Techniques for Islet Preparation

Protease Activity in Pancreatic Islet Isolation by Enzymatic Digestion

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Commercial Collagenase* prepared from *Clostridium histolyticum* is widely used in isolation of pancreatic islets. It is known that the enzyme is very impure and that there are substantial variations in effectiveness between batches. Our studies suggest that one of the impurities of importance in islet isolation is a protease that has not been very well characterized. Comparison of two batches of enzyme, one of which was known to give good yields of islets and the other poor yields, showed that they had very similar activity against collagen (measured by digestion of insoluble collagen followed by assay of soluble products with ninhydrin) but substantially different activities against azocasein as measured by optical density increase (measured by release of dye). Eighteen batches of Collagenase were examined for efficiency in islet isolation, and the yields obtained correlated with manufacturer's data of activity against casein. The data show that low caseinase activity is associated with performance in islet isolation (r = .5 after adjusting for collagenase activity). The effect of supplementing a batch of collagenase, known to be poor in isolating islets, with proteolytic enzymes was investigated. Trypsin and papain had apparently no effect, but dispase significantly increased yield. Dispase alone failed to digest pancreas. Size-exclusion high-performance liquid chromatography identified a peak associated with high protease activity and efficiency in islet isolation, having an M_r of ~30,000, compared to 78,000 for collagenase. The protease, like collagenase, is inhibited by EDTA. Increased Ca^{2+} and Mg^{2+} (up to 10 mM) did not affect activity. Both the protease and collagenase are stable under normal use but are inactivated by heating at 56°C. Other properties, e.g., effect of temperature, pH, and dithiothreitol, were also investigated. *Diabetes* 38 (Suppl. 1):126–28, 1989

Commercial Collagenase* prepared from *Clostridium histolyticum* is widely used in isolation of pancreatic islets. It is known that the enzyme is very impure and that there are substantial variations in effectiveness between batches. We present evidence that some of this variation is due to the activity of a neutral protease, which was initially described by Mandl et al. (1) and has not been thoroughly investigated. This enzyme may be measured by its activity against casein and azocasein. We also describe some properties of this enzyme and of activity against collagen.

MATERIALS AND METHODS

"Collagenase" prepared from *Clostridium histolyticum* was obtained from Sigma (types I, II, V, and XI; St. Louis, MO) and from Boehringer Mannheim (Indianapolis, IN). Collagen (insoluble, collagen type I from bovine Achilles tendon), ninhydrin, hydrindantin, papain, trypsin, and lithium acetate were also obtained from Sigma. Dispase was obtained from Boehringer Mannheim. Dimethyl sulfoxide (DMSO), 1-propranol, and other reagents were obtained from BDH (Poole, Dorset, UK). Inbred DA rats bred on-site were used for islet isolation.

Islets were isolated by the method of Sutton et al. (2). "Collagenase" was used at a concentration of 3 mg/ml; variations are described below.

Activity of collagenase was measured by the digestion of native collagen, followed by filtration and estimation of the amino groups in the filtrate by reaction with ninhydrin. Digestion was performed during a 5-h incubation at 37°C in phosphate-buffered saline at pH 7.1–7.2 with 77 mM NaCl and 0.36 mM $CaCl_2$ unless otherwise stated. The method was supplied by Sigma. Ninhydrin reagent was made up in DMSO. Optical density (OD) was measured at 570 nm, and

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*Throughout this article, *Collagenase* (with uppercase C and quotation marks if needed) refers to commercially available enzyme, whereas *collagenase* (lowercase c) refers to activity specifically against collagen.

activity was expressed relative to 2 mM L-leucine standard, after subtracting the OD produced by a reagent blank.

Activity of neutral protease was assayed by liberation of dye from azocasein, measuring OD at 360 nm (4). The conditions for the assay were incubation for 3 h at 37°C, pH 7.2, and 2.5 mM $CaCl_2$ unless stated otherwise.

Enzyme solutions were made up at 0.05–3 mg/ml for assay against collagen and 0.005–0.1 mg/ml for activity against azocasein. Enzyme activities were compared in various batches, and the effects of temperature, pH, Ca^{2+}, Mg^{2+}, EDTA, and dithiothreitol (DTT) were investigated. Solutions of dispase and trypsin were used in these studies for comparison.

High-performance liquid chromatography (HPLC) separations were performed on 14 batches of Collagenase with a Beckman automated system with a DEAE SPW column for ion-exchange chromatography and a TSK 4000 SW column for size exclusion. Fractions from HPLC were assayed for activity against azocasein and collagen.

Data on activity of collagenase, clostripain, tryptic activity, and caseinase provided by Sigma on 18 batches of Collagenase were compared with a grading of efficiency in islet isolation on a scale of 1 (poor) to 5 (very good).

Attempts were made to improve the activity of a batch of enzymes with low activity against azocasein and poor performance in islet isolation by adding proteolytic enzymes. Islets were isolated as described above, with either unaltered or supplemented Collagenase solutions, the tubes used for isolation were coded and randomized during the procedure, and the islet yield was counted under a low-power microscope before breaking the code.

Statistical analysis. Regression and correlation methods were used to relate islet isolation performance of different batches to enzyme activity, and in some cases to relate different measurements of activity and HPLC results. These studies were analyzed with the Minitab computerized analysis package (Pennsylvania State Univ., University Park, PA).

Islet yields with and without added protease were compared by Student's *t* test for paired data.

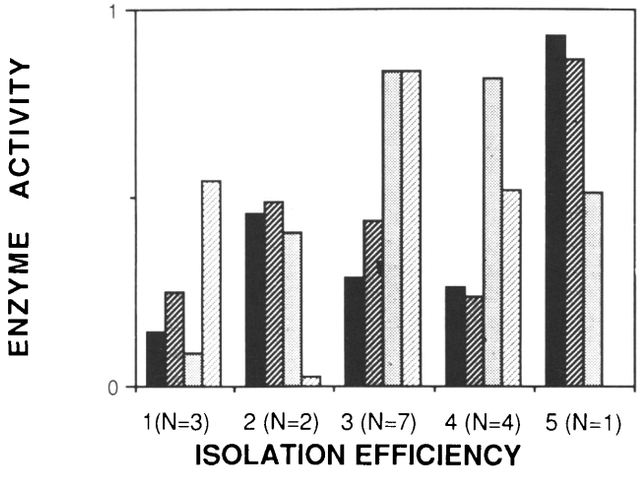

FIG. 1. Mean relative activity of enzymes by isolation grade. *Solid bars*, collagenase; *dark hatched bars*, clostripain; *stippled bars*, caseinase; *light hatched bars*, tryptic.

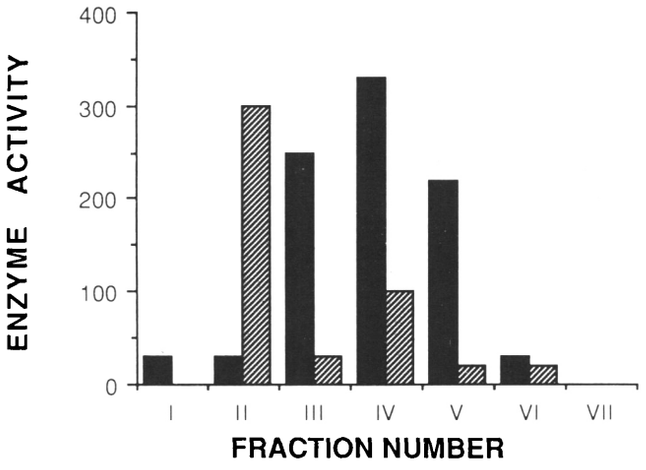

FIG. 2. Relative enzyme activities of fractions obtained by size-exclusion high-performance liquid chromatography. Fraction I, highest-molecular-weight (earliest) fraction. *Solid bars*, protease; *hatched bars*, collagenase.

RESULTS

Batch comparisons. In initial experiments we compared two batches of type I Collagenase, one of which was known from previous experience to be very efficient in islet isolation and the other to be very poor. Both were found to be of similar activity against collagen (0.55 ± 0.049 vs. 0.53 ± 0.053 μmol leucine equivalent under standard conditions). However, they differed substantially in activity against azocasein (OD increase 450 ± 26 vs. 65 ± 8 × 10^{-3} OD units at 0.05 mg/ml for the efficient and poor batches, respectively). A similar association was shown between activities and efficiency after analysis of other batches.

Analysis of 18 batches provided further evidence for the role of caseinase. In multiple regression analysis, collagenase and caseinase activities (manufacturer's data) were associated with isolation efficiency ($P < .05$). They were negatively correlated with each other; after controlling for this, the partial correlation between caseinase activity and isolation grade was $r = .5$ ($P < .05$). Tryptic and clostripain activities showed no significant effect. The mean activity of each enzyme for the different isolation grades is shown in Fig. 1. Seven batches were assayed for activity against azocasein, which correlated well with manufacturer's data of caseinase ($r = .94$, $P < .01$).

Effect of added protease. Initial experiments with adding trypsin and papain to poor batches of collagenase showed no improvement in islet yield; trypsin produced an overdigested appearance. Dispase (2.5 mg/ml), however, did improve yield significantly (with dispase, 328 ± 131 islets/pancreas; without dispase, 239 ± 117; $n = 6$, $t = 3.76$, $P < .02$). Dispase alone did not digest the pancreas.

HPLC studies. Size-exclusion HPLC showed two major peaks. One peak corresponded to ~78,000 M_r; the other was a broad peak ~30,000 M_r. In addition, a "shoulder" could sometimes be seen on the lower side of the first peak. Testing of fractions showed that the collagenase activity was chiefly associated with the 78,000-M_r peak, whereas protease was associated with the 30,000-M_r peak (Fig. 2, fractions II and IV, respectively). Both dispase and bovine serum

albumin produced single peaks. Ion-exchange separation produced a more complex pattern, with five identifiable peaks. It was not possible to detect enzyme activity in fractions after ion-exchange chromatography; this may be the result of removal of ions from the active site (1). In both cases the position of peaks was reasonably constant between batches, but the height varied considerably.

Activity studies. Both collagenase and protease activities were almost completely inhibited by 0.1 mM EDTA, as was dispase activity. The concentration of Ca^{2+} and Mg^{2+} in the medium did not appear to affect activity. Both collagenase and protease activities of Collagenase and the activity of dispase were stable if left at room temperature for 24 h and also if refrozen and rethawed. Both activities increased with temperature up to 43°C; at 46°C, the activity of collagenase lessened. Heating for 30 min at 56°C removed at least 75% of the activity of these enzymes and of dispase. Activity did not vary greatly over the pH range 6.1–8.7, the optimum activity being ~7; in contrast, trypsin showed the expected increase with pH. Addition of DTT at concentrations between 0.25 and 2.5 mM inhibited protease activity as well as that of dispase and trypsin. The inhibition of collagenase was less; indeed, activity was increased at 0.25 mM.

DISCUSSION

The impurity of Collagenase preparations has long been known, but the possible importance of contaminants in digestion has not been thoroughly studied. Neutral protease activity has long been recognized (1) but has not been studied as thoroughly as have some other bacterial proteases, including clostripain from *C. histolyticum* (4). The number of different enzymes involved in nonspecific protease activity is unknown, as are their specificities. Several different enzymes are known to be involved in activity against collagen (5,6). The molecular weight of some of these is close to that found in this study; the failure to identify forms with higher molecular weight may be due to insufficient resolution, or they may have been absent in our preparation.

The precise role of protease in digestion of the pancreas is unclear. Because both caseinase activity and isolation efficiency were found to vary between batches with constant collagenase activity, it is unlikely to be simply a result of protease enhancing activity against collagen. Note that the assay used in this study uses type I collagen as substrate, whereas the pancreas contains both type I and type III (reticulin). We know of no studies comparing activities of bacterial collagenase against different types of collagen. This study demonstrates that islet isolation with the use of Collagenase is affected by enzymes other than true collagenase. Greater understanding of the role of the various enzymes in islet isolation may lead to improved performance and to resolving the problems of batch variation, which have long been a source of difficulty in experimental islet isolation.

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