

Tissue Dissociation and Primary Cells Isolation Using Recombinant Collagenases Class I and II

Monica Salamone^{a,c}, Silvia Saladino^b, Mariangela Pampalone^b, Simona Campora^b, Giulio Gheresi^{a,b}

^a ABIEL S.r.l., Via del Mare 3, Campobello di Mazara (TP)-Italia;

^b Dipartimento di Scienze e Tecnologie Biologiche, Chimiche e Farmaceutiche (STEBICEF), Università di Palermo, Viale delle Scienze, edificio 16, Palermo-Italia.

^c IAMC-CNR., Via del Mare 3, Campobello di Mazara (TP)-Italia;
giulio.ghersi@unipa.it

Collagenases class I (Col G) and class II (Col H) currently available for tissue dissociation are produced from *Clostridium histolyticum* (human pathogen) strains. In the processes of extraction of the cells from the tissue, combined activity of both classes of enzymes is required. CI and CII are complementary in degrading collagen. ABIEL recently produced the collagenase class I and II using the recombinant DNA technologies (PCT WO 2011/073925 A9). The enzymes were produced in *E. coli* and purified by affinity chromatography. The method of production adopted allows absolute control of the final composition of these enzymes, as well as their stability, purity, activity, absence of toxicity and higher reproducibility of batches of collagenase. The two collagenases produced separately have been used in conjunction according to precise proportions to dissociate calvaria and liver of the BALB/c mouse and bovine hoof. The analysis carried out on all isolated cell populations suggest that the cells maintain the structural and functional integrity of specific tissues/organs originating. Recombinant Col G and Col H enzymes represent a promising tool for tissue dissociation.

1. Introduction

The isolation of vital organs, as well as the dissociation of tissues and recovery of cells that constitute them, are procedures that can be ultimately the field of cell transplantation and tissue engineering. Many of the enzymes currently used in tissue dissociation procedures have limited power, and the incomplete knowledge of their mechanism of action does not help in choosing between different mining. In addition, the parameters that can affect the outcome of an extraction protocol are varied: the type of tissue or organ of departure, the species of origin, the age of the donor, and the "medium" of dissociation used, the enzymes used and their concentrations, the temperature, incubation times, etc.

Primary cells can be used for different applications. For example the Osteoblastic cells are used to study bone metabolism and biomaterial/cell interactions essential for bone tissue engineering, Chondrocyte treatment of osteochondral disorders, Hepatocyte for toxicology studies. Collagens, which distribute in skin, scale, bone, tendon, teeth, and blood vessels are the most important protein constituents of the extracellular matrix of the tissue. Collagens are resistant to most proteases except for a limited number of collagenolytic proteases. Collagenolytic proteases include metalloproteinases, serine proteases, and other proteases. *Clostridium histolyticum* is a Gram positive anaerobic pathogenic to humans as involved in the formation of gangrene. Produces a variety of collagenase in large quantities, which efficiently degrade the collagen present in the connective tissue. The *clostridium histolyticum* collagenase are metalloproteases belonging to the family M9 having collagenolytic activity (Matsushita 1994).

Different isoforms of collagenase from clostridium are divided into two classes class I (col G) e class II (col H). The various isoforms were determined by analyzing their relative activities against the native insoluble collagen and synthetic substrates. The enzymes of class I have high collagenase activity and a modest activity against peptides FALGPA and PZ, while the enzymes of class II have a modest activity against collagen and a high activity of the peptides FALGPA and PZ (Matsushita 1994). Commonly used crude enzyme preparations contain several proteases in variable concentrations, as well as a variety of polysaccharidases, nucleases and lipases. This variability is reflected in lot-to-lot variability and it represents one of the obstacle of tissue dissociation and is consequence of the lot-to-lot variability. The evaluation of the efficiency of digestion is a very delicate phase, there is not a substrate that enables accurate measurement of the activity against the collagen in the form perfectly natural, with the exception of the experiments in vivo (Brandhorst H, et al. 2008). Our goal is to obtain an enzyme blend both efficient for tissue dissociation and at the same time characterized by lot-to-lot reproducibility. On this purpose, recombinant collagenases class I and II were synthesized as described elsewhere (PCT WO 2011/073925 A9). Recombinant Col G and Col H are produced separately in fermentation process and chromatography purified. In this paper we analyzed the composition and the activity of their recombinant Col G and Col H in order to investigate the different role of class I and class II collagenases in tissue dissociation..

2. Method

2.1 Enzymatic activity assay

To quantified the Pz activity of col G and col H we use the synthetic peptide Carbobenzoxy-Gly-Pro-Gly-Gly-Pro-Ala-OH that is specifically hydrolyzed by collagenase in two fragments: the Carbobenzoxy-Gly-Pro-Gly and Gly-Pro-Ala, which reacts with the ninhydrin can be quantified by a spectroscopic method (W. Grassmann, A 1960). In this assay 1 U catalyzes the hydrolysis of 1micromol of Gly-Pro-Ala from Z-Gly-Pro-Ala (Flucka 27673) in 1 minute a pH 7,4 a 37°C. to quantified activity on insoluble collagen for tissue dissociation to we used insoluble collagen from rat tail(C-9879) The digested fragments were quantified using the ninhydrin colorimetric method (Moore, S. and Stein, W.H. 1948). In this case one CDU unit liberates peptides from collagen (C-9879) equivalent in ninhydrin color to 1.0 micromole of leucine in 5 hours at pH 7.4 at 37°C in the presence of calcium ions.

2.2 Isolation and culture of Osteoblasts

Oseoblastic cells were enzymatically isolated from calvaria of mice by method of Bakker A.D. (2012) with some modifications. Breaflly the pieces of bone derived from 2 mouse were digested sequentially with trypsin and collagenases solution. The first step consist in bone incubation for 30 minutes with 4 ml of collagenase solution ColG/ColH (2/1) 1 mg/ml in DMEM 10% FBS. Remove and add equal volume of the same solution of collagenases, for a second treatment of 30 minutes. Remove and add 4 ml of trypsin (5 mg/ml in PBS, EDTA 0.2 g / ml). After 30 minutes, replace again with 4 ml of collagenase solution for another half hour. Remove and wash 3 times the bone fragments with DMEM 10% FBS. Transfer the pieces of bone in a clean plate (100x20 mm) with 4 ml of complete DMEM, both in the presence and absence of ascorbic acid (100 microg/ml). After 15 days in culture, cells are trypsinized and plated in 24 well plate to quantified the alkaline phosphatase activity. When cell riched the confluence they were washed 3 time in PBS and incubated with 1 ml of alkaline phosphates solution (BIO-RAD, No.172-1063) for 30 min. at 37°C. After incubation plate were readed at 405 nm.

2.3 Isolation and culture of Hepatocytes

Hepatocytes were isolated from one mice by two stap collagenase perfusion using applyinf the protocol of (James R. Klaunig et al. In Vitro Vol. 10: 913-925. 1981) with same modifications: animal was anesthetize and dissect the abdominal cavity, a canula was inserted into PV and start perfusion (2 ml/min). After the first Perfusion, a second perfusion was performed with 70 ml of digestive medium with 0.01 mg/ml of Thermolysin (SIGMA) and 1 mg/ml of a mix of Collagenase G and H in rapport 2:1. The liver was collected in a petri dish with digestive medium and in a tissue culture hood dissociate the liver lobes using two pairs of forceps. Therefore, incube al 37°C for 15 minutes. After purification we count cells in a hemocytometer, calculate viability based on Trypan Blue staining and suspended pelleted cells in DMEM/F-12 medium. The cells were plated in collagen coated culture dishes and place them in incubator 5% CO2 at 37°C.

2.4 Isolation and culture of Chondrocytes

To dissociate chondrocyte from bovine hoof we use the protocol (Current Protocols in Pharmacology (2001) 12.2.1-12.2.18) with same modification. For the first step of dissociation the cartilage shavings was

weighed and transfer in a petri dishes. We digest 1,5 gr of tissue with pronase 10 mg/ml (total volume 15 ml) for 1 hr. For the second step of dissociation we use collagenases G-H blend with the optimal ratio without adding proteases . We calculate the CDU (collagen digestion unit) necessary to efficiently digest 1,5 gr cartilage shavings. We found that to digest 1,5 gr of cartilage shavings the best concentration was 3,5 mg/ml (G/H = 2/1) (total volume of 15 ml). After 3 hrs of digestion cell were purified and quantified. We obtain 13×10^6 cells from bovine hoof. Using high clostripain bacterial collagenase of Worthington Biochemical the number of cells obtained are $3 \times 10.000.000$ for bovine hoof. Obtained chondrocyte were cultivated in 2D and 3D method (Curret Protocol in Parmacology).

3. Results

3.1 Enzymatic activity on different substrates

To monitorate the activity of the purified enzyme we use different method. We found that the collagenases Col G and col H have a different affinity and efficient depending on used substrate. The Col G is more efficient to degrade native collagen and more important role in ex vivo dissociation. To analyzed the function of the single enzyme produced separately we use pz test. As Show in figure1, the Col H digests Pz peptide about 2.7 time more efficiently of Col G. Differently when we analyzed the activity against native collagen Col G show a more efficient compared with Col H (Figure 2). Moreover was interesting to see that the in enzyme blend in a G/H 2:1 ratio the enzyme showed a collaborative effect, a synergy, of the two enzyme (Figure 2)

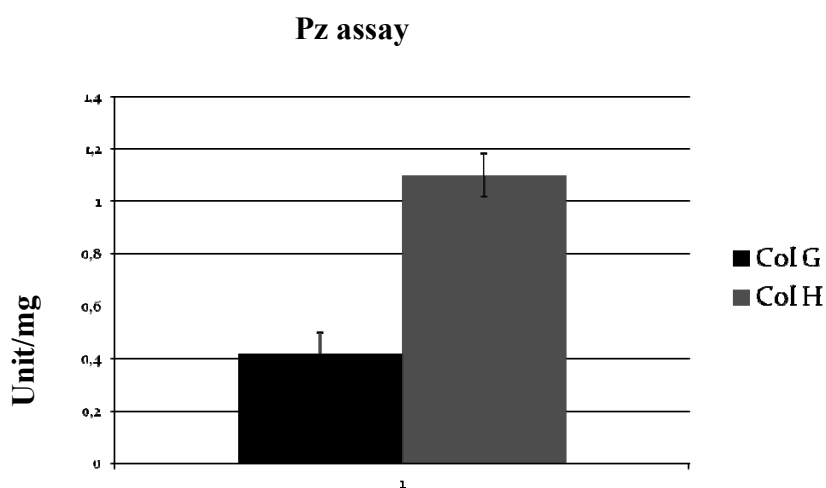


Figure 1: Enzymatic activity of recombinant collagenases on PZ peptide. One unit equals one micromole di Gly-Pro-Ala from Z-Gly-Pro-Gly-Gly-Pro-Ala in 1 minute at pH 7,5 at 37 °C.

All this data suggest that to optimized the tissue dissociation we must used a combination of Col G and Col H at 2:1 ratio in order to obtain a optimal collagen insoluble digestion. We found that the best ratio G/H was 2/1 corresponding 900 CDU/mg (Figure 2) (One CDU unit liberates peptides from collagen (C-9879) equivalent in ninhydrin color to 1.0 micromole of leucine in 5 hours at pH 7.4 at

Activity on native collagen

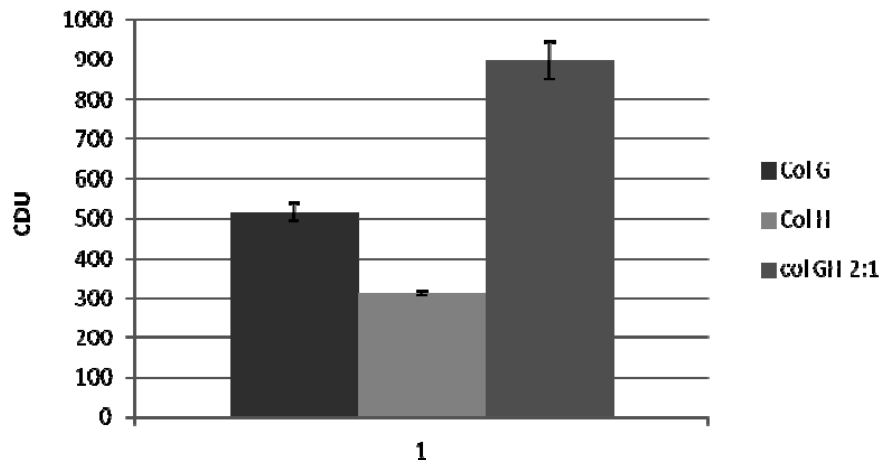


Figure 2: Enzymatic activity of recombinant collagenases bovine tendon collagen "native collagen" and Recombinant class I-II collagenases. One unit equals one micromole of L-leucine equivalents released from collagen in 5 hours at 37°C, pH 7.5, under the specified conditions

37°C in the presence of calcium ions. This data confirm the collaborative effect of Col G and Col H and predominant role of G in *in vivo* digestion.

3.2 Primary cells cultures.

To obtain an efficient tissue dissociation, we use recombinant Col G-H blend (G/H=2:1) and we add different proteases based on the specific tissue. The chondrocytes was extract using pronase as described on method. We obtain 13x 10.000.000 cells from bovine hoof. After purification cells were cultured in 2D in presence of ascorbic acid (Figure 3b). For osteoblast extraction from calvaria mouse the proteases added to collagenases was trypsin as described in method. After 30 days osteoblastes were trypsinized and transferred in 24 well plate and cultivated in presence or absence of ascorbic acid. The differentiation was evaluated by alkaline phosphatase activity. This enzyme is crucial in the synthesis of the extracellular bone matrix, because it contributes to mineralization. As show in Figure 4 we found a strong different in alkaline phosphates activity in cell grown in presence of ascorbic acid. We also use preosteoblastic cell line MC3T3 as control. Finally the hepatocytes are extracted use termolysin as proteases as show in method. After extraction cells were cultivated on collagen coated plate and showed the classical morphology with two nucleus for cell (Figure 3a).

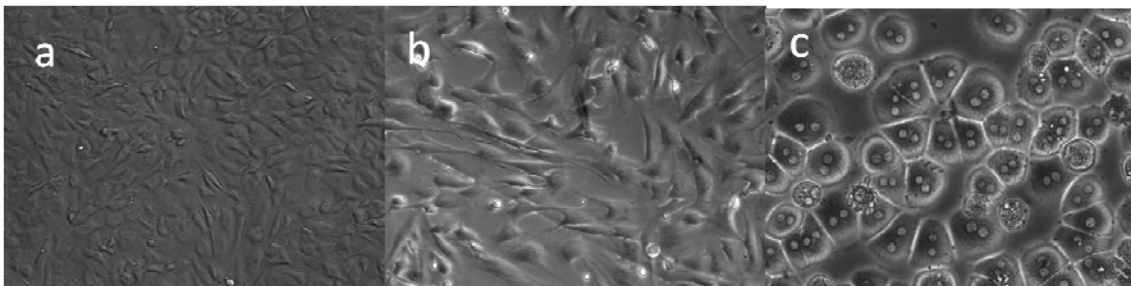


Figure 3: optical imagine of cell isolated and plated in 2D: a) Condrocyte (10X); b) Osteoblast (10X); Hepatocyte c) (10X).

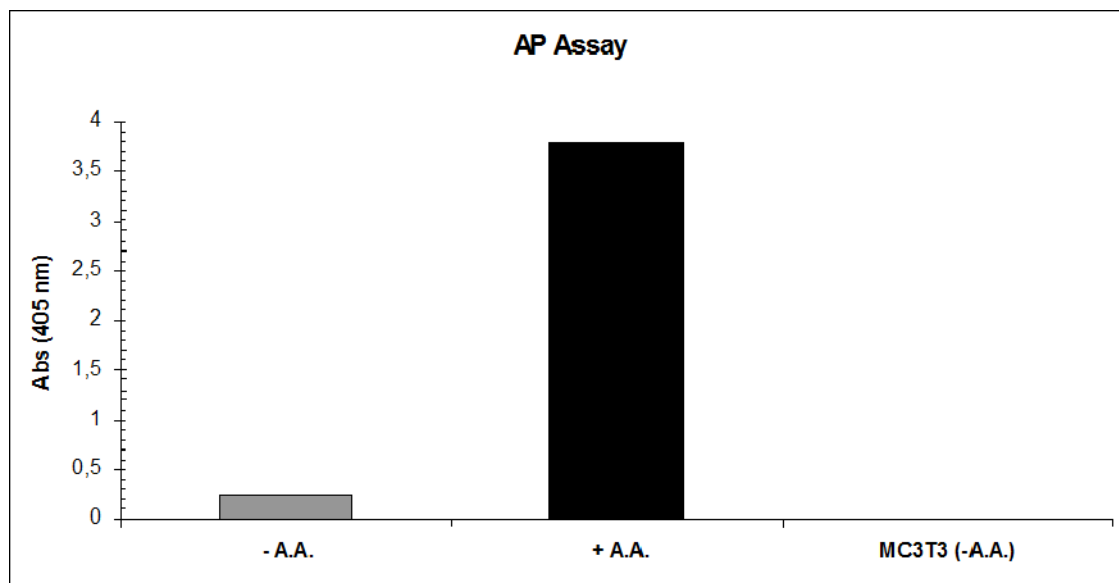


Figure 4: AP (alkaline phosphatase) Assay. AP levels measured in a spectrophotometer at of 405 nm, after 30 days osteoblasts culture of (135,000 cell/1ml in wells of a 24-well) grown in the absence of ascorbic acid (AA-); cultured in presence of ascorbic acid (AA +). MC3T3, murine pre-osteoblast cells, grown in the absence of ascorbic acid used as control.

4. Conclusion

We analyze the compositions and the activity of recombinant collagenase G and H which are produced separately in fermentation and are chromatography purified. We confirm the different specificity and the collaborative effect on in vivo digestion process. The evaluation of the efficiency of digestion is high sensitive phase, there is not a substrate that enables accurate measurement of the activity against the collagen in the form perfectly natural, with the exception of the experiments in vivo. We found that the collagenase G is more important of H in ex vivo extraction and that the best ratio to dissociate tissue is G/H= 2:1. We analyzed the best combination of recombinant COL-G and COL-H and proteases (thermolysin, pronase or trypsin) in order to obtain the best tissue dissociation. With the purpose of obtaining useful cell sources for tissue engineering studies, chondrocytes were purified from bovine hoof, and osteoblasts isolated from skullcap (calvaria) of the mouse. Moreover we optimized the procedure of extraction of hepatocyte for murine liver.

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