β -D-Fucosidase in the Limpet, *Patella vulgata*

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The visceral hump of the limpet, *Patella vulgata*, is a good source of several glycosidases (Conchie & Levvy, 1957). It has now been observed to contain an enzyme that hydrolyses *p*-nitrophenyl β -D-fucoside, and it was necessary to determine the relationship between this enzyme and the β -D-galactosidase known to be present in the preparation. Among other methods of discrimination, the actions of the inhibitors, D-galactonolactone and D-fuconolactone, were examined. Levvy, McAllan & Hay (1962) found that the effects of these inhibitors on β -D-galactosidase preparations are largely, if not entirely, dependent on the proportion of the (1 $\rightarrow$ 5)-lactones formed in aqueous solutions of the crystalline (1 $\rightarrow$ 4)-lactones. Since this proportion varies rapidly and extensively with the pH, solutions of the lactones were treated immediately before use to give maximum inhibition.

EXPERIMENTAL

Substrates and inhibitors. D-Fucose was obtained commercially or made as follows from D-galactose. The method of Schmid & Karrer (1949) was used to reduce 6-tosyl-1,2:3,4-di-isopropylidene-D-galactose (Raymond & Schroeder, 1948) with lithium aluminium hydride (lithium tetrahydroaluminate) to 1,2:3,4-di-isopropylidene-D-fucose, and to hydrolyse the reduction product. The reduction was carried out with 64.3 g. of the starting material, and on this scale is approached the limit for safety with ordinary laboratory equipment. After final quenching of the reduction mixture with water, it was poured into a mixture of m-Na₂CO₃ and ether, and stirred until the residual insoluble material became granular. The reduction product in the ether layer was worked up in the usual way.

p-Nitrophenyl β -D-fucoside was prepared as described by Levvy & McAllan (1961). *p*-Nitrophenyl tri-O-acetyl- β -D-fucoside was obtained crystalline for the first time. It was crystallized from methanol and recrystallized from ethanol, m.p. 165–166° (corr.), $[\alpha]_D^{25} - 5.6^\circ$ (c in acetone, 1.0) (Found: C, 52.3; H, 5.1; N, 4.0. C₁₈H₂₁O₁₀N requires C, 52.6; H, 5.1; N, 3.4%). The overall yield of the fucoside was increased by the use of the crystalline triacetate, and also by applying the deacetylation procedure of Leaback (1960).

o-Nitrophenyl β -D-galactoside was made by the method of Seidman & Link (1950), the procedure of Leaback (1960) being used to decompose the triacetate.

D-Galactono-(1 $\rightarrow$ 4)-lactone and D-fucono-(1 $\rightarrow$ 4)-lactone were converted immediately before use into the corresponding 'most inhibitory solutions' (Levy *et al.* 1962) as follows. After opening of the (1 $\rightarrow$ 4)-ring with NaOH, the

solution was brought to pH 2 with HCl and heated at 100° for 7.5 min. It was then cooled and adjusted to pH 4.

Enzyme preparation and assay. The limpet preparation, made by ammonium sulphate fractionation of an aqueous extract as described by Conchie & Levvy (1957), had a β -D-fucosidase activity of 58000 μ g. of *p*-nitrophenol/g. of tissue and a β -D-galactosidase activity of 50000 μ g. of *o*-nitrophenol/g. under the following standard assay conditions.

The incubation mixture contained 1 ml. of 0.2M-Na₂HPO₄–0.1M-citric acid buffer (McIlvaine, 1921), 0.5 ml. of enzyme preparation and 2 ml. of 5 mM-*p*-nitrophenyl β -D-fucoside or 5 mM-*o*-nitrophenyl β -D-galactoside. The volume was made up to 4 ml. with water or inhibitor solution, and the final pH was 4.7 for β -D-fucosidase and 3.1 for β -D-galactosidase assay. (The lactone solutions are reasonably stable at these pH values.) After 1 hr. at 37°, 4 ml. of 0.4M-glycine–NaOH buffer, pH 10.5, was added, the mixture was centrifuged and the liberated nitrophenol was measured on the Spekker photoelectric absorptiometer with Ilford no. 601 violet filters (peak transmission at 430 m μ). The colours were quite stable in the alkaline medium, and the usual enzyme and substrate controls were made.

RESULTS

Variation in activity with pH. As shown in Fig. 1, the pH optima for β -D-fucosidase and β -D-galactosidase were different. After inactivation of the latter, as described below, the pH-activity curve for the former was unchanged in shape. When β -D-fucosidase was inactivated, the pH-activity curve for β -D-galactosidase became sharper. At first sight therefore it might appear that, whereas the β -D-galactosidase has little action on the β -D-fucoside, the β -D-fucosidase contributes considerably to the hydrolysis of the β -D-galactoside by the original preparation. From other experiments, however, it seems more probable that a component of β -D-galactosidase was incidentally lost when β -D-fucosidase was removed (see below). Conchie & Levvy (1957) suggest that limpet β -D-galactosidase contains more than one component.

Variation in stability with pH. This is shown in Fig. 2. Specimens were adjusted to the required pH at 0° with sodium hydroxide or hydrochloric acid, incubated for 1 hr. at 37°, cooled to 0° and brought back to the original pH (6.1). The activities of both enzymes were measured under the standard conditions and compared with those of an untreated

control that had been kept at 0°. The two enzymes could be clearly distinguished, and the following experiments on their relative heat-stabilities were carried out at the limits of their pH-stabilities, in an attempt to separate them.

Heat inactivation. The unbuffered limpet preparation (50 ml./g. of tissue) was adjusted to the required pH with sodium hydroxide or hydrochloric acid, heated at different temperatures for various periods, cooled to 0° and brought back to the original pH. Activities were compared with those of an untreated control in the usual way. β -D-Fucosidase and β -D-galactosidase behaved as different enzymes, and the best conditions for achieving separation are illustrated in Fig. 3. The pH optima for the individual enzymes thus prepared are shown in Fig. 1. Adjustment to pH 8.5 caused an immediate fall in the β -D-galactosidase activity, with only a small fall in β -D-fucosidase activity. The preparation was maintained at 25° for 2 hr. to ensure minimum β -D-galactosidase activity. It fell to 5% compared with the control, whereas 80% of the β -D-fucosidase activity was retained

after this time (Fig. 3). If the residual β -D-galactosidase activity in this treated β -D-fucosidase preparation were in fact due to the latter enzyme, the effect was too small to account for the lost component in the pH-activity curve for β -D-galactosidase when β -D-fucosidase was inactivated (Fig. 1).

Fig. 1. Effect of pH on the rate of hydrolysis of 2.5 mM-*o*-nitrophenyl β -D-galactoside by: $\circ$, untreated limpet preparation; $\bullet$, preparation after 10 min. at pH 3.0 and 50°; and of 2.5 mM-*p*-nitrophenyl β -D-fucose by: $\triangle$, untreated limpet preparation; $\blacktriangle$, preparation after 2 hr. at pH 8.5 and 25°. The results for each preparation and substrate are expressed as percentages of the maximum observed. Maximum values in terms of μ g. of nitrophenol/g. of tissue were: $\circ$, 48000; $\bullet$, 32000; $\triangle$, 56000; $\blacktriangle$, 46000.

Fig. 2. Effect of incubation at various pH values for 1 hr. at 37° on ($\circ$) the β -D-galactosidase and ($\triangle$) the β -D-fucosidase activity of the limpet preparation. The initial activities were 50000 μ g. of *o*-nitrophenol/g. ($\circ$) and 58000 μ g. of *p*-nitrophenol/g. ($\triangle$).

Fig. 3. Residual activity in the limpet preparation of ($\circ$) β -D-galactosidase and ($\triangle$) β -D-fucosidase after various periods at pH 3.0 and 50°, and ($\bullet$) β -D-galactosidase and ($\blacktriangle$) β -D-fucosidase after various periods at pH 8.5 and 25°. The initial values were as in Fig. 2.

Effects of varying inhibitor and substrate concentrations. Each enzyme was inhibited by both lactones, but the relative power of the two inhibitors differed greatly, and was reversed on passing from one substrate to the other (Fig. 4). The inhibition was greatest when substrate and lactone were derived from the same sugar (Table 1). After combined treatment of the limpet preparation with heat and acid or alkali to free each enzyme from the other, curves indistinguishable from those shown in Fig. 4 were obtained in every case: this confirmed the view that neither enzyme caused much hydrolysis of the other type of substrate.

Inhibition was always competitive and values for K_m , the Michaelis constant, and K_i , the inhibitor constant, calculated by the method of Lineweaver & Burk (1934), are given in Table 1. Michaelis constants were also determined for each enzyme after

inactivation of the other, but the values were identical with those obtained with the untreated limpet preparation.

When untreated freshly prepared solutions of the (1→4)-lactones were tested at a concentration of 1 mM with mM-substrate at pH 5.2 in 0.05M-acetic acid-sodium hydroxide buffer, fuconolactone caused 40% inhibition of β -D-galactosidase and 78% inhibition of β -D-fucosidase, whereas galactonolactone gave corresponding values of 71% and 23%; results obtained under the standard conditions of assay described above were similar.

Other D-fucosidases. This section deals with miscellaneous preliminary trials of potential interest. The limpet extract caused barely perceptible hydrolysis of *p*-nitrophenyl α -D-fucoside (Levy & McAllan, 1961); this may well have been a side action of the α -D-galactosidase known to be present (Conchie & Levy, 1957), being inhibited by galactono-(1→4)-lactone, but not by fucono-(1→4)-lactone. A *Trichomonas foetus* extract, supplied by Miss Winifred Watkins, liberated 13000 μ g. of *p*-nitrophenol/g. of dried extract from 2 mM- β -D-fucoside in 1 hr. at pH 5.2 (acetate buffer), and 1200 μ g. from 2 mM- α -D-fucoside. This extract is known to possess α - and β -D-galactosidase activities (Watkins, 1959). The *T. foetus* β -D-fucosidase activity was inhibited by galactone-(1→4)-lactone, but not by fucono-(1→4)-lactone.

Fig. 4. Inhibition of the enzymes in the untreated limpet preparation by various concentrations of lactones: 2.5 mM-*o*-nitrophenyl β -D-galactoside with (○) galactonolactone and (●) fuconolactone; 2.5 mM-*p*-nitrophenyl β -D-fucoside with (△) galactonolactone and (▲) fuconolactone. The inhibitor concentrations are on a logarithmic scale.

DISCUSSION

It is evident that the β -D-fucosidase activity of limpet extracts is due to an enzyme distinct from β -D-galactosidase. Although there was cross-inhibition by the lactones derived from the two sugars, such effects were relatively small, and after inactivation by combined treatment with heat and acid or alkali neither enzyme caused much hydrolysis of the other type of substrate. Ox-liver β -D-galactosidase on the other hand was more powerfully inhibited by fuconolactone than by galactonolactone (Levy *et al.* 1962). It is possible that the

Table 1. Values obtained with the untreated limpet preparation for K_m for both substrates and K_i for both lactones

The experiments were done at pH 3.1 (β -D-galactosidase) and 4.7 (β -D-fucosidase) in phosphate-citrate buffer. The amount of lactone required for 50% inhibition was read from Fig. 4.

Substrate	Inhibitor K_m (mM)	Galactonolactone		Fuconolactone	
		K_i (mM)	Concn. for 50% inhibition (mM)	K_i (mM)	Concn. for 50% inhibition (mM)
<i>o</i> -Nitrophenyl β -D-galactoside	2.0	0.019	0.046	2.3	2.8
<i>p</i> -Nitrophenyl β -D-fucoside	0.63	1.9	11	0.012	0.057

limpet encounters D-fucose residues in its diet, but the possibility that one or other of the two enzymes under discussion may hydrolyse α -L-arabinosides has still to be studied (see Wallenfels & Malhotra, 1960).

SUMMARY

1. The limpet, *Patella vulgata*, contains an enzyme that hydrolyses β -D-fucosides. This enzyme and β -D-galactosidase can be separated from each other by heat inactivation at suitable pH values.

2. Both enzymes are inhibited by galactonolactone and fuconolactone, but inhibition is much more pronounced when the inhibitor and substrate are derived from the same sugar.

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Penicillinase from *Klebsiella aerogenes*

A COMPARISON WITH PENICILLINASES FROM GRAM-POSITIVE SPECIES

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The introduction of α -aminobenzylpenicillin (Penbritin, ampicillin), an antibiotic active against Gram-negative organisms (Rolinson & Stevens, 1961*a*), has revived interest in the penicillinases [penicillin amidohydrolases, EC 3.5.2.6, called β -lactamases by Pollock (1961)] produced by members of the Enterobacteriaceae.

During a clinical trial of ampicillin carried out by Trafford *et al.* (1962) several penicillinase-producing coliform organisms were isolated, one of which is the subject of the present paper. The penicillinase from this organism was markedly different from the penicillinases of Gram-positive bacteria.

METHODS AND MATERIALS

Reagents. The following penicillins were used: sodium penicillin G, obtained from Glaxo Laboratories, Greenford, Middlesex; penicillin V (phenoxymethylpenicillin), phenoxethylpenicillin (Broxil, phenethicillin), phenoxypentylpenicillin (propicillin), 2,6-dimethoxyphenylpenicillin (Celbenin, methicillin), α -aminobenzylpenicillin (Penbritin, ampicillin), 4-isoxazolyl-5-methyl-3-phenylpenicillin (BRL 1400) and 6-aminopenicillanic acid, all gifts from Beecham Research Laboratories, Brockham Park, Betchworth, Surrey; and phenoxymethylpenicillin (Penspek), a gift from The Distillers Co. (Biochemicals) Ltd., Speke.

Phosphate-buffered saline was made by diluting 100 ml. of 0.2M-phosphate buffer, pH 7.4 [81 ml. of $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ (35.6 g./l.) plus 19 ml. of $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ (31.2 g./l.)], with 300 ml. of 0.9% NaCl and 400 ml. of water.

Organisms. A motile variant of *Klebsiella aerogenes* (laboratory number 418) was isolated from a patient with a post-operative chest infection. It gave a positive reaction in the filter-membrane test of Knox & Smith (1961*a*) and a negative penicillin-amidase test (carried out by the butyl acetate-extraction technique of Batchelor, Chain & Rolinson, 1961*b*), indicating that it produced a penicillinase. The organism produced acid and gas from the following compounds: glucose, lactose, sucrose, maltose, mannitol, salicin, adonitol and inositol. Dulcitol was not fermented, nor was H_2S or indole produced. It was weakly urease-positive, Voges-Proskauer-negative, and methyl red- and malonate-positive. Growth occurred on citrate as the sole carbon source. Gelatin was not liquefied.

Bacillus cereus NRRL 569 and the following strains of penicillinase-producing *Staphylococcus aureus* were used as representative of Gram-positive penicillinase-producing organisms: E3 (Knox, 1960), 13137 (Jevons, 1961), 8354/6/1 (Gorrell, 1957) and 110 (a recently isolated clinical strain).

Organisms were maintained on agar slopes and stored at 4° between subcultures.

Penicillinase preparations. Organisms were inoculated into infusion broth (Southern Group Laboratories, Hither Green, London, S.E. 13) and incubated at 37° overnight

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