Vol. 30(4):467-472 | Ocean and Polar Research | December 2008

Review

Possible Roles of Antarctic Krill Proteases for Skin Regeneration

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Abstract : Antarctic krill has a strong proteolytic enzyme system, which comes from a combination of several proteases. This powerful activity can be easily detected by krill's superior *post mortem* autolysis. Mammalian skin consists of epidermis and dermal connective tissue, and functions as a barrier against threatening environments. A clot in a wound site of the skin should be removed for successful skin regeneration. Epithelial cells secrete proteases to dissolve the clot. In previous studies Antarctic krill proteases were purified and characterized. The proteolytic enzymes from Antarctic krill showed higher activity than mammalian enzymes. It has been suggested that these krill clean up the necrotic skin wound to induce a natural healing ability. The enzymes exhibited additional possibilities for several other biomedical applications, including dental plaque controlling agent and healing agent for corneal alkali burn. Considering that these versatile activities come from a mixture of several enzymes, discovering other proteolytic enzymes could be another feasible way to enhance the activity if they can be used together with krill enzymes. Molecular cloning of the krill proteases should be carried out to study and develop the applications. This review introduces possible roles of the unique Antarctic krill proteases, with basic information and suggestion for the development of an application to skin regeneration.

Key words : Antarctic, krill, proteases, skin regeneration, debridement

1. Introduction

Antarctic crustacean krill (*Euphausia superba*) has been considered an abundant food reservoir because of its high nutritional value (Sidhu *et al.* 1970; Fedotova *et al.* 1977; Kunachowicz *et al.* 1978; Piekarska and Rutkowska 1978; Rys and Koreleski 1979; Mroz 1981; Rehbein 1981; Zaleska-Freljan and Cywinska 1991; Tou *et al.* 2007; Clarke and Tyler 2008). Besides these nutritional studies, few other particular interests have been suggested except for astaxanthin and omega-3 oils (Venkatraman *et al.* 1994; Sampalis *et al.* 2003; Takaichi *et al.* 2003; Bunea *et al.* 2004; Grynbaum *et al.* 2005; Moretti *et al.* 2006; Kidd 2007; Tou *et al.* 2007). In many attempts to discover other applications, krill has been exploited for biomedical developments since krill enzymes have been shown to have a superior proteolytic activity (Nishimura *et al.* 1983; Turkiewicz *et al.* 1986; Ellingsen and Mohr 1987; Anheller *et al.* 1989; Karlstam *et al.* 1991; Turkiewicz *et al.* 1991; Mekkes *et al.* 1997; Mekkes *et al.* 1998; Sangwan *et al.* 1999). It has been suggested that these krill enzymes clean up the necrotic skin wound to induce the natural healing ability (Hellgren *et al.* 1986; Turkiewicz *et al.* 1991; Mekkes *et al.* 1997; Mekkes *et al.* 1998; Sangwan *et al.* 1999; Berg *et al.* 2001).

Mammalian skin consists of epidermis and dermal connective tissue. Hairs and glands are derived from the epidermis. The main function of the skin is to protect the body from threatening environments, such as heat, cold, mechanical stress, microorganisms, and dryness. Once this barrier is defective it must be rebuilt rapidly. A clot plugs the defect initially and a series of recovery steps are followed to regenerate the lost tissues. Inflammatory cells migrate into the clot followed by fibroblast and capillaries to build a contractile granulation tissue to shrink in size in order to pull the wound margins together. Wound healing process continues in collaboration of many different tissues and cell lineages for cell proliferation, migration, matrix synthesis, and contraction by growth factor signals (Martin 1997).

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This review summarizes Antarctic krill proteases and current relevant research in biomedical applications, including skin wound remedy. First, we briefly introduce why proteases are important in the wound healing process. We then review the proteases found in Antarctic krill in terms of their functional properties and higher activities in comparison with mammalian enzymes. The last part of the review provides examples of how proteases from Antarctic krill can be used for biomedical treatments, such as enzymatic debridement of necrotic skin wounds, controlling dental plaque formation, and wound healing after corneal alkali burn. To conclude, we attempt to relate Antarctic krill proteases to its application as a wound healing agent, and take a look at future prospects in the field.

2. Proteases in Wound Healing

Overall Mechanisms of Skin Regeneration

Wound sites of the skin in most case cause blood leakage from damaged vessels. A clot formation is necessary to seal the damaged region and supplies a matrix for cell migration during the recovery. The clot functions as a pool of cytokines and growth factors (Martin 1997). Neutrophils reach the wound location within minutes after damage to get rid of bacterial infection and to release pro-inflammatory cytokines for the activation of fibroblasts and keratinocytes. Neutrophils are phagocytosed by macrophages after their duties are finished. Macrophages are accumulated to remove other damaged cells and matrix debris, as well as to secrete growth factors and cytokines into the wound site. Previous studies summarized functions of growth factors and cytokines as motogens and mitogens in wound healing process (Martin 1997). The released growth factors and cytokines activate neighboring keratinocytes to migrate to the wound site. The basal keratinocyte monolayer contacts a layer of basal lamina, reconstitutes the collagen matrix, and reestablishes the link of basal lamina to underlying connective tissues by anchoring fibrils (Compton *et al.* 1989). Mesenchymal cells transform into fibroblasts. They lay fibrin to build a framework for cell migration. The fibroblasts generate ground substance and later collagen. This secretion induces wound strength, resulting in reorganization of collagen fibers. Lost blood supply is delivered by capillary migration (Fisher *et al.* 1994).

Functions of Proteases in Wound Healing

The leading-edge keratinocytes digest the fibrin clot in order to dissolve the clot barrier and reach to the healthy dermis beyond the clot (Grondahl-Hansen *et al.* 1988). The enzyme secreted from the clot itself for this digestion is plasmin, which is a fibrinolytic enzyme. The enzyme synthesis is positively regulated by the migrating keratinocytes (Grondahl-Hansen *et al.* 1988). The keratinocytes up-regulate the level of members of matrix metalloproteinase (MMP) family, including MMP-1 (interstitial collagenase), MMP-9 (gelatinase B), and MMP-10 (stromelysin-2) (Saarialho-Kere *et al.* 1992; Saarialho-Kere *et al.* 1994; Salo *et al.* 1994). Functioning together with fibrinolysin, MMP family members are necessary to generate high proteolytic activity for controlling unregulated healing (Grinnell *et al.* 1992; Tarnuzzer and Schultz 1996).

3. Proteases found in Antarctic Krill

Antarctic krill have a strong proteolytic enzyme system that comes from a combination of several protease enzymes (Osnes 1985; Osnes and Mohr 1985; Hellgren *et al.* 1986; Osnes 1986; Osnes *et al.* 1986). This powerful activity can be easily detected by their superior *post mortem* autolysis (Konagaya 1980; Ellingsen and Mohr 1987). In an early krill enzyme study, total extract of whole body was used for the enzyme purification (Hellgren *et al.* 1986; Turkiewicz *et al.* 1986). The species of krill proteases were evidently determined based on experiments using substrate specificity, sensitivity to each protease inhibitor, molecular weight, and isoelectric point (Nishimura *et al.* 1983). They fractionated the total extract and determined substrate specific protease activity, as well as sensitivity to inhibitors. They defined carboxypeptidase A and B, aminopeptidase, trypsin, and cathepsin A from Antarctic krill (*Euphausia superba*). Later, thiol-dependent serine proteinase was identified from the digestive tract of the krill (Turkiewicz *et al.* 1986). Further study clarified more specifically each component of the krill protease group, referred to as Krillase (Hellgren *et al.* 1986; Anheller *et al.* 1989; Berg *et al.* 2001). Another research group was successful in purifying a total of eight proteolytic enzymes out of three trypsin-like proteinases: a chymotrypsin-like proteinase, two carboxypeptidase A, and two carboxypeptidase B from the same krill species (Sjodahl *et al.* 2002). Another study showed that the presence of metalloprotease in Antarctic krill, originated from an Antarctic marine bacterium *Psychrobacter proteolyticus*, which was isolated from the stomach of the Antarctic krill (Denner *et al.* 2001).

4. Antarctic Krill Proteases in Human Skin Regeneration

Enzymatic Debridement of Necrotic Skin Wounds

Effective removal of necrotic debris, fibrin or blood crusts is considered an important factor in the skin regeneration process. A balanced cocktail of endo and exopeptidases from Antarctic krill was proposed for management of necrotic skin wounds (Hellgren *et al.* 1986). A protease extract prepared from Antarctic krill showed more effective proteolytic activity than a common component of commercial enzymatic debrider, bovine trypsin (Anheller *et al.* 1989). Another research group published a krill serine proteinase as a fibril-reconstituting agent of calf skin collagen and type I and V of Achilles tendon collagens (Turkiewicz *et al.* 1991). Isolated krill enzyme preparation was applied to digestion of whole pig tissue specimens and showed significantly more effective activity *in vitro* than papain and Elase (fibrinolysin/DNAse), which is the most common commercially available enzyme product (Mekkes *et al.* 1997). The same research group showed an *in vivo* result presenting more effective wound debridment activity of the krill enzyme than that of the commercial products (Mekkes *et al.* 1998).

Properties of Antarctic Krill Proteases

A three-dimensional protein structure of the recombinant krill serine protease expressed in yeast *Pichia pastoris* expression system was 81% identical to the known crystal structure of crab collagenase I (Perona *et al.* 1997; Benjamin *et al.* 2001). This result implies that the cold-adapted krill protease possibly has broad substrate specificity, including collagen cleavage activity, which is essential in wound healing steps. The trypsin-like proteinases purified from Antarctic krill revealed about 12 and 60 times higher activity than bovine trypsin at 37°C and 1-3°C, respectively (Sjodahl *et al.* 2002).

5. Other Aspects on Biomedical Applications

Dental Plaque Control

Salivary pellicle is a layer of negatively charged salivary glycoprotein that adheres to the tooth surface's enamel, allowing cariogenic bacteria to adhere to the tooth surface. Therefore, protease application can be a feasible way to inhibit the attachment of the bacteria to the glycoprotein for a dental plaque control. In a previous study, the glycoprotein was degraded efficiently by krill proteases and resulted in successful removal of oral biofilms *in vivo* and *in vitro*, showing that the krill enzymes can be used for this purpose (Berg *et al.* 2001).

Wound Healing After Corneal Alkali Burn

A preclinical experiment using rabbits was conducted to examine the effect of krill proteases on corneal ulceration after alkali burning (Sangwan *et al.* 1999). The krill enzymes were applied topically to the 4N alkali-induced corneal defects, and showed statistically significant reepithelialization efficiency in 28 days of post treatment. The result indicates that krill proteases can be applied to corneal wound healing.

6. Conclusion

Wound debridement plays a key role in skin regeneration since it exposes the healthy dermis, so that the recovery can be accelerated. Therefore, the debridement can be considered as a wound bed preparation. For this treatment, sometimes, medically cleaned maggots have been used in the initial phase of the wound debridement to remove dead cell debris, slough, and necrotic tissues. Surgical debridement has been conducted as well. However, these surgical treatments have been regarded as a painful means, and require well-trained skill. Antarctic krill proteases suggest a non-surgical enzymatic debridement option for skin regeneration (Hellgren *et al.* 1986; Anheller *et al.* 1989; Mekkes *et al.* 1997; Mekkes *et al.* 1998). Antarctic krill enzymes showed much better proteolytic activity than mammalian proteases, and revealed broad active temperature spectrum (Gudmundsdottir 2002). Furthermore, the enzymes exhibited superior activity to commercially available enzyme products, such as Elase, papain, and others (Mekkes *et al.* 1997; Mekkes *et al.* 1998).

Several other enzymes for wound debridement can be considered, such as bromelain, cathepsin, chymotrypsin, collagenase, deoxyribonuclease, elastase, fibrinolysin, hyaluronidase, papain, pepsin, staphylolysin, streptokinase, streptodornase, subtilisin, and trypsin (Westerhof *et al.* 1990). Considering that the krill enzyme preparation is a mixture of several enzymes in an optimal ratio, it might be a feasible idea to develop another combination of the krill enzyme with other enzymes mentioned above.

Another possibility of utilizing krill enzymes for biomedical application is that it could be used to generate selenium organic species from biomass of Antarctic krill. Other research was carried out and showed that enzymatic digestion of the krill protein produced 1 to 8 μg/g of organic

selenium in forms of selenomethionine, selenocystine, and its derivatives (Siwek *et al.* 2005; Siwek *et al.* 2006). This recovery of the selenium organic species after enzymatic hydrolysis by krill proteases suggests its application to another biomedical use. Selenium is a cofactor of glutathione peroxidase and involved in the immune system, including the thyroid gland activity. It antagonizes against heavy metals (Siwek *et al.* 2005).

To develop the Antarctic krill proteases for the skin regeneration including wound healing, enough enzymes should be available to be used in many researches. The enzyme preparation procedure should be optimized in a large scale and the activity can be enhanced if the enzymes are engineered. Further preclinical and clinical experiments should be conducted to obtain the efficiency and the safety of the enzyme application. Constructing recombinant Antarctic krill proteases would be another promising strategy for the successful enzyme development. In conclusion, this review introduces possible roles of the unique Antarctic krill proteases, supplies basic information, and makes suggestions for the development of applications toward skin regeneration.

Acknowledgements

This work was supported by the Korea Polar Research Institute (Grant PE08050).

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Received Aug. 8, 2008
Revised Oct. 21, 2008
Accepted Nov. 3, 2008