



Sara Priscila Lopes de Abreu **Collagenase for biotechnology applications**

Colagenase para aplicações biotecnológicas

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Colagenase para aplicações biotecnológicas

Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Molecular e Celular, realizada sob a orientação científica da Doutora Ana Sofia Duarte Professora Auxiliar Convidada do Departamento de Biologia da Universidade de Aveiro, e sob a coorientação científica da Doutora Ana Cristina Esteves, Professora Auxiliar Convidada do Departamento de Biologia da Universidade de Aveiro.

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palavras-chave

Colagénio, Colagenases, FALGPA, Purificação

resumo

O colagénio é uma proteína abundante em todos os animais, exercendo funções estruturais e reguladoras. Está presente em diversos locais e executa funções específicas, nomeadamente na manutenção dos tecidos e na conservação da sua estrutura celular, da sua força e integridade estrutural. Possui uma elevada importância na formação da matriz extracelular do tecido conjuntivo ao contribuir para as suas propriedades físicas.

Colagenases são enzimas, mais especificamente metaloproteinases, com um característico domínio de ligação a zinco característico

Atualmente, as colagenases bacterianas possuem diversas aplicações industriais. Por exemplo, na clínica são utilizadas no tratamento de doenças associadas a acumulação excessiva de colagénio, como a Doença de Dupuytren, no desbridamento de queimaduras e feridas e também em terapia génica oncológica.

O objetivo deste trabalho é a comparação e a otimização de dois métodos de purificação, para avaliar qual dos processos será mais rentável. Para tal, foi utilizado um método de purificação de proteínas recombinantes, que foram marcadas com histidinas, utilizando um método de cromatografia de afinidade com ião metálico imobilizado (IMAC). Para comparação, a purificação foi estabelecida utilizando um método em coluna, HisTrap FF e um método em *batch*.

Os resultados obtidos através da quantificação de proteína, usando técnica de quantificação por densitometria demonstraram que a purificação quando realizada por coluna, apresenta maior rendimento. Através de ensaios realizados posteriormente foi possível demonstrar a atividade da ColAh por zimografia de gelatina, e quantificar a atividade colagenolítica contra o péptido sintético N-(3-[2-Furyl]acryloyl)-Leu-Gly-Pro-Ala (FALGPA). Os resultados também demonstram que a proteína recombinante apresentou estabilidade, quando armazenada ao longo do tempo, a diferentes temperaturas (temperatura ambiente; 4°C; -20°C).

keywords

Collagen, Collagenases, FALGPA, Purification

abstract

Collagen is an abundant protein in all animals and is the main component of numerous tissues, having also structural and regulatory roles. Collagen can be found in various numerous locations presenting specific functions, such as the establishment of the cellular shape, its strength and structural integrity, and is also involved in tissue maintenance. Moreover, collagen has an important role in the formation of the extracellular matrix of the connective tissue, by contributing to its physical properties.

Collagenases are enzymes, more specifically metalloproteinases, with a zinc-binding domain. These enzymes are able to digest the triple helical region of native collagen, cleaving its peptide bonds. In recent years, bacterial collagenases have been gaining interest for industry proposes, by offering a variety of applications. In the clinical area, collagenases are used as therapy for diseases associated with excessive deposition of collagen, as for example, in patients suffering from Dupuytren's disease. The enzymes can also be used to help the debridement of wounds and burns, and also for cancer gene therapy applications.

The objective of this work is the comparison and optimization of two methods of recombinant collagenase purification, evaluating which method provides the most cost-effective purification. For that purpose, a simple and high resolution method for purification of histidine-tagged recombinant protein was used.

Immobilized metal ion affinity chromatography (IMAC) with a Ni Sepharose 6 Fast Flow, through a column HisTrap FF 1ml or in batch were used.

Results obtained through protein quantification by densitometry technique, showed that the purification using a column has higher yield. By zimography was possible determine the gelagenolytic activity of ColAh, and was also possible quantify the collagenolytic activity of ColAh, using a synthetic peptide N-(3-[2-Furyl]acryloyl)-Leu-Gly-Pro-Ala (FALGA). Obtained results also showed that the obtained recombinant protein is stable to storage over time and when submitted to different temperatures (room temperature; 4°C; -20°C).

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IV. List of abbreviations

ABS - Absorbance

ATCC - American type culture collection

BCA - Bicinchoninic acid

BGP – Batch/gravity-flow purification

BSA – Bovine serum albumin

ColAh – Purified recombinant collagenase

CV – column volume

DMEM - Dulbecco's modified eagle medium

ECM – Extracellular matrix

EDTA - Ethylenediaminetetraacetic acid

FALGPA - N-(3-[2-Furyl]acryloyl)-Leu-Gly-Pro-Ala

FBS – Fetal bovine serum

FPLC – Fast protein liquid chromatography

IMAC - Immobilized-metal affinity chromatography

IPTG - Isopropyl β -D-1-thiogalactopyranoside

LA - Luria agar

LB - Luria broth

MMP – Matrix metalloproteinases

SPB - Sodium phosphate buffer

SDS-PAGE – Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

CHAPTER I

1. INTRODUCTION

1.1. COLLAGEN

Collagen is a structural protein, abundant in all animals [1, 2], that exists in all shapes and sizes [3]. Approximately 30% of total proteins in mammalian bodies are various types of collagens, being essential to the structural components of connective tissues [4]. This protein is highly important in the formation of the extracellular matrix (ECM) of connective tissue and has diverse locations and specific functions [1, 5]. It is the major constituent of skin, tendons, blood vessels, and cartilage - being part of the organic component of bones, dentin, cornea and others structures of body, contributing for much of their physical properties [6-10]. As the major structural element of connective tissues, collagens are also found in the interstitial tissue contributing to stability of the tissues and organs and maintain their structural integrity [8]. Fibril-forming collagens, such as collagen type I and III, provide a major biomechanical platform for cell attachment and anchorage of macromolecules [4, 6, 9]. It is a long and rod-like molecule and is composed by three polypeptide chains (figure 1.1). Each chain contains 1050 amino acids wound around one another [7], and contains one or more regions characterized by the repeating amino acid motif Gly-Xaa-Yaa triplets, where Xaa and Yaa can be any amino acid, allowing to form a right-handed triple helical structure [3, 6, 7, 9-11].

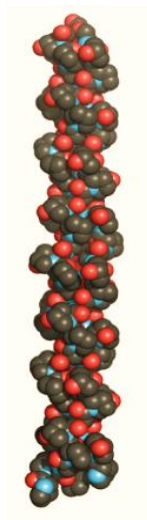


Figure 1.1. Overview of triple helix of collagen. First high-resolution crystal structure of collagen.
(Adaptated from Shoulders and Raines, 2009)

There are several types of collagens with the same function: help tissues withstand stretching. In the body, 80-90 % of collagen consists of collagen types I, II and III [7, 9]. These three types of collagen molecules pack together and form a long thin fibril with a similar structure [7].

The different types of collagens are distinguished by the ability of their helical and non-helical regions to associate into fibrils, to form sheets, or to cross-link different collagen types [7]. They are in general associated to particular tissues, and this heterogeneity may reflect the differential and specific physiological functions of each type [10].

The triplet Gly-Xaa-Yaa can occur in all types of collagen. This types can be categorized in fibrillar and non-fibrillar, however in case of nonfibrillar collagen, triple helical region it is disrupted at some locations within the triple-helical domain, resulting in a nonfibrillar architecture of the connective tissue core [2, 6, 9]. The most non-fibrillar collagen type is type IV and VI [2, 3]. Type IV collagen molecules are longer than the fibrillary collagen and it is the primary component of basement membranes, which represents another type of supramolecular organization which forms three dimensional networks [4, 6]. This membranes are specialized structures, present on the borders of tissues, underlying epithelial, endothelial, fat, muscle and nerve cells [3]. Classical fibrillary collagens account for 80-90% of all collagens in mammals and includes types I, II and III [7, 9]. Collagen type I is the most widely occurring collagen found in skin, tendon, bone, cornea, lung and the vasculature. Type II collagen, has a specific tissue distribution being limited essentially to cartilage, and type III is found in relatively elastic tissues such as embryonic skin, lung and blood vessels [3, 8].

Fibrillar collagens are synthesized and secreted into the extracellular matrix as procollagen, with N- and C- terminal propeptide extensions (figure 1.2) [2]. The triple helix are the basic unit of fibrillary collagen. Have approximately 3000 Å in length and 15 Å in diameter [9]. As in all collagens, each fibrillar collagen molecule consists of three polypeptide chains, called α chains [3].

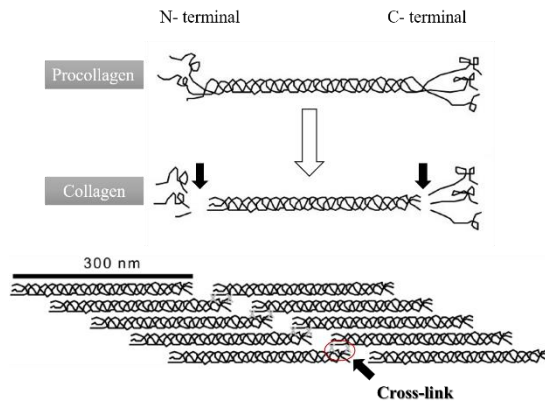


Figure 1.2. Fibrillar procollagens and fibril assembly. *N- and C-terminal processing leads to spontaneous assembly of collagen fibrils, which are subsequently stabilized by the formation of covalent cross-link. (Adapted from Hulmes, 2008).*

Fibrillar collagens are synthesized in form of procollagens, with N- and C- terminal propeptide domains. Usually, C-propetides are removed through specific metalloproteinases. N-terminal extent processing depends the type of collagen [3].

Interstitial collagen is assembled by three polypeptide strands [12]. If the residues in the position X and Y are Proline and Hydroxyproline the stability of triple helix increases. Thus, the amino acid sequence is related to the protein stability and their molecular interactions [9].

1.2. COLLAGEN DEGRADATION

Collagen is resistant to most common proteases due to its special structure and only can be degraded by a few types of proteases from mammals or bacteria [4]. During the past decades, the number of microorganisms capable to produce enzymes that degrade collagen have been increasing [7]. Degradation of collagen, occurs during various physiological and pathological conditions. However, only a limited number of proteases with unique characteristics can trigger collagen degradation [10, 13].

1.2.1. Bacterial collagenases

Over the years, substantiating evidence has been assembled to the hypothesis that growth and proliferation of pathogenic bacteria is dependent on the proteolytic enzymes action [5, 10]. Collagenases are endopeptidases that degrade native collagen in the triple helix region [11, 14]. They have a great ability to disaggregate connective tissues [15, 16] and have a characteristic multidomain MMP structure which is composed by a signal peptide, a propeptide, a catalytic domain, a hinge region, and a hemopexin domain [17].

Clostridial collagenases are large multi-modular zinc metalloproteases consisting of four to six domains and they belong to the MEROPS peptidase family M9, which comprises microbial metalloproteases with presumable collagenolytic activity [12, 18] . The first ones to be identified and characterized are the reference enzymes for comparison of newly discovered collagenolytic enzymes [9].

The pioneer studies about clostridial collagenases' characteristics were carried by Mandl, Seifter, Harper and their associates [19] and later by Van Wart and Bond [20] the classification work, and the first commercially available collagenase isolated from *Clostridium histolyticum* appeared in 1959 [14]. When the study of collagenases began, researchers have recognized the involvement of proteolytic enzymes from *Clostridium* in tissue putrefaction. Consequently, a *Clostridium histolyticum* extracellular enzyme, which was capable to digest tendons was isolated [10]. Thus, collagenases expressed by *Clostridium histolyticum* are preferred to others, since they are active practically on all mammalian collagen isoforms, even in their native conformation [15, 16]. These enzymes also completely degrade the obtained fragments into a mixture of small oligopeptides [12]. Based in kinetic and structural characteristics, clostridial collagenases can be classified into two classes: class I and II. This was established according its activities towards collagen, gelatin and synthetic substrates, freeze-thawing stability and the primary, secondary and tertiary structures [20, 21]. Collagenases from class I are highly active against collagen and moderately active against the synthetic peptide FALGPA used to kinetic studies of collagenases due to its characteristics of other proteinases are not capable of hydrolyzing it [22]. Class II enzymes show moderate activity towards collagen but high activity against synthetic peptides [9].

Collagenases can be further classified into two groups. The first are the 'classic' collagenases as the enzymes that cleave interstitial collagens, and the other group in 'type-specific' collagenases, which is constituted by enzymes with capacity to degrade one or more specific collagens [23]. Collagenases are capable to degrade native triple helical collagen [12]. The enzymes demonstrate preference towards different fibrillar collagen substrates cleaving only at well-defined site [12]. Under physiological conditions of pH and temperature, true collagenases cleave helical regions of collagen molecules in fibrillar form [4, 10, 16].

The degradation of native triple helical collagen, for animal collagenases is crucially dependent to type of collagen and the species of origin. True collagenases, gelatinases or other bacterial proteases, are difficult to differentiate between them [9]. However, vertebrate gelatinases and collagenases are very easy to distinguish. First, vertebrate gelatinases do not hydrolase collagen fibers, only denatured collagen (gelatin). Vertebrate collagenases cut the collagen molecule at a single peptide bond, across the three α chains, forming specific 3/4 and 1/4 fragments. The fragments tend to uncoil into random polypeptides, being more susceptible to other proteases [9]. Therefore, vertebrate collagenases differ from bacterial collagenases in that they exhibit broader substrate specificity [14]. Bacterial collagenases can digest all collagen types, being capable to make multiple cleavages within triple helical regions, and degrade water-insoluble native collagens and water-soluble denatured ones [12, 14].

1.3. APPLICATIONS OF COLLAGENASES

Nowadays, important progresses have been made in the identification of new bacterial collagenases, and their mechanisms of collagen degradation [4].

Microbial collagenases have several applications. These enzymes are used in diverse areas such as food industry [24], cosmetics, pharmaceutical and clinical areas [4] or in preparation of cells [9].

1.3.1. Life science research

A. Cell culture

Cell culture has been widely used as an essential tool in diverse areas, as in molecular biology, toxicology and biotechnology, to address important scientific, technical and economic issues in biology [9].

Enzymes are frequently used for tissue disaggregation in the first step of cell isolation and for cell dissociation during subculturing procedures to maintain in vitro cell cultures. Trypsinization is a procedure commonly used for dissociation and disaggregation of adherent cells from solid substrates during subculturing or in order to harvest cells to being extensively used in laboratory experiments to re-suspend cells adherent to the Petri dish wall during the process of harvesting cells [9, 25]. Trypsin, originally was isolated from pancreas, and has functions on the digestion of food and in the degradation of proteases inhibitors [26]. Furthermore, trypsin cleaves peptide bonds at the carboxyl side of lysine

and arginine residues, and its proteolytic activity may damage cells by cleaving the cell surface growth factor receptors or membrane proteins [9].

The choice of the most suitable enzymes in the appropriate concentration, in order to obtain a maximum performance of functionally viable single cells, is crucial. Furthermore, fibrous tissues, with high collagen contents, are especially resistant to the action of trypsin digestion. In this case where trypsin would not be effective or may be damaging, collagenases are especially valuable [9].

1.3.2. Food industry

In food industry, the meat tenderness is one of the most concerning characteristics. Tenderness is an important sensory quality in the meat. This can be influenced by various factors, as the length of the sarcomeres, the integrity of myofibrils that affect the actomyosin toughness and the connective tissues integrity which contribute to the background toughness [24].

Over the years, different ways to improve physical and chemical methods for meat tenderization have been investigated. Proteolytic enzymes constitute one of the most progressive methods to increase meat tenderness. Nowadays, the enzymes as mesophilic and thermophilic proteases are used in meat tenderness [24].

Collagen constitutes 80% of the connective tissue and it is partly responsible by the toughness of red meats, being a significant factor influencing the texture of meat. Therefore, collagen digestion is an inducer of meat tenderness [9, 24]. Due to their characteristics, bacterial collagenases can be profitable in the process of meat tenderization [9].

However, pathogenicity concerns have limited the industrial use of bacterial collagenases in the process of meat tenderization. To be an ideal meat tenderizer, the enzyme should be specific, with high activity at room temperature and easily inactivated during cooking [9, 24].

In food industry, productions of recombinant collagenases in non-pathogenic strains would simplify their purification and avoid the presence of undesired virulence factors in collagenases.

1.3.3. Health industry

The health industry is probably the most relevant area for bacterial collagenases' application, due to their ability matrix remodelling ability useful to treat several human diseases. These enzymes are successfully applied in the debridement treatment of wounds and burns, cancer genetic therapy or electro-genetic therapy, treatment of lumbar disc herniation, to the treatment of chronic total occlusions, and Dupuytren's disease [4, 9]

Collagenases from *Clostridium histolyticum* are widely used in the clinical area, due to their ability to disaggregate connective tissues, to facilitate the isolation of cells of interest from multiple tissues, and used as a therapeutic drug for the removal of necrotic wound tissues for several decades [4]. These enzymes are preferred to others, because of their proteolytically active practically on all mammalian collagen isoforms, even in native conformation [15, 18]. Currently, clostridial collagenases were approved to be used for Peyronie's disease as a non-surgical treatment [4].

A. Wound debridement

For more than half century, enzymatic debridement was suggested as a therapy and has been continuously applied till the current days [9, 27].

Wound healing can be delayed if necrotic tissues are left in the wound site. Therefore, wound debridement is necessary to remove this necrotic tissue, cellular waste, and harmful exudate. Among various debridement methods, enzymatic wound debridement uses proteolytic enzymes to hydrolyze the denatured proteins (mostly collagens) in wound scar tissue [28].

According to the study of Waycaster and Milne, which purpose was to determine the cost-effectiveness of enzymatic debridement using a collagenase ointment derived from *C. histolyticum*, but the cost of collagenase ointment is high [27].

Nonetheless, the use of a collagenase ointment, in a long-term care setting treatment of necrotic pressure can result in substantial cost savings compared to autolysis with a hydrogel dressing [27].

1.3.4. Others applications

Collagenases have a variety of applications as described above. In addition, collagenases have also applications in the leather industry [9].

In the leather industry there are two processes where the action of enzymes is useful. One is in the dyeing process, which is an important process in the leather industry that uses a large number of colorants. Other process is the *unhairing* process [9].

Dyeing process uses synthetic colorants which are an important pollution source [9]. This process is an inevitable step of imprinting color to the leather, carried out in post-tanning operations. Leather is a difficult substrate to dye due to the unique nature of the raw material that has variations within the matrix [29]. Employing collagenases in this process can be an advantage due to their application, aiming pollution reduction. After leather tanning, the use of collagenases results in opening-up of the fibrous leather network enhancing the diffusion of dyes into the leather matrix. This approach is more eco-friendly, uptaking up to 99% of the dye in leathers with improved bulk properties like softness, fullness, grain smoothness, feel and general appearance [9, 29].

Unhairing is the first step in leather making, which consists in the removal of hair and epidermis from the pelt and the keratinous material that needs to fill the hair follicles. Since this process is highly pollutant, the use of enzymes has been studied, showing that they are capable to reduce the loaded pollution. Application of enzymes in the skin-unhairing step accomplishes many weighty advantages, mainly on the final leather product quality and over comes all the environmental pollution by chemicals [30]. However, the costs of enzymes production, scale up and bioprocessing, suitability and efficacy of enzyme preparation to process different raw materials are concerning factors to implement enzymes in dehairing process [31].

1.4. RELEVANCE OF NOVEL COLLAGENASES

Currently, bacterial collagenases are still largely unknown and understudied. However, the identification of novel collagenases is extremely important to a variety of applications in many fields, as the wide application in research, medicine, food processing and their enormous biotechnological potential [4, 5, 9].

A large number of potential “true collagenases” is still unknown. With that, the characterization of novel bacterial collagenases will certainly expand the range of applications of these enzymes [9].

1.5. PURIFICATION OF RECOMBINANT PROTEINS

Proteins have many potential applications in therapeutic, diagnosis and industry. A high throughput screening approaches are being performed in order to identify proteins with these potentials and applications [32, 33].

In several areas as genomics, or proteomics, the number of experiments produced using recombinant techniques have been grown [32, 34]. Recombinant proteins' production and purification are intimately related [34]. Nowadays, there are a large number of alternative methods for the same purpose. For example, the competition method is widely used to identify specific binding proteins among the proteins retained on an affinity resin [35].

His-tag is the most commonly purification tag used worldwide and a variety of different techniques using this purification method have been developed [33]. Purification of recombinant proteins by affinity chromatography is one of the most efficient strategies due to the high recovery yields and purity achieved [32, 34, 36].

Recombinant proteins can be manual purified or by using a chromatographic system, in order to save time and efforts [37].

Chromatography techniques are used to purify proteins, having the purpose to separate the proteins according to the differences in specific properties. Affinity chromatography is designed to specifically recognize proteins, namely recombinant tagged proteins [37].

Affinity tags are useful for purifying individual proteins and especially protein complexes, allowing diverse proteins to be purified in a single step [36, 37]. Usually, affinity purifications involve less chromatography steps resulting in a decrease of time to perform the methodology (figure 1.3). Affinity chromatography can have high yields, making this alternative economically favorable [32].

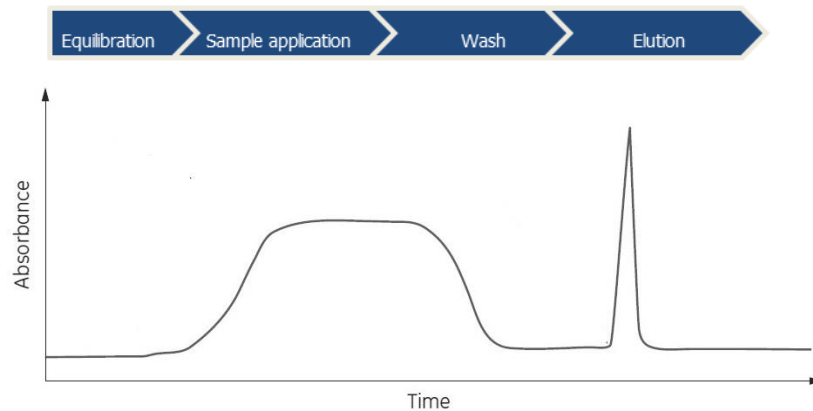


Figure 1.3. General workflow of affinity purification of tagged recombinant proteins. (Adapted from recombinant protein purification principles and methods – GE Healthcare)

Developments in affinity chromatographic methods, such as Immobilized Metal Ion Affinity Chromatography (IMAC), are thus extremely important if the desired proteins are to be isolated in high yield and purity from complex biological feed stocks generated by fermentation or cell culture techniques [38]. IMAC is a simple and fast technique which has been successfully used for the purification of many recombinant proteins, produced by different microorganisms [39].

Affinity tags can be defined as exogenous amino acid sequences with a high affinity for specific biological or chemical ligand [32]. Purification methods using affinity tags is an advantageous method due to its stability of the immobilized metal ions, high capacity of protein loading and reduced costs [40].

These tags (namely the His-tag) usually have a high selective affinity towards a metal ion such as Ni^{2+} [37]. In general, Ni^{2+} is the preferred metal ion for purification of histidine-tagged proteins [30]. His-tagged proteins are enriched by interaction with the immobilized metal ions [40]. The tagged protein will be selectively bound to a metal ion charged media such as Ni Sepharose High Performance (HP) or Ni Sepharose 6 Fast Flow (FF) while others proteins will bind weakly. Generally, histidine-tagged proteins are the strongest binder among all the proteins in a crude sample extract.

Currently, *Escherichia coli* is still the preferred organism for large-scale production of recombinant proteins. Fusion proteins have helped considerably in enhancing the solubility of heterologous proteins and their purification with affinity chromatography [41]. Histidine-tagged proteins expressed in *E. coli* can accumulate in two main forms: i) as inclusion bodies that are aggregates of denatured or partly denatured protein that lack biological activity but often allow high expression levels of the recombinant protein, or ii) as biologically functional soluble proteins[37].

Figure 1.4 shows an overview of a common purification workflow for histidine-tagged protein, under native conditions.



Figure 1.4. Scheme of Affinity chromatography. 1) *Equilibration: IMAC medium are equilibrated in binding buffer containing a low concentration of imidazole; 2) Samples application: sample applied under conditions favoring specific binding of tagged protein to the ligand, and unbound material are washed away 3) Elution: concentration of imidazole increase to promote elution of the tagged protein.* (Adapted from recombinant protein purification principles and methods – GE Healthcare)

As referred above, purification can be performed in many scales, having a number of columns with different sizes, and the columns can be prepacked with a chromatography medium or can be manually packed [37]. Ni Sepharose 6 Fast Flow is a bio process IMAC medium which contains 90 μm beads of highly cross-linked agarose, to which a chelating group has been coupled and after charged with Ni^{2+} . Its properties of high flow makes it an excellent choice for scale-up and also its low Ni^{2+} leakage, high protein binding capacity, and its compatible with a wide range of additives used in protein purification [30].

In summary, engineered purification tags can facilitate very efficient purification of recombinant proteins, resulting in high yields and purities in a few standard steps [33].

1.6. AIM OF STUDY

In the present work, the main purpose was to

- i) Optimize a purification protocol for the recombinant collagenase ColAh
 - a. Comparedifferent purification methods
- ii) Characterize the recombinant protein – ColAh.

In order to accomplish these objectives was also evaluated:

- Gelatinolytic and collagenolytic activity;
- ColAh application in the dissociation of cells in culture;
- Performance of ColAh in collagen hydrolysis;
- Storage stability at different temperatures of incubation.

CHAPTER II

2. MATERIAL AND METHODS

2.1. EXPRESSION OF RECOMBINANT COLAH

For the expression of the recombinant collagenase (ColAh), 10 µl of a positive transformant (clone A stored in 15% glycerol, -80°C) was used to inoculate a LA plate containing kanamycin (50 µg/ml) and grown overnight at 37°C. Clone A was previously obtained by cloning in *E. coli* and consists of an open reading frame that codes for a putative *Aeromonas* collagenase [42].

Once clone A was grown, 15 ml of LB+50 µg/ml kanamycin were inoculated with a single bacterial colony from a fresh LB-agar plate and incubated at 37 °C with shaking (170-180 rpm) overnight. 50 µl of the overnight cultures were diluted 1:1.000 in fresh LB medium, containing the appropriate antibiotics, and incubated at 37 °C with shaking in 250-ml baffled flasks until the bacterial cultures reached the planned optical density.

One milliliter of the overnight culture was diluted in 50 ml LB+50 µg/ml kanamycin in a 250-ml baffled flasks and incubated at 37°C (170–180 rpm) until the bacterial cultures reached the planned optical density ($OD_{600nm} = 0.6$).

After induction with 0.5 mM IPTG, cultures were transferred to 26°C (130rpm) and allowed to grow for additional 18 h.

Cells were separated from the supernatant by centrifugation at 4°C, for 10 minutes, at 10.000 rpm. After centrifugation, the supernatant was collected and immediately processed for buffer exchange.

2.2. PREPARATION OF THE CRUDE EXTRACT

2.2.1. Addition of salts

Buffers

100mM Sodium Phosphate Buffer (SPB)¹

Table 2.1. Sodium Phosphate Buffer (100mM)

Final Volume	500 ml
<i>NaH₂PO₄*H₂O</i>	1.55 g
<i>Na₂HPO₄</i>	5.45 g

The pH was adjusted to 7.4 and the final volume was brought to 500 mL with ultrapure water.

¹ Sodium Phosphate Buffer: *NaH₂PO₄*H₂O*; *Na₂HPO₄*

Crude extracts were supplemented with the appropriated volume of SPB stock solution (Table 2.1) containing salts (Table 2.2.), to the final concentrations: 20 mM SPB, 0.5 M NaCl and 5 mM imidazole.

The mixture was homogenized by stirring for 30 minutes and the pH was adjusted to 7.4. Afterwards, the samples were filtered using a 0.22 μ m of cellulose acetate membrane syringe filter (sterile).

Table 2.2. Example of contents for salts addition. Final volume of 625ml (500ml of supernatant – 125ml stock salt solution (100mM SPB))

Final Volume (Crude extract)	625 ml
<i>SPB 20mM</i>	125 ml
<i>NaCl 0.5M</i>	18.26 g
<i>Imidazole 5mM</i>	0.43 g

2.2.2. Dialysis

Alternatively to salt addition, crude extracts (prepared, as described in section 2.2) were dialyzed in cellulose dialysis tubing with a molecular weight cut-off =14.000Da (Sigma-Aldrich) against binding buffer 10 mM². For efficient buffer exchange, dialysis was performed in a large stirred beaker overnight at 4°C with at least 2 buffer changes.

2.3. PURIFICATION METHODS OF THE HISTIDINE-TAGGED RECOMBINANT COLAH

2.3.1. Immobilized-metal affinity chromatography (IMAC)

Buffers

Stripping Buffer³

Table 2.3. Stripping Buffer

Final volume	500 ml
Sodium phosphate (100mM)	100 ml

² Binding Buffer 10mM: 20mM sodium phosphate; 0.5M NaCl; 10mM imidazole; pH 7.4.

³ Stripping Buffer: 20 mM Sodium Phosphate, 0.5 M NaCl, 50 mM EDTA, pH 7.4

EDTA (500mM)	50 ml
<hr/>	
NaCl	14.61 g

The pH was adjusted to 7.4 and the final volume was brought to 500 mL with ultrapure water.

Elution Buffer I⁴

Table 2.4. Elution Buffer I

Final volume	500 ml
<hr/>	
Sodium phosphate (100mM)	100 ml
<hr/>	
NaCl	14.61 g
<hr/>	
Imidazole	17.02 g

The pH was adjusted to 7.4 and the final volume was brought to 500 mL with ultrapure water.

Elution Buffer II⁵

Table 2.5. Elution Buffer II

Final volume	500 ml
<hr/>	
Sodium phosphate (100mM)	100 ml
<hr/>	
NaCl	14.61g
<hr/>	
Imidazole	2.553g

The pH was adjusted to 7.4 using and the final volume was brought to 500 mL with ultrapure water.

⁴ Elution Buffer I: 20mM sodium phosphate; 0.5M NaCl; 500mM imidazole; pH 7.4

⁵ Elution Buffer II: 20mM sodium phosphate; 0.5M NaCl; 75mM imidazole; pH 7.4

Binding Buffer¹

Table 2.6. Binding Buffer

<u>Final Volume</u>	<u>500 ml</u>
Sodium phosphate (100mM)	100 ml
NaCl	14.61g
Imidazole	0.341g

The pH was adjusted to 7.4 and the final volume was brought to 500 mL with ultrapure water.

Purification was carried out in a pre-equilibrated (Binding buffer², 1ml/min) HisTrap FF 1ml column (GE Healthcare), in an AKTA FPLC system using the Unicorn 5.11 software. Crude extracts (20ml), pre-prepared in the appropriated salt conditions (prepared by dialysis or salt addition) were injected onto the resin. The target protein was eluted with 75mM imidazole using a step gradient of imidazole (75, 100 and 500 mM imidazole) at a flow rate of 1 ml/min. A blank run was established before every purification procedure. All the water and buffer solutions were degassed [with Helium(g)] and filtrated (0.22 µm pore) before applied to the system. Collected fractions were stored at -20°C, until further characterization.

A. Procedure of stripping and recharging the column of 1 ml.

To improve protein purification, the column was stripped and recharged between every purification procedure, according to the manufacturer's instructions.

The prepacked column was washed with at least five column volumes with ultrapure water, buffer containing imidazole (10 mM imidazole), stripping buffer and at last with ultrapure water. In the second part of this step, the resin was recharged with NiSO₄ 0.1M, washed with ultrapure water and the column was equilibrated with binding buffer¹.

2.3.2. Batch/gravity-flow purification (BGP)

A. Resin equilibrations and sample binding

Batch purification was carried out with pre-equilibrated (binding buffer¹) Ni Sepharose 6 Fast Flow resin (GE Healthcare). Crude extract was loaded onto the resin with binding

buffer. The resin was washed with buffer containing imidazole (Binding Buffer 10mM imidazole). The target protein was eluted with elution buffer II⁵ with 75 mM imidazole.

B. Stripping and recharging with nickel sulfate

After each purification, the resins were submitted to stripping and recharging with NiSO₄. First the resins were washed at least twice 5-10 column volumes with stripping buffer², then 5-10 column volumes of binding buffer¹ and finally 5-10 column volume with ultrapure water (three - four times).

The resins were recharged with NiSO₄ 0.1M and washed at least three times with ultrapure water.

2.4. DESALTING (HIPREP 26/10 DESALTING COLUMN)

Desalting procedure was performed according to the manufacturer's instructions. Desalting was performed using a Sephadex G-25 Fine column (HiPrep 26/10 – GE Healthcare) coupled to an ÄKTA FPLC system. Before loading the sample, the system and column were washed with ultrapure water. The filtrated protein sample was loaded onto the column and the target protein was collected when the ABS (mAU) values started to increase. Sample collection was stopped when the conductivity reached 0.040 mS/cm. All the solutions (water and buffers) used in the HiPrep 26/10 desalting column were degassed [with Helium(g)] and filtrated (0.22 µm) before used onto the column.

2.5. LYOPHILIZATION

As final step, the samples were concentrated by lyophilization. This is a process where the water is removed from a product after it is frozen (-80°C) and placed under a vacuum, allowing the ice to change directly from solid to water vapor without passing through a liquid phase. The samples had approximately 15/20 ml each one. In this step a Snijders scientific type 2040 lyophilizer was used.

2.6. PROTEIN QUANTIFICATION

2.6.1. Colorimetric methods

A. Pierce 660nm Protein Assay

Pierce 660nm Protein Assay from Thermo Scientific was used to quantify the total protein concentration. This methodology is compatible with high concentrations of many

detergents, reducing agents and other commonly used reagents. Is a methodology based on the binding of a proprietary dye-metal complex to protein in acidic conditions. This causes a shift in the maximum absorption of the dye at 660nm, reproducing a colorimetric response, dye-metal complex is reddish-brown and changes to green upon protein binding [43]. In this method bovine serum albumin (BSA) was used as standard.

A standard curve was prepared with BSA final concentration from 25 µg/ml to 2000 µg/ml and was measured with an absorbance at 660nm. The 25 µg/ml standard was prepared by mixing 10 µl of the 1000 µg/ml standard with 390 µg of 0.9% saline. 0.1ml of each replicate of standard, unknown sample and blank sample was added into an appropriately labeled test tube. Protein assay reagent (1.5ml) was added to each tube and vortexed to mix. The labeled test tubes were cover and incubated for 5 minutes at room temperature. Afterwards, total protein concentration was determined by measuring the absorbance at 660nm.

B. BCA Protein Assay

To determine the protein concentration of ColAh samples, the BCA Protein Assay Reagent Kit, from Thermo Scientific, was used. Bovine serum albumin (BSA) was used as a standard with a final concentration from 5 µg/ml to 250 µg/ml and was measured with an absorbance at 562nm, following the manufacturer's instructions.

This is a high-precision and detergent-compatible assay reagent, based on bicinchoninic acid (BCA) for the colorimetric detection of the total protein concentration compared to a protein standard. It consists in the reduction of Cu^{2+} into Cu^{1+} by proteins in an alkaline medium creating a biuret reaction. The amount of Cu^{2+} reduced is proportional to the protein concentration present in the samples. This method has a high working range (5-250 µg/ml) [44].

Total protein concentration of each sample was determined using a standard curve. The standards were prepared as described in the following table 2.7. The protocol was based on the manufacturer instructions.

Table 2.7. Diluted Albumin (BSA) Standards: (2mg/ml) used in the BCA protein assay.

	Volume (µl)	BSA volume (µl)	Final concentration of BSA (µg/ml)
A	700	100 µl of stock	250
B	400	400 µl of A dilution	125
C	450	300 µl of B dilution	50
D	400	400 µl of C dilution	25
E	400	100 µl of D dilution	5
F	400	0	blank

2.6.2. Spectrophotometry assay

The NanoDrop spectrophotometer software (Thermo Scientific) uses the absorbance at 280nm in combination with the molar extinction coefficient to calculate the protein concentration. The system was calibrated using 2 µl of ultrapure water, and loading 2 µl of sample gently homogenized, avoiding bubbles when mixing and pipetting. The concentration of the ColAh was calculated using the Beer-Lambert Law:

$$c = \frac{A}{\epsilon \cdot l}$$

c - molar concentration

A - absorbance (280nm)

ε - molar extinction coefficient (units $M^{-1}cm^{-1}$)

l - pathlength of the cuvette (units cm)

The ColAh molar extinction coefficient (ϵ) was estimated using ProtParam – ExPASy [45].

2.6.3. Densitometry analysis

Densitometry analysis on SDS-PAGE (10% of acrylamide gel) was also used to quantify protein. The gel analysis was performed after staining the gel with Coomassie Brilliant Blue R-250 and scanning on a GS-800 Calibrated Densitometer (Bio-Rad). To estimate the quantity of ColAh, the software Quantity One 5.11 (Bio-Rad) was used, using

known concentrations of BSA (1 µg, 2 µg, 3 µg, 4 µg, 5 µg, and 10 µg) as standard which were loaded to the same SDS-PAGE gel. For each gel were performed replicates.

2.7. EVALUATION OF PURITY AND ENZYMATIC ACTIVITY OF COLAH

2.7.1. SDS-PAGE – Polyacrylamide gel electrophoresis

This assay is adapted from the method described by Laemmli (1970). It is an electrophoretic separation of proteins in a polyacrylamide gel on the presence of SDS.

In this methodology, the presence of ColAh was characterized on a 10% polyacrylamide gel. The samples were denaturated at 100°C for 5 minutes. Mini-Protean 3 Cell (Bio-Rad), linked to an electrical potential difference generator [PowerPac 300 (Bio-Rad)] were used. The gels were electrophorized for 100-120 minutes at 120Volts. After the run, the gel was emerged into a stain solution, Coomassie Brilliant Blue R-250 for 1 hour. The staining solution contains 0.25% (w/v) of staining, 10% of acetic acid (v/v) and 50% of ethanol (v/v). After, the gel was incubated into a destaining solution with 25% of ethanol (v/v) and 5% of acetic acid (v/v), until the stains become visible. Afterwards, the gel was scanned on a Gel Image Analyzer GS-800 Calibrated Densitometer (Bio-Rad) using the software Quantity One (Bio-Rad). For each assay, were tested three replicates for each sample.

2.7.2. Zymography

Zymography is an electrophoretic assay, which implies a co-polymerization of a substrate (gelatin) to the 10% polyacrylamide gel, allowing detection of enzymes and of their activity. The samples were partially and reversibly desaturated by SDS, but not reduced, and applied into a gel for separation. The run was performed at 4°C, 120 volts, for 120 minutes. When the electrophoresis ends, the gel was washed with a non-ionic detergent, Triton X-100 (0.25%, v/v). Afterwards, the gel was incubated with a reaction buffer⁶ for 2 hours, at 37°C, allowing the digestion of substrate by ColAh. The result was visualized after emerged the gel into a staining solution, Coomassie Brilliant Blue R-250. The gel was incubated in a staining solution with 0.25% (w/v) of staining, 10% of acetic acid (v/v) and 50% of ethanol (v/v), for 1 hour. Destaining step was performed by soaking the gel in 25% of ethanol (v/v) and 5% of acetic acid (v/v). The gel was incubated until digested areas become visible. For each assay, were tested three replicates for each sample.

⁶ *Reaction Buffer: Tris 1.5M; NaCl 1M; CaCl₂ 1M; ZnCl₂ 1µM.*

2.8. ENZYMATIC DETERMINATION OF COLLAGENASE ACTIVITY

2.8.1. FALGPA assay

Collagenase activity was determined according to Van Wart and Steinbrink (1981). This assay is used to determine the activity of collagenases and is based on the hydrolysis of synthetic peptide: 2-furanacryloyl-leu-gly-pro-ala (falgpa).

For each assay, FALGPA reaction buffer⁷ and FALGPA were preincubated on a water bath at 37°C, for approximately 20 minutes. Before each assay, a recommended test to the peptide was performed (negative control) [12, 22].

FALGPA reaction buffer was used as a blank to calibrate the spectrophotometer. The assays mixtures were prepared by adding the FALGPA peptide to the FALGPA reaction buffer. The reaction was started by adding the adjusted volume of the enzyme sample (table 2.8). The continuous spectrophotometric rate determination (A345, Light path = 1 cm) for each enzymatic assay was recorded for 10 minutes. Were performed three replicates for each sample.

Table 2.8. Contents for activity assay using FALGPA.

ABS	Molar extinction coefficient (mM ⁻¹ cm ⁻¹)	[FALGPA] (mM)	FALGPA (µl)	FALGPA Buffer (µl)	ColAh (µl)	Vfinal (µl)
324 nm	e324=2.250	0.031	28.1	968.5	3.3	1000
		0.062	56.3	940.3	3.3	1000
		0.124	112.7	883.9	3.3	1000
345 nm	e345=0.530	0.248	225.4	771.2	3.3	1000
		0.496	450.9	545.7	3.3	1000
		0.744	676.3	320.3	3.3	1000
		0.992	901.8	94.8	3.3	1000

2.8.2. Collagen hydrolysis

Collagen type I was prepared according to Duarte *et al.*, 2005, and used as complex substrate for evaluating ColAh collagenolytic activity. ColAh was purified through a prepacked HisTrap FF 1 ml column, as previously described (section 2.3). NanoDrop

⁷ FALGPA buffer: 50 mM Tricine buffer; 10 mM CaCl₂; 400 mM NaCl. Adjust to pH 7.5

spectrophotometer software (Thermo Scientific) was used to quantify the ColAh. Hydrolysis of collagen was carried out by incubating type I collagen (148 µg) with ColAh (2.5 µg) for 24h at temperature between 25-30°C. After digestion, the samples were analyzed by SDS-PAGE polyacrylamide gel 6% of acrylamide, allowing to the detection of the hydrolysis of collagen by ColAh. The gel was running for 80 minutes at 100volts. Clostridial collagenase SERVA was used as positive control [46].

2.9. STORAGE STABILITY ASSAY

To evaluate the storage stability of ColAh, it was incubated at different temperatures: room temperature, 4°C and -20°C, over time. At day 1 and 18 of storage, SDS-PAGE gels (7.5% of acrylamide) was performed, loaded with the samples (three replicates each) stored at different temperatures. The gels were running for 45minutes at 80Volts.

The assay was performed accordingly the conditions established by the Patent NO.: *US 8,725,985 B2 – Clostridium histolyticum recombinant collagenase and method for the manufacture thereof* [47].

2.10. COLAH APPLICATION ON CELL CULTURE PROCEDURE

C2C12 cell line derives from Mouse C₃H muscle myoblasts. This cell line was used in order to evaluate the capacity of the recombinant collagenase (ColAh) for detachment of adherent cells. The maintenance of the cell line was carried out according to the protocol recommended by American Type Culture Collection (ATCC). Cells were grown in culture flasks at 37°C in a humidified atmosphere with 5% CO₂ and maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 3.7g/l NaHCO₃, 1% of antibiotic and 10% of Fetal Bovine Serum (FBS). Cell detachment was performed by using trypsin (control) or the ColAh in 0.1mg, 0.25mg, 0.5 mg and 1mg were added to each well. The culture plate was placed one minute at 37°C. To inhibit the action of trypsin and the ColAh, DMEM with 10% of FBS was added. Finally, cell culture morphology was evaluated by TC10 Automated Cell Counter, Bio-Rad Laboratories, Hercules,

CHAPTER III

3. RESULTS AND DISCUSSION

In the present study, the main purpose was to evaluate the possible biotechnological potential of a recombinant protein – ColAh. For that, several assays were performed to evaluate different parameters, such as collagenolytic activity and purification yield.

3.1. ACTIVITY AND STABILITY OF COLAH

After ColAh expression and salt addition, different methodologies were implemented until reaching its pure (and still active) form. Immobilized metal affinity chromatography purification was carried out through batch procedure with an equilibrated Ni Sepharose 6 Fast Flow resin (nominated batch gravity protocol - BGP) (figure 3.1).

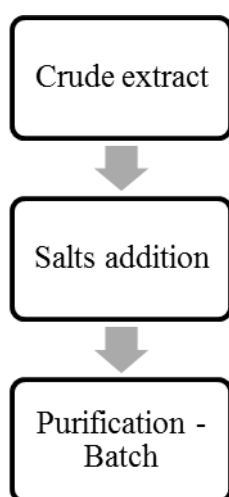


Figure 3.1. Scheme of the process to obtain ColAh until purified

One of the steps to evaluate and characterize the purified recombinant collagenase – ColAh, is to verify its activity. In this case, after purification by batch, ColAh was tested to assess its collagenolytic activity against a synthetic peptide.

The assay requires the use of a spectrophotometer, able to automatically make readings over time, and with thermostated cell. To measure collagenolytic activity, the synthetic peptide 2-furanacryloyl-Leu-Gly-Pro-Ala (FALGPA) [22], that mimics the primary structure of the collagen molecule was selected and its hydrolysis was monitored over time., The Serva Collagenase NB 1 from *Clostridium histolyticum* was used as control [48]. The concentration of FALGPA degradation was determined spectrophotometrically and calculated using the Beer- Lambert Law.

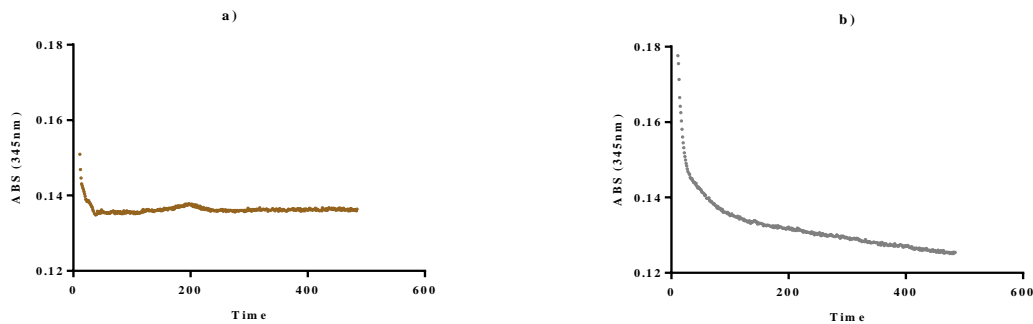


Figure 3.2. Collagenolytic activity determination using the synthetic peptide FALGPA. a) Pure recombinant protein – ColAh - with salts addition and purification through batch. b) Commercial collagenase from Serva as control.

Synthetic peptide FALGPA mimics the primary structure of the collagen molecule. It is considered a good substrate for collagenases [22], which hydrolyzes FALGPA results in a decrease in spectrophotometric absorbance at 324 nm [49].

Figure 3.2 shows that ColAh is able to degrade the synthetic peptide, demonstrating that this recombinant protein retains collagenolytic activity. However, the collagenolytic activity of ColAh (1.16×10^{-04} mM/sec) is lower than the control (3.39×10^{-04} mM/sec), indicating that peptide degradation is lower than expected.

3.2. CELL CULTURE

In this assay, the detachment abilities of ColAh were assayed using C2C12 cell line from Mouse C3H muscle myoblast. Trypsin was used as control.

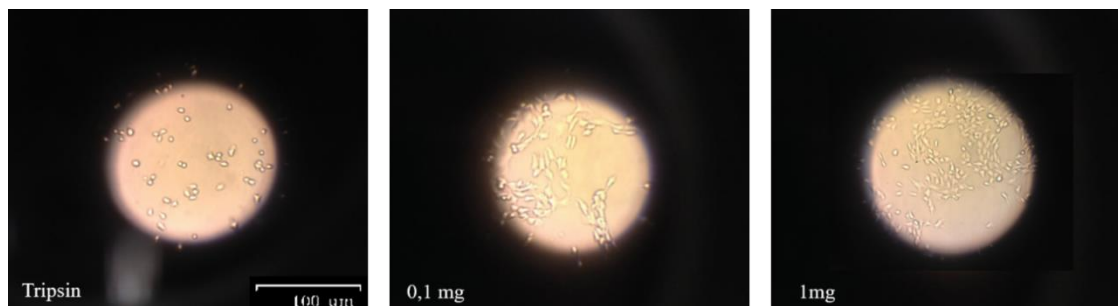


Figure 3.3. C2C12 cells detachment by ColAh and trypsin.

Trypsinization is a frequently used for the dissociation of adherent cells, in order to allow their harvest and use for a wide range of analysis [25]. Trypsin can be ineffective in when tissues are too fibrous or may cause damage in sensitive cells. The use of commercial collagenases for the cell detachment influences yield, viability and cells function. Viability and function of isolated cells are essential for several applications, as in diabetes therapy, pharmacological tests, scientific research and others [48].

The purpose was to evaluate the cell release performance of ColAh from the culture plate and to compare its effects with trypsin's performance.

Figure 3.3 shows that, contrasting to trypsin, the recombinant protein was unable to completely dissociate C2C12 cells, even using higher concentrations of pure ColAh. Nevertheless, the results revealed slightly cell detachment by using 1mg of ColAh. This observation suggests that the ColAh added to the cells might not be at the required concentration to obtain a full detachment of the cells.

One possible explanation is that the concentration used may not be sufficient to obtain the expected result. However, if higher doses of ColAh were used (higher than the commercial collagenases concentration required) the process would not be rentable. This may be due to the fact that the protein quantification (made using the BCA Protein Assay Reagent Kit) [44] was not the appropriate, estimating a larger protein concentration than the real purified amount of ColAh.

Therefore, other methods for protein quantification were tested, such as the Pierce 660 nm Protein Assay (Thermo Scientific) [43] and absorbance measurement at 280nm (using

a NanoDrop) [50]. However, again the protein quantification obtained from these methods was not in accordance with the corresponding analysis by SDS-PAGE (figure 3.4). The SDS-PAGE gel allows separating proteins according to their molecular weight [51], as well confirming the quantity of protein electrophoresed on the gel.

The protein quantification methods used differ in several parameters. BCA Protein Assay is a method based in the reduction of Cu^{2+} to Cu^{1+} by proteins in an alkaline medium, the called biuret reaction. It is a highly sensitive and selective colorimetric detection of Cu^{1+} by bicinchoninic acid (BCA). The amount of Cu^{2+} reduced is proportional to the sample protein concentration. This method is highly sensitive to the presence of reducing agents [52].

The Pierce 660 nm Protein Assay is also based in a colorimetric protein quantification. Bovine serum albumin was used as standard. This method is compatible with most detergents even in high concentration, reducing agents and other commonly reagents. However, it is known that certain substances interfere with the method [43]. This is the case of imidazole: the maximum compatible concentration is 200mM and EDTA (maximum compatibility concentration is 20mM) [43].

The quantification by absorbance measurement (in a NanoDrop) allows to quantify purified proteins that contains Trp, Tyr residues or Cys-Cys disulphide bonds [50, 53]. All the methods performed did not corroborate with the quantities added to the SDS-PAGE gels. This is well illustrated in figure 3.4, where the same protein quantity, determined by absorbance measurement (NanoDrop) is not confirmed by SDS-PAGE analysis.

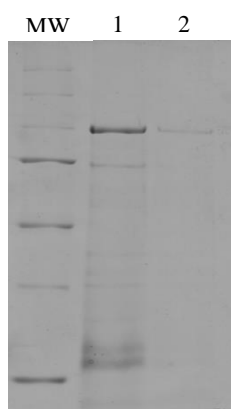


Figure 3.4. SDS-PAGE gel with two samples of purified ColAh from two different expressions. 1) Colah 10 μg - expression 19-05-15; 2) Colah 10 μg - expression 22-04-15

In figure 3.4, the SDS-PAGE gel shows the presence of ColAh, to relate the SDS-PAGE with protein quantification by NanoDrop. After protein quantification, 10 μg of ColAh

were load. As it is possible to observe, the amount loaded onto the gel did not match the quantification result.

The results obtained until this stage of the work suggest that the ColAh maybe co-purified with an interferent. From this point of view, the interferent might have also been quantified, giving erroneous values of ColAh concentration. This explains the divergent result obtained by SDS-PAGE analysis.

Until here, the protein purification was carried by batch gravity purification (BGP). So, it is possible that the purification of ColAh by this method does not yield a pure enzyme at the end of the process. With that arises another hypothesis for the unexpected results, demonstrated in the first tasks. The method used for ColAh purification is not effective. In order to clarify this hypothesis collagenolytic activity of crude extract was assayed.

3.3. ACTIVITY ASSAYS OF CRUDE EXTRACT

In the previous tasks, it was demonstrated that the recombinant protein, in its pure form, did not exhibit the expected activity (figure 3.2), suggesting that during some phase of the protocol, expression or purification, the protein is losing activity. For that reason, a hypothesis that the purification process may not be carried as predictable was emerged. In this task, gelatinolytic and collagenolytic activity of crude extract was tested.

Firstly, aiming to assess the deviation from the initial planning, a zymography assay was performed. This technique allows determining the apparent molecular weight and the gelatinolytic activity of a protein. Two samples of centrifuged crude extracts from two independent expression assays were loaded into a gelatin zymogram gel. In this way, it is possible to verify if the recombinant protein expression is being successful, and if not, this test allows the identification of the problem that was observed on the first presented results.

Zymography is an electrophoretic assay with an appropriate substrate, in this case gelatin [13].

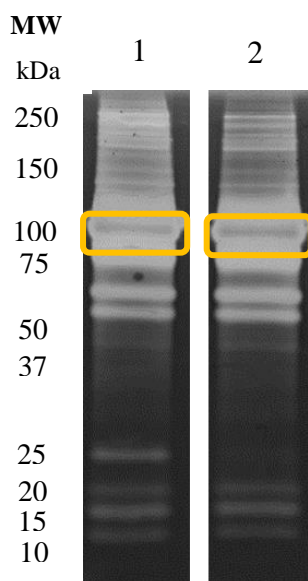


Figure 3.5. Zymography assay. 1- Centrifuged crude extract – Expression of day 17.04.15
2- Centrifuge crude extract – Expression of day 20.04.15

Figure 3.5 shows the two samples of crude extract from different expression and purification procedures. This result confirms that crude extract has gelatinolytic activity, shown in the zymogram as clear bands (substrate degradation) against dark background [13]. Thus, the expression step was being successful. This result, may suggest that the protein may be losing its activity in some part of the purification process. Collagenolytic activity of the crude extract was further assessed. As described above, the activity was measured using the synthetic peptide FALGPA; as control the commercial collagenase from Serva was used. This assay was performed using a spectrophotometer with readings over time, with a thermostated cell.

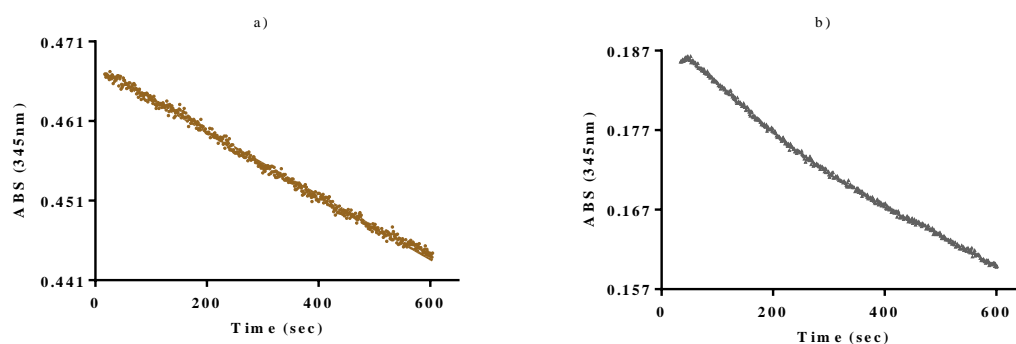


Figure 3.6. Collagenolytic activity determination using the synthetic peptide: FALGPA. a) Centrifuged crude extract of ColAh; b) Commercial collagenase Serva as control.

Figure 3.6 shows that the crude extract has collagenolytic activity presenting a degradation velocity of 1.78×10^{-05} mM/sec and the commercial collagenase shows a velocity of degradation of 2.18×10^{-05} mM/sec. These results validate the results obtained by zymography (figure 3.5). Therefore, through the zymography gel (figure 3.5) and the determination of collagenolytic activity (figure 3.6.), it is confirmed that the crude extract has activity, leading to the conclusion that the absence of expected performance in protein's purified form relies in the purification process.

For that reason, the next task of this study was to analyze different purification methods to determine the most suitable method to purify the protein without having deleterious effect on its activity.

3.4. COMPARISON BETWEEN DIFFERENT PURIFICATION METHODS

As demonstrated in the previous subsections, the expression process was effective, but the purification process failed.

The use of recombinant proteins has been increasing in recent years, highlighting their use in numerous applications [14]. Tagged recombinant protein are advantageous since they facilitate the purification while allowing its detection [15]. The purification of recombinant proteins can be performed using a chromatography system or manually. Ni Sepharose 6 Fast Flow is designed to scale up the purification of histidine-tagged proteins [15]. This method has a very high protein binding capacity, leading to lower costs per prep as a lower quantity of medium is required [16].

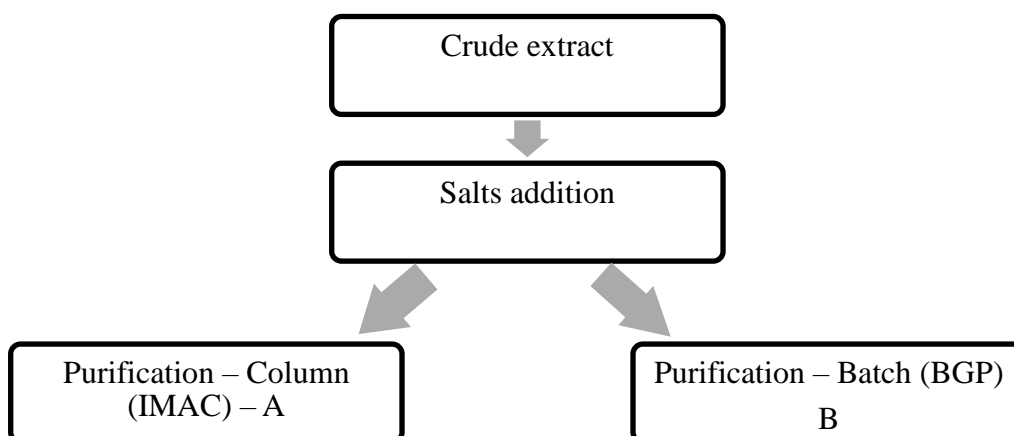


Figure 3.7. Different purification schemes used for ColAh

The purpose of the next task was to characterize the purification method according to the ColAh activity. Two methods were tested, to optimize the process (higher yields of pure ColAh). The ColAh was purified (figure 3.7.) by IMAC Sepharose 6 Fast Flow, using HisTrap FF 1ml prepacked column (A) and by batch purification with gravity flow (B), in order to choose the more profitable purification method.

Protein yield in the different steps of each method of purification was determined by ColAh quantification using SDS-PAGE (10% of acrylamide). Known concentrations of BSA were loaded on gels to prepare a standard curve.

Table 3.1. Values of the used volumes in the purification process and the obtained amount of protein.

	Volume	Protein quantity
Inoculum	400 ml	563.9 µg/ml
Centrifuged crude extract	315 ml	83.0 µg/ml
Salts addition	390 ml	81.8 µg/ml

Table 3.2. Affinity purification by batch and column procedures: Elution volumes and ColAh yield.

	Batch (BGP)		Column (IMAC)	
	Obtained volume	Protein quantity	Obtained volume	Protein quantity
Purification	350 ml	0.7 µg/ml	70 ml	9.6 µg/ml
Desalting	500 ml	0.04 µg/ml	140 ml	0.6 µg/ml

Table 3.1 and 3.2, shows the sample volume and protein quantity of each step. It is possible observe the course of ColAh during the different processes to obtain ColAh in its pure form.

The following results present the SDS-PAGE made to quantify the ColAh. Image was acquired and analysed using a GS-800 densitometer (Bio-Rad) and Quantity One 1-D analysis software. This software allows quantifying, calculating and comparing the optical density of bands.

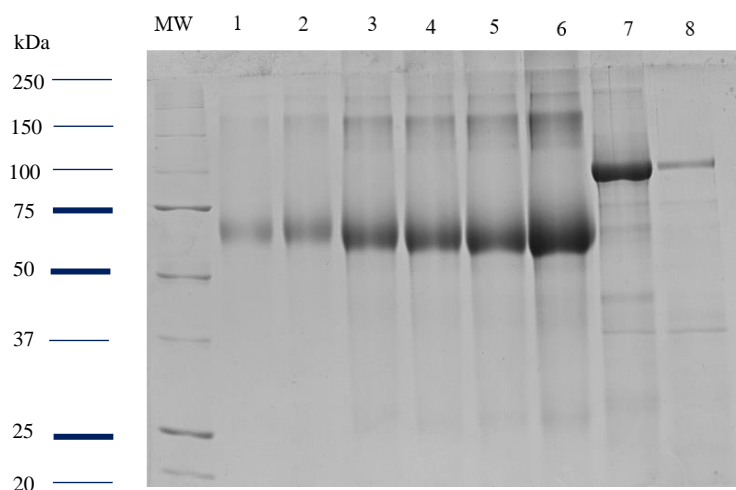


Figure 3.8. Example of an SDS-PAGE for protein quantification through densitometry, with known concentration of BSA as standard. 1) 1 μ g of BSA; 2) 2 μ g of BSA; 3) 3 μ g of BSA; 4) 4 μ g of BSA; 5) 5 μ g of BSA; 6) 10 μ g of BSA; 7) ColAh purified with column; 8) ColAh purified in Batch.

In figure 3.8 it is possible observe the ColAh present on the gel. The loaded samples correspond to pure ColAh. ColAh sample obtained from the purification using column procedure (lane 7) and from batch purification procedure (lane 8).

Table 3.3. Values of the obtained quantity of total protein.

	Total protein quantity	
	Batch	Column
ColAh - lyophilized	48.2 μ g	97.7 μ g

Based on the densitometry method of quantification, the total protein concentration was calculated. The comparison between both methods, demonstrated that column chromatography is more effective purification process with a total protein quantity of 97.7 μ g, as it yielded more pure protein than purification by batch, which obtained 48.2 μ g of total protein.

After the quantity determination of total pure protein, ColAh activity was determined by zymography, besides assessing the differences between both purification methods, and its gellatinolytic activity.

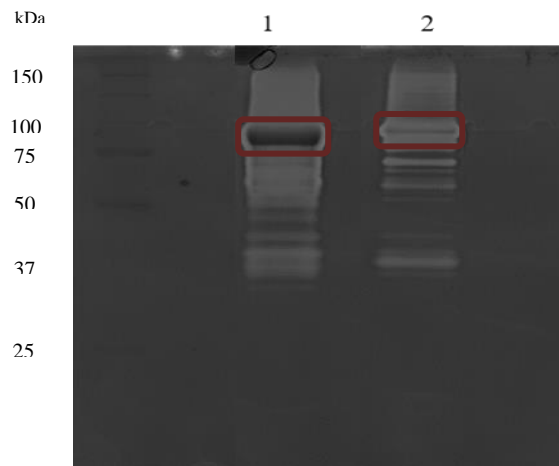


Figure 3.9. Zymogram (10%) of pure ColAh. 1) ColAh purified by column; 2) ColAh purified by batch.

In figure 3.9, it is possible to observe that the recombinant collagenase retains activity and that the different methods of purification result in different yields of protein.

The next step is to evaluate the collagenolytic activity of the ColAh, to assess the different effects on the collagenolytic capacity of this protein.

3.4.1. Determination of collagenolytic activity

The following procedure consisted in the quantification of collagenolytic activity, using a synthetic peptide FALGPA of pure ColAh obtained either by column or batch chromatography.

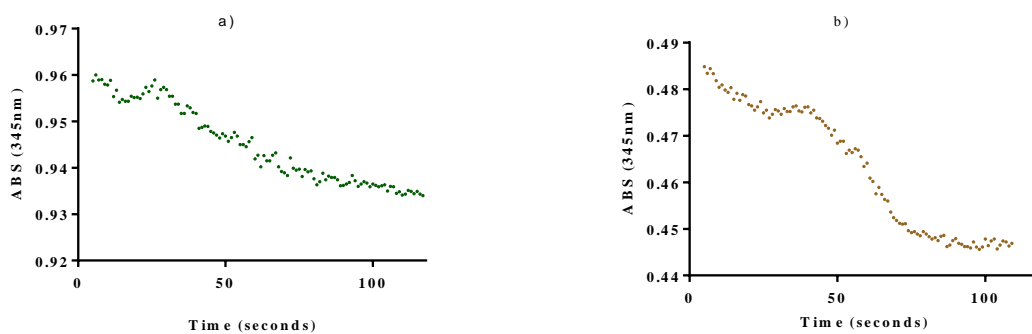


Figure 3.10. Collagenolytic activity determination of pure ColAh using the synthetic peptide: FALGPA. a) Recombinant protein purification by batch; b) Recombinant protein purification through column.

Figure 3.10, shows a graph representing the two different purification methods. The concentration of FALGPA was determined spectrophotometrically using $\epsilon_{324}=2.250 \text{ mM}^{-1} \text{ cm}^{-1}$. Graph a) represent the purification of ColAh by batch, presenting a collagenolytic

activity of 1.10×10^{-04} mM/sec. Collagenolytic activity of ColAh purified by column, represented in graph b) is 1.90×10^{-04} mM/sec. With the comparison between purification methods, the results show that the purification using the column procedure allows obtaining enzyme of higher activity than that obtained by batch purification method.

3.5. COMPARISON BETWEEN DIALYSIS AND SALTS ADDITION IN PREPARATION OF CRUDE EXTRACT

In order, to obtain ColAh in its pure form with higher activity, others parameters was tested. Preparation of crude extract was tested with dialysis and salts addition, as it is possible observe in the following representative scheme in figure 3.11.

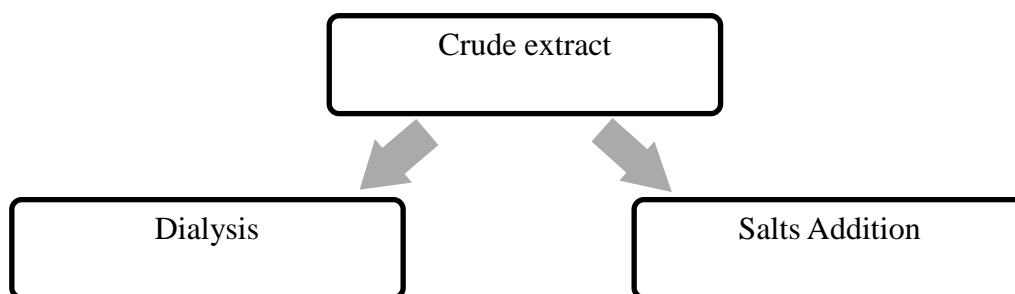


Figure 3.11. Scheme of two different purification processes tested.

The following results represent the amount of total ColAh determined by densitometry quantification method, from the step with dialysis and salts addition.

Table 3.4. Values of the used volumes in the purification process and the obtained amount of protein

	Volume	Protein quantity
Inoculum	150.0 ml	630.5 µg/ml
Centrifuged crude extract	135.0 ml	108.5 µg/ml
Dialysis	67.5 ml	48.0 µg/ml
Salts addition	84.5 ml	161.6 µg/ml

Results presented on table 3.4, demonstrate the course of ColAh and the differences between dialysis and salts additions to the crude extract. The amount of ColAh obtained from salts addition is higher than the amount obtained when it is performed dialysis. In sum, in the preparation of crude extract the yielded step is the salts addition.

However, ColAh samples were also tested from dialysis and salts addition steps in the different purification processes, as is demonstrated in the following scheme (figure 3.12).

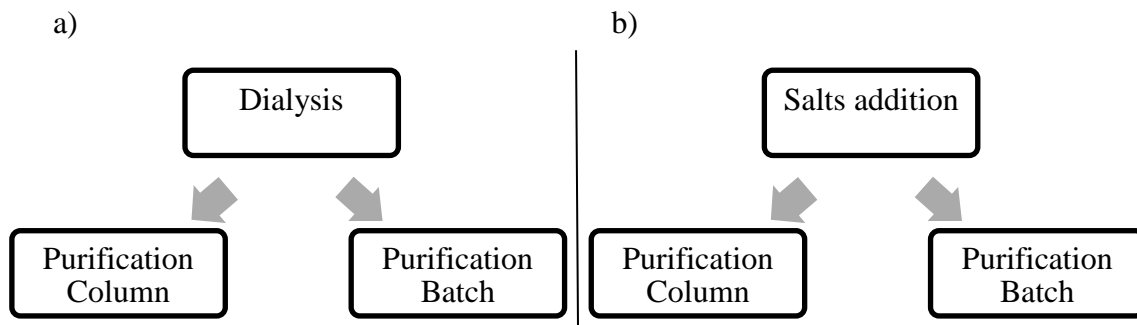


Figure 3.12. Scheme of crude extract preparation with Dialysis a) and Salts addition b), through two different methods of purification

Table 3.5. Total protein quantity – comparison between column and batch purification and salts addition versus dialysis in which purification method.

Total protein quantity					
		Dialysis		Salts addition	
		Column	Batch	Column	Batch
ColAh	-	2.6 μg	22.2 μg	54.1 μg	25.2 μg
lyophilized					

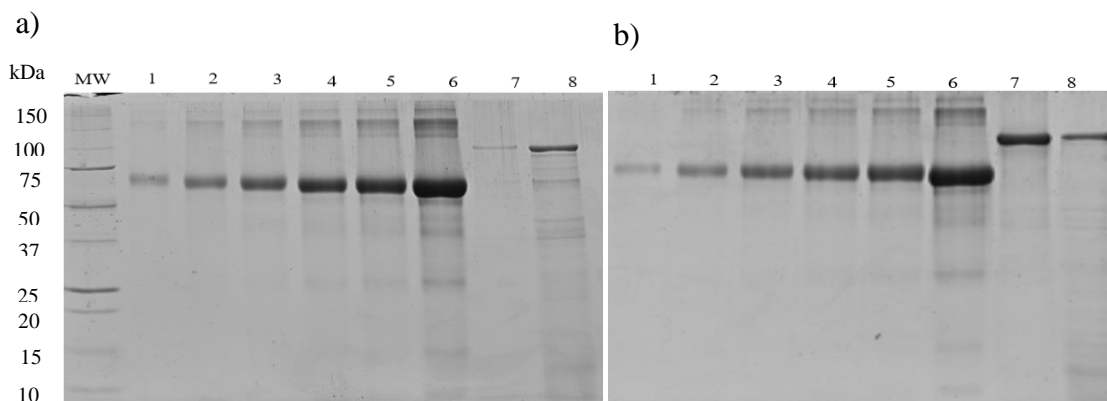


Figure 3.13. SDS-PAGE for protein quantification by densitometry, with known concentration of BSA as standard. a) & b): 1) 1 μg of BSA; 2) 2 μg of BSA; 3) 3 μg of BSA; 4) 4 μg of BSA; 5) 5 μg of BSA; 6) 10 μg of BSA; a) 7) ColAh dialyzed and purified by through column; 8) ColAh dialyzed and purified by batch purification; b) 7) ColAh with salts addition and purified through column; 8) ColAh with salts addition and purified by batch purification.

After purification of samples from dialysis and salts addition steps, the protein quantity was quantified in order to understand the yield of each one.

Based on the obtained results of protein quantification (table 3.5), using a densitometry approach (figure 3.13), it is possible to observe that the purification yield from dialyzed samples is higher for batch gravity protocol. Besides, the samples were performed with salts additions present higher yield for purification using a column.

However, from all tested samples, the ColAh which presented higher quantity was the sample performed with salts addition and purified through a column with 54.1 μg of ColAh in the end of the process.

The following result shows a chromatogram from the purification using a column (figure 3.14).

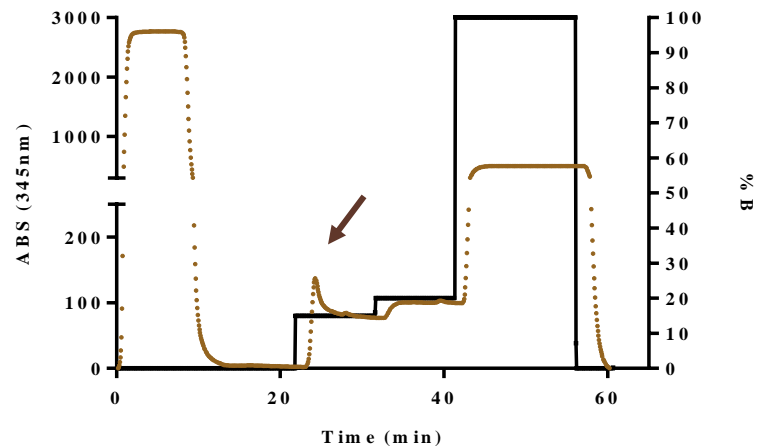


Figure 3.14. Chromatogram of ColAh purification. Recombinant protein sample after salts addition.

Figure 3.14 shown a chromatogram of purification performed using a column. The sample composition was adjusted to the composition and pH of the binding buffer by adding buffer (SPB), NaCl and imidazole. It is possible to observe a peak corresponding to ColAh with 137 mAU in a 15% gradient of eluent B (marked with an arrow).

Afterwards, collagenolytic activity was assayed using a synthetic peptide FALGPA of pure ColAh prepared either with dialysis or salts addition, obtained by batch chromatography.

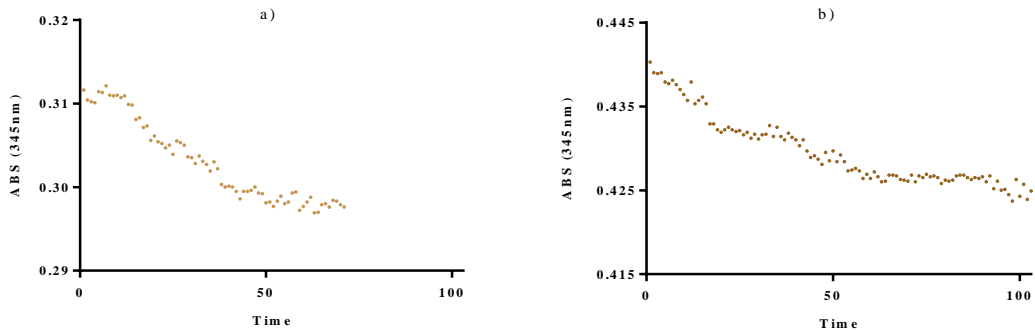


Figure 3.15. Collagenolytic activity of ColAh determined by FALGPA hydrolysis. a) Recombinant protein purification with dialysis and purified in batch; b) Recombinant protein purification with salts addition purified in batch.

Figure 3.15 shows the collagenolytic activity of recombinant ColAh after a) dialysis and after salts addition b). Both were purified using batch gravity protocol (BGP).

These results show that ColAh obtained from the procedure that includes the salts addition step presents a higher collagenolytic activity (1.01×10^{-4} mM/sec) than that obtained using dialysis (5.73×10^{-5} mM/sec). Thus, the purification method using the addition of salts seems to be the most effective method for purifying ColAh.

Batch and column purification of recombinant protein with salts addition were also compared.

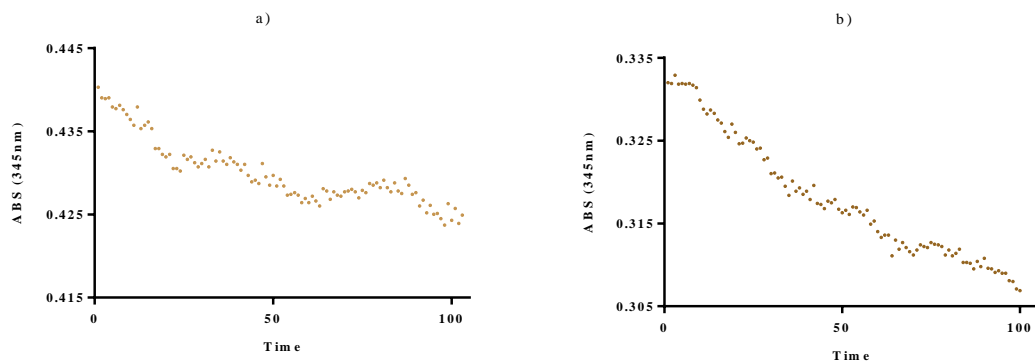


Figure 3.16. Collagenolytic activity of ColAh determined by FALGPA hydrolysis. a) Recombinant protein purification with salts additions and purified in batch. b) Recombinant protein purification with salts addition purified in column.

Figure 3.16 shows the performance of ColAh purified by the two different methods. Purification using the column procedure presents higher collagenolytic activity (1.10×10^{-04} mM/sec) than that performed using batch protocol (5.13×10^{-05} mM/sec).

The comparison between column and batch purification reveals that the suitable method of purification is the column chromatography as it yielded higher amounts of purified protein, when salts are added.

The preferred organism for large-scale production of recombinant proteins still is *E. coli* [54]. In a study performed by Karakus *et al* were they use *E. coli* to produce recombinant proteins, they also obtained a higher efficient of purification using affinity chromatography [55]. This technique has several advantages because it is fast and reliable what makes it a suitable method for purification of histidine-tagged recombinant proteins[41].

3.6. COLLAGEN HYDROLYSIS

As described before, type I collagen is the major protein component of ECM. Thus, its hydrolysis is an important subject to address. Collagen degradation plays a key role in several clinical problems (e.g. in diseases where excessive deposition of collagen occurs – DD and PD) [18], in meat tenderization [19], leather processing [20], research and other many areas of application.

The purpose of this assay was the evaluation of human type I collagen hydrolyses by ColAh. Human type I collagen was incubated at temperature between 25-30°C with the ColAh, for 24 hours. The reactions were stopped by adding the same volume of denaturing solution. The digestion reactions were analysed by SDS-PAGE (6% of acrylamide gel). In the SDS-PAGE gel four control samples were loaded, to evaluate all the components used in this assay. Collagen digestion was carried out according to Duarte *et al.* 2005. After running the electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250.

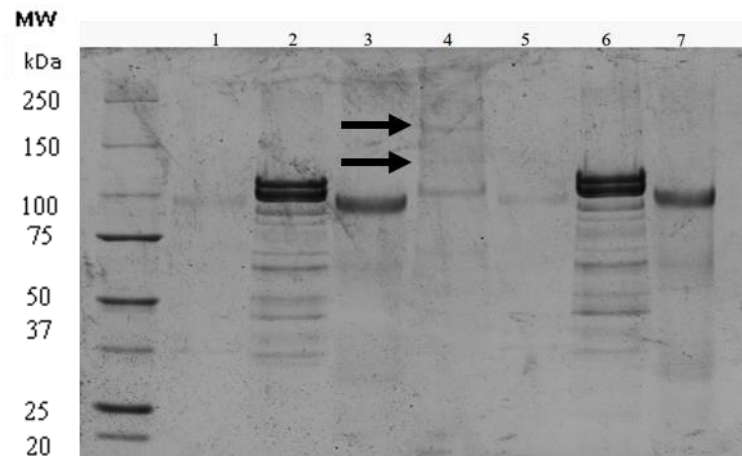


Figure 3.17. SDS-PAGE 6% Collagen type I hydrolysis. 1- Control I: Crude extract + ultrapure water; 2- Control II: Commercial collagenase: Serva + ultrapure water; 3- Control III: ColAh + ultrapure water; 4- Control IV: Collagen + ultrapure water; 5- Collagen + centrifuged crude extract; 6- Collagen + commercial collagenase: Serva; 7- Collagen + ColAh

The capacity to hydrolyze collagen is a mechanism of pathogenicity of diverse microorganisms that is very important [4]. Collagen molecules are composed of three helical chains. Each chain is characterized by the presence of two telopeptides, in both terminals.[18]

In figure 3.17, the control IV (lane 4) which was incubated in the same conditions but without ColAh, shows intact human type I with its α chains ($\alpha 1$ and $\alpha 2$). The sample with

ColAh and human type I collagen (lane 7), shows the disappearance of human type I collagen α chains. The same result was obtained for collagen digestion by crude extract (lane 5), suggesting that ColAh in its pure form and crude extract, can hydrolyze collagen molecules. Commercial collagenase from SERVA, used as control, also hydrolyzed the human type I collagen as can be observed in the figure 3.17 (lane 6).

Accordingly to Duarte *et al.*, the hidrolisis of collagens by a proteinase under similar experimental conditions agree with the results obtained in this work [5].

3.7. STORAGE STABILITY

In this assay, storage stability of ColAh was tested, in order to evaluate the stability in function of storage temperature and time. Times of incubation and different temperatures were the selected parameters to accomplish the aim of this assay. ColAh was incubated for 1 and 18 days at three different temperatures: room temperature, 4°C and -20°C. The samples were then analysed by SDS-PAGE using a 7.5% acrylamide gel. The samples were electrophoresed for 45 minutes at 80 volts (constant).

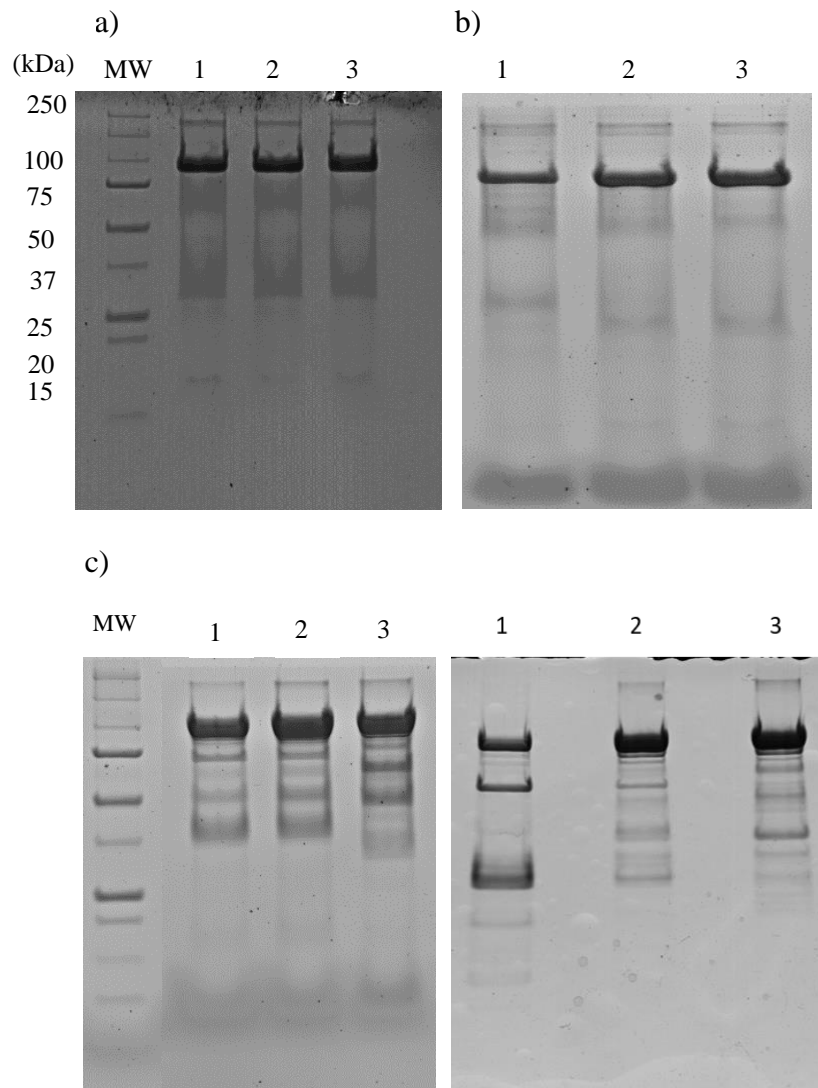


Figure 3.18. Samples of ColAh and commercial collagenase SERVA during different storage periods. a) 1 and b) 18 days of storage. 1) Room temperature; 2) 4°C; 3) -20°C

Figure 3.18 shows the ColAh in the 1st (a) and 18th day (b) of storage, incubated at room temperature (lane 1), 4°C (lane 2) and at -20°C (lane 3). As it is possible observe, the ColAh at room temperature present low differences between 1st and 18th day and the samples at 4°C and at -20°C show no differences between the different periods of storage. Besides, the commercial collagenase SERVA shows more differences between day 1 and day 18 at room temperature. Thus, with this results is possible conclude that ColAh is stable for long periods of storage and at different temperatures.

Study carried by Salome *et al*, tested the storage stability of different recombinant collagenases and the obtained results present low differences between the days of storage [16, 47].

The present results and the literature suggest that recombinant enzymes present stability over days and at different temperatures of incubation. With that, it is possible to use the ColAh in highly reproducible protocols.

4. CONCLUSION AND FUTURE PERSPECTIVES

This study allowed attaining several conclusions, concerning the appropriated method for purification of ColAh and its characterization. This characterization includes the gelatinolytic and collagenolytic activity of ColAh as well its stability and capacity to hydrolyze collagens. Also, the application of this recombinant collagenase in the dissociation of human cells *in vitro* was assessed. However, more studies should be performed to optimize the conditions to detachment of human cells.

By different methods to purify ColAh, we can conclude that Ni Sepharose 6 Fast Flow column HisTrap FF 1ml represents the assays with the higher yield. Furthermore, the purified ColAh has a collagenolytic activity that equals the activity observed in the commercial collagenase, according with results obtained though FALGPA assay. Also, ColAh shows to be stable for long periods of time and even at different temperatures, what leads to the development of a wide range of possible applications, namely in the meat tenderization, in the treatment of diseases with excessive collagen deposition.

In this way, this work provides a major evidence for the applicability of the recombinant collagenase, ColAh, as an alternative to the commercial enzymes.

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INFORMAÇÃO PESSOAL

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EXPERIÊNCIA PROFISSIONAL

2015- até ao presente

Prestação de serviços

Aveiro Rossio Hostel

2010–2011

Participação Programa "5 Ao Dia"

MARB (Mercado Abastecedor de Braga), Braga (Portugal)

2010–2011

Participação no Programa "Voluntariado Jovem para as Florestas"

IPJ (Instituto Português da Juventude), Braga (Portugal)

2007–2010

Monitora nos Programas "Tempos Livres" e "Férias em Movimento"

IPJ (Instituto Português da Juventude), Braga

EDUCAÇÃO E FORMAÇÃO

2013–2015

Mestrado em Biologia Molecular e Celular

Universidade de Aveiro, Aveiro (Portugal)

Especialização em Ciências Biomoleculares

- Biologia do Desenvolvimento;
- Neurobiologia;
- Fisiologia e Genética Microbiana
- Biologia do Genoma;
- Bioempreendedorismo;

Tese a desenvolver, intitulada de: "Colagenases para Aplicações Biotecnológicas"

- Aprendizagem de diversificadas técnicas de laboratório: Expressão e purificação de proteínas (AKTA - FPLC), fotosspectrometria, técnicas de electroforese e de determinação de atividade enzimática.

2009–2013

Licenciatura em Biotecnologia

IPVC-ESA (Instituto Politécnico de Viana do Castelo – Escola Superior Agrária de Ponte de Lima), Viana do Castelo (Portugal)

Especialização em Ciências Biotecnológicas

- Biotecnologia Ambiental, Alimentar e Agrícola;
- Engenharia Genética;
- Biologia Molecular e Celular;
- Cultura de Tecidos;

- Tecnologia Enzimática

Participação no Programa Erasmus durante o segundo ano de licenciatura, na Universidade de Ciências da Vida e do ambiente em Wroclaw (Polónia)

03/2013–07/2013

Estágio curricular de final de licenciatura, pelo Programa ERASMUS

IBMCP-UPV (Universidade Politécnica de Valencia - Instituto Biología Molecular e Celular de Plantas), Valencia (Espanha)

Tema de estágio: "Estudo da produção de compostos antioxidantes em resposta ao stress abiótico, pela espécie *Juncus*."

Aprendizagem de técnicas de Espectrometria e extração. Técnicas na área da Bioquímica e Biologia Molecular.

COMPETÊNCIAS PESSOAIS

Língua materna português

Outras línguas

	COMPREENDER		FALAR		ESCREVER
	Compreensão oral	Leitura	Interação oral	Produção oral	
inglês	B2	B2	B2	B2	B2
espanhol	B2	B2	B2	B2	B2
francês	A1	A1	A1	A1	A1

Níveis: A1 e A2: Utilizador básico - B1 e B2: Utilizador independente - C1 e C2: Utilizador avançado
 Quadro Europeu Comum de Referência para as Línguas

Competências de comunicação

- Boa capacidade de comunicação e facilidade de trabalho em equipa.
- Capacidade de criar e manter boas relações e de fácil integração em diferentes ambientes devido a experiências associativas realizadas.
- Atitude proativa.

Competências de organização

Senso de organização e habilidade em gestão de projetos. Experiência adquirida ao longo do percurso académico e na participação dos vários programas mencionados.

Competências relacionadas com o trabalho

Diversificadas técnicas de laboratório adquiridas durante a licenciatura e o mestrado.
 Dirigente associativa da ECOESA (Associação de Ecologia da Escola Superior Agrária de Ponte de Lima–Instituto Politécnico de Viana do Castelo), em 2010.
 Relações públicas da TUNESA (Tuna Feminina da Escola Superior Agrária de Ponte de Lima), em 2009-2010

Competência digital

AUTOAVALIAÇÃO				
Processamento de informação	Comunicação	Criação de conteúdos	Segurança	Resolução de problemas

Utilizador avançado	Utilizador avançado	Utilizador avançado	Utilizador avançado	Utilizador independente
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Competências digitais - Grelha de auto-avaliação

- Domínio do Microsoft Office™ (Word™, Excel™ e PowerPoint™)
- Domínio do software STATGRAPHICS CENTURION XVI.I
- Conhecimento básico da aplicação IBM SPSS Statistics software.

Outras competências

- Aulas de música (piano e canto) durante cinco anos.
- Participação num grupo de teatro durante cinco meses.
- Aulas de danças de salão durante três anos.
- Membro da “Tuna Feminina da Escola Superior Agrária de Ponte de Lima” 2008-2013

Carta de Condução

B