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SHORT COMMUNICATION

In Vitro* Tissue-digesting Properties of Krill Enzymes Compared with Fibrinolysin/DNase, Papain and Placebo*J. R. MEKKES,* I. C. LE POOLE, P. K. DAS, A. KAMMEYER, W. WESTERHOF***Department of Dermatology, Academic Medical Centre, University of Amsterdam, Meibergdreef 9, 1105 AZ, Amsterdam, The Netherlands*

Wound debridement, the removal of necrotic tissue, can be achieved with proteolytic enzymes. Recently, a new multi-enzyme preparation, krill enzyme, isolated from Antarctic shrimp-like organisms (*Euphausia superba*), was reported to possess powerful proteolytic activity towards protein substrates. In this paper, we study the *in vitro* digestive properties of krill enzymes towards whole tissue, compared with placebo, papain, and fibrinolysin/DNase. Freshly obtained skin specimens were exposed for 3 days to krill enzymes (3; 0.6 and 0.06 U/ml), papain (120; 60; 6 and 0.6 U/ml), fibrinolysin/DNase (2.5/1500 E and 1/600 E), and phosphate-buffered saline control solution. Tissue digestion was estimated by measuring wet wt, dry wt, and histological examination. After 72 hr of exposure to 3 U/ml krill enzymes, the dry wt of the specimens was reduced to $2.7\% \pm 1.9$ (SEM, $n = 5$), compared with $31.0\% \pm 2.7$ for placebo, $25.7\% \pm 2.5$ for 120 U/ml papain, and $24.5\% \pm 3.3$ for 2.5/1500 E/ml fibrinolysin/DNase. The differences between krill enzymes and fibrinolysin/DNase, papain, and control solution were statistically significant ($p < 0.007$). These data suggest that krill enzymes are more active than other commonly available proteolytic agents used for wound debridement. © 1997 Elsevier Science Ltd

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INTRODUCTION

Wound debridement, the removal of necrotic tissue from a wound, is an essential first step in the process of wound healing (Haury *et al.*, 1978). The human defense system itself can remove non-vital tissue and bacteria by means of specialized cells (leucocytes, macrophages) that are able to secrete enzymes in their micro-environment and are capable of phagocytosis (Clark, 1985).

When the extent of necrosis is too large, or when additional compromising conditions, such as lack of arterial blood supply or infections, are present, the natural defense system may be

inadequate and additional debridement techniques are indicated. The simplest technique, surgical excision, is not always appropriate, especially not in superficial ulcers that contain both necrotic areas and healthy granulating areas in the same excisional level, or that are located directly above vital structures (joints, tendon sheets, nerves). In this case, more selective and conservative cleaning techniques are used, such as the repeated application of moistened gauzes, gels, gel-sheets, alginates, hydrocolloid occlusive dressings, or proteolytic enzymes.

ENZYMATIC DEBRIDEMENT

Several enzymes have been used for wound cleaning, including bromelain, cathepsin, chy-

*To whom all correspondence should be addressed.
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mopapain, collagenase, deoxyribonuclease, elastase, fibrinolysin, hyaluronidase, papain, pepsin, staphylolysin, streptokinase, streptodornase, subtilisin, and trypsin (Westerhof and Mekkes, 1990). The most frequently used commercial product is Elase (fibrinolysin/DNase).

Krill enzymes, isolated from the intestine of a small shrimp-like organism (*Euphausia superba*) that lives in the Antarctic ocean, have been described as potentially effective for wound debridement (Anheller *et al.*, 1989; Hellgren and Vincent, 1989). Krill enzymes consist of a mixture of endopeptidases and exopeptidases. Among the main endopeptidases, three trypsin-like serine proteases were identified. One of these trypsin-like enzymes also exerts exopeptidase activity. Five exopeptidases were identified: two carboxypeptidases of the A-type, two carboxypeptidases of the B-type, and one aminopeptidase. The exact composition and properties of the mixture has been reviewed in detail (Osnes, 1985a, 1985b, 1986; Osnes and Mohr, 1985; Osnes *et al.*, 1986). The entire mixture, the crude extract, is more powerful than its individual components, pointing to a synergy between these enzymes (Osnes, 1986).

In this study, we describe the digestive activity of krill enzymes towards full-thickness tissue specimens, compared with placebo and two other enzymes designed for use as a solution in wounds, papain and fibrinolysin/DNase.

MATERIALS AND METHODS

Sixty full-thickness skin specimens, 10 mm in diameter and approximately 10 mm thick, ranging in weight from 134 to 186 mg, were removed from the thighs of a New Yorkshire pig, under sterile conditions. The specimens contained epidermis, dermis and subcutaneous fat. After weighing, they were placed in sterile containers, containing 10 ml of the test solutions. The test solutions were krill enzymes (Medisan AB, Uppsala, Sweden) in concentrations of 3, 0.6 and 0.06 casein Units/ml in PBS (phosphate-buffered saline); fibrinolysin/DNase (Warner-Lambert, Amsterdam, The Netherlands) in concentrations of 2.5 E/1500 E and 1 E/600 E/ml in PBS; papain (Sigma, Zwijndrecht, The Netherlands) in concentrations of 120, 60, 6 and 0.6 U/ml in PBS; and a control solution, PBS. The concentrations were selected on the basis of the recommended concentrations for clinical use as a solution in

necrotic ulcers: 6 U/ml for krill enzymes (Westerhof *et al.*, 1990), 1–2.5 E/ml for fibrinolysin (Westerhof *et al.*, 1987) and 60–100 U/ml for papain (Avakyan *et al.*, 1987). Penicillin (100 U/ml) and streptomycin (100 µg/ml) were added. The containers were rotated slowly at 1 rpm at 37°C for 3 days. After 24, 48, and 72 hr, the skin specimens were removed from their containers and weighed (wet wt); the enzyme solutions were refreshed every day. After 72 hr also, the dry wt was measured, and one of the six biopsies per test solution was examined histologically using routine haematoxylin–eosin staining and picosirius red staining for collagen (James *et al.*, 1986).

RESULTS

Already macroscopically (see Fig. 1) it could be observed that the skin specimens exposed to 3 U/ml krill enzyme for 3 days were nearly completely digested, while the specimens exposed to PBS control, papain or fibrinolysin/DNase seemed still to be intact. Figure 1 shows the average dry wt ($\pm$ SEM) of the skin specimens, measured after 72 hr of exposure to the enzyme solutions, and expressed as a percentage of the original weight. With 3 U/ml krill enzymes, the dry wt was $2.7\% \pm 1.9$ (SEM, $n = 5$), compared with $31.0\% \pm 2.7$ for placebo, $25.7\% \pm 2.5$ for papain, and $24.5\% \pm 3.3$ for fibrinolysin/DNase. The differences between 3, 0.6 and 0.06 U/ml krill enzymes and PBS control were statistically significant (p -values < 0.0004 ; Student's t -test). Krill enzymes, even in the lowest concentration (0.06 U/ml), were significantly more effective than 2.5 E/1500 E/

Fig. 1. Digestion of whole tissue specimens after 72 hr of exposure to krill enzymes (3, 0.6 and 0.06 U/ml), Elase (2.5 E fibrinolysin and 1500 E/ml DNase), papain (120 U/ml), and control (PBS). Dry wt after 72 hr was measured. The results are expressed as a percentage of the original weight before exposure to the different enzymes.

Fig. 2. Tissue specimens after 72 hr of exposure to control solution (PBS, phosphate-buffered saline), Elase (fibrinolysin and DNase), papain, and krill enzymes (at concentrations of 0.06, 0.6 and 3 U/ml). (A) Control (PBS). (B) Elase (2.5 U/ml); showed little digestive activity. (C) Papain (120 U/ml); the epidermis was removed completely. (D) Krill enzymes (0.06 U/ml); the epidermis became detached. (E) Krill enzymes (0.6 U/ml); the specimen was digested to 64% of its original weight. (F) Krill enzymes (3 U/ml); this is 50% of the recommended concentration for clinical use, and in this case, all tissue components except some collagen fibres had disappeared. Picrosirius red stain for collagen. The original size of the specimens was 10×10 mm.

ml fibrinolysin/DNase ($p = 0.007$) and papain ($p = 0.002$). The lower concentrations of papain (60, 6, 0.6 U/ml) and fibrinolysin/DNase (1 E/600 E/ml) were not effective at all and are not further shown or mentioned in the text. The highest concentrations of papain and fibrinolysin/DNase had some digestive properties, but the difference compared with control was not significant.

Histological examination of the skin specimens confirmed that, after 72 hr of exposure to 3 U/ml krill enzymes, all tissue components except collagen fibres had disappeared, and even the collagen fibres were disintegrating [see Fig. 2(F), picrosirius red stain for collagen]. The tissue specimens exposed to control solution

(PBS), papain and fibrinolysin/DNase, showed little morphologic change, except for detachment of the epidermis of the specimens exposed to 120 U/ml papain [Fig. 2(C)].

The daily measurements of wet wt after 24, 48, and 72 hr showed that, initially, because of hydration, most specimens increased in weight, but not those exposed to krill: after 24 hr of exposure to 3 U/ml krill enzyme, the average weight was reduced to 64%; after 48 hr, to 32%; and after 72 hr, to 18% of its original weight. The effect of krill enzymes was dose dependent.

DISCUSSION

Enzymatic debridement of necrotic wounds is a subject that has regained interest during the

last 3 years (Davidson, 1996). Several pharmaceutical companies now are developing and testing new and more powerful proteolytic enzyme preparations. An interesting feature of enzymatic debridement is that, apart from the wound cleaning process, which is evidently beneficial for wound healing, the healing process may be enhanced indirectly because breakdown products of extracellular matrix proteins such as collagen can stimulate the attraction of polymorphonuclear leucocytes and monocytes/macrophages to the wound bed (Scher, 1987; Ahlstedt *et al.*, 1983; Norris *et al.*, 1982; Postlethwaite and Kang, 1976; Senior *et al.*, 1980).

Results of *in vitro* studies cannot be extrapolated directly to the *in vivo* situation, where serine protease inhibitors present in serum and wound fluid may inhibit enzymatic activity. It is not known whether krill enzymes are more susceptible to inhibition by human protease inhibitors than fibrinolysin or papain. Investigating effectivity of wound-care products in human studies can be difficult, because of the considerable variability in wounds and patients. Large randomized controlled trials are needed to give conclusive results. The first step in the development of a new product is to show its activity *in vitro*.

This *in vitro* experiment showed that krill enzymes are very potent, and are capable of digesting nearly all tissue components. The *in vitro* effect of 1–10% of the proposed concentration for clinical use was superior to the recommended maximum concentrations of other enzymatic products like papain and fibrinolysin/DNAse. The origin of the krill enzymes may explain their good properties: these enzymes are operative in the digestive tract of an organism that through evolution has managed to survive in a low temperature environment, where nutrients are available only episodically.

In conclusion, the tissue-digesting capacities of krill enzymes *in vitro* are superior to collagenase/DNAse and papain.

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