Biochemical Studies on Carbohydrates.

CXI. On Snail Enzymes.

Second Report: Mannosidases.

By

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α -Mannosidase was first discovered in 1921 by Hérissé¹⁾ in legume seeds and later on exhaustive investigations were accomplished by Helferich and co-workers²⁾ regarding the ones in almond and alfalfa seeds. However, of β -mannosidase and, in animal tissues, also of α -mannosidase we have never before been informed. I found out these enzymes in the snail liver extract, though not equally active. α -Phenol- and α -methyl-mannoside were cleft at the maximum at pH's 4.1 and 4.5 respectively and β -phenolmannoside at pH 4.5, namely, the optimum pH's of the α - and β -mannosidase resembled each other. On the contrary, however, the resistance of the enzymes to heat showed remarkable divergency which enables one to gain a solution containing α -mannosidase alone. The details concerned will be seen in the experimental part. Tissues of higher animals appear to be devoid of these enzymes.

EXPERIMENTAL.

Enzyme Solutions.

I. The snail enzyme solution.

It was prepared in a like manner as communicated in the first report³⁾ taking the same stock porridge. But, this time, to dissolve the half dried alcoholic enzyme precipitate, the acidified water was used as much as 3/2 the volume instead of double the volume of the clarified stock solution and the extract obtained was further decolorized by treatment with purified⁴⁾ "charcoal, animal" E. Merck (0.1 gm. per cc.). The solution, which was still tinged yellow, was kept for the use at 0°C under saturation with toluene. It gave Molisch reaction but was almost biuret-negative.

Throughout the whole course of the present investigation one and the same enzyme solution was employed.

II. *Canine pancreatic juice and the extracts of bull liver and rabbit liver, pancreas and kidney.*

The pancreatic juice collected from a dog with a fistula was evaporated to dryness under reduced pressure and preserved. For the examination it was dissolved in water in the proportion of 32 mgm. in 2 cc., and the centrifuged clear supernatant liquid, either as such⁵⁾ or after acidifying to pH 4.5⁶⁾ (with 2 drops of 1% acetic acid), was adapted to incubation with the substrates. The organs were minced by means of a masticator, mixed with an equal weight of water and after addition of 12.5 cc. (in the case of ox liver) or 1.25 cc. (in the cases of rabbit organs) of toluene for 100 gm. tissue, maintained at 37°C for 48 hours with occasional shaking. After acidification and centrifugation, the dark brown supernatant fluids were precipitated by the use of alcohol and subsequent operations were carried out as when the snail enzyme solution was prepared. Decolorization with charcoal was omitted, when the extent of the enzymic digestion was intended to estimate by the iodometric titration alone.

Substrates.

α - and β -phenolmannoside (Helferich and Winkler⁷⁾) and α -methylmannoside (E. Fischer⁸⁾) were employed. Under the conditions, which will be described below, the substrates rotate D-light:

	α (degree)	
	Before scission	When totally hydrolyzed
α -Phenol-d-mannoside ($[\alpha]_D = +113.5^\circ$)	+1.22	+0.11
α -Methyl-d-mannoside ($[\alpha]_D = +79.2^\circ$)	+0.61	+0.10
β -Phenol-d-mannoside ($[\alpha]_D = -71.6^\circ$)	-0.77	+0.11

Methods.

Substrate-buffer solutions were prepared by dissolving 0.054 mol of phenolmannosides or 0.051 mol of α -methylmannoside in 1 cc. of a buffer solution (pH ≤ 2.9 : Sørensen's 0.1 n. citrate solution, pH 3.2-6.2: Michaelis' 0.1 n. acetate solution, pH ≥ 7.0 : Michaelis' 0.1 n. phosphate solution) saturated with toluene. 1 cc. of them and 0.3 cc. of an enzyme solution were mixed and incubated in a water thermostat at 37°C. When

the digestion was optically traced, the mixtures were alkalized by addition of 10 mgm. of potassium carbonate and filled up in a 1 dm. long tube, and for iodometry (Macleod and Robison⁹) they were cooled in ice and 0.5 cc. was taken out.

In the control runs the substrate-buffer solutions were replaced with the buffer solutions containing no substrate.

Results.

I. On the snail enzymes.

The constant of reaction velocity was calculated out by the formula $k = \frac{1}{t_{min}} \times \ln \frac{100}{100 - \% \text{cleavage}}$. In the controls the optical rotatory power (-0.01°) showed no change.

1. Optimum pH's for the action of the mannosidases are seen in Table I.

TABLE I.

Optimum pH's of the Snail Mannosidases.

α_1 : initial rotation. α_2 : rotation after incubation.

Substr.	α -Phenol-d-mannoside (Incub.: 10 min.)			α -Methyl-d-mannoside (Incub.: 60 min.)			β -Phenol-d-mannoside (Incub.: 10 min.)		
	$\alpha_1 - \alpha_2$	Cleavage %	$k \times 10^3$	$\alpha_1 - \alpha_2$	Cleavage %	$k \times 10^3$	$\alpha_1 - \alpha_2$	Cleavage %	$k \times 10^3$
pH									
3.2	+0.10	9.0	9.42	+0.01	2.0	0.30			
3.8	+0.13	11.7	12.43	+0.07	13.7	2.35	-0.02	2.3	2.33
4.1	+0.15	13.5	14.49	+0.09	17.6	3.32	-0.03	3.4	3.45
4.5	+0.10	9.0	9.42	+0.14	27.5	5.35	-0.04	4.5	4.60
5.0	+0.06	5.4	5.55	+0.11	21.6	4.05	-0.01	1.1	1.15
5.8	+0.05	4.5	4.60						
6.2				+0.07	13.7	2.35			

2. The hydrolysis velocity diminished with the progress of digestion, suggesting the occurrence of the reverse reaction. Table II shows the courses of cleavage at the optimum pH's.

3. Inactivation by heat. Parts of the enzyme solution were heated in stoppered tubes for 30 minutes at various temperatures and after cooling, 0.3 cc. was pipetted out from each for determining the residual activity. Opalescence appeared, when heated at 60°C . At higher temperatures flocculi precipitated, but the precipitates were subjected to the use together with the mother liquid after shaking to equal dispersion. (Table III).

TABLE II.
Hydrolysis Velocity of Mannosides by the Snail Enzymes.

Time of incub. min.	Substr.	α -Phenol-d-mannoside (Digest. at opt. pH, 4.1)			α -Methyl-d-mannoside (Digest. at opt. pH, 4.5)			β -Phenol-d-mannoside (Digest. at opt. pH, 4.5)		
		$\alpha_1-\alpha_2$	Cleavage %	$k \times 10^3$	$\alpha_1-\alpha_2$	Cleavage %	$k \times 10^3$	$\alpha_1-\alpha_2$	Cleavage %	$k \times 10^3$
5		+0.08	7.2	14.93						
10		+0.15	13.5	14.49				-0.04	4.5	4.60
15					+0.06	11.8	8.38			
20		+0.21	18.9	10.46				-0.07	8.0	4.16
30					+0.10	19.6	7.26			
40		+0.32	28.8	8.48				-0.10	11.4	3.02
60		+0.45	40.5	8.64	+0.14	27.5	5.35			
90					+0.16	31.4	4.18			

TABLE III.
Heat Inactivation of the Snail Mannosidases.
Incubated for 10 minutes at the opt. pH's (4.1 and 4.5).

Temp. of heating °C	Substr.	α -Phenol-d-mannoside			β -Phenol-d-mannoside		
		$\alpha_1-\alpha_2$	Cleavage %	$k \times 10^3$	$\alpha_1-\alpha_2$	Cleavage %	$k \times 10^3$
50		+0.14	12.6	13.45			
60		+0.13	11.7	12.43	-0.02	2.3	2.33
70		+0.10	9.0	9.42	0.0	0.0	0.0
80		0.0	0.0	0.0			

β -Mannosidase was destroyed at 70°C entirely, whereas more than half of the α -enzyme still remained active at this temperature.

4. The activity of α -mannosidase was found not to decrease within a month of preservation at 0°C. For the measurement α -phenolmannoside was employed. pH of the incubation mixtures was 4.1 and the estimation was made every two days at first and afterwards every three days, but always $k \times 10^3$'s of 14.93 and of 14.49 were given respectively in 5 and 10 minutes' incubation.

II. On the extracts of mammal organs and pancreatic juice.

The cleavage of α -phenolmannoside was examined at pH 4.5 in a like manner as above. The time of incubation was 60 minutes for bull liver,

60 and 120 minutes for the pancreatic juice and 30 minutes for rabbit organs. Measured optically as well as iodometrically for bull liver, optically for the pancreatic juice¹⁰⁾ and iodometrically for rabbit organs. In any case neither the optical power nor the iodine use underwent alteration.

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Bibliography and Notes.

- 1) Hérissé, C. r. Acad. Sci. Paris, 1921, **172**, 766.
 - 2) Helferich et al., Z. physiol. Chem., 1932, **209**, 269; 1933, **214**, 139; 1933, **221**, 252. Hill, Ber. sächs. Akad. Wiss., 1934, **86**, 115.
 - 3) Utusi et al., Tohoku J. Exp. Med., 1949, **50**, 175.
 - 4) Ogata and Kondo, Kagakuzikken Sosaho (Chemical Experimental Methods), 12th Ed., Nankodo Co., Tokyo, 1944, p. 42.
 - 5) Precipitation occurred when the liquid was mixed with the substrate-buffer solution.
 - 6) When acidified, the solution became turbid, but it was adapted to incubation immediately as such.
 - 7) Helferich and Winkler, Ber. dtsh. chem. Ges., 1933, **66**, 1556.
 - 8) E. Fischer, *ibid.*, 1896, **29**, 2927.
 - 9) Macleod and Robison, Bioch. J., 1929, **23**, 517.
 - 10) Optical measurement was made after centrifuging off of the precipitates.
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