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EVALUATION OF KERATINASE DEPOSITION AND PERMEABILITY IN THE SKIN

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ABSTRACT: The skin acts as a protective barrier shielding our body from external aggression. This fact hinders the entry of substances into the various layers of the skin. One of the skin components involved in this defense function is keratin. Keratin is a fibrous structural protein very resistant to proteolysis but susceptible to be hydrolyzed by keratinases; however, the mechanisms of keratinolysis is still unsolved. This article aims to provide further knowledge about the interaction between keratinase and skin. Skin samples were exposed to different concentrations of keratinase, 1000 U, 5000 U and 10000 U, and they are compared with control group. It was observed the formation of intercellular fissures in all the dilutions of keratinase. Transdermal absorption tests were used to verify the amount of sodium ascorbate as an active ingredient that is deposited and penetrates through the skin. It was observed that pretreatment with keratinase 5000 U induced a significant deposition ($p < 0.05$), resulting in up to 9 times greater retention of sodium ascorbate, as compared to control group and also facilitates the permeation of the sodium ascorbate into the different layers of the skin. These results could be the primary approach for further research of keratinase regarding the development of interesting applications in the cosmetic industry.

INTRODUCTION: The skin serves as a multifaceted barrier separating the human body from the external environment. It shields the body from exogenous chemical and physical agents, acts as the primary defense against harmful microorganisms, participates in immunological functions, contributes to metabolic processes, regulates temperature and absorption, being a modulator of the permeation of active ingredients into the skin and their penetration through it [1, 2]. The skin's barrier function is accomplished by the stratum corneum mostly [3].

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This extremely thin skin layer is the ultimate stage in the epidermal differentiation process. Its structure and composition are crucial for its barrier function properties and its nearly impermeable characteristics.

It Consists of:

1. Corneocytes, thin and flat highly specialized dead cells, which produce and are filled with keratin to provide strength and protection to the skin. They are arranged as a laminate of compressed keratin-filled corneocytes, giving fortified networks of keratin[4].
2. Corneodesmosomes, which function as "rivets" to hold corneocytes together.
3. Mortar lipids filling the space between stacked corneocytes. They are a complex mixture of

ceramides, cholesterol, and free fatty acids, which generate the permeability barrier.

4. Pro-barrier lipids, secreted by lamellar bodies that give rise to the multiple lipid lamellae of the stratum corneum.

5. Lipolytic enzymes, involved in processing pro-barrier lipids; and proteolytic enzymes, in charge of degrading desmosomes, structures responsible for joining the different sub-layers of the epidermis. Each of these elements plays a critical role in the integrity of the stratum corneum barrier, posing a hurdle to transdermal molecule delivery. Any interference with or modification of the functional attributes of these components could compromise the barrier's strength and its permeability [5].

Keratin is a fibrous structural protein and the major component in epidermal tissues of vertebrates. It can be also found in hair, feathers, nails, horns, hoofs, scales and wool. Keratin structure constitutes a challenging biopolymer resistant to conventional proteolytic degradation [6]. The best-known function of keratins and keratin filaments is to provide a scaffold for epithelial cells and tissues to provides a formidable defense against physical trauma, pathogens, and environmental stressors, maintaining their structural and functional integrity [7-9]. Keratins typically consist of three domains, each of which contains different subdomains characterized by their secondary structure[7]. Both the domains and subdomains interact with those of adjacent keratins, forming heterodimers, tetramers, and ultimately keratin fibers [10]. Human keratins have a molecular weight ranging from approximately 44 to 66 kDa and are classified into types: type I (“acidic”) and type II (“basic to neutral”). They constitute their filaments by heteropolymeric pair formation of type I and type II (1:1) molecules [11]. The formation of these fibers is influenced by hydrophobic interactions, hydrogen bonds and disulfide bonds, hydrogen, which also are involved in the rigidity and resistance of keratin molecules [12]. The keratin present in the human body is alpha-keratin and depending on the number of cysteines and disulfide bonds, we can refer to (i) hard keratin, when the protein containshigh amount of cysteine and disulfide bonds. They have greater resistance and are present in hairand nails. Or (ii) soft keratin, when the protein has low amount of cysteine and disulfide bonds. They have greater flexibility and are present in the skin [11]. There are 28 genes encoding type I keratins, which include 17 epithelial keratins and 11 hair keratins, and 26 genes encoding type II keratins, comprising 20 epithelial keratins and 6 hair keratins [11]. The primary keratin pair in keratinocytes of stratified squamous epithelia consists of type-II keratin K5 and type-I keratin K14 [13].

Keratinases, a distinct class of proteolytic enzymes, have emerged as a key player in the enzymatic realm, particularly due to its ability to hydrolyze keratin-rich substrates [14]. They are robust enzymes with a wide temperature and pH activity range and are largely serine or metalloproteases [15]. They possess a unique ability among proteases to adhere to complex and insoluble substrates such as feathers, wool, silk, collagen, elastin, horns, stratum corneum, hair, azokeratin, and nails [15]. While the precise mechanism of enzyme adsorption remains unclear, it is established that increased adsorption capacity correlates with higher levels of keratin hydrolysis [16]. Following enzyme binding and disulfide bond cleavage, keratin undergoes conformational changes, exposing numerous sites for enzymatic hydrolysis. Nonetheless, most of the purified keratinases known to date cannot completely solubilize native keratin [17, 18], the mechanism of keratinolysis is highly complex and still an enigma in the world of proteases.

The potential of keratinases for various biotechnological applications has long been recognized, offering sustainable solutions for industrial and environmental processes but also for biomedical, pharmaceutical, and cosmetic applications [19]. It has been claimed to have application in the elimination of keratin in acne, psoriasis, elimination of human callus and degradation of keratinized skin, depilation, preparation of vaccine for dermatophytosis therapy, pharmaceutical enhancement of nail treatment and treatment of scars, and epithelium regeneration [9]. However, the knowledge on these enzymes, its function, production, and optimization still need to be clarified to bring to light their importance and direct research into the probable applications of these enzymes.

On the way to provide more information, this article aims to understand the interaction between keratinase and skin, exploring its effect with *in-vitro* studies and determining whether keratinase increases the permeability of the skin.

MATERIALS AND METHODS:

Protein Expression and Purification: A recombinant *E. coli* BL21_T7 bacterial strain was used to overexpress the native keratinase protein cloned from *Bacillus licheniformis* strain PWD-1. It was purified by a HiLoad 26/60 Superdex 200pg gel filtration column running on a fast protein liquid chromatography (AKTA-FPLC). The protein was greater than 95% pure as judged by SDS_PAGE electrophoresis and high-performance liquid chromatography (HPLC). Samples were extensively dialyzed with 20 mM Tris buffer and 150 mM NaCl (pH 8.0), frozen and stored at -45°C. Protein concentration was calculated from the absorbance measured at 280 nm.

Effect on Skin Surface: Lyophilized keratinase P70 was provided by Proteos Biotech SL. The assay was performed with human skin from abdominoplasty of one donor (supplied by Biopredic International). The skin was received frozen, not dermatomized and stored at -20°C until use. For the experiment, the skin was maintained in culture medium supplemented: Dulbecco modified eagle culture media (DMEM) with glutamine (4mM g/L), glucose (4.5g/L), sodium pyruvate (1mM), sodium bicarbonate (1.5g/L). This was renewed daily until the start of the assay. The skin was cut in circles and mounted on Franz cells with the stratum corneum facing up. The recipient compartment was filled with culture medium taking care that no bubbles appeared. Keratinase reconstituted with DMEM medium or control was added to the donor compartment so that it completely covered the skin and was sealed with Parafilm to prevent evaporation. The samples were incubated for 16 h at 5% CO_2 and 37°C. After this time the sample was removed and stored, together with the surface wash, for subsequent analysis of the activity. The skin was kept in culture medium (not completely covered) until evaluation under the microscope. For the latter, each skin sample was sputtered and then imaged. The images of the skin were taken using Scanning Electron Microscopy.

Absorption Activity: Transdermal absorption assays were performed using an adaptation of OECD 428 [20]. For this purpose, dermatomized skin explants (supplied by Biopredic International) and modified Franz cells were used. Explants were acquired frozen from one donor. Once thawed and tempered with PBS, the skin was dermatomized to a thickness of 400 µm. Dermatomized skin explants were placed between the (i) donor and (ii) receptor compartments with the stratum corneum facing up. The receptor compartment was filled with a receptor solution, which simulated physiological conditions (PBS, 0,5% (w/v) EDTA and 0,0025% (w/v) Na_2SO_4) and allowed to equilibrate for at least 30 minutes.

Receptor solution was then added to the donor compartment and skin integrity was determined by EVOM™ Epithelial Volt/Ohm Transcellular and Paracellular Pathway of Ion or Electrical Current Flow (TEER) Meter 3. After removing the solution and drying the donor, keratinase pretreatment solutions, reconstituted with PBS, were administered into the donor compartment and covered with Parafilm. After 16 h of incubation at 5% CO_2 and 37°C, the solutions were removed to administer a formulation with the substrate sodium ascorbate (20% (w/v) sodium ascorbate, 0,5% (w/v) EDTA and 0,0025% (w/v) Na_2SO_4). Samples were taken from the receptor compartment at predetermined times to obtain the amount of permeated substrate. These samples were filtered on 0.2 µm pore size cellulose acetate filters and immediately analyzed by high performance liquid chromatography (Agilent HPLC). Franz cells were disassembled, after taking the last sample. The diffusion area of the skin was cut into small pieces and placed in extraction liquid (75% (v/v) KH_2PO_4 and 25% (v/v) methanol) under agitation for 1 hour. The samples were filtered and analyzed by HPLC to obtain the amount of substance retained in the skin. Experiment was repeated 4 times.

Statistical Analysis: Data are represented as mean ± SEM. Statistical analyses were carried out with GraphPad Prism 9 (GraphPad Software, La Jolla, CA). Comparisons between the groups were performed using a non-parametric test, the Kruskal-Wallis for non-normal distributed data. P-values < 0.05 were considered significant.

RESULTS:

Effect on Skin Surface: Keratinase was studied at 3 dilutions: 1000 U, 5000 U and 10000 U. In the "control" and "vehicle control" conditions, it is shown the arrangement of the overlapping cells creating a solid barrier for their defense. Keratinase 1,000U, 5,000U and keratinase 10,000U treatments showed the formation of intercellular fissures **Fig. 1.**

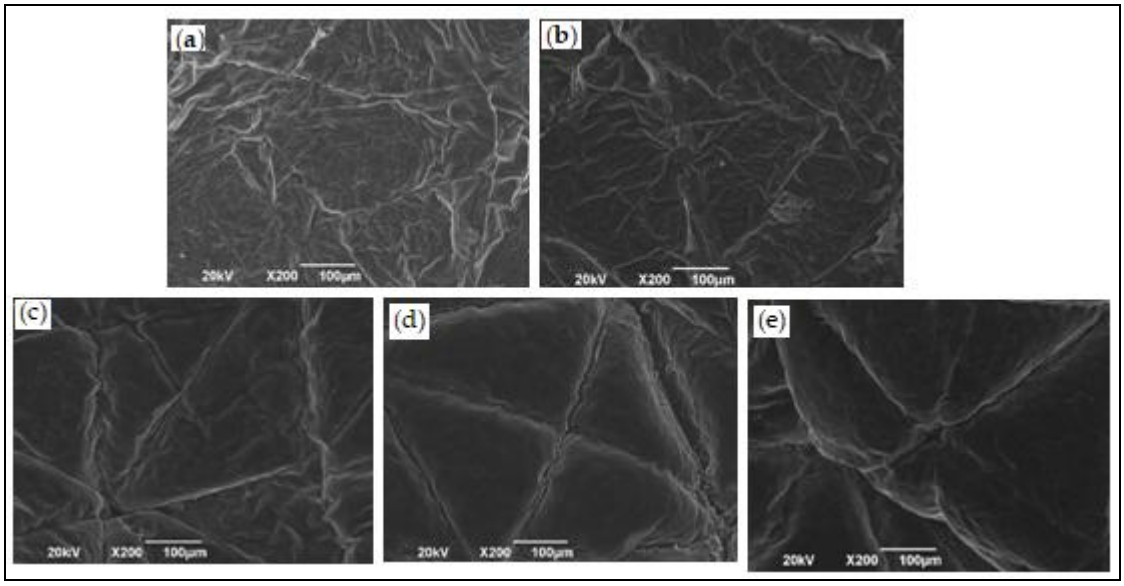

FIG. 1: IMAGES OF THE SKIN SURFACE BEFORE AND AFTER TREATMENT WITH KERATINASE AT DIFFERENT CONCENTRATIONS TAKEN WITH SCANNING ELECTRON MICROSCOPY AT X200.(A) CONTROL. (B) CONTROL VEHICLE. (C) SKIN TREATED WITH KERATINASE 1000 U. (D) SKIN TREATED WITH KERATINASE 5000 U. (E) SKIN TREATED WITH KERATINASE 10000 U.

Absorption Activity:

Skin Deposition: For the skin deposition variable, the value of the amount of the sodium ascorbate retained in the skin was analyzed at 1000 and 5000 U of keratinase. Pretreatment with keratinase induced an increase in the amount of sodium ascorbate retained in the skin with respect to the control. Pretreatment with keratinase 1000 U induced a non-significant increase and keratinase 5000 U induced a significant increase ($p < 0.05$), resulting in up to 9 times greater retention of sodium ascorbate, as compared to control group **Fig. 2.**

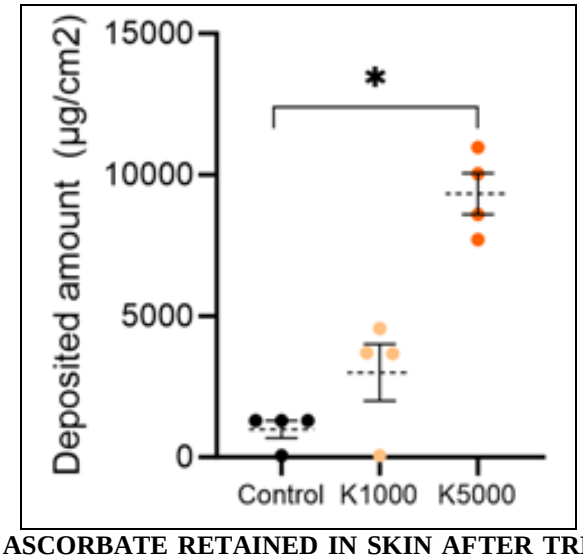

FIG. 2: AMOUNT OF SODIUM ASCORBATE RETAINED IN SKIN AFTER TREATMENT WITH KERATINASE 1000 U AND KERATINASE 5000 U COMPARED TO THE CONTROL GROUP (DATA IS REPRESENTED AS A MEAN ± SEM). SYMBOL SHOWS STATISTICAL DIFFERENCES BETWEEN GROUPS ANALYZED BY THE KRUSKAL-WALLIS TEST (*P < 0.05VS CONTROL)

Skin Permeation: For the skin permeation analysis, keratinase activity was evaluated at 5000 U. The values of permeation were monitored at different times: initial (T0), after 2 h (T2), 6 h (T6) and 24 h (T24). Pretreatment with keratinase 5,000 U induced a slight increase in sodium ascorbate permeation at 6 and 24 hours **Fig. 3.**

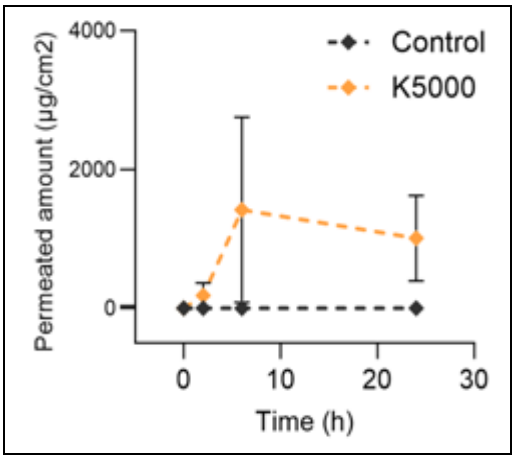

FIG. 3: TRANSDERMAL ABSORPTION OF SODIUM ASCORBATE CHANGE CURVE FOR CONTROL AND KERATINASE 5000UMEASURED AT DIFFERENT TIME POINTS (DATA IS REPRESENTED AS A MEAN ± SEM).

DISCUSSION: The efficiency delivery into the skin of pharmaceutical compounds or cosmetic formulations, profoundly influences the efficacy of these applications. Skin interaction with the different ingredients depends mostly on the health status of the skin, the size, flexibility and chemistry of the molecule, and the total composition of the pharmaceutical or cosmetic formula [21, 22]. Advancements in nanotechnology, transdermal delivery systems, vesicles and other formulation strategies have become pivotal in overcoming the barriers presented by the skin, ensuring that active ingredients reach their intended targets within the different layers of the skin [23, 24].

In this study it was observed that keratinase acted on the skin surface producing furrows and eliminating the cellular overlapping. Our studies suggested that keratinase could producecellular renewal and separation of keratinocytes, interacting with corneodesmosomes and desmosomes, forming these intercellular fissures. This effect was already observed by Dobson and Bosley in 1962 [25]. They exposed a healthy subject to different concentrations of keratinase, both on intact skin with the stratum corneum and without it. Their conclusion was that this proteolytic enzyme does not have a direct effect on keratin, but rather its action is acantholytic. In the skin with the stratum corneum, no effects were observed; however, when the epidermis was directly exposed to keratinase, clusters of free-floating cells were observed, and in some areas, the epidermis was entirely absent. Similar changes were also noted in the external root sheath of the hair follicle and the basal cells of the sebaceous gland. However, lipid-containing sebaceous cells were unaffected. Eccrine sweat gland cells were also not altered.

The disruption caused by keratinase in the skin weakens its action as a barrier, thus potentially enhancing permeability and consequently increasing penetration of the active ingredients through them. This effect was shown in the transdermal absorption or skin absorption test, which allows quantifying the amount of one or more active ingredients that have penetrated through the skin and thus, establishing the levels of exposure to these substances [26]. The values of permeation give the amount of active substance that has penetrated through the skin. The values of deposition give the amount of active substance that is retained in the skin, considering a local, superficial skin distribution [21]. In this study we showed that keratinase favored the deposition in the skin of sodium ascorbate and increased skin permeability. We have used this molecule because it is one of the most commonly used active ingredients in cosmetics and due to its multifunctionality [30]. This finding can be very important for the potential use of keratinase as a delivery booster in cosmetic formulations or preparations were the ingredients need to reach properly their site of action in the skin.

There is evidence in the literature where keratinase acts on more complex keratinized structures, as it is in ungual processes. It was observed how keratinase acted on the intercellular matrix of the nail and separated corneocytes on the dorsal surface from one another, disrupting the integrity of the nail plate. Permeation studies using hoof membranes as a model for the nail plate showed that the enzyme enhanced drug permeation through the hoof membrane. The permeability was found to be significantly increased in hoof membrane and nail plate in the presence of the enzyme [27].

Detection of these approaches may become promising applications of keratinases. Considering this effect, it can have significant importance the use of keratinase in the treatment of certain dermatological disorders or in skincare practices characterized by a high accumulation of keratin. In such scenarios, it is crucial for the drug or active ingredient intended to alleviate these effects to effectively penetrate the deeper layers of the skin. Examples of these applications are: (i) treating hyperkeratosis skin lesions such as calluses, corns, or psoriasis; (ii) treating skin disorders produced by keratin mutations; (iii) treating scars and fibrotic processes or (vi) improving ungual drug delivery [28-32]. Furthermore, the effect of keratinase on enhancing cellular renewal can also be beneficial in addressing other cutaneous processes, such as: (i) acne, promoting a cellular renewal, cleansing the area and allowing the acne treatment to reach the site of action more effectively; and (ii) replace the dry or squamous skin. Recognizing the importance of precise ingredient delivery and understanding the complex interplay between skin, keratin and the enzymatic activities of keratinases not only unravels more aspects about the skin biology but also enhances the therapeutic potential of skincare products and holds the promise of dermal therapies and advanced skin delivery systems.

CONCLUSIONS: Keratinase at 5000 U enhanced the deposition of the active ingredient, sodium ascorbate, in the skin ($p < 0.05$) up to 9 times compared to the control and increased the skin permeability. This study provides additional knowledge about the activity of keratinase in skin, being a primary approach to discover and develop interesting applications of these enzymes in the pharmaceutical and cosmetic industries.

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