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CHITINASE AND β-*N*-ACETYLGLUCOSAMINIDASE FROM THE DIGESTIVE FLUID OF THE SPIDER, *CUPIENNIUS SALEI*

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Summary

Two different enzymes which cause the breakdown of nutritional chitin in the hunting spider *Cupiennius salei* were purified to homogeneity and characterized. One chitinase (EC 3.2.1.14) was enriched about 80-fold (molecular weight 48 000). Its action upon solubilized chitin yields *N,N'*-diacetylchitobiose (88%), trimers (5%) and monomers (7%) of *N*-acetylglucosamine, respectively. The enzyme has a pH-optimum around pH 7.2 and it liberates about 30 nmol of *N,N'*-diacetylchitobiose from chitin per mg protein per s. The enthaply of activation is 9.5 kcal/mol at 30°C. *N,N'*-Diacylchitobiose, in turn, is cleaved by a specific β-*N*-acetylglycosaminidase (EC 3.2.1.30), which hydrolyzes *N,N'*-diacetylchitobiose, the 4-methylumbelliferyl β-derivatives of *N*-acetylglucosamine and *N*-acetylgalactosamine, and *p*-nitrophenyl-β-*N*-acetylglucosaminide at nearly equal rates (approx. 250 nkat/mg protein). The pH optima range around pH 5.4 for the chromogenic substrates and around pH 6.5 for *N,N'*-diacetylchitobiose. Heat stability for the enzyme is better at pH 6.5 than at pH 5.4; activity is not inhibited by any of the substrates used. β-*N*-Acetylglucosaminidase was purified about 160-fold (molecular weight 108 000) and is characterized by kinetic and thermodynamic parameters and inhibitors. Through inhibition studies with *N*-acetylglucosamine-1 → 5 lactone, it could be shown that all activities pertain to only one enzyme and bind to an identical active centre.

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Abbreviations: E_a, activation energy; e.u., entropy units; GalNAc, *N*-acetylgalactosamine; GlcNAc, *N*-acetylglucosamine; GlcNAc-β-1-4 GlcNAc, *N,N'*-diacetylchitobiose; 4-MeUmb, 4-methylumbelliferyl-; *p*-NP, *p*-nitrophenyl-.

Introduction

Chitin degradation in arthropod tissues has been shown to be brought about by an enzyme system consisting of chitinase (EC 3.2.1.14) and either β-*N*-acetylglucosaminidase (chitobiase, EC 3.2.1.30) or β-*N*-acetylhexosaminidase (EC 3.2.1.52) [1—4]. The existence of endogenous chitinase/β-*N*-acetylglucosaminidase in intestinal fluids of arthropods has been shown by several authors [1,3,5], yet the function and further analysis of the enzymes involved have mostly been followed for chitinoclastic action associated with moulting, especially in insects [3—6].

As chitin is a major component, not only of the cuticle of arthropods, but also of the lining of the fore and hindgut, and the tracheae of insects, it should constitute a source of carbohydrate for arthropod-hunting animals. In a previous report [7], it was shown that chitinase and β-*N*-acetylglucosaminidase indeed were among the most active hydrolytic enzymes contained in the digestive fluid of a web-building spider. The scope of this work was to investigate the chitin digesting capability in a hunting spider (*C. salei*), and to thoroughly analyze the enzymes involved.

Materials and Methods

The spiders of the genus *C. salei* (Ctenidae) came from the laboratory stock of the Zoology Institute at the University of Munich. Uncontaminated digestive fluid was collected as outlined previously [8] from subadult or adult female spiders only, after the animals had been starved for at least 5 days. The collected fluid (max. vol. approx. 80 µl per specimen) was pooled and centrifuged for 5 min at approx. 10 000 × *g*. Fractionation of the supernatant was started within 5 h after collection. All steps were carried out at 0—4°C. Chemicals were purchased from Sigma Chem. Co. (St. Louis, U.S.A.), except for the 4-methylumbelliferyl (MeUmb) derivatives which came from Serva (Heidelberg, F.R.G.).

Chitin from crab shells was solubilized in ice cold concentrated HCl [9], and chitinase activity measured as suggested by Jeuniaux [1]. After the incubation period (up to 60 min) in a total vol. of 250 µl (2.5—5 mg chitin/ml), the reaction was terminated by heating, undigested chitin and proteins were centrifuged off, and 100 µl portions of the supernatant were mixed with 20 µl of either β-glucosidase from almonds (5 mg/ml), or partially purified β-*N*-acetylglucosaminidase from either *Cupiennius* (0.05 mg/ml) or *Helix pomatia* digestive fluid, and incubated at 37°C for up to 2 h. Amounts of GlcNAc were estimated by a modified Morgan-Elson test [10], calibrated with GlcNAc. β-*N*-Acetylglucosaminidase, β-*N*-acetylgalactosaminidase, β-glucosidase, β-glucuronidase, and α-glucosidase activities were all measured with the appropriate *p*-NP substrates, either by continuous spectrophotometry at 340, 346 or 366 nm [11], or after termination of enzymic reaction by addition of 400 µl glycine/NaOH buffer (0.5 M, pH 10.2) at 405 nm [12]. Incubation assays consisted of 200 µl substrate solution in buffer and 20 µl enzyme solution in the same buffer. β-*N*-Acetylglucosaminidase and β-*N*-acetylgalactosaminidase were also measured with their appropriate 4-MeUmb derivatives by

continuous photometry at 350 nm [13] in a total volume of 490 µl. Chitobiase activity was determined using *N,N'*-diacetylchitobiose in an assay volume of 220 µl, and estimating the GlcNAc liberated as above [10]. The substrate was purified as mentioned below.

All enzymic reactions were run at 30°C unless otherwise indicated. Controls contained heat denatured enzyme. Protein was measured by the Lowry method [14]. Data given in Results represent the mean of three independent determinations in duplicates. Data for the determination of K_m and V were fitted using a computer programme for Woolf-plots (S/v versus S) without weighting [15]. Inhibition studies were performed using three different concentrations of inhibitors in triplicates, and analyzing Woolf plots as before. Molecular weights were estimated from gel filtration and from acrylamide slab gel electrophoreses [16].

Deacetylase (EC 3.5.1.33) was tested in citrate buffers (pH 5.5—7.5, 5 values) using GlcNAc as substrate (0.5 mM). 10 µl of undiluted digestive fluid were added to 200 µl of buffered substrate solution. After 1 to 4 h at 30°C, the remainder of substrate was determined prior to and after acetylation with acetic anhydride [10]. The 1 → 5 lactone of GlcNAc was obtained by oxidation of GlcNAc with HgO according to Findlay et al. [17]. Sodium phosphate-citrate buffers (60 mM, pH 6.5) was used for all chromatographic steps, sodium citrate buffers (60 mM, variable pH) for all glucosidase-like activities, and sodium phosphate-acetate or sodium phosphate buffers (60 mM, variable pH) for determination of chitinase.

Analysis of products of chitinase action. 1 ml portions of solubilized chitin (5 mg/ml final conc.) were added to purified chitinase and incubated in ammonium/acetate buffer (50 mM, pH 6.5) for up to 8 h. After the incubation, assays were heated on a boiling water bath for 3 min, samples were centrifuged for 5 min at 10 000 × *g*, and 0.5 ml of the supernatant were freeze dried. The lyophilisate was taken up in 0.5 ml sodium acetate buffer (30 mM, pH 4.8) or water, and portions of 100 µl were applied to a Bio-Gel P-2 column (115 × 0.9 cm). Effluents were monitored at 215 nm and 0.8 ml fractions were collected. Other 100-µl samples were mixed with purified β-*N*-acetylglucosaminidase from *Cupiennius* (10 µl, 0.2 mg/ml), incubated for 3 h at 37°C, and applied to the Bio-Gel P-2 column after heating and centrifugation. Carbohydrates in the column effluents were quantitatively determined by the Morgan-Elson procedure [10], and by a reducing sugar method [18].

Results

1 ml portions of digestive fluid from *C. salei* were submitted to ammonium sulphate fractionation. The fraction from 40 to 75% saturation, containing 83.5% of the β-*N*-acetylglucosaminidase and 66% of the chitinase activity, was taken up in buffer, dialyzed overnight and applied to a Sephadex G-100 column (118 × 0.9 cm). The elution profile is given in Fig. 1. β-*N*-Acetylglucosaminidase elutes as a single peak in the fractions 43 through 48, which were combined. The volume was reduced to about 1 ml in an Amicon concentration chamber and the concentrate was applied to a Sephadex G-150 column (60 × 0.9 cm). The chitinase(s) elute as very broad peaks from the Sephadex

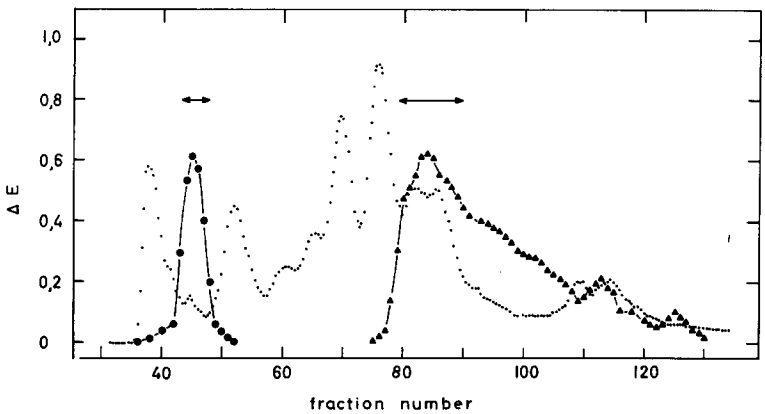

Fig. 1. Gel filtration of ammonium-sulphate fractionated digestive fluid on Sephadex G-100. Flow rate was 3.0 ml/h, and 1.2 ml fractions were collected. Protein (·, E_{280}); β-*N*-acetylglucosaminidase activity was assayed with *p*-NP-β-GlcNAc (●), and chitinase upon solubilized chitin as mentioned in Materials and Methods (▲). 20-μl portions were tested, incubation times were 3 min for β-*N*-acetylglucosaminidase, and 45 min for chitinase, respectively. All fractions were assayed; fractions beneath the arrows were used for further purification steps.

G-100 column, well separated from the β-*N*-acetylglucosaminidase. The fractions with the highest specific activities (Nos. 79—90) were pooled, their volume reduced to about 1 ml by dialysis against polyethyleneglycole (20%, w/v), and the concentrate applied to a Sephadex G-50 column (55 × 0.9 cm). The active fractions in the eluate were concentrated again and reapplied to the same column. After these steps, both enzymes were obtained in a purified state and could be located as single bands after polyacrylamide gel electrophoresis. Chitinase gave a single but slightly tailing band under different electrophoretic

TABLE I

PURIFICATION SCHEME OF *CUPIENNIUS SALEI* CHITINOLYTIC SYSTEM *

Step	Chitinase			β-*N*-Acetylglucosaminidase		
	Specific activity **	Purification (*n*-fold)	Yield (%)	Specific activity ***	Purification (*n*-fold)	Yield (%)
Digestive fluid	0.37	1.0	100	1.64	1.0	100
Ammonium sulphate fractionation (40—75% saturation)	0.52	1.4	66	3.21	2.0	84
Sephadex G-100	7.8	21	21	63.61	40	52
Sephadex G-50	29.2	80	13	—	—	—
Sephadex G-150	—	—	—	264.2	160	32

* Five purifications were done; maximum specific activities reached were 34 nkat/mg for chitinase and 312 nkat/mg for β-*N*-acetylglucosaminidase, respectively. Yields ranged from 12 to 16% for chitinase and from 25 to 34% for β-*N*-acetylglucosaminidase.

** Specific activity given as nmol of *N,N'*-diacetylchitobiose liberated from solubilized chithin per s and mg protein at 30°C.

*** Specific activity given as nmol of *N,N'*-diacetylchitobiose hydrolyzed per s and mg protein at 30°C.

separation regimes. The tailing could be reduced by passing the enzyme through the Sephadex G-50 column again, but the specific activity of the preparation did not increase. Overall purification of both enzymes is given in the fractionation scheme in Table I.

Chitinase

The one chitinase analyzed shows a molecular weight of 48 000 ± 4000; its pH-optimum lies in the slightly alkaline region (Fig. 2). When purified, the enzyme liberates 29.2 nmol of *N,N'*-diacetylchitobiose from solubilized chitin per s and mg protein at 30°C and pH 7.2. An analysis of the sugars produced by chitinase is given in Fig. 3. When the effluents of the Bio-Gel P-2 column were assayed for GlcNAc without further treatment, only peak No. 5 gave a positive reaction. After further incubation of the eluted fractions with purified β-*N*-acetylglucosaminidase from *Cupiennius* or partially purified β-*N*-acetylglucosaminidase from *H. pomatia*, positive Morgan-Elson reactions were obtained for peak Nos. 2 and 3 as well. By this method and by calibrating the column with raffinose, *N,N'*-diacetylchitobiose, and GlcNAc, the peaks 2, 3, and 5 could be identified as the trimer, dimer and monomer of GlcNAc, respectively. Calculated from the Morgan-Elson reaction, the following amounts of the three sugars on a molecular basis can be given: trimer 5.2%, dimer 87.9%,

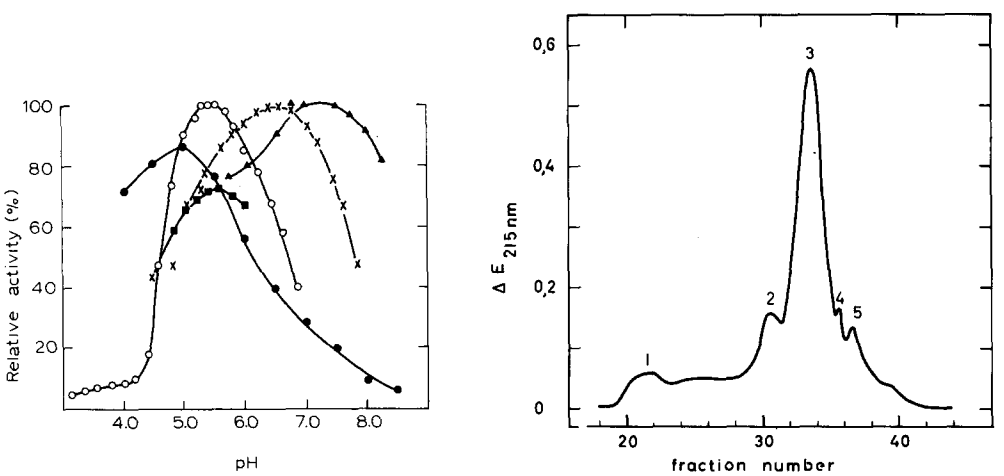

Fig. 2. pH-profiles of purified spider chitinase and β-*N*-acetylglucosaminidase. Chitinase (▲) was measured in acetate-phosphate buffer, incubation time with solubilized chitin was 1 h. Prior to the addition of β-*N*-acetylglucosaminidase, the pH was adjusted to pH 6.5. β-*N*-Acetylglucosaminidase was assayed with *N,N'*-diacetylchitobiose in citrate buffer (X———X, 60 mM), and with *p*-NP-β-GlcNAc in citrate buffer (○———○, 60 mM), universal buffer [20] (●———●), and acetate buffer (■———■, 60 mM). Substrate concentrations were 10 mM throughout; incubation times up to 8 min. Identical results are obtained for all chromogenic substrates in citrate buffer. pH-values were controlled prior to and after incubation.

Fig. 3. Separation of chitinase products on Bio-Gel P-2. For conditions see Materials and Methods. Flow rate was 3.6 ml/h. Peak Nos. 2, 3, and 5 correspond to the β-1,4trimer, β-1,4dimer, and monomer of *N*-acetylglucosamine, respectively. All three peaks gave positive reactions when tested for aminosugars [10] after treatment with β-*N*-acetylglucosaminidase from *Cupiennius salei* or *Helix pomatia*. Only peak No. 5 gave positive reaction without the addition of β-*N*-acetylglucosaminidase. Peak Nos. 1 and 4 remained unidentified.

TABLE II

KINETIC DATA OF β-*N*-ACETYLGLUCOSAMINIDASE ACTION UPON DIFFERENT SUBSTRATES AT THE RESPECTIVE pH-OPTIMA *

Substrate	K_m ± S.D. (mM/l)	*n*	*V* (nkat/mg) **	Relative activity	pH-optimum
N,N'-diacetylchitobiose	0.93 ± 0.10	3	63.61	1.0	6.5
p-NP-β-GlcNAc	0.35 ± 0.02	10	67.61	1.06	5.4
p-NP-β-GalNAc	1.05 ± 0.04	4	6.42	0.1	5.4
4-MeUmb-β-GlcNAc	0.77 ± 0.01	3	52.62	0.89	5.4
4-MeUmb-β-GalNAc	1.23 —	2	90.33	1.42	5.4

* Substrate concentrations (minimum eight values) ranged from 0.05 to 2.0 mM, or, where feasable, to 10 mM/l. Incubation times up to 5 min. Regression coefficients of Woolf-plots above |0.996|.

** Maximum velocities are given in nmol substrate cleaved per s and mg protein at 30°C after the first gel filtration step.

and monomer 6.9%. When the products of the chitinase action were digested with *Cupiennius* β-*N*-acetylglucosaminidase prior to application to the column, only peaks 1 and 5 could be found. Peak 1, which has a molecular weight above the exclusion limit of the gel (i.e. >1800), and which gave no reaction with reagents for reducing sugars, probably consists of polymers of GlcNAc which fail to sediment in the centrifugation step and which in turn are too big to be attacked by β-*N*-acetylglucosaminidase. Longer periods of incubation did not change the elution patterns. Peak 4, which could not be found on all chromatogrammes, remained unidentified.

The chitinase fails to further hydrolyze *N,N'*-diacetylchitobiose or *N,N',N''*-triacetylchitotriose, as the relative percentage of the three sugars remained constant over different incubation periods, and no GlcNAc was liberated when chitinase was tested with the dimer or trimer of GlcNAc. The enzyme does not attack any of the chromogenic substrates for β-*N*-acetylglucosaminidase used in this study and displays no chitosanase activity when tested upon chitosan according to Monaghan et al. [19]. The temperature dependence of the reaction rate for *Cupiennius* chitinase was followed from 10 to 45°C, and the resulting Arrhenius plot was continuous between 10 and 33°C, the energy of activation being 10.1 ± 0.16 kcal/mol.

Although only one chitinase is characterized here, it is more than likely that about two to three other chitinases are present in the digestive fluid as well, since all fractions Nos. 79 to 125 in Fig. 1 show chitinase activity, displaying three to four different peaks.

β-N-Acetylglucosaminidase

After gel filtration on Sephadex G-150, one single protein was detectable on polyacrylamide slab gels, the molecular mass of which is 108 000 ± 6500. The enzyme readily hydrolyzed *N,N'*-diacetylchitobiose, and the *p*-NP and 4-MeUmb derivatives of GlcNAc and GalNAc. The ratio of enzyme activity with these five substrates remained constant throughout the purification steps. Kinetic data are given in Table II. In addition to the activities listed, the enzyme revealed β-glucosidase (2% relative to *N,N'*-diacetylchitobiose as sub-

TABLE III

INHIBITION OF β-*N*-ACETYLGLUCOSAMINIDASE ACTION BY COMPETITIVE INHIBITORS *

Inhibitior	K_i (mM/l)	S.D.	*n*	Substrate
N-Acetylglucosamine	12.0	±2.0	5	*p*-NP-β-GlcNAc
N-Acetylgalactosamine	7.3	±1.2	3	*p*-NP-β-GlcNAc
N-Acetylmannosamine	30.5	—	2	*p*-NP-β-GlcNAc
Glucosamine-HCl	8.0	±2.2	4	*p*-NP-β-GlcNAc
N-Acetylleucine **	24.5	—	2	*p*-NP-β-GlcNAc
N,N'-Diacetylchitobiose	1.01	±0.07	4	*p*-NP-β-GlcNAc
Gluconolactone	no inhibition	no inhibition	3	*p*-NP-β-GlcNAc
N-Acetylglucosamine 1 → 5 lactone	$23.9 \cdot 10^{-3}$	$\pm 2.9 \cdot 10^{-3}$	4	*p*-NP-β-GlcNAc
N-Acetylglucosamine 1 → 5 lactone	$24.8 \cdot 10^{-3}$	$\pm 2.5 \cdot 10^{-3}$	4	*p*-NP-βGalNAc
N-Acetylglucosamine 1 → 5 lactone	$22.8 \cdot 10^{-3}$	$\pm 1.8 \cdot 10^{-3}$	3	4-MeUmb-β-GlcNAc
N-Acetylglucosamine 1 → 5 lactone	$23.6 \cdot 10^{-3}$	$\pm 2.2 \cdot 10^{-3}$	4	4-MeUmb-β-GalNAc

* Substrate concentrations ranging from 0.1 to 2.5 mM (minimum six values) in citrate buffer (60 mM, pH 5.4); 3 different inhibitor concentrations. Incubation time up to 3 min (continuous registration) or up to 5 min (one point determination). Calculated from Woolf-plots.

** Mixed type inhibition.

state) and α-glucosidase (1.5%) side activities, but no β-glucuronidase activity when tested with the appropriate *p*-NP derivatives at pH 5.4.

β-*N*-Acetylglucosaminidase was purified about 160-fold (cf. Table I). This value is not falsely increased, as no deacetylase was detectable in the digestive fluid. No charge or molecular weight isomers of the enzyme could be found after gel electrophoreses under different separation regimes or after gel chromatography.

The pH-optima differ depending on the substrate used as presented in Fig. 2. Maximum activities for the four chromogenic substrates are reached around pH 5.4, whereas the optimum lies considerably higher (pH 6.5) for the cleavage of the natural substrate *N,N'*-diacetylchitobiose. The pH optimum for the enzymic cleavage of *p*-NP-β-GlcNAc is slightly dependent on the buffer used, as shown in Fig. 2 as well. Davies universal buffer [20] and sodium acetate buffer both shift the optimum, but in both cases, the overall activity drops. The enzyme optima are not shifted, nor are the activities changed, if the ionic strength of the assays is increased by the addition of NaCl (80 mM). Enzymic activity is not inhibited by any of the substrates used, but is competitively inhibited by one of the products formed, i.e. GlcNAc or GalNAc. Data for these and several other competitive inhibitors are given in Table III. In order to ascertain whether all of the activities found pertained to one enzyme and one identical active site, the inhibition of enzyme the powerful competitive inhibitor 2-deoxy-2-acetamido-D-glucono-1 → 5 lactone was studied. The results given in Table III indicate that the dissociation constants (K_i) for the enzyme-lactone complex are independent of the substrate used. The β-*N*-acetylglucosaminidase fails to liberate any GlcNAc from chitin, whereas it hydrolyzes *N,N'*-diacetylchitobiose, and it could be shown qualitatively that it does hydrolyze the trimer of GlcNAc as well (cf. Fig. 3).

Activation kinetics for the β-*N*-acetylglucosaminidase were followed for three substrates at temperature adjusted constant pH, and the resulting

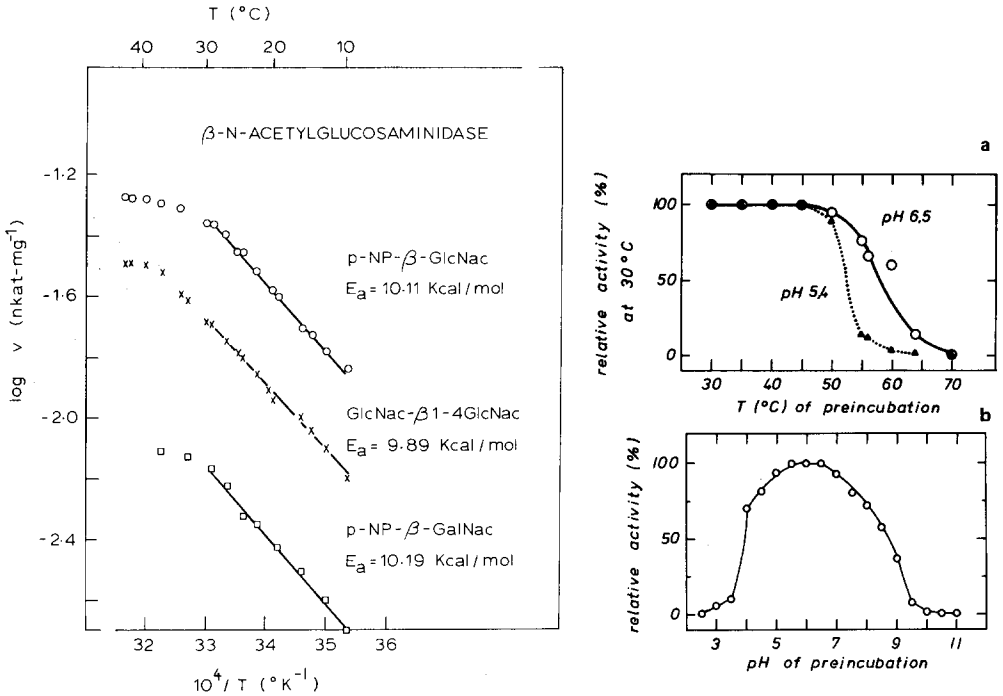

Fig. 4. Effect of temperature on purified spider β-*N*-acetylglucosaminidase (Arrhenius plot). Enzyme assays were incubated for 5 to 20 min after a preheating period of 15 min. Assay conditions: *p*-nitrophenyl-β-*N*-acetylglucosaminide (*p*-NP-β-GlcNAc, ○———○, 8 mM, pH 5.45); *N,N'*-diacetylchitobiose (GlcNAc-β-1-4GlcNAc, X———X, 8 mM, pH 6.5); *p*-nitrophenyl-β-*N*-acetylgalactosaminide (*p*-NP-β-GalNAc, □———□, 3 mM, pH 5.45), all in citrate buffers (60 mM). The pH was adjusted at each temperature. Correlation coefficients of regression lines were above |0.992|.

Fig. 5. (a) Heat stability of β-*N*-acetylglucosaminidase. The enzyme was preincubated in citrate buffer (○———○, 60 mM, pH 6.50, and ▲———▲, pH 5.40) at the given temperature for 20 min and assayed with *N,N'*-diacetylchitobiose as substrate in citrate buffer (120 mM, pH 6.5) for 10 min at 30°C. (b) Effect of preincubation pH on β-*N*-acetylglucosaminidase. Enzyme solutions were incubated for 100 min at 30°C in diluted (1 : 10) universal buffer [20], and assayed with *N,N'*-diacetylchitobiose for 12 min in citrate buffer (120 mM, pH 6.5). Identical curves were obtained for the chromogenic *p*-NP substrates assayed at pH 5.45.

TABLE IV

SOME THERMODYNAMIC PARAMETERS OF β-*N*-ACETYLYGLUCOSAMINIDASE ACTION UPON DIFFERENT SUBSTRATES

Calculated for 30°C from the data in Fig. 5 and Table II, using the equations of Low et al. [36].

Substrate	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} M^{-1})	(cal/mol)			$\Delta S^{\ddagger}$ (e.u.)
			$\Delta H^{\ddagger}$ ± S.D.	$\Delta G^{\ddagger}$	ΔG [b]	
GlcNAc-β-1 → 4GlcNAc	28.5	30.6	9289 ± 150	15 739	−4205	−21.28
p-NP-β-GlcNAc	30.2	86.6	9508 ± 395	15 701	−4793	−20.43
p-NP-βGalNAc	2.8	2.7	9586 ± 282	17 122	−4131	−24.84

Arrhenius plots are presented in Fig. 4. In all cases, a continuous Arrhenius plot was obtained. The slope of the straight line is not significantly different for the three substrates. The average enthalpy of activation can be calculated from the values of E_a (activation energy) as 9.46 kcal/mol at 30°C (cf. Table IV).

Furthermore, the heat stability of the purified enzyme at two different pH-values, and the pH-stability were investigated. Results are given in Figs. 5a and b. The enzyme is more labile to heat inactivation at pH 5.4 than it is at pH 6.5. On the other hand, the enzyme is rather stable at 30°C in buffers ranging from pH 5.0 to 7.0. Below and above these values, rather rapid irreversible inactivation occurs.

Discussion

The chitinolytic system from the digestive fluid of *Cupiennius* consists of two enzymes which successively break down chitin to yield GlcNAc. The first enzyme acts upon chitin, and the second further hydrolyzes the products of the first.

Chitinase

The first enzyme can be classified as a true chitinase (poly [1,4-β-(2-acetamido-2-deoxy-D-glucoside)] glycanohydrolase). It liberates mainly *N,N'*-diacetylchitobiose from solubilized chitin and it cannot act as a β-*N*-acetylglucosaminidase and does not attack chitosan. The molar activity of the homogenous spider enzyme (1.4 kat/mol) is more than ten-fold higher than the values calculated from the data of Skujinš et al. [21] and Cornelius et al. [22] for purified chitinases from *Streptomyces* and *Perodicticus*, respectively. The results on spider chitinase agree well with the data given by Berger and Reynolds [23], who reported that *Streptomyces* chitinase has no chitosanase activity and liberates mainly dimers and monomers of GlcNA. Streptomyces chitinase produces longer oligomers of GlcNAc when tested upon soluble chitodextrins, indicating that the enzyme displays random attack on soluble polymers, whereas it acts only as an exochitinase on its insoluble substrate [23]. The same behaviour can be anticipated for the *Cupiennius* enzyme.

Considering the fact that chitinase is a soluble enzyme which acts on surfaces of insoluble substrate particles, it is unlikely that the ration will follow Michaelis-Menten kinetics; furthermore, it has been shown that a hysteresis exists in the adsorption and desorption of *Streptomyces* chitinase to solubilized chitin [24]. Therefore the dependence of *N,N'*-diacetylchitobiose liberation on chitin concentration for the spider chitinase was not further investigated. The pH-optimum of the chitinase lies near the pH value of the digestive fluid (pH 7.35), and thus is very similar to the chitinase studied in a web-building spider [7], but lies considerably above the pH-optima for chitinases from other sources [1,3,4]. Lysozyme (EC 3.2.1.17, mucopeptide *N*-acetylmuramoyl-hydrolase), too, is effectively adsorbed by chitin, but its hydrolytic activity upon chitin is 600 times lower than that of *Streptomyces* chitinase [24].

The products of chitinase action upon chitin have been insufficiently investigated, rendering comparison with other chitinases difficult. Jeuniaux [2] pointed out that the bulk of chitinase action upon solubilized chitin yields

N,N'-diacetylchitobiose, which gives very low colour reaction with Morgan-Elson reagent for GlcNAc (Refs. 1, 3 and Results), yet many authors quantify chitinase activity with this reagent without prior hydrolysis of polymers of GlcNAc. *Cupiennius* chitinase liberates mono-, di- and trimers of GlcNAc from chitin, in contrast to the chitinase from *Bombyx mori* moulting fluid, which yielded 95.5% dimers but no trimers [30]. Jeuniaux [1] suggested that the amount of monomers released is rather a function of the chitin preparation than of the enzyme.

The molecular weight of the spider enzyme at 48 000 is intermediate compared with chitinases from other sources [5,21,22,25—27]. The amount of active chitinase contained in the digestive fluid of *Cupiennius* is approx. 1.2% of total protein. As in most other animal sources, more than one chitinase is present in the spider, too; the chitinase with the highest molecular mass has been characterized here.

β-N-Acetylglucosaminidase

The spider enzyme can be classified by its properties as a *β-N*-acetylglucosaminidase rather than a *β-N*-acetylhexosaminidase. As five different substrates have been used throughout this study, the question arose, whether all activities were brought about by the same active centre of the enzyme. If an identical active centre is involved in the cleavage of several substrates, a specific inhibitor should give identical K_i-values when assayed with the single substrates. This could be well confirmed for the highly specific inhibitor GlcNAc-1 → 5-lactone. For practical reasons, this kind of assay could not be carried out for *N,N'*-diacetylchitobiose as substrate. To get the information needed for this substrate as well, mixed substrate analysis was performed with *N,N'*-diacetylchitobiose and *p*-NP-*β*-GlcNAc. As no additive effect was evident, but purely competitive inhibition instead (cf. Table III), resulting in a K_i for *N,N'*-diacetylchitobiose not significantly different from its K_m, it can be concluded that this activity, too, is governed by the same active centre. The suggestion of only one active site is also supported by the identical pH-sensitivity displayed by the enzyme when testing the pH-stability with both *p*-NP-*β*-GalNAc and *p*-NP-*β*-GlcNAc (Fig. 5b).

The substrate binding is apparently influenced by the chemical nature of the aglycon, as binding of GlcNAc is tighter than of GalNAc, yet quite unexpected differences are apparent in the actual catalytic efficiencies (cf. Table II). Some thermodynamic parameters were calculated to allow an interpretation of the data of Table II and Fig. 4 (Table IV). When comparing *p*-NP-*β*-GalNAc and *N,N'*-diacetylchitobiose, binding to the enzyme is equally tight in both cases, the rate constant, however, is ten-fold higher for the natural substrates, reflected in the differences in $\Delta G^{\ddagger}$ and $\Delta S^{\ddagger}$. The differences observed in the values of k_{cat} can be accounted for by the increase in free energy of activation and drop in free entropy of activation.

GlcNAc cannot be funnelled directly into anabolic pathways but first has to be deacetylated [28]. As no GlcNAc-deacetylase (EC 3.5.1.33) could be found in *Cupiennius* digestive fluid, it can be anticipated that such an enzyme will act upon GlcNAc after its uptake from the digestive tract. The *β-N*-acetylglucosaminidase present in the digestive fluid can be calculated to amount to about

0.6% of total protein. The rate limiting step in the degradation of nutritional chitin, as already found elsewhere [7], seems to be the chitinase rather than the following β-*N*-acetylglucosaminidase at the pH of the digestive fluid (pH 7.35).

In contrast to most β-*N*-acetylglucosaminidases, the *Cupiennius* enzyme is not inhibited by any of its substrates. For a *Sclerotina* enzyme, possibly associated with chitinase, substrate inhibition was observed for *p*-NP-β-GlcNAc but not for *N,N'*-diacetylchitobiose at 0.5 mM [29].

Although the β-*N*-acetylglucosaminidases seem rather flexible in their substrate specificities, the enzymes from various sources are quite similar in their molecular weights, all ranging between 110 000 and 125 000 [4,7,30,31]. Kimura [30] could show by dodecyl sulphate electrophoresis that the *B. mori* haemolymph enzyme is composed of two identical subunits.

A feature apparently not yet thoroughly investigated seems to be the different pH optima for the chromogenic substrates on one side and *N,N'*-diacetylchitobiose on the other. The optimum pH for the cleavage of the presumably 'natural' substrate lies 1 pH unit above the pH which is optimal for the hydrolysis of the chromogenic substrates. This fact is also reflected in the pH stability which is markedly increased at pH 6.5. Without testing optima for non-chromogenic substrates, Berkeley et al. [32] and Li and Li [33], found similar discrepancies for pH-optima and the apparent optimum pH stability for *Bacillus subtilis* and Jack bean meal β-*N*-acetylglucosaminidase, respectively. Although temperature optima have been given by several authors [4,21,24,32], activation kinetics have not been followed extensively. The enthalpies of activation of the spider enzyme (Table IV) are only slightly lower than the value of 9.89 kcal/mol given for human hexosaminidases A and B [34]. The side activities of the purified β-*N*-acetylglucosaminidase reported here, have also been found for an *Aspergillus niger* β-*N*-acetylglucosaminidase [35].

Finally, it seem that the spider β-*N*-acetylglucosaminidase with all its properties and its association with chitinase is well adapted to function in vivo as a true '*N,N'*-diacetylchitobiase' with a rather unique feature of a β-*N*-acetylglucosaminidase not to be inhibited by substrates.

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