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BRIEF COMMUNICATION

Collagenase Isoforms for Pancreas Digestion

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The available information concerning the characteristics and composition of collagenase batches, which are effective in the digestion of human pancreas for islet transplants, is scarce and incomplete. A large inter- and intrabatched variability in activity and efficiency of blend enzymes available for isolation has been observed. The aim of this study was to characterize enzyme blend components. Liberase batches were characterized by SDS-PAGE analyses, microelectrophoresis, and then by MALDI-TOF MS analysis. Three main bands were detected by SDS-PAGE analysis and submitted to MALDI-TOF MS analysis. Two bands were found to correspond to class I (isoform β and another of 106 kDa) and one to class II (isoform δ) collagenase. These results represent an important step towards a complete characterization of enzymes, with the final aim of identifying key components for a standardized product.

Key words: Islet isolation; Purification; Collagenase; Proteases

INTRODUCTION

Efficient islet isolation represents a necessary requirement for successful islet transplantation as a treatment for type 1 diabetes (12). Although more than 20 years have passed since the first isolation of human pancreas, organ digestion is not yet a fully standardized procedure, mainly due to variable enzyme efficacy.

The enzymes used, collagenase and neutral protease, present different characteristics from batch to batch, and from vial to vial, due to problems of instability and of production (14). Collagenase available for isolation of human pancreas is currently extracted from in vitro bacterial cultures of *Clostridium hystoliticum*. Minimal differences in culture conditions as well as possible aspecific bacterial contamination induce great variability between enzyme batches. Subsequent purification does not succeed in batch homogenization and therefore their composition and efficacy are variable (1,2,6,14).

The criteria proposed to characterize in vitro enzyme composition and efficacy before use have been unsuccessful (11). Variable clinical conditions of pancreas donors and therefore of pancreas characteristics further complicate any attempts at identifying standardized procedures (11). The final result is that often many pancreases need to be digested to achieve isolated islets of sufficient quality and quantity for transplantation in a single recipient.

The standardization of the enzyme composition represents the logical base and the necessary first step for the final real standardization of isolation procedures. It was described that the collagenase necessary for human pancreas digestion includes class I and class II collagenase (5,6,8). Each class includes some collagenase isoforms, identified by the Greek letters (α, β, γ, δ, ϵ, ζ) (3,10,15). Nothing is known about the role of the different isoforms of collagenase within the same class in terms of successful pancreas digestion and therefore the

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ideal composition of each enzyme blend for pancreas digestion remains unknown. The first experiments with a type of recombinant collagenase have not provided additional information (7). The role of other enzymes in pancreas digestion (i.e., the neutral proteases) appeared to be complementary to that of collagenase (9), but less important mainly due to its relative low concentration in the final enzymatic blend used in isolation (1).

The aim of our studies was to characterize the content of collagenase isoforms in enzyme blends available for pancreas digestion.

MATERIAL AND METHODS

Liberase™ HI (Roche, Indianapolis, IN, USA; seven batches) from *C. histolyticum* used in human isolation was analyzed. Each aliquot of Liberase™ was collected before enzyme reconstitution from a new vial, after proper equilibration on ice and mixing of the vial contents (2). Four samples were reconstituted from each batch with 10 mM EDTA solution (Sigma-Aldrich Corporation, St. Louis, MO, USA) on ice and quickly loaded for characterization using different analyses. A protocol for the preparation of enzyme samples was tested and standardized to prevent uncontrolled degradation prior to biochemical analysis and to be used in isolations. The samples' final concentration was 10 μg/ml (mg of powder mass provided by the manufacture). The samples from lyophilized separated components, collagenase class I (CI), collagenase class II (CII), and thermolysin (Roche, Indianapolis, IN, USA), were similarly collected and reconstituted.

Samples were characterized by SDS-PAGE electrophoresis. Polyacrylamide gel electrophoresis experiments were carried out with 7.5% acrylamide gels. To prevent proteolysis of samples during denaturation, 10 mM EDTA (Sigma-Aldrich Corporation) was added to the denaturing buffer; Coomassie blue (Sigma-Aldrich Corporation) was used to stain the protein bands. The same batches were analyzed also by microelectrophoresis analysis, as previously reported (1). The Protein 200 Plus assay (Agilent Technologies, Palo Alto, CA) allows sizing of proteins ranging from 14 to 200 kDa. Sensitivity of Protein 200 Plus assay is 20 ng/μl bovine serum albumin (BSA) in phosphate-buffered solution (PBS). The chip-based separations were performed on the Agilent 2100 Bioanalyzer in combination with the Protein 200 LabChip kit and the dedicated Protein 200 assay software (Agilent Technologies).

For protein identification, the bands of interest were excised from Coomassie-stained gels, reduced, alkylated, and digested overnight with bovin trypsin as described elsewhere (13). Supernatant (1 μl) of the digestion was used for MALDI-TOF MS analysis using the dried droplet technique and cyano-4-hydroxycinnamic acid (HCCA) as matrix. All analyses were performed using a Voyager-DE STR (Applied Biosystems, Framingham, MA, USA) time of flight (TOF) mass spectrometer operated in the delayed extraction mode. Peptides were measured in the mass range from 750 to 4000 Da; all spectra were internally calibrated and processed via Data Explorer software. Proteins were unambiguously identified by searching a comprehensive nonredundant protein database using the program ProFound (16).

RESULTS

Liberase samples were analyzed by three different techniques: SDS-PAGE electrophoresis, microelectrophoresis, and by MALDI-TOF MS. By SDS-PAGE electrophoresis three main bands (Fig. 1a, A, B, C) were observed for Liberase™ with the corresponding apparent molecular weights of 100, 110, and 115 kDa. When separate components, collagenase CI and collagenase CII were analyzed with the same method, we observed that bands of 115 and 100 kDa correspond to CI and band of 110 kDa corresponds to CII (data not shown).

The three main bands revealed by SDS-PAGE electrophoresis appeared to correspond to the three main peaks obtained by microelectrophoresis (Fig. 1b). In particular, when the separate fractions produced by Roche were analyzed by the two methods, collagenase CI corresponded to band A and C of SDS-PAGE electrophoresis and of peak A and C of microelectrophoresis; collagenase CII corresponded to band B of SDS-PAGE electrophoresis and of peak B of microelectrophoresis.

The analysis by MALDI-TOF MS analysis of the three main bands of proteins separated by SDS-PAGE analysis allowed further characterization of their composition. The first band (A) was confirmed to match with collagenase CI (total score 389, 52 peptides out of 79 measured peptides matched sequences 1, accession number CAB69454), with a molecular weight of 114 kDa, similar to the molecular weight of CI isoform β (molecular weight 115 kDa). The second band (B) matches with collagenase CII (total score 281, 41 peptides out of 57 measured peptides matched sequences 1, accession number CAA02888, 50 ppm of mass accuracy), with a molecular weight of 101 kDa, similar to the molecular weight of CII isoform δ (100 kDa). The third band (C) matches with collagenase CI (total score 440, 53 peptides out of 67 measured peptides matched sequence 1, accession number CAB69454, 50 ppm of mass accuracy), with a molecular weight of 106 kDa, which appears not to correspond with any molecular weight of CI isoforms previously described (β = 115 kDa, α = 68 kDa, γ = 79 kDa).

DISCUSSION

There is poor information on the composition of enzymatic blends available for human islet isolation and

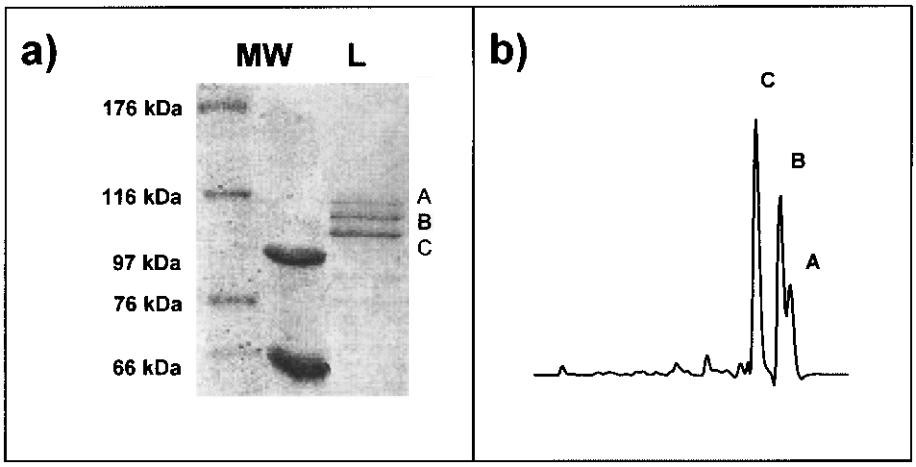

Figure 1. Liberase analyses by SDS-gel electrophoresis (a) and microelectrophoresis (b). The sodium dodecyl sulphate polyacrylamide electrophoresis gel of a Liberase™ batch representative of the batches studied (L) allowed the identification of three main bands (a: A, B, C). See their molecular weights in the scale on the left (MW). Similarly, three main peaks (b: A, B, C) in the same batch enzyme were revealed by microelectrophoresis.

this is the main obstacle for a real standardization of pancreas digestion. We isolated and sequenced here for the first time the molecular structure of the main enzymes necessary for pancreas digestion. In this analysis it appears clear that the structure of collagenase CII commonly present in an enzymatic blend for islet isolation corresponds to isoform δ (100 kDa). Questionable indeed is the interpretation of the structures of the two molecules of class I collagenase. By MALDI-TOF MS analysis the first and third bands matched with the same protein (collagenase CI), thus suggesting that the smaller protein (band C) might derive by proteolysis from the larger one (band A), as previously suggested (2). Alternatively, they correspond to two different isoforms of collagenase CI: β and one isoform with 106 kDa molecular weight (1). The existence of different isoforms of CI has been debated: it is not clear whether these isoforms are all functional, if their appearance is due to maturation of the protein or to a degradation process, or whether or not this is physiological (4). The evidence that only two collagenase genes in *C. histolyticum* (*ColG* for CI and *ColH* for CII) have been identified without any alternative-splicing transcripts thus seems to confirm the hypothesis of CI isoforms as degradation products (4).

These data should be taken together with those recently published about the relative role of each component in terms of isolation outcome (1). In fact, on the same enzyme batches we previously identified class II as determinant for isolation success, with the contribution of the bigger isoform of class I directly correlated with the purification of islet preparation. All together this information represents the real basis for a standardization of isolation procedures. We can now hypothesize the production by recombinant technique of separate enzymes and their separate storage. The expected results will be a reduction of the auto degradation process, an improved purification of enzyme blend (proper reconstituted just before the isolation), and the possibility to modify the composition of the enzyme blend according to specific protocols or organ characteristics.Final results will be the optimization of the isolation procedures, the reduction of costs, the reliability of the results, and, at the final end, the promotion of clinical islet allotransplantation activity.

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