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Biochemistry of the autolytic processes in Antarctic krill *post mortem*

Autoproteolysis

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1. Autoproteolysis *post mortem* was examined at 0 °C by following the changes in the major classes of krill (*Euphausia superba* and *Euphausia crystallorophias*) proteins and by liberation of peptides and free amino acids, and was based on experiments conducted on board expedition vessels in the Antarctic. 2. Primarily salt-soluble proteins were broken down during the first week of incubation, whereas water-soluble and insoluble proteins were degraded to a much smaller extent. The enzymes responsible for the hydrolysis presumably originate primarily from the digestive apparatus of the krill. 3. In general, the individual amino acids were released at rates corresponding to their relative occurrence in the bulk protein of the krill. Alanine was liberated in larger amounts than would be expected from the composition of the krill protein, and was evidently formed also by reactions other than proteolysis. Glutamic acid, and certain amino acids which presumably occur with high frequency adjacent to glutamic acid residues in the krill protein, were liberated only to a limited extent, and accumulated in smaller peptides. 4. During proteolysis, arginine seemed to be converted to some degree into ornithine, and on prolonged incubation conversion of arginine and lysine into their corresponding decarboxylation products, agmatine and cadaverine, appeared to take place.

INTRODUCTION

Antarctic krill comprise a group of shrimp-like crustaceans which occupy an unique position in the ecosystem of the Antarctic Oceans (Everson, 1977). The krill represent the major food source for most higher forms of life in the Antarctic region, including fishes, birds, and mammals such as seals and whales. The ecological situation of krill is highly special. During much of the year the krill apparently experience a very limited supply of food. Food only becomes available in abundance during the short Antarctic summer, when sunlight allows the primary production in the Ocean to accelerate.

As a result, the survival of krill seems to depend on their ability to utilize available food and build up energy reserves during a short period of food plenty in the Antarctic. This is probably a major reason why Antarctic krill seem to possess an unusually effective digestive apparatus. The autoproteolysis of krill *post mortem* is particularly striking, and results in extensive breakdown of the krill proteins in largely free amino acids and shorter peptides (Ellingsen & Mohr, 1979; Ellingsen, 1982). Considerable information is starting to accumulate on the nature of the peptide hydrolases responsible for this hydrolysis (see Osnes & Mohr, 1985*a*, for review), and the principal enzymes involved have been purified to a substantial degree and characterized (Osnes & Mohr, 1985*a*,*b*, 1986; Osnes *et al.*, 1986).

In the present paper we report on the distinctive features of the biochemistry of the autoproteolysis of the main krill species in the Antarctic, *Euphausia superba*, as well as another species, *Euphausia crystallorophias*, which is characterized by having a more southerly distribution than *E. superba*. Particular effort was taken to obtain material which allowed detailed examination of the composition and changes *post mortem* in the krill, and the studies were based on sampling and laboratory work carried out on board the expedition vessels in the Antarctic. The krill examined originate from two expeditions and were obtained from widely different locations in the Antarctic. Since also two different species are represented, we consider that the studies provide a representative and comprehensive picture of the autoproteolytic processes typical of Antarctic krill.

MATERIALS AND METHODS

Krill samples

The studies were carried out on four catches of krill obtained during the Norwegian Antarctic Expedition 1976–77 and one obtained during the expedition in 1978–79 (Table 1). The krill obtained during the first expedition were caught with an Isaacs–Kidd Midwater Trawl as described by Fevolden (1979), the trawl time being 10–60 min. During the second expedition a simple conical net and a trawl time of 30 min were employed (Fevolden, 1980).

Immediately after catching, samples of krill were incubated at 0 °C on board the expedition vessel to study proteolysis *post mortem* as described below. To obtain reference samples, either krill were placed in 0.6 M-$HClO_4$ 10–20 s after catching, and were frozen and kept at −80 °C, or they were frozen within 10–15 min of catching in a specially designed solid-CO_2 plate freezer,

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Table 1. Krill catches

Data are taken from Fevolden (1979, 1980): –, not determined.

Catch no.	Date	Location	Species	Length (mm)	Sexually mature krill (%)	Male (%)	Female (%)
8	17.01.77	Weddell Sea	*E. crystallorophias*	15–35	–	–	–
10	17.01.77		*E. crystallorophias*	18–34	100	57	43
20	12.02.77		*E. superba*	18–30, 30–40, 40–48	6	2	4
29	23.02.77	Bouvet Island	*E. superba**	40–60	100	50	50
7	31.12.78	0.5 nautical mile E of Bouvet Island	*E. superba*†	19–35	0	0	0

* 95% of the catch was *E. superba* and 5% *E. triacantha*.
† 99.6% of the catch was *E. superba* and 0.4% *Thysanoessa macrura*.

in which polythene bags containing krill were placed between aluminium boxes filled with solid CO_2. Studies showed that the temperature in the middle of the krill block fell to −3 °C within 1 min, and to −60 °C within 5–7 min (Ellingsen, 1982). The samples were subsequently transferred to a low-temperature freezer, and stored at −80 °C for 13–15 months before being used for analysis.

Incubation and sampling

Portions of krill (30 and 100 ml respectively) were distributed in a series of 100 ml and 500 ml polythene bottles with screw caps, and incubated in an ice/water bath at 0 °C on board the expedition vessel within 15–40 min of catching. In separate experiments krill were homogenized in an Ultra-Turrax TP18-10 instrument (Janke and Kunkel K.G., Staufen, West Germany) operated at 20000 rev./min for 1–2 min. Portions (5 ml) of the homogenate were incubated in closed glass test tubes (13 mm diam., length 10 cm) at 0 °C as above.

At intervals, samples of whole and homogenized krill were removed and immediately frozen and stored at −80 °C for 13–19 months before analysis in the laboratory. In addition, samples of whole krill were homogenized on board the expedition vessel to prepare $HClO_4$ extracts. The entire contents of the polythene flasks were first homogenized as outlined above; 1 vol. of homogenate (usually 10 ml) was added to 2 vol. of 0.6 M-$HClO_4$ in 100 ml polythene flasks. After 5 min the flasks were placed at −80 °C and stored for 3–19 months before analysis in the laboratory.

$HClO_4$ extracts

In the laboratory, the frozen samples of krill homogenate in $HClO_4$ were thawed in a water bath at 60 °C. The time of thawing was approx. 5 min. To the thawed samples were added 20 ml of 0.6 M-$HClO_4$ (20 °C), and the samples were then filtered (Schleicher and Schüll no. 597). The filter cake was suspended in 40 ml of 0.6 M-$HClO_4$, homogenized for 20–30 s in an Ultra-Turrax TP18-10 homogenizer at maximum speed, and filtered as above. The filter cake was finally washed with 10 ml of 0.6 M-$HClO_4$.

To the combined $HClO_4$ extracts was added 5 M-KOH to give pH 6.5, and the samples were kept overnight at 4 °C. Precipitated $KClO_4$ was removed by filtration, and washed with 5 ml of cold saturated $KClO_4$ solution. The neutralized extract was diluted with water to a total volume of 110 ml.

A 60 ml portion of the neutralized extract was evaporated to dryness in a rotary evaporator at 25–40 °C, and the residue dissolved in 40 ml of 0.067 M-sodium citrate buffer, pH 2.2. The solution was frozen and stored at −80 °C for later analysis of free amino acids, peptides and diamines.

The entire contents of the test tubes with frozen homogenized krill were thawed after the addition of 3 ml of 0.6 M-$HClO_4$. Subsequently 35 ml of 0.6 M-$HClO_4$ was added, and the further procedure was as described above.

Preparation of main classes of protein

The extraction procedure was based on that described by Dyer *et al.* (1950) for the extraction of fish proteins. The preparation of the main classes of protein was carried out on board the expedition vessel. Krill homogenate (4 ml) was added to 80 ml of 0.05 M-

phosphate buffer (0.05 M-KH_2PO_4/0.035 M-NaOH, $I = 0.12$, pH 7.0), and the mixture was centrifuged at 1800 *g* ($r_{av.}$ 12.5 cm) for 15 min at 20 °C. The supernatant, which is referred to as 'water-soluble fraction', was decanted. The insoluble residue was further extracted with 80 ml of 0.6 M-KCl buffered with 0.05 M-phosphate buffer (0.6 M-KCl/0.05 M-KH_2PO_4/0.035 M-NaOH, $I = 0.72$, pH 7.0), and centrifuged as described above. The supernatant, which is referred to as 'salt-soluble fraction', was decanted. The water- and salt-soluble and insoluble fractions were immediately transferred to a freezer, and stored at −35 °C for 1–2 months until analysis.

Amino acids and diamines

Free amino acids in $HClO_4$ extracts dissolved in citrate buffer, or acid hydrolysates of krill protein, were analysed on a Beckman automatic amino acid analyser 120C, equipped with a u.v. detector (Uvicord type 4701A; LKB, Bromma, Sweden). A standard program and Beckman Spherical ion-exchange resin (type M82) were employed. Asparagine and glutamine were determined with the same resin, but a different elution program. The serine values obtained by the standard program were about 40% higher than those found with the latter program, owing to poor separation from asparagine and glutamine. The serine values given in the Results section were obtained by the standard program.

Diamines were analysed on a Bio-Rex 70 −400 mesh column (Bio-Rad Laboratories, Richmond, CA, U.S.A), which was eluted with 0.055 M-pyridine/0.05 M-NaCl, pH being adjusted to 4.21 with acetic acid.

Amino acid composition of protein

Whole fresh krill were frozen and stored at −80 °C for 1–2 years, and subsequently homogenized in the frozen state. Approx. 40 mg of the homogenate was hydrolysed in 4 ml of 6 M-HCl for 110 °C for 48 h under vacuum. HCl was removed under vacuum at 42 °C, and the sample dissolved in 0.067 M-sodium citrate buffer, pH 2.2. The samples were frozen and kept at −80 °C until analysed for amino acid composition on the amino acid analyser as outlined above.

Gel filtration

$HClO_4$ extracts dissolved in citrate buffer were analysed by gel chromatography on Sephadex G-15 (fine grade; 40–120 mesh) and G-50 (fine grade, 20–80 mesh) (Pharmacia, Uppsala, Sweden), with 0.05 M-phosphate buffer/0.035 M-NaOH, pH 7.0, as the eluent. The effluent was monitored with a u.v. monitor (Uvicord type 4701A).

Determination of peptides and protein

$HClO_4$ extracts dissolved in citrate buffer, or fractions from gel chromatography, were analysed for peptide content by the biuret method modified as described by Klungsøyr (1969).

Dry weight

Dry weights were determined after drying for 24 h at 105 °C.

RESULTS

Changes in main classes of proteins *post mortem*

The changes in the main classes of protein *post mortem* are shown in Fig. 1, and are expressed as the amino acid contents of each fraction. The values are not corrected for water added during hydrolysis. For the water-soluble fraction, the values were obtained after subtracting the

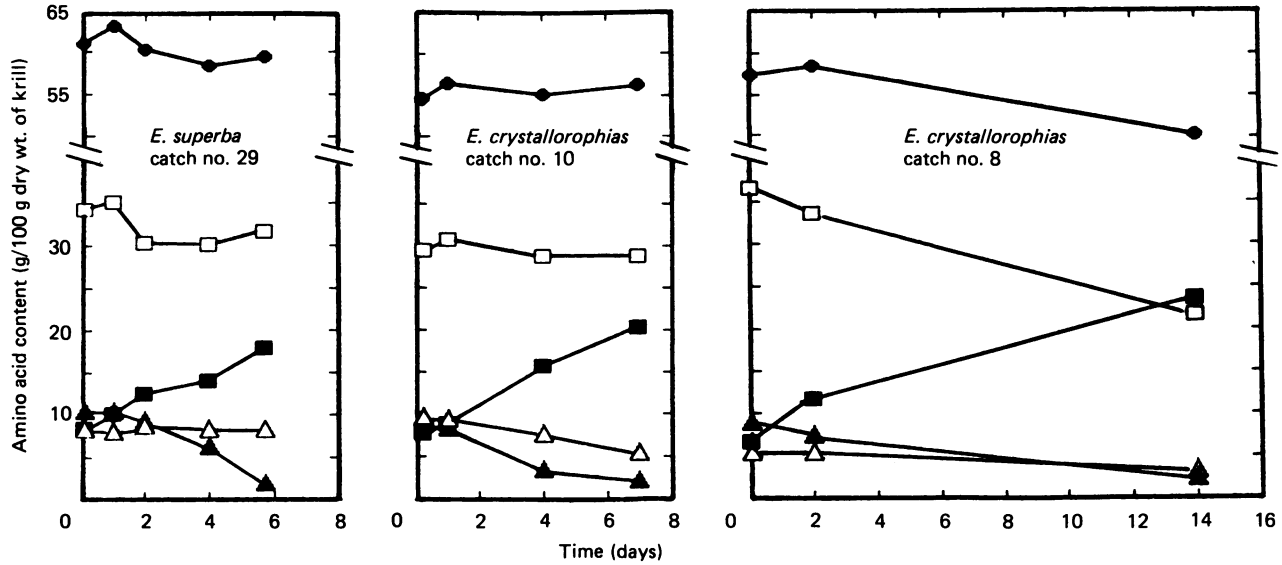

Fig. 1. Changes in the main classes of proteins in krill stored at 0 °C *post mortem* (Antarctic expedition 1976–77)

□, Water-soluble fraction; ▲, salt-soluble fraction; △, insoluble fraction; ■, free amino acids; ●, sum of fractions. Portions (100 ml) of krill were placed in 500 ml polythene bottles with screw caps and incubated at 0 °C immediately after catching. Samples were removed at various times, and the main classes of proteins were prepared by successive extractions as outlined in the Materials and methods section. The amino acid content of each fraction was determined after acid hydrolysis. The values are not corrected for water added during hydrolysis. For the water-soluble fraction, the data are corrected for free amino acid content before hydrolysis. The free amino acid content does not include ornithine and sarcosine.

free amino acid contents. The results indicate that the free amino acids which are formed during the first week of incubation originate mainly from hydrolysis of salt-soluble proteins. On prolonged storage (14 days), the water-soluble proteins of *E. crystallorophias* also seemed to be hydrolysed. In *E. superba* the content of insoluble proteins was nearly constant during the storage period of 1 week, whereas the corresponding proteins in *E. crystallorophias* appeared to be broken down to a substantial extent during the first week of incubation.

In broad terms, the total amino acid content of the krill seemed to change comparatively little during the first week of incubation, indicating that the amino acids released during proteolysis were not converted into other products to any appreciable extent (Fig. 1). However, for *E. crystallorophias*, the sum of the amino acid contents of the fractions declined on prolonged storage.

The proteins of the water-soluble, salt-soluble and insoluble fractions appeared to have very similar amino acid compositions (Table 2), although the proteins of the insoluble fractions had a lower content of lysine, leucine and isoleucine residues, and a higher proportion of glycine and serine, than did the proteins of the water- and salt-soluble fractions. The water-soluble proteins appeared to have the lowest content of arginine and proline residues.

During incubation at 0 °C *post mortem*, the amino acid composition of the salt-soluble and insoluble fractions changed very little during the course of the first week. However, it should be pointed out that the protein content of these two fractions diminished in the same period (Fig. 1). Thus it appeared from these studies that the amino acid composition of the proteins which were broken down was similar to that of the proteins which remained in the salt-soluble and insoluble fractions. For the water-soluble proteins, the proportion of aspartic and glutamic acid residues increased during incubation, and on prolonged storage of *E. crystallorophias* a characteristic decrease was observed in the content of lysine, arginine and histidine residues in the water-soluble and salt-soluble fractions.

The amino acid composition of whole krill seemed to change very little during the first week of incubation (Table 3). Consequently, the amino acids that were liberated during this phase were evidently not converted into other products. However, the proportion of alanine residues showed a certain increase *post mortem*, and on prolonged storage of *E. crystallorophias* there was a

Table 2. Amino acid compositions of the water-soluble, salt-soluble and insoluble fractions of krill

Portions (100 ml) of krill were placed in 500 ml polythene bottles with screw caps and incubated at 0 °C for up to 6 h immediately after catching. Samples were removed at different times, and the main classes of proteins prepared by successive extractions as outlined in the Materials and methods section. The amino acid content of each fraction was determined after acid hydrolysis (–, not determined). For the water-soluble fraction, the data are corrected for free amino acid content before hydrolysis.

	Content (residues/100 amino acid residues)								
Fraction...	Water-soluble			Salt-soluble			Insoluble		
Krill...	*E. superba*	*E. crystallorophias*		*E. superba*	*E. crystallorophias*		*E. superba*	*E. crystallorophias*	
Amino acid	Catch no. 29*	Catch no. 18†	Catch no. 10‡	Catch no. 29*	Catch no. 8†	Catch no. 10‡	Catch no. 29*	Catch no. 8†	Catch no. 10‡
Alanine	8.8	8.9	8.8	8.6	8.5	8.2	8.7	8.5	8.2
Valine	6.6	6.8	6.9	6.1	5.9	5.7	6.5	6.3	6.5
Leucine	7.7	8.0	7.7	8.1	7.7	7.8	6.8	7.1	6.9
Isoleucine	5.4	5.7	5.4	6.1	5.5	5.6	5.0	5.1	5.0
Proline	4.4	4.5	3.6	5.5	5.6	6.5	5.3	5.5	5.5
Phenylalanine	3.7	3.6	3.3	4.2	4.1	3.8	3.9	4.0	4.1
Tryptophan	–	–	–	–	–	–	–	–	–
Methionine	2.5	2.5	2.5	3.2	2.6	2.7	2.3	2.9	3.0
Glycine	9.0	9.1	8.9	9.6	9.0	8.0	10.9	10.5	9.6
Serine	5.7	5.9	5.5	5.3	5.7	5.3	6.3	5.9	6.4
Threonine	5.2	5.1	5.6	4.7	5.3	5.1	5.0	5.0	5.0
Tyrosine	3.3	3.2	3.2	3.2	3.0	2.7	3.5	3.8	3.7
Cysteine	–	–	–	–	–	–	1.2	1.0	1.0
Aspartic acid	11.2	10.5	10.8	9.5	9.4	10.4	10.2	10.3	10.3
Glutamic acid	13.4	12.3	13.6	11.1	12.5	12.0	12.4	12.2	12.1
Lysine	7.1	7.6	8.2	7.2	7.6	7.3	5.1	5.3	5.9
Arginine	3.9	4.3	4.3	5.5	5.5	5.9	4.7	4.7	4.9
Histidine	2.3	2.1	1.8	2.1	2.0	3.1	2.4	2.1	2.2
Sum	100.2	100.1	100.1	100.0	99.9	100.1	100.2	100.2	100.2

* Stored 4 h at 0° C.
† Stored 1 h at 0° C.
‡ Stored 6 h at 0° C.

Table 3. Amino acid compositions of krill stored at 0 °C *post mortem* (Antarctic expedition 1976–77)

The data represent the sum of the amino acid contents of the water-soluble, the salt-soluble and the insoluble fractions (–, not determined). The water-soluble fraction includes free amino acid content. The 10 min samples refer to the amino acid content of whole frozen and thawed krill

	Content (residues/100 amino acid residues)											
	E. superba					*E. crystallorophias*						
	Catch no. 29					Catch no. 8				Catch no. 10		
Amino acid	10 min	4 h	25 h	2 days 1 h	5 days 18 h	10 min	1 h	2 days	14 days 2 h	6 h	24 h	7 days 1 h
Alanine	8.5	8.8	9.3	9.3	10.0	8.4	8.7	9.1	12.2	8.7	8.9	10.8
Valine	5.8	6.0	6.2	5.7	6.4	6.0	6.2	6.2	6.7	6.1	5.8	6.1
Leucine	7.0	7.1	7.1	7.3	7.5	7.1	7.3	7.6	7.9	7.0	7.5	7.1
Isoleucine	4.8	5.1	5.1	4.9	5.3	5.0	5.2	5.4	5.9	5.0	5.5	5.3
Proline	6.0	5.9	6.2	5.8	5.3	7.9	7.5	7.5	7.4	7.8	8.0	7.7
Phenylalanine	3.5	3.6	3.8	3.5	4.0	3.4	3.5	3.4	3.5	3.2	3.5	3.4
Tryptophan	–	–	–	–	–	–	–	–	–	–	–	–
Methionine	2.4	2.4	2.5	2.4	2.4	2.4	2.4	2.4	2.7	2.5	2.4	2.4
Glycine	12.0	12.0	11.7	11.3	11.8	10.0	10.2	9.8	10.6	9.8	9.5	9.8
Serine	5.5	5.5	4.9	5.7	4.5	5.5	5.7	4.6	4.4	5.3	5.2	4.3
Threonine	4.9	4.7	4.7	4.9	4.6	5.0	4.9	4.7	5.1	5.0	4.8	5.1
Tyrosine	3.2	3.1	3.1	3.2	3.0	3.0	3.0	3.0	3.1	3.2	3.1	3.0
Cysteine	–	–	–	–	–	–	–	–	–	–	–	–
Aspartic acid	10.2	9.7	9.1	9.6	9.4	10.0	9.5	10.0	8.3	9.7	9.2	8.7
Glutamic acid	12.1	11.5	11.7	11.5	11.7	12.2	11.3	11.6	12.5	11.7	11.3	11.5
Lysine	7.1	6.7	6.8	7.1	7.0	7.2	7.0	7.1	4.6	7.1	7.2	7.5
Arginine	5.2	6.0	5.9	5.8	5.1	5.3	5.7	5.7	3.5	6.1	6.1	5.7
Histidine	1.9	2.1	1.9	2.0	2.0	1.8	2.0	2.1	1.5	2.0	2.1	1.8
Sum	100.1	100.2	100.0	100.1	100.0	100.2	100.1	100.2	100.1	100.1	100.1	100.2

distinct decrease in the total content of lysine and arginine residues, and to a lesser extent in the content of aspartic acid, suggesting that these residues were undergoing reactions *post mortem*.

Release of free amino acids

Further insight into the nature of the autoproteolytic process was obtained from detailed studies of the amino acids liberated *post mortem*. In fresh krill, immediately after catching, free amino acids accounted for 6.6–7.5% of the dry weight of the animal, which probably reflects the content of free amino acids in living krill. Proteolysis *post mortem* resulted in a rapid increase in the free amino acid content, which reached approx. 20% after 1 week of storage at 0 °C. Judging from these results, there were comparatively small differences between the initial rates of release of free amino acids in the different catches.

Curves illustrating the release of the individual amino acids are shown in Fig. 2. From these curves it emerges that the liberation of free amino acids is near-linear with time during the first week of incubation. However, on extended storage of *E. crystallorophias*, a decrease in the rate of release was apparent for all amino acids. For aspartic acid, lysine and arginine, a distinct decrease in the content of these amino acids was detected in samples of *E. crystallorophias* stored for more than 1 week (Fig. 3). As was pointed out above, analyses of acid hydrolysates of whole krill revealed that the total content of these amino acids in fact decreased on prolonged storage (Table 3), suggesting that they were converted into other products.

In separate analyses it was established that the decrease in lysine and arginine content was accompanied by the formation of the diamines cadaverine and agmatine, in amounts of 46 and 62 μmol/g dry wt. of krill respectively. Analyses also revealed a gradual increase in the content of ornithine throughout the storage period (Fig. 2). Apart from a decrease in the amount of serine and histidine on prolonged storage of *E. crystallorophias*, the total content of the other amino acids remained virtually constant during storage (Table 3), providing evidence that the general decrease in the rate of release of amino acids after the initial linear phase probably cannot be explained by the conversion of amino acids into other products. This is partly supported by the fact that the rate of ammonia production during this period was relatively low, and did not increase (cf. Fig. 2), indicating that deamination of amino acids took place only to a very limited extent.

The studies on the autoproteolysis of krill were confirmed and extended during the Norwegian Antarctic Expedition 1978–79. In addition to studies on whole krill, proteolysis *post mortem* was followed also in homogenates of the animal. The results (Fig. 4) provide new insight into some of the phenomena observed previously. Firstly, the data failed to show a distinct initially linear phase in the release of the individual amino acids, but rather revealed a progressive decrease in the rate of

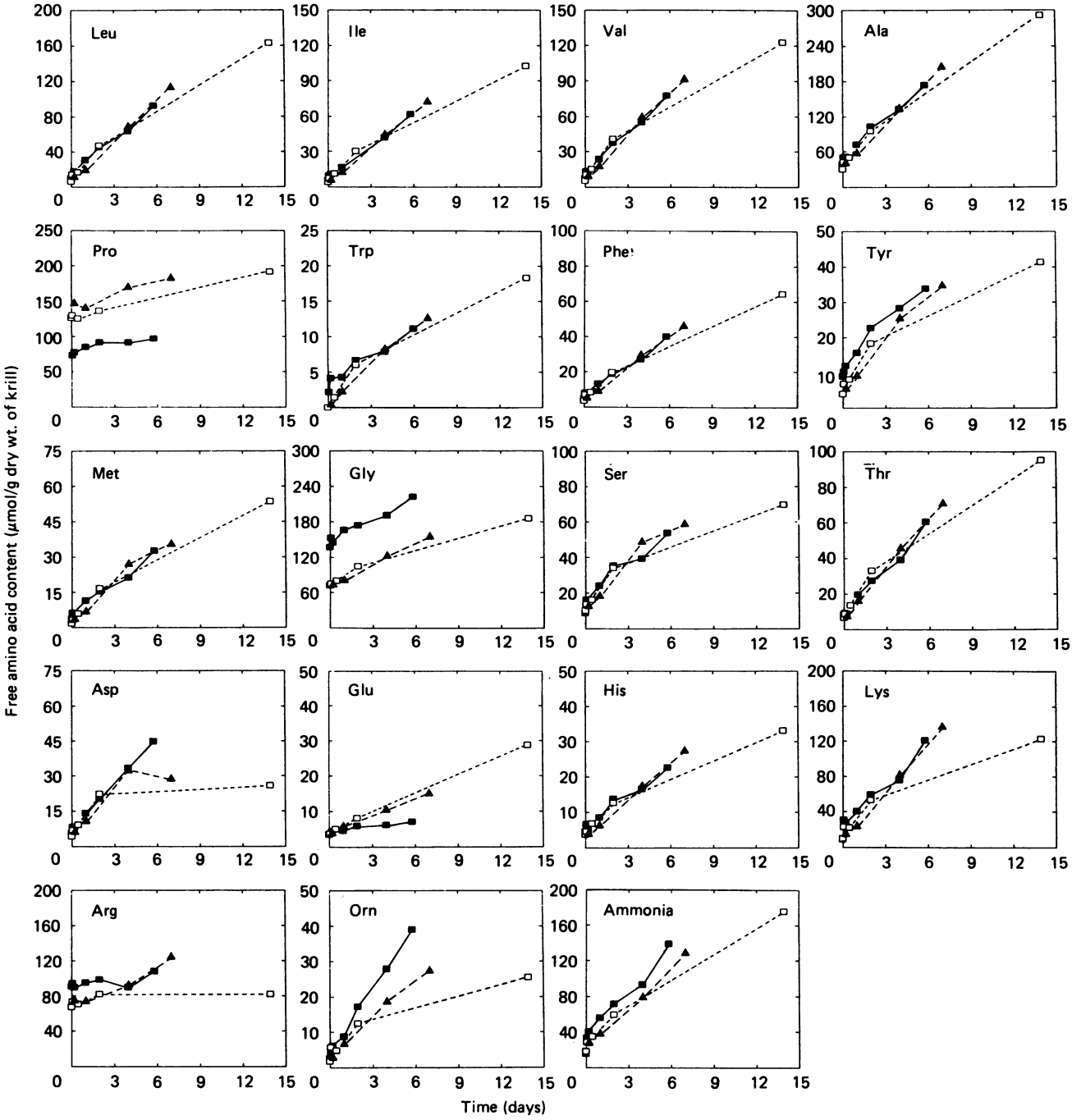

Fig. 2. Formation of free amino acids and ammonia in krill stored at 0 °C *post mortem* (Antarctic expedition 1976–77)

■, *E. superba*, catch no. 29; □, *E. crystallorophias*, catch no. 8; ▲, *E. crystallorophias*, catch no. 10. Portions (100 ml) of krill were placed in 500 ml polythene bottles with screw caps and incubated at 0 °C immediately after catching. Samples were removed at various times, homogenized, 0.6 M-$HClO_4$ was added, and they were frozen and stored at −80 °C before analysis of the free amino acids in the laboratory. The data refer to the sum of the contents of the individual amino acids. Ornithine and sarcosine are not included in the data.

liberation of amino acids with time during the initial stage of proteolysis. Secondly, the experiments clearly demonstrated that the initial rate of liberation of amino acids is considerably higher in homogenates than in whole krill. However, the total amount of free amino acids found at the end of the incubation period was roughly the same in homogenates as in whole krill. A clear exception was glutamic acid, which was released in much higher amounts in homogenates than in whole krill (Fig. 4).

In accord with the results of the previous expedition, the content of free arginine fell drastically on prolonged storage of whole krill, evidently as a result of conversion into other product(s) (Fig. 4). Most probably agmatine was formed, since the kinetics of the production of ornithine in whole krill failed to suggest a conversion of

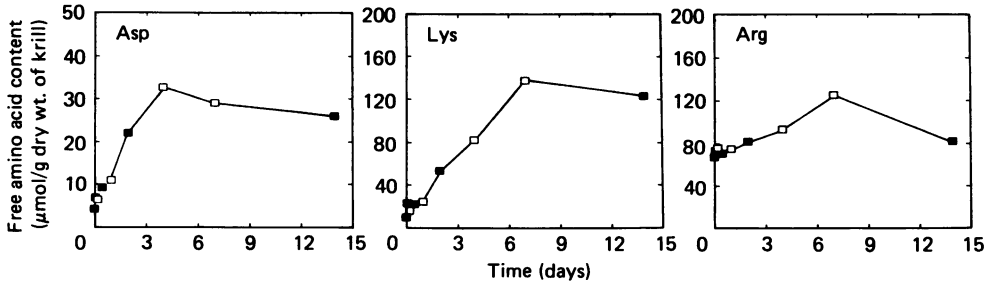

Fig. 3. Content of aspartic acid, arginine and lysine in *E. crystallorophias* stored at 0 °C *post mortem* (Antarctic expedition 1976–77)

■, Catch no. 8; □, catch no. 10. The experimental conditions are outlined in Fig. 2.

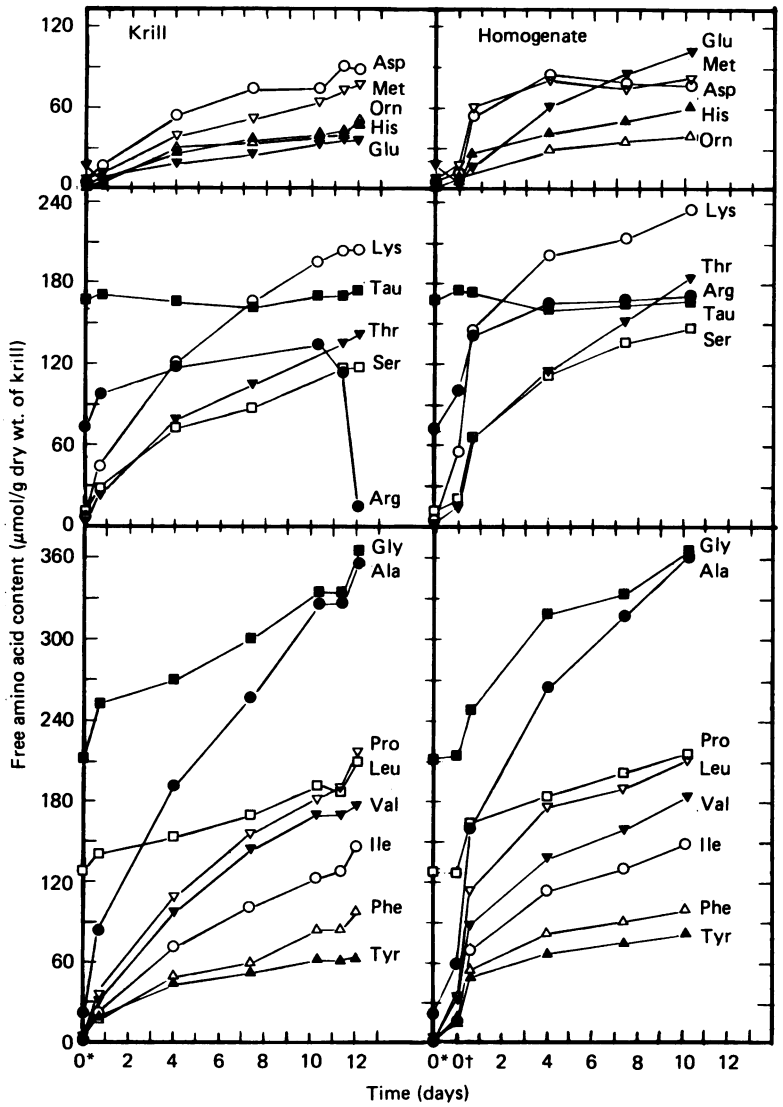

Fig. 4. Formation of free amino acids in whole krill and homogenates of krill stored at 0 °C *post mortem* (*E. superba*, catch no. 7, Antarctic expedition 1978–79)

Portions (80 ml) of krill were placed in 500 ml polythene bottles with screw caps and incubated at 0 °C immediately after catching. Samples were removed at various times, homogenized and 0.6 M-$HClO_4$ was added. The samples were frozen and stored at −80 °C for 18–27 months before being analysed for free amino acid content. Similarly, 5 ml portions of krill homogenate were dispensed in 13 ml glass test tubes with screw caps and incubated as above. *Krill to which $HClO_4$ added 10–20 s after catching. †Krill stored *post mortem* at 0 °C for 75 min, then homogenized, and $HClO_4$ was added 145 min after catching.

Table 4. Relationship between the rate of release of individual amino acids and their proportion in the krill protein

The values are obtained from the data presented in Fig. 2. The rate of release of amino acids is expressed on a molar basis relative to that of leucine (100). The amino acid composition of the krill protein is given after subtraction of free amino acid content, and is expressed on a molar basis relative to that of leucine (100): –, not determined.

	E. superba				*E. crystallorophias*		
Storage period . . .	Catch no. 20 1–125 h		Catch no. 29 10 min–138 h		Catch no. 8 4–48 h		Catch no. 10 6–97 h
Amino acid	Relative rate of release	Relative content in total protein	Relative rate of release	Relative content in total protein	Relative rate of release	Relative content in total protein	Relative rate of release
Alanine	135	110	163	113	163	109	164
Valine	83	84	85	83	91	83	88
Leucine	100	100	100	100	100	100	100
Isoleucine	68	68	68	68	69	70	67
Proline	45	73	27	62	20	69	40
Phenylalanine	34	51	43	49	38	48	42
Tryptophan	–	–	13	–	18	–	14
Methionine	32	25	35	34	40	34	41
Glycine	92	131	86	133	91	118	84
Serine	68	66	54	70	62	68	63
Threonine	76	68	58	67	73	69	67
Tyrosine	28	38	30	43	35	42	35
Aspartic acid	49	140*	47	146*	46	142*	46
Asparagine	41	140*	33	146*	28	142*	28
Glutamic acid	16	182†	3	172†	10	174†	20
Glutamine	19	182†	8	172†	19	174†	9
Lysine	91	94	134	101	91	100	116
Arginine	26	49	20	49	26	53	29
Histidine	20	22	21	26	24	23	23

* Asparagine + aspartic acid.
† Glutamine + glutamic acid.

arginine into ornithine. A similar decrease in the arginine content was not observed in homogenates. Furthermore, lysine did not seem to be converted into other products either in homogenates or in whole krill.

In all catches examined, alanine was released at the highest rate (Figs. 2 and 4). The rate of liberation of the other amino acids decreased in the order Ala > Lys, Leu > Gly, Val > Thr, Ile > Ser > Asp > Phe, Met, Asn, Pro, Tyr, Arg > His > Trp > Gln > Glu. Approximately the same ranking was observed in homogenates. However, the initial rate of release of amino acids was considerably higher in homogenates than in whole krill.

In broad terms, the individual amino acids were released in amounts corresponding to their relative proportions in the krill protein (Table 4). However, there were some notable exceptions. Alanine was released in amounts which were clearly higher, and proline, glycine, arginine and, in particular, glutamic acid/glutamine and aspartic acid/asparagine, were set free in quantities considerably lower, than would be expected from the content of these residues in the bulk protein of the krill.

Formation of peptides

The release of peptides during proteolysis was examined on the basis of analyses of both total peptide content and content of oligopeptides and dipeptides. The total peptide content was determined by estimating the amino acid composition of $HClO_4$ extracts after acid hydrolysis, and subtracting the free amino acid content of the extracts, corrections being made for added water during hydrolysis. The content of oligopeptides was determined by the modified biuret method (Klungsøyr, 1969). The content of dipeptides was calculated as the difference between the peptide content determined by amino acid analysis (corrected for free amino acid content before hydrolysis and for water added during hydrolysis), and the value obtained by the modified biuret method (Klungsøyr, 1969).

The content of oligopeptides showed characteristic variations during incubation of the krill *post mortem*. During the first 1 h of incubation of whole krill, there seemed to be a rapid increase in the peptide content. Subsequently the amount of oligopeptides stayed virtually constant for the remainder of the storage period (see Fig. 5). In homogenates of krill a different picture emerged, in the sense that the initial increase in peptide content was succeeded by a gradual decline in the proportion of peptides during the storage period (Fig. 5).

The molecular size of the peptides released was examined by gel chromatography. The studies were made on samples of *E. crystallorophias* incubated for 1 h and 14 days at 0 °C. The elution profiles of the peptides on Sephadex G-15 and G-50 suggested that the average molecular size of the peptides decreased somewhat during the incubation (Fig. 6). Thus a major proportion of the

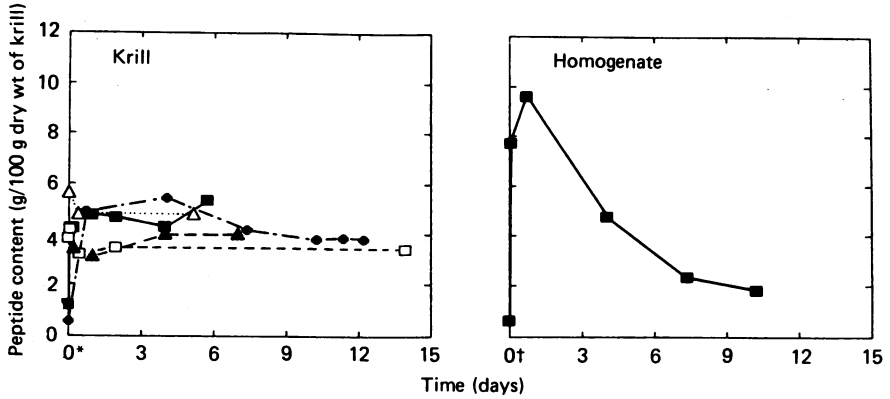

Fig. 5. Content of $HClO_4$-soluble peptides in whole krill and krill homogenates stored at 0 °C *post mortem* (Antarctic expeditions 1976–77 and 1978–79)

Portions (80–100 ml) of krill were placed in 500 ml polythene bottles and incubated at 0 °C immediately after catching. In a separate experiment krill were homogenized 75 min after catching, and portions (5 ml) of homogenate were incubated at 0 °C in closed glass test tubes. Samples were removed at various times. Immediately after sampling, the preparations of whole krill were homogenized, 0.6 M-$HClO_4$ was added, and they were frozen and stored at −80 °C for 3–19 months before analysis of the content of $HClO_4$-soluble peptides. The preparations of homogenized krill were immediately after sampling frozen and stored at −80 °C for 19 months. The samples were subsequently thawed in the presence of 0.6 M-$HClO_4$ and analysed for peptide content by the modified biuret method (Klungsøyr, 1969). Krill: □, *E. crystallorophias*, catch no. 8 (1976–77); ▲, *E. crystallorophias*, catch no. 10 (1976–77); △, *E. superba*, catch no. 20 (1976–77); ■, *E. superba*, catch no. 29 (1976–77); ●, *E. superba*, catch no. 7 (1978–79). *Krill stored *post mortem* at 0 °C for 60 min (△, □), 6 h (▲), 10–15 min (■), 10–20 s (●). Homogenate: ■, *E. superba*, catch no. 7 (1978–79). †Krill stored *post mortem* at 0 °C for 10–20 s.

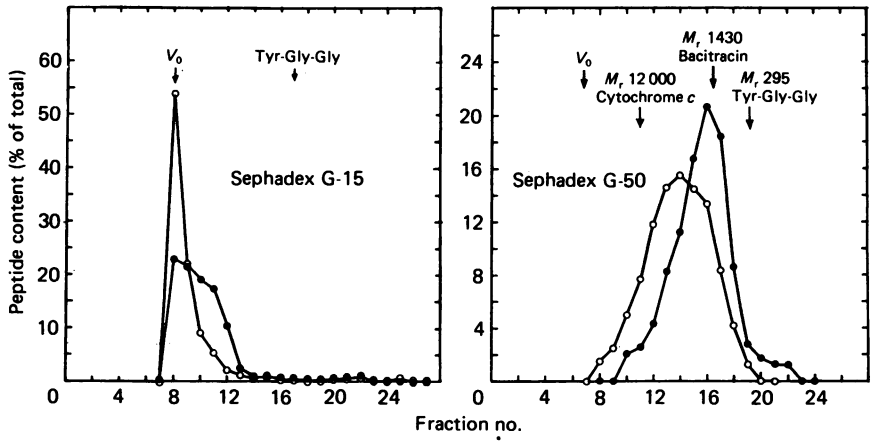

Fig. 6. Gel chromatography on Sephadex G-15 and G-50 of $HClO_4$ extract from *E. crystallorophias* (catch no. 8 Antarctic expedition 1976–77)

Portions (100 ml) of krill were placed in 500 ml polythene bottles with screw caps and incubated at 0 °C immediately after catching. Samples were removed at various times, homogenized, 0.6 M-$HClO_4$ was added, and they were stored at −80 °C. $HClO_4$ extracts were chromatographed on Sephadex G-15 and G-50 as described in the Materials and methods section. The void volume (V_0) was established with Blue Dextran as a marker; positions of M_r standards are also shown. The peptide content of the fractions was determined by the modified biuret method (Klungsøyr, 1969).

peptides in the 1 h sample had an M_r of 1500–12000, whereas a considerable proportion of those after 14 days of storage had M_r values less than 1500. Further analyses indicated that the content of dipeptides was low during the early stages of storage (approx. 0.5% of the dry weight), but that dipeptides accumulated as incubation proceeded, and accounted for approx. 3.6% of the dry weight after 14 days.

As pointed out above, proline, glycine, arginine and, in particular, glutamic acid/glutamine and aspartic acid/asparagine were liberated in much smaller quantities during autoproteolysis *post mortem* than would be expected from the occurrence of these residues in the bulk protein of the krill. Furthermore, these amino acids did not seem to be enriched to any significant extent in the salt-soluble or insoluble fraction during proteolysis. Therefore it was of considerable interest to see if $HClO_4$-soluble peptides rich in these residues accumulated during storage. For this purpose the amino acid composition of the $HClO_4$-soluble peptides was determined, as well as that of the 'large' peptides in the void volume after gel filtration on Sephadex G-15, and

Table 5. Amino acid composition of total protein and of peptides formed during storage of *E. crystallorophias* at 0 °C *post mortem* (Antarctic expedition 1976–77)

Portions (100 ml) of krill were placed in 500 ml polythene bottles with screw caps and incubated at 0 °C immediately after catching. Samples were removed as a function of time, 0.6 M-$HClO_4$ was added, and the mixtures were frozen and stored at −80 °C for 3–4 months before analysis of free amino acids in the laboratory. $HClO_4$ extracts dissolved in citrate buffer were also analysed by gel chromatography on Sephadex G-15 as outlined in the Materials and methods section. After gel chromatography, the amino acid compositions of the 'large' peptides of the void volume and of the 'small' peptides were determined (–, not determined). The composition of the 'small' peptides was expressed as the difference between the amino acid content of the total $HClO_4$-soluble peptides added to the gel-chromatography column, and that of the peptides of the void volume. The amino acid composition of the total protein of whole krill and that of the total $HClO_4$-soluble peptides, after subtracting free amino acid content in the samples, is given as a reference.

	Content (residues/100 amino acid residues)						
	Total amount of $HClO_4$-soluble peptides		'Large' $HClO_4$-soluble peptides		'Small' $HClO_4$-soluble peptides		
Amino acid	t = 1h	t = 14 days 2h	t = 1h	t = 14 days 2h	t = 1h	t = 14 days 2h	Total protein in fresh krill
Alanine	10.8	1.6	12.0	10.0	10.4	0.7	8.4
Valine	4.8	1.0	6.3	5.9	4.3	0.5	6.4
Leucine	7.2	0.6	5.8	5.5	7.5	0.1	7.7
Isoleucine	4.9	1.6	3.4	4.9	5.3	1.2	5.4
Proline	3.4	6.5	6.9	7.7	2.5	6.3	5.3
Phenylalanine	2.2	0.7	1.2	1.4	2.5	0.6	3.7
Tryptophan	–	–	–	–	–	–	–
Methionine	2.3	0.9	Traces	Traces	2.9	1.0	2.6
Glycine	10.0	11.5	9.1	10.7	10.2	11.6	9.1
Serine	5.2	1.0	6.5	6.2	4.8	0.4	5.2
Threonine	4.1	2.7	5.5	5.5	3.7	2.4	5.3
Tyrosine	0.5	0.1	0.7	0.7	0.5	0.1	3.2
Aspartic acid	11.6	23.2	10.6	11.9	11.9	24.4	10.9
Glutamic acid	20.7	45.3	18.1	20.0	21.5	48.0	13.4
Lysine	8.9	1.7	8.9	5.9	8.8	1.3	7.7
Arginine	2.1	0.3	3.2	1.9	1.8	0.3	4.1
Histidine	1.5	1.2	1.9	1.9	1.4	1.2	1.8
Sum	100.2	99.9	100.1	100.1	100.0	100.1	100.2

the 'small' peptides eluted at volumes larger than the void volume on the same column.

Table 5 shows that the amino acid composition of the 'small' and 'large' peptides is very similar in the 1 h sample. Compared with the bulk protein of the krill, these peptides seemed to be somewhat enriched in glutamic acid residues. On prolonged storage the composition of the 'large' peptides changed comparatively little, whereas the 'small' peptides exhibited a drastic increase in the content of, particularly, glutamic and aspartic acid residues. The data also suggest that a considerable proportion of the peptides containing these acidic residues are dipeptides.

DISCUSSION

The exact anatomical origin of the enzymes responsible for the autoproteolysis in krill is as yet not completely clear. However, on the basis of histological work, Ellingsen (1982) has established that the liquefaction of the krill tissues *post mortem* starts in the region of the stomach and hepatopancreas, and gradually spreads to adjoining tissues. Taken together with the fact that the peptide hydrolases detected in krill include typical digestive enzymes such as trypsin-like enzymes and carboxy- and amino-peptidases (Osnes *et al.*, 1986), available evidence suggests that a major proportion of the autoproteolytic activity in krill is due to enzymes of the digestive apparatus. However, cathepsins have been detected in the muscle tissue of the tail (Doi *et al.*, 1978), and may play some role in the autolytic process.

Studies of the main classes of proteins in krill showed that water-soluble and salt-soluble proteins accounted for 65–72% and 18–20%, respectively, of the total protein, whereas the remaining 11–15% of the protein remained insoluble under the conditions examined. The high content of water-soluble proteins in krill accords with the results of Suzuki & Kanna (1977) and Watanabe *et al.* (1979), working with frozen and thawed samples. The high content of water-soluble proteins in krill contrasts with the situation in many marine fishes, in which water-soluble sarcoplasmic proteins typically make up 20%, salt-soluble myofibrillar proteins approx. 75%, and insoluble stroma proteins only 5% of the total proteins of muscle tissue (Dyer *et al.*, 1950; Hamoir, 1955; Aitken & Connell, 1979).

A factor that may contribute to the high proportion of water-soluble proteins determined in krill is the fact that large amounts of lipid, including phospholipid, become

dispersed in the aqueous phase upon extraction of krill with dilute buffer or salt solution. The lipid phase, which probably also contains particles of insoluble protein, is not easily separated from the aqueous solution and insoluble residue on centrifugation, and may thus add to the protein content of the extract. Although such phenomena may have some influence on the results, the data clearly suggest that during proteolysis *post mortem* the free amino acids formed during the first week of incubation originate mainly from salt-soluble proteins, and that water-soluble and insoluble proteins are degraded to a much smaller extent.

In broad terms, the individual amino acids seemed to be released at rates corresponding to the content of each amino acid in the krill protein. However, there were exceptions to this rule. Alanine was released at a rate which was considerably higher than would be expected from the content of alanine in the bulk protein of krill. An explanation for this phenomenon could be that not only alanine is formed by hyrolysis of protein, but it arises also by other reactions. This contention is supported by the observation that the total amount of alanine seems to increase during incubation. A likely explanation is that alanine may arise by transamination of pyruvate formed during glycolysis, as this reaction has been demonstrated in other crustaceans (Huggins, 1966; Huggins & Munday, 1968; Gilles, 1970; Schoffeniels & Gilles, 1970; Lange, 1972). Alanine may also be formed from pyruvic acid via other pathways (Huggins & Munday, 1968; Gilles, 1970).

The very limited release of certain amino acids, particularly glutamic acid, during autolysis of krill is characteristic and interesting. Studies on the effect of temperature on autoproteolysis (Ellingsen, 1982) have revealed that the activation energy of the liberation of glutamic acid is approx. 30–50% higher than the average for the other amino acids in the krill protein. This provides a plausible explanation why glutamic acid is liberated only to a limited extent, and tends to accumulate in small peptides during proteolysis. The reason why aspartic acid/asparagine, glycine and proline are also liberated in lower quantities than would be expected from the content of these amino acids in the bulk protein of krill is probably because they occur frequently in positions adjacent to glutamic acid residues in the krill protein, and are thus enriched together with glutamic acid in small peptides during proteolysis, as shown in this study.

A characteristic feature of the autoproteolysis of krill is the decrease in arginine content during incubation. This phenomenon may be due to a conversion of arginine to ornithine and urea *post mortem*, as the content of ornithine has been shown to increase as storage of the krill proceeds. We have reason to believe that urea was further converted into ammonia, and that this reaction is responsible for the formation of 30–50% of the ammonia produced *post mortem*.

In *E. crystallorophias* both arginine and lysine appeared to be converted into their corresponding decarboxylation products, agmatine and cadaverine, on prolonged storage. These conversions are probably due to bacterial activity. In separate studies it has been demonstrated that the total viable bacterial count of krill stored at 0 °C remains very low during the first week of incubation, and subsequently shows a steep increase with time (Fevolden & Eidså, 1981).

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