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Chitinase and β-*N*-Acetylglucosaminidase in the Digestive Juice of *Helix pomatia*

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A β-*N*-acetylglucosaminidase from *Helix pomatia* digestive juice was separated and partly purified by gel chromatography. The optimal pH for the degradation of *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide was 3.4. The molecular weight was around 160 000 and the pI = 4.95. In the same gel chromatography run two chitinase active peaks were also obtained. These chitinases, with molecular weights around 26 000 and 13 000, had somewhat different pH activity curves with optima at 4.2 and 4.3. By isoelectric focusing the first peak with molecular weight around 26 000 was divided in two chitinase active regions with pI at 5.7 and 3.8. The second peak with molecular weight around 13 000 had a pI at 7.3.

The digestive juice of the snail, *Helix pomatia* has been used as a source for different enzymes. During studies of lysozyme and glycol chitinase activities in different animal species it was found [1] that *Helix* digestive juice had a strong glycol chitin splitting activity when assayed viscosimetrically and a very weak lysozyme (muramidase) activity when tested as a *Micrococcus*-lysing enzyme. Further studies of the *Helix* juice showed a strong β-*N*-acetylglucosaminidase* activity, earlier reported [2-4] but not characterized. Cellulase [5] and chitinase [2,5] have also been reported in *Helix* juice and a muramidase has been separated from a chitinase from the same material.[6] In the present investigations two glycol chitin splitting enzymes were separated from a β-*N*-acetylglucosaminidase. These enzymes were preliminary studied.

* *Abbreviation.* β-*N*-Acetylglucosaminidase: NAGase. *Enzymes*: NAGase (EC 3.2.1.30), chitinase (EC 3.2.1.14), β-glucosidase (EC 3.2.1.21), lysozyme (EC 3.2.1.17).

MATERIAL AND METHODS

Materials. The *Helix pomatia* digestive juice (batch No. 61310) was purchased from Koch-Light Lab., Colnbrook, Bucks., England. The glycol chitin was from Seikagaku Kogyo Co., Tokyo (lot 4701, mol.wt. 20 000 – 60 000). *p*-Nitrophenyl-*N*-acetyl-β-D-glucosaminide, grade III, was purchased from Sigma, USA.

Chitinase assay. The activity was determined with glycol chitin as substrate using the viscosimetric method of Hultin [7] as described earlier.[1,8] The chitinase activity is expressed in viscosimetric units called Hultin units (HU) as defined from Hultin's formula.[8] The incubation mixture consisted of 1.0 ml enzyme, 1.5 ml 0.1 M McIlvaine buffer and 1.5 ml 0.35 % glycol chitin in 0.9 % NaCl. The incubation was made at 35.5 °C and pH 4.1 if not otherwise reported.

β-N-*Acetylglucosaminidase* (NAGase) assay was performed according to Verpoorte [9] as follows. Twentyfive μl of the enzyme sample was mixed with 2.0 ml of 2 mM *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide in 0.05 M Na-citrate buffer, pH 4.5, and incubated at 37 °C. After 10 min the reaction was stopped by increasing the pH to about 10.5 by the addition of 2 ml of 0.5 M glycine-NaOH buffer pH 10.5. The absorbance was measured at 430 nm in a Beckman DB-G spectrophotometer and the NAGase activity expressed as absorbance in the figures. The absorbance, $A_{430}(1\ \text{cm}) = 0.500$, corresponded to 0.33×10^{-3} μkat of *p*-nitrophenol liberated under the conditions of the experiments.

Gel chromatography. The Sephadex gel (Pharmacia Fine Chemicals, Uppsala, Sweden) and the Bio-Gel (Bio Rad, USA) were equilibrated with the elution medium always containing 2 % 1-butanol as sterilizing agent.

Isoelectric focusing. The material was also studied isoelectrophoretically in a 110 ml column, type 8100-1 (LKB-Beckman Instrument AB, S-162 11 Vällingby 1, Sweden). For further details see Ref. 1.

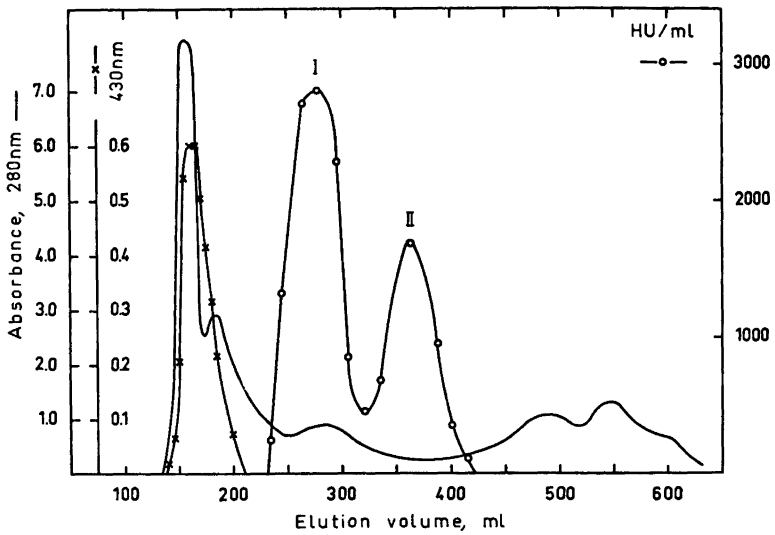

Fig. 1. Gel chromatography of *Helix pomatia* digestive juice. A 1.9 ml sample of the juice was applied to a P-150 column (1.9 × 178 cm) in 0.15 M ammonium acetate, pH 6.0 and 0.5 M NaCl and eluted in the same medium. The effluent was collected in 5 ml fractions at a flow rate of 23 ml/h at + 5 °C. ×, NAGase activity; O, chitinase activity.

RESULTS

Gel chromatography. The *Helix pomatia* digestive juice was run through Sephadex G-100, Sephadex G-150, and Bio-Gel P-150 columns. The best fractionation was obtained in a Bio-Gel P-150 column (Fig. 1). A full separation of NAGase from chitinase was obtained here and the glycol chitin splitting activity appeared in two peaks. The NAGase activity was assayed at pH 4.5 and the (glycol) chitinase activity at pH 4.1. These pH values were chosen from preliminary pH activity curves.

β-*N*-Acetylglucosaminidase

Influence of pH. The optimal pH of NAGase, purified 10 times by means of gel chromatography, was about 3.4 as seen in Fig. 2. At this pH 1.20×10^{-3} μkat of *p*-nitrophenol was liberated per ml reaction mixture. At pH values below 3, the enzymatic activity decreased rapidly, which was also the case for pH values higher than 5.

Kinetics. The effect of time on the NAGase reaction at constant enzyme concentration was linear for incubation times from 10 to 60 min (Fig. 3 A). The effect on the reaction rate of a 50-fold variation in the enzyme concentration was also linear when assayed after a constant time interval (Fig. 3 B). Both these series were studied at pH 4.5.

Isoelectric focusing. Fig. 4 demonstrates an isoelectric focusing of NAGase purified 6 times by gel chromatography. A homogeneous peak of enzymatic activity was obtained at a pI =

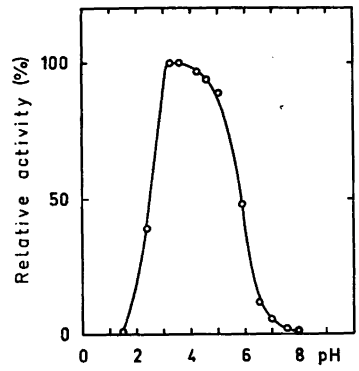

Fig. 2. Effect of pH on the activity of β-*N*-acetyl-glucosaminidase. The *Helix pomatia* NAGase was purified 10 times by gel chromatography.

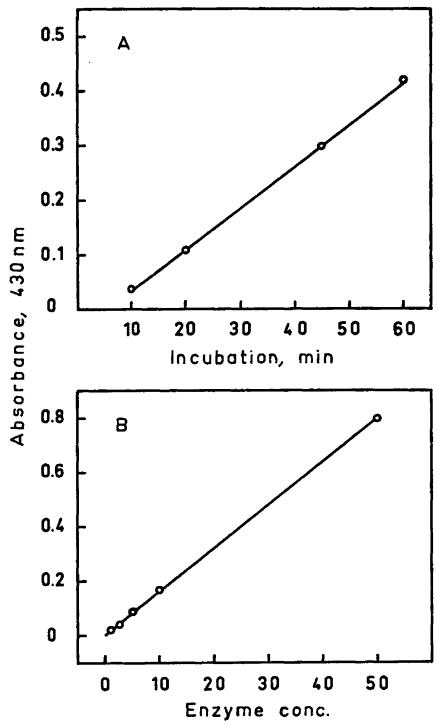

Fig. 3. Activity of NAGase. A. Effect of time of incubation on the degree of hydrolysis. B. Effect of enzyme concentration on the reaction velocity.

4.95 ± 0.1. The procedure resulted in a final 28-fold purification of the enzyme with a recovery of 30 % in the top fraction.

Molecular weight. By means of molecular sieving through a Bio-Gel A-1.5 m column in 0.15 M ammonium acetate, pH 6.0 and 0.5 M NaCl, the molecular weight could be estimated to be around 160 000 ± 20 000 with IgG (Tika), ceruloplasmin (Kabi) and albumin (Kabi) as marker proteins.

Chitinase

Influence of pH. Degradation of glycol chitin by the unpurified *Helix pomatia* digestive juice gave an optimum at pH 4.3 in McIlvaine buffer. The active interval was found between pH 3.2 – 5.7. By means of gel chromatography (Fig. 1) two active peaks were obtained. The effect of pH for each of these active regions is shown in Fig. 5.

Isoelectric focusing. Fig. 6 demonstrates an isoelectric focusing of the purified chitinase I from Fig. 1. One chitinase peak appeared at pI 5.7 ± 0.1 and another peak, at pI 3.8 ± 0.1. Repeated isoelectric focusing of peak I from Fig. 1 gave essentially the same results. The chitinase peak II (Fig. 1) gave one sharp distinct peak (Fig. 7) at pI 7.3. The maximum enzyme activities for these chitinase peaks

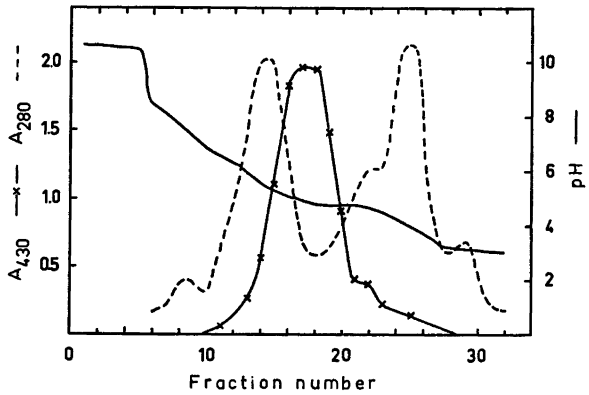

Fig. 4. Isoelectric focusing of *Helix* NAGase. The purified enzyme was dialyzed against 1.0 % glycine for 20 h at +4 °C and the dialyzed solution (20 ml) was applied to the column in 0.85 % Ampholine (LKB No. 8141 pH 3 – 10). The medium was stabilized with a sorbitol gradient (0 – 55 %). After 47 h running at 400 – 1100 V (2.4 – 1.7 W) and at +4 °C the column was drained in 3.3 ml fractions and the pH was measured at +4 °C. After dialysis against 0.9 % NaCl the enzymatic assay was performed.

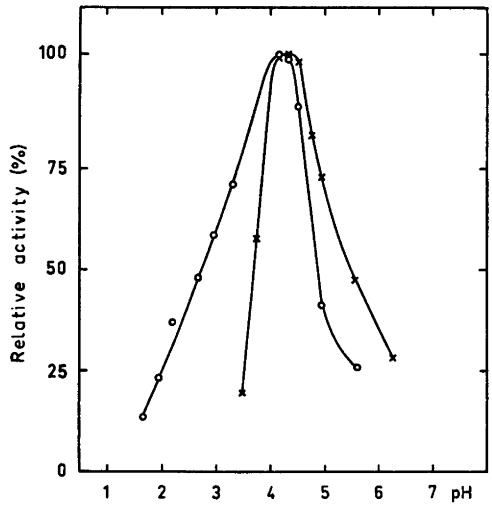

Fig. 5. Effect of pH on the activity of *Helix* chitinase. The purified chitinase pools I and II (Fig. 1) were used. Chitinase from pool I, O; and from pool II, ×.

were 880, 540, and 2480 HU/ml for the peaks with pI values at 5.7, 3.8, and 7.3, respectively. The recovery of the pI 7.3 enzyme (from peak II, Fig. 1) was 77 % of which the top fraction contained 43 % with a purification factor of 72 (Fig. 7, fraction 10).

Molecular weight. The molecular weights for chitinase I and II estimated by gel chromatography were 26 000 ± 2000 and 13 000 ± 1000, respectively. A Bio-Gel A-1.5 m column in 0.15 M ammonium acetate, pH 6.0 and 0.5 M NaCl was used and ovalbumin (Schwartz/Mann), myoglobin (whale skeletal muscle, Sigma, 17 800) and cytochrome *c* (horse heart Sigma) were used as marker proteins.

DISCUSSION

NAGase. For the assay of the enzyme *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide was used as substrate. This assay is convenient and the substrate has been used in studies of NAGase from *inter alia* ram testis,[10] *Helix pomatia* juice,[4] Jack bean meal,[11] beef spleen,[9] human parotid saliva,[12] silkworm [13] and cock spermatozoa.[14] In the present investigation the pH optimum found of purified *Helix* NAGase was 3.25 which is near the pH optimum 3.5 of cock spermatozoa.[14] In earlier studies of *Helix* NAGase,[2-4] pH optima were not reported. For most NAGases the pH optima with this substrate are usually higher, *e.g.*, around 4.5 in many studies,[3,10,12,15,16] 5 – 6 for NAGase from Jack bean,[11] 5.0 from silk worm blood,[12] and 5.8 from moulting fluid of this animal.[13] The isoelectric point for Helix NAGase was 5.0. Very few pI values have been reported for this enzyme. A purified endo-NAGase from *Staphylococcus aureus* [17] possessed a pI = 9.6 ± 0.1 and NAGase from human plasma [18] had a pI = 4.73. Compared to the molecular weight of 160 000

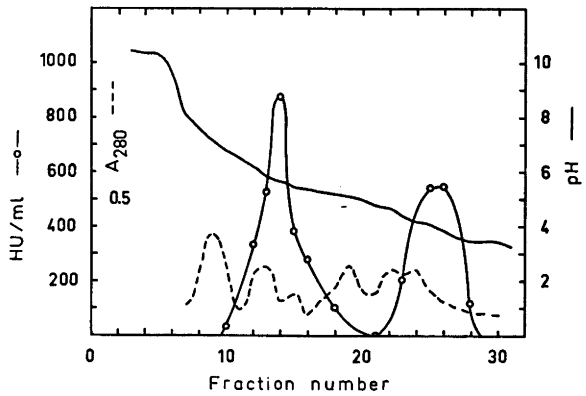

Fig. 6. Isoelectric focusing of *Helix* chitinase I. From peak I (Fig. 1) a pool was dialyzed against 1 % glycine and the dialyzed solution (10 ml) was applied to the column in 0.85 % Ampholine (LKB no 8141 pH 3 – 10). The medium was stabilized with a sorbitol gradient (0 – 55 %). After 94 h running at 380 – 1020 V (2.2 – 1.0 W) and at +4 °C the column was drained in 3.2 ml fractions and the pH was measured at +4 °C. After dialysis against 0.15 M ammonium acetate, pH 6 and 0.5 M NaCl the viscosimetric assay was performed.

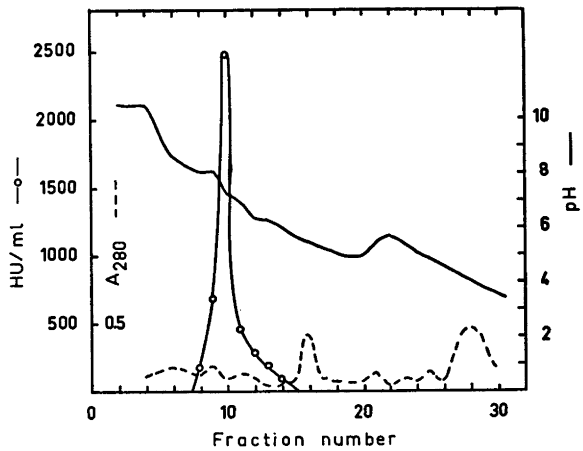

Fig. 7. Isoelectric focusing of *Helix* chitinase II. From peak II (Fig. 1) a pool was dialyzed against 1 % glycine and the dialyzed solution (25 ml) was applied to the column. After 43 h running at 435 – 1000 V (2.6 – 1.8 W) and at + 4 °C the column was drained in 3.2 ml fractions and the fractions dialyzed as described in Fig. 6.

of our NAGase, values of 100 000,[11] 105 000,[18] 140 000,[9] and 153 000 [12] have been reported for other species.

Chitinase. The pH optima in this paper were close to 4.25 for both the separated enzymes, but the curves were not coincident (Fig. 5). This optimum is close to those of *Staphylococcus aureus* chitinase [19] at pH 4.5 and commercial *Streptomyces* chitinase [20] at pH 4.2. More acid pH optima were found for an *Aspergillus niger* extract [21] at 3.6 and for bovine serum chitinase, as studied by the present authors,[22] at pH = 2.0. An extremly low pH optimum of 0.7 was recently found in Leydig's organ (lymphomyeloid oesophageal tissue) from the fish *Raja radiata.*[23]

The activity of chitinase II (Fig. 1) was confined to a narrow peak at pI 7.3, whereas the activity of chitinase I appeared in two peaks, one at pI 5.7, the other at pI 3.8.

For chitinases of different origin, molecular weights of 30 000 for *Streptomyces antibioticus* [24] and 90 000 for chitinase isoenzymes contained in pancreas of the gastric mucosa of the frog [25] were found.

The estimated molecular weights of 26 000 and 13 000 for the two chitinase activities, I and II, see Fig. 1, rises the question whether we are dealing with one or two enzymes. In view of the isoelectrophoretic separation of activity peak I, Fig. 6, into two chitinase active peaks with pI-values of 5.7 and 3.8, suggesting the presence of two isoenzymes, it seems reasonable to regard the two chitinase activity peaks I and II of Fig. 1 to be ascribed to two different enzymes, or perhaps more correctly three enzymes thereby accounting for the two suggested isoenzymes of molecular weight 26 000.

It has been discussed if the *Helix* chitinase is of bacterial origin, but the extensive studies by Jeuniaux [26] have stated that the enzymatic activity derives from the hepato-pancreas.

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