N-Acetyl-β-D-hexosaminidases of the Brine Shrimp *Artemia*: Partial Purification and Characterization

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Artemia, N-Acetyl-β-D-hexosaminidases, Isoenzymes, Kinetics

N-Acetyl-β-D-hexosaminidases (EC 3.2.1.52) from *Artemia* nauplii were isolated and characterized. Three different enzymes I, II_1 and II_2 were separated according to their behaviour on anion exchange chromatography and gel filtration columns. Their apparent molecular masses were 83,000 ± 7000, 110,000 ± 10,000 and 56,000 ± 5000 Da with corresponding S-values of 8.6, 11.9 and 7.9. All three enzymes also differ in their apparent pH-optima (5.1, 4.5 and 6.1) and they all bind to concanavalin A.

The three enzymes have about the same affinities (app. K_m between 0.16 and 0.72 mmol/l) for the three substrates (*p*-nitrophenyl-N-acetyl-β-D-glucosamine or *p*-nitrophenyl-N-acetyl-β-D-galactosamine and N,N′-diacetyl-chitobiose) and are therefore N-acetyl-β-D-hexosaminidases. In contrast, the three enzymes behave quite differently, both in terms of their inhibitor constants and the type of inhibition. The substrates inhibit both enzymes II_1 and II_2 but not enzyme I. On the other hand, N-acetyl-β-D-galactosamine inhibits enzyme I in a non-competitive way but not enzymes II_1 and II_2. All three enzymes are inhibited by the end product N-acetyl-β-D-glucosamine, enzyme I in a competitive manner, both enzymes II_1 and II_2 in a non-competitive way. 2-Acetamido-2-deoxy-D-galactonolactone is a strong inhibitor for enzyme I (K_i = 13 μmol/l) with much lower affinities towards enzymes II_1 and II_2 (K_i = 0.63 and 1.03 mmol/l). All three enzymes are inhibited in a dose-dependent way and completely reversible by α-methyl-mannoside.

Introduction

In addition to their various functions in the metabolism of glycoproteins, glycolipids, sphingolipids and gangliosides, N-acetyl-β-D-hexosaminidases together with chitinases also play an important role in the degradation of chitin [1–4]. The presence of both enzymes has already been described in *Artemia* and the involvement of these enzymes not only in moulting processes but also for the hatching of the nauplii has been reported in preceeding papers [5, 6]. However, so far only chitinase was characterized in *Artemia* [7, 8]. When studying the levels of N-acetyl-β-D-hexosaminidase and of chitinase during development from cysts to adults, it was necessary to separate these two enzymes by anion exchange chromatography and we found that, specifically during earliest time of development, a second peak of N-acetyl-β-D-hexosaminidase activity appeared [6]. In order to understand better the physiological roles of the N-acetyl-β-D-hexosaminidases at certain developmental stages, a characterization of these enzymes was necessary. We selected nauplii for this investigation, because they contain the two clearly separated enzymatic activities.

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Materials and Methods

Animals and preparation of cytosol

Cysts from Sera Aquaristic were used for rearing of the nauplii; 2 g dry cysts/l medium (400 mM NaCl, 10 mM $MgCl_2$, 10 mM $CaCl_2$) were incubated at 25 °C with continuous aeration. After 2 days nauplii were collected (making use of the positive phototactic behaviour), thoroughly washed with distilled water and shock-frozen. For all experiments described here only nauplii were used. The material was lyophilized, powdered and stored at −20 °C for several months without loss of activity. The dry powder was extracted with acetone at −10 °C, centrifuged at −10 °C (5 min, 7000 x *g*), the supernatant decanted and the pellet reextracted 3 to 4 times and the final pellet was dried at 4 °C. This dry powder could be stored at −20 °C for several months. For all chromatographic purposes, this powder was suspended in 10 mM sodium-potassium phosphate buffer, pH 6.2 and homogenized with an Ultra-Turrax (18 times for

10 sec each, under cooling with ice). This homogenate was centrifuged at 4 °C for 1 h at 150,000 × *g*. The supernatant was filtered, the pellet reextracted as described and the supernatants combined. For comparison homogenate from freshly collected nauplii was used also prepared as described above. Such crude extracts are very stable at 4 °C; after two months although only about 10% of the original protein is still present, 80% of the total N-acetyl-β-D-hexosaminidase activity remain.

Purification of N-acetyl-β-D-hexosaminidases

Anion exchange chromatography was performed on DEAE-Sepharose CL-6 B (Pharmacia) as described elsewhere [6, 7]. N-Acetyl-β-D-hexosaminidase activity can be separated by this procedure into two enzymes, I and II. The pooled fractions of enzymes I and II are free of chitinase, which is also present in the crude homogenate [7].

All other chromatographic steps as well as the density-gradient centrifugation were performed essentially as described previously [7].

N-Acetyl-β-D-hexosaminidase assay

N-Acetyl-β-D-hexosaminidase activity was determined as follows: between 50 and 100 µl of the samples were mixed with 50 µl of 0.3% *p*-nitrophenyl-N-acetyl-β-D-glucosamine (*p*NPGlcNAc) or *p*-nitrophenyl-N-acetyl-β-D-galactosamine (*p*NPGalNAc) (both from Serva, Heidelberg, B.R.D.) in 200 mM sodium citrate-phosphate buffer, pH 5.5 and incubated at 40 °C. After 30 min the reaction was stopped by addition of 2.5 ml 10 mM NaOH and the absorbance measured at 410 nm. The standard assays were performed with *p*NPGlcNAc as substrate. If N,N′-diacetyl-chitobiose ($GlcNAc_2$) (Sigma, St. Louis, U.S.A.) was used as a substrate, the resulting endproduct N-acetyl-glucosamine (GlcNAc) was determined as already described [9].

Protein determination

Protein was determined according to Bradford [10] using bovine serum albumin as a standard.

Results

During purification (see Table I) enzymatic activity can be separated into 3 clearly distinguishable fractions. On anion exchange columns there are 2 peaks of enzymatic activity, enzyme I and II [6, 7]. Enzyme II consists of 2 active fractions, which are different in size. The apparent molecular masses as determined by gel permeation chroma-

Table I. Purification scheme of N-acetyl-β-D-hexosaminidases from *Artemia* nauplii. I, II_1, and II_2 are the three fractions with enzymatic activity.

Purification step	Total protein [mg]		Activity µM/min		%		Spec. activity µM/min × mg protein^{-1}		Purification fold	
Crude extract (day 0)	103.0		1136		100		11.0		–	
Crude extract (day 3	62.9		1291		114		20.5		1.9	
	I	II	I	II	I	II	I	II	I	II
Anion exchange chromatography	2.3	2.4	678	344	60	30	289	142	26	13
Gelfiltration	0.5	*	264	*	23	*	524	*	48	*
Concanavalin-A sepharose-chrom.	0.07	*	127	*	11	*	1814	*	165	*

* Data on a separate sheet, since the activity of enzyme II splits into two isoenzymes.

*

Purification step	Total protein [mg]		Activity µM/min		%		Spec. activity µM/min × mg protein^{-1}		Purification fold	
	II_1	II_2	II_1	II_2	II_1	II_2	II_1	II_2	II_1	II_2
Gelfiltration	0.31	0.22	52	39	4.6	3.4	170	177	15	16
Concanavalin-A sepharose	0.03	0.026	22	27	1.9	2.4	733	1056	67	96

tography are 110,000 ± 10,000 for enzyme II_1 and 56,000 ± 5000 for enzyme II_2, whereas enzyme I has an apparent molecular mass of 83,000 ± 7000 (means ± S.D., $n = 10$). These differences in apparent molecular mass as determined by gel filtration are also reflected by density-gradient centrifugation which reveals S-values of 8.6 for enzyme I and 11.9, and 7.9 for enzymes II_1 and II_2, respectively. In Fig. 1 one representative example of a density-gradient centrifugation is shown.

The activities of all three enzymes are not influenced by high salt concentrations (NaCl up to 15 M), Ca^{2+}- and Cu^{2+}-ions (1 and 3 mM), N-ethylmaleimide (1 mM) or sucrose (0.4 M). α-Methyl-mannoside, which is used for the desorption of the three enzymes from a Concanavalin A column, strongly inhibited the enzymatic activity of all three enzymes in a dose-dependent way (Fig. 2). This inhibition was fully reversible.

The 3 enzymes can also be distinguished by other criteria. The isoelectric points of the enzymes II_1 and II_2 are 5.9 (Fig. 3), those of enzyme I is 5.4. The pH optima and the shape of the optimum-curves are different for all 3 enzymes (Fig. 4). The apparent pH optimum for enzyme I is 5.1, those for enzymes II_1 and II_2 are 4.5 and 6.1, respectively.

In addition to the physico-chemical differences there are also pronounced differences in the kinetic properties of the 3 enzymes. The following parameters were determined: K_m-values for 3 substrates,

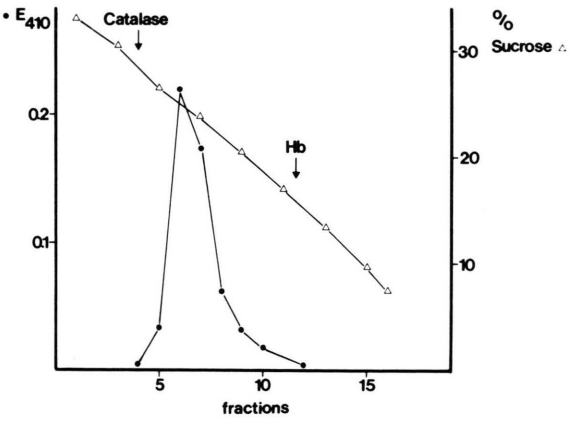

Fig. 1. Density gradient centrifugation of N-acetyl-β-D-hexosaminidase I from *Artemia* nauplii (4 °C, 50,000 rpm, rotor TST 54, Kontron, 13 h). Catalase and hemoglobin (Hb) were used as calibration markers.

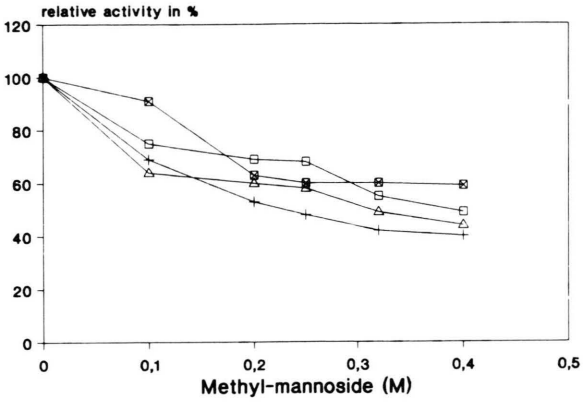

Fig. 2. Influence of α-Methyl-mannoside on N-acetyl-β-D-hexosaminidase I (+), II_1 (□), II_2 (△) and chitinase (⊠) from *Artemia* nauplii.

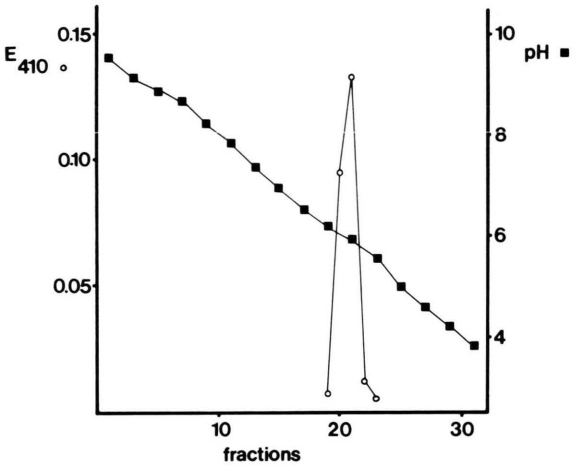

Fig. 3. Determination of the isoelectric point of N-acetyl-β-D-hexosaminidase II_2 from *Artemia* nauplii by preparative isoelectric focussing (Ultrodex, LKB). Activity could be detected in only 5 fractions.

inhibitor constants and the type of inhibition for the substrates, products and inhibitors according to Ahlers *et al.* [11].

Enzyme I has a higher affinity for all 3 substrates used as compared to the enzymes of type II (Table II) and in addition, enzyme I is not inhibited by its substrates (Fig. 5), in contrast to the enzymes II (Fig. 6). The constants K_{ss} for the formation of the catalytically dead-end-complex ESS were determined by a Dixon-plot, as shown in Fig. 6c for enzyme II_1. The complex pattern of inhibition of the 3 enzymes is demonstrated in Fig. 7 and 8 and summarized in Table III.

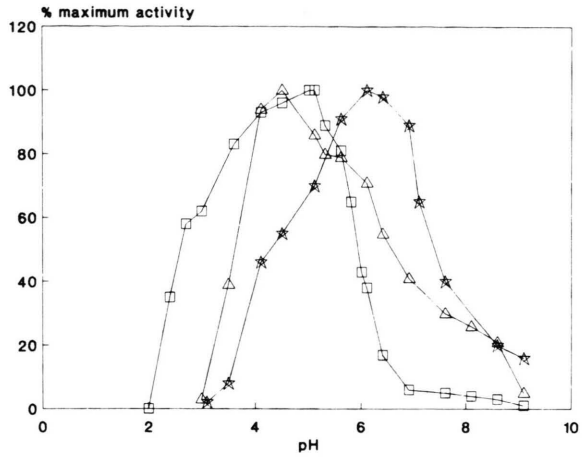

Fig. 4. Effect of pH on the activity of N-acetyl-β-D-hexosaminidases I (□), II_1 (△) and II_2 (☆) from *Artemia* nauplii. The values are means of 4 independent determinations. S.D. are < 5%.

Table II. Apparent K_m-values of the N-acetyl-β-D-hexosaminidases from *Artemia* nauplii for 3 substrates. All experiments were performed four times, the standard deviations are less than 5%. The values were calculated from Lineweaver-Burk plots.

Substrate	K_m-values (mmol/l)		
	Enzyme I	Enzyme II_1	Enzyme II_2
*p*NPGlcNAc	0.16	0.72	0.63
*p*NPGalNAc	0.21	0.31	0.40
$GlcNAc_2$	0.23	0.65	0.62

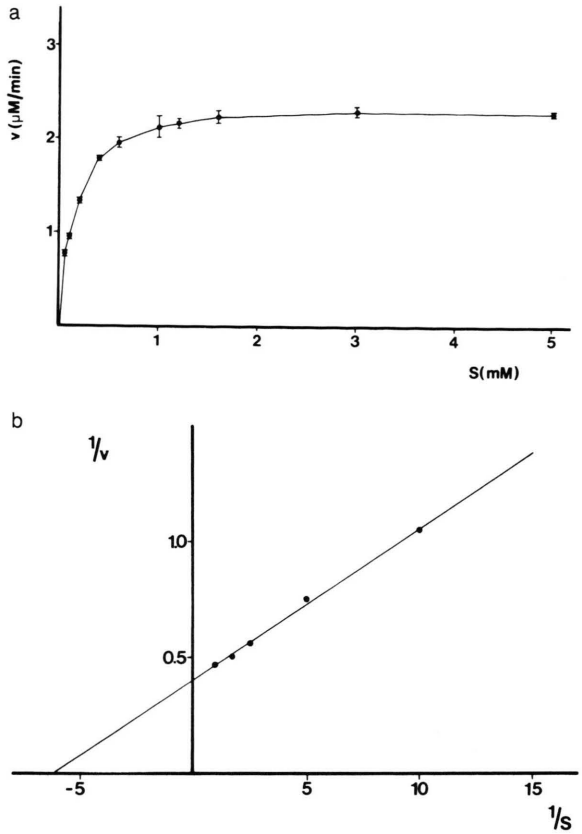

Fig. 5. Effect of the concentration of the substrate (*p*NPGlcNAc) on the activity of N-acetyl-β-D-hexosaminidase I from *Artemia* nauplii (means ± S.D., $n = 4$). In b) the result is shown as a Lineweaver-Burk plot (regression lines).

Table III. Inhibition constants and type of inhibition for the three N-acetyl-β-D-hexosaminidases from *Artemia* nauplii.

Inhibitor	Enzyme I	Enzyme II_1	Enzyme II_2
	Type of inhibition Inhibition constants [mmol/l]		
GlcNAc	competitive $K = 5.28$	non-competitive $K_i = 10.76$ $K_{ii} = 8.68$	non-competitive $K_i = 10.42$ $K_{ii} = 9.50$
GalNAc	non-competitive $K_i = 0.62$ $K_{ii} = 2.66$	no inhibition	no inhibition
*p*NPGlcNAc	no inhibition	$K_{ss} = 0.20$	$K_{ss} = 0.23$
$GlcNAc_2$	no inhibition	$K_{ss} = 0.41$	$K_{ss} = 0.52$
2-Acetamido-2-deoxy-D-galactonolactone	non-competitive $K_i = 0.013$ $K_{ii} = 0.013$	non-competitive $K_i = 1.03$ $K_{ii} = 0.90$	non-competitive $K_i = 0.72$ $K_{ii} = 0.63$

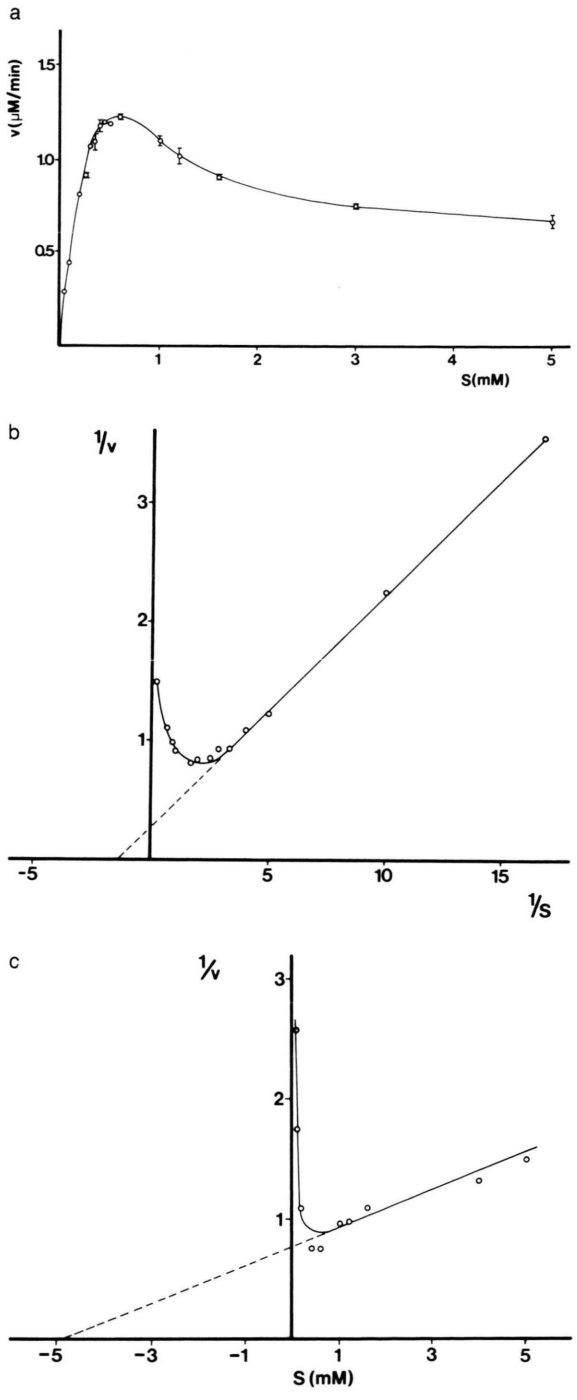

Fig. 6. Effect of the concentration of the substrate (*p*NPGlcNAc) on the activity of N-acetyl-β-D-hexosaminidase II_1 from *Artemia* nauplii (means ± S.D., $n = 4$). In b) the result is shown as a Lineweaver-Burk plot (regression lines), in c) as a Dixon-plot in order to determine K_{ss}.

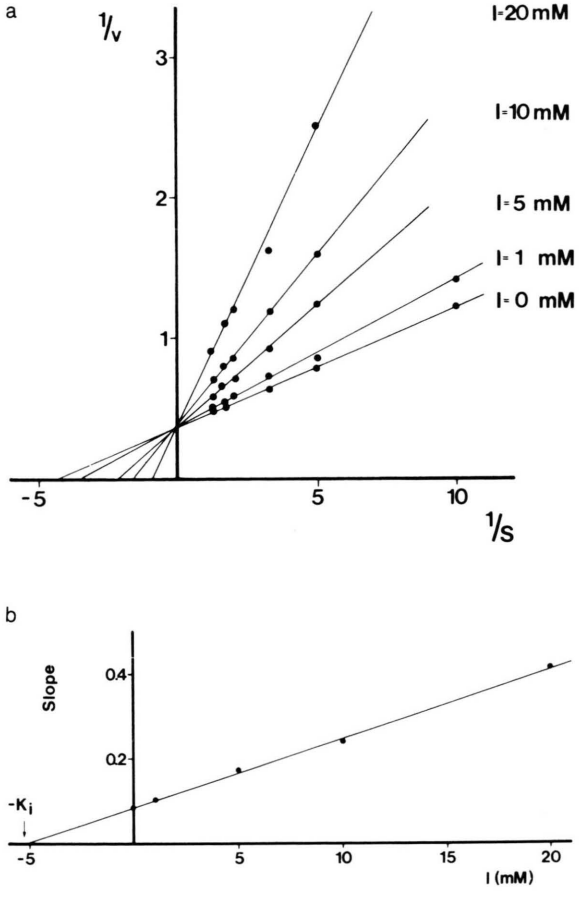

Fig. 7. Competitive inhibition of N-acetyl-β-D-hexosaminidase I from *Artemia* nauplii by the product GlcNAc. a) Lineweaver-Burk plot, b) secondary diagram from a). All lines are regression lines.

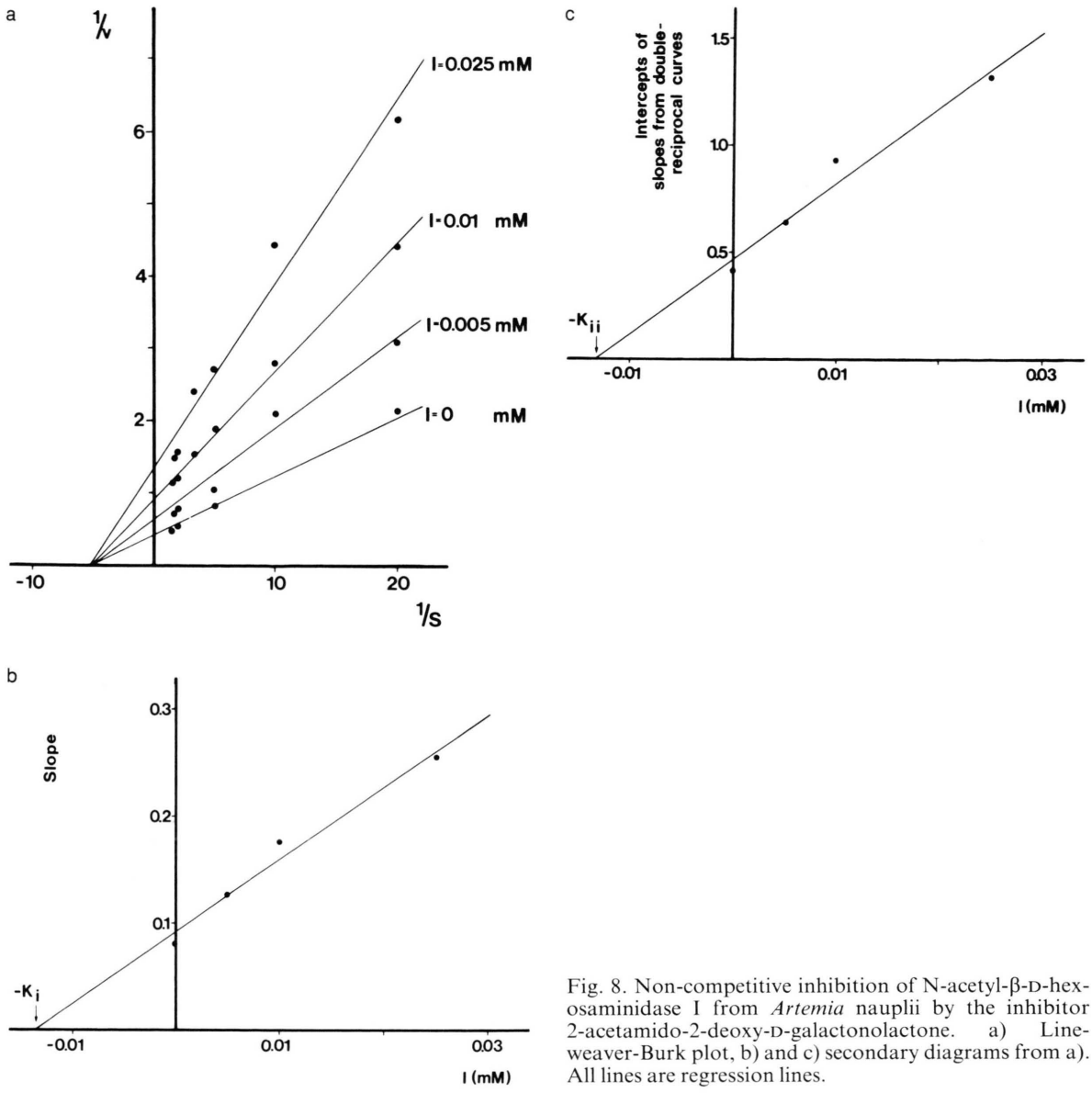

Fig. 8. Non-competitive inhibition of N-acetyl-β-D-hexosaminidase I from *Artemia* nauplii by the inhibitor 2-acetamido-2-deoxy-D-galactonolactone. a) Lineweaver-Burk plot, b) and c) secondary diagrams from a). All lines are regression lines.

Discussion

The N-acetyl-β-D-hexosaminidase activity from the nauplii of *Artemia* can be separated into three distinct forms according to their electric charge and size. The presence of N-acetyl-β-D-hexosaminidase isoenzymes is quite common and has been demonstrated, *e.g.*, in the insects *Culex quinquefaciatus* [12], *Locusta migratoria* [1], *Bombyx mori* [13, 14], *Manduca sexta* [15–18] and in *Drosophila melanogaster* K_c-cells [19]. Within the crustaceans, only the two Krill species *Euphausia superba* and *Meganyctiphanes norvegica* have been investigated under this aspect to a certain degree [20].

If we compare the physico-chemical properties of the *Artemia* N-acetyl-β-D-hexosaminidases with those of other species, some common features appear: all three enzyme forms from *Artemia* are acid hydrolases and glycoproteins. The molecular

masses of about 120,000, 80,000 and 56,000 Da are not unusual for N-acetyl-β-D-hexosaminidases. Especially the largest size is the most common one from invertebrates to vertebrates [*e.g.* 13, 20–24]. A size of 75–80,000 Da has only been found in *Euphausia superba* [20], whereas the smaller size of 56,000 Da is similar to one isoenzyme from *Manduca sexta* [15, 18]. The activities of all three enzymes from *Artemia* are not influenced by ionic strength, which is also the case in the two Krill species [20] and in the lepidopteran *Manduca* [25], but different from a mollusc [26] and the human aorta [27].

The 3 enzymes from *Artemia* nauplii hydrolyse N,N′-diacetyl-chitobiose, *p*NPGlcNAc and *p*NPGalNAc, but they have no activity against chitin nor against *Micrococcus luteus* and are therefore neither lysozymes nor chitinases, the latter being also present in *Artemia* [7]. All three enzymes have about the same affinities against *p*NPGlcNAc and *p*NPGalNAc and are therefore true N-acetyl-β-D-hexosaminidases (EC 3.2.1.52). From their kinetic behaviour, enzymes II_1 and II_2 cannot be distinguished but they are clearly separate from enzyme I, especially if we compare the influence of various inhibitors on these enzymes. Three aspects should be mentioned: 1) Both enzymes II_1 and II_2 are inhibited by the substrates, in contrast to enzyme I. Inhibition of N-acetyl-β-D-hexosaminidases by the substrate has been demonstrated in fungi [28], insects [14, 21, 25, 28, 29] and a crustacean [20]. 2) Enzyme I is inhibited by the product GalNAc in contrast to the enzymes II_1 and II_2. The other end product, GlcNAc, inhibits both enzymes I, II_1 and II_2, but with a different sensitivity and in a different manner. Inhibition by the end product is not universal for the N-acetyl-β-D-hexosaminidases, but it has often been demonstrated in plants and mammals [15, 26, 28, 30–33]. 3) All three enzymes from *Artemia* are inhibited by a N-acetamidolactone but again, enzymes I, II_1 and II_2 can be clearly distinguished by the sensitivity against this inhibitor. Enzyme I is at least 50-fold more susceptible than enzymes II_1 and II_2.

Our results clearly demonstrate that *Artemia* nauplii possess 3 different N-acetyl-β-D-hexosaminidases which can be distinguished according to their size, electric charge and kinetic properties when different substrates and inhibitors are used. A comparison of these data with the pattern of isoenzymes throughout development of *Artemia* may lead to the following conclusion: Enzymes II_1 and II_2 might have special functions since they are only present during early development, whereas enzyme I might be involved in chitin digestion together with chitinase, both during the hatching process and also during the moulting cycles which occur not only at larval stages but also in the adults. Enzyme I is present from cysts to adults and it shows pronounced changes in concentration during the moulting cycle [6]. On the other hand digestion of chitin must also occur during the hatching process, as demonstrated earlier [5, 6]. The higher affinity of enzyme I as compared to enzymes II_1 and II_2 towards N,N′-diacetyl-chitobiose which is one of the end products of chitinase action [7] supports this view.

Acknowledgements

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