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Debriding ability of a novel multi-enzyme preparation isolated from Antarctic krill *(Euphausia superba)*

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Summary. The wound-debriding activity of various types of proteolytic enzymes and proteases from Antarctic krill (multi-enzyme system consisting of both endo- and exopeptidases) was evaluated. The results, based on the enzymatically achieved weight reduction of a necrotic animal material (excised rat skin) in vitro, clearly showed that the multi-enzyme system (krill) had a higher degrading activity than the single enzyme preparation, or that with only a few enzymes. The debriding effect of the krill enzymes was markedly related to the enzyme concentration, resulting in 70–100% substrate degradation after 24 h. The digesting capacity of trypsin reached about 50%, but an increase in concentration of this enzyme did not substantially influence its overall activity. The effect of streptokinase-streptodornase, collagenase and plasmin-desoxyribonuclease was weak (10–20% digested).
Key words. Enzymatic debridement; proteolytic enzymes; Antarctic krill *(Euphausia superba)*; trypsin; streptokinase-streptodornase; collagenase; plasmin-desoxyribonuclease.

The objective of enzymatic debridement in the secondary healing leg ulcers is to decompose the slough and/or necrotic tissue in the wound in order to achieve a 'red' granulating and epithelializing wound surface.

Proteolytic enzymes used for this purpose are heterogeneous as regards their origin, structure and substrate specificity. For evaluation of their in vitro activity a careful choice of clinically relevant substrates is of great importance. Therefore, in previous studies, we used substrates originating from human secondary ulcers (necroses, fibrin, blood clots)[1–3].

In the present report a necrotic animal model (excised rat skin) was chosen as a representative and reliable material for testing the potency of various enzymes. This tissue is very similar to, although not identical with the human necrosis which exists in different stages of dehydration/degradation.

The effects of the main enzymatic debriders used in Scandinavia, crystalline trypsin (Trypure®, Novo), streptokinase-streptodornase (Varidase®, Cyanamid), collagenase (Iruxol®, Knoll) and plasmin-desoxyribonuclease (Fibrolan®, Parke-Davis), were compared with that of a novel multi-enzyme preparation originating from Antarctic krill *(Euphasia superba)*.

One of the most characteristic features of this reddish shrimp-like crustacean is its rapid autolytic degradation post mortem. This self-deterioration is mediated by the activity of endogenous enzymes, especially the peptide hydrolases. These enzymes have recently been isolated and characterized, and their relevance in protein degradation reviewed[4]. The enzymes hitherto identified include three trypsin-like serine proteases of which one seems to possess a significant exopeptidase effect in addition to endopeptidase activity. The other two trypsin-like enzymes are true endopeptidases. Moreover, five enzymes exhibiting exopeptidase activity were identified and purified: two carboxypeptidases of the A-type, two carboxypeptidases of the B-type, and an aminopeptidase.

Material and methods. Enzyme preparations: The krill *(Euphausia superba)* raw material, originating from Japanese commercial catches (Taiyo Fishery Co.), was frozen aboard and kept at −20 °C until used. The krill proteases were isolated from a defatted aqueous extract, gel chromatographed and freeze-dried.

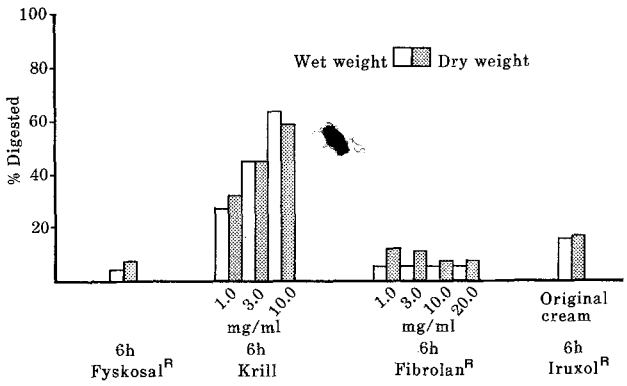
Commercially available crystalline trypsin (Trypure®, Novo), streptokinase-streptodornase (Varidase®, Cyanamid), collagenase (Iruxol®, Knoll) and plasmin-desoxyribonuclease (Fibrolan®, Parke-Davis),were used for comparison.

Determination of proteolytic activity: Comparisons of the proteolytic activity between Trypure® and the krill enzyme preparation were established by using two different biochemical enzyme assays.

The total proteolytic activity was determined with denatured casein as a substrate[5]. The enzymatic reaction – performed in 0.1 M Tris-HCl buffer, pH 7.5 – was terminated after 20 min by the addition of 5% trichloroacetic acid for precipitation of protein. After high-speed centrifugation and removal of the sediment, the supernatant was measured spectrophotometrically at 578 nm for the analysis of free aromatic amino acids using the Folin-Ciocalteau reagent. A tyrosine standard was used as reference

Enzymatic degradation of animal necrotic tissue (excised rat skin) in vitro at 37 °C and for 24 h (wet weight)

	20 mg/ml	10 mg/ml	5 mg/ml
Krill			
Weight before	66 mg	60 mg	69 mg
Weight after	0	0	18 mg
	−66 mg	−60 mg	−51 mg
Digested	100%	100%	74%
Trypure®			
Weight before	69 mg	63 mg	58 mg
Weight after	29 mg	29 mg	29 mg
	−40 mg	−34 mg	−29 mg
Digested	58%	54%	50%
Varidase®			
Weight before	69 mg	58 mg	59 mg
Weight after	61 mg	47 mg	46 mg
	− 8 mg	−11 mg	−13 mg
Digested	12%	19%	22%



Digestion rate in an animal necrotic model (30 mg of excised rat skin) incubated at 37 °C with 1–10 mg protein/ml of krill enzymes, Fibrolan®, Iruxol® and Fyskosal® (saline) after 6 h. The values are based on wet/dry weight determinations.

solution. One unit of enzyme activity causes the liberation of 1 μmole of tyrosine/ml/min at 35 °C.

The ester-hydrolyzing activity was based on the hydrolysis of p-toluenesulfonyl-L-arginine methyl ester (TAME) as a substrate dissolved in 46 mM Tris-HCl buffer pH 8.1, containing 11.5 mM $CaCl_2$[6]. The substrate was quickly mixed with the enzyme preparation at 25 °C and the absorbancy was monitored continuously at 247 nm for 3–4 min. One unit of enzyme activity causes the hydrolysis of 1 μmole of substrate/min.

Determination of protein: The protein content of Trypure® and the krill enzyme preparation was established by two different methods; UV absorbancy and the Folin method[7], respectively. This is due to the fact that the Folin method gives protein values for trypsin that are too high (unpublished data). The cause of this phenomenon is the higher content of aromatic amino acids (tryptophan, tyrosine and phenylalanine) in the trypsin molecule than in the standard used (bovine serum albumin). The molar extinction coefficient ($\varepsilon_{280}^{1\%}$) for bovine trypsin of 14.3 was used when measuring the absorbancy of the Trypure® samples.

Evaluation of debriding effects in vitro: The experiments were performed by incubating pieces of excised rat skin of approximately 30 mg or 60 mg wet weight with the enzyme solutions in varying concentrations for 6–24 h at 37 °C. After incubation the remaining pieces were carefully blotted with filter paper and weighed immediately (wet weight) and after drying to constant weight for 90 h at 60 °C (dry weight).

Results and discussion. Experimental data regarding the debriding effect of different enzymes in the animal necrotic model (excised rat skin) are shown in the table and the figure. The results clearly demonstrated that the krill enzyme preparation had a higher level of activity than the other enzyme preparations. Even at the lowest concentration of krill protein (5 mg/ml) around 70% of the excised rat skin was digested in 24 h, while trypsin managed around 50% in the same period. With increasing concentration the debriding effect of the krill enzyme increased up to 100%. For trypsin, however, the debriding activity remained around 50% for 5 mg/ml and higher concentrations. The effect of streptokinase-streptodornase, collagenase and plasmin-desoxyribonuclease resulted in approx. 10–20% degradation, practically unaffected by concentration.

The same pattern was found when either wet or dry weight data were compared (fig.). The wet weight figures showed a clear correlation with the dry weight determinations, but were somewhat higher throughout. This is due to the tissue hydration, which is especially pronounced in the samples exposed to streptokinase-streptodornase (liquefaction)[8].

The results reported here accord well with data from biochemical assays, where one single enzyme preparation (Trypure®) was compared with krill enzymes (multi-enzyme system). In spite of the fact that the proteolytic activity of the krill sample, using casein or p-toluene-sulfonyl-L-arginine methyl ester (TAME) as a substrate, was 6-8 times less than that of Trypure® the debriding effect of the latter was weaker even though the amount of enzyme was increased. One possible explanation of this fact could be that this mammalian enzyme has a too high specificity compared with the krill enzymes which originate from a primitive organism in an early evolutionary stage with a broad spectrum of activity.

In conclusion, our experimental in vitro data support the idea that it is more important to develop an enzymatic debrider containing co-operative individual enzymes than one containing pure single enzymes or few enzyme systems, with limited and too-specific degrading effects on complex biological substrates. Krill proteases possess these unique properties, associated with good stability and a low autodigestion rate.

Based on the broad safety margin shown by current toxicity/allergenicity studies, a first phase II clinical trial was performed in our clinic. One week of treatment with 0.1% protein solution (krill enzymes) on 19 necrotic secondary ulcers resulted in a significant reduction of necroses and complete removal of fibrin coat. The pain was ameliorated and no side effects were observed (unpublished data).

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