Purification and Properties of β -*N*-Acetylhexosaminidase from the Mollusc *Helicella ericetorum* Müller

By PEDRO CALVO, ANGEL REGLERO and JOSE A. CABEZAS

Department of Biochemistry, Faculties of Sciences and Pharmacy,
University of Salamanca and Consejo Superior de Investigaciones Científicas,
Salamanca, Spain

(Received 18 April 1978)

1. A β -*N*-acetylhexosaminidase was purified 330-fold from the digestive gland of the terrestrial mollusc *Helicella ericetorum* Müller. 2. Its pH optimum is 4.5 for both β -*N*-acetylglucosaminidase and β -*N*-acetylgalactosaminidase activities in two buffer solutions; it is fully stable at 37°C for 2 h in the pH range 3.8–4.6 and shows one isoelectric point (pH 4.83). 3. The estimated mol.wt. is between 120000 and 145000. 4. The enzyme shows an endo- β -*N*-acetylhexosaminidase activity on natural substrates such as ovalbumin, ovomucoid, chondroitin 4-sulphate, chitin and hyaluronic acid. 5. Two forms of the enzyme were separated by preparative polyacrylamide-gel electrophoresis. 6. K_m and V_{max} for *p*-nitrophenyl 2-acetamido-2-deoxy- β -D-glucopyranoside and *p*-nitrophenyl 2-acetamido-2-deoxy- β -D-galactopyranoside are 0.43 mM, 30.1 μ mol of *p*-nitrophenol/min per mg and 0.19 mM, 8.6 μ mol of *p*-nitrophenol/min per mg respectively. 7. It is inhibited by Hg²⁺, Fe³⁺, acetate, some lactones, *N*-acetylgalactosamine, *N*-acetylglucosamine and mannose. 8. Mixed-substrates analysis and K_i values for competitive inhibitors indicated that β -*N*-acetylglucosaminidase and β -*N*-acetylgalactosaminidase activities are catalysed by the enzyme at the same active site.

β -*N*-Acetylhexosaminidase (EC 3.2.1.52) is an enzyme that catalyses the hydrolysis of β -*N*-acetylglucosaminyl or β -*N*-acetylgalactosaminyl moieties. This enzyme is widely distributed in Nature. It has been purified from mammalian tissues (Walker, 1966; Calvo, 1977), plants (Bahl & Agrawal, 1968; Li & Li, 1970; Meyer & Bourrillon, 1973), bacteria (Wadström & Hisatsune, 1970a; Koide & Muramatsu, 1974), protozoa (Edwards *et al.*, 1975), slime moulds (Every & Ashworth, 1973; Dimond & Loomis, 1974; Kilpatrick & Stirling, 1977) and marine molluscs (Muramatsu, 1968; Egami, 1974; Pérez & Cabezas, 1977). Robinson & Stirling (1968) were the first to separate the β -*N*-acetylhexosaminidase of human spleen into two components, A and B, by starch-gel electrophoresis and by DEAE-cellulose chromatography.

The catabolism of the glycoproteins, glycolipids and glycosaminoglycans has been considered to involve such enzymes and, in addition to this physiological significance, enzymes that split *N*-acetylhexosamine linkages may be useful as analytical tools for study of the structures of some *N*-acetylhexosamine-containing glycoconjugates.

We have found that the digestive gland of the terrestrial snail *Helicella ericetorum* Müller is a very rich source of β -*N*-acetylhexosaminidase. This enzyme acts as an endoglycosidase toward natural

substrates, and it is also very active against both *p*-nitrophenyl 2-acetamido-2-deoxy- β -D-glucopyranoside and *p*-nitrophenyl 2-acetamido-2-deoxy- β -D-galactopyranoside. We decided to study the kinetics of the enzyme to find out whether the enzyme catalyses the hydrolysis of both substrates at the same active site or at different sites. Here we describe the kinetic characteristics of the enzyme toward *p*-nitrophenyl glycosides and some competitive inhibitors. Furthermore, we have also observed that this enzyme shows several characteristics very different from those of other β -*N*-acetylhexosaminidases.

Preliminary report of some of this work was presented at the 10th FEBS Meeting (Calvo *et al.*, 1975).

Materials and Methods

Materials

The snails *Helicella* (*Helicella*) *ericetorum* Müller, 1774, were collected in November from a field in Salamanca, and were used immediately or stored at –20°C.

p-Nitrophenyl glycosides, neutral carbohydrates, standard proteins, ovalbumin, ovomucoid, chondroitin 4-sulphate, chitin, hyaluronic acid, Tris and NaN₃ were from Sigma Chemical Co., St. Louis, MO, U.S.A. Standard monosaccharides for paper-

chromatography experiments were obtained from Merck, Darmstadt, West Germany. 2-Acetamido-2-deoxy-D-gluconolactone and 2-acetamido-2-deoxy-D-galactonolactone were from Koch-Light Laboratories, Colnbrook, Bucks., U.K. Blue Dextran, Sephadex G-150 (superfine grade), G-200 (superfine grade), G-100, and G-200 were from Pharmacia, Uppsala, Sweden. Ampholines were from LKB Produkter A.B., Bromma, Sweden. All other reagents were of analytical grade.

Enzyme assays

Glycosidase activities were determined essentially as described by Reglero & Cabezas (1976). The reaction mixture consisted of 1.4 ml of 0.1 M-sodium citrate buffer, pH 4.5, containing 4.2 μ mol of appropriate *p*-nitrophenyl glycoside (or 1.4 μ mol for *p*-nitrophenyl 2-acetamido-2-deoxy- β -D-galactopyranoside) and 100 μ l of enzyme preparation. After incubation at 37°C for a preset time (generally 15 min), 1.5 ml of 0.2 M- Na_2CO_3 was added to stop the reaction. The released *p*-nitrophenol was measured at 400 nm. All the buffer solutions contained 0.02% NaN_3 as preservative. One unit of enzyme was defined as the amount of enzyme which hydrolyses 1 μ mol of *p*-nitrophenyl glycoside/min at 37°C under the assay conditions; specific activity was expressed as units/mg. Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Enzyme purification

All procedures were carried out at 4°C unless otherwise indicated.

Step 1: preparation of the crude extract. Digestive glands were homogenized with water (1:15, w/v) in a mortar with sand. The suspension was left to settle for 1 h and the crude extract was centrifuged at 12000 *g* for 20 min; the precipitate was discarded.

Step 2: $(\text{NH}_4)_2\text{SO}_4$ fractionation. Solid $(\text{NH}_4)_2\text{SO}_4$ was added with stirring to the supernatant obtained from step 1, to give 30% saturation. The resulting precipitate was removed by centrifugation as above, and discarded. The supernatant was adjusted to 50% saturation with solid $(\text{NH}_4)_2\text{SO}_4$ added with stirring and the suspension was centrifuged at 12000 *g* for 20 min; the supernatant was discarded.

Step 3: dialysis at pH 4.0. The precipitate from step 2 was dissolved in 10 ml of 0.1 M-sodium citrate buffer, pH 5.0, and dialysed for 16 h against 10 litres of 0.1 M-sodium citrate buffer, pH 4.0. Afterwards it was centrifuged at 12000 *g* for 20 min and the resulting precipitate was discarded.

Step 4: gel filtration on Sephadex G-150 (superfine grade). The supernatant fraction from step 3 was applied to a column (3.5 cm $\times$ 100 cm) of Sephadex

G-150 (superfine grade) equilibrated and eluted with 0.1 M-sodium citrate buffer, pH 4.0; 5 ml fractions were collected. Those containing β -N-acetylhexosaminidase activity (fraction nos. 69–84) were pooled and concentrated by vacuum ultrafiltration through collodion bags (Sartorius-Membranfilter G.m.b.H., Göttingen, Germany).

Step 5: gel filtration on Sephadex G-200 (superfine grade). The concentrated solution from step 4 was applied to a column (3.5 cm $\times$ 100 cm) of Sephadex G-200 (superfine grade) equilibrated and eluted with 0.1 M-sodium citrate buffer, pH 4.0; 3 ml fractions were collected.

Step 6: preparative isoelectric focusing. Fractions containing β -N-acetylhexosaminidase activity from step 5 (fraction nos. 124–149) were pooled and electrofocussed by the method of Vesterberg & Svenson (1966) in a 110 ml electrofocusing column (LKB Instruments, Bromma, Sweden). The ampholyte concentration was 1% (v/v), with a pH range from 3.5 to 5.0 in a sucrose gradient (0–50%, w/v); voltage was 800 V. The run was performed in 48 h after which 2 ml fractions were collected and the pH of each fraction was measured at 2°C with a Radiometer pH M-62 digital pH-meter.

Electrophoresis

Analytical polyacrylamide-gel disc electrophoresis was performed by the method of Ornstein (1964) and Davis (1964) with 7 or 5% polyacrylamide gels, 2–3 mA per column, and Tris/glycine buffer, pH 8.3 (0.4 M-glycine was adjusted to pH 8.3 with Tris solution and diluted to 0.2 M); after electrophoresis the gels were stained for protein by using Coomassie Brilliant Blue (Weber & Osborn, 1969) or Amido Black (0.5%, w/v, in 7% acetic acid). For glycoproteins the gels were stained with periodic acid/Schiff reagent, as described by Porto & Cabezas (1970). Enzyme activity was located in the gels by slicing them into 1 mm slices, which were later tested for β -N-acetylhexosaminidase activity by using *p*-nitrophenyl substrates as above.

Preparative polyacrylamide-gel electrophoresis was carried out in a Shandon Apparatus SAE (Shandon Scientific Co., London N.W.10, U.K.) with 5% polyacrylamide gel, essentially as described in the manual supplied by Shandon: 3 ml fractions were collected, at a flow rate of 12 ml/h.

Molecular weight

The molecular weight of β -N-acetylhexosaminidase was determined on columns (2 cm $\times$ 90 cm) of Sephadex G-200 or G-100, equilibrated and eluted with 0.1 M-sodium citrate buffer (pH 4) in 0.1 M-NaCl, by using the following standards: γ -globulin (mol.wt. 160000), bovine serum albumin (mol.wt.

67000), ovalbumin (mol.wt. 45000), pepsin (mol.wt. 34500), trypsin (mol.wt. 23800), myoglobin (mol.wt. 17000) and cytochrome *c* (mol.wt. 12400).

Hydrolysis of natural substrates by β -N-acetylhexosaminidase

Ovomucoid, ovalbumin, chitin, hyaluronic acid and chondroitin 4-sulphate were used in these studies: 12mg of ovomucoid or ovalbumin, or 6mg of chitin, hyaluronic acid or chondroitin 4-sulphate was incubated with 1.4 units of enzyme (previously determined with *p*-nitrophenyl 2-acetamido-2-deoxy- β -D-glucopyranoside) in a total volume of 4ml (containing 0.02% NaN₃) for 38h at 37°C. The reaction was stopped by heating at 100°C for 5min and the products liberated were separated by ultrafiltration through collodion bags.

Descending chromatography on Whatman no. 1 paper was used for identifying the products liberated by enzymic hydrolysis. The solvent was butan-1-ol/pyridine/0.1M-HCl (5:3:2, by vol.). The reducing products were located by the method of Trevelyan *et al.* (1950).

Determination of K_m

The Michaelis constant and maximal velocities were determined for each synthetic substrate from the Lineweaver-Burk (1934) plots. Enzyme preparation was incubated with the substrates at concentrations ranging from 66.6 μ M to 5mM for *p*-nitrophenyl 2-acetamido-2-deoxy- β -D-glucopyranoside and 66.6 μ M to 3.3mM for *p*-nitrophenyl 2-acetamido-2-deoxy- β -D-galactopyranoside in 0.1M-sodium citrate, pH 4.5, for 10min at 37°C.

Determination of inhibitor constants

Inhibitor constants, K_i , were determined by the method of Dixon (1953), with *p*-nitrophenyl glycosides as substrates at concentrations ranging from 67 to 467 μ M for *p*-nitrophenyl 2-acetamido-2-deoxy- β -D-glucopyranoside and from 133 to 467 μ M for *p*-nitrophenyl 2-acetamido-2-deoxy- β -D-galactopyranoside.

N-Acetyl-lactones were used as competitive inhibitors at concentrations ranging from 0 to 40 μ M for 2-acetamido-2-deoxy-D-gluconolactone and from 0 to 27 μ M for 2-acetamido-2-deoxy-D-galactonolactone.

The enzyme preparation was incubated with the substrate and inhibitor in 0.1M-sodium citrate for 10min at 37°C.

Determination of common or separate active centres

The active centre(s) of enzyme were determined as described by Dixon & Webb (1964a), by using an

equimolecular mixture of the substrates *p*-nitrophenyl 2-acetamido-2-deoxy- β -D-glucopyranoside and *p*-nitrophenyl 2-acetamido-2-deoxy- β -D-galactopyranoside at concentrations ranging from 66.7 to 466.7 μ M for each substrate. The experimental maximal velocity of the enzyme incubated with both mixed substrates was calculated from the Lineweaver & Burk (1934) plots.

The theoretical maximal velocity of enzyme incubated with both mixed substrates, V^{A+B} , was calculated by the equation $K_m^A/\alpha \cdot K_m^B = (V^A - V^{A+B})/(V^{A+B} - V^B)$, where K_m^A , K_m^B , V^A , V^B are the Michaelis constants and the maximal velocities determined by reaction of the enzyme with the separated substrate A (*p*-nitrophenyl 2-acetamido-2-deoxy- β -D-glucopyranoside) and B (*p*-nitrophenyl 2-acetamido-2-deoxy- β -D-galactopyranoside), respectively, and α is the concentration ratio of the substrates; in this case $\alpha=1$.

Enzyme preparation was incubated in 0.1M-sodium citrate, pH 4.5, for 10min at 37°C.

Results

Purification of β -N-acetylhexosaminidase

Fractions 15 and 16 from step 6 were pooled. The enzyme was purified approx. 330-fold; purification was attained with a recovery of about 8%. The specific β -N-acetylglucosaminidase activity of this pool was 33 units/mg.

A summary of the purification is given in Table 1.

Purity at various stages in the purification of the enzyme was monitored by polyacrylamide-gel disc electrophoresis. Two bands were observed in the pool of fractions obtained after electrofocusing. The position of enzyme activity, determined in one gel parallel run, completely coincided with both protein bands.

The main glycosidase contaminant was β -D-galactosidase, present at 2% of the total activity of β -N-acetylhexosaminidase; all other glycosidase activities (α -D-mannosidase, α -D-glucosidase, α -L-fucosidase, β -D-glucuronidase, β -D-glucosidase, β -D-galactosidase, β -D-fucosidase and β -D-xylosidase) were less than 0.5%. The ratio of the β -N-acetylglucosaminidase to β -N-acetylgalactosaminidase activities, 4.2 ± 0.3 , remained constant in all steps of the purification procedure.

Separation of different forms of the enzyme

Two forms of the purified enzyme from step 5, both with β -N-acetylglucosaminidase and β -N-acetylgalactosaminidase activities, were separated by preparative polyacrylamide-gel electrophoresis, as shown in Fig. 1.

Table 1. *Purification of β -N-acetylhexosaminidase from digestive gland of Helicella ericetorum Müller*
 One unit of enzyme was defined as the amount of enzyme which hydrolyses 1 μ mol of *p*-nitrophenyl glycoside/min at 37°C under the assay conditions; specific activity was expressed as units/mg. For full experimental details see the Materials and Methods section.

Step and procedure	Total protein	Specific activity (units/mg)				Total activity (units)		Purification (-fold)			Recovery (%)		Specific-activity ratio β -N-Acetyl-glucos-aminidase
		β -N-Acetyl-glucos-aminidase	β -N-Acetyl-galactos-aminidase	β -N-Acetyl-aminidase	β -N-Acetyl-glucos-aminidase	β -N-Acetyl-galactos-aminidase	β -N-Acetyl-aminidase	β -N-Acetyl-glucos-aminidase	β -N-Acetyl-galactos-aminidase	β -N-Acetyl-aminidase			
1. Crude homogenate	3508	0.1	0.024		350	84		1	1	100	100		4.1
2. (NH ₄) ₂ SO ₄ precipitation (30–50% saturation)	753	0.64	0.14		481	105		6	6	138	126		4.5
3. Dialysis (pH 4)	215	1.6	0.36		344	77		16	15	99	91		4.5
4. Sephadex G-150 (fractions 69–84)	17.6	12.0	2.9		211	51		120	118	64	63		4.2
5. Sephadex G-200 (fractions 124–149)	7.8	20.8	5.4		162	42		208	225	52	55		3.9
6. Isoelectric focusing (fractions 15 and 16)	0.8	33.0	7.8		26	6		330	326	8	8		4.2
(fractions 14–17)	1.7	30.9	7.3		52	12		309	304	15	15		4.2

Fig. 1. Preparative polyacrylamide-gel electrophoresis of β -N-acetylhexosaminidase

○, β -N-Acetylglucosaminidase; ●, β -N-acetyl-galactosaminidase; □, A_{280} . Experimental conditions are indicated in the text.

These forms were also separated by slicing the gels after disc electrophoresis, their location being coincident with that of both bands of protein corresponding to the final step of purification. However, this was not a convenient purification procedure, with a yield of only 20% of the applied activity.

pH optimum, pH stability and isoelectric point

The pH optimum of the enzyme obtained from step 5 is 4.5, with both *p*-nitrophenyl 2-acetamido-2-deoxy- β -D-glucopyranoside and *p*-nitrophenyl 2-acetamido-2-deoxy- β -D-galactopyranoside as substrates. Similar profiles are obtained in two different buffer solutions.

Enzyme stability at various pH values, ranging from 2.7 to 9.2, was tested by previous incubation of the enzyme preparation from step 5 for 2 h at 37°C in buffer solutions obtained by appropriate mixtures of 0.2M-citric acid and 0.2M-Tris. The remaining activity was measured as described in the Materials and Methods section, and showed that the enzyme is stable in a narrow range of pH from 3.8 to 4.6. The enzyme loses catalytic activity outside this range, and at pH 3.3 and 6.3 50% of the β -N-acetylhexosaminidase was lost.

The enzyme preparations from step 1 and step 5 were subjected to isoelectric focusing. The column profiles are shown in Fig. 2. In both cases, one major peak at pH 4.83 was detected.

Thermal stability

The enzyme activity is stable to previous incubation for 100 min at 50°C. Similar profiles are obtained by using two synthetic substrates (Fig. 3).

Fig. 2. Isoelectric focusing of β -N-acetylhexosaminidase (a) Enzyme from step 1. (b) Enzyme from step 5. ○, β -N-Acetylglucosaminidase; ●, β -N-acetyl-galactosaminidase; ▲, pH.

Estimation of the molecular weight

The mol.wt. of both forms of β -N-acetylhexosaminidase was estimated to be between 118000 and 147000 by gel filtration on Sephadex G-200, and between 120000 and 145000 on Sephadex G-100.

Effect of ions, chelator, acetate, NaN_3 and some carbohydrates and derivatives

The effect of several cations (Ni^{2+} , Mg^{2+} , Zn^{2+} , Cu^{2+} , NH_4^+ , Na^+ and Fe^{3+} , as sulphates; Mg^{2+} , NH_4^+ , Mn^{2+} , Ca^{2+} and Hg^{2+} , as chlorides), CN^- , EDTA, sodium acetate and NaN_3 , at 10mM concentration, was investigated. Hg^{2+} , Fe^{3+} and sodium acetate were effective inhibitors (97%, 85% and 20% inhibition, respectively). D-Glucose, D-galactose, D-mannose, D-fructose, L-sorbose, D-fucose, L-fucose, D-ribose, L-arabinose, D-xylose, D-maltose, D-lactose, D-sucrose, D-raffinose, D-glucosamine, D-galactosamine, N-acetyl-D-glucosamine, N-acetyl-D-galactosamine, δ -D-gluconolactone, γ -D-galactonolactone, 2-acetamido-2-deoxy-D-gluconolactone and 2-acetamido-2-deoxy-D-galactonolactone were tested at 100mM concentration. Inhibition was found with 2-acetamido-2-deoxy-D-galactonolactone (100%), 2-acetamido-2-deoxy-D-gluconolactone (100%), N-acetyl-D-galactosamine (80%), N-acetyl-D-glucosamine (80%) and D-mannose (75%).

Hydrolysis of natural substrates

The purified enzyme (step 6) shows an endo- β -N-acetylhexosaminidase activity. N-Acetylglucosamine-

Fig. 3. Thermal stability of β -N-acetylhexosaminidase

(a) Samples of enzyme were heated in 0.1 M-sodium citrate buffer, pH 4.5, between 0 and 100 min at the following temperatures: $\circ$, 50°C; Δ , 55°C; ∇ , 60°C; $\square$, 65°C; $\blacklozenge$, 70°C. Remaining activity was measured as described in the Materials and Methods section. (b) Samples of enzyme were heated in 0.1 M-sodium citrate buffer, pH 4.5, for 5 min at temperatures ranging between 40 and 70°C. Remaining activity was measured as described in the Materials and Methods section.

containing oligosaccharides from ovalbumin, ovomucoid, chitin and hyaluronic acid, and N-acetylgalactosamine-containing oligosaccharides from chondroitin 4-sulphate, were liberated. β -N-Acetylhexosaminidase also splits the β -N-acetylhexosamines from the above-mentioned substrates, but at a low rate.

Kinetic characteristics of the enzyme

The rate of hydrolysis of *p*-nitrophenyl 2-acetamido-2-deoxy- β -D-glucopyranoside and *p*-nitrophenyl 2-acetamido-2-deoxy- β -D-galactopyranoside by the purified β -N-acetylhexosaminidase was measured. The enzyme was found to follow typical Michaelis-Menten kinetics.

The Lineweaver-Burk plots (Fig. 4) gave the following values for the apparent K_m : 0.43 mM for β -N-acetylglucosaminidase activity and 0.19 mM for β -N-acetylgalactosaminidase activity with the *p*-nitrophenyl glycosides as substrates. These values are similar to the K_m values of other β -N-acetylhexosaminidases (Li & Li, 1970; Verpoorte, 1972; Tallman *et al.*, 1974; Pérez & Cabezas, 1977) with the same substrates.

The purified enzyme shows maximal velocities of 30.1 and 8.6 μ mol of *p*-nitrophenol/min per mg of protein respectively for the above-mentioned substrates.

The kinetic of competition for the enzyme was studied with mixed substrates to determine whether the enzyme has one or two active sites for the hydrolysis of β -N-acetylglucosaminides and β -N-acetylgalactosaminides. A maximum velocity of 15.3 μ mol of *p*-nitrophenol/min per mg was calculated for competition between substrates according to the

Fig. 4. Lineweaver-Burk plots for β -N-acetylhexosaminidase

Enzyme was incubated with *p*-nitrophenyl glycosides as described in the Materials and Methods section. $\circ$, *p*-Nitrophenyl 2-acetamido-2-deoxy- β -D-glucopyranoside; $\bullet$, *p*-nitrophenyl 2-acetamido- β -D-galactopyranoside; $\blacksquare$, mixture of both substrates.

theoretical rate expression (see the Materials and Methods section). The experimental maximal velocity, determined by the Lineweaver-Burk plots (Fig. 4), is also 15.3 μ mol of *p*-nitrophenol/min per mg. This finding means that both substrates compete for a single active site. A corollary of this theoretical rate law for a single active site with multiple substrates is that the total rate of the reaction will be

Table 2. Kinetic constants for the study of competition between β -N-acetylglucosaminide and β -N-acetylgalactosaminide substrates for the active site of the purified β -N-acetylhexosaminidase

The enzyme activities were assayed with *p*-nitrophenyl 2-acetamido-2-deoxy- β -D-glucopyranoside and/or *p*-nitrophenyl 2-acetamido-2-deoxy- β -D-galactopyranoside as substrates, under the conditions described in the text. K_m values are the Michaelis constants; V , the maximal velocities; V_{max} , the maximal velocity of the enzyme acting on the mixture of both substrates; K_i , the inhibitor constants with *N*-acetylgluconolactone; $K_{i'}$, the inhibitor constants with *N*-acetylgalactonolactone.

Activity	K_m (mM)	V (μ mol/min per mg)	$V^A + V^B$ (μ mol/min per mg)	V_{max} (μ mol/min per mg)		K_i (μ M)	$K_{i'}$ (μ M)
				Theoretical	Experimental		
β -N-Acetylglucosaminidase (A)	0.43	30.1	38.7	15.3	15.3	172	6.3
β -N-Acetylgalactosaminidase (B)	0.19	8.6				168	6.2

less than the sum of the rate of the reactions measured separately (Dixon & Webb, 1964b). The total rate of the reaction in our case is 15.3 μ mol of *p*-nitrophenol/min per mg, which is less than the sum of the rate of the reactions separately: 30.1 + 8.6 = 38.7 μ mol of *p*-nitrophenol/min per mg.

Additionally, if an enzyme catalyses the hydrolysis of two substrates at the same active site, a competitive inhibitor must have the same K_i when it is assayed with either substrate (Dixon & Webb, 1964b). 2-Acetamido-2-deoxy-D-gluconolactone has practically the same K_i values with respect to both the β -N-acetylglucosaminidase (172 μ M) and the β -N-acetylgalactosaminidase (168 μ M) activities of the purified enzyme. K_i values are also practically identical for both activities with 2-acetamido-2-deoxy-D-galactonolactone as inhibitor (6.3 μ M and 6.2 μ M respectively).

All the kinetic parameters found in the study of competition between both substrates for the same active site of the enzyme are summarized in Table 2.

Discussion

There is experimental evidence for the presence of β -N-acetylhexosaminidase activity in a large number of tissues. In some cases, this enzyme has been purified. Li & Li (1970) have obtained β -N-acetylhexosaminidase from jack-bean meal in crystalline form. Molluscs have scarcely been investigated as sources for this enzyme, yet they are rich in this and other glycosidases (Muramatsu, 1968; Egami, 1974; Reglero & Cabezas, 1976; Cabezas & Reglero, 1977; Pérez & Cabezas, 1977). We have not found any reference to the presence of this enzyme in the terrestrial mollusc *Helicella ericetorum* Müller.

It was difficult to obtain β -N-acetylhexosaminidase preparations free from other glycosidases, but, after electrofocusing, the final preparation was shown to be practically without contamination, and had a specific activity of 33.0 units/mg. This compares

favourably with that recorded for purified enzyme from other sources, e.g. mammalian tissues.

This enzyme is only stable in a narrow range of pH (from 3.8 to 4.6), so its stability was low under the conditions of the preparative electrophoresis.

Only one major isoelectric point, pH 4.83, was found in both unpurified (step 1) and purified (step 5) enzyme preparations. (Unpurified fraction was used with a view to avoiding the risk of artifacts.) Several peaks in the unpurified enzyme preparation profile and a shoulder in the purified fraction profile are also apparent (Fig. 2); perhaps they correspond to multiple forms of the enzyme that may be due to a different sialic acid content.

The mol.wt. of this enzyme is similar to those of β -N-acetylhexosaminidases from other sources, which generally range between 100000 and 150000.

Polyacrylamide-gel disc electrophoresis shows two bands for the purified preparation of the enzyme, suggesting different molecular forms. Each band has both β -N-acetylglucosaminidase and β -N-acetylgalactosaminidase activities at the same ratio. Besides, the ratio of the β -N-acetylglucosaminidase and β -N-acetylgalactosaminidase activities remains constant at all stages of the enzyme purification. Furthermore, the enzyme assayed on both synthetic substrates has the same pH optimum, the same stability against different pH values and heat, and the same molecular weight for both activities. These evidences suggest that both activities reside in the same enzyme molecule, as expected. A similar behaviour was observed by Robinson & Stirling (1968) for β -N-acetylhexosaminidase from human spleen. Also, the behaviour of the enzyme with different effectors and the identical isoelectric point for both activities indicate that these two activities are catalysed by the same enzyme protein.

From our kinetic assays, it may be deduced that β -N-acetylhexosaminidase from *Helicella* catalyses the hydrolysis of both β -N-acetylglucosaminides and β -N-acetylgalactosaminides at the same active

site. These results agree with those reported for β -*N*-acetylhexosaminidases from jack-bean meal (Li & Li, 1970), ox spleen (Verpoorte, 1972) and the plant *Trigonella foenum graecum* (Bouquelet & Spik, 1976).

The enzyme hydrolyses not only synthetic substrates but also natural ones. In this case, it acts mainly as an endo- β -*N*-acetylhexosaminidase on β 1 $\rightarrow$ 4-linkages. An endo- β -*N*-acetylglucosaminidase activity has been observed by Korman *et al.* (1970) and by Wadström & Hisatsune (1970b) in *Staphylococcus aureus*, by Koide & Muramatsu (1974) in *Diplococcus pneumoniae*, and by Trimble & Maley (1977) in *Streptomyces plicatus*. Yet, a human α -*N*-acetylglucosaminidase, purified, by von Figura (1977), acts exclusively as an exoglycosidase specific for *N*-acetylglucosamine residues. On the other hand, Stirling (1974) has described a chitobiase activity associated with human liver β -*N*-acetylhexosaminidase.

Finally, a β -*N*-acetylhexosaminidase from a marine mollusc, *Chamelea gallina* L. (old name, *Venus gallina* L.), also hydrolyses natural substrates (Pérez & Cabezas, 1977), but it differs from that of the terrestrial mollusc *Helicella* since it has two pH optima (one for *p*-nitrophenyl 2-acetamido-2-deoxy- β -D-glucopyranoside, another for *p*-nitrophenyl 2-acetamido-2-deoxy- β -D-galactopyranoside), and it shows only one band on polyacrylamide-gel disc electrophoresis. So it is evident that there are significant differences in the characteristics of *N*-acetylhexosaminidases from even very closely related sources.

This research, supported by the Ministerio de Educación y Ciencia and Consejo Superior de Investigaciones Científicas and by a grant of the Presidencia del Gobierno, fulfils, in part, requirements for a Ph.D. for P. C. We thank Dr. S. Bouquelet for his advice during the stay of P. C. in the Laboratory of Biochemistry of the University of Lille, France (Professor J. Montreuil), and Professor D. Robinson (Queen Elizabeth College, University of London) for critical reading of the manuscript. The secretarial work by Mrs. Rosario Reglero is acknowledged.

References

- Bahl, O. P. & Agrawal, K. M. L. (1968) *J. Biol. Chem.* **234**, 98–102
- Bouquelet, S. & Spik, G. (1976) *FEBS Lett.* **63**, 95–101
- Cabezas, J. A. & Reglero, A. (1977) in *Avances de la Bioquímica* (Cornudella, L., Oró, J., Heredia, C. F. & Sols, A., eds.), pp. 145–155, Salvat, Barcelona
- Calvo, P. (1977) Ph.D. Thesis, University of Salamanca
- Calvo, P., Reglero, A. & Cabezas, J. A. (1975) *Abstr. FEBS Meet. 10th Abstract* 947
- Davis, B. J. (1964) *Ann. N.Y. Acad. Sci.* **121**, 404–427
- Dimond, R. L. & Loomis, W. F. (1974) *J. Biol. Chem.* **249**, 5628–5632
- Dixon, M. (1953) *Biochem. J.* **55**, 170–171
- Dixon, M. & Webb, E. C. (1964a) *Enzymes*, 2nd edn., pp. 54–90, Longmans, London
- Dixon, M. & Webb, E. C. (1964b) *Enzymes*, 2nd, Ed. 201–209.
- Edwards, R. G., Thomas, P. & Westwood, J. H. (1975) *Biochem. J.* **151**, 145–148
- Egami, F. (1974) *Colloq. Int. CNRS* **221**, 289–297
- Every, D. & Ashworth, J. M. (1973) *Biochem. J.* **133**, 37–47
- Kilpatrick, D. C. & Stirling, J. L. (1977) *Biochem. J.* **161**, 149–157
- Koide, N. & Muramatsu, T. (1974) *J. Biol. Chem.* **249**, 4897–4904
- Korman, R. Z., Lis, H. & Sharon, N. (1970) *Biochim. Biophys. Acta* **201**, 380–383
- Li, S. C. & Li, Y. T. (1970) *J. Biol. Chem.* **245**, 5153–5160
- Lineweaver, H. & Burk, D. (1934) *J. Am. Chem. Soc.* **45**, 658–666
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Meyer, D. & Bourrillon, R. (1973) *Biochimie* **55**, 5–10
- Muramatsu, T. (1968) *J. Biochem. (Tokyo)* **64**, 521–531
- Ornstein, L. (1964) *Ann. N.Y. Acad. Sci.* **121**, 321–349
- Pérez, N. & Cabezas, J. A. (1977) *Biochimie* **59**, 729–733
- Porto, E. & Cabezas, J. A. (1970) *Rev. Esp. Fisiol.* **26**, 335–338
- Reglero, A. & Cabezas, J. A. (1976) *Eur. J. Biochem.* **66**, 379–387
- Robinson, D. & Stirling, J. L. (1968) *Biochem. J.* **107**, 321–327
- Stirling, J. L. (1974) *FEBS Lett.* **39**, 171–175
- Tallman, J. F., Brady, R. O., Quirk, J. M., Villalba, M. & Gal, A. E. (1974) *J. Biol. Chem.* **249**, 3489–3499
- Trevelyan, W. E., Procter, D. P. & Harrison, J. S. (1950) *Nature (London)* **166**, 444–445
- Trimble, R. B. & Maley, F. (1977) *Biochem. Biophys. Res. Commun.* **78**, 935–944
- Verpoorte, J. A. (1972) *J. Biol. Chem.* **247**, 4787–4793
- Vesterberg, O. & Svenson, H. (1966) *Acta Chem. Scand.* **20**, 820–834
- von Figura, K. (1977) *Eur. J. Biochem.* **80**, 171–175
- Wadström, T. & Hisatsune, K. (1970a) *Biochem. J.* **120**, 725–734
- Wadström, T. & Hisatsune, K. (1970b) *Biochem. J.* **120**, 735–745
- Walker, P. G. (1966) in *Aminosugars* (Balazs, E. A. & Jeanloz, R. W., eds.), vol. 2B, pp. 165–169, Academic Press, New York
- Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406–4412