Standardization of a Digestion-filtration Method for Isolation of Pancreatic Islets

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SUMMARY

Standardization of a technic for isolating large numbers of pancreatic islets is described. This procedure employed collagenase digestion of rat pancreatic tissue in a cylindrical wire screen in order to separate isolated islets from undigested pancreas. From this basic protocol the following conditions were established: (1) the duration of the initial digestion period was found to be optimal at six minutes; (2) three subsequent digestions of one minute each effected maximum islet yield; (3) the optimal initial collagenase concentration was found to be 1,000 U. (Worthington)/ml.; and (4) proper reductions of collagenase concentrations during the three subsequent digestions were found to be 50 per cent of each preceding incubation period. This method, combined with Ficoll gradient separation, yielded a mean of 800 islets per two rat pancreases. The isolated islets appeared morphologically intact, contained 0.36 ± 0.05 μg. protein/islet, and demonstrated a normal biphasic release of insulin in response to stimulative levels of D-glucose. The present method provides a means for obtaining a large mass of viable islet cell tissue in a short time. DIABETES *25*:667-72, August, 1976.

Isolation of intact islets has been accomplished by either freehand dissection[1] or by collagenase digestion procedures.[2] The advantage of the collagenase digestion procedures is that they allow a fairly rapid technic for isolation of a large quantity of islets for biochemical and morphologic studies. However, difficulties still persist in these isolation procedures in determining definite endpoints to digestion and in obtaining a reproducible maximum yield of islets from an individual pancreas. Recently, a digestion-filtration method[3] has been described that permits graded digestion of the pancreas by using a wire mesh screen for filtration of isolated islets during the digestion process. This procedure allows removal of isolated islets from the collagenase-treated tissue by washing the released islets through a wire mesh screen at intervals during the digestion period and results in an increased yield of islets by preventing overdigestion of individual islets.

The purpose of the present investigation was to standardize the digestion-filtration method in order to obtain a maximum reproducible yield of viable pancreatic islets without depending on an arbitrary endpoint to digestion. The basic parameters studied consisted of the following: (1) optimal duration of the initial collagenase digestion period, (2) optimal initial collagenase concentration and optimal concentrations of collagenase during subsequent digestion periods, and (3) an evaluation of the biphasic pattern of insulin release following D-glucose stimulation of islets isolated by this procedure, while maintained in vitro by perifusion.

MATERIALS

Male Wistar rats (200-300 gm.), fed ad libitum, were used. Collagenase (type IV) was purchased from Worthington Biochemical Co. (Freehold, N.J.). Four different batches of collagenase ranging in specific activity from 160 to 202 units of collagenase activity/mg. protein were used. The collagenase was dissolved in Hanks' solution, which consisted of 136 mM NaCl, 5.36 mM KCl, 1.26 mM $CaCl_2$, 0.8 mM

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$MgSO_4$, 0.33 mM Na_2HPO_4, and 0.44 mM KH_2PO_4 and was adjusted to pH 7.4 with 7.5 per cent $NaHCO_3$. Ficoll (Sigma Chemical Co., St. Louis, Mo.) was dialyzed against distilled water for 30 hours and lyophilized before use.

The digestion apparatus was a modification of that described by Sharp et al.[4] It consisted of a stainless-steel wire cylinder (1.7 cm. diameter x 4.5 cm.) placed in the barrel of a 50-ml. plastic syringe (B-D Plastipak) cut off to 8 cm. in length, with the rubber end of the plunger used as a stopper (figure 1). The cylinder, open at one end, was made of no. 60 mesh stainless-steel wire screen (type 316) with stainless-steel reinforcing collars at each end. Eight small, L-shaped, stainless-steel flanges were attached to the outer surface of the screen to insure clearance from the surface of the syringe barrel. A three-way stopcock was placed at the end of the syringe.

In the perifusion studies of isolated islets, the medium used was a modified Krebs-Ringer bicarbonate solution containing 115 mM NaCl, 5 mM KCl, 24 mM $NaHCO_3$, 1.0 mM $MgCl_2 \cdot 6H_2O$, 2.5 mM $CaCl_2$, 0.5 per cent W/V crystallized bovine plasma albumin (Armour Pharmaceutical Co., Chicago, Ill.), and D-glucose (1.0 mg./ml. or 5.0 mg./ml.). The medium was maintained at 37° C. in a water bath and was continuously gassed with 95 per cent O_2 and 5 per cent CO_2, to pH 7.4. All glassware was siliconized.

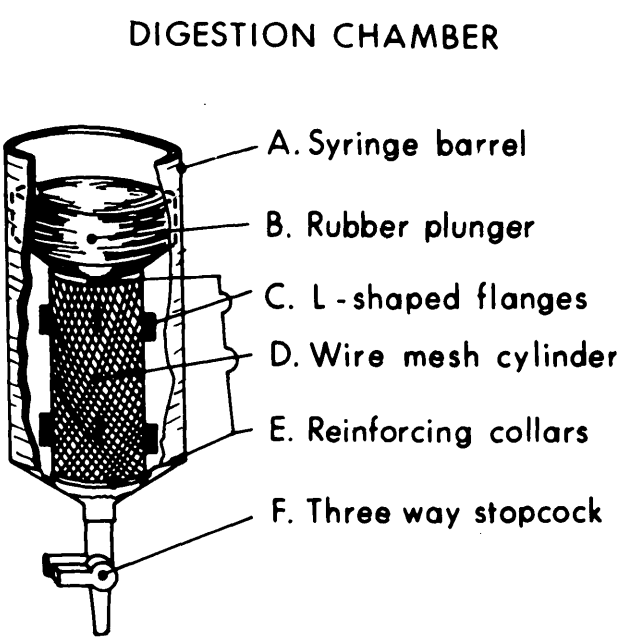

FIG. 1. Digestion-filtration apparatus.

METHODS AND RESULTS

Preparation of the Pancreas

In each experiment the pancreases of two rats were distended by the method of Lacy and Kostianovsky.[2] Rats were first anesthetized with Nembutal, the common bile duct of each animal was cannulated with P.E. 50 tubing, the distal end of the duct was clamped, and the pancreas was distended by injecting Hanks' solution (20-30 ml.). The pancreas was excised quickly, minced finely with sharp scissors, and washed twice with Hanks' solution. The tissue was then poured into a graduated culture tube (16 x 75 mm.), the supernatant was decanted, and the volume of suspended tissue was recorded (ranges 3.8-4.8 ml.).

Digestion-filtration of the Pancreas

The suspended pancreatic tissue was poured into the cylindrical screen with the stopcock open; the rubber plunger was then pushed into the syringe barrel until it fitted snugly against the open end of the screen. A freshly prepared stock solution of collagenase was injected into the chamber by using a syringe and 20-gauge needle. The digestion chamber was then placed in a water bath (37° C.) and shaken by hand at a rate of approximately 40 oscillations per 10 seconds during the incubation period. At the end of each incubation period, the islets were flushed from the chamber by injecting Hanks' solution (7-10 ml.), gently inverting the chamber three or four times, and then forcing the mixture from the chamber by injecting air (20 ml.). This washing procedure was repeated three times for each interval, and the fluid from each wash was collected in individual centrifuge tubes (15 ml.). To determine the number of islets, each tube was spun in a clinical centrifuge at 1,470 × g for two minutes, the supernatant was discarded, and the pellet of islets was resuspended in Hanks' solution and poured into a Petri dish. The number of islets present in each dish was counted with the aid of a dissecting microscope and is enumerated in the text as the mean ± S.E.M.

Optimal Time for Initial Digestion

In order to determine the optimum time period for the initial digestion interval, the tissue was incubated in the chamber for periods of three, four, five, six, and seven minutes, which were followed by three additional incubation periods of one minute each. The total volume of digestion fluid during each interval was maintained at a constant value of 1.5 times the original volume of the suspended, chopped pancreas.

In these experiments, the concentration of collagenase for the initial incubation interval was 800

units/ml. of digestion fluid and was decreased to 75 per cent, 56 per cent, and 42 per cent of the initial concentration of collagenase for the three succeeding one-minute intervals. The numbers of islets obtained with different initial periods of incubation are shown in table 1. The highest yield of islets (461 ± 43) was obtained with the seven-one-one-one-minute protocol. Morphologically, the islets appeared overdigested, with ragged and frayed edges on their surfaces when observed with a dissecting or phase-contrast microscope. The yield with the six-one-one-one-minute protocol was slightly less (329 ± 25); however, the islets appeared intact without evidence of overdigestion. Thus, the latter protocol was used in subsequent studies. The yield of islets with only three minutes of incubation initially was extremely low (123 ± 9).

Since the amount of pancreatic tissue remaining in the wire cylinder decreased following each incubation period, a series of nine studies were accomplished to determine the optimum collagenase concentration to be used in the one-minute incubation periods following the initial digestion period of six minutes. The concentration of collagenase during the six-minute period of initial digestion was 1,000 units/ml. and the per cent decrease of this initial concentration for the three subsequent one-minute intervals was as follows: (A) 75 per cent, 56 per cent, 42 per cent; (B) 50 per cent, 25 per cent, and 12.5 per cent; (C) 25 per cent, 6.25 per cent, and 1.6 per cent. The total yield of islets was essentially the same for these three protocols (546 ± 48, 535 ± 49, and 556 ± 32, respectively, for protocols A, B, and C; n = 3 for each protocol). Morphologically, the islets obtained with protocol A

TABLE 1

Yield of islets following different initial periods of digestion with collagenase

Initial incubation Minutes	Incubation Intervals Number of Islets Released 1st*	2nd*	3rd*	4th*	Total
3	0	2 ± 1	46 ± 5	75 ± 3	123 ± 9
4	2 ± 1	40 ± 13	95 ± 20	118 ± 2	274 ± 34
5	2 ± 0.2	90 ± 27	133 ± 28	70 ± 4	297 ± 49
6	56 ± 17	166 ± 15	86 ± 12	20 ± 9	329 ± 25
7	58 ± 21	145 ± 30	212 ± 13	43 ± 11	461 ± 43

*Mean ± standard error of the mean of islets released following incubation for three to seven minutes in the first interval and subsequent incubation for one minute each in the second, third, and fourth intervals. Three experiments were performed for each of the initial incubation intervals.

Optimum Collagenase Concentration

The effect of different concentrations of collagenase obtained from three different commercial lots was determined by the six-one-one-one-minute protocol. The concentrations of collagenase used in the initial six-minute digestion period were 500, 800, 1,000, and 1,300 units/ml. of digestion fluid. In each instance, the concentration of collagenase was decreased to 75 per cent, 56 per cent, and 42 per cent of the initial concentration in each of the succeeding one-minute intervals of incubation. The total volume of digestion fluid was maintained at 1.5 times the original volume of the suspended chopped pancreas for each digestion interval. The total number of islets obtained per two rat pancreases was significantly greater with a concentration of 1,000 units of collagenase/ml. of digestion fluid as compared to 800 units/ml. (table 2). The yield of islets obtained with 1,300 units/ml. was essentially the same as that obtained with 1,000 units/ml. However, in the presence of the higher concentration of collagenase, the islets had a translucent appearance, indicating overdigestion. Thus, the optimum initial concentration of collagenase was 1,000 units/ml.

TABLE 2

Yield of islets following incubation with different concentrations of collagenase during the initial incubation interval*

Collagenase concentration Units/ml. of digestion fluid	Total number islets released Mean ± S.E.M.
500	313 ± 29 (3)
800	346 ± 18 (9)
1,000	531 ± 21 (6)
1,300	532 ± 48 (3)

*Digestion-filtration was accomplished with the six-one-one-one-minute protocol.

appeared overdigested and in protocol C many of the islets were still attached to capillary fragments; whereas in protocol B the islets were isolated and appeared normal morphologically. Thus, optimum collagenase concentrations for the three subsequent one-minute digestion periods appeared to be 50 per cent, 25 per cent, and 12.5 per cent of the initial concentration of collagenase.

In these experiments, the digestion screen was washed three times with Hanks' solution following each incubation period and the number of islets was determined in each of the washes. Since the yield of islets in the third wash was extremely low (five to 10), this wash could be eliminated without seriously affecting the total yield of islets.

Mass Isolation of Islets–Optimum Protocol

A protocol based on the preceding experiments was established that would provide an optimum and reproducible yield of islets. This procedure was combined with the Ficoll technic, which has been used for separation of the islets by centrifugation.[4] The optimum protocol for mass isolation of islets is as follows: The volume of suspended, minced pancreatic tissue is recorded, and this tissue and suspending fluid are poured into the digestion chamber and the rubber stopper is inserted into the chamber. A stock solution of collagenase (3,000 units/ml.) is prepared and a volume (50 per cent of the initial tissue volume) of the stock solution is injected into the digestion chamber with a resultant final concentration of 1,000 units of collagenase/ml. of digestion medium. The digestion mixture is shaken in a water bath (37° C.) for six minutes and the digestion chamber is flushed with 7-10 ml. of Hanks' solution. The chamber is gently inverted four or five times to wash islets from the screen and to dilute the collagenase; the mixture is then forced from the chamber by injecting air and is collected in a centrifuge tube. The washing procedure is repeated, the two tubes are centrifuged as soon as possible, and the supernatants are discarded.

The subsequent one-minute digestion period is initiated by injecting collagenase stock solution (50 per cent of the volume used in the initial digestion) and Hanks' solution to maintain a total digestion volume of 1.5 times the volume of the original suspended, minced pancreatic tissue. The digestion apparatus is then shaken for one minute in a water bath and the digestion chamber is washed as described above. This procedure is repeated for two subsequent one-minute digestion periods, the volume of stock collagenase solution being reduced by 50 per cent for each successive incubation. Thus, the concentration of collagenase per milliliter of digestion mixture is 1,000 units/ml. in the initial six-minute interval and 500, 250, and 125 units/ml., respectively, in the subsequent one-minute digestion intervals.

Pellets of islets from the washes are resuspended in Hanks' solution, and any dark viscid material is removed from the upper portion of the pellets. The remainders of the pellets are collected into four tubes (16 × 100 mm.) and centrifuged at 1,470 × g for two minutes; the supernatants are then discarded and 4 ml. of 25 per cent (W/V) Ficoll solution is added to each tube and vortexed gently to resuspend the islets. Two milliliters of 23 per cent, 20.5 per cent, and 11 per cent Ficoll solutions are layered on top in descending density and the gradient is centrifuged at 800 × g for 10 minutes. Isolated islets are collected from the 20.5 per cent–11 per cent interface by means of a siliconized pipette. The total isolation procedure can be accomplished in 45 minutes.

This procedure was used in five experiments, and the average number of islets obtained was 793 ± 49 per two rat pancreases. Islets isolated by this procedure were hand-picked and perifused in vitro by the technic described previously.[5] As shown in figure 2, a distinct biphasic pattern of secretion was obtained fol-

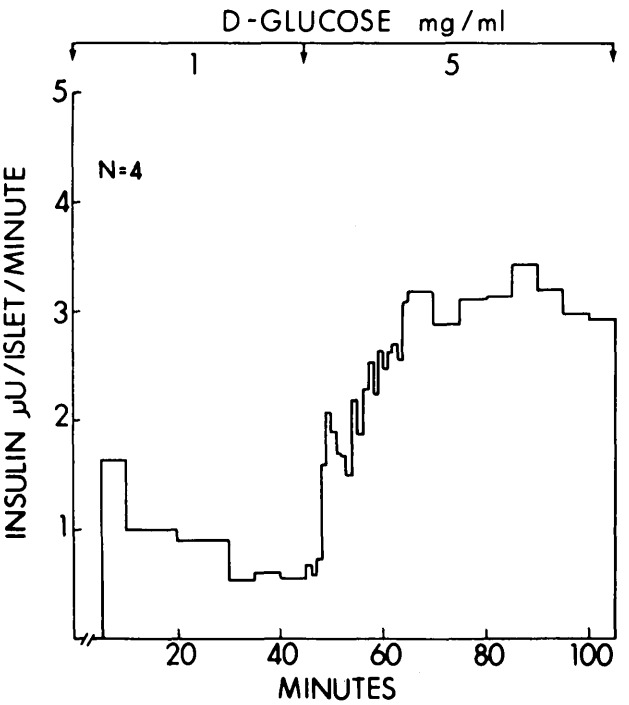

FIG. 2. Insulin response to D-glucose (5 mg./ml.) in perifused islets isolated by the digestion-filtration technic (mean of four experiments).

lowing stimulation with glucose (5.0 mg./ml.), and the rate of secretion was comparable to rates observed previously.[5]

The average protein content of each total islet population obtained from four separate preparations with the digestion-filtration method was 0.36 ± 0.05 μg./islet. This value was derived from counting the islets from each experiment and then measuring the protein content of these islets by the method of Lowry et al.[6] In comparison, protein concentrations of 0.85 ± 0.13 μg./islet were observed in hand-selected islets obtained by a conventional collagenase-digestion procedure.[2] In a series of four experiments employing a larger-volume screen (1.7-cm. diameter × 8.0 cm.), the digestion-filtration method was repeated twice, with five pancreases per procedure. This protocol yielded 4,404 ± 376 islets per 10 pancreases as calculated from the total protein values. Digestion of seven pancreases in a single preparation with the larger screen produced a calculated islet yield of 2,539 ± 584 (n=4).

DISCUSSION

A major problem in isolating islets by the collagenase technic is determination of the endpoint to digestion of the chopped pancreas.[3] Development of a digestion-filtration technic using a wire-mesh screen for incubation of the pancreas with collagenase permits a graded digestion of the pancreas and eliminates the need for an arbitrary endpoint to digestion.[3] In the present study, optimal digestion conditions were defined as those which produced the maximal yield of islets exhibiting an intact gross morphologic appearance when observed under a phase-contrast microscope. When this optimal protocol was used in conjunction with the Ficoll technic[4] for separation of the islets, it was possible to obtain approximately 800 islets per two rat pancreases. Use of a larger-volume screen resulted in a yield of approximately 4,000 islets when the digestion-filtration method was repeated twice with five pancreases per procedure, and 2,500 islets were obtained from seven pancreases in a single isolation. Employment of larger-volume screens with this technic allows a flexibility for processing considerably more pancreatic tissue than could be handled by conventional islet isolation procedures in the same short time period (approximately one hour).

The functional viability of isolated islets obtained by digestion-filtration was determined from observation of a prompt, biphasic, insulin release following glucose stimulation during perifusion in vitro (figure 2). This characteristic response to a glucose challenge is considered a reliable index of islet viability[7] and suggests that islets isolated by this technic are suitable for use in experimental systems designed to investigate islet-cell function.

Currently, digestion-filtration can provide numbers of isolated islets an order of magnitude greater than could be collected in the same time using other technics, thereby eliminating the need for multiple repetitions of an isolation procedure. When small numbers (200-400) of isolated islets are required, such as for acute insulin-release experiments, the conventional collagenase islet-isolation technics of Lacy and Kostianovsky[2] and Lindall et al.[8] are preferred because they efficiently accommodate the necessary amount of tissue. However, for procedures requiring more than a few hundred islets, in particular those which include organelle isolation, a large mass of islet tissue is generally needed to provide adequate starting material. In this type of application, the digestion-filtration method is particularly advantageous since fresh islets are obtained from a single procedure rather than collected from multiple isolations over a period of several hours and stored prior to use.

The observed disparity between islet protein concentrations in the present method (0.36 ± 0.05 μg./islet) and earlier isolation protocols in which islets are hand-picked (0.85 ± 0.13 μg./islet) is probably explained by a preference toward harvesting larger islets in the latter procedure. Since the volume of these predominantly spherical islets varies with the third power of their radii, selection of islets with only a 25 per cent greater diameter would result in a twofold increase in islet volume and a correspondingly greater cellular mass. When the Ficoll separation procedure is used following digestion-filtration, the entire islet population (rather than the more easily selected large islets) was harvested and counted, and the total protein determined. Thus, the value of 0.85 ± 0.13 μg. protein/islet for hand-selected islets does not appear to accurately estimate the average protein content of the entire range of islet sizes but instead represents the protein content of a select portion of the total islet population. The digestion-filtration method provides a continuum of islet sizes with a total protein yield approximately 150 μg. of islet protein per pancreas.

In order to maintain optimal conditions for this technic, it is necessary to select a screen with dimensions adequate to contain sufficient pancreatic tissue for the desired islet yield (assuming a recovery of 300-400 islets per pancreas) in a single procedure.

Although a thorough study was not conducted to determine an optimum ratio of tissue volume to screen volume, it appears that a value of 1:3 will provide a good islet yield. When the tissue volume approached one half of the screen volume, islet yield was reduced. Such factors must be assessed more thoroughly if screens designed to contain large amounts of pancreatic tissue are contemplated.

Standardization of the digestion-filtration technic serves as a point of departure for investigators who desire to develop expanded pancreatic-islet-isolation procedures. This method can provide a large mass of viable islet tissue in a short time and has the flexibility to meet specifications of particular islet-isolation demands that may be required for biochemical or islet-transplantation studies.

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