Short Communications

A Preliminary Characterization of a Novel Recombinant Clostridial Collagenase Blend

(collagenase / recombinant / *Escherichia coli)*

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Abstract. Clostridial collagenases are essential biotechnological tissue dissociation agents owing to their ability to cleave different types of collagen. Standardization of collagenase-based protocols has been hampered by impurities in products manufactured from *Clostridium histolyticum*. To enhance the purification process, we produced recombinant collagenase classes G and H, taking advantage of the *Escherichia coli* expression system. The respective gene sequences were derived from *C. histolyticum* and modified by addition of a C-terminal polyhistidine tag. Harvested bacteria were lysed and the collagenase protein was affinity purified using a His-tag column. The purity, identity, integrity of the eluted collagenases G and H were determined by SDS electrophoresis and Western blot. The proteolytic activity of the collagenase G and H blend (rColGH) was determined by the standard FALGPA assay. The tissue dissociation activity was verified using a standardized method for isolation of rat pancreatic islets. Biocompatibility of the blend was validated by a standardized viability assay on the isolated islets. Two batches of rColGH were produced and compared to a commercially available collagenase. Based on our results, we conclude that rColGH is a functional and non-toxic novel recombinant collagenase worth further characterization and blend optimization in order to make it a competitive commercial product.

Introduction

The enzymatic tissue dissociation techniques continue to be developed for study or further utilisation of cells or cell clusters liberated from the surrounding extracellular matrix. Collagens represent a major component of the matrix (Gelse et al., 2003). These fibrillar molecules comprise a triple helix structure, which in its native state is resistant to most proteolytic enzymes, including pepsin, trypsin or chymotrypsin (Gelse et al., 2003). However, the collagen triple helix can be specifically cleaved by mammalian matrix metalloproteinases and cysteine proteinases (Zhang et al., 2015) involved in tissue remodelling. In addition, several bacterial strains produce bacterial collagenases serving as a virulence factor (Duarte et al., 2016). Since the first isolation of collagenase (Mandl et al., 1953), *Clostridium histolyticum,* re-classified as *Hathewaya histolytica* in 2016 (Lawson, 2016), became an important biotechnological source of tissue dissociation enzymes for research and for therapeutic usage, including relief from Dupuytren's contracture, enzymatic debridement of wounds or burns (Alipour et al., 2016), and isolation of pancreatic islets for diabetes treatment (Johnson et al., 1996).

The structure and function of bacterial collagenases were recently reviewed (Zhang et al., 2015; Duarte et al., 2016). Clostridial collagenases are multidomain me-

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Abbreviations: ColG – collagenase G, ColH – collagenase H, FALGPA – N-[3-(2-furyl)acryloyl]-Leu-Gly-Pro-Ala, HBSS – Hanks' balanced salt solution, PVDF – polyvinylidene fluoride, SDS-PAGE – sodium dodecyl-sulphate polyacrylamide gel electrophoresis, rColGH – recombinant collagenase G + H blend.

talloproteins that have a single catalytic (collagenase) domain, linked by one or more polycystic kidney disease-like domains to one or more collagen-binding domains. A total of six distinct collagenase enzymes were identified in commercial crude collagenase preparations from *C. histiolyticum* (Van Wart et al., 1985). They were functionally classified into two classes, C1 (collagenases α, β, γ) and C2 (collagenases δ, ε, ζ), on the basis of differential enzymatic activity towards the substrates of native collagen and N-[3-(2-furyl)acryloyl]-Leu-Gly-Pro-Ala (FALGPA) peptide (Van Wart et al., 1985). Two collagenase genes were cloned from *C. histiolyticum, colG* for collagenase G (Matsushita et al., 1999) and *colH* for collagenase H (Yoshihara et al., 1994). All six collagenases appear to be encoded in *C. histiolyticum* by only the two genes *colG* and *colH*, which correspond to the class 1 and class 2 collagenases, respectively (Matsushita et al., 2001). Within each class, the collagenases possess an identical N-terminus encoding the catalytic domain and various truncations of the C-terminus, which is responsible for the size differences.

colG encodes a polypeptide comprising 1118 amino acids with predicted molecular weight of 126 kDa, which subsequently matures into a 114 kDa enzyme, comprising one collagenase domain, one linking domain, and two collagen-binding domains (Matsushita et al., 2001; Breite et al., 2011). *colH* encodes a polypeptide consisting of 1021 amino acids with predicted molecular weight of 116 kDa, which subsequently matures into a 112 kDa enzyme, which contains, besides the catalytic domain, two linking domains and one collagen-binding domain (Matsushita et al., 2001; Breite et al. 2011). The predicted sizes correspond well with the size determination by SDS electrophoresis (Bond et al., 1984). The domain organization of the immature collagenases is depicted in Fig. 1.

Collagenases of both classes are necessary for successful isolation of pancreatic islets (Wolters et al., 1995;

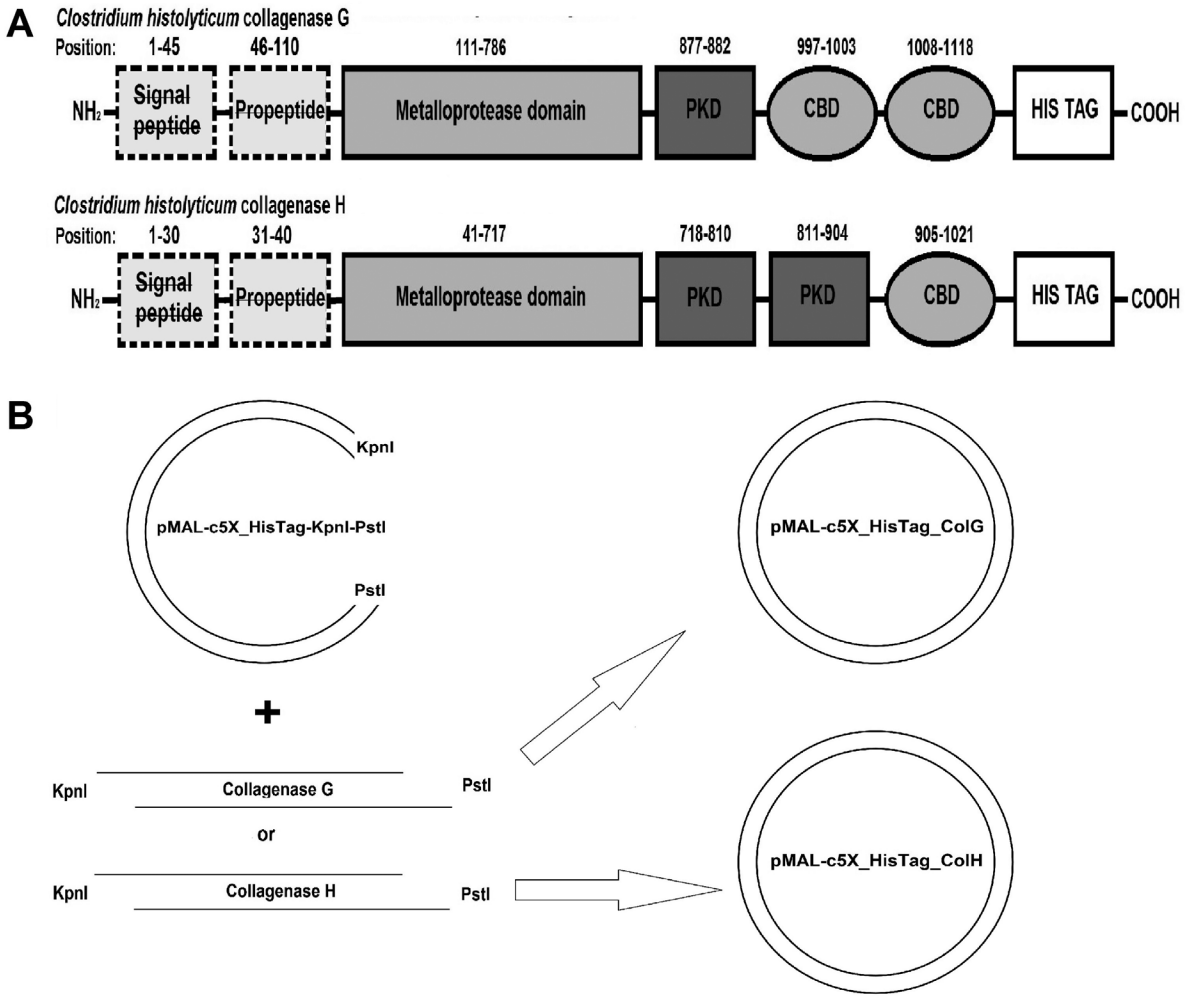

Fig. 1. Domain organization of collagenases class G (114 kDa) and H (112 kDa), with C-terminal modification for affinity purification. PKD – polycystic kidney disease-like domain; CBD – collagen-binding domain. Signal peptide and propeptide were not used in our DNA inserts. Position – position in the natural sequence; His-Tag – polyhistidine tag (**A**) and collagenase gene insertion into the appropriate plasmid (**B**).

Fujio et al., 2014). The crude collagenase from *C. histolyticum* is a complex mixture of different collagenases, neutral protease and other enzymes with tryptic-like activity. Seven types of preparations of the crude collagenase have been developed and optimized for particular tissues, differing in the relative content of non-collagenase enzymes. Because the tryptic-like activity contributes to pancreatic islet isolation, a standardized composition of the collagenase blend is crucial for obtaining reproducible results in terms of the yield and quality of the islets. Degradation of native collagenases was described during fermentation and purification steps (Balamurugan et al., 2010; Brandhorst et al., 2017; Berkova et al., 2018). The inconsistent presence of other proteolytic activities of clostripain (Loganathan et al., 2018) also contribute to the batch-to-batch variation. The batch variability was reduced but not completely eliminated by refinement of the manufacturing process (Loganathan et al., 2020), and by detailed quality testing of the enzyme blends (Jahr et al., 1999; Brandhorst et al., 2010).

Development of recombinant collagenases may theoretically overcome the above-mentioned problems. The bacterial strains tested so far for the enzyme production include *Grimontia hollisae* (Tanaka et al., 2020) or *Escherichia coli* (Ducka et al., 2009). The separate expression of ColG and ColH polypetides in *E. coli* facilitated the blend optimization (ratio between class I and II collagenases, addition of neutral proteases), thus improving the product performance in comparison to the crude collagenase from *C. histolyticum* (Balamurugan et al., 2016). It was possible to manufacture milligram amounts of high-grade protein free of contaminants (Ducka et al., 2009). A recombinant collagenase was also suitable for isolation of human islets (Brandhorst et al., 2003).

Our aim is to produce a blend of recombinant clostridial collagenases optimized for isolation of pancreatic islets. The purpose of this study was to achieve separate expression and basic biochemical and functional characterization of the protein.

Material and Methods

Preparation of DNA sequences

Each collagenase gene was subcloned into pMAL-c5x plasmid (New England Biolabs, Ipswich, MA) with added His-tag. See Supporting information for DNA sequences of PCR primers and collagenase inserts. The genomic DNA of *C. histolyticum* CCM 8656 was isolated by a NucleoSpin Microbial DNA kit (Macherey-Nagel, Düren, Germany). The genes for collagenases G (3357 bp) (Matsushita et al., 1999) and H (3066 bp) (Yoshihara et al., 1994) without signal peptide and propeptide and with His-tag (with predicted molecular weight of ColG 115 kDa and ColH 113 kDa) were amplified by PCR amplification using gene-specific primers and Q5 High-Fidelity DNA Polymerase, Q5 Reaction Buffer and Deoxynucleotide solution mix, all from New England Biolabs, and PCR thermal cycler (Veriti 96-Well Thermal Cycler, Applied Biosystems, Foster City, CA), while introducing the restriction sites for *Kpn*I and *Pst*I. The PCR products *Kpn*I-ColG-*Pst*I and *Kpn*I-ColH-*Pst*I were purified using a QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). pMAL-c5x plasmid was modified between the restriction sites *Nde*I and *Sbf*I by subcloning the His-tag together with the restriction sites *Kpn*I and *Pst*I using an In-Fusion HD Cloning Kit (Clontech, Mountain View, CA), resulting in the pMAL-c5X_HisTag-*Kpn*I-*Pst*I construct. The individual collagenase PCR products *Kpn*I-ColG-*Pst*I and *Kpn*I-ColH-*Pst*I were cloned using the In-Fusion enzyme into pMAL-c5X_HisTag-*Kpn*I-*Pst*I, resulting in pMAL-c5X_HisTag_ColG and pMAL-c5X_HisTag_ColH plasmids. NEB Express competent *E. coli* cells (Clontech) were transformed with the respective plasmids. Four positive clones (ColGa-d and ColHa-d) were verified for each construct by full insert length sequencing. The clones ColGa and ColHa were used for the protein expression.

Protein expression and purification

Bacterial clones ColGa and ColHa were cultured in LB medium (Carl Roth, Karlsruhe, Germany) with 2% glucose (Sigma-Aldrich, St. Louis, MO) and 50 µg/ml ampicillin (Serva, Heidelberg, Germany) at 37 °C, and agitated at 260 rpm. Protein expression was induced at absorbance A = 0.8 by addition of 0.5 or 0.25 mM IPTG (Sigma-Aldrich) for 4 h at 30 °C or 25 °C, respectively. Bacterial cells were sonicated in a His-Tag-binding buffer containing 50 µg/ml DNase (Roche, Rotkreuz, Switzerland) and 1 mg/ml lysozyme (Serva). The supernatant was purified on a His-Tag Purification Resin, cOmplete (Merck, Munich, Germany). The collagenase in the eluent was quantified using the BCA protein assay (Thermo Fisher Scientific, Waltham, MA). Transfer of the purified protein into Hanks' balanced salt solution (HBSS) was performed using a 50 kDa Amicon filter (Merck, Darmstadt, Germany). The protein solutions of ColG and ColH were stored in aliquots at −20 °C for one month.

SDS PAGE and Western blot

Samples were mixed with 4x Laemmli loading buffer containing 8% SDS, 40% glycerol, 0.02% bromophenol blue, 250 mM Tris, and 20% 2-mercaptoethanol (all from Sigma-Aldrich), pH 6.8, heated at 95 °C for 3 min, and run in 15% polyacrylamide gel and transferred to PVDF membranes (Merck Millipore, Burlington, MA) using a Pierce G2 electroblotter (Thermo Fisher Scientific). The membranes were blocked with 3% BSA (Sigma-Aldrich). Primary polyclonal antibody rabbit anti-collagenase ab182916 (Abcam, Cambridge, UK) was used at 1 : 10,000 dilution. The secondary antibody goat anti-rabbit IgG-HRP (Merck Millipore) diluted to 1 : 50,000 was used. Chemiluminescent SuperSignal West Pico Plus chemiluminescent substrate (Thermo Fisher Scientific) was used for detection. The signals

were acquired using G:BOX Chemi XR5 (Syngene, Cambridge, UK).

Assessment of collagenase activity in vitro

The collagenase activity was measured using the FALGPA substrate reaction. One hundred µl of 2mM FALGPA substrate was mixed with 90 µl of reaction buffer (50 mM Tricine, 10 mM $CaCl_2$, 400 mM, pH = 7.5) and 10 µl of collagenase sample at the following concentration (rColGH-1, 0.42 mg/ml; rColGH-2, 0.44 mg/ml; Control 1 mg/ml). Reaction kinetics was measured using an Epoch ELISA reader (Biotech, Prague, CZ) for 30 min under the following conditions: in 20 s intervals at 345 nm and 37 °C, according to the manufacturer's protocol. Factor 3.9 was used to convert FALGPA units into PZ units (Wünsch) according to Lockhart et al. (2015). The measurements of collagenase concentration and FALGPA activity were carried out in three replicates (N = 3).

Isolation of rat pancreatic islets

All experimental protocols were approved by the Experimental Animal Welfare Committee of the Institute for Clinical and Experimental Medicine and the Ministry of Health of the Czech Republic in accordance with the European Union Council Directive 86/609/EEC. Male outbred rats (Wistar or Sprague-Dawley) 5–6 months old were used in this study. Rat islet isolation was performed using the method standardized in our laboratory at the Institute for Clinical and Experimental Medicine (Kriz et al., 2011; Girman et al., 2015). Briefly, the pancreas was distended with intraductal injection of 15 ml collagenase in HBSS. A commercial collagenase (C9263, Sigma-Aldrich) was used as a standard at the concentration 1.0 mg/ml. Digestion was carried out at 37 °C and the digestion time was measured as the one of the endpoints to evaluate the efficiency of collagenase. Determination of digestion end was defined as the time when the distended pancreas disintegrates to very small pieces homogenously dispersed throughout the whole volume. After purification on a discontinuous gradient of Ficoll (Sigma-Aldrich), the purity and the number of isolated islets was assessed. Purity of isolated islets was assessed under the microscope before counting the islet yield as the ratio of exocrine tissue and pancreatic islets expressed as a percentage. Viability was determined using dead and live cell staining with acridine orange (2.5 µg/ml) and propidium iodide (50 µg/ml), respectively, in three replicates (N = 3).

Results and Discussion

The recombinant collagenases, classes G and H, were affinity purified and blended (1 : 1 ratio mg/mg). Two different batches were produced and tested. Each batch was characterized and compared to a commercially available product, as described below.

Identity and integrity of purified recombinant clostridial collagenases G and H

The collagenases classes G and H were each produced by separately transformed bacteria and subsequently affinity purified. For each class, the SDS PAGE revealed only a single strong band of the expected molecular size for ColG (115 kDa) and ColH (113 kDa), as depicted in Fig. 2A. The identity of the collagenase was further verified by a specific polyclonal anti-collagenase antibody on Western blot, where the correct sizes were found, as shown in Fig. 2B. No proteolytic degradation products were observed, demonstrating the integrity of the respective purified proteins.

Enzymatic activity of blended collagenase rColGH in vitro

Both affinity purified recombinant collagenases classes G and H were produced and purified separately and then blended (1 : 1 ratio mg/mg) with a final concentration to serve as two different batches. Both batches were

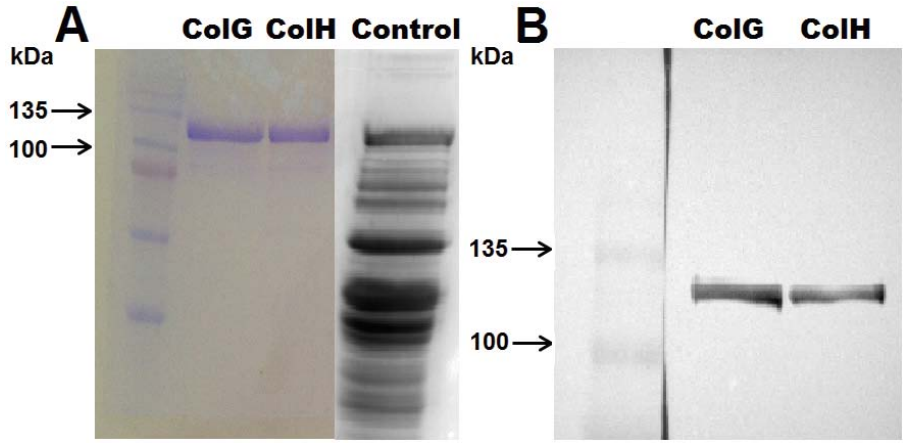

Fig. 2. Characterization of the His-tag column eluents of the lysates from the clones ColG (115 kDa), ColH (113 kDa) and control crude collagenase by SDS-electrophoresis (**A**) and Western blot (**B**).

produced, characterized, and compared to commercially available products, as described below. Final concentrations for isolation of rat pancreatic islets was rColGH-1 0.42 mg/ml; rColGH-2 0.44 mg/ml; Control 1 mg/ml. In the first experiments, we used a Collagenase Activity Colorimetric Assay Kit (Biovision, Milpitas, CA) at 37 °C according to the manufacturer's protocol. The temperature for pancreas digestion in the islet isolation protocol was also 37 °C. The specific activities of the blends rColGH-1 and -2 were 2.5 and 4.3 FALGPA U/mg protein, respectively (i.e., 0.28 and 0.46 U PZ, Wünsch)/mg protein, respectively).

Application of rColGH to isolation of pancreatic islets

A standard procedure for isolation of rat pancreatic islets was selected to demonstrate the tissue dissociation capacity of the blended recombinant collagenase. Two subsequent collagenase blends rColGH-1 and rColGH-2 were compared to a commercially available standard, which is routinely used for islet isolation in our lab. The final concentrations for isolation of rat pancreatic islets were 0.42 mg/ml (rColGH-1), 0.44 mg/ml (rColGH-2), and 1 mg/ml (Control). Four criteria were assessed, including the time necessary for the pancreas tissue digestion, the total yield of islets, the islet purity, and the islet viability. The results are summarized in Table 1. In comparison to the standard 12 min digestion time, a slower release of islets (17 and 20 min) was observed with the respective blends rColGH-1 and rColGH-2. Similarly, a lower islet yield per rat body weight of islets per rat was obtained using rColGH-1 and rColGH-2 (400 and 550 islets) as compared to the standard (750 islets). Also, the viability of the islets was lower with rColGH-1 and rColGH-2 (83 and 93 %) compared to the standard collagenase (98 %). On the other hand, semi-quantitatively assessed purity of the islets isolated by the recombinant collagenases was higher (+++) in comparison to the control (++) (Table 1).

These results were obtained for two subsequent batches that were blended at the same ratio (1 : 1), which, however, may not be optimal for the purpose of islet isolation (Loganathan et al., 2019). Moreover, the prepared collagenase blends contained no other proteolytic enzymes such as clostripain and neutral protease, which are present in commercial collagenases from *C. histolyticum*. Specification of the control collagenase was 2 FALGPA U/mg; 175 caseinase U/mg; 1.2 clostripain U/mg; 0.28 tryptic activity U/mg (datasheet information). Addition of other proteolytic enzymes to the collagenase blend significantly improves the islet yield and quality (Stahle et al., 2015; Balamurugan et al., 2016). It may therefore be expected that optimization of the enzyme blend can produce better results in the future.

Conclusion

Here, we present our first biochemical and functional characterization of two batches of new recombinant, affinity purified collagenases G and H. Our preliminary data demonstrate the basic integrity of the two constituent collagenase proteins and their effectiveness in isolation of pancreatic islets. We conclude that these novel recombinant collagenases are worth further characterization and blend optimization, which are under way.

Author contributions

Conceived project and designed experiments: TK, ZB, FS. Performed experiments: IL, KB, ZB, AL. Analysed the data: TK, ZB, DH, IL. Wrote the manuscript: DH, IL, TK, ZB. Contributed chemical reagents: MB.

Acknowledgement

Clostridium histolyticum CCM 8656 (Patent No. CZ2017537A3) at the Industrial Property Office, Czech Republic, related to *C. histolyticum* DSM 2158 (Leibniz Institute, DSMZ – German Collection of Microorganisms and Cell Cultures, GmbH, Braunschweig, Germany), was obtained from MB Pharma, Prague, Czech Republic.

Table 1. Functional comparison of rColGH to a commercial collagenase

Collagenase	**Control**	**rColGH-1**	**rColGH-2**
Specific activity [(FALGPA U)/mg protein]	2.0*	2.6	4.1
Activity in final solution [FALGPA U/ml]	2.0*	1.1	1.8
Concentration of final solution [mg/ml]	1	0.42	0.44
Digestion time [min]	12	17	20
Number of islets per rat	750	400	550
Islet purity	++	+++	+++
Viability [%]	98	83	93

*datasheet information

Supplemental information

Primers:
Collagenase1FUS_F1 – CAGGGTTCGGCATCGATAGCGAATACTAATTCTGAGA
Collagenase1FUS_R1 – ATGGTGACCAGAACCTTTATTTACCCTTAACTCATAG
Collagenase2FUS_F1 – CAGGGTTCGGCATCGGTACAAAATGAAAGTAAGAG
Collagenase2FUS_R1 -ATGGTGACCAGAACCTCTTCCTACTGAACCTTCTATATTAATTCTATATGG

Collagenase G:
ATAGCGAATACTAATTCTGAGAAATATGATTTTGAGTATTTAAATGGTTTGAGCTATACTGAACTTACAA
ATTTAATTAAAAATATAAAGTGGAATCAAATTAATGGTTTATTTAATTATAGTACAGGTTCTCAAAAGTT
CTTTGGAGATAAAAATCGTGTACAAGCTATAATTAATGCTTTACAAGAAAGTGGAAGAACTTACACTG
CAAATGATATGAAGGGTATAGAAACTTTCACTGAGGTTTTAAGAGCTGGTTTTTATTTAGGGTACTATA
ATGATGGTTTATCTTATTTAAATGATAGAAACTTCCAAGATAAATGTATACCTGCAATGATTGCAATTCA
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CAGAATAACTTACGGACAAGTTGATGGTAATAAGGTATCTAATAAAGTTAAATTAAGACCAGGAAAATA
TTATCTACTTGTTTATAAATACTCAGGATCAGGAAACTATGAGTTAAGGGTAAATAAA

Collagenase H:

GTACAAAATGAAAGTAAGAGGTATACAGTATCATATTTAAAGACTTTAAATTATTATGACTTAGTAGATT
TGCTTGTTAAGACTGAAATTGAGAATTTACCAGACCTTTTTCAGTATAGTTCAGATGCAAAAGAGTTCT
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CGGAGGAGCTACTTGGGTAGTATATGATGAAAATAATAATGCAGTATCTTATGCCACTGATGATGGGCA
AAATTTAAGTGGAAAGTTTAAGGCAGATAAACCAGGTAGATATTACATCCATCTTTACATGTTTAATGG
TAGTTATATGCCATATAGAATTAATATAGAAGGTTCAGTAGGAAGA

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