The β -Glucosidase in the Gut Contents of the Snail *Achatina achatina* ENERGY-DEPENDENT MODIFICATION OF STRUCTURE AND ACTIVITY

By GABRIEL M. UMEZURIKE

Department of Biochemistry, University of Nigeria, Nsukka, Nigeria

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1. The enzyme β -glucosidase (β -D-glucoside glucohydrolase, EC 3.2.1.21) from the gut contents of active *Achatina achatina* exists in two molecular forms, β -glucosidase C (mol.wt. about 82000) and D (mol.wt. about 41000). 2. Only the lower-molecular-weight species was found in the gut contents of aestivating snails or in extracts from their digestive glands and washed gut walls. 3. On re-activation of some aestivating snails, β -glucosidase C re-appeared in the gut contents. 4. Addition of ATP and Mg^{2+} to the isolated gut contents or to extracts from washed gut walls led to the formation of higher-molecular-weight forms of the enzyme, β -glucosidase A (mol.wt. about 329000) and β -glucosidase B (mol.wt. about 165000). 5. All these forms of the enzyme have similar pH optimum (pH 5.0–5.6). 6. The Michaelis constants (K_m) and heat stability of the enzyme increased with increasing molecular complexity.

The enzyme β -glucosidase occurs in kidney, liver and intestinal mucosa of animals (Robinson, 1956; Robinson *et al.*, 1967*a,b*; Abrahams & Robinson, 1969) and also in fungi, yeasts and bacteria (Jermyn, 1955*a,b*, 1962; Duerksen & Halvorson, 1958; Hu *et al.*, 1960; Pettersson, 1963; Mahadevan & Eberhart, 1964; Horikoshi & Yonosuke, 1965; Li *et al.*, 1965; Fleming & Duerksen, 1967*a,b*; Marchin & Duerksen, 1968*a,b*; Legler, 1968; Han & Srinivasan, 1969; Wood, 1971; Umezurike, 1971, 1975). The β -glucosidases from the various sources are all probably aggregates of a monomeric form with a mol.wt. of about 40000, that isolated from *Botryodiplodia theobromae* existing in a range of aggregates. The monomer, dimer, tetramer and octamer, with mol.wts. of about 40000, 80000, 160000 and 320000 respectively, have been examined (Umezurike, 1975). The β -glucosidase from other organisms has also been reported to exist in multiple molecular forms (Jermyn, 1955*a,b*, 1962; Pettersson, 1963; Mahadevan & Eberhart, 1964; Horikoshi & Yonosuke, 1965), though such multiplicity has often been attributed to the formation of stable complexes between a single enzyme and a variety of polysaccharides in the medium (Jermyn, 1955*a,b*, 1962).

Only the octameric form of β -glucosidase could be detected in young cultures of *B. theobromae*, whereas appreciable amounts of the lower-molecular-weight species were found in older cultures (Umezurike, 1975). The factors responsible for this effect are not known.

It was decided to investigate the structure of β -glucosidase as a function of the nutritional state of the tropical gastropod *Achatina achatina* which, like other herbivorous gastropods, starts aestivating when

food (leaves and vegetable matter) and water are scarce.

Materials and Methods

Organism

A number of snails (*Achatina achatina*), bought from the local market, were maintained in the laboratory (22–25°C) in a perforated metal box. The animals were fed on fresh paw-paw (*Carica papaya*) leaves. The box was moistened with tap water at regular time-intervals. When aestivating animals were required, aestivation was induced by transferring the animals to a dry perforated metal box without food and water.

Chemicals

o-Nitrophenyl β -D-glucopyranoside and the protein markers used for gel filtration were obtained from Koch-Light Laboratories, Colnbrook, Bucks., U.K. ATP was purchased from Sigma (London) Chemical Company Ltd., Kingston-upon-Thames, Surrey, U.K. All other chemicals and materials were as described previously (Umezurike, 1971).

Source of β -glucosidase

The digestive tracts were removed immediately after de-shelling the snails. In earlier experiments, the digestive tracts were stored frozen at –15°C. When required, the thawed digestive tracts were gently pounded in a pre-cooled mortar to release their contents and digestive juice. The released gut contents and digestive juice were taken up in a minimal

amount of McIlvaine buffer, pH 5.0 (McIlvaine, 1921), containing 0.1 M-NaCl. In later experiments, the gut contents of each gut were drained and diluted with the desired amount of the same buffer. The extracted gut contents were then centrifuged at 10000 g for 30 min at 4°C and the supernatants were stored frozen at -15°C until required.

When the β -glucosidase in washed gut walls was required, corresponding sections of the gut wall (crop, stomach and intestine) of a number of snails were separately opened up and washed four times with about 50 ml of 0.05 M-sodium acetate buffer (pH 5.0). The gut walls were blotted to remove excess of liquid. About 7–8 g (fresh wt.) of the various sections of the gut wall was homogenized manually in a minimal amount of 0.05 M-sodium acetate buffer (pH 5.0), and the homogenates were then centrifuged at 10000 g for 15 min at 4°C. The supernatant solutions were mixed with twice their volume of cold acetone and left at 4°C for 30 min. The precipitates obtained after centrifugation at 10000 g for 15 min were separately dissolved in 0.05 M-sodium acetate buffer (pH 5.0). After centrifugation to remove undissolved particles, the supernatant solutions were made up to 8 ml with the above buffer and portions of these solutions were used for enzyme assay. β -Glucosidase activity was expressed as nmol of *o*-nitrophenol liberated/min per g fresh wt. of tissue. Preliminary experiments showed that β -glucosidase is completely precipitated from solution in the presence of 2 vol. of acetone.

Enzyme assay

β -Glucosidase activity was determined as described previously (Umezurike, 1971), except that *o*-nitrophenyl β -D-glucopyranoside in 0.05 M-sodium acetate buffer (pH 5.0) was used as substrate.

Gel filtration

Columns of Sephadex G-100 and G-200 (superfine) were prepared as described elsewhere (Umezurike, 1970). The eluting buffer was McIlvaine buffer (phosphate/citrate buffer) containing 0.1 M-NaCl, pH 5.0 (McIlvaine, 1921). All chromatographic separations were performed at 4°C. Estimation of molecular weight by gel filtration, by the method of Andrews (1964, 1965), was carried out with Blue Dextran (mol. wt. 2000000), β -galactosidase (540000), catalase (250000), bovine serum albumin (monomer, 65000; dimer, 130000), β -lactoglobulin (35000), cytochrome *c* (12270) and glucose (180) as markers.

Carboxamidomethylation of reduced enzyme protein

Reduction of enzyme protein with 8 M-urea and 0.1 M-2-mercaptoethanol was followed by alkylation with 0.05 M-iodoacetamide (Umezurike, 1971).

Protein determination

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Results

Estimation of molecular weights of β -glucosidase components

Gel filtration of the gut contents of active *Achatina achatina* revealed the presence of two enzymically active forms of β -glucosidase. The molecular weights of these two forms of β -glucosidase were estimated by the chromatographic method of Andrews (1964, 1965) by comparison of their behaviour with those of standard protein markers. The elution volumes of the higher-molecular-weight enzyme (designated β -glucosidase C) and the lower-molecular-weight enzyme (designated β -glucosidase D) on a column (30 cm $\times$ 2.5 cm) of Sephadex G-100 were 72.5 ml and 87.5 ml respectively. The corresponding values of standards were for bovine serum albumin (dimer), 60 ml; bovine serum albumin (monomer), 77.5 ml; β -lactoglobulin, 90 ml; and cytochrome *c*, 115 ml. The molecular weight of β -glucosidase C was estimated to be 78000–86000 (mean value 82000) and that for β -glucosidase D, 38000–44000 (mean value 41000). Similar results were obtained when the gut contents of snails fed for 7 days on Whatman filter paper or on leaves of *Carica papaya* were used for gel filtration, β -glucosidase C being the major component.

Effect of starvation/aestivation on β -glucosidase structure

Active snails were divided into two batches. One batch (batch 1) was maintained in a perforated metal box and fed with fresh leaves of *Carica papaya*. The box was watered at regular time-intervals to keep the environment moist. The other batch (batch 2) was also kept in a perforated metal box without food and water. After 3–4 days the snails in batch 2 had started aestivating, whereas those in batch 1 remained active. The snails were maintained in these states for another 6–7 days before they were de-shelled.

Gel filtration of the gut contents of active snails of batch 1 showed the same two species of β -glucosidase with mean mol. wts. of 82000 (β -glucosidase C) and 41000 (β -glucosidase D). On the contrary, the gut contents of the aestivating snails of batch 2 contained only one enzymically active β -glucosidase, with a mean mol. wt. of 41000 (Fig. 1). When aestivating snails were re-activated by watering the environment at regular time-intervals and by supplying fresh leaves, and maintained in the activated state for 6 days, the usual two species of the enzyme (β -glucosidases C and D) found in the gut contents of active

Fig. 1. Elution profile on a column of Sephadex G-100 (88cm $\times$ 2.5cm) of β -glucosidase in the gut contents of aestivating snails

β -Glucosidase activity (●) was determined with *o*-nitrophenyl β -D-glucopyranoside as substrate, and protein distribution (○) was estimated as E_{280} . The fraction size was about 5.0ml.

snails were obtained on gel filtration of the gut contents of these snails.

It thus appears that during aestivation the higher-molecular-weight enzyme dissociates into the lower-molecular-weight species. A possible polymerization of the lower-molecular-weight species into the higher-molecular-weight enzyme in re-activated snails is also indicated by these results.

Effects of Mg^{2+} and ATP

The results presented above suggested that the change in molecular weight of β -glucosidase found in the gut of *Achatina achatina* may be reflecting the energy state of the snails. To test this possibility, the gut contents from active snails were made 10mM with respect to ATP and $MgSO_4$, incubated at 30°C for

Fig. 2. Gel filtration of the gut contents of active snails on a column (88 cm $\times$ 2.5 cm) of Sephadex G-100 (a) before treatment, (b) after incubation at 30°C for 45 min in the presence of 10mM-ATP and 10mM- $MgSO_4$ and (c) after incubation at 30°C for 45 min in the presence of 10mM- $MgSO_4$

The gel was equilibrated with 0.1 M-citric acid/0.2 M- Na_2HPO_4 buffer (pH 5.0) containing 0.1 M-NaCl. Elution was with the same buffer. The volume of each fraction was about 5.0ml. β -Glucosidase activity (●) was determined with *o*-nitrophenyl β -D-glucopyranoside as substrate (see the Materials and Methods section) and total protein (○) was estimated as E_{280} .

Fig. 3. Gel filtration of pooled and concentrated fractions (a) 17–19 and (b) 20–23 of the separation shown in Fig. 2(b) on a column (33 cm $\times$ 2.5 cm) of Sephadex G-200 (superfine grade)

β -Glucosidase activity (●) and protein distribution (E_{280} , ○) were determined as described in the legend to Fig. 2.

45 min and used for gel filtration. Three molecular species (designated β -glucosidases A, B, and C) were found (Fig. 2b). β -Glucosidase A was eluted at the void volume of the column (Sephadex G-100). When the same sample was made 10 mM with respect to MgSO_4 and incubated for 45 min at 30°C, only β -glucosidase C and D were found (Fig. 2c). For comparison, Fig. 2(a) shows the result obtained when the same sample was used for gel filtration without pretreatment with ATP or MgSO_4 . Fig. 3 shows the results obtained when fractions 17–19 and 20–23 of the separation shown in Fig. 2(b) were separately pooled, concentrated and each sample was used for gel filtration on a Sephadex G-200 column. The mol. wts. of the four species estimated by the method of Andrews (1964, 1965) were $329\,000 \pm 5\,000$ (β -glucosidase A), $165\,000 \pm 2\,000$ (β -glucosidase B), $82\,000 \pm 4\,000$ (β -glucosidase C) and $41\,000 \pm 3\,000$ (β -glucosidase D). No high-molecular-weight form larger than β -glucosidase A was found, but carboxamidomethylation of the reduced form of each of the four species (A–D) gave rise to complete inactivation. The β -glucosidase in the gut contents of

aestivating snails gave similar results to those presented above on treatment with Mg^{2+} -ATP.

β -Glucosidase in washed gut wall

In view of the uncertainty of the origin of certain carbohydrases found in the gut contents of some other molluscs (Florkin & Lozet, 1949; Galli & Giese, 1959; Parnas, 1961), extracts from washed gut walls were examined for β -glucosidase activity. β -Glucosidase activity in extracts from the crop, stomach and intestine from active snails was 60.6, 65.1 and 6.5 nmol of *o*-nitrophenol liberated/min per g of tissue respectively. The corresponding values of β -glucosidase activity in extracts from the crop, stomach and intestine from aestivating snails were 49.3, 54.6, and 4.0 nmol of *o*-nitrophenol liberated/min per g of tissue respectively. The β -glucosidase activity of unwashed digestive glands from active and aestivating snails was 58.9 and 49.1 nmol of *o*-nitrophenol liberated/min per g of tissue respectively. In a similar experiment in which de-veined leaves (15 g) of *Carica papaya* were used, the activity of β -gluco-

sidase was 0.1 nmol of *o*-nitrophenol liberated/min per g of leaf tissue, a much smaller value than in snail tissues.

Examination of portions of the extracts from the crop, stomach and digestive glands of active and aestivating snails by gel filtration showed that the extracts contained only β -glucosidase D (mol.wt. approx. 41 000). When the extracts were made 10 mM with respect to ATP and MgSO_4 and incubated at 30°C for 45 min before gel filtration on a column (33 cm $\times$ 2.5 cm) of Sephadex G-200, higher-molecular-weight forms of β -glucosidase (species A, B and C) were found.

Kinetic properties of the various molecular species

In these experiments, fraction 14 of the separation shown in Fig. 3(a) and fraction 18 of that shown in

Fig. 3(b) were used as source of β -glucosidase A and B respectively, and fraction 34 of the separation shown in Fig. 2(b) and fraction 37 of the separation shown in Fig. 2(a) were used as source of β -glucosidase C and D respectively. These fractions were re-chromatographed, and pooled fractions were exhaustively dialysed against several changes of water and then against three changes of 0.05 M-sodium acetate buffer (pH 5.0) at 4°C before use. Any precipitate formed was removed by centrifugation.

(i) *Michaelis constants*. Lineweaver-Burk plots (Lineweaver & Burk, 1934) of the results obtained when velocity was measured as a function of substrate concentration showed that the four species of β -glucosidase (species A–D) were inhibited by the substrate in the presence of more than 0.2 mM-*o*-nitrophenyl β -D-glucopyranoside. The data obtained in the presence of 0.04–0.2 mM-*o*-nitrophenyl β -D-glucopyranoside are plotted as v versus $v/[S]$ in Fig. 4. The K_m values calculated from the slope of the lines in Fig. 4 were 0.16 mM-, 0.12 mM-, 0.10 mM- and 0.09 mM-*o*-nitrophenyl β -D-glucopyranoside for β -glucosidase A, B, C, and D respectively. Thus the affinity of the various species of β -glucosidase appears to increase slightly with decreasing molecular complexity. Similar results have been reported for corresponding forms of β -glucosidase of *Botryodiplodia theobromae* (Umezurike, 1975).

(ii) *Heat-stability*. The effect of pre-incubating the various β -glucosidase species in 0.05 M-sodium acetate buffer (pH 5.0) at 59°C for various lengths of time

Fig. 4. Plots of v versus $v/[S]$ of results obtained when various β -glucosidase species were used

β -Glucosidase species A (●) and B (○) were obtained from fractions 14 and 18 of separations similar to those shown in Figs. 3(a) and 3(b) respectively, and species C (■) and D (□) were obtained from fractions 34 and 37 of separations similar to those shown in Figs. 2(b) and 2(a) respectively. The various fractions were re-chromatographed and appropriate fractions were pooled, concentrated and exhaustively dialysed against water and then against several changes of 0.05 M-sodium acetate buffer (pH 5.0) before use. The substrate (*o*-nitrophenyl β -D-glucopyranoside) was dissolved in 0.05 M-sodium acetate buffer (pH 5.0).

Fig. 5. Semilogarithmic plots showing the heat-stability of the various β -glucosidase species

Solutions of the various species, obtained as shown in the legend to Fig. 4, were dialysed against 0.05 M-sodium acetate buffer (pH 5.0) and pre-incubated at 59°C. At zero time, and at regular time-intervals, samples were removed for enzyme assay. The plots are for species A (●), B (○), C (■) and D (□).

Fig. 6. Effect of pH on the activity of β -glucosidase species C in 0.1 M-citric acid/0.2 M- Na_2HPO_4 buffers

Assay mixtures contained 0.8 mM *o*-nitrophenyl β -D-glucopyranoside (final concentration) in buffers of different pH values, and enzyme activity is expressed as nmol of *o*-nitrophenol liberated per min at 40°C. See the Materials and Methods section for further details.

before enzyme assay is shown as semilogarithmic plots in Fig. 5. The results suggest that heat-stability of the various species of β -glucosidase increases with molecular complexity. The non-linearity of the plots for the higher-molecular-weight forms here observed was also reported for the corresponding β -glucosidase species of *Botryodiplodia theobromae* and the probable cause discussed (Umezurike, 1975).

(iii) *Effect of pH.* The pH/activity curves for the various species of β -glucosidase were similar. All showed a broad pH optimum between pH 5.0–5.6 in McIlvaine buffer (McIlvaine, 1921). A representative pH activity curve is shown in Fig. 6.

Discussion

The giant tropical mollusc *Achatina achatina* is a terrestrial animal which usually remains hidden in shady and moist environments by day. They are found more abundantly in areas with low vegetation littered with decaying vegetable matter. They feed mainly at night, or after the rains, on leaves and decaying vegetable matter, like other land pulmonates (Morton, 1963). Since cellulose thus forms a major source of carbohydrate for this animal, it is not surprising that β -glucosidase, which is always associated with cellulase, is found in large amounts in

their gut contents. Thus β -glucosidase action appears to play a major role in providing the glucose used by this animal for metabolic processes. Two molecular-weight species of β -glucosidase (species C, with mol.wt. about 82000, and species D, with mol.wt. about 41000) were found in the gut contents of active snails, whereas only the lower-molecular-weight form (species D) was present in the gut contents of aestivating snails. The effect of aestivation on the structure of β -glucosidase appears to be the conversion of species C into species D. On re-activating the snails, there appears to be some polymerization of species D into species C. In Nature, aestivation occurs mainly as an adaptation to seasonal drought and is an effective mechanism for surviving periods when food and water are scarce (Hughes, 1974). During aestivation, respiratory activities and the heart-beat in snails decrease, though the rate of the heart-beat is closely dependent on temperature (Borradaile *et al.*, 1963). It is therefore conceivable that the nutritional state of the animal determines the molecular form in which β -glucosidase exists at any time. This idea is strongly supported by the finding that, in the presence of added ATP and Mg^{2+} , higher-molecular-weight forms (i.e. β -glucosidase A, with mol.wt. about 329000, and β -glucosidase B, with mol.wt. about 165000) are formed in the isolated gut contents. If species D is accepted as the monomeric form, since no enzymically active species with a lower molecular weight was ever found, then, as in the β -glucosidase of *Botryodiplodia theobromae* (Umezurike, 1975), species C may be regarded as dimer, species B as tetramer and species A as octamer. It is noteworthy that no higher polymeric species larger than species A was formed in the presence of added ATP and Mg^{2+} .

The results obtained from kinetic experiments show that the affinity of the four active β -glucosidase species (A–D), derived from the isolated gut contents, for the substrate increases with decreasing molecular complexity. In aestivating snails, where respiratory activity is low and the amount of substrate present in the gut is necessarily being depleted, it is an advantage to the snails that the species of β -glucosidase present (species D) has a high affinity for the substrate. That the higher polymeric forms of β -glucosidase (species A and B) were not found in the gut contents of active snails is apparently due to the absence of enough ATP. Thus it appears that the energy requirements of active snails are satisfied by the activity of β -glucosidase C.

It is noteworthy that a β -glucosidase with mol.wt. about 40000 has been found in animal tissues (Robinson *et al.*, 1967a,b; Abrahams & Robinson, 1969) and that β -glucosidase activity appears to be lower in tissues of carnivorous animals than in those of herbivorous animals (Robinson, 1956). The detection of high β -glucosidase D activity in the digestive

glands and washed gut walls from *Achatina achatina* may thus be regarded as suggesting that β -glucosidase in the gut juice from this animal is probably secreted by the animal tissues in the low-molecular-weight form (β -glucosidase D).

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