Collagen and Leather

REVIEW

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Collagenases and their inhibitors: a review

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Abstract

Hide and skin are complex tissue where the most abundant component is collagen. Matrix metalloproteinases and bacterial collagenases are two kinds of collagenases that can cleave the triple-helical domain of native fibrillar collagens. In this paper, the family members and domain composition of matrix metalloproteinases and bacterial collagenases are summarized. The catalytic mechanism of collagen hydrolysis by collagenases is described, and the methods adopted to date for investigating and regulating collagenases and their inhibitors are reviewed. Furthermore, the applications of collagenases and their inhibitors in biomedicine, food processing and the enzymatic unhairing process in the leather-making industry are presented.

Keywords Matrix metalloproteinases, Bacterial collagenase, Inhibitor, Enzymatic unhairing

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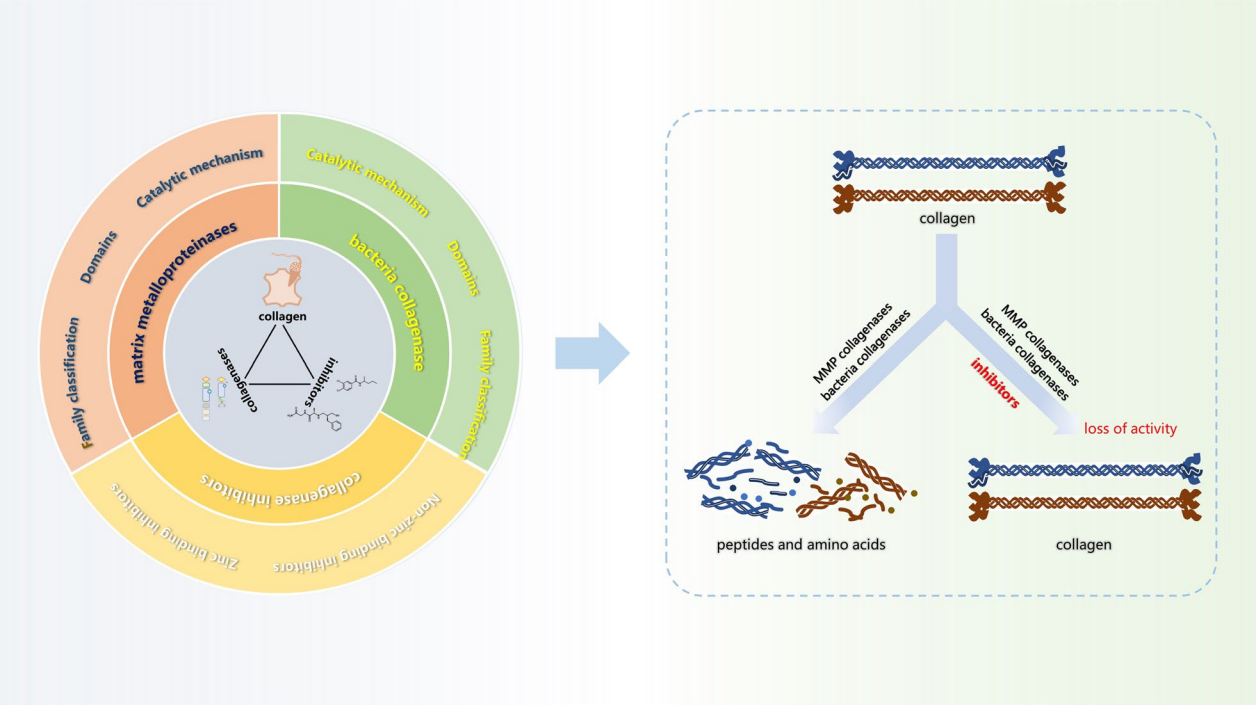
Graphical Abstract



1 Introduction

Collagens are important components of the extracellular matrix (ECM) in mammalian tissues such as skin, ligaments and tendons [1]. To date, 40 vertebrate collagen genes have been identified. These genes form at least 28 different collagen molecules [2–4], fibrillar or non-fibrillar molecules, which are sequentially numbered using Roman numerals (I–XXVIII). Fibrillar collagens have high thermal stability and strong mechanical strength [5–9].

The hide and skin of mammalians consist of various type of collagens, such as types I, III, IV and VII, among which type I and type III collagens are the major types of fibrillar collagen [10, 11]. The most abundant collagen is type I collagen, whose molecule consists of three α polypeptides (two α_1(I)-chains and one α_2(I)-chain) that are wound around each other to form a triple helix, and at either end of the triple helix are non-helical moieties, called telopeptides (Fig. 1) [12–14]. Native type I collagen is highly resistant to common proteases, such as trypsin and chymotrypsin, while enzymes with collagenolytic activity, such as matrix metalloproteinase-1 (MMP-1; also known as interstitial collagenase), show the ability to cleave type I collagen at the triple-helical domain [15].

The most widely studied collagenases are matrix metalloproteinases (MMPs) from animals and bacterial collagenases from micro-organisms. MMPs are a family of multi-domain proteolytic enzymes containing zinc ions [16]. They have the potential to decompose polypeptides in the ECM and play an important role in physiological and pathological processes [17–21]. Bacterial collagenases are mainly secreted by micro-organisms such as *Clostridium* and *Vibrio*. In the Merops peptidase database, bacterial collagenases and MMPs belong to the M9 family and the M10 family, respectively.

Unhairing is an essential process in leather manufacturing. However, the conventional hair-burning unhairing process will cause seriously pollution problem. As a reliable alternative to the conventional lime-sulfide process, unhairing by proteases (enzymatic unhairing) in the leather-making process has been employed over decades [22, 23]. A wide range of enzymes have been investigated for their potential applications in enzymatic unhairing, such as alkaline proteinases from *Bacillus* [24], keratinases from *Actinomadura keratinilytica* [25], and serine proteases from *Caldicoprobacter algeriensis* [26]. It has been noted that the main risk of grain damage during enzymatic unhairing is due to the over-hydrolysis of collagens by collagenases. To fully exploit the potential

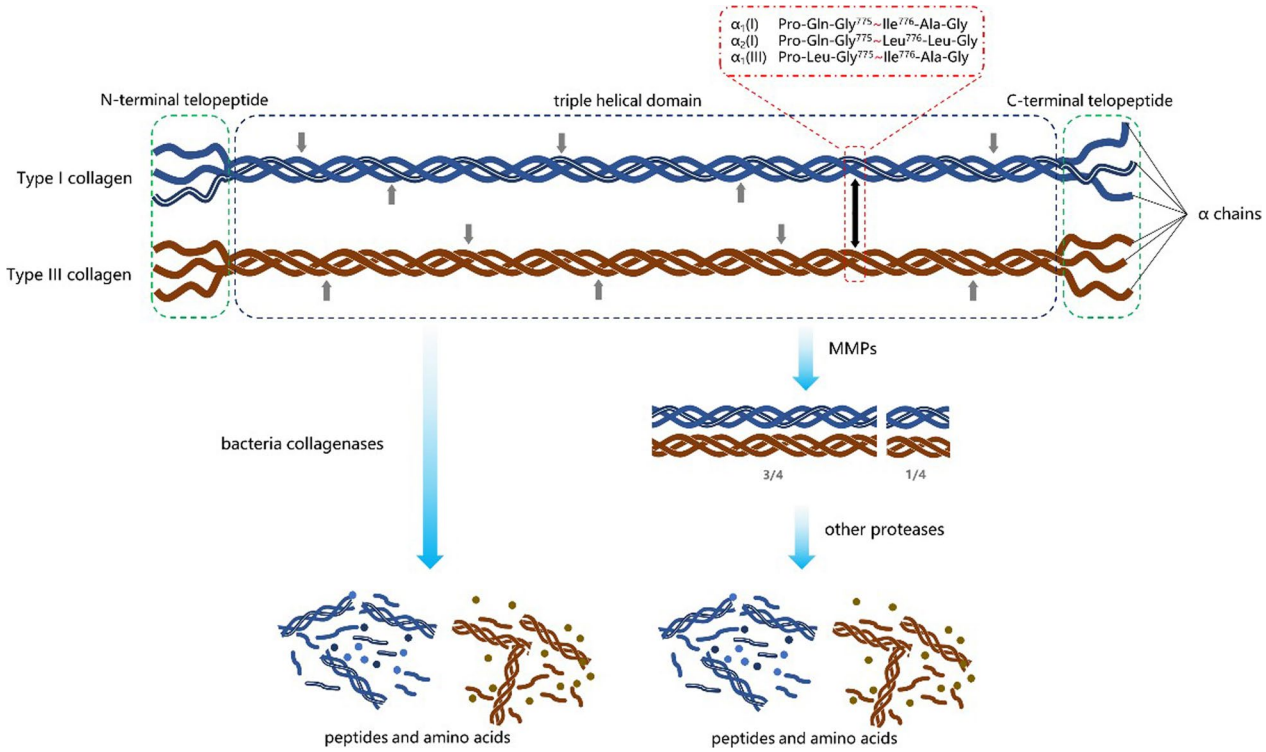


Fig. 1 Triple-helical structures of type I and type III collagens. There are three α-chains in each collagen molecule. Type I collagen includes two α_1(I)-chains and one α_2(I)-chain and type III collagen includes three α_1(III)-chains. The triple-helical domain can be degraded by collagenases into peptides

of enzymatic unhairing, it is crucial to understand the action and effect of components with collagenase activity on different collagen types in the hide and skin. Herein, the family classification and structural characteristics of collagenases (MMPs and bacterial collagenases) are summarized. The catalytic mechanism of collagen hydrolysis by collagenases is described. Furthermore, the methods so far employed for investigating and regulating collagenases and their inhibitors are reviewed.

2 Progress of research on collagenases

Proteases are a group of hydrolytic enzymes that can cleave the peptide bonds of protein molecules and degrade them into small peptides and amino acids. Collagenases are proteases possessing the ability to degrade various types of collagens at the specific site [24] and are predominantly found in animal and micro-organisms, which differ in substrate specificity [21].

2.1 MMPs

2.1.1 Classification of MMPs

MMPs are a family of proteolytic enzymes that have different substrate preferences, while share the same zinc-dependent active site and have similar structural features [27]. They are grouped into collagenases, gelatinases, stromelysins, matrilysins and membrane-type (MT)MMPs and others, which are characterized by their domain organization and substrate preference (Fig. 2 and Table 1) [28–33].

Collagenases cleave ECM proteins and other soluble proteins, however, the most important role of this type of MMPs is that they can digest fibrillar collagens of types I, II, III, IV and XI into characteristic 3/4 and 1/4 fragments [34–36]. In addition to MMP-1, MMP-8, MMP-13 and MMP-18, other specific MMPs may also cleave fibrillar collagens such as MMP-2 and MMP-14. They can digest type I, II and III collagens in a manner similar to that of collagenases but are divided into other subgroups due to their domain organization [37].

2.1.2 Domains of MMPs

Although different subgroups of MMPs have different domain organizations, several important domains are common to almost all MMPs. They are (from N-terminal to C-terminal positions) a propeptide (Prop), a catalytic domain (Cat), a linker of variable length, and a hemopexin-like domain (Hpx), as shown in Fig. 3A [38].

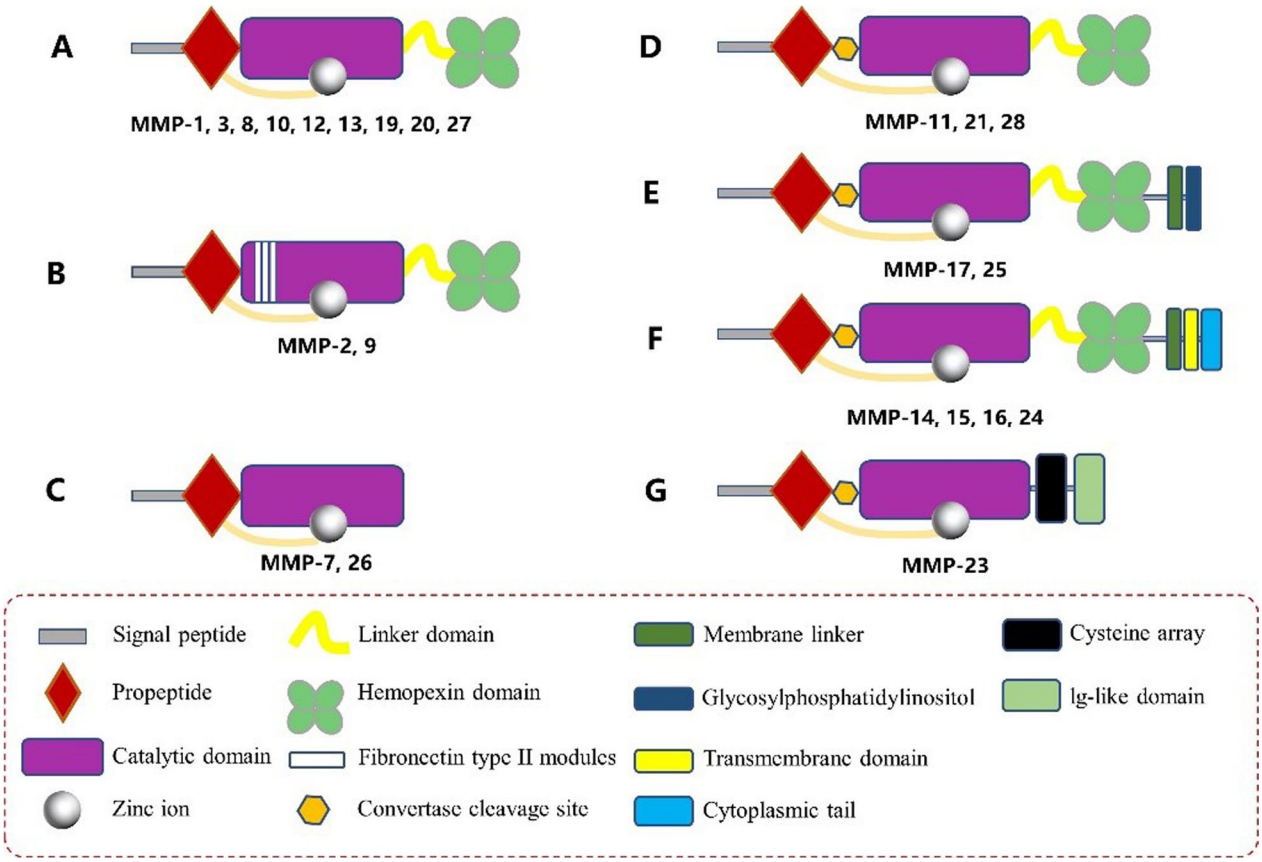


Fig. 2 Schematic representation of the various domains of different MMPs

2.1.3 Propeptide

The propeptide consists of about 80 amino acid residues forming three α-helices [39]. The α(3)-helices are followed by a "cysteine switch", a very conserved region (PRCGXPD) [40, 41], where the sulfhydryl group is coordinated with the catalytic Zn(II) ion, forming a tetrahedral coordination sphere. This keeps the MMPs in a latent state until the cysteine-Zn^{2+} interaction is disrupted; then, the Zn(II) ion can combine with water molecules which are necessary for the hydrolysis of polypeptides.

2.1.4 Catalytic domain

The catalytic domain consists of about 160 amino acid residues, including a five-stranded β-sheet (four parallel(β2–β1–β3–β5) and one anti-parallel(β4) components), three α-helices (α1, α2 and α3) (Fig. 3A), two Zn(II) ions (one catalytic ion and one structural ion, about 80–100 nm apart), and at least one Ca(II) ion (usually three) [28, 36]. These structures are assembled into a sphere with a diameter of about 400 nm [39–41]. The catalytic Zn(II) ion is essential for the catalytic process of MMPs. It coordinates with three the His residues in the **H**EXG**H**XXGXX**H** sequence (Fig. 3C) [34, 41–44], which is conserved among all the MMPs. In the activated free enzyme, the catalytic Zn(II) ion will also be coordinated by a water molecule hydrogen-bonded to the Glu residue flanking the active site [32]. Moreover, the catalytic Zn(II) ion flanked by a hydrophobic cavity (S1′ pocket) contains mainly hydrophobic residues and is comprised of a Ω-loop, which includes the loop linking helices α2 and α3. The loop holds a hydrophobic Met residue which is part of the highly conserved 1,4-β-turn referred to as the "Met-turn" (Fig. 3B).The structural Zn(II) ion and the Ca(II) ions play crucial roles in maintaining the conformation of proteins [43].

2.1.5 Linker region

The catalytic domain and hemopexin-like domain are connected by a proline-rich linker region (also called hinge region) (Fig. 3A). This region is flexible to a certain extent due to its variable length, which gives interdomain flexibility to the structure of MMPs, retains enzyme stability, and participates in the hydrolysis of some complex substrates [45]. Therefore, the linker region is important for MMPs to express collagenolytic activity.

Table 1 Classification of MMP family with their domain organization and substrates preference

MMP	Name of class	Domain organization	Substrates preference
MMP-1	Collagenases	A	Collagens (I, II, III, VII; VIII; X); gelatin; aggrecan, nidogen, perlecan, proteoglycan link protein, serpins, tenascin-C, versican
MMP-8	Collagenases	A	Collagens (I, II, III, V; VII; VIII; X); gelatin; aggrecan, elastin, fibronectin, laminin, nidogen
MMP-13	Collagenases	A	Collagens (I, II, III, IV); gelatin; aggrecan, fibronectin, laminin, perlecan, tenascin
MMP-18	Collagenases	A	Collagens (I, II, III, IV); gelatin
MMP-2	Gelatinases	B	Collagens (I, II, III, IV; V; VII; XI); gelatin; aggrecan, elastin, fibronectin, laminin, nidogen, proteoglycan link protein, versican
MMP-9	Gelatinases	B	Collagens (IV; V; VII; X; XIV); gelatin; aggrecan, elastin, fibronectin, laminin, nidogen, proteoglycan link protein, versica
MMP-3	Stromelysins	A	Collagens (II; III; IV; IX; X; XI); gelatin; aggrecan, decorin, elastin, fibronectin, laminin, nidogen, perlecan, proteoglycan, proteoglycan link protein, versican
MMP-10	Stromelysins	A	Collagens (III; IV; V); gelatin; aggrecan, elastin, fibronectin, laminin, nidogen
MMP-11	Stromelysins	D	Aggrecan, fibronectin, laminin
MMP-7	Matrilysins	C	Collagens (IV; X); gelatin; aggrecan, elastin, enactin, fibronectin, laminin, proteoglycan link protein
MMP-26	Matrilysins	C	Collagens (IV); gelatin; fibrinogen, fibronectin, vitronectin
MMP-14	MT-MMPs	F	Collagens (I; II; III); gelatin; aggrecan, elastin, fibrin, fibronectin, laminin, nidogen, perlecan, proteoglycan, tenascin, vitronectin
MMP-15	MT-MMPs	F	Collagens (I); gelatin; aggrecan, fibronectin, laminin, nidogen, perlecan, tenascin, vitronectin
MMP-16	MT-MMPs	F	Collagens (I); aggrecan, fibronectin, laminin, perlecan, vitronectin
MMP-17	MT-MMPs	E	Gelatin; fibrin
MMP-24	MT-MMPs	F	Gelatin; chondroitin sulfate, dermatin sulfate, fibrin, fibronectin, N-cadherin
MMP-25	MT-MMPs	E	Collagen (IV); gelatin
MMP-12	Other MMPs	A	Collagen (IV); gelatin; elastin, fibronectin, laminin
MMP-19	Other MMPs	A	Collagen (I; IV); gelatin; aggrecan, fibronectin, laminin, nidogen, tenascin
MMP-20	Other MMPs	A	Collagen (V); aggrecan, cartilage; oligomeric protein, amelogenin
MMP-21	Other MMPs	D	-
MMP-22	Other MMPs	A	Gelatin
MMP-23	Other MMPs	G	Gelatin
MMP-27	Other MMPs	A	–
MMP-28	Other MMPs	D	–

2.1.6 Hemopexin-like domain

The hemopexin-like domain has the shape of an ellipsoidal disc (Fig. 3A). It consists of approximately 210 amino acid residues and contains a four-bladed β-propeller structure with a single disulfide bond between the 1st and 4th blade, [46]. Each blade is composed of four antiparallel β-strands and has a Cys residue at each end. A calcium ion and a chloride ion are usually present in the center of the four-bladed β-propeller [47, 48]. Although it has been found that the catalytic domain is sufficient for degradation of non-collagenous substrates, the hemopexin-like domain is inevitable for recognition and hydrolyzation of fibrillar collagen [49–51].

2.1.7 Catalytic mechanism of MMPs with collagen-hydrolyzing activity

The catalytic action of MMPs on collagen has been explored for decades. To understand the catalytic mechanism, triple-helical peptides (THPs) have been used as models of native collagens [51–53]. Moreover, computer simulation using molecular dynamics has been applied for exploring the interaction between MMPs and collagens [54–58]. Both approaches can help us to better discover the interaction mode between collagenases and their substrates.

MMP-1 serves as the prototype for all interstitial collagenases. MMP-1(activated form) cleaves type I collagen molecules at a site three-quarters from the N-terminus, after which collagen becomes a poor substrate of collagenase due to denaturation, and it continues to be degraded by gelatinase or common cathepsin [59].

2.1.8 Interdomain communications modulate the activity of MMP-1

The effective hydrolysis of collagens by MMP-1 requires synergy between the substrates and multiple domains. Although the catalytic domain is essential for substrate hydrolysis, it alone can only degrade non-collagen

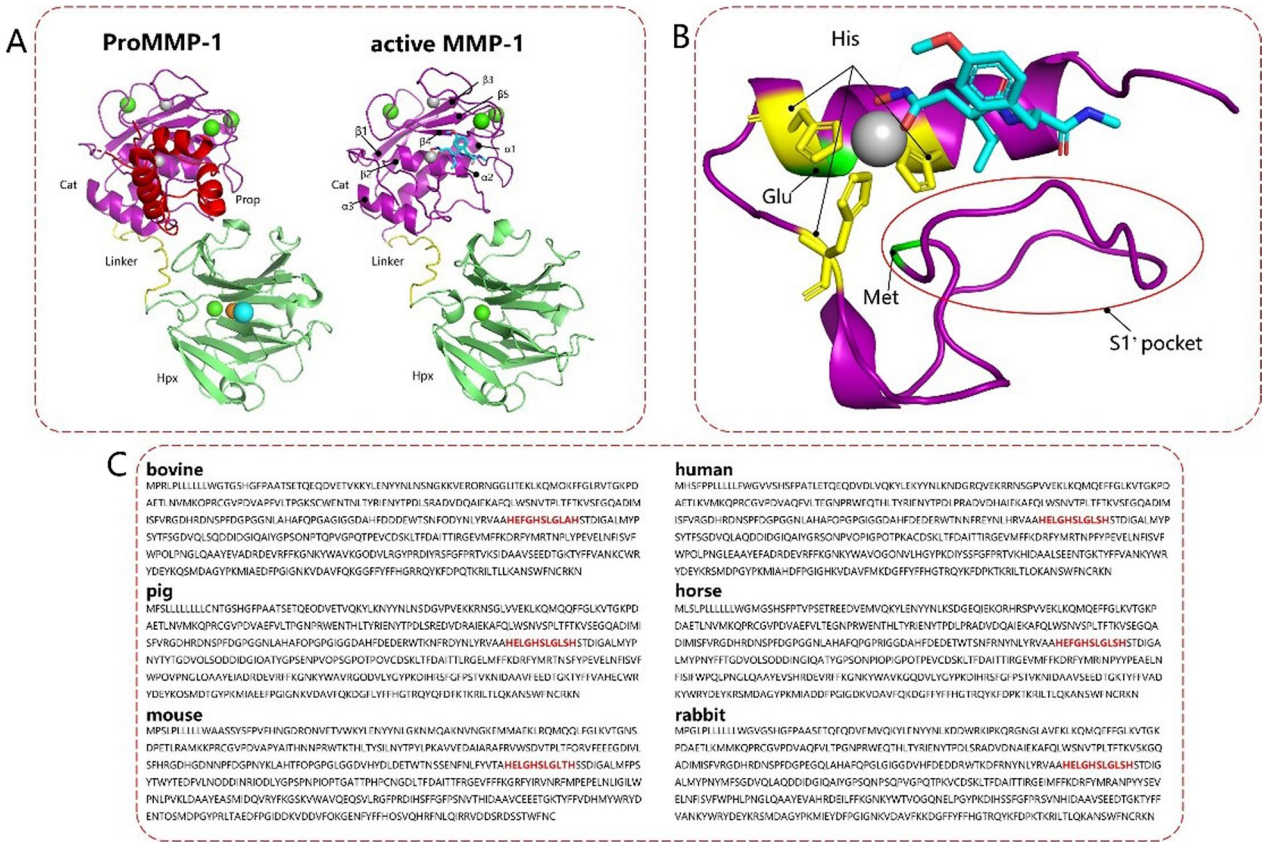


Fig. 3 Diagram of the structure of MMP-1 and the sequence of MMP-1 between different organism species. **A** Ribbon diagram of proMMP-1 (human) and active MMP-1 (pig). From N-terminal to the C-terminalpositions: a propeptide, a catalytic domain, a linking region and a hemopexin-like domain. The catalytic zinc(II) ion is represented by a blue sphere. **B** Ribbon diagram of active site of MMP-1. The propeptide is shown in red; the catalytic domain, in purple; the linker region, in yellow; the hemopexin domain, in lime; zinc ions, in gray; calcium ions, in green; the sodium ion, in cyan; and the chloride ion, in orange. The propeptide is removed when the MMPs are activated. The images were prepared with Bookhaven Protein Data bank entries 1SU3 (proMMP-1) and 1FBL(MMP-1) using Pymol. C. The sequence of MMP-1 between different organism species. The conserved sequences are marked in red

substrate (gelatin or heat-denatured collagen). The binding process between enzyme and substrate is initiated by the hemopexin-like domain, which can interact with triple-helical substrates with residues at specific positions [60–66], including residues Ile290, Arg291, Phe301, Val319, and Asp338. Moreover, through the allosteric communication between the catalytic domain and the hemopexin-like domain, the catalytic domain is located at the position of the scissile bond, thus initiating the degradation of collagen [53, 56, 67].

2.1.9 Collagen hydrolysis mechanism induced by catalytic domain

It is suggested with the three-dimensional structure of MMP-1 that its substrate binding site is too small to accommodate the triple-helical structure of collagen. When the MMP locates the scissile bond between collagen and active site with interdomain flexibility, the catalytic domain begins to hydrolyze the substrate. However, it was found that the closely intertwined triple-helical structure of collagen (about 150 nm in diameter) could not match the gap in the active site of the catalytic domain of MMP-1 (about 50 nm in diameter). Chung et al. [68] found that it is necessary to locally unwind the collagen triple-helical structure before the enzyme cleaves collagen. After unwinding, the single-strand collagen polypeptide chain can be surrounded by the catalytic domain of MMP-1; thus, hydrolysis occurs. During this process, the conformation changes for both collagen and MMP-1.

MMP-1 requires the catalytic Zn(II) ion, three His residues, a Glu residue and a water molecule to exhibit its catalytic ability. Futhermore, there is a Met residue as a hydrophobic base, supporting the structure around the catalytic Zn(II) ion [69]. The breaking process of the scissile bond is shown in Fig. 4. When the scissile bond of the substrate is located at the active site, the catalytic Zn(II) ion carries out penta-coordination with the

carbonyl oxygen atom of the scissile bond, an oxygen atom from Glu-bound water and three His residues. At the beginning of hydrolysis, the water molecule polarized by the Glu residue carries out nucleophilic attacks on the carbonyl carbon of the scissile bond [45]. Subsequently, protons are transferred to amino nitrogen atoms with Glu residues, promoting the formation of an intermediate with tetrahedral geometry [35, 70], resulting in the cleavage of the scissile bond. The whole catalytic process can be divided into four steps: (a) the substrate enters the active site and the water molecules between the Zn(II) ion and the Glu residue are polarized; (b) the polarized water molecules carry out nucleophilic attacks on the carbonyl group of the scissile bond to form a tetrahedral structure (which is unstable); (c) the tetrahedral structure collapses, and the scissile bond breaks; (d) the substrate broken by the scissile bond leaves the active site, and the MMP begins to hydrolyze the next substrate.

2.2 Bacterial collagenases

2.2.1 Members of bacterial collagenase

Collagenases produced by *Clostridium histolyticum* are the most studied bacterial collagenases, which can be categorized into class I (encoded by gene ColG) and class II (encoded by gene ColH) [71]. Class I collagenases show high activity with collagen and moderate activity with FALGPA (a synthetic peptide used for studying the kinetics of collagenases), while class II collagenases are highly active with synthetic peptides and moderately active with collagen [72]. These two types of collagenases belong to the M9B subfamily in the Merops peptidase database. Proteases produced by *Vibrio* are divided into three classes (I, II and III) characterized by their function and structure. Proteases in class I possess no collagenolytic activity, while classes II and III members are able to degrade collagenous substrates.

2.2.2 Domain of bacterial collagenase

The main literature source on bacterial collagenase is the extensive research on the enzymes produced by *Clostridium histolyticum*. Collagenases produced by *Clostridium* are multi-domain zinc metalloproteinases, possessing a signal peptide, the N-terminal collagenase unit and the C-terminal recruitment domain [17], as shown in Fig. 5. The N-terminal collagenase unit contains an activator domain (AD) and a peptidase domain (PD). The C-terminal recruitment domain usually contains one or two collagen-binding domains (CBDs) and one or two polycystic kidney disease-like domains (PKDs).

2.2.3 Collagenase unit

The molecular weight of a collagenase unit is about 78 kDa. The activator domain and peptidase domain show a saddle-shaped architecture [17]. There is a conserved Zn(II) ion in the peptidase domain, which coordinates with two His residues in a conserved motif (-**H**EXX**H**), a Glu residue downstream of the motif and a water molecule [73, 74]. In addition to the Zn(II) ion, there is also a Ca(II) ion in the active site. These two ions are indispensable for enzyme activity [75].

2.2.4 Recruitment domains

The molecular weight of both the CBD and PKD is about 10 kDa. Based on the studies of the CBD of two kinds of *Clostridium* collagenase, it was found that the CBD can recognize and bind to the triple-helical structure of collagen, which is necessary for the hydrolysis of insoluble collagen fibers by *Clostridium* collagenase, and the binding can be enhanced in the presence of calcium ions [76, 77]. The PKD, which consists of 80–90 amino acid residues, is the alignment platform between the collagenase unit and the CBD, but its specific role in collagen hydrolysis is still unclear [78]. It was found that the PKD of some special collagenases not only can bind to collagen but can also swell microfibrils to improve the collagenolytic efficiency [79]. The Ca(II) ions in the PKD can stabilize the conformation of the domain, thus stabilizing the overall stability of collagenase [80].

2.2.5 Catalytic mechanism of bacteria collagenase

MMPs show strict substrate specificity for collagen hydrolysis, cleaving collagen only at well-defined recognition sites. However, *Clostridium* collagenases have no obvious preference for any type of collagen and can hydrolyze collagen in multiple locations and completely decompose it into small peptides [81].

The information regarding the structure and hydrolysis mechanism of bacterial collagenases is scarce, partly owing to their multi-domain organization. Attempts have been made to sovle the three-dimensional structure of the ColG collagenase from *Clostridium histolyticum* [72]. In ColG, the recruitment domain locates and anchors the collagen by specifically recognizing their triple-helical conformation, while the collagenase unit hydrolyzes the prepared collagen. Similar to MMPs, collagen changes its configuration before being hydrolyzed by bacterial collagenases. Eckhard called this a "chew-and-digest mechanism" [74]. The hydrolysis process of bacterial collagenases can be divided into two steps: At the beginning of binding with collagen, the collagenase gradually changes its configuration from open to closed; then, the collagenase changes its configuration to semi-open. Meanwhile, the triple helices of collagen are unlocked, and the scissile bond is exposed to the active site to complete hydrolysis. Once this part of collagen is hydrolyzed,

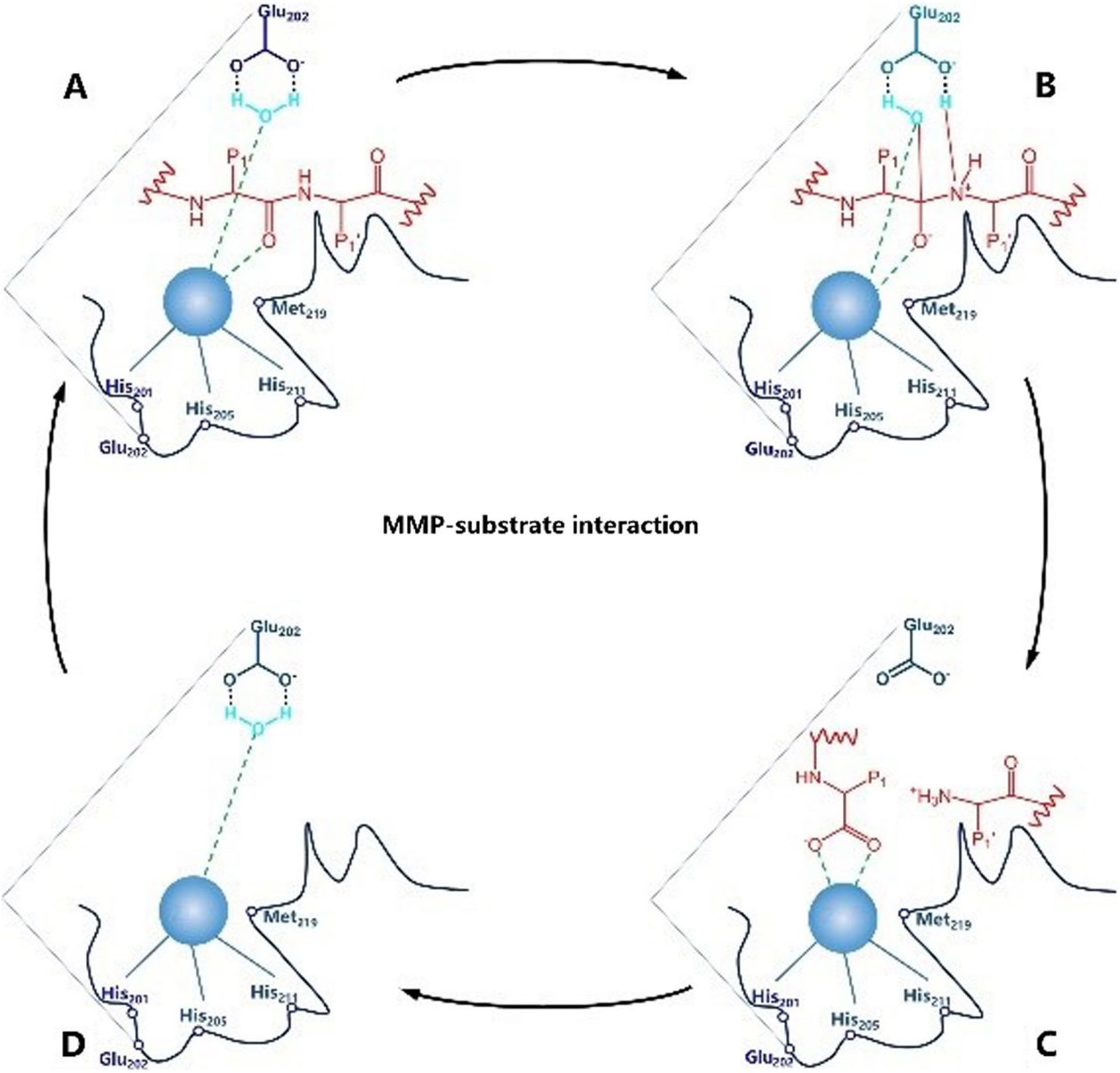


Fig. 4 Catalytic mechanism of hydrolysis of scissile bond of collagen at the active site of the MMP. **A** The substrate enters the active site, and the Zn(II) ion becomes penta-coordinated. **B** The glutamate-bound water molecule is polarized and attacks the carbonyl carbon of the scissile bond to form an unstable tetrahedral intermediate. **C** The structure of the intermediate collapse and the scissile bond breaks. **D** The cleaved substrate leaves the active site and the MMP starts the next cycle. The catalytic Zn(II) ion is represented by a blue sphere

the collagenase returns to the open configuration so that another collagen molecule can be degraded.

2.3 Applications of collagenases

The structure and catalytic mechanism of collagenases have been clearly identified, therefore, a wide range for applications of collagenases have been investigated in fields such as biomedicine, food processing and leather manufacturing. The following section focuses on collagenases applications.

2.3.1 Biomedical field

Collagenases can be directly applied to clinical treatment [82–84]. Collagens are the most abundant proteins in the ECM and have numerous functions. However, the

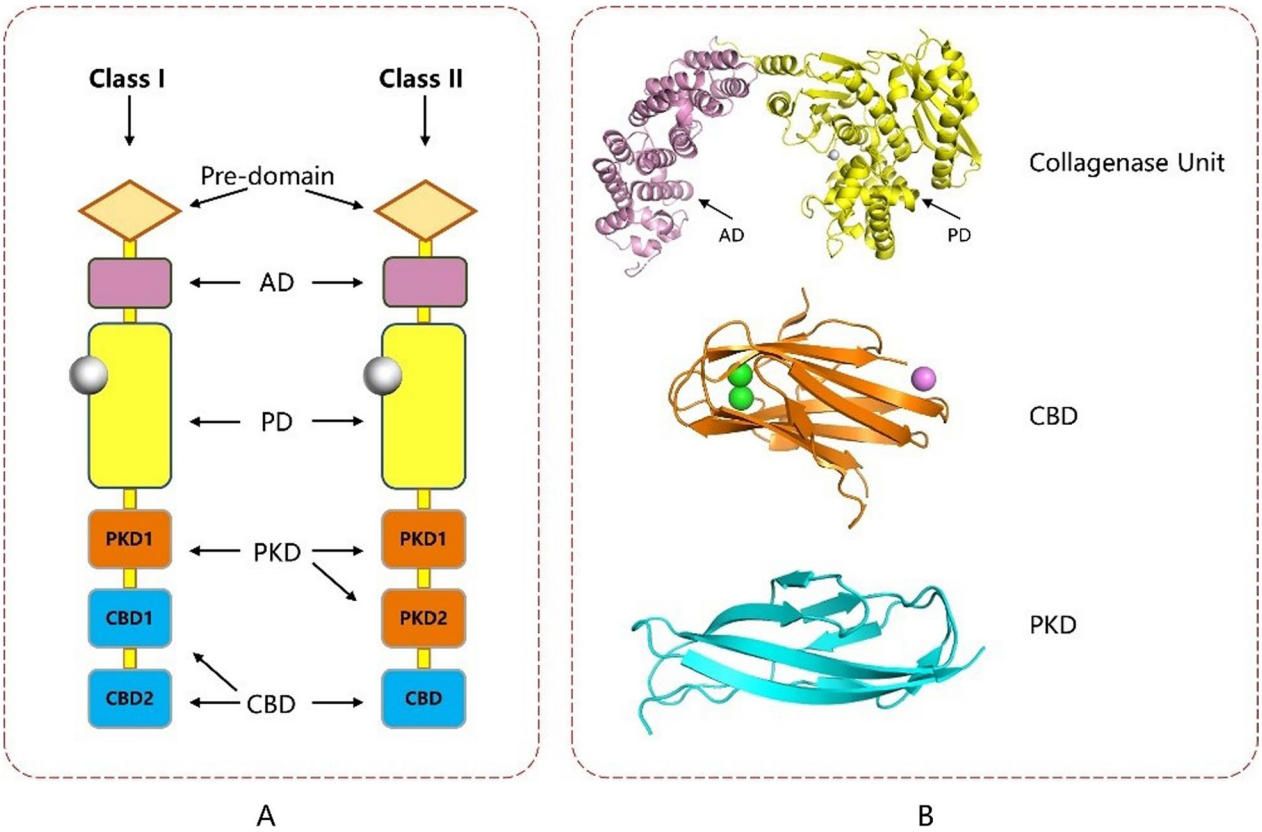


Fig. 5 Diagram of structure of bacteria collagenases. **A** Schematic diagram of the structure of two kinds of collagenase from *Clostridium histolyticum*. Class I collagenase(left) and class II collagenase(right). The catalytic Zn(II) ion is represented by a blue sphere; **B** Ribbon diagram of collagenase unit, collagen-binding domain (orange) and polycystic kidney disease-like domain (cyan) of ColG. The collagen unit contains an activator domain (pink) and a peptidase domain (yellow). The zinc ion is shown in gray; calcium ions, in green; and the chloride ion, in violet. The images were prepared with Bookhaven Protein Data bank entries 4ARE (collagenase unit), 2O8O (CBD) and 4TN9 (PKD) using Pymol

anomalous expression or degradation of collagens might cause some diseases.

One of the applications of collagenases is related to the enzymatic debridement of wounds and other injuries [85]. Debridement is applied to the treatment of chronic wounds, which is stalled in the inflammatory phase [86]. The use of collagenases in wound debridement is considered as a safe and effective choice [87, 88]. Karagol et al. [89] reported a successful case of enzymatic debridement, showing that collagenases can be used not only for the removal of eschar but also for the avoidance of the progression of necrotic tissue. The *Clostridium* collagenase ability to minimize burn progression was evaluated using the classic burn comb model in pigs. It was found that collagenase treatment had multiple effects including providing an early and improved inflammatory response and preventing the destruction of dermal collagen [90]. Recently, Francesco and coworkers performed enzymatic debridement on 70 patients with chronic wounds of different etiologies using ointment (Bionect Start®) based on hyaluronic acid and collagenase. Most of the patients (62 of 70) healed completely within 8 weeks [91]. It has been confirmed that with the aid of collagenase, the complications of surgery can be partly avoided, and the progress and enlargement of necrotic tissue can be limited [92].

Another application of collagenases is related to the treatment of Dupuytren's disease (DD), a disorder caused by the immoderate deposit of collagen (mainly types I and III) [93]. Before the production of collagenase, the only treatment for DD was surgery [94, 95]. In the early 1990s, Badalamente et al. began to investigate therapeutic treatment for DD with collagenases, and they finally obtained the approval of the Food and Drug Administration (FDA) in 2010 [96, 97]. Reports have indicated that collagenase treatment is effective and simple and promotes quick recovery in comparison with surgical treatment [98]. However, collagenase treatment has shown a certain recurrence rate and might be accompanied by some complications. Nayar and coworkers investigated 34 patients for the rates of contracture resolution and recurrence who went through collagenase treatment. It

Table 2 Applications of collagenases in the biomedical field

Disease	Cause of Disease	Treatment	Reference
Chronic wounds	Chronic wounds stalled in the inflammatory phase of wound healing	Collagenase injection	[89]
Dupuytren's disease	Immoderate deposit of collagen (mainly types I and III)	Collagenase injection	[99]
Cancers	Tumor ECM primarily composed of collagen and hyaluronic acid obstructs drug delivery	Collagenase injection	[105]
Peyronie's disease	Deposition of excessive collagen in the soft tissue of the penis	Collagenase injection	[183]
Glaucoma	Fluid drainage channels of the eye are closed because of abnormal production of collagen	Collagenase injection	[184]
Intervertebral disc herniation	Aggregation of collagen tissue and reduction in the distance between the vertebral spines	Collagenase injection	[185]
Keloids	Excessive growth of granulation tissue (collagen type III) at the site of a cured skin injury	Collagenase injection	[186]
Chronic total occlusions	Various degrees of fibroatheromatous plaque and thrombus	Collagenase injection	[187]
Uterine fibroid	Altered, disordered, and crosslinked collagens (types I, III, and V)	Collagenase injection	[188]
Burn wound	Hot liquid, steamand electrical burns and other forms of burns	Collagenase ointment	[189]

was observed that 42 of 44 metacarpophalangeal joints and 14 of 33 proximal interphalangeal joints performed immediate contracture resolution (improving from 50° to 1.5° with $p < 0.001$ and from 44° to 16° with $p = 0.182$, respectively). Moreover, they re-evaluated the patients at different time (up to 2 years) and found that reoccurrence may occur within 2 years after treatment [99]. Coleman et al. summarized the efficiency of collagenase treatment and adverse events on post-injection day 30. The results indicated that complications following collagenase treatment are common, among which the most (>75% of patients) were contusion, local peripheral edema, and pain in the extremities. But the severity of most adverse events is mild to moderate [100]. Nonetheless, studies of patient-reported outcome measures suggested that more than 85% of patients were satisfied following collagenase treatment for DD [101].

Collagenases are applied in genetic therapy, which has been viewed as a promising approach to treat cancers [102]. Cemazar and coworkers treated the tumors with enzymes and evaluated the transfection efficiency in 3, 9, and 15 days post-transfection. It was shown that a tenfold increase in the percentage of transfected area of GFP and a tenfold increase in functional luciferase within the tumor occurred after treatment with both collagenase and hyaluronidase, which suggested that the administration of two enzymes active against collagen and hyaluronan may be required to substantially increase gene titers within the tumor [103]. Increased interstitial fluid pressure (IFP) in tumors is an obstacle to the accumulation of systemically delivered nanocarriers [104]. The intravenous injection of MMP-1 was utilized to investigate whether this collagenase could reduce IFP in tumors. The results showed that IFP and the amount of collagen in the tumor are significantly reduced by MMP-1 at 1 h post-injection, supporting the potential use of collagenase has the ability to improve systemic gene delivery into tumors [105]. The applications of collagenases in the biomedical field are shown in Table 2.

2.3.2 Food processing

Collagenases have been applied to meat tenderization. The toughness of meats is partly due to the presence of collagen. Accordingly, its digestion may result in meat tenderness. The tenderization effect and the action of a cold-adapted collagenolytic enzyme MCP-01 on beef meat were investigated. The shear force of meat was reduced by 23%, and the relative myofibrillar fragmentation index of meat was increased by 91.7% at 4 ℃, while the fresh color and the moisture content of meat remained unchanged [106]. Ekram et al. isolated strains producing collagenases from slaughterhouse waste and applied them to the beef tenderization process. The results showed that the enzyme produced by the isolated strains was able to tenderize meat [107]. It is worth noting that safety concerns about pathogenicity and other unfavorable effects have limited the industrial use of microbial collagenases in the process of meat tenderization. One possible approach is the production of recombinant collagenases in non-pathogenic micro-organisms, which may avoid the existence of virulence factors. A recombinant metallopeptidase of *Aeromonas salmonicida* was produced using *Pichia pastoris* transformation for further meat tenderization. The pure peptide "Pro-Leu-Gly-Met-Trp-Ser-Arg" was used to determine the activity of recombinant collagenase. The concentration of the substrate (peptide) after 180 min was two times lower than that of the control. Meanwhile, the result of histological studies

of beef shank samples revealed a marked detachment of the perimetry from the muscle bundles and the disintegration of collagen fibrils, while the muscle fibers remained unchanged [108].

Collagen hydrolysates are bioactive and have been approved by the FDA as Generally Recognized as Safe (GRAS), which makes them widely exploited in the pharmaceutical, food and cosmetic industries [109–112]. Collagenases can be used for the preparation of collagen hydrolysates due to the fact that the hydrolysates obtained using enzymatic hydrolysis are environmental-friend and safe. A multitude of works have focused on the isolation of collagenases produced by various micro-organisms and their use for the hydrolysis of collagen tissues. Lima et al. described a simplified strategy to hydrolyze type I collagen using a collagenolytic protease produced by *P. aurantiogriseum*. The maximum value of the degree of collagen hydrolysis was achieved at 7.5 mg/mL collagen concentration, pH 8.0 and 25 °C [113]. Furthermore, some byproducts in the food industry have been used for the preparation of collagen hydrolysates. Recombinant bacterial collagenase mining using *Bacillus cereus* was successfully performed and applied to hydrolyze bovine bone to obtain collagen-soluble peptides. The collagenase exhibited optimal collagen hydrolytic capacity under the conditions of 110.0 μg/mL collagenase concentration, 35 ℃, pH 8.0 and 6.0 h. [114]. Recently, marine bacteria have become an important source for the identification of novel collagenases. Collagenases produced by marine bacteria are usually more efficient in the catalysis of marine collagen in fish skin and bone than collagenases from land animals [108]. Yang and coworkers used *Pseudoalteromonas* sp. SJN2 to optimize the method for producing marine collagenases. The experimental collagenase production was 322.58 U/mL under the optimal fermentation conditions [115].

3 Regulatory approaches using collagenase inhibitors

MMPs are involved in the degradation of ECM proteins. Ever since their first description, MMPs and their inhibitors have been in the focus of pharmaceutical research. To regulate the enzyme activity of MMPs, researchers have screened and developed MMP inhibitors (MMPis) in recent decades [116]. Over time, the overuse of antibiotics led to a high number of bacterial populations resistant to multiple antimicrobials. A promising way of overcoming the problem is to design compounds that target virulence factors rather than bacteria. As a prominent virulence factor, it is necessary to design inhibitors against bacterial collagenases. Inhibitors can be divided into two categories: zinc-binding inhibitors and non zinc-binding inhibitors.

3.1 Zinc-binding inhibitors

The first zinc-binding inhibitors were small molecular mimics of native peptide substrates; the design idea was to bind the small molecular mimics of these peptides with a zinc-binding group (ZBG) represented by hydroxamic acid and chelate the catalytic Zn(II) ion. The ZBGs in these inhibitors serve to replace the zinc-bound water molecule, lock the inhibitor in the active site and direct the backbone of the inhibitor to the substrate binding pocket to inactivate the enzyme [117, 118]. These inhibitors have a wide range of inhibition and show a strong inhibitory effect because the catalytic Zn(II) ion is conserved in MMPs and bacteria collagenases. Hydroxamic acid (–CONHOH) emerged as a preferred zinc-binding group, as it can effectively interact with the catalytic Zn(II) ion in a bidentate fashion (Fig. 6) and is relatively easy to synthesize, which makes hydroxamic acid-based compounds account for a large part of the inhibitors [119–122]. Compounds **1** (Marimastat), **2** (Batimastat) and **3** (Ilomastat) are typical examples of such inhibitors (Table 3) [123–125].

However, there has been some concern about the use of strong chelation of Zn(II) ions with hydroxamic acid as the ZBG, such as low selectivity for different enzymes and a possible off-target effect [126]. Accordingly, researchers have been looking for other relatively weak ZBGs to replace hydroxamic acid. Many functional groups have been investigated, such as carboxylic acid, phosphonate, phosphinate, and thiol (compounds **4**, **5**, **6** and **7**; Table 3) [127–130]. Figure 5 shows the chelation of these groups with the zinc ion. The application of these weaker ZBGs reduces the effect of metal chelation on binding affinity and enhances the supramolecular interaction between ligands and proteins [131, 132].

Due to the occupancy of the catlytic Zn(II) ion by the zinc-binding inhibitors, the substrates cannot be hydrolyzed by collagenases. As a result, these inhibitors are all competitive inhibitors.

3.2 Non zinc-binding inhibitors

3.2.1 Catalytic domain (non zinc-binding) inhibitors

In addition to the catalytic Zn(II) ion binding site, there are several other binding sites (recognition pockets) in the catalytic domain of MMPs. Some of them are on the right side of the catalytic Zn(II) ion (called primed side, with pockets being named S1′, S2′ and S3′); others are on the left side of the catalytic Zn(II) ion (called unprimed side, with pockets being named S1, S2 and S3) [133]. The interaction with these pockets has been found to be

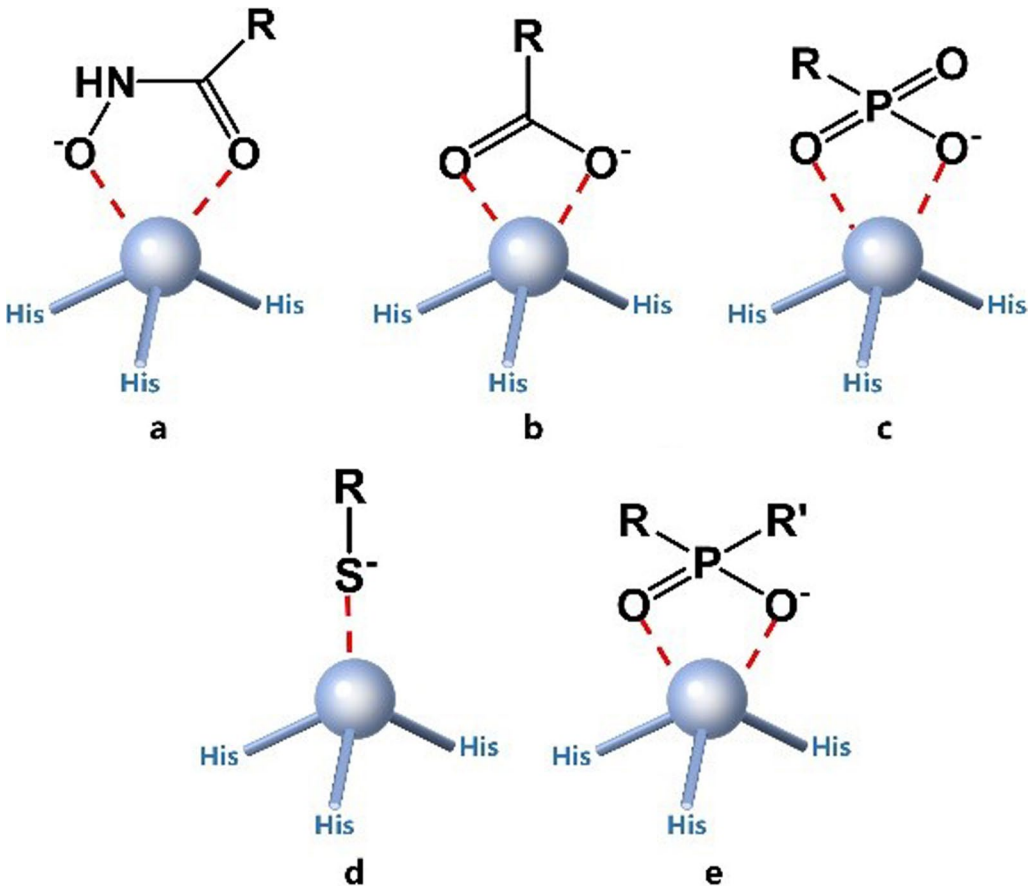


Fig. 6 Diagram of chelation of ZBGs (**a** hydroxamate; **b** carboxylate; **c** phosphonate; **d** thiol; **e** phosphinate) with the catalytic Zn(II) ion (represented by a blue sphere)

able to guide the development of new species of inhibitors. Among them, it has been proved that the S1' subsite (pocket) presents the greatest difference in amino acid sequence and depth among different MMPs, which is very important for substrate selectivity [134]. Based on the depth of the S1' pocket, MMPs can be divided into shallow (such as MMP-1), intermediate (such as MMP-8) and deep (such as MMP-13) [135]. Learning from the limitations of the former MMPis, researchers began to shift their attentions to the catalytic domain of MMPs and have developed several non zinc-binding inhibitors targeting the catalytic domain (compounds **8, 9** and **10**; Table 3) [134–140]. Most of these inhibitors have long molecular structures and have aromatic or planar connecting ring structures [141].

It is notable that different MMP inhibitors can be designed according to the depth of the pocket, but it is not the only issue to consider. For example, the X-ray structure of MMP-1 binding to an inhibitor (compound **11**; Table 3) with a diphenyl ether sulfone substituent indicates that the group can penetrate the S1′ pocket with an induced matching mechanism. This indicates that the consideration of enzyme kinetics is equally crucial in the process of collagenase binding with the substrate [142].

3.2.2 Inhibitors binding to the hemopexin-like domain

Apart from the catalytic domain, MMPs also contain other distal structures, such as a propeptide domain and a hemopexin-like domain. Previous works have not devoted much attention to them, because these domains are far from the catalytic domain. It is now recognized that the hemopexin-like domain can allosterically manipulate enzyme activity during collagen hydrolysis, which can be applied for the development of novel inhibitors (compound **12** and **13**, Table 3) [143–149]. With increasing studies being conducted on the overall structure of MMPs, these non-catalytic domains may be further utilized for designing selective MMPis. Unlike inhibitors that employ Zn(II) chelation, these compounds generally display a non-competitive mechanism of inhibition [141]. Although, some inhibitors

Table 3 List of inhibitors with different inhibitory principles

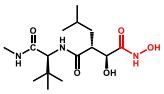
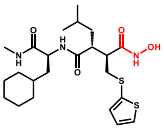
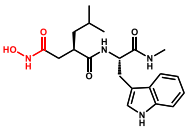
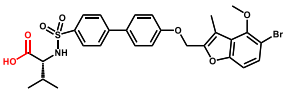
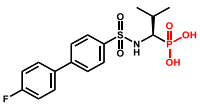
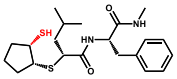
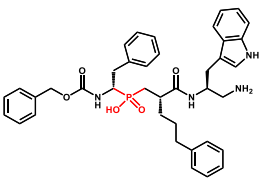
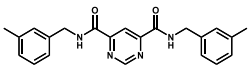
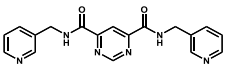
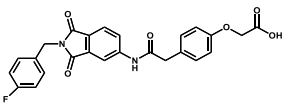
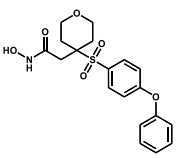
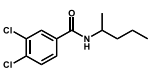
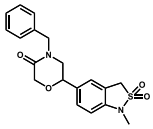
ID	Structure	Inhibitory principles	Type of inhibitor	Reference
1	 IC50: MMP-1 = 5 nM	Zinc binding (hydroxamic acid)	Competitive	[123]
2	 IC50: MMP-1 = 5 nM	Zinc binding (hydroxamic acid)	Competitive	[124]
3		Zinc binding (hydroxamic acid)	Competitive	[125]
4	 IC50: MMP-13 = 0.5 nM	Zinc binding (carboxylic acid)	Competitive	[127]
5	 Ki: MMP-8 = 0.6 nM	Zinc binding (phosphonate)	Competitive	[128]
6	 Ki: MMP-8 = 2.5 nM	Zinc binding (phosphinate)	Competitive	[129]
7	 IC50: MMP-8 = 1.2 μM	Zinc binding (thiol)	Competitive	[130]
8	 IC50: MMP-13 = 400 nM	Catalytic domain binding (non-zinc)	Non-competitive	[190]
9	 IC50: MMP-13 = 6.6 nM	Catalytic domain binding (non-zinc)	Non-competitive	[190]
10	 IC50: MMP-13 = 6 nM	Catalytic domain binding (non-zinc)	–	[136]
11		Catalytic domain binding (non-zinc)	–	[142]
12		Hemopexin-like domain binding	–	[143]

Table 3 (continued)

ID	Structure	Inhibitory principles	Type of inhibitor	Reference
13	 IC50: MMP-13 = 0.56 nM	Hemopexin-like domain binding	–	[144]

still have a competitive inhibition mechanism [150, 151]. As a consequence, the absence of a ZBG is not an obvious guarantee of a non-competitive/uncompetitive mechanism, and a detailed kinetic characterization is required.

3.3 Applications of collagenase inhibitors

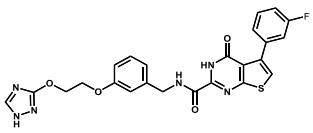
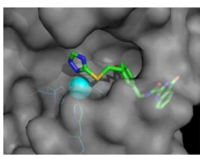
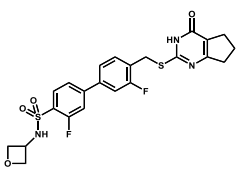
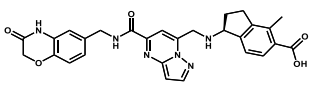
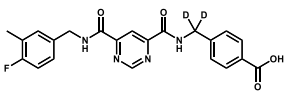
3.3.1 Biomedical field

MMPs have the potential to degrade different components of the ECM, including collagen, and play a role in cell proliferation and apoptosis, as well as physiological processes such as immune function and tissue healing [152]. The over-expression of MMPs may give rise to quite a few diseases, such as osteoarthritis (OA) and cancer [153]. Consequently, the use of MMPis becomes a promising therapeutic tool for these diseases.

The inhibition of MMP-13 is one of the most promising approaches for the treatment of cartilage degradation in OA, which is an age-related chronic disease characterized by the destruction of articular cartilage and progressive degradation due to mechanical stress [154]. The fact that type II collagen (the major structure protein of the cartilage matrix) can be degraded by MMP-13 makes the enzyme a pivotal role in OA formation [155, 156]. Several strategies, such as high-throughput screening (HTS) and natural-product-derived fragments (NPDFs) [140], have been used for discovering of MMP-13 inhibitors and have yielded a number of MMP-13 inhibitors, including zinc-binding inhibitors and non zinc-binding inhibitors. A variety of ZBGs have been used in zinc-binding inhibitors, such as hydroxamic acids, reverse hydroxamic acids, pyrimidinetriones and carboxylic acids [127, 157–159].

A series of compounds that possess a 1,2,4-triazol-3-yl group as a ZBG were designed and synthesized as MMP-13 inhibitors. Among these evaluated compounds, compound **14** (Table 4) exhibited outstanding potency against MMP-13 (IC_{50} = 0.036 nM) and selectivity (> 1500-fold) for other MMPs and tumor necrosis factor-α converting enzyme (TACE). Further evaluation demonstrated that compound **12** was effective in preventing in vitro degradation (70.8% inhibition of cartilage degradation at 1 μM) [160]. Recently, a series of novel inhibitors based on a previously developed MMP-13 inhibitor that interacts with the S1′ subsite and reaches over the catalytically

Table 4 List of MMP-13 inhibitors for the treatment of OA

ID	Structure	IC_{50} against MMP-13 (nM)	Cocrystal structure	Reference
14		0.03		[160]
15		42	–	[161]
16		0.03	–	[163]
17		4.8	–	[164]

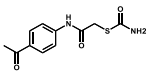
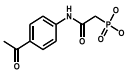
active Zn(II) ion within the active site were also designed and synthesized, among which oxetane-containing compound **15** (Table 4) was found to exhibit favorable inhibition of MMP-13 (IC_{50} = 42 nM) in in vitro studies with an excellent selectivity among other MMPs. Furthermore, in vivo pharmacokinetic studies within the synovial fluid of the rat knee joint were performed and demonstrated that the compound is a promising lead compound for osteoarthritis [161].

Apart from binding to the Zn(II) ion, it is found that the introduction of fully hydrophobic interaction with its S1′ pocket is another common strategy for obtaining inhibitory potency and selectivity against MMP-13 [162]. The synthesis and evaluation of a non zinc-binding MMP-13 selective inhibitor (compound **16**; Table 4) were reported. The compound possessed outstanding potency against the catalytic domain of recombinant human MMP-13 (IC_{50} = 0.03 nM) with great selectivity for other MMPs, TACE and Aggrecanase 1 (> 20,000-fold). Furthermore, it could penetrate and attain high, sustained concentrations (≥ 2 μM for 8 weeks) in cartilage and demonstrated 100% inhibition for 3 weeks [163]. Bendele reported a potent pyrimidine dicarboxamide derivative, compound **17** (Table 4), as a potential disease-modifying OA drug (DMOAD). The potency of compound **17** against the catalytic domain of recombinant human MMP-13 was determined to be 4.8 nM, and it showed high selectivity, with little inhibition for other MMPs (IC_{50} > 100,000 nM for MMP-1, -2, -3, -7, -12, and -14) [164].

In addition to the treatment of arthritis, MMPis also play a role in other fields of medicine, such as cancer [165, 166], idiopathic pulmonary fibrosis [167], cardiac disease [168] and acute lung injury [169].

Alternative non-antibiotic therapy is urgently needed with the gradual rising in bacterial drug resistance and the slow discovery of new antibiotics [170]. The pharmacological inhibition of bacterial collagenase is a promising strategy to block bacterial virulence without exerting selective pressure [171]. Several compounds have been designed that bind closely to the active site of bacterial collagenase using different ZBGs to obtain the expected inhibitory potency [172–178], some of which, while being reasonably effective inhibitors of *clostridium* collagenase, demonstrate negligible activity against human MMPs. Schönauer and coworkers discovered an N-aryl mercaptoacetamide-based inhibitor scaffold using surface plasmon resonance-based screening complemented with enzyme inhibition assays. Compound **18** (Table 5), containing a thiocarbamate unit, showed submicromolar affinity (IC_{50} = 0.01 μM) for ColH from the human pathogen *Clostridium histolyticum* with more than 1000-fold selectivity for MMPs [177]. Recently, they substituted the thiol moiety with phosphonate, which is not prone to oxidation or degradation, while keeping the *N*-arylacetamide core structure intact. This novel compound, compound **19** (Table 5), retained the binding mode of compound **18** with reasonably potent inhibition (IC_{50} = 7 μM) against the *clostridial* collagenase ColH remained. Therefore, it still showed remarkable selectivity for other MMPs and even better selectivity for other human off-targets (histone deacetylases (HDACs) and TACE) [178]. These studies may pave the way for the development of selective bacterial collagenase inhibitors with potential therapeutic applications in humans.

Table 5 List of bacterial collagenases (ColH) inhibitors

ID	Structure	IC_{50} against ColH (μM)	Reference
18		0.010	[177]
19		7	[178]

3.3.2 Leather manufacturing

Collagenase inhibitors are applied in leather manufacturing. The conventional unhairing process generates and discharges a wide range of pollutants [179], urging people to look for novel materials and processes. Compared with traditional methods, enzymatic unhairing shows advantages in reducing the pollution load [180]. However, the collagenase component in enzyme preparations hydrolyzes the collagens of the hide and skin, which may cause grain damage. Bivalent metal ions Mn(II), Mg(II), and Zn(II) were introduced to inhibit the activity of AS1.398 protease of hydrolyzing collagen during the enzymatic unhairing of bovine hide. The presence of Mn(II), Mg(II), and Zn(II) ions at 10 mM led to the AS1.398 protease hydrolysis of collagen being inhibited by 10%, 35% and 55%, respectively. Compared with metal ion-free enzymatic unhairing, the addition of certain bivalent metal ions resulted in the same unhairing rate with less damage to the grain and hair pores [181, 191]. Furthermore, Li et al. [182] investigated the mixture inhibitors system based on Cu(II) ions for the enzymatic unhairing of bovine hide. The results inferred that the addition of 5 mmol/L Cu(II) and 20 mmol/L sodium succinate could maintain the unhairing rate while performing efficient inhibition of collagenolytic activity; thus, the leather was subjected to less grain damage and maintained an intact pore structure.

Currently, the study of collagenases and their inhibitors mainly focuses on the medical field, and only some

studies investigated enzymatic unhairing in the leather-making process. The inhibitory effect of different inhibitors on enzyme preparations for unhairing also needs to be urgently solved. Insights into collagenases and their inhibitors could bring new impetus to the innovation and promotion of the enzymatic unhairing process.

4 Conclusion

MMPs and bacterial collagenases are two kinds of collagenases that can cleave the triple-helical domain of native fibrillar collagens. They have different domain compositions and catalytic mechanisms on hydrolysis of collagens. This review presents approaches on the applications of collagenases and their inhibitors in biomedicine, food processing, and the enzymatic unhairing process in leather-making industry. MMPs and bacterial collagenases are recognized as targets for the treatment of a variety of diseases, therefore their inhibitors are desperately needed and the achievements about the collagenases and their inhibitors derived from previous studies are helpful for the future research.

Abbreviations

ECM	Extracellular matrix
MMP	Matrix metalloproteinase
MT-MMPs	Membranetype-MMPs
Prop	Propeptide
Cat	Catalytic domain
Hpx	Hemopexin-like domain
AD	Activator domain
PD	Peptidase domain
CBD	Collagen binding domain
PKD	Polycystic kidney disease-like domain
FDA	Food and Drug Administration
DD	Dupuytren's disease
GFP	Green fluorescent protein
IFP	Interstitial fluid pressure
GRAS	Generally recognized as safe
ATPS	Aqueous two-phase system
ABTS	2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)
DPPH	1,1-Diphenyl-2-picryl-hydrazyl
ORAC	Oxygen radical absorbance capacity
MMPi	MMP inhibitor
ZBG	Zinc binding group
OA	Osteoarthritis
HTS	High-throughput screening
NPDFs	Natural-product-derived fragments
TACE	Tumor necrosis factor-α converting enzyme
DMOAD	Disease modifying OA drug
HDAC	Histone deacetylases

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Author contributions
Conceived and designed the review, and critical revised the manuscript: HMC; Wrote and revised the manuscript: SJW, XWZ and ZCJ. All authors read and approved the final manuscript.

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Availability of data and materials
Not applicable.

Declarations

Competing interests
The authors declare that they have no competing interests.

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