CARBOHYDRASES OF THE SNAIL Eulota maakii

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By means of a total preparation of the stomach of the snail E. maakii it is possible to obtain the native triterpene genins of panaxoside A (PA) and of caulosides D and E (CD and CE) [1] and the progenins of the steroidal polygonatoside D (PG_D) [2]. On testing the combined preparation, it was found that it contained enzymes hydrolyzing synthetic glycosides of betulin [3]. It was also found that the preparation acts on the pectin of seaweeds – zosterin [4] – and possesses laminarinase (β-1,3-glucan glycanohydrolase) activity. The present paper gives the results of the purification of the carbohydrases from E. maakii.

The preliminary determination of the optimum pH values of the action of the enzymes hydrolyzing zosterin, betulin glucoside (BG), and panaxoside C (PC) showed that the optimum pH is in the acid region. For the investigation we selected a 0.1 M succinate buffer with pH 5.6 ("working buffer").

The combined extract was subjected to gel filtration on a column of Biogel P-200 previously equilibrated with the working buffer. All the fractions were analyzed for their protein contents and their carbohydrase activities with respect to the following substrates: p-nitrophenyl α- and β-glucosides and α- and β-galactosides, laminarin, zosterin, PG_D, PC, CE, BG, cholesterol glycoside (CG), and panaxadiol diglucoside (PDDG). Compounds PC, CE, and PG_D contain glucose, rhamnose, and arabinose in various combinations. Consequently, the columns were also tested with methyl β-D-arabinoside and rutin, which contains rhamnosylglucose.

The results of the fractionation are shown in Fig. 1. Most of the glucosidase activities are associated with the high-molecular-weight peak A (fractions 2-8) and the peak C following it (fractions 10-16). Glycosides containing oligosugars (CE, PG_D, rutin) are hydrolyzed by several fractions, which are apparently responsible for the splitting off of different sugars from them. In actual fact, the TLC behavior of the products of enzymatic hydrolysis shows the formation of two possible progenins from CE under the action of the fractions of peak A and of one of these progenins and the genin by the fractions of peak C. Similarly, from PG_D the fractions of peak C form one of the progenins, and fraction 26 gives two possible progenins. The enzymatic hydrolysis of PC (fraction 14) leads to the formation of two progenins. Synthetic CG and BG form the aglycones – cholesterol and betulin – with the fractions of peak A. Synthetic PDDG (at C_3 and C_{12}), on enzymatic hydrolysis with fractions 5 and 15, gives panaxadiol glucoside at C_{12}. The fractions of peak B split off rhamnose from rutin; glucose and rhamnose are formed from rutin on incubation with fraction 31.

On fractionation on Biogel P-200, two peaks were observed that possessed "zosterinase" activity, one of which (Z_2) caused a considerable fall in the viscosity of solutions of zosterin and the other (Z_1) a rise in the amount of reducing sugars with no change in the viscosity (see Fig. 1). It has been established that the fragmentation of zosterin with Z_1 and Z_2 takes place differently from that with a commercial preparation of pectinase.

As can be seen from Fig. 1, the laminarinase of E. maakii was separated into two fractions the relative activities of which varied according to the time of collection of the snail.

The products of the hydrolysis of laminarin with the laminarinases were investigated by paper chromatography. Only glucose was found as the product of enzymatic hydrolysis; it is possible that these enzymes with the exo type of action.

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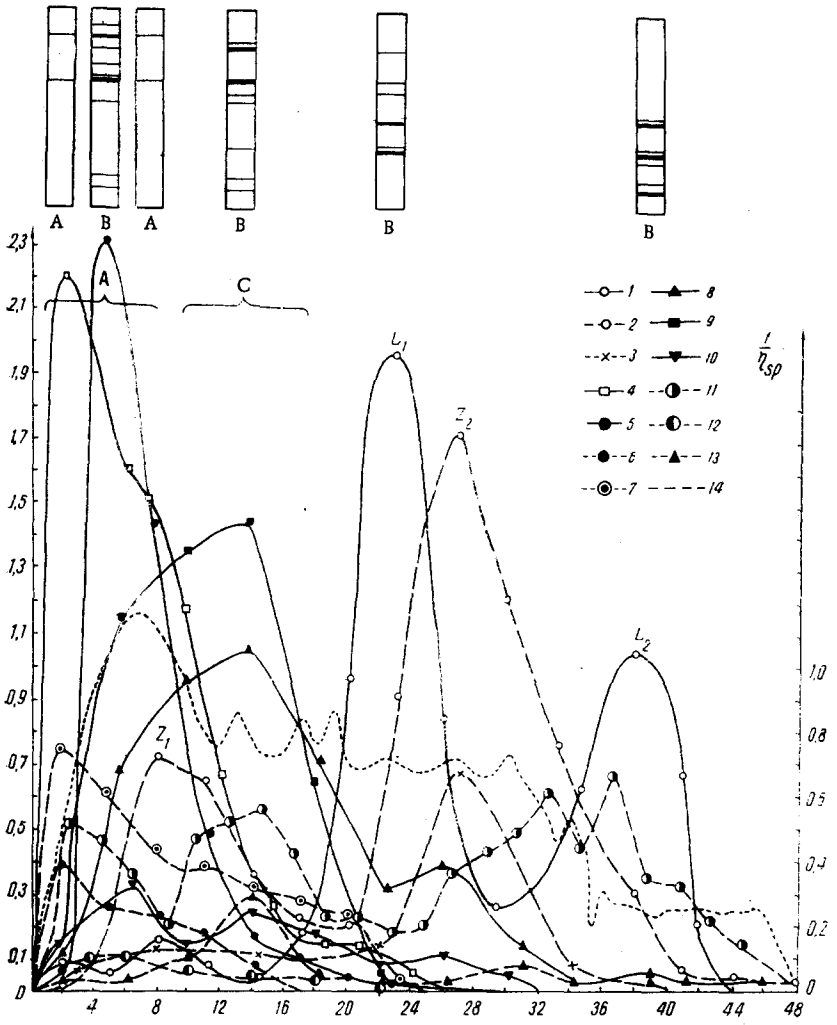

Fig. 1. Gel filtration on Biogel P-200 of the total extract of the gastrointestinal tract of E. maakii: carbohydrase activities with respect to: 1) laminarin; 2) zosterin; 3) zosterin (from the viscosity); 4) p-nitrophenyl β-D-glucoside; 5) p-nitrophenyl α-D-glucoside; 6) p-nitrophenyl β-D-galactoside; 7) p-nitrophenyl α-D-galactoside; 8) PG_D; 9) CE; 10) methyl β-D-arabinoside; 11) rutin; 12) BG; 13) PC; 14) protein. At the top: zymograms of the corresponding types: A – activity; B – protein.

Simultaneously with the appearance of protein, there was a localization of activities for a number of glucosidases (with the corresponding 2-naphthyl glycosides as substrates). By this method we found at least two bands in each case for the β-glucosidases and β-galactosidases that coincided in position, and two bands of α-galactosidases, one of which coincided in position with one of the bands of the β-glucosidases mentioned above.

For all the carbohydrases tested, an increase in specific activity by a factor of not more than 2 was achieved by gel filtration. For the fractions investigated, the rates of enzymatic hydrolysis of the p-nitrophenyl glucosides exceeded the rates of enzymatic hydrolysis of the steroid glycosides (PG_D and CG) by an order of magnitude and those of the triterpene glycosides even more. The glycosidases found in E. maakii cannot apparently be considered as specific for these steroid or triterpene glycosides.

EXPERIMENTAL

Production of a Total Enzyme Preparation. The snails were collected in July-September in regions of the Maritime Territory. The work was carried out at +4°C. The gastrointestinal tracts of the snails were carefully ground with silica gel and extracted with the working buffer (two volumes). The resulting homogeneous mass was centrifuged for 20 min at 12000 ppm. The supernatant was used as the initial preparation.

Substrates. The 2-naphthyl and p-nitrophenyl glucosides were commercial preparations, and so were the rutin and the methyl β-D-arabinoside. Betulin and cholesterol glucosides and panaxadiol diglucoside were synthesized [3, 5]. PC, CE, and PG_D isolated from natural sources were kindly given to us by workers in the Laboratory of the Chemistry of Triterpene and Steroid Compounds of TIBOKh [Pacific-Ocean Institute of Bio-organic Chemistry], and zosterin by workers of the Laboratory of Carbohydrate Chemistry of TIBOKh. Soluble laminarin was obtained from the seaweed Laminaria cycharioides by Black's method [9].

Determination of the Activities of the Enzymes. The activities of the majority of the carbohydrases were determined from the increase in the amount of reducing sugars in the incubation medium. The increase in the reducing capacity of the sugars was measured by Nelson's method [7]. Below we give the compositions of the incubation mixtures and the times of incubation for the various substrates: a) for rutin, zosterin, and methyl β-D-arabinose, 0.4 ml of a 0.2% solution and 0.1 ml of the fraction, 30 min; b) for laminarin, 0.25 ml of a 0.05% solution, 0.2 ml of water, and 0.05 ml of the fraction, 10 min; c) for PG_D, PC, CE, BG, CG, and PDDG, 0.3 ml of a 0.1% solution and 0.2 ml of the fraction, 24 h. The reactions were stopped by the addition of 0.5 ml of a mixture of Nelson's reagents A and B [7].

For p-nitrophenyl α- and β-glucosides and galactosides the activities were measured from the amount of p-nitrophenol liberated: 0.25 ml of a 0.05% solution of the substrate, 0.2 ml of water, and 0.05 ml of the fraction (10 min) with the addition of 2 ml of 1 M Na_2CO_3 at 400 nm. For all the reactions mentioned, corresponding blanks on the substrate and the enzyme were performed.

To determine zosterinase activity with respect to viscosity, the fall in viscosity was measured in an Ostwald viscometer with a 0.56-mm diameter capillary. The incubation mixture consisted of 10 ml of a 0.5% solution of zosterin and 0.5 ml of the fraction, and the time of incubation was 3 h. The results were calculated to $1/\eta_{sp}$. The concentration of protein was determined by Lowry's method [8]. In calculation of the specific activity, the activity obtained (mg of glucose) was referred to 1 mg of protein.

Gel Filtration on Biogel P-200. Columns (2 × 50 cm) of biogel P-200 were prepared in accordance with the standard procedure recommended by the manufacturers and were equilibrated with the working buffer. Then 160 mg of the total enzyme preparation was deposited on a column. The rate of elution was 12 ml/h.

Disk electrophoresis was performed in 7.5% polyacrylamide gel and tris-glycine buffer, pH 8.9. The protein was revealed by means of a published procedure [9]. The localization of some glycosidases was determined in the following way. After incubation of the gel columns (20 h) with a 0.025% solutions of naphthyl glycosides the liberated β-naphthol was stained by reaction with Diazo Blue B (0.1% solution in water, 30 min.), the excess of dye being washed out with a 7.5% solution of acetic acid.

Paper Chromatography. The products of the enzymatic hydrolysis of laminarin and of rutin were chromatographed on FN-1 paper in the butan-1-ol–pyridine–water (6:4:3) system. The spots were revealed with aniline phthalate.

The thin-layer chromatography of the products of the enzymatic hydrolysis of the triterpene and steroid glycosides was performed in the chloroform–ethanol (6:1) system for the progenins and the benzene–ethyl acetate (5:2) system for the genins. The spots were revealed with sulfuric acid.

CONCLUSIONS

A complex combination of carbohydrase activities has been demonstrated in the digestive tract of the inland snail E. maakii. Gel filtration on biogel P-200 enables partially purified preparations of several glycosides (including those acting on some triterpene and steroid glycosides), two laminarinases, and a pectinase–"zosterinase"–to be obtained.

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