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Two distinct forms of the chitin-degrading enzyme *N*-acetyl-β-D-glucosaminidase in the Antarctic krill: specialists in digestion and moult

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Abstract In the Antarctic krill *Euphausia superba* two forms of the chitinolytic enzyme *N*-acetyl-β-D-glucosaminidase (NAGase, EC 3.2.1.52) have been described, previously identified as NAGase B and NAGase C. Here, we demonstrate the organ-specific distribution and physiological relevance of both forms using a polyclonal antibody preparation which allows them to be distinguished immunologically. While NAGase B was localized in the integument and displayed a pattern of activity related to the moult cycle, the activity of NAGase C was independent of the moult cycle and was predominantly found in the gastrointestinal tract. Accordingly, NAGase B played a significant role in chitin degradation during the krill's moult, whereas NAGase C participated in the digestion of chitin-containing dietary components. Chromatographic elution profiles of isolated organs confirmed the immunological results by displaying characteristic organ-specific patterns in NAGase activity. The molecular characteristics of the moulting form, NAGase B, may further indicate a vesicular transport of moulting enzymes from the epidermis into the ecdysial space. Based on our results we develop a hypothesis explaining the concurrent processes of simultaneous chitin degradation and chitin synthesis occurring during moult.

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Introduction

Chitin-degrading enzymes are widely distributed in nature and can be found in most animal taxa (Jeuniaux 1961, 1966; Elyakova 1972; Flach et al. 1992). Their different function in arthropods for hatching, moulting and digestion has been investigated in several studies (see e.g. Mommsen 1980; Arnould and Jeuniaux 1982; Funke and Spindler 1989; Lynn 1990; Spindler and Spindler-Barth 1996). In crustaceans, the enzyme *N*-acetyl-β-D-glucosaminidase (NAGase, EC 3.2.1.52) is involved in the breakdown and re-utilization of the old cuticle during moult (Buchholz 1989). In concert with endochitinases, which cleave the chitin chain into oligosaccharides, NAGases catalyze the hydrolytic cleavage of terminal *N*-acetyl-glucosamine monomers from these smaller breakdown products. This facilitates their resorption through the epidermis for further metabolic processing, i.e. primarily synthesis of the new cuticle (Speck and Urich 1972). In the Antarctic krill *Euphausia superba* NAGase has also been shown to act as a digestive enzyme which is involved in the digestion of chitin contained in the diet (Buchholz 1989; Buchholz and Saborowski 1996; Saborowski and Buchholz 1999).

However, though different patterns of NAGase activity have been described in the digestive tract and in the integument in the course of the moult cycle (Buchholz 1989; Spindler-Barth et al. 1990), there is still a lack of information as to whether the enzymes involved in moulting are identical to the digestive enzymes or not. Chromatographic studies have shown that at least two different NAGase forms exist in krill (Spindler and Buchholz 1988), and the significance of such isoforms in temperature adaptation processes has been discussed (Spindler and Buchholz 1988; Buchholz and Vetter 1993). Accordingly, in a previous study we established a purification protocol to chromatographically separate both forms, termed NAGase B and NAGase C, present in the Antarctic krill *Euphausia superba* (Peters et al. 1998). Purified NAGase C had been used to produce

polyclonal antibodies which proved to be highly specific, thus allowing the two NAGases to be distinguished immunologically.

Prior to the establishment of routine quantification of NAGase isoforms in temperature adaptation studies, it was necessary to determine the anatomical location of both isoforms in krill in order to identify target organs for analysis. This is a prerequisite for an assessment of the potential influence of endogenous physiological factors which might, apart from temperature, additionally affect the enzyme concentration and must consequently be taken into account.

In the present study we utilized the antibody to investigate the distribution of both NAGase forms in the digestive organs, including stomach and hepatopancreas, and in the integument. Furthermore, we investigated whether the activities of both enzymes were regulated during the moult cycle.

Materials and methods

Origin of samples

Euphausia superba were caught during the austral summer of 1991/92 from Admiralty Bay, King George Island, South Shetlands, with a ringtrawl (1 m^2 opening and 500 µm mesh size) and a rectangular trawl (1.5 m^2 opening and 3 mm mesh size) at depths of 50 to 80 m. Duration of hauls was kept to a minimum (< 10 min) in order to obtain krill in good condition. In specimens used for studies related to the moult cycle the following parameters were recorded: length, mass, sex and maturity stage; the moulting stage was determined according to Buchholz (1982). Samples were subsequently frozen and stored at −80 °C.

Sample preparation

For the determination of enzyme activity during the moult cycle, whole animal extracts were prepared from individual krill. These were homogenized in 2 ml citrate phosphate buffer (CPB, 0.2 mol l^{-1}, pH 5.5) per gram wet weight using an ultraturrax (IKA 18/19, Jahnke & Kunkel, Stauffen, Germany) while being cooled on ice. The extract was centrifuged for 5 min at 15 000 ×*g*, and the supernatant was subjected to further analysis.

Different organs were dissected to study the tissue-specific distribution of respective NAGase forms. Stomachs were excised from deep-frozen krill under a stereomicroscope. The krill was placed on a cold (< −20 °C) ice pack. The dorsal part of the carapace was cut off with a scalpel and the stomach, still frozen, was carefully excised with a preparation needle. Thereafter, adhesive tissue and the pigmented ectodermal lining of the stomach was removed by carefully blotting the thawing stomach on a filter paper. Organs were weighed and, four to six of them were pooled in a micro reaction tube (Eppendorf 3810).

The midgut gland (hepatopancreas) was subsequently excised from the still frozen body after the remaining cuticle of the carapace was carefully removed. Non-hepatopancreatic tissue (gonads, heart, connective tissue, etc.) was cut away with a scalpel, and the midgut glands were quickly transferred into reaction tubes.

The stomach and the midgut gland samples were homogenized manually in a twofold (v/w) amount of ice-cold CPB using a small teflon pestle (which fit into the reaction tubes; Eppendorf). After centrifugation for 5 min at 15 000 ×*g* the supernatants were used for analysis. Integument samples (cuticle and epidermis) were prepared from the abdominal segments of freshly thawed krill according to Buchholz (1989). The telson and the cephalothorax were removed, and the ventral part of the abdomen, including the pleopods and the ventral cuticle, were cut off. The remaining abdominal tissue, by now covered only by the dorsal and lateral parts of the cuticle, was placed on a medium-soft rubber pad. The muscle tissue was squeezed out of the cuticle by rolling a glass rod from the dorsal to the ventral side. The remainder consisted almost exclusively of the cuticle with epidermis attached, as was indicated by the pigmentation. Several integuments were pooled and a fivefold volume (v/w) of CPB added. The tissue was homogenized with an ultraturrax while being ice-cooled and centrifuged for 5 min at 15 000 ×*g* to obtain the supernatant for analysis.

Chromatography

Tissue extracts (stomach, midgut gland and integument) were applied to an FPLC anion exchange chromatography on Q-Sepharose HP XK 16/10 (16 mm diameter, 100 mm bed height, Pharmacia). The elution buffer used was 0.01 mol l^{-1} imidazole/HCl, pH 6.8 with a NaCl-gradient from 0 to 0.85 mol l^{-1} (Peters et al. 1998).

Enzyme activity

The activity of NAGase was determined according to Kimura (1974): 50 µl of 0.2 *M* CPB, pH 5.5, was incubated with 50 µl substrate solution [0.3% (w/v) of p-nitrophenyl-*N*-acetyl-β-D-glucosaminide (Sigma N-9376) in CPB] and 50 µl of sample for 15 min at 35 °C. The reaction was stopped with 2.5 ml of 0.01 *M* NaOH. The absorbance of samples and blanks was read at 410 nm (Uvicon 810, Kontron Instruments).

Immunotitration

The proportion of the two NAGase forms, B and C, in the respective samples was determined with an immunotitration method following Mentlein et al. (1985). Supernatants of crude extracts were diluted 1:10 with CPB. Fifty microliters of extract were supplemented with an increasing amount (1 to 10 µl) of antiserum [anti-(*Euphausia superba*-NAGase C)-IgG, diluted 1:20; Peters et al. 1998] and made up to 100 µl with Tris-buffered saline (TBS) containing 0.05% Triton X-100. During subsequent incubation for 4 h at 25 °C the antibody was allowed to react completely with the antigen. Approximately 5 mg of hydrated, washed and drained Protein A-Sepharose (Pharmacia, Heidelberg) was added to the mixture so as to bind IgGs quantitatively. After gently shaking for 30 min the IgGs, now adsorbed to Protein A-Sepharose, were removed by centrifugation (5 min at 15 000 ×*g*). The resulting supernatant was subjected to a determination of enzyme activity.

Calculation of specific activity

The respective absolute activities of NAGase B and NAGase C in whole animal extracts were assessed as follows: the total specific activity of NAGase (U_{spec}) was expressed in relation to the fresh mass of whole animals (µmol p-nitrophenol min^{-1} g^{-1}, at 35 °C). The proportion of activity of each isoform was then calculated by multiplying the total specific activity by the percentage of each form as determined by simultaneous immunotitration.

Statistics

Differences in the respective enzyme activities between different moult stages were tested using a Kruskal–Wallis ANOVA followed by a pairwise multiple comparison procedure using the parametric Student–Newman–Keuls method (SigmaStat 1.0, Jandel). Previous to testing of the latter, the data were square-root-transformed in order to ensure normal distribution. The significance level was set at $p < 0.05$.

Results

Chromatographic separation

The chromatogram of whole body extracts displayed two distinct peaks of NAGase activity (Fig. 1a). The first (NAGase B) eluted between Fraction 28 and 55 at a NaCl-gradient of 20 to 40% and appeared comparatively inhomogeneous. In contrast, the second one (NAGase C) eluted within a sharp and homogeneous peak between Fraction 70 and 83 at about 70% of the NaCl-gradient.

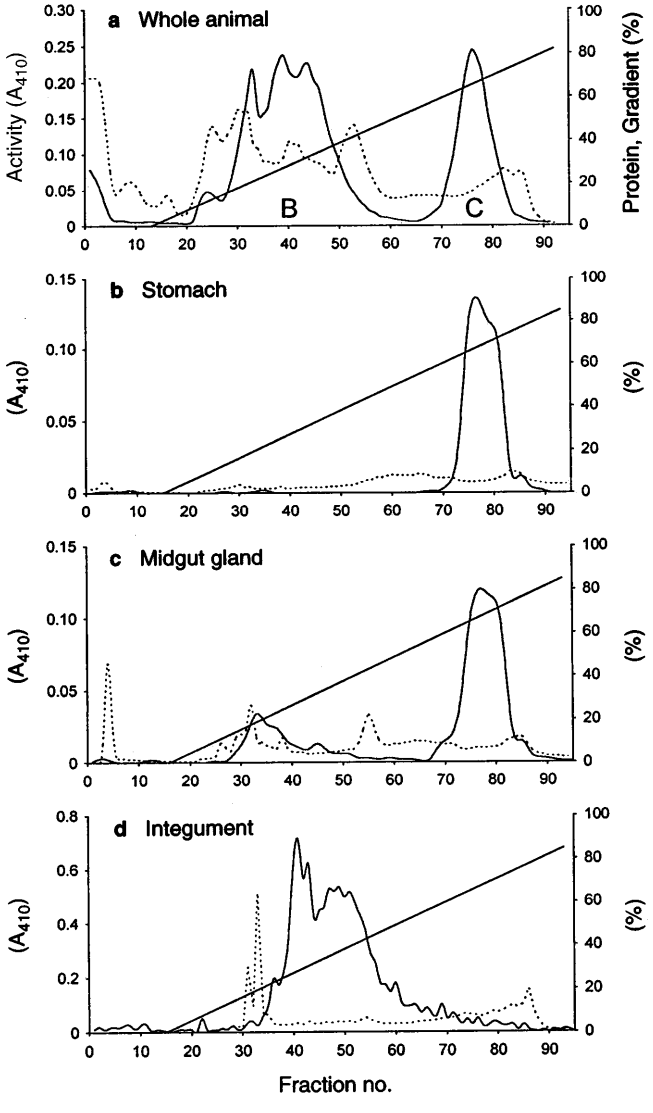

Fig. 1 *Euphausia superba*. Chromatograms of extracts prepared from krill whole animal (**a**), stomach (**b**), midgut gland (**c**), and integument (**d**). Extracts were derived from 19 stomachs (30 mg), 19 midgut glands (526 mg) and 48 integuments (940 mg). Chromatography was performed on an anion exchanger Q-Sepharose HP XK 16/10 (Pharmacia) with a salt gradient from 0 to 0.85 *M* NaCl (*straight line*). Continuous lines indicate NAGase activity (A_{410}), dotted lines indicate the protein profile (A_{280}, expressed as percent of full range) (*B* NAGase B; *C* NAGase C)

The chromatogram of the stomach extracts (Fig. 1b) showed one single sharp activity peak of NAGase C between Fraction 72 and 85. No NAGase B activity was found. The midgut gland extract displayed a similar chromatogram (Fig. 1c). Again, a dominant and homogeneous NAGase C peak appeared. Additionally, a small amount of NAGase B eluting between Fraction 30 and 50 with the maximum activity at Fraction 35 was found in the midgut gland. However, it was considerably reduced compared to the whole animal extract (cf. Fig. 1a).

The extract prepared from integuments displayed an entirely different pattern (Fig. 1d). Here, NAGase B eluted between Fraction 35 and 60, while no NAGase C peak was apparent. The NAGase B peak was not as well defined in the integument as in the whole body extract. Specifically, that portion of activity found between Fraction 20 and 35 in the whole body extract was not present in the integument extract.

Immunological differentiation

The polyclonal antibody allowed both NAGase forms to be reliably differentiated. Our previous study (Peters et al. 1998) revealed that NAGase C was quantitatively precipitated by the IgG, while less than 9% of cross-reactivity occurred with NAGase B. Thus, for the calculation of respective proportions of the two NAGases in the organ and whole body extracts we had to correct for this slight interference. The resulting activity in the supernatant after immunotitration (U_{IT}) was only $0.91 \times U_{NAGaseB}$. Therefore, the activity of NAGase B was calculated as $U_{NAGaseB} = U_{IT}/0.91$. Accordingly, $U_{NAGaseC}$ was U_{tot} (before immunotitration) $- U_{NAGaseB}$.

The proportions of both NAGases were markedly different between the stomach and the integument. In the stomach we found almost exclusively NAGase C (Fig. 2a), while NAGase B amounted to only 8.5%. In contrast, in the integument more than 90% of the NAGase activity was apportioned to NAGase B (Fig. 2b).

NAGase activity in relation to the moult cycle

The morphometric data of krill used in the analyses related to the moult cycle are summarized in Table 1. The specific activities of the two NAGases in whole body extracts showed different activity patterns throughout the moult cycle. The activity of NAGase B showed pronounced moult cycle-dependent variations which were significant ($p < 0.0001$, Kruskal–Wallis ANOVA, Fig. 3a). In contrast, NAGase C did not express any relationship to the moult cycle ($p > 0.1$) and partially showed a high degree of scatter (Fig. 3b). During the early premoult stage (D_1' onward) the activity of NAGase B rose significantly to stabilize at a higher level following Stage D_1'''. Thereafter, the activity remained constant at this elevated level until ecdysis (Fig. 3a).

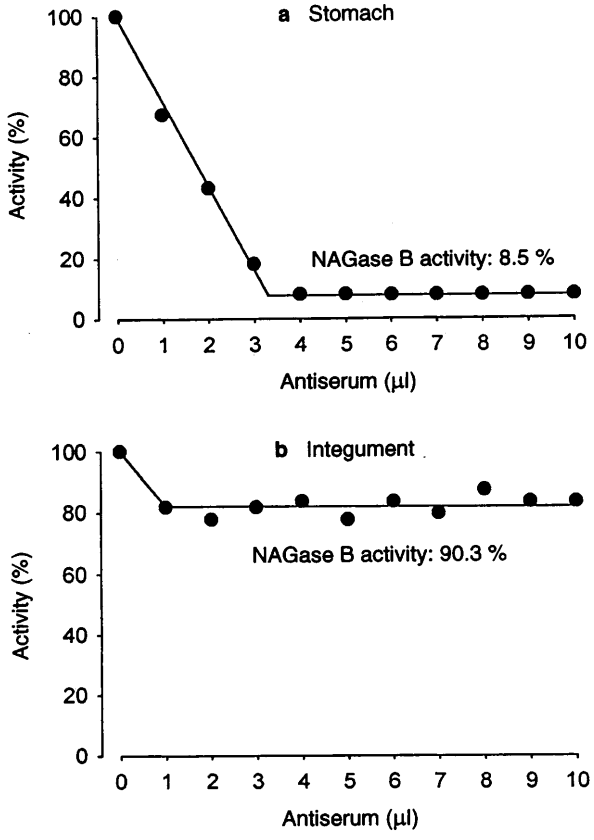

Fig. 2 *Euphausia superba*. Immunotitration of an extract prepared from Antarctic krill stomachs (**a**) and integuments (**b**) with anti-(*E. superba*-NAGase C)-IgG. Activity was calculated from those values lying on the plateau phase

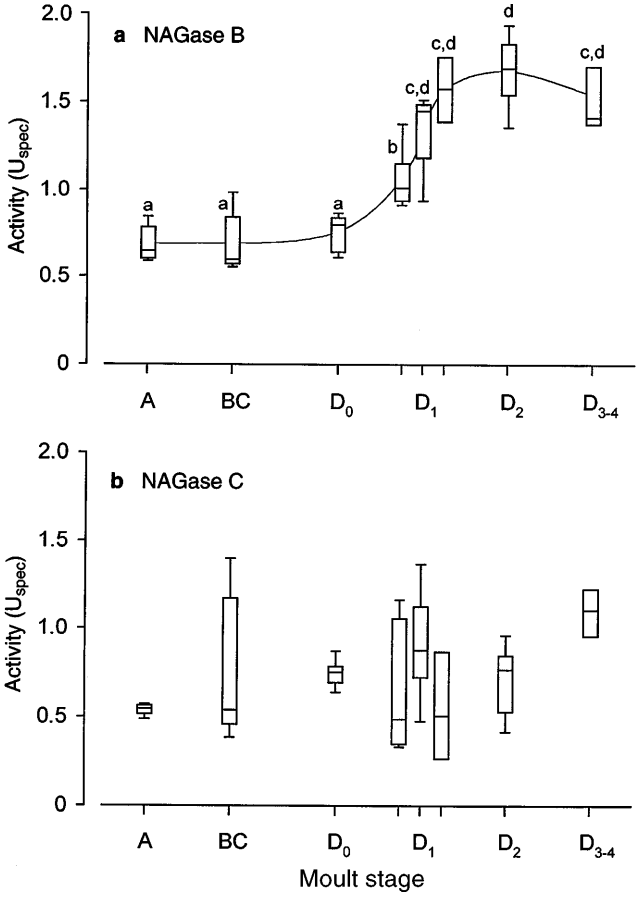

Fig. 3 *Euphasia superba*. Specific activities of NAGase B (**a**) and NAGase C (**b**) in individual krill of different moult stages. Boxes indicate the median, the 25th and 75th percentiles, error bars indicate the 10th and 90th percentiles. The continuous line in **a** connects the respective means. Identical letters affixed to single moult stages in **a** indicate where enzyme activities were not significantly different ($p > 0.05$). Sample size was between four and eight krill per moult stage. Ordinate scaling represents the average duration of different moult stages (the three ticks at stage D_1 represent D_1', D_1'' and D_1''', respectively)

Pairwise multiple comparison (Student–Newman–Keuls method) showed that in NAGase B the level of premoult activity was significantly higher than the postmoult level (Fig. 3a), with the transition between both phases being characterized by a steep rise in enzyme activity.

Discussion

Our results demonstrate that the two NAGase forms found in krill are related to different functions within the organism. The chromatographic separation of whole animal extracts displayed prominent peaks of both NAGase forms, B and C. Chromatographic analyses of selected organs, however, resulted in single peaks of NAGase B in integument extracts and NAGase C in stomach extracts. In the midgut gland, the predominant form was NAGase C, although a substantial amount of NAGase B was found. Accordingly, NAGase B turned out to be the isoform involved in moulting while NAGase C was the enzyme associated with digestion. This was confirmed by the moult-related course of enzyme activity in whole animal extracts after immunological differentiation between both NAGase forms. During postecdysis the titre of NAGase B was low but increased steadily during Phases D_1' to D_1''' (compare Buchholz 1989). The highest activities of NAGase B appeared during the Premoult Stages D_1''' and D_2. The increasing NAGase activity, in concert with other enzymes such as endochitinases and proteinases, accelerates the decomposition of the old cuticle during the last stages of the

Table 1 *Euphausia superba*. Morphometric data and sex distribution of *E. superba* used for moult-related studies ($n = 46$). Length was measured from the front of eyes to the base of telson. Within each moult stage, males and females were evenly distributed. Most of the specimens were mature, and some carried a spermatophore

	Mean	SD	Range
Length (mm)	34.5	3.0	26–39
Mass (mg)	517	145	182–859
	Males		Females
Sex ratio (%)	67		33
Spermatophore (%)	23		47

moult cycle. The liberated chitin oligomers and, in particular, the aminosugars are resorbed via the epidermis (Buchholz and Buchholz 1989) and channelled into anabolic pathways such as the re-synthesis of chitin (Speck and Urich 1972).

A similar course of chitinolytic activity was found in the shrimp *Palaemon serratus* (Spindler-Barth et al. 1990; Spindler and Spindler-Barth 1996). Since the onset of elevated chitinolytic enzyme activity at Stage D_1''' coincides with the highest titre of moult hormones (Baldaia et al. 1984; Buchholz 1989, 1991), it is likely that the enzymatic activity in krill may also be controlled by ecdysteroid levels as was found in the barnacle *Balanus amphitrite* (Freeman and Costlow 1979; Freeman 1980). However, this regulation only applies to the moult-related NAGases, while the digestive NAGases appear to be totally unaffected by the moult cycle, as can be deduced from the present study (see also Buchholz 1989; Spindler-Barth et al. 1990).

Similarly, a recent study employing molecular biological techniques on the Kuruma prawn *Penaeus japonicus* indicated the presence of two different chitinase-like proteins in the integument and hepatopancreas (Watanabe et al. 1996; Watanabe and Kono 1997). However, only indirect evidence was given that the catalytic specificity is chitinolytic, by comparing isolated cDNA with known sequences encoding chitinolytic enzymes. Here, we could directly demonstrate that two distinct NAGases were involved in the processes of moulting and digestion, each under control of a different regulating factor.

Furthermore, the glycoprotein nature of the moulting enzyme NAGase B (Peters et al. 1998) leads us to suspect a different functional mechanism in the secretion of this enzyme compared to the digestive enzyme NAGase C, which is a non-glycoprotein. Since glycoproteins are principally non-cytosolic, located in the plasma membrane, endoplasmic reticulum, or lysosomes (Alberts et al. 1983), a mechanism of vesicular transport of the moulting NAGase from the epidermis into the ecdysial space might be considered. The digestive NAGase, however, seems to be cytosolic. As such, it is probably released into the lumen of the midgut gland by exocytosis, a mechanism described for the F-type cells in the midgut gland of Crustacea (Brunet et al. 1994).

The appearance of vesicle-like bodies or "exolysosomes" in the ecdysial space of premoult crabs has recently been described by Compère et al. (1997). Assuming a secretion and transport of moulting enzymes via such exolysosomes in krill, and considering the pattern of enzymatic activity during the moult cycle (Fig. 3a), we suggest the following explanation for the coincident functioning of the conflicting processes of resorption of the old cuticle and the simultaneous synthesis of the new one. Discharge of moulting enzymes from the epidermal cells into the exuvial cleft starts prior to Stage D_1 (Buchholz and Buchholz 1989). The synthesis and deposition of the new cuticle begins at Stage D_1'. From Stage D_1''' onward, the fully developed, new epicuticle constitutes a barrier for further secretion of moulting enzymes (Buchholz and Buchholz 1989). Although extensive pore channels pass through the procuticle (Buchholz and Buchholz 1989; Buchholz et al. 1989; Pütz and Buchholz 1991), they were never found to penetrate the epicuticle (C. Buchholz personal communication). Thus, although the new epicuticle restricts the outward (and inward, see below) passage of moulting enzymes (note: NAGase B, M_r = 120 000), the epidermal resorption of the low-molecular-mass breakdown products (*N*-acetyl-glucosamine, M_r = 18.4) from the old cuticle is not obstructed by the new cuticle; the concentration of glucosamine in the haemolymph peaks at Stage D_{3-4} (Buchholz and Buchholz 1989), i.e. immediately before ecdysis. At this time even the new exocuticula is almost completely synthesized. The resorption of small molecules might then be facilitated by the pore channels which initially contain epidermal cell processes (Buchholz and Buchholz 1989). Later, during the synthesis of the endocuticle, they become narrower and disappear as the endocuticle is completed (Buchholz et al. 1989).

The constant NAGase B activity from Stage D_1''' onward seen in the present study (Fig. 3a) plainly reflects these physiological and structural changes in the cuticle. Thus, after secretion, enzymes persist within the exuvial cleft, acting on the inner side of the old endocuticle until the process of degradation is terminated by the ecdysis. A vesicular release of moulting enzymes prior to the completion of the epicuticle would explain the long-lasting and constant NAGase B activity following Stage D_1''' (Fig. 3a).

In crabs, exolysosomes are purported to play a role in protecting the acid hydrolases contained in these vesicles from digestion by earlier secreted alkaline hydrolases (Compère et al. 1997), which act on the disintegration of the subcuticular membranous layer (O'Brien and Skinner 1987). However, in krill, lacking this membranous layer (Buchholz et al. 1989), a vesicular secretion of certain moulting enzymes might primarily aid in achieving a *time lag* in chitinolysis. This would allow the new epicuticle to be completed before chitinases could act upon it. After completion, the new epicuticle containing a high amount of sclerotic glycoproteins (Buchholz et al. 1989) protects the underlying exocuticle from chitinolysis by the moulting fluid. Thus, this mechanism might explain how the old cuticle can be broken down whilst at the same time the new one is being deposited.

This hypothesis of a vesicular secretion, combined with a delayed release of enzymes from these vesicles, is supported by the observation that by far the highest concentration of resorbed glucosamine in the haemolymph and the main reduction in cuticular laminae of the old cuticle occur only during Stage D_{3-4} (Buchholz and Buchholz 1989). But all chitinases are present within the ecdysial space already from Stage D_1''' onward, following which no further secretion of enzymes can occur (see Fig. 3a). In addition, the ultrastructural examinations

in decapod crustaceans by Compère et al. (1997) indicate that the observed exolysosomes appear to gradually lose their contents during Stage D_2, thus presumably releasing enzymes into the ecdysial space. Furthermore, the course of the development of exolysosomes, appearing firstly at D_0 inside epidermal cells, and from late D_1''' onward exclusively in the ecdysial space (Compère et al. 1997), parallels the NAGase activity described in this work, thus indicating, albeit indirectly, that the NAGases might be secreted by such exolysosomes, although these structures have not yet been observed in krill.

Our observations on the course of NAGase activity during the moult cycle do not closely reflect those of Buchholz (1989). While we found a significant and constant activity of NAGase during the postmoult stages, Buchholz (1989) reported an almost complete cessation of activity. The main reason for this may be due to the different preparation procedures applied: Buchholz (1989) investigated the isolated integument, while we analyzed the whole body. Therefore, the remaining activity during postmoult might be due to a constitutive form of NAGase, found neither in the integument nor in the digestive tract, which does not react with the anti-(*Euphausia superba*-NAGase C)-IgG and which therefore was included in the assessment of NAGase B. The activity of this enzyme remained remarkably constant and did not drop below 0.55 U g^{-1}. Such a constitutive NAGase was described by Kimura (1974, 1976) in the haemolymph of insects and showed little variation during the larval moulting period. The physiological role of such NAGases in the haemolymph is still being discussed, but there is some indication that they are involved in the hydrolysis of glycoproteins or glycolipids (Flach et al. 1992).

The direct immunological determination of the digestive enzyme NAGase C revealed that it was virtually unaffected by the moult cycle and, as such, does not seem to be regulated by ecdysteroids. In support of earlier results (Buchholz 1989), we did not find any moult-related pattern but a rather high variability in the enzyme titre at single moult stages (Fig. 3b). This variability may reflect the individual nutritional state of krill taken from the field due to an induction of NAGase synthesis by appropriate chitinous diet (Buchholz and Saborowski 1996; Saborowski and Buchholz 1999). In this regard a bacterial contribution to the NAGase pool can be essentially ruled out due to earlier microbiological studies from our laboratory on *Meganyctiphanes norvegica* (Donachie et al. 1995), the boreal congener of *Euphausia superba*.

The lack of a moult-related activity in this enzyme further suggests that food ingestion and the digestive processes involved do not stop substantially around ecdysis. The high energy demand of krill (Kils 1982) might prohibit periods of extended fasting associated with ecdysis, forcing these animals to resume feeding immediately after moult. This necessity is further facilitated by the extremely rapid ecdysis which takes no more than a few seconds (Buchholz 1991).

In conclusion, we identified two different NAGase proteins in krill: a moult-related (ecdysone-regulated) form in the integument and a second, diet-induced form in the digestive tract. Additionally, indication is given for a constitutive NAGase which, most probably, is situated in the haemolymph. The presence of a similar NAGase system in the boreal congener *Meganyctiphanes norvegica* indicates a very conservative evolution of this enzyme system, at least in the Euphausiacea (cf. Peters et al. 1998). Knowledge on regulation processes in such enzymes involved in chitinolysis should provide a broad basis for further studies on the physiological adaptations of euphausiids in response to different environmental parameters. The moult-related form, NAGase B, appears favorable in this context due to its low variability, if changes occurring during the moult cycle are taken into account. Here, Moult Stage D_2 could be used as a standard reference because at this stage the complete amount of enzymes has been synthesized, possibly being still preserved inside the exolysosomes. The immunological identification of NAGase C provides a tool for dietary studies on animals taken from the field and thus may be useful to address ecological questions. Our study has shown that, although both enzymes catalyze the same reaction, they are regulated by highly different processes and play different roles in the metabolism of krill.

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