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Biochemical Comparison Between Clostridium Hystoliticum Collagenases G and H obtained by DNA Recombinant and Extractive Procedures

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One of the main success practices in cell therapy is the transplantation of allogeneic pancreatic islets cells in patients with type 1 diabetes. However, at the moment less than 50 % of procedures are successfully completed; this is because the procedure of pancreatic islet isolation/purification, are not well standardized. This low percent of success can be ascribed to several factors, including: - the variability in donor characteristics, - pancreas recovery and preservation, - not the last, an unpredictable enzymatic blend efficiency.
At present, the process of pancreatic islets isolation, sees the use of proteolytic enzymes, collagenases (Class I and Class II) produced by Clostridium hystoliticum bacteria, and purified by extractive procedures. Limit of these enzymes lies in composition variability, such as their concentration and the presence of toxic components, together autocatalytic processes and the presence of other proteolytic enzymes.
To reduce the variability in collagenase composition in the blends usable in cell isolation, and to minimize the variability in enzyme composition, we produced the major classes of C. hystoliticum collagenases: collagenase G and collagenase H by DNA recombination techniques. Using biochemical/ezymologic approaches, we compared composition, enzymatic activity and auto-digestion processes, of the C. hystoliticum collagenases obtained using extraction procedures versus the recombinant ones.

1. Introduction

The *Clostridium hystoliticum* collagenases are enzymes which are able to digest collagen fibres and are used widely within the field of medicine as a result of their ability to disaggregate connective tissues and to facilitate the isolation of cells of interest from diverse tissues. In particular, collagenases produced from the bacteria *C. hystoliticum* are preferred to others, since they are proteolytically active practically on all mammalian collagen isoforms, even in native conformation (Breite et al, 2011; Philominathan et al, 2009). The catalytic properties of *C. hystoliticum* collagenases have been assessed, highlighting the presence of at least six main isoforms. In function to the recognized

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substrates and amino acid sequence homology, the different isoforms were grouped into class I collagenases or ColG (α, β, γ) and class II collagenases or ColH (δ, ε, ξ) (Shi et al., 2010; Matsushita et al., 1999). The isoforms having the higher molecular weight in class I and II are the β and δ respectively; from these ones is supposed other forms to be generated by autocatalytic processes.
Enzyme activity, purity, and formulation, strongly influence the outcome of the islet isolation. The composition of collagenase and other enzymes in each lot must be ideal to the task of isolating islets specifically. (Wolters et al., 1995.). Formulations with increased collagenase activity, a specific range of both neutral proteases and clostripain, and with low levels of trypsin activity may produce the most viable islets (de Haan et al., 2004).
The tryptic-like activity in enzyme blends may work in concert with the other enzymes to increase the activity of the digestion, although there is some debate about the damage induced by tryptic-like activity on the islets (Brandhorst et al., 2008).
The procedures currently used for the production of collagenases for medical use are based on the culture of C. hystoliticum and the subsequent purification thereof from all the bacterial proteins produced, by ultra filtration and chromatographic procedures. This kind of approach, however, is not sufficiently controlled due to autocatalysis processes and to deterioration of the same collagenases during storage (Johnson et al., 1996). This variability is currently considered to be one of the major obstacle for effective isolation of cells usable in cell therapy and tissues engineering; in particular, in human pancreatic islets transplantation (Kin et al., 2007). The problem regarding the variability of batches seems to be closely linked to the procedure actually used to obtain collagenases by C. histolyticum culture. In fact, this method does not allow effective control, in terms of composition of the various mixtures obtained with a consequent presence of various batches (Fermo et al., 2007). Moreover, the presence of several contaminants due to purification method, together to no uniform percentage ratios between the different collagenase classes involves little homogeneity in the protocols used for the extraction of cells and therefore a calibration of the process every time the batch change, with the consequent loss of precious biological material. In addition, even the minimal hydration accompanying the freezing-thawing cycles of the lyophilized product between different cellular isolation procedures, may cause deterioration of the collagenase function.
Recently by DNA recombinant technique we have generate class I-ColG and classII-ColH collagenases in Escherichia coli host. The recombinant molecules can be produced separately, to minimize autocatalytic processes. Moreover the enzymes contains at their amino-terminal portion the maltose binding protein (MBP) to favor stability, and can be purify by affinity chromatography (PCT WO 2011/073925 A9). The MBP-collagenases maintains their function. In this work we compared stability of recombinant collagenases to commercial ones, obtained by classical production processes, previously described.

2. Experimental

2.1 Materials

Recombinant collagenases class I and class II (Abiel srl); collagenases class I and class II obtained by extractive procedures Liberase, New Liberase, Collagenase P (Roche) and NB-1 (Serva); Termolisin (Roche) and Neutral protease (Serva).

2.2 Gelatine zymography

The different enzymes were mixed in an electrophoresis buffer devoid of reducing agents (2β-mercaptoethanol or the like); the samples also are not subjected to boiling in order to avoid the loss of enzyme activity. The samples thus prepared were stratified in polyacrylamide gel containing gelatine at a concentration of 1 mg/mL. After the electrophoresis run at 100 V for 45 min the gels were washed with a 2 % TRITON X-100 solution in H_2O containing 0.02 % NaN_3 (3 times for 20 min for each wash) (Piñeiro-Sánchez et al., 1997). They were then incubated overnight at 37 °C and then dyed with H_2O-acetic acid-methanol solution in the ratios 5:1:5 containing 0.8 % Coomassie Brilliant Blue R-250 for 3 h with stirring. The excess dye was removed using a 5 % CH_3COOH solution.

2.3 Analysis of the densitometric profiles of the lytic activity demonstrated by gelatine substrate zymography

The recombinant collagenases G and H produced by Abiel and the collagenases produced by Roche and Serva, more specifically: Liberase HP (first generation collagenases G and H, Roche), NB1 (collagenases G and H, Serva) were separated using gelatine zymography by loading between 1.25 and 10 μg/well. The various collagenases were resuspended in PBS at a concentration of 1 mg/ml and incubated at various temperatures (-20°C +4°C and at room temperature) for various numbers of days (1, 4, 7 and 18 days); any type of collagenase scarcely re-suspended (from the lyophilized form) at a concentration of 1 mg/mL in PBS was used as a control. After the electrophoretic run the gels were dyed as described above and the profiles of the lytic bands of the various collagenases obtained at various incubation times at various incubation temperatures were compared using Image 1.42 software from NIH in order to show the variability of the peaks and the pore disappearance over time. Based on these two parameters it is possible to determine greater or lesser stability of a sample compared to the others.

3. Results and Discussion

3.1 Protein and gelatinolytic activities present in analyzed samples

To evaluate the protein composition and enzymes having gelatinolytic activity several commercial samples (from Roche and Serva) and collagenase produced by us using the recombinant DNA technique were analyzed in polyacrilamide gel electrophoresis in presence of SDS (SDS-PAGE) and by gelatin zymography.

SDS-PAGE and gelatin zymography analyses showed very complex pattern, composed by several components, compared with a very limited number of peaks observed in HPLC analyses showed, by vendors, in the data sheet accompanying the specific samples. As shown in Figure 1 both the different beaches of Liberase, together the collagenase P (lines 1, 2 and 3) and NB1 (line 4) presented several proteins bands and many gelatinolytic activities, compared to recombinant ones. Surprising, the protease neutral (line 5) show gelatynolitic activities in non-canonical position; while, thermolysin seems to be very clear with only its specific enzymatic activity.

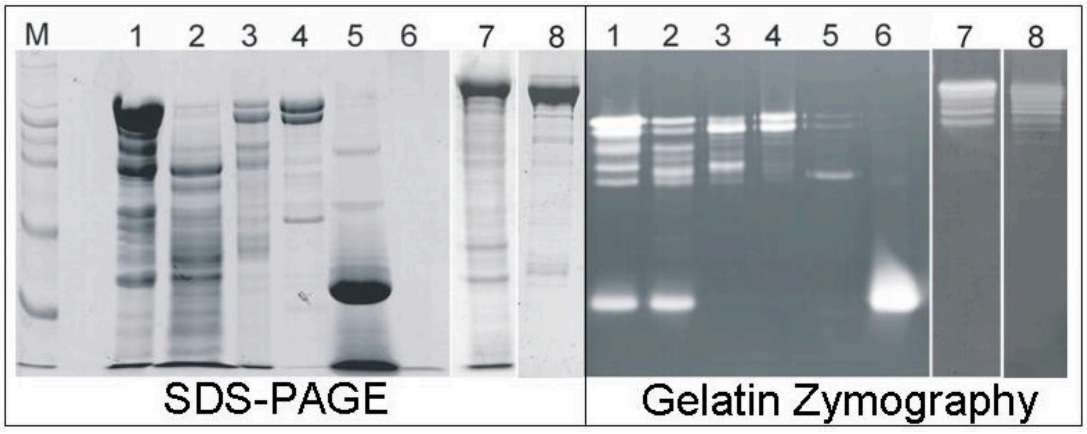

Figure 1. SDS-PAGE and gelatin zymography analyses of some commercial ezymes used in islets purification. In [A] are shown the proteins electrophoresis patters and in [B] the gelatin zymography lytic bands of the followed samples: 1- Liberase (Roche); 2- New Libersae (Roche); 3- Collagenase P (Roche); 4- Collagenase NB-1 (Serva); 5- Protease neutral (Serva); 6- Termolisine (Roche); 7- Coll G recombinant (Abiel); 8- Coll H recombinant (Abiel). In each line were loaded 20 μg of proteins for SDS-PAGE and 0.2 μg in gelatin zymography analyses. In the figure M are the markers that have a m.w (from the top to the bottom) of 205; 116; 97.4; 66; 54 and 45 kDa respectively

3.2 Different temperature stability of recombinant and extractive collagenases

In order to evaluate the stability in function of temperature the extractive commercial collagenases, already described, versus recombinant ones were analyzed by gelatin zymography when incubated at different temperature. To better value the difference of each sample in different incubation conditions the densitometric analysis of zymographic profiles were determinated.

Recombinant ColG and ColH (Abiel) together the collagenases class I and II, LIberase (Roche) and NB1 (Serva) were tested in gelatin zymography after that they were in solution at -20 °C, 4 °C or r.t. for different days. In Figure 2 are shown the gelatin zymographyes lytic patterns observed.

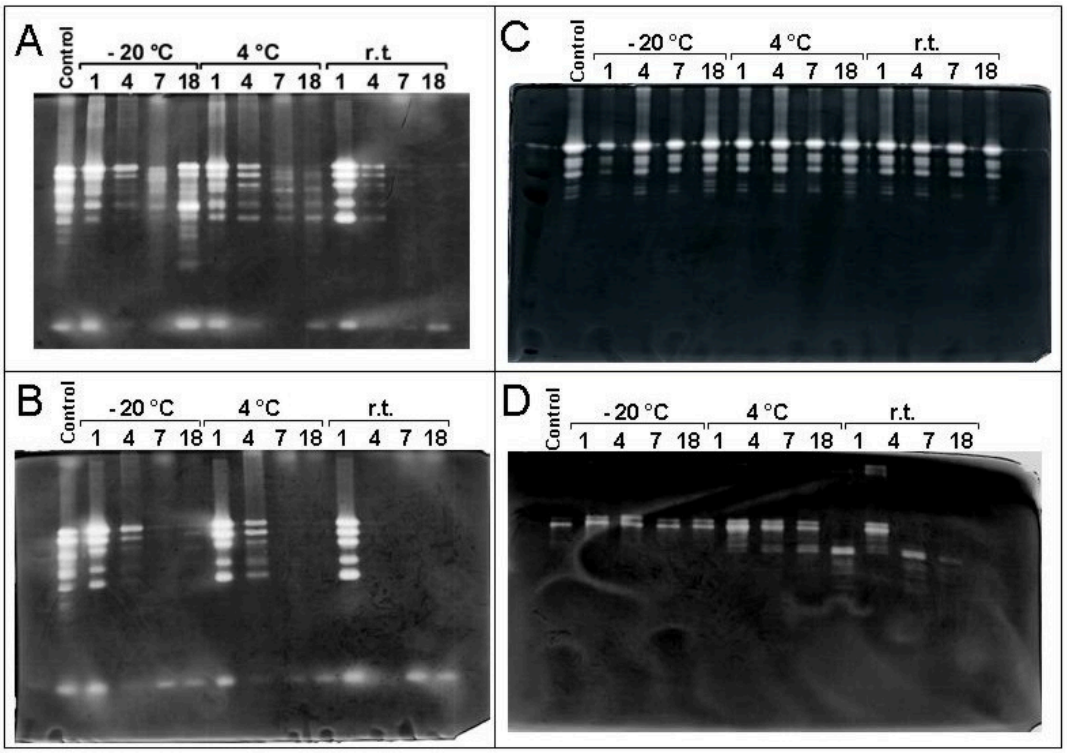

Figure 2. Gelatinolytic activities present in Liberase, NB-1, Coll G and Coll H recombinants

In [A] NB-1 (Serva); in [B] Liberase (Roche); in [C] Coll G recombinant (ABIEL Srl) and in [D] Coll H recombinant (ABIEL Srl). The experiments were performed by samples incubation at -20°C; +4°C and r.t. for different times (1; 4; 7; and 18 days). Gelatin zymographies were developed in Tris/Triton X-100 buffer containing Ca^{2+} ions at 37°C o.n.

The profiles obtained from densitometry analysis of the bands present in zymography, were compared in order to evaluate the variation of catalytic activity in each analyzed sample; such as, the presence of auto-catalysis processes, generating smaller fragments having enzymatic activity, were evaluated .
Figure 3 shows the different profiles of analyzed enzyme in the different conditions and times.

4. Conclusions

The patterns of proteins and the gelatinolytic activities observed in the different samples showed a low number of bands in recombinant collagenases respect to those obtained using extractive protocols, thus suggesting an improved enzyme stability in recombinant ones. Improved recombinant Class I stability is confirmed by its profile obtained after 18 days in solution, in which low enzymatic degradation occurs. Indeed collagenase class II appears to be little more instable, however to 4 °C didn't show any variability in enzymatic lytic profile, in long time of treatment. The reason can be found

on the different roles played by the different classes of enzymes; in fact, the class I acts on native, three dimensional substrate, and in well condition of purification is not probably for it to found permissive substrate (native collagen fibers). The class II, differently, works on linear substrate sequences and therefore is easier to found permissive substrate and induce auto catalytic processes. Moreover, the presence of maltose binding protein, especially in recombinant class II collagenase, pprobably mitigates the processes of autocatalysis that physiologically occur on enzyme present together in samples obtained from extractive processes.
This study, although preliminary, strongly suggests more stability in recombinant enzymes and the possibility to use them in highly reproducible protocols, such as the possibility to use recombinant collagenases in cell therapy and tissue engineering application.

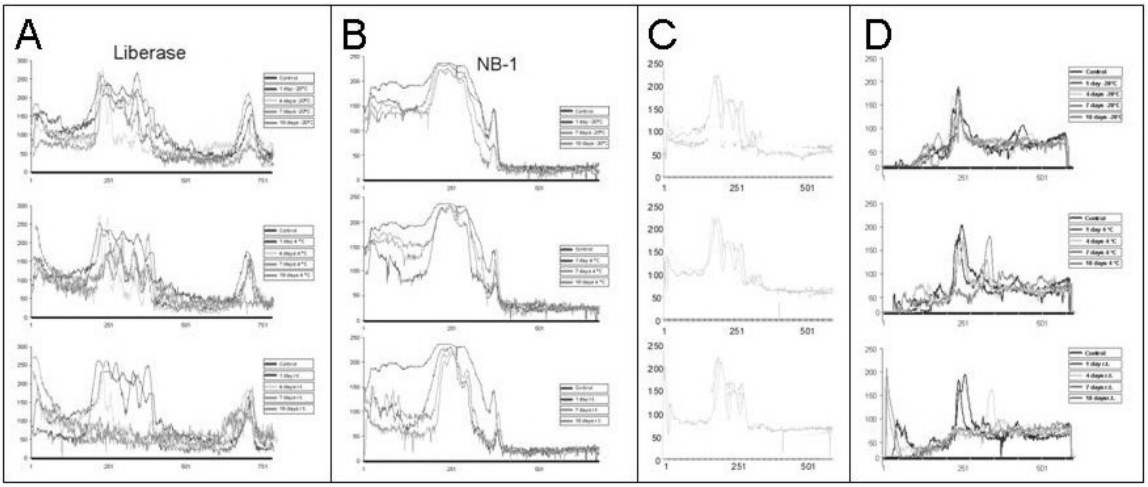

Figure 3. Densitometry profiles of gelatinolytic activities present in Liberase, NB-1, Coll G and Coll H recombinants. In [A] Liberase (Roche); in [B] NB-1 (Serva); in [C] Coll G recombinant (ABIEL Srl) and in [D] Coll H recombinant (ABIEL Srl). Densitometry analyses were performed using Image 1.42 software from NIH

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