University of Alberta

Assessment and Use of Collagenase Blends in Human Islet Isolation

by

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Abstract

Background: A better understanding of activities of each component of the collagenase blends for effective release of islets from the surrounding extracellular matrix will help improve efficacy of collagenase blend performance. This study retrospectively examined the samples of the collagenase blend used in the human islet isolation.

Methods: High performance liquid chromatography and kinetic measurements of collagenase and protease activity were used to assess potency. Mass normalization was achieved by total protein measurement.

Results: We obtained a cutoff point of 0.204 in CII/CI from the Receiver Operating Characteristic (ROC) curve for distinguishing between successful and unsuccessful isolation outcome. A significant increase in islet recovery was observed using a vial with CII/CI<0.204 (249,782±16,020 IEQs vs. 156,209± 20,833IEQs, respectively P=0.001).

Conclusions: Since manufacturer provide CII and CI in separate form, the predeterminate optimal CII/CI activity ratio would yield reproducible and effective collagenase cocktail to and potentially retrieve enough islets for human transplantation from one donor to one or more recipients.

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Dedication

To my parents for their continual encouragement, steadfast support, love and understanding through the years.

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ANOVA: Analysis of variance

BMI: Body mass index

BCA: Bicinchoninic acid

CDU: Collagenase degradation units

CI: Class I collagenase

CI: Confident interval

CIb :Class I collagenase isoform

CII: Class II collagenase

CIT: Cold ischemia time

CofA: Certificate of analysis

DCCT: Diabetes control and complications trial

DF: Dilution factor

DI: Dilution time

EC: Enzyme nomenclature

ECM: Extracellular matrix

EDTA: Ethylediamine tetra acetic Acid

FPG: Fasting plasma glucose

FPIR: First phase insulin response

GAD: Glutamic acid decarboxylase autoantibodies;

GDM: Gestational diabetes mellitus

HAS:Human serum albumin

HBSS: Hank's balanced salt solution

HOPE: Human organ procurement exchange

HPLC: High performance liquor chromatograph

IAA: Insulin autoantibodies

ICA: Islet cell autoantibodies

IDDM: Insulin-dependent diabetes mellitus

IE/kg: Islet equivalent per kilogram of body weight

IGT: Impaired glucose tolerance

IL2: Anti-Interleukin2

ITN: Immune tolerance network

ITR: Islet transplant registry

IVGTT: Intravenous glucose tolerance test

mL: Milliliter

NIDDM: Non-insulin-dependent diabetes mellitus

NP: neutral protease

PBS: Phosphate buffered saline

PES: Polyethersulfone

PFC: Perfluocarbons

PVDF: Polyvinylidene fluoride

PW: Pancreas weight

ROC: Receiver operating characteristic

RT: Room temperature

SE: Standard error

SFCA: Surfactant-free cellulose acetate:

SPK: Pancreas-kidney

TLM: Two layer method

UW: University of Wisconsin

WHO: World health organization

Chapter One

Introduction

Part 1) Diabetes Mellitus

Diabetes mellitus is a clinical disorder of intermediary metabolism characterized by hyperglycemia and glucosuria due to the inadequate secretion and/or utilization of insulin. Defect in carbohydrate lipid and protein metabolism are also present. Such deficiency results in increased concentration of glucose in blood, which in turn leads to serious damage to the different systems of the body, such as heart disease, blindness, and kidney failure and nerve damage to the lower legs (1).

The History of Diabetes

In 1869 Paul Langerhans, a German medical student announced in a dissertation that the pancreas contains two systems of cells. One set secretes the normal pancreatic juice; the function of the other was unknown. Several years later, these cells are identified as the 'islets of Langerhans' (2). In 1889 Von Mering and Minkowski discovered the vital link between the pancreas and diabetes when they observed hyperglycemia and glucosuria in a pancreatectomized dog (3). In 1893, the first clinical attempt to transplant the pancreas was performed at the Bristol Royal Infirmary in England. Williams and Harsant buried three pieces of freshly slaughtered sheep's pancreas, which size was about a Brazil nut, under the skin of a 13-year old boy dying of diabetic ketoacidosis (4). After the discovery of insulin in 1921, diabetes entered a new era (5), people with diabetes did not die, and many initially thought that diabetes had been cured. More into recent times, diabetes by the mid-1990's, was already considered as a major international health problem (6) and a costly clinical public health disorder (7).

Classification and Diagnosis

Two major types of diabetes were recognized: type 1 (insulin-dependent) diabetes and type 2 (non-insulin-dependent) diabetes. The first test used to distinguish between these two main forms of diabetes was the response to insulin, when Wilhelm Falta and other investigators in Vienna drew attention to the existence of insulin-sensitive and –resistant, forms of diabetes (8). However, the World Health Organization and American Diabetes Association classifications are currently shifted to a system that defines diabetes in terms of pathogenesis. The four main groups are: 1) Insulin-dependent diabetes mellitus (IDDM), which has been replaced by type 1 (immune mediated or idiopathic β-cell dysfunction leading to absolute insulin deficiency). These patients have β-cell destruction, which is usually immune-mediated; most develop absolute insulin deficiency and are ketosis-prone, 2) non-insulin-dependent diabetes mellitus (NIDDM) has been replaced by type 2 diabetes, which encompasses the most prevalent form of the disease. Most patients with type 2 diabetes exhibit insulin resistance and ultimately develop a concomitant insulin secretory defect, 3) the third class includes genetic defects in insulin secretion and action, diseases of the exocrine pancreas, endocrinopathies that induce hyperglycemia, drug-induced forms of diabetes, infectious causes, and genetic syndromes that are associated with an increased incidence of diabetes, and 4) gestational diabetes mellitus, which remains a separate class as before (1, 9, 10).

New criteria for the diagnosis of diabetes reflect an appreciation of the fact that the oral glucose tolerance test is rarely used in practice. Using the revised criteria, there are three ways to diagnose diabetes mellitus: 1) random, or casual plasma glucose ≥200 mg/dL (11.1 mmol/L), associated with symptoms (polyuria, polydipsia, unexplained

weight loss); 2) fasting plasma glucose (FPG) ≥126 mg/dL (7.0 mmol/L); 3) 2-h glucose ≥200 mg/dL (11.1 mmol/L) after a 75-g glucose load. Any of these criteria are sufficient for diagnosis, but each should be confirmed on a separate day (1, 9).

The decrease in FPG threshold from 140 mg/dL to 126 mg/dL is the major change in these diagnostic criteria. The previous FPG level of 140 mg/dL was not as sensitive as the new criteria potentially missing a group of patients, which remained undiagnosed(1, 9). A category of impaired glucose tolerance (IGT) is retained for individuals with fasting plasma glucose ≥110 mg/dL but <126 mg/dL. Patients in this group are at increased risk for the development of diabetes and cardiovascular disease (11).

Etiology

Type 1 diabetes mellitus is perceived as a chronic autoimmune disease with a sub-clinical prodrome characterized by selective loss of insulin producing beta cells in the pancreatic islets in genetically predisposed subjects. Because clinical type 1 diabetes typically does not present until approximately 80%-90% of the beta cells have already been destroyed (12). Various exogenous triggers, such as certain dietary factors and viruses, are thought to induce the autoimmune process leading in some individuals to extensive β-cell destruction and ultimately to the clinical manifestation of type 1 diabetes (13). There is a remarkable gap between the onset of autoimmunity and the onset of diabetes (Figure 1-1).

Epidemiology

Type 1 diabetes is one of the most common chronic diseases of childhood. Even with the recent epidemic of type 2 diabetes, type 1 accounts for approximately two thirds of all cases of diabetes in children (12). An estimated 177 million people suffer from

diabetes mellitus type 1 and type 2 worldwide, and that number may well double by the year 2025 reported by World Health Organization 's fact sheets. More than 2 million Canadians have diabetes. By the end of the decade, this number is expected to rise to 3 million (14, 15).

Between 1995 and 2025, the World Health Organization projects that the number of adult population affected by diabetes mellitus in developing countries will grow by 170%, from 84 to 228 million. Worldwide, a rise is projected to make a total of 300 million people. Much of this increase will occur in developing countries and will be due to population ageing, unhealthy diets, obesity and sedentary lifestyle (16).

The incidence of type 1 diabetes among children age <19 years in the U.S. is an estimated 18.2 per 100,000/year, that is 13,171 new cases each year. In adults an estimated 16,542 cases of type 1 diabetes arise each year in persons age >20 years in the U.S. (17, 18). In Montreal, Canada, there are large differences among ethnic groups. In contrast, Japan has a very homogeneous population and shows little variation among studies (19). Of the 16 million people with diabetes in North America, an estimated 1.7 million are type 1 and the rest are patients with type 2 diabetes (20). Although many autoimmune disease disproportionately affect women, type 1 diabetes seems to equally affect men and women (12), differences in disease prevalence and changes in incidence rates suggest that a combination of multiple genetic and environmental factors contribute to type 1 diabetes risk (12).

Costs

Due to its chronic nature, the severity of its complications and the means required to control them, diabetes is a costly disease, not only for the affected individual and his/her family, but also for the health authorities. In the USA, the total health costs of a person with diabetes are several times more than for people without the condition. As

of 2002, the per capita annual costs of health care for people with diabetes rose from $10,071 in 1997 to $13,243, an increase of more than 30%. In contrast, health care costs for people without diabetes amounted to $2,560. Intangible costs also have a great impact on lives of patients and their families, and are the most difficult to quantify. The direct costs of diabetes include medical care, drugs, insulin, and other supplies, representing a burden to society of approximately $92 billion. The indirect costs come from the number of diabetic patients that may not be able to continue working or work as effectively as they could before the onset of their condition, which represent approximately $40 billion. Sickness absence, disability, premature retirement or premature mortality can cause loss of productivity (21). Altogether, indirect and direct costs were calculated to be $132 billion in 2002 (22). This represents 19% of total personal health care expenditures in the U.S. However, diagnosed diabetes patients account for only 4.2% of the total U.S. population. In Canada, an estimated 2 million people have diabetes at the current time, and this is expected to reach 3.0 million by the year 2010 (23). Given that the Canadian population is approximately one tenth the size of the US population and has roughly similar diabetes prevalence rates, the direct economic cost of diabetes in Canada may be as high as $9 billion US annually (i.e. 10% of $92 billion) (24).

Part 2) Endocrine Replacement Therapies

Although the exact mechanism of pancreatic islet cell destruction in type 1 diabetes mellitus remains to be clarified, the autoimmune destruction of beta cells led by T cells seems to be the most likely explanation. Environmental and genetic factors certainly play a role but have not been fully identified (12, 25). The administration of exogenous insulin has been the primary treatment for type-1 diabetes since the

discovery of insulin in 1921 by Banting and Best (5, 20). Although the survival rates for diabetes have increased substantially, exogenous insulin therapy does not represent a cure for this disease. Moreover, data from the Diabetes Control and Complications Trial (DCCT) in 1993 showed that intensive insulin therapy could significantly reduce the risk of neurovascular complications, (50% reduction in eye, nerve, and kidney complications), but increases the risk of hypoglycemia by three times (26). Therefore, the only way to ensure long-term health in patients with diabetes is to ensure a way of maintaining constant normoglycemia. The two strategies proposed to replace total pancreatic endocrine function are pancreas transplantation and islet (insulin-producing tissue) transplantation.

Pancreas Transplantation

The first successful pancreas transplantation in conjunction with simultaneous kidney transplantation was performed by Richard Lillehei, MD, from the University of Minnesota in 1966. Until about 1990, the procedure was considered experimental. Now it is a widely accepted therapy. The pancreas comes from a cadaveric organ donor. However, select cases of living-donor pancreas transplantations have been performed. About 100 transplant centers in the United States perform pancreas transplantations. About 1400 cases are performed annually in the United States (27).

About 75% of pancreas transplantations are performed with kidney transplantation (both organs from the same donor) in patients with renal failure who are diabetic. This is referred to as simultaneous pancreas-kidney transplantation. About 15% of pancreas transplantations are performed after previously successful kidney transplantation. This is referred to as a pancreas-after-kidney transplantation. The remaining 10% of cases are performed as pancreas transplantation alone in non-uremic patients with very labile and problematic diabetes (27). It remains a significant surgical

procedure with substantial morbidity and occasional mortality (28). A recent controversial report by Verstrom group has raised the possibility that patient survival could be compromised after pancreas transplant alone or pancreas after kidney transplant, compared with patients awaiting this procedure in the United States (29). This study has recently been brought into question in that the group, which excluded patients with modestly impaired renal function, might be expected to have an increasing risk of mortality on the waiting list.

Because of the risks associated with surgery and long-term immunosuppressive drug therapy, pancreas transplantation is largely reserved for patients with diabetes with clinically significant complications, where the severity of their disease justifies accepting the risks of the procedure and immunosuppression. Therefore, with the exception of rare patients with severe, labile forms of diabetes, pancreas transplantation is not a practical option for young patients with diabetes who have not yet developed complications.

Islet Transplantation

Compared to pancreas transplantation, Islet transplantation is technically much simpler (Figure 1-2), has low morbidity, and offers the opportunity of storage of the islet graft in tissue culture or cryopreservation for banking or islet shipping. Moreover, the fact that islets can be kept in culture offer a unique opportunity to immunologically manipulate the islet graft, as well as optimize recipient conditioning prior to transplantation, thereby facilitating tolerance induction. The low morbidity of the procedure and the potential for tolerance induction make islet transplantation a promising strategy for treating young patients, including children, before the establishment of secondary complications (30).

While enthusiasm for clinical islet transplantation began in the early 1970s (31), its application was significantly limited, largely because of poor quality, low-yield islet

preparations and ineffective immunosuppression. Ricordi et al developed a new method for pancreas dissociation using the Ricordi digestion chamber in 1988 (32). Robertson et al improved islet purification using the COBE continuous purification system in 1993 (33). Lakey et al developed controlled pancreatic distension with the digestive enzyme collagenase in 1999 (34) and Roche provided a purified enzyme blends, Liberase ™ with low endotoxin levels in 1997 (35). All of these advances contributed to improvements in obtaining higher-yield, better-quality islet preparations.

After many years of intensive research and extensive collaborations between islet groups worldwide, in 2000, Shapiro and collaborators reported that they achieved 100% success (insulin-independence) one year after transplantation in a series of 7 patients performed at the University of Alberta in Edmonton, Canada (36). Thus, there was a remarkable shift in clinical success with the introduction of the so-called "Edmonton Protocol". Over 850 patients with type 1 diabetes mellitus have now received islet transplants at more than 50 institutions worldwide in the past 5 years than in the entire preceding 30-year history of islet transplantation. Rates of insulin independence at 1 year with the Edmonton protocol are impressive (37).

The Edmonton Protocol

The success of the "Edmonton Protocol" has been attributed to several key points: 1) sufficient number of islets with high quality 2) a new steroid-free immunosuppressive therapy combination formed by Sirolimus, a low dose calcineurin inhibitor (Tacrolimus), and induction with an anti-Interleukin2 (IL2)-receptor antibody (Daclizumab), and 3) recipient selection (Table 1-1).

The potential causes of failure of islet transplants had been determined and included failure of initial engraftment, inflammatory response at the transplant site, allo- or autoimmune response, and immunosuppressive drug-induced β-cell toxicity (38). The

Edmonton Protocol not only addressed these issues, but further optimized islet function by controlled delivery of a purified low-endotoxin collagenase enzyme, immediate graft processing, and transplantation of a total average of 800,000 IE (just over 11,000 IE per kg recipient body weight) into the liver via the portal vein (36). The experience of the Edmonton Trial has been reproduced in other centers through the Immune Tolerance Network (ITN) Trial, which started in the year 2000 and included Centers in Edmonton, Minneapolis, Miami, Seattle, St. Louis, Boston, Geneva, Giessen and Milan. The objectives in the creation of this trial were to replicate the Edmonton Protocol at multiple sites, to provide a base of qualified islet centers for future ITN tolerance trials, and to explore mechanisms of islet acceptance/rejection.

The protocol could be replicated at times to a very high level of success depending on the experience of the site. Three of the most experienced centers achieved a 90% insulin-free rate after 1 year (39). In fact, 5 of the first 13 ITN patients (38%) achieved insulin-independence with a single donor islet transplant, and single donor success was as high as 75% at one of the centers (39). Preliminary analysis suggests that the two most significant factors affecting clinical success are 1) the center's skills in obtaining islets with high quality and quantity following identical isolation protocol, and 2) the center's ability to maintain the recipient's immunosuppressant levels within a specified target range after transplantation (40). Despite these encouraging reports, the results of islet transplantation among different centers continue to be variable, and show limitations of this procedure (41). To date, after 5 years follow-up of patients in the Edmonton Protocol, we have learned that most patients have reverted to using some insulin, although C-peptide secretion (regarded as islet graft survival) has been maintained in the recipients over this period, which is still beneficial for the internal regulation of blood glucose levels (glucose homeostasis) (42).

Worldwide, there have now been over 850 patients treated in the past 5 years and increasing momentum and focus on the remaining challenges of pancreas procurement, islet isolation, alternative insulin-secreting regulated sources, islet engraftment, better immunosuppression with fewer side-effects, and the possibility of immunological tolerance continue to drive the field forward. Further improvements are necessary to make islet transplantation a routine clinical treatment.

Part 3) Normal Human Pancreas and Islets of Langerhans

Pancreas Architecture

The pancreas is an organ in the digestive system that serves two major functions: 1) exocrine - it produces pancreatic juice containing digestive enzymes. 2) endocrine - it produces several important hormones, including insulin (43).

In humans, the pancreas is a 6-10 inch elongated organ in the abdomen located retroperitoneally. Shape is compared to the letter J turned sideways, with loop of J around the duodenum. It is often described as having three regions: a head, body and tail. The pancreatic head abuts the second part of the duodenum while the tail extends towards the spleen. It consists of islets of Langerhans which are round, compact, and highly vascularized min-organs with scanty connective tissue. Human Islet composition is as follows (43).

- Alpha cells (20%) produce glucagon.
- Beta cells (68%) produce insulin.
- Delta cells (10%) produce somatostatin, which represses release of insulin and glucagon.

- PP cells (2%) produce pancreatic polypeptide, which stimulates secretion of gastric and intestinal enzymes and inhibits intestinal motility.

The Number and the Distribution of Islets in one Pancreas

The number of islets in a human pancreas has been determined using thorough morphometrical analysis. In a study involving seven pancreases, the total number of islets varied between 3.6 and 14.8 million (44), and the total islet volume was 0.5 to 1.3 cm^3. In that study, all islets were included, and a large number of small islets dominated the reported value. Hellman et al had previously reported similar findings in a series of publications (45, 46). In these studies, the total number of islets with a diameter of >=23 μm in a human pancreas could be estimated to be about 1.5 million. Volumetric distribution analysis showed a maximum corresponding to islets with a diameter of about 140 μm, with a remarkable degree of regularity between the pancreases examined. The average percentage (v/v) of islets in the pancreas was 1.3%, with a progressive increase from the caput to the cauda. These data were referred in Table 1-2, showing the distribution of islets and their relative contribution to the total islet volume in a normal pancreas. Islets with a diameter of 100 μm or more constituted only about 20% of the total number of islets but almost 80% of the total islet volume (47). Theoretically, about 500,000 islets with a diameter of 150 μm (the diameter of the standardized islet; IEQ) comprise the total islet volume in a normal 70g pancreas (≈1 cm^3) (47). Wittingen et al examined nine normal pancreases with concentration of islets in head, body tail (Table 1-3) (48). They counted numbers of islets in the human pancreas on a circular cross section 6mm in diameter and found no consistent relationship between the islet concentrations of the pancreas or its parts to donor age, sex, body weight or the weight of the pancreas. However, the islet concentration of the tail was significantly greater than the concentration in the head and body (48).

Part 4) Pancreatic Islet Isolation Procedure

Islet isolation is one of the most critical procedures, which remains highly, labor intensive, expensive, and relatively inconsistent. Current islet isolation methods are based on the principles described by Gray et al (49) in 1984.

The procedure of islet isolation consists of three main steps:

Pancreas distention with collagenase enzyme: Intraductal administration allows the collagenase, an enzyme from Clostridium histolyticum, to be distributed throughout the whole pancreas, which, in turn, results in acinar disruption, breakdown of the interstitial matrix and enhances islet separation. Enzyme solution is continuously perfused into the pancreas under a controlled pressure. This step is performed at cold temperatures (approx. 4°C). This controlled perfusion via the pancreatic duct allows the effective delivery of the enzyme, achieving maximal distension to all regions of the pancreas and leading to an increased recovery of islets (34).

Pancreas digestion: The digestion step comprises a mechanical dissociation (shaking of the pancreas pieces in the Ricordi chamber with steel marbles) and a chemical dissociation (enzyme activity at 37°C). The digestion is stopped by dilution with cold solutions and quenching of the enzyme with concentrated human albumin solutions. The principals behind the automated method introduced by Ricordi et al (32) are minimal physical trauma and continuous collagenase digestion of the pancreas during which islets are continually removed from the injurious action of collagenase (32).Nonetheless, the large variability in collagenase activity adds another uncertainty to the digestion phase (50).

Islet Purification: After the washing and collection of the digested tissue, the islets are physically separated in the preparation from the exocrine tissue with continuous density

gradients (1.100 and 1.019) using a refrigerated COBE 2991 cell processor. Because of the limiting factor of density gradients, namely the minimal difference of density between islets and exocrine tissue, density gradients are still the standard procedure.

Part 5) Factors Influencing Islet Isolation

One of the main goals currently in islet transplantation is the achievement of insulin independence with islets from 1 donor pancreas, and this requires a consistent, successful islet isolation (51). Although islet isolation has been progressively improved, islet yields are still highly variable. Donor selection (donor characteristics), conditions of pancreas procurement and storage, and the inconsistency of enzyme blends used for digestion are some of the parameters that contribute to this variability (52-54).

Donor Selection

Factors affecting the success of islet isolation have been extensively studied. (52, 53, 55, 56) Donor-related factors are uncontrollable, therefore careful donor selection may help to improve islet yields. Previous studies found that age more than 20 years old, body mass index (BMI) over 25 kg/m^2, no cardiac arrest, no severe hypotension, and short intensive care stay have positive impact on islet isolation outcome. Efforts are ongoing in the search of a predictive system for results of the isolation procedure (57-60).

Pancreas Procurement and Storage

Human Islet isolation outcome is critically affected by all stages that precede it. Atraumatic manipulation of the pancreas, and immediate *in situ* cooling of the pancreas have been shown to minimize both warm and cold ischaemic injury, stabilize endogenous enzyme activity, and lead to significantly improved islet yields and viability

(61, 62). A further concern that has a significant impact on islet yield is the duration of cold ischaemia, given that the donor pancreas typically requires to be transported over a long distance to islet isolation centers. It has been demonstrated that a long cold ischaemia time reduces post-transplant islet function (63). One of the most remarkable advances to overcome this concern has been the introduction of a "two-layer" cold storage method using perfluorocarbons (PFCs) and standard University of Wisconsin preservation solution(64-66). PFCs have an extremely high affinity for oxygen, which diffuses into the preserved pancreas, thereby maintaining membrane integrity and reducing ischaemic cell swelling (67, 68) The two layer method has been shown to reverse the damaging effects of warm ischaemia, increase islet yields and improve islet engraftment (69, 70).

Inconsistency of Enzyme Blends

Another important factor to determine islet isolation success would be efficacy of collagenase blends (50, 71). However, there is a remarkable variation between collagenase blends. Lakey et al. reported that there was a variation in the enzyme formulation (Figure 1-3) and in the enzymatic efficacy between lots of commercially available enzyme blends (71). To achieve effective dissociation is highly dependent on the use of stable enzyme blends of known potency and formulation. Roche Applied Science (Indianapolis, IN) recently introduced class II (CII) and class I (CI) collagenase in a separated form, providing flexibility to independently dose the components in the pancreas. In the near future, the collagenase digestion phase would be standardized. In this stage, the priority would be to determine the role of CII and CI in the human islet isolation with the multitude of pancreatic variables present. There could be different combinations of CII and CI to match the donor factors of each pancreas for isolation.

Part 6) Connective Tissue and Collagenase

Structure and Properties of Collagen

As successful human islet isolation depends upon effective separation of islets from exocrine tissue. Collagen proteins building collagen fibrils are the main components of the supporting tissue of connective tissue. The smallest integral structural unit of collagen is the tropocollagen molecule (M.W.300, 000). It consists of three parallel polypeptide chains, called a-chains, of approximately 1000 residues each. Nearly one third of the amino acids in these chains are glycine and another fifth are either proline or hydroxyproline. The composition of these chains is a consequence of their unusual primary structure in which every third residue is glycine. Thus, each a-chain is composed of a series of repeating Gly-X-Y triplets, where X and Y are most often proline and hydroxyproline, respectively (Figure 1-4). The unique sequence of tropocollagen gives rise to its unique three-dimensional structures. Each a-chain exists as a left-handed, poly-proline-type helix and the three parallel a -chains are wound around each other into a tightly coiled right-handed super helix. This triple helical conformation gives tropocollagen a rod-like tertiary structure, which is the basis for its structural role in tissue. In addition, its triple helical structure makes the molecule extremely resistant to attack by general tissue proteases. The tropocollagen triple helix melts (denatures) near 37°C, the precise temperature being determined by the type of collagen and other environmental conditions such as pH, ionic strength, etc. Melting produces a mixture of unwound, randomly coiled a-chains, referred to as gelatin, which is appreciably more susceptible to proteolysis than native triple helical collagen,

In tissue, collagen exists as fibrils in which the tropocollagen monomers are stacked head to tail and side to side to form long rows, with a one-quarter stagger

between rows. The fibrils are much more stable to thermal denaturation than tropocollagen in solution and remain intact up to about 55°C. Depending on the type and age of the tissue, variable numbers of covalent crosslinks may form between adjacent tropocollagen molecules in these fibers. Interchain cross-linking of hydroxylysyl residues stabilizes the collagen complex and makes it more insoluble and resistant to hydrolytic attack by most proteases. The repeating (Gly-X-Y) triplet has short, nonhelical regions at the carboxyl and amino termini called telopeptides. The abundance of collagen fibers and the degree of cross-linking (Figure1-5) tend to increase with advancing age, making cell isolation more difficult (72). The mechanisms of crosslink formation have been comprehensively reviewed (72, 73). Initial fibril orientation is unstable because collagen molecules associate only via noncovalent interactions in the immature fibril. Collagen molecules can slide past one another and the immature fiber is more subject to disruption by collagenolysis, variations in ionic strength, and temperature. Tensile strength and functionality of the collagen fibril are due primarily to the formation of crosslinks. Crosslinking is initiated immediately upon fibril aggregation by the oxidative deamination of specific lysine or hydroxylysine residues by the enzyme lysyl oxidase. Although there is generally an increase in mature intramuscular connective tissue crosslinks with chronological age, it is also clear that the rate of crosslink formation and directional shifts in the concentration of mature crosslinks, regardless of age, can be altered (72).

The primary component of connective tissue is collagen, a structural protein that accounts for one third of all body protein. Because of its unique triple-helical structure, collagen is highly resistant to attack under physiological conditions by all known digestive and intracellular proteinases. The only enzymes capable of hydrolyzing collagen at an appreciable rate are specific collagenases [EC 3.4.23.3], which are defined as enzymes that catalyze the hydrolytic cleavage of the undenatured, triple-

helical portion of collagen at physiological pH and temperature. The most potent known system for the degradation of connective tissue is the culture filtrate of Clostridium histolyticum, which contains a mixture of collagenases and other proteinases. There is evidence to suggest that the enzymes in this mixture act synergistically to degrade collagen (74-76).

Collagenase for Tissue Dissociation

The Mechanisms of Collagenase for Tissue Dissociation

Collagenases are enzymes that are able to cleave the peptide bonds in the triple helical collagen molecule under physiological condition(50).

Bacterial collagenase [EC 3.4.23.3] is a crude complex containing a collagenase more accurately referred to as clostridiopeptidase A which is a protease with a specificity for the X-Gly bond in the sequence Pro-X-Gly-Pro, where X is most frequently a neutral amino acid. Such sequences are often found in collagen, but only rarely in other proteins. While many proteases can hydrolyze single-stranded, denatured collagen polypeptides, collagenases are unique among proteases in their ability to attach and degrade the triple-helical native collagen fibrils commonly found in connective tissue. True collagenase may cleave simultaneously across all three chains or attack at a single strand. Mammalian collagenase split collagen in its native triple-helical conformation at a specific site yielding fragments, with ¾ and ¼ lengths of the tropocollagen molecule. After fragmentation the pieces tend to uncoil into random polypeptides and are more susceptible to attack by other protease (50).

Bacterial collagenases are usually extracted from host invasive strains. These enzymes differ from mammalian collagenase in that they attack many sites along the helix. Collagenases from Clostridium histolyticum, first prepared by Mandl et al.(77), have been most thoroughly studied. Commercially available collagenase has been

limited primarily to that from Clostridium histolyticum; although, other sources have recently become available.Clostridal collagenase also degrades the helical regions in native collagen preferentially at the X-Gly bond in the sequence Pro-X-Gly-Pro where X is most frequently a neutral amino acid. This bond in synthetic peptide substrates may also be split (78).

Purified clostridiopeptidase A alone is usually inefficient in dissociating tissues due to incomplete hydrolysis of all collagenous polypeptides and its limited activity against the high concentrations of non-collagen proteins and other macromolecules found in the extracellular matrix (75, 76). Crude collagenase is suited for tissue dissociation since it contains the enzyme required to attack native collagen in addition to the enzymes which hydrolyze the other proteins, polysaccharides and lipids in the extracellular matrix of connective and epithelial tissues (79). There are several modifying factors listed in Table.1-4. Exposure of the enzyme to divalent cation chelators removes zinc and calcium, rendering the enzyme inactive. The inhibition by EDTA is reversed by addition of excess calcium, but not by adding excess zinc(50). The optimum pH and temperature range are 7.5-8.0 and 35-37 °C, respectively. Although lower temperatures may be used, enzyme activity (and the rate of tissue dissociation) will be slowed.

Characteristics of Collagenase Enzymes

Six isoforms of collagenase [EC 3.4.24.3] acting on native collagen can be isolated from the medium of Clostridium histolyticum(80).CI has forms α,β,γ.; CII has δ,ε,ζ (Table 1-5).The two classes are immunologically crossreactive, but have significantly different sequences, and different specificities such that their actions on collagen are complementary(80). CI is less active against various synthetic peptide substrates than CII. CI has greater activity towards high molecular weight collagen, and while CI exhibits a preference for low molecular weight collagen fragments. Each of the

purified collagenase is completely homogeneous. It has been suggested that one class evolved from another by gene duplication followed by independent evolution by point mutation to yield enzymes with different substrate specificities (81). Moreover the amino acid sequence for both CI and CII collagenases have been determined(80). CI and CII collagenases are encoded by genes on ColG and ColH, respectively (82, 83).

Liberase Collagenase vs. Traditional Collagenase

Traditional collagenase preparations are concentrated from a crude and variable fermentation by-product of Clostridium histolyticum. These preparations are heterogeneous, containing as many as 30 different enzymes, cellular debris, pigments and endotoxin. High endotoxin levels and variability of enzyme activities are the most significant liabilities of traditional collagenase. As the name implies, the primary enzyme constituents are collagenases (CI and CII, as described by Bond et al (80))

Traditional collagenases are characterized by many limitations, which seriously limit their use particularly in demanding applications, such as .high endotoxin levels which is as high as 13,000 EU/ml in working concentrations of traditional collagenase. Endotoxin contamination of cell isolation reagents (84) has been suggested as responsible for the failure of isolated cells to survive (85). Compared to traditional collagenase, the endotoxin levels in working concentrations of Liberase™ enzymes average less than 10 EU/ml.

The components of traditional collagenases are highly variable from lot to lot, and manufacturer to manufacturer. Enzyme activities and concentrations are variable due to bacterial strain, culture condition, and age of the culture. Therefore, it requires time-consuming lot testing. Moreover, once a lot has been selected, there is no guarantee that the collagenase will not degrade over time. Traditional collagenase losses tissue dissociation efficacy over time regardless of storage conditions, and at varying rates (86).

Liberase™ enzymes have been most widely used for human islet isolation. Liberase enzyme technology comprises the methods (87, 88) for purifying Clostridial collagenase isoforms (CI and CII collagenases) to high specific activity, and for blending them together with neutral protease in optimal ratios for effective dissociation of the pancreas. Roche starts with a traditional collagenase, which is manufactured to have a specific activity as much as 3-5 times higher than most other traditional collagenases and screens their collagenase raw material by High Performance Liquid Chromatography (HPLC) (Figure 1-6) to ensure that the collagenase isoforms in that product are intact. Then the raw material collagenase is purified over a series of three columns, which remove pigment, endotoxin, unwanted cellular debris and unnecessary enzyme activities. Finally Roche collects highly purified (>95% homogeneity) collagenase CI and CII collagenases. These two collagenase isoforms are blended in a precise ratio with each other, and with a non-Clostridial neutral protease, thermolysin (MW 34KDa). Thermolysin is purified from the fermentation of Bacillus thermoproteolyticus. All these procedures ensure that Liberase™ enzyme has consistently low levels of endotoxin and toxicity (89).

Enzyme Handling

Liberase™ enzymes are white lyophilizates consisting of aseptically filled, blended enzymes, and a small quantity of buffer salts (90).

Lyophilized proteins tend to be very hygroscopic so they should not be opened in humid areas. It is recommended that any vial be brought to room temperature before opening. Ideally, the vials should be taken from the refrigerator at least a half hour before opening and they should be left in a desiccator.

Once diluted with medium or buffer, proteolytic enzymes can undergo autolysis. Therefore it is recommended that enzyme should be dissolved immediately before use

and stored at 2-8^{O}C once they are reconstituted. If necessary reconstituted enzyme can be aliquoted and frozen at -20^{O}C. Repeated freeze-thaw cycles should be avoided (79).

Although Liberase^{TM} enzyme products are filtered through a 0.2 μm filter, then dispensed and lyophilized under aseptic condition, the sterility of the lyophilized preparations is not verified as part of Roche quality control (90). In order to guarantee sterility of the solution, it should be filtered through either cellulose acetate or low-protein-binding polysulfone membranes of 0.22-μm porosity. It is not recommended to use nylon or nitrocellulose membrane filters (90). Over a period of time, enzymes will be deactivated even at moderate temperatures (79). Storage of enzymes at 5^{O}C or below is generally the most suitable. Some enzymes lose their activity when frozen (79).

Part 7) Purpose of this Study

It can be seen that the collagenase digestion phase is a complex process with many interrelated variables, especially the efficacy of collagenase blends. There is marked variation of the ratio of the different component in the commercially available collagenase blends. A better understanding of the characteristics and specific activities of each component of the collagenase blends for effective release of islets from ECM will help improve efficacy of collagenase blend performance. The overall hypothesis states that increasing the proportion of CI collagenase in current commercially available collagenase blends may yield greater number of intact islets for transplantation. This study will retrospectively examine the samples of the commercially available collagenase blend used in the human islet isolation.

The aims of this thesis are therefore:

I. To identify inter-lot variations in collagenase blend enzyme components
II. To investigate the correlation between the ratios of the components of collagenase blend enzyme and islet isolation outcome.

- Sufficient number of high-quality pancreatic islets
    - Refined pancreas procurement techniques
    - Improved enzyme digestion and purification process
    - Multiple donors as needed for adequate islet-cell mass

    (approximately 12 000 IE/kg islet mass, based on the recipient body weight)
- Recipient selection
    - Brittle type 1 diabetic patients
    - Normal kidney function, no severe cardiovascular disease
    - No insulin resistance and moderate insulin requirements (< 0.7 U/kg)
- Tailored immunosuppression
    - Sirolimus based and steroid free
    - Induction with anti-IL2 receptor antibody
    - Low-dose calcineurin inhibitor

Table 1-1. Key points for the success of the Edmonton Protocol.
(Modified from Oberholzer J, Shapiro AM, Lakey JR, Ryan EA, Rajotte RV, Korbutt GS, Morel P, Kneteman NM Current status of islet cell transplantation. Adv Surg 2003; 37:253-82)

Islet diameter(μm)	Number of islets	% of total number of islets	% of total islet volume
< 50	782,000	52%	5.4
50-100	416,000	28%	17
101-150	203,000	14%	30
151-200	54,000	3.6%	26
201-250	20,000	1.3%	15
>250	3,000	0.2%	6.3

Table 1-2. The human endocrine pancreas.
(From: Korsgren: Transplantation, Volume 79(10).May 27, 2005.1289-1293)

	Mean	Standard deviation
Head	25.54	9.89
Body	28.07	8.80
Tail	45.70	17.04

Table 1-3. Islet concentration in the different region of the pancreas.
(Modified from Wittingen J, Frey CF. Islet concentration in the head, body, tail and uncinate process of the pancreas Ann Surg 1974; 179(4):412-414)

Modification	Factor
Inhibitors	0.1M EDTA Cysteine Mercaptoethanol Protease inhibitors Serum Albumin
Stabilizers	Calcium
Cofactors	Zinc,calcium

Table 1-4. Modifying factors on collagenase enzyme.
(From Roche Liberase Instruction Manual)

	Mr	Isoelectric point
α	68,000	5.85,5.90
β	115,000	5.55,5.60,5.75
γ	79,000	6.10,6.20
δ	100,000	5.80
ε	110,000	5.90,5.95
ζ	125,000	5.35

Table 1-5. Physicochemical properties of Clostridium histolyticum collagenase.
(Modified from Bond MD, Van Wart HE. Characterization of the individual collagenase from Clostridium histolyticum. Biochemistry 1984;23(13):3085-3091.)

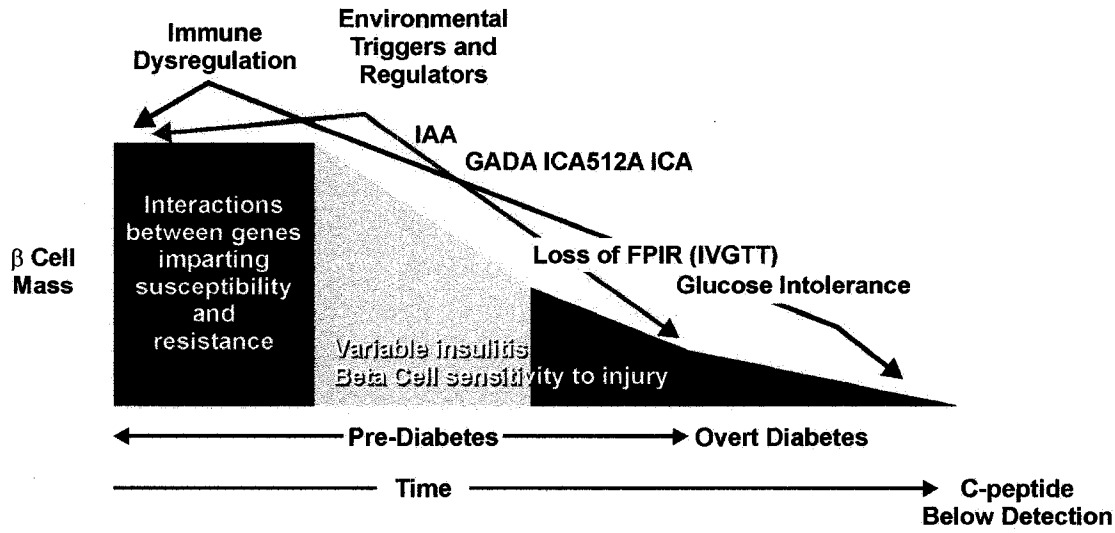


Figure 1-1. Model of the pathogenesis and natural history of type 1 diabetes.
The modern model expands and updates the traditional model by inclusion of information gained through an improved understanding of the roles for genetics, immunology, and environment in the natural history of Type 1 diabetes., first phase insulin response (FPIR); glutamic acid decarboxylase autoantibodies; (GAD), insulin autoantibodies (IAA); islet cell autoantibodies (ICA); ICA 512, autoantibodies against the islet tyrosine phosphatase; intravenous glucose tolerance test (IVGTT). (From Haller MJ, Atkinson MA, Schatz D Type 1 diabetes mellitus: etiology, presentation, and management Pediatr Clin North Am. 2005 Dec;52(6):1553-78).

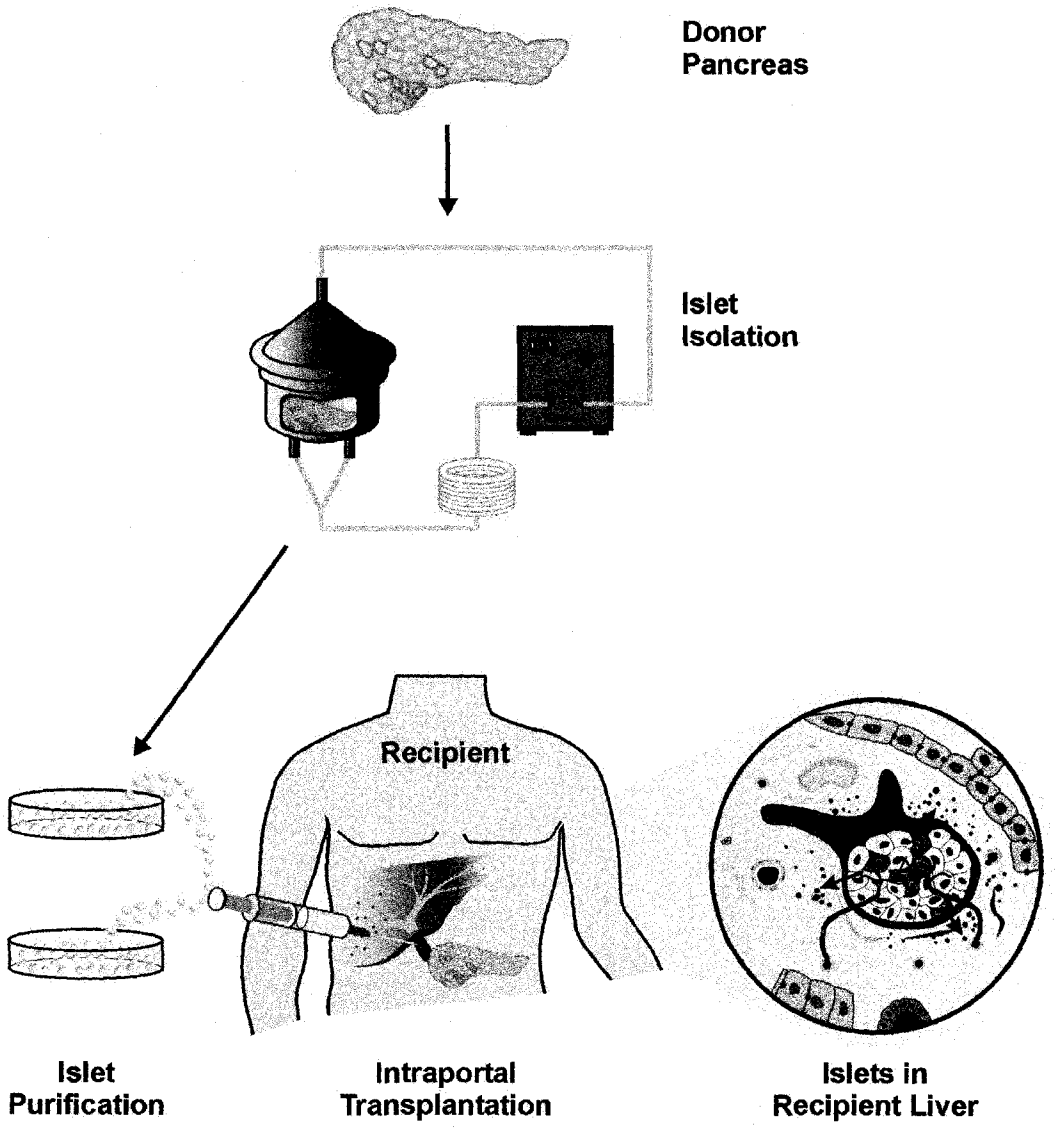


Figure 1-2. Islet transplantation.
The steps involved in the preparation of islets from donor pancreas organs through to implantation into the portal vein of patients with Type 1 diabetes. (From Nanji SA; Shapiro AMJ Advances in pancreatic islet transplantation in humans Diabetes, Obesity and Metabolism, 8(1), 15-25).

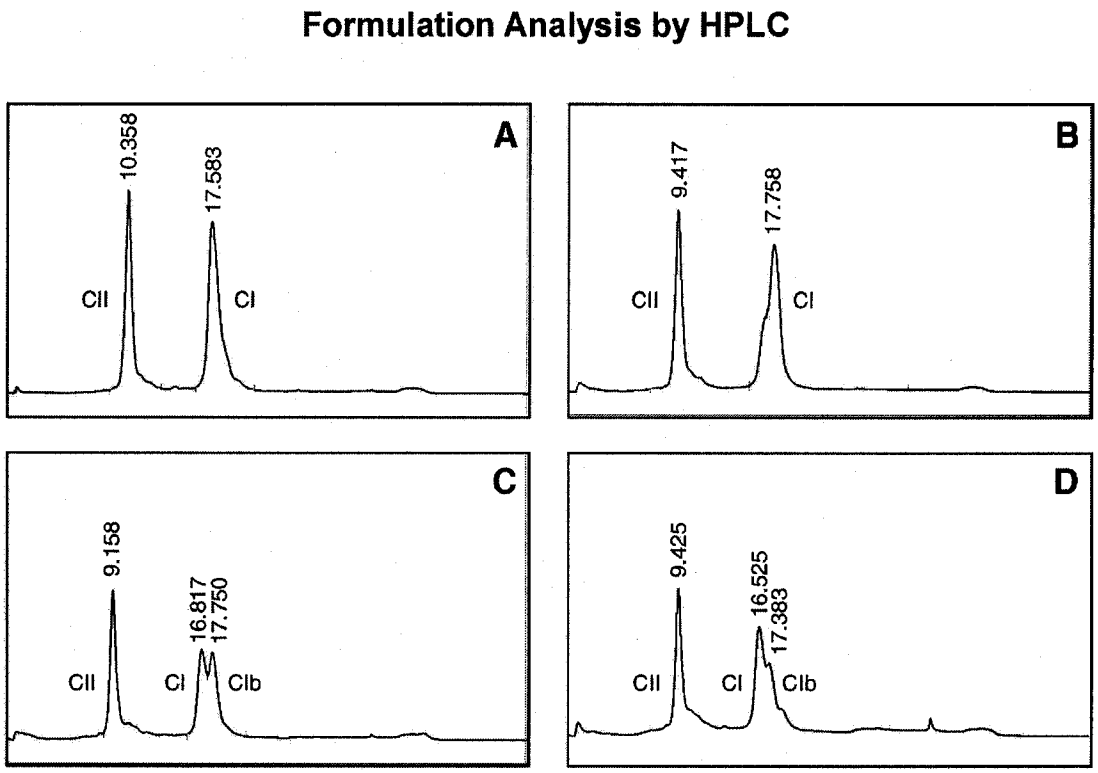


Figure 1-3. Chomatograms of Liberase™ HI. (A) Lot 93096820 (B) Lot 93096820 with a peak shoulder. (C-D) Lot 93131620 and 93134620 had distinct proportions of CIb.
(From Barnett MJ, Zhai X, LeGatt DF, Cheng SB, Shapiro AM, Lakey JR. Quantitative assessment of collagenase blends for human islet isolation. Transplantation 2005; 80(6):723-728)

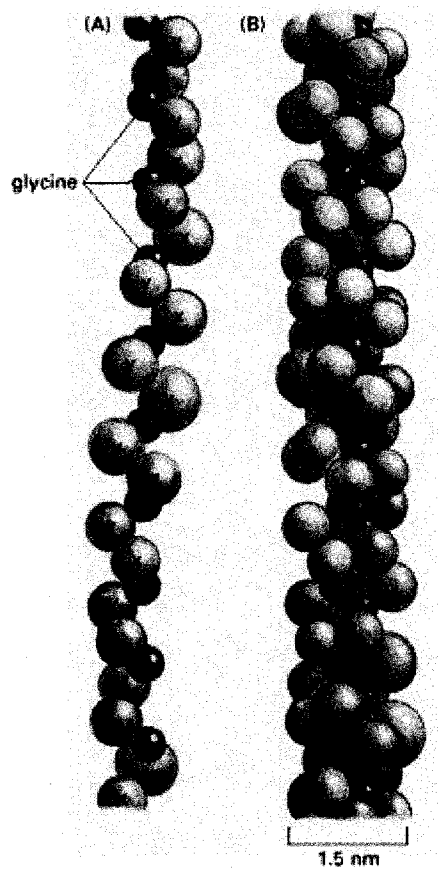


Figure 1-4. The structure of a typical collagen molecule. (A) A model of part of a single collagen chain in which each amino acid is represented by a sphere. The chain is about 1000 amino acids long. It is arranged as a left-handed helix, with three amino acids per turn and with glycine as every third amino acid. Therefore, a chain is composed of a series of triplet Gly-X-Y sequences, in which X and Y can be any amino acid (although X is commonly proline and Y is commonly hydroxyproline). (B) A model of part of a collagen molecule in which three chains, each shown in a different color, are wrapped around one another to form a triple-stranded helical rod. Glycine is the only amino acid small enough to occupy the crowded interior of the triple helix. Only a short length of the molecule is shown; the entire molecule is 300 nm long. (From model by B.L. Trus in the Molecular Biology of the Cell, 4th edition)

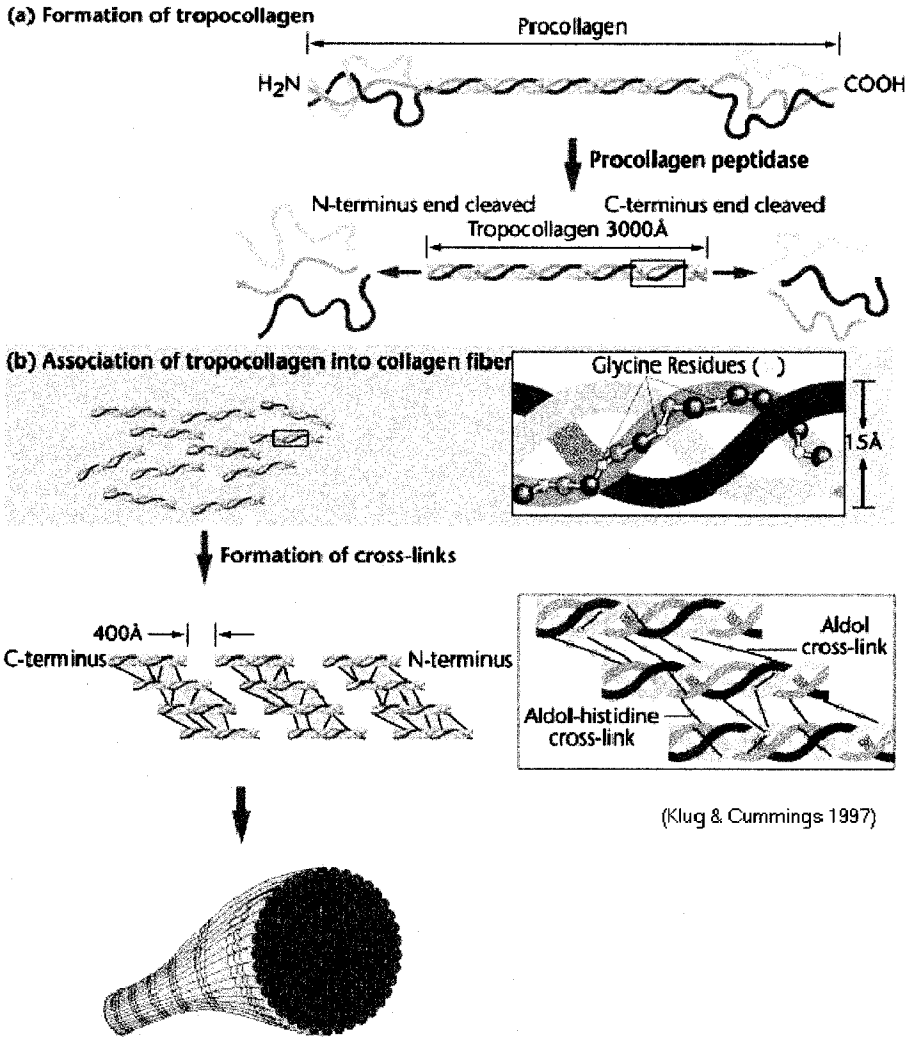


Figure 1-5. The diagram of formulation of collagen fiber.

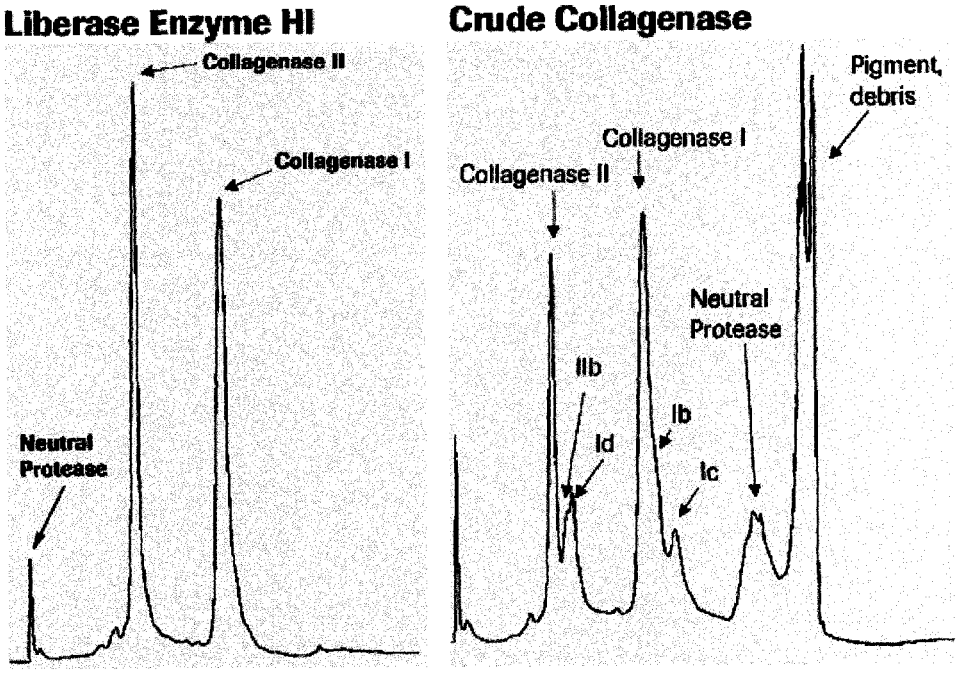


Figure 1-6. HPLC profile comparison between LiberaseTM enzyme and traditional enzyme.
(Source from Roche Applied Science)

Chapter Two

Materials and Methods

Enzyme Sample collection

The enzyme vial was removed from storage at -80°C. It was allowed to equilibrate to ambient temperature. To begin the reconstitution process, Hanks Balanced Salt Solution (HBSS) is transferred from a vial and poured into a sterile bottle. To minimize the enzyme loss, the vial is rinsed several times with HBSS. The resulting solution in combination with the initial transfer is prepared to a final volume of 350 ml which constitutes the perfusion solution. The perfusion solution is put on ice for subsequent human islet isolation. The samples were taken from perfusion solution samples for HPLC analysis and collagenase, protease activity analysis and total protein measurement.

Mono Q HPLC Analysis

Solutions

Buffer A: 20mM Tris, 1mM calcium chloride,pH 7.5:.

Buffer B: 20mM Tris, 1mM calcium chloride and 1.0 M NaCl,pH 7.50

Both buffers filter through a 0.45 micron filter and degas under vacuum for at least 10 minutes.

Procedures

To setup HPLC, a MonoQ column (Amersham Biosciences, Uppsala, Sweden) is connected to the Beckman System Gold HPLC (Beckman coulter,Fullerton,CA). The column is a strong anion exchanger with particle size of 10μm and bed volume of 1ml. The HPLC pump is primed by flushing it with high purity water. The HPLC system is

initialized with wavelength set at 280nm, flow rate at 1.5 mL/min and pressure upper limit at 1.5 kpsi.

To prime the system for analysis, 10 mL of water is allowed to flow through the pump. To monitor for any leaks or bubbles, the system is allowed to run at 1.5 mL /min with 50% Buffer B. Prior to equilibration, the pump is flushed at 3.0 mL/min with 15 mL of running buffer with 50% of Buffer B for 3 minutes. Subsequently, the column is equilibrated with Buffer A at 1.5 mL/min for 40 minutes.

For the maintenance purpose, the pump volumes and pump pressure would be checked and recorded on the daily maintenance log. Room temperature would be maintained between 21-24°C.

The system gradients are linear changes, not step (Figure 2-1). Roche Applied Science provided the background method for separating component Class I (CI) and Class II (CII) from Liberase™ enzymes (71).

Sample Analysis

A Liberase™ HI control pool sample is always run at the beginning of the analysis to ensure the column has been properly equilibrated. The control is evaluated qualitatively for characteristic peak signature. Samples are prepared from powder dissolved in Buffer A, or clinical samples diluted 1/5 with Buffer A. A minimum volume of 800 µL is required in all autosampler vials. For multiple samples, create a Sequence, and submit after the control is verified

The final sample is HPLC Water. This serves to flush the autosampler needle prior to shutdown of the system

SC 100 Fractionator system (Beckman Coulter, Fullerton,CA) is on, set to the fraction program (Table 2-1) for CII and CI collection separately. There is a total of 6 mL collected in the CI and CIb peak range and 4 mL collected in CII peak range. The

sample collections are based on HPLC profiles. Injection volume for all samples is 499 µL.

Definition of peaks

Peak	Elution time
CII peak	8 to 10 minutes
CI peak	15 to 20minutes
CIb peak	2 to 3 minutes after CI peak

Since there are lot to lot differences in column and day to day differences in buffers and temperatures, it is to be expected that the exact location of peaks will vary by day. CII peak is the largest peak in the specified region and the CI peak is the first peak in the specified region. The CI and CIb could not be separated completely and could not be achieved by minor adjusting the flow rate and gradient protocol at this point. Our difficultly in achieving full separation of CI and CIb peaks has been encountered in previous studies, due to the very similar physical properties of these isoforms (91).

Calculations

Calculate the ratio of CI to total collagenase:

$$\frac{\text{Peak area of CI}}{\text{Peak area CI (+CIb*) + peak area of CII}}$$

Calculate the ratio of CII to total collagenase:

$$\frac{\text{Peak area of CII}}{\text{Peak area CI (+CIb*) + peak area of CII}}$$

Calculate the ratio of CIb to total collagenase:

$$\frac{\text{Peak area of CIb*}}{\text{Peak area CI (+CIb*) + peak area of CII}}$$

*Total peak area includes CIb as well if it appears in the HPLC profile.

System Shutdown

The system would shut down after flushing it with HPLC water for 30min.

Collagenase kinetic assay

This kinetic enzyme assay (Molecular Probes E-12055) will quantitate the activity of collagenase type I and II. The assay uses fluorescein conjugated gelatin (denatured collagen type I), with the increase in fluorescent signal measured by standard FITC filtersets (485 nm excitation, 530 nm emission). The cleavage of collagen (gelatin) by collagenase releases highly quenched fluorescein, with the increase in signal directly proportional to the mean velocity of the reaction with excess substrate zero-order kinetics.

This system has been optimized to a 26 min reaction time, and interpolation of activity within a linear range of 0.08125 – 1.0 Collagenase Degradation Units (CDU)/ mL. The assay uses a commercially supplied standard, and the unit of measure has been validated against Collagenase I-S and II-S (Sigma) of known CDU activity.

Considerations for use:

The CDU measurement does reflect combined activity from the CI and CII isoforms. We have observed (and confirmed by third-party investigation), that destabilization in the CI isoform does produce artifact in the total collagenase result. Under conditions of induced denaturation of CI and CIb, there can appear to be an intermediate increase in activity, even above baseline. The imbalance in substrate specificity in a collagenase blend where intact CII competes with destabilized CI isoforms is reflected in an overall increase in CDU activity until CII is itself destabilized in those same conditions. In that case, the overall CDU activity will decrease to reflect denaturation in all isoform populations. Confirmation of isoform mass stability by HPLC,

and CII and CI specific activity is necessary to discern when this artifact is present. For this reason, the CII and CI components have been separated to avoid cross reaction for enzyme activity assay.

This assay shows a strong substrate specificity of CI preference over CII. This observation confirmed the finding described by Van Wart et al. (80).

Preparation of Reagents

Allow buffer and substrate to reach room temperature (RT) before reconstituted. The DQ® Gelatin substrate tube must be PROTECTED FROM LIGHT.

1x Reaction Buffer

1.0 mL of stock 10x reaction buffer from the Molecular Probes E-12055 kit is mixed with 99 mL of HPLC water and labeled: "Collagenase Assay 1x Reaction Buffer, Lot#, date of preparation, and initials". This tube is stored at RT between assays to facilitate stable reaction temperatures.

DQ® Gelatin Substrate

1000 µL of HPLC water and 10 µL of 200mM sodium azide (Caution: Toxic) solution as a preservative are added to the substrate tube. The mixture is allowed to sit for 30 min and inverted several times in the dark to maximize reconstitution. The mixture is inverted once again prior to usage. Note the date prepared on the tube.

Preparation of Standards

Thaw the Collagenase Standard from the kit 1 hour before reconstitution. The vial is 500 units total by mass. 1000 µL of HPLC water is transferred into the tube, and let reconstitute for 1 hour before aliquotting. The resultant concentration is 500 CDU/mL.

And the aliquots of 50 µL of stock standard would be stored at -80°C. Record the required information on the Master List.

Dilution of Standards

Prepare the working standards as follows:

- **1.0 CDU/mL**

  998 µL of 1X buffer + 2 µL of stock standard

- **0.5 CDU/mL**

  500 µL of 1X buffer + 500 µL of "1.0" standard.

- **0.25 CDU/mL**

  500 µL of 1X buffer + 500 µL of "0.5" standard.

- **0.125 CDU/mL**

  500 µL of 1X buffer + 500 µL of "0.25" standard

Standards are run with every assay. Standards are run in duplicate.

Preparation of the Control

Control material is prepared as a 1 mg/mL solution in 1x Reaction Buffer of Sigma Collagenase II-S (cat# C-1764) or Sigma Collagenase I-s (cat# 1639) of known CDU activity. Sample for total protein is used to trend the mass. Record lot#, expiry, and assay value on the master list. Aliquot in 50 µL portions, and store at –80˚C.The control is diluted 1/1000 prior to loading of the plate, in duplicate. Stability of standards and controls is generally limited to 8 weeks as frozen aliquots.

Preparation of Samples

Samples are diluted in 1X reaction buffer. Samples are diluted as follows:

- 1 mg/mL stock samples are diluted 1/10,000

- Total Collagenase from human digests is diluted to a total of 1/10,000, with any previous dilution factors taken into account.
- CI/CIb fractions are diluted 1/100, with any results outside of the linear range to be appropriately diluted, and repeated.
- CII fractions are diluted 1/50, with any results outside of the linear range to be appropriately diluted, and repeated.

Samples are run in duplicate.

Preparing the Plate

80µL of 1x reaction Buffer is transferred into each well first and then 100µL of all blanks, standards, control and samples and add 20 µL of substrate to each well at last. The plate is mixed well, and placed in the FLX800 fluorometer for 26 min kinetic measurement.

Completing Run and Interpretation of Results

The R^2 value must be greater than 0.97. % coefficient of variation (CV) for standards must not exceed 10%, with exception of the 0.125 standard, which may have a %CV of up to 15%. The control must be within range for the run to be valid, with a %CV of less than 10%. A failure of either of these conditions invalidates the run, and it would be repeated with new working standards and control. Samples must have a %CV less than 15%, or that individual sample must be repeated on a future run. All samples with results outside the assay range would be repeated with an appropriate dilution.

Calculations

Record the control value on the quality control (QC) log. Record any corrective actions required.

Samples values are multiplied by the dilution factor (DF).

Protease kinetic assay

This kinetic enzyme assay (Molecular Probes E-6638) will quantitate the activity of proteases, namely the serine and neutral (acid) proteases. The representative enzymes from these groups are Trypsin, and Thermolysin, respectively. The assay uses Bodipy conjugated casein, with the increase in fluorescent signal measured by standard FITC filter sets (485 nm excitation, 530 nm emission). The cleavage of casein by the protease releases highly quenched Bodipy, with the increase in signal directly proportional to the Mean V of the reaction with excess substrate zero-order kinetics.

This system has been optimized to a 30 min reaction time, with a standardized mass normalization of a linear range of 0.005 – 0.02 µg/mL for Sigma Thermolysin cat# T7902. The corresponding specific activity of the standard protease solution of 1 mg/mL provides the absolute activity value in units/volume. As each pool of thermolysin standard is made, the actual activity based on the protein mass and certificate activity value from the manufacturer is entered into the assay platemap in order to derive the standard curve. The standard dilutions are DF 50, DF100, and DF 200 in triplicate.

Preparation of Reagents

Allow buffer and substrate to reach RT before reconstitution. The Bodipy casein substrate tube must be protected from light.

1x Reaction Buffer

Take 5 mL of stock 20x reaction buffer from the kit. and fill up to 100 mL with HPLC water and labeled: "Protease Assay 1x Reaction Buffer, Lot#, date of preparation, and initials". This tube is stored at RT between assays to facilitate stable reaction temperatures.

Bodipy-Casein Substrate

Add 200 µL of 1X buffer to the substrate tube. Allow to reconstitute for 30 min in the dark after inverting several times and mix it again prior to use.

Take 19.0 mL of 1X buffer into a 50 mL conical labeled "Protease Assay Substrate, Lot#, date of preparation". Transfer the 200 µL of substrate into the conical tube, followed by 2x400 µL rinses with 1X buffer to a final volume of 20 mL.

Preparation of Standards

Protease standards are prepared as 1.0 mg/mL solutions in 1X Buffer. Take sample for total protein assay. Multiply the total protein result in mg/mL by the activity stated on the Sigma vial in units per mg protein. Divide this number by the corresponding dilution factors of 50,100,200 in order to enter in the actual activity values for the pool in KC4 software. Aliquot 1mg/mL solution in 50 µL each and then store aliquots at –80^{O}C. Record the required information on the Master List.

Dilution of Working Standards (std)

Prepare the working standards as follows:

- **0.02 mg/mL**

  784 µL of 1X buffer + 16µ L of stock 1 mg/mL std.

- **0.01mg/mL**

  400 µL of 1X buffer + 400 µL of "0.02" std.

- **0.005 mg/mL**

  400 µL of 1X buffer + 400 µL of "0.01" std.

Standards are run with every assay. Standards are run in triplicate.

Preparation of the Control

Control material is prepared as a 1 mg/mL solution in phosphate buffered saline (PBS) of Serva Thermolysin (powder). Prepare 10 mg in a conical tube, adding 10 mL of 1X buffer. Verify total protein for sample. Record lot#, expiry, and assay value. Aliquot it in 300 µL each, and store at –80˚C. One control is used straight for each run in duplicate.

Preparation of Samples

Samples are diluted in 1X buffer. Samples are diluted as follows:

- 380 µL of 1X buffer + 20 µL of sample for a dilution factor of 20. This is used for "Pre".

Samples are run in duplicate.

Preparing the Plate

100 µL of 1X Buffer is put in the "Blanks". 100 µL of samples are added in the plate. Then 100 µL of substrate is added to each well. Mix it well, and place in the FLX800 fluorometer for 30 min kinetic measurement.

Completing Run and Interpretation of Results

The R^2 value should be greater than 0.97. %CV for standards must not exceed 10%.The control must be within range for the run to be valid, with a %CV of less than 10%. A failure of either of these conditions invalidates the run, and it would be repeated with a new standard and control. Samples must have a %CV less than 15%, otherwise it would be repeated on a future run. All samples with results outside the assay range must be repeated with an appropriate dilution

Calculations

Record the control value on the QC log. Record any corrective actions required.

The final result is the assay value x DF.

BCA Protein Assay

Protein measurement uses a Micro bicinchoninic acid (BCA) protein Assay (Pierce, Rochford, IL). This assay is a detergent-compatible formulation based on bicinchoninic acid for the colorimetric detection and quantitation of total protein. This method combines the well-known reduction of Cu^{2+} to Cu^{1+} by protein in an alkaline medium (the biuret reaction) with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu^{1+}) using a unique reagent containing bicinchonic acid

Preparation of Reagent

Calculate # wells required. All samples, controls, and standards are run in duplicate. The working reagent total volume required for the run would be calculated by the numbers of wells multiplied 150 µL plus 500 µL to account for dead volume. It is mixed by reagent A (0.50 x of total volume), reagent B (0.48 x total volume) and reagent C (0.02 x of total volume) together.

Preparation of Standards

Prepare the standards from the 2 mg/mL stock diluted with 1x PBS as follows:

- **(200 µg/mL)**

  Take 100 µL of stock standard from ampule (BCA kit) + 900µL of PBS

- **(100 µg/mL)**

  Take 500 µL of the "200" std + 500 µL of PBS

- **(50 µg/mL)**

Take 500 µL of the "100" std + 500 µL of PBS

- **(10 µg/mL)**

  Take 100 µL of the "50" std + 400 µL of PBS

Preparation of the Control

Human Serum Albumin (HSA) Control is prepared from a single vial of 25% HSA by performing a 10,000-fold dilution with PBS. Prepare 20 mL of control solution by adding 2 µL of human albumin to 19,898 µL of PBS. Then add 100 µL of 200mM sodium azide solution. The lot #, expiry, and date of preparation would be recorded on the QC Production Log. the aliquots of 500 µL would be stored at 4°C. Record the required information on the Master List.

Preparation of Samples

Samples are diluted 1:20 in PBS pH 7.4 to a degree appropriate for the assay range (0 – 200 µg/mL).

Preparation of the Plate

The blanks are PBS only. 150 µL of all stds, controls, and samples in duplicate, and then add 150 µL of reagent into each well.Mix the plate well on a rotator at medium-low speed "3" for 30 sec. After mixing, incubate the plate at 37 °C for 2 hours.

Completing Run and Interpretation of Results

The R^2 value must be greater than 0.99.The %CV for standards must be below 5%, with 10% maximum for 1 standard only (the lowest std). The control must be within the posted range. Any failure of the above requires a repeat run to be performed.

Samples should have a %CV of less than 10%, with > 15% requiring a re-run for that sample. Any samples above the linear range would be diluted and repeated.

Filtration Study

0.2-μm pore-size membrane sterile filtration is the method of choice used for sterilizing enzyme preparation before use. Four common types of filter membrane with low-binding of protein have been used in this study. They were polyethersulfone (PES) (Millipore), polyvinylidene-fluoride (PVDF) (Millipore), nylon (Fisher) and surfactant-free cellulose acetate (SFCA) (Corning).Stock solution of Liberase ™ HI was prepared at a concentration of 1.0mg/mL. A total of 20 mL of the solution was passed through each of the following 0.2-μm pore-size membranes: SFCA, Nylon, PES, or PVDF. Pre- and post-filtration samples were collected and analyzed by High Performance Liquid Chromatography, and for collagenase and protease activities, and protein concentration. The samples were also separated into CI and CII collagenase components using an anion exchange column.

Statistical Analysis

Data were analyzed using SPSS for Windows ™ (SPSS Inc., Chicago, Illinois).All results were expressed as mean± standard error (SE) unless otherwise stated. Differences between means of different groups were compared using one-way analysis of variance (ANOVA) with post hoc analysis by the Bonferroni test, and an unpaired, two-tailed Student-t test was utilized where appropriate. Correlation was analyzed using Pearson's bivariate test. Receiver operating characteristic (ROC) curve was applied to determine cutoff point of variable (at which the sum of the sensitivity and specificity values is highest) for distinguishing between successful and unsuccessful isolations. Over 300,000 IEQs[1] (islet equivalent based on a mean diameter of 150 μm) after purification was considered as successful isolation. Odds ratios for factors predicting

[1] Dithzone-stained samples were evaluated in duplicate to determine islet yield. Islets were counted and sized to normalized yields of islet equivalent to 150 μm under the microscope.

successful isolation outcome were estimated using univariate logistic regression analysis. P-values < 0.05 were considered statistically significant.

Type	Window peak
Fraction by time	1min
Flow delay	0.02
Peak detector	1V
Peak threshold	1%
Peak width	4 mins
Window 1 start	9:00
Window 1 end	12:00
Window 2 start	16:00
Window 2 end	20:00
Window 3 start	Off
Non-peak/window	Divent
Restart	Inject, skip tube

Table 2-1. Fraction program for CII and CI.

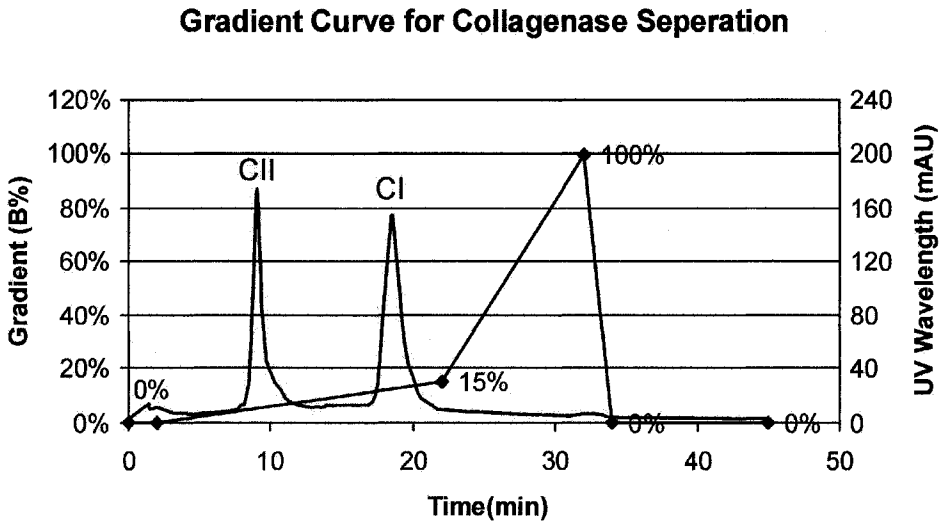


Figure 2-1. HPLC gradient curve for collagenase separation.

Chapter Three

Results

Effect of Neutral Protease (NP) and Wunsch (WU) on the outcome of Islet isolation

Roche Applied Science provides certificate of analysis (CofA) of enzyme product, namely collagenase activity expressed as Wunsch units (WU)/vial and thermolysin (neutral protease) activity expressed as caseinase units (NP)/vial. First of all, we investigated if these values are useful to predict islet isolation outcome. We analyzed 251 human islet isolation where11 different lots were used (Table 3-1).

When thermolysin activity per gram pancreas was categorized into quartiles, the mean post-purification islet yield showed an inverse U-shaped curve, with lower islet yield in the 1st and 4th quartiles; both low and high doses of thermolysin resulted in poor yields (Figure 3-1A). The similar inverted U-shaped curve was seen in relation to collagenase activity per gram pancreas with post-purification islet yield; a decrease in islet yield was found with both lower and higher dosage of collagenase (Figure 3-1B).

ROC curves were constructed on the thermolysin activity per g pancreas to determine the optimal cutoff value for distinguishing between successful and unsuccessful isolation outcome, with pooled data of the 1st and 2nd quartiles for a lower point and with pooled data of the 3rd and 4th quartiles for an upper point. From these analyses, we obtained a lower and upper cutoff point of 624.3 and 987.9 NP/g pancreas, respectively. Thus, we selected the optimal range of thermolysin between 624.3 and 987.9 NP/g pancreas. There were 127 cases within this range, 124 outside of this range. Post-purification islet yield was significantly higher in cases within the optimal dosage

than in those outside of the range (266,243±15,998 IE vs. 188,491±12,245 IE, P<0.001). The odds of successful isolation were 3.45-fold higher in optimal dosage cases than in suboptimal cases (95% confidence interval 1.82-6.56, P=0.0001).

Similarly, the optimal cutoff values of collagenase dosage were determined using ROC curves on the subgroup of the 1st and 2nd quartiles for a lower point, and on the subgroup of the 3rd and 4th quartiles for an upper point. From these analyses, we obtained a lower and upper cutoff point of 21.0 and 32.3 WU/g pancreas, respectively. Thus, we selected the optimal range of collagenase, being 21.0-32.3 WU/g pancreas. There were 135 cases within this range, 116 outside of this range.

Figure 3-2 shows the rate of successful isolation stratified by both thermolysin and collagenase dosage ranges. The result (35.7%) obtained in cases with optimal dosage in thermolysin and over dosage in collagenase, while the result (0%) observed at the combination of under dosage of thermolysin and over dosage of collagenase. When the optimal dosage of thermolysin was employed for isolation, the successful rate was always greater than 30% regardless of collagenase dosage range. When both thermolysin and collagenase dosages were entered into a logistic regression equation with successful isolation as the dependent variable, only thermolysin dosage was retained as a significant predictor of successful isolation (P=0.002); collagenase dosage was not a significant indicator (P=0.763).

These results indicate that collagenase activity measured by the manufacture is not a major determinant for islet isolation outcome.

Since neutral protease to collagenase activity ratio has been suggested playing an important role in islet isolation (92), we assessed NP/WU ratio for each lot. The mean of NP/WU ratio for each lot varied from 15.2 to 52.6 (Figure 3-5A). Figure 3-5B showed success rate of islet isolation in each lot of enzyme product. The rate of successful isolation fluctuated between 0% and 75%. However, NP/WU ratio did not explain this

fluctuation: there was no correlation observed between NP/WU ratio and post-purification islet yield (r=0.05, P=0.46).

These results emphasize a need for a more sensitive and reliable indicator of enzyme product performance. The followings are results from our own assay.

Variability of HPLC Profile of Enzyme Products

First, seven different lots were assessed by Mono-Q HPLC method. There were two major peaks identified after injecting the samples in the column. The peaks and retention time have been confirmed by the manufacturer (Roche). Based on the elution order, CII, CI and CIb appeared in the chromatograms (Figure 3-3). The index of each peak area to the total area was calculated to demonstrate differences in their proportion to the blend in each of these seven lots (Figure 3-4A and Table 3-2). CII peak area index was fairly consistent among lots: the mean CII peak area index of each lot ranged from 0.37 to 0.44. CIb substantially existed in five of seven lots but was barely detectable in the remaining two lots. This resulted in a considerable variation in both CIb and CI peak area indexes among lots comparing to CII area index: the mean CIb and CI indexes ranged from 0.01 to 0.36 and from 0.25 to 0.62, respectively.

We assessed HPLC Area indexes of enzyme components regarding to predicting isolation outcome. Table 3-2 showed there was no significant difference in these indexes between successful group and unsuccessful group. There was no significant correlation observed between CII, CI or CIb index and post-purification islet yield (r=-.0.06, 0.101,-0.06 and P=0.53, 0.31, 0.50 respectively).

Variability of Activity of Enzyme Products

There was a possibility that the lyophilized enzyme pellets were not homogeneous, CII and CI fractions of these seven lots have been collected for

collagenase assay as well. After the separation, we were able to determine the activity of each component without cross reactivity between CII and CI. The figure 3-4B and Table 3-2 showed that CII specific activity and CI specific activity have significant difference among these seven lots (P<0.001). Mean of CII specific activity was 1.22 ± 0.04, ranging from 0.54 to 2.69. Mean of CI specific activity was 5.83 ± 0.11, ranging from 3.36 to 8.42.

Next, we investigated if there were any differences in potency between these two fractions and if any significant difference could be correlated to IE yield. Table 3-5 showed that the mean CI specific activity for the successful vs. unsuccessful groups was significant by using a two-tailed unpaired-t test (5.43±0.24 CDU/μg 6.09±0.13, respectively, P=0.015). In contrast, the mean CII specific activity for the successful vs. unsuccessful yield group was not significantly different (1.13±0.07CDU/μg vs.1.28±0.05, respectively, P= 0.119). These results suggest that CI specific activity has played a significant role on islet isolation outcome.

Effect of CII/CI activity ratio on the outcome of Islet Isolation

Next, we assessed the CII/CI collagenase activity ratio from each vial used in the latest 133 isolations. The mean of CII/CI activity ratio of each lot varied from 0.148 to 0.237 and this variability was likely to explain the fluctuation in success rate. Figure 3-6A-B showed success rate of isolation and CII/CI ratio for each lot used in the latest 133 isolations; higher CII/CI ratio resulted in lower success rate. Furthermore, CII/CI ratio was weakly but significantly inversely related to the post-purification islet yield (r=-0.21, P=0.04). We obtained a cutoff point of 0.204 in CII/CI from the ROC curve for distinguishing between successful and unsuccessful isolation outcome. The odds of successful isolation was 8.67 times higher when a vial with CII/CI ratio<0.204 was used than when a vial with CII/CI ratio>=0.204 was used (95%CI 1.93-38.93, P=0.005). A significant increase in islet recovery was, therefore, observed when a vial with

CII/CI<0.204 was used (249,782±16,020 IEQs vs. 156,209± 20,833IEQs, P=0.001). These results suggest that CII/CI ratio may be a useful marker to determine "bad" vs. "good" lot.

Correlation Between Digestion Time and Islet Equivalent Yield

It has been suggested that long digestion time has an adverse effect on the islet yield. The digestion time must be minimized. Digestion time was negatively associated with islet yield (r=-0.360, P<0.001). We obtained a cutoff point of 12.91 in digestion time for distinguishing between successful and unsuccessful isolation. The odds of successful isolation was 5.28 times higher when DT<=12.91 than DT>12.91. (95% CI 2.15-12.97, P<0.001).There was significant increase in islet yield when DT<= 12.91(Post-purification: 265322±20,723IEQs (n=46) vs.112, 881±13212 IEQs (n=73), P<0.001).

Influence of Sterile Filtration Membrane on Enzyme Product

Collagenase activity losses were observed after filtration through SFCA, PVDF, Nylon and PES membrane filter. The collagenase activity (Figure 3-7) losses were 20.8%, 24.3%, 34.8% and 33.4% of the pre filtration level, respectively. There was a significant loss in the activity after filtration with Nylon or PES ($p<0.05$, n=6). Interestingly, no significant effect on protease activity (Figure 3-8) or protein binding (Figure 3-9) and enzyme component by HPLC (Figure 3-10) was observed in any type of the filters evaluated. This study demonstrates that certain types of membranes used for sterile filtration of enzyme solutions can substantially reduce the collagenase activity of the solution. SFCA and PVDF are acceptable membranes for sterile filtration of Liberase ™ HI using for human islet isolation.

	Lot No.	# Isolation	Wunsch units/vial	Caseinase units/vial
A	93213920	10	2318	-
B	93290120	40	2070	-
C	93315520	20	2265	75907
D	93026020	6	2240	71708
E	93121020	5	2029	60626
F	93237620	10	2156	63917
G	93477120	10	2427	-
H	93131620	39	2550	75029
I	93134620	31	2386	69983
J	93167720	13	2529	-
K	90329821	2	2147	60550
L	93096820	3	2074	56626
M	93080820	8	2289	62318
N	93103520	7	2148	57190
O	90344420	4	2466	63749
P	93257320	16	2479	-
Q	93393420	27	2614	-

Table 3-1. Roche certificate of value of enzyme blend in the lots used in human isolation.

	Lot	N	Mean	Std. Error	Minimum	Maximum
CII Index	I [a]	10	0.371	0.003	0.358	0.383
	F	8	0.382	0.009	0.353	0.426
	P	12	0.400	0.006	0.373	0.426
	B [a]	39	0.374	0.005	0.349	0.551
	C [a]	19	0.372	0.012	0.348	0.580
	Q	22	0.438	0.022	0.387	0.872
	G	11	0.376	0.004	0.348	0.394
CI Index	I [a,b]	10	0.294	0.002	0.285	0.303
	F [a,b]	8	0.274	0.007	0.246	0.297
	P [a,b]	12	0.251	0.005	0.226	0.274
	B [a,b]	39	0.310	0.003	0.276	0.393
	C [a,b]	19	0.272	0.009	0.242	0.420
	Q [b]	22	0.516	0.027	0.128	0.613
	G	11	0.619	0.005	0.594	0.652
CIb Index	I [a,b]	10	0.335	0.003	0.326	0.354
	F [a,b]	8	0.345	0.007	0.318	0.369
	P [a,b]	12	0.349	0.003	0.326	0.367
	B [a,b]	39	0.316	0.007	0.056	0.348
	C [a,b]	19	0.356	0.020	0.000	0.401
	Q	22	0.046	0.019	0.000	0.210
	G	11	0.005	0.003	0.000	0.026
CII Activity (CDU/µg)	I	10	1.254	0.101	0.580	1.820
	F	8	1.566	0.073	1.340	1.930
	P [b]	11	0.855	0.057	0.540	1.080
	B	39	1.191	0.049	0.680	2.010
	C [b]	19	1.725	0.096	1.150	2.690
	Q [b]	22	1.098	0.065	0.670	1.820
	G	11	0.810	0.073	0.540	1.300
CI Activity (CDU/µg)	I [b]	10	5.494	0.368	4.180	7.630
	F [b]	8	6.439	0.330	5.540	8.130
	P [b]	12	4.430	0.182	3.460	5.520
	B [b,c]	39	5.982	0.177	3.930	8.420
	C [b]	19	6.705	0.250	4.840	8.330
	Q [b]	21	6.242	0.138	5.070	7.340
	G	11	4.360	0.343	3.360	6.340

Table 3-2. Enzyme component characteristics on HPLC profile and activity in seven lots.

Statistical analysis was performed using one-way factorial ANOVA followed by the Bonferroni post hoc test. a: significance between these lots vs. Q. b: significance between these lots vs. G. C: significance between B vs.C. $P<0.05$ was considered statistical significantly.

Lot	Age(yr)	PW(g)	CIT(hr)	DT(min)	CII/CI ratio
I	45±3	92.98±4.27	10.02±0.91	16.2±0.6	0.22±0.01
F	47±5	91.33±8.32	9.81±0.84	17.9±1.7	0.23±0.01
P	45±4	114.94±12.74	8.38±0.73	15.5±1.1	0.19±0.01
B	47±2	94.34±3.36	7.29±0.52	13.5±0.5	0.17±0.01
C	42±3	91.53±4.56	9.65±0.91	13.7±0.8	0.24±0.01
Q	49±2	98.34±7.05	7.41±0.67	15.5±0.7	0.16±0.01
G	40±4	97.05±6.72	6.83±0.95	13.7±1.4	0.15±0.01
P-value	0.586	0.223	0.017[a]	0.019[b]	0.001[c]

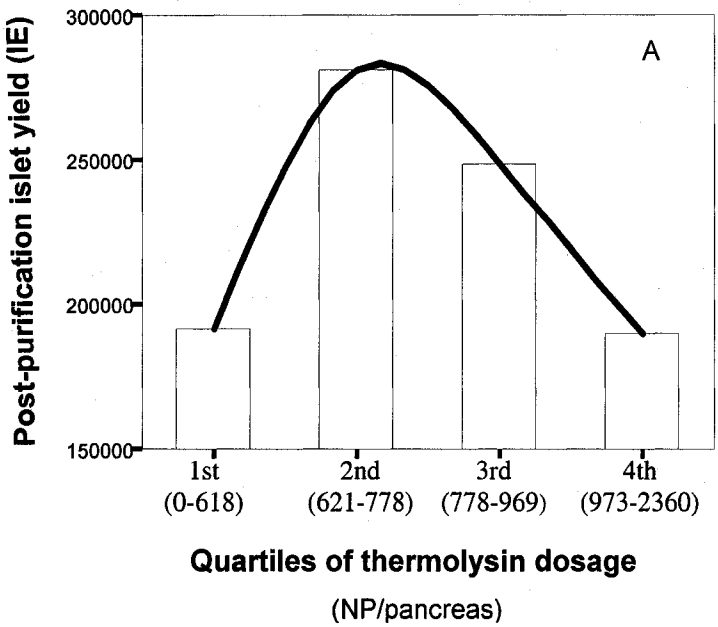
Table 3-3. Donor and organ procurement and isolated-related variables on outcome of isolation in different lots of enzymes.
Statistical analysis was performed using one-way factorial ANOVA following Bonferroni post hoc test. a: significance between I vs. G. b: significance between F vs.B c:significance between B vs.I,F,C;C vs.P,B,Q,G;Q vs.I,F,C;and G vs.I,F,C.$P<0.05$ was considered statistical significantly.

Lot	IEQs	IEQs/g	Viability (%)	Purity (%)	Success Rate (%)
I	196,505±24,462	2,081±264	77.51±3.2	47.61±4.12	19.4(1/10)
F	135,034±25,606	1,620±309	75.50±5.53	63.21±6.31	0(0/10)
P	215,513±34,811	2,455±890	74.51±4.60	53.90±4.29	18.8(3/16)
B	249,410±21,131	2,765±264	81.22±2.41	55.16±2.78	32.5(13/40)
C	166,667±23,941	1,877±250	76.25±3.39	55.19±4.69	5(1/20)
Q	204,184±29,693	2,407±372	79.70±3.18	48.80±4.02	18.5(5/27)
G	273,944±48,248	2,920±504	75.10±6.85	52.25±6.01	50(5/10)
P-value	0.034	0.215	0.744	0.408	0.02

Table 3-4. Outcome of isolation using different enzyme lots.

Variable	Successful group (n=28)	Unsuccessful group (n=105)	P-Value
Post IE(IEQ)	402,745±13,882	150,953±8,053	0.001
Purity (%)	63.55±2.88	49.84±1.98	0.001
Viability (%)	83.73±2.37	76.38±1.66	0.026
Age(yr)	45±2	46±1	0.985
CIT(hr)	6.73±0.57	8.74±0.38	0.012
PW(g)	98.38±5.46	96.90±3.06	0.821
CII Index	0.39±0.01	0.39±0.01	0.429
CI Index	0.39±0.03	0.35±0.01	0.130
CIb Index	0.23±0.03	0.26±0.02	0.289
CII/CI activity ratio	0.166±0.005	0.193±0.005	0.005
CI Activity (CDU/μg)	5.43±0.24	6.09±0.13	0.015
CII Activity (CDU/μg)	1.13±0.07	1.28±0.05	0.119
Protease Activity (DMCU/mL)	17.31±0.90	15.98±0.60	0.273
DT(min)	12.91±0.71	15.03±0.37	0.009

Table 3-5. Analysis of affecting factors for human islet isolation.



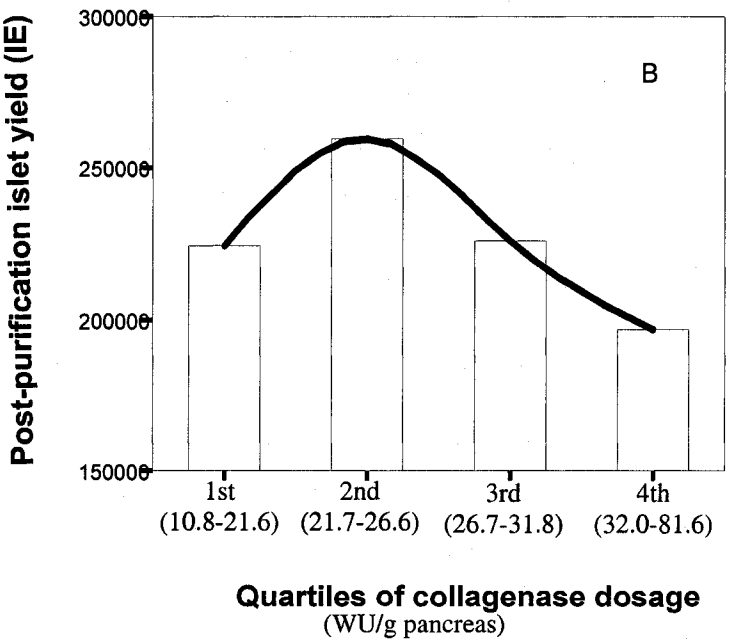


Figure 3-1A-B. Islet yield distribution group by collagenase and protease dosage.

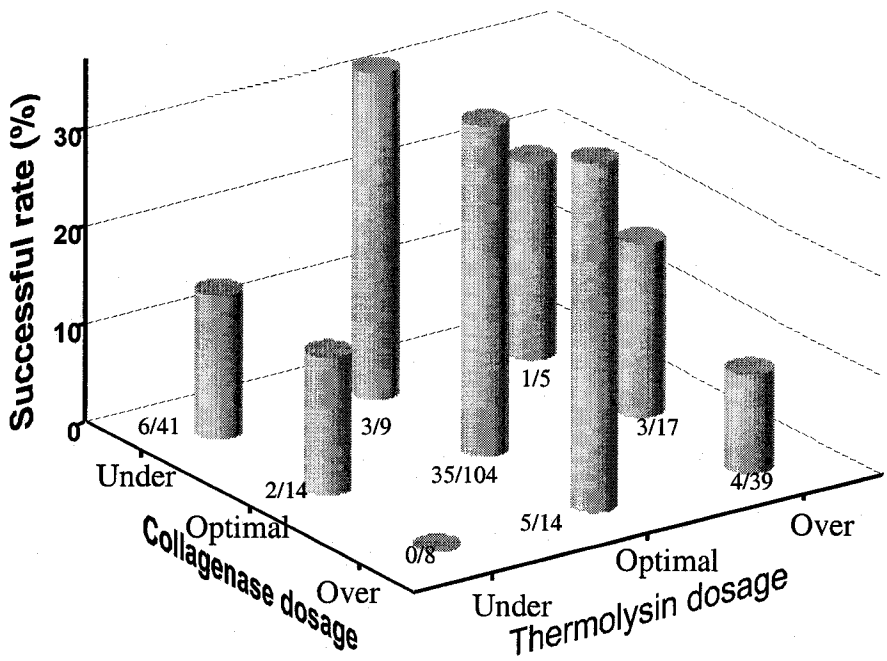


Figure 3-2. Association between enzyme dosage and successful rate.

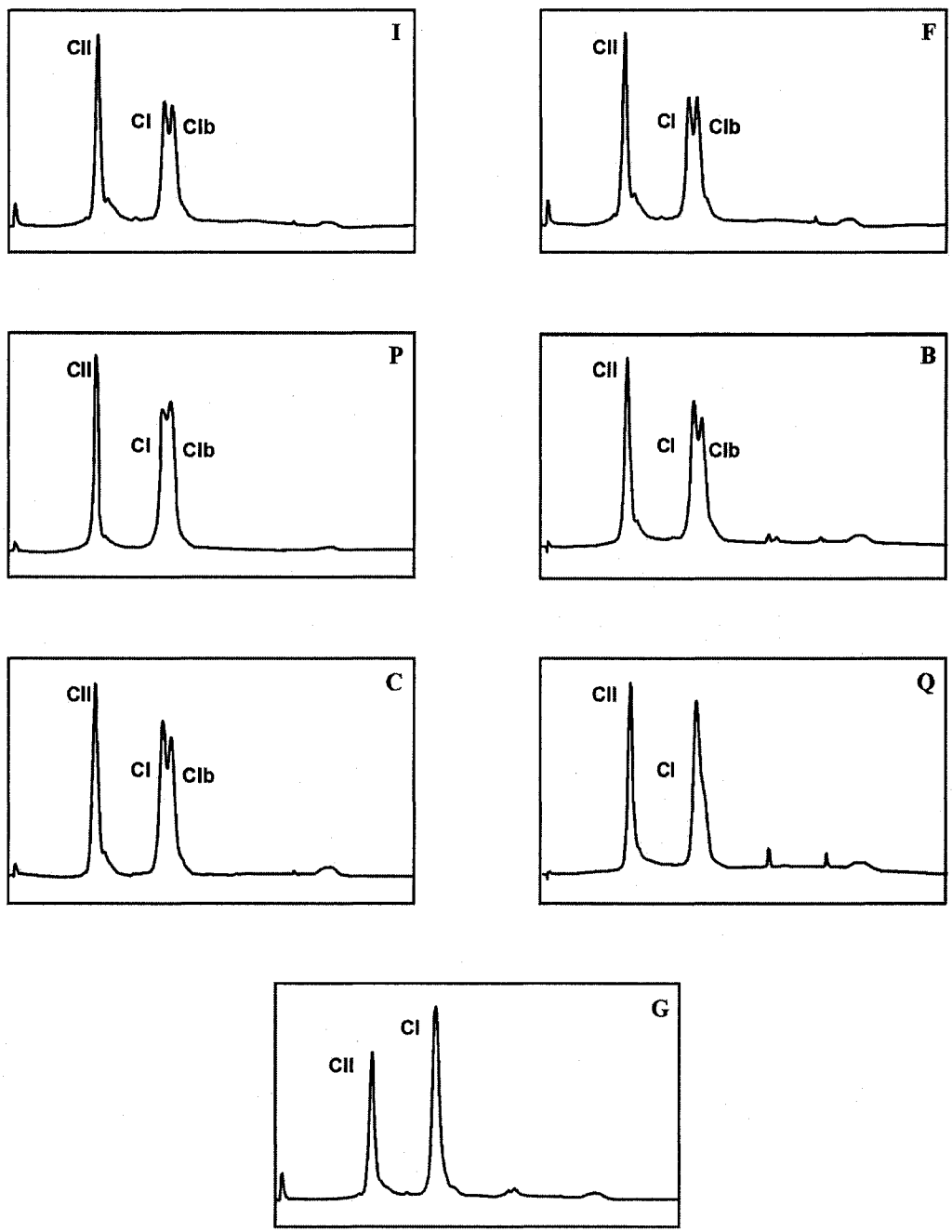


Figure 3-3. HPLC analysis of seven lots of Liberase ™.

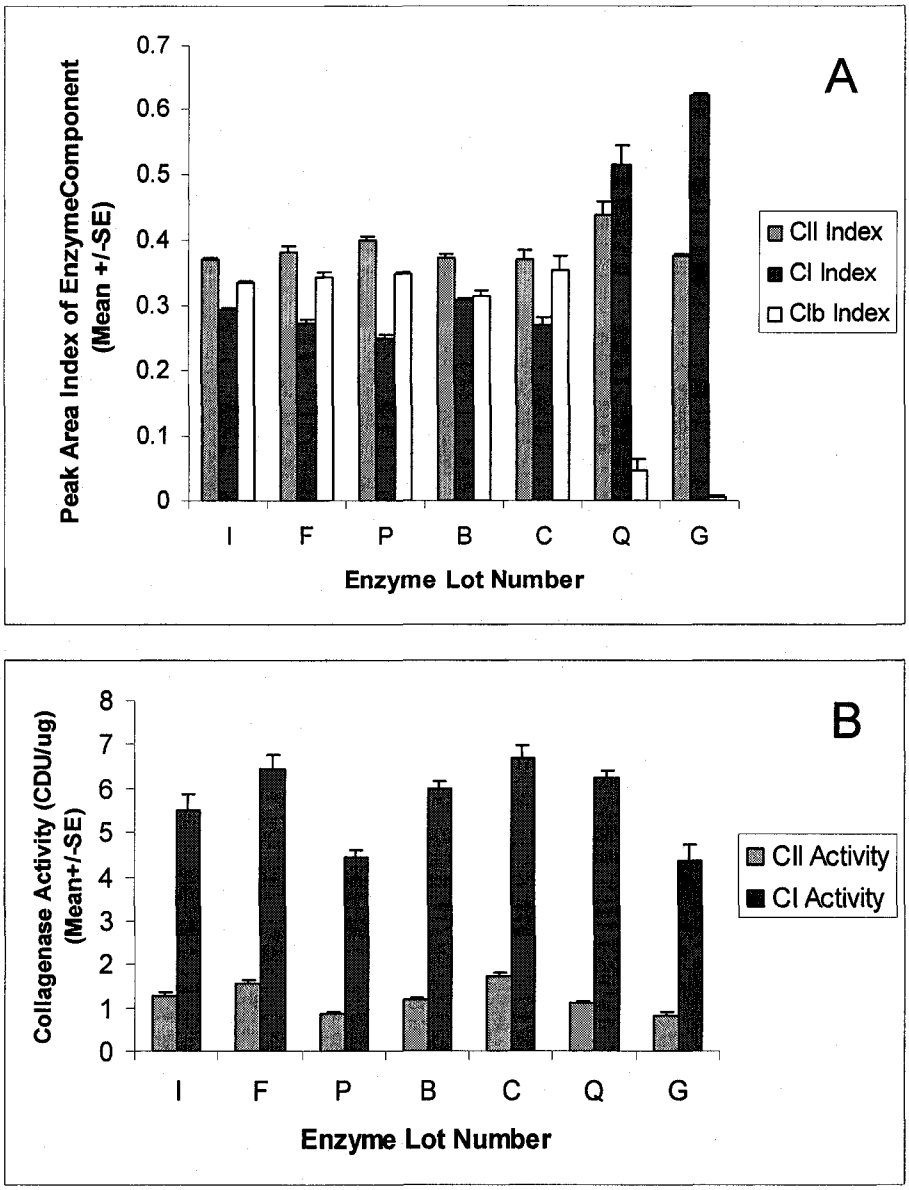


Figure 3-4A-B. Variations of enzyme components in HPLC profile and enzyme activity in different lots.

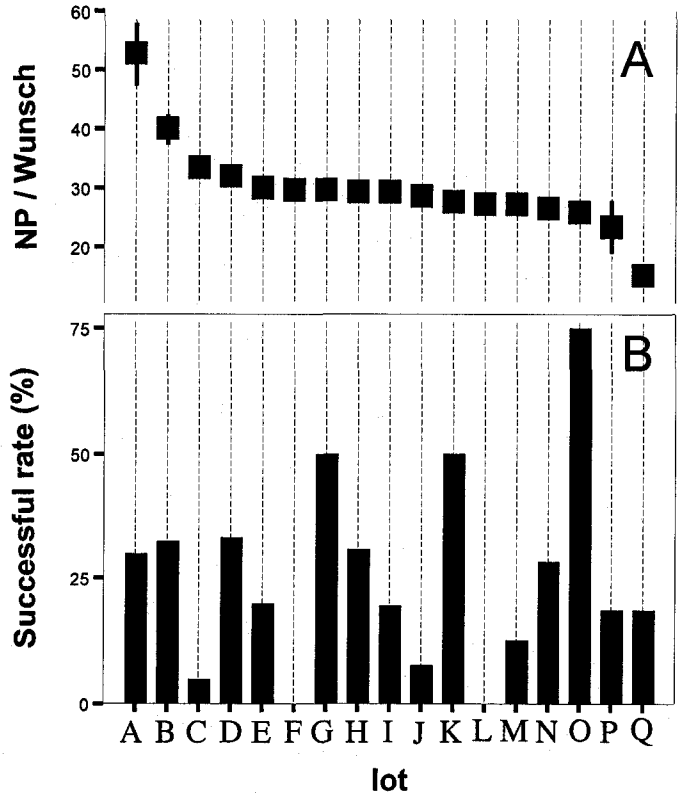


Figure 3-5 A-B. Correlation of ratio of NP/Wunsch of Roche value with isolation success rate.

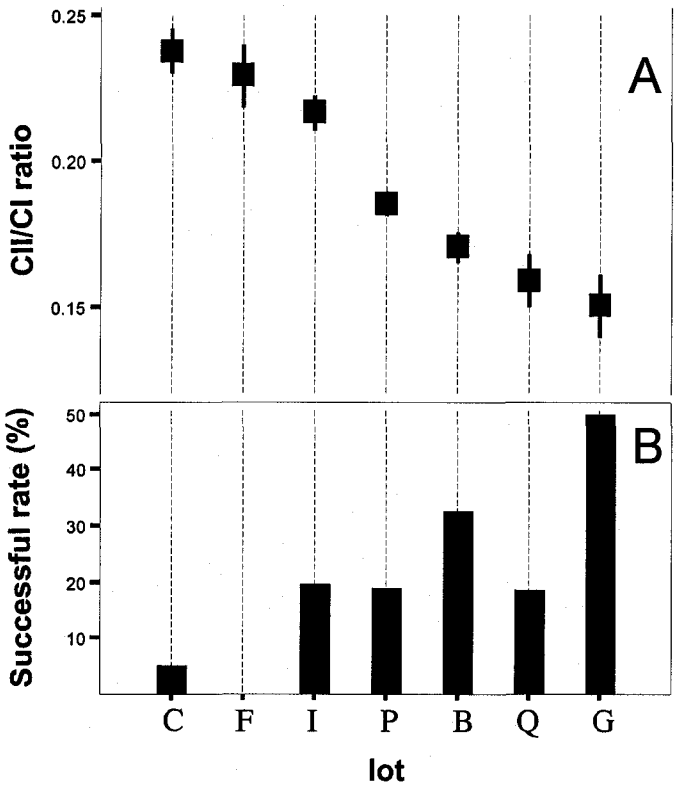


Figure 3-6 A-B. Correlation of activity ratio of CII and CI in enzyme component with isolation outcome.

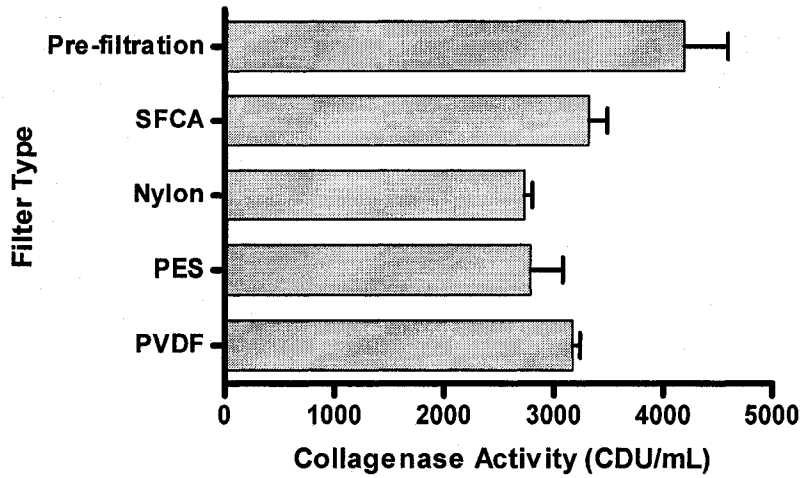


Figure 3-7. Collagenase activity comparison in different type membrane filters.

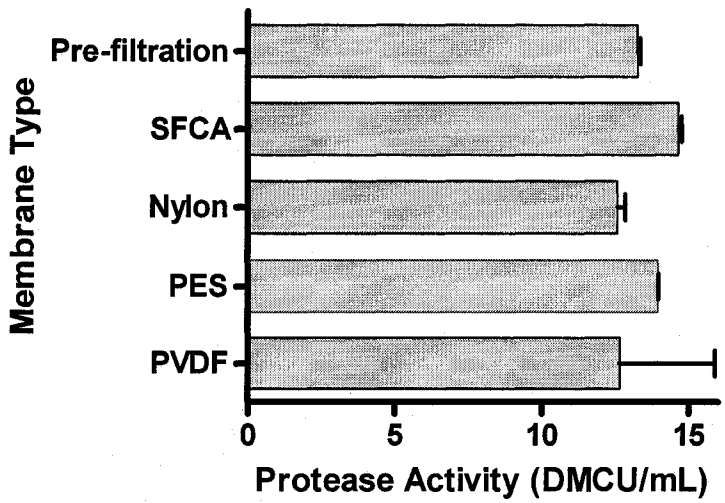


Figure 3-8. Protease activity comparison in different type membrane filters.

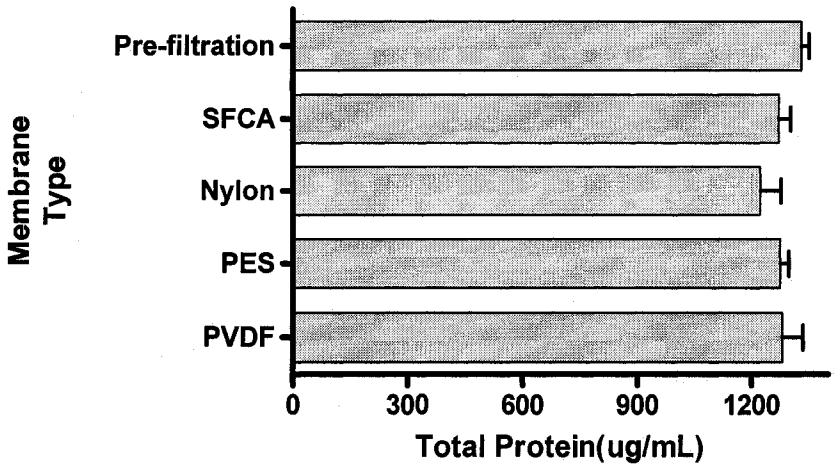


Figure 3-9. Protein comparison in different type membrane filters.

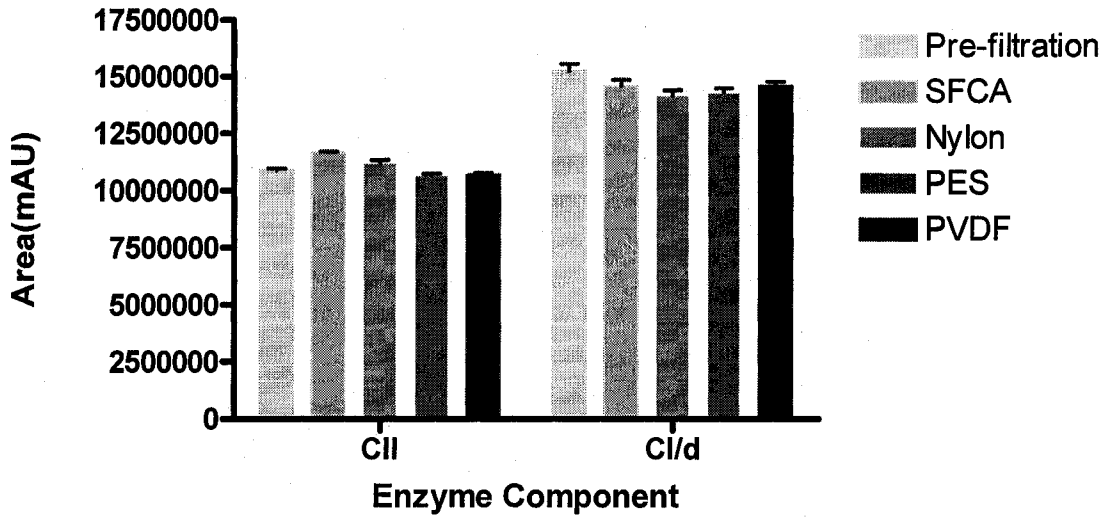


Figure 3-10. HPLC area comparison in different type membrane filters.

Chapter Four

Discussion

Although tremendous advances have been made in islet transplantation, cellular replacement therapy for the treatment of diabetes faces distinct challenges ahead. Recent improvements made in this field can be summarized and include the optimization of organ procurement techniques to reduce warm ischemia, organ storage through the routine use of PFC's and the TLM during pancreas transportation, more purified enzyme blends to decrease the variability in pancreas digestion and islet isolation, the use of culture media supplements to improve islet survival in culture, the use of modified immunosuppressive therapies aimed to improve graft survival and function after transplantation, and the development of transplantation procedures that decrease the risks of the intraportal approach.

However, the future of human islet transplantation is in part dependent on solving problems in the process of islet isolation, which is riddled with inconsistencies, where multiple factors potentially affect the final product, the islets. After the advent of new immunosuppression therapies and subsequent reduction in rejection rates, islet availability is one of the main obstacles for success in islet transplantation.

The collagenase digestion phase of islet isolation has been previously reported to cause morphological disruption of both acinar and islet endocrine cells (93). Islet cells are fragmentized and dispersed into single beta cells with prolonged pancreas digestion and high intraductal collagenase perfusion pressures (34, 94). Endogenous pancreatic proteases are activated during islet isolation. In the automated islet isolation, it is perhaps most likely to be neutral protease (thermolysin) within commercial collagenase

rather than the collagenase itself (94). Most studies on pancreatic protease effect during isolation have concentrated on the damaging effects of trypsin. Low islet yields are associated with high trypsin activity but trypsin inhibitor with Pefabloc improve better islet yield. It is perhaps that trypsin is known to cause islet fragmentation and also it is capable to activate other pancreatic proenzymes.However, there is endogenous enzymes activation and effect on isolation outcomes are extremely unpredictable.

It is not surprising that many other factors account for the activation such as donor factors and cold ischemia time and the batch of collagenase and digestion time (95-97). It showed as Table 3-3 and 3-4.

The development of Liberase™ led to major improvements in the reproducibility of the islet isolation outcome. Therefore, Liberase™ is currently the enzyme blend of choice for the human isolations (35). However, Liberase™ lots have shown marked variability in their efficiency in isolation (71).

This study has focused on the roles of the components of the commercially available enzyme in pancreas digestion and islet isolation. It explored the ratio of the components of collagenase blends to be efficient to release viable free islets in a short period time. First of all, we identified the variation of inter- lots of Liberase™ enzymes (Roche), which are widely used in human islet

In our study, we have retrospectively assessed the efficiency of Liberase™ enzymes in terms of islet yield. It must be mentioned that a substantial difference was observed between collagenase activity measured by the manufacturer and that measured by us. This could be because of differences in the assay method. The wunsch assay, which involves the hydrolysis of 4-phenyl-azo-benzyl-oxycarbonyl-L-prolyl-L-leucyl-glycyl-L-prolyl-D-arginine, is favor of CII. Our CDU assay which involve denatured collagen type I shows a strong substrate specificity of CI preference over CII. This observation confirmed the finding described by Van Wart et al. (80). From our HPLC

profiles of Liberase™, Table 3-2 showed that CII peak area index was relatively constant compared to CI and CIb peak area indexes. CI peak area index fluctuated between 0.25 and 0.62. CII index fluctuation was between 0.37 and 0.44. CIb index fluctuation was between 0.01 and 0.36. Our study showed that a substantial portion of CIb appeared beside CI peak section in five of seven lots. Thus, it suggested that CDU assay would better monitor the variation in collagenase enzyme. The emergence of CIb is possible reason for difficulty we observed in our isolations. Increased CIb may also be linked to proteolytic or degradative influences happened when production process and may decrease available CI specific activity necessary for higher IE yields. Enzymatic activities of CII and CI were measured in parallel in the same laboratory with the same method to be able to compare enzyme-lot characteristics. We observed that there were variation of CII and CI specific activity among lots.

Several groups have tried to elucidate the roles of CII, CI collagenase, and neutral protease in islet isolation. However, no consensus has been obtained as to the ideal combination, as some favor a major role for certain ratios of CII vs. CI collagenase while others favor the importance of neutral protease activity.(75, 76, 92, 98, 99). However, there is no conclusive result yet. In order to define the role of CII/CI and neutral protease, we retrospectively assessed the latest 133 isolations. In Figure 3-5A-B showed the possibility of working with enzyme blends at the exact neutral protease to collagenase activity ratio was not limited to reproducibility issues. Moreover, there was no correlation observed between neutral protease to collagenase activity ratio and post-purification islet yield. Neutral protease activity did not show significant difference between successful and unsuccessful group in our study. However, there was significant difference of CII/CI activity ratio between successful and unsuccessful group (Table 3-5). Introduction of CII and CI in separate form has provided some flexibility to independently dose the components in the pancreas to standardize collagenase efficacy. This study

has contributed to determine the activity ratio of CII/CI for achieving islet isolation success. In this study, Table 3-5 also showed there was significant difference of dilution time between successful group and unsuccessful group.

The overall strategy governing the optimization of the automated method for islet isolation was the notion that the islets must be librated from surrounding exocrine tissues by enzymatic and not mechanical force in short time.

It has been shown that, during the isolation process, proteolytic activity gradually increase as proteases are activated and released from pancreatic acinar cells (94) while collagenase activities were continually decreasing (100). Proteolytic enzymes are considered to have dual roles in pancreatic digestion. On one hand, without collagenase, these proteases can not break down native collagen but they can degrade protease-sensitive area on proteoglycans and glycoproteins., which then allows collagenase access to collagen (76). On the other hand, proteases can damage islet cells irreversibly by fragmentation of the free islets. To limit adverse effects on the islets during the isolation process the processing time must be minimized.

Moreover, prolongation of enzyme digestion time may lead to destruction of those free islets released at earlier stages and maintained in the circuit (over digestion of free islets).Gray et al. reported that prolonged contact with enzyme solutions produces toxic effects resulting in tissue damage and decreased islet yield (101). This appears to be in line with studies reporting that extensive acinar cell destruction mediated by collagenase may be responsible for the release of a " hypotensive factor" (pancreatic shock factor) (93). The complexity of occurrence leading to islet cell destruction after isolation involves oxidative stressful events initiated at the time of organ procurement and further triggered during the isolation phase. Studies have also demonstrated that destruction of the extracellular matrix during enzymatic digestion of the pancreas leads to islet cell apoptosis. Shorter yet sufficient exposure of pancreatic cells to warm enzyme

appears to be beneficial and prevent damage to the islet morphology (102, 103).Our study confirmed that there was significant difference of digestion time between successful and unsuccessful group (12.91±0.71 min and 15.03±0.37 min, respectively. P=0.009). It was also suggested the lower CII/CI activity ratio would be more effective to release islets in shorter period time.

Our filtration study showed that there were collagenase activity losses through these low-protein binding filters. Interestingly, there were no significant loss in neutral protease activity and protein content. The damage of collagenase three-dimensional structure through the membranes might result in a decrease in collagenase activities.

In conclusion, we identified the basis of these variations of collagenase. This understanding will guide improvements to manufacturing methods. We also found optimal CII/CI activity ratio would improve islet isolation outcome. Since manufacturer provide CII and CI in separate form, the optimal determination of CII/CI activity ratio would yield reproducible and effective collagenase cocktail and potentially retrieve enough islets for human transplantation from one donor to one or more recipients.

Until we have a complete understanding of the components of Clostridium histolyticum collagenase that are required for the full spectrum of different pancreata that we receive in our islet laboratories (including those from donors younger than 20 yrs), the separate forms of CII and CI would appear to be a welcome improvement rather than the complete solution to the problem. One useful future study would be to fully determine which of the components of collagenase are required for human islet isolation at both a biochemical and histological level. The data obtained from this study could then be used to prepare a reproducible, stable enzyme preparation. With many interrelated variables present, it is unlikely that one blend can be used for all human pancreata. Instead, we could then match to the donor factors of each pancreas that we receive. Another study would be to determine the role of the pancreatic endogenous proteases. By using

selective protease inhibitors together with an effective collagenase blend, it can be envisaged that the collagenase digestion phase of human islet isolation would be a much better standardized and controlled procedure.

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