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Review

Collagenolytic enzymes produced by fungi: a systematic review

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ABSTRACT

Specific proteases capable of degrading native triple helical or denatured collagen have been required for many years and have a large spectrum of applications. There are few complete reports that fully uncover production, characterization and purification of fungi collagenases. In this review, authors searched through four scientific on line data bases using the following keywords (collagenolytic OR collagenase) AND (fungi OR fungus OR fungal) AND (production OR synthesis OR synthesize) AND (characterization). Scientific criteria were adopted in this review to classify found articles by score (from 0 to 10). After exclusion criteria, 21 articles were selected. None obtained the maximum of 10 points defined by the methodology, which indicates a deficiency in studies dealing simultaneously with production, characterization and purification of collagenase by fungi. Among microorganisms studied the non-pathogenic fungi *Penicillium aurantiogriseum* and *Rhizoctonia solani* stood out in volumetric and specific collagenase activity. The only article found that made sequencing of a true collagenase showed 100% homology with several metalloproteinases fungi. A clear gap in literature about collagenase production by fungi was verified, which prevents further development in the area and increases the need for further studies, particularly full characterization of fungal collagenases with high specificity to collagen.

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Introduction

Collagen is a fibrous protein found in skin, tendons, bones, teeth, blood vessels, intestines and cartilage, corresponding to 30% of the total protein, whose main function is structural.[1,2] There are more than 26 genetically distinct types of collagens, characterized by considerable complexity and diversity in their structure, their splice variants, presence of additional, non-helical domains, their assembly and their function.[3,4] Each collagen molecule is a small, hard stick formed by interlacing in a triple helix of three polypeptide chains called alpha chains (Fig. 1).

Specific proteases capable of degrading native triple helical or denatured collagen have been required for many years.[5] Collagenases have been isolated and characterized from different sources, as digestive tracts of fish and invertebrates including: tadpole tailfin,[6,7] Atlantic cod,[8] land snail (*Achatina fulica*),[9] tropical shrimp (*Penaeus vannamei*),[10,11] catfish (*Parasilurus asotus*),[10,12] mackerel (*Scomber japonicas*)[13]; besides plants (*Zingiber officinale*)[14]; bacteria as: *Bacillus cereus* and *Klebsiella pneumoniae*,[15] *Bacillus pumilus*,[16] *Bacillus licheniformis*[17–19] and fungi, shown in this review.

Proteases, in general, from microbial sources are preferred to the enzymes from plant and animal sources for its biochemical diversity and genetic manipulation possibility.[20,21] Microbial collagenase have been recovered from pathogenic micro-organisms, especially *Clostridium histolyticum*, which is the most widely used commercial source.[22] Other studies reported collagenase producing fungi of genera *Aspergillus*, *Cladosporium*, *Penicillium* and *Alternaria*.[23]

Among microorganisms that produce collagenolytic enzymes, filamentous fungi have great advantages such as high productivity and low production cost, rapid development, and the resulting enzyme may be modified and recovered more easily.[24] Enzyme production occurs extracellularly, which makes it particularly easier to recover afterwards.[25] As fungal proteases are capable of hydrolyzing many other proteins besides collagen, the demand for collagenases from fungi with suitable characteristics, namely high specificity, is a very significant research direction to be taken.[26] Collagenases are capable of hydrolyzing both native and denatured collagen, and are becoming increasingly important commercially.[27]

Collagenases have been used in medical, pharmaceuticals, food, cosmetics and textiles segments and have applications in fur and hide tanning to help ensure the uniform dying of leathers.[28,29] In medical applications, it can be used in burns and ulcers treatment,[30,31] to eliminate scars,[32] for Dupuytren's disease treatment in addition to various types of fibrosis such as liver cirrhosis, to preparing samples for diagnosis,[33] for production of peptides with antioxidant and antimicrobial activities,[34] and play an extremely important role in the transplant surgery success of some specific organs.[32]

Fig. 1 – Collagen molecule: intertwining three alpha chains triple helix.

The rules for vertebrate collagenase classification are very clear, but the same does not apply to microbial enzymes. It is difficult to distinguish between true collagenases and gelatinases or other proteases, which leads to controversy and imprecision in the classification and nomenclature of these enzymes. Microbial collagenases are capable of degrading triple-helical collagen and denatured fragments in various sites and are less specific. Although several proteases can hydrolyze denatured collagen, they cannot be mistaken with true collagenases, able to hydrolyze the native collagen as found in connective tissues.[35,36]

The search for new microbial collagenases has increased over the years and its production currently represents one of the biggest enzyme industries.[37,38] The development of new production methods, including the search for producing micro-organisms, alternative sources of substrates, and better extraction conditions and purification of collagenase, has been of great importance, since it has a wide application spectrum with high biotechnological potential. Besides, the main published review papers concerning microbial collagenolytic enzymes are limited to bacterial source.[22,35,39] In this context, the authors felt the need to better understand the state of the art regarding production, characterization and purification of collagenolytic enzymes by fungi.

Material and methods

The first step on this process, was to make electronic searches in the Scopus (http://www.scopus.com/), ScienceDirect (http://www.sciencedirect.com/), ISI Web of Science (http://apps.isiknowledge.com) and PubMed (http://www.ncbi.nlm.nih.gov/pubmed) databases, using the following keywords: (collagenolytic OR collagenase) AND (fungi OR fungus OR fungal) AND (production OR synthesis OR synthesize) AND (characterization).

This procedure allowed selecting published papers on the production and characterization of collagenolytic enzyme produced by fungi. Papers that did not report on the enzyme production process were excluded. There were no limitations regarding the year and date of publication, due to lack of publications about this issue. No restrictions were made for methodology used, types of analysis and quantification of results. In addition, there were no restriction on type of micro-organism, collagenolytic activity methodology, culture conditions and characterization assays.

Two independent searches were made and the conformity of the selected papers validated, considering the inclusion criteria described. In case of divergence among the papers, all of the criteria were reviewed and discussed. When in the article title only protease production was mentioned, lacking collagen related terms, researchers proceeded to summary evaluation, looking for methodologies for activity determination involving collagen or gelatin as substrate.

Papers selection criteria were defined to evaluate both better conditions for collagenolytic enzyme production by fungus with biotechnological potential applicability and methodological quality in the characterization of the enzyme. Scientific criteria adopted in this review were according to the ones proposed by Greenhalgh.[40] The parameters were classified on the scale: adequate (score: 2), partially adequate (score: 1) and inadequate (score: 0) or adequate (score: 1) and inadequate (0).

Production process: Papers that studied the best growing conditions for producing collagenolytic enzyme received a score of 2, papers that did not conduct studies to improve growing conditions, using collagen or gelatin as substrate, received score of 1, and those which used nonspecific means for collagen production received a score of 0. *Characterization of the enzyme*: papers that reported biochemical characterization of enzyme and included other tests as well as optimum pH and temperature and enzyme inhibition tests, received a score of 2. Those which evaluated only optimum pH and temperature and the effect of inhibitors received a score of 1. Papers that did not have at least these three factors in enzyme characterization were considered inadequate and received a score of 0. *Quantification method of collagenolytic activity*: methods that used chromogenic substrates (OrangeCollagen or Azocoll) for quantification of collagenolytic activity, received a score of 2. Papers with other quantitative methodologies for collagenolytic activity, received a score of 1, and those that held only qualitative analysis activity, received a score of 0. *Purification*: purification by chromatography methods received a score of 2, those which used other purification methods, received a score of 1, and those that did not do any kind of purification, received 0. *Micro-organism*: articles that used non-pathogenic fungi for collagenolytic enzyme production received a score of 1, while those using pathogenic fungi were considered inadequate and received a score of 0. *Substrate specificity*: enzymes with specific activity over collagen, received a score of 1; those who presented a wide hydrolysis spectrum or have not been tested, received a score of 0.

Maximum overall score was 10 points. Other parameters such as production time, year of publication, satisfactory collagenolytic activity, among others, did not scored but were taken into consideration, as they were relevant to subsequent discussion. The parameters scored are summarized in Table 1.

A table was assembled with a summary of selected articles relevant data according to criteria adopted on the review, including some features as optimum pH and temperature, inhibitors, enzyme nature (true collagenase or not) and enzyme sequence.

Results and discussion

By applying the established search procedure, a total of 1346 articles were found in Science Direct database, 678 articles in Scopus database, 45 articles in PubMed, and 5 articles in Web of Science, totaling 2074 articles. Based on defined inclusion and exclusion criteria, 21 articles were selected for this review, distributed as shown in Fig. 2.

Regarding the scores obtained for each selected article, none obtained the maximum of 10 points defined by the methodology. According to the distribution in Table 2, only one article hit a score of 9 (4.77% of selected articles), two articles obtained the score of 8 (9.52%), and three articles reached the score of 7 (14.29%). 71.43% of the articles achieved scores below 7, which indicates a deficiency in studies dealing simultaneously with production, characterization and purification of collagenase by fungi. Where the enzyme obtained should present specificity to substrate and have its activity quantified by the method adopted as the most appropriate (Azocoll).

As described in the methodology, no time interval has been defined. However, only 11 articles have been published in the last 10 years. Of these 11 articles, only 4 were published in the last 5 years, clearly indicating a need for further research related to the production of collagenase by fungi.

Microorganism

Based on this systematic review, 21 articles were selected, of which 17 were carried out with 10 different genera of filamentous fungi (*Penicillium*, *Aspergillus*, *Arthrobotrys*, *Monacrosporium*, *Trichophyton*, *Microsporum*, *Lecanicillium*, *Entomophthora*, *Micromycetes* and *Lagenidium*). Two genera found were classified as dimorphic (*Coccidioides* and *Paracoccidioides*), and only one had a yeast morphology (*Zygosaccharomyces*).

From the industrial point of view, pathogenicity can negatively influence microorganism choice for bioprocess development. Interestingly, approximately 40% of fungi cited in selected articles are described as classic pathogens. The non-pathogenic species that were associated with good collagenolytic enzyme production were *Rhizoctonia solani* with a production of 212.3 U/mL[55] and *Penicillium aurantiogriseum* with 231 U/mL[24] and 164 U/mL[58].

A great diversity of collagenolytic enzymes producing fungi could be observed (more than 20 different taxa). Most belonging to phylum *Ascomycota*, other to phyla *Basidiomycota* (*R. solani*), *Entomophthoromycota* (*Conidiobolus coronatus*) and *Oomycetes* (*Lagenidium giganteum*). Filamentous fungi are clearly more studied in comparison to yeasts for collagenolytic enzyme production. Many articles contain pathogenic fungi in order to better understand its pathogenesis mechanisms and not in order to study enzymatic production itself. The genus *Aspergillus* was the most frequent, followed by *Penicillium* and *Entomophthora* genres. Considering pathogenesis, enzyme activity and specificity, the fungi better qualified for enzyme production were the filamentous fungus *P. aurantiogriseum* and *Zygosaccharomyces rouxii* yeast.

Culture medium

Culture medium selection is of great importance for collagenase production, since this factor will directly affect final process cost. As said earlier, one of the advantages of working with microorganisms is the possibility to vary the composition of the culture medium, using lower cost materials, such as byproducts of the fishing industry, for example, as substrate. Nine of the selected papers presented a culture medium containing collagen or gelatin in its composition, other studies used other sources of carbon and nitrogen, mainly yeast extract. Some studies involving bacteria indicate that adding gelatin or casein in the medium increases the collagenase yield. However, the work of Ok and Hashinaga[48] with *Z. rouxii* yeast, observed that adding gelatin in YPG medium was not essential for the production of collagenase. Lima et al.[24] reported the use of a inexpensive culture medium for

Table 1 – Score of selected parameters for critical evaluation of the systematic review.

Criteria for determining the scores	Pointing		
	2	1	0
(A) Production	Specific for collagenase, with controlled variables	Specific for collagenase, with uncontrolled variables	No specific for collagenase
(B) Characterization	Complete	Partial	Absent
(C) Microorganism		Non-pathogenic	Pathogenic
(D) Collagenolytic activity method	Azocoll or OrangeCollagen	Others	Absent
(E) Purification	Complete	Partial	Absent
(F) Substrate		Collagenase (specific)	Non-Specific

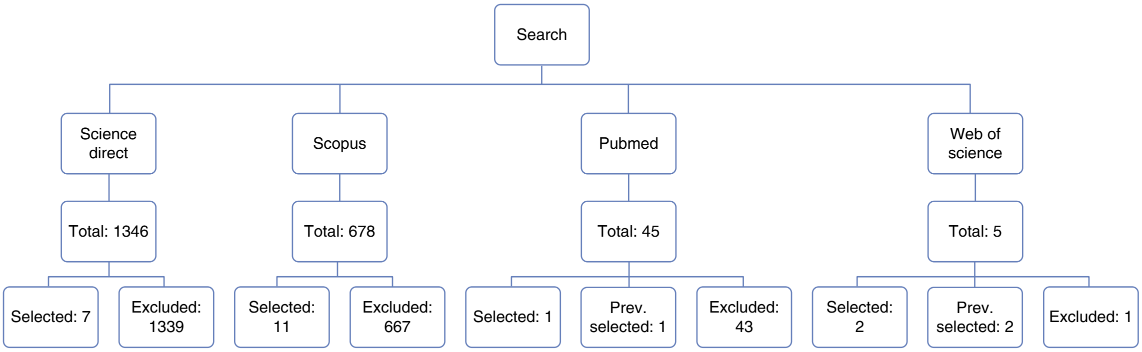

Fig. 2 – Total articles selected in four different databases using the described methodology.

Table 2 – Scores distribution of selected articles.

Authors	(A)	(B)	(C)	(D)	(E)	(F)	Total
Hurion et al. (1977)[41]	1	0	0	2	2	0	**4**
Hurion et al. (1979)[42]	1	0	0	2	2	0	**4**
Olutiola and Nwaogwugwu (1982)[43]	0	2	1	0	0	0	**4**
Dean and Domnas (1983)[44]	0	2	1	1	1	0	**6**
Zhu et al. (1990)[45]	0	0	0	0	0	0	**0**
Tomee et al. (1994)[46]	1	0	0	0	0	0	**3**
Ibrahim-Granet et al. (1996)[47]	0	1	0	2	2	0	**4**
Ok and Hashinaga (1996)[48]	2	1	1	1	1	1	**7**
Benito et al. (2002)[49]	0	2	1	2	2	0	**7**
Minglian et al. (2004)[50]	1	2	1	2	2	0	**8**
Yang et al. (2005)[51]	1	2	1	1	1	0	**6**
Wang et al. (2006)[52]	1	2	1	2	2	0	**7**
Mahmoud et al. (2007)[53]	2	1	0	2	2	0	**6**
Viani et al. (2007)[54]	1	0	1	0	0	0	**3**
Hamdy (2008)[55]	2	2	1	2	2	0	**8**
Lopes et al. (2008)[56]	0	1	0	0	0	0	**1**
Voltan et al. (2008)[57]	1	0	0	1	1	0	**4**
Lima et al. (2011a)[24]	2	2	1	1	1	1	**9**
Lima et al. (2011b)[58]	2	0	1	0	0	0	**5**
de Siqueira et al. (2014)[59]	0	2	1	1	1	0	**6**
Sharkova et al. (2015)[26]	0	0	1	0	0	0	**3**

(A) *Production*: Specific for collagenase production with controlled variables (score 2), specific for collagenase production with uncontrolled variables (score 1), non-specific for collagenase (score 0).
(B) *Characterization*: Complete characterization (score 2), partial characterization (score 1), absent (score 0).
(C) *Microorganism*: Non-pathogenic microorganism (score 1), pathogenic microorganism (score 0).
(D) *Collagenolytic activity*: Chromogenic substrate for collagenolytic activity method (score 2), others quantitative methods (score 1), qualitative (score 0).
(E) *Purification*: Purification by chromatography (score 2), partial purification (score 1), absent (score 0).
(F) Substrate Specificity: Collagenase with specificity for collagen (score 1), non-specific (score 0)

P. aurantiogriseum collagenase production, using soy flour as main substrate, and the same medium was used by authors Lima et al.,[58] reaching one of the best collagenolytic activity values found during this review (Table 3).

According to Hamdy,[55] the use of different batch or collagen types may interfere in enzymes production (enzyme activity) and collagenases from different microorganisms have affinity for specific types of collagen.[60] The production of different fungi in different media must be the subject of extended studies.

Culture conditions

Process development is a factor to be considered since optimization of culture conditions can promote an increase in the yields of protease and reduction in production costs, a major issue from an industrial point of view.[58,61]

Culture medium initial pH influences many enzymatic processes, such as enzyme production, cell transport across membranes and extracellular proteases expression.[62,63] The pH of the culture medium used in the selected articles ranged from 5.5 to 8.0, while temperature ranged from 18 to 37 °C. Regarding agitation, only Hurion et al.[42] showed non-mixed enzyme production, with microorganism *E. coronata*. In most of the works, ranged an agitation was in the range 100–200 rpm.

Fermentation time to collagenase production varied widely, from 24 h to 14 days, a time of 6–7 days being reported by 8 papers. Several studies showed activity decay after the 7th day of fermentation. Zhu et al.[45] demonstrated that, in medium containing insoluble collagen, after 2 weeks, fungus grows only to half the mass obtained in milk medium for 1 week. Articles that studied time influence on enzyme production reported higher production during stationary phase.

The work of Lima et al.[24] presented a factorial design to define the best growing conditions for the production of collagenase. Authors stated that initial pH, temperature and concentration of substrate are significant factors for collagenase production by *P. aurantiogriseum* using soybean flour medium.

Temperature influence on protease production by microorganisms is an important factor.[64] Temperature can regulate some components as enzymatic synthesis, enzyme secretion and length of the enzyme's synthesis phase, besides the properties of cell wall[63,65]. In general, studies used temperatures between 18 and 37 °C during production. The papers that studied different temperatures showed 30 °C as the optimum temperature for collagenolytic protease production. According to de Siqueira et al.,[59] incubation temperature interferes with fungus growth and metabolism, and consequently, peptidase production, the best temperature being 30 °C, according to Hamdy.[55] Lima et al.[24] reported that the best conditions for volumetric collagenolytic activity and biomass production were 24 °C and pH 7.0.

Among works that discriminated the shaking speed, 150–200 rpm were most used, except for Yang et al.,[51] that used 100 rpm. Hamdy[55] showed in his results that although there is little difference, the agitation of 175 rpm was the best for enzyme production.

Collagenolytic activity

Collagenolytic activity can be described as collagen hydrolysis by collagenase with peptides or amino acids release. Different methods are described in literature to measure this activity: colorimetric, fluorescence, turbidity and viscometry or radioactivity, among others. All these methods are quite time-consuming, the time needed ranging from 3 to 18 h. On the other hand, their major advantage is that most of them use native collagens.[22,66]

The radioactive or fluorescent methods require more time to produce substrate and more specific measuring equipment, as well as immunological methods. Moreover, synthetic oligopeptide is not an entirely specific substrate for collagenase.[66] Another used technique was developed by Mandl et al.,[60] using collagen *in natura* as substrate and ninhydrin as coloring reagent. The ninhydrin method measures free amino acids release, which difficult continuous activity monitoring or may underestimate enzymes activity if it releases peptides and not free amino acids. Besides, in this method the ninhydrin can react with free amino acids existing in solution, which limits the technique sensitivity.[67]

Among colorimetric methods, there is the Azocoll based.[68] The Azocoll is an azo dye-impregnated collagen, which is a specific substrate for collagenase, since it allows observing hydrolysis by release of dye-impregnated soluble peptides that are measured by spectrophotometry, increasing the method sensitivity.

All 21 articles selected in this review have different methodologies to quantify collagenase activity. Eight of the articles used Azocoll as a substrate for measurement of collagenolytic activity. Other papers used other quantitative methods, such as: ninhydrin (4 items), Folin (1 item), synthetic peptide (4 items) and OrangeCollagen (1 item).

Regarding the specific activity, less than half the articles quantify this parameter. Interestingly Hamdy[55] reported a specific activity value well above the others (18,064.7 × 10^3 U/mg). Another article that presented a good specific activity was Lima et al.,[24] with 319 U/mg. In general, the specific activity varied significantly (from 0.37 to 18,064.7 × 10^3 U/mg). The highest activities were observed in studies involving production optimization. However, effectiveness of production tends to be evaluated by volumetric collagenolytic activity due to the industrial relevance of this parameter Lima et al.[58]

Enzyme characterization

Isoeletric point

From selected articles, only two values for isoelectric point of collagenolytic enzyme were reported. The values found by Minglian et al.[50] and Wang et al.[52] were respectively 4.9 to an enzyme produced by *A. oligospora* and 6.8 to another produced by *M. microscaphoides*. However, in these studies no significant collagenolytic activity was reported when compared to other activities found, as can be seen in Table 3.

pH and temperature optimal

The optimum pH for enzyme activity varied considerably (pH 5–10). For the best results regarding collagenolytic activity,

Table 3 – Summary of selected articles relevant data according to the criteria adopted on the review.

Purif.	Enzyme nature	Enzyme sequence	Substrate	Specific activity	Inhibitors	pH and temper.	Isoelectric point
Chromatography	Gelatinolytic	X	BAE (trypsin-like), Elastin, Synthetic Peptides	0.088 nkat/mg	X	X	X
Ultrafiltration, Sephadex G-25 column	Gelatinolytic	X	Casein, Elastin, Synthetic Peptides	X	EDTA, DFP, TLCK, TPCK	X	X
Ammonium sulfate	Collagenolytic	X	Casein, Elastin, Collagen, Gelatin, p-nitrophenol caprylate	3.6 U/mg	Ca^{2+}, Na^{+}, EDTA, 2,4-DNP	pH 7, 35°	X
X	Collagenolytic	X	BAPA, TAME	X	PMSF, TPCK, IAA2-mercaptoethanol, cysteine HCl, Zn, Ca, Mg, EDTA, Ca, Mg	pH 8.4, 60°	X
(NH$_4$)$_2$SO$_4$ Ammonium Sulfate, Sephadex G25, Biogel A	Collagenolytic	X	Azocasein, Type I Collagen, Elastin	X	EDTA, fenantrolin, PA, PMSF, elastina, NEM	X	X
Cation exchange chromatography	Collagenolytic	X	Casein, Elastin, Orange Collagen	0.39 U/mg	X	X	X
GF, Orange 3, Yellow 1, HA, TSK	Collagenolytic	VFLGREPKPDAFY	Synthetic Peptides, Collagen	X	Fenantrolina, EDTA,	X	X
X	Collagenolytic	X	Synthetic Collagen, Peptides	70.4 U/mg	X	pH 8.2	X
Ammonium sulfate and cation exchange chromatography	X	AEQTDSTWGL	Casein, BSA, Skimmed milk, Gelatin, Collagen, Denatured Collagen, Nematode cuticle	0.37 U/mg	PMSF, EDTA, Pepstatin A, Leupeptin, Aprotinins	pH 10, 55°	X
Ammonium sulfate, Q Sepharose FF, Sephacryl S-100	Gellatonolytic	X	Casein, Gelatin, Nematode cuticle, Azocoll	1.12 U/mg	PMSF e SSI	pH 6–8, 45°	4.9
Ultrafiltration, HITrap SP FF, HiPrep phenyl FF	Gellatonolytic	AITQQQGAPW	Casein, BSA, Gelatin, Collagen, Nematode cuticle	48 U/mg	Leupeptin, Aprotinin, EDTA, Pepstatin A, PMSF	pH 10, 70°	X
Source 15Q, Phenyl Superose	Gellatonolytic	AEQLDSTWGL	Casein, BSA, Skimmed milk, Gelatin, Hydrolyzed Collagen	X	PMSF	pH 9, 60°	6.8
Ammonium Sulfate, Sephadex G-25 e DEAE-cellulose	Collagenolytic	X	X	92.17 U/mg	Cetrimide	X	X
X	Gelatinolytic	X	Keratin, Elastase, Synthetic Peptide	X	X	X	X
Ammonium sulfate, DEAE-cellulose, Sephadex G150	Collagenolytic	X	Collagen, Casein, elastin	18,064.7x10^{3} U/mg	EDTA, Iodoacetate, Sodium arsenate, arsenito, Cysteine	pH 5, 40°	X
X	X	X	Casein, Gelatin, Keratin, Albumin, Hemoglobin	X	PMSF	X	X
X	Collagenolytic	X	Casein, Elastase. Azocoll	X	PMSF, EDTA, Phenanthroline	X	X
X	Collagenolytic	X	Azocoll, Type I collagen, Gelatin, Azocasein	319 U/mg	PMSF, iodoacetic acid, EDTA e Pepstatin A	pH 9, 37°	X
X	Collagenolytic	X	X	X	X	X	X
X	Collagenolytic	X	Casein, Keratin	X	PMSF, EDTA, IAA	pH 6.5, 55°	X
X	Collagenolytic	X	Plasmin, Plasminogen, Azocoll	X	X	X	X

Table 3 – (*Continued*).

Purif.	Molecular weight (kDa)	Col. activ.	Col. activ. method.	Culture conditions	Culture medium	Microorg.	Authors
Chromatography	23–40	X	Synthetic peptide	pH 5.6, 30° C, without agitation, 15 days	Casamino acids, Dextrose, CaCl2, YE and Berthelot solution	*E. coronata*	Hurion et al. (1977)[41]
Ultrafiltration, Sephadex G-25 column	X	X	Synthetic peptide	pH 5.6, 30° C, without, 15 days	Casamino acids, Dextrose, CaCl2, YE and Berthelot solution	*E. coronata*	Hurion et al. (1979)[42]
Ammonium sulfate	X	X	Achilles tendon bovine	7 days, 30 °C	Glucose, salts, l-cysteine, tryptone, biotin, thiamine	*A. aculeatus*	Olutiola and Nwaogwugwu (1982)[43]
X	X	8 U/mL	Azocoll	Gyrotory shaker (20–24 °C), sob luzes fluorescents	Peptone, yeast extract and glucose (PYG) broth	*L. giganteum*	Dean and Domnas (1983)[44]
$(NH_4)_2SO_4$ Ammonium Sulfate, Sephadex G25, Biogel A	X	X	SDS-PAGE	2 weeks, T.A.	M9 (without NH_4Cl) + collagen	*A. flavus*	Zhu et al. (1990)[45]
Cation exchange chromatography	32	X	Orange collagen e Synthetic Peptide	5 days, 37 °C, 150 rpm	Yeast carbon base + collagen type I	*Aspergillus*	Tomee et al. (1994)[46]
GF, Orange 3, Yellow 1, HA, TSK	82	X	Rat native type I collagen	25 °C, pH 6, 7 days	Sabouraud	*T. schoenleinii*	Ibrahim-Granet et al. (1996)[47]
X	X	70.4 U/mL	Ninhydrin	pH 7, 25 °C, 50 h, with agitation	YPG	*Z. rouxii*	Ok and Hashinaga (1996)[48]
Ammonium sulfate and cation exchange chromatography	35	1% and 2%	Azocoll	26 °C, 200 rpm, 7 days	LMZ	*P. chrysogenum*	Benito et al. (2002)[49]
Ammonium sulfate, Q Sepharose FF, Sephacryl S-100	38	0.0134 U/mL/m	Azocoll	25–18 °C, 6 days,150-200 rpm pH 6.5	LMZ – with gelatin	*A. oligospora*	Minglian et al. (2004)[50]

Table 3 – (*Continued*).

Purif.	Molecular weight (kDa)	Col. activ.	Col. activ. method.	Culture conditions	Culture medium	Microorg.	Authors
Ultrafiltration, HITrap SP FF, HiPrep phenyl FF	32	14%	Non described	26 °C, 100 rpm, 6 days	Glucose, gelatin and salts	*L. psalliotae*	Yang et al. (2005)[51]
Source 15Q, Phenyl Superose	39 kDa	Collagen 15.9%, Denatured Collagen 48.1%,	Folin	6 days, 26 °C, 200 rpm	Protease inducing – with gelatin	*M. microscaphoides*	Wang et al. (2006)[52]
Ammonium Sulfate, Sephadex G-25 e DEAE-cellulose	72–92 kDa	82.95 U/mL	Ninhydrin	6 days, 37 °C	Gelatin, glucose, yeast extract, and native bovine collagen	*A. flavus*	Mahmoud et al. (2007)[53]
X	X	1 Unit of collagenase	Synthetic peptide	14 days	Medium with type I collagen	*M. canis*	Viani et al. (2007)[54]
Ammonium sulfate, DEAE-cellulose, Sephadex G150	66 kDa	212.33 U/mL	Ninhydrin	108 h, 175 rpm, pH 5.5, 30 °C	Sabouraud-glucose-collagen	*R. solani*	Hamdy (2008)[55]
X	25 kDa	X	Zymogram	pH 5.5, 9 days, T.A.	Czapek	*C. immitis*	Lopes et al. (2008)[56]
X	20–200 kDa	1.2 U/mL	Azocoll	150 rpm, 35 °C, for 7, 14, 21 e 28 days	Yeast carbon base + collagen + vitamin solution; neopeptoneBHI + elastin	*P. brasiliensis*	Voltan et al. (2008)[57]
X	X	164 U/mL	Azocoll	0.75% gelatin, 200 rpm, pH 8.0 and 28 °C	Soybean flour, glucose and mineral solution	*P. aurantiogriseum*	Lima et al. (2011a)[24]
X	X	231 U/mL	Azocoll	pH 7.0, 24 °C, 24 h	Soybean flour, Glucose and mineral solution	*P. aurantiogriseum*	Lima et al. (2011b)[58]
X	X	0.165 OD/mL	Azocoll	2.0×10^5 espores, 72 h, 30 °C, 75% humidity	Solid medium of wheat bran	*A. terreus*	de Siqueira et al. (2014)[59]
X	X	113.2 and 332×10^{-3} U/mL	Azocoll	200 rpm, 28 °C, 4 days	Several	*Micromycetes*	Sharkova et al. (2015)[26]

Lima et al.[58] and Mahmoud et al.,[53] the optimum pH of the enzyme was not evaluated. Ok and Hashinaga[48] evaluated the optimal pH (8.2) of the enzyme produced by *Z. rouxii* yeast. Lima et al.[24] found that pH of 9.0 was the best for collagenolytic enzyme produced by *P. aurantiogriseum*. Only the enzyme produced by *R. solani* presented an acid optimum pH, 5.0.[55] As pH, optimum enzyme activity temperature also varied greatly (from 35 to 70 °C). Only one of the works have produced a *in natura* collagen specific collagenase and evaluated optimum temperature, 37 °C.[24]

Inhibitors

Enzyme inhibitors are molecules that interact with enzyme or compounds that chelate metal ions required by the enzyme to maintain its conformation.[22] Some compounds can inactivate irreversibly to collagenase, such as dithiothreitol (DTT) and mercaptoethanol.[69,70] Other inhibitors tested are phenylmethylsulphonyl fluoride (PMSF) for serine proteases, ethylenediaminetetraacetic acid (EDTA) for metalloproteases, and iodoacetic acid (IAA) for cysteine proteases.[58]

Of the 21 selected articles, most conducted inhibitors tests (14 articles). Six concluded that the enzyme belongs to serine proteases group, four concluded belongs to metalloproteinases, two articles to both of the groups and in the remainder articles no conclusion were obtained. The collagenolytic enzyme produced by *R. solani* was inhibited by Hg^{2+}, iodoacetate, arsenate, arsenite, cystein and EDTA.[55] Lima et al.[24] reported the inhibition of the collagenase enzyme produced by *P. aurantiogriseum* by PMSF, indicating that the enzyme is a serine protease.

Substrate specificity

For certain industrial applications, such as medical and cosmetic areas, the enzyme specificity is one of the most important parameters to consider. From the 21 selected articles, 15 conducted substrate specificity tests using other protein sources. None performed specificity tests using different types of collagen. Hamdy[55] tested the enzyme produced by *R. solani* on collagen, casein and gelatin, and the best results were obtained with collagen. Lima et al.[24] reported enzyme specificity tests produced by *P. aurantiogriseum* on Azocoll, type I collagen, gelatin and azocasein, where the best results were found for the first substrate, Azocoll.

Molecular weight

The identified size of collagenolytic enzymes found in the different papers ranged from 25 to 82 kDa. However, the majority of the values (5 of 11 papers) are between 32 and 39 kDa. None of the two studies that have specific activity for collagen succeeded in obtaining the precise enzyme molecular weight. Among the articles that presented largest enzymatic activity, only Hamdy[55] determined the enzyme size by electrophoresis, reporting a value of 66 kDa, with 212.33 U/mL of enzyme activity.

Molecular analysis

Only two articles found performed sequencing of gene responsible for enzyme production. Both studies were about collagenolytic proteases from nematode-trapping fungi.[50,52] However, these enzymes have low activity for native collagen, which prevents its characterization as a true collagenase.

The enzyme sequence of true collagenase produced by *Tricophyton schoenleinii* (VFLGREPKPDAFY) had homology with rat protease thimet oligopeptidase and YscD oligopeptidase from yeast *Saccharomyces cerevisiae* and was classified as subfamily of zinc-metalloproteinases. It was found homology to various fungi, suggesting that the enzyme may be involved in cellular mechanism for conserved.[47]

It was conducted a Standard Protein BLAST, available on the NCBI (National Center for Biotechnology Information) website, was possible to find, with 100% homology, a wide variety of sequences of fungal proteases from the following genres: *Trichophyton* (accession numbers OAL69080.1, EGD96548.1, XP_003234056.1), *Coccidioides* (accession numbers XP_012213938.1, KMU73771.1, XP_003065029.1), *Microsporum* (accession number XP_003170328.1) and *Arthroderma* (accession number: XP_002849330.1) and *Paemoniella* (accession number KKY24142.1). In addition, a putative conserved domain of Peptidase Gluzincin family (thermolysin-like proteinase, TLPs) could be found, that includes Several zinc-dependent metallopeptidases (accession number cl14813), as Fungalysin that hydrolyzes extracellular matrix proteins, such as elastin, keratin and collagen.[71] Family of Gluzincin is included among families dependent zinc metalloproteinase with skills to hydrolyze collagen and present waste critical role in assisting the connection and opening (unwinding) of collagen.[35]

Enzyme nature

Bacterial proteases can be divided into two groups according to the ability to hydrolyze native or denatured collagen, being considered as gelatinolytic and collagenolytic, respectively.[35,60] With regard to the fungal collagenase, this classification is not well understood. However, adopting the same parameters used by Duarte et al.,[35] systematic review found articles 13 (61.10%) who described produced enzymes as true collagenases, six articles (28.57%) with enzymes classified only as gelatinolytic and only two articles (9.52%) could not be identify the nature of the enzyme (Table 3). A proper enzyme characterization must include confirmation of this activity, so it can be identified the real potential of the studied enzymes.

Purification

Once a crude collagenase extract is recovered, it must be purified using one of several chromatographic methods that can be classified as: gel filtration, ion exchange, hydrophobic interaction or affinity.[22] Furthermore, there are traditional enzymatic extraction methods, such as ammonium sulfate precipitation, ultrafiltration, Tris–HCl buffer extraction, with sodium bicarbonate buffer, among others.[22,72]

From the 21 articles selected, 12 had some kind of purification, 11 of them using chromatographic techniques and only one exclusive by ammonium sulfate.[43] Mahmoud et al.[53] purified the enzyme produced by *A. flavus* using the DEAE-Cellulose column and obtained a yield of 39.43%. Hamdy[55] could yield 60.49% with the purification using gel filtration chromatography, but the enzyme activity had reduced the amount to 128.4 U/mL.

The others papers reporting good enzymatic activities did not undergo any purification activities.[24,48,58] Other selected articles showed no significant amount of enzyme nor quantify the collagenase produced.

Conclusions

From the 21 select papers, 11 were published in the last 10 years and only four in the last 5 years. According to the scoring methodology criteria, only five studies showed score ≥7. This paper summarized the main findings on production of fungal collagenase. Only two studies reported enzymes with high specificity to collagen over other protein substrates. Among microorganisms studied the *P. aurantiogriseum* and *R. solani* stood out in volumetric and specific collagenase activity, and are non-pathogenic filamentous fungi and extracellular enzyme producers. In the culture medium composition the use of collagen-based compounds seems not essential for collagenolytic enzymes production. For enzymes characterization, articles found differed a lot regarding parameters analyzed. The articles with better scores did not undergo an appropriate purification process. Six of selected articles presented enzymes that could not be considered true collagenases. Although two of the articles have found the gene responsible for enzyme production, both enzymes showed low activity against native collagen. The only article found that made sequencing of a true collagenase showed 100% homology with several metalloproteinases fungi. It was possible to observe a gap in literature about collagenase production by fungi and its characterization, which prevents further development in the area and increases the need for further studies, particularly for full characterization of fungal collagenases with high specificity. It was also observed that studied fungal collagenases presents promising and competitive biotechnology characteristics when compared with bacterial enzymes, most used commercially.

Conflicts of interest

The authors declare no conflicts of interest.

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