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Original Research

Low Cost, Enriched Collagenase-Purified Protease Enzyme Mixtures Successfully Used for Human Islet Isolation

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Abstract

***Background*:** Purified bacterial collagenase and protease enzymes are commonly used to recover human islets from clinical grade pancreata for subsequent clinical islet transplantation. The high cost of purified enzymes can be prohibitive for recovering human islets from research

pancreata used for translational research or pre-clinical studies. In this study, we successfully isolated islets from human research pancreata using enriched collagenase products supplemented with BP Protease (DE Collagenase 800 or Collagenase Gold-BP Protease mixtures) that cost significantly less than purified collagenase-protease enzymes used for clinical islet isolation.

***Methods*:** Defined, enriched collagenase products (DE Collagenase 800 and Collagenase Gold) with a purity comparable to purified clinical collagenase blends (> 85% purity) were prepared from *Clostridium histolyticum* culture supernatants. These products contained primarily intact class I (C1) and class II (C2) at about a 75:25 C1:C2 ratio as determined by analytical anion exchange chromotography. Enriched collagenase-BP Protease mixtures were used at either 19,000-48,000 or 32,500-78,800 neutral protease U (NP U) per g pancreas, and hereafter referred to as low or high protease enzyme mixtures. Each enzyme mixture was diluted up to 350 mL of HBSS and used in a modified Ricordi procedure with isolations performed at three different islet processing centers.

***Results*:** Comparison of the biochemical characteristics of enriched collagenase to the purified collagenase products showed the enriched product is comparable and in some cases superior to those higher priced purified collagenase products. The mean post-purification islet yields from three different centers were 4444 and 4261 IEQ/g pancreas when using the high and low protease enzyme mixtures, respectively. The mean stimulation indices calculated after analysis of glucose-stimulated insulin release were 2.68 and 2.11 when using the high and low protease enzyme mixtures, respectively.

***Conclusions*:** The use of a low cost enriched collagenase products with biochemical characteristics comparable to purified collagenase enzymes were shown to be as effective in human islet isolation as higher priced products. These data indicate that using enzyme mixtures with high Wunsch activity (measurement of C2 collagenase) may not be required for successful human islet isolation. The amount of Wunsch activity/g tissue can be reduced to 25-50% of the amount currently used by some islet transplant centers for isolating human islets. Further studies are needed to validate enzyme compositions required for successful human islet isolation.

Keywords

Islet isolation; islet transplantation; low-cost enzyme; enriched; intact C1 collagenase; C1/C2 collagenases ratio; research pancreas; collagenase; thermolysin; neutral protease; BP-protease

1. Introduction

The success of human islet transplantation as a therapy to manage adult patients with type 1 diabetes is dependent on recovering a sufficient number of islets from a human pancreas for transplant [1, 2]. The major constraint to recover islets was the inability to find a reliable source of *Clostridium histolyticum* collagenase required to release islets from pancreatic tissue. Ricordi and colleagues successfully used Collagenase P [3], a crude collagenase initially manufactured and sold

by Boehringer Mannheim Biochemicals in 1990. However, there were limitations for using this product. Only about 30% of these lots could be used for this application and the shelf life ranged from 12 to 18 months before the ability to recover islets was lost [4]. This resulted in many laboratories spending significant amounts of time to prescreen new lots of collagenase products prior to purchase.

The development of Liberase-HI purified enzyme blend by Boehringer Mannheim Biochemicals in 1994 and its subsequent modifications showed that a consistent collagenase product could be manufactured for this application [5]. The product manufactured after 2002 contained approximately 2000 Wunsch Units (WU) of purified collagenase that was mixed in a 60:40 class I: class II collagenase ratio with purified thermolysin. Liberase-HI set a new standard for enzymes used to isolate human islets and contributed to the success of Edmonton Protocol [6]. The high cost of the Liberase-HI product reflected the cost when a multi-step process was required for enzyme purification. The first step resulted in enrichment of collagenase; the second, separation of the contaminating clostripain from the collagenase; and the third, separating class 1 (C1) from class II (C2) collagenase [7]. The final enzyme mixture was prepared by adding purified C1:C2 in a 60:40 ratio to purified thermolysin [5].

New knowledge of the collagenase requirements for human islet isolation indicate that the requirements can be relaxed for the following reasons. A recent study showed a wide range of C1:C2 ratios were equally effective in human islet isolation [8]. The structure-function of C. *histolyticum* collagenase is known and the ability of different molecular forms of collagenase to degrade native collagen has been defined. And lastly, a hypothetical model has been proposed describing how collagenase and protease release cells from tissue. The model emphasizes the use of intact C1 and C2 collagenase will be the most efficient reagent for degrading the extracellular matrix and that the narrow specificity of enriched or purified collagenase enables excess collagenase to be used in the cell isolation procedure [9].

The new knowledge was incorporated into the manufacture of the enriched collagenase products used in the DE Collagenase 800 and Collagenase Gold products used in this report. The results from this report confirm an earlier report demonstrating the effectiveness of collagenase Gold and BP Protease (BPP) to recover islets from human tissue [10]. It extends this report by showing the biochemical characteristics of these enzymes compared with the current purified enzymes and demonstrates the effectiveness of DE-800 and Gold-BPP enzyme mixtures which were tested at three different islet processing centers to confirm the efficacy of low cost enriched collagenase on islet yield and functions.

2. Materials and Methods

2.1 Donor Pancreas

Human cadaveric donor pancreases (n=15) were obtained through organ procurement organizations from brain-dead donors after informed consent had been obtained as part of multiorgan procurement. The procured pancreases were shipped in the cold University of Wisconsin solution or histidine tryptophane ketoglutarate from the donor center to the islet isolation laboratory. Islet isolations were performed at three different islet isolation centers to study the efficacy of low-cost enriched collagenase with a fixed amount of either low or higher dose of BPP

on islet yield. The islet isolation centers are University of Louisville (UL), University of Arizona (UA), and University of California, San Francisco (UCSF).

2.1.1 Islet Isolation Enzymes

Enriched *C. histolyticum* collagenase and purified BPP were obtained from a commercial supplier (VitaCyte, Indianapolis IN). The enriched collagenase was prepared by minimal processing of a *C. histolyticum* culture supernatant that contained predominately collagenase and low amounts of *C. histolyticum* neutral protease and clostripain. The neutral protease was removed in a subsequent processing step, resulting in minimal contamination of this preparation with clostripain. Two different enriched collagenase products, DE Collagenase 800 (DE-800) and Collagenase Gold, 1 g pack size (Gold), were used in this study. Both products used the same collagenase preparation for manufacturing the product with the only difference being the addition of 18mg (≈ 2.88 million NP Units) of purified BPP to the DE-800 product. The enriched collagenase used in either product contained approximately 420 mg of enriched collagenase at a ≈ 75:25 C1:C2 ratio and a small amount of clostripain contamination. These products also contained a non-mammalian peptide excipient. The excipient protected the collagenase from proteolytic degradation during storage and provided additional mass that enabled the product to be readily weighed out prior to use.

VitaCyte also supplied CIzyme Collagenase HA (Collagenase HA), a purified natural *C. histolyticum* collagenase product with a 60:40 class I (C1) to class II (C2) collagenase ratio. BPP is a purified neutral protease obtained after purification from a *Paenbaccillus polymyxa* culture supernatant (1.1 million NP U per bottle). *P. polymyxa* is an animal origin free product, using culture media containing only plant-based media components. Both products were supplied as lyophilized proteins.

Liberase MTF (Roche Diagnostics, Indianapolis IN) and Collagenase NB-1, Premium Grade (Nordmark Biochemicals, Utersen, Germany) were obtained from other islet isolation groups as reconstituted frozen enzyme solutions for enzyme characterization.

2.1.2 Enzyme Characterization

The samples used for enzymatic analysis were diluted to 1 A_{280} U/mL so that equivalent specific enzyme activities (U/A_{280} U) could be determined. Collagenase activity was measured by a fluorescent microplate collagen degradation activity assay [11], the Wunsch assay [12], and a spectrophotometric microplate modification to the FALGPA Assay [13]. Neutral protease activity was measured by a fluorescent kinetic microplate assay using FITC-human serum albumin as substrate [14] or by a spectrophotometric assay using succinyl casein as substrate [15]. In the latter assay, enzyme units are expressed as µmoles of amino groups liberated per min. Analytical anion exchange chromatography was performed as previously described [16]. Purified *C. histolyticum* C1 and C2 internal standards were used to verify the location of the intact C1 and C2 peaks detected in the collagenase products.

2.2 Human Islet Isolation

Islet isolations were performed using standard protocol as previously reported [17]. On arrival, the pancreas was trimmed, cannulated, and distended with digestive enzymes. DE-800 or Gold-BPP enzyme mixtures were used to digest the pancreas and are referred to below as high protease or

low protease enzyme mixtures. This designation is based on the amount of protease product used per g of pancreas. DE 800 contains 2.88 million NP U whereas the Gold-BPP mixture contains 2.2 million NP U. Each enzyme mixture was diluted in 350 mL in Hanks balanced salt solution for whole pancreas isolation. After ductal perfusion of the enzymes, the pancreas was digested in a Ricordi chamber using a modification of Ricordi's semi-automated method. The digested tissue was then purified by continuous iodixanol (OptiPrep; Axis-Shield, Oslo, Norway) density gradient with a COBE-2991 cell processor (Terumo BCT, Lakewood, CO). The purified islets were cultured in CMRL-1066 supplemented medium (Corning/Mediatech Inc., Manassas, VA) for the indicated number of days prior to functional testing. The research pancreata were either untreated or were persufflated (Persufflation is a way of delivering oxygen to the entire organ throughout preservation by using its native vasculature) prior to performing the islet isolation procedure. These isolations were performed at three different institutions.

2.3 Islet Quality Assessment

Isolated islets from both enzyme products (DE-800 and Gold) were assessed for viability using fluorescein diacetate/propidium iodide [18]. Islet potency was monitored by oxygen consumption rate (OCR/DNA) measurement [19]. A glucose-stimulated insulin release (GSIR) test was used to assess the islet functional quality and subsequently the stimulation index was calculated [20].

2.4 Statistical Analysis

All results were expressed as the mean ± standard deviation, except for OCR/DNA measurements were expressed as mean ± standard error. Islet yield obtained from low or high dose protease enzyme mixtures were analyzed by paired or unpaired student t-test. Statistical significance for the analysis was set at $p<0.05$.

3. Results

3.1 Characterization of the Enriched Collagenase (DE 800 and Collagenase Gold) Products and Comparison to Other Purified Collagenase Products

Figure 1 presents the anion exchange chromatographic profile, the C1:C2 ratio derived from this analysis, and the specific CDA and Wunsch activities for Collagenase Gold, Collagenase HA, purified collagenase from a Liberase MTF kit, and NB-1 Collagenase. The first major peak from this analysis is C2 collagenase followed by a second major C1 collagenase peak. Symmetrical peaks indicate a uniform molecular form of collagenase in the product. Multiple molecular forms of C1 are resolved in this analysis whereas C2 collagenase typically is a symmetrical peak. These results show symmetrical collagenase peaks were obtained with the Collagenase HA and Collagenase Gold products (Figure 1A and 1B). The similar specific CDA reflects differences in the C1:C2 ratios. Collagenase HA is prepared by combining purified C1 and C2 at a 60:40 mass ratio whereas C1:C2 ratio in Collagenase Gold reflects the ratio of collagenase present in the C. *histolyticum* culture supernatant. Here, there is a lower percentage of C2 collagenase as reflected by the lower specific Wunsch activity (Figure 1B).

The Roche MTF collagenase has a doublet C2 peak and a symmetrical C1 peak. This same pattern was observed in a different lot of Roche MTF collagenase (Figure 1C). The specific Wunsch activity is similar to that found in Collagenase HA while the specific CDA was about 25% lower than that found in the Collagenase HA or Gold products.

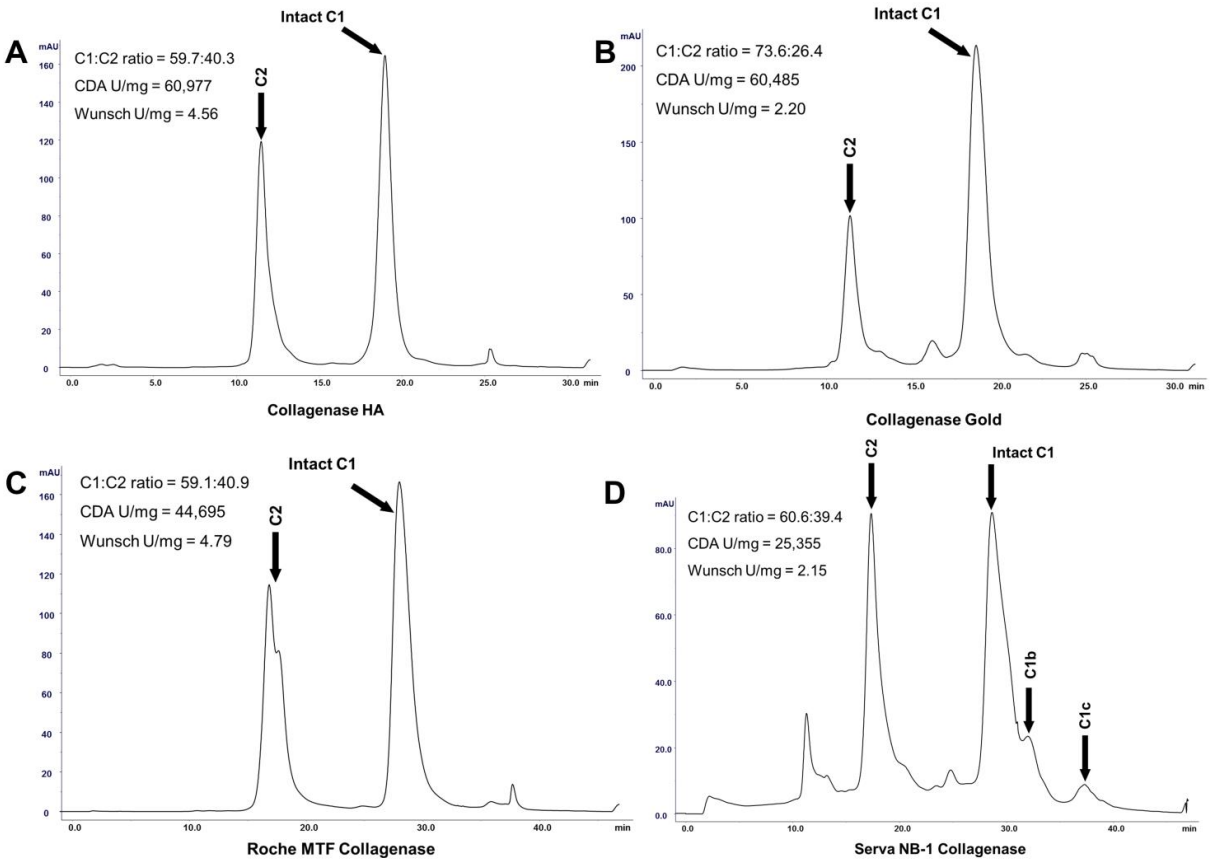

Figure 1 High pressure liquid chromatography (HPLC) analysis illustrates differences between collagenase obtained from VitaCyte, Roche, and Serva enzyme suppliers. (A) Collagenase HA; (B) Collagenase Gold from VitaCyte; (C) Mammalian Tissue Free (MTF) Collagenase from Roche; (D) NB-1 Collagenase from Serva.

The NB-1 Collagenase had an unknown peak migrating ahead of the C2 peak. The asymmetrical C1 collagenase peak indicates the presence of C1b on the back shoulder of the intact C1 peak and an adjoining smaller peak that likely represents C1c collagenase (Figure 1D). The C1b and C1c peaks contain truncated C1 where the carboxy terminal collagen binding domain is lost by proteolytic degradation [21]. Truncated C1 forms also have a lower specific CDA as reflected by the lower specific CDA found in the NB-1 Collagenase product. The lower specific Wunsch activity in the NB-1 Collagenase product likely reflects the presence of other contaminants in this product.

3.2 Efficiency of Using Enriched Collagenase with BP Protease for Human Islet Isolation

Islet isolation outcomes from three different centers, utilizing enriched collagenase with high and low doses of protease are summarized in Table 1 and Table 2. Table 1 summarizes the results from 8 islet isolations utilizing DE 800 enzyme containing a high protease dose (18 mg of BPP per vial) to process pancreases from donors ranging from 31 to 69 years. A very low Wunsch activity per g

pancreas was used for isolation, ranging from 5.9 to 14.2 per g pancreas. The characteristics of these digests (digestion time, islet morphology, pellet size) were comparable to those using a standard dose of purified collagenase enzymes. The average post-purification islet yield obtained was 4444 IEQ/g pancreas (ranges from 3,590 to 8,820 IEQ/g pancreas).

Table 1 Islet isolation outcome using collagenase DE 800 (high dose of BP-protease).

No. of isolations	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**Average**
Age (yrs.)	69.0	33.0	58.0	37.0	31.0	32.0	40.0	31.0	42.8 ± 14.1
Height (cm)	178.0	160.0	156.0	155.0	168.0	180.0	175.0	157.0	165.7 ± 9.8
Body weight (kg)	88.1	79.5	54.0	70.0	51.7	88.0	47.0	75.0	70.1 ± 15.6
Body mass index (kg/m^2)	27.8	31.1	22.2	29.1	18.3	27.1	15.4	30.4	25.6 ± 5.6
Persufflation time (hr)	20.4	10.8	19.0	NA	NA	NA	NA	NA	16.7 ± 5.1
Cold ischemia time (hr)*	21.4	12.8	20.5	10.3	6.2	12.0	6.8	7.2	12.0 ± 5.5
Trimmed pancreas weight (g)	126.2	81.9	56.6	52.0	104.0	109.0	83.2	106.0	92.0 ± 25.3
Wunsch Unit/g tissue	5.9	9.0	13.0	14.2	7.1	6.8	8.9	7.0	8.97 ± 3.6
Neutral Protease Unit/g pancreas	32,472	50,006	72,398	78,788	39,394	37,587	49,225	38,651	49,815 ± 17,043
Digestion time (Phase I) (min)	17	20	16	15	26	22	16	13	18.3 ± 4.0
Undigested tissue (g)	33.6	24.7	11.0	1.2	23.0	20.0	31.9	43.3	30.3 ± 23.5
Undigested tissue (%)	26.6	30	19.4	2.3	22	18.3	38.3	40.8	30.4 ± 20.5
Digest islet number/pancreas	351,000	512,000	370,000	364,000	378,000	499,000	324,000	270,000	383,500 ± 82,742
Digest islet equivalent/pancreas	572,200	722,600	497,400	340,751	456,271	391,260	314,400	540,000	479,360 ± 134,434
Digest islet equivalent/g pancreas	4,535	8,820	8,790	6,553	4,387	3,590	3,777	5,094	5,693 ± 2,124
Embedded Islet (%)	2.0	0.0	1.0	20.0	25.0	30.0	1.0	10.0	11.0 ± 11.4
Tissue volume (ml)	15.0	22.0	15.0	20.0	35.0	45.0	23.0	25.0	23.8 ± 10.1
Post-purification islet equivalent/pancreas	663,700	454,600	343,700	309,358	460,933	387,217	224,900	215,500	382,488 ± 146,398
Post-purification islet equivalent/g pancreas	5,260	5,549	6,074	5,949	4,432	3,552	2,702	2,033	4,444 ± 1,533
Viability by FDA/PI (%)	NA	NA	NA	86	89	80	98.6	98.4	92.0 ± 7.7
GSIR Stimulation index	1.76	NA	1.74	2.9	3.2	2.7	2.28	4.18	2.69 ± 0.7
OCR/DNA ± SEM	212.0 ± 2.8	172.4 ± 14.9	134.9 ± 8.0	NA	NA	125.5 ± 7.2	133.8 ± 3.9	140.4 ± 4.7	153 ± 5.0

Table 2 summarized the results from 7 islet isolations using Gold enzyme with a low protease dose (13.8 mg of BPP per vial) to process pancreases from donors ranging from 34 to 56 years. A low Wunsch activity/g pancreas was used for isolation, on average 9.8 ± 4.2 (ranging from 6.2 to 19.0). The characteristics of these digests were comparable to those using a standard dose or purified collagenase enzymes. The average post-purification islet yield obtained was 4261 IEQ/g tissue (ranging from 2,867 to 8,465 IEQ/g pancreas).

Table 2 Islet isolation outcome using collagenase gold with low dose of BP-protease.

No. of isolations	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**Average**
Age (yrs.)	44.0	36.0	52.0	34.0	37.0	56.0	42.0	43.0 ± 8.3
Height (cm)	170.2	173.0	152.0	183.0	162.5	170.0	167.0	168.2 ± 9.5
Body weight (kg)	59.4	118.0	44.9	126.1	73.0	121.0	97.7	91.4 ± 32.5
Body mass index (kg/m^2)	20.6	39.4	19.4	37.7	26.8	41.9	34.2	31.4 ± 9.1
Persufflation time (hr)	Na	NA	NA	3.3	18.7	3.0	3.0	7.0 ± 7.7
Cold ischemia time (hr)*	8.8	14.5	15.5	16.3	24.8	9.4	11.0	14.3 ± 5.4
Trimmed pancreas weight (g)	69.0	82.0	40.0	121.7	77.3	147.2	92.9	90.0 ± 35.2
Wunsch Unit/g tissue	11.0	9.3	19.0	6.2	7.8	8.2	7.4	9.84 ± 4.2
Neutral Protease Unit/g pancreas	27,698	23,307	47,779	19,146	24,110	25,343	28,868	28,036 ± 9,261
Digestion time - Phase I (min)	19.0	20.0	18.0	21.0	18.0	14.0	14.0	17.7 ± 2.75
Undigested tissue (g)	10.0	10.0	5.0	70.3	31.4	28.1	30.0	26.4 ± 22.2
Undigested tissue (%)	14.4	12.1	12.5	57.7	40.5	19.0	32.3	26.9 ± 17.3
Digest islet number/pancreas	307,000	207,000	166,000	210,500	227,000	346,500	309,000	253,286 ± 67,033
Digest islet equivalent/pancreas	452,892	291,923	338,600	381,500	346,030	509,420	266,183	369,507 ± 86,481
Digest islet equivalent /g pancreas	6,563	3,560	8,465	3,134	4,474	3,462	2,867	4,646 ± 2,094
Embedded Islet (%)	15.0	10.0	10.0	5.0	10.0	7.5	5.0	8.9 ± 3.4
Tissue volume (ml)	50.0	40.0	15.0	25.0	15.0	30.0	20.0	27.9 ± 13.1
Post-purification islet equivalent/pancreas	414,402	286,656	321,000	312,200	327,495	425,010	242,800	332,795 ± 65,806
Post-purification islet equivalent/g pancreas	6,005	3,496	8,025	2,564	4,234	2,888	2,615	4,261 ± 2,052
Viability by FDA/PI (%)	92.1	83.6	84.7	72.7	74.3	90.3	87.2	83.6 ± 7.4
GSIR Stimulation index	3.0	1.8	1.9	1.2	1.2	2.6	3.1	2.1 ± 0.7
OCR/DNA ± SEM	123.4 ± 5.3	NA	NA	132.7 ± 1.5	135.7 ± 5.0	154.4 ± 2.4	147.5 ± 4.8	138.6 ± 12.3

Overall, our results from both enriched collagenase (DE 800 and Gold) combined with high and low dose BPP recovered greater than 4000 IEQ/g from research pancreases, irrespective of donor characteristics. Similarly, there was no impact on islet yield for organs that had been persufflated, and the results obtained from this study were comparable to studies utilizing purified enzymes for human islet isolation (Table 3).

Table 3 Summary of human islet yields from selected reports.

Institution	**Reference**	**n**	**Mean IEQ ± 1 SD* or SEM#/g pancreas**	**Enzyme used (N/S = Nordmark/Serva)**
University of Louisville & others	This report	8	4444 ± 1533*	DE Collagenase 800
		7	4261 ± 2052*	Collagenase Gold/ BP Protease
City of Hope	Khiatah, B. et al., 2018 [10]	8	3503 ± 729#	Collagenase Gold/BP Protease
		48	3551 ± 312#	Roche MTF
		15	2918 ± 276#	N/S NB-1 Collagenase/NB Protease
University of Minnesota	Balamuruga n, A.N. et al., 2012 [17]	24	2202 ± 1403*	N/S GMP Grade NB-1 Collagenase & NB Protease
		5	1635 ± 726*	N/S Premium Grade NB-1 Collagenase/NB Protease
		4	2217 ± 1405*	Roche MTF
		37	3467 ± 1698*	VC Collagenase HA/Thermolysin
		12	5329 ± 2519*	NEM: VC Collagenase HA & Serva NB Protease
University of Alberta	O'Gorman, D. et al., 2010 [22]	17	4249 ± 424#	Roche MTF
		24	3836 ± 390#	N/S GMP Grade NB-1
University of Uppsala	Brandhorst, H. et al., 2009 [23]	101	4010 ± 232#	Liberase HI
		96	2979 ± 149#	N/S NB-1 collagenase & NB Protease

3.3 Functional Assessment of Islets Isolated Using Enriched Collagenase

Quality control results for islets isolated from enriched collagenase with high and low dose protease showed the following (Table 1 and Table 2): the average islet viability by the fluorescein diacetate/propidium iodide method were 92±7.7 and 84±7.4, and the average stimulation index in glucose stimulated release assay were 2.7±0.8 and 2.1±0.8. The average oxygen consumption rates of the islet preparation were 153±7 and 139±12, respectively.

4. Discussion

The need for sufficient number of functional islets for successful allo-islet transplantation led commercial enzyme manufacturers to develop multi-step purification procedures to separate collagenase from other contaminants found in C. *histolyticum* culture supernatants [24-26]. These additional processing steps led to sale of purified collagenase that was priced three to four times higher than the traditional crude or enriched collagenase products. The open question does this increased cost required for human islet isolation add value for those researchers who require human islets for translational research or pre-clinical research studies? And, if no, are there other alternative products that can be used for this application?

The islet isolation group at the University of Illinois Chicago, frustrated by the inconsistency in performance of higher priced, purified collagenase enzymes, pre-screened and pre-filtered lots of Sigma Type V crude collagenase for human islet isolation [27]. Four different lots of Type V collagenase were pretreated by passing the reconstituted enzyme through 0.8, 0.45, and 0.22 µm filters. Forty percent of these materials could not pass through the 0.8 µm filter and were not used for islet isolation. All other materials passed through all the filters and were used for human islet isolation. Endotoxin contamination was determined on the diluted enzyme solution [27].

This group compared results from 42 and 52 human islet isolations using the Nordmark/Serva NB-1 Collagenase and NB Protease and pre-filtered Sigma Type V collagenase, respectively and found no difference in the human islet isolation results as assessed by islet yield per pancreas or per g of tissue; islet size distribution; islets isolated in the high purity fraction after density gradient purification; islet viability; or glucose stimulated insulin secretion [27]. Type V had significantly lower endotoxin contamination and was significantly more efficient in digesting human pancreas than the Nordmark/Serva enzymes. All lots of Type V collagenase worked for this application but there was no mention of the relationship of enzyme activities listed on the Certificate of Analysis to the islet isolation results. Nor is it clear what is meant by 40% rejection of bottles used in the study which will impact the cost effectiveness of using this enzyme for human islet isolation [27].

The present report confirms and extends an earlier report that showed Collagenase Gold and BPP were as effective as collagenase-protease enzymes supplied by Roche's MTF kit or Nordmark/Serva NB-1 Collagenase/NB Protease enzymes [10]. These authors found the Collagenase Gold-BPP mixtures recovered islets with significantly higher viability than those obtained using the Roche or Nordmark/Serva products. The authors recommended the use of this product for isolating islets for human research studies based on product performance and cost per isolation [10].

The human islet yields from this study and prior reports are listed in Table 3. The human islet yields obtained with low-cost DE 800 or Collagenase Gold-BPP enzyme mixtures are comparable and in some cases superior to those obtained using higher priced, purified collagenase-protease enzyme mixtures for human islet isolation. The surprising finding is that in six of eight islet isolations using DE 800 and five of seven isolations using Collagenase Gold/BPP, < 10 WU/g tissue were used to digest the tissue. A prior report using purified collagenase and BPP found that 12 WU/g tissue at a 60:40 C1:C2 ratio gave significantly lower islet yields per g tissue and a higher percentage of undigested tissue (n=5) when compared to results from those isolations using 20 WU/g tissue [20]. The result in the present report may reflect the higher C1:C2 ratio (75:25) where the majority of the C1 is intact enzyme. A prior report using recombinant collagenase and BPP found that a wide range

of C1:C2 ratios (55:45 to 38:62) had no influence on islet yields [8]. Further experiments will need to be performed to confirm this observation.

The effectiveness of low-cost DE 800 and Collagenase Gold/BPP enzyme mixtures reflects the biochemical characteristics of these enzymes which are comparable and in some cases superior to purified enzymes supplied by other manufacturers (Figure 1). This analysis was performed on reconstituted-frozen purified collagenase from other suppliers and was sent to VitaCyte by other islet isolation labs. In our experience, reconstitution and freezing of purified collagenase has no impact on the biochemical or enzymatic activity of this enzyme.

The consistency of collagenase used in the DE 800 or Gold products is the result of VitaCyte's focus to improve the collagenase raw material. New knowledge of collagenase structure-function by Matsushita's group was translated into a hypothetical model of tissue dissociation that proposed that the primary function of collagenase is to degrade native and denatured collagen [9]. The restricted specificity of this enzyme for these substrates means that an excess amount of collagenase with minimal bioactive contaminants will not affect the outcome of any cell isolation procedure. Consequently, if collagenase is in excess, then control of the neutral protease activity is critical for successful cell isolation. The Collagenase Gold can be used to replace any purified collagenase enzyme product used for human islet isolation.

The enriched collagenase is also manufactured as a GMP grade product (Collagenase Gold +).This product is sold at a higher price because of the performance of additional assay to ensure consistency and safety of the final product. The Gold + product does not contain excipient and is as stable as the Collagenase Gold product.

In summary, the data generated from three different islet isolation centers using this new enriched collagenase resulted in comparable outcomes as those obtained utilizing with the current GMP grade enzymes, used for human islet isolation.

Abbreviations

DE800 - DE 800 Collagenase; Gold - Collagenase Gold; BPP - BP-Protease; DOE - Design of experiment; rC1 - Purified recombinant class I collagenase; rC2 - Purified recombinant class II collagenase; U - Units of enzyme activity ; WU - Wunsch Units; GSIR - Glucose stimulated insulin release; OCR - Oxygen consumption rate

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Author Contributions

G.L., G.L.S., K.E.S., and A.N.B., participated in all experiments and G.L., R.C.M. and A.N.B, participated in article preparation. F.E.D., D.R.C., and M.L.G. performed the biochemical analysis on collagenase. G.L., A.N.B., S.V., and M.G.H., participated in study design and statistical analysis of data. G.L., M.L.G participated in data collection. G.L., G.L.S., M.L.G., K.K.P., F.E.D., R.C.M. and A.N.B.

provided expert opinion in data analysis. The authors M.L.G., F.E.D., D.R.C. and R.C.M of this manuscript have conflicts of interest to disclose. R.C.M. and F.E.D. are employees and own shares of VitaCyte LLC. D.R.C. and M.L.G. are employees of VitaCyte All other authors declare no conflicts of interest.

Competing Interests

The authors have declared that no competing interests exist.

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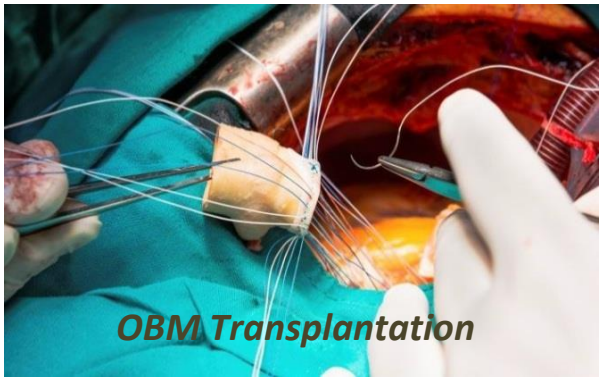

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