thermal incubation, the protein samples were re-constituted with 20 mL of 0.01% (v/v) acetic acid, placed in a dialysis membrane (6-8 kDa cutoff) and dialyzed against a 3000 mL volume of distilled water acidified with 0.100 mL glacial acetic acid (2 exchanges) and then subsequently with only dH_2O (3 exchanges) over a period of 24 h before final re-lyophilization for storage at 4°C.

3-2.3 Non-denaturing acidic polyacrylamide gel electrophoresis

Native polyacrylamide gel electrophoresis (PAGE) was carried out with modifications of the acid/urea/Triton (AUT) gels described by Bonner et al. (1980) but modified by leaving out urea and Triton X-100. The gel solution (10 mL of 30% (29:1) (w/v) acrylamide/bis-acrylamide; 7.3 mL dH_2O; 1.2 mL of 100% glacial acetic acid; 65 μL of NH_3OH; and 2 x 667 μL 0.006% (w/v) riboflavin) was combined and degassed. After mixing, 100 μL tetramethylethylenediamine (TEMED) was added, and the resulting solution was immediately poured to prepare a 1.5 mm thick minigels of 15% acrylamide pH 3.5. n-Butanol (100 μL) was layered on top and the gels polymerized under a bright light. A 5% acrylamide stacking gel solution was used (2 x 760 μL of 30% acrylamide/bis-acrylamide; 2 x 100 μL of 2.5% (w/v) bis-acrylamide; 650 μL of glacial acetic acid; 40 μL of NH_3OH; 7.0 mL of dH_2O; 1000 μL of 0.006% riboflavin; and 60 μL of TEMED). Running buffer was 100 mM glycine (pH 3.5) prepared by dissolving 7.5 g glycine and 10 mL of glacial acetic acid to a total volume of 1000 mL with dH_2O. A sample buffer was prepared with 100 mM glycine, 1% (v/v) acetic acid,

0.1% (w/w) bromophenol blue, and 10% (v/v) glycerol in distilled water. After electrophoresis at constant current of 5 mA for 225 volts-hour (VH), the gel was stained with Coomassie Brilliant Blue R-250.

3-2.4 Quantification of tryptic activities

The activity of trypsin was determined by measuring the rate of hydrolysis of BAEE at pH 7.5. The activity 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. A 5.00-mL aliquot of substrate solution (1.00 mM BAEE or ATEE; 100 mM KCl; and 1.00 mM $CaCl_2$) was transferred to the reaction vessel with a constant stream of N_2 air blown over the solution surface and subsequently titrated to a pH of 7.5 using 0.020 N NaOH added via a micro-syringe. A 100 μL sample containing 5.00 μg of the enzyme was added to the reaction vessel, and the volume of base (0.020 N NaOH) added per unit time to maintain a constant pH of 7.5 (endpoint) by the autotitrator was calculated as the rate of hydrolysis.

3-2.5 Quantification of tryptic and chymotryptic activities after incubation at 45°C

Trypsin (0.1 mg/mL) in 5.00 mL volume (20 mM Tris, pH 8; 2 mM $CaCl_2$) was incubated at 45°C. Aliquots of 100 μL were removed at timed intervals and diluted to a concentration of 0.050 mg/mL by the addition of 100 μL of 0.05% (v/v) acetic acid and placed on ice. The enzyme's estereolytic activity was measured as described above and the rates were reported as percentage of the initial rates.

3-2.6 Measurement of autolysis of trypsin by pH-stat

A sample of either native, dimethylated or glycated porcine trypsin enzyme was weighed and reconstituted in 0.100 M KCl to a concentration of 6.00 mg/mL. An aliquot of 500 μL of the 6.00 mg/mL enzyme solution (3.00 mg of enzyme) was added to 2.50 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 in the same manner as in the activity measurement (section 3-2.4). 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 8.5. The reaction solution containing the enzyme was titrated to this pH by adding 0.010 N NaOH 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 accurately determine 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 997.538 kg/m3 for water at 23°C it was calculated that a volume of 1.770 μL ± 0.003 μL (n=5) was delivered to the reaction solution per millimetre deflection on the autotitrator recorder.

3-3 RESULTS

Glycated trypsin has a significantly decreased electrophoretic mobility than native trypsin on an acid native PAGE (**Figure 3-1**). This shows that the glycation has significantly increased the size of the protein resulting in a decrease in elctrophoretic mobility due to the effect of molecular sieving. As glucose is small relative to the protein, this indicates that extensive glycation has taken place. The glycated trypsin appears to be relatively homogenous as a distinct band is observed and no un-modified trypsin or no glycated intermediate products are present. The band is only slightly broader than the native trypsin band, indicating that the vast majority of the 14 lysine residues (Nakamura and Matsushima 1969) present have been glycated. This is confirmed by the mass spectrum of the glycated bovine trypin in **Figure 3-2** which shows that species with 9 to 14 glycated lysine residues are present, but most contains 11 to 14 glycated residues.

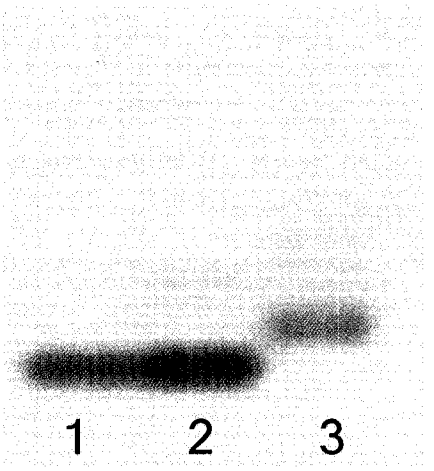


Figure 3-1. Non-denaturing PAGE of native and *in vacuo* glycated trypsin. Samples of trypsin were lyophilized with glucose and trehalose in a weight ratio of 5:1:0.1 and incubated under vacuum at 85°C for a period of approximately 30 hours. A 5.00 μg of each enzyme sample was subjected to non-denaturing PAGE. Lane 1: native trypsin; lane 2: control trypsin; lane 3: *in vacuo* glycated trypsin. Gel is stained with Coomassie Blue R-250.

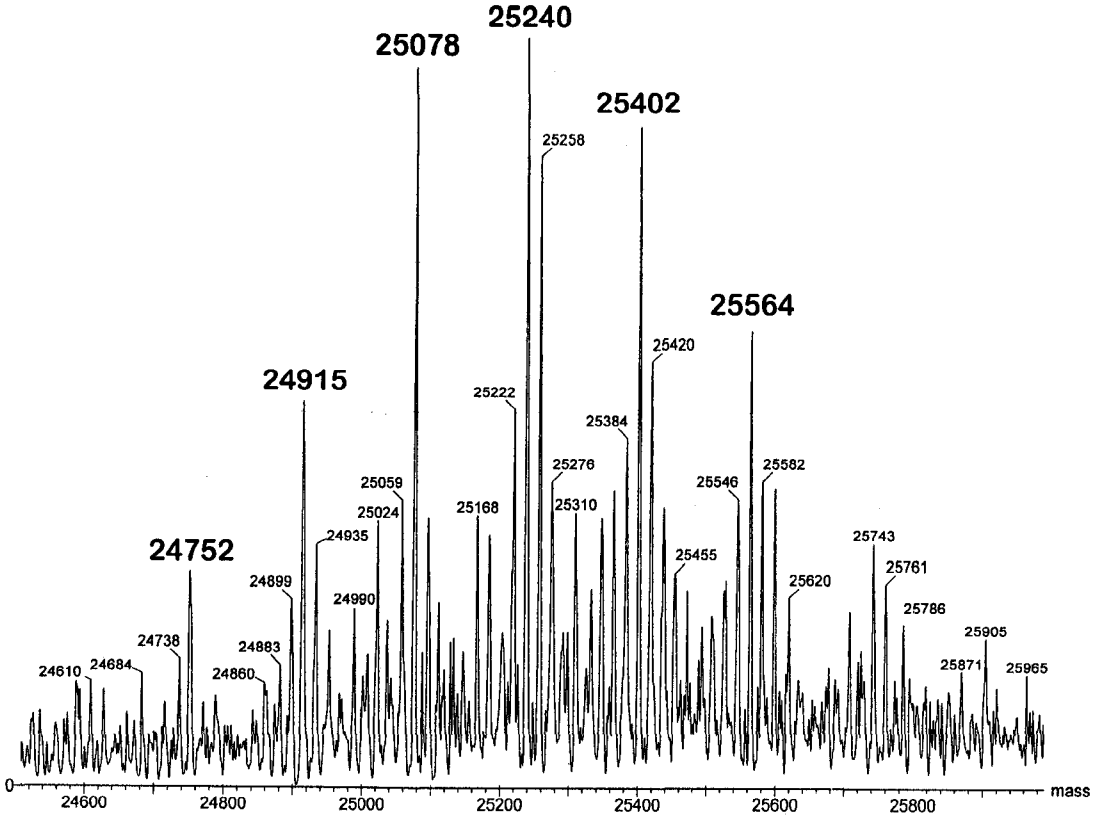


Figure 3-2. Deconvoluted electrospray mass spectrum of glycated bovine trypsin.

The enzymatic activity of trypsin towards the BAEE at 25°C does not appear to be altered significantly. However, at 45°C and pH 8, the native trypsin rapidly loses activity (**Figure 3-3**). This has been attributed to autolytic cleavage of peptide bonds at lysine and arginine. In sharp contrast to this, the glycated trypsin loses little of its activity over a 3 h period and glycation of the lysine residues which removes potential autolytic cleavage sites is consistent with a decrease in autolysis. This is confirmed by the direct measurement of autolysis using a pH stat in which the release of protons on cleavage of peptide bonds at pH 8.5 is detected (**Figure 3-4**). Native trypsin, incubated at its pH optimum, releases protons more rapidly than the glycated trypsin and hence has a higher rate of autolysis. Dimethylated trypsin also blocks potential autolytic cleavage sites and our direct measurement of autolysis confirms that it also reduces autolysis but less than the glycated trypsin. The extent of autolysis relative to native trypsin at various

time intervals is tabulated in **Table 3-1**. The initial relative rates of autolysis over the first two minutes are native trypsin (1.0):DM trypsin (0.60):glycated trypsin (0.40).

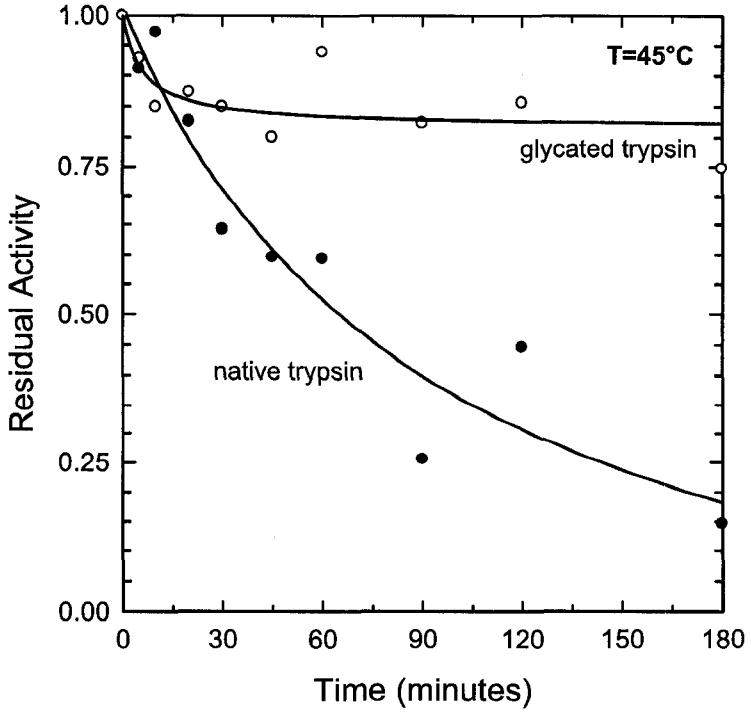


Figure 3-3. Effect of glycation on the activity of bovine trypsin. The rate of BAEE hydrolysis by native trypsin (•) and glycated trypsin (○) was measured after incubation of the enzyme sample for various periods of time at different elevated temperature of 45°C. The activity is shown as % values relative to the initial activity of the samples.

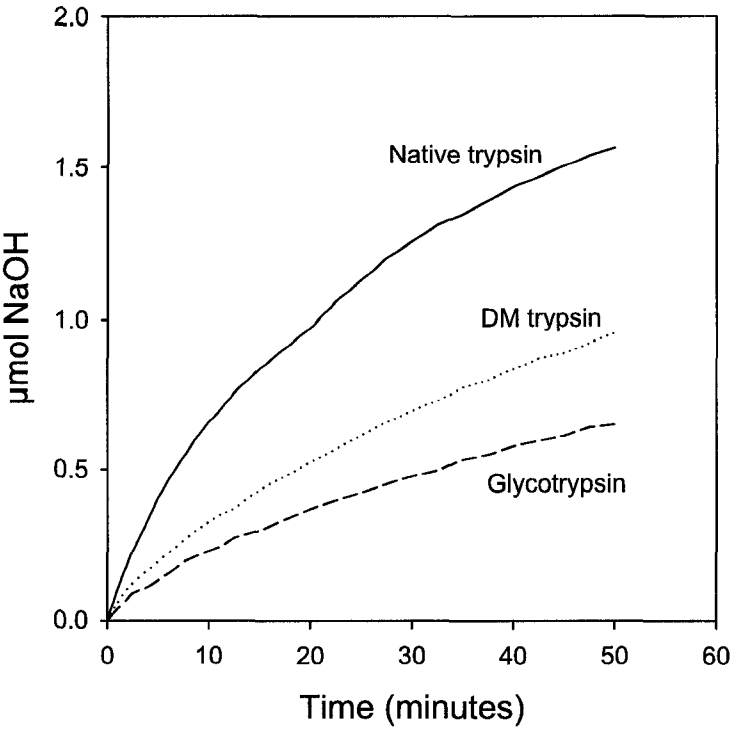


Figure 3-4. Autolysis of native porcine trypsin, dimethylated (DM) porcine trypsin, and glycated porcine trypsin at 37°C. Enzyme samples were incubated at a concentration of 1 mg/mL at 37°C and the extent of autolysis was measured on a pH-stat at pH 8.5 by addition of a volume of 0.01N NaOH to compensate for a pH drop resulting from the release of acid by cleavage of a peptide bond.

Table 3-1. Extent of autolysis exhibited by DM trypsin and glycated porcine trypsin. The extent of autolysis at each time interval is given relative to that of native porcine trypsin (**Figure 3-4**).

Time (minutes)	DM Trypsin	Glycotrypsin
5	0.50	0.33
10	0.50	0.35
20	0.54	0.38
30	0.55	0.38
40	0.58	0.40
50	0.61	0.41

3-4 DISCUSSION

The results obtained demonstrate that trypsin can be efficiently glycated without loss of activity using the *in vacuo* glycation methodology developed by Kaplan and coworkers (Kaplan *et al.* 2002; 2006). Bovine trypsin has 14 lysine residues and the mass spectrometry of the glycated trypsin shows that only species containing 9 to 14 glucose residues with the majority containing 12 glucose residues. Another advantage of *in vacuo* glycation is that the repeated glycation can readily be carried out and this would result in one species containing 14 glycated residues. This appears to be unnecessary as the small amount of additional glycation is unlikely to significantly alter the autolysis-resistance of the glycated trypsin used in this study. The results also confirm the stability of the ketoamine derivative formed as in all operations at low and high temperatures there is no evidence of the loss of the covalently attached sugar moiety.

Solid state glycation has been previously carried out on a mixture of reducing sugar and lysozyme under an atmosphere of 40 to 60% humidity at 40°C for 5 days

(Yeboah *et al.* 2004). Under these conditions, 10% of lysozyme was unmodified and the rest contained 1 to 5 modified residues out of the possible 6. Also, a significant amount of AGEs were formed and these were minimized by incubation at a low temperature of 40°C. Similarly, Kato *et al.* (1993) reported 60% glycation of lysine on bovine trypsin after 4 days. In both studies, the nature of the glycosylic linkage they obtained is not clear as no chemical characterization was carried out, but based on the observation of AGEs formation, it is likely a variety of linkages were present [Yeboah MS maillard product refs]. Carrying out the glycation process under vacuum in the absence of oxygen and water produces only a single ketoamine product (Kaplan *et al.* 2002; 2006) and no AGEs are formed. The *in vacuo* glycation process is also much more rapid, requiring hours instead of days, and the extent of glycation is much greater.

Bovine and porcine trypsins are commercially available. Porcine trypsin is currently the most widely used trypsin in proteomic studies because it is reported to be more stable and autolysis-resistant than bovine trypsin (Nakamura and Matsushima 1969). In the first part of this study, bovine trypsin was used because of its high degree of autolysis and this would therefore give us a more rigorous test of the effect of glycation on the autolysis. The quantification of the effect of glycation on autolysis was carried out with porcine trypsin so that it could be compared with methylated porcine trypsin which is the most widely used autolysis-resistance trypsin.

A novel approach using a pH-stat was used to quantify the relative rates of autolysis of trypsin, methylated trypsin, and glycated trypsin. On hydrolysis of a peptide bond, a free carboxyl and a free amino group are generated. As the α-amino group of a polypeptide has a pKa of ~8.5 and the α-carboxyl group pKa of ~3.5, at neutral pH values, the proton on the carboxyl group will be transferred to the amino group and there will be no net proton release. However at pH 8.5, the pH at which we carried out our autolysis measurement, approximately half a mole of proton will be released per mole of peptide bond hydrolysed. Although, the different α-amino groups generated will have slightly different pKa values, the fact that we are using the same trypsins means that the same peptide bonds will be cleaved and the relative rates of autolysis we obtained will therefore be accurate.

The rate of autolysis of the glycated porcine trypsin at pH 8.5 and 37°C was 40% of that of native form. It was also significantly less than that of the reductively methylated trypsin which is the most widely used form of autolysis-resistant trypsin commercially available. The rate of autolysis of the reductively methylated trypsin was 60% that of the native trypsin. Although the autolysis rates change over time, the integration or relative amount of total base consumed at the plateau region or after 50 minutes incubation are 100%, 60%, and 40% for native trypsin, methylated trypsin, and glycated trypsin respectively (**Table 3-1**). Therefore, it appears from our data that reductive methylation which was carried out in solution is less effective than *in vacuo* glycation. Since porcine trypsin contains 14 possible autolytic sites, 10 lysine and 4 arginine residues, the number of protected sites in the glycated trypsin is $(1.0 - 0.4)\text{x}14 = 8.4$. This means that on average 8.4 lysine residues out of the 10 (i.e. 84%) are glycated, this corresponds well with bovine trypsin mass spectrum (**Figure 3-2**) which shows that on average 12 out of 14 (i.e. 85%) lysine side chains are glycated.

3-5 CONCLUSION

In vacuo glycation of trypsin is an effective approach for the production of an autolysis-resistant trypsin. To our knowledge, glycated trypsin is the most autolysis-resistant form of trypsin produced by chemical modification.

Chapter 4: Application of *in vacuo* glycation for improving thermostability of proteins

4-1 INTRODUCTION

There is substantial evidence that naturally glycosylated proteins in which polysaccharides are attached enzymatically to asparagine, serine, and threonine side chains have increased structural stability (Spiro 1973; Paulson 1990; Matthews 1993; Imperiali and O'Connor 1999; Spiro 2002; Sinha and Surolia 2007). It would be advantageous if improved structural stability could be conferred on non-glycosylated proteins by the covalent attachment of sugars or polysaccharides. *In vitro* glycosylation of proteins is difficult, and for many proteins not even possible, due to the fact that highly specific enzymes requiring complex carbohydrates and specific recognition sequences on the protein are necessary (Werner *et al.* 2007). Non-enzymatic attachment of carbohydrates to the amino groups of proteins, i.e. glycation, can be achieved in solution for any soluble protein. However, glycation in aqueous solution is thermodynamically unfavourable and as a consequence, the glycation process is slow and yields heterogeneous reaction products (Khalifah *et al.* 1996; Cho *et al.* 2007). The use of high temperatures to increase the rate of glycation usually results in irreversible protein unfolding. Attachment of carbohydrates to amino groups using chemically activated glycans has been used to overcome these difficulties (Sola *et al.* 2006; Sola and Griebenow 2006b; a; Sola *et al.* 2007). This approach for the covalent attachment of carbohydrates to proteins has been referred to as "chemical glycosylation". Griebenow and coworkers (Sola *et al.* 2006) have recently reported a new method for attaching various sizes of carbohydrate by reacting the amino groups on the protein with succinimidyl suberate to which the carbohydrate has been coupled, thus forming a covalent cross-link between the carbohydrate and protein with suberate as a linker.

There is evidence that limited glycation increases the thermostability of proteins (Marshall and Rabinowitz 1976; Liang and Rossi 1990; Kato *et al.* 1993; Morgan *et al.* 1999; Yeboah *et al.* 2004). In the case of trypsin (Kato *et al.* 1993), limited glycation carried out over 4 days was reported to increase the thermostability as measured by

differential scanning calorimetry; however, the increase as measured by retention of activity after incubation at elevated temperatures (60°C to 70°C) was modest and measured for only a 1 minute incubation. It is not clear from their data to what extent the observed increase in retention of activity was due to reduced autolysis or increased structural stability. Interpretation of their results is also complicated by the fact that the glycation was incomplete and a variety of products from the Maillard reaction appeared to be present. Nevertheless, these results indicate that if complete glycation could be achieved, there would be a substantial effect on the thermostability of trypsin.

In vacuo glycation overcomes some of the most serious difficulties encountered in solution glycation of proteins, namely, incomplete glycation, production of advanced glycation end products (AGEs) by the Maillard reactions, protein inactivation due to solvent induced denaturation, thermal induced unfolding and enzymatic degradation. This glycation procedure provides a simple and convenient method for testing the effect of attaching monosaccharides on the thermal stability of a protein. In the present study, trypsin and chymotrypsin were employed as model proteins because of the extensive studies carried out to improve their stability and the interest in their therapeutic and industrial applications (Marshall *et al.* 2003; Werner *et al.* 2007).

4-2 MATERIALS AND METHODS

4-2.1 Materials

Bovine pancreatic trypsin, porcine pancreatic trypsin, bovine pancreatic α-chymotrypsin, chicken egg lysozyme, and bovine ribonuclease A were purchased from Sigma-Aldrich Chemical Company. N-benzoyl-L-arginine ethyl ester (BAEE), N-acetyl-L-tyrosine ethyl ester (ATEE), and tosyl-L-phenylalanine chloromethyl ketone (TPCK) were from Sigma-Aldrich Chemical (St. Louis, Missouri). Performic acid oxidized bovine ribonuclease A (pfo-RNase) was prepared acid according to procedure described by Hirs (Hirs 1967). Standard 0.100 N NaOH was from Canada VWR (Montreal, Quebec). D-(+)-glucose was purchased from the BDH Chemical Company. Whatman 3MM paper was purchased from Sigma-Aldrich Chemical Company. All other chemicals, reagents and solvents were high purity preparations obtained from commercial sources.

4-2.2 Preparation of *in vacuo* glycated enzymes

Trypsin and chymotrypsin were glycated with glucose as previously described (Kaplan *et al.* 2002). In the case of the trypsin preparations, these samples were treated with TPCK to inactivate any pseudotrypsin or chymotrypsin present in the stock preparations. All enzyme samples were dialyzed (3.5 kDa cutoff) before use to remove any traces of lower molecular weight impurities. Enzyme (50 mg) was suspended in 10 mL in distilled water (dH_2O) and glucose (10 mg) was added. The pH was adjusted to 7.0 with 0.100 N NaOH and 2.0 mL aliquots (~10 mg protein) were lyophilized in Pyrex borosilicate tubes (size 16x100 mm). The glass tubes were then narrowed and sealed under a vacuum of 50 mTorr, and subsequently placed in an oven at 85°C for 40 h. After thermal incubation, the protein samples were re-constituted with 20 mL of 0.01% (v/v) acetic acid, placed in a dialysis membrane (6-8 kDa cutoff) and dialyzed against a 3000 mL volume of distilled water acidified with 0.100 mL glacial acetic acid (2 exchanges) and then subsequently with only dH_2O (3 exchanges) over a period of 24 h before final re-lyophilization for storage at 4°C.

4-2.3 Non-denaturing acidic polyacrylamide gel electrophoresis

Glycated trypsin and chymotrypsin were analyzed by an acidic non-denaturing polyacrylamide gel electrophoresis (PAGE) with the Bio-Rad Mini-Protean II electrophoresis system. The electrophoresis was carried out with the acid/urea/Triton (AUT) gels described by Bonner et al. (1980) but modified by leaving out urea and Triton X-100. Protein (5 to 10 μg) was loaded onto an acidic 15% acrylamide gel (1.5-mm thickness) with 100 mM glycine containing 1% (v/v) glacial acetic acid as running buffer. After electrophoresis at constant current of 5 mA for 180 to 225 volts-hour (VH), the gel was stained with Coomassie Brilliant Blue R-250.

4-2.4 Quantification of tryptic and chymotryptic activities

The activities of trypsin and chymotrypsin were determined by measuring the rate of hydrolysis of BAEE and ATEE respectively. The activity 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. A 5.00-mL aliquot of substrate solution (1.00 mM BAEE or ATEE; 100 mM

KCl; and 1.00 mM $CaCl_2$) was transferred to the reaction vessel with a constant stream of N_2 air blown over the solution surface and subsequently titrated to a pH of 7.5 using 0.020 N NaOH added via a micro-syringe. A 100 μL sample containing 5.00 μg of the enzyme was added to the reaction vessel, and the volume of base (0.020 N NaOH) added per unit time to maintain a constant pH of 7.5 (endpoint) by the autotitrator was calculated as the rate of hydrolysis.

4-2.5 Quantification of tryptic and chymotryptic activities after incubation at elevated temperatures

Trypsin or chymotrypsin (0.1 mg/mL) in 5.00 mL volume (20 mM Tris, pH 8; 2 mM $CaCl_2$) was incubated at elevated temperatures (50°C, 55°C, and 60°C). Aliquots of 100 μL were removed at timed intervals and diluted to a concentration of 0.050 mg/mL by the addition of 100 μL of 0.05% (v/v) acetic acid and placed on ice. The enzyme's estereolytic activity was measured as described above and the rates were reported as percentage of the initial rates. Bovine trypsin and chymotrypsin (native and glycated) were compared at 50 and 55°C. Bovine and porcine trypsins were compared at 60°C.

4-2.6 High voltage paper electrophoresis (HVPE)

HVPE was carried out by using the Hunter Thin Layer Electrophoresis equipment (HTLE 7002; CBS Scientific, Ins., Del Mar, CA).

4-2.7 Digestion of performic oxidized ribonuclease A

Pfo-RNase was solubilised (1 mg/mL) with 0.1% (w/v) ammonium bicarbonate solution. Aliquots (1 mL) of the pfo-RNase were transferred to microcentrifuge tubes and incubated with 20 μg bovine glycated trypsin and 20 μg bovine native trypsin for 24 h at 25°C. After digestion the samples were dried under vacuum using a Speed-Vac apparatus before analysis by HVPE. The pfo-RNase samples (100 μg each) were spotted on 3 MM Whatman paper and subjected to electrophoresis for 35 min at 1200 V (60 V/cm) in pH 2.1 formic acid-glacial acetic acid-water (20:80:900 v/v/v). The electrophoretogram was stained with cadmium/ninhydrin.

4-2.8 Digestion of native ribonuclease A

Native bovine ribonuclease A (5 mg/mL; 400 μL) and native porcine trypsin or glycated porcine trypsin (0.02 mg/mL; 100 μL) in buffer (10 mM Tris; 1 mM $CaCl_2$; pH 8) were combined and incubated at 70°C for 24 h. The ratio of protein to enzyme was 1000:1. The digests were analyzed by HVPE with a loading of 80 μg of RNase for each lane (lane 1: no enzyme; lane 2: glycated trypsin; lane 3: native trypsin). The electrophoresis was carried out at 1200 V for 25 min (or 500VH) in pH 2.1 buffer.

4-2.9 Differential scanning calorimetry

Differential scanning calorimetry (DSC) scans were obtained on a Calorimetry Sciences 6300 nano-ITC. Proteins were dissolved in 200 mM acetate buffer, pH 5.0, at a concentration of 0.3 mg/mL and degassed under vacuum for 10 min. The reference cell was filled with 0.3 mL of degassed buffer, and the sample cell with 0.3 mL of protein. Samples were scanned at 1°C/min from 20 to 100°C at 3 atmospheres pressure.

4-2.10 Circular dichroism

A Jasco® 810 circular dichroism (CD) spectrometer (Jasco Inc., Easton, MD, USA) was used to monitor the far-UV region (180 nm to 260 nm) in order to assess the secondary structures and thermal stability of the native and glycated proteins. Trypsin and chymotrypsin were treated with PMSF immediately upon dissolution in order to prevent autolysis during the analysis. After extensive dialysis (3.5 kDa cutoff), samples containing approximately 1.0 mg/mL of protein in buffer (10 mM Tris acetate, 5 mM calcium acetate, pH 7.5) and protein concentrations used to calculate mean residue ellipticity were determined from standard curves based on absorption at 280 nm of the native protein and/or the dry weight of the protein taking into consideration the average number of sugar residues attached to the glycated forms as determined by mass spectroscopy (Ewing 2006; Kaplan *et al.* 2007). CD spectra were recorded in a cell with a 0.2 cm optical path. In the thermal denaturation experiments, spectra were recorded at 5°C increments as the sample was heated from 25 to 95°C; each spectrum represented the average of 5 accumulations. The mean residue ellipticities at 215 nm were monitored at

each temperature and used to determine the fraction folded following the method of Pace and coworkers (Pace and Shirley 1997).

4-3 RESULTS

Figure 4-1 shows the relative mobilities of native and glycated bovine trypsin and bovine chymotrypsin on acid non-denaturing gels. The band widths of the native and glycated proteins are approximately equal indicating that the glycated proteins are relatively homogeneous. Data from mass spectrometry (Chapter 3, **Figure 3-2**) confirms the homogeneity of glycated bovine trypsin in which, on average, at least 12 of the 14 possible lysine residues have reacted with glucose (Ewing 2006; Kaplan *et al.* 2007). The activity of the glycated enzymes appears, within the limits of the experimental accuracy, to be unaltered (**Table 3-1**). The proteolytic specificity, as determined by the electrophoretic pattern of peptides generated by digestion of performic acid oxidized ribonuclease with native and glycated trypsin, also appears to be unaltered (**Figure 4-2**).

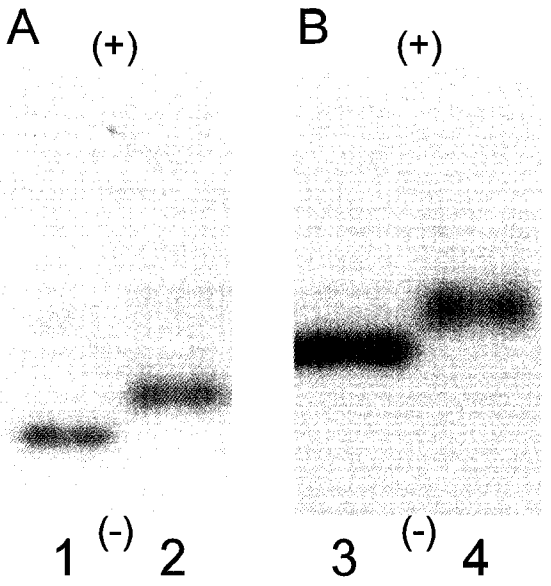


Figure 4-1. PAGE of glycated trypsin and glycated chymotrypin.

A) Acidic (pH 3.5) non-denaturing 1.5-mm 15% PAGE gel of bovine trypsin run at constant current of 5mA for 225 VH and stained with Coomassie Brilliant Blue R. Lane 1: native trypsin 5 μg; Lane 2: glycated trypsin 10 μg.

B) Acidic (pH 3.5) non-denaturing 1.5-mm 15% PAGE gel of bovine chymotrypsin run at constant current of 5 mA for 180 VH and stained with Coomassie Brilliant Blue R. Lane 3: native chymotrypin, 5 μg; Lane 4: glycated chymotrypsin, 5 μg.

Table 4-1. Activities of glycated bovine trypsin and bovine glycated chymotrypsin derived from the hydrolysis of BAEE and ATEE respectively.

	Relative Activity	
Enzyme	Native	Glycated
Bovine Trypsin	1.00	0.98 ± 0.05
Bovine Chymotrypsin	1.00	0.94 ± 0.05

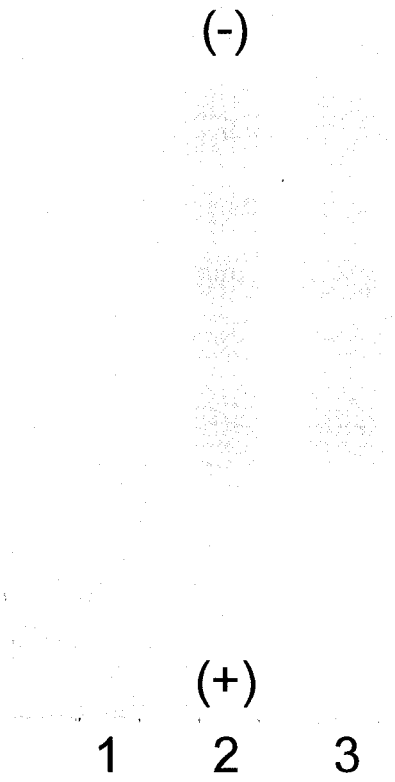


Figure 4-2. HVPE of 24 hour tryptic digestion at 25°C of performic oxidized bovine ribonuclease A (pfo-RNase), enzyme/pfo-RNase ratio of 1:50 (w/w). Each lane contained 100 μg RNase and HVPE was carried out at pH 2.1 at 60 V/cm for 35 minutes (700 VH). The electrophoretogram was stained with cadmium/ninhydrin.
Lane 1: pfo-RNase; Lane 2: pfo-RNase + glycated trypsin; Lane 3: pfo-RNase + native trypsin.

In order to test the thermal stability of the glycated proteins, both native and glycated enzymes were incubated at elevated temperatures. Aliquots were removed at various time intervals and the residual activity at 25°C determined (**Figure 4-3**). The results show that the glycated enzymes have a marked increase in retention of enzymatic activity. Native bovine trypsin and native bovine chymotrypsin rapidly lose activity when incubated at either 50 or 55°C (**Figure 4-3**). Their glycated counterparts also lose activity, but at a much reduced rate. Glycated bovine trypsin retains 50% of its activity even after two hours incubation at 55°C, whereas the native form is inactivated

(**Figure 4-3b**). Similarly, glycated bovine chymotrypsin retains approximately 30% of its activity after two hours incubation at 55°C, while the non-glycated native form is inactivated.

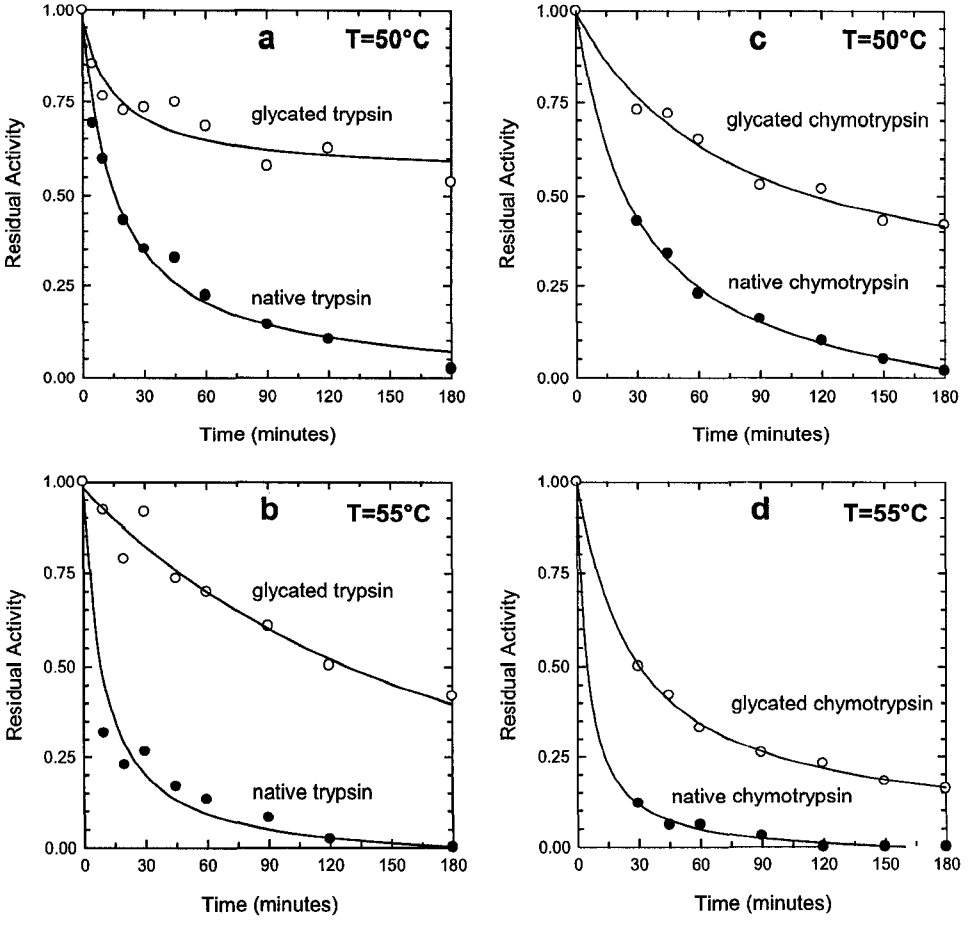


Figure 4-3. Activities of bovine trypsins and bovine chymotrypsins after incubation for various time periods at elevated temperatures: a) glycated and native trypsin incubated at 50°C; b) glycated and native trypsin incubated at 55°C; c) glycated and native chymotrypsin incubated at 50°C; d) glycated and native chymotrypsin incubated at 55°C.

Porcine trypsin is preferred in proteomic applications because it is more stable than bovine trypsin (Vithayathil *et al.* 1961; Buck *et al.* 1962; Nakamura and Matsushima 1969). Bovine trypsin was used because of its poorer stability and it thus provides a more rigorous test of the effects of glycation on stability. **Figure 4-4** shows a comparison of the thermal stabilities, as measured by retention of enzymatic activity, of the glycated and non-glycated forms of bovine (4a) and porcine (4b) trypsin at 60°C. These results also demonstrate the increased thermostability of the porcine enzyme over the bovine enzyme. Glycation, however, also significantly increases the stability of porcine trypsin beyond that of the native enzyme to yield a remarkably stable enzyme. The glycated porcine trypsin retains more than 50% of its original activity even after three hours incubation at 60°C at its pH optimum where maximum autolysis occurs. It appears that the glycated enzymes, especially the glycated porcine trypsin, are sufficiently thermostable that they can be used to carry out proteolytic digestion at elevated temperatures. Glycated porcine trypsin does indeed digest native RNase at 70°C even at the extremely low enzyme to protein ratio of 1:1000 (**Figure 4-5**, lane 2). In comparison, there is very little digestion of native RNase with the non-glycated trypsin (**Figure 4-5**, lane 3) under the same conditions.

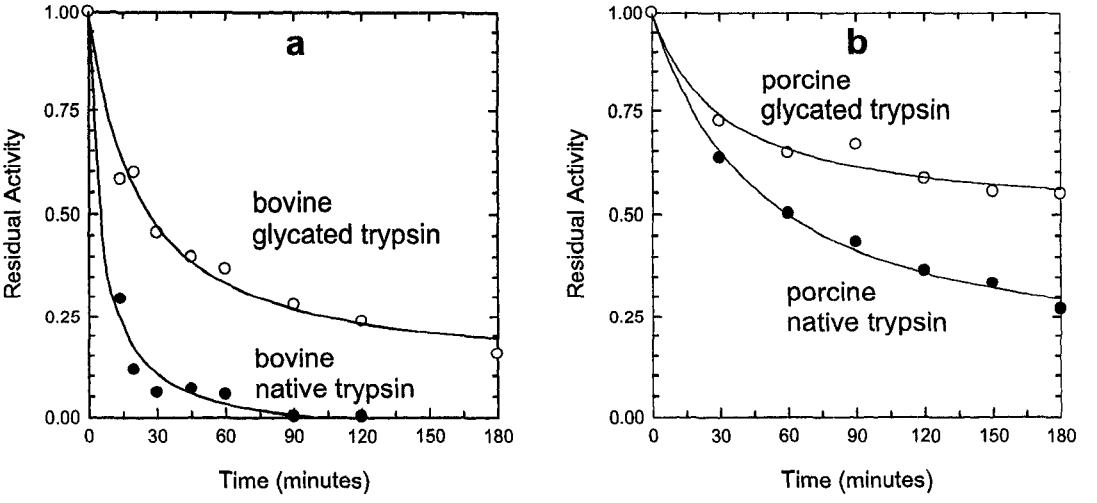


Figure 4-4. Activities of bovine trypsins and porcine trypsins after incubation for various time periods at 60°C: a) glycated and native bovine trypsin; b) glycated and native porcine trypsin.

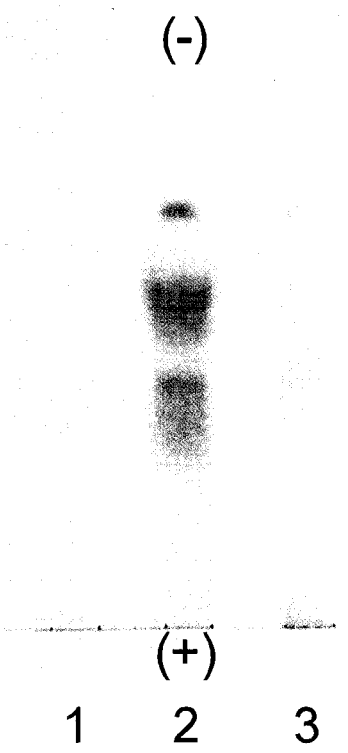


Figure 4-5. HVPE of 24-hour tryptic digestion of native RNase A at 70°C. Each lane contained 80 μg RNase and HVPE was carried out at pH 2.1 at 60 V/cm for 25 minutes (500 VH). The electrophoretogram was stained with cadmium/ninhydrin.
Lane 1: RNase; Lane 2: RNase + glycated trypsin; Lane 3: RNase + native trypsin.

Thermal stability of the glycated porcine enzymes was further examined by both differential scanning calorimetry (DSC) and circular dichroism (CD) (**Table 4-2**). Trypsin and chymotrypsin are homologous proteins and could be expected to respond similarly to glycation. Bovine RNase A was added to provide a structurally different protein. The data from the CD and DSC, while quantitatively somewhat different, are qualitatively similar and both techniques indicate that glycation significantly increases thermostability.

Table 4-2. T_m values of glycated and native proteins determined by CD and DSC.

	T_m (°C) CD			T_m (°C) DSC		
Protein Sample	Native	Glycated	ΔT_m	Native	Glycated	ΔT_m
Porcine Chymotrypsin	54.3	59.6	5.3	54.3	55.8	1.5
Porcine Trypsin	67.7	72.2	4.5	56.8	60.8	4.0
Bovine RNase A	58.0	63.6	5.6	59.1	62.9	3.8

4-4 DISCUSSION

Glycosylation of proteins *in vivo* usually attaches a complex sugar chain to a relatively small number of specific sites in the protein. In contrast, the *in vacuo* glycation procedure used here adds a single sugar moiety to a relatively large number of sites (14 lysine residue in the case of bovine trypsin and 10 in the case of porcine trypsin). The results obtained demonstrate that *in vacuo* glycation with glucose is an effective means of increasing the thermostability of trypsin and chymotrypsin without altering the activity or specificity of the enzyme. CD spectra of the native and glycated enzymes at ambient temperature (data not shown) confirm that glycation with glucose does not significantly alter the overall conformation of the proteins.

Proteolytic enzymes such as trypsin and chymotrypsin provide extremely sensitive systems for testing the thermostability of proteins. This is because protein unfolding at elevated temperatures results in increased exposure of susceptible sites for autolytic cleavage and prevents refolding of the denatured protein molecules. The activity results demonstrate that glycation with glucose can confer a significant increase in the apparent thermostability of trypsin and chymotrypsin. This increase in stability is in fact more than one would expect based upon the significant, yet modest, trends observed in the increase in T_m values obtained by either CD or DSC (**Table 4-2**). While glycation does increase the resistance to unfolding at higher temperatures, this effect is magnified because of the decreased rate of autolysis in the glycated enzymes. The CD,

DSC and activity results taken together are particularly significant because they demonstrate that a modest increase in thermal stability can have a very large effect on the biological properties of a protein.

In the case of trypsin, it is known that blocking of lysine residues decreases the rate of autolysis (Rice *et al.* 1977; Finehout *et al.* 2005). This, in part, may account for the large increase in the apparent thermostability of the glycated enzyme. However, in the case of chymotrypsin, where modification of lysine residues does not decrease the number of potential sites for autolytic cleavage, a large increase in apparent thermostability is still observed. These data, therefore, indicate that much of the observed increase in stability of both the glycated trypsin and the glycated chymotrypsin is due to an increase in conformational stability which in turn results in a decreased rate of autolysis in both enzymes because potential autolytic sites remain buried in the folded protein. The DSC and CD results for ribonuclease indicate that the effect of *in vacuo* glycation on thermostability is not confined to proteolytic enzymes and is likely general in its applicability.

Griebenow and coworkers (Sola *et al.* 2006), working with bovine chymotrypsin, have demonstrated that the attachment of more complex carbohydrates to lysine through a linker can result in a 5 to 6°C increase in thermostability. That increase in thermostability, however, was accompanied by a decrease in k_{cat}. In the case of the *in vacuo* glycated trypsin and chymotrypsin, our data indicate that there is no decrease in activity (and hence k_{cat}) in the glycated enzymes. We do, however, observe a similar (~ 4 to 5°C) increase in T_m. It is somewhat surprising that the modification of lysines with only a single monosaccharide moiety gives a comparable increase in thermal stability. It is possible that the lack of a linker allows the sugar to interact more strongly with the nearby structures on the protein. Preliminary studies on the *in vacuo* glycation of proteins using other hexose monosaccharides indicate that there is very little difference in the observed increase of thermostability. Other preliminary studies have shown that polysaccharides with a terminal reducing sugar can be attached to proteins by the *in vacuo* glycation procedure. It is possible that such glycation will have an even greater effect on thermostability, but this has yet to be determined.

Trypsin is the most widely used protease for studies on proteins (Finehout *et al.* 2005), especially in proteomic applications involving the use of mass spectrometry to analyze the peptides generated. One of the problems encountered is that native proteins are not readily digested by proteolytic enzymes, so they must be denatured to achieve efficient digestion. The need to denature proteins, usually by the addition of chemicals, adds additional steps and further complicates analytical procedures. Another problem is that trypsin autolysis generates peptides that may also complicate the analysis of MS spectra. This latter problem has been addressed by the use of autolysis-resistant trypsin; however, this does not address the problems caused by the use of denaturants. The glycated trypsin described in this study undergoes much less autolysis than native trypsin (see Chapter 3) and this alone would be very advantageous in proteomic applications. Digestion with trypsin is usually carried out at 20°C to 40°C, whereas the *in vacuo* glycated trypsin provides the possibility of carrying out digestions at higher temperatures. This could have the advantage of shorter incubation times or less enzyme and possibly less autolysis. Also, if sufficiently high temperatures can be used, the need for chemical denaturation prior to digestion may not be required, as the proteins of interest will be heat denatured *in situ*. The glycated porcine trypsin was found to be remarkably stable at 60°C and therefore has the potential for such an application. Indeed, it was found to digest native ribonuclease (**Figure 4-5**) at 70°C, whereas with the native enzyme there was virtually no digestion. The ratio of enzyme to protein was 1:1000 as compared to the 1:50 or 1:100 usually used. Thus, in addition to the advantage of eliminating the need for denaturants, the very small amount of enzyme required would in itself greatly reduce the amount of potential autolytic peptides from trypsin that can contaminate the analysis. This result indicates that the glycated porcine trypsin could be very useful for applications at high temperatures and further studies to establish the optimum conditions for its use as well as similar studies on other *in vacuo* glycated proteins could prove to be very useful.

4-5 CONCLUSIONS

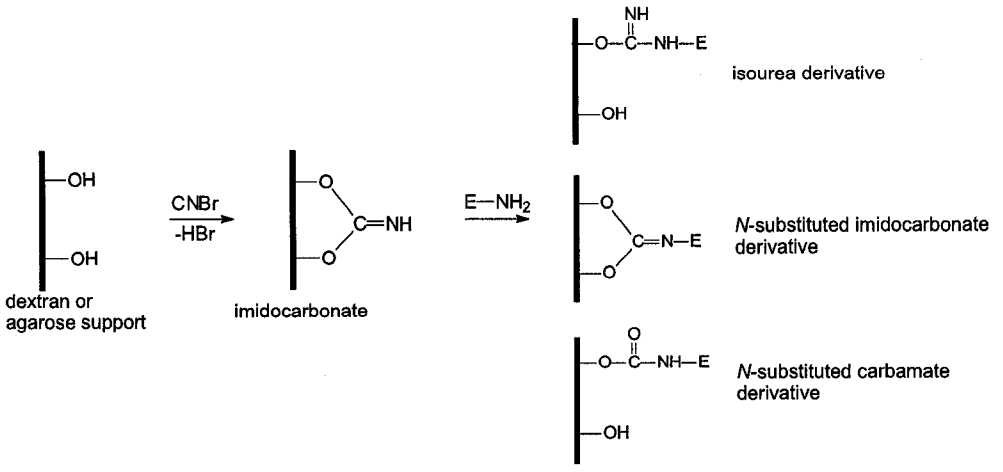
Glycation with monosaccharide can significantly increase the thermostability of proteins. The *in vacuo* glycated trypsin and chymotrypsin retain full activity and enzymatic specificity indicating that there is no significant structural changes in the proteins. It appears that the increased structural stability results from interactions of the covalently attached sugar moiety with nearby elements of the protein structure. The phenomenon may be general in applicably as glycated ribonuclease, which is structurally unrelated to trypsin and chymotrypsin, also shows increased structural stability compared to native ribonuclease.

Chapter 5: A novel protein immobilization strategy without the use of chemical reagents

5-1 INTRODUCTION

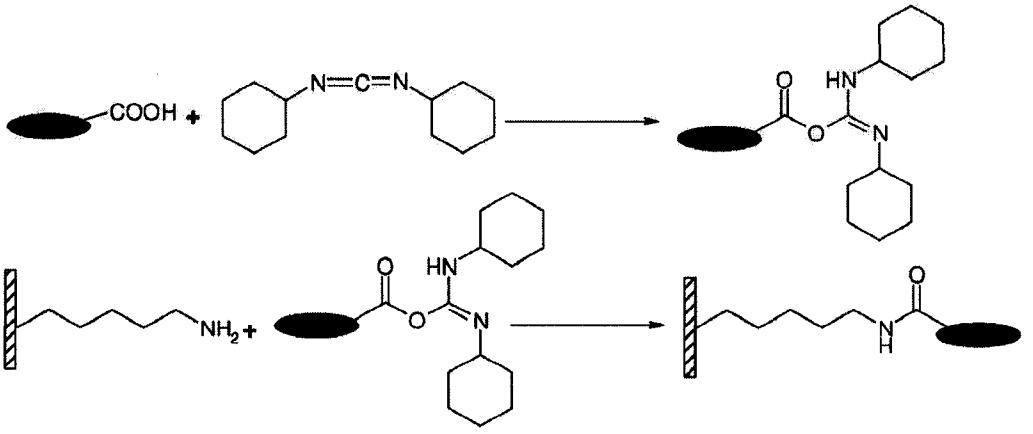
Protein immobilization is the attachment of protein molecules to a solid surface resulting in a loss of mobility. There are three immobilization categories: physical, bioaffinity, and covalent immobilization. Physical adsorption to surfaces is often used because of its relative experimental simplicity, but has the disadvantage that proteins often lose activity due to interactions with the surface. Bioaffinity immobilization, which is a highly specific form of adsorption, overcomes such difficulties but has the disadvantage that the affinity tag must first be attached to the surface and affinity tags for most proteins do not exist. Covalent attachment is usually the method of choice if practicable.

There is a wide variety of solid supports and activation processes that can be used to immobilize proteins. One of the most common is the activation of agarose and dextran, namely Sepharose® and Sephadex®, by cyanogen bromide (Palomo at al., 2004; Yang and Chase, 1998; Zaborsky, 1973). The activation involves the creation of a reactive imidocarbonate which will undergo reaction with the protein amino groups, hence coupling the protein to the support.



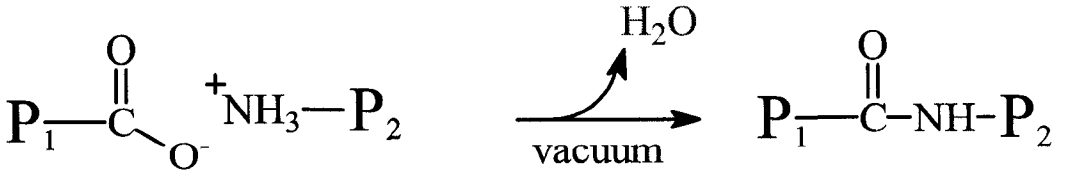
Also widely used are solid supports such as glass beads and polystyrene containing carboxyl groups which can be activated by water soluble carbodiimides.

There are a number of procedures in which there is either a functional group covalently attached to the solid surface that is chemically activated and which subsequently reacts with a functional group on the protein or a functional group on the protein which is chemically activated and which subsequently reacts with the functional group covalently attached to the solid surface. The two examples given above are cases where the groups on the solid surface are chemically activated. An example where a protein functional group is activated is given below (Rusmini *et al.* 2007):



In this case, the carboxyl groups on the protein are activated by carbodiimide and react with free amino groups covalently attached to a solid surface.

Recently, Simons et al. (2002) described a procedure for the cross-linking of proteins that does not require the use of chemical activating reagents. In this procedure, proteins of interest are simply lyophilized together and incubated *in vacuo* at 80 to 100°C. An amide bond is formed between the interacting carboxyl and amino groups on different protein molecules, resulting in a zero-length covalent cross-link.



There is still a need for efficient procedures for the covalent attachment of proteins to surfaces and the current challenge is to develop immobilization methodologies that give high loading and retention of biological activity (Rusmini *et al.* 2007). The *in*

vacuo cross-linking procedure has the potential to provide a novel approach for cross-linking proteins to solid supports in which the likelihood of protein inactivation is greatly reduced because no activating chemicals or solution reactions are required. The objective of the present study was to determine whether the *in vacuo* protein cross-linking procedure developed by Simons *et al.* (2002) could be applied to cross-link proteins to a functionalized solid support, i.e. immobilize proteins.

Two proteins, trypsin and porcine zona pellucida (ZP), were used to test our hypothesis for the *in vacuo* immobilization of proteins to solid supports. Trypsin was chosen because of its industrial and pharmaceutical applications and also because of the experiences acquired during the research on the effect of glycation on autolysis and thermostability (Chapters 2 and 3). The zona pellucida (ZP) is a matrix containing three proteins (ZP1, ~75 kDa; ZP2, ~75 kDa; ZP3, ~44 kDa) (Skinner *et al.* 1999; Wassarman *et al.* 1999). The proteins are extensively glycosylated and are believed to be involved in the recognition of sperm in the fertilization of eggs. Dr. Nongnuj Tanphaichitr at the Ottawa Health Research Institute (OHRI) brought the ZP protein to our attention and informed us of the difficulties encountered in the immobilization of this protein by conventional immobilization procedures. This ZP protein therefore provides a good test of the proposed immobilization methodology.

5-2 MATERIALS AND METHODS

5-2.1 Materials

Porcine pancreatic trypsin and chicken lysozyme were purchased from the Sigma-Aldrich Chemical Company (St. Louis, Missouri). The amine functionalized 3 μm glass beads were purchased from Varian Incorporated. Porcine zona pellucida ovary was purchased from Calbiochem. All other chemicals, reagents and solvents were high purity preparations obtained from the indicated commercial sources.

5-2.2 Immobilization of trypsin

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% (w/v) NH_4HCO_3 solution and then washed twice with a volume of

ddH_2O and resuspended in ddH_2O. A sample of glycated porcine trypsin was dissolved 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 0.10 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 centrifuged to collect the pellets. Upon retrieval, the pellets were 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 10.0 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 resuspended in 3 mL of ddH_2O and lyophilized as before.

5-2.3 Thermostability of immobilized trypsin at elevated temperatures

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 made by the addition of a pH 8.00 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 h) 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 h, 0.5 h, 1 h, 2 h, 4 h and 24 h) 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 to 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 8.0 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.

5-2.4 Digestion of native lysosyme at 60°C

Hen egg white lysozyme was digested by either native, glycated or immobilized porcine glycotrypsin, where the digestion ratio was 1000:1 (lysozyme to trypsin). A 0.200 mg/mL solution of the native and glycated porcine tryspsins 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 calculated that one milligram of the immobilized glycotrypsin sample 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 (corresponding to 10.0 mg of the immobilized product which has the equivalent activity of 2.00 μg of native soluble porcine trypsin) of the immobilized

glycotrypsin sample in NH_4HCO_3 buffer was taken and placed in a 20 mL screw-capped vial. All of the trypsin samples were incubated for a five 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 lysozyme taken from a stock 10.0 mg/mL solution in NH_4HCO_3 buffer was added to each of the trypsin samples. The lysozyme digestion 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 buffer (8% acetic acid and 2% formic acid) and freezing by immersion in dry ice. The samples were dried on a speed vac. The samples were reconstituted with 50.0 μL of pH 2.1 buffer. A 40.0 μL aliquot of each sample (80.0 μg of protein) was applied to a 3.3 cm wide strip of a 20 cm x 20 cm piece of Whatman chromatography paper. In order to ensure proper elelctrophoresis 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 to visualize the peptides generated by lysozyme digestion.

5-2.5 Immobilization of Zona Pellucida

Zona pellucida from porcine ovary (1 mg) was solubilised in 500 μL borate buffer (200 mM, pH 9.2) in a microvial and incubated at 70°C for 45 minutes. After incubation, the solution was clarified by centrifugation and the supernatant was microdialysed (MWCO 12k-14k) against 0.1% ammonium bicarbonate buffer for 3 h. The dialysed sample was then mixed with 100 μL volume of amidated ceramic beads, Sigma-Aldrich ceramic HyperD F Hydrogel beads (ca. 50 μm) functionalized with lysine residues, and lyophilized in borosilicate glass tube (size 13x100 mm). The tube was then sealed under

vacuum and heated at 85°C for 48 h in a similar procedure developed for trypsin immobilization (section 5-2.2). The ZP-beads were extensively washed with PBS buffer (10 times) and 0.02% ammonium bicarbonate buffer (5 times) before re-lyophilization for storage.

Characterization of ZP-beads

Characterization of the bounded ZP was performed by Dr. Nongnuj Tanphaichitr at the OHRI. ZP-beads were incubated (30 min, room temperature) with polyclonal rabbit anti-pig ZP3 IgG antiserum (provided by Dr. E Yurewicz) at 1:1000 dilution in PBS and washed successively in PBS. The washed beads were then incubated (30 min, room temperature) with FITC-conjugated goat anti-rabbit IgG (20 μg/mL) followed by thorough washing in PBS. The beads were viewed under a fluorescent microscope. Alternatively, ZP-beads were incubated with normal rabbit serum (NRS) IgG at 100 μg/mL instead of anti-ZP3 antiserum, or they were incubated with only FITC-secondary antibody alone without any primary antibody.

The ZP-beads and control beads were incubated with spermatozoa (1 M/mL) in PBS buffer and the localization of the spermatozoa was view under a microscope.

5-3 RESULTS

5-3.1 Immobilization of Trypsin

The results obtained demonstrate that when trypsin was lyophilized with amino functionalized glass beads and subjected to the *in vacuo* cross-linking procedure, it did indeed become immobilized on the glass surface. After multiple washings, the beads retain esterolytic activity toward BAEE and preotolytic activity toward denatured RNase. The amount of immobilized trypsin was estimated based the activity of the native trypsin in solution. It was found that 100 μm beads have a solution equivalent of 0.35 μg trypsin / mg of glass beads and the 3 μm glass beads have a solution equivalent of 2.5 μg trypsin / mg of glass beads. This result is expected as there is more surface area per gram of glass in the smaller beads. The ratio of surface areas is 100/3 = 33.3 and the ratio of the activities is 7.1, thus although the activity per gram of bead is greater for the smaller beads, the amount of trypsin immobilized per unit of surface area is greater on the larger

beads. **Table 3-1** demonstrate that the beads retain their tryptic activity after multiple washings and this data confirms the effectiveness of the immobilization. **Figure 2-2** shows the peptide pattern obtained on electrophoresis of CM-RNase digested at 37°C with native trypsin and immobilized trypsin. The immobilized trypsin gives a much more complete digestion as evidenced by the number of sharp peptide bands.

Table 5-1. Retention of Activity* by Glass-Immobilized Bovine Trypsin on Repeated Use.

Number of trials (washing)	Percent Original Activity
	100%
1	94%
2	93%
3	95%
4	95%

*The glass-immobilized trypsin has a solution equivalent of 0.35 μg of native trypsin per mg of glass beads. After each trial the glass-immobilized trypsin (15 mg) was recovered by centrifugation and washed with 10 mL 10mM Tris pH 8 and follow by 2 x 10 mL of distilled water. The activity was quantified by the rate of hydrolysis of BAEE.

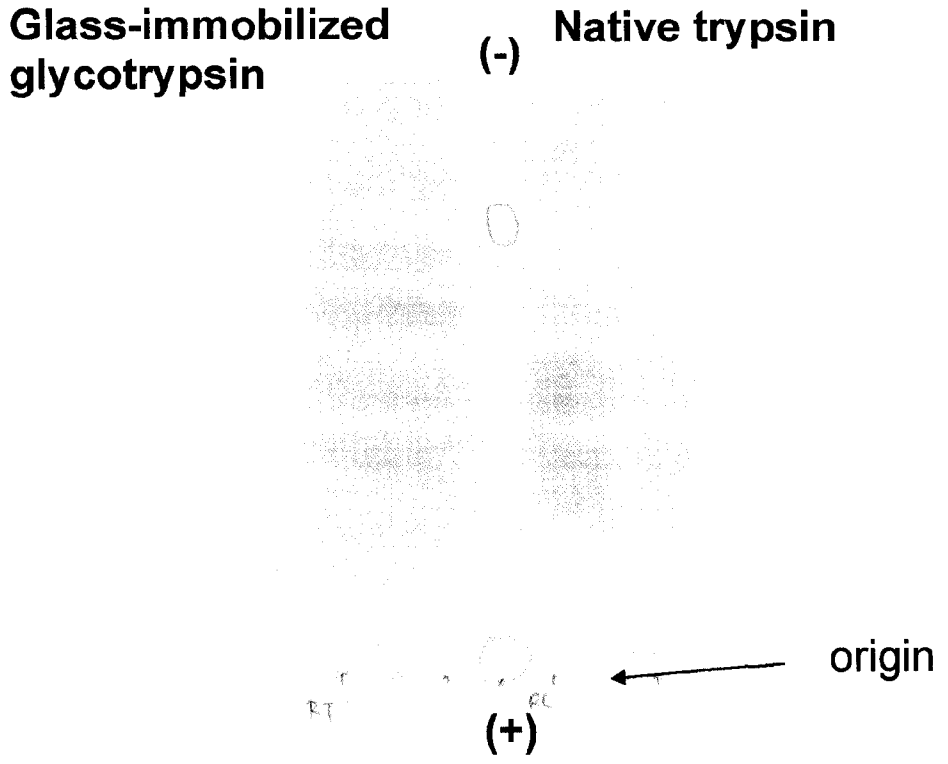


Figure 5-1. Digestion of CM-RNase was carried out with native trypsin at a ratio of 50:1 w/w and with glass-immobilized trypsin also at a ratio of 50:1 w/w (CM-RNase/Glass 1:25 w/w) at 37°C and pH 8.0 for 18h. HVPE was carried out at pH 2.1 at 20V/cm for 2 hour and the chromatogram stained with cadmium-ninhydrin. The positions of the fluorescent markers, dansyl sulfonic (+0) and dansyl arginine (+1), are outlined in pencil.

Figure 5-2 demonstrates that the immobilized trypsin has greatly increased thermostability. After 2 hours at 60°C, it has retained a 100% of its activity whereas in comparison the free native trypsin in solution has lost 80% of its activity. Immobilized glycotrypsin is remarkably thermostable retaining 100% of its activity after 4 hours incubation at 60°C and approximately 90% after 8 hours (**Figure 5-3**). At 70°C the immobilized glycotrypsin retains 50% of its activity after 4 hours incubation (**Figure 5-4**). In comparison, free glycotrypsin in solution at 60°C loses 50% of its

activity in 3 hours (**Figure 4-4**). The proteolytic specificity and efficiency of immobilized glycotrypsin at 60°C was determined by comparing electrophoretic peptide patterns obtained on digestion of CM-RNase with a complete digestion of CM-RNase with native trypsin at 37°C (**Figure 5-5**). After one 1 h digestion, the glycotrypsin generates the same peptides as the 24 h native trypsin digestion. The immobilized glycotrypsin also generates the same peptides as the free glycotrypsin.

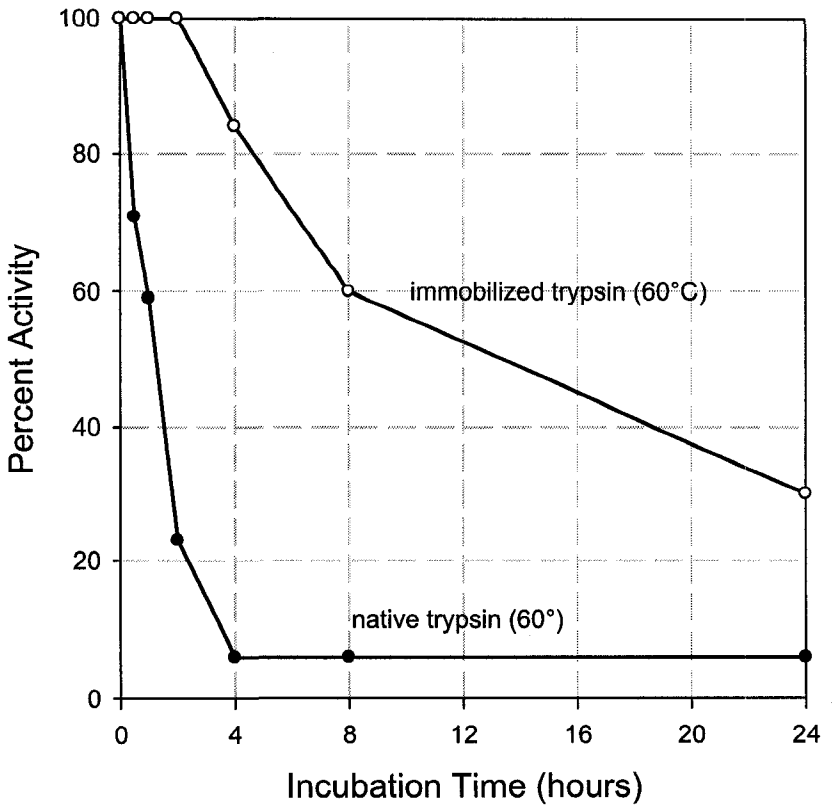


Figure 5-2. Effect of temperature on native porcine tryspin (●) and immobilized porcine trypsin (○). The rate of BAEE hydrolysis by native and immobilized porcine trypsin was measured at a temperature of 60°C after continuous incubation for various periods of time. The activity is shown as % values relative to the initial activity of the samples.

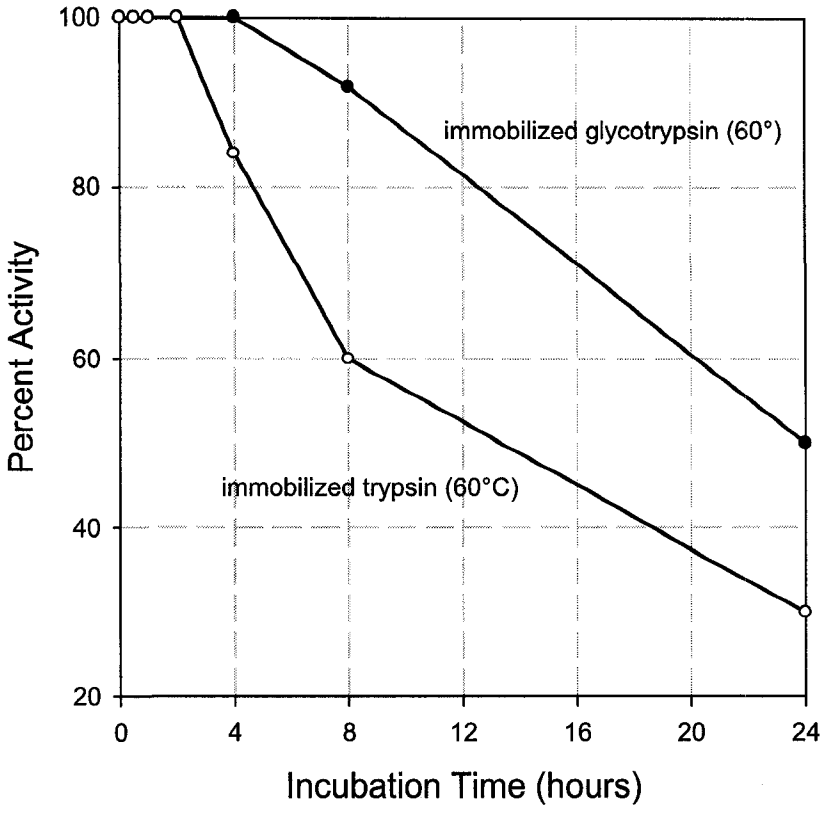


Figure 5-3. Comparison of the effect of glycation on trypsin heat stability of immobilized native porcine tryspin (○) and immobilized porcine glycated trypsin (●). The rate of BAEE hydrolysis by immobilized porcine trypsin was measured at a temperature of 60°C after continuous incubation at that temperature for various periods of time. The activity is shown as % values relative to the initial activity of the samples.

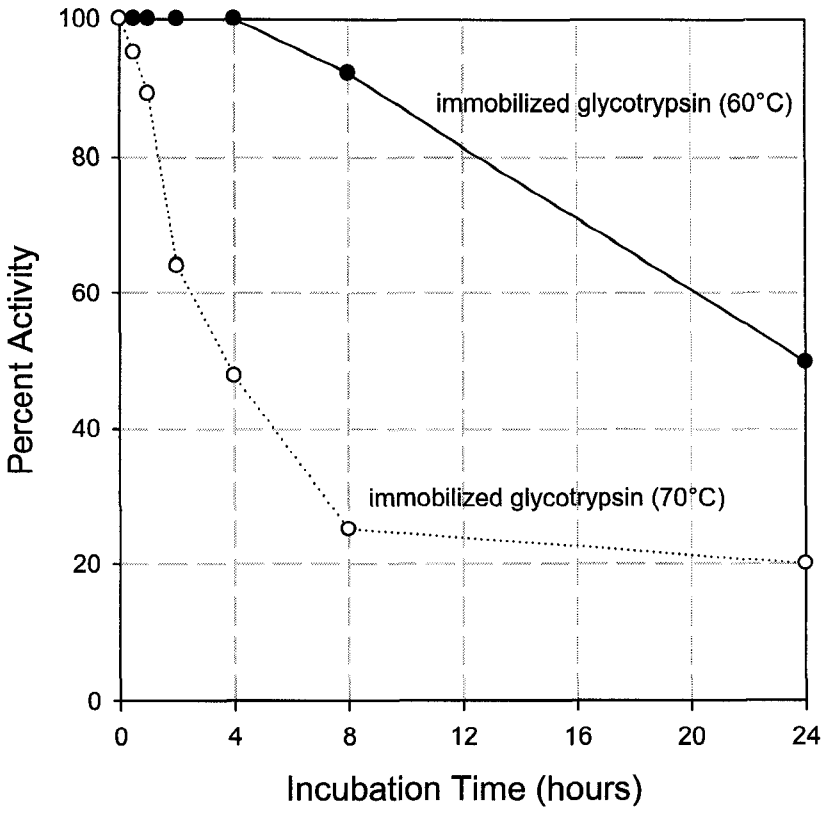


Figure 5-4. Comparison of the effect of temperature on immobilized porcine glycotryspin at 60°C (●) and 70°C (○). The activity is shown as % values relative to the initial BAEE hydrolysis activity of the samples.

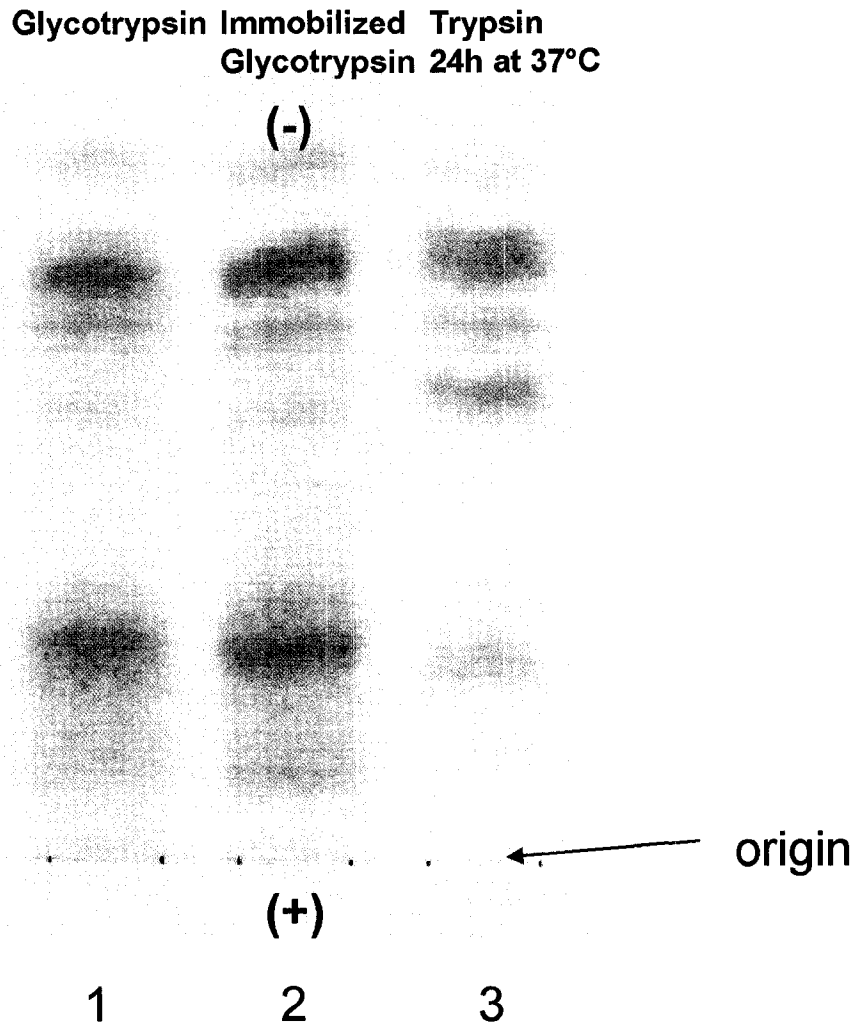


Figure 5-5. Digestion of CM-RNase was carried out with native porcine trypsin, glycotrypsin, and glass-immobilized glycotrypsin at a ratio of 200:1 for 1h at 60°C. For a control, digestion of CM-RNase was carried out with native porcine trypsin at a ratio of 50:1 (w/w) at 37°C for 24h. HVPE was carried out at pH 2.1 at 20V/cm for 2 hour and the chromatogram stained with cadmium-ninhydrin.

The ability of the immobilized glycotrypsin to digest native proteins without the usual prior denaturation was tested by incubation with native lysozyme at 60°C for 24 h. As can be seen in **Figure 5-6**, extensive digestion has taken place as evidenced by the

number of strong peptide bands generated (lane 2). Native trypsin (lane 1) in comparison generates no peptide bands whatsoever. Free glycotrypsin (lane 3) also generates peptide bands, but the digestion is not as extensive.

5-3.2 Immobilization of Zona Pellucida (ZP) Protein

Solubilized porcine ZP was lyophilized at LpH 8.0 with Sigma-Aldrich ceramic HyperD F Hydrogel beads (ca. 50 μm) functionalized with lysine residues and subjected to the *in vacuo* cross-linking procedure by incubation at 85°C for 48 h. Subsequent characterization of the immobilized ZP was carried by Dr. Nongnuj Tanphaichitr. The beads were first tested for ZP immobilization by the binding of fluorescent antibodies. **Figure 5-7** demonstrates that the beads subjected to the *in vacuo* immobilization bind fluorescent antibodies (panels A and B) while the control untreated beads do not (panels C and D). The beads were incubated with sperm and observed under a microscope for the sperm-binding properties. The results are shown in **Figure 5-8**. In panel A, spermatozoa are observed to be concentrated in the regions close to the beads, and panel B is a close-up of one of the beads. The control beads do not show any concentration of spermatozoa in the neighborhood of the beads (panels C and D).

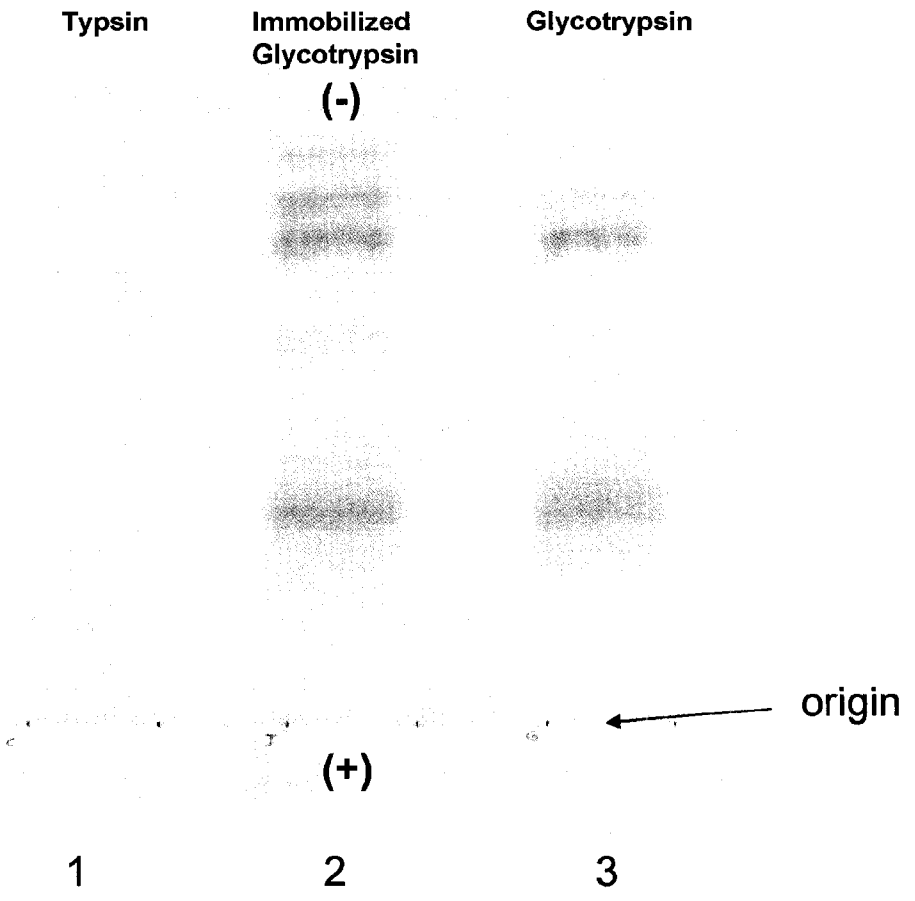


Figure 5-6. Digestion of non-denatured lysozyme was carried out with native porcine trypsin, glass-immobilized glycotrypsin, and glycotrypsin at a ratio of 1000:1 for 24h at 60°C in 50mM ammonium bicarbonate. HVPE was carried out at pH 2.1 at 20V/cm for 2 hour and the chromatogram stained with cadmium-ninhydrin.

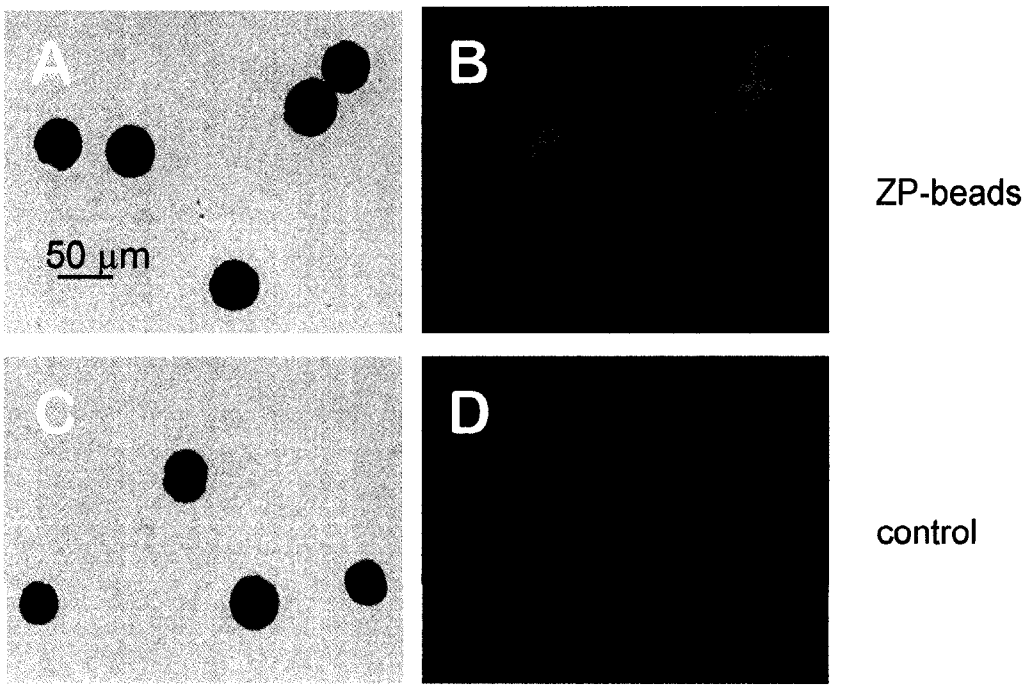


Figure 5-7. Immunofluorescent staining of immobilized Zona Pellucida matrix protein. ZP-beads (A, B) or control beads (C, D) were incubated with polyclonal rabbit anti-pig ZP3 IgG antiserum at 1:1000 dilution in PBS, then followed by FITC-conjugated goat anti-rabbit IgG (20 µg/mL). The beads were viewed under a fluorescent microscope.

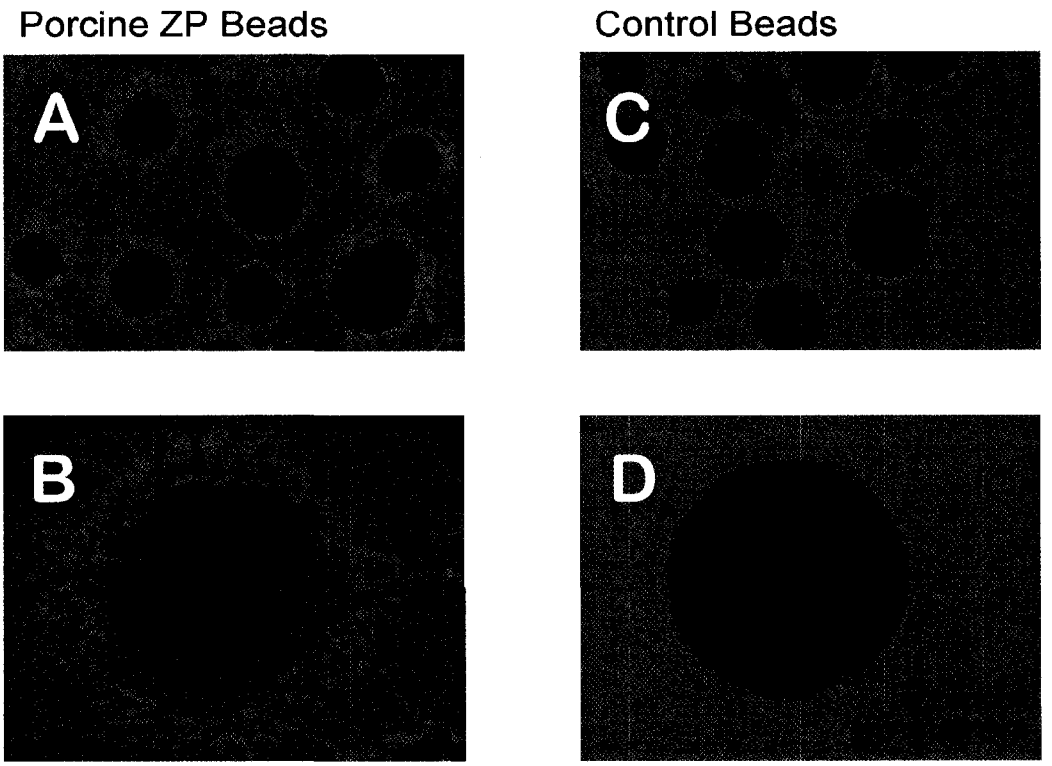


Figure 5-8. Sperm-binding: porcine ZP-beads (A, B) bind sperms where as the control beads (C, D) do not. ZP protein was immobilized on Sigma-Aldrich ceramic HyperD F Hydrogel beads (ca. 50 μm) functionalized with lysine residues. The beads were incubated with sperm and observed under the microscope for the sperm-binding properties.

5-4 DISCUSSION

The *in vacuo* cross-linking procedure requires that there be a strong interaction between ammonium and carboxylate ions in the lyophilized state. Initial studies by Simons *et al.* (2002) were concerned with cross-linking of protein molecules and it was demonstrated that such interactions did exist between protein molecules in the lyophilized state. In the case of ribonuclease, it was found that the interaction between the amino and carboxyl groups between molecules is highly specific with only one cross-link formed between Lys-66 on one molecule and Glu-9 on the other (Simons *et al.* 2007). It seemed likely that if a protein was lyophilized with a solid support functionalized with amino groups that there would be sufficient interaction with carboxyl groups on the protein in the lyophilized state to effect the *in vacuo* formation of an amide linkage thereby immobilizing the protein on the solid surface. Extensive washing of the glass beads after subjection to the *in vacuo* procedure with trypsin or ZP did not result in loss of protein activity.

In the case of trypsin, the protein had greatly improved stability consistent with covalent immobilization. The conformational stability of the trypsin molecule is unlikely to be altered by the cross-linking. Therefore the observed increased thermostability most likely resulted from a decrease in autolysis as contact between enzyme molecules is greatly restricted and thermally unfolded molecules can refold to an active conformation. Glycotrypsin has been shown to have a greater thermostability than native trypsin (Chapter 4) and the immobilized glycotrypsin also showed a greater thermostability than immobilized native trypsin. In fact the immobilized glycotrypsin for all practical purposes retained its full activity for 8 h at 60°C. The proteolytic specificity of the immobilized trypsin and glycotrypsin is unaltered indicating that there are no alterations in the active site of the enzyme.

Native proteins are resistant to proteolytic enzymes when folded in their native state. In order to obtain efficient enzymatic hydrolysis of peptide bonds, proteins are denatured prior to digestion. This denaturation is most often accomplished by the addition of denaturants such as urea or chemical modification or both. These chemicals interfere with subsequent analysis of the proteolytic fragments generated and must be

removed. This is problematic in proteomic applications using MS analysis as these chemicals not only interfere but also can be a source of significant contaminations (Xu *et al.* 2003). The immobilized trypsins described in this thesis offer the possibility of mitigating these problems.

To our knowledge, the *in vacuo* immobilized glycotrypsin is the most stable form of trypsin ever described. As has been demonstrated, glycotrypsin has greatly improved resistance to autolysis (Chapter 3) and thermostability (Chapter 4). The immobilized glycotrypsin has a remarkable thermostability and retains its full activity at elevated temperatures where native trypsin is completely inactivated. One advantage of this is that proteolytic digestions can be carried out at elevated temperatures with much less enzyme and for shorter period of time. For example, tryptic digestions are usually carried out at trypsin/protein ratio (w:w) of 1:50 to 1:100 at 37°C for 8h to 24h (Burlingame *et al.* 1998; Roepstorff 2000). In the case of glycotrypsin (Chapter 4), it was demonstrated such digestions can be carried out with a glycotrypsin/protein ratio (w:w) of 1:1000 at 70°C. With the immobilized glycotrypsin, complete digestion of a denatured protein can be carried in 1 h at 60°C and a ratio of 1:200 (**Figure 5-5**). Furthermore, immobilized glycotrypsin will digest native lysosyme at 60°C at a immobilized glycotrypsin/lysozyme ratio (w:w) of 1:1000 without any requirement for prior denaturation. Lysozyme has a melting point of 68°C and is much more thermostable than most proteins (Sartor *et al.* 1994). Nevertheless, the results show that it is sufficiently unfolded at 60°C for the immobilized glycotrypsin to digest it and it is likely this will be true for most proteins.

5-5 CONCLUSIONS

In summary, digestion with immobilized trypsin has several advantages over the free enzyme. Digestions can be carried out at elevated temperatures with less enzyme for shorter periods of time. Furthermore, the immobilized enzyme can be easily removed by centrifugation. These two advantages should significantly reduce the extent of contamination of the peptide samples and facilitate the analysis of fragmentation patterns by MS.

ZP is a complex of several proteins and it is likely that conventional immobilization procedure requiring the use of activated chemical components would

disrupt and inactivate this structure. The successful immobilization of ZP with retention of biological activity demonstrates the advantage of the *in vacuo* immobilization methodology. The procedure requires lyophilization which is one of the most used methods for preserving the activity of proteins on storage. Proteins are much more thermostable in the dry state (Klibanov 1995) and under vacuum they are even more thermostable so that the incubation at 85°C does not result in any loss of activity. With both test proteins, there was full retention of activity and this is likely to be true for most proteins. Indeed in all proteins tested to date, there has been no loss of activity (Taylor *et al.* 2005; Simons *et al.* 2006a; Simons *et al.* 2007).

Chapter 6: Conclusions and future research

6-1 GENERAL CONCLUSIONS

The theme of the work presented in this thesis has been the application of the *in vacuo* chemical modification to the development of new and improved methodologies for the study of proteins. *In vacuo* methylation is a new methodology for chemical modification of proteins and has not as yet been widely applied in other laboratories. However in the last year, several laboratories have recognized the advantages of this methodology and applied it in their research (Simons *et al.* 2006b; Johnson *et al.* 2007). There are several general experimental advantages of modifying proteins *in vacuo*: 1) Modifications are carried out on the proteins in a lyophilized state where proteins are much more stable than in aqueous solution. 2) Modifications can be carried out with water insoluble reagents to achieve modifications that are difficult or impossible in aqueous solution. 3) In the lyophilized state there is no equilibrium between the protonated and deprotonated species, i.e. pH-memory effect (Klibanov 1995). Therefore, Le Chatelier's principle is not fully operative during the chemical modification and the specificity of the modifications can be controlled much more effectively by the choice of the pH at which proteins are lyophilized (LpH). There are also several technical procedural advantages: 1) Purification of the modified proteins is much simpler as no buffer or reagents have to be removed. 2) As there is no competition from water, much smaller amounts of reagents are required, typically 1/100 to 1/1000 of that needed for aqueous modifications. 3) The use of labelled reagents, e.g. radio-labelled or isotopically-labelled, is much more economical than in water and in addition any unreacted reagents can be recovered and reused. 4) Modification on much smaller amounts of proteins can be carried out as, unlike solution based modifications, sample size requires no alterations whatsoever in the modification procedure. 5) In the lyophilized state, proteins are very thermostable, often at temperatures greater than 100°C (Klibanov 2001), so that reactions *in vacuo* can be greatly accelerated. 6) In some cases, e.g. glycation and protein cross-linking, no chemical activation or chemical activating reagents are required to achieve a chemical modification.

Every methodology has its advantages and disadvantages. The major disadvantage of *in vacuo* chemical modification is that reactions must be carried out under vacuum and this requires that the proteins and reagents be sealed under vacuum. Most laboratories are not setup to carry this out and most chemists and biochemists have not been trained in the experimental skills required. This makes it difficult for researchers to apply this methodology. The skills cannot be acquired by reading descriptions of the methodology, but can only be learned by hands-on experience. Another disadvantage is that reagents must be volatile or able to be volatilized and this limits the use of many reagents. Proteins must be soluble and stable to lyophilization, which most proteins are, but there are some proteins whose properties will not permit *in vacuo* modification. In our opinion, the advantages greatly outweigh these disadvantages and in time the *in vacuo* chemical modification of proteins and peptides will be more widely used. Other laboratories have already successfully applied the *in vacuo* methodology described in this thesis, some examples of which are Laremore *et al.* 2005, Simons *et al.* 2006, and Johnson *et al.* 2007.

6-2 *IN VACUO* METHYLATION

It was demonstrated that *in vacuo* methylation could be used to selectively isolate N and C-terminal peptides from protein digests. This proof of principle was made possible by the ability to incorporate economically a ^{14}C-labelled methyl group into the proteins. This permitted the visualization of the peptides on the diagonal (Chapter 2) and provides the evidence that N and C-terminal peptides do indeed lie along a diagonal line. The cost of obtaining such evidence by radio-labelling in water would have been prohibitive. Further research is required for the MS application of this methodology. In preliminary experiments, it was found that C-terminal peptides were indeed present by ESI-MS; however, the analysis was complicated by the presence of non-peptide impurities in the sample eluted from the diagonal. It was not possible to carry out further research to solve this problem because of the lack of access to MS facilities, but there are several reasons to expect that this difficulty can be resolved. The presence of impurities in peptide samples is not uncommon and methods have been developed to address this problem (Xu *et al.* 2003; Saavedra and Barbas 2007). Also the bulk of the non-peptide

impurities in the paper can be removed before electrophoresis; therefore, any subsequent sample cleanup by established methods should be more effective. The trimethylated peptides are also detected with much greater sensitivity in MALDI-MS and likely ESI-MS than unmodified peptides, thus improving the ability to differentiate them from non-peptide impurities.

In the study of the reaction of the protonated amino group with iodomethane (Chapter 2), it was observed that there was greater than an order of magnitude increase in the sensitivity of detection with ESI-MS of the type A allatostatin III peptide on trimethylation. The sensitivity of the detection was not the focus of these experiments, but nevertheless it is an interesting and potentially useful observation. Further studies on a variety of peptides need to be carried out to determine the generality of this observation and its potential utility.

6-3 MS ANALYSIS OF PEPTIDES

The ability to introduce economically a double isotopic label with iodomethane (ICH_3 and ICD_3) into peptides provides interesting possibilities for research into differentiating peptides from non-peptides impurities in MS spectra and improving the MS analysis of peptides. By carrying out the methylation of the protein on equal amounts of sample, one with ICH_3 and the other with ICD_3, and on combining the samples, the N-terminal peptides in the MS spectrum should appear as two peaks 9 mass units apart with equal intensities. This, in principle, should serve to differentiate peptides from non-peptides impurities. In the case of C-terminal peptides where only one methyl group is incorporated, following the same procedure there should be two peaks with equal intensities separated by 3 mass units. Another possibility for further research is to carry out the labelling *in vacuo* with acetic anhydride [$(CH_3CO)_2O$ and $(CD_3CO)_2O$], in which case the peptides could be detected as two peaks with equal intensities 3 mass units apart.

Tandem MS of the trimethylated peptides is governed by a mechanism that differs from peptides with a mobile proton (Gross 2000; Wysocki *et al.* 2000). It is expected that the major ion series (a, b, and c) will be predominate in MS/MS fragmentation of the peptide backbone and not the xyz ion series, as a permanent charge resides on the

N-terminal side. Since the peptide bond is the major fragmentation site, it is expected that an enhanced b ion series will be observed in tandem MS, and indeed this has been observed (Poon *et al.* 2004; Simons *et al.* 2006b). Therefore, trimethylation offers the possibility of facilitating sequence determination of the isolated peptides. Another favourable feature of *in vacuo* methylation is that histidine and lysine residues are permethylated giving them a permanent positive charge. This should facilitate the sequence determination of peptides containing lysine and histidine residues as it will prevent preferential fragmentation at these residues (Gross 2000; Wysocki *et al.* 2000). Another possible avenues of future investigation are the use of *in vacuo* methylation of a peptide digest as a general procedure to differentiate peptides from non-peptides impurities, to increase the sensitivity of peptide detection, to facilitate sequence determination, and to quantify the amount of individual peptides.

6-4 *IN VACUO* GLYCATION

Aqueous glycation has not been widely used as an approach for glycation of proteins because of the low extent of glycation, heterogeneous glycation products and production of AGEs. On the other hand, the results obtained for *in vacuo* glycation (Chapter 3) demonstrate that this methodology produces a high degree of glycation, a homogeneous ketoamine linkage and no AGEs. It was found that extensive glycation significantly increases the thermostability of proteins (Chapter 4). Glucose was used as the primary glycating agent, and preliminary work indicates that the effect of glycation on protein properties does not differ greatly with other reducing monosaccharides. However, these results are not conclusive and it is possible that other monosaccharides will show even greater effects. More research needs to be carried out with different monosaccharides and a variety of different proteins to fully characterize the effects of such glycation.

In vacuo glycation is very efficient with monosaccharides and it would be of interest to determine how well this glycation process works with larger polysaccharides having a terminal reducing sugar. It is expected that it will be more difficult to achieve the same degree of glycation as with monosaccharides because specific interaction of amino groups with the reducing end of the terminal sugar in the lyophilized state will be

decreased. Glycation with di, tri, and tetrasaccharides and even larger polysaccharides can be carried out to determine the extent of glycation and optimal conditions. It may be possible to achieve high extents of glycation by repeated *in vacuo* glycation. With larger polysaccharides a significant effect on protein properties may be observed even with a low degree of glycation. The glycation studies described here were carried out on a limited number of proteins and additional studies should be carried out on other proteins to establish firmly the generality of the results obtained. There is always the possibility that other glycated proteins will have novel properties not observed with the proteins studied here.

6-5 *IN VACUO* PROTEIN IMMOBILIZATION

It was discovered by Simons *et al.* (2002) that amide bonds could be formed *in vacuo* between interacting carboxylate and ammonium ions to yield covalently cross-linked proteins. This discovery was extended to show that proteins could be cross-linked to solid supports containing functionalized amino groups to yield proteins covalently immobilized by an amide bond. An advantage of this approach is that no chemical activating reagents are required so that the possibility of protein inactivation is reduced. This is particularly important in the immobilization of protein complexes involving several interacting proteins where chemical modification usually disrupts such structures. ZP is such a protein complex consisting of three proteins and the successful covalent immobilization of this complex (Chapter 5) demonstrates the potential utility of the *in vacuo* immobilization procedure. Protein immobilization is widely used for many purposes and future research on the *in vacuo* immobilization of other proteins, especially protein complexes, is likely to yield novel theoretical and practical applications.

6-6 *IN VACUO* REACTION MECHANISM(S)

Another issue of interest is the mechanism(s) by which reactions occur *in vacuo* with proteins. In our studies, no systematic effort was made to investigate the effect of the *in vacuo* pressure (~ 50 to 70 mTorr) or to the amount of water vapour present in the samples. Perhaps these are important factors and investigations in which these factors are precisely controlled should be carried out. Reactions that are unfavourable at

atmospheric pressure, such as the formation of amide bonds between interacting carboxylate and ammonium groups on proteins, take place *in vacuo*. Proton transfers in the solid state are obviously involved and it is likely that water vapour participates in facilitating this, and it would be mechanistically interesting to determine the optimal vapour pressure of water and the effect of pressure on this reaction. Similarly, in the case of *in vacuo* methylation of proteins proton transfers are involved. It is unlikely that the conditions adopted for the work described in this thesis were optimal and further research needs to be carried out to establish optimal conditions.

Claims to original research

1. The development of a diagonal electrophoretic procedure for the selective isolation of peptides derived from the carboxyl terminus that is not sequence dependent.
2. The development of a diagonal electrophoretic procedure for the selective isolation of peptides derived from a free or blocked amino terminus that is not sequence dependent.
3. Demonstration that peptides with a trimethylated α-amino group are detected with an increased sensitivity in ESI-MS.
4. Evidence that water mediated proton transfers may be important in *in vacuo* chemical modifications of proteins and peptides.
5. Preparation of a glycated form of trypsin that is more autolysis resistant than any previously reported forms of soluble trypsin.
6. Preparation of an immobilized glycotrypsin that retains full activity for up to 8 hours at 60°C and is more thermostable than any form of trypsin previously reported.
7. Demonstration that glycation of proteins with glucose significantly increases their stability.
8. Demonstration that the immobilized glycotrypsin can digest native folded proteins at elevated temperatures without the requirement for prior denaturation.
9. Demonstration that proteins can be immobilized on functionalized solid supports without the use of any activating chemicals by *in vacuo* cross-linking.
10. Demonstration that complex structures composed of several interacting proteins can be immobilized on functionalized solid supports without loss of activity by *in vacuo* cross-linking.

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