Purification and partial characterization of a novel hyaluronic acid-degrading enzyme from Antarctic krill (*Euphausia superba*)

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Summary. A novel enzyme degrading hyaluronic acid has been isolated, purified and characterized from Antarctic krill (*Euphausia superba*). A combination of affinity chromatography (Con A-Sepharose), gel filtration (Superose 6) and fast protein liquid chromatography (Mono Q) was used for the purification. The hyaluronidase activity was determined by a radial diffusion method based on hyaluronic acid incorporated into an agarose gel. Moreover, the beta-glucuronidase and endo-(1,3)-beta-D-glucanase activities were also followed through the process using phenolphtalein mono beta-glucuronic acid and laminarin as substrates. After the final purification step on Mono Q column, the chromatogram showed three main peaks designated A, B and C. Peak C contained high hyaluronidase activity undetectable in peak A and B. The beta-glucuronidase activity was associated with peak A, while the endo-(1,3)-beta-D-glucanase activity was found in peak B and slight in peak C. The hyaluronidase was purified about 85-fold. It had a pH optimum of 5.3, a temperature optimum of 37°C and a molecular weight of 80 000 Daltons. On polyacrylamide gradient gel electrophoresis the enzyme fraction showed one major band associated with hyaluronic acid decomposition, slightly contaminated with a few other components. Isoelectric focusing in combination with a hyaluronic acid zymogram demonstrated one major band at pH 6.7 with high enzyme activity. Preliminary data on enzyme specificity suggest that krill hyaluronidase is a new endo-beta-glucuronidase and support the concept of krill enzymes as a remarkable and unusually effective digestive system adapted to the Antarctic marine ecosystem.

Introduction

*Euphausia superba*, the Antarctic krill is one of the dominant species in the Antarctic Ocean. Krill is characterized by a rapid post mortem autolysis caused by high levels of digestive enzymes. Several reports demonstrating high proteolytic activity in krill have been published (for review Osnes and Mohr 1985). Since krill feed mainly on the abundant phytoplankton which has a high content of various carbohydrates in addition to proteins, it has also been assumed that krill may contain highly active polysaccharide-degrading enzymes in its digestion tract. However, little is known about these enzymes with the exception of a few published papers. A specific endo-(1,3)-beta-D-glucanase possessing high activity with laminarin as substrate has been purified and characterized (Turkiewicz et al. 1985). Other studies have revealed the presence of exo-(1,3)-beta-D-glucanase and beta-D-glucosidase (Chen and Lian 1986; McConville et al. 1986) proving explicitly, that *E. superba* produces the system of enzymes which may be responsible for decomposition of (1,3)-beta-D-glucans in its diet. Recently chitin-degrading enzymes have been shown to be involved in the digestion of chitin-containing food (Spindler and Buchholz 1988). The breakdown products, essentially aminosugars, will be channeled into the metabolic pool and become precursors for the synthesis of new cuticle. Further data on degradation of acidic sulphated polysaccharides like carrageenan and polygalacturonic acid indicated very low or no activity in krill (Chen and Gau 1981; McConville et al. 1986).

The aim of our studies was to investigate whether any enzyme activity for hyaluronic acid does exist in *E. superba* and secondly examine the synergistic action of enzymes involved in the degradation of the substrate. The present study deals with the purification and characterization of a krill hyaluronidase. Furthermore, the beta-glucuronidase and endo-(1,3)-beta-D-glucanase activities were followed through the process and the enzymes partially purified.

Materials and methods

Krill samples

Antarctic krill (*E. superba*), caught in 1986, was obtained from Taiyo Fishery, Co. Ltd. (Tokyo, Japan). The samples of krill were delivered
in frozen blocks and were kept at −20°C until preparation of a defatted crude aqueous extract.

**Reagents, chemicals and equipments**

Phenolphtalein mono-beta-glucuronic acid (P-0376), laminarin (L-9634), methyl-alpha-D-mannopyranoside (M-6882) and hyalurondase from bovine testes (H-3631) were obtained from Sigma (St. Louis, MO). Healon® (10 mg/ml) was manufactured by Pharmacia (Uppsala, Sweden). Tris-(hydroxymethyl)-amino methane (TRIS), 3,5-dinitrosalicylic acid, potassium-sodium tartrate (C₄H₄O₆) and acrylamide (PAA 4/30) were obtained from Merck (Darmstadt, FRG).

Con A-Sepharose, Sepharose 6 prep grade, Agarose A, polyacrylamide gradient gels (PAA 4/30), gels filtration calibration kit, isolectric focusing calibration kit (pH 3-10) and Pharmalyte (pH 3-10) were from Pharmacia. Superose 12 HR 10/30 gel filtration and Mono Q HR 5/5 anion exchange columns connected to a FPLC-system were also from Pharmacia as well as other chromatographic and electrophoretic equipments. Ultrafiltration cell and membranes (YM 10) were obtained from Amicon Corporation (The Netherlands).

**Purification of hyaluronidase**

**Krill extract.** Freshly frozen krill was thawed in a cold room and defatted with acetone. The defatted residue, after removing the solvent by centrifugation, was homogenized in distilled water and centrifuged at 10 000 x g for 30 min at 4°C. The supernatant was collected, defatted and concentrated through an Amicon YM 10 membrane filter. This concentrate was used for isolation of krill hyaluronidase.

**Affinity chromatography on Con A-Sepharose.** Commercially available Con A-Sepharose gel was washed with the appropriate buffer (20 mM TRIS-HCl, pH 7.5, 1 mM Mn²⁺, 1 mM Mg²⁺, 1 mM Ca²⁺, 1 M NaCl) and packed in a K 16/20 column to a height of 15.5 cm. The column was further equilibrated with the same buffer at room temperature. Fifteen to 30 ml of the crude krill aqueous extract, with addition of solid salt to the same concentration as the elution buffer itself, was applied to the column and eluted with 200 ml of buffer at a flow rate of 13 ml/h. The column was subsequently desorbed with buffer containing 5% methyl-alpha-D-mannopyranoside. The elution was continuously monitored at 280 nm until no UV-absorbing material was detected in the effluent volume. The fractions collected (2 ml/fr) were assayed for hyaluronidase activity and the active fractions were pooled and concentrated by using an Amicon YM 10 membrane filter.

**Gel filtration.** Approximately 4 ml of the concentrated Con A-pool was applied to a Superose 6 prep grade column (1.6 cm x 100 cm), equilibrated with 50 mM TRIS-HCl buffer, pH 7.5, at room temperature. The column was eluted with buffer at 12 ml/h and the elution was followed spectrophotometrically at 280 nm. The fractions (2 ml/fr) were assayed for hyaluronidase activity and the active fractions were pooled.

**Fast protein liquid chromatography.** The pool from the gel filtration step was further purified using a strong anion exchange column (Mono Q HR 5/5) equilibrated with 50 mM TRIS-HCl buffer, pH 7.5, at room temperature. Four ml of sample was applied to the column and the proteins were eluted with a stepwise gradient of NaCl from 0-0.18 M, 0.18-0.34 M and 0.34-1.0 M. The fractions (1 ml/fr) were assayed for hyaluronidase, beta-glucuronidase and endo-(1,3)-beta-D-glucanase activities and the active fractions of each enzyme were pooled, respectively.

**Enzyme assays**

**Assay of hyaluronidase activity.** A simple plate assay for determination of hyaluronidase activity was used according to Richman and Baer (1980). Briefly hyaluronic acid was incorporated into a 1.5% agarose gel and the enzyme, 8µl of different concentrations, was allowed to diffuse from punched wells at 37°C for 20 h. The undigested hyaluronic acid was then precipitated by addition of cetylpyridinium chloride and clear circles appeared after approximately 30 min. The diameters of the circles were proportional to the logarithm of the enzyme concentration applied to the well. Testicular hyaluronidase was used as a reference standard enzyme preparation for making the standard curve (Fig. 1).

**Assay of beta-glucuronidase.** Phenolphtalein mono-beta-glucuronic acid was used as a substrate for detection of beta-glucuronidase, as described by Fishman et al. (1948). A series of tubes was filled with 0.8 ml of 0.1 M NaAc, pH 4.5 + 0.1 ml of phenolphtalein glucuronic acid, pH 7.0 and incubated at 37°C for 5 min. At timed intervals each sample was added 0.1 ml of enzyme solution and the blank 0.1 ml of distilled water. The tubes were incubated at 37°C for 30 min. The reaction was stopped by adding 5.0 ml of 0.2 M glycine, 0.2 M NaOH and the samples were cooled to room temperature and read at 540 nm. The number of micromoles phenolphtalein liberated were calculated by means of a standard curve. One unit equals one micromole of phenolphtalein glucuronate/min (37°C, pH 4.5).

**Assay of endo-(1,3)-beta-D-glucanase.** Laminarin, a polysaccharide consisting of beta-D-glucose residues joined mainly through 1:3 linkages, was used as a substrate for detection of endo-(1,3)-beta-D-glucanase activity as described by Turkiewicz et al. (1985). The enzyme activity unit is expressed as micromoles of glucose released/min.

The glucose released was assayed with dinitrosalicylic acid (Bernfeld 1955). 0.5 ml of enzyme solution, buffered with 10 mM sodium phosphate buffer, pH 6.2, was incubated with 0.5 ml of substrate (1 mg laminarin/ml in buffer as above) for 10 min at 50°C. One ml of dinitrosalicylic acid reagent was added immediately. The tubes were put in a boiling water bath and heated for 5 min. Finally, the samples were cooled and 8.0 ml of distilled water was added. The absorbance was read immediately at 540 nm. The number of micromoles of glucose liberated was calculated from a standard curve and the enzyme activity was expressed as units/min.

![Fig. 1. The hyaluronidase activity was determined by using a simple plate assay based on hyaluronic acid as substrate incorporated in 1.5% agarose. The zone diameter, as a result of digested substrate, was read after 20 h at 37°C and was proportional to the logarithm of the activity. Testicular hyaluronidase was used as a reference for the standard curve.](image-url)