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Collagenase-assisted wound bed preparation: An in vitro comparison between *Vibrio alginolyticus* and *Clostridium histolyticum* collagenases on substrate specificity

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Bacterial collagenase from the aerobic non-pathogenic *Vibrio alginolyticus* chemovar *iophagus* is an extracellular metalloproteinase. This collagenase preparation is obtained through a fermentation process and is purified chromatographically, resulting in a highly purified 82-kDa single-band protein that does not contain non-specific proteases or other microbial impurities. *V. alginolyticus* collagenase was added to a hyaluronan (HA)-based device to develop a novel debriding agent to improve the treatment of ulcers, necrotic burns, and decubitus in the initial phase of wound bed preparation. In this study, an in vitro biochemical characterisation of *V. alginolyticus* collagenase versus a commercial preparation from a *Clostridium histolyticum* strain on various dermal extracellular matrix (ECM) substrates was performed. *V. alginolyticus* collagenase demonstrated its ability to carry out the enzymatic cleavage of the substrate, allowing a selective removal of necrotic tissues while sparing healthy tissue, as reported in clinical studies and through routine clinical experience. in vitro tests under physiological conditions (pH, presence of Ca++, etc.) have demonstrated that *V. alginolyticus* collagenase exhibits very poor/limited non-specific proteolytic activity, whereas the collagenase preparation from *C. histolyticum* is highly active both on collagen and on non-collagenic substrates. This finding implies that while the *V. alginolyticus* enzyme is fully active on the collagen filaments that anchor the necrotic tissue to the wound bed, it does not degrade other minor, but structurally important, components of the dermal ECM. This feature could explain why collagenase preparation from *V. alginolyticus* has been reported to be much gentler on perilesional, healthy skin.

KEYWORDS

bacterial collagenase, clostridium histolyticum, hyaluronic acid, Vibrio alginolyticus, wound

1 | INTRODUCTION

Collagenase belongs to a family of zinc-dependent metalloproteinases that degrade collagen substrates that consist of a protein structure featured by a rigid left-handed triple-helical configuration[1] rich in proline, hydroxyproline, and glycine.[2,3] In humans, collagenases (matrix metallo-proteinases, MMPs) play an essential role in the modulation of various aspects of inflammation.[4]

Such enzymes are made not only by humans[5,6] but also by several bacteria, such as *Clostridium histolyticum*,[7,8] *Nocardiopsis dassonvillei*,[9] and *V. alginolyticus*.[10,11]

Bacterial collagenase preparations, such as ointments, have been successfully used in clinical practice for more than 25 years as debriding agents in chronic wounds.[12] These agents allow cleansing to remove cellular "*debris*," which often constitutes an obstacle for the migration of epidermal cells in wound re-epithelialisation.[13] In fact, bacterial collagenases perform lytic functions and selective removal of necrotic tissue,[14,15] with only minor involvement of the perilesional, healthy skin.[16,17]

Historically, the most widely used collagenase preparation was derived from a highly pathogenic strain of *C. histolyticum*.[18] This preparation represents the reference standard compared with other recently discovered collagenases. However, although *C. histolyticum*-derived collagenase is regularly used, its production is generally reported to be limited because of low purity.[19] The collagenase preparation from *C. histolyticum* (Santyl, Noruxol) is available as a hydrophobic ointment indicated for topical application of cutaneous lesions of various origins, such as chronic wounds, bedsores, and burns.

The bacterial collagenase ("Achromobacter" collagenase, EC 3.4.24.3) derived from the non-pathogenic aerobic *V. alginolyticus* chemovar *iophagus* strain has also attracted much interest within the medical community and has been recently used in the debridement of wounds in a similar ointment formulation.[20-22] *V. alginolyticus* collagenase is an 82-kDa extracellular metalloproteinase highly specific for collagen; its amino acid sequence does not bear significant similarity to other collagenases.[23]

We have recently developed a process for the isolation of such a collagenase. The aerobic fermentation process allows for the production of a highly purified enzyme lacking in non-specific proteases or other microbial contaminants, making this preparation particularly useful in clinical settings.[24]

Hyalo4 Start (Fidia Farmaceutici S.p.A, Abano Terme, Padova, Italy) is a semi-solid preparation consisting of a hydrophobic ointment containing *V. alginolyticus* collagenase and low-molecular weight hyaluronic acid (LMW-HA, 100-400 kDa). The product combines the collagenolytic activity of the novel collagenase with the wound-healing properties of hyaluronic acid for the topical treatment of wounds, bedsores, burns, and ulcers.[25-27] Furthermore, from a clinical standpoint, the product has been reported as a safer formulation compared with its competitors because it does not damage the perilesional skin and does not cause dermatitis, pain, or redness.[21,22] In fact, to avoid any damage to the perilesional skin, competitor products envisage confining the wound area where the product is applied with a zinc-oxide paste to create a physical barrier that

Key Messages
- in this study, in vitro biochemical characterisation of various dermal extracellular matrix (ECM) substrates of *Vibrio alginolyticus* collagenase versus a commercial preparation from a *Clostridium histolyticum* strain was performed
- *V. alginolyticus* collagenase demonstrated its ability to carry out enzymatic cleavage, allowing a selective removal of necrotic tissues while sparing healthy tissue, as reported in clinical studies and by routine clinical experience
- in vitro tests under physiological conditions (pH, presence of Ca++, etc.) have demonstrated that *V. alginolyticus* collagenase exhibits very poor/limited non-specific proteolytic activity
- while the enzyme is fully active on the collagen filaments that anchor the necrotic tissue to the wound bed, it does not degrade other minor, but structurally important, components of the dermal ECM

prevents the contact of the ointment preparation with the surrounding healthy skin.[28-31]

To investigate findings that support clinical evidence of Hyalo4 Start in improving the wound-healing process and the protection of perilesional skin, we evaluated the efficacy of available *V. alginolyticus* and *C. histolyticum* collagenase preparations.[32] The working hypothesis is that both enzymes are equally active on the specific substrate (i.e., type I-III collagen) but not as active on other minor components of the dermal extracellular matrix (ECM). Therefore, by preserving such structurally important elements because of the less-aggressive behaviour of *V. alginolyticus* collagenase, a lower rate of clinical complications in the healing of chronic ulcers would be expected.

To test this hypothesis, under the same physiological conditions, we used in vitro testing to assess the purity, enzymatic activity, and specificity towards each selected substrate of the dermal ECM of purified soluble preparations/extracts of the two enzymes from *V. alginolyticus* and *C. histolyticum*. Then, we compared the outcomes of each enzymatic digestion.

2 | MATERIALS AND METHODS

2.1 | Bacterial strain and collagenases

Microbial collagenase ("Achromobacter" collagenase, EC 3.4.24.3) is produced by the non-pathogenic aerobic

bacterium *V. alginolyticus* chemovar *iophagus* (NCIMB Number: 11038, synonym LMG 3418, hereinafter referred to as *Vibrio alginolyticus*). The manufacturing process results in high yields of collagenase with a stable, reproducible, and economical fermentation.[24]

A standard soluble *C. histolyticum* collagenase preparation (NB4, cat. no. 17454, SERVA Electrophoresis GmbH, Heidelberg, Germany) was used in this study, and this preparation is assumed to be representative of the product currently available on the market.

Given that we are dealing with purified enzymes, the comparison of the two preparations has been made on a weight-to-weight basis (same mg of the enzyme/test sample).

2.2 | *V. alginolyticus* collagenase manufacturing process

Production and purification of collagenase from *V. alginolyticus* cultures has been described previously[24] and is briefly summarised in the following sections.

2.2.1 | Preparation of inoculum

A 1.5-mL ampoule of *V. alginolyticus* (from Working Cell Bank) is inoculated into a 5-L Erlenmeyer flask containing 2 L of culture medium consisting of a solution of 1.21 g/L TRIS, 23.4 g/L NaCl, 0.29 g/L $CaCl_2$, and 15 g/L peptone of non-animal origin dissolved in distilled water (Millipore milliQ). The pH of the culture medium is adjusted to 7.1 with HCl and sterilised in the autoclave at a temperature of up to 122°C for 30 minutes. The culture is grown at 30°C under stirring at 150 rpm until the optical density at 600 nm (OD600) reaches a value of 3 (approximately 16 hours). The inoculum is then ready to be transferred to the main 1500 L fermenter (TECNinox s.r.l, Noceto [PR], Italy).

2.2.2 | Fermentation

The fermentation broth is the same as that used for the preparation of inoculum with the addition of 0.25 g/L of antifoam 204 (Sigma, St. Louis, Missouri) and sterilised as previously noted. A volume of 2 L of inoculum is transferred into the fermenter containing 800 L of fermentation broth. The following fermentation parameters are employed: temperature 30 ± 1°C, stirring 100 rpm, air 10 to 80 Nm^3/h, dissolved oxygen greater than 50%, pH 7.1 ± 0.1, and pressure 0.4 bar. Collagenase is an extracellular enzyme; therefore, it is secreted in the culture medium. Collagenase activity is determined spectrophotometrically using the modified Wunsch–Heidrich method.[33] When the final enzymatic activity is greater than 25 000 nkat/L (on average after

16 hours), the fermentation is terminated, and $CaCl_2$ is added to a final concentration of 1.47 g/L to stabilise the enzyme. Finally, the temperature is lowered to 8°C under stirring at 300 rpm for approx. 20 minutes.

2.3 | *V. alginolyticus* collagenase purification

Briefly, 800 L of fermented culture broth is transferred to a Holder Sartocon II ultrafiltration system (Sartorius, Gottingen, Germany) equipped with five cassettes of modified PolyEtherSulphone (PES) membrane (Sartorius) with a MW cut-off of 300 kDa. The clarified culture medium is concentrated by ultrafiltration using an UF-A-P0971 ultrafiltration system (PALL, Port Washington, New York) equipped with 10-kDa cut-off PES membrane (PALL). The 20-fold concentrated medium (40 L) was dialysed against 10 mM $CaCl_2$, 25 mM TRIS–HCl, and pH 7.1 buffer.

The solution from the previous step underwent a first purification by weak anion-exchange chromatography using a column prefilled with DE-52 resin (DEAE: diethylaminoethyl cellulose, Whatman, Maidstone, UK). Collagenase activity is recovered within 3 to 5 bed-column volumes (BV) eluted with 700 mM NaCl, 10 mM $CaCl_2$, 300 mM TRIS–HCl, and pH 7.1 buffer. The raw enzyme then undergoes a strong anion-exchange chromatography by using a column pre-filled with Source 15Q resin (GE Healthcare, Chicago, Illinois). The collagenase is eluted from the column with 10 mM $CaCl_2$, 300 mM Tris–HCl, and pH 7.1 buffer. All chromatography runs were monitored using a UV–vis detector (GE Healthcare) at 280 nm. The individual fractions eluted from each chromatographic column were subjected to an enzyme activity assay[33,34] and analysed by Sodium Dodecyl Sulphate–PolyAcrylamide Gel Electrophoresis (SDS-PAGE).

Pooled fractions exhibiting collagenolytic activity underwent ultrafiltration by the Cogent system (Millipore, Burlington, Massachusetts) equipped with 30 kDa cut-off modified PES membranes (Millipore) against 10 mM $CaCl_2$, 25 mM TRIS–HCl, and pH 7.1 buffer. Routinely, a purified *V. alginolyticus* collagenase preparation from an 8-L solution exhibits 700 nKat/mL enzymatic activity.

2.4 | Ultra-high-pressure fast size exclusion chromatography (UPLC-SEC)

UPLC-SEC (ACQUITY UPLC H-Class Bio System equipped with ACQUITY UPLC BEH200 SEC 200 Å, 1.7-μm column, Waters, Milford, Massachusetts) was employed to trace enzyme dimers, higher molecular weight products, and collagenase degradation products (altogether considered as impurities) using 99.5% purified *V. alginolyticus* collagenase as an internal standard.

Separation was accomplished using isocratic elution in 150 mM NaCl, 20 mM Na-Phosphate buffer pH 6.8. The following parameters were used: sample injection volume, 5 μL (0.5 mg/mL); flow rate, 0.5 mL/min; temperature, 30 ± 1°C; total run time, 8 minutes; and wavelength, 214 nm (TUV ACQUITY UPLC Detector, Waters).

2.5 | Electrophoresis on polyacrylamide gel (SDS-PAGE)

Protein content and purity were verified using the Laemmli method[35] with 12% polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE) using a Mini-PROTEAN 3 (BIO-RAD, Hercules, California) following the supplier's instructions. The molecular weight of the purified *V. alginolyticus* enzyme was estimated by comparison with high molecular weight standard proteins (250 to 10 kDa, All Blue Standards, BIO-RAD). SDS-PAGE gels were stained with Coomassie Brilliant Blue G-250 (BIO-RAD). Images were acquired by ImageQuant 300 TL (GE Healthcare), whereas qualitative and quantitative analyses were performed using the instrument's software.

2.6 | Collagenase assay

2.6.1 | Wunsch–Heidrich modified method

The collagenase activity is determined by the enzymatic reaction according to the Wunsch–Heidrich modified method[33] against a synthetic peptide (Sigma). The reaction of aqueous solution of collagenase with the synthetic substrate PZ-L-prolyl-L-leucyl-glicyl-L-prolyl-D-arginine (where PZ = 4-phenyl azobenzyloxycarbonyl) under controlled conditions (pH, temperature, and time) produces two fragments: PZ-L-prolyl-L-leucine and glicyl-L-prolyl-D-arginine. The first fragment is determined spectrophotometrically at 320 nm after acidified ethyl acetate extraction. This reaction is specific for the collagenolytic activity, which is expressed as nanomoles of reacted substrate per second per mL of solution (nkat/mL). The specification of purified *V. alginolyticus* collagenase is 500 to 700 nKat per mL of solution.

2.6.2 | Mandl's modified method

Collagenase activity is determined according to the Mandl's modified method.[34] Briefly, 25 mg of bovine collagen (Sigma) were solubilised in 5.0 mL of 0.05 M TES ([Tris (hydroxymethyl)-methyl-2-aminoethane sulphonate]), 0.36 mM $CaCl_2$, and pH 7.5 buffer; the reaction mixture was pre-warmed at 37°C for 15 minutes. Consequently, 0.1 mL of enzyme dilution was added to the pre-warmed reaction buffer. After 5 hours, 0.2 mL of reaction mixture was transferred in a test tube containing 1.0 mL of ninhydrin-citric acid mixture (50 mL of 4% ninhydrin in methyl cellosolve together with 50 mL of 0.2 M sodium citrate, 0.71 mM stannous chloride, and pH 5.0). This mixture was heated for 20 minutes in a boiling water bath. After cooling on ice, the test sample was diluted with 5 mL of 50% n-propanol. After 15 minutes, the absorbance was read at 600 nm. The free L-leucine micromoles produced by ninhydrin reaction from an L-leucine standard curve were determined.

The collagenase activity in the sample is calculated using the following equation:

$$\text{Units/mg} = \frac{\text{micromoles L-leucine equivalents liberated}}{\text{mg enzyme in digestion mixture}}.$$

2.6.3 | Caseinase activity

Caseinase activity in collagenase solution was determined as described by Anson, M.L.[36] and by the Folin Ciocalteu Method[37] using casein (Sigma) as a substrate and evaluating the production of tyrosine following substrate digestion. One caseinase unit definition is the amount of enzyme/protein able to hydrolyse casein to produce 1.0 μmole of tyrosine at pH 7.5 and 37°C for 5 hours (colour produced by Folin & Ciocalteu reagent). *V. alginolyticus* specifications are less than 1.0 U/mL of purified collagenase preparation.

2.6.4 | Experimental conditions for specific enzymatic activity on selected dermal substrates

Bovine Achilles tendon collagen (Fluka), pig skin gelatine, human plasma fibronectin, and bovine decorin (all from Sigma) were each brought to a final concentration of 1 mg/mL with a buffer consisting of 10 mM $CaCl_2$, 25 mM TRIS–HCl, and pH 7.1.

To 50 μg of each substrate, 0.8 μg of *V. alginolyticus* collagenase or 0.8 μg of *C. histolyticum* collagenase preparation was added to yield a final volume of 60 μL. The reaction mix was incubated at 37°C for 90 minutes. Control tubes containing substrates only or collagenase preparations only were run in the same final volume of 60 μL. Tubes in which pigskin gelatine was present were inactivated and used for the Mandl's[34] modified colorimetric test. The tubes containing bovine Achilles tendon collagen were duplicated. One sample was diluted in loading buffer up to 120 μL, and 25 μL of sample/well were employed for SDS-PAGE analysis. The other sample was inactivated and used for Mandl's[34] modified colorimetric test.

A portion of the content of decorin and fibronectin tubes after 1:1 dilution in loading buffer was loaded on SDS-PAGE wells.

3 | RESULTS

3.1 | Production and purification system of the soluble autologous enzyme

A process for collagenase isolation from a selected *V. alginolyticus* bacterial strain was developed. The process uses a culture medium free of animal components, so the purification process results in a highly pure and stable end product for clinical use, as demonstrated by the single peak in the UPLC-SEC chromatogram (Figure 1). In addition, a 99% pure, 82-kDa single band can be detected by SDS-PAGE (Figure 2A). On average, the biological activity of each production batch is set as >1000 nkat/mg of protein.

3.2 | Electrophoretic profile of *V. alginolyticus* versus *C. histolyticum* collagenase

The electrophoretic profile of *V. alginolyticus* collagenase has been proven to be highly pure (>98%) and does not show contaminants even after heavy loading of the enzyme preparation in the 12,5% SDS-PAGE gel (Figure 2A). In contrast, the electrophoretic profile of the *C. histolyticum* NB4 collagenase preparation shows a double band at 110 to 100 kDa, which is identified as the collagenase enzyme, in addition to several contaminant bands at lower MW (Figure 2B).

In fact, *V. alginolyticus* collagenase employed in this study shows caseinase activity less than 1.0 U/mL. In contrast, the preparation of *C. histolyticum* collagenase (NB4) exhibits caseinase activity of approximately 250 U/mL,
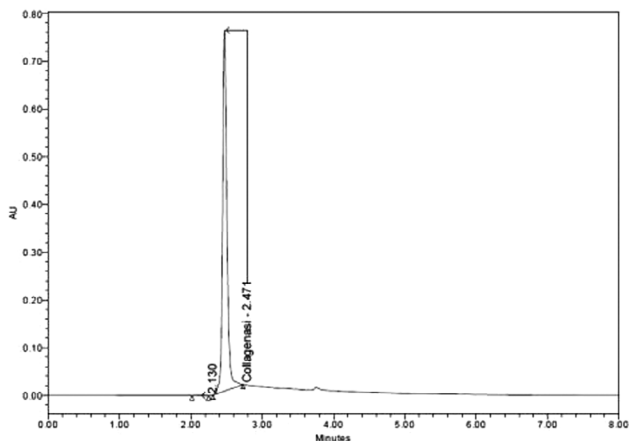
confirming the aspecific proteolytic activity of the contaminant protein pool.

The comparative results demonstrate how *V. alginolyticus* collagenase exhibits a better safety profile in pharmaceutical/biomedical applications with a considerably higher purity grade compared with other commercially available collagenase from *C. histolyticum*.

3.3 | Comparison of the specific collagenolytic activity on selected dermal substrates: collagen

In the electrophoretic profile of type I collagen in lane 1, two main bands are observed at 120 and 110 kDa. Such bands disappeared; in fact, the bands are completely digested separately by both *V. alginolyticus* and *C. histolyticum* collagenase preparations as shown in lines 2 and 4, respectively (Figure 3A).

Of note, the NB4 collagenase preparation could undergo digestion of itself in the presence of the specific collagen substrate. In fact, the characteristic 110 to 100-kDa double band is no longer detected at the end of the reaction (lane 4, Figure 3A). The test result obtained (line 4, Figure 3A) suggests the presence of an intermolecular pathway in the process of autolysis of the collagenase preparation NB4 in the presence of its digestible substrate at low concentrations, as previously reported for calcium-activated neutral protease (CANP).[38] In fact, various studies on the production of collagenase in *Achromobacter iophagus* have demonstrated how the synthesis of the enzyme itself is induced by its specific collagen substrate present in the medium. In particular, the maximum quantity of enzymatic activity that resulted was proportional to the final concentration of the inductor present in the culture medium.[39-41] Furthermore, in neutral solutions, it was reported that the pure collagenase from bacteria produces partially degraded enzymatic forms through autodigestion that are still active towards the native collagen.[42] In addition, this process of autodigestion of collagenase seems to be similar to that described for some proteins, which exhibit proteolytic activity accompanied by inherent autolytic serine protease-like activities.[43-45] On the other hand, the process of the autodigestion of the collagenase enzyme NB4 in the presence of collagen type I could also result in the enzymatic activity of aspecific proteases present in the analysed preparation.[46]

According to the colorimetric assay, both collagenase preparations digest type I collagen from bovine Achilles tendon and collagen in pig skin gelatine to the same extent (Figure. 3B). Therefore, such enzymes are equally active on the native substrate and on the denatured, more complex matrix of pigskin gelatine.
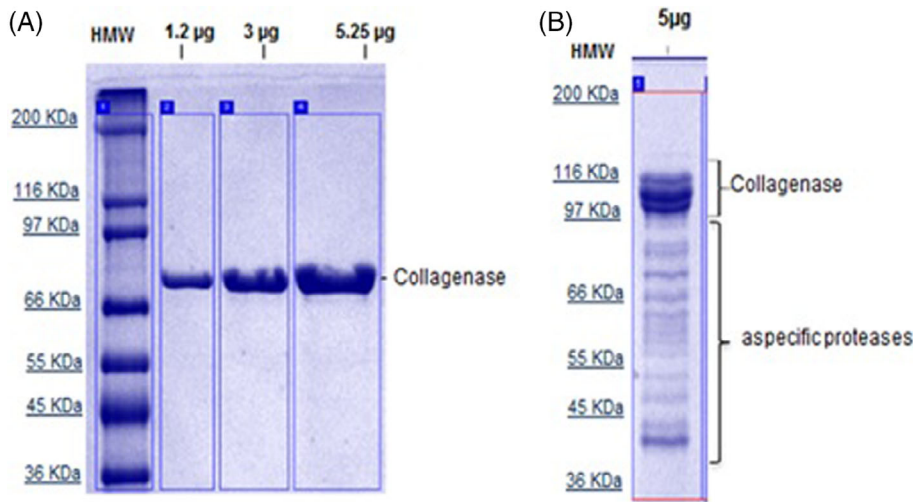

FIGURE 1 UPLC-SEC chromatogram of the *V. alginolyticus* collagenase product after purification

FIGURE 2 Purity by 12,5% SDS-PAGE and detection in Coomassie Gel Blue stain. A, Electrophoretic profile of *Vibrio alginolyticus* collagenase; B, Electrophoretic profile of *Clostridium histolyticum* collagenase
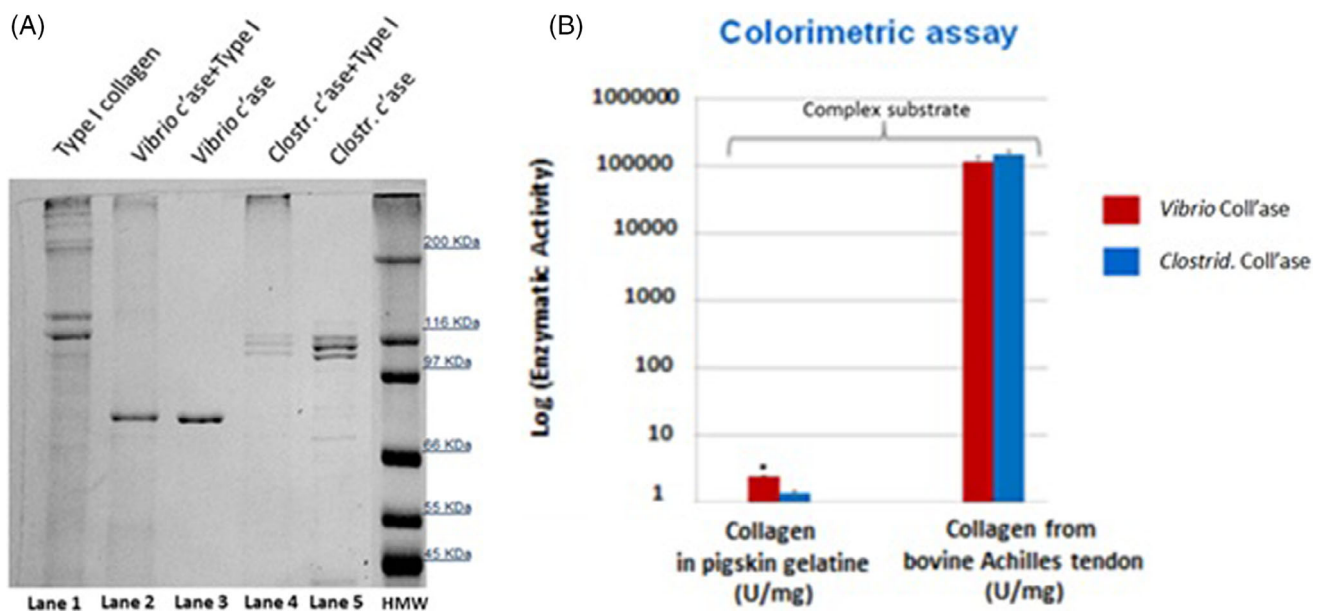

FIGURE 3 Comparison of the specific collagenolytic activity on collagen substrate. A, 7,5% SDS-PAGE after 90 minutes of incubation with Type I collagen (bovine). *Lane 1*: Control test tubes run with 50 μg Type I collagen; *Lane 2*: 50 μg Type I collagen combined with 0.8 μg *Vibrio alginolyticus* collagenase; *Lane 4*: 50 μg Type I collagen combined with 0.8 μg *Clostridium histolyticum* collagenase; and *Lanes 3 and 5*: Control test tubes run with collagenase preparations only (0.8 μg). B, Specific activity by collagenase assay on pigskin gelatine and bovine Achilles tendon, which represents complex natural substrates, using the Mandl's colorimetric method. Data are expressed as mean (SD) from three different experiments (*$P < 0.05$, Student's *t*-test, $n = 3$)

3.4 | Comparison of the specific enzymatic activity on selected dermal substrates: decorin

Here, 4% to 15% SDS-PAGE after decorin digestion with both collagenase preparations shows that *V. alginolyticus* collagenase degrades decorin only to a (very) minor extent (five main bands in the range 50-15 kDa were produced), whereas the *C. histolyticum* collagenase preparation degrades decorin completely with no remnants of the original 75-kDa smeared band (Figure 4).

3.5 | Comparison of the specific enzymatic activity on selected dermal substrates: fibronectin

Regarding fibronectin, 4% to 15% SDS-PAGE shows only a partial degradation of the substrate by *V. alginolyticus* collagenase, resulting in the production of a main band at 220 kDa (58% of the total amount). Specifically, 42% of the starting material was still present (lane 2, Figure 5), and the lower bands collectively comprised <1%. This activity is
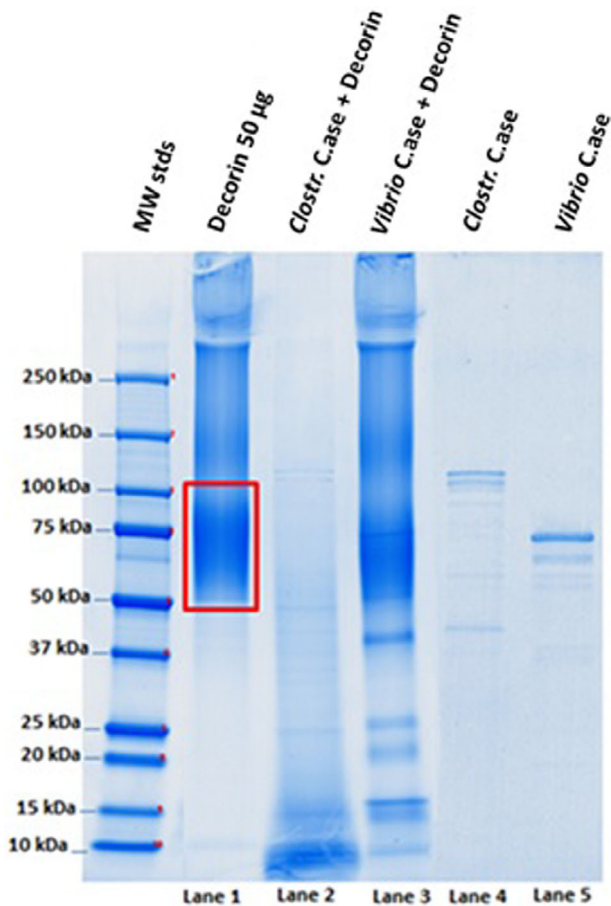
FIGURE 4 Electrophoresis by 4% to 15% SDS-PAGE. Experimental conditions: 90 minutes incubation at 37°C, pH 7.1; *Lane 1*: Control test tubes run with 50 μg decorin; *Lane 2*: 50 μg decorin combined with 0.8 μg *Clostridium histolyticum* collagenase; *Lane 3*: 50 μg decorin combined with 0.8 μg *Vibrio alginolyticus* collagenase; and *Lanes 4 and 5*: Control test tubes run with collagenase preparations only (0.8 μg)

FIGURE 5 Electrophoresis by 4% to 15% SDS-PAGE. Experimental conditions: 90 minutes incubation at 37°C, pH 7.1; *Lane 1*: Control sample lane run with 50 μg fibronectin; *Lane 2*: 50 μg fibronectin combined with 0.8 μg *Vibrio alginolyticus* collagenase; *Lane 3*: 50 μg fibronectin combined with 0.8 μg *Clostridium histolyticum* collagenase; *Lane 4*: Control sample lane run with *Clostridium histolyticum* collagenase preparation only (0.8 μg); and *Lane 5*: Control sample lane run with *Vibrio alginolyticus* collagenase preparation only (0.8 μg)

3.6 | Influence of pH on collagenase activity

In vitro collagenase activity was assessed at different pH values, which were characteristic of healthy skin and a healing wound environment. Pure collagenase preparations lose activity at acidic pH. This finding is particularly true for *V. alginolyticus* collagenase. At pH values of 5.6, *V. alginolyticus* collagenase exhibited 20/25% activity. However, at pH of 5.0, the activity was completely lost. In contrast, the *C. histolyticum* preparation still exhibits 60% activity at pH 5.6 and 30% activity at pH 5.0 (Figure 6).

4 | DISCUSSION

Hyalo4 Start is a class III medical device based on 0.2% LMW-HA and a novel collagenase preparation purified from a non-pathogenic aerobic *V. alginolyticus* bacterial strain. This combination forms a novel debriding agent where the two components act in a synergistic manner to speed up the healing process by an effective wound bed preparation for ulcers, necrotic burns, and bed sores.[47] The novel *V. alginolyticus* collagenolytic enzyme is characterised by a

evaluated using a camera-based imaging system CCD where the quantitative analysis is achieved by applying ImageQuant TL image analysis software (GE Healthcare). In contrast, the *Clostridium* preparation degrades 100% of fibronectin, resulting in many bands at considerably lower MWs (lane 3, Figure 5).

Therefore, the specific enzymatic activity of the two collagenases on selected dermal substrates was compared. Our findings demonstrate that with the same concentration of the analysed substrate (and equal protein concentrations of the two collagenases), *V. alginolyticus* collagenase completely degrades only the collagen substrate. Thus, *V. alginolyticus* collagenase exhibits not only a high catalytic activity but also a high specificity of action. In contrast, *C. histolyticum* collagenase completely degrades all the three analysed protein substrates.
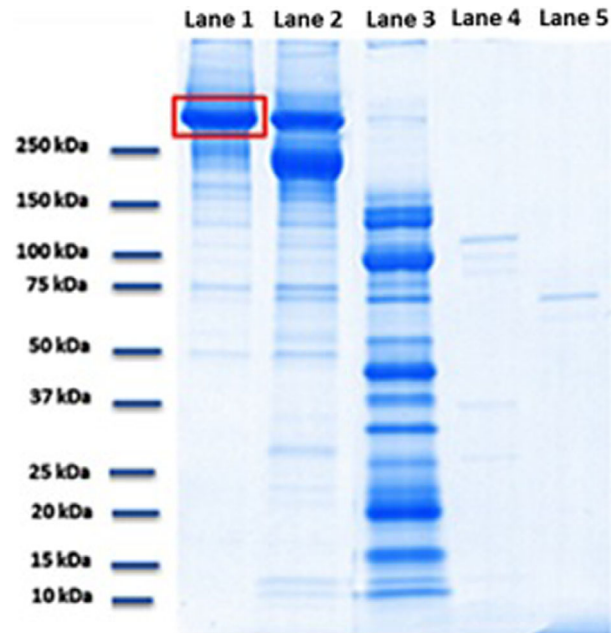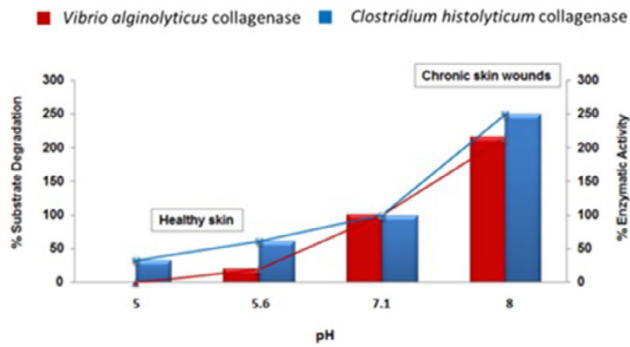
FIGURE 6   In vitro collagenase activity was determined at different pH values by quantifying the fragment produced by the enzymatic reaction according to the Wünsch method

single, highly purified 82-kDa protein. A key factor in achieving this result was the use of a culture medium for bacterial growth completely lacking protein inducers (either from animal or non-animal sources). Typically, approximately 1000 nkat/mg (batch size approximately 5 g) of collagenase are recovered from each biosynthetic preparation.

Clinical experience has demonstrated the effectiveness of this device (Hyalo4 Start) along with a lower rate of perilesional skin complications compared with collagenase-based products derived from *C. histolyticum*.[22,32] The latter preparation showed two major bands at 110 and 100 kDa via SDS-PAGE in addition to other bands of lower MW. We assumed that a different substrate specificity, particularly in the case of minor structural components of the dermis, might explain such a lower complication rate. Therefore, in our in vitro study, a biochemical characterisation of *V. alginolyticus* collagenase versus a commercial preparation from *C. histolyticum* strain on collagen and various dermal ECM substrates was performed. The same amounts of enzyme preparations were used in the assays; therefore, such amounts reflect the quantity of product/cm^2 commonly employed for wound management (activity).[48,49] in vitro studies of the enzymatic preparations were not performed regarding comparable enzyme activity of the collagenase given the presence of *C. histolyticum* proteases associated with the preparation. Although these proteases represented a minimal portion of the preparation, they reacted with the collagenase in the degradation of collagen.

In vitro studies showed that the *V. alginolyticus* enzyme is as effective as the commercial preparations of *C. histolyticum* collagenase on many types of collagen.[50] Collagen digestion performed with each enzyme preparation was examined by SDS-PAGE, probing type I collagen from bovine Achilles tendon, or a colorimetric assay probing either collagen type I or pig skin gelatine, which is mainly composed of denatured dermal collagen. In both cases, the two preparations were equally active in substrate digestion.

In contrast, when other minor components of the dermal ECM were probed with the *V. alginolyticus* or *C. histolyticum* collagenase, different behaviours were observed.

V. alginolyticus collagenase degrades fibronectin to a limited extent, mainly resulting in a major band at 220 kDa. In addition, 42% of the starting substrate was not digested. In the case of *C. histolyticum* digestion, the 250-kDa fibronectin band completely disappeared, and only lower MW bands in the range 130 to 15 kDa were detected.

When probed with decorin, the same results were observed. Only partial degradation was observed with the *V. alginolyticus* preparation, and most of the original protein band remained undigested. In contrast, *C. histolyticum* enzyme completely digested the substrate. This evidence is crucial because decorin and fibronectin, although present in small amounts, are structurally fundamental components of the dermal ECM.[51] In fact, decorin is a proteoglycan that plays a role in the control of fibrosis and scar formation as a tissue healing physiological regulator, and the lack of expression or the absence of this protein in skin tissues reduces the regenerative ability after skin damage.[52-54] On the other hand, fibronectin, a high molecular weight glycoprotein of the ECM, facilitates dermal fibroblast migration during normal wound healing.[55]

The different substrate specificity can be explained taking into account that *Vibrio* collagenase cleaves the Y-Gly bond of the collagen sequence –Pro-Y-Gly-Pro–.[56] Preparations of *C. histolyticum* collagenase are actually made of six enzymes with both collagenolytic and proteolytic activity.[57] The latter enzyme preparation cleaves the collagen strand at the Y-Gly bonds in the repeating Yaa-Gly-Xaa-Yaa collagen sequence (MEROPS, Summary for family M9). It is important to recall that the collagenolytic activity refers to the cleavage of any collagen substrate measured in nkat (amount of a collagen peptide digested/time/mL),[58] whereas the proteolytic activity refers to the cleavage of any protein present in the reaction mixture measured in mg protein digested/time. Therefore, proteases present in the *C. histolyticum* collagenase preparation are responsible for non-collagenic substrate digestion, such as decorin or fibronectin, whereas pure collagenase preparations (such as the *V. alginolyticus* one) degrade non-collagenic substrates to a minor extent. This finding is confirmed by the caseinase assay results. The *C. histolyticum* enzyme pool promptly degrades casein, whereas *V. alginolyticus* collagenase does not.

Normal healthy skin is characterised by pH values in the mildly acidic range (5.0-5.6),[59] and similar pH values are noted in spontaneously healing, acute wounds. Non-healing, chronic wounds are characterised by higher pH values in the mild basic range (8.0-9.5).[59] *V. alginolyticus* collagenase

loses 90% activity at pH 5.6 (compared with 100% activity at pH 7). In contrast, *C. histolyticum* collagenase preparation still retains 60% and 30% activity at pH values of 5.6 and 5.0, respectively, therefore implying that this enzyme pool is still able to degrade the ECM matrix in healthy skin. At mild basic pH values (7.4-8.5), typical of chronic, non-healing wounds, both preparations are equally effective on the collagenic substrate.

In conclusion, a difference in aggressiveness between the two products containing different collagenases that act on periwound skin was demonstrated.[32] Actually, the wound edges in patients treated with *V. alginolyticus*-derived collagenase were in better condition than the wounds treated with *C. histolyticum*-derived enzyme. In fact, the *V. alginolyticus* enzyme is gentler on healthy tissue and allows an improvement of the surrounding skin. It can be speculated that the protective action is mediated by the presence of 0,2% hyaluronic acid in the product; hyaluronan (HA) allows the formation of the ECM matrix, promotes cell migration, modulates the inflammatory response, and improves collagen deposition.[32,60,61] However, the purpose of this comparative study was to evaluate the effectiveness and safety of enzymatic debriders, *V. alginolyticus*-derived collagenase, and *C. histolyticum*-derived collagenase, by in vitro biochemical characterisation of various dermal ECM substrates.

Therefore, we demonstrated that the *V. alginolyticus* collagenolytic enzyme is fully active on the collagen filaments that anchor the necrotic tissue to the wound bed, whereas the same collagenase is inactive on other minor but structurally important components of the dermal ECM. In addition, regarding the influence of pH on *V. alginolyticus* collagenase activity, the analysis demonstrated that this enzyme performs its lytic activity exclusively on necrotic tissue. This could be the reason why collagenase preparation from *V. alginolyticus* has been reported to be much more gentle on perilesional, healthy skin.[22,32,47,62,63]

Finally, these findings support ever more the clinical evidence that the effect of Hyalo4 Start is not as aggressive on perilesional skin as the commercial *C. histolyticum*-based products.[32]

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