INSTITUTO TECNOLOGICO DE ESTUDIOS SUPERIORES DE MONTERREY
CAMPUS MONTERREY
SCHOOL OF ENGINEERING AND SCIENCE

Characterization of serine-proteases from *P. hypophthalmus* epithelial mucus as a potential feedstock for biocosmetic applications

A THESIS PRESENTED BY

María Isabela Avila Rodríguez

Submitted to the

School of Engineering and Sciences

in partial fulfillment of the requirements for the degree of

Master of Science
In Biotechnology

Monterrey, Nuevo León, México

Instituto Tecnológico de Estudios Superiores de Monterrey
Campus Monterrey
School of engineering and Science

The committee members, hereby, certifies to have read the thesis presented by María Isabela Avila Rodríguez and declares that it is fully adequate in scope and quality as a partial requirement for the degree of Master of Science in Biotechnology.

PhD. Jorge Alejandro Benavides Lozano
Tecnológico de Monterrey
School of Engineering and Science
Advisor

PhD. Mirna Lorena Sánchez
Universidad Nacional de Quilmes
Science and Technology Department
Co-Advisor

PhD. Laura Romero
Tecnológico de Monterrey
Committee member

PhD. Daniel Jacobo
Tecnológico de Monterrey
Committee member

PhD. Rubén Morales Menéndez
Dean of Graduate Studies
School of Engineering and Science

Monterrey Nuevo Leon,
28th de November de 2019

Declaration of Authorship

Through the present declaration, I María Isabela Avila Rodríguez state that the composition of the present work titled as "Characterization of serine-proteases from *P. hypophthlamus* epithelial mucus as a potential feedstock for biocosmetic applications" was produced by my own, from which the information and procedures from other sources are properly cited. As well, it is declared that no prior publication has been submitted with the information contained in it, as well as when procedures have been accomplished by teamwork, it is well stated in which parts the contribution was external and in which the contribution was by my work. All the presented data was obtained wholly or mainly through the candidature of the degree in the present university.

María Isabela Avila Rodríguez
Monterrey, Nuevo León, México
November 6th of 2019

Dedication

To my parents and my brother who gave me strength to be. For you an eternal hug and *abracaribe.*

To my grandmothers Carmen and Rosario who are always watching my back.

To the universe for creating nature and such beautiful figures to explore them with respect.

To science for letting me do what I love the most, learn.

And finally, to all the curious ones who came before me to seed doubts and to all the ones that will be brave enough to continue doubting.

Acknowledgements

In first instance, thankyou mom and dad for making grow into a free woman, with own criteria and strong values. I can't express enough how much I care for you and how thankful I am for you love, care and teachings. You have always been my guide and my light; I deeply love you. To my brother, you are a great example of how you can overcome anything that comes in the battlefield, thank you for being by my side in spite of the long distance.

I would also like to thank the Tecnológico de Monterrey and CONACYT for tuition and economical support during my postgraduate studies. With this support I was able to grow as a professional and create knowledge.

As well I would like to thank my advisors. You both believed in me and took me under your guidance to discover what the word investigation really means. For that I am eternally grateful. Thank you both for teaming up into such interesting custody of a child who liked to play with fishes.

Dr. Jorge I thank you for visualizing the positive side of every unfortunate event, this is a virtue that made me laugh and smile when all went wrong on the lab. Also thank you for making me feel welcomed into our group when I came from volcano land (Puebla), I never felt alone or in between an earthquake afterwards.

Dr. Mirna I thank you for not letting me give up in this heavy but satisfying road of knowledge. With your vision I learned that its only matter of discipline and good will to be able to do something and that no goal is too big for someone. To coryta, I thank you for giving me a truthful friendship on the way along. Keep dreaming high as you always do but remember, always give yourself some space to charge batteries, breath and have a good laugh, those ones that heal your soul.

Dale, thank you for teaching me about enzymes, but most importantly I thank you for always giving me a hand when my heavy head sank into the deepest negative thoughts. For always letting me know I am not a hen, instead I am a falcon whose sight gets blinded by fear. I hope life gives us time to continue arguing about science, cooking and thinking about how matter needs to come back together in some point, remember Σ 'αγαπώ για πάντα.

Jesus, I thank you for teaching me most of the things I know, including life. Please continue being that light that unites most of the people you know. You have a gift and that's for sure. Just have some faith in humanity, is difficult but what is life without a little mistake.

Gaby, thank you for making me feel at home and giving me your hand when I was a stranger. I know life is waiting for us to grow into those strong women we once talked about. Keep shining with that humble way of yours of seeing good in every human being. We need it.

To David and Jorge, thank you for starting this dream with me and continue to fight for what you believe. Hope this road keeps us together to share our fears, mistakes and good times as often as we like to.

To my gang, what would have been of me without you. Guys your laughter, our lunch times, even our lab discussions made my studies one of the bests moments of my life. Coming to a different city was a fearful experience at the beginning. I felt out of the water (yes the joke is on me), but you made me feel at home, like a second family who cared for me as a sister. Ñoñiatis funny name but yes, nerdiness will keep us young and happy. Keep shining as you do, I admire you all and I am really happy to have found you.

List of abbreviations

KLK	Kallikrein type serine proteases
SDS-PAGE	Sodium Dodecyl Sulphate polyacrylamide gel electrophoresis
2D-PAGE	Bidimensional polyacrylamide gel electrophoresis
US	United States
ASAPS	American Society of Aesthetic Plastic Surgery
TCA	Trichloroacetic acid
ER-YAG	Erbium:yttrium-aluminum-garnet
rpm	Revolutions per minute
nm	nanometers
UI/mg	International activity units per milligram
U/ml	Activity units per milliliter
kg	kilograms
cm	centimeters
pH	Hydrogen potential
º C	Celsius
MMPs	matrix metalloproteases
ADAMs	metalloprotease-desintegrins
ECM	extra cellular matrix
TEMED	N, N, N', N'-Tetramethylethylenediamine
IEF	Isoelectrofocusing
IPG	Immobilized pH gradient
HPLC	High pressure liquid chromatography
DTT	Dithiothreitol
CHAPS	3- ((3-Cholamidopropyl)dimethylammonio)-1-Propanesulfonic Acid
BCA	Bicinchoninic acid
FAO	Food and Agriculture Organization
ME	Mucus extract
EME	Evaporated mucus extract
DME	Desalted mucus extract
PMSF	Phenylmethylsulfonyl fluoride
EDTA	Ethylene Diamine Triacetic Acid
V/h	Volts per hour
mL	Milliliters
uL	Microliters
ug/mL	Micrograms per milliliter
kDa	Kilodaltons
M	Molar
mM	Millimolar

Characterization of serine-proteases from *P. hypophthalmus* epithelial mucus as a potential feedstock for biocosmetic applications

by

Avila Rodríguez, María Isabela

Abstract

Chemical peeling is a cosmetical treatment that promotes skin renewal, by the remotion of skin layers through the appliance of corrosive compounds. It has proven to be successful for the removal of acne, scars, photoaging, and pigmentary lesions. Yet this procedure is aggressive and can produce several complications, among them infections, eruptions, erythema or scarring. As an alternative, enzymatic peelings have been proposed. Enzymes lead natural desquamation processes, principally by serine proteases (SP). SP have also been identified in fish epithelial mucus. As well, empirical evidence has shown that direct contact with Iridescent shark (*Pangasius hypophthalmus*) epithelial mucus, promotes skin regeneration. Hence, through the present study, the characterization of the proteases present in *P. hypophthalmus* epithelial mucus was held, in order to identify new SP with potential cosmetical use. Epithelial mucus was extracted by rinsing specimens in extraction buffer (NaCl 50mM pH 7.4) in polyethylene bags and held back to tank. The obtained extracts were pooled and centrifuged. Supernatant was concentrated and desalted using vacuum evaporation and PD-10 columns. Protease activity was evaluated through caseinolytic activity and zymography using casein and gelatin universal protease substrates. Also in-gel inhibition (PMSF, benzamidine, EDTA, O-phenanthroline, and iodoacetamide) and activation (Zn^{2+}, Ca^{2+}, K^{+}, Na^{+} and no ion) for specific protease families were evaluated. Caseinolytic activity was detected (5.33 ± 0.37 U/mg at 25 ºC). As for zymography, active bands within 130-15 kDa were identified for gelatin, while only one active band of 63 kDa was identified for casein. Compared to control treatment (Zn^{2+}), K^{+} and Na^{+} enhanced gelatinolytic activity of medium weight bands (63, 58 and 48 kDa), while $Ca2^{+}$ depleted most protease activity. Serine and cysteine protease inhibitors, PMSF and iodoacetamide, excerpted similar inhibition by reducing 63 kDa and inhibiting 58, 56, 30 kDa activity. MMP inhibitors exerted slight inhibition to superior weight bands (114, 90 and 71 kDa). Benzamidine only depleted 45 kDa activity. The present study proves the presence of the MMPs and SPs within *P. hypophthalmus* epithelial mucus. This positive result opens the possibility for further protease characterization and isolation for their evaluation as feasible agents for biocosmetic treatments.

Keywords: serine proteases, *Pangasius hypophthalmus*, enzymatic peeling, desquamation, epithelial mucus

List of Figures

List of Tables

Table of Contents

Chapter 1

Introduction

1. Introduction

Cosmetical industry has been in constant development for the renewal of efficient products and methods to give a healthier and younger skin (1). Since its early beginnings, in 10,000 BC in ancient Egypt (2), to its boom after cosmetic surgery development in the 1900´s (3), the tendency of facial treatments marked a path by passing from: creams with herbal extracts, then to surgical methods and currently to non-invasive methods. The classification cosmetical treatments is currently stablished by surgical and non-surgical, were surgical are defined as invasive procedures in which a professional practitioners modifies patients body, in order to improve its appearance (4). While non-surgical methods, also known as minimally-invasive, using less drastic methods in order to perform body modifications, this includes the use of apparatus, substances and injections (5).

Just in 2015, 15.9 millions of surgical and minimally-invasive cosmetic procedures were performed in the US (6). Among them, minimally invasive alternatives including Botox®, hyaluronic acid fillers, photo-rejuvenation and chemical peelings, are gaining leadership over the user demand. This has been a consequence of the need of more pain-free and quick recovery treatments (7). From the cluster of minimally invasive procedures, chemical peelings constitute one of oldest and most commonly used alternative, especially for the treatment of acne, scars, age marks and hyperpigmentation (6).

Through chemical peelings, the epidermis or dermis is artificially removed by the appliance of corrosive substances to achieve a new and better texture (8). Depending on the skin depth removal, chemical peels are classified as: A) very superficial only reaching the stratum corneum; B) superficial which partially or totally removes the epidermis; C) medium which removes epidermis and part of papillary dermis; and D) deep, which removes up to the reticular dermis (9). Depending on the depth needed on the treatment, corrosives substances like alpha-hydroxyacids (glycolic acid), resorcinol, trichloroacetic acid, phenol or jessner solution, are used in concentrations from 20% to 50% in superficial to medium peelings and up to 70 to 88% on medium to deep procedures (10).

This approach has proved to be successful in the removal of acne, acne scarring, photoaging, and pigmentary lesions (11). Nevertheless, chemical peelings are aggressive, non-specific and require high concentrations of reagents to be effective. Also, several complications like infections, acneiform eruptions, hyper- and hypo-pigmentation, erythema, herpetic lesions and scarring (10,12,13) are prone to appear after the treatment application as part of the skin healing process.

To overcome the prior mentioned risks of chemical peeling, enzymatic components are starting to be used as active agents for dermatological treatments, including peeling procedures (14–16). For this, topically formulated proteases, including collagenases, bromelain and papain, have been applied to the affected area to enhance skin exfoliation, debridement or to stimulate desmolysis (17,18), with low concentrations of the active agent. It has been proved that the use of topic enzymatical formulations ameliorates several dermatological pathologies, such as: contact dermatitis, pruritus, debridement of chronic wounds, psoriasis, skin flaking as well as increasing moisturizing or chemical peelings effects when used as a pretreatment (17,19).

Concerning these applications, vegetal, bacterial and animal proteases have been characterized and included in to several commercial and clinical formulations. Among the common proteases found as part of cosmetical and dermatological applications, bromelain (20), papain (21), collagenases (22), keratinases (23), elastases (24), or serine proteases (25) are the most common proteases found in wound healing and cosmetic compositions.

For instance, human skin natural peeling (desquamation) is mediated by several enzymes (**Figure 1.1**). Through this process, the outer layer of skin epithelium, stratum corneum, is constantly renewed by the detachment of dead skin cells called corneocytes (26). Corneocyte detachment occurs as a consequence of the balance among the synthesis and degradation of specific cellular attachments (corneodesmosomes) (24), mainly cathepsin-like and serine proteases (25).

Among the serine protease family, kallikrein type 5 and 7 have been reported to be expressed in stratum corneum and participate in the desquamation process (27). Serine proteases have been also, described to be present in fish epidermal mucus. Fish epidermal mucus is a protective matrix that is constantly secreted through goblet cells to serve as a physicochemical barrier that permits osmoregulation, respiration, nutrition, locomotion and innate immune response against pathogenic agents (28,29).

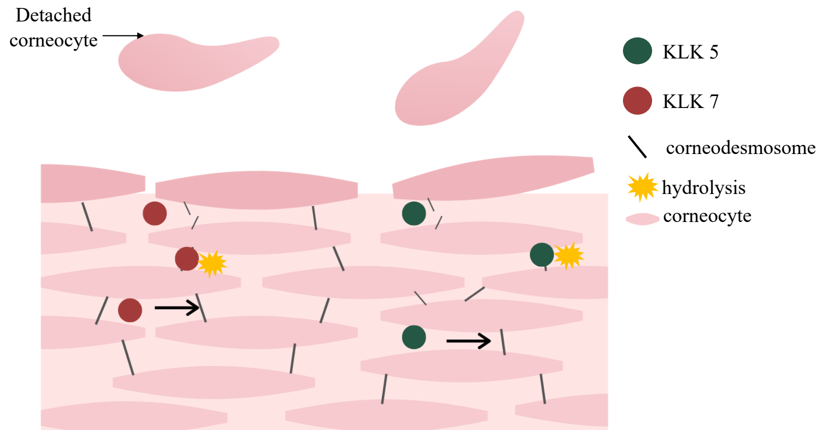

Figure 1.1 Schematic representation of human *stratum corneum* desquamation process (30)

This barrier is normally composed by glycosaminoglycans, lysozyme, immunoglobulins, complement, carbonic anhydrase, lectins, C-reactive protein, calmodulin, crinotoxins, pheromones and proteases (31). Within the proteolytic enzymes present in epidermal mucus, serine proteases have been reported to be in major proportion on the epithelial mucus of several fish species such as *Cirrhinus mrigala*, *Labaeo rohita*, *Catla catla*, *Rita rita* and *Channa punctate* (28).

In the case of the Iridescent shark (*Pangasius hypophthalmus*), no information has been described on its mucosal protease profile. This specie, native from Vietnam, is processed in a rate of 3,500 tons daily and distributed to over 80 countries around the world (32). As part of the production process, when specimens are reared, they have to be moved to bigger tanks by manual manipulation. After performing this exercise, workers from an aquaculture farm from Cuernavaca, México, evidenced softer skin texture in their hands, in spite of hard labor.

Hence through this study, the characterization of the protease profile present within *P. hypophthalmus* epithelial mucus is presented. Through this analysis, the identification and comparison of the found serine proteases to human KLK5-7, led to proposal of a candidate protease to be tested as a feasible agent for selective desquamation of human skin.

1.1 Hypothesis

The epithelial mucus secreted by *P. hypophthalmus* contains one or several proteases with similar family class and substrate preference to human serine protease kallikrein 5 and 7, then they can be a feasible feedstock for biocosmetic applications.

1.2 General objective

To identify and characterize which from the proteases present within the secreted epithelial mucus, belongs to the serine protease family in order state their homology to KLK 5 and 7, and propose their evaluation for its use as suitable agents for cosmetic enzymatic peelings.

1.3 Specific objectives

1.3.1 Screen the protein and protease profile of *P. hyphophthalmus* secreted epithelial mucus by SDS-PAGE and zymography.

1.3.2 Identify which of the proteases belong to the serine protease family through inhibition zymography.

1.3.3 Isolate and analyze the secreted serine protease isoforms and isoelectric points through 2D-PAGE and 2D zymography.

Chapter 2

Background

2. Background

2.1 Facial cosmetic treatments

Beauty treatments have been part of human well-being since ancient times. Records of such go back to the 1500 b.c, where Egyptians employed alabaster dust, animal mineral oils, sandpaper and milk baths to exfoliate facial and body skin (33). As well, Eritreans and Hokkaido inhabitants used natural solutions such as camel milk or soy flour facial masks for skin cleansing (34). With the advance of technology, the knowledge over chemistry, human physiology and biology, became available to the cosmetic industry. This opened a window of possibilities over the topic of beauty and skin management, and sat base for the world´s cosmetic industry, with an approximate worth of tens of billions of US dollars (35) .

Currently the demand for cosmetical procedures and products is still on the rise. According to the “Cosmetic Europe” association reports, users manifest an increasing necessity of achieving good health and confidence by taking care of their appearance (36). Only in 2018, over 1.5 and 3.3 millions of surgical and non-surgical procedures where performed in the United States. In **Figure 2.1**, the annual expense in US billion dollars for the top 5 surgical and non-surgical cosmetic procedures is depicted.

Non-surgical procedures constituted the first option for facial improvement, where the top four alternatives were botulinum toxin (Botox®), hyaluronic acid, photo rejuvenation and chemical peels. On the other hand, from the top surgical performed procedures, only eyelid surgery was the only one that target facial correction (37).

By comparing all of the mentioned procedures (**Figure 2.1**), most of the methodologies used for facial skin treatments are non-surgical. This agrees with the necessity of consumers to have quick and painless methods for a healthier appearance (7), and explains why the trend over less invasive and more cost-effective procedures is pushing innovation in to functional cosmetics that can cope with this necessity (38). As well, from the four mentioned alternatives, Botox®, hyaluronic acid and photo-rejuvenation are treatments that need to be performed by a qualified practitioner. While chemical peelings can be found in professional and household formats, which makes this technique even more available to consumers.

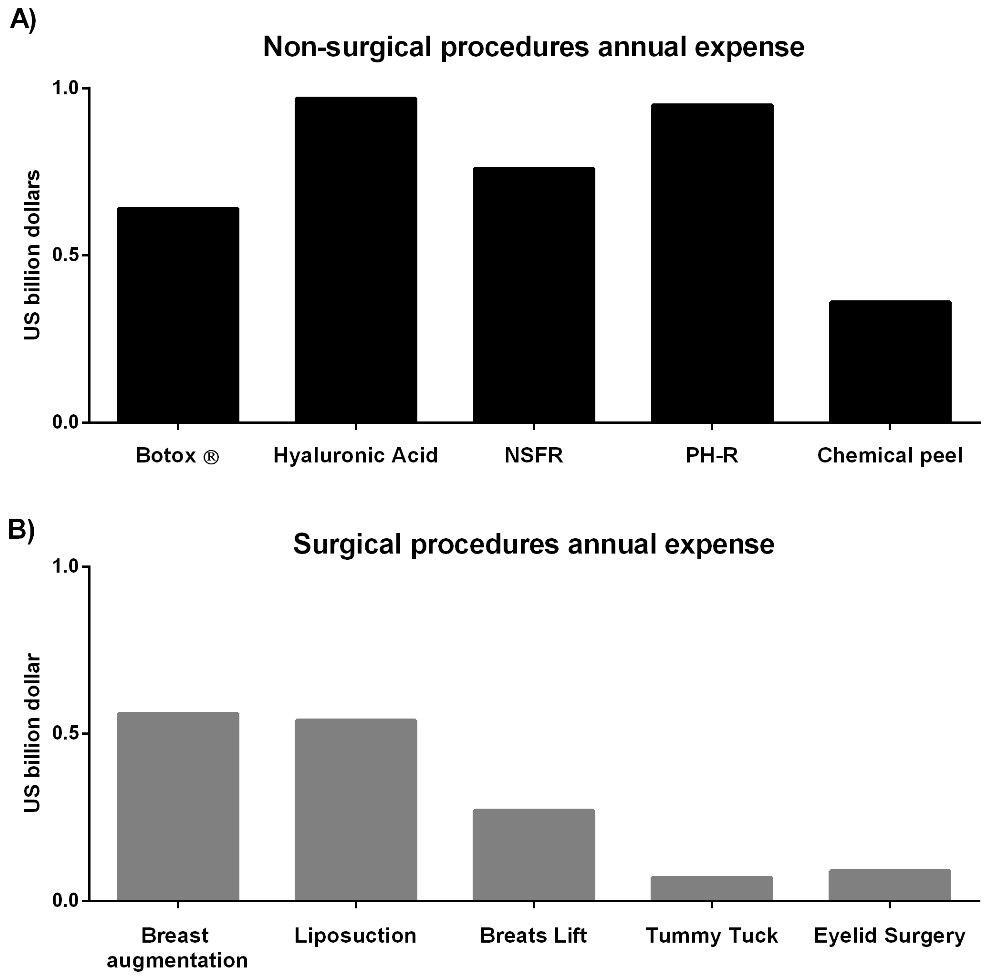

Figure 2.1 Total annual expense of the top five A) non-surgical and B) surgical procedures performed in the USA during 2018 * Information adapted from ASAPS statistics 2018, NSFR: non-surgical fat reduction, PH-R: photo rejuvanation

Nevertheless, chemical peelings represent an aggressive treatment as it requires the unselective ablation of facial skin in order to obtain the desired results (8). As variants of chemical peelings, systematic and enzymatic peelings have been proposed in order to provide a more controlled facial skin removal. On the following sections these three main types of facial peelings will be described.

2.1.1 Chemical peelings

A chemical peeling is a process in which the destruction of epidermis to papilar dermis is endorsed, mainly through the application of corrosive substances. This in order to eliminate imperfections and stimulate the growth of healthier skin (39). The first register of this practice goes back to the 1900s, where doctor George Mackee used phenol for periods of 30 to 60 seconds in order to remove wrinkles and acne scars from its patients (40).

In actuality, chemical peelings continue to be used with a slightly changed format, in which skin removal depths were characterized in relation to the concentration of the corrosive used. The four stablished depth categories for this procedure are very superficial, superficial, medium-depth and deep peelings (13), which target different cell layers of human skin.

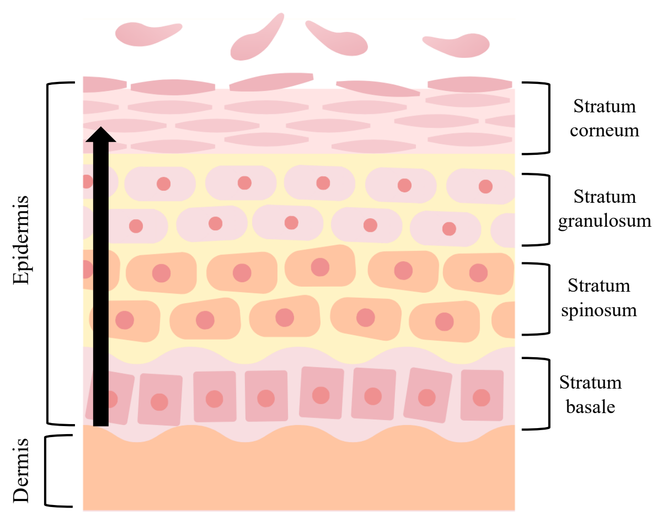

Figure 2.2 Schematic diagram of the anatomy of human skin

Human skin is comprehended by three main layers: epidermis, dermis and hypodermis (**Figure 2.2**). Epidermis in particular, is divided in other sublayers denominated as: A) stratum corneum, B) stratum lucidum, C) stratum granulosum, D) stratum spinosum, and E) stratum basale (41). When superficial peelings are performed, the stratum corneum cells, also called corneocytes (27), get detached by the degradation of their cell junctions (corneosomes) generating epidermolysis and exfoliation.

As for medium-depth peelings, coagulation of membrane proteins occurs, devitalizing the cells of the entire epidermis. In the case of deep peelings, a complete epidermolysis and protein coagulation occurs. This leads to total dermal restoration and restructure of basal layer (12) Several caustic agents have been reported for skin artificial degradation, including α/β-hydroxyacids, β-lipohydroxy acids or combinations depending of the goal depth (11). The most common classification and peeling solutions used for chemical peelings are described in **Table 2.1**.

Table 2.1 Type of chemical peelings classified under target depth (9–11,13)

Type	Corrosive agent	Removal Depth	Therapeutic indication	Time and frequency of treatment
Very superficial	Glycolic acid (30-50%) *Jessner solution (1 to 3 coats) Low-concentration resorcinol (20-30%) *TCA (10% and 1 coat)	Stratum corneum	Acne, wrinkles, melasma and photo aging	1 up to 10 min Repeated applications
Superficial	Glycolic acid (50-70%) Jessner solution (4 to 10 coats) Resorcinol (40-50%) TCA (10-30%)	Epidermis		2 to 60 min Repeated applications
Medium-depth	Glycolic acid (70%) Jessner solution (4 to 10 coats) Resorcinol (40-50%) TCA (35-50%) Augmented TCA (CO_2 + 35% TCA) Jessner + TCA 35% Glycolic acid 70% + TCA 35%	Epidermis and part of papillary dermis	Photoaging, lentigos, pigment lesions and atrophic scars	3 to 30 minutes 5 days of desquamation One-off procedure
Deep	Phenol 88% *Baker -gordon phenol formula	Up to reticular dermis	Severe photo aging, scarring	Depends on method 10-14 days for re-epithelization Single session

*TCA: Trichloroacetic acid; Jessner solution (14 % salicylic acid, 14% lactic acid and 14% resorcinol in 95% ethanol (42); Baker Gordon phenol formula (Phenol USP 88%, liquid soap and croton oil (43)

Despite the good results and versatility of corrections that chemical peelings can offer (**Table 2.1**), several complications can appear as part of the final result. This includes: prolonged erythema, infection by bacteria (*Stafilococcus aeureus*) or virus (Herpes simplex), acne induction, blistering, hypo/hyper-pigmentation, and milia (10). In the case of deep peelings, the patient requires of cardiopulmonary monitoring due to phenol cardiotoxicity in circulatory system (44). By which the effectiveness of this treatment plays a risk with well-being of the patient, as the re-appearance of certain undesired facial defects is feasible even though these were the main motive to start treatment in the first place.

2.1.2 Physical peelings

A physical peeling is defined as the process in which the removal of the epidermis achieved by means of abrasive materials. At initial stages, abrasion was opposed through salts and sterile sandpaper. This methods permitted skin exfoliation, yet there was no control over the process and sand paper in particular lead to erythema, hemorrhages and proinflammatory pigmentations (33). Physical peelings may be classified as ablative, where a detachment of skin layers occurs, and non-ablative where modification of skin surface is obtain without generating deep wounds (45).

Among the most common non-ablative procedures it may be found micro-needling (46), microdermabrasion (33), radiofrequency and non-fractionated low intensity lasers (45). The microinjuries generated by these treatments stimulate formation of new collagen in order to heal the wound, and as a desired effect a more youthful skin. In the case of ablative procedures, dermabrasion or laser resurfacing are the gold standard (47). This types of procedures are considered mainly for deep skin removal and for the treatment of similar conditions treated by chemical peelings suchlike facial rhytids, scars, acne, photo aged skin, lentigo or melasma (48,49).

Dermabrasion uses diamond fraises to excerpt a cut force to epidermal tissue in velocity ranges from 10,000 to 85,000 rpm. The patient must be sedated and the area to treat most be previously cryo-anesthetized (49). As for laser resurfacing, several types of beams and modes have been utilized, including pulsed and continues wave CO2 and ER:YAG (erbium:yttrium-aluminum-garnet) (50) in wavelength ranges between 10,600 nm and 2940 nm respectively (51). CO_2 treatments removed tissue and promoted immediate coagulation, yet unspecific thermal necrosis was the main drawback of this technology (51).

As chemical deep peelings, total removal of epidermis leaves to edema, exudation and sloughing in the first three days of treatment (48). Also this procedure is considered chirurgical, in most of the cases with elevated costs as it requires a medical staff, an operating room and antimicrobial prophylaxis during the whole process (52).

2.1.3 Enzymatical peelings

Enzymatical peelings appear as an emerging variant of chemical peels, as the exfoliation in this case is catalyzed by proteases, conferring the advantage of selective degradation of skin. In this case exfoliation happens by the enzymatically enhanced desmolysis of corneocytes (17,18) through the use of proteases from animal, vegetal of bacterial sources through topical administration.

In normal conditions, corneocyte detachment happens through skin desquamation process. Within a turnover period of two to four weeks, stratum corneous layer is continuously shed by the degrading action of kallikreins and cathepsins endogenous proteases (27) over corneocyte specialized junctions (corneodesmosomes) (53). This allows the proper conversion of keratinocytes to corneocytes and maintain the proper layer thickness (54,55).

In several studies, enzymatical peelings have proved accelerated skin cell remotion. This was evidenced by Lopes (2008) who tested papain 0.2% (w/v) solution over human breast skin culture. After treatment, a decreased corneosome crosslinking was evidenced (56). As well, cathepsins were tested as treatment in a sample of 30 subjects, within an age range of 30-45 years old. These were randomly separated in two testing groups, where one group used serum with 15% cathepsin D (10,000 UI/mg) and the other just serum.

As a result, patients that used topical formulation containing cathepsin D, resulted in significant improvement over epidermal properties compared against the placebo group (57). Also, it was demonstrated that cod trypsin was effective in degrading keratin from human plantar callus *in vitro* (58). Topically applied proteases such as subtilisin and trypsin have proved to be successful agents for epidermal ablation through murine and human *in vitro* and in vivo skin samples (59).

Enzymatic peelings apart of skin exfoliation, has been proposed as therapeutic agents for other dermatological conditions. Including the treatment of actinic keratosis, epidermal neoplasm, acne vulgaris, anti-aging, and debridement in wound healing (59). Debridement comprises the second most used application for proteases, as enzymatical formulations help to eliminate dead scaffold promoting the healing of chronic wounds. For example, a clinical trial carried out on 32 patients with 50 pressure ulcers demonstrated that cod trypsin hydrogel (5 U/mL) acted on a superior manner over other four conventional treatments (60).

Even though enzymes have started to be used in cosmetic products because of good consumer appeal and improved performance (35), little information is known over the cosmetical application of the already mentioned proteases. Nor information of different techniques apart from topical and injectable applications. Most of the information that is offered in present literature only evaluates proteolytic activity.

As well, no structural or sequence analyzes of the resemblance of used protases with the ones present in human skin has been done. As well no scientific validation over secondary effects for cosmetical approach of enzymatic peelings have been assessed. By which this type of treatments could be study in closer detail in order to prove its effectivity as cosmetical feed stock and offer a less aggressive non-invasive treatments to the consumer.

Other types of proteases that have been used and proposed for cosmetical have been found to provide by marine sources. Also, animal secretions rich in proteases, including skin epithelial mucus of fishes, have proved therapeutic and exfoliating action over human skin, including the species *Netuma barba* (61), *Channa striatus* (62), and *Clarias gariepinus* (63).

In the case of the fish *Pangasius hypophthalmus*, no information on its mucosal protease profile has been reported. As part of their production process, specimens are handled to bigger tanks. After performing this exercise, the fish secreted mucus. Workers from an aquaculture farm from Cuernavaca, México, after prolonged exposition to this secretion evidenced softer skin texture in their hands, in spite of hard labor.

2.2 General description of *Pangasius hyphophthalmus*

Pangasius hypophthalmus, also known as stuchi catfish, iridescent shark-catfish, and striped catfish (**Figure 2.3**), is a riverine freshwater specie from the pangasidae family original from the Mekong River in Vietnam (64). It is well known because of its great spawning capacity and big size of 130 cm and almost 44 kg. This specie is benthopelagic, which means that lives in the nearest water layer of the bottom of the body of water in which it lives (65,66). typically at temperatures of 22-26 °C within pH ranges of 6.5 to 7.5 (67).

Figure 2.3 Picture of the Iridescent shark (*Pagnasius hypophthalmus*) (68)

P. hypophthalmus lacks of scales, by which its first defense mechanism against environmental burdens is the epithelial mucus (66). This secretion is continuously produced through specialized cells identified as goblet, club and sacciform cells (69), which are evenly distributed through the *stratum espinosum* of fish epidermis.

Once goblet cells releases mucosal secretion, they are not able to discharge new mucosal content, by which a continuous turnover of epidermis occurs in order to maintain an adequate thickness of the mucosal barrier (70). The mucus secretion could increase or diminish depending on the external stimuli, this includes pathogen presence, handling and wounding (71).

2.2.1 Fish epithelial mucus and its relation to proteases

Epithelial mucus is a barrier that constitutes an important component of fish innate immune system as: 1) it avoids microbial colonization by its continues re-motion, and 2) it confers protection as the present immune molecules within the matrix have antibacterial effects (28). The innate components that have been described to be present within fish epithelial mucus are principally: mucins, glycosaminoglycans, lysozyme, immunoglobulins, complement, carbonic anhydrase, lectins, C-reactive protein, calmodulin, crinotoxins, pheromones and proteolytic enzymes (31).

Proteolytic enzymes act against pathogenic agents (72). Among the reported proteases present within epithelial secreted mucus, trypsin (serine-protease), cathepsin B/L (cysteine-protease), cathepsin D (aspartic-protease) and metalloproteases (73) have been extensively identified. From this mentioned cluster, serine and metalloproteases have proved to be in greater proportion within the epithelial mucus of several fish species (74).

Proteases are a type of enzymes that belong to the hydrolase class, with specific action over peptidic bonds (75). Depending on their catalytic core, these enzymes can be classified as metalloproteases, cysteine proteases, threonine proteases and aspartic proteases (76). Concomitant, the general characteristics and involvement of the previously mentioned proteases in skin remodeling processes, will be described.

Metalloproteases exist in two tightly related families, which are matrix metalloproteases (MMPs) and metalloprotease-desintegrins (ADAMs) (77).

The MMPs family is characterized by its dependence to Zn^{+2} in order to permit catalysis. As well, MMPs are the main participants in skin remodeling processes, as they mediate extra cellular matrix (ECM) modification through several stages of skin homeostasis and in all of the steps of skin wound healing(78,79).

This family has also been detected in fish mucosal secretions as fish matrix metalloproteinases (FMMPs) 9, 13 and meprins (FMM) (80,81). ADAM family is similar to MMPs, but it counts with a trans-membranal union site (77). This family has been described to be strongly involved in fertilization processes and regulating of shedding activity in humans (82). Yet, this type of proteases has not been reported for fish epithelial mucus.

In contrast, serine, cysteine and aspartic proteases are characterized by having a well-established catalytic triad, with a marked nucleophilic amino acid, by which the name of the protease is given. The most common catalytic triads for these enzymes are: Asp-His-Ser for serine proteases (83), Cys-His-Asn for cysteine proteases (84) and Asp-Ser-Gly for aspartic proteases (85). As previously mentioned, cathepsins L, B, D and trypsin proteases have been described to be present in fish epithelial mucus.

These types of enzymes, are also present in human organisms and participate in several cellular events, including human skin remodeling and wound healing. In the case of cathepsins, it has been reported that they have a strong roll in skin desquamation (27). As well they participate in hemostasis (86), ECM remodeling (87), and keratinocyte migration (88). As for serine proteases, they have been described as regulators of miscellaneous physiological processes (89), including signaling cascades, epidermal homeostasis and hemostasis (90,91), ECM remodeling for cellular proliferation (92) and desquamation among other functions.

In the case of skin desquamation, kallikrein (KLK) serine proteases are involved in this process. This type of enzymes has been identified in human body with more than 15 variants. From this cluster, it has been described that all of chymotrypsin-like and 50% of the trypsin-like activity in stratum corneum can be described by KLK 7 (chymotrypsin-like) and KLK 5 (trypsin-like) (53). Serine proteases also appear to be present in mucosal secretions (96). Yet poor substrate and structure characterization over these specific variants is available.

After contrasting the current state of the art, it is clear that the anectodical event of skin softening by *P. hypophthalmus* skin secretions, is a consequence of the presence of proteases within this colloid. As well, skin desquamation and remodeling processes are highly mediated by several type of proteases, yet the current cosmetic techniques, more in specific, peelings, have not been well characterized in order to take advantage of this mechanisms to develop less aggressive methodologies.

Through this research, the characterization of the protease profile and the identification of the serine proteases present in *P. hypophthalmus* epithelial mucus, is presented. This study is the first one to describe the proteases content within this specie skin secretions, and to describe its serine protease structural homology to KLK 5 and KLK 7. Giving notions of applicability of proteases in biocosmetic applications.

Chapter 3

Materials and methods

3. Materials and Methods

In order to describe which from the total protein components belonged to the protease family, more in specific to serine proteases, three fundamental steps were endorsed. These were sample collection and adequation, were total protein and protease activity is confirmed. Protein and protease profile screening were total protease bands of fish epithelial mucus can be screened. And protease profile characterization, were specific protease family features can be obtained through inhibition, ion zymography and bidimensional electrophoresis analysis. In the following chapter, the description of each of the methods used for the previously stablished steps will be described.

3.1 Reagents

Sodium chloride, sodium hydroxide, chloridric acid, zinc chlorine, calcium chlorine, sodium chlorine, potassium chlorine, β-casein, gelatin, L-tyrosine, sodium azide (NaN_3), sodium tartrate, trichloroacetic acid, copper sulphate pentahydrate, folin-ciocalteu reagent 2 N, ammonium persulphate, TEMED, sodium thiosulphate, formaldehyde solution 36.5-38%, acetic acid, tricine, aluminum sulfate hydrated, orthophosphoric acid, Triton X 100 and tergitol were acquired from Sigma Aldrich in reagent grade. 30% Acrylamide:Bisacrylamide 29:1 (3.3% crosslinker concentration), 10 X Tris-glycine-SDS, Tris-HCL, Tris, Tricine, rehydration buffer (8 M urea, 2% CHAPS, 50 mM DTT, 0.2% Bio-Lyte ® ampholyte, 0.001% bromophenol blue), ReadyPrep ™ Reduction-Alkylation Kit, ReadyStrip™ IPG strips pH 3-10, bromophenol blue, Xdual Pro protein ladder, IEF standardad (pH range 4.45–9.6, catalog no. #1610310), carrier ampholytes (40% Biolyte 3/1º ampholyte), silver nitrate, coomassie blue G-250, low fusion point agarose, running buffer 10 X were acquired from BioRad ®, United States. Sodium carbonate anhydrous, pierce BCA Protein Assay Kit and sodium dodecyl sulphate were acquired from Thermo Fisher scientific ®, United States. Acetonitrile HPLC grade was acquired from J.T Baker. Glycerol, Isopropyl alcohol, methanol, ethanol, mineral oil was acquired from DEQ, México.

3.2 Animal Maintenance

Four iridescent sharks (*Pangasius hypophthalmus*) specimens with average weight of 20 ± 0.3 g of an approximate age of one to six months, were acquired from a local pet store. The care conditions were held under the guidelines of the FAO sanity pisciculture manual (93). Animals were reared at 25 ºC within an 80 L freshwater tank and an oxygen pump coupled with an active carbon filter (Bio-Bag ®, Tetra). Fishes were fed *ad libitum* with commercial pellet brand (Tetrafin ®, Tetra).

3.3 Sample collection and preparation

Mucus extraction from *P. hypophthalmus* was performed according to Subramanian et al. (2007) (94) protocol with modifications. The protocol with modifications will be described below. Unanesthetized specimens were transferred one per one into polyethylene bags (Ziplock ®) containing 10 mL of extraction buffer (NaCl 50 mM, pH 7.4). Then, these were gently massaged within a period of 1 minute, in order to trigger mucus excretion due to handling stress response (95). This protocol was authorized by the bioethics committee CICUAL from *Tecnologico de Monterrey* University under protocol number 2019-017.

The mucus solubilized in extraction buffer, was pooled and centrifuged at 5000 g for 15 minutes at 4 ºC. The obtained supernatant was denominated crude mucus extract (ME). ME was aliquoted in fractions of 10 mL and concentrated through centrifugal vacuum evaporation for 5 hours (GeneVac EZ-2 ® series, SP Scientific, New York, United States). Once this process was finished, a second pooling of concentrated samples was performed. The resulting sample was denominated evaporated mucus extract (EME).

For SDS-PAGE, EME samples were desalted by Sep-Pak C18 cartridge (96) and PD-10 column (5 kDa MWCO) under supplier instructions (Amersham Bioscience). For 2D SDS-PAGE and 2D-zymography, EME was desalted through PD-10 column only. Total protein concentration was determined through bicinchoninic acid colorimetric essay as described by supplier (BCA Protein Assay kit, Thermo Fisher). All samples were stored at 4 º C until use until one month. Sample collection was repeated with a minimum period of one month between each extraction in order to allow total goblet cells reconstitution in skin epithelium (70).

3.4 Protein and protease activity screening

3.4.1 SDS-PAGE and 2D-PAGE

Total protein profile, molecular weight estimation and total isoform screening of epithelial mucus was described through this method. For gel electrophoresis, 1.5 ug of ME, EME and desalted samples through Sep-Pak and PD-10 columns (C18ME and DME) were loaded in to a 12% Tircine SDS-PAGE gel (97). The essay was run at 30 V for 30 minutes, and then at 90 V until the running front touched bottom of the gel. For 2D SDS-PAGE electrophoresis, 10 ug of DME in 125 uL rehydration buffer, were loaded through passive rehydration overnight into a 7 cm immobilized pH strip (IPG) of broad range (pH 3-10). After, Isoelectrofocusing (IEF) was done by an Ettan IPGphorTM 3 (GE Healthcare, Uppsala, Sweden) with the following voltage ramp: step of 500 V for 4 hours, a linear gradient of 500 to 1000 V for 1 hour, and a linear gradient from 1000 V to 8000 V until 49,880 V/h where reached. IPG strips were reduced and alkylated under supplier instructions (ReadyPreo(™) 2-D Starter kit). Then, strips were submerged six times in running buffer and casted into a 12% Tricine SDS-PAGE gel, which was ran at 90 V using a mini-PROTEAN tetra cell (Biorad, Hercules, CA, United States). Both type of gels were stained with silver nitrate staining method (98).

3.4.2 1D and 2D Zymography

Protease activity profile of *P. hypophthalmus* epithelial mucus was evaluated through zymography, using gelatin and casein as substrates. As well, the effect of reported KLK activator ions and different reported protease inhibitors were evaluated. For this essay, 1.5 ug of EME were loaded with non-reducing loading buffer (12% SDS, 30% glycerol, 0.05% Coomassie blue G-250 and 150 mM Tris-HCl) in to a 12% polyacrylamide gel copolymerized with 0.3% w/v of the mentioned substrates. Gels were run at 4ºC at 30 V for 30 minutes and then, at 90 V until the running front touched bottom of the gel. After, these were treated with 50 mL of washing buffer (2.5% Triton X 100, 50 mM Tris-HCl, 5 mM $CaCl_2$, 0.02% NaN_3; pH 7.5) twice for 30 minutes. Concomitant, gels were treated with 50 mL of incubation buffer (50 mM Tris-HCl, 5 mM $CaCl_2$, 0.02% NaN_3, 1% Triton X 100 , 1 µM $ZnCl_2$; pH 7.6) overnight (16 h) at 37 ºC and 60 rpm (99,100). For ion effect evaluation, 1 µM of $CaCl_2$, NaCl, KCl, $ZnCl_2$ and no metal salt were stablished as treatments. Inhibition assay was held by adding 5 mM benzamidine, 2 mM PMSF (serine proteases), 5mM iodoacetamide (cysteine proteases), 5 mM EDTA, and 5 mM O-phenanthroline (metalloproteases) during the incubation step (101) (102). Gels were washed from incubation solution with distilled water twice per 30 minutes and stained with colloidal Coomassie (103).

Total active isoforms of epithelial mucus were identified through gelatin 2D-zymography analysis. For this assay, 10 ug of DME in 125 uL non-reducing rehydration buffer (1% tergitol, 5% glycerol, 0.001% bromophenol blue and 0.25% carrier ampholytes - 40% Biolyte 3/1º ampholyte Biorad), were loaded through passive rehydration overnight into a 7 cm immobilized pH strip (IPG) of broad range (pH 3-10). IEF was performed using the previously described voltage ramp, until 50,118 V/h were reached. Then, strips were incubated in 1.5 mL equilibrium buffer (0.375 M Tris-HCl, 2% SDS, 20% glycerol) for 10 minutes. After, it was submerged 6 times in running buffer and casted into a 12% polyacrylamide gel co-polymerized with 0.3% of gelatin. The resulting gel processed as previously described 1D zymography methodology (104). Gel was then washed with distilled water twice per 30 minutes and stained with colloidal Coomassie blue (103). Active isoforms were compared with the obtained spots of 2D-SDS PAGE through Image J (Fiji) software (105). The identified spots were cut out from the SDS-PAGE gel with sterile scalpel and forceps. Excised pieces were placed in sterile microcentrifuge tubes and preserved in 1 ml of ultrapure water at - 20 ºC.

3.5 Molecular weight and optical densitometry analysis

Image Lab software (Biorad ®) was employed for the estimation of the apparent molecular weight and densitometry analysis. Molecular weight calculated by selecting the “lanes and bands” command. Then, in the “Lane” tab, the manual selection of lanes was performed by adjusting the pre-defined frame to the image. Then in the “Band” tab, the “Add band” command was selected in order to mark the visible bands present in each of the lanes. After, the size of the bands was adjusted to its correct width. Once this process was finished, the “molecular weight analysis” command was selected. Within this menu, the “Bio-rad X dual pro” option for “standard” was chosen. As well, the lane containing the ladder was ticked in order to identify it as the standard. Then the general report command in the main menu was executed in order to obtain the resulting molecular weights.

Densitometry analysis was held with the same program by using "quantity tools command". For this, the bands within the same molecular weight range were previously selected through the already mentioned protocol. Once bands and lanes were defined, in the "Relative" tab, the "select" button was activated. Once this tab was blue, the band that was defined as control treatment was clicked. Then the general report command in the main menu was executed in order to obtain the resulting relative quantities. These data were then graphed through Prisma software.

3.6 Caseinolytic activity

Protease activity of ME, EME was evaluated through caseinolytic activity under Das & Col 2013 protocol with modifications (106). Using a 1:3 sample to substrate proportion, reaction tubes were constituted by preheating 100 uL of sample (5 ug) at 25 º C and 37 º C for 5 minutes. Then, 200 uL of substrate (casein 1% w/v in Tris-HCl 20 mM, pH 7.4) were added to start the reaction. Samples were subsequently incubated and agitated at 25 º C and 37 º for 1 hour. To stop the reaction, 700 uL of ice-cold TCA 0.4 M were added. Samples were centrifuged at 2000 rpm for 15 minutes, and supernatant was measured by Lowry colorimetric assay (107) using L-Tyrosine as standard. Each reaction was measured by triplicate. Negative control was considered as substrate with sample solvent. A unit per milliliter of caseinolytic activity was defined by equation 1 (108).

$$protease\ activity\ \left(\frac{U}{ml}\right) = \frac{Tyr_{eq}\, V_t V_{cr}}{V_e\, t} \quad \textbf{(eq. 1)}$$

Tyr_{eq} = L-Tyrosine equivalents (µmol)
V_T = total reaction volume (ml)
V_{CR} = colorimetric reaction volume (ml)
V_e = Enzyme volume (ml)
t = time of assay (min)

3.7 Isoelectric point estimation

Isoelectric point estimation of the identified active variant of *P. hypophthalmus* epithelial mucus was done by using Fiji software (Image J), Photoshop CC 2019 ® and RF vs pI correlation. Through photoshop, pI standard gel (pI2D) and sample gel (S2D) images in RGB format, were normalized in size and color by using routes: Image>Image size and Image>adjustments> match color. Once this was accomplished these images were settled in a same plane and saved as TIFF format. The obtained file was then opened on Fiji application, were line marks for the beginning and ending of total running front were fixed. Spots of each gel were marked with circular and line tools. Once each of the spots were identified, line tool was used to measure the distance of the spot to the initial boundaries of the horizontal dimension with an angle of 180 ºC for each measurement. The obtained distances were passed to Rf (eq.2) and transformed to a logarithmic scale in order to calculate the pI of S2D according to pI 2D pattern.

$$Rf = \frac{Migration\ distance\ of\ protein}{Migration\ distance\ of\ dye\ fron}\ x\ 100 \quad \textbf{(eq. 2)}$$

3.8 Statistical analysis

All of the measurements for the experiments here presented, where held at least by triplicate. The statistical analysis was performed with central tendency methods, except for optical densitometry and molecular weight analysis. All numerical measurements are expressed as mean and standard deviations. As for molecular weight analysis, a correlation coefficient was used to state accuracy upon the molecular weight and migration distance of the obtained bands.

Chapter 4

Results and discussion

4. Results and Discussion

Through the present chapter the discussion of the obtained results will be held. After proceeding with the previously stated experimental strategy (Chapter 3), serine protease activity as well as kallikrein characteristic features could be confirmed. Through gelatin zymography a protease profile of ten bands could be screened. From inhibition and ion activity tests, one potential candidate of 63 kDa with only one isoform of pI 6.8 was identified. The comparison and analysis of each of the obtained results will be developed in the following subsections.

4.1 Sample collection and preparation

In order to obtain the highest amount of analyte and maintain animal welfare according to the three Rs for animal research (109), refinement of mucus extraction process was held. Epithelial mucus was extracted (ME), clarified and concentrated through vacuum evaporation (EME). Protease activity and total protein concentration was measured for both of sample states (ME and EME). After performing the previously described method, protease activity could be confirmed for ME and EME by caseinolytic activity at 25 ºC, temperature of *P. hypophthalmus* habitat, and 37 ºC, human physiological temperature. Specific total activity for samples ME and EME at different temperatures are shown in **Table 4.1**.

Table 4.1 Temperature effect over mucus extract caseinolytic activity

Sample	Protease activity (U/mg)	
	25 ºC	37 ºC
Crude extract (ME)	504.80 ± 93.31	21.31 ± 18.46
Vacuum evaporation (EME)	5.33 ± 0.37	1.67 ± 0.31

Total specific activity diminishes after vacuum evaporation process in 25 ºC and 37 ºC conditions, and in greater extent at 37 ºC. This decrease could be explained as in first instance, *P. hypophthalmus* is a specie that grows in temperatures of 22-26 ºC (28). Even though 37ºC is a common condition to test, as it is considered a physiological temperature for humans (107), is outside the range of the normal temperature of *P. hypophthlamus*.

Normally, when proteases are exposed to non-optimal temperatures, their activity could be inhibited due to destabilization (108).

The inhibitory effect of vacuum evaporation occurs as a consequence of the presence of different molecules, apart from proteases, in the mucus extract. These molecules represented contaminants, that when they are not removed, a significative decrease of the specific activity occurs (110). Yet, in other studies when no concentration step was performed, no detectable levels of proteases activity could be characterized on rainbow trout, Coho salmon and Atlantic salmon mucus samples (111).

Hence in order to make suitable this process contaminant molecules must be removed from the total mixture. As an example, Ong (1976) reported an increase of 53.4 U/ml to 261 U/ml and of 6.92 mg/ml to 31.68 mg/ml when concentrating thermocyclase from filtrated crude media through vacuum evaporation at 45 ºC (112). It is important that a first titration of sample is performed before any purification process to avoid over-manipulation of the analyte, and misguided results. Despite of the decrease of specific activity, vacuum evaporation was a necessary step as mucus extract solubilization (ME) yielded low protein concentrations that were not suitable for the sensitivity of gel electrophoresis. The total protein profile of ME and EME can be observed in **Figure 4.1**.

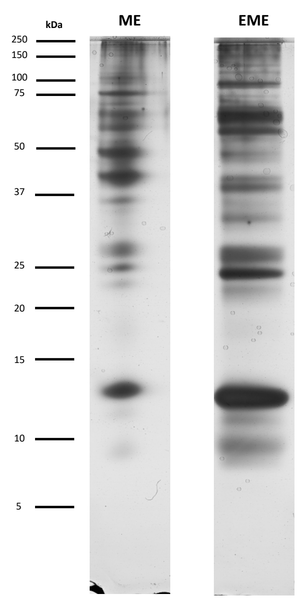

Figure 4.1 Protein profile of *P. hypophthalmus* epithelial mucus where ME (2 µg), EME (1.9 µg) were loaded to a 12% SDS-tricine PAGE stained with silver nitrate. No change on the number of bands is appreciable within profiles.

Most of SDS-PAGE methodologies rely on increasing protein concentration or the sensitivity of the detection method (113), in order to avoid sample underloading. This problem is one of the most common troubleshooting's on electrophoresis, in which minor components and major bands become too faint for reproducible screening (114). It can be solved by loading 40 to 60 ug of total protein of crude samples when staining with Coomassie Blue (115) or 100 fold less when using silver nitrate as its sensibility at nanogram range (116). In this case, the increase of protein concentration was a useful step to improve band resolution, as it allowed the proper screening of the protein profile of *P. hypophthlamus* epithelial mucus. As shown in **Figure 4.1**, an increase on band definition could be achieved after sample pretreatment, increasing total protein concentration from 51.50 ± 1.67 ug/mL to 257.5 ± 11.77 ug/mL.

4.2 Protease profile of *P. hypophthlamus* epithelial mucus

Protease profile of *P. hypophthalmus* epithelial mucus was screened through Zymography analysis, using universal protease substrates, gelatin and casein (117). In **Figure 4.2**, EME protein (Lane 2) and protease (lane 2 and 3) profile is shown.

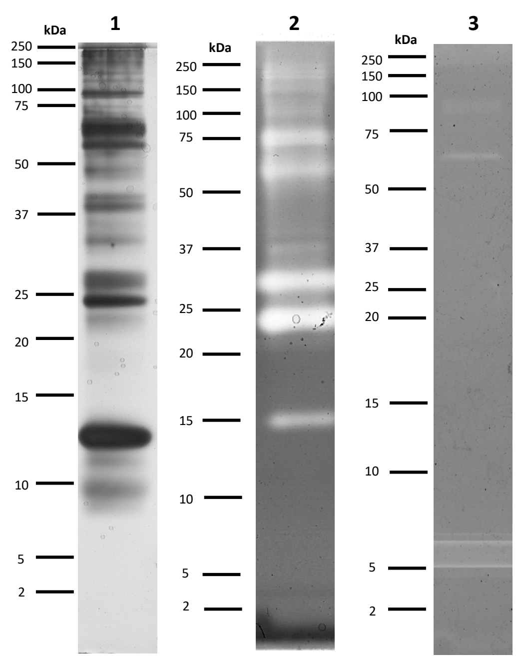

Figure 4.2 Protein and protease activity profile of EME from *P. hypophthalmus*. 1) EME 1.9 µg in a 12% SDS-tricine PAGE stained with silver nitrate. 2) Zymography of EME (1.5 µg) in 12% SDS-PAGE gel copolymerized at 0.3% w/v gelatin. 3) EME (1.5 µg) in 12% SDS-PAGE gel copolymerized at 0.3% w/v casein. Both lanes were incubated with at 37 ºC in pH 7.6 and revealed in coomassie blue.

From the total profile (16 bands) of *P. hypophthalmus* epithelial mucus (**Figure 4.2** lane 1), 10 bands (179, 114, 90, 71, 63, 58, 56, 48, 36 and 30 kDa) presented gelatinase activity (**Figure 4.2** lane 2). The obtained pattern is similar to the one reported by Salles et al 2007, who describes seven proteases of similar molecular weights (130, 100, 73, 69, 53, 43, 36 and 15 kDa) in Tambacu hybrid fish mucus (118).

As well, protease profile of epithelial mucus of the species *Channa punctata*, *Cirrhinus mrigala*, *Labeo rohita*, *Catla catla* and *Rita rita* display similar molecular weights to the ones obtained by the present study (75, 60, 50, 40, 22, and 17 kDa) (101). The case is the same for *Oncorhynchus mykiss*, *O. kisutch* and *Salmo salar* epidermal mucus (102,111) and *Myxine glutinosa* mucus protease activity (119).

In the case of caseinolytic activity (**Figure 4.3** lane 3), a 63 kDa band revealed activity. The obtained band does not match with the ones reported for *Paralichtys olivaceus* mucus (24 kDa) (120), Tambacu mucus (73 and 100 kDa) (118) and *Sparus aurata* mucus (12, 15, 76 and 80 kDa) (121). This could lead to the consensus that similar protease profile is shared among several fish species when looking at gelatin zymography.

Yet this profile may have variations due to the conditions and niche in which the specie develops (101). On the other hand, only one band had substrate selectivity for casein and gelatin. These substrates have different structural conformations, as casein is globular (122) and gelatin, composed mainly by collagens, which are fibrillar proteins (123). By which it could be presumed that the 63 kDa protease could participate in two roles, as remodeling and activator agent. This behavior is generally attributed to multifunctional enzymes, which contain various catalytic activities (124). By which it could be presumed that *P. hypophthalmus* does present a protease activity with a dual substrate proteases. This reinforces the initial hypothesis as KLK 5 and 7 can as well catalyze globular (125,126) and fibrillar substrates (127,128).

4.3 Effect of ions over protease activity

The role of ions over protease activity has been well characterized, as these can regulate, stabilize and activate them. Metal ions have been classified in two types, where type I refer to cofactor-like behavior and type II as allosteric effector behavior (129). The selected type II ions have been reported as activators of KLK 5 serine proteases (130,131). While Zn^{+2} is a well-known cofactor in metalloprotease catalysis (79). Through the present assay, the enhancing and inhibitory effects ions type I (Zn^{+2}), type II (Ca^{2+}, K^{+} Na^{+}) and no ions were tested. For this assay, Zn^{+2} was taken as control treatment as this ion has been reported to enhance activity for hydrolases in higher frequency for both ion behaviors (132). In **Figure 4.3, Figure 4.4** and **Figure 4.5** the modulating effects of the previously mentioned ions over universal protease substrate activity (gelatinolytic and caseinolytic) of EME is shown.

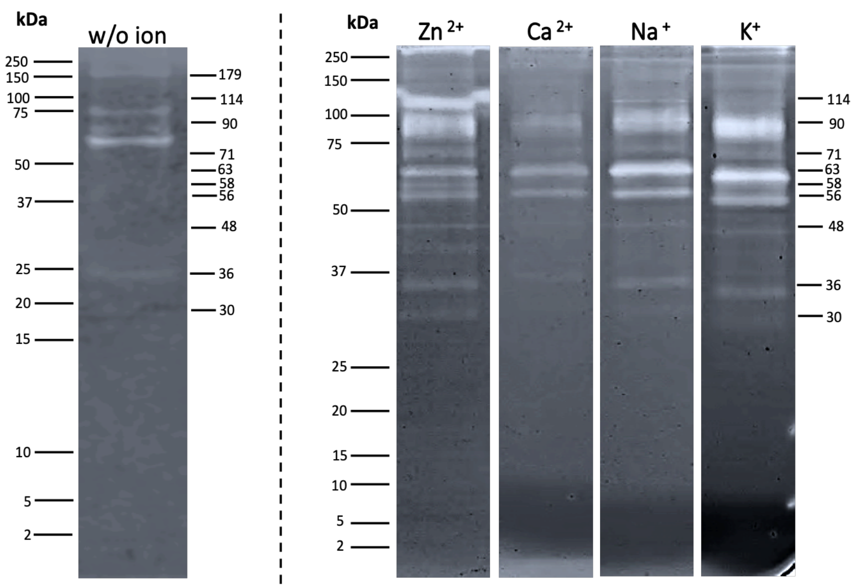

Figure 4.3 Ion effect on gelatin protease profile. EME (1.5 µg) ran in a 12% polyacrylamide gel copolymerized with 0.3% w/v of gelatin incubated without (w/o) and with different ions ($ZnCl_2$, $CaCl_2$ NaCl and KCl) in incubation solution at 37 ºC and pH 7.6. Gels were stained with Coomassie blue to reveal protease activity.

As a result, protease profile of the control treatment (Zn^{2+}) showed 10 active bands (**Figure 4.3**), mentioned previously in section *4.2 Protease profile of P. hypophthlamus epithelial mucus*. This ion has been reported to intervene in redox and non-redox catalysis. In both mechanisms enzymes increase their activity as Zn^{2+} could increase nucleophilicity, act as an activator or an electrostatic stabilizer (132). This could explain the increased number of bands when using Zn^{2+} as modulator agent. When no ion treatment was endorsed, gelatin zymography revealed 5 bands within the high molecular weight range (179-71 kDa) of the protease profile bands (**Figure 4.3**). This result shows that most of the proteases within the cluster interact with ions in order to stabilize or modulate their activity. In most of the cases, enzyme activity is enhanced through weak interaction of large monovalent cations by kosmotropic structural stabilization (118). A more detrimental effect was obtained when treating the samples with Ca^{2+}, as only 4 active bands appeared. On the other hand, K^+ and Na^+ affected lesser extent the catalytic activity, as 7 and 6 bands gave signal (**Figure 4.3**). Nevertheless, band intensity through these two treatments was greater than the ones form the control sample, by which it could be presumed that the proteins present in EME could belong to KLK class. In order to quantitatively measure and compare the change on band intensity, a densitometry analysis (133) per band on gelatinolytic and caseinolytic activity was held. In **Figure 4.4** the densitometry analysis of the gelatinolytic activity of EME is shown.

According to densitometry analysis (**Figure 4.4**), K^+ treatment favored the gelatinase activity of several proteases (90, 63, 58, 36, and 58 kDa). In several cases it has been describes K^+ as an allosteric promoter of substrate binding through conformational modifications (129). Na^+, also enhanced protease activity with greater extent in the 30 kDa band. These results resemble the ones reported by Olivera (2010), were alkaline protease activity was enhanced up to 220% when testing 2 mM NaCl (134). Such increment was also reported for subtilase with and increment of 280% when using 600 mM (135). By which Na^+ acts as a type I ion by enhancing activity, nevertheless K^+ excerpts a greater aid to protease activity.

In contrast, Ca^{2+} treatment exerted higher decrease on band activity in comparison to control treatment, where 71, 48, 36 and 30 kDa were the proteases that showed less activity in comparison to the control. This disagrees with the results obtained by several authors, who report activation of proteases when using calcium as modulating agent. Such was the case for colicin V (CvaB) cysteine protease (136), subtilase serine protease (135), and cathepsin D aspartic protease (137). As for metalloproteases, Ca^{2+} ions are needed for correct structural conformation, as these count with a calcium binding domain (138).

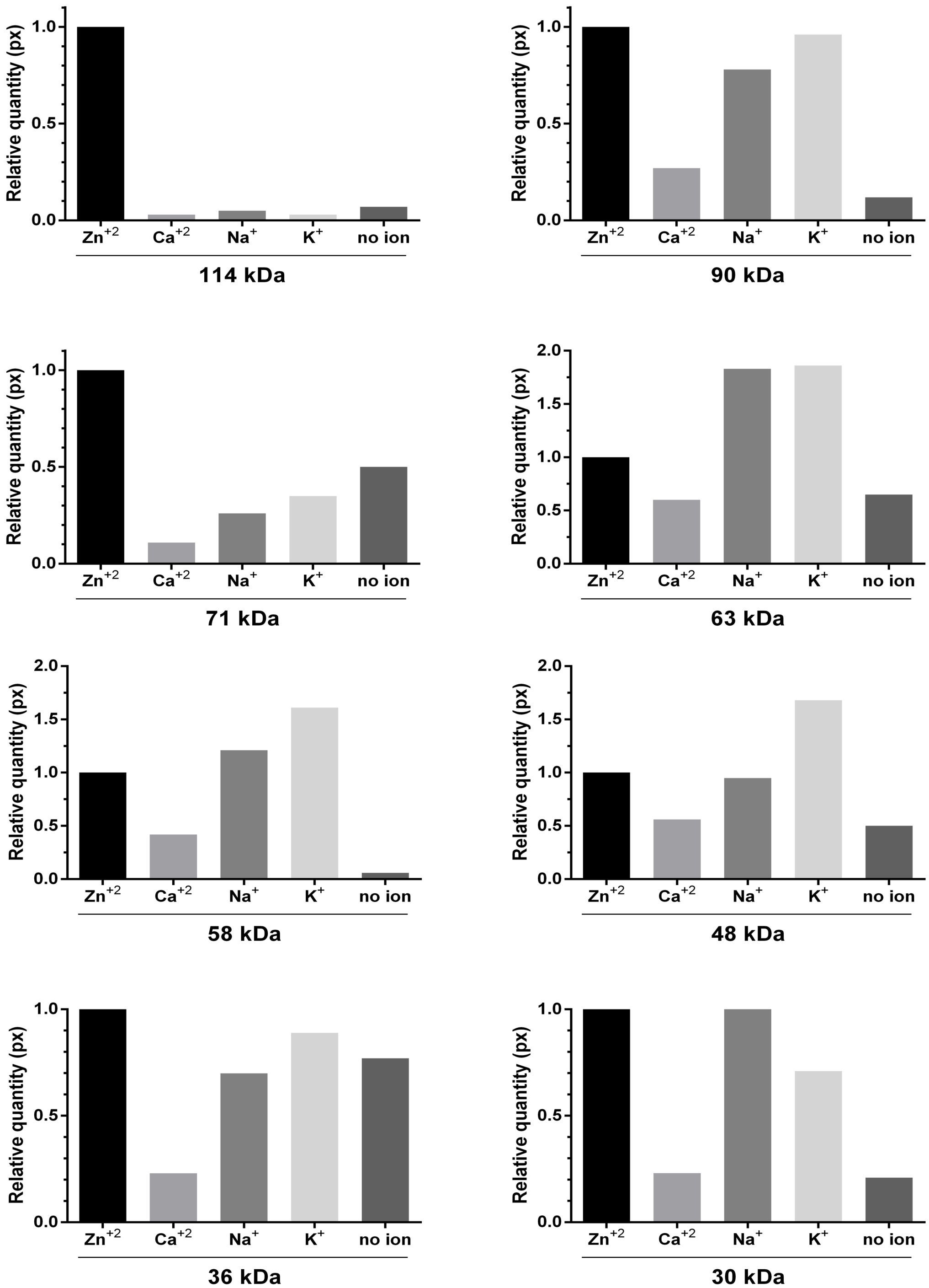

Figure 4.4 Densitometry of the ion effect on gelatin protease profile. Densitometry analysis of each of the ion per band where Zn+2 is considered as the 100 % of relative activity

The obtained effect could translate to a change in conformation of the protease substrate. In general, Ca^{2+} has been reported to stabilize protein structure due to its ionic force (139,140). As a consequence, an increase on catalytic activity in proteases happens (141). As part of the washing steps of zymography, $CaCl_2$ is used to enhance protein refolding (142,143). Yet, it has been demonstrated that $CaCl_2$ could act as mild denaturant, loosening substrates structure (144). Hence by adding this ion in greater proportion, substrates conformation could not have been in an adequate state for proper reaction.

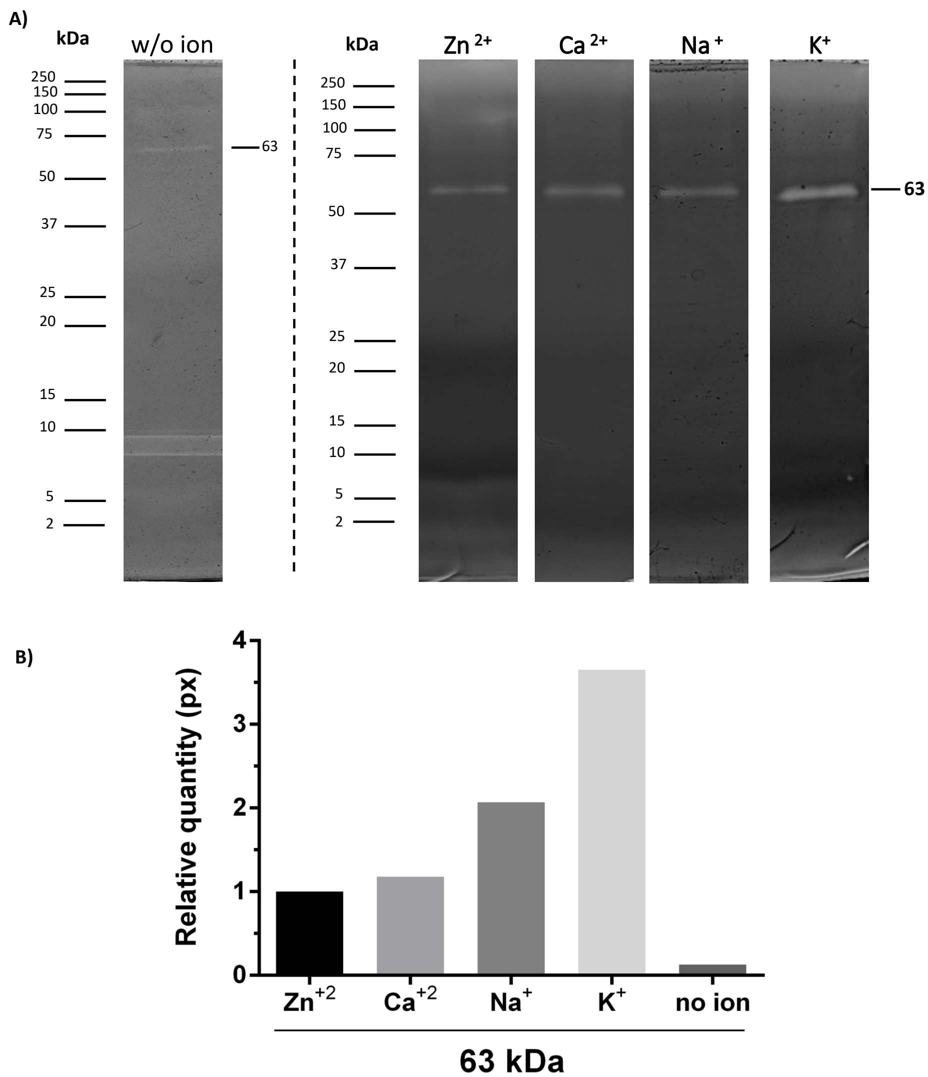

Figure 4.5 Ion effect on protease profile A) EME (1.5 µg) ran in a 12% polyacrylamide gel copolymerized with 0.3% w/v of gelatin incubated without (w/o) and with different ions ($ZnCl_2$, $CaCl_2$ NaCl and KCl) in incubation solution at 37 ºC and pH 7.6. Gels were stained with Coomassie blue to reveal protease activity. **B)** Densitometry analysis of each of the treatments were Zn^{+2} is considered as the 100 % or relative activity.

In the case of casein zymography (**Figure 4.5**-A), the 63 kDa protease did not lose activity with ion treatments, while no ion almost reduced its total signal. As well, its relative quantity increased 3.7 times when treated with potassium (**Figure 4.5**-B), similar behavior to the one obtained with gelatin activity, where this same protease increased its relative intensity up to

2.0 in comparison to control treatment. As well sodium treatment enhanced in the same quantity caseinolytic and gelatinolytic activity.

The obtained results confirm that K^+ and Na^+ act as activators of several proteases within *P. hypophthalmus* epithelial mucus. More in specific, 63, 58 and 48 kDa bands had higher relative quantity in comparison to the control treatment. By which it could be presumed that these bands could be possible kallikrein type protease candidates. Yet, serine protease activity had to be confirmed in order to relate these to the KLK family. For which inhibition assays were proposed.

4.4 Effect of inhibitors over protease activity

Proteases are hydrolyses that have been classified in four principal categories due to its catalytic core (metalloproteases, serine protease, cysteine proteases and aspartic proteases) (145). In order to identify which type of enzymes exists in a complex mixture, specific substrates or specific inhibitors for these protease families can be tested. In zymography analysis, the copolymerization of specific peptides for protease families is not feasible as most of these are artificial peptides with short stability at normal incubation temperatures (25-37ºC). By which inhibition assays are more suitable for zymography screening.

In the specific case of skin epithelial mucus, trypsin (serine-protease), cathepsin B/L (cysteine-protease), cathepsin D (aspartic-protease) and metalloproteases (70) have been identified (119). Hence through inhibition zymography, EME protease profile classification was endorsed by using PMSF, benzamidine (serine protease), EDTA, O-phenanthroline (metalloprotease) and iodoacetamide (cysteine protease) inhibitors as treatments (94,101). In **Figure 4.6**, the inhibition effect of well characterized protease inhibitors over the activity pattern of EME is shown.

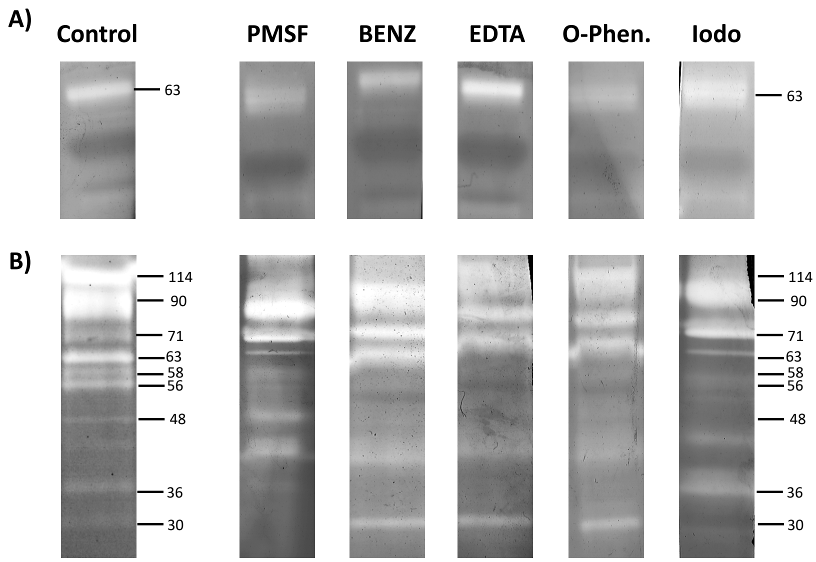

Figure 4.6 Effect of different inhibitors on protease profile of EME (1.5μg) ran in a 12% polyacrylamide gel copolymerized with 0.3% (w/v) of A) casein and B) gelatin incubated with different inhibitors, including PMSF 2mM, Benzamidine 5mM, EDTA 5mM, O-Phenantroline 5 mM and Iodoacetamide 5 mM. Incubation was held at 37 ºC and pH 7.6. Gels were stained with Coomassie blue to reveal protease activity. Control was sated as sample incubated without inhibitor.

The effect of different inhibitors over the protease profile or *P. hyphophthalmus* epithelial mucus were evaluated as describe in section 3.4.2 of materials and methods. This in order to identify the possible protease families present in the extract (**Figure 4.6**). Inhibition tests were held by incubating EME gelatin and casein zymograms with PMSF (serine proteases and papain), Benzamidine (trypsin, trypsin-like and serine proteases), iodoacetamide (cysteine proteases), O-phenantroline (metalloproteases) and EDTA (metalloproteases) (139,146). PMSF and iodoacetamide treatment inhibited 58, 56, 30 kDa bands and reduced 63 kDa gelatinolytic activity **(Figure 4.6**).

As for 114, 90 and 71 kDa bands, slight inhibition could be detected after metalloprotease inhibitor (EDTA and O-phenanthroline) treatments. Through caseinolytic activity, the 63 kDa protease got strongly inhibited with PMSF, benzamidine and O-phenanthroline. Slight inhibition was exerted with iodoacetamide and no inhibition was exerted by EDTA. The inhibition through O-phenantroline in the bands that classify as serine proteases could be attributed to their need of ions to induce structure stabilization as previously explained in ion assays. The obtained results, regarding the bands that previously were described as potential kallikrein type proteases (63, 58, and 48 kDa), indicate that these could be classified as cysteine or serine proteases. Such inhibitions and molecular weights are similar to the ones reported in several fish species epithelial mucus. Serine proteases with 60, 62 and 66 kDa have been describe in *L. rohita* and *C. migrala* epithelial mucus (101). As well, serine proteases within molecular weight of 55, 45 and 44 kDa were identified in *Salmo salar* and *Oncorhynchus mykiss* species (102,111). In the case of cysteine proteases, 73 and 40 kDa proteases were described for Hag fish and Tambacu species (94,118). With the obtained results it could be confirmed that the most probable kallikrein candidates are the bands within the molecular weight range of 63, 58, and 48 kDa. Hence to continue proper characterization, it is important to see if these protease bands are composed by one of more isoforms, as well as their pI. The knowledge of these parameters facilitates further characterization analysis including mass sequencing, purification strategies and kinetic characterization. By which isoelectrofocusing and bidimensional zymography represent powerful alternatives to determine these parameters from complex mixtures.

4.5 Isoform and isoelectric point determination of candidate serine protease

Isoelectric point (pI) is the pH in which the net charge of a molecule, such like a protein, is equal to zero (147). Through pI, the estimation and understanding of different protein states and behaviors is possible including: stability, solubility, enzyme-substrate interaction, or isoform diversity (148). IEF is normally coupled to SDS-PAGE in order to visualize all the present isoforms of a complex mixture in a bidimensional manner.

Yet when this second dimension is a zymography, bidimensional analysis is simplified as only protease isoforms are screened avoiding overlapping activity that normally happen on 1D zymography (149,150). Through 2D-PAGE and 2D-zymography, pI and total protease isoforms of *P. hypophthalmus* epithelial mucus was obtained. These profiles are depicted on **Figure 4.7**. Few 2D-zymography studies of fish epithelial mucus can be found in literature, 2D PAGE studies are more common and available, by which comparison of the obtained results will be contrast with both techniques.

From the observed cluster (**Figure 4.7**-A), the obtained pI range for all the identified isoforms was within 5.15-9.02 pH. As for serine protease bands (63, 58, and 48 kDa), the identified isoelectric point pH was of 6.62, 7.15 and 6.3. Protease activity was also screened at pH 6.8 (**Figure 4.7**-B). In the case of the high molecular weight bands (179, 114, 90, and 71 kDa) only clear spots could be identified for 71 kDa in both bidimensional screenings. Similar results were obtained by Wilkesman S. (2007), who characterized marine sponge proteases obtaining a 66 kDa protease with pI 8.0 on gelatin substrate. As well higher molecular weight bands (>150 kDa) could not be detected on bidimensional analysis. According to this author, two main causes could induce this shift: A) Aggressive sample treatment for isoelectrofocusing and/or B) high molecular weight proteases are composed by subunit that get scattered after 2D-electrophotesis (151).

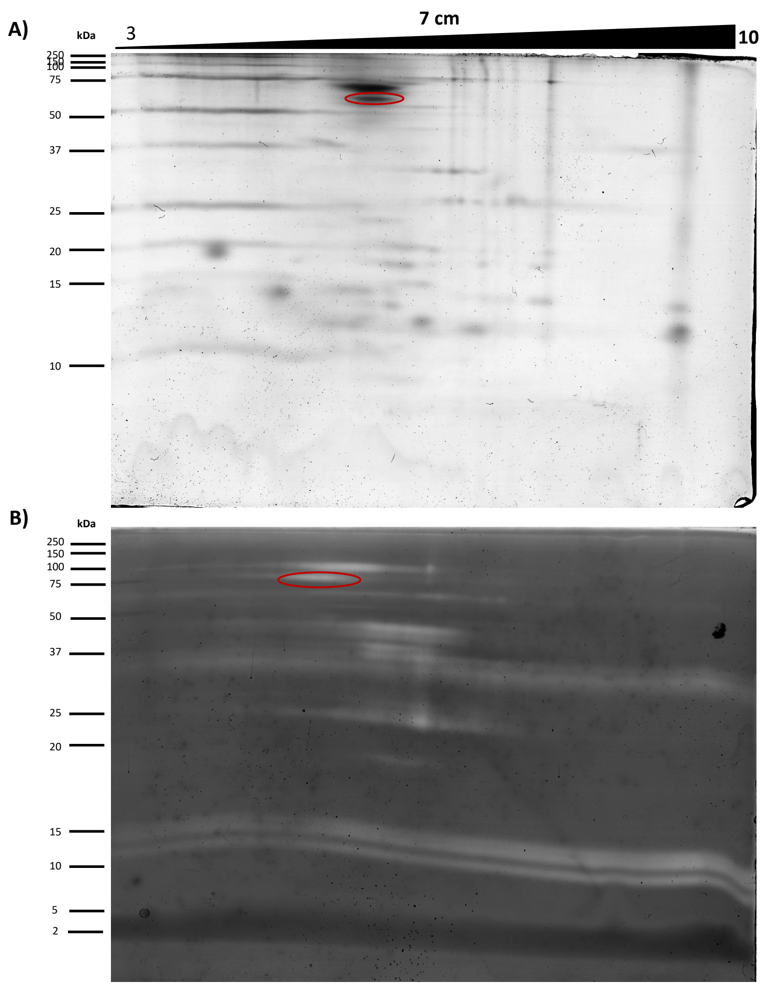

Figure 4.7 Protease isoform screening of E-ME (80 μg) in a bidimensional electrophoresis elaborated with an IPG of 7 cm (pH 3-10) in a 12% polyacrylamide gel, where **A)** depicts a tris-tricine SDS PAGE and **B)** depicts a gelatin zymography (0.3% w/v). Gel was incubated with 50 mL of incubation solution with Zn^{+2} as ion at 37 ºC and pH 7.6. Spots marked in red circles are the ones identified as serine proteases. Both gels were stained with coomassie blue.

Subunit scattering has been detected for matrix metalloproteases, which in most of the cases have represented the high molecular bands present in several fish epithelial mucus (94,102,118). MMP complexes can be detected on 1D zymography with molecular weight sizes of 240 kDa to 130 kDa (133), explaining why the presence to absence from 1D to 2D electrophoresis screening of high molecular weight bands happened.

When comparing with other fish mucosal extracts, *Paralichtys olivaceus* (olive flounder) presented a shorter pI range (4.2-6.8 pH) of proteases within 24-165 kDa in comparison to the obtained results (120). Similar was the case of *Sparus aurata* (gilthead seabream) skin mucus with a pI range of 4.0-6.5 (152). On the contrary, *Godus morhua* (Atlantic cod) presented a similar pI range of 4.0-8.0 (153). This variability could be normally explained due to the niche and environmental conditions in which the specie lives in (101). In the case of the specie *P.hypophtalmus,* in contrast to the previously mentioned species, inhabits fresh water basis on benthopelagic behavior (32).

With the obtained results, the complete mapping of serine protease presence in *Pangasius hyphophthalmus* could be confirmed. The presence of single isoforms facilitate further proteomic characterization as only one spot will be needed to characterize the mapped protease on the sequence level.

Chapter 5

Conclusions and final remarks

5. Conclusion and final remarks

The concept of beauty has always been an important feature for human wellbeing. Nowadays, off the shelf concepts, also known as non-surgical procedures, are gaining more strength due to quicker results with less pain and less investment. From these, chemical peelings are still one of the most commonly used in spite of the uncomfortable process and secondary effects. As a solution, enzymatic peelings have started to appear as a more suitable approach, yet few efforts have been done to improve or develop a concise enzymatic agent for this method.

Through the present study, the preliminary identification and characterization of serine proteases in *P. hyphophthalmus* epithelial mucus was achieved. Among the obtained protease profile of ten active bands, the presence of a 63 kDa serine protease was demonstrated. This protease could be classified as such due to its positive results on three characteristic tags for the serine protease family, including: positive inhibition by PSMF and benzamidine, activation by reported KLK activating ions K^+ and Na^+ and multi-substrate specificity for gelatin and casein, where in casein it was the only active band. Also, several metalloproteases could be identified as part of the mucus extract through inhibitory and substrate assays. As well, through 2D-PAGE vs 2D-zymography, it could be stated that this protease is present in just one isoform with an estimated pI of 6.62. Furthermore, homology analysis of the identified serine protease with human KLK 5 and 7 needs to perform to confirm similarity and comprehend feasible catalytic mechanisms to state target substrate and catalytic rate. This data is necessary for the development of a topical enzymatic treatment.

From the obtained data, it could be stated that serine proteases are conserved among species and are located on similar tissue levels. Metalloproteases and serine proteases on normal basis, participate as principal mediators of skin remodeling processes, which could explain the presence of these proteases in epithelial fish mucus and the exfoliating action of it over the skin of the aquacultures who manipulated the specie for rearing purposes.

Even though the presence of serine proteases was confirmed in the epithelial mucus of the iridescent shark, further studies must be done to evaluate and prove its potential as an effective enzymatic agent for cosmetical peeling treatments. In order to follow through, a heterologous production of the characterized serine protease must be accomplished, this in order to propose an adequate model of its catalytic activity, stability, production and formulation process for dermatological applications.

The development of this bioprocess will lead to further feasibility evaluation from the technical and economical point of view. For the technical validation, the heterologous production of the enzyme from upstream to downstream must be achieved within local regulations to assure process acceptance by law entities. As well kinetic characterization of the obtained enzyme must be performed, as to comprehend within which parameters the active agent could work. This includes physicochemical characterization of pure and in-formulation enzyme, as well as the evaluation of its catalytic mechanism on skin cell lines in order to comprehend which are the target substrate of the enzyme when applied to skin, hence the mechanism in which the developed formulation will enhance skin peeling. These assays also will allow to estimate total treatment time of the formulation, identify cell migration and how much this process could be enhanced by the appliance of both enzymatic presentations (pure and formulated). Once bench scale process is validated, an economic analysis of the bioprocess most be endorsed, in order to detect if the formulation development can become a sustainable product for the actual economical state of cosmetic industry market.

Also, other epithelial treatment applications could be explored. As previously mentioned, the two main groups of enzymes present in tissue remodeling processes are metalloproteases and serine proteases. By which other type of regeneration mechanisms from different epitheliums such as eyes, organs or skin disruptions (wounds) could be feasible targets for enzymatic treatment. Such application could be managed from catalysis or modulation perspectives.

Modulatory effects of ions over enzymatic catalysis has mainly been evaluated under the focus of understanding kinetics and possible molecular switches that could induce changes over enzymatic behavior. Nevertheless, no attempt on using salt as a therapeutic application has yet been proposed. By which modeling of enzymatic activity in function of ions could be implemented in order to enhance endogenous KLK activity and avoid external protease applications.

6. References

[1] Taofiq O, González-Paramás AM, Martins A, Barreiro MF, Ferreira ICFR. Mushrooms extracts and compounds in cosmetics, cosmeceuticals and nutricosmetics—A review. Industrial Crops and Products. 2016 Nov;90:38–48.

[2] Chaudhri S, Jain NK. History of cosmetics. In 2009.

[3] Rogers BO. A Brief History of Cosmetic Surgery. Surgical Clinics of North America. 1971 Apr 1;51(2):265–88.

[4] Dean NR, Foley KL, Ward PR. Defining cosmetic surgery. In 2018.

[5] Frucht CS, Ortiz AE. Nonsurgical Cosmetic Procedures For Men: Trends And Technique Considerations. J Clin Aesthet Dermatol. 2016 Dec;9(12):33–43.

[6] Minimally-Invasive Cosmetic Procedures Market Trends and Forecast the Upcoming Opportunities and Demand | With Growth Prospects by Regions and Segmentation till 2023 - MarketWatch [Internet]. [cited 2019 Aug 14]. Available from: https://www.marketwatch.com/press-release/minimally-invasive-cosmetic-procedures-market-trends-and-forecast-the-upcoming-opportunities-and-demand-with-growth-prospects-by-regions-and-segmentation-till-2023-2018-05-16

[7] Bowler PJ. Impact on facial rejuvenation with dermatological preparations. Clin Interv Aging. 2009;4:81–9.

[8] López Martín-Prieto S, Sánchez Conejo-Mir J. Peeling químico con ácido tricloroacético. Un peeling clásico de máxima actualidad. Actas Dermosifiliogr. 2001 Dec 1;92(12):537–47.

[9] Hassan KM, Benedetto AV. Facial skin rejuvenation: Ablative laser resurfacing, chemical peels, or photodynamic therapy? Facts and controversies. Clinics in Dermatology. 2013 Nov;31(6):737–40.

[10] Clark E, Scerri L. Superficial and medium-depth chemical peels. Clinics in Dermatology. 2008 Mar 1;26(2):209–18.

[11] Sadick N. Nonsurgical Facial Rejuvenation. Advances in Cosmetic Surgery. 2018 Jun;1(1):99–107.

[12] Ganceviciene R, Liakou AI, Theodoridis A, Makrantonaki E, Zouboulis CC. Skin anti-aging strategies. Dermatoendocrinol. 2012 Jul 1;4(3):308–19.

[13] O'Connor AA, Lowe PM, Shumack S, Lim AC. Chemical peels: A review of current practice. Australas J Dermatol. 2018 Aug;59(3):171–81.

[14] Li S, Yang X, Yang S, Zhu M, Wang X. TECHNOLOGY PROSPECTING ON ENZYMES: APPLICATION, MARKETING AND ENGINEERING. Computational and Structural Biotechnology Journal. 2012 Sep;2(3):e201209017.

[15] European Comission. Collection of information on enzymes. Office for Official Publications of the European Communities, Luxembourg [Internet]. 2002; Available from: https://ec.europa.eu/environment/archives/dansub/pdfs/enzymerepcomplete.pdf

[16] Gupta PL, Rajput M, Oza T, Trivedi U, Sanghvi G. Eminence of Microbial Products in Cosmetic Industry. Nat Prod Bioprospect. 2019 Aug 1;9(4):267–78.

[17] Del Rosso JQ. Application of protease technology in dermatology: rationale for incorporation into skin care with initial observations on formulations designed for skin cleansing, maintenance of hydration, and restoration of the epidermal permeability barrier. J Clin Aesthet Dermatol. 2013 Jun;6(6):14–22.

[18] Ramundo J, Gray M. Enzymatic Wound Debridement: Journal of Wound, Ostomy and Continence Nursing. 2008 May;35(3):273–80.

[19] Enzymatic pre-treatment enhances beneficial effects of glycolic acid peel in Japanese skin. Journal of the American Academy of Dermatology. 2007 Feb;56(2):AB26.

[20] Analytics for US Patent No. 8540983, Debriding composition from bromelain and methods of production thereof [Internet]. [cited 2019 Aug 14]. Available from: http://www.patentbuddy.com/Patent/8540983

[21] Shaikh SAM, Rashid H ur. Role of Papaya Dressings in the Management of Diabetic Foot Ulcers. In 2014.

[22] Collagenase SANTYL Ointment enzymatic debrider | Smith & Nephew - Corporate [Internet]. [cited 2019 Aug 14]. Available from: https://www.smith-nephew.com/key-products/advanced-wound-management/collagenase-santyl-ointment/

[23] Vidmar B, Vodovnik M. Microbial Keratinases: Enzymes with Promising Biotechnological Applications. Food Technol Biotechnol. 2018 Sep;56(3):312–28.

[24] Fein H. Selective enzyme treatment of skin conditions [Internet]. US20030026794A1, 2003 [cited 2019 Aug 14]. Available from: https://patents.google.com/patent/US20030026794A1/en

[25] Bjarnason J. Fish serine proteinases and their pharmaceutical and cosmetic use [Internet]. US20020141987A1, 2002 [cited 2019 Aug 12]. Available from: https://patents.google.com/patent/US20020141987A1/en?oq=US20020141987A1+

[26] Egelrud TR. Desquamation in the Stratum Corneum. :2.

[27] Cosmeceuticals: Function and the Skin Barrier [Internet]. Clinical Gate. 2015 [cited 2019 Aug 14]. Available from: https://clinicalgate.com/cosmeceuticals-function-and-the-skin-barrier/

[28] Komatsu N, Takata M, Otsuki N, Toyama T, Ohka R, Takehara K, et al. Expression and Localization of Tissue Kallikrein mRNAs in Human Epidermis and Appendages. Journal of Investigative Dermatology. 2003 Sep;121(3):542–9.

[29] Dash S, Das SK, Samal J, Thatoi HN. Epidermal mucus, a major determinant in fish health: a review. IJVR [Internet]. 2018 Jun [cited 2019 Jul 17];19(2). Available from: http://doi.org/10.22099/ijvr.2018.4849

[30] Sanahuja I, Ibarz A. Skin mucus proteome of gilthead sea bream: A non-invasive method to screen for welfare indicators. Fish & Shellfish Immunology. 2015 Oct;46(2):426–35.

[31] Shephard KL. Functions for fish mucus. Rev Fish Biol Fisheries. 1994 Dec 1;4(4):401–29.

[32] FAO Fisheries & Aquaculture - Cultured Aquatic Species Information Programme - Pangasius hypophthalmus (Sauvage, 1878) [Internet]. [cited 2019 Aug 12]. Available from: http://www.fao.org/fishery/culturedspecies/Pangasius_hypophthalmus/en

[33] Sánchez ELG, Arias LF, Olivera RMP, Castellanos AP. Revisión de técnicas de abrasión cutánea. 2015;6.

[34] Secchi G. Role of protein in cosmetics. Clinics in Dermatology. 2008 Jul;26(4):321–5.

[35] Sunar K, Kumar U, Deshmukh SK. Recent Applications of Enzymes in Personal Care Products. In: Agro-Industrial Wastes as Feedstock for Enzyme Production [Internet].

Elsevier; 2016 [cited 2019 Jul 17]. p. 279–98. Available from: https://linkinghub.elsevier.com/retrieve/pii/B9780128023921000125

[36] Consumer Insights [Internet]. Cosmetics Europe - The Personal Care Association. [cited 2019 Aug 26]. Available from: https://cosmeticseurope.eu/cosmetic-products/consumer-insigths

[37] News Releases - Statistics, Surveys & Trends - ASAPS Newsroom - The American Society for Aesthetic Plastic Surgery Reports That Modern Cosmetic Procedures are on the Rise [Internet]. [cited 2019 Aug 26]. Available from: https://www.surgery.org/media/news-releases/the-american-society-for-aesthetic-plastic-surgery-reports-that-modern-cosmetic-procedures-are-on-the-rise

[38] Terabe Y. Comments on the Functional Cosmetics from a Medical Doctor. In: Sugibayashi K, editor. Skin Permeation and Disposition of Therapeutic and Cosmeceutical Compounds [Internet]. Tokyo: Springer Japan; 2017 [cited 2019 Jul 17]. p. 419–21. Available from: http://link.springer.com/10.1007/978-4-431-56526-0_38

[39] Soleymani T, Lanoue J, Rahman Z. A Practical Approach to Chemical Peels. J Clin Aesthet Dermatol. 2018 Aug;11(8):21–8.

[40] Mackee GM, Karp FL. The Treatment of Post-Acne Scars with Phenol. British Journal of Dermatology. 1952;64(12):456–9.

[41] Yousef H, Alhajj M, Sharma S. Anatomy, Skin (Integument), Epidermis. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2019 [cited 2019 Aug 13]. Available from: http://www.ncbi.nlm.nih.gov/books/NBK470464/

[42] Pinto JMN, Delorenze LM, Vasques W, Issa MC. Jessner's Peel. In: Issa MCA, Tamura B, editors. Chemical and Physical Peelings [Internet]. Cham: Springer International Publishing; 2016 [cited 2019 Aug 13]. p. 1–6. (Clinical Approaches and Procedures in Cosmetic Dermatology). Available from: https://doi.org/10.1007/978-3-319-20252-5_4-1

[43] Chisaki C, Horn G, Noriega LF. Phenol Solutions for Deep Peelings. In: Issa MCA, Tamura B, editors. Chemical and Physical Peelings [Internet]. Cham: Springer International Publishing; 2016 [cited 2019 Aug 13]. p. 1–27. (Clinical Approaches and Procedures in Cosmetic Dermatology). Available from: https://doi.org/10.1007/978-3-319-20252-5_10-1

[44] Nikalji N, Godse K, Sakhiya J, Patil S, Nadkarni N. Complications of Medium Depth and Deep Chemical Peels. J Cutan Aesthet Surg. 2012;5(4):254–60.

[45] Preissig J, Hamilton K, Markus R. Current Laser Resurfacing Technologies: A Review that Delves Beneath the Surface. Semin Plast Surg. 2012 Aug;26(3):109–16.

[46] Skroza N, Proietti I, Potenza C, Dessy LA. Mechanic Resurfacing, Needling, Dermoabrasion and Microdermoabrasion. In: Scuderi N, Toth BA, editors. International Textbook of Aesthetic Surgery [Internet]. Berlin, Heidelberg: Springer Berlin Heidelberg; 2016 [cited 2019 Aug 14]. p. 1167–82. Available from: https://doi.org/10.1007/978-3-662-46599-8_80

[47] Roy D. Ablative Facial Resurfacing. Dermatologic Clinics. 2005 Jul 1;23(3):549–59.

[48] Alexiades-Armenakas MR, Dover JS, Arndt KA. The spectrum of laser skin resurfacing: Nonablative, fractional, and ablative laser resurfacing. Journal of the American Academy of Dermatology. 2008 May;58(5):719–37.

[49] Serrano Falcón C, Serrano Ortega S. Indicaciones actuales de la dermoabrasión. Piel. 2008 Nov 1;23(9):514–8.

[50] Kaufmann R, Beier C. Laser Skin Ablation: An Update on Aesthetic and Medical Indications. Medical Laser Application. 2004 Jan 1;19(4):212–22.

[51] Buzina D, Lipozenčić J, Bukvic Mokos Z, Ceovic R, Kostovic K. Ablative Laser Resurfacing: Is It Still the Gold Standard for Facial Rejuvenation? Acta dermatovenerologica Croatica: ADC / Hrvatsko dermatolosko drustvo. 2010 Sep 1;18:190–4.

[52] Shah S, Alam M. Laser Resurfacing Pearls. Semin Plast Surg. 2012 Aug;26(3):131–6.

[53] Rawlings AV. Trends in stratum corneum research and the management of dry skin conditions. Int J Cosmet Sci. 2003 Apr;25(1–2):63–95.

[54] Egelrud T. Desquamation in the stratum corneum. Acta dermato-venereologica Supplementum. 2000;208:44–5.

[55] Honari G, Maibach H. Chapter 1 - Skin Structure and Function. In: Maibach H, Honari G, editors. Applied Dermatotoxicology [Internet]. Boston: Academic Press;

2014 [cited 2019 Aug 14]. p. 1–10. Available from: http://www.sciencedirect.com/science/article/pii/B9780124201309000013

[56] Lopes PS, Ruas GW, Baby AR, Pinto CAS de O, Watanabe I, Velasco MVR, et al. In vitro safety assessment of papain on human skin: A qualitative Light and Transmission Electron Microscopy (TEM) study. Rev Bras Cienc Farm. 2008 Mar;44(1):151–6.

[57] Smith WP, Bishop M, Gillis G, Maibach H. Topical proteolytic enzymes affect epidermal and dermal properties. Int J Cosmet Sci. 2007 Feb;29(1):15–21.

[58] Bjelland S, Hjelmeland K, Volden G. Degradation of human epidermal keratin by cod trypsin and extracts of fish intestines. Arch Dermatol Res. 1989 Jan;280(8):469–73.

[59] Fornbacke M, Clarsund M. Cold-Adapted Proteases as an Emerging Class of Therapeutics. Infect Dis Ther. 2013 Jun;2(1):15–26.

[60] Gudmundsdóttir Á, Hilmarsson H, Stefansson B. Potential Use of Atlantic Cod Trypsin in Biomedicine. BioMed Research International. 2013;2013:1–11.

[61] Costa-Neto EM. Implications and applications of folk zootherapy in the state of Bahia, Northeastern Brazil. Sustainable Development. 2004;12(3):161–74.

[62] Jais AMM. Pharmacognosy and pharmacology of Haruan (Channa striatus), a medicinal fish with wound healing properties [Internet]. Boletín Latinoamericano y del Caribe de Plantas Medicinales y Aromáticas. 2007 [cited 2019 Aug 14]. Available from: http://www.redalyc.org/articulo.oa?id=85660303

[63] Akunne TC, Okafor SN, Okechukwu DC, Nwankwor SS, Emene JO, Okoro BN. Catfish (Clarias gariepinus) Slime Coat Possesses Antimicrobial and Wound Healing Activities. UK Journal of Pharmaceutical Biosciences. 2016 Jun 1;4(3):81.

[64] Kristanto AH, Slembrouck J. FfRsr sExuAL MATURATlON AND BREEDTNG cycLE oF pangasius Hypophthalmus (stLuRtFoRMEs, pANGASIDAE) REARED tru Foruo. :6.

[65] Dunlop KM, Jarvis T, Benoit-Bird KJ, Waluk CM, Caress DW, Thomas H, et al. Detection and characterisation of deep-sea benthopelagic animals from an autonomous underwater vehicle with a multibeam echosounder: A proof of concept and description of data-processing methods. Deep Sea Research Part I: Oceanographic Research Papers. 2018 Apr 1;134:64–79.

[66] Jayaneththi HB. Record of Iridescent shark catfish Pangasianodon hypophthalmus Sauvage, 1878 (Siluriformes: Pangasiidae) from Madampa-Lake in Southwest Sri Lanka. Ruhuna J Sci. 2016 Feb 23;6(2):63.

[67] Emer J, Levy L. Complications of minimally invasive cosmetic procedures: Prevention and management. J Cutan Aesthet Surg. 2012;5(2):121.

[68] Neilson ME, Loftus WF, Benson A. iridescent shark (Pangasianodon hypophthalmus) - Species Profile [Internet]. NAS - Nonindigenous Aquatic Species. 2019 [cited 2019 Aug 23]. Available from: https://nas.er.usgs.gov/queries/FactSheet.aspx?SpeciesID=2603

[69] Reverter M, Tapissier-Bontemps N, Lecchini D, Banaigs B, Sasal P. Biological and Ecological Roles of External Fish Mucus: A Review. Fishes. 2018 Oct 9;3(4):41.

[70] Rakers S, Niklasson L, Steinhagen D, Kruse C, Schauber J, Sundell K, et al. Antimicrobial peptides (AMPs) from fish epidermis: perspectives for investigative dermatology. J Invest Dermatol. 2013 May;133(5):1140–9.

[71] Jakowska S. MUCUS SECRETION IN FISH-A NOTE*. Annals of the New York Academy of Sciences. 2008 Jun 28;106(2):458–62.

[72] Bragadeeswaran S, Priyadharshini S, Prabhu K, Rani SRS. Antimicrobial and hemolytic activity of fish epidermal mucus Cynoglossus arel and Arius caelatus. Asian Pacific Journal of Tropical Medicine. 2011 Apr 1;4(4):305–9.

[73] Beck B, Peatman E. Mucosal Health in Aquaculture. 2015. 1 p.

[74] Ángeles Esteban M. An Overview of the Immunological Defenses in Fish Skin. ISRN Immunology. 2012;2012:1–29.

[75] López-Otín C, Bond JS. Proteases: Multifunctional Enzymes in Life and Disease. J Biol Chem. 2008 Nov 7;283(45):30433–7.

[76] Mótyán JA, Tóth F, Tőzsér J. Research applications of proteolytic enzymes in molecular biology. Biomolecules. 2013 Nov 8;3(4):923–42.

[77] Chang C, Werb Z. The many faces of metalloproteases: cell growth, invasion, angiogenesis and metastasis. Trends Cell Biol. 2001 Nov;11(11):S37–43.

[78] Cui N, Hu M, Khalil RA. Biochemical and Biological Attributes of Matrix Metalloproteinases. Prog Mol Biol Transl Sci. 2017;147:1–73.

[79] Pelmenschikov V, Siegbahn PEM. Catalytic Mechanism of Matrix Metalloproteinases: Two-Layered ONIOM Study. Inorg Chem. 2002 Nov;41(22):5659–66.

[80] Krasnov A, Skugor S, Todorcevic M, Glover KA, Nilsen F. Gene expression in Atlantic salmon skin in response to infection with the parasitic copepod Lepeophtheirus salmonis, cortisol implant, and their combination. BMC Genomics. 2012 Apr 5;13:130.

[81] Schütte A, Lottaz D, Sterchi EE, Stöcker W, Becker-Pauly C. Two alpha subunits and one beta subunit of meprin zinc-endopeptidases are differentially expressed in the zebrafish Danio rerio. Biol Chem. 2007 May;388(5):523–31.

[82] Giebeler N, Zigrino P. A Disintegrin and Metalloprotease (ADAM): Historical Overview of Their Functions. Toxins (Basel) [Internet]. 2016 Apr 23 [cited 2019 Aug 24];8(4). Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4848645/

[83] Di Cera E. Serine Proteases. IUBMB Life. 2009 May;61(5):510–5.

[84] Richau KH, Kaschani F, Verdoes M, Pansuriya TC, Niessen S, Stüber K, et al. Subclassification and Biochemical Analysis of Plant Papain-Like Cysteine Proteases Displays Subfamily-Specific Characteristics. Plant Physiology. 2012 Apr 1;158(4):1583–99.

[85] Miller M, Jaskólski M, Rao JKM, Leis J, Wlodawer A. Crystal structure of a retroviral protease proves relationship to aspartic protease family. Nature. 1989 Feb;337(6207):576–9.

[86] Wolters BK. Cathepsins L and V in human keratinocytes [Internet]. Jacobs University Bremen; 2006 [cited 2019 Aug 24]. Available from: http://nbn-resolving.org/urn:nbn:de:101:1-201305226106

[87] Vidak E, Javoršek U, Vizovišek M, Turk B. Cysteine Cathepsins and their Extracellular Roles: Shaping the Microenvironment. Cells. 2019 Mar 20;8(3):264.

[88] Reinheckel T, Hagemann S, Dollwet-Mack S, Martinez E, Lohmüller T, Zlatkovic G, et al. The lysosomal cysteine protease cathepsin L regulates keratinocyte proliferation by control of growth factor recycling. J Cell Sci. 2005 Aug 1;118(Pt 15):3387–95.

[89] Leyvraz C, Charles R-P, Rubera I, Guitard M, Rotman S, Breiden B, et al. The epidermal barrier function is dependent on the serine protease CAP1/Prss8. J Cell Biol. 2005 Aug 1;170(3):487–96.

[90] Ovaere P, Lippens S, Vandenabeele P, Declercq W. The emerging roles of serine protease cascades in the epidermis. Trends in Biochemical Sciences. 2009 Sep 1;34(9):453–63.

[91] Coughlin SR. Thrombin signalling and protease-activated receptors. Nature. 2000 Sep 14;407(6801):258–64.

[92] Perona JJ, Craik CS. Structural basis of substrate specificity in the serine proteases. Protein Sci. 1995 Mar;4(3):337–60.

[93] Meza J, Galeano A. Dr. Alejandro Flores Nava, Oficial Principal de Acuicultura y Pesca para América Latina y el Caribe, FAO. :52.

[94] Subramanian S, MacKinnon SL, Ross NW. A comparative study on innate immune parameters in the epidermal mucus of various fish species. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology. 2007 Nov;148(3):256–63.

[95] Barton BA, Iwama GK. Physiological changes in fish from stress in aquaculture with emphasis on the response and effects of corticosteroids. Annual Review of Fish Diseases. 1991 Jan;1:3–26.

[96] Conlon JM, Leprince J. Identification and Analysis of Bioactive Peptides in Amphibian Skin Secretions. In: Soloviev M, editor. Peptidomics [Internet]. Totowa, NJ: Humana Press; 2010 [cited 2019 Jul 17]. p. 145–57. Available from: http://link.springer.com/10.1007/978-1-60761-535-4_12

[97] Shägger H. Tricine–SDS-PAGE | Springer Nature Experiments [Internet]. [cited 2019 Aug 20]. Available from: https://experiments.springernature.com/articles/10.1038/nprot.2006.4

[98] González-González M, Mayolo-Deloisa K, Rito-Palomares M. PEGylation, detection and chromatographic purification of site-specific PEGylated CD133-Biotin antibody in route to stem cell separation. J Chromatogr B Analyt Technol Biomed Life Sci. 2012 Apr 15;893–894:182–6.

[99] Green MR, Sambrook J, Sambrook J. Molecular cloning: a laboratory manual. 4th ed. Cold Spring Harbor, N.Y: Cold Spring Harbor Laboratory Press; 2012. 3 p.

[100] Monteiro-dos-Santos J, Conceição K, Seibert CS, Marques EE, Ismael Silva P, Soares AB, et al. Studies on pharmacological properties of mucus and sting venom of Potamotrygon cf. henlei. International Immunopharmacology. 2011 Sep;11(9):1368–77.

[101] Nigam AK, Kumari U, Mittal S, Mittal AK. Comparative analysis of innate immune parameters of the skin mucous secretions from certain freshwater teleosts, inhabiting different ecological niches. Fish Physiol Biochem. 2012 Oct;38(5):1245–56.

[102] Firth KJ, Johnson SC, Ross NW. CHARACTERIZATION OF PROTEASES IN THE SKIN MUCUS OF ATLANTIC SALMON (SALMO SALAR) INFECTED WITH THE SALMON LOUSE (LEPEOPHTHEIRUS SALMONIS) AND IN WHOLE-BODY LOUSE HOMOGENATE. para. 2000 Dec;86(6):1199–205.

[103] Dyballa N, Metzger S. Fast and sensitive colloidal coomassie G-250 staining for proteins in polyacrylamide gels. J Vis Exp. 2009 Aug 3;(30).

[104] Kannan M, Ramya T, Anbalagan S, Suriya J, Krishnan M. Proteomic analysis of pupal gut serine protease of Silkworm, Bombyx mori : Partial purification and biochemical characterization. Biocatalysis and Agricultural Biotechnology. 2017 Oct;12:159–65.

[105] Natale M, Maresca B, Abrescia P, Bucci EM. Image Analysis Workflow for 2-D Electrophoresis Gels Based on ImageJ. Proteomics□Insights. 2011 Jan 1;4:PRI.S7971.

[106] Das D, Urs N, Hiremath V, Vishwanath BS, Doley R. Biochemical and biological characterization of Naja kaouthia venom from North-East India and its neutralization by polyvalent antivenom. J Venom Res. 2013 Nov 6;4:31–8.

[107] Waterborg JH, Matthews HR. The Lowry method for protein quantitation. Methods in molecular biology. 1994;32:1–4.

[108] Anson ML. The Estimation of Pepsin, Trypsin, Papain, and Cathepsin with Hemoglobin. The Journal of General Physiology. 1938 Sep 20;22(1):79–89.

[109] Fenwick N, Griffin G, Gauthier C. The welfare of animals used in science: How the “Three Rs” ethic guides improvements. Can Vet J. 2009 May;50(5):523–30.

[110] Robinson PK. Enzymes: principles and biotechnological applications. Essays Biochem. 2015 Nov 15;59:1–41.

[111] Fast MD, Sims DE, Burka JF, Mustafa A, Ross NW. Skin morphology and humoral non-specific defence parameters of mucus and plasma in rainbow trout, coho and Atlantic salmon. Comp Biochem Physiol A Mol Integr Physiol. 2002 Jul;132(3):645–57.

[112] Ong PS, Gaucher GM. Production, purification and characterization of thermomycolase, the extracellular serine protease of the thermophilic fungus Malbranchea pulchella var. sulfurea. Can J Microbiol. 1976 Feb;22(2):165–76.

[113] Vallejo-Illarramendi A, Marciano DK, Reichardt LF. A novel method that improves sensitivity of protein detection in PAGE and western blot. Electrophoresis. 2013 Apr;34(8):1148–50.

[114] Grabski A, Burgess R. Preparation of protein samples for SDS-polyacrylamide gel electrophoresis: Procedures and tips. inNovations. 2001 Jan 1;13:10–2.

[115] Kurien B, Scofield R. Common Artifacts and Mistakes Made in Electrophoresis. Methods in molecular biology (Clifton, NJ). 2012 Apr 9;869:633–40.

[116] Chevallet M, Luche S, Rabilloud T. Silver staining of proteins in polyacrylamide gels. Nat Protoc. 2006;1(4):1852–8.

[117] Vandooren J, Geurts N, Martens E, Van den Steen PE, Opdenakker G. Zymography methods for visualizing hydrolytic enzymes. Nature Methods. 2013 Mar;10(3):211–20.

[118] Salles CMC, Gagliano P, Leitão SAT, Salles JB, Guedes HLM, Cassano VPF, et al. Identification and characterization of proteases from skin mucus of tambacu, a Neotropical hybrid fish. Fish Physiol Biochem. 2007 Apr 27;33(2):173–9.

[119] Subramanian S, Ross NW, Mackinnon SL. Comparison of the biochemical composition of normal epidermal mucus and extruded slime of hagfish (Myxine glutinosa L.). Fish Shellfish Immunol. 2008 Nov;25(5):625–32.

[120] Palaksha KJ, Shin G-W, Kim Y-R, Jung T-S. Evaluation of non-specific immune components from the skin mucus of olive flounder (Paralichthys olivaceus). Fish & Shellfish Immunology. 2008 Apr;24(4):479–88.

[121] Sanahuja I, Fernández-Alacid L, Sánchez-Nuño S, Ordóñez-Grande B, Ibarz A. Chronic Cold Stress Alters the Skin Mucus Interactome in a Temperate Fish Model. Front Physiol. 2019 Jan 11;9:1916.

[122] Donato L, Guyomarc'H F. Formation and properties of the whey protein/κ-casein complexes in heated skim milk - A review. Dairy Science & Technology. 2009;89(1):3–29.

[123] Rodríguez MIA, Barroso LGR, Sánchez ML. Collagen: A review on its sources and potential cosmetic applications. Journal of Cosmetic Dermatology. 2018;17(1):20–6.

[124] Multifunctional Enzymes. In: Encyclopedic Reference of Genomics and Proteomics in Molecular Medicine [Internet]. Berlin, Heidelberg: Springer Berlin Heidelberg; 2006 [cited 2019 Aug 26]. p. 1213–1213. Available from: https://doi.org/10.1007/3-540-29623-9_7909

[125] de Veer SJ, Furio L, Swedberg JE, Munro CA, Brattsand M, Clements JA, et al. Selective Substrates and Inhibitors for Kallikrein-Related Peptidase 7 (KLK7) Shed Light on KLK Proteolytic Activity in the Stratum Corneum. Journal of Investigative Dermatology. 2017 Feb 1;137(2):430–9.

[126] Furio L, Pampalakis G, Michael IP, Nagy A, Sotiropoulou G, Hovnanian A. KLK5 Inactivation Reverses Cutaneous Hallmarks of Netherton Syndrome. PLoS Genet [Internet]. 2015 Sep 21 [cited 2019 Oct 8];11(9). Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4577096/

[127] Yu Y, Prassas I, Dimitromanolakis A, Diamandis EP. Novel Biological Substrates of Human Kallikrein 7 Identified through Degradomics. J Biol Chem. 2015 Jul 17;290(29):17762–75.

[128] Furio L, de Veer S, Jaillet M, Briot A, Robin A, Deraison C, et al. Transgenic kallikrein 5 mice reproduce major cutaneous and systemic hallmarks of Netherton syndrome. J Exp Med. 2014 Mar 10;211(3):499–513.

[129] Vašák M, Schnabl J. Sodium and Potassium Ions in Proteins and Enzyme Catalysis. Met Ions Life Sci. 2016;16:259–90.

[130] Webster ME, Prado ES. [50] Glandular kallikreins from horse and human urine and from hog pancreas. In: Methods in Enzymology [Internet]. Academic Press; 1970 [cited 2019 Aug 20]. p. 681–99. (Proteolytic Enzymes; vol. 19). Available from: http://www.sciencedirect.com/science/article/pii/0076687970190550

[131] Girolami JP, Pecher C, Bascands JL, Cabos G, Adam A, Suc JM. Purification of human active urinary kallikrein: comparative inhibition studies of kininogenase and amidolytic activities. Prep Biochem. 1989;19(2):75–90.

[132] Andreini C, Bertini I, Cavallaro G, Holliday G, Thornton J. Metal ions in biological catalysis: From enzyme databases to general principles. Journal of biological inorganic chemistry : JBIC : a publication of the Society of Biological Inorganic Chemistry. 2008 Aug 1;13:1205–18.

[133] Snoek-van Beurden PAM, Von den Hoff JW. Zymographic techniques for the analysis of matrix metalloproteinases and their inhibitors. BioTechniques. 2005 Jan;38(1):73–83.

[134] Oliveira AN de, Oliveira LA de, Andrade JS. Production and some properties of crude alkaline proteases of indigenous Central Amazonian rhizobia strains. Braz arch biol technol. 2010 Oct;53(5):1185–95.

[135] Zeng J, Gao X, Dai Z, Tang B, Tang X-F. Effects of Metal Ions on Stability and Activity of Hyperthermophilic Pyrolysin and Further Stabilization of This Enzyme by Modification of a Ca2+-Binding Site. Applied and Environmental Microbiology. 2014 May 1;80(9):2763–72.

[136] Wu K-H, Tai PC. Cys 32 and His 105 Are the Critical Residues for the Calcium-dependent Cysteine Proteolytic Activity of CvaB, an ATP-binding Cassette Transporter. J Biol Chem. 2004 Jan 9;279(2):901–9.

[137] Jiang ST, Wang YT, Chen CS. Purification and characterization of a proteinase identified as cathepsin D from tilapia muscle (Tilapia nilotica .times. Tilapia aurea). J Agric Food Chem. 1991 Sep 1;39(9):1597–601.

[138] Tallant C, Marrero A, Gomis-Rüth FX. Matrix metalloproteinases: Fold and function of their catalytic domains. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research. 2010 Jan 1;1803(1):20–8.

[139] Eijsink V, Matthews B, Vriend G. The role of calcium ions in the stability and instability of a thermolysin-like protease. Protein Sci. 2011 Aug;20(8):1346–55.

[140] Milles LF, Unterauer EM, Nicolaus T, Gaub HE. Calcium stabilizes the strongest protein fold. Nat Commun. 2018 Nov 12;9(1):1–10.

[141] Sipos T, Merkel JR. Effect of calcium ions on the activity, heat stability, and structure of trypsin. Biochemistry. 1970 Jul 7;9(14):2766–75.

[142] Bushmarina NA, Blanchet CE, Vernier G, Forge V. Cofactor effects on the protein folding reaction: Acceleration of α-lactalbumin refolding by metal ions. Protein Sci. 2006 Apr;15(4):659–71.

[143] Toth M, Fridman R. Assessment of Gelatinases (MMP-2 and MMP-9) by Gelatin Zymography. Methods Mol Med [Internet]. 2001 [cited 2019 Oct 8];57. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3845455/

[144] Head DL, Yankeelov JA. The Effect of Calcium Chloride on the Activity and Inhibition of Bacterial Collagenase. International Journal of Peptide and Protein Research. 1976;8(2):155–65.

[145] Gomis-Rüth FX, Trillo-Muyo S, Stöcker W. Functional and structural insights into astacin metallopeptidases. Biological Chemistry [Internet]. 2012 Jan 1 [cited 2019 Jul 17];393(10). Available from: https://www.degruyter.com/view/j/bchm.2012.393.issue-10/hsz-2012-0149/hsz-2012-0149.xml

[146] Aldrich S. Protease Inhibition and Detection. :32.

[147] Cleaves HJ (Jim). Isoelectric Point. In: Gargaud M, Amils R, Quintanilla JC, Cleaves HJ (Jim), Irvine WM, Pinti DL, et al., editors. Encyclopedia of Astrobiology [Internet]. Berlin, Heidelberg: Springer Berlin Heidelberg; 2011 [cited 2019 Sep 24]. p. 858–858. Available from: https://doi.org/10.1007/978-3-642-11274-4_819

[148] Xia X, editor. Protein isoelectric point. In: Bioinformatics and the Cell: Modern Computational Approaches in Genomics, Proteomics and Transcriptomics [Internet]. Boston, MA: Springer US; 2007 [cited 2019 Sep 24]. p. 207–19. Available from: https://doi.org/10.1007/978-0-387-71337-3_10

[149] Grudkowska M, Lisik P, Rybka K. Two-dimensional zymography in detection of proteolytic enzymes in wheat leaves. Acta Physiol Plant. 2013 Dec;35(12):3477–82.

[150] Kaberdin VR, McDowall KJ. Expanding the Use of Zymography by the Chemical Linkage of Small, Defined Substrates to the Gel Matrix. Genome Res. 2003 Aug;13(8):1961–5.

[151] Wilkesman JG, Schröder HC. Analysis of serine proteases from marine sponges by 2-D zymography. ELECTROPHORESIS. 2007 Feb 1;28(3):429–36.

[152] Jurado J, Fuentes-Almagro CA, Guardiola FA, Cuesta A, Esteban MaÁ, Prieto-Álamo M-J. Proteomic profile of the skin mucus of farmed gilthead seabream (Sparus aurata). Journal of Proteomics. 2015 Apr;120:21–34.

[153] Rajan B, Fernandes JMO, Caipang CMA, Kiron V, Rombout JHWM, Brinchmann MF. Proteome reference map of the skin mucus of Atlantic cod (Gadus morhua) revealing immune competent molecules. Fish & Shellfish Immunology. 2011 Aug 1;31(2):224–31.

Vita

María Isabela Avila Rodríguez was born in August 27th of 1994, in Bogotá Colombia. Through her early years, she was keen on the curious events of scientific phenomena with special interest on how genetic science worked. Later on, she left her country and started living on México, where she obtained her bachelor and undergraduate studies in Biotechnology engineering with focus on Bioprocesses in December of 2017. During this period, she participated as member of the Biotechnology Engineering alumni committee, where she helped on the logistics of several academic events. As well, from 2016 to 2017 she was part of the innovation and research academic program, in which she gained great interest over the proteomics field. As well as part of this modality, the publication of the article "Collagen: a review on its sources and potential cosmetical applications" in the Journal of Cosmetic Dermatology as first author was done. After this period, enzymology became one of her greatest passions, more in specific their effect over human skin as biocosmetic products. By which she entered the Master's in science program with focus in Biotechnology in Tecnológico de Monterrey. Within this period, she was part of the organizing committee of international conference Tec.nano 2018. As well she participated on poster sessions of national and international congress of the field. Proteomics is still her field of interest, in which she desires to continue her research in the near future. Within the field, her vision is to achieve sustainability through this catalyst with their optimized appliance on bioprocesses and biocosmetic fields.

This document was written using Microsft Word by María Isabela Avila Rodríguez

FORMATO DE DECLARACIÓN DE ACUERDO PARA USO DE OBRA

Por medio del presente escrito, **Maria Isabela Avila Rodríguez** (en lo sucesivo EL AUTOR) hace constar que es titular intelectual de la obra titulada **Characterization of serine-proteases from P. hypophthalmus epithelial mucus as a potential feedstock for biocosmetic applications** (en lo sucesivo LA OBRA), en virtud de lo cual autoriza al Instituto Tecnológico y de Estudios Superiores de Monterrey (en lo sucesivo el ITESM) para que efectúe resguardo mediante copia digital o impresa para asegurar su conservación, preservación, accesibilidad, disponibilidad, visibilidad, divulgación, distribución, transmisión, reproducción y/o comunicación pública con fines académicos o propios al objeto de la institución y sin fines de lucro como parte del Repositorio Institucional del ITESM.

EL AUTOR reconoce que ha desarrollado LA OBRA en su totalidad de forma íntegra y consistente cuidando los derechos de autor y de atribución, reconociendo el trabajo intelectual de terceros. Esto incluye haber dado crédito a las contribuciones intelectuales de terceros que hayan participado como coautores, cuando los resultados corresponden a un trabajo colaborativo.

De igual manera, EL AUTOR declara haber dado reconocimiento y crédito de autoría a cualquier parte de LA OBRA que haya sido previamente sometida, para obtener un grado académico, titulación y/o certificación en ésta o cualquier otra universidad. Incluyendo la debida atribución a través de cita y/o referencia bibliográfica en LA OBRA a conceptos, escritos, imágenes y cualquier representación intelectual al consultar publicaciones académicas, científicas, culturales o artísticas de otros autores, así como la fuente de su obtención.

EL AUTOR establece su deseo de conceder esta autorización de forma voluntaria y gratuita, y que de acuerdo a lo señalado en la Ley Federal del Derecho de Autor y la Ley de Propiedad Industrial, el ITESM se compromete a respetar en todo momento la autoría y a otorgar el crédito correspondiente en todas las actividades mencionadas anteriormente de LA OBRA.

De la misma manera, EL AUTOR manifiesta que el contenido académico, literario, la edición y en general cualquier parte LA OBRA presentada es de su entera responsabilidad, por lo que deslinda al ITESM por cualquier violación a los derechos de autor y/o propiedad intelectual o cualquier responsabilidad relacionada con LA OBRA frente a terceros.

María Isabela Avila Rodríguez

Nombre completo y firma autógrafa de EL AUTOR