ELSEVIER

Comparative Biochemistry and Physiology Part B 120 (1998) 743–751

CBP

Isoforms of an *N*-acetyl-β-D-glucosaminidase from the Antarctic krill, *Euphausia superba*: purification and antibody production

Gerrit Peters [a,*], Reinhard Saborowski [b], Rolf Mentlein [c], Friedrich Buchholz [b]

[a] *Institut für Meereskunde, Abteilung Zoologie, Düsternbrooker Weg 20, D-24105 Kiel, Germany*
[b] *Biologische Anstalt Helgoland, Meeresstation, D-27498 Helgoland, Germany*
[c] *Anatomisches Institut der Universität Kiel, Olshausenstrasse 40, D-24118 Kiel, Germany*

Received 25 January 1998; received in revised form 5 June 1998; accepted 1 July 1998

Abstract

Two forms of the chitinolytic enzyme *N*-acetyl-β-D-glucosaminidase (NAGase, EC 3.2.1.52) have been isolated from the Antarctic krill, *Euphausia superba*, in order to study their potential role in temperature adaptation processes. A chromatographic protocol was developed that allowed complete separation of the two enzyme forms, named NAGase B and NAGase C. The latter was purified to homogeneity with 600-fold enrichment and a yield of 17%. The molecular mass was 150 kDa. NAGase B showed characteristics of a glycoprotein due to affinity towards concanavalin A sepharose, while NAGase C did not. Highly specific polyclonal antibodies to NAGase C [anti-(*E. superba*-NAGase C)-IgG] showed only negligible cross-reactivity with NAGase B isoforms. A comparison with the Northern krill, *Meganyctiphanes norvegica*, revealed a corresponding chromatographic pattern with two main activity peaks, for differentiation named NAGase II and NAGase III. Application of the antibody on *M. norvegica* revealed a high specificity toward NAGase III and a low cross-reactivity with NAGase II. First indication is given that the two forms are no isoenzymes in a strict sense but instead may have different functions in the metabolism of krill.

Keywords: Antibody; Chitinase; Chromatography; Digestion; Enzyme purification; Krill; Meganyctiphanes norvegica; Molting; NAGase; Temperature adaptation

1. Introduction

The Antarctic krill *Euphausia superba* Dana (Crustacea, Euphausiacea), is unique among other crustaceans living in the cold environment of the Southern Ocean in its particularly active way of life. As a filter-feeding planctivorous organism, this species inhabits, at least during the summer, the eupelagial where it grazes, constantly swimming, on patchily distributed phytoplankton [11]. Due to its high specific mass, krill is forced to compensate the negative buoyancy by steady swimming to avoid sinking into food and oxygen depleted deep water layers. In earlier studies *E. superba* has been characterized to express high growth rates on sufficient food supply [4] and to be capable of high swim and escape velocities [17,18].

This energy-demanding life style with exceptional high metabolic rates gave rise to questions for the kind of physiological adaptations which might compensate the rate-limiting extreme low temperatures enabling these animals to inhabit the cold oceans. In recent years, several studies were performed on the temperature-dependent regulation of metabolic pathways in Antarctic krill [5,29]. Such regulations in the course of long and short term adaptations, are likely to be related to alterations on the catalytic and thus on the enzymatic level. Accordingly, the investigation of enzymatic properties may provide valuable information on mechanisms that are involved in the adaptation of invertebrates to extreme climatic environments.

* Corresponding author. Tel: +49 431 5973939; fax: +49 431 5973994; e-mail: gpeters@ifm.uni-kiel.de

0305-0491/98/$19.00
PII S0305-0491(98)10073-1

We focused our work on an enzymatic model system, the hexosaminidase *N*-acetyl-β-D-glucosaminidase (NAGase, EC 3.2.1.52, formerly EC 3.2.1.30 in earlier publications of our laboratory), which is involved in digestion and molting processes in krill [3,6,28]. It has two main characteristics: firstly, it shows high activities throughout the class of Crustacea which is important for comparative investigations. Secondly, it has a high in vitro stability which enables extended laboratory procedures to be performed without significant loss of activity. In previous investigations on krill two potential isoforms of NAGase were distinguished chromatographically [5,28]. Their simultaneous occurrence may indicate a physiological adaptation utilizing a mechanism of altering isoenzyme concentrations [12].

In order to study the ecophysiological adaptations in the krill we purified the NAGase of Antarctic krill and compared the enzymatic characteristics of the isolated forms. Moreover, as a basis for a quantitative determination of NAGase protein, polyclonal antibodies were prepared against one of the isoforms.

Our work complements another study on *E. superba* which focuses on the kinetic properties of NAGases in relation to environmental temperatures [5].

2. Material and methods

2.1. Origin of samples

E. superba were caught during the austral summer of 1991/1992 in Admiralty Bay, King George Island, South Shetlands. Sampling devices were a ringtrawl with an opening of 1 m^2 and a mesh size of 0.5 mm and a rectangular trawl with an opening of 1.5 m^2 and a mesh size of 3 mm. Hauls lasted on average only 10 min in order to obtain the krill in good condition.

For comparative studies the Northern krill, *Meganyctiphanes norvegica* was caught in June 1991 in the Gulmarnfjord (South Sweden) using an Isaacs–Kidd midwater trawl [14], opening 0.5 m^2.

Animals were frozen at -80°C immediately after capture and stored at this temperature until analysis.

2.2. Homogenates

Frozen animals were homogenized with an Ultraturrax (IKA 18/10, Jahnke and Kunkel, Staufen, Germany) in 2 ml of citrate–phosphate buffer (CPB, 0.2 $mol \cdot l^{-1}$, pH 5.5; McIlvain) per gram of krill, permanently cooled on ice [5]. After centrifuging for 30 min at $80000 \times g$ (5°C), the supernatant was applied onto G 25-sephadex PD-10 gel filtration columns (Pharmacia, Uppsala, Sweden) where it was desalted and simultaneously rebuffered into the appropriate buffer for the subsequent chromatographic step. An upscaled process utilized a single XK 50/30 column packed with 500 ml of G 25-gel instead of several PD-10 units. The extract was applied with a peristaltic pump at a flow rate of ≈ 5 $ml \cdot min^{-1}$. The capacity of the column was sufficient to easily process extracts prepared from up to 70 g of krill.

2.3. Determination of protein and enzyme activity

Protein was determined with the Micro BCA-Assay (Pierce, Rockford, IL). In order to eliminate interferences with different buffer systems used in each of the chromatographic steps the samples were washed as follows prior to routine assays: Proteins were precipitated by addition of 20 μl of 3 $mol \cdot l^{-1}$ trichloroacetic acid (TCA) to up to 500 μl of sample. After thorough mixing and centrifugation at $15000 \times g$ for 5 min the supernatant was discarded and the protein-pellet was re-dissolved in 50 μl of 0.1 $mol \cdot l^{-1}$ NaOH. Thereafter, the sample was supplemented with distilled water to 500 μl and the standard test procedure was run ([24]).

The activity of NAGase was determined photometrically [19,28]. Briefly, 50 μl of 0.2 $mol \cdot l^{-1}$ CPB, pH 5.5, were 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 were incubated for 15 min at 35°C. The reaction was stopped by addition of 2.5 ml of 0.01 $mol \cdot l^{-1}$ NaOH. The absorbance of samples and blanks was read at 410 nm.

2.4. Enzyme purification

All chromatographic steps [2,15] were performed at room temperature using a FPLC-system and chromatography columns from Pharmacia. The fraction sampler was cooled with ice during the operation. Sampled fractions were assayed for NAGase-activity and activity peaks were pooled. Prior to each new chromatographic step, samples were rebuffered into the respective starting buffer for the next chromatography by ultrafiltration (Amicon chamber 8050 with membrane Diaflo PM 10, exclusion size 10 kDa).

2.4.1. Anion exchange chromatography

Anion exchange chromatography on *Q*-sepharose HP XK 16/10 (16 mm diameter, 100 mm bed height) was run with a linear NaCl-gradient from 0 to 0.85 $mol \cdot l^{-1}$ at a flow rate of 3 $ml \cdot min^{-1}$. After upscaling, an XK 26/40 column was used with a stepwise increase in the NaCl-concentration and a flow rate of 4 $ml \cdot min^{-1}$. The elution buffer was 0.01 $mol \cdot l^{-1}$ imidazole/HCl, pH 6.8.

2.4.2. Hydrophobic interaction chromatography

Hydrophobic interaction chromatography (HIC) was performed on phenyl sepharose HP XK 16/10 using 0.01 mol·l^{-1} imidazole/HCl, pH 6.8 as elution buffer. A linear gradient was applied extending over 200 ml with decreasing ammonium sulphate (40–0% saturation) and increasing ethylene glycol concentrations (0–50% v/v). The flow rate was 1 ml·min^{-1}.

2.4.3. Affinity chromatography

Affinity chromatography was performed on concanavalin A (con A) sepharose XK 16/10 containing lectin ligands which specifically bind to glycosylic and mannosylic derivatives. The column was equilibrated with 0.02 mol·l^{-1} HEPES/NaOH, pH 7.4 containing 0.14 mol·l^{-1} NaCl, 1 mmol·l^{-1} $MnCl_2$ and 1 mmol·l^{-1} $CaCl_2$. After application the column was washed with the starting buffer to remove non-binding substances. Bound proteins were eluted with 0.02 mol·l^{-1} HEPES/NaOH, pH 7.6 containing 0.14 mol·l^{-1} NaCl and 0.2 mol·l^{-1} methyl-α-D-mannopyranoside. After use the column was reactivated with 0.1 mol·l^{-1} Na-acetate-buffer, pH 6.0 containing 1 mol·l^{-1} NaCl, 1 mmol·l^{-1} $CaCl_2$, 1 mmol·l^{-1} $MgCl_2$ and 1 mmol·l^{-1} $MnCl_2$.

2.4.4. Gel filtration

Gel filtration (size exclusion chromatography) was carried out on a superdex 200 prep grade XK 16/60 column with a separation range between M_r 10000 and M_r 1000000 corresponding to an elution volume of 100–40 ml, respectively. The elution buffer was 0.01 mol·l^{-1} imidazole/HCl, pH 6.8 containing 0.15 mol·l^{-1} NaCl. The sample was concentrated down to 1 ml prior to application. Molecular mass calibrations were performed with a gel filtration calibration kit (Pharmacia) containing: thyroglobulin (660 kDa), ferritin (440 kDa), aldolase (158 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa), and ribonuclease A (13.7 kDa). Due to the small fraction size of 0.5 ml, a relative resolution of 4% of the respective molecular mass was achieved.

2.4.5. High resolution anion exchange chromatography

High resolution anion exchange chromatography on mono *Q*-sepharose HP 5/5 was the final purification step. The same buffers were used as described for (Section 2.4.1). A gradient from 0 to 1 mol·l^{-1} NaCl extending over 30 ml was performed at a flow rate of 1 ml·min^{-1}.

2.5. Electrophoretic procedures

In parallel with the chromatographic separations the purification process was controlled by gel electrophoresis.

2.5.1. Native PAGE

A horizontal thin-layer (0.5 mm) polyacrylamide gel electrophoresis (PAGE, [26]) was conducted with a pore gradient from 5.1 to 18% T (Acrylamide). The gel was cast on a covalently binding polyester foil. The electrophoresis was performed under native conditions (without heating samples under reducing conditions and without use of SDS) in a LKB 2117 Multiphor II electrophoresis chamber with a programmable power supply (LKB 2303 Multidrive XI) under the following running parameters: 1200 V, 2 mA cm^{-1} gel width, max. 40 W, duration 1200 Vh, 4°C. The anode buffer was 0.25 mol·l^{-1} Tris–HCl, pH 8.4, the cathode buffer additionally contained 2 mol·l^{-1} glycine. In order to obtain a well detectable protein and enzyme content each sample was concentrated and desalted prior to application with ultrafiltration units (Millipore Centrifree UFC3LGC, cut-off 10 kDa). Due to the native conditions no MW-markers were used.

2.5.2. Native IEF

Isoelectric focusing [25] was performed using a rehydrated gel (Ampholine pH 3.5–9.5; LKB 1818–101) cast with a homogeneous pore matrix (30% T) on a polyester support [27]. On both sides of the electrodes pieces of filter paper were placed, soaked with 1 mol·l^{-1} NaOH, to absorb CO_2 which may cause a pH drift [15,25]. Concentrated and desalted samples were applied onto the gel surface using small rectangles of filter paper (5 × 10 mm). Markers (LKB 1860–203) were applied in the pI-range of 5.65–8.3. After a prefocusing phase of 30 min (1500 V, 8 mA, 8 W max) samples were applied and focused at 4°C in three phases: (a) 30 min at 500 V, 10 mA, 5 W; (b) 75 min at 2000 V, 8 mA, 8 W; (c) 15 min at 2000 V, 8 mA, 10 W.

2.5.3. Gel staining

All samples were applied onto the gel in duplicate sets. After electrophoresis the gel was cut into two pieces. One set was stained for proteins and the other set was stained for NAGase activity, allowing a direct comparison of proteins and enzyme activities.

2.5.4. Protein

Gel slabs were fixed for 30 min in a 20% TCA solution and then stained for 1.5 h in a solution of 40% methanol, 10% acetic acid and 0.1% coomassie blue (Servablue R 250). The background staining was removed by washing in a solution of 25% methanol and 10% acetic acid (1–3 h, controlled by observation). All steps were carried out in a water bath at 60°C under continuous agitation.

Activity staining was performed according to Hourdry ([13]) with the following modifications: 13 mg of naphthol-AS-Bi-*N*-acetyl-β-D-glucosaminide (Sigma N-4006) dissolved in 2 ml ethylene glycol monomethyl

Table 1
Purification of NAGase C from *E. superba* using 60 g of deep frozen krill (total and specific activity were multiplied by 10^{-3})

Purification step	Total protein (mg)	Total activity (E 10^{-3})	Specific activity (E·10^{-3} mg^{-1})	Purification factor	Yield (%)
Crude extract	3500	862	0.246	1.0	100
G25-superdex	2300	692	0.301	1.2	80
Q-sepharose	51.8	37.1*	0.716	2.9	4.3
Phenyl sepharose	12.4	434	35.0	142	50
Superdex 200	3.1	225	72.6	295	26
Mono-*Q*	1.04	154	148.0	601	17

* The apparent loss of activity after use of *Q*-sepharose was probably due to a co-chromatographing of an enzyme inhibitor which had been removed during the subsequent steps.

ether (Serva 11310), and 45 mg Fast Garnet GBC (Sigma F8761) dissolved under vigorous shaking in 2 ml 0.2 mol·l^{-1} CPB, pH 5.5 were given to 50 ml of 0.2 mol·l^{-1} CPB, pH 5.5. Gels were incubated in this solution for 15–30 min at 35 °C until enough red azo-dye developed by enzymatic activity. Thereafter, the gel was incubated for 5 min in a destaining solution (25% methanol, 10% acetic acid) to remove the slight yellow background.

2.6. Immunological techniques

A rabbit (White Newzealand) was immunized with *200* μg of purified NAGase C dissolved in 500 μl of 0.14 mol·l^{-1} NaCl and emulsified with 500 μl of Freund's complete adjuvans. The first immunization was followed by three booster injections at intervals of 3–4 weeks. In the latter cases the enzyme was emulsified in Freund's incomplete adjuvans. After 8 weeks the serum was tested for antibodies by Dot-Blot-test. The specificity of the antibody was determined using an immunotitration method ([21]):

Fifty µl of respective NAGase preparations (sample) were supplemented with an increasing amount (1–14 μl) of antiserum (diluted 1:20), filled up to 100 μl with TBS buffer (50 mmol·l^{-1} Tris–HCl, pH 7.6 + 0.14 mol·l^{-1} NaCl + 1 mmol·l^{-1} EDTA) containing 0.05% Triton X-100 and incubated for 4 h at 25°C. A few milligrams of hydrated protein A sepharose (Pharmacia) were then added to the mixture and after shaking for ≈30 min the reaction caps were centrifuged (5 min at 15000). A 'control' containing 50 μl sample and 50 μl buffer only was incubated in parallel (without addition of antiserum and protein A sepharose) and compared to the '0 μl antiserum' sample (with addition of protein A sepharose) in order to assess the amount of unspecified precipitation which might have been caused by the addition of protein A sepharose to the samples. A blank consisting of 14 μl antiserum and 86 μl buffer served to correct for the background colour. Since protein A binds specifically to IgGs the supernatant becomes totally depleted of IgGs if protein A is added in excess. Subsequent testing of the supernatant for NAGase activity revealed whether the antibody bound to the NAGase.

Preparations of NAGases from *E. superba* and also from *M. norvegica* were tested for immunospecificity.

3. Results

3.1. NAGase purification

Anion exchange chromatography of crude extracts of *E. superba* resulted in two main NAGase peaks similar to those that have been previously described [5,28]. They were named NAGase B and NAGase C (Fig. 1(a)) according to the established terminology ([5]). NAGase B was less homogeneous than NAGase C. Further separation of the NAGase B pool on phenyl sepharose (HIC) gave three distinct peaks termed B_1, B_2, and B_3 (Fig. 1(b)). After gel filtration only B_1 eluted within a single peak (Fig. 1(c)) with a molecular mass of 160 kDa. The pools of B_2 and B_3 were further separated into three (120, 160 and 240 kDa) and two peaks (70 and 140 kDa), respectively (Fig. 1(d, e)). Accordingly, NAGase B proved to be a mixture of active enzymes or/and enzyme fragments with different molecular masses (Table 2).

In contrast to NAGase B, NAGase C was more homogeneous and eluted within one single sharp activity peak after anion exchange chromatography (Fig. 1(a)). Chromatography on HIC also gave a distinct peak (Fig. 1(f)). Accordingly, NAGase C proved to be more suitable for further chromatographic processing so as to obtain the high purity desired for antibody production. The subsequent chromatographic steps clearly demonstrate the purification process. After gel filtration a large peak of unspecific protein was separated (Fig. 1(g)) and the mono–*Q* profile displayed a nearly identical pattern of NAGase activity and protein absorbance (Fig. 1(h)). With this purification protocol NAGase C was enriched ≈600-fold with a final yield of approximately 17% (Table 1).

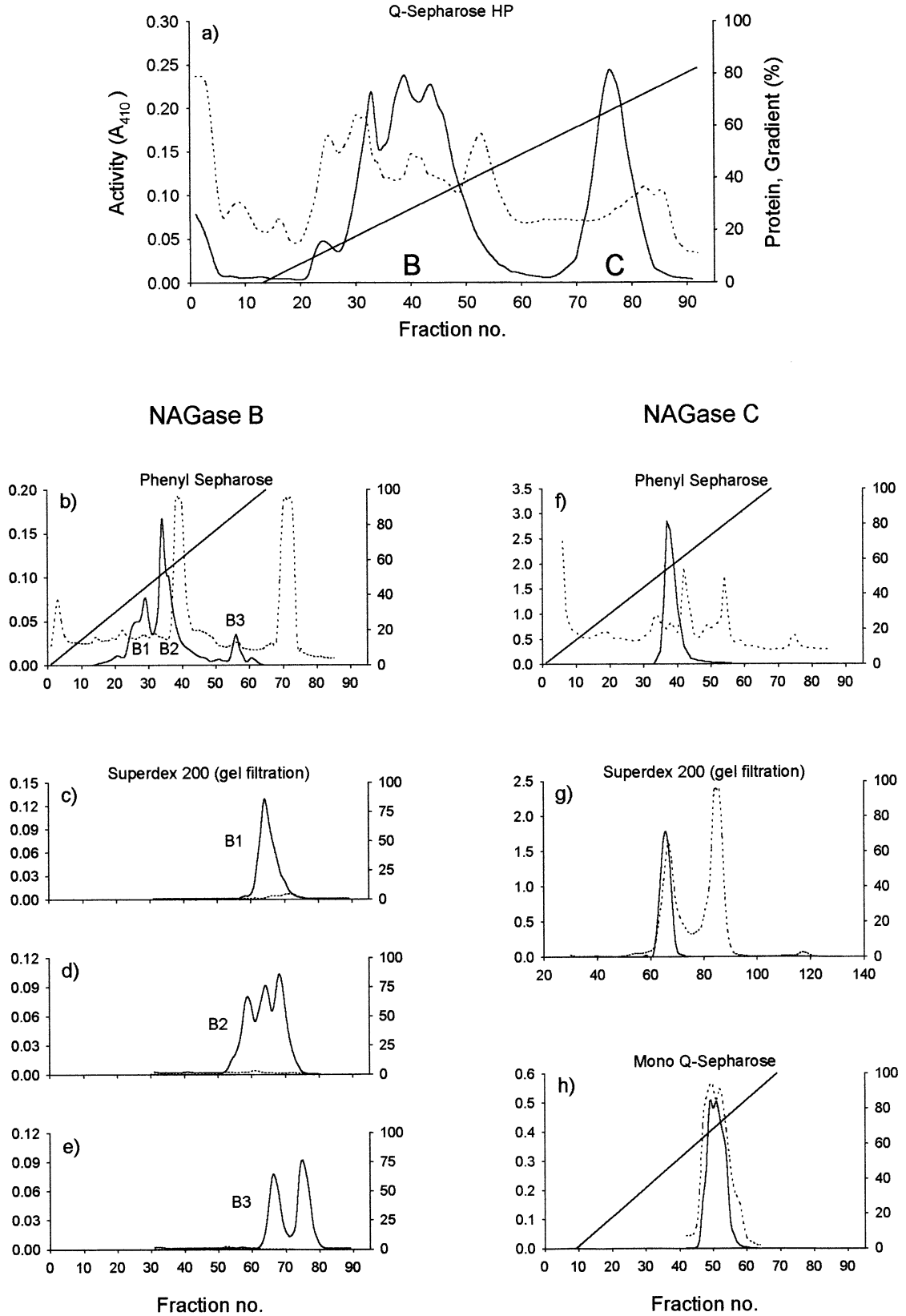

Fig. 1. Chromatographic purification of *N*-acetyl-β-D-glucosaminidases B and C from *E. superba* crude extracts. Following anion exchange chromatography (a), NAGase B was further purified using HIC (b) and gel filtration (c–e). NAGase C was purified to homogeneity using HIC (f), gel filtration (g) and high resolution anion exchange chromatography (h). Solid lines indicate NAGase-activity, broken lines indicate the protein profile (A_{280}).

3.2. Electrophoretic properties

The chromatographic results were supported by the native PAGE (Fig. 2(a)): only small amounts of contaminating proteins were visible in the protein trace of the mono-*Q* eluate. The NAGase C showed a single sharp band. The apparently increasing protein content and some additional protein bands over the first three chromatographic steps resulted from the increasing concentration of the samples applied to the PAGE.

On the native IEF, NAGase C split into four main bands with pIs of 5.6–5.9 after purification on *Q*-sepharose and gel filtration (Fig. 2(b)).

3.3. Characteristics of NAGase isoforms

A main difference between the two NAGase fractions B and C was found when being chromatographed on con A-sepharose: While NAGase B completely bound to con A, NAGase C proved to have no affinity at all toward the lectin ligands.

The molecular masses of the NAGase B-isoforms ranged between 70 and 240 kDa, while NAGase C displayed a homogeneous mass of 150 kDa (summarized in Table 2). None of the masses reported for NAGase B had a common divisor with the mass reported for NAGase C. Furthermore, both NAGases eluted widely separated on anion exchange chromatography. A supplement study on the kinetic properties of NAGase B and C (unpublished data, compare [5]) revealed different Michaelis-constants (K_M) and activation energies for both enzyme pools (Table 2).

3.4. Immunology

Eight weeks after the first immunization of the rabbit, antibodies which reacted specifically with NAGase C (purified after HIC) were detected by Dot-Blot-tests.

Specificity determinations by the immunotitration-technique revealed a high ability of the antibody anti-(*E. superba*-NAGase C)-IgG to discriminate between both enzyme forms: immunotitration of NAGase C with the antibody resulted in a total loss of activity in the supernatant after precipitating the antibody complex with protein A sepharose (Fig. 3). While the antibody bound quantitatively to NAGase C, only $8.9 \pm 1.5\%$ of cross-reactivity occurred with NAGase B.

Table 2
Characteristics of NAGase B and NAGase C from *E. superba*

Property	NAGase B	NAGase C
Elution from *Q*-sepharose at NACl-gradient ($mol \cdot l^{-1}$)	0.2–0.35	0.7
Affinity to concanavalin A	+	–
Molecular mass (kDa)	70, 120, 140, 160, 240	150
Reaction with the antibody anti-(*E. superba*-NAGase C)-IgG (%)	<9	100
K_M value ($mmol \cdot l^{-1}$)	0.06 ± 0.011	0.11 ± 0.03
Activation energy ($kJ \cdot mol^{-1}$)	64.9 ± 0.4	59.8 ± 2.0

3.5. Comparison between Euphausia superba and Meganyctiphanes norvegica

The chromatographic properties of the NAGases from the Antarctic krill, *E. superba*, were compared with those of the Northern krill, *M. norvegica*, using anion exchange chromatography (*Q*-sepharose). In both cases two peaks appeared and the profiles matched closely (results not shown). The two resulting enzyme pools obtained from *M. norvegica* were named NAGase II and NAGase III, corresponding to NAGases B and C from *E. superba*.

Comparative immunotitration of *M. norvegica* NAGases with anti-(*E. superba*-NAGase C)-serum revealed a very similar binding pattern. The antibody bound with 98% to NAGase III and only with 25% to NAGase II. The higher cross-reactivity with NAGase II and the incomplete reaction with NAGase III might have occurred because the enzyme preparations in *M. norvegica* were not performed to maximum purity but passed only the first purification step (anion exchange). Therefore, cross-contamination might have impaired the result.

4. Discussion

The investigation of enzymatic properties is an essential tool to study physiological adaptations expressed by organisms in response to different environmental conditions, since they in turn determine the rate of biochemical reactions. In general, enzymatic activities can be regulated in two ways [12]: the first one is to alter the kinetic properties of enzymes for obtaining different catalytic rates. This qualitative regulation may take place either by the expression of different isoforms with characteristics that better meet the new demands, or by modulation of existing enzymes through effectors inducing changes in the katalytic properties of an enzyme. The other way, the quantitative regulation, is altering the enzyme concentration by means of synthesis and degradation.

Recent investigations on krill have shown that there are few substantial differences in the kinetic properties of NAGases between the polar *E. superba* and the boreal *M. norvegica* [5,28] and that those differences found may not sufficiently compensate for the different temperature regimes these animals are exposed to (-1–2°C for *E. superba* and 4–16°C for *M. norvegica* [8]). However, for maintaining the energetically

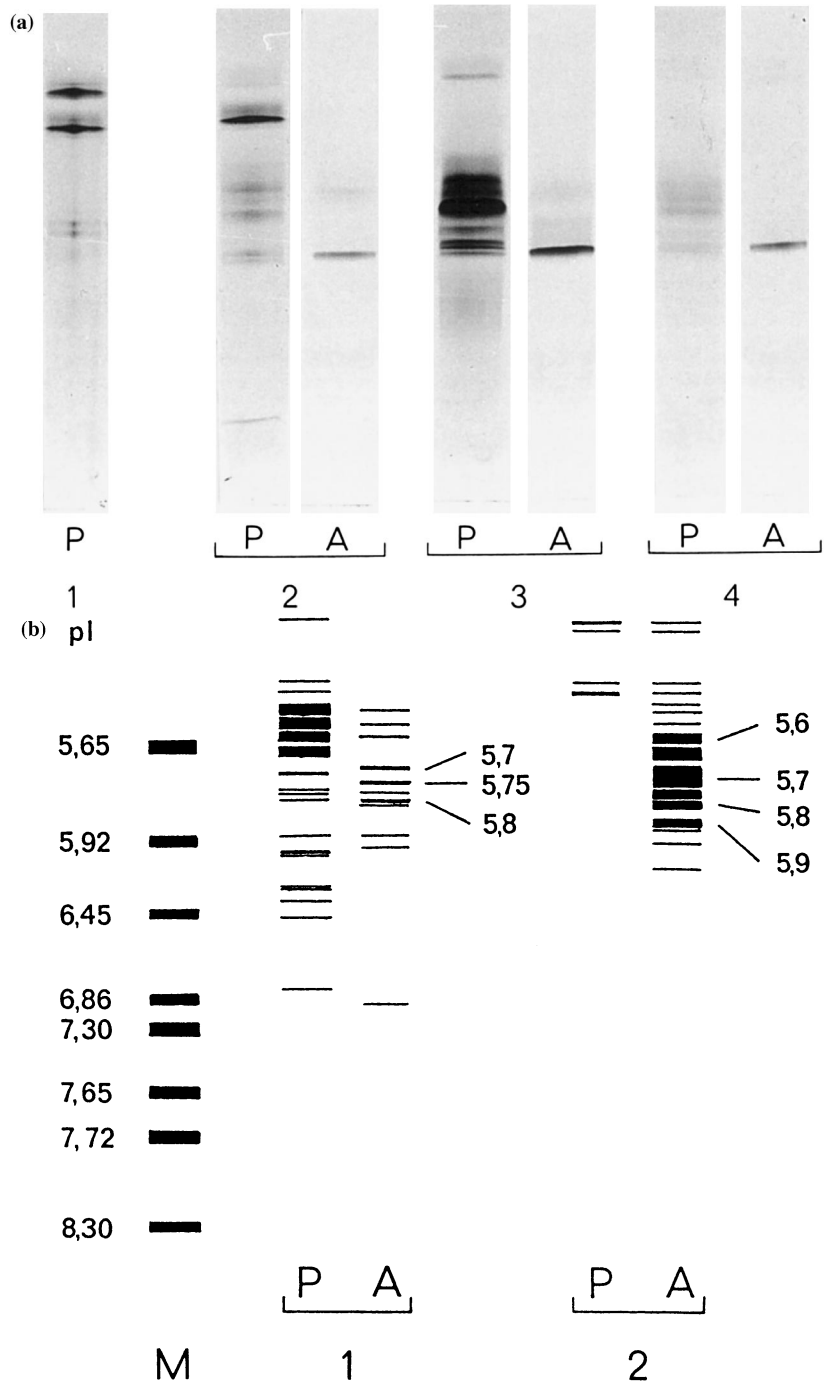

Fig. 2. a) Native PAGE of NAGase C after passing the following purification steps: (1) *Q*-Sepharose; (2) HIC; (3) Superdex 200; (4) Mono-*Q*-Sepharose. b) Native isoelectric focusing with rehydrated gel: *M*, pI-marker; NAGase C after (1) *Q*-Sepharose, (2) Gelfiltration (Superdex). *P* = protein, *A* = NAGase activity (in 2(b) redrawn from the gel).

demanding way of life expressed by these constantly swimming ectothermes, some adaptive regulation must occur. We, therefore wanted to examine whether this enzyme system might be regulated qualitatively by expression of different isoenzymes or quantitatively by adjustments in the concentration of one single enzyme. A prerequisite for this approach was the purification of the enzyme and the production of antibodies against this protein for a reliable immunological identification.

The chromatographic and immunological properties show that the two NAGases from *E. superba* differ to a great extent. In the first place, there seems to be a pronounced difference in the molecular charge which is indicated by the wide elution distance between NAGase B and NAGase C on ion exchange chromatography (Fig. 1). The HIC also revealed differences in the

hydrophobic properties of both forms. Furthermore, NAGase B expressed a very inhomogeneous pattern on HIC. This result was confirmed by the gel filtration in that one single molecular mass could be addressed to NAGase C while five different molecular masses were found for NAGase B. This elution pattern suggests that at least some of the activity peaks were probably fragments of one or more oligomeric proteins.

Partial defolding of labile enzymes is known to frequently occur after HIC [9]. In our study this effect may have resulted in a disintegration of the protein on gel filtration chromatography or even earlier on HIC, resulting in fragments which still retained their enzymatic activity, e.g. the B_3-fraction split into two portions of M_r 70000 and 140000, one of which might be a monomer, the other a dimer. The same may be valid for NAGase B_2 with its M_r 120000 and M_r 240000 fractions. The remaining M_r 160000 fraction may have been caused by a contamination with NAGase B_1 which eluted close to B_2 after passing HIC. Since there was no common divisor for all of the fragments, we conclude that NAGase B consisted of at least two, or even three (M_rs 140000, 160000 and 240000), original proteins. To our knowledge, however, a such high molecular mass of 240000 (NAGase B_2) has not been reported yet for NAGases or other hexosaminidases [10]. In the literature, molecular masses of hexosaminidases range from M_r 60000 to M_r 150000 [7,20,22,23] corresponding to our results on NAGase C.

The occurrance of several activity bands of the generally homogeneous NAGase C on IEF indicated the presence of differently charged isomers of this enzyme. This is also supported by the shape of the activity maximum when chromatographed on the high resolution mono-*Q* column. Although eluting homogeneously during the previous purification steps, on mono-*Q* a double-peaked maximum appeared which was reproduced in several chromatographic runs. Such variations in the molecular charge of the same enzyme might result from post-translational modifications, affecting only a fraction of the enzyme molecules [16].

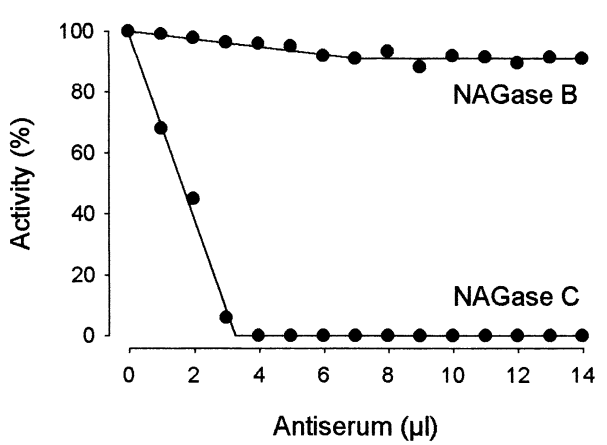

Fig. 3. Immunotitration of NAGase B and NAGase C after purification on *Q*-sepharose and phenyl-sepharose. An increasing amount of antiserum lead to a total precipitation of NAGase C, whereas only 9% of cross-reactivity occurred with NAGase B.

An additional interesting difference between NAGase B and NAGase C was their opposite reaction towards con A sepharose. The binding of all NAGase B fractions characterized this enzyme as a glycoprotein. In contrast, NAGase C showed no affinity, thus indicating that it does not possess oligosaccharide chains, at least none of those saccharides specifically binding to Con A, α-D-glucose and α-D-mannose.

This result suggests that the two isoforms might be located at different sites within the cell. Glycoproteins are mainly bound to membranes or restricted to compartments like the endoplasmic reticulum or lysosomes whereas cytosolic proteins never show glycosylation [1]. If the two enzymes actually do have different sites of activity this may indicate different metabolic functions and gives rise to the question, whether it would still be correct to use the term isoenzyme in the case of the two NAGase-forms. As a consequence the enzymes would not be functionally interchangeable and it would therefore be very unlikely that alteration of their ratios in response to temperature variation could regulate metabolic functions.

The immunological properties clearly differentiated the two NAGase forms, emphasizing the structural differences we found using chromatographic techniques. The unrestricted affinity of the antibody towards NAGase C and the very low cross-reactivity with NAGase B provides the basis for further investigations, where both enzymes shall be examined separately. Furthermore, the similar affinity pattern displayed by the corresponding NAGases from *M. norvegica* enables the use of the antibody for studies in *M. norvegica* as well.

Another important conclusion which can be drawn from the immunological results is that the NAGases from *E. superba* and *M. norvegica* resemble each other structurally very closely. This is also supported by the similar elution pattern found after chromatography on *Q*-sepharose. Previous studies [5,28] support this finding in that they revealed only marginal differences in energies of activation, temperature- and pH-profiles, thermal stability and substrate affinities in the corresponding NAGases from the two euphausiids. Furthermore, the molecular mass of M_r 150000 determined for NAGase C corresponds very well with the mass of M_r 152000 of NAGase III from *M. norvegica* [5]. Thus, although both euphausiid species have evolved from a common ancestor some million years ago and now are geographically segregated, inhabiting different climatic zones, their NAGases still retained profound similarities. This indicates that this essential chitinolytic enzyme system is inherited conservatively within the euphausiids and potentially within the entire class of Crustacea.

The fact that the properties of the NAGases from *E. superba* and *M. norvegica* show such a high degree of similarity, both structurally and catalytically, favours the assumption that each of the two enzymes might be regulated in a quantitative way by means of synthesis and degradation. Thus, different environmental temperatures might be compensated by adjustments in the enzyme concentration alone. Additionally, there is a wide range of variation of NAGase activity in the integument of *E. superba* in relation to the molt cycle [3] thus pointing to the fact that not only temperature- but also molt-dependent regulation takes place. This has to be taken into account as mechanisms of temperature adaptation are to be considered.

The prepared antibody now provides the possibility not only to quantify the contribution of each enzyme fraction to the total NAGase activity but also to elucidate their tissue-specific distribution within the organism in the context of molting and digestive processes.

Acknowledgements

G.P. and F.B. are indebted to S. Rakusa-Suszczewski, and the members of the 15th Polish Antarctic expedition for their hospitality during a stay at the Polish Antarctic Station 'Henrik Arctowski'. The Alfred-Wegener-Institut, Bremerhaven, provided logistic support and facilitated the transportation of scientific equipment on RV 'Polarstern'. We are grateful to D. Adelung for providing laboratory space and facilities. Thanks are further due to R.A.H. Vetter for fruitful discussions, and S. Böhm, M. Ehmke-Kasch and V. Stenzel for their assistance. This work has been supported by a grant from the German Research Council (DFG Bu 548/2).

References

[1] Alberts B, Bray D, Lewis J, Raff M, Roberts K, Watson JD. Molecular Biology of the Cell. New York: Garland, 1983:345–76.

[2] FPLC ion exchange and chromatofocusing. Uppsala: Pharmacia Technical Booklet No. 17-0558-01, Pharmacia Laboratory Separation Division; 1985:171.

[3] Buchholz F. Moult cycle and seasonal activities of chitinolytic enzymes in the integument and digestive tract of Antarctic krill, *Euphausia superba*. Polar Biol 1989;9:311–7.

[4] Buchholz F. Moult cycle and growth of Antarctic krill, *Euphausia superba* in the laboratory. Mar Ecol Prog Ser 1991;69:217–29.

[5] Buchholz F, Vetter RAH. Enzyme kinetics in cold water: characteristics of *N*-acetyl-β-D-glucosaminidase activity in the Antarctic krill, *Euphausia superba*, compared to other crustacean species. J Comp Physiol B 1993;163:28–37.

[6] Buchholz F, Saborowski R. A field study on the physiology of digestion in the Antarctic krill, *Euphausia superba*, with special regard to chitinolytic enzymes. J Plankton Res 1996;18:895–906.

[7] Dziadik-Turner C, Koga D, Mai MS, Kramer KJ. Purification and characterization of two β-*N*-acetylhexosaminidases from the Tobacco Hornworm, *Manduca sexta* (L). Arch Biochem Biophy 1981;212:546–60.

[8] Einarsson H. Euphausiacea. I. North Atlantic species. Dana Rep 1945;5:1–185.

[9] Eriksson KO. Hydrophobic interaction chromatography. In: Janson JC, Rydén L, editors. Protein Purification. New York: VCH Publishers, 1989:207–26.

[10] Flach J, Pilet PE, Jollès P. What's new in chitinase research? Experientia 1992;48:701–16.

[11] Hamner WH, Hamner PP, Strand SW, Gilmer RW. Behavior of Antarctic krill, *Euphausia superba*: chemoreception, feeding, schooling and molting. Science 1983;220:433–5.

[12] Hochachka PW, Somero GN. Strategien Biochemischer Anpassung. Stuttgart: Thieme Verlag, 1980:403.

[13] Hourdry J. Analyse électrophorétique de la *N*-acétyl-β-glucosaminidase intestinale, au cours du développement larvaire et de la métamorphose de *Discoglossus pictus*. CR Acad Sc Paris 1977;284:2023–5.

[14] Isaacs JE, Kidd LW. Isaacs-Kidd midwater trawl. Uni Calif Scripps Instit Ocean SCIO 1953;53:1–18.

[15] Janson JC, Rydén, L., editors. Protein Purification, New York: VCH Publishers, 1989:502.

[16] Karlsson E, Rydén L, Brewer J. Ion exchange chromatography. In: Janson JC, Rydén L, editors. Protein Purification. New York: VCH Publishers, 1989:107–48.

[17] Kils U. Swimming behaviour, swimming performance and energy balance of Antarctic krill, *Euphausia superba*. BIOMASS Sci 1982;3:1–121.

[18] Kils U. Swimming and feeding of Antarctic krill, *Euphausia superba*-some outstanding energetics and dynamics, some unique morphological details. Ber Polarforsch Sonderh 1983;4:130–55.

[19] Kimura S. The β-*N*-acetylglucosaminidase from *Bombyx mori* (L). Comp Biochem Physiol 1974;49B:345–51.

[20] Lynn KR. Chitinases and chitobiases from the American lobster (*Homarus americanus*). Comp Biochem Physiol 1990;96B:761–6.

[21] Mentlein R, Berge RK, Heymann E. Identity of purified monoacylglycerol lipase, palmitoyl-CoA hydrolase and aspirin-metabolizing carboxyesterase from rat liver microsomal fractions. Biochem J 1985;232:479–83.

[22] Mommsen TP. Chitinase and β-*N*-acetylglucosaminidase from the digestive fluid of the spider, *Cupiennius salei*. Biochem Biophys Acta 1980;612:361–72.

[23] Mozo PS, Rama MF, Pernas RV, Amil MR. Purification and properties of two enzymatic forms of β-N-acetylglucosaminidase from *Mytilus edulis* L. hepatopancreas. Comp Biochem Physiol 1977;58B:29–34.

[24] Pierce Chemical Company. Micro BCA protein assay reagent. Technical Bulletin 23235, Rockford, IL, 1989:14.

[25] Righetti PG. Isoelectric Focusing. Amsterdam: Elsevier Biochemical Press, 1983:386.

[26] Schickle HP, Theßeling G, Westermeier R. Pharmacia Technical Leaflet No. SDRE-58. Freiburg: Pharmacia LKB Biotechnologie, 1989:23.

[27] Schickle HP, Westermeier R. Pharmacia Technical Leaflet No. SDRE-01. Freiburg: Pharmacia LKB Biotechnologie, 1989:16.

[28] Spindler KD, Buchholz F. Partial characterization of chitin degrading enzymes from two euphausiids, *Euphausia superba* and *Meganyctiphanes norvegica*. Polar Biol 1988;9:115–22.

[29] Vetter RAH, Saborowski R, Peters G, Buchholz F. Temperature adaptation and regulation of citrate synthase in the Antarctic krill compared with other crustaceans from different climatic zones. In: Battaglia B, Valencia J, Walton DWH, editors. Antarctic Communities: Species, Structure and Survival. London: Cambridge University Press, 1997:295–9.