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A note on the use of protease inhibitors during chromatin fractionation on hydroxyapatite columns

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Summary. It was found that $NaHSO_3$ present in the eluents enabled full and reproducible recovery of chromosomal proteins from a hydroxyapatite column. Another protease inhibitor, PMSF, did not have that effect.
Key words. Chromatin proteins; hydroxyapatite; protease inhibitors.

Hydroxyapatite (HAP) chromatography is a very widely used method of obtaining chromatin proteins. It makes it possible to obtain nonhistone proteins free from histones and nucleic acids by a single-column procedure, without long ultracentrifugation[1,2]. The major disadvantage of the method is the changing amount of proteins eluted from the column. Rickwood and MacGillivray[1] recommended washing nuclei with sodium deoxycholate for chromatin with totally 'elutable' proteins. Unfortunately, the nuclei treated with deoxycholate were found by the authors to be devoid of some proteins especially of high mol. wt.

Materials and methods. White Wistar male rats (120–150 g) were killed by decapitation and livers were quickly excised, perfused with ice-cold saline and immediately used for further experiment. All solutions used for obtaining cell nuclei and chromatin contained phenylmethylsulphonylfluoride (PMSF) added freshly as an 0.1 M solution in isopropanol[3]. All preparative and chromatographic work was carried out at 4 °C. Cell nuclei were obtained by the method of Widnell and Tata[4] with an additional washing with 0.5% Triton X-100 in isotonic sucrose. Chromatin was prepared according to Spelsberg and Hnilica[5]. The nuclei were washed twice with saline-EDTA and swollen in 1.5 mM NaCl – 0.15 mM sodium citrate. The chromatin was sonicated, adjusted to about 0.25 mg DNA/ml and applied onto a column. The column was washed with 1 mM potassium phosphate buffer (pH 7.5) and then the total chromatin proteins were eluted with a buffer containing 2 M KCl, 5 M urea, 80 mM potassium phosphate (pH 7.5). The solutions for washing and elution contained 5 mM $NaHSO_3$ (analytical grade, P.O.Ch.) or 1 mM PMSF (Sigma).

Protein concentration was estimated in the effluent by the dye binding method of Bradford[6] using bovine serum albumin as a standard.

Results and discussion. Samples of chromatin obtained in the same preparation cycle were run simultaneously on HAP columns with eluents containing one of the two protease inhibitors: $NaHSO_3$ or PMSF. The results given in the table showed that the amount of protein is 2.61–2.75 times higher when sodium bisulfite is added to the eluents.

Since Carter and Chae[3] showed that PMSF is a powerful protease inhibitor it has been widely used in chromatin studies ever since, though there has recently been critical discussion concerning its inhibitory properties[7]. We found that it cannot replace $NaHSO_3$ when total recovery of proteins from HAP is expected. Sodium bisulfite was used as an inhibitor of proteolytic activity by Chiu and his coworkers[8] during the removal of the bulk of nonhistone proteins with 5 M urea and by Fredericq and Hacha[9] in sequential elution of histones and nonhistone proteins from HAP columns. The eluents were composed of 2 M KCl and various concentrations of phosphate buffer only. The use of $NaHSO_3$ may have contributed to the successful separation without applying any strong solubilizing agents (urea or guanidine hydrochloride). Later, bisulfite was used by Bloom and Anderson[10] who reported a quantitative separation of chromatin

Total chromatin proteins (mg) recovered from an HAP column with 2 M KCl – 5 M urea – 80 mM potassium phosphate buffer (pH 7.5) supplemented with either 1 mM PMSF or 5 mM $NaHSO_3$. 1 mg chromatin DNA was applied onto a 2 ml column

No. of sample	Eluent + PMSF	Eluent + $NaHSO_3$
1	0.68	1.79
2	0.71	1.85
3	0.65	1.79

proteins on HAP columns. The 'releasing' activity of sodium bisulfite can be explained by its inhibiting a thiol protease insensitive to PMSF which produces protein fragments tending to form insoluble complexes. In spite of the absence of $NaHSO_3$ throughout the preparation of cell nuclei and chromatin we were able to recover the chromatin proteins almost completely, which would suggest that the protease is activated only when the chromatin complex has been dissociated.

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Proteases of Antarctic krill – a new system for effective enzymatic debridement of necrotic ulcerations

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Summary. The Antarctic krill *(Euphausia superba)* possesses an 'over-dimensioned' digestive system, which is of vital importance for the survival of this euphaucean shrimp in the extreme marine environment. The isolated enzymes contain a well-balanced mixture of both endo- and exopeptidases, assuring fast and complete breakdown of proteinaceous material. These unique properties have now been shown to be extremely valuable for the effective removal of necrotic debris, fibrin or blood crusts in vitro. Therefore the krill enzymes should be considered as an important resource in the future management of necrotic wounds.
Key words. Antarctic krill *(Euphausia superba)*; proteases; enzymatic debridement in vitro.

Tissue necroses in secondary ulcers due to skin damage of diverse etiology such as burns, leg ulcers, decubitus is a common clinical problem. Although many factors are important in the healing of wounds, there is no doubt that the correct strategy in determinig the appropriate therapeutical approach must be first of all to attempt to identify and treat the main cause of the ulcers as well as the other underlying, negative factors. Adequate treatment of the systemic factors and disorders causing deterioration is thus a prerequisite for successful local wound care.

The local treatment of leg ulcers includes not only the removal of factors preventing healing (necroses, pus, fibrin, infections, etc.) but also the addition of those that improve the milieu of the healing wound, such as moisture.

The devitalized material, such as necroses, pus, blood crusts and fibrin, which accumulates in the wound delays the healing process. Therefore, one of the most important objectives of topical treatment is a rapid and efficient removal of necrotic debris, pus and fibrin so that a normal sequence of events, such as granulation and reepithelialization can take place.

Initially, the ulcerated area can be debrided either enzymatically or surgically. The main proteolytic enzyme preparations available at present in Scandinavia are stabilized crystalline trypsin (Trypure®, Novo), streptokinase-streptodornase (Varidase®, Lederle), bovine fibrinolysin combined with deoxyribonuclease (Elase®, Park-Davis) and collagenase (Iruxol®, Knoll). All of these enzyme preparations differ in their effectiveness as regards different substrates. However, it is generally agreed that their effect is not sufficient to permit a rapid debridement of the kind which is desirable in modern wound-care.

Previously we have reported in vitro and in vivo observations regarding the effect of stabilized, crystalline trypsin (Trypure®, and streptokinase-streptodornase (Varidase®) as debriding agents[1,2]. In connection with this work we also screened other enzymes of animal, bacterial and plant origin including some enzyme preparations of marine origin. One of these, obtained from Antarctic krill *(Euphausia superba)* was shown to possess outstanding debriding properties.

Krill is a marine crustacean which occurs in very large numbers in the Antarctic region. The common Antarctic krill, *E. superba*, attains a length of approximately 60 mm in the adult stage and lives at temperatures approaching that of freezing sea water. The distribution and biology of Antarctic krill have been reviewed by Everson[3]. Krill has developed a particularly efficient digestive

Table 1. The influence of proteolytic enzymes on different substrates originating from leg ulcers. The effects are expressed as percentages of the initial values (dry weight). The figures within brackets represent intermediate stages

– – – –	Weight decrease	76–100%
– – –	Weight decrease	51–75%
– –	Weight decrease	26–50%
–	Weight decrease	1–25%
0	No change	0%

Table 2. The influence of trypsin (Trypure®), streptokinase-streptodornase (Varidase®), krill enzymes and saline (control) on blood crusts isolated from leg ulcers. The data (dry wts) are expressed as percentages of the initial values, and ranked according to the scale in table 1

Enzyme	Concentration mg/ml	Time of exposure (h) 1	2	4
Trypsin	1	–	– –	– – (–)
	5	– –	– – (–)	– – –
	10	– – –	– – –	– – – –
Streptokinase-streptodornase	1	0	(–)	–
	5	(–)	–	–
	10	(–)	(–)	– (–)
Krill	1	– –	– – –	– – – (–)
	5	– – (–)	– – –	– – – –
	10	– – – (–)	– – – –	– – – –
Saline		0	(–)	(–)