南極産オキアミの筋肉部プロテアーゼの部分精製とその性質

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Partial Purification and Characterization of Proteinases from Abdomen Part Muscle of Antarctic Krill

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Proteinase activities of abdomen muscle of antarctic krill were very low compared with those in cephalothorax portion. From the badomen muscle extract of antarctic krill *Euphausia superba*, three types of proteinases were separated by DEAE-cellulose ion exchange chromatography, and designated proteinases I, II and III, respectively. Each proteinases were further purified by gel filtration on Sephadex G-100, DEAE-cellulose (2nd) and hydroxyapatite chromatography. Proteinase I hydrolyzed hemoglobin maximally at pH 4.0 and azocasein at pH 5.0. Since proteinase I was inhibited by leupeptin, antipain, monoiodoacetic acid and PCMB, it was classified into thiol proteinase including cathepsin L (EC 3.4.22.15). The molecular weight was determined to be 80,000, which is higher than that of the muscle of cathepsin L so far reported.

Proteinase II hydrolyzed azocasein maximally at pH 6.0. Proteinase II was inhibited by soybean trypsin inhibitor, PMSF and chymostatin. Enzymatic property of proteinase II was similar to that reported for chymotrypsin-like (EC 3.4.21.1) proteinase in krill cephalothorax, but its molecular weight was higher than that of the latter.

Since proteinase III hydrolyzed hemoglobin maximally at pH 3.0 and was inhibited by pepstatin, it was a typical acid proteinase.

Euphausia superba, a kind of krills inhabit the Antarctic Ocean, is considered to be one of promissing source of human foods in the future because of its nutritive value[2,3] and enormous population.[4] However, the rapid tissue decomposition and freshness decline during cold storage of krill is empirically known. Proteinases are most likely responsible for the deterioration. Konagaya[5] reported the proteinases which effected on the autolysis of antarctic krill and Noguchi *et al.*[6] and Seki *et al.*[7] purified and characterized some proteinases of antarctic krill.

The muscle portion of fish is important for food protein, because in Japan, it is extensively utilized as food materials for fish paste such as Kamaboko. Therefore, the usage of muscle portion of antarctic krill is also expected as food. Kawamura *et al.*[8] studied on the autolysis in abdomen muscle portion of antarctic krill and suggested the presence of some proteinases. There are many reports about the proteinases from the whole body of antarctic krill but few reports about them from the muscle portion. We planned to isolate and characterize as much proteinases as possible in *E. superba* in order to obtain the basic information with the reference to quality of krill and have reported studies of proteinases from the whole body of *E. superba*.[9-12] This paper describes separation and characterization of proteinases of muscle portion of antarctic krill.

Materials and Methods

Euphausia superba

E. superba were obtained in the Antarctic Ocean, frozen immediately at −30°C and stored at −60°C until use.

Materials

Soybean trypsin inhibitor (STI), azocasein, *N*-*p*-toluenesulfonyl-1-lysine-chloromethyl-ketone (TLCK) and *N*-*p*-toluene-sulfonyl-2-phenylalanine-chloromethyl-ketone (TPCK) were obtained from Sigma Chemical Co. Leupeptin, antipain, pepstatin and chymostatin were from the Protein Research Foundation. DEAE-cellulose (DE-52) was from whatman Co. Sephadex G-100 was from Pharmacia Fine Chemicals. All other chemicals and materials were of reagent grade.

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Extraction of Proteinases

The whole body of antarctic krill was divided into cephalothorax portion and abdomen portion. Each portion was homogenized in three volumes of 0.02 M phosphate buffer, pH 7.0, centrifuged at 10,000×g for 30 min, and the enzyme solutions from each portion were obtained.

Proteinase Assay

A reaction mixture containing 0.2 m*l* of enzyme solution and 1 m*l* of acid denatured hemoglobin[13] (1% in 0.2 M glycine-HCl buffer, pH 3.0) was incubated at 37°C for 3 h. The reaction mixture contained 0.1% toluene. After the addition of 1 m*l* of 10% TCA, the reaction mixture was filtered and the absorbance of the filtrate at 280 nm was measured. The assay of azocasein-hydrolyzing activity was made as described above, except that incubation time and pH were 20 h and pH 6.0, and the absorbance of the filtrate was measured at 410 nm. One unit was defined as the absorbance of 1.000 (10 mm light path) per incubation time (h).

Protein Concentration

The protein concentration was measured spectrophotometrically at 280 nm, assuming that the absorbance of 1 mg/m*l* of solution is 1.000.

Determination of Molecular Weight

Molecular weights were determined by gel filtration on Sephadex G-100. Aldolase (MW, 140,000), bovine serum albumin (MW, 68,000), ovalbumin (MW, 45,000) and myoglobin (MW, 17,000) were used as molecular weight markers.

Results

Proteinase Activities

In abdomen portion, acid proteinase activity at pH 3.0 was one fifth, and azocasein-hydrolyzing activity at pH 6.0 was one twentieth, when compared with those in the cephalothorax portion (Table 1). Benzoyl-DL-arginine-*p*-nitroanilide (BAPA)-hydrolyzing activity was not detected in the abdomen muscle porion. The proteinase activities in abdomen portion were very low but might participate more or less proteolysis in the abdomen portion.

Separation and Purification of Proteinases from Abdomen Part

Proteinase in the abdomen extract were separated into three components by DEAE-cellulose

Table 1. Proteinase activities of cephalothorax and abdomen protions of *E. superba*

Substrate	pH*	Cephalothrax portion	Abdomen portion
Hemoglobin	3	6.2 units/m*l*/h	1.2 units/m*l*/h
Azocasein	6	6.6 units/m*l*/h	0.17 units/m*l*/h
BAPA	8	7.3 units/m*l*/h	0

* pH value of the assay mixture

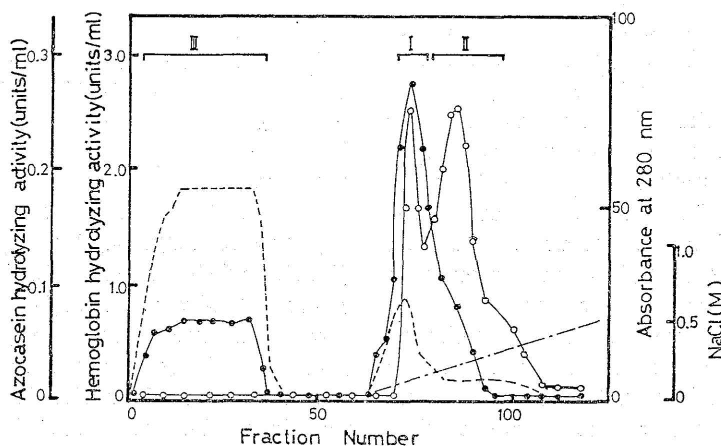

Fig. 1. Chromatography of proteinases from abdomen portion of antarctic krill on DEAE-cellose column.

The extract was applied to a DEAE-cellulose column (2.2×25 cm), previously equilibrated with 0.02 M phosphate buffer, pH 7.0. This column was washed with the same buffer and eluted with a 1,000 m*l* linear gradient of 0–1 M NaCl in the same buffer. ●—●, hemoglobin hydrolyzing activity; ○—○, azocasein hydrolyzing activity; ---, protein concentration.

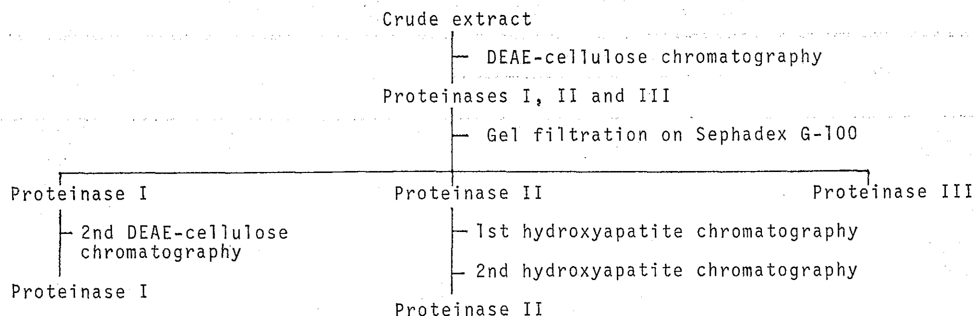

Fig. 2. Purification step of proteinases I, II and III.

Table 2. Purification of proteinases I, II and III

Proteinase	Procedure*	Yield	Specific activity (unit/mg protein)	Purification (fold)
Proteinase I	2nd DEAE-cellulose chromatography	37	0.74	426
Proteinase II	2nd hydroxyapatite chromatography	13	0.99	124
Proteinase III	Sephadex G-100 gel filtration	34	0.17	97

* The final purification step

Table 3. Enzymatic properties of proteinases I, II and III*

	Proteinase I		Proteinase II	Proteinase III
Substrate	Hemoglobin	Azocasein	Azocasein	Hemoglobin
Optimum pH	4.0	5.0	6.0	3.0
Optimum temperature	45°C	45°C	45°C	45°C
Range stable to pH	3–8	3–8	5–8.5	3–7
Range stable to temperature	0–40°C	0–40°C	0–40°C	0–40°C
Molecular weight	80,000		52,000	58,000

* After enzyme solution was preincubated at 37°C and various pH for 15 min, the remaining activity was measured for pH stablity test. Range stable test to temperature was measured the enzyme solution had been stood for 15 min at pH 6.0 and various temperatures.

ion exchange chromatography, being designated proteinases I, II and III, respectively (Fig. 1). Proteinases I and III hydrolyzed hemoglobin at pH 3.0. Proteinases I and II hydrolyzed azocasein at pH 6.0. Fractions of each proteinase activity eluted were combined and concentrated with Toyo Ultra Filter UK-10.

Each of proteinases I, II and III fraction was applied to a Sephadex G-100 gel filtration. Each active fractions of proteinases I and II from gel filtration were collected and further purified as follows (Fig. 2). Fraction of proteinase I was further chromatographed by second DEAE-cellulose column. Both proteinase activities against hemoglobin at pH 3.0 and azocasein at pH 6.0 were eluted at the same position and were observed to give the same peak. Two different activities may be due to the same enzyme. Proteinase II was further chromatographed twice on hydroxyapatite columns. Purification of final preparations, proteinases I, II and III, was shown in Table 2. These proteinase preparations were stored at −30°C for the following characterization.

Enzymatic Properties of Proteinases I, II and III.

Proteinase I and II hydrolyzed azocasein maximally at pH 5.0 and 6.0, respectively, while maximum hydrolyses of hemoglobin by proteinases I and III were observed at pH 4.0 and 3.0, respectively. Optimum temperature of the three proteinases were 45°C. Proteinases I, II and III were stable between pH 3–8, pH 5–8.5 and pH 3–7, respectively, below 40°C. The molecular weight of proteinases I, II and III were estimated to be 80,000, 52,000 and 58,000, respectively, by

Table 4. Effect of inhibitors on proteinase activities*1

Inhibitors	Proteinase I*2 (PH 3.0)	Proteinase I*2 (pH 5.0)	Proteinase II	Proteinase III
None	100%	100%	100%	100%
PMSF	98	96	21	92
DFP	94	95	10	99
STI	97	90	24	95
Pepstatin	90	100	100	6
Antipain	17	40	92	95
Leupeptin	10	30	81	40
Chymostatin	2	41	1	92
Monoiodoacetic acid	31	56	80	88
TLCK	17	47	90	86
TPCK	98	99	96	100
PCMB	40	50	83	81
EDTA	100	100	95	94
Dithiothreitol	100	100	100	100

*1 0.2 m*l* of enzyme solution was preincubated with 0.02 m*l* of each inhibotor at 37°C for 15 min. The remaining activity was assayed. The concentrations of all inhibitors were 1 mM in the preincubation mixture except for STI which was 1 mg/m*l*.
*2 Activities against hemoglobin at pH 3.0 and azocasein at pH 5.0 were measured.

gel filtration on Sephadex G-100. These molecular weights were higher than that of any of proteinases A_1, A_2, B and C separated previously[10,12] from the whole body of antarctic krill. Results were collectively given in Table 3.

Effect of Inhibitors on Proteinases from Abdomen Portion

Proteinase I was neither inhibited by soybean trypsin inhibitor, diisopropyl fluorophosphate (DFP) and phenylmethylsulfonyl fluoride (PMSF) which normally inhibited krill proteinases,[19,12] nor by pepstatin which inhibits acid proteinase. Wherese, it was inhibited by leupeptin, antipain and monoiodoacetic acid which inhibit thiol proteinases. As shown in Table 4, the inhibition profiles of hemoglobin-hydrolyzing activity at pH 3.0 and azocasein hydrolyzing activity at pH 6.0 were very similar to each other. These findings suggested that proteinase I had two hydrolysis activities towards hemoglobin and azocasein. Proteinase II was inhibited by PMSF, soybean trypsin inhibitor, and chymostatin but not antipain, leupeptin and TLCK which inhibit trypsin (EC 3.4.21.4)-type proteinase. Proteinase II was similar to chymotrypsin-like proteinase from cepharothorax portion of antarctic krill,[12] but this enzyme's moleular weight was higher than that of each cepharothrax proteinase. Proteinase II was classified into chymotrypsin-like proteinase.

Since Proteinase III was inhibited strongly by pepstatin, it was concluded to be a typical acid proteinase.

Discussion

The krill extracts was confirmed not to be contaminated by cepharothorax proteinases, by the findings that benzoyl-DL-arginine-*p*-nitroanilide hydrolysis activity, being very high in cephalothorax portion, was not detected at all in the abdomen extract.

From the abdomen extract, three types of proteinases, designated as proteinases I, II and III, were separated and characterized. Proteinase I was tentatively classified into cathepsin L type, since it was inhibited by leupeptin, antipain and monoiodoacetic acid but not by DFP and PMSF, and it hydrolyzed the natural substrates of azocasein and hemoglobin. Proteinase I had higher molecular weight than that reported for cathepsin L from rat liver[14] and rabit skeltal muscle,[15] but its enzymatic properties above mentioned seemed to be similar so far reported cathepsin L.[1] Proteinase III was classified into cathepsin D (EC 3.4.23.5) type by the inhibition by pepstatin. Since it is known that cathepsin L and D[1,16-19] degrade the muscle protein, proteinase I and III are possibly respon sible for the muscle degradation of antarctic krill. Kawamura *et al.*[8] reported that the autolysis in abdomen part of antarctic krill was inhibited by leupeptin, monoiodoacetic acid and pepstatin. Properties of proteinases I and III support their statement. Proteinase II was similar to chymotrypsin-like proteinase from antarctic krill cephalothorax,[8] judged from the enzymatic properties. However, proteinase II was different

from the cephalothorax chymotrypsin-like proteinase, because the former has higher molecular weight than the latter. Therefore, it was assumed that in abdomen part, chymotrypsin-like proteinase having high molecular weight, was present other than that reported in cephalothorax portion. Proteinase II is also likely responsible for the autolysis in abdomen portion. Further study is still necessary as their behavior in the tissue. Calcium-activated proteinase which is well known to be present in the muscle of various species was not detected in this material.

The muscle of antarctic krill is expected to be utilized as food but it is known to be very fragile, compared with that of other fishes.[20] Proteinase in the abdomen portion seem to accelerate the fragility and deteriolation of the muscle protein of antarctic krill. Moreover, their presence cannot be considered to have any beneficial effects on processing products.

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