

**Unraveling the Molecular Foundations Behind the Diverged Behaviors
of Mouse Insulin 1 and Insulin 2, Connecting Diabetes Risk with
Glucocorticoid Treatment and Chronic Migraine through the Analysis
of Islet Chemistry, and Capturing Key Posttranslational Modifications
All Through the Application of a Novel LC-IMS-MS Workflow**

An Honors Thesis Presented for the

Bachelor of Science

Degree

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ABSTRACT

We display the capabilities of our established liquid chromatography-ion mobility spectrometry-mass spectrometry (LC-IMS-MS) workflow in the investigations of islet chemistry at the sub-single-islet level. We begin by characterizing the structural differences of Ins1 and Ins2 to present novel insights as to why their behaviors diverge. We then examine the effects of the stress hormone corticosterone, the rodent equivalent of human cortisol that is often used as a therapeutic, on pancreatic peptide hormone secretion. We also uncover the molecular connection behind the inverse relationship between type 2 diabetes (T2D) risk and chronic migraine via the neuropeptides CGRP and PACAP. Lastly, we apply our technique to the detection of posttranslational modifications (PTMs).

Through our studies, we elucidate how corticosterone-induced beta-cell proliferation may lead to insulin resistance. Our results show that the level of amylin, a peptide co-secreted with insulin from the beta-cells, increased significantly compared to the control samples, correlating well with an increase in beta-cell population observed in immunoassays. Furthermore, while the corticosterone-treated mice might produce a high level of insulin due to the proliferation of insulin-positive cells, the hormones were quickly degraded. Because the truncated form of insulin may not be as effective as the intact form, our data help rationalize insulin resistance induced by corticosterone.

We show that alpha-CGRP remodels amylin fibrillization, a process implicated in T2D. CGRP and/or amylin monomers reduce the secretion of both mouse Ins1 and Ins2 proteins, however, CGRP oligomers have a reverse effect on Ins1. Beyond the mechanistic

insights, our data suggest that CGRP regulates insulin secretion and lowers the risk of T2D, thus rationalizing how migraine might be protective against T2D.

We observe that PACAP-38 enhances Ins1 secretion under glucose stimulation yet has no significant effects on Ins2 *in-vitro*. We address the need for future studies with transgenic mice to determine if PACAP's remodeling of islet morphology can serve therapeutic purposes in T2D patients.

We demonstrate that DTIMS can capture important PTMs that can serve as biomarkers—Asn deamination and *IsoAsp* formation. Finally, we use DTIMS analysis to show that spontaneous *cis/trans* proline isomerization can occur under a different mobility type.

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CHAPTER ONE

PANCREATIC ISLETS AND DISEASE

1.1 Pancreatic Islets of Langerhans

Islets of Langerhans are collections of endocrine cells that produce and secrete signaling molecules called hormones. Islets are spread throughout the pancreas and play a vital role in the homeostasis of many bio-physiological processes. They consist of five main cell types— α -, β -, γ -, δ -, and ϵ -cells. α -cells produce the peptide hormone glucagon. Glucagon is implicated in glucose homeostasis and increases blood glucose levels. To achieve this, glucagon promotes glycogenolysis (the breakdown of glycogen to monomeric glucose) and gluconeogenesis (the *de novo* synthesis of glucose) while also inhibiting glycolysis (the breakdown of glucose for energy) and glycogenesis (the storage of glucose into glycogen) [1]. β -cells produce insulin, whose functions oppose those of glucagon. Insulin lowers glucose levels by inhibiting gluconeogenesis and glycogenolysis while upregulating glycolysis and glycogenesis. Insulin also promotes protein synthesis and lipogenesis—the synthesis of fatty acids and triglycerides from glucose [2]. γ -cells secrete pancreatic polypeptide (PP). PP functions to reduce food intake, inhibit glucagon secretion, and increase energy use [3, 4]. Somatostatin is secreted from δ -cells. Somatostatin inhibits both insulin and glucagon release while also impeding β -cell proliferation—a process that increases cell number [5]. Finally, ϵ -cells release ghrelin, a hormone that increases hunger, inhibits insulin secretion, and reduces energy expenditure [6].

The percentage of each cell type compared to the overall number of endocrine cells within islets differs between species. Human islets are comprised of 50-70% β -cells, 20-40% α -cells, and less than 10 percent of γ -, δ -, and ϵ -cells [7]. Comparatively, laboratory mouse (*Mus musculus*) islets contain 60-80% β -cells, 15-20% α -cells, <10% γ -cells and <1% δ - and ϵ -cells [8]. Human and mouse islets also differ in cell arrangement, morphology, and nerve fiber innervation patterns [7]. While these differences exist, endocrine signaling pathways are highly conserved between mice and humans, and the necessity of mouse models for human disease research is apparent.

1.2 Type 2 Diabetes as a Protein Misfolding Disease

Type 2 diabetes (T2D) is a degenerative metabolic disorder characterized by insulin resistance and progressive β -cell dysfunction and/or death. T2D accounts for the vast majority (~90%) of diabetes cases and affects more than 415 million people worldwide. Its prevalence is expected to grow to ~642 million by the end of 2040 [9]. The aggregation of human amylin (also islet amyloid polypeptide; hIAPP), has been implicated as the causative agent of T2D [10]. Amylin is a 37-residue peptide that is synthesized in the pancreas and co-released with insulin from pancreatic β -cells in a 1:100 ratio in healthy patients [11]. Amylin is hydrophobic and contains an intramolecular disulfide bridge and amidated C-terminus [12]. Amylin has physiological functions in glucose regulation via gastric emptying, increasing satiety, and reducing gastric secretion [13]. It also inhibits insulin secretion to lower blood glucose levels as well as prevents both the synthesis of glycogen and glucose uptake [12].

Amylin monomers are soluble and intrinsically disordered, meaning they lack a well-defined structure in their native states. These monomers quickly aggregate at low micromolar concentrations and accumulate into insoluble protein deposits called amyloid, which is found in over 90% of T2D patients [14]. Normally, insulin binds amylin monomers and inhibits their self-assembly and subsequent aggregation [15]. However, under T2D pathology, initial glucose intolerance leads to increased insulin secretion, and therefore, increased amylin secretion [16]. High levels of amylin lead to amylin nucleation and subsequent aggregation. Amylin aggregation occurs via the conversion of helix-coil conformations to helix- β -strand conformations in homodimers. These homodimers propagate the β -sheet motif to other monomers [17]. The β -sheet motif allows for further formation of oligomeric species and, ultimately, insoluble fibrils. Both oligomers and fibrils induce cytotoxic effects, with oligomers being the primary culprits. Mechanisms of amylin-induced toxicity include mitochondrial dysfunction, membrane pore formation and dysfunction, formation of reactive oxygen species (ROS), initiation of apoptosis, DNA damage, and islet inflammation [18]. All-in-all, excessive amylin aggregation results in the early β -cell dysfunction and eventual β -cell death observed under T2D pathology.

1.3 Glucocorticoids and Diabetes Development

Cholesterol-derived steroid hormones called glucocorticoids (GCs) are synthesized and released from the adrenal gland in response to emotional and physiological stress. GCs are typically under hypothalamic-pituitary-adrenal (HPA) axis control [19]. GCs bind the GC receptor, a nuclear receptor, and are implicated in a variety of physiological processes such as immune response, metabolism, development, cognitive functions, and mood [20-

23]. Administration of GCs is commonly used in the treatment of inflammation, cancer, and autoimmune disease [24]. Despite its considerable therapeutic success, GC treatment can result in side effects such as new-onset hyperglycemia. T2D patients are particularly susceptible to a severe worsening of hyperglycemia [25]. Hyperglycemia involves an excess of glucose in the bloodstream. Its onset can promote both insulin resistance and β -cell dysfunction. Insulin resistance is characterized by the insensitivity of insulin in target tissues, whereas β -cell dysfunction is more severe and displays impaired insulin secretion [26]. Both insulin resistance and β -cell dysfunction further promote hyperglycemia and, therefore, T2D. In addition to insulin, GCs can also affect glucagon, somatostatin, amylin, and other islet peptides [27, 28].

Humans and mice utilize different GCs. Corticosterone is found primarily in rodents, while in humans it is produced in low amounts (10-20-fold lower concentrations than the primary GC secreted in humans, cortisol) [29-33]. Both cortisol and corticosterone bind to the same mineralocorticoid receptors (MR) in the brain with high affinity, and the glucocorticoid receptors (GR) with an approximately 10-fold lower affinity [34]. Both molecules have 21 carbons; the only difference is an extra hydroxyl group at the 17th carbon of cortisol (circled red in Figure 1). Various direct and indirect effects of cortisol/corticosterone treatment have been reported with distinctions between acute and chronic exposure [35]. Cushing syndrome, for example, is characterized by endogenous hypercortisolism and can lead to T2D development [36]. Moreover, corticosterone treatment has been shown to promote the early markers of β -cell proliferation that correlate to insulin resistance and hyperinsulinemia without increasing total body mass [37]. Overall,

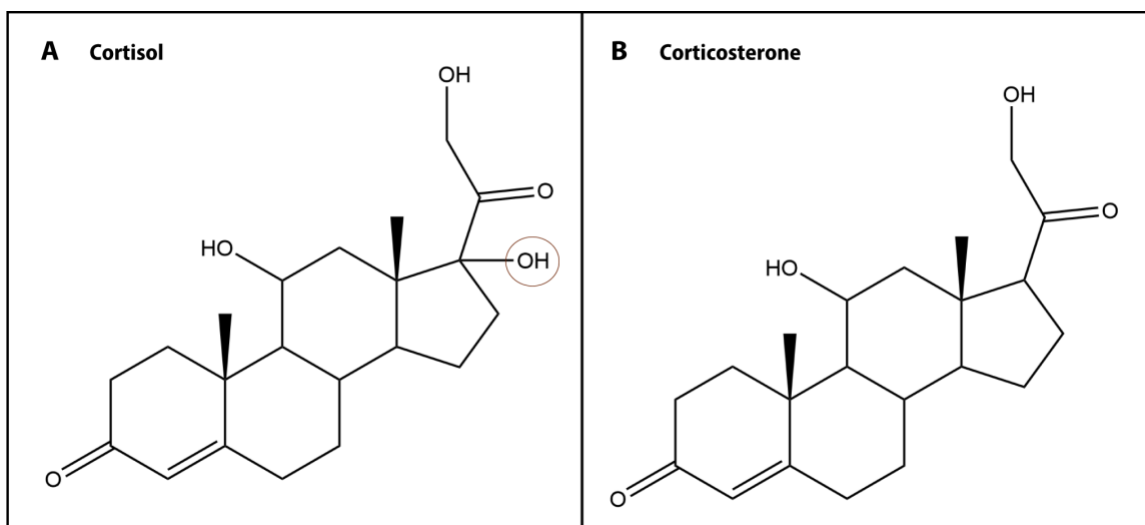


Figure 1. Chemical structures of (A) cortisol and (B) corticosterone.

stress is highly connected to metabolic processes through the secretion of GCs. As such, the effects of GCs on the islet environment are important in understanding both how stress can lead to disease development and how GC treatment can result in disastrous side effects like diabetes.

1.4 Neuropeptides Link Type 2 Diabetes Risk with Chronic Migraine

Conditions

Migraine is a disabling neurovascular disorder affecting more than 12% of the general population [38]. Migraine involves enhanced cortical excitability that leads to the activation of the trigeminal sensory pathway and results in an inflammatory response that causes pain [38]. Two epidemiological studies propose that patients with chronic migraine have a much lower susceptibility to developing T2D [39, 40]. The inverse relationship between the two diseases stems from a molecular connection mediated by migraine-associated neuropeptides that are either produced or have receptors in pancreatic cells. There are a variety of these peptides including pain sensory (calcitonin gene-related polypeptide [41]; CGRP, pituitary adenylate cyclase-activating polypeptide [42]; PACAP, and substance P [43]; SP), sympathetic (neuropeptide Y [44]; NPY), and parasympathetic (vasoactive intestinal peptide [45]; VIP) neuropeptides. CGRP and PACAP are both implicated in migraine-associated vasodilation and nociception and have been reported to modulate insulin secretion in pancreatic islets [46-49]. CGRP has been found to both promote and inhibit insulin secretion, while PACAP stimulates insulin secretion in a glucose-dependent way without inhibiting insulin sensitivity. Another neuropeptide, NPY inhibits insulin secretion [50] with a feedback mechanism [51].

By evaluating the effects of these migraine-associated neuropeptides on the islet environment, a better understanding of the connection between these diseases is achievable and may lead to better therapeutic and preventative strategies. For example, the inhibition of insulin secretion by CGRP and/or NPY would result in less amylin secretion. Decreased amylin secretion would prevent excessive amylin aggregation and its associated cytotoxicity. Furthermore, lower levels of circulating insulin are protective of T2D while high levels of circulating insulin are a risk factor for insulin insensitivity and pancreatic cancer progression [52, 53].

At first, the stimulation of insulin and amylin release by PACAP may seem contradictory to the concept that chronic migraine is protective of T2D, however, PACAP also stimulates the secretion of glucagon [54] and catecholamines [55]. The dual effect of PACAP on elevating both glucagon and insulin levels allows for the increase in glucose turnover without changing plasma glucose levels and inducing hyperglycemia [56]. Additionally, catecholamines mediate the increase of glucagon secretion while also inhibiting insulin secretion, meaning the direct effects of insulin on the islet may be suppressed by other mechanisms of PACAP [57]. Despite higher levels of insulin under PACAP conditions, glucose elimination rates are unaffected [57]. PACAP also exerts bidirectional effects on islet morphology depending on pathological status. For example, PACAP significantly suppresses islet density and exhibits a reduction in islet mass in transgenic mice with severe T2D with PACAP overexpression ($A^y/+;PACAP/+$) compared to $A^y/+$ mice [58]. The same study showed that PACAP may inhibit ductal islet neogenesis, a mechanism implicated in the increased β -cell mass observed in T2D [58]. Alternatively,

in T1D-induced mice, β -cell proliferation is observed under increased PACAP levels [59]. Increasing β -cell proliferation is proposed to counter the near-total loss of β -cells observed under T1D pathology [60]. Finally, PACAP displays anti-diabetic effects through the protection of β -cells against glucotoxicity, calcium toxicity, and lipotoxicity [59].

1.5 Project Goals

The overarching goals of this project are extensive. We aim to prove the robust and reliable nature of our established LC-IMS-MS workflow [61] in detecting pancreatic peptide hormones at the single-islet and sub-single-islet levels. We will apply this method to observe the effects of stress-induced GC release and/or GC treatment on metabolism, as well as to elucidate the molecular mechanism behind the inverse relationship between T2D and chronic migraine. Particularly, the effects of acute corticosterone treatment on peptide hormone secretion and enzymatic activity will be assessed to unravel the mechanisms in which stress and/or steroid treatment may induce insulin insensitivity, β -cell dysfunction, and eventual T2D. The link between chronic migraine and T2D will be explored through the effects of migraine-associated neuropeptides, namely CGRP and PACAP, on pancreatic islet chemistry. Along the way, we will determine the structural differences between the previously uncharacterized mouse insulin 1 (Ins1) and insulin 2 (Ins2) proteins and explore how differences in their primary sequences affect insulin aggregation propensities and functions. Finally, the power of LC-IMS-MS in the analysis of biological samples will be assessed with a separate peptide system and compared to a different IMS-MS instrument type. Key posttranslational modifications will be explored in this peptide

system which reveals novel abilities of drift tube (DT)IMS-MS to pinpoint asparagine deamidation and subsequent *isoaspartate* formation to specific residue positions.

CHAPTER TWO

APPROACH AND INSTRUMENTATION

2.1 Pancreatic Perfusion and Islet Isolation

For experiments involving mouse pancreatic islets, mouse treatment conditions varied between projects and will be listed in each project's representative section. Despite this, a standardized pancreas perfusion and islet isolation method was performed, with the steps outlined below.

To begin the pancreas perfusion, the desired mouse is anesthetized prior to cervical dislocation. The surgical field is set up and the proper steps are taken to prepare the pancreas for perfusion. A tiny hole is made in the common bile duct and a needle cannula attached to the syringe of injection enzyme solution is inserted. 6 mL of prepared enzyme injection solution is injected. The pancreas is then removed and placed in a Falcon tube with ~10 mL enzyme injection solution. The serum is then processed, and the top layer is extracted.

Digestion of the pancreas is then performed. The tube containing the pancreas and enzyme is placed into a 37 °C water bath with oxygenation (95/5) for 5 minutes. Post-oxygenation, the tube is placed in a water bath for 25 minutes and shaken every 5 minutes. After digestion, the tube is removed from the water bath and a prepared cold quenching buffer solution is added to stop the digestion of the enzyme. The solution is homogenized, and undigested exocrine tissue is filtered out. The tube undergoes centrifugation at 1100

RPM for 3 minutes at 4 °C. Finally, the supernatant is decanted, and excess liquid is removed via gravity.

Extraction of islets is initiated with the addition of 7 mL of 25% polysucrose 400 Ficoll (Fisher 156328, MP Biomedicals), a hydrophilic polysaccharide utilized in gradient centrifugation. Sequentially, 7 mL of 23%, 20%, and 11% Ficoll is added to separate the sample into four distinct layers. The tube is centrifuged at 2100 RPM for 20 minutes. The mouse islets are localized to the interface between the 20% and 11%, as well as between the 23% and 20% Ficoll layers. Islets are removed and transferred to a Falcon tube containing 30 mL quenching buffer before being centrifuged at 1100 RPM for 3 minutes at 4 °C. Supernatants are then removed and washed with quenching buffer and another round of centrifugation. New supernatants are removed and 10 mL of room temperature RPMI 1640 with glutamine (Fisher 10040CV, Mediatech) are added to the pellet. All media is then removed, and islets are placed in a tissue culture dish. An inverted microscope is used to assess the quality and purity of islets. Lastly, islets are placed in an incubator with 5% CO₂ at 37 °C.

The day after isolation, islets are removed from the incubator. Islets are cleaned until no ductal or exocrine tissue remains before they are counted and evenly distributed into microcentrifuge tubes containing fresh media. The islets are subsequently stimulated with IL-1B. First, 1 µL IL-1B is added to ~1 mL pure islet solution before incubation for 3 hours with 5% CO₂ at 37 °C. After incubation, most of the media is removed and 300 µL RLT (Qiagen 1015750) buffer is added. The islet solution is then placed in a -80 °C freezer. Additional information can be found in a previous work [61].

2.2 Liquid Chromatography-Ion Mobility Spectrometry-Mass-Spectrometry

The advent of ion mobility spectrometry-mass spectrometry (IMS-MS) has allowed researchers and companies to apply the technique to a variety of fields such as explosives, environmental analysis, food science, and (bio)chemical studies [62-65]. MS functions to determine and quantitate analyte identities by their mass-to-charge ratios (m/z) [66]. IMS enables the separation of gas-phase ions based on their charge, size, and shape to give structural information about molecules [66]. The coupling of these techniques yields a high resolving power proving useful to many diverse fields of research. Analyte ionization is a key part of IMS-MS. Our primary instrument, the Agilent 6560 IMS-QTOF (Agilent, Santa Clara), utilizes an electrospray ionization (ESI) source which transfers intact biological samples into the gas phase [67]. ESI is considered a “soft” ionization method ideal for biological samples. When the aqueous sample is introduced to the ESI-jet stream source, a high voltage is applied to the emitter needle. The liquid is eluted as a fine mist of charged droplets. This is called the Taylor cone. Solvent molecules subsequently evaporate from the charged droplets. Charge repulsion promotes the ejection of smaller charge droplets until there is only one ion per droplet. Ions are then pulsed into the ion mobility cell that is under an electric field and filled with inert gas, typically helium (He) or nitrogen (N₂). Ions in the drift cell collide with the chosen buffer gas and separate based on their charge, size, and shape. Upon reaching the end of the drift cell, ions move through a quadrupole time-of-flight (Q-TOF) mass spectrometer before reaching the detector. This results in arrival time distribution (ATD) data that yields information on the size and shape of analytes. Ion

abundance can also be obtained from ATD data. The Q-TOF separates ions based on their m/z ratios. The detector allows for the generation of a mass spectrum that pairs each m/z ratio to an ion and its relative intensity. Lastly, a collisional cross section (CCS) can be determined for analytes. CCS enables further identification of analytes across various ion mobility instruments. CCS represents the shape of an ion of a specific compound in a particular buffer gas environment. CCS (σ) is related to the ion's reduced mobility (K_0) which is calculated using arrival times and the Mason-Schamp equation [68]:

$$\sigma_{exp} \approx \frac{3ze}{16N_0} \frac{1}{K_0} \sqrt{\frac{2\pi}{\mu k_B T}}$$

Equation 1: Mason-Schamp Equation

where z is the charge of the ion, e is the elementary charge, N_0 is the gas number density of the drift-gas, K_0 is the reduced mobility, μ is the reduced mass of the ion and drift-gas, k_B is the Boltzmann constant, and T is the temperature of the drift gas. CCS is a powerful tool, as it can be compared across multiple measurement modalities including nuclear magnetic resonances (NMR), x-ray crystallography, circular dichroism (CD), computational modeling, and electron microscopy [69].

A liquid chromatography (LC) system can be coupled to the Agilent 6560 IMS-MS instrument. LC can further identify analytes in complex solutions based on their retention time—the time it takes for a sample to elute from the column. This property depends on the polarity of the analytes in the sample. Our workflow utilizes an Agilent 1260 Infinity LC with a 2.1x50 mm Zorbax Extended C18 reverse-phase column (Agilent, Santa Clara).

Drift Tube IMS-MS Method

DTIMS experiments were performed on an Agilent 6560 IMS-QTOF (Agilent Technologies, Santa Clara). Samples were ionized with an ESI source and ions were pulsed through a nitrogen-filled drift cell (3.94 Torr) in a multifold experiment. Upon exiting the drift cell, ions were guided through the QTOF mass spectrometer by a hexapole ion guide. Data were collected in positive polarity with $\Delta V = 1490, 1390, 1290, 1190, \text{ and } 1090 \text{ V}$. ATDs and mass spectra were analyzed with Agilent IM-MS Browser software (v.10.0). CCSs were determined using **Equation 1**.

LC-IMS-MS Method

Per each run, 4.0 μL of each sample was injected into a 2.1 x 50 mm Zorbax Extended C18 reverse-phase column (Agilent, Santa Clara). The mobile phase consisted of an aqueous solution of 0.1% formic acid in LC-grade water (solvent A) and acetonitrile (solvent B). The mobile phase gradient is as follows: 98% solvent A for 3 minutes, 80% solvent A (minutes 3-5), 65% solvent A (minutes 5-18), 50% solvent A (minutes 18-23), 25% solvent A (minutes 23-26), 10% solvent A was held for 2 minutes and then followed by a rise to 98% (minutes 35-40) and kept for 5 minutes (minutes 40-45) for column calibration. The column was set to 40 °C.

2.4 LC-IMS-MS vs. Traditional Immunoassays

Traditionally, researchers use immunoassays like the enzyme-linked immunosorbent assay (ELISA) to measure insulin secretion. ELISA functions by exploiting the reactions between antigens and antibodies. First, antigens are put into a solid phase and allowed to react with antibodies. The addition of a second, enzyme-labeled antibody detects this reaction [70]. When using chromogenic substrates that induce color

changes in the particular enzymes added, color development arises where the antigen is present and can be detected and quantified using a microtiter plate reader [70].

There are two major advantages of our LC-IMS-MS workflow to ELISA methods. First, ELISA requires ~30 islets to measure secreted insulin levels. Our LC-IMS-MS workflow can reliably detect insulin levels at the sub-single-islet level. In our sub-single-islet experiments, a single islet is suspended in 50 μ L of solvent. Only 4 μ L of the islet solution is injected into the instrument, meaning we measure approximately 1/10th of a single islet which includes ~150 cells. We can detect heterogeneity within islet cell populations by collecting multiple technical replicates of these ~150 cells from a single islet. Furthermore, we can utilize multiple islets from the sample animal per experiment. This limits variations between animals and limits animal-related costs.

Secondly, ELISA is unable to distinguish between isoforms of insulin in animals possessing more than one insulin gene. Mice, for instance, have a two-gene system encoding mouse insulin 1 (Ins1) and mouse insulin 2 (Ins2). Our LC-IMS-MS method can distinguish between mouse Ins1 and Ins2 and has shown these proteins have different structures and actions [61]. We propose the ability/inability to distinguish these isoforms is vital to research efforts that aim to correlate mouse insulin functions to those of human insulin functions. These features highlight the advantage LC-IMS-MS has over traditional methods of islet chemical analysis.

2.4 Trapped Ion Mobility Spectrometry-Mass Spectrometry

Trapped ion mobility spectrometry (TIMS)-MS is a mobility type that can achieve a much higher resolving power ($R \sim 200$) than the drift tube (DT)IMS instrument ($R \sim 40$)

[71, 72]. The TIMS instrument employs an electric field to hold ions stationary against a moving gas [69]. This setup is the inverse of the DTIMS instrument. Despite their differences, TIMS operates under the same physics as DTIMS within the drift cell, meaning ATD and CCS data can still be obtained accurately. ESI, among other ionization methods, can be applied to the TIMS instrument, similar to the DTIMS instrument.

2.5 Circular Dichroism

Circular dichroism (CD) is used to investigate the secondary structure of proteins and peptides. CD arises from the unequal absorption of left-handed and right-handed circularly polarized light [73]. These two polarized waves can be separated by prisms or devices that use Pockel's effect. Asymmetric molecules differentially absorb left- and right-handed circularly polarized light, which is detected by CD instruments. Each secondary structural element— β -sheet, loop, α -helix, etc.—has a unique CD spectrum. This enables researchers to predict the secondary structure of uncharacterized peptides and explore conformational changes due to aging, heat, interactions, and mutations [73]. In this study, we will use CD to probe differential conformations of Ins1 and Ins2 to observe how those structures change over time.

2.6 Scanning Transmission Electron Microscopy

Scanning transmission electron microscopy (STEM) is a tool used to view samples at the atomic level, enabling microstructural characterization. STEM focuses a high energy beam of electrons, at a high angle unlike the perpendicular angle in TEM, from an electron gun onto a small probe at the specimen plane. This happens while the instrument is pulled

under a vacuum. The electron probe is then scanned across the specimen. Transmitted inelastically scattered electrons, as opposed to elastically scattered electrons in TEM, are collected by the detectors for each point [74]. The result is the formation of Z-contrast images based on the angular distribution of transmitted electrons where the scattering angle depends on the mean atomic number Z of the specimen [75].

CHAPTER THREE

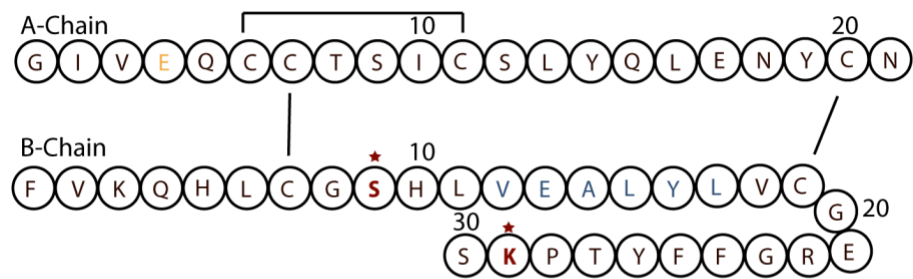
CHARACTERIZATION OF MOUSE INSULIN 1 AND INSULIN 2

Unlike humans, mice have a two-gene system that encodes two insulin proteins—insulin 1 (Ins1) and insulin 2 (Ins2). The *Ins2* gene is the orthologue of human insulin, while the *Ins1* gene is a retrogene that originated from a partially processed *Ins2* mRNA strand that was reverse-transcribed and incorporated into the murine genome [76]. It is proposed that the *Ins1* retrogene was positively selected for in mice and its preservation in the genome served as an evolutionary advantage during ancient times due to the lack of available food resources [76]. Ins1 was shown to be more sensitive to glucose stimulation than Ins2 [77], making it advantageous under scant food conditions. However, as food became more abundant, *Ins1* left mice with a greater susceptibility to T1D development. *Ins2* was also positively selected for, most likely due to its ability to protect mice from T1D. Unlike most retrogenes, *Ins1* contains regulatory regions similar to those of *Ins2*. mRNA expression levels of Ins1 and Ins2 are similar (57:43% of the total insulin mRNA) [77]. However, Ins1 and Ins2 have diverged functions in metabolism. For instance, a study on non-obese diabetic (NOD) mice lines showed that male NOD mice with only one copy of *Ins1* develop type 1 diabetes (T1D) before reaching 10 weeks of age, and female NOD mice with only one copy of *Ins1* have a high prevalence of T1D and islet inflammation (insulinitis). Alternatively, male and female NOD mice with only one copy of *Ins2* exhibit normal insulin levels and do not experience T1D pathologies [78]. Unlike *Ins1* NOD-knockout (KO) mice, *Ins2* NOD-KO mice displayed lower levels of insulin content than

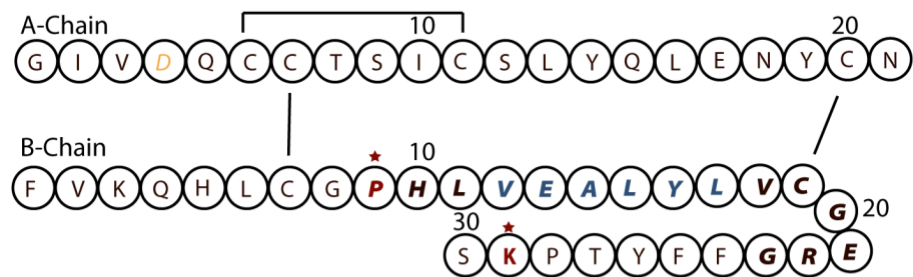
wild-type (WT)-NOD mice in both males and females [78]. These results suggest that *Ins1* may be a defective gene due to the lack of compensatory mechanisms.

Apart from diverged genetic regulation and functions, *Ins1* and *Ins2* also differ in structure. The insulin genes encode preproinsulin which includes the N-terminal signaling peptide, A-chain, B-chain, and connecting peptide (C-peptide). Preproinsulin is then cleaved to proinsulin consisting of the A-chain, B-chain, and C-peptide. Here disulfide bonds form between the A-chain and B-chain at residues A7-B7 and A20-B19. An additional interchain disulfide bond is created at A6-A11. Proinsulin is cleaved at either side of the C-peptide leaving the mature insulin protein containing only the A-chain and B-chains with the disulfide bonds intact. The C-peptides differ at three residues, and *Ins1* displays two gaps in the sequence compared to *Ins2*. In mature insulin, *Ins1* and *Ins2* differ at two residues in the B-chain—positions B9 and B29. *Ins1* incorporates a proline at B9 and a lysine at B29, whereas *Ins2* has a serine at B9 and a methionine at B29. Note, that human insulin includes a serine at B9 and a lysine at B29. Mouse and human insulins differ at one position in the A-chain (AspA4 in mice and GluA4 in humans). The variation in primary sequences between *Ins1* and *Ins2* results in structural differences that may lead to differential aggregation propensities and diverged molecular actions. Figure 2 compares human insulin, mouse *Ins1*, and mouse *Ins2* sequences. Red stars indicate residue differences in the B-chains, lines indicate disulfide bonds, and the B9-23 segment is indicated by bold and italic font. The blue VEALYL sequence highlights the amyloidogenic region. Dissimilar to biological assays, our LC-IMS-MS workflow can

A Human Insulin



B Mouse Ins1



C Mouse Ins2

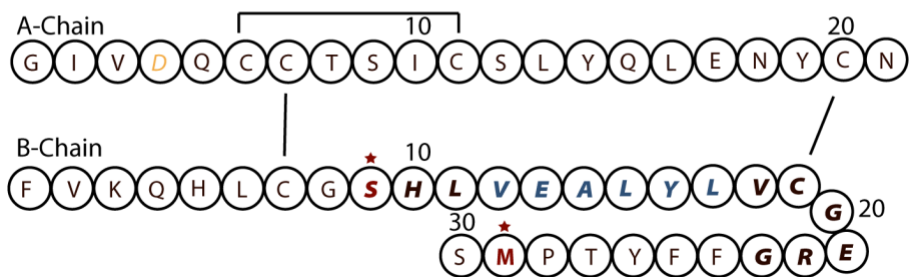


Figure 2. Comparison of the primary sequences of human insulin and mouse Ins1 and Ins2.

distinguish between Ins1 and Ins2 in isolated mouse islets, making it a powerful tool for their characterization. Our data on isolated islets show that Ins2 is degraded more readily than Ins1, as seen in Figure 3. Intact Ins2 levels are lower than intact Ins1 levels and truncated forms of Ins2 are prominent. We hope to characterize Ins1 and Ins2, for the first time, with LC-IMS-MS to explain degradation patterns observed in mouse islets. We focus our attention on Ins1 and Ins2 B-chains, as the only differences in the primary sequences occur there. We also investigate the B9-23 segments, which have been demonstrated to elicit different immune responses [79]. Ins1 B9-23 was suggested to be diabetogenic, while Ins2 B9-23 protects against spontaneous diabetes in NOD mice [79]. The proline in Ins1 B9-23 may be the driving factor behind these results. Furthermore, the B9-23 segments are particularly prone to aggregation and include the amyloidogenic region—VEALYL [80]. The effects of proline substitution within the B9-23 segment will also be explored.

3.1 Materials and Methods

The Ins1 and Ins2 synthetic B-chains used for this study were synthesized by LifeTein, LLC and used without any further purification. Ins1 and Ins2 B9-23 peptides were synthesized with the help of Dr. Kristi Cantrell and Ethan Hawke from Westmont College. The peptides were pretreated with hexafluoroisopropanol (TCI America). Aliquots of the peptide stocks were left to evaporate in a desiccator overnight. All insulin B-chains and B9-23 segments were reconstituted in LC-grade water (VWR) in 20 mM ammonium acetate buffer, pH = 6.8, for final peptide concentrations of 10 μ M. All DT

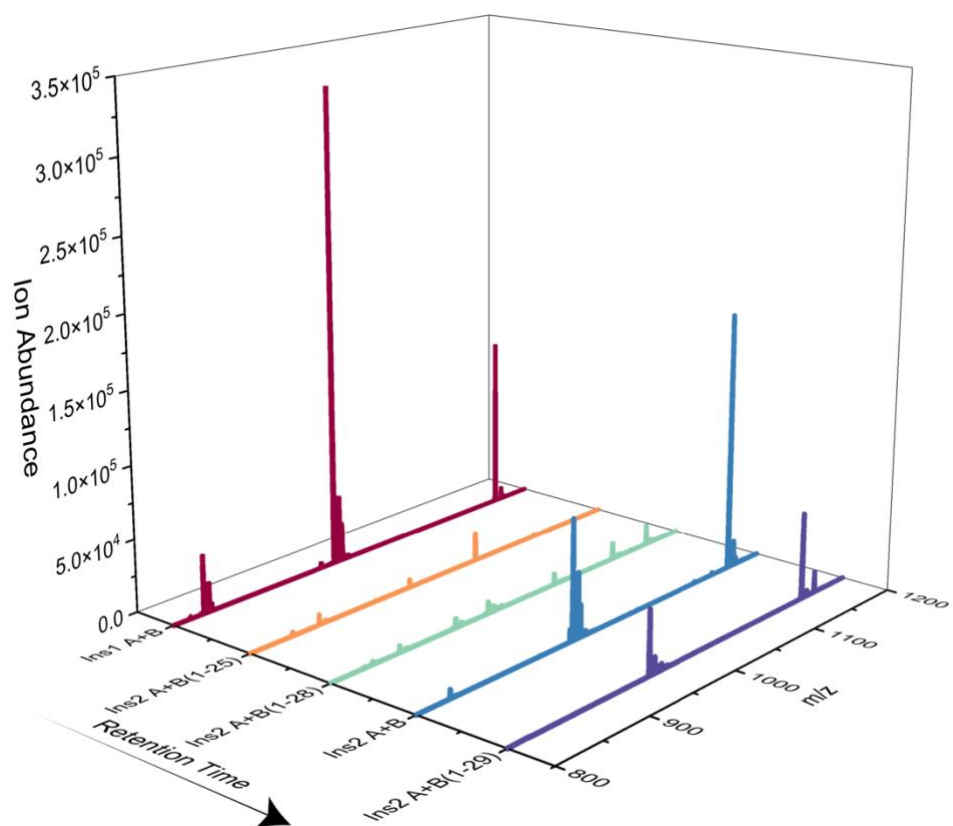


Figure 3. 3D plot of the ion abundances of intact Ins1 (red), intact Ins2 (blue), and truncated Ins2 peptides (yellow, green, and purple).

IMS-MS and LC-IMS-MS experiments were carried out according to the methods outlined in **Chapter 2.2**.

Far-UV Circular Dichroism

Ins1 B9-23 and Ins2 B9-23 were diluted to a concentration of 100 μM in 10 mM sodium phosphate, pH = 7.0. The samples were filtered using a 0.20 μm , 4 mm Titan syringe regenerated cellulose filter to remove insoluble materials. Samples were diluted to 100 μM using the extinction coefficient for the tyrosine residue ($\epsilon_{275} = 1420 \text{ cm}^{-1} \text{ M}^{-1}$). The absorbances were measured on a Thermo Electron Corporation Nicolet Evolution 200 UV-Visible Spectrometer. CD data were collected in a Jasco J-810 CD spectrometer. The CD measurements were collected at 1 nm intervals from 260 to 190 nm with a 4 s response time and a 1 nm bandwidth. All spectra were collected at 22 $^{\circ}\text{C}$ in a 1 mm quartz cuvette. Fifteen scans were collected, the data were averaged, a baseline generated from a sample containing only the solvent was subtracted from the averaged scans, and the curves were baseline corrected. Spectra were taken of samples quickly following their preparation and incubated in a 37 $^{\circ}\text{C}$ water bath for 1 week before collecting the final spectra to test for β -sheet formation.

Scanning Transmission Electron Microscopy

Ins1 B9-23 and Ins2 B9-23 peptides were diluted to a concentration of 100 μM in 10 mM sodium phosphate, pH = 7.0. Samples were stored in a water bath at 37 $^{\circ}\text{C}$ for 1.5 weeks. The samples were then spun down for fifteen minutes at 10,000 rpm. The Ins B9-23 samples formed a pellet upon centrifugation, and a pellet was not observed with the full-length B chain peptides. Samples (5 μL) were applied to a glow discharged, 400-mesh,

carbon-coated support film and stained with 1% uranyl acetate. Images of fibrils at a magnification of 21,000X and 137,200X were recorded using an FEI Tecnai F30 scanning transmission electron microscope (STEM) operated at 300 kV and equipped with a Gatan charge-coupled device (CCD) digital micrograph.

3.2 Ins2 Adopts a More Compact Conformation than Ins1

We began by performing an LC-IMS-MS analysis of the synthetic Ins1 B-chain. The acquired chromatogram displays two distinct peaks indicating unique different features, as seen in Figure 4. The mass spectrum of the Ins1 B-chain ($z = +5$) in each LC feature shows that the monoisotopic m/z ratio shifts to the left by ~ 2 Da. This mass shift is attributed to the oxidation/reduction of an intramolecular disulfide bond between the cysteine residues at B6 and B19. The LC peaks were labeled either oxidized (with disulfide bond) or reduced (without disulfide bond). The mass shift of ~ 2 Da stems from the loss of two hydrogens when the disulfide bond is oxidized. To confirm the oxidation/reduction reaction was indeed taking place, H_2O_2 was added to samples in a 1:10 H_2O_2 :Ins1 B-chain ratio. H_2O_2 has the propensity to exert both oxidizing and reducing actions. Figure 5 displays the results of time-course H_2O_2 experiments in which the addition of H_2O_2 initially ($t = 1$ hour) shifts the sample towards the reduced form without the disulfide bond before rearranging into the oxidized form at $t = 24$ and $t = 68$ hours. Peptide aging in the absence of H_2O_2 shifts the B-chain to the oxidized form. The Ins2 B-chain mass spectrum also displays the ~ 2 Da mass shift indicating disulfide bond formation. Note that even in oligomers, disulfide bonds were identified as intramolecular, not intermolecular, bonds.

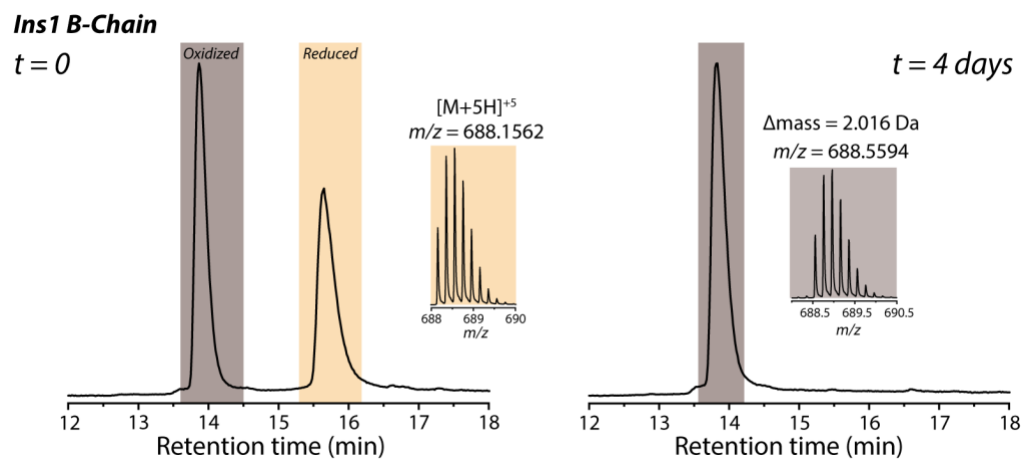


Figure 4. LC chromatogram of Ins1 B-chain showing disulfide bond formation with associated mass spectra.

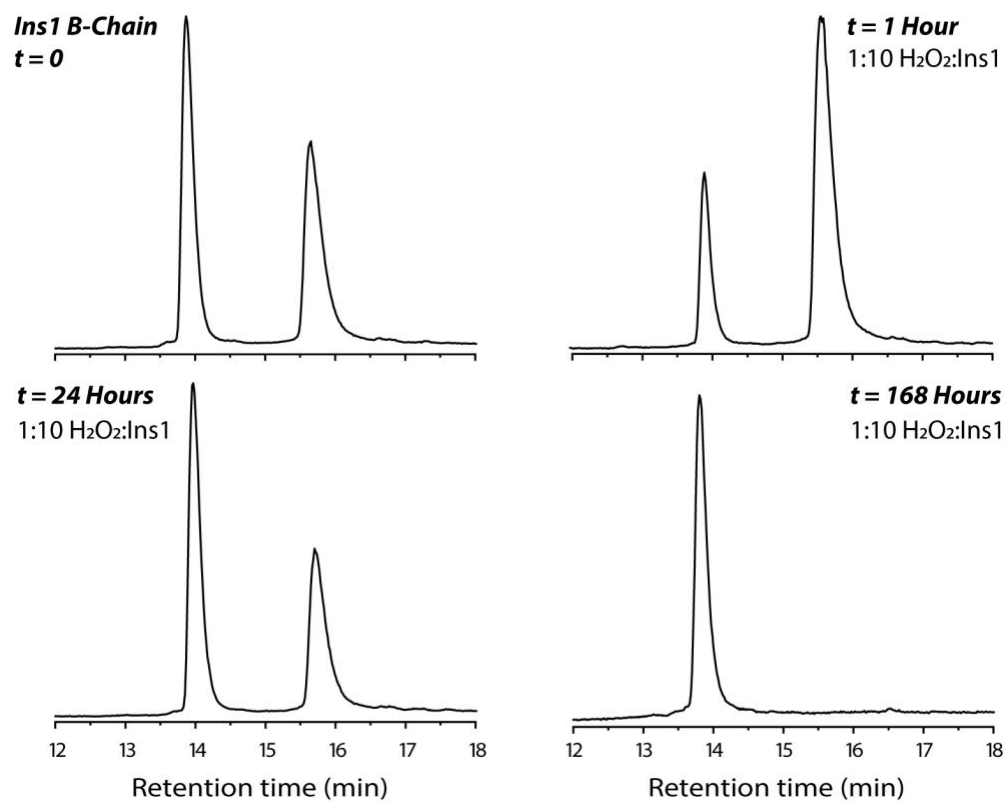


Figure 5. Time-course LC experiments of Ins1 B-chain with H₂O₂.

The presence of these non-native disulfide bonds may affect insulin B-chain aggregation and structure. Turning to mobility data, the collected CCS_{N2} values of oxidized Ins1 B-chain and Ins2 B-chain were $782.37 \text{ \AA}^2$ and $741.70 \text{ \AA}^2$ ($z = 4$) respectively. CCS_{N2} values of reduced Ins1 B-chain and Ins2 B-chain were $898.60 \text{ \AA}^2$ and $817.85 \text{ \AA}^2$ ($z = 4$). ATDs were obtained with LC-IMS-MS, as seen in Figure 6, which shows that Ins2 B-chain has a smaller arrival time ($z = 4$) in both oxidized and reduced forms than Ins1 B-chain. These results indicate that Ins2 B-chain is more compact and folded than Ins1 B-chain.

Synthetic Ins1 and Ins2 B9-23 segments were also evaluated with LC-IMS-MS in similar conditions to insulin B-chains. Figure 7 represents the mass spectra, ATDs, and 2D plots of the B9-23 segments ($z = 2$). The presence of a non-native intermolecular disulfide bond is indicated by a mass shift of $\sim 2\text{Da}$. Because the B9-23 segments contain only one cysteine residue per monomer, we observe two monomers linking covalently to form a dimer. The CCS_N of Ins1 B9-23 was found to be $431.1 \text{ \AA}^2$ ($z = 2$). The CCS_{N2} of Ins2 B9-23 was $428.3 \text{ \AA}^2$ ($z = 2$). The main conformer in Ins1 B9-23 arrives at 28.80 ms, while the primary Ins2 B9-23 conformer arrives at 28.67 ms. These results indicate that, as expected, Ins2 B9-23 is more compact compared to Ins1 B9-23. The observation that both Ins2 B-chain and Ins2 B9-23 adopt more compact conformations than Ins1 B-chain and Ins1 B9-23 correlates well with our data on mouse islets that show mature Ins2 also adopts a more compact and folded conformation. We speculate that Ins2 is better at retaining its native folded state than Ins1 given that most proteins start to unfold/denature as the charge state increases.

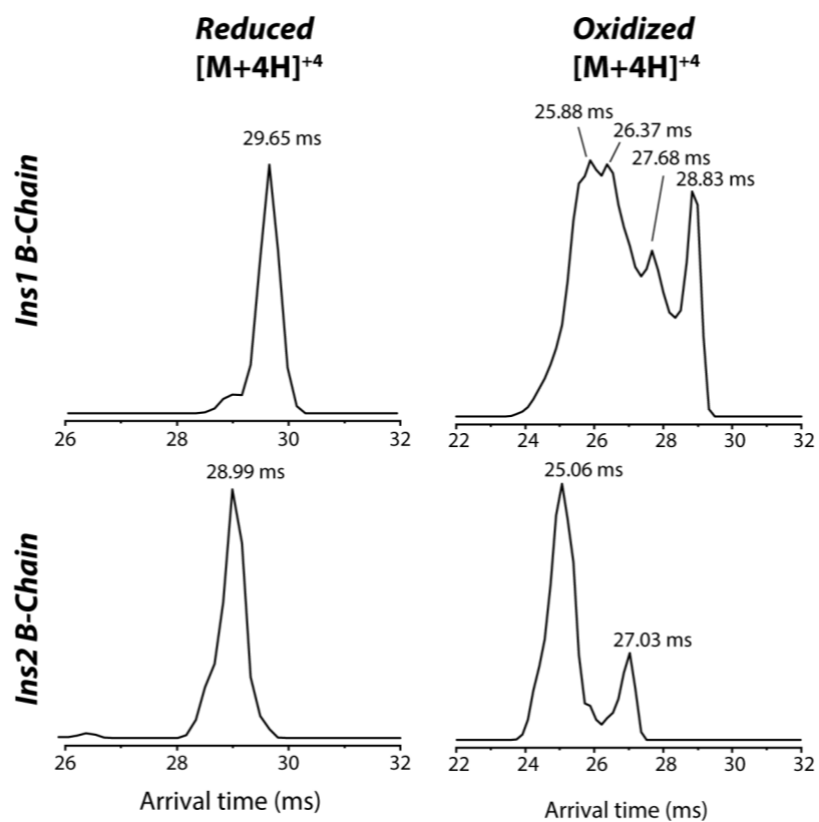
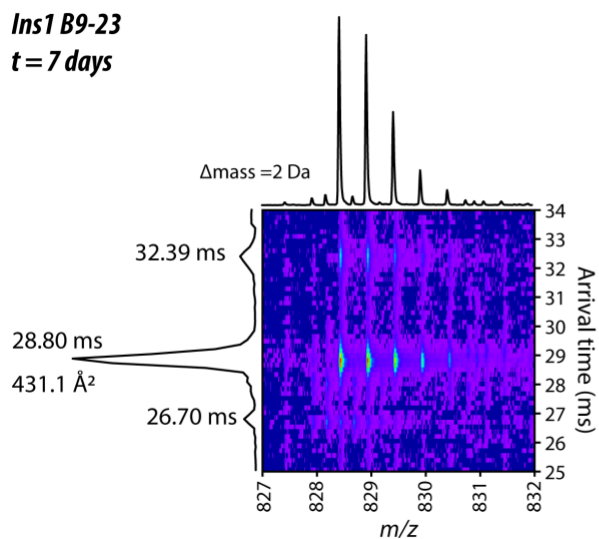


Figure 6. ATDs of Ins1 and Ins2 B-chains with reduced and oxidized disulfide bonds ($z = +4$).

Ins1 B9-23
t = 7 days



Ins2 B9-23
t = 7 days

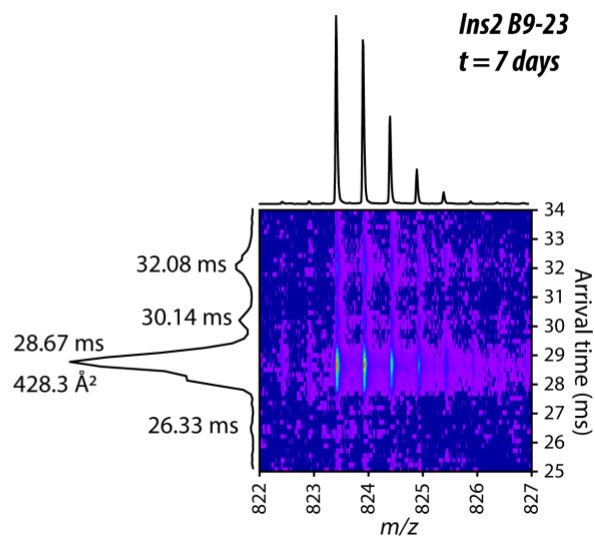


Figure 7. IMS-MS characterization of Ins1 and Ins2 B9-23 segments.

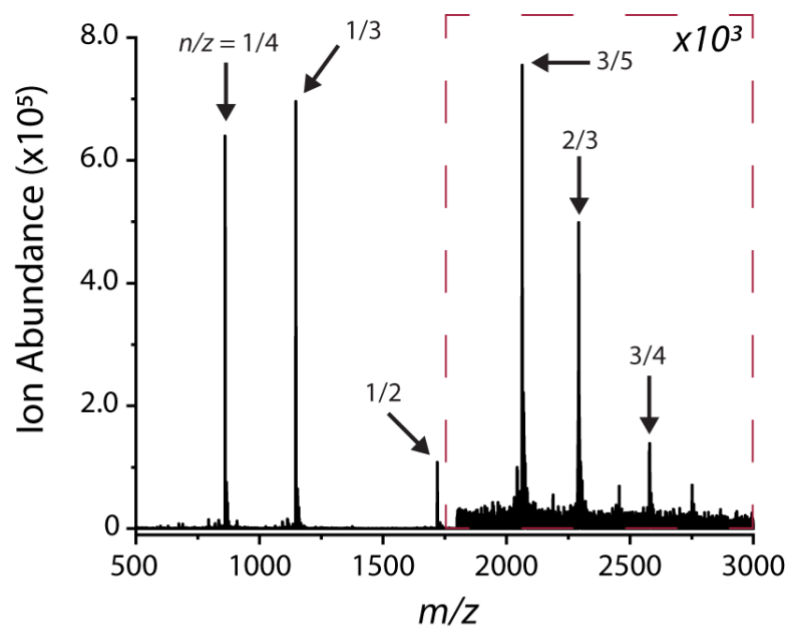
3.3 Ins1 Oligomerizes More Readily than Ins2

Direct infusion IMS-MS on synthetic insulin B-chains show that oligomerization is not apparent at $t = 0$ but occurs by $t = 96$ hours. Figure 8 includes mass spectra of Ins1 B-chain (top) and Ins2 B-chain (bottom) with normalized intensity. Peaks are indicated with the oligomer state/charge ratios (n/z). Monomers ($n/z = 1/4, 1/3, 1/2$), dimers ($1/2, 2/3$), trimers ($3/5, 3/4$), and tetramers ($2/3$) are all observed in both Ins1 B-chain and Ins2 B-chain. Ins1 B-chain portrayed a greater propensity to form oligomers than Ins2 B-chain, as evidenced by the higher intensity for dimeric and tetrameric species in Figure 8.

Similar to the B-chains, direct infusion IMS-MS on the synthetic insulin B9-23 segments shows that oligomerization is apparent at $t = 7$ days. Figure 7 shows that the mass spectra, ATD, and 2D plot are more intense for the Ins1 B9-23 dimer than for the Ins2 B9-23 dimer (both at $n/z = 1/2$), indicating Ins1 B9-23 oligomerizes more readily, much like the full Ins1 B-chain. Figure 9 displays the mass spectra of Ins1 and Ins2 B9-23 dimers ($n/z = 2/3$) at $t = 7$ days, showing the dimer is more intense in Ins1 B9-23 than Ins2 B9-23.

Our results show that both Ins1 B-chain and Ins1 B9-23 oligomerize more readily than Ins2 B-chain and Ins2 B9-23. Mature human insulin dimerization is thought to proceed through interactions between newly formed β -strands of natively disordered residues (B24-B28) between two monomers [81]. Van der Waals contacts between hydrophobic residues of the B-chain α -helix and β -strand are also thought to be implicated in this process. The 3D structure of the human insulin dimer is shown in Figure 10. The proline present in Ins1, but not Ins2, may drive the differences in oligomerization observed

Ins1



Ins2

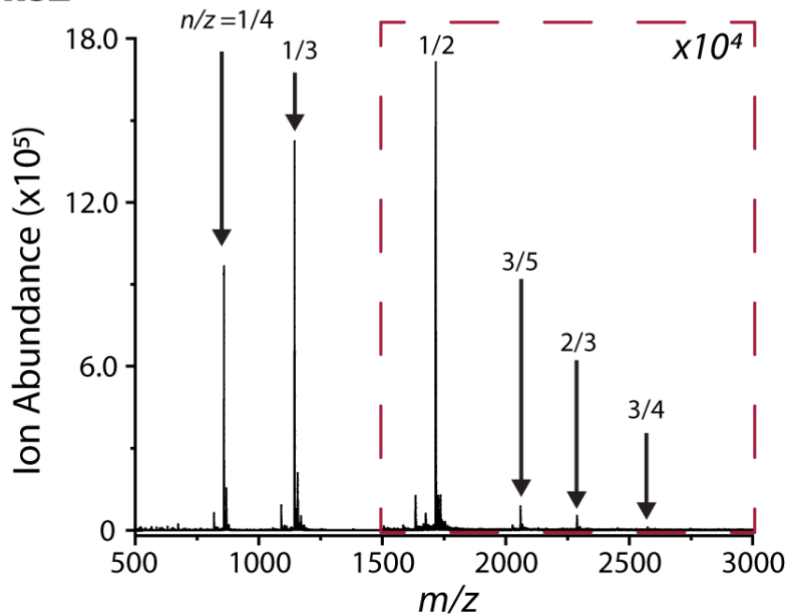


Figure 8. Mass spectra indicating oligomeric species in Ins1 and Ins2 B-chains.

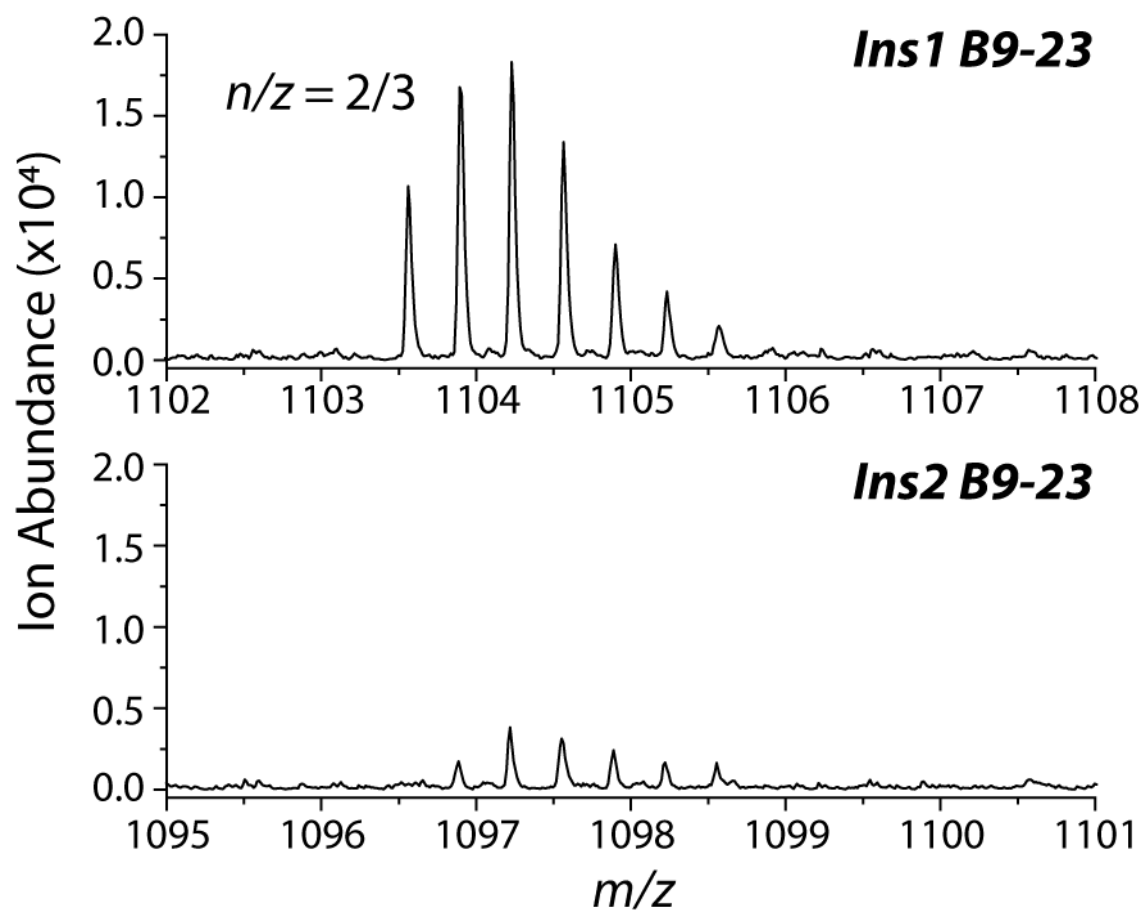


Figure 9. Mass spectra of Ins1 B9-23 and Ins2 B9-23 showing the dimer ($n/z = 2/3$).

Human Insulin Dimer

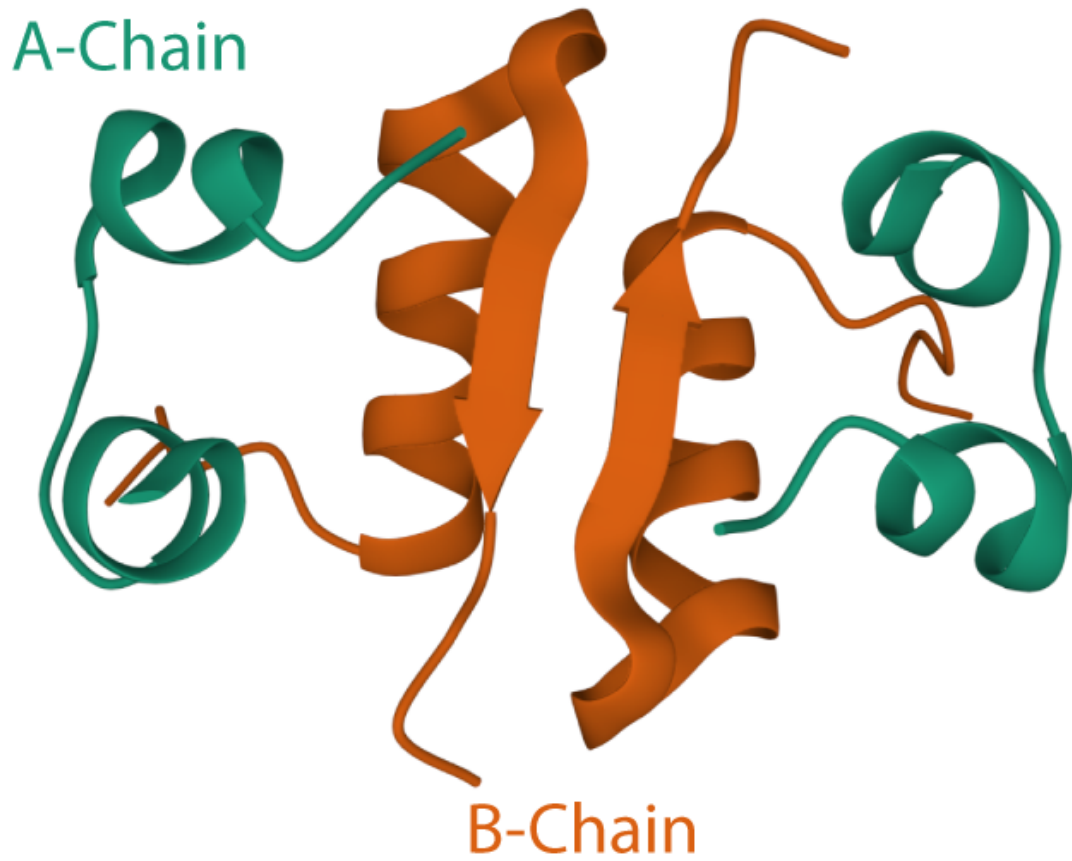


Figure 10. 3D structure of the human insulin dimer and pH = 2 (PDB = 1GUJ) [81, 82].

in our experiments. In human insulin dimers, SerB9 has been shown to interact with GluB'13 and TyrB'16 of the opposite monomer [83]. Furthermore, SerB9 is implicated in a pathway leading to insulin dimer dissociation [83]. ProB9 in Ins1 may form stronger contacts with residues of the opposite monomer as well as disrupt the dimer dissociation pathway. Proline is more hydrophobic than serine and can form van der Waals with hydrophobic residues within the hydrophobic core of the dimer. Furthermore, if ProB9 can interact with TyrB'16, favorable CH/ π interactions can form between the two residues [84].

Our direct infusion IMS-MS results show that the residue difference in the B9 position can alter oligomerization rates, a cytotoxic pathway. We incorporated circular dichroism (CD) and scanning transmission electron microscopy (STEM) to look at aggregation propensities. Figure 11 represents CD data of Ins1 B9-23 and Ins2 B9-23 obtained at $t = 0$ days and $t = 7$ days after incubation. Both peptides display primarily random coil conformations at $t = 0$ days but shift to β -sheet conformation at $t = 7$ days. The α -helix to β -sheet transition is implicated in the insulin fibrillization mechanism [85].

Fibrillization is a cytotoxic pathway in which oligomers are disassembled into monomers that become partially misfolded, undergo the transition to a β -sheet conformation, and aggregate into highly stable rod-like structures. Figure 12 presents TEM images of Ins1 B9-23 and Ins2 B9-23 segments incubated for 1.5 weeks. Both Ins1 and Ins2 B9-23 segments show substantial fibrillization, however, Ins1 B9-23 was observed to form fibrils more extensively across the sample. Again, the proline at residue B9 may alter the 3D structure of the peptide and be implicated in the fibrillization process.

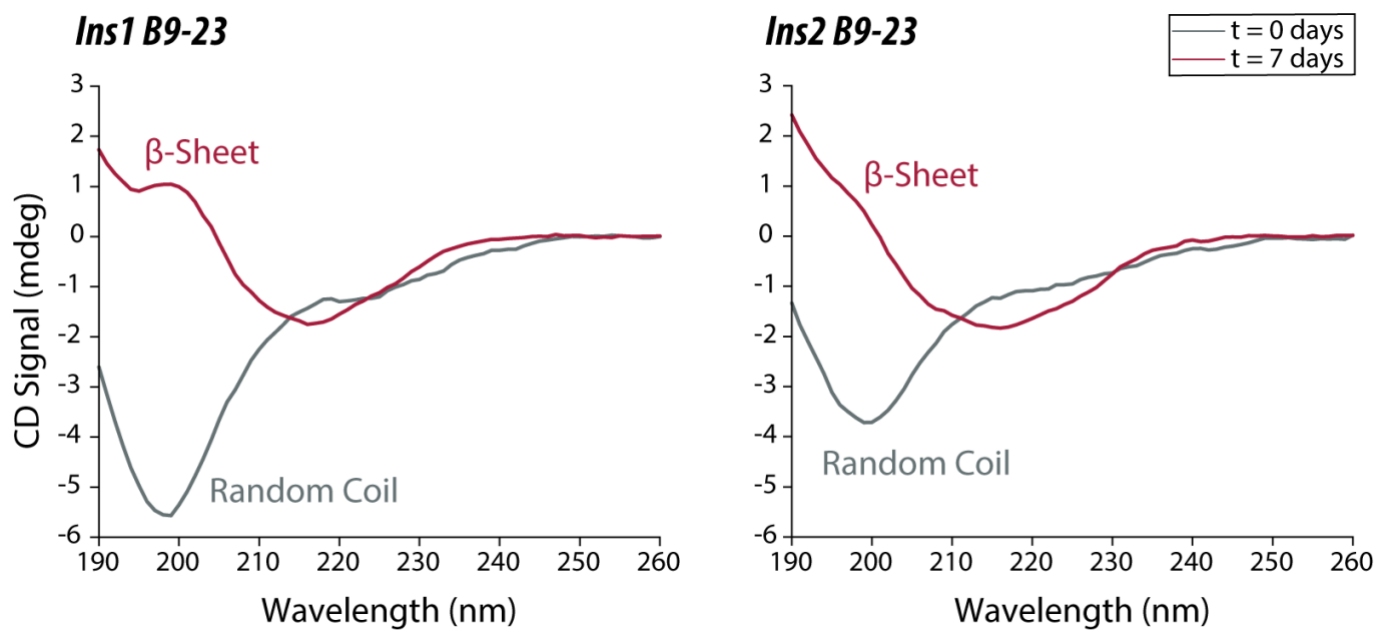
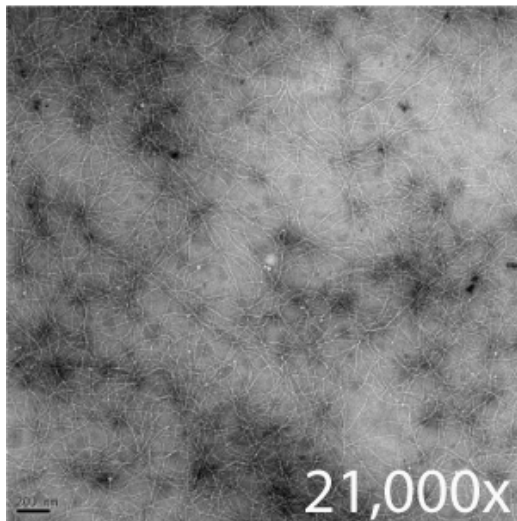


Figure 11. CD data of Ins1 B9-23 and Ins2 B9-23.

Ins1 B9-23



Ins2 B9-23

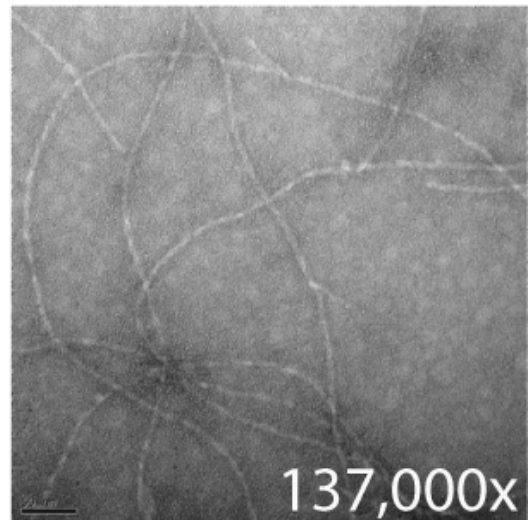
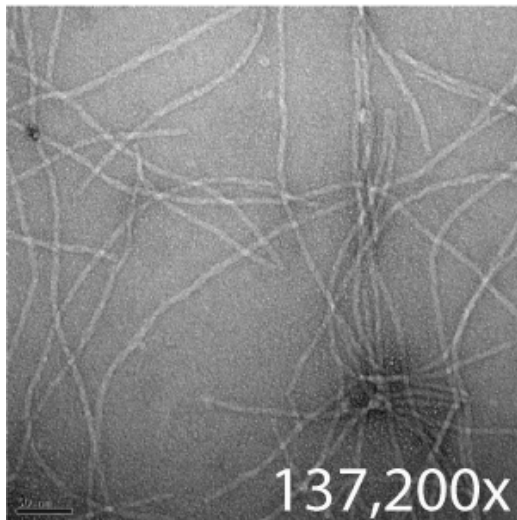
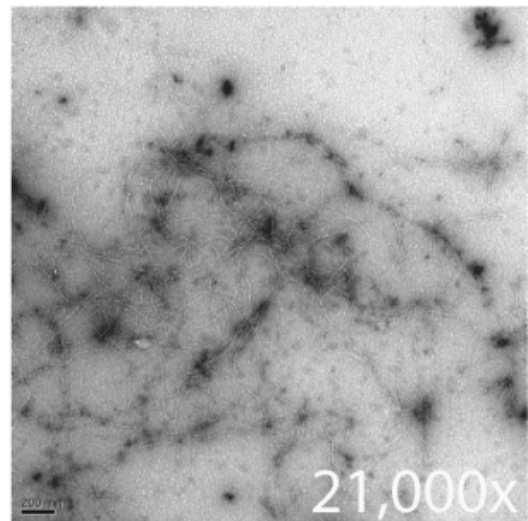


Figure 12. TEM images of Ins1 B9-23 and Ins2 B9-23.

3.4 Non-Enzymatic Peptide Cleavage Gives Insight into Ins1 and Ins2

Degradation Patterns

Despite using ESI, a “soft” ionization method that is thought to minimize fragmentation, we observe considerable non-enzymatic peptide cleavage (fragmentation) in LC-IMS-MS experiments on synthetic insulin B-chains without utilizing collision-induced dissociation (CID) capabilities. ESI fragmentation proceeds through the mobile proton model which states that when protons are added during ESI, they can travel across the peptide backbone and attach to amide nitrogen [86]. This allows for nucleophilic attack by the C-terminally located carbonyl oxygen on the carbonyl carbon and leads to either a b_x -ion or a y_z -ion and a neutral fragment as seen in Figure 13 [87].

The general assumption is that peptide bonds from a more folded region are protected from fragmentation, while peptide bonds from unstructured regions are more susceptible to it. Typically, the weakest peptide bonds in a particular peptide are affected by fragmentation. Our data shows substantial fragmentation only under LC-IMS-MS conditions and not when samples are directly infused into the IMS-MS instrument. This implies the LC system denatures the peptides. It has been reported that reverse-phase LC disrupts hydrophobic interactions in peptides due to the hydrophobicity of the matrix [88]. This would disrupt the secondary structure of the peptides and allow for fragmentation near aromatic/hydrophobic residues. Figure 14 shows the mass spectra obtained using LC-IMS-MS of aged Ins1 and Ins2 B-chains with reduced disulfide bonds and normalized intensity. Ins1 B-chain displays a multitude of very low-intensity fragments, whereas Ins2 B-chain shows more intense fragments. This tells us that Ins2 B-chain has a higher propensity to

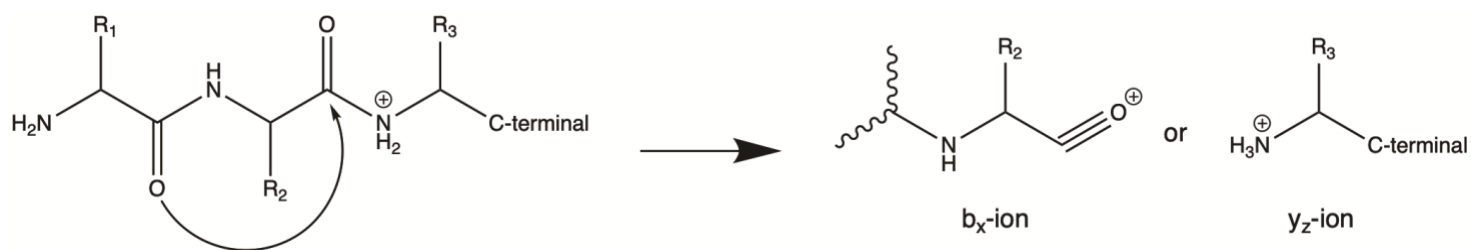


Figure 13. Peptide fragmentation mechanism.

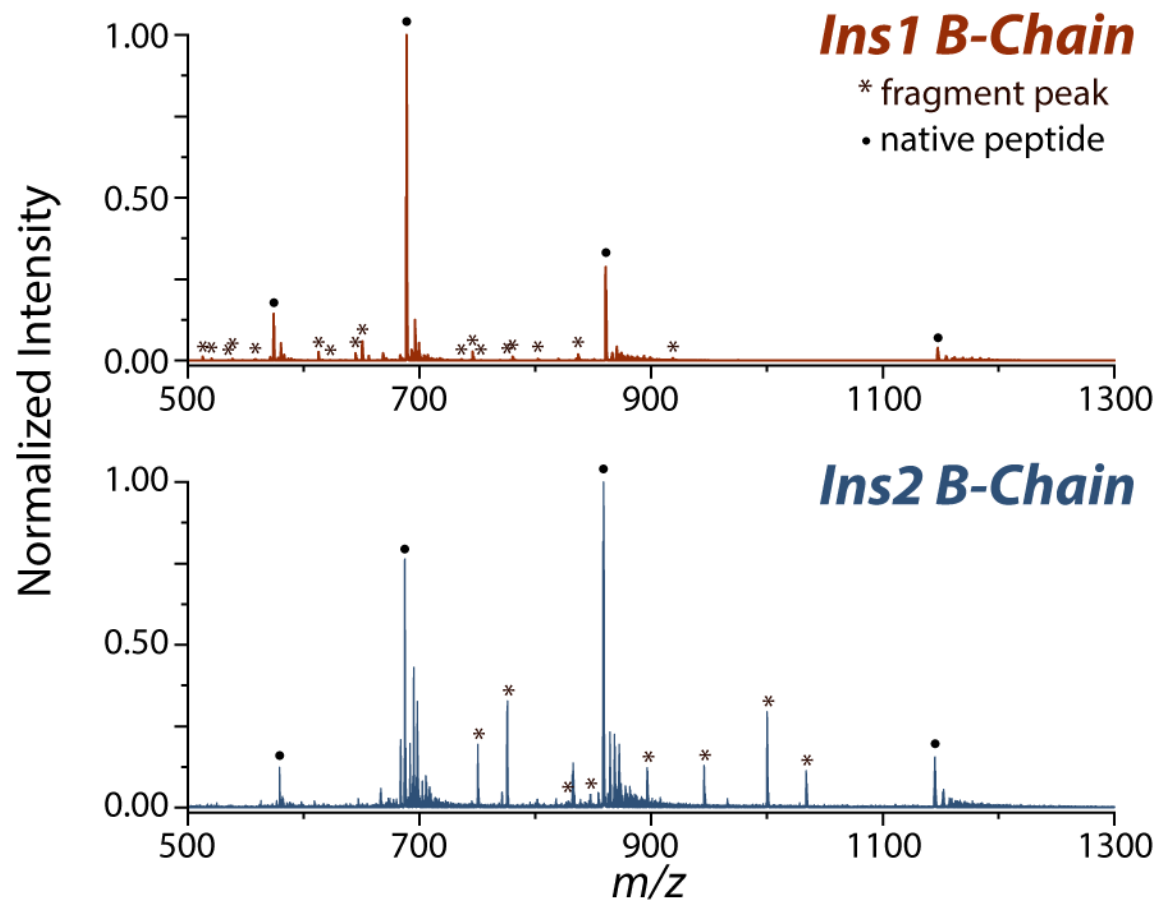


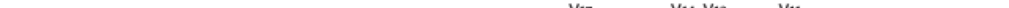
Figure 14. Mass spectra of Ins1 and Ins2 B-chains showing fragmentation.

fragment and may include weaker peptide bonds than Ins1 B-chain. Looking at the fragmentation pattern in Figure 15, we can see that most fragmentation for the reduced form of Ins1 B-chain occurs in the middle of the peptide while most fragmentation in the reduced form of Ins2 B-chain takes place towards the C-terminal. When we look at the structure of human insulin in Figure 16, we can see that the A-chain interacts with the middle region of the B-chain. We propose the A-chain protects the weakest peptide bonds in Ins1 through these interactions. On the other hand, the C-terminal portion where the weakest bonds of Ins2 are located is highly exposed. Correlating the fragmentation patterns with the 3D structure allows us to rationalize the increased degradation of mature Ins2 compared to mature Ins1 seen in Figure 3.

3.5 Conclusions

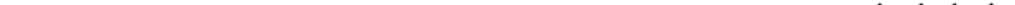
This is the first study to characterize the differences in structure and oligomerization propensities of mouse Ins1 and Ins2. We investigated synthetic insulin B-chains and B9-23 segments with LC-IMS-MS. We conclude that a) Ins2 B-chain is more compact than Ins1 B-chain, b) Ins1 oligomerizes more readily than Ins2, c) Ins2 B-chain has a higher propensity to fragment and may be a less stable peptide than Ins1 B-chain, and d) fragmentation patterns of the B-chains provide a rationale for the increased degradation of Ins2 observed in biological samples. Overall, we demonstrate that Ins1 and Ins2 have different structures and functions. Given this, distinguishing between mouse Ins1 and Ins2 is vital for making conclusions about human insulin behavior. Our LC-IMS-MS workflow has proven to be robust in that it can easily distinguish between Ins1 and Ins2, unlike traditional methods.

Ins1 B-Chain

Reduced F V K Q H L C G P H L V E  A L Y L V C G E R G F F Y T P K S
b₁₄ b₁₅ b₁₆ b₁₇ b₁₈ b₁₉ b₂₀ b₂₅ b₂₆ b₂₇

Oxidized FVKQHLCPHLVEALYLCGERGFFSYTSPKS
b25 b26 b27

Ins2 B-Chain

Reduced F V K Q H L C G S H L V E A L Y L V C G E R 

Oxidized FVKQHLCGSHLVEALYLVCGERGFFY**Y****T****P****M****S**
b₂₆ b₂₇ b₂₈ b₂₉

Figure 15. Fragmentation patterns of Ins1 and Ins2 B-chains.

Disulfide Bonds

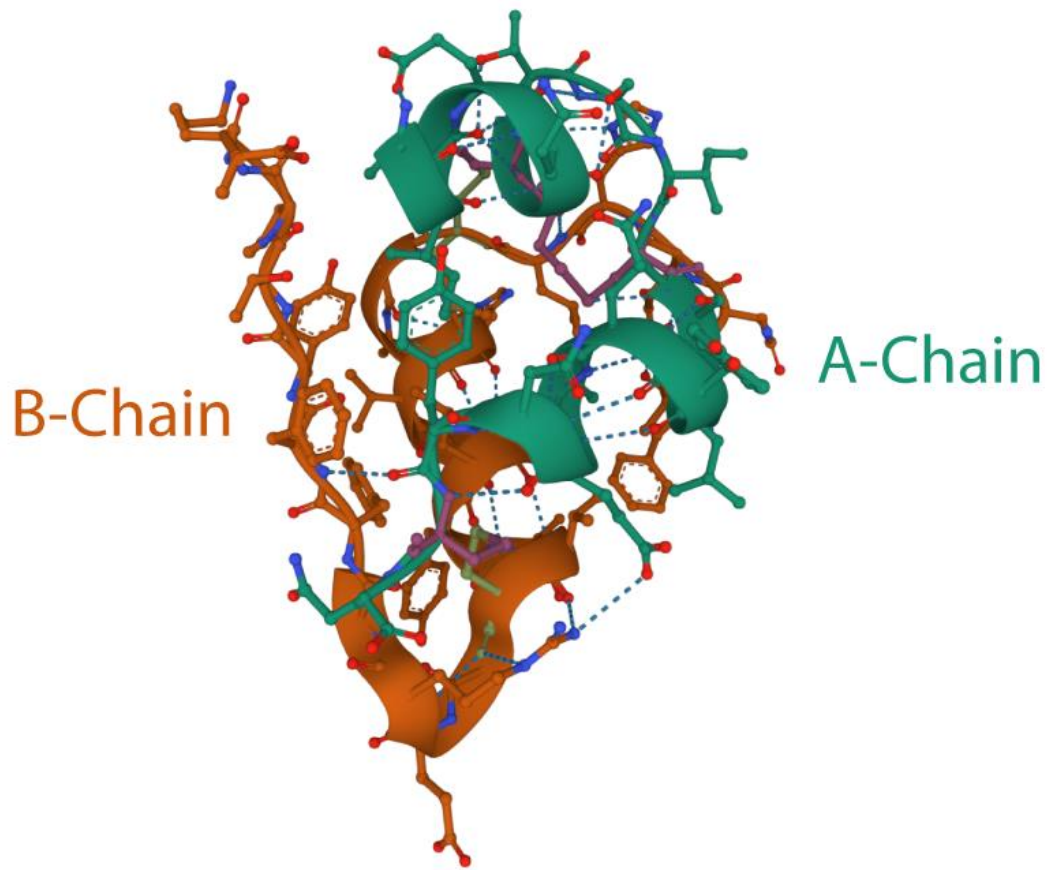


Figure 16. 3D structure of human insulin (PDB 3I40) [82, 89].

CHAPTER FOUR

THE EFFECTS OF ACUTE CORTICOSTERONE TREATMENT ON MOUSE PANCREATIC PEPTIDE HORMONES PROBED AT THE SUB-SINGLE-ISLET LEVEL

4.1 Materials and Methods

Pancreatic Perfusion and Islet Isolation

Twelve 8-week-old male C57BL/6J mice (stock #000664, The Jackson Laboratory, Bar Harbor, Maine) were obtained and allowed to acclimate to animal facility light (12 h light/12 h dark) and temperature ($22 \pm 1^{\circ}\text{C}$) conditions for two weeks. Mice had access to standard chow and drinking water *ad libitum* until they reached 10 weeks of age. Then, mice were evenly distributed between treatment or control drinking water groups randomly. Treatment group mice were given drinking water containing 100 $\mu\text{g/ml}$ corticosterone in 1% ethanol. Control group mice were given drinking water containing 1% ethanol. Upon reaching 12 weeks of age, the mice were euthanized via CO_2 asphyxiation and cervical dislocation. Pancreatic perfusion was performed, and islets were isolated in the method explained in **Chapter 2.1**.

All live animal procedures were approved by the University of Tennessee Institutional Animal Care and Use Committee. After islet isolation, islets were incubated at 37°C and 5% CO_2 for 24 h in RPMI + D-glucose. After incubation, islets were transferred into Eppendorf tubes containing 5% MeOH/0.1% formic acid solution. This study used six

mice for two sets of experiments. First, three islets each from three corticosterone-treated mice were used. Secondly, one islet each from three control mice was used.

Drift Tube IMS-MS

All chemicals utilized were reagent grade and were used without further purification. Rodent amylin peptides were obtained from AnaSpec (California, USA) at 98% purity. Glucagon peptides were obtained from Millipore Sigma. Peptide powders from both amylin and glucagon peptides were pretreated with hexafluoroisopropanol (TCI America) and solvents were left to evaporate in a desiccator overnight. Prior to IMS-MS experiments, peptide stock solutions were prepared by reconstituting the lyophilized peptides in LC-grade water (Mercedes) and diluting it to 10 μ M in a buffer of 20 mM ammonium acetate (VWR), pH = 6.8. Drift tube IMS-MS experiments were carried out according to the method outlined in **Chapter 2.2**.

LC-IMS-MS

Isolated islets were placed in a 50 μ L solution of 5% MeOH/1% formic acid. Three technical replicates were obtained from each sample, with two consecutive blank injections between each sample for column cleaning. LC-IMS-MS experiments were run according to the method outlined in **Chapter 2.2**.

4.2 Sampling of Pancreatic Hormones from Single Mouse Islets

Our established LC-IMS-MS workflow allows for the accurate detection of various peptide hormone levels from approximately one-tenth of a single islet as described in **Chapter 2.4**. This allows for the collection of multiple technical replicates on the same islet sample and the investigation of several conditions on the same animals. Here, we

compared pancreatic hormone levels in control and corticosterone-treated male mice. Corticosterone treatment in this study was carried out for two weeks and is considered to be acute. Ins1, Ins2, glucagon, amylin, and truncated insulin and amylin peptides were detected in islet samples and their abundances were compared per treatment group. Tables 1 and 2 summarize the LC-IMS-MS characterization of peptide hormones from control and corticosterone-treated mice.

Figure 17A shows an extracted, partial LC chromatogram of a single islet from a control mouse. The retention times of major islet hormones were annotated. Figure 17B displays the mass spectra and ATDs of the peptide hormones. Ins1 was detected at four different charge states which were identified unambiguously with the isotope spacings with a mass accuracy of -2.58 ppm. Ins2 was detected at the same four charge states with a mass accuracy of 0.69 ppm. To verify the mass spectral peaks of endogenous Ins1 and Ins2, we performed glucose-stimulated insulin secretion (GSIS) experiments on batches of 10 control mouse islets. Islets were treated with both 3 mM and 16 mM glucose. The media after each treatment was collected and analyzed with LC-IMS-MS. Secreted insulin accounts for less than 5% of the total insulin within islets. GSIS experiments on three batches of islets showed that when islets were treated with 16 mM glucose, a higher level of mouse insulin was detected than when treated with 3 mM glucose, as seen in Figure 18. This data is consistent with the expected behavior of glucose-responsive β -cells.

Using IMS-MS, Ins1 and Ins2 structures can be compared via ion mobility and CCS measurements. Note, that the three disulfide bonds within Ins1 and Ins2 are assigned based

Table 1. LC-IMS-MS characterization of pancreatic peptide hormones in control mice.

Peptide		Retention time (min)	Theoretical Mass	Observed Mass	Δ ppm	z	m/z
Ins1	GIVDQCCTSCISLYQLENVCFVKQHLGPHLVEALVCGGFFYPKS	15.793-16.217	5799.673	5799.658	-2.58	6	967.6175
							1159.5186
Ins2	GIVDQCCTSCISLYQLENVCFVKQHLGSHLVEALVCGGFFTPMS	16.455-16.914	5792.589	5792.593	0.69	5	1016.2782
Ins1: A+B(1-24)	GIVDQCCTSCIPLYQLENVCFVKQHLGPHLVEALVCGGERGF	15.134-15.796	5081.355	5081.391	7.08	5	1014.2679
Ins2: A+B(1-24)	GIVDQCCTSCISLYQLENVCFVKQHLGSHLVEALVCGGERGF	14.879-15.338	5071.334	5071.340	1.18	5	1043.676
Ins2: A+B(1-25)	GIVDQCCTSCISLYQLENVCFVKQHLGSHLVEALVCGGERGFF	16.217-16.608	5218.402	5218.382	-3.83	5	1115.9181
Ins2: A+B(1-28)	GIVDQCCTSCISLYQLENVCFVKQHLGSHLVEALVCGGFFYTP	16.102-16.323	5579.566	5579.591	4.48	5	1142.1283
Ins2: A+B(1-29)	GIVDQCCTSCISLYQLENVCFVKQHLGSHLVEALVCGGFFYTPM	16.679-17.325	5710.606	5710.642	6.30	5	870.1590
Glucagon	HSQGTFTSDYSKYLDSRRAQDFVQWLMMIT	15.385-15.657	3480.616	3480.634	5.03	4	980.4942
Amylin	KCNTATCATQRANFLVRSSNNLGPVLPPTNVGSNTY	13.704-14.247	3921.994	3921.977	-4.33	4	

Table 2. LC-IMS-MS characterization of pancreatic peptide hormones in mice under corticosterone treatment.

Peptide		Retention time (min)	Theoretical Mass	Observed Mass	Δ ppm	z	m/z
Ins1	GIVDQCCTSCISLYQLENVCNFKQHLGPHLVEALVLCGERGFFYPK	15.051-15.306	5799.673	5799.675	0.42	6	967.6204
Ins2	GIVDQCCTSCISLYQLENVCNFKQHLGSHLVEALVLCGERGFFTPMS	16.036-16.512	5792.589	5792.605	2.76	5	1159.5289
Ins1: A+B(1-24)	GIVDQCCTSCIPLYQLENVCNFKQHLGPHLVEALVLCGERGF	14.728-15.000	5081.355	5081.355	-2.85	5	1016.2681
Ins2: A+B(1-23)	GIVDQCCTSCISLYQLENVCNFKQHLGSHLVEALVLCGERG	13.760-14.100	4924.265	4924.273	1.62	5	984.8545
Ins2: A+B(1-24)	GIVDQCCTSCISLYQLENVCNFKQHLGSHLVEALVLCGERGF	14.371-14.609	5071.334	5071.329	-0.99	5	1014.266
Ins2: A+B(1-25)	GIVDQCCTSCISLYQLENVCNFKQHLGSHLVEALVLCGERGFF	15.425-15.697	5218.402	5218.412	1.92	5	1043.682
Ins2: A+B(1-28)	GIVDQCCTSCISLYQLENVCNFKQHLGSHLVEALVLCGERGFFTP	15.747-15.951	5579.566	5579.598	5.74	5	1115.9196
Ins2: A+B(1-29)	GIVDQCCTSCISLYQLENVCNFKQHLGSHLVEALVLCGERGFFTPM	16.359-17.004	5710.606	5710.605	-0.17	5	1142.1209
Amylin	KCNTATCATQRANFLVRSSNNLGPVLPPTNVGSNTY	13.234-13.522	3921.994	3921.988	-1.53	4	980.4969

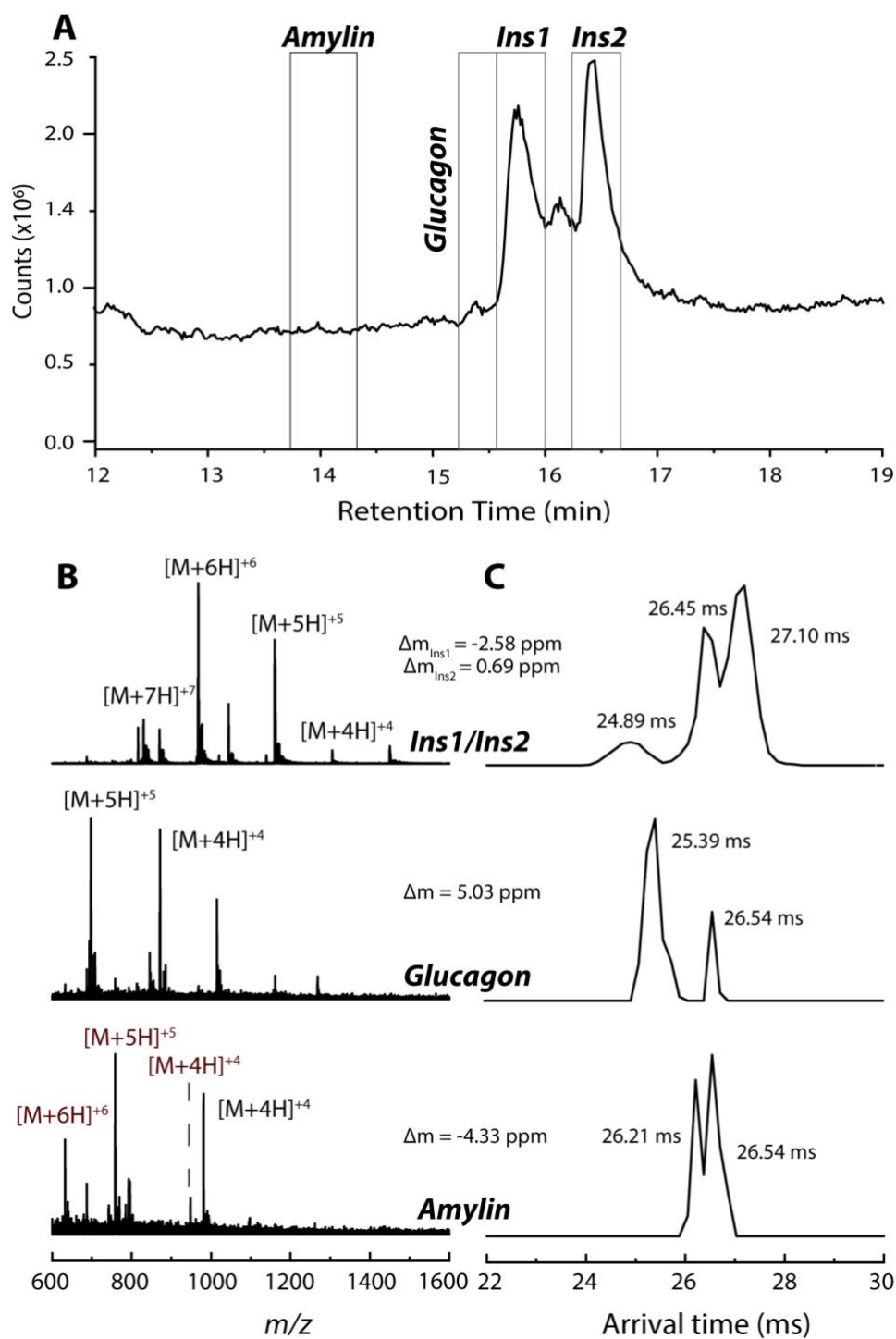


Figure 17. Extracted chromatogram (A), mass spectra (B), and ATDs (C) showing pancreatic peptide hormones.

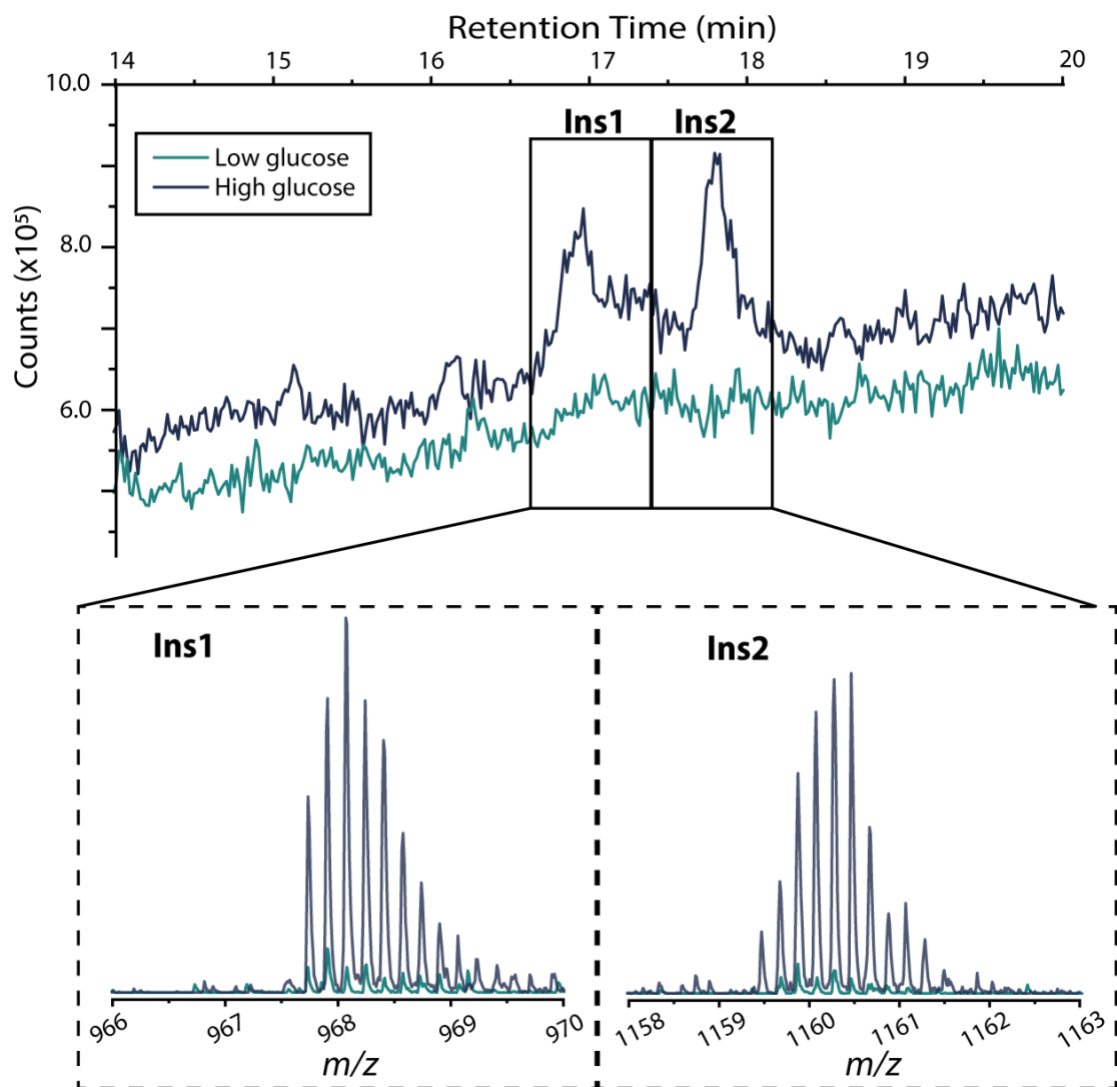


Figure 18. Summary of GSIS experiments.

on the crystal structures of porcine [89] and human [90] insulins, but since there is no crystal structure of Ins1, we cannot eliminate the possibility that the disulfide bonds of Ins1 were scrambled. Previously, we have established that the experimental CCS of Ins2 is in good agreement with human insulin [61]. Furthermore, we showed that Ins2 adopts a more compact and folded conformation than Ins1 does. This suggests that Ins2 is better at retaining its native folded state than Ins1 and stems from the structural differences in the B-chain, as discussed in **Chapter 3.2**.

Glucagon was detected at two charge states with a mass accuracy of 5.03 ppm. Figure 17C shows the ATD of endogenous glucagon in which two primary, well-resolved features are seen—one at 25.39 ms and the other at 26.54 ms. LC and direct infusion IMS-MS experiments were performed on synthetic glucagon. Synthetic glucagon displayed similar mobility features to endogenous glucagon at 25.56 ms and 26.86 ms, as seen in Figure 19. The experimental CCS_{He} of synthetic glucagon were $620 \text{ \AA}^2$ and $645 \text{ \AA}^2$. CCS_{He} of endogenous glucagon were $616 \text{ \AA}^2$ and $641 \text{ \AA}^2$. Glucagon CCS is reported here for the first time.

Amylin was detected at one charge state with a mass accuracy of -4.33 ppm. Our amylin assignment was confirmed with experiments on synthetic mouse amylin peptides. Experiments of both endogenous and synthetic amylin display two mobility features at 25.55 ms and 26.62 ms with corresponding CCS_{He} of $618 \text{ \AA}^2$ and $638 \text{ \AA}^2$. Helium CCSs were reported here to compare them to another study in which our CCS values of endogenous amylin are in strong agreement with ($619 \text{ \AA}^2$ and $644 \text{ \AA}^2$) [91]. In addition to intact amidated amylin, the peaks labeled red in the amylin mass spectrum of Figure 17B

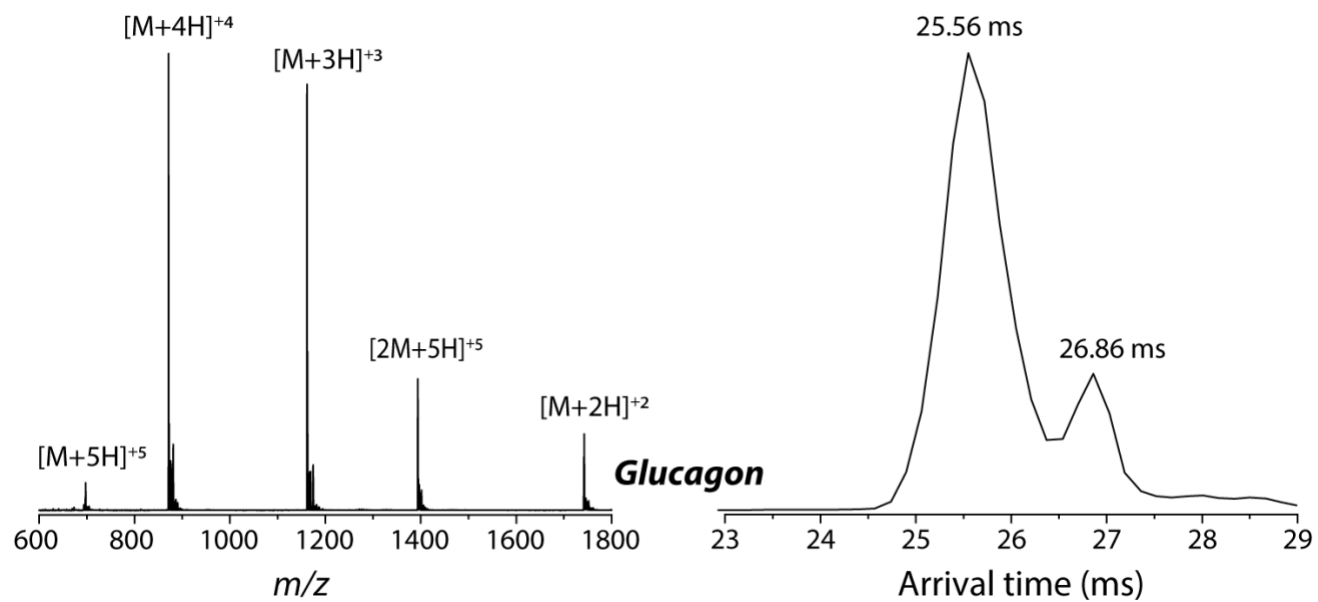


Figure 19. Characterization of synthetic glucagon.

represent amylin(2-37). The truncated amylin peptide was observed at three different charge states. The robust nature of our workflow is apparent in our detection of Ins1, Ins2, glucagon, and amylin. While insulin can be detected by MSI experiments on rodent pancreatic tissues or by high-throughput single-cell analyses, only one insulin isoform (Ins1) is often reported. Furthermore, in a healthy pancreatic islet, the ratio of insulin to amylin is approximately 100:1. Since insulin concentration in a single islet is approximately ~1 mM (~10 ng in 1.0 nL volume), an analytical technique with a limit of detection (LOD) of femtomole or better would easily detect insulin from a single cell (~1 pL). However, given amylin concentration is 100 times lower (~10 μ M), detecting this important peptide would require a LOD of attomole or better. Therefore, the advantages of our method include shorter acquisition time and improved analyte coverage at the expense of tissue contexts and individual cell identities [92].

4.3 Effects of Acute Corticosterone Treatment on Insulin Chemistry

Probed at the Single-Islet Level

Our experiments showed an inhibitory effect on glucagon within islets of mice treated with corticosterone. This correlates with another study that demonstrated islet incubation in 50 nM corticosterone for two hours corresponded to a reduction in glucagon following low glucose stimulation [93]. All other peptides excluding the truncated amylin (residues 2-37) and one truncated form of Ins2 [A+B(1-23)], which, like glucagon, were not observed in all islets of both conditions, were analyzed with a one-way ANOVA to determine the statistical significance of our data. This was followed by a Tukey test between the control and treatment conditions. Figure 20 shows the statistical analyses.

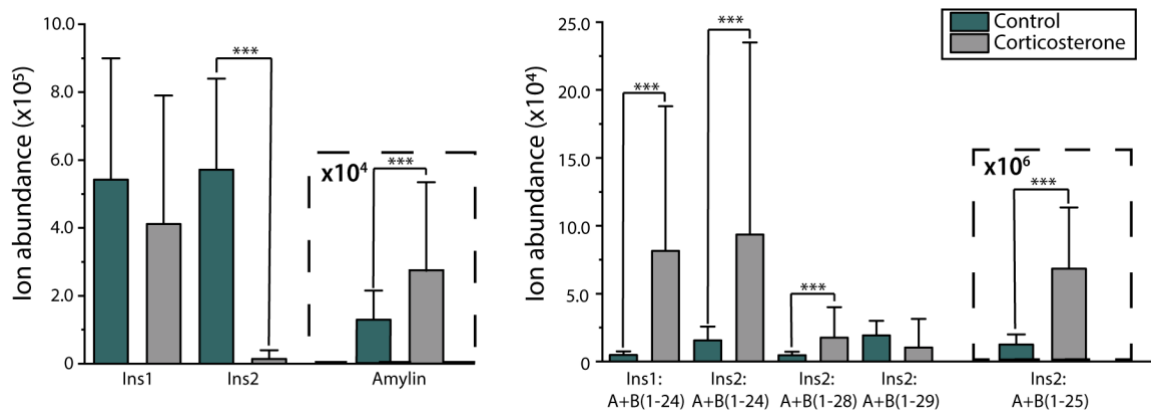


Figure 20. Statistical analysis of the effects of corticosterone treatment on pancreatic hormones.

We observed that while both intact Ins1 and Ins2 levels dropped in corticosterone-treated mice, only the Ins2 levels dropped significantly compared to the control mice. The opposite trend was observed with the putative truncated insulin peptides, in which all but the Ins2 A+B(1-29) peptide increased significantly. Amylin is co-secreted with insulin from pancreatic β -cells and its levels were analyzed under corticosterone treatment. We observed that amylin levels were significantly increased only in corticosterone-treated mice.

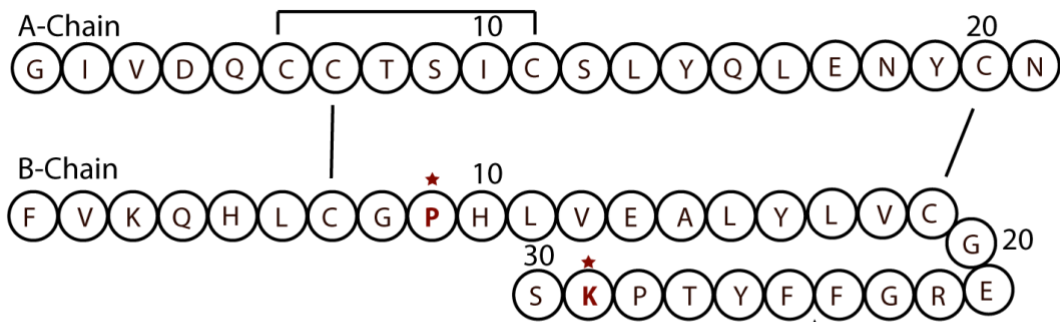
These observations have interesting implications for understanding acute glucocorticoid treatment and the effects of stress on the islet environment. First, the differential effects of corticosterone on Ins1 and Ins2 indicate the need to differentiate these proteins in biological samples to reliably make conclusions on human insulin behavior. Here, only Ins2—the orthologue of human insulin—levels decreased significantly under corticosterone treatment. Furthermore, the enzymatic hydrolysis products of Ins2 increased significantly and will be discussed thoroughly below. Finally, the significant increase of amylin levels in corticosterone-treated mice implies that either the healthy ratio of insulin:amylin (100:1) was impaired or the level of amylin increased proportionally with an increase in insulin production. Similar results are reported when a synthetic GC, dexamethasone is used [94-96]. Specifically, both the plasma amylin level and amylin secretion from isolated pancratia were increased in dexamethasone-induced insulin resistant (IR) rats. These findings suggest that GC administration may aid in amylin release. The human amylin version is amyloidogenic and has long been linked to T2D. Studies have shown that amylin fibrils have been found in the pancreas of 95% of T2D patients [97, 98].

The amylin aggregates are toxic to the pancreatic β -cells. Therefore, our experiments here provide a molecular basis linking a high level of corticosterone/cortisol with T2D in humans.

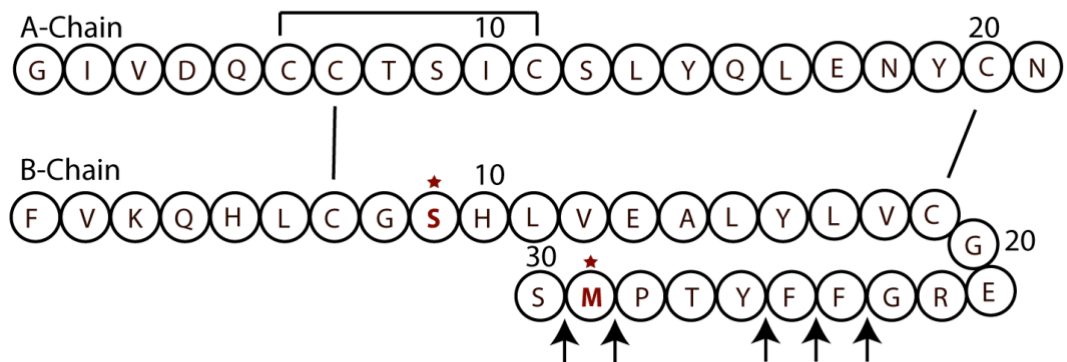
4.4 Ins2 is Enzymatically Degraded at a Much Faster Rate than Ins1

Our LC-IMS-MS workflow was able to detect multiple truncated Ins1 and Ins2 peptides. This truncation occurs towards the C-terminal of the B-chains and is due to enzymatic hydrolysis. We observe only a single truncated Ins1 peptide—Ins1 A+B(1-24). Alternatively, we detect five truncated Ins2 peptides including Ins2 A+B(1-23), A+B(1-24), A+B(1-25), A+B(1-28), and A+B(1-29). The differential cleavage patterns are most likely due to the differences in secondary structures between Ins1 and Ins2, specifically within the B-chains. The cleavage patterns are indicated by arrows in Figure 21. The retention times, observed masses, and mass accuracy of the truncated Ins1 and Ins2 peptides are displayed in Table 1. We note that the MS ions we detected correspond to the complete peptides (with NH₂ N-terminus and COOH C-terminus) and not MS fragments (b- and y-ions). This excludes the possibility that the peptide cleavages were due to MS-induced in-source fragmentation. Furthermore, the truncated peptides have distinct retention times from the intact proteins, indicating that they existed in solution. Lastly, different rates of degradation were found for Ins1 compared to Ins2, suggesting that the truncated forms are formed due to enzymatic activities rather than MS-induced processes. Furthermore, Grasso and co-workers observed a similar enzymatic cleavage pattern in bovine insulin: A+B(1-24), A+B(1-25) to name among others [99]. Note that the bovine insulin has a serine at the B9 position much like Ins2. Many other studies show that insulin-

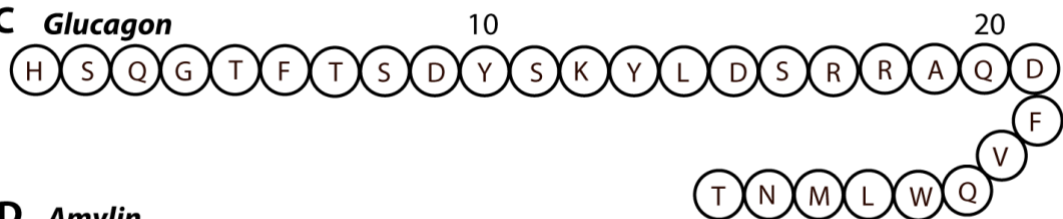
A *Ins1*



B *Ins2*



C *Glucagon*



D *Amylin*

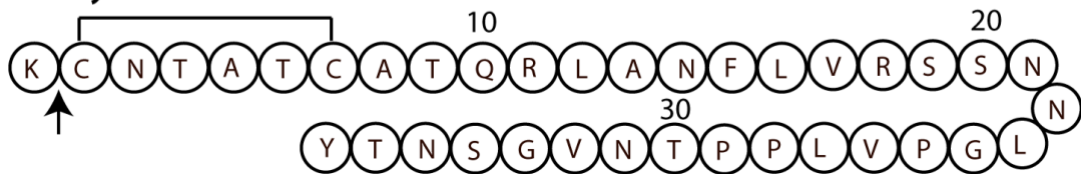


Figure 21. Peptide sequences and cleavage patterns.

degrading enzyme (IDE) has a high affinity to hydrolyze insulin between the two phenylalanine residues at B24 and B25. Congruently, our data show that both the A+B(1-24) Ins1 and Ins2 truncated peptides are the highest in intensity in the islets of the corticosterone-treated mice.

Each truncated insulin peptide that was detected in the corticosterone-treated mice was also identified in the control samples, except for Ins2 A+B(1-23). As stated in **Chapter 4.3**, the levels of all but one truncated insulin peptide were significantly increased in the corticosterone-treated mice while intact Ins1 and Ins2 levels dropped, as seen in Figure 20. These results may stem from differential binding propensities of Ins1 and Ins2 to the IDE. IDE is a zinc metallothiolproteinase and serves as the major endopeptidase for insulin degradation in the pancreas. IDE also binds and cleaves other peptide hormones like glucagon [100], amylin [101], and somatostatin [102], albeit with a much lower affinity [103]. A study by Wei-Jen Tang and co-workers on human insulin showed that residues B1-B4 and B13-B20 convert from an α -helix into a β -strand conformation and form β -sheets with IDE strands β -12 and β -6, respectively [104]. However, residues B6-B12 were not observed in the crystal structure, and the impacts of S9P (Ins2 $\rightarrow$ Ins1) on the IDE binding mechanisms have not yet been explored. We rationalize that given Ins1 includes a proline at residue B9 compared to serine at B9 in Ins2 and human insulin, the disproportionate hydrolysis of Ins2 in islets may be due to structural changes in Ins1 that disfavor IDE binding compared to Ins2. This is supported by the role of proline in the disruption of secondary structures. Additionally, the same study found that peptides that do not have a significant positive charge at the C-terminal and avoid the repulsion from

IDE-C are better substrates. By looking at the Ins1 and Ins2 peptide sequences, we see that Ins2 includes a neutral methionine residue towards the C-terminal, whereas Ins1 has a positively charged lysine residue.

As mentioned above (and seen in Figure 17B), we were also able to detect a truncated form of amylin as well (2-37). Since IDE has an approximately 4-fold greater affinity for insulin than for amylin, the amylin degradation will be proportionally impaired [101]. This increased amylin production coupled with lower levels of degradation may allow for sufficient accumulation of amylin, which could lead to islet amyloid formation [101].

Overall, our results indicate that corticosterone actions increase the enzymatic activity of endopeptidases like IDE within mouse islets. Previous studies on similar mice showed that after two-week exposure to corticosterone, a high level of serum insulin was observed together with an increase in insulin-positive cells. It was suggested that during this period, corticosterone promoted β -cell proliferation. From the same study, it was shown that insulin sensitivity was reduced, evident by the high level of serum glucose compared to control mice. Our data here provide a reasonable explanation. Despite increased insulin production in corticosterone-treated mice, the mature insulins are more readily degraded leading to high abundances of truncated and biologically less-active peptides. Eventually, the β -cells produce more insulin leading to reduced insulin sensitivity. Because Ins2 is more prone to IDE degradation than Ins1, this leads to a condition where intact Ins1 becomes dominant. Essentially, the mice would behave more similarly to those with only the Ins1 gene. As mentioned above, transgenic mice without

the Ins2 gene develop diabetes as early as 10-weeks old. The IDE resistance property of Ins1, compared to Ins2, may explain why Ins1 is a defective form of insulin and why the diabetes risk is increased with this form.

CHAPTER FIVE

ELUCIDATING THE MOLECULAR CONNECTION BETWEEN TYPE 2 DIABETES AND CHRONIC MIGRAINE PART I: CGRP

Calcitonin gene-related peptides (CGRPs), just like amylin, are part of the calcitonin gene family. CGRP exists in two major isoforms— α -CGRP and β -CGRP. α -CGRP is expressed in the CNS and PNS, whereas β -CGRP is expressed in the enteric nervous system [105]. Both isoforms of CGRP are released from sensory nerves [106] and δ -cells within pancreatic islets [107]. CGRP has been implicated in both inhibiting [46, 108] and stimulating [109] insulin secretion. Here, our LC-IMS-MS workflow will be applied to single isolated mouse islets to observe the effects of CGRP on mouse Ins1 and Ins2. Furthermore, we will investigate how CGRP affects amylin fibrillization and amylin-associated cytotoxicity. Note, that three residues are different in mouse α -CGRP compared to the human peptide, as seen in Figure 22.

5.1 Materials and Methods

Chemicals were purchased and used without further purification. Human amylin and human α -CGRP peptides were purchased from AnaSpec (California, USA) at 98% purity. Peptide powders were pretreated with hexafluoroisopropanol (TCI America). Solvents were left to evaporate in a desiccator overnight. Prior to IMS-MS analysis, stocks of each peptide were prepared by reconstituting the lyophilized peptide in LC-grade water (Honeywell) and diluting it to 10 μ M in a buffer of 20 mM ammonium acetate (VWR), pH = 6.8. The amylin:CGRP mixture was prepared by mixing the two peptide stocks in a 1:1

	1	10	20	30
Human α -CGRP	ACDTATCVT	HRLAGLLSRS	GGVVKNNFVP	TNVGSKAF
Human β -CGRP	ACNTATCVT	HRLAGLLSRS	GGMVKSNFVP	TNVGSKAF
Mouse α -CGRP	SCNTATCVT	HRLAGLLSRS	GGVVKDNFVP	TNVGSEAF
Mouse β -CGRP	SCNTATCVT	HRLADLLSRS	GGVLKDNFVP	TDVGSEAF
Human Amylin	KCNTATCAT	QRLANFLVHS	SNNFGAILSS	TNVGSNTY
Mouse Amylin	KCNTATCAT	QRLANFLVRS	SNNLGVLPP	TNVGSNTY
PACAP	HSDGIFTDS	YSRYRKQMAV	KKYLA AVL GK	RYKQRVKNK

Figure 22. Primary sequences of pancreatic peptides.

ratio and diluting the solution with 20 mM ammonium acetate to the final concentration of each peptide of 5 μ M. For the peptides containing ZnCl₂, we used 50:50 methanol:H₂O in place of ammonium acetate.

Mouse Islet Culturing and Harvesting

Six 10-week-old male C57BL/6J mice (stock #000664) were ordered from the Jackson Laboratory (Bar Harbor, Maine) and allowed to acclimate to the animal facility conditions on a 12 h light cycle at 22 $\pm$ 1 °C. The mice were provided standard chow and drinking water *ad libitum* until they reached 12 weeks of age. At 12 weeks of age, the mice were euthanized via CO₂ asphyxiation followed by cervical dislocation. All procedures were approved by the University of Tennessee Institutional Animal Care and Use Committee. Additional details regarding pancreatic perfusion and islet isolation can be found in the ESI [61]. Following isolation, the islets were incubated at 37 °C and 5% CO₂ for 24 h in RPMI + D-glucose. After the 24-hour incubation, the islets were individually pipetted into Eppendorf tubes containing the blank solvent, solutions of individual peptides, or a mixture of the peptides.

Here we used a total of six mice for two sets of experiments. In the first set of experiments (3 mice), islets from the same mouse, except for those used as controls, were incubated with fresh amylin, fresh CGRP, or a fresh mixture of amylin and CGRP. We used at least 3 islets from different mice per peptide condition. Because our LC-IMS-MS workflow was optimized for sub-single-islet measurements, we can test multiple conditions on the same mouse, and obtain multiple (at least three) technical replicates from a single islet. In the second set of experiments (3 mice), we followed the same procedure

as above. The only difference is that the islets were incubated with 4-day-old amylin, CGRP, or the 1:1 mixture.

DTIMS-MS and LC-IMS-MS

DTIMS experiments were performed on an Agilent 6560 IMS-QTOF (Agilent Technologies, Santa Clara). Samples were ionized with an ESI source and ions were pulsed through a helium-filled drift cell (3.94 Torr). Upon exiting the drift cell, ions were guided through the QTOF mass spectrometer by a hexapole ion guide. Data were collected in positive polarity with $\Delta V = 890, 790, 690, 590, \text{ and } 490 \text{ V}$. Additional instrument parameters can be found in a previous paper [61]. ATDs and mass spectra were analyzed with Agilent IM-MS Browser software (v.10.0). CCSs were determined using **Equation 1**—the Mason-Schamp equation.

Stocks of amylin and CGRP were prepared as previously described and diluted with a 5% MeOH/0.1% formic acid solution to a final concentration of 10 μM . The concentration of each peptide in the amylin:CGRP mixture was 10 μM . Aliquots of 50 mL of each peptide solution (amylin, CGRP, and amylin:CGRP) were placed into Eppendorf tubes, wherein single islets were manually placed. Two sets of peptide solutions were used: a fresh solution, where the peptide solutions were prepared approximately one day before acquiring the islets and contains primarily peptide monomers, and then an “aged” solution, where peptide solutions were left at room temperature for four days before islet acquisition and consist primarily of peptide oligomers. The “aged” solution was characterized with IMS-MS to determine the relative ratios of oligomers to monomers. This was used to

evaluate the effect of oligomers on the same islet system. LC-IMS-MS analysis was carried out according to **Chapter 2.2**.

5.2 CGRP Disrupts Amylin Fibrillization

We utilize IMS-MS, TEM imaging, and cell viability assays in the characterization of CGRP and amylin aggregation, an event that is cytotoxic to cells. IMS-MS shows that amylin has a higher propensity to form oligomers or aggregates than CGRP. We carried out additional studies with the addition of ZnCl_2 , as Zn^{2+} plays a vital role in the islet environment and has previously been shown to inhibit amylin fibrillization at low extracellular concentrations [110, 111] yet promote aggregation at high intracellular concentrations [111]. IMS-MS shows that when Zn or the $\text{Zn}(\text{H}_2\text{O})_5$ adduct complex with amylin, the β -hairpin conformation of amylin is promoted and stabilized [61]. The β -hairpin conformation is implicated in the fibrillization of amylin. The same studies were performed on CGRP. Much like amylin, the addition of the Zn or the $\text{Zn}(\text{H}_2\text{O})_5$ adduct to CGRP mediates the transition to the β -hairpin conformation. However, this transition occurs to a lesser extent, and the α -helix (predicted with REMD) prevails as the primary conformation [61]. The propensity for CGRP to maintain the α -helix motif prevents its aggregation.

TEM imaging shows that amylin forms fibrils with and without the addition of ZnCl_2 , whereas CGRP does not form aggregates or fibrils in either condition [61]. Interestingly, TEM shows that CGRP prevents amylin fibrillization when the two are placed in a 1:1 mixture. Cell viability assays using cultured β -cells exposed to amylin, CGRP, or the mixture were utilized to determine the cytotoxicity of the aggregates. The

release of an intracellular enzyme marker of death, adenylate kinase, was investigated. No significant direct cytotoxicity on β -cells was observed in any of the conditions [61]. This implies a non-direct mechanism of amylin-associated toxicity through inflammation that is mediated by islet resident macrophages [112].

5.3 Effects of CGRP and Amylin on Insulin Secretion

LC-IMS-MS analysis of isolated mouse islets allowed us to distinguish and detect Ins1 (observed mass of 5799.668 Da, 0.276 ppm difference from theoretical mass) and Ins2 (observed mass of 5793.5974, 2.261 ppm difference from theoretical mass) across multiple charge states. CCS values for Ins1 and Ins2 at $z = 4$ are 753 $\text{\AA}^2$ and 783 $\text{\AA}^2$ in helium and 902 $\text{\AA}^2$ and 936 $\text{\AA}^2$ in nitrogen respectively. CCS values for Ins1 and Ins2 have not been reported before; however, our observed values agree well with the CCS of the human insulin monomer at $z = 4$ (772 $\text{\AA}^2$) [113].

The structural differences between intact Ins1 and Ins2 can be seen in the 2D plots of Figure 23, which shows that Ins2 adopts an additional conformation that Ins1 does not. This conformation has a smaller arrival time, indicating it is more folded and compact. These structural differences served as the basis for the investigations of Ins1 and Ins2 B-chains in **Chapter 3**.

Figure 24 displays the statistical analysis, comprised of a one-way ANOVA analysis followed by a Tukey test between each condition, of Ins1 and Ins2 levels in control islets and islets incubated in either amylin, CGRP, or a mixture of amylin and CGRP. Experiments were performed in either fresh (A & C) or aged (B & D) media. Fresh media

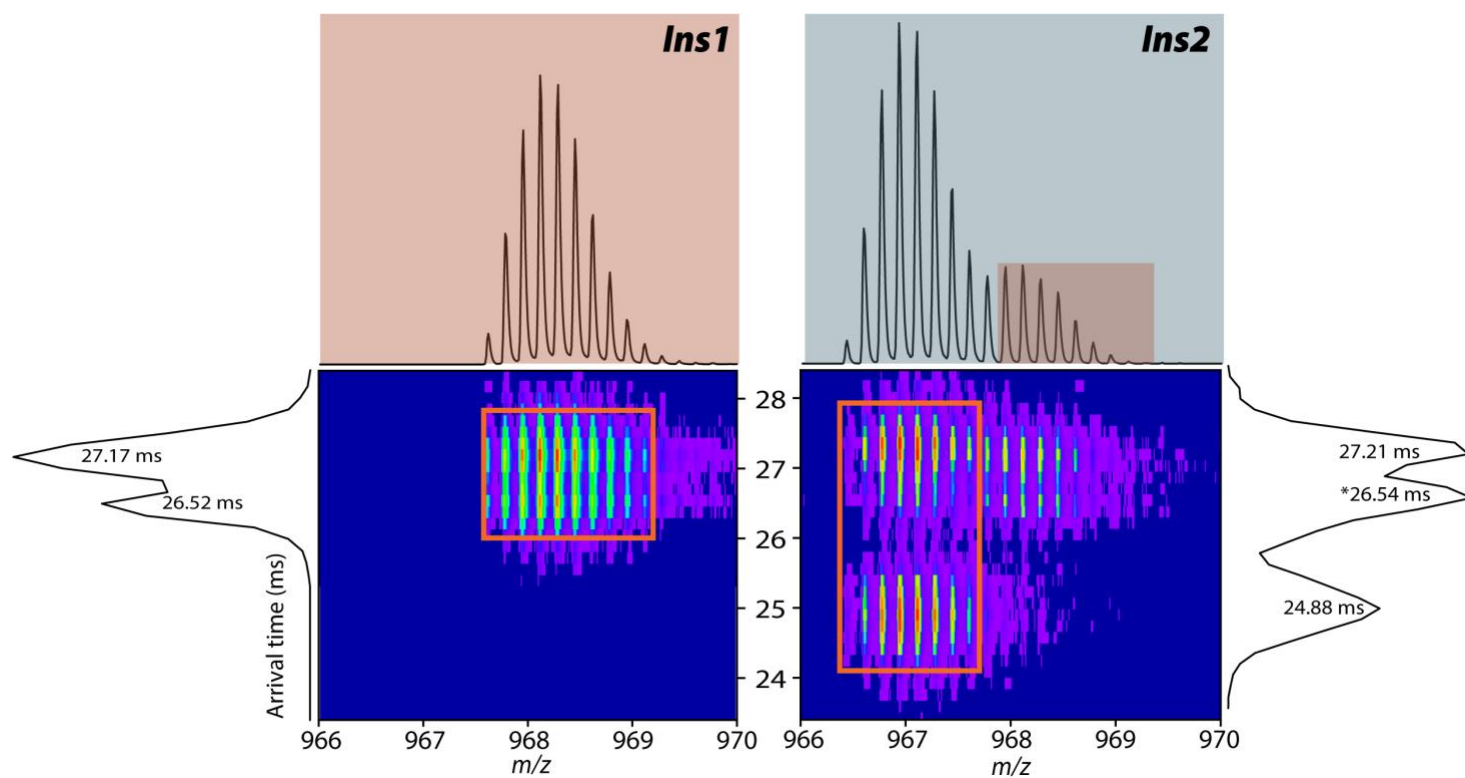


Figure 23. 2D plots of arrival time vs. m/z of Ins1 and Ins2 at $z = +6$. Adapted from Gray et al. *Chem. Sci.* 2021.

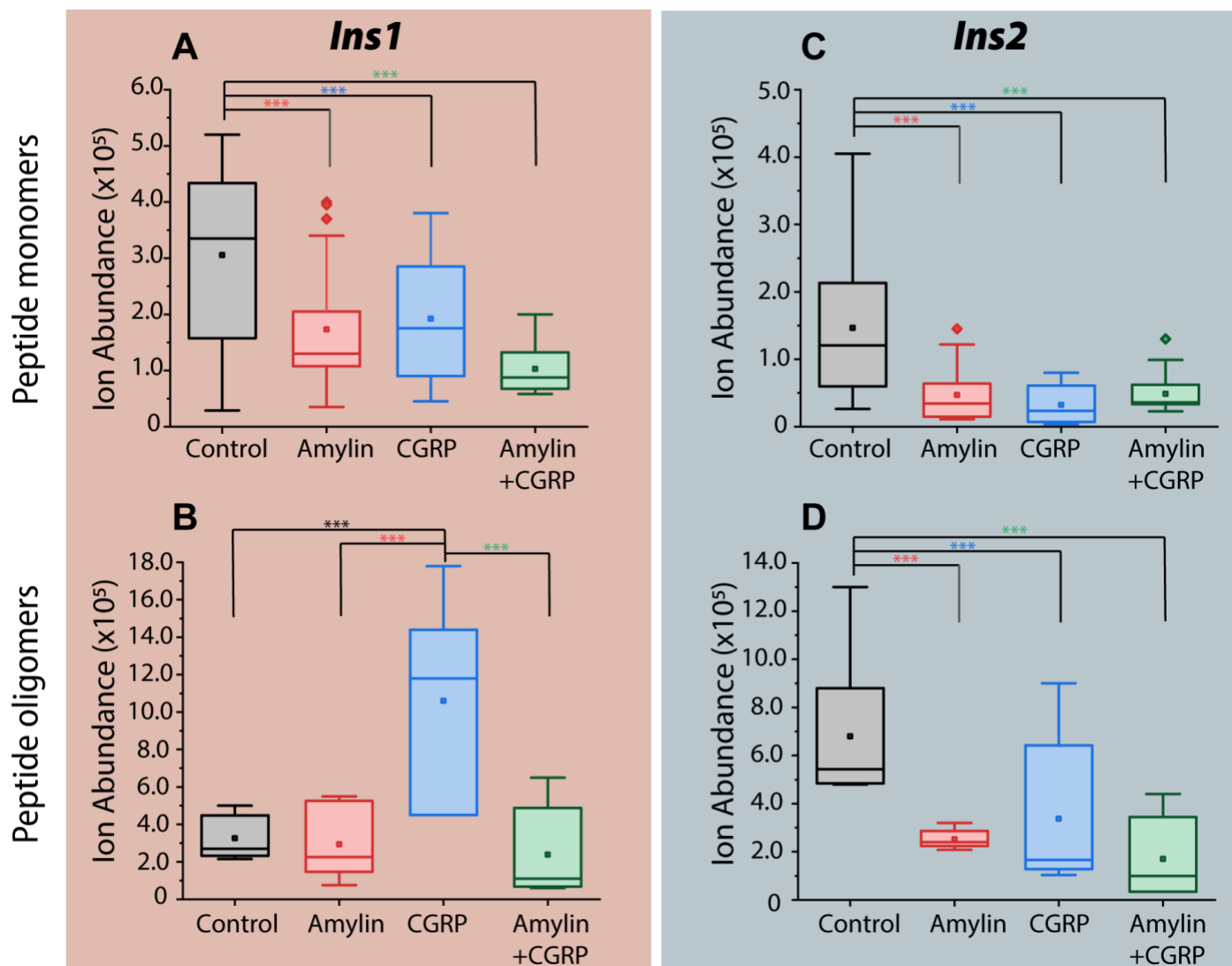


Figure 24. Comparisons of insulin secretion obtained from islets. Adapted from Gray et al. *Chem. Sci.* 2021.

is thought to contain CGRP monomers, whereas aged media consists of more CGRP oligomers. The presence of monomers or oligomers is confirmed by LC-IMS-MS analysis [61]. Ins1 and Ins2 levels are differentially affected. For Ins1, levels decrease significantly in fresh media of all conditions and increase significantly after aged CGRP incubation. The observed dual effect of CGRP on insulin secretion is founded on the behavior differences between CGRP monomers and oligomers. For Ins2, all three conditions inhibit its secretion in both fresh and aged samples.

Ultimately, CGRP alone can effectively reduce the secretion of Ins2, the human orthologue, but is less effective on Ins1. Given the fact that amylin is co-secreted with insulin from β -cells, lowering insulin secretion is a viable strategy to prevent amylin aggregation and the progression of T2D. Additionally, CGRP hinders the fibrillization of amylin, a mechanism implicated in T2D. Lastly, CGRP is shown to be nontoxic to β -cells. Ultimately, CGRP serves as a primary component in the molecular connection behind the inverse relationship between T2D risk and chronic migraine conditions, and exogenous CGRP treatment could be an effective strategy to combat T2D.

CHAPTER SIX

ELUCIDATING THE MOLECULAR CONNECTION BETWEEN TYPE 2 DIABETES AND CHRONIC MIGRAINE PART II: PACAP

Pituitary adenylate cyclase-activating polypeptide (PACAP) is a vasodilator that plays a role in migraine pathophysiology through its actions within the trigeminovascular system as an activator of the nociceptive pathway and its modulation of neurotransmission and neuro-inflammation in other areas of the brain [114]. PACAP exists in two bio-active forms—PACAP-38 (the most abundant form) and the result of its internal cleavage, PACAP-27—and binds the G-protein coupled receptors (GPCRs) PAC1, VPAC1, and VPAC2, all three of which are expressed in pancreatic islets [38]. PACAP has many different functions in various organ systems such as the urinary, reproductive, gastrointestinal, endocrine, central nervous, and peripheral nervous systems [38]. In islets, PACAP signaling results in the activation of adenylate cyclase which increased cyclic AMP (cAMP) levels to activate protein kinase A (PKA) [115]. PKA promotes the exocytosis of insulin-containing granules and the opening of L-type calcium channels. PACAP binding to PAC1 also activates phospholipase C (PLC) which releases calcium from calcium stores to increase cytosolic calcium levels. Lastly, PACAP elevates cytoplasmic sodium levels. Increases in intracellular calcium and sodium, coupled with activation of PKA, enhance insulin secretion in a glucose-dependent manner [115]. As mentioned in **Chapter 1.4**, the physiological role PACAP plays is quite complex. In this study, we will characterize PACAP using IMS-MS for the first time. Furthermore, because PACAP is often co-localized with CGRP [116], we will determine whether the two

peptides form oligomeric complexes. We will explore the idea that PACAP might complex with amylin, similar to CGRP as discussed in **Chapter 5.2**. Finally, we will investigate the *in-vitro* effects of PACAP on glucose-stimulated Ins1 and Ins2 secretion from pancreatic islets. Note, that the primary sequence of PACAP is identical in mice and humans (Figure 22).

6.1 Material and Methods

Chemicals were purchased and used without further purification. The PACAP-38 peptide was purchased from GenScript (USA) and used without further purification. Peptide powders were pretreated with hexafluoroisopropanol (TCI America). Solvents were left to evaporate in a desiccator overnight. Prior to IMS-MS analysis, a stock solution of PACAP was prepared by reconstituting the lyophilized peptide in LC-grade water (Honeywell) and diluting it to 10 μ M in a buffer of 20 mM ammonium acetate (VWR), pH = 6.8.

Mouse Culturing and Harvesting and GSIS Protocol

Four 10-week-old male C57BL/6J mice (stock #000664) were ordered from the Jackson Laboratory (Bar Harbor, Maine) and allowed to acclimate to the animal facility conditions on a 12 h light cycle at 22 ± 1 °C. The mice were provided standard chow and drinking water *ad libitum* until they reached 12 weeks of age. At 12 weeks of age, the mice were euthanized via CO₂ asphyxiation followed by cervical dislocation. All procedures were approved by the University of Tennessee Institutional Animal Care and Use Committee. Additional details regarding pancreatic perfusion and islet isolation can be found in **Chapter 2.1**. Following isolation, the islets were incubated at 37 °C and 5% CO₂

for 24 h in RPMI + 11.1 mM D-glucose. After the 24-hour incubation, the islets were transferred to a clean plate with 5 mM glucose in RPMI media for 18 hours. Then islets were transferred to an Eppendorf tube containing 0.1 mL of 3 mM glucose in HBSS. After 1 hour, the supernatant was removed and measured for secreted insulin. The same islets were then incubated in 0.1 mL of 15 mM glucose in Hank's balanced salt solution (HBSS). After 1 hour, the supernatant was removed again and measured for secreted insulin. Islets were partitioned into tubes containing 1, 10, or 30 islets. Islets were placed into either control or PACAP treated (10 μ M) groups. Three technical replicated were obtained from each sample, with two consecutive blank injections between each sample for column cleaning. LC-IMS-MS experiments were run according to the method outlined in **Chapter 2.2**. DTIMS-MS experiments on PACAP were carried out according to the method outlined in **Chapter 2.2**.

6.2 IMS-MS Characterization of PACAP-38 and its Interactions with CGRP and Amylin

Using IMS-MS, PACAP-38 is detected at seven different charge states (see Figure 25), which were identified unambiguously with isotopic spacing. The most intense peak was $m/z = 907.3132$ (charge = +5, Δ ppm = 17.63). Mobility data of PACAP shows 2 distinct features at 28.85 ms and 30.09 ms ($z = +5$). The CCS_{N_2} of these conformers are 1070.59 $\text{\AA}^2$ and 1138.46 $\text{\AA}^2$ respectively. The main conformer of PACAP has a quicker arrival time and smaller CCS, indicating it is more folded than the other conformer.

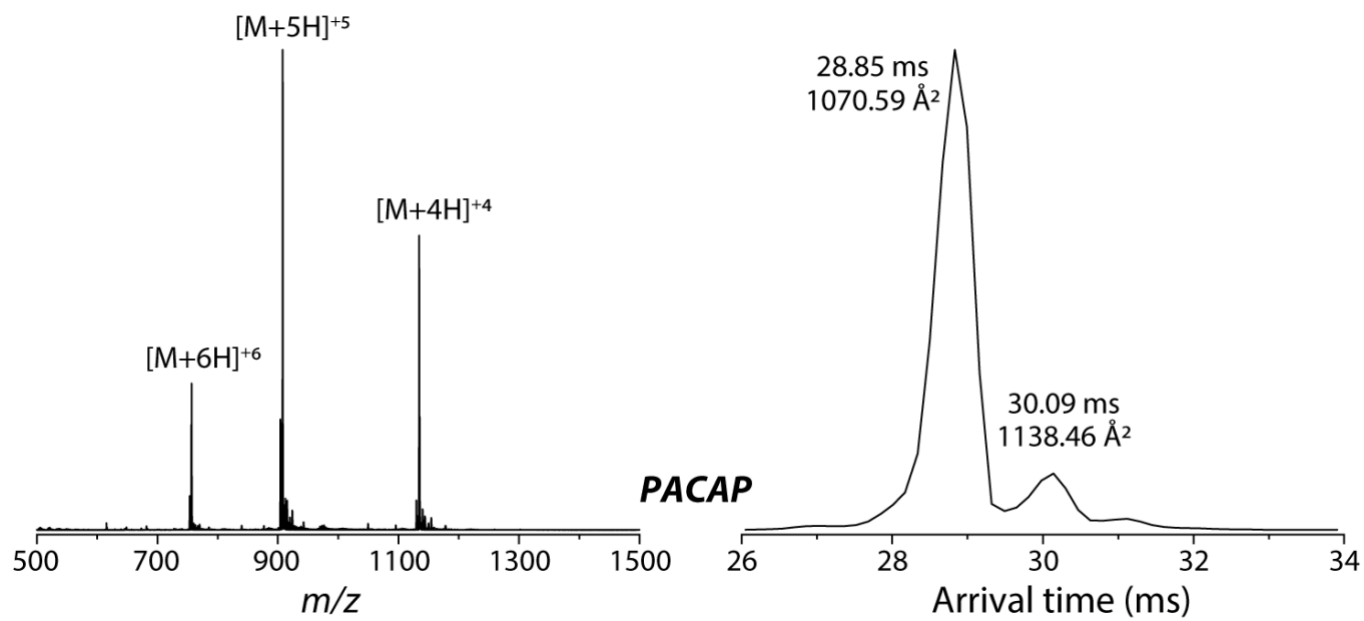


Figure 25. Characterization of PACAP.

Next, we investigate PACAP oligomerization and its complexation with CGRP and amylin. Figure 26 shows the mass spectra of samples containing either PACAP and CGRP or PACAP and amylin. PACAP displayed low levels of dimerization at $n/z = 1/2$ and $2/5$. CGRP formed both dimers and trimers ($n/z = 1/2$ and $3/5$). PACAP:CGRP 1:1 complexes were found at charge states +4 and +5. Amylin formed dimers and trimers at $n/z = 2/5$ and $3/7$. Various complexes of amylin and PACAP were observed, including 1:1, 1:2, and 1:3 PACAP:Amylin. Like CGRP, PACAP interacts readily with amylin in solution. Studies with TEM will be conducted in the future to determine if PACAP exhibits prevention of amylin fibrillization, as was seen with CGRP.

6.3 PACAP Exhibits a Small Effect of Ins1 Secretion In-Vitro

Our GSIS protocol allows us to measure secreted versus overall insulin levels in isolated mouse islets using LC-IMS-MS. Measurements of secreted insulin were obtained after stimulation with 15 mM glucose. The amount of secreted insulin is about $1.5 \pm 0.04\%$ of the total insulin, as seen in Figure 27. With this in mind, we can apply this method to measure the effects PACAP has on secreted and total insulin levels. A one-way ANOVA was performed to determine the statistical significance of our data. This was followed by a Tukey test between the control and treatment conditions. The statistical analysis is summarized in Figure 27. Our data show that treatment with 10 μM PACAP only has a significant effect on secreted Ins1 levels, but not Ins2 levels, in which PACAP increased Ins1 secretion. This data is interesting, as we initially expected Ins2 levels to increase with PACAP exposure as well. As discussed previously, PACAP actions are seemingly quite complex. In the future, we hope to conduct studies using LC-IMS-MS to study the *in-vivo*

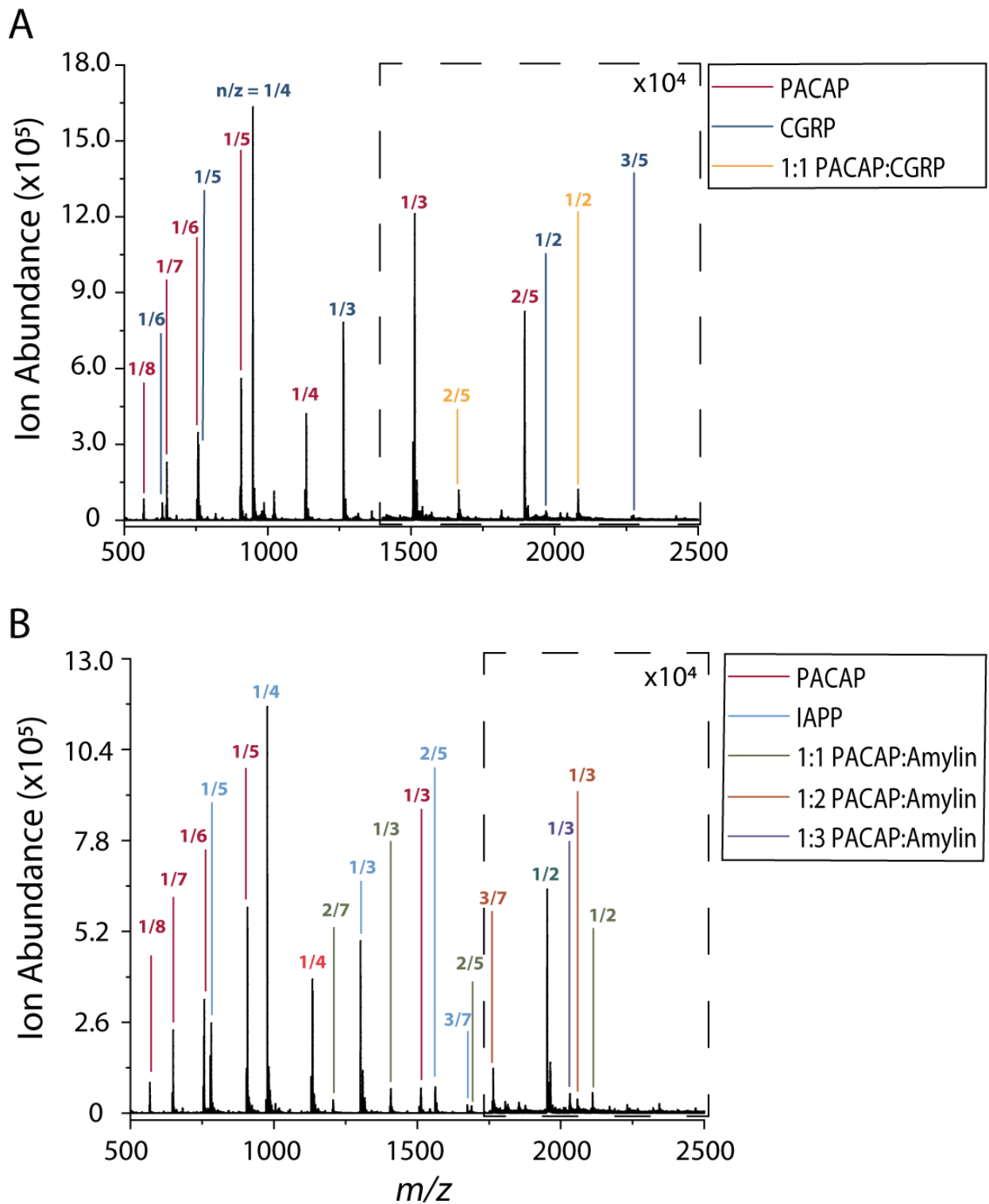


Figure 26. PACAP oligomerization and complexation with CGRP and Amylin.

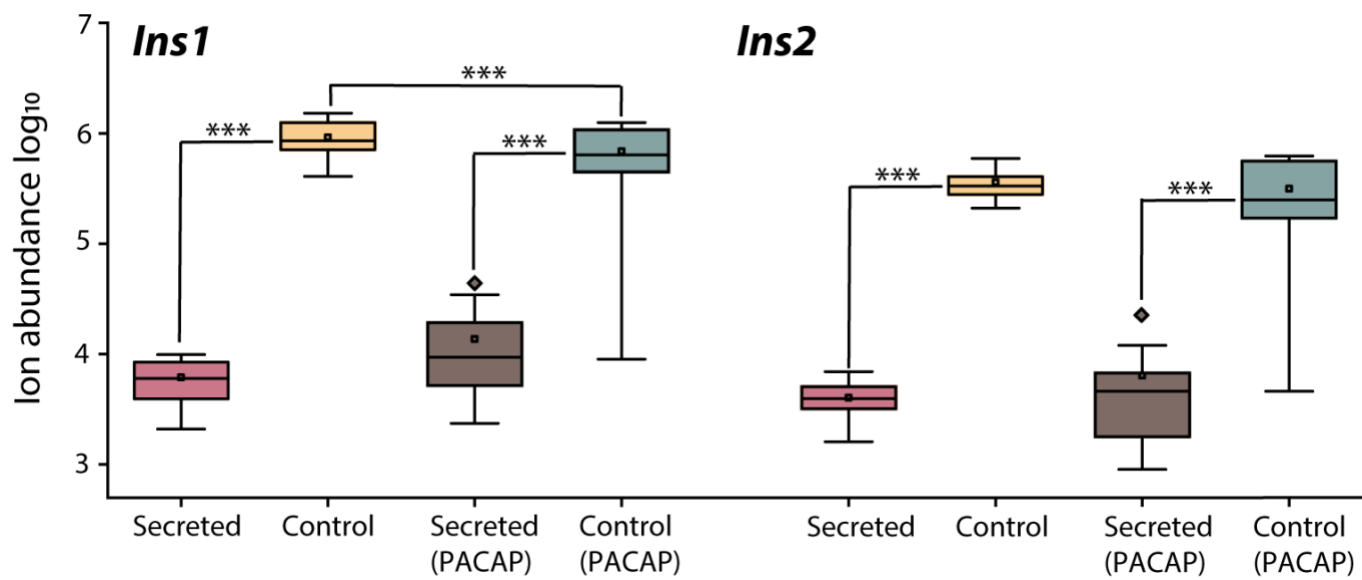


Figure 27. Statistical analysis of PACAP GSIS experiments.

effects of PACAP with transgenic mice to give us a better idea of the ramifications PACAP has on the islet environment. We also hope to identify PACAP's effects on other pancreatic hormones like amylin and glucagon. Lastly, we plan to explore the changes in morphology that PACAP exerts on β -cells using microscopy-guided single-cell profiling MS (MicroMS).

CHAPTER SEVEN

LC-IMS-MS FOR BIOCHEMICAL ANALYSIS OF POSTTRANSLATIONAL MODIFICATIONS

7.1 Non-Enzymatic Posttranslational modifications and Peptide Cleavages

Observed in Peptide Epimers

Posttranslational modifications (PTMs) allow organisms to maintain cellular homeostasis by modulating protein function and stability. PTMs regulate protein turnover, localization, enzymatic activity, protein-protein interactions, and expression levels [117-122]. More than 600 different PTMs are currently listed in the UniProt database [123]. A posttranslational modification is executed through the addition, removal, exchange, and rearrangement of functional groups to amino acid side chains [124] and the peptide backbone [125]. These modifications occur both spontaneously and through enzymatic activity. Given the vital role of PTMs in the cellular environment, they serve as biomarkers for many disease states including cancer [126], neurodegeneration [127], aging [128], diabetes [129], and cardiovascular disease [130].

Traditional methods of PTM identification include isotopic labeling, immunochemistry, and amino acid analysis [131]. Recent advances in MS have proven useful for PTM quantitation and have led to the identification of many new PTMs. MS has many advantages over traditional techniques due to increased sensitivity and the ability to identify PTMs in complex solutions, study volume-limited samples, identify specific PTM sites, and discover novel PTMs [132]. Coupling IMS to MS techniques further enhances

their capabilities to identify PTMs that are isomeric/isobaric through the additional separation of ions in the gas phase based on their electrophoretic mobility in buffer gas [133].

This work utilizes both trapped ion mobility spectrometry (TIMS)-MS and drift tube IMS (DTIMS)-MS to explore PTMs in the pleurin peptide system. Pleurin peptides are found endogenously in the central nervous system (CNS) of *Aplysia californica* and contain D-amino acids in the N-terminal regions. Recent studies show differential isomerization of pleurin peptides in specific regions of *A. californica*'s CNS and demonstrate the relevance of L- to D-residue isomerization [134, 135]. We follow up these studies by exploring three important PTMs that can occur in the absence of enzymes—proline *cis/trans* isomerization, asparagine (Asn) deamidation to aspartate (Asp), and Asp isomerization to *IsoAsp*. We direct our focus to the Plrn2 peptides, namely, L-Plrn2, D3-Plrn2, and P12A-Plrn2 (the sequences are shown in Figure 28) to capture these PTMs and determine whether they are biological, or instrument driven. This contribution will speak to the capabilities of TIMS and DTIMS in the study of PTMs.

7.2 Material and Methods

Peptide Sample Preparation

All samples of the Plrn2 peptides used for this study were purchased from GenScript and used without any further purification. The peptides were pretreated with hexafluoroisopropanol (TCI America). Aliquots of the peptide stocks were left to evaporate in a desiccator overnight. The three Plrn2 peptides were then reconstituted in LC-grade water (VWR) in 20 mM ammonium acetate buffer at pH 6.8, or phosphate-buffered saline

L-Plrn2	SFYTTGNGNHYPRI-NH2
D3-Plrn2	SF _d YTTGNGNHYPRI-NH2
P12A-Plrn2	SFYTTGNGNHYARI-NH2

Figure 28. Peptide sequences of Plrn-2 peptides.

(PBS), for final peptide concentrations of 10 μ M. All DT IMS-MS and LC-IMS-MS experiments were carried out according to the methods outlined in **Chapter 2.2**.

7.3 P12A Mutation Confirms Cis/Trans Proline Isomerization in TIMS that is not observed in DTMS

Cis/trans proline isomerization can occur through peptidyl-proline isomerases [136] or spontaneously. However, spontaneous isomerization happens on the order of microseconds to seconds which is slower than protein folding [137]. Proline is unique compared to other amino acids in that there is a similar steric clash in both the *cis* and *trans* conformations of peptide bonds, resulting in a potential energy difference between isomers of only ~2.1 kJ/mol, compared to ~10.5 kJ/mol in other non-proline peptide bonds, and a transition barrier of ~54.4 kJ/mol [138]. Like non-proline-containing peptide bonds, there is still a preference for the *trans* proline isomer [139]. The *cis* conformer is rare in native proteins, yet can be found more frequently in the denatured state [140]. *Cis/trans* proline isomerization is implicated in protein folding dynamics [139, 141] cell signaling [142], and the regulation of protein stability [143].

Figure 29A shows the ATD obtained with the TIMS instrument of the P12A-Plrn2 monomer ($z = +2$). As seen from the primary sequences in Figure 28, P12A-Plrn2 has alanine in place of proline. A single intense peak is observed in the ATD, indicating one major conformer present. The inset of Figure 29A shows the mass spectrum of the monomer, whereas Figure 29B shows the ATD obtained with the TIMS instrument of the L-Plrn2 monomer ($z = +2$). Two distinct peaks are observed in the ATD, indicating

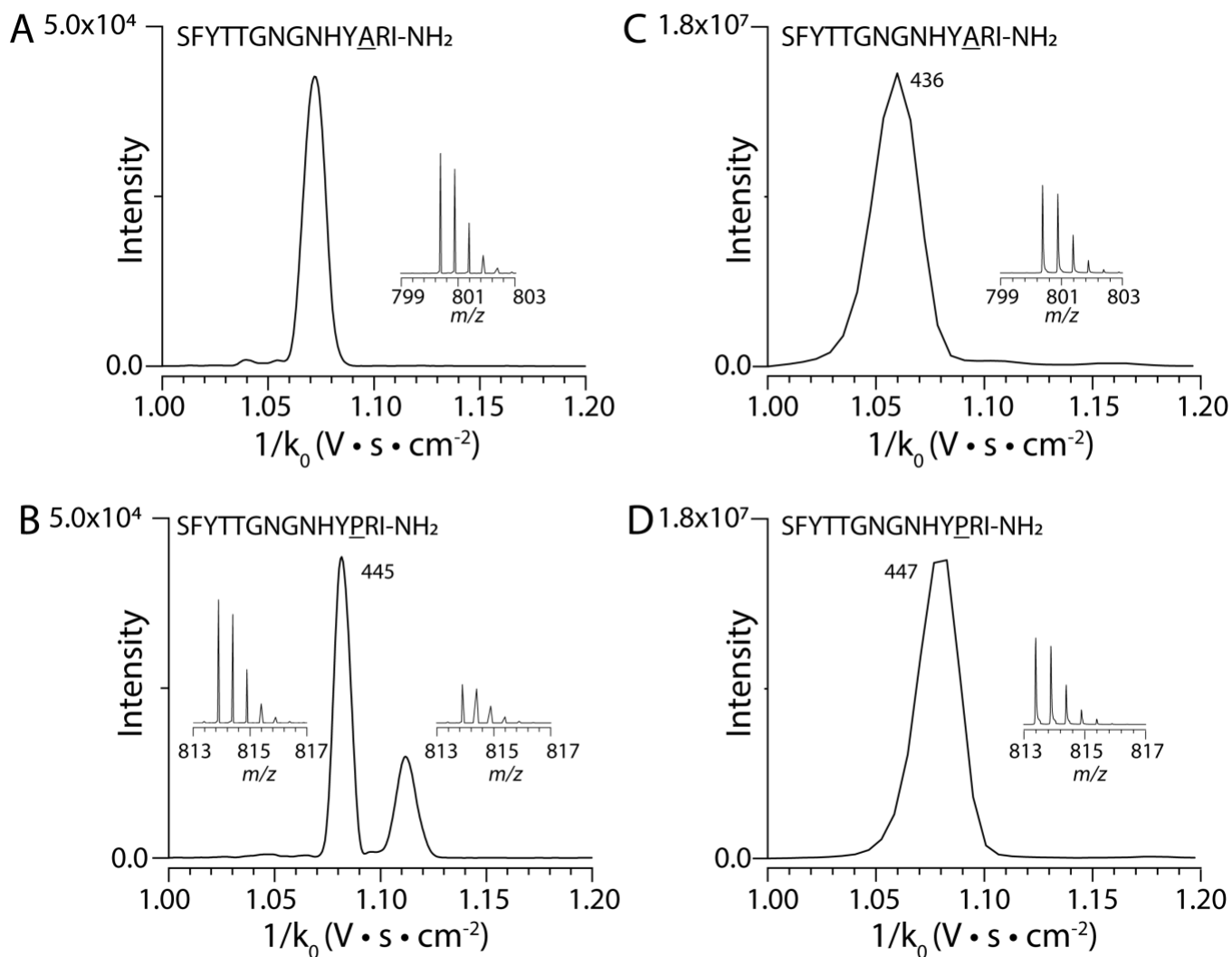


Figure 29. Mobility data collected with the TIMS instrument of P12A-Plrn2 (A), L-Plrn2 (B), and the DTIMS instrument of P12A-Plrn2 (C), and L-Plrn2 (D).

two conformers in the sample. The less intense peak has a higher reduced ion mobility ($\frac{1}{K_0}$), representing a more elongated 3D structure than the more intense first peak. The first peak has similarly reduced ion mobility to the peak in Figure 29A. The inset of Figure 29B shows the mass spectra of the two conformers which verify they share the same m/z ratio. The disappearance of the second peak in the P12A-mutated peptide indicates that the conformers in the L-Plrn2 ATD represent the *cis/trans* isomerization of the proline.

Additional ion mobility measurements were acquired using a DTIMS instrument to determine whether the *cis/trans* proline isomerization observed in Figure 29B was biological or instrument-driven. Figure 29C shows the ATD obtained with the DTIMS instrument of the P12A-Plrn2 monomer ($z = +2$). A single intense peak is observed, which was similarly observed in the TIMS data. The ATD obtained with the DTIMS instrument of the L-Plrn2 monomer ($z = +2$) is shown in Figure 29D where a single major peak is observed, indicating only one conformer is present in the sample.

These results indicate that the DTIMS instrument did not capture *cis/trans* proline isomerization. Even though the TIMS instrument has higher resolving power (R typically around ~ 200 compared to ~ 40 in DTIMS) [71, 72], the DT instrument should have sufficient resolution to distinguish these peaks given the large differences in CCS data. While TIMS is an efficient method to investigate the conformational dynamics of biomolecules and “soft” settings can be employed to study native folding [144], structural rearrangements have been observed when higher DC voltages or longer measurement time scales are utilized [145]. The successive build-up of internal energy over time allows ions to overcome isomerization barriers [145] which is most likely the case with the TIMS data

on Plrn2 in which an accumulation time of 350 ms was used. “Softer” TIMS parameters include accumulation times as low as ≤ 10 ms [144] but decrease resolving power at the same time [146].

7.4 Non-Biological Asn Deamidation and IsoAsp Formation

Since Plrn2 peptides contain two asparagine (Asn) residues in their sequence, we investigated the ability of the DTIMS instrument to capture Asn deamidation, a PTM that is occurring here in the absence of a biological environment. Samples of L-Plrn2 were run through the DTIMS instrument via direct infusion as soon as the sample was prepared ($t = 0$ days; fresh), stored at ~ 4 °C, and re-ran about five months later ($t = 159$ days; aged). The 2D plots of m/z vs. arrival time of L-Plrn2 ($z = +2$) are shown in Figure 30. The 2D plot of the fresh sample shows a single distinct peak that represents one major species. Evidence of a second species arises in the aged sample 2D plot that coincides with a mass shift of +0.984 Da which denotes Asn deamidation [147].

Asn deamidation is a spontaneous PTM that proceeds non-enzymatically through either direct hydrolysis or a succinimide intermediate. Initially, Asn cyclizes to the gem-hydroxylamine tetrahedral intermediate resulting from the nucleophilic attack of the ($N + 1$) residue’s amide nitrogen to the amide carbon of the Asn side chain [148]. Deammoniation of the gem-hydroxylamine intermediate forms succinimide. Figure 31 shows that the succinimide intermediate is detectable in the mass spectrum of L-Plrn2 at $z = +2$ as evidenced by a mass shift of -17.02 Da. LC-MS/MS confirmed the identification of the succinimide intermediate (Figure 32). The succinimide ring is then hydrolyzed

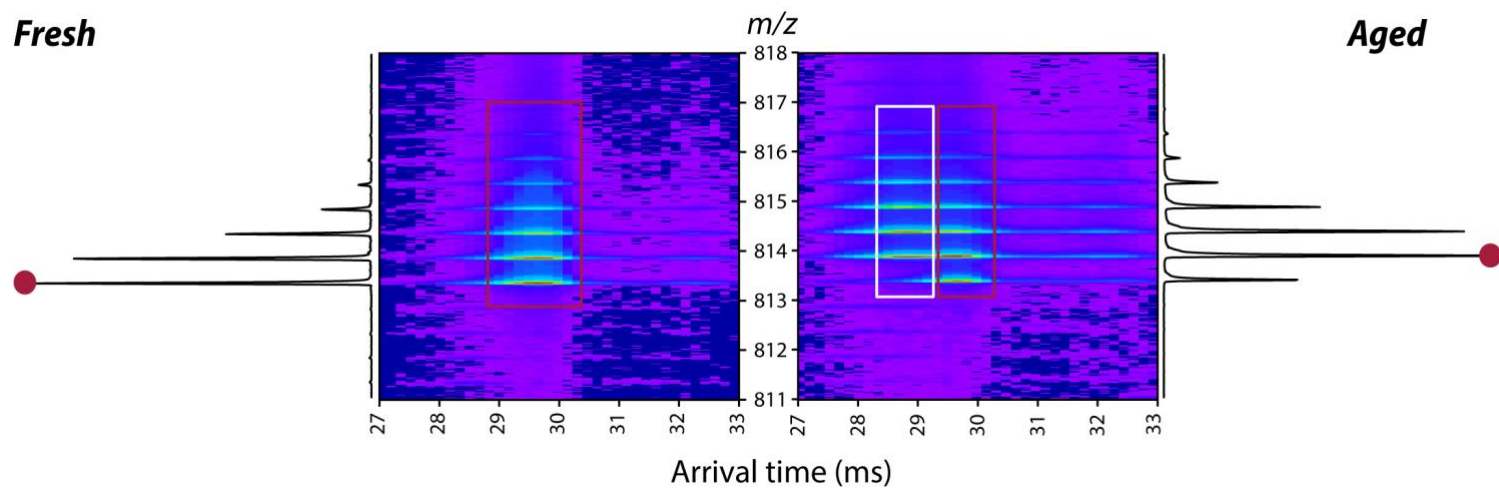


Figure 30. 2D plots and mass spectra of fresh and aged L-Plrn2.

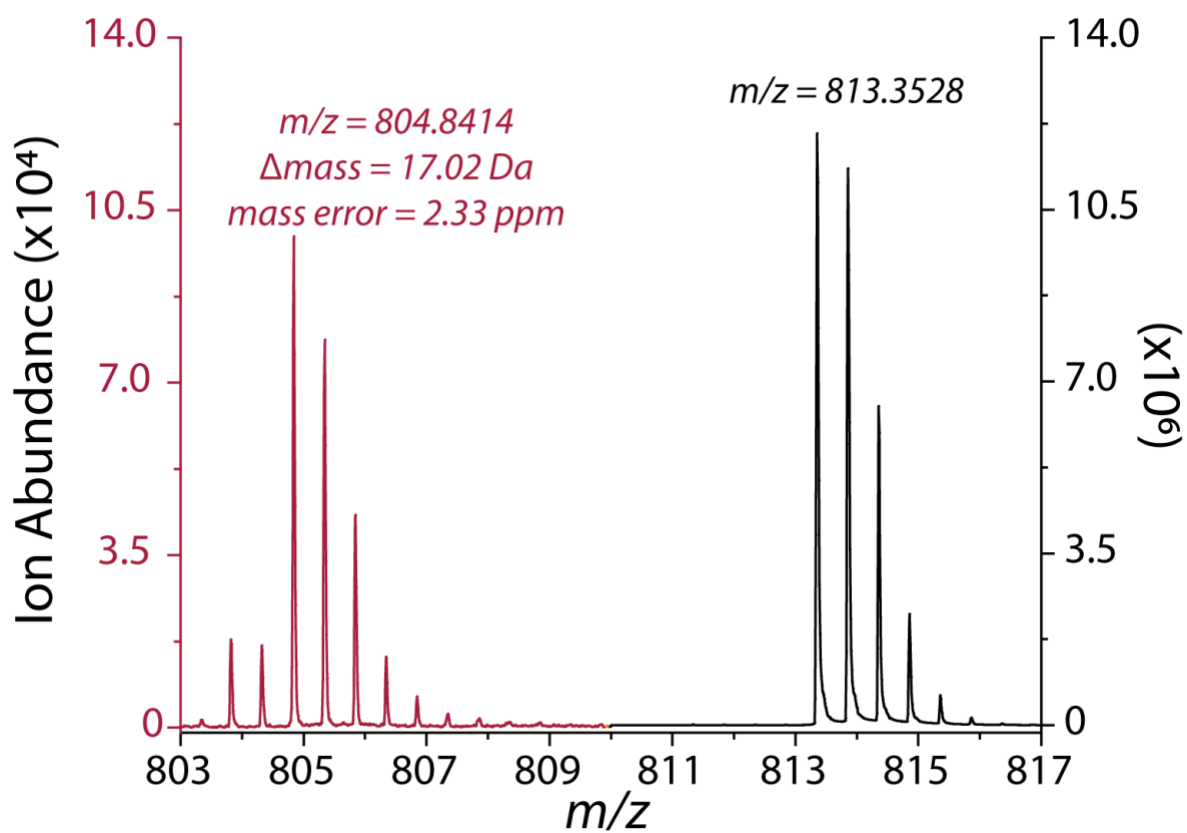


Figure 31. Mass spectrum displaying the detection of the succinimide intermediate in red.

Succinimide MS/MS

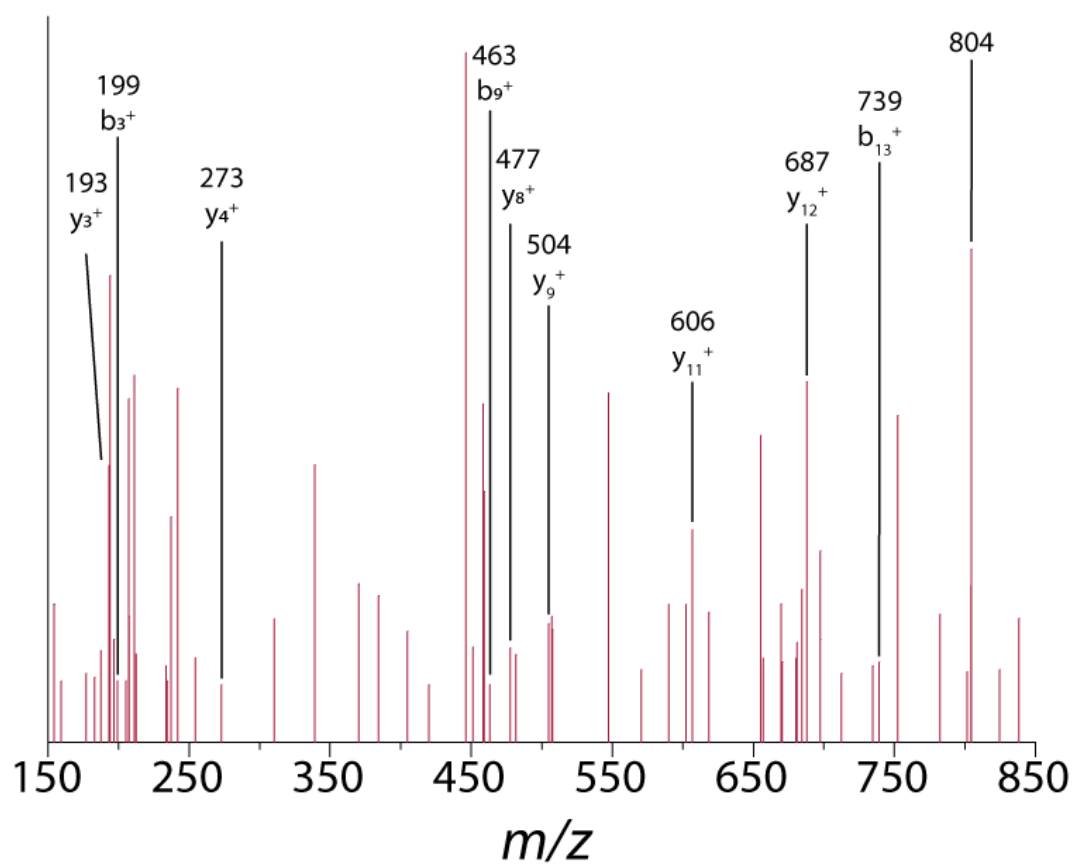


Figure 32. MS/MS of succinimide intermediate.

resulting in Asp formation [147]. Depending on which carbonyl of the ring is hydrolyzed, Asp or *IsoAsp* is produced with *IsoAsp* typically predominating in a 3:1 ratio [149].

IsoAsp formation was further investigated with LC-IMS-MS experiments. Figure 33A shows the LC chromatograms of the L-Pln2 peptide run at $t = 0$ days and $t = 2$ days. Only one peak is observed in the $t = 0$ days sample, whereas three distinct peaks are observed in the $t = 2$ days sample. The peaks from left to right were identified as the native asparagine-containing peptide, *IsoAsp*-containing peptide, and Asp-containing peptide, respectively. Figure 33B presents the 2D plots of m/z vs. drift time for Asn, *IsoAsp*, Asp, and succinimide-containing peptides ($z = +2$). Figure 33B shows that Asn deamidation causes the peptide structure to become more compact, while the isomerization to *IsoAsp* loosens the peptide structure. The mobility of the succinimide intermediate is similar to that of Asp and *IsoAsp*, which is expected given its similar molecular geometry.

Using low-energy collision-induced dissociation (CID) through ESI-MS/MS, diagnostic b- and y-type ions have been identified to distinguish Asn, Asp, and *IsoAsp* [150]. The internal rearrangement of *IsoAsp* yields $[b_{n-1} + H_2O]$ and $[y''_{i-n+1} - 46]$ ions [151]. Figure 34 shows the MS/MS data for P12A-Pln2. The b-ions were detected at m/z 772, 829, and 944 indicating that both Asn7 and Asn9 can be deamidated. The presence of the $[y_8^+ - 46]$ and $[b_7^+ + 18]$ ions but not $[y_6^+ - 46]$ and $[b_9^+ + 18]$ ions show that only the first Asn (N7) is deamidated and subsequently isomerized into *IsoAsp*. *IsoAsp* formation tends to occur more readily in flexible protein regions and when the C-terminal residue is glycine, which is why we only see *IsoAsp* in the 7th position and not the 9th position [152]. Asn deamidation and *IsoAsp* formation are associated with many biological and pathological

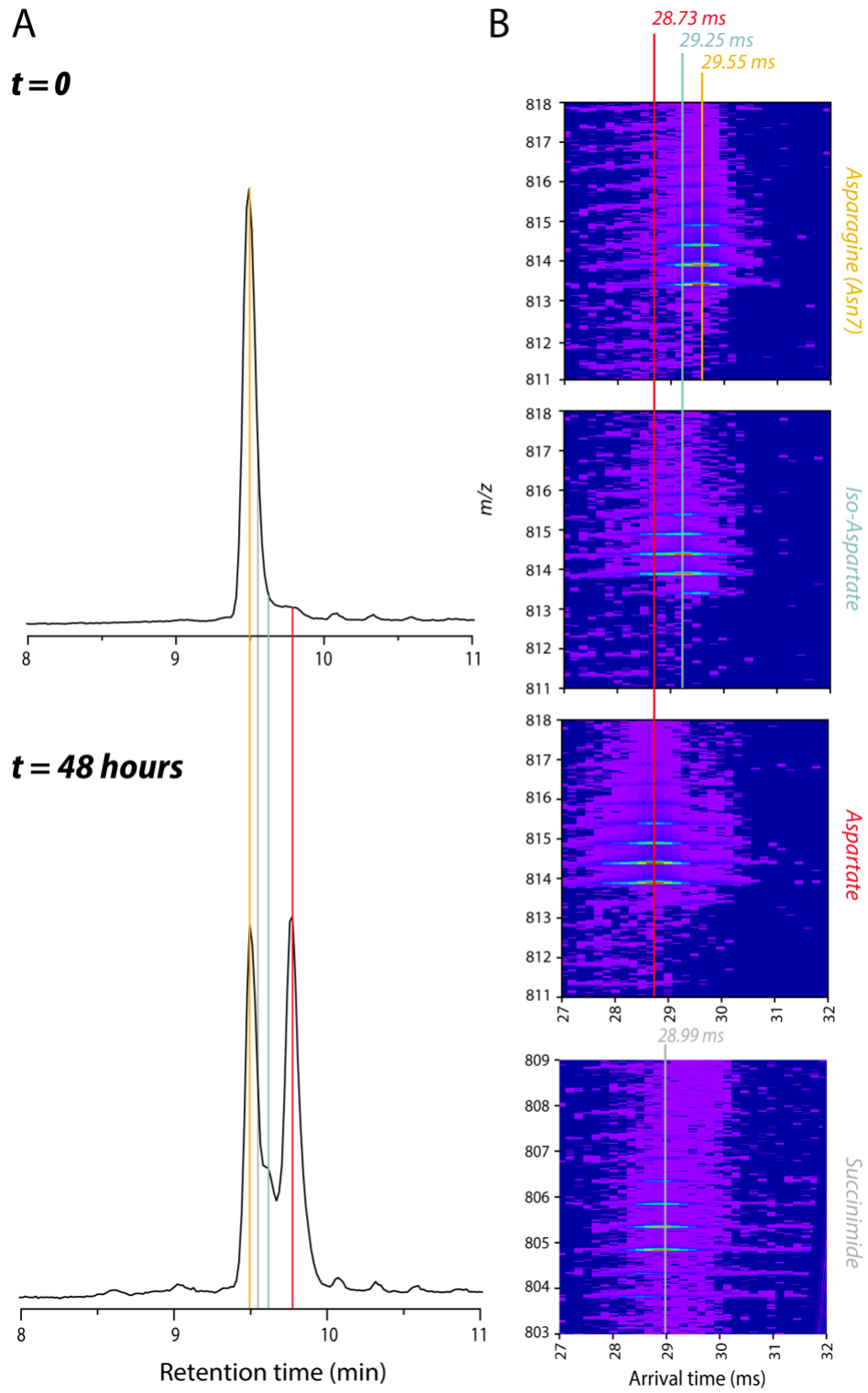


Figure 33. A) LC chromatographs of L-Plrn2 at $t = 0$ and $t = 48$ hours. B) 2D plots of observed peptides.

L-Plrn2 MS/MS

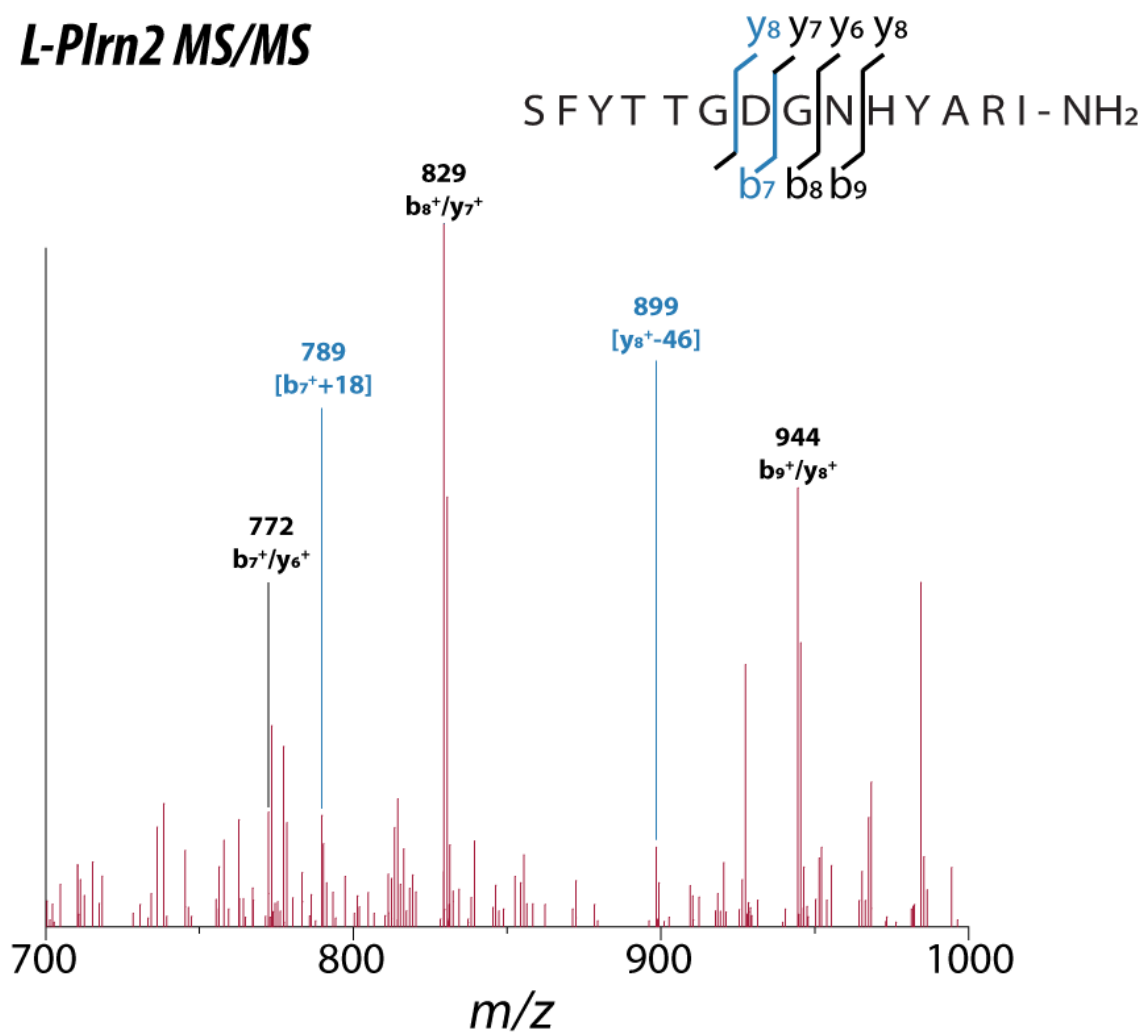


Figure 34. MS/MS of deamidated L-Plrn2.

processes and act as a molecular clock [153]. Asn deamidation is implicated in age-related diseases such as cataracts [154, 155], macular degeneration [156], osteoarthritis [157], and neurodegeneration [158]. Asn deamidation also has roles in type 2 diabetes [159], autoimmune disease progression [160], gene regulation [161], protein stability [162], and cancer [163]. Given this, Asn deamidation and *IsoAsp* formation serve as biomarkers for many of these diseases. Our work shows that the DTIMS instrument can effectively capture both Asn deamidation and its subsequent formation of *IsoAsp*. Furthermore, the precise location of Asn deamidation and *IsoAsp* formation can be probed with MS/MS, and we found that *IsoAsp* formation was more likely to occur at the Asn residue between two glycine residues (N7) compared to a glycine and histidine residue (N9).

7.5 Non-Enzymatic Peptide Cleavage in Pleurin Peptides

Like with the insulin peptides in **Chapter 3**, extensive non-enzymatic peptide cleavage was observed in LC-IMS-MS experiments of the Plrn2 peptides despite the use of a “soft” ionization method. The mass spectrum obtained from the LC-IMS-MS experiments is shown in Figure 35A. The peaks corresponding to the y-ions, y_{11} , and y_{12} , are intense, with the y_{12} ions corresponding to (d)YTTGNGNHYPRI and YTTGNGNHYARI being the most intense. This indicates that the bond most susceptible to hydrolysis is between Phe2 and Tyr3, the two residues that exist in both L- and D-configurations [135]. Figure 35B shows the 2D plot and mass spectrum of the y_{11} ion. The presence of two distinct features with a mass shift of +0.984 Da indicates that fragmentation is not induced through Asn deamidation. Similar results are obtained for L-

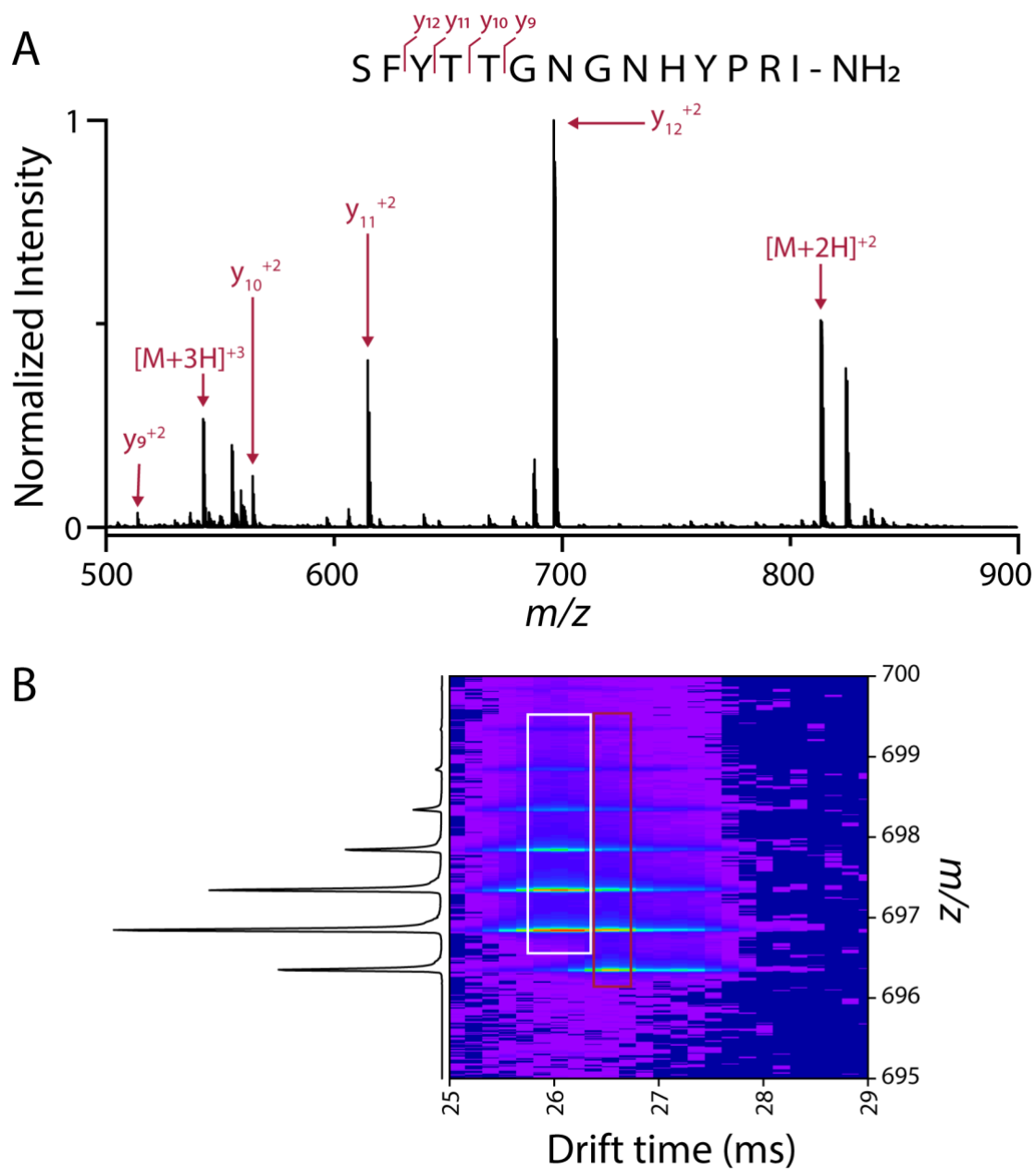


Figure 35. A) Mass spectrum of LC-IMS-MS data of L-Plrn2. Labeled y-ions provide evidence for non-enzymatic peptide cleavage. B) 2D plot and mass spectrum of the y₁₁ ion.

Plrn2 in the TIMS instrument but not for direct infusion DTIMS-MS experiments. Furthermore, similar fragmentation patterns (between hydrophobic and aromatic residues) are captured when other pleurin peptides (Plrn1 and Plrn3) are analyzed with the TIMS instrument [134].

Contamination of the LC column with proteases such as chymotrypsin from prior experiments was proposed to explain this cleavage. However, proteases tend to have increased specificity for L-amino acids and do not readily cleave D-amino acids due to a lack of interactions in the active site [164]. There was not a significant difference between the fragmentation rates of L-Plrn2 and D3-Plrn2, indicating that the observed cleavage is not due to enzymatic activity.

As stated in **Chapter 3.4**, the mobile proton model states that in MS, protons move across the peptide backbone and can lead to non-enzymatic cleavage via nucleophilic attack resulting in b- and y-ions [165]. The general assumption is that peptide bonds of a folded and protected region are less likely to be cleaved than those from unstructured or flexible regions. Given that extensive fragmentation is only observed in LC experiments and not direct infusion, the LC component of the instrument might denature the peptides. It has been reported that reverse-phase LC disrupts hydrophobic interactions in peptides due to the hydrophobicity of the matrix [88]. This would disrupt the secondary structure of the peptides and allow for fragmentation near aromatic/hydrophobic residues. Non-enzymatic peptide bond cleavage specific to aromatic residues has not been thoroughly investigated. We have been studying other peptide systems, such as insulin B-chains in **Chapter 3**, to determine whether the peptide cleavages are primary sequence-mediated or

conformation-specific. These findings are important for better understanding the implications of peptide fragmentation in LC and give insight into non-enzymatic peptide cleavage *in-vivo*.

CHAPTER EIGHT

CONCLUSIONS AND FUTURE DIRECTIONS

8.1 Conclusions

This work extensively displays how our established LC-IMS-MS workflow can be applied for the analysis of pancreatic islets at the single-islet and sub-single-islet levels. Our method can reliably detect pancreatic peptide hormones to elucidate their behaviors under various conditions, such as corticosterone treatment and under the effects of the chronic migraine-associated neuropeptides—CGRP and PACAP. We prove that LC-IMS-MS possesses the ability to distinguish between mouse Ins1 and Ins2, unlike traditional methods that fail to separate them. Our studies further reveal the need to distinguish these insulins, as they display diverged functions and differing behaviors under different conditions. For instance, corticosterone treatment only significantly affects Ins2 levels, CGRP exhibits a dual effect on Ins1 depending on oligomeric content, compared to an inhibitory effect on Ins2, and PACAP only impacts Ins1 levels under glucose stimulation *in-vitro*. Additionally, we characterized Ins1 and Ins2, and show they exist in differing secondary structures and exhibit different aggregation rates due to sequence differences in the B-chain.

In our work with corticosterone, we showed that Ins2 is better at preserving a folded conformation that is not found in Ins1. The structural differences between the two isoforms are likely responsible for their rates of enzymatic degradation in which Ins2 may be a better substrate for IDE. With our LC-IMS-MS procedure, we reveal new insights into the acute

corticosterone actions on mouse islets. We show that Ins2, the orthologue of human insulin, levels decrease significantly with acute corticosterone treatment. Corticosterone actions also increase the enzymatic activity of endopeptidases within mouse islets, with Ins2 being hydrolyzed more readily than Ins1. To our knowledge, this is the first study that reports the differential enzymatic patterns of mouse Ins1 and Ins2 *in vivo*. Lastly, we show that amylin levels are increased under corticosterone conditions, implicating the role of corticosterone in promoting β -cell dysfunction and the development of new-onset hyperglycemia. These findings provide a molecular basis for the different levels of diabetes risk associated with the two insulin isoforms. In addition, we showed that short-term exposure to corticosterone further impairs the level of intact functional insulin. Finally, insulin resistance and subsequent diabetes development induced by chronic GCs regimens may be due to fragmentation of insulin, resulting in reduced efficacy of the hormone and/or the overproduction of amylin, which in humans can aggregate and become toxic to the β -cells.

Investigations into the lessened risk of T2D development in patients with chronic migraine led to the conclusion that the neuropeptide CGRP is a prime peptide in the molecular connection between the two diseases. We show that CGRP does not readily aggregate, and its aggregation is not significantly provoked by Zn^{2+} complexation. Moreover, we demonstrate that CGRP can interfere with amylin fibrillization. We show that neither amylin nor a amylin:CGRP mixture causes direct toxicity to β -cells, but most likely recruits islet resident macrophages which induce toxicity via inflammation. We suggest that lowering insulin, and therefore amylin, secretion could lower amylin accumulation and aggregation thereby preventing cytotoxicity and T2D progression. We

find that CGRP can independently reduce the secretion of Ins2 but is less effective against Ins1 to which it can stimulate secretion, a process linked to early diabetic development.

Here, we characterize PACAP for the first time with IMS-MS. Preliminary studies with PACAP demonstrate that the peptide exists in two main conformers at physiological pH and readily complexes with amylin and, to a lesser extent, CGRP. Studies of the effects of PACAP on islet chemistry show that PACAP significantly increases Ins1 secretion under the stimulation of glucose while having no significant effect on Ins2. *In-vivo* studies are proposed to obtain a clearer view of the effects of PACAP on pancreatic peptide hormones and islet morphology, which will lead to a better understanding of PACAP's role in protecting against T2D.

Finally, we show the power of LC-IMS-MS in the detection and characterization of important posttranslational modifications. Our DTIMS workflow was utilized to determine whether proline *cis/trans* isomerization was occurring biologically or due to TIMS instrumental parameters. Furthermore, we demonstrate the ability of DTIMS to identify Asn deamidation to Asp and its subsequent isomerization into *IsoAsp*. Using MS/MS, we probe the exact Asn (one of two in the peptide studied) that was deamidated and isomerized. This study demonstrates the capabilities of DTIMS in the study of posttranslational modifications.

8.2 Future Directions

The work accumulated here serves as a foundation for numerous follow-up studies. Moving forward, we plan to obtain further structural information and aggregation propensities on Ins1 and Ins2 and their B-chains through X-ray crystallography, 2D-

infrared spectroscopy (2D-IR), atomic force microscopy (AFM), thioflavin T (ThT) fluorescence assays, and computer simulations. We also plan to pursue subsequent studies on the effects of PACAP using transgenic mice overexpression PACAP. MicroMS cell profiling will also be applied to determine the effects of PACAP on islet morphology. Finally, we aim to establish an MS-based method to accelerate the discovery of drug molecules for pancreatic diseases starting with T2D. Our measurements will be performed at single-islet or sub-single-islet levels where structurally and functionally diverged mouse Ins1 and Ins2 can be readily distinguished and complemented by high-throughput single-cell profiling and subcellular imaging. Starting with migraine-associated neuropeptides with activities in pancreatic islets, we will identify peptide analogs that regulate insulin secretion and remodel human amylin aggregation.

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VITA

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