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Almond-Emulsin β-D-Glucosidase and β-D-Galactosidase

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The existence of different types of β-glucosidase and β-galactosidase with variations in C-4 specificity has clearly emerged from investigations of many sources by various authors.

Horikoshi (1942) investigated some preparations in which only one of these activities could be detected. A β-glucosidase from the sorghum group of grasses and the β-galactosidase from pig spleen were each inhibited by derivatives of the corresponding hexose only, but the β-glucosidase of *Proteus vulgaris* and the β-galactosidase of *Escherichia coli* were each inhibited by derivatives of both hexoses. Cohn & Monod (1951) reported an induced bacterial β-galactosidase with no activity on β-glucoside and no inhibition by glucose. Duerksen & Halvorson (1958) found an induced β-glucosidase in yeast with no β-galactosidase activity or inhibition by galactose.

Several preparations containing both activities have been studied. Miwa (1939) changed the ratio of β-glucosidase activity to β-galactosidase activity of Taka-diastase by adsorption, heat inactivation and solvent precipitation; Ezaki (1940) and Horikoshi (1942) showed that each was inhibited only by derivatives of the corresponding hexose. Dahlqvist (1961) failed to separate the activities of pig intestinal mucosa by chromatography or heat inactivation and concluded that they were associated with a single active protein. Conchie & Levvy (1957) showed that the activities in a preparation from rumen micro-organisms were each inhibited only by the corresponding hexonolactone, whereas those in limpet visceral hump were inhibited by both glucono- and galactono-1→4-lactone.

Previous studies of fractionation and inhibition of β-glucosidase and β-galactosidase in sweet-almond emulsin have led to conflicting results (see Discussion section). This paper describes a study of the inhibition of emulsin β-glucosidase and β-galactosidase by glucose, galactose, glucono- and galactono-1→4-lactone and of the competition between substrates of each kind. A preliminary account of part of this work has been published (Heyworth & Walker, 1961).

MATERIALS AND METHODS

Substrates. *p*-Nitrophenyl β-D-glucoside was supplied by California Corp. for Biochemical Research.

p-Nitrophenyl 2,3,4,6-tetra-*O*-acetyl-β-D-galactoside was prepared by condensing 2·7 g. of 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide (Ohle, Marecek & Bourjau, 1929) with 1·1 g. of *p*-nitrophenol in 38 ml. of acetone and 7·9 ml. of N-sodium hydroxide. After 24 hr. the mixture was extracted with 75 ml. of chloroform, washed with sodium hydroxide and water, and dried over anhydrous magnesium sulphate. The syrup obtained on evaporation

was crystallized from ethanol to give 1·2 g. (41% yield) of *p*-nitrophenyl 2,3,4,6-tetra-*O*-acetyl-β-D-galactoside, m.p. 138°, $[\alpha]_D^{23}$ −12° (*c* 0·5 in chloroform) (Found: C, 51·2; H, 4·9; N, 2·7. $C_{20}H_{23}NO_{12}$ requires C, 51·2; H, 4·9; N, 3·0%). De-*O*-acetylation in 10 ml. of dry methanol and 0·25 ml. of N-sodium methoxide gave 374 mg. (41% yield) of *p*-nitrophenyl β-D-galactoside (Aizawa, 1939), which on recrystallization from water had m.p. 181–182°, $[\alpha]_D^{21}$ −84° (*c* 0·3 in water) (Found: C, 46·9; H, 5·1; N, 4·4. $C_{12}H_{15}NO_8$ requires C, 47·9; H, 5·0; N, 4·6%).

Buffers. These were constant-ionic-strength (*I* 0·05) sodium butyrate–hydrochloric acid buffers.

Enzyme preparation. Partially defatted sweet-almond meal (1 g.) was shaken with 20 ml. of 0·05M-butyrate buffer, pH 4·9, for 15 min., kept for 2 hr. at 4° and filtered to give the clear stock enzyme solution.

Estimation of enzyme activity. Except where indicated, 0·1 ml. of enzyme solution diluted from the stock solution in 2% Triton X-100 was incubated in duplicate with substrate in a total volume of 1·0 ml. of 0·05M-butyrate buffer, pH 5·6, for 20 min. at 37°. Liberated *p*-nitrophenol was measured after adding 2 ml. of 0·2M-borate buffer, pH 9·8 (Woollen, Heyworth & Walker, 1961) by reading at 420 mμ (for minimal absorption by substrates). The maximum hydrolysis of substrate in any determination was 10%.

RESULTS

Effect of enzyme concentration. Good duplicate activity measurements were obtained and activity was proportional to enzyme concentration in the presence of 0·2% of Triton X-100 when stock enzyme at concentrations of 0·05–0·4% (v/v) was incubated with 1·6 mM-*p*-nitrophenyl glucoside at pH 5·2 for 15 min. or when stock enzyme at concentrations of 0·1–0·8% (v/v) was incubated with 2 mM-*p*-nitrophenyl galactoside at pH 5·3 for 12 min.

The effect of Triton X-100 was similar to the protection of enzyme activity given by Triton X-100 on dilution of testicular *N*-acetylglucosaminidase (Woollen *et al.* 1961).

Effect of time of incubation. Activities were proportional to time of incubation for 20 min. when stock enzyme at a concentration of 0·2% was incubated with 2 mM-*p*-nitrophenyl glucoside at pH 5·2 or when stock enzyme at a concentration of 0·8% (v/v) was incubated with 8 mM-*p*-nitrophenyl galactoside at pH 5·6.

Effect of pH. Fig. 1 shows the effect over the pH range 4–6 on the rates of hydrolysis of the two substrates. Both rates are at a maximum between 5·5 and 5·6. All subsequent experiments were carried out at pH 5·6.

Fractionation with ammonium sulphate. A solution of the enzyme at pH 5·6 was treated with successive amounts of saturated ammonium sulphate. The precipitated fractions were redissolved in water and dialysed for 2 days at 4° against water. Samples were made 2% (v/v) with respect to Triton X-100 and the two activities measured (Table 1). No clear indication of a separation of the activities was obtained.

Partial heat inactivation. Enzyme solutions [4% (v/v) stock enzyme] in 2% Triton X-100 were preincubated for various times at 65° and then the activities measured (Table 2). The activities had been progressively decreased to one-quarter of the original with no change in the ratio of the two activities.

Kinetic experiments. D-Glucose, D-galactose, D-glucono-1→4-lactone and D-galactono-1→4-lactone were found to be competitive inhibitors of the two activities. Figs. 2 and 3 describe typical experiments from which K_m and K_i values (given in Table 3) were determined by the method of Lineweaver & Burk (1934). The concentrations of

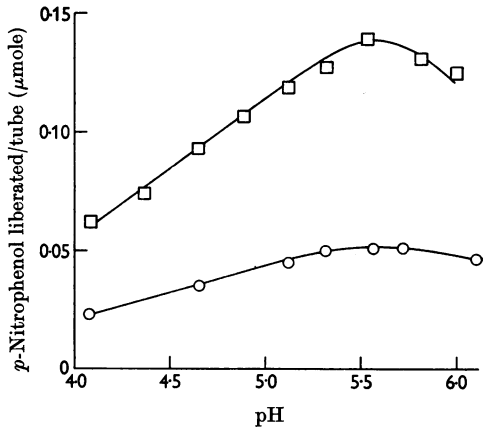

Fig. 1. Effect of pH on glucosidase and galactosidase activities. Activities were measured under standard conditions with 0·4% (v/v) stock enzyme and 2 mM-*p*-nitrophenyl glucoside (□) or 8 mM-*p*-nitrophenyl galactoside (○).

Table 1. *Effect of ammonium sulphate fractionation on glucosidase and galactosidase activities*

The enzyme was fractionated as described in the text, and the activities of the fractions (between the indicated limits of percentage saturation with ammonium sulphate) were measured under standard conditions with 8 mM-*p*-nitrophenyl glucoside or galactoside for 10 min. The glucosidase activity of the whole extract was equivalent to 0·14 μmole of *p*-nitrophenol liberated by 0·2% (v/v) stock enzyme.

Enzyme fraction	Total glucosidase in fraction (% of whole)	Glucosidase activity / Galactosidase activity
Whole	100	4·6
0–30% $(NH_4)_2SO_4$	65	4·3
30–60% $(NH_4)_2SO_4$	5	4·6
Supernatant	11	5·0

the substrates were 1–7 mM for *p*-nitrophenyl glucoside and 4–28 mM for *p*-nitrophenyl galactoside. The K_i values are the average of two determinations. The K_i values for the lactones are comparative only; they were used 15–39 days after solution to allow equilibration (see Conchie, 1954). The agreement of the K_i values for each inhibitor when measured against the respective substrates suggests that the hydrolysis of the two substrates occurs at the same enzyme site.

Competition between the substrates was tested in two ways. The total enzyme hydrolysis of equimolar mixtures was measured and compared with the theoretical hydrolyses on the respective assumptions of two independent sites and of one site (Thorn, 1949) calculated from the K_m and $V_{max.}$ values obtained at the same time with the separate substrates (Fig. 4). Also the total hydrolysis with a high concentration of glucoside and various concentrations of galactoside was compared

Table 2. *Effect of partial heat inactivation on glucosidase and galactosidase activities*

The enzyme solution was preincubated at 65° and pH 5·6 for various times and then activities were measured under standard conditions for 10 min. with 8 mM-*p*-nitrophenyl glucoside or galactoside. The initial glucosidase activity was equivalent to 0·168 μmole of *p*-nitrophenol liberated.

Period of preincubation (min.)	Glucosidase activity (% of initial)	Glucosidase activity / Galactosidase activity
0	100	4·6
5	90	4·5
15	53	4·0
30	26	4·4

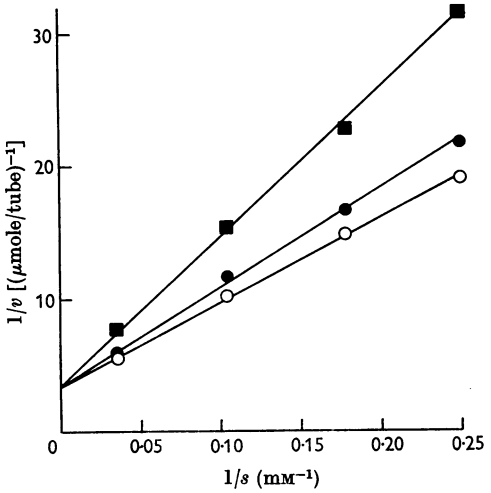

Fig. 2. Effect of glucose or galactose on hydrolysis of *p*-nitrophenyl galactoside. The activities were measured under standard conditions with 0·4% (v/v) stock enzyme and *p*-nitrophenyl galactoside alone (○), or with 0·25M-glucose (■), or with 0·14M-galactose (●) also. *v*, *p*-Nitrophenol liberated (μmole/tube); *s*, concentration of *p*-nitrophenyl galactoside (mM).

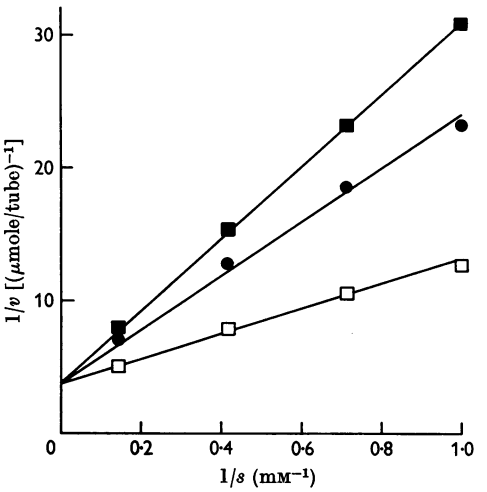

Fig. 3. Effect of glucono-1→4-lactone and galactono-1→4-lactone on hydrolysis of *p*-nitrophenyl glucoside. The activities were measured under standard conditions with 0·2% (v/v) stock enzyme and *p*-nitrophenyl glucoside alone (□); or with mM-glucono-1→4-lactone (■), or with 10 mM-galactono-1→4-lactone (●) also. *v*, *p*-Nitrophenol liberated (μmole/tube); *s*, concentration of *p*-nitrophenyl glucoside (mM).

Table 3. K_m *values for* p-*nitrophenyl* β-D-*glucoside and* β-D-*galactoside and* K_i *values for competitive inhibitors*

Values of K_m and K_i were calculated by the method of Lineweaver & Burk (1934) from results similar to those of Figs. 2 and 3 (see text). Results for K_m are given as means ± S.E. with the number of results in parentheses.

Constant	Inhibitor	K_m and K_i values (mM): *p*-Nitrophenyl glucoside	*p*-Nitrophenyl galactoside
K_m	—	2·59 ± 0·07 (10)	20·2 ± 0·8 (6)
K_i	D-Glucose	210	330
K_i	D-Galactose	640	790
K_i	D-Glucono-1→4-lactone	0·49	0·39
K_i	D-Galactono-1→4-lactone	7·4	6·6

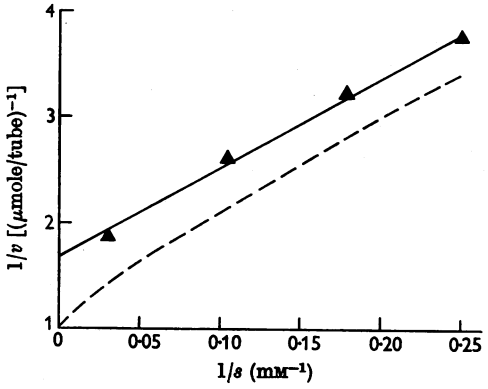

Fig. 4. Enzymic hydrolysis of equimolar mixtures of *p*-nitrophenyl glucoside and *p*-nitrophenyl galactoside (▲). *v*, *p*-Nitrophenol liberated (μmole/tube); *s*, total substrate concentration (mM). The lines show the hydrolysis expected on the assumptions of competition of substrates at one site (full line) or of two independent sites (broken line) (see Thorn, 1949). The K_m and $V_{max.}$ values respectively were for the glucoside 2·89 mM and 0·636 μmole; and for the galactoside, 18·7 mM and 0·339 μmole.

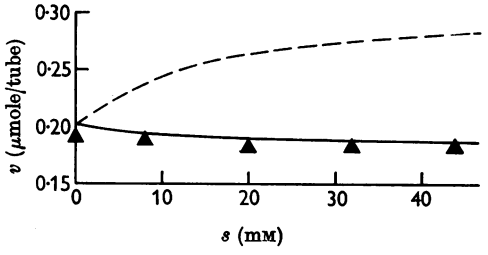

Fig. 5. Enzymic hydrolysis of mixtures of *p*-nitrophenyl glucoside and *p*-nitrophenyl galactoside (▲). *v*, *p*-Nitrophenol liberated (μmole/tube) in 15 min.; *s*, concentration of *p*-nitrophenyl galactoside (mM); 20 mM-*p*-nitrophenyl glucoside was present throughout. The lines show the hydrolysis expected on the assumptions of competition at one site (full line) or of two independent sites (broken line), with the K_m values of Table 3 and mean $V_{max.}$ values of 0·223 μmole for *p*-nitrophenyl glucoside and 0·127 μmole for *p*-nitrophenyl galactoside.

with similar theoretical plots based on mean K_m and $V_{max.}$ values (Fig. 5). In both cases the experimental points lie near to the theoretical plot for one site.

DISCUSSION

Helferich and his co-workers (see Veibel, 1950) could demonstrate no separation of β-glucosidase and β-galactosidase in emulsin by extensive purification, salt and solvent fractionation, adsorption or partial inactivation by heat, ozone, osmium tetroxide, u.v. light and formalin. The attempted separation of the activities in a simple extract of almond meal by ammonium sulphate fractionation and by partial heat inactivation described here is supplementary to this considerable body of work on partially purified preparations; the results support the conclusion that the two activities are associated with the same enzyme protein.

Helferich & Jung (1958) reported that treatment of emulsin preparations with polymers of *p*-hydroxystyrene β-glucoside and *p*-hydroxystyrene α-galactoside produced a change in the relative activity of β-glucosidase to β-galactosidase. In each case the partial separation appeared to be due to preferential adsorption of β-glucosidase. The existence of different specific glycosidases could not, however, be directly demonstrated since the adsorbed enzyme was not recoverable. It is not known if K_m values for the glucoside and galactoside were altered by the treatment of the enzyme.

Ezaki (1940) and Horikoshi (1942) showed that each activity in emulsin was inhibited by both hexonolactones but not by either hexose. Veibel, Wangel & Østrup (1947) were unable to detect inhibition of β-glucosidase by galactose but found different K_i values for glucose measured against the two *o*-cresyl glycosides. This was taken as evidence of different active sites. Alterations in the relative β-glucosidase and β-galactosidase activities of emulsin produced by modifying the structure of substrates (Helferich, 1938; Wagner & Kuhmstedt, 1957) or the conditions of assay (Veibel *et al.* 1947) do not necessitate the assumption of specific sites.

The kinetic experiments described here show that both hexoses and both lactones are competitive inhibitors of β-glucosidase and β-galactosidase with similar K_i values when measured against the two substrates. This makes it unnecessary to postulate and unlikely that there are two separate breakdown sites in our preparation. The experiments with mixed substrates also showed that these competed for a single site.

These results support the conclusion that the β-glucosidase and β-galactosidase activities of sweet-almond emulsin are due to one enzyme. The lack of specificity for configuration at C-4 is similar to that of limpet and mammalian β-glucuronidase (Marsh & Levvy, 1958) and testicular *N*-acetylglucosaminidase (Woollen *et al.* 1961). These findings are not in agreement with recently expressed views on the high specificity of glycosidases in general for the substrate glycone (Webb, 1960).

SUMMARY

1. β-D-Galactosidase and β-D-glucosidase activities have been measured in sweet-almond emulsin with the *p*-nitrophenyl hexosides.

2. The pH optima in hydrochloric acid–sodium butyrate buffers were between 5·5 and 5·6.

3. No separation of the two activities was achieved by ammonium sulphate fractionation or by partial heat inactivation.

4. D-Glucose, D-galactose, D-glucono-1→4-lactone and D-galactono-1→4-lactone were competitive inhibitors with similar K_i values when measured against the two substrates.

5. Mixed substrate experiments supported the conclusion that one enzyme site is responsible for both activities.

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Carbohydrates in Protein

4. THE DETERMINATION OF MANNOSE IN HEN'S-EGG ALBUMIN BY RADIOISOTOPE DILUTION*

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There are a number of earlier reports that hen's-egg albumin contains carbohydrate (see Needham, 1931). Although Hofmeister (1890) and Hopkins & Pincus (1898) obtained the protein in crystalline form, it was not until Sørensen & Høyrup (1916) showed that the protein after three crystallizations was free from 'mucoids' that such observations could have real significance. As a result of experiments carried out with 10% barium hydroxide, i.e. under conditions that may have resulted in at least partial destruction of the carbohydrate, Levene & Mori (1929) expressed the opinion that egg albumin does not contain carbohydrate. That the Carlsberg workers did not share this view is evident from the careful work of Sørensen & Haugaard (1933), who critically examined the orcinol–sulphuric acid procedure for the determination of carbohydrates and showed that egg albumin contained mannose by following the rate of formation of the coloured product; their results suggested a content of 1·7% of this sugar (Sørensen & Haugaard, 1933; Sørensen, 1934). It was not established, however, how this carbohydrate was attached to the protein. In Part 1 (Neuberger, 1938) it was shown that the bonding was almost certainly covalent and that the mannose content of the protein was at least 1·2%, as shown by actual isolation of a derivative. The orcinol–sulphuric acid procedure has also been reported to give 1·8% mannose by Neuberger (1938), Hewitt (1938) and Kaverzneva & Bogdanov (1961), and 2·0% by Johansen, Marshall & Neuberger (1960). Montgomery (1961) has reported 2·0% as the mannose content using a phenol–sulphuric acid procedure (Dubois, Gilles, Hamilton, Rebers & Smith, 1956) on the whole protein. Later Lee & Montgomery

* Part 3: Johansen, Marshall & Neuberger (1961).

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