A SURVEY OF THE ENZYMES FROM THE GASTRO-INTESTINAL TRACT OF *HELIX POMATIA*

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INTRODUCTION

The digestive tract of *Helix pomatia* is known to contain many digestive enzymes. Holden & Tracey (1950) list a wide variety of substrates which are attacked by *Helix* sp. digestive juices, but their data are mainly qualitative and are drawn from publications by a number of authors.

Extracts from the gastro-intestinal tract of *H. pomatia* are particularly rich in cellulase and have frequently been used in cytological work to digest plant cell walls because of this cytase activity. The present investigation was carried out both to assess the relative amounts of cellulase and other enzymes in such an extract before attempting to purify cellulase from it, and to see how the efficacy of snail cytase is correlated with the range of carbohydrases present.

MATERIALS AND METHODS

(*a*) *Preparation of an enzymically active extract from* Helix pomatia

Specimens of *H. pomatia* in a resting condition, with a thick calcareous epiphragm closing the shell, were dissected by the method of Keilin (1956). The digestive tracts were excised and cut into 1–2 cm. lengths. They were homogenized with the gastro-intestinal tract contents and an equal volume of ice-cold distilled water in a Potter homogenizer (Potter & Elvehjem, 1936) which was fitted with a Perspex pestle and surrounded by ice. The homogenate was centrifuged at 19,000 *g* at 4° C. and the clear brown supernatant decanted. It was dialysed for 16 hr. in cellophane dialysis tubing against a large volume of ice-cold distilled water and any precipitate which formed was removed by centrifuging. The resulting solution was used in the enzyme assays.

200 snails yielded about 100 ml. of this solution, which contained approx. 10 mg./ml. protein N, and was stored for up to 6 months at $-15°$ C. The solution showed three boundaries when examined in a Tiselius electrophoresis apparatus (Perkin Elmer Model 38). At pH 6·8 and I 0·2, the mobilities of these boundaries in the descending limb of the apparatus were $0{\cdot}372 \times 10^{-5}$ cm.^{2} V.$^{-1}$ sec.$^{-1}$, $0{\cdot}854 \times 10^{-5}$ cm.^{2} V.$^{-1}$ sec.$^{-1}$ and $2{\cdot}45 \times 10^{-5}$ cm.^{2} V.$^{-1}$ sec.$^{-1}$.

(*b*) *Preparation of substrates*

Tween 20 was freed from free fatty acids by the method of Archibald (1946).

β-Methyl cellobioside was prepared by a four-stage process. Cellobiose octa-acetate was prepared from 20 g. cotton-wool (Dorée, 1947) and was converted to hepta-acetyl β-methyl cellobioside by the method of Pacsu (1930). This was deacetylated (Zemplén, 1926) and the product recrystallized. 3 g. of crystalline β-methyl cellobioside were obtained, m.p. 191° C. (uncorr.); $[\alpha]_D$ −18·4°. Zemplén & Gerecs (1930) give m.p. 193° C. and $[\alpha]_D$ −19·1° for this compound.

Chitosan acetate was prepared by the method of Meyer & Wehrli (1937).

Cellofas B, a sodium carboxymethyl cellulose derivative, was supplied by I.C.I. Ltd. The degree of substitution of a sample labelled 'low' viscosity grade was found to be 0·46 by a colorimetric method (Eyler, Klug & Diephuis, 1947). An approximately 1% solution was prepared. The powder was added to distilled water, stirred mechanically for about 4 hr. and the mixture centrifuged at 800 *g* for 30 min. to remove traces of insoluble material. The amount of Cellofas B in the supernatant was found by a dry-weight determination on a small sample and the concentration adjusted by the addition of distilled water to give a 0·5% solution which could be stored up to 2 months at 2° C.

Bleached cotton linters were kindly given by Lansil Ltd., Shipley, Yorks. They were swollen with phosphoric acid at 0° C. for 10 min. (Walseth, 1952). The degree of polymerization of the treated linters was estimated by the method of Atchison (1943) and was found to be 500.

Laminarin was kindly given by Dr D. J. Manners.

Wheat straw xylan and *walnut xylan* were prepared by the method of Dorée (1947).

Snail galactan was prepared by the method of May (1934).

Yeast mannan was prepared by the method of Haworth, Heath & Peat (1941), and *ivory nut mannan A* and *ivory nut mannan B* by the method of Aspinall, Hirst, Percival & Williamson (1953).

Yeast glucan was prepared by the method of Bell & Northcote (1950). The other substrates used were supplied commercially.

(*c*) *Methods of measuring enzymic activities*

Proteinases. These were assayed by the method of Anson (1938).

Carbohydrases. Invertase and *maltase* were estimated by the manometric method of Keilin & Hartree (1948) using a glucose oxidase preparation supplied by the Sigma Chemical Company and labelled 'Crude, on inert base'. *Lactase* was estimated, using the same impure notatin preparation, by the method described by Hestrin, Feingold & Schramm (1955).

Cellobiase was estimated by a reducing sugar method, since a glucose oxidase preparation free from cellobiase could not be obtained. 5 ml. of 0·5% β-methyl

cellobioside solution were incubated in a stoppered boiling tube at 37° C. with 0·5 ml. M buffer at an appropriate pH and 4·0 ml. distilled water. 0·5 ml. of the enzyme solution was added and the mixture was incubated with shaking for 20 min. 2 ml. of the incubation mixture were then added to 2 ml. of alkaline copper-sulphate solution (Somogyi, 1952) and heated on a boiling-water bath for 15 min. The solution was cooled rapidly to room temperature, 2 ml. arseno-molybdate reagent (Nelson, 1944) added, the volume of the solution made up to 20 ml. with distilled water and the colour intensity at 620 mμ read on a Unicam colorimeter (Model S.B. 350) against a blank of boiled enzyme incubated in the same way. The glucose in the sample was then calculated from a calibration curve, using 50–200 μg. samples of glucose.

α-Amylase and β-amylase were estimated by the method of Noelting & Bernfeld (1948).

Xylanase, yeast mannanase, laminarinase and *galactanase* were estimated in the same way as cellobiase activity, the amount of reducing sugar being calculated in each case using a calibration curve prepared from the appropriate monosaccharide. *Ivory nut mannan A* was used in 0·1% solution, since it was insoluble at higher concentrations.

Pectinase was estimated viscometrically. 5 ml. of 5% apple pectin solution were mixed in an Ostwald viscometer with 0·5 ml. M buffer of the required pH and 4·0 ml. distilled water and placed in a constant-temperature bath at 30° C. When the contents of the viscometer had reached the temperature of the bath, the time for the liquid to flow through the capillary of the viscometer was noted. 0·5 ml. of the enzyme solution was added and the solution mixed by blowing air through it. The time taken for the solution to flow through the capillary of the viscometer was measured at intervals from the time of mixing.

Chitosanase was measured viscometrically by a similar method to that used for pectinase.

Cellulase was assayed by measuring the reducing sugar formed from Cellofas B solutions, as in the determinations of cellobiase, and also by the production of reducing sugars from phosphoric acid-swollen cotton linters (an insoluble substrate). For these assays, 0·2000 g. samples of oven-dried, phosphoric acid-swollen cotton linters were shaken at 25° C. in 20 ml. screw-capped bottles with 1 ml. M buffer at the required pH value and distilled water to give a total volume of 19 ml. In some cases 2 ml. 0·1% crystalline bovine serum albumin (Whitaker, 1952) were added before the water. 1 ml. of the enzyme solution was added and the incubation continued for 17 hr.; 2 ml. of the mixture were then added to 2 ml. alkaline copper reagent (Somogyi, 1952) and the assay completed in the same way as that of cellobiase activity.

Yeast glucanase and activity towards *ivory nut mannan B* were estimated by similar procedures to that used for cellulase on insoluble substrates. Calibration curves were prepared from the appropriate monosaccharide and were used to calculate the degradation.

Chitinase was measured in a similar way to cellulase on the insoluble substrate,

but the incubation period was extended to 40 hr. and the acetyl glucosamine formed was estimated by the method of Aminoff, Morgan & Watkins (1952).

Lipases were measured on Tween 20 by the method of Bier (1955).

(*d*) *Reagents*

All chemicals used were of A.R. quality.

Phosphate buffers were used between pH 8·0 and 5·75, acetate between pH 5·75 and 3·5 and glycine/HCl between pH 3·5 and 2·0.

(*e*) *Nitrogen determinations*

Micro-Kjeldahl digestion was carried out as described by Chibnall, Rees & Williams (1943) followed by distillation and titration.

EXPERIMENTAL RESULTS

(*a*) *Proteinases*

Table 1 shows the proteinase activity of the enzymically active extract from the digestive tract of *Helix pomatia* in the presence and absence of potassium cyanide solution. An enzyme solution which liberated 1 mM. of tyrosine when it was incubated with nine times its volume of 2·2% denatured haemoglobin was given an activity of 1 (Anson, 1938).

Table 1. *Proteinase activity of* Helix pomatia *digestive tract extract*

pH value of incubation in 0·05 M buffer	2 M-KCN added/10 ml. incubation mixture (ml.)	Enzymic activity/ml. digestive tract extract
7·5	—	41·6
7·5	0·1	41·6
5·6	—	3·0
5·6	0·1	3·0
2·5	—	16·2
2·5	0·1	16·2

(*b*) *Carbohydrases*

Table 2 gives the results of the quantitative determinations of the activity of the carbohydrases in the extract from *H. pomatia* digestive tract.

β-Methyl cellobioside was used to assay cellobiase activity and Cellofas B to assay cellulase activity. It was necessary to confirm that the enzymic degradations of these substrates proceeded in the same way as those of cellobiose and cellulose, by cleavage of 1:4-β-glycosidic bonds between adjacent glucose units.

9·5 ml. of the assay medium were incubated for 16 hr. at 37° C. with 0·5 ml. undiluted snail digestive tract extract. The solution was then evaporated at 60° C. under reduced pressure, to give 0·1 ml. of a thin oil.

The concentrated solution from the hydrolysis of β-methyl cellobioside was investigated chromatographically and electrophoretically. 5μl. were examined

chromatographically on Whatman No. 1 filter-paper which had been sprayed with saturated sodium borate solution and allowed to dry. The chromatogram was developed for 16 hr. at 25° C., using propanol azeotrope, which had been saturated with sodium borate, as the solvent system. The paper was dried and spots coloured by the silver nitrate reagent of Trevelyan, Proctor & Harrison (1950). It showed spots at 14 and 19·5 cm. from the origin. A methyl glucose marker ran 19 cm. and β-methyl cellobioside and glucose markers ran 14 cm. from the origin. In the electrophoretic examination, 5 μl. were used and the electrophoresis carried out in 0·5 M sodium borate solution (Fuller & Northcote, 1956). After 1 hr. at 33 V./cm. and 20 mA., the spots were coloured by silver nitrate. Two spots were seen from

Table 2. *Carbohydrase activities of* Helix pomatia *digestive tract extract*

Substrate	Main monosaccharide constituents	Links in substrate	pH value for optimum activity	Temperature of assay (° C.)	mg. reducing sugar formed/hr./ml. extract
Sucrose	Glucose, fructose	1:2-αβ	5·6	37	1·1
Lactose	Galactose, glucose	1:4-β	7·25	37	0·9
Maltose	Glucose	1:4-α	5·0	37	1·1
β-Methyl cellobioside	Glucose	1:4-β	4·75	37	3
Wheat straw xylan	Xylose	1:4-β	4·75	37	40
Walnut xylan	Xylose	1:4-β	4·5	37	40
Snail galactan	Galactose	—	4·0–7·5	37	0
Yeast mannan	Mannose	1:2-α 1:3-α	5·0	37	30
Ivory nut mannan A	Mannose	1:4-β	4·5	37	80
Ivory nut mannan B	Mannose	1:4-β	4·5	25	7*
Yeast glucan	Glucose	1:2-β 1:3-β	5·5	25	2*
Laminarin	Glucose	1:3-β	5·1	37	160
Soluble starch	Glucose	1:4-α	6·8	25	0·78
		1:6-α	4·8	25	2·5
Snail glycogen	Glucose	1:4-α	6·8	25	1·9
		1:6-α	4·8	25	11·1
Chitin	Acetyl glucosamine	1:4-β	4·75	25	12
Cellofas B	Glucose, Carboxymethyl glucose	1:4-β	5·5	37	1090
Phosphoric acid-swollen cotton linters	Glucose	1:4-β	5·5	25	11*

* Crystalline bovine serum albumin added to give a final concentration of 2 mg./20 ml. assay medium.

the hydrolysate, at 0·5 and 5·0 cm. from the origin towards the anode. The latter corresponded to the glucose marker and the former to β-methyl glucose and β-methyl cellobioside markers, these last two running together in this experiment. No evidence that cellobiose was present was found from either the electrophoretic or the chromatographic experiments.

The concentrated solution from the enzymic hydrolysis of Cellofas B was examined electrophoretically. The buffer used was pyridine/ethyl acetate/water at pH 6·5 (Michl, 1951). The field strength was approx. 50 V./cm., the current of about 35 mA. being passed for 1 hr. After electrophoresis the paper was dried and sprayed with aniline phthalate (Consden & Stanier, 1952) to detect reducing sugars or with a 1% solution of bromphenol blue to detect acidic spots. Reducing sugar

spots were seen at distances 1 cm. from the origin towards the cathode and at 7, 11 and 17 cm. towards the anode; a glucose marker moved 1 cm. towards the cathode. The spots which migrated towards the anode also stained with bromphenol blue, and were attributed to carboxymethyl sugar derivatives. This interpretation was confirmed by examining a partial acid hydrolysate of Cellofas B by electrophoresis, when the spots which stained with both aniline phthalate and bromphenol blue were seen to move the same distances towards the anode as in the enzymic hydrolysate.

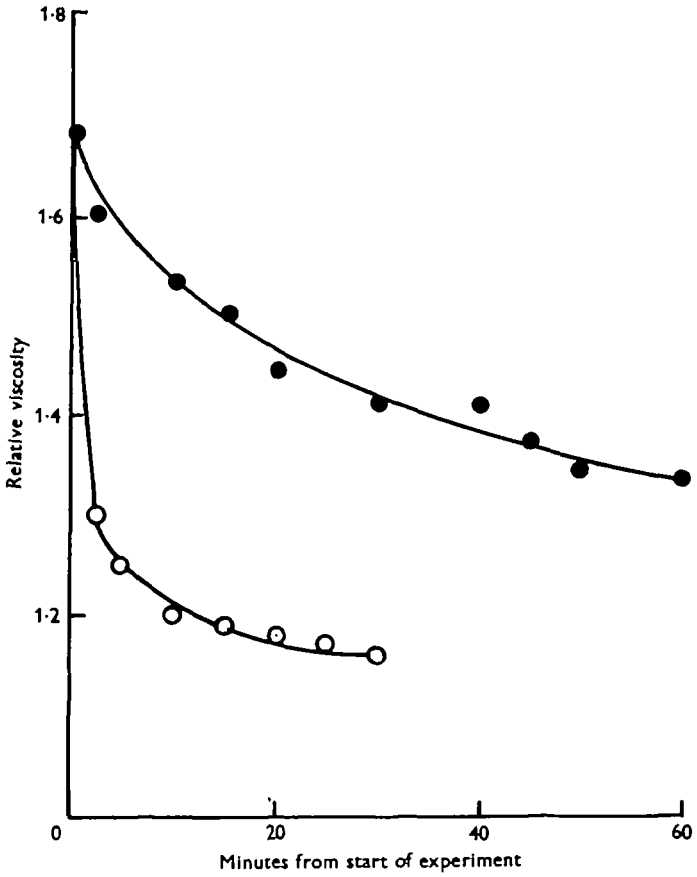

Fig. 1. Enzymic degradation of apple pectin. Full circles—*Helix pomatia* digestive tract extract. Open circles—Pectinol 100 D. Experiments were carried out at 30° C. In the final solutions there were 5 mg./ml. apple pectin.

Fig. 1 shows the decrease in viscosity of an apple-pectin solution when incubated with snail digestive tract extract and compares the rate of fall with that found when a fungal pectinase solution (Pectinol 100 D, Rohm & Haas) of similar protein content was incubated with a similar solution. For both pectinase solutions, optimum activity was found at pH 4·0.

Fig. 2 illustrates the fall of viscosity of a chitosan acetate solution when it was incubated with undiluted *H. pomatia* digestive tract solution at the pH for optimum activity, pH 5·6.

(*c*) *Lipases*

There were 1050 units of the lipase activity in each ml. of undiluted digestive tract extract. The definition of the unit of lipase activity was that used by Bier (1955).

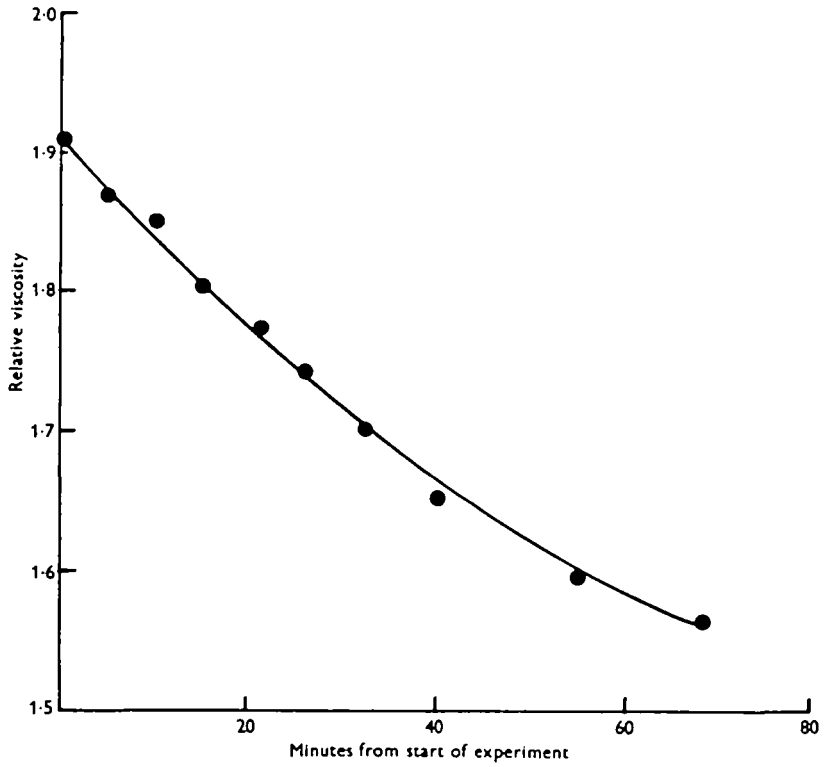

Fig. 2. Enzymic degradation of chitosan acetate solution by *Helix pomatia* digestive tract extract. The experiment was carried out at 30° C. There were 5 mg./ml. chitosan acetate in the final solution.

DISCUSSION

The experiments which have been reported show that a variety of substrates are attacked by a preparation from the digestive tract of *H. pomatia*. All these substances are present in the snail's diet under natural conditions or are closely similar to such compounds. The enzyme preparation showed three boundaries in the Tiselius electrophoresis apparatus, so that there were not less than three soluble proteins in the solution.

The proteinases present were mainly of the intercellular, digestive type; their

activities were not stimulated by the addition of cyanide to the assay system, although the prosthetic groups of cathepsins often contain sulphydryl groups and are activated by -SH reagents (Smith, 1951). The relative lack of proteinases in the extracts was perhaps not surprising, since the snail is herbivorous. The stability of the carbohydrases when the solution was kept for long periods must derive, at least in part, from the small amounts of proteinases present, for even native carbohydrases would be attacked to some extent by proteinases.

Since a carbohydrase is usually specific with reference both to the linkage and to the monosaccharide constituents of the polymer it splits, it is probable that most of the carbohydrates chosen were degraded by discrete enzyme systems. However, the possibility that a number of substrates were attacked by the same enzyme cannot be ignored. A number of the polysaccharide substrates were broken down much more rapidly than any of the disaccharides tested.

The linkages in yeast mannan are mainly α (Haworth, Hirst & Isherwood, 1937) and those in ivory nut mannans are β (Klages, 1934*a*, *b*) which could account for the observed differences in pH optima for the two substrates and for the different extents of degradation. The use of ivory nut mannan A and ivory nut mannan B enables a direct comparison to be made of the amount of degradation of soluble and insoluble molecules. Both these mannans are made up from two types of molecules, one consisting of chains of 1:4-β linked mannopyranose units and the other of identical chains terminated by a galactopyranose residue, the only difference between the two mannans being the lengths of the chains (Aspinall *et al.* 1953). From Table 2 it can be seen that reducing sugar is released from ivory nut mannan B at 8% of the rate at which it is released from ivory nut mannan A. A larger difference was found when cellulase was assayed on Cellofas B and phosphoric acid-swollen cotton linters, possibly because the latter substance was enzymically degraded without the production of reducing sugars and because the two substrates are of different chemical compositions. It is interesting that bleached cotton linters were not degraded by *H. pomatia* digestive tract extract with the release of reducing sugars until they had been swollen with phosphoric acid.

The only substrate which could not be degraded under the experimental conditions used was snail galactan. This observation is in contrast to the result of Weinland (1953) who found a weak galactanase activity which was optimum at pH 4·0.

From the results given, it is clear that cellulose and some hemicellulose components of plant cell walls will be attacked by snail cytase preparations. Care is necessary, therefore, in the interpretation of structural studies using such preparations; the study of lignified material is further complicated by the report (Holden & Tracey, 1950) that ligninase is present in *H. pomatia* digestive tract.

The significant lipase activity of the digestive tract extract is probably an important factor in its utility as a cytase, contributing to the breakdown of lipoprotein structures in cell walls.

SUMMARY

1. The digestive tract of the snail has been isolated and a soluble extract prepared from it and its contents. A quantitative analysis of the proteinases, lipases and twenty different carbohydrase activities present in this extract has been made.
2. Snail galactan was not degraded by the enzymic preparation.
3. The use of the preparation for cytological studies has been discussed.

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