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Enzymes of *Trichomonas foetus*

SEPARATION AND PROPERTIES OF TWO β-GALACTOSIDASES

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The β-galactosidase activity in extracts of *Trichomonas foetus* is separable into two fractions by gel filtration on Sephadex G-200. When *o*-nitrophenyl β-D-galactoside is used as substrate the first fraction to be eluted, β-galactosidase 1, has 50 times the activity (units per mg of protein) of the crude preparation. This fraction is activated by Mn^{2+} and Co^{2+} and inhibited by Hg^{2+} and EDTA. In the presence of Mn^{2+} the pH optimum for the hydrolysis of *o*-nitrophenyl β-D-galactoside or lactose is 5.8–6.0. β-Galactosidase 1 is an exoglycosidase that releases β-linked galactose joined to aliphatic and various carbohydrate aglycones. Hydrolysis is prevented, however, by a substituent on either the subterminal sugar or the terminal non-reducing β-galactosyl residue in an oligosaccharide. The second fraction, β-galactosidase 2, is not activated by metal ions or inhibited by EDTA and has a broad pH optimum from 4.5 to 6.0.

Extracts of the protozoan *Trichomonas foetus* contain enzymes that destroy the serological activity of the human A, B, H and Le^a blood-group-specific glycoproteins (Watkins, 1959). Characterization of these enzymes demonstrated that they are glycosidases that destroy blood-group activity by the removal of terminal non-reducing sugars from the carbohydrate chains in the glycoproteins (Watkins, 1956, 1960; Tyler & Watkins, 1960; Harrap & Watkins, 1964). The *T. foetus* extract also contains galactosidases, hexosaminidases, fucosidases (Watkins, 1959) and neuraminidases (Romanovska & Watkins, 1963) that bring about further degradation of blood-group-specific substances after the initial change, which leads to loss of serological specificity, has occurred. This organism therefore offers a source from which to obtain purified glycosidases needed for the further stepwise enzymic degradation of the carbohydrate chains in the blood-group substances and for the determination of the anomeric linkages of sugars in oligosaccharides, whether isolated from the chemical degradation products of the glycoproteins (cf. Aston, Donald & Morgan, 1968) or synthesized by glycosyltransferase reactions (cf. Ziderman, Gompertz, Smith & Watkins, 1967). Attempts to isolate a purified β-galactosidase from *T. foetus* revealed that the activity is separable into at least two parts and the preparation and properties of these two β-galactosidase fractions are described in this paper.

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MATERIALS AND METHODS

Chemicals. *o*-Nitrophenyl β-D-galactoside, *p*-nitrophenyl α-D-galactoside and *p*-nitrophenyl β-*N*-acetyl-D-glucosaminide were purchased from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K. The oligosaccharides from human milk, 2′-fucosyl-lactose, lacto-*N*-tetraose, lacto-*N*-neotetraose, lacto-*N*-fucopentaose I, lacto-*N*-fucopentaose II, lacto-difucotetraose, lacto-*N*-difucohexaose I and lacto-*N*-difucohexaose II were the gift of Dr Adeline Gauhe. Sephadex G-200 (140–400 mesh) was obtained from Pharmacia, Uppsala, Sweden. DEAE-cellulose (0.83 mg/g, medium mesh, lot 14B-1220) was purchased from Sigma (London) Chemical Co. Ltd., London, S.W.6, U.K.

Buffers. Tris–maleate (0.2 M) and McIlvaine (0.2 M-disodium phosphate–0.1 M-citric acid) buffers were prepared as described by Gomori (1955). Phosphate buffer (0.1 M) was made up as described by Sörensen (1909).

Growth of organisms. Cells of *T. foetus* var. Belfast were grown at the Microbiological Research Establishment, Porton Down, Wilts, U.K. The organisms, grown in batch culture in a glucose–serum broth medium (Kerr & Robertson, 1947) at 37°C for 48 h were harvested by centrifuging, washed once with 0.15 M-NaCl and stored at −18°C until required.

Protein determination. The protein concentration of the enzyme preparations was determined by the method of Lowry, Rosebrough, Farr & Randall (1951) with purified human albumin as a standard.

Measurement of enzyme activity. When *o*-nitrophenyl β-D-galactoside was used as substrate, β-galactosidase activity was measured by one of two methods. In the first method, which is essentially similar to that described by Cohn & Monod (1951), *o*-nitrophenyl β-D-galactoside (0.5 ml of an aq. 6.7 mM soln.) was mixed with 2.8 ml

of 0.67 M-sodium phosphate buffer, pH 7.0, and 0.2 ml of enzyme solution in a 1 cm optical glass cell. The change in extinction at 420nm was measured at room temperature in a Unicam SP. 600 spectrophotometer. The control cell contained substrate (0.5ml) and buffer solution (3.0ml). Readings were taken at intervals of 1 min for a total of 5 min. The unit of activity was defined as the amount of activity that hydrolysed 1 μmol of substrate/min under these conditions. In the second method β-galactosidase activity was assayed by incubating 6.7mM-*o*-nitrophenyl β-D-galactoside (0.2ml) and 0.67M-phosphate buffer, pH7.0 (3ml) with 0.1ml of enzyme solution at 37°C. The reaction was stopped by the addition of 1M-Na_2CO_3 (1ml) and the extinction was read in 1cm light-path cells at 420nm in a Unicam SP. 600 spectrophotometer.

In tests for β-galactosidase activity with lactose as a substrate the liberation of reducing sugars was measured by the method of Nelson (1944).

Fractions from column chromatography were assayed for α-galactosidase and β-*N*-acetylglucosaminidase activity by the second method described for β-galactosidase assay except that *p*-nitrophenyl α-D-galactoside and *p*-nitrophenyl β-*N*-acetyl-D-glucosaminide respectively were used as substrates.

Enzymic destruction of A, B, H and Le^a serological activity was assayed as described by Watkins (1966*b*).

Neuraminidase activity was measured with a sialic acid-containing blood-group Le^a-active glycoprotein (Pusztai & Morgan, 1961) as substrate. An 0.1% solution of the Le^a substance (0.1ml in 0.2M-sodium acetate buffer, pH5.0) was incubated at 37°C for 1h with 0.1ml of enzyme solution and then analysed for free *N*-acetylneuraminic acid by the method of Warren (1959).

RESULTS

Purification of β-galactosidases 1 *and* 2

Preliminary experiments indicated that extraction of β-galactosidase from *T. foetus* organisms stored at −18°C could be achieved by stirring the thawed protozoa in dilute salt solution. The enzymes in the extract were concentrated by ammonium sulphate precipitation and fractionated on columns of Sephadex G-200 and DEAE-cellulose. All procedures were carried out at 4°C.

Step 1: *extraction of the enzymes.* *T. foetus* organisms (75ml of concentrated deposit) were thawed, suspended in 0.01M-sodium phosphate buffer, pH7.0, and the volume was made up to 200ml with buffer. The pH was adjusted to 6.5 with sodium hydroxide, the suspension stirred for 2h and the cellular debris was then removed by centrifugation at 5000*g* for 30min.

Step 2: *precipitation with ammonium sulphate.* The supernatant was treated with solid ammonium sulphate to bring the concentration to 40% saturation. The solution was stirred during the addition and stirring was continued for another 2h. The precipitate was removed by centrifugation for 15 min at 29000*g* and discarded. The supernatant was brought to 60% saturation with solid ammonium sulphate, stirred for 2h and the precipitate collected by centrifugation at 29000*g* for 15min.

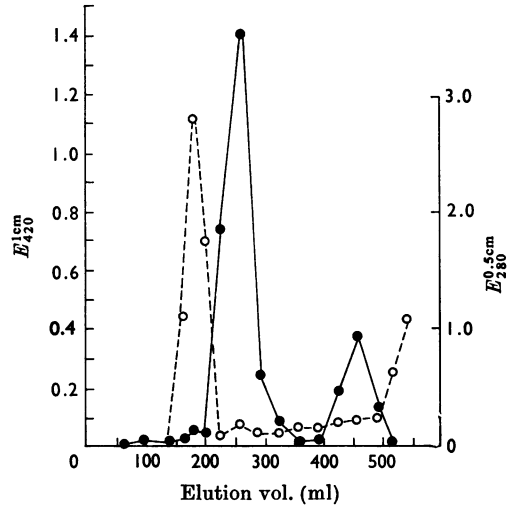

Fig. 1. Elution of β-galactosidase activity from a Sephadex G-200 column. ●, β-Galactosidase activity (E_{420}); ○, protein (E_{280}).

Step 3: *fractionation on Sephadex G-200.* The precipitate from 40–60% saturation with ammonium sulphate was dissolved in 0.15M-sodium chloride (12ml) and applied to a column of Sephadex G-200 (3cm × 100cm) previously equilibrated with 0.15M-sodium chloride. The column was eluted with a solution of 0.15M-sodium chloride saturated with toluene and the emerging fractions (5ml) were tested for u.v. absorption at 280nm and for β-galactosidase activity. A typical elution profile is given in Fig. 1. Two well-separated peaks of β-galactosidase activity were obtained; the first, β-galactosidase 1, emerged t 200–300ml elution volume and the second, β-galactosidase 2, at 400–500ml elution volume. The fractions corresponding to each peak were pooled separately and concentrated by ultrafiltration to about 5ml and stored at 4°C. The two β-galactosidases retained their distinct chromatographic behaviour when refractionated on a smaller column of Sephadex G-200 (1cm × 34cm) and were again eluted as single peaks with different K_d values (Flodin, 1962).

At this stage in the purification, β-galactosidase 1 was contaminated with neuraminidase, but was essentially free from enzymes that destroy the serological specificity of A, B, H and Le^a blood-group-active glycoproteins and from α-D-galactosidase and β-*N*-acetyl-D-glucosaminidase activity. β-Galactosidase 2 was contaminated with all these enzymes and no further attempt was made to purify

Table 1. *Recovery of β-galactosidase activity at different stages of purification*

For details see the text. Protein was determined by the method of Lowry *et al.* (1951).

Step	Purification stage	Units of β-galactosidase activity	Recovery of activity (%)	Specific activity (units/mg of protein)
1	Extraction of *T. foetus* organisms	106	100	0.09
2	40–60% $(NH_4)_2SO_4$ precipitate	62	59	0.31
3	Fractionation on Sephadex G-200			
	(a) β-galactosidase 1	36	34	4.5
	(b) β-galactosidase 2	11	10	0.45

this preparation. The recoveries of β-galactosidases 1 and 2 after ammonium sulphate fractionation and chromatography on Sephadex G-200 are given in Table 1.

Step 4: *fractionation on DEAE-cellulose.* A solution of β-galactosidase 1 in 0.15M-sodium chloride was dialysed for 24h at 4°C against 5mM-sodium phosphate buffer, pH 6.9, and 2.3ml (10.7 units of β-galactosidase activity) was loaded on to a DEAE-cellulose column (1.5cm × 31cm) previously equilibrated with the same buffer. The column was eluted with 120ml of 5mM-sodium phosphate buffer, pH 6.9, and then with a convex concentration gradient (Alm, Williams & Tiselius, 1952) obtained with 5mM-sodium phosphate (550ml) in the mixing chamber and 0.1M-sodium phosphate buffer, pH 6.9, containing 0.3M-sodium chloride in the reservoir. The emerging fractions (8ml) were examined for absorption at 280nm and for β-galactosidase and neuraminidase activities. Separation of β-galactosidase and neuraminidase activities was achieved (Fig. 2) although both peaks showed some tailing. Concentration of the active fractions yielded only 2.4 units of β-galactosidase (22% yield) and, in view of the considerable loss of activity, the purification on DEAE-cellulose was not attempted unless freedom from neuraminidase activity was required. The experiments described below on the properties of β-galactosidase 1 were carried out with preparations obtained by fractionation on Sephadex G-200.

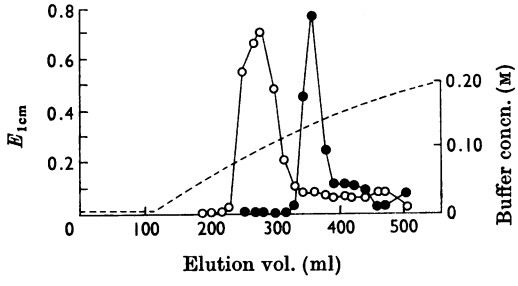

Fig. 2. Elution of β-galactosidase 1 and neuraminidase activities from a DEAE-cellulose column. ●, β-Galactosidase activity (E_{420}); ○, neuraminidase activity (E_{549}); ----, molarity of buffer.

Properties of β-galactosidases 1 *and* 2

Stability. Both β-galactosidases 1 and 2 are stable to dialysis and ultrafiltration. Repeated freezing and thawing (up to ten times) has little or no effect on the activity of either enzyme, but 98% of the activity of β-galactosidase 1 and 40% of the activity of β-galactosidase 2 was lost on freeze-drying. The solutions can be stored at either 4°C or −10°C. A preparation of β-galactosidase 1 retained 57% of its original activity after 5 months at 4°C and 40% of its activity after 7 months at −10°C.

Effect of pH *and metal ions.* Attempts to determine the optimum pH for β-galactosidase 1 activity in the absence of added metal ions revealed considerable differences in the optima with different buffer systems when either *o*-nitrophenyl β-D-galactoside or lactose was used as substrate. The effects of various bivalent cations and of EDTA on the activity of the β-galactosidases were therefore examined. β-Galactosidase 1 is activated by Mn^{2+} and Co^{2+} and to a lesser extent by Mg^{2+} (Table 2) and is inhibited by Hg^{2+} and EDTA. The effects of various concentrations of Mn^{2+} and of EDTA are shown in Table 3. With the same assay system the inhibiting effect of EDTA (0.1ml; 6mM soln.) on β-galactosidase 1 (58m-units) is reversed by the addition of Mn^{2+} (0.9ml; 6mM soln.) or Co^{2+} (0.9ml; 6mM soln.). β-Galactosidase 2 is not activated by metal ions or inhibited by EDTA but Hg^{2+} completely inactivates the enzyme (Table 2).

pH–activity curves for β-galactosidase 1, with three different buffer systems, McIlvaine (1921), tris–maleate (Gomori, 1955) and phosphate (Sörensen, 1909), were determined in the presence of Mn^{2+}. The discrepancies in the pH optima in the

Table 2. *Effect of metal ions and EDTA on the activities of β-galactosidases* 1 *and* 2

Enzyme (0.1 ml containing 58 m-units of β-galactosidase 1 in 0.15 M-NaCl or 20 m-units of β-galactosidase 2 in 0.15 M-NaCl) was incubated at 37°C with buffer (2 ml), *o*-nitrophenyl β-D-galactoside (0.2 ml; 6.7 mM soln.) and metal salt solution or EDTA (1 ml; 6 mM soln.). The reaction was stopped by the addition of 1 M-Na_2CO_3 (1 ml) and the solution centrifuged to remove any precipitate formed. Extinctions were read in 1 cm light-path cells at 420 nm. The results are expressed relative to the activity of the enzyme in the absence of added metal ions or EDTA taken as 100.

Buffer system	Addition	Enzyme activity: β-Galactosidase 1	Enzyme activity: β-Galactosidase 2
Sodium phosphate, pH 5.8 (0.1 M)	None	100	100
	$MnCl_2$	200	105
	$CoCl_2$	192	97
	$MgCl_2$	150	102
	$CaCl_2$	90	101
	$HgCl_2$	3	0
	$BaCl_2$	105	—
	EDTA	18	112
Tris–maleate, pH 6.4 (0.2 M)	None	100	—
	$MnCl_2$	130	—
	$MgCl_2$	100	—
	$HgCl_2$	1	—
	$CaCl_2$	74	—
	EDTA	8	—

Table 3. *Effect of* Mn^{2+} *and EDTA concentration on the activity of β-galactosidase* 1

β-Galactosidase 1 (100 μl; 58 m-units) was incubated with 0.1 M-sodium phosphate buffer, pH 5.8 (2 ml), $MnCl_2$ or EDTA solution (1 ml) and *o*-nitrophenyl β-galactoside (0.2 ml; 6.7 mM soln.) at 37°C for 7 min. The reaction was stopped by the addition of 1 M-Na_2CO_3 (1 ml) and the extinctions were read at 420 nm in 1 cm light-path cells. Activities are expressed as in Table 2.

Addition	Concentration of added solution (mM)	Enzyme activity
None	—	100
$MnCl_2$	0.0006	112
	0.006	164
	0.06	192
	0.6	220
	6.0	213
EDTA	0.06	29
	0.6	33
	6.0	31

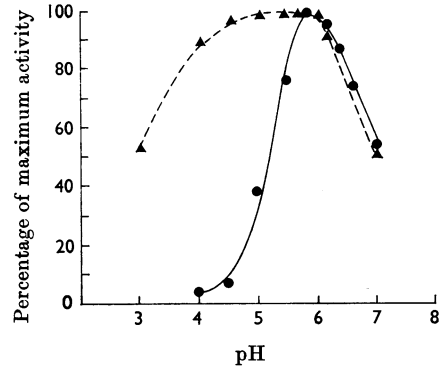

Fig. 3. Variation of β-galactosidase activity with pH value. β-Galactosidase 1 (100 μl; 58 m-units) was incubated at 37°C with McIlvaine buffer (2 ml), 6 mM-$MnCl_2$ (1 ml) and 6.7 mM-*o*-nitrophenyl β-D-galactoside (0.2 ml). β-Galactosidase 2 (100 μl; 19 m-units) was incubated at 37°C with McIlvaine buffer (3 ml) and 6.7 mM-*o*-nitrophenyl β-D-galactoside (0.2 ml). The reactions were stopped by the addition of 1 M-Na_2CO_3 (1 ml) and the extinctions read at 420 nm. ●, β-Galactosidase 1; ▲, β-galactosidase 2.

absence of added ions were no longer observed and a consistent pH optimum of 5.8–6.0 was obtained for the hydrolysis of both *o*-nitrophenyl β-D-galactoside (Fig. 3) and lactose. β-Galactosidase 2 has a broad pH optimum between 4.5 and 6.0 when either *o*-nitrophenyl β-D-galactoside (Fig. 3) or lactose is used as substrate.

Influence of variation of enzyme and substrate concentration on the activity of β-galactosidases 1 *and* 2. Figs. 4 and 5 illustrate the variation with time and with enzyme concentration in the rate of hydrolysis of *o*-nitrophenyl β-D-galactoside by β-galactosidases 1 and 2. The variation in the rate of hydrolysis with changes in substrate concentration was also determined for the two β-galactosidases; the plot of $1/v$ versus $1/s$ yielded a K_m value

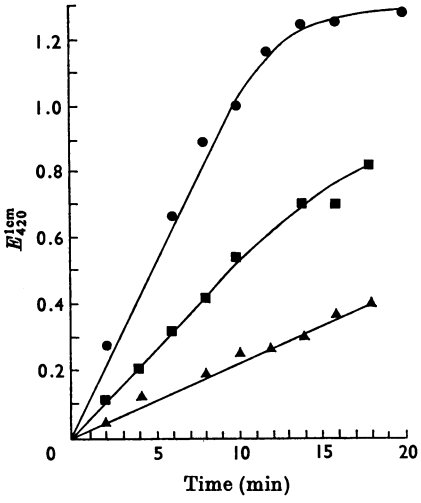

Fig. 4. Progress curves for β-galactosidases 1 and 2. The incubation mixtures for β-galactosidase 1 contained 0.1M-sodium phosphate buffer, pH5.8 (2ml), 6mM-$MnCl_2$ (0.1ml), 6.7mM-*o*-nitrophenyl β-D-galactoside (0.2ml), water (0.8ml) and enzyme in 0.15M-NaCl (950m-units/ml). The incubation mixtures for β-galactosidase 2 (196m-units/ml) contained McIlvaine buffer, pH5.2, in place of the phosphate buffer and $MnCl_2$ solution. The mixtures were incubated at 30°C and samples withdrawn at given time-intervals. The reactions were stopped by the addition of 1M-Na_2CO_3 (1ml) and the extinctions read at 420nm. ●, 100μl of β-galactosidase 1; ■, 50μl of β-galactosidase 1; ▲, 100μl of β-galactosidase 2.

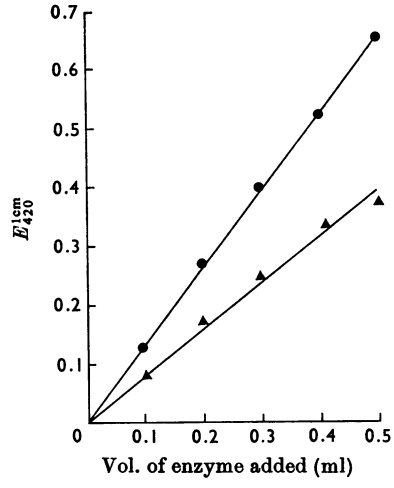

Fig. 5. Effect of enzyme concentration on the rate of hydrolysis by β-galactosidases 1 and 2. The incubation mixtures were the same as in Fig. 4 except that different amounts of enzyme (β-galactosidase 1, 95m-units/ml; β-galactosidase 2, 39m-units/ml) were added and the final volume of each mixture was adjusted to 3.2ml with water. The mixtures were incubated at 30°C for 10min (β-galactosidase 1) or 15min (β-galactosidase 2). ●, β-Galactosidase 1; ▲, β-galactosidase 2.

of 2.4×10^{-4}M for β-galactosidase 2 (Fig. 6). The plot for β-galactosidase 1 (Fig. 6) flattened at higher substrate concentrations, but if this is assumed to be the result of substrate inhibition, as has been suggested for the β-galactosidase from ram testis (Caygill, Roston & Jevons, 1966), a K_m value of 2×10^{-3}M may be estimated.

Substrate specificity of β-galactosidases 1 and 2. Experiments with lactose as a substrate for β-galactosidase 1 indicated that Co^{2+} was a more effective activator than Mn^{2+} and that the pH optimum was slightly higher (6–6.5) in the presence of Co^{2+}. For tests on the specificity of the enzyme with glycosides and oligosaccharides as substrates β-galactosidase 1 (950m-units/ml) was therefore dialysed against 0.1M-sodium phosphate buffer, pH6.2, containing 3mM-Co^{2+} and β-galactosidase 2 (196m-units/ml) was dialysed against phosphate–citrate buffer (Gomori, 1955), pH5.2. Each enzyme was then incubated with a number of simple glycosides and galactose-containing disaccharides and the products were examined by paper chromatography. Under the incubation conditions used low-molecular-weight carbohydrate fragments were not liberated from either enzyme preparation in the absence of substrate. All the β-linked galactosides tested were hydrolysed by β-galactosidase 1, whereas the α-linked galactosides were not attacked (Table 4). Cellobiose [*O*-β-D-glucopyranosyl-(1→4)-D-glucose] was not attacked by β-galactosidase 1 under conditions in which lactose was completely hydrolysed, indicating the importance of the configuration of the hydroxyl group at C-4 in the glycone. The β-galactosidase 2 preparation also hydrolysed all the β-linked galactosides, but did not show anomeric specificity (Table 4), indicating the presence of a contaminating α-galactosidase. When all the fractions in the β-galactosidase 1 peak emerging from the Sephadex G-200 column were pooled some preparations had slight α-galactosidase activity on more prolonged incubation of the enzyme with the substrates. However, when only those fractions with maximal β-galactosidase activity, together with those fractions on the ascending portion of the peak (Fig. 1), were pooled, the preparation was specific for β-linked galactosides.

Further information about the specificity of β-galactosidase 1 was obtained by using certain oligosaccharides isolated from human milk (for references see Kuhn, 1957; Neuberger & Marshall, 1966) as substrates. None of the fucose-containing

compounds was hydrolysed by β-galactosidase 1 (Table 5), but the two tetrasaccharides that did not have fucose substituents, i.e. lacto-*N*-tetraose and lacto-*N*-neotetraose, each gave galactose and a trisaccharide, $R_{lactose}$ 0.67, which was probably lacto-*N*-triose II [*O*-β-(*N*-acetyl-D-glucosaminyl)-(1→3)-*O*-β-D-galactosyl-(1→4)-D-glucose]. This trisaccharide was not available for comparison, but has a reported $R_{lactose}$ value of 0.72 (Kuhn & Gauhe, 1962) in the same solvent system as used in our experiments.

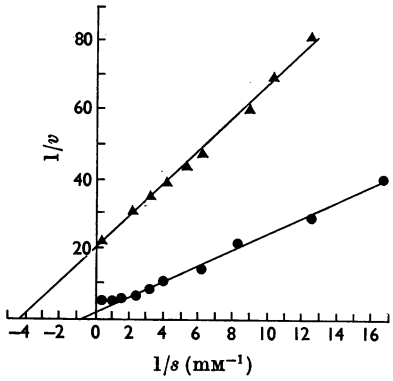

Fig. 6. Determination of K_m values for β-galactosidases 1 and 2. Plot of the reciprocal velocity $1/v$ against the reciprocal *o*-nitrophenyl β-D-galactoside concentration $1/s$. Reaction mixtures for β-galactosidase 1 contained 0.1M-phosphate buffer, pH5.8 (2ml), 6mM-$MnCl_2$ (0.1ml), enzyme (0.5ml; 20m-units) and various amounts of *o*-nitrophenyl β-D-galactoside in water (0.5ml). The mixtures were incubated for 10min at 30°C. Reaction mixtures for β-galactosidase 2 contained McIlvaine buffer, pH5.2 (2ml), enzyme (0.5ml; 20m-units) and various amounts of *o*-nitrophenyl β-D-galactoside in water (0.5ml) and the mixtures were incubated for 15min at 30°C. The reaction was stopped by the addition of 1M-Na_2CO_3 (1ml) and the extinctions were read at 420nm in 1cm light-path cells. The rate of hydrolysis (v) is expressed as μmol of *o*-nitrophenyl β-D-galactoside hydrolysed/min by 20m-units of enzyme. ●, β-Galactosidase 1; ▲, β-galactosidase 2.

All the oligosaccharides tested with the β-galactosidase 1 preparation were extensively hydrolysed by the β-galactosidase 2 preparation, largely to the constituent monosaccharides, so that no further conclusions can be drawn from these experiments about the specificity of the enzyme.

Transferase activity of β-galactosidases 1 *and* 2. To test whether the two β-galactosidases had transferase activity, in addition to their hydrolytic properties, 10μl samples of each (9.5m-units of galactosidase 1 in 0.1M-sodium phosphate buffer, pH6.2 containing 3mM-Co^{2+}; 1.96m-units of β-galactosidase 2 in phosphate–citrate buffer, pH5.2) were incubated with 100μl of 5% lactose solution at 37°C. Samples (20μl) were withdrawn after 20, 40, 60, 120 and 180min, heated at 100°C to stop enzyme action and run on a paper chromatogram in butan-1-ol–pyridine–water (6:4:3, by vol.). Reducing sugars were detected with benzidine–trichloroacetic acid spray reagent (Bacon & Edelman, 1951). There was evidence of transferase activity in both preparations. After 20min incubation three spots running more slowly than lactose were detected on the chromatogram of the products given with β-galactosidase 1. The number of spots running more slowly than lactose increased to five on further incubation. The chromatogram of the products obtained with β-galactosidase 2 had one

Table 4. *Action of β-galactosidases* 1 *and* 2 *on simple glycosides and disaccharides*

Enzyme solution (10μl) was incubated at 37°C with aq. 2% solution of substrate (10μl). Incubation times of 30min for β-galactosidase 1 and 90min for β-galactosidase 2 were selected after preliminary tests indicated complete hydrolysis of lactose under these conditions. The reactions were stopped by heating the mixtures for 5min at 100°C and the products were run on paper chromatography in the solvent mixture 'amyl alcohol' (Analak; BDH Chemicals Ltd.,)–dioxan–water–acetic acid (43:34:23:0.6, by vol.) and detected with benzidine–trichloroacetic acid spray reagent (Bacon & Edelman, 1951). +, Hydrolysis; −, no hydrolysis.

Substrate	Hydrolysis	
	β-Galactosidase 1	β-Galactosidase 2
Methyl α-D-galactopyranoside	−	+
Phenyl α-D-galactopyranoside	−	+
Methyl β-D-galactopyranoside	+	+
O-β-D-Galactosyl-(1→3)-D-glucose	+	+
O-β-D-Galactosyl-(1→4)-D-glucose	+	+
O-β-D-Galactosyl-(1→3)-*N*-acetyl-D-glucosamine	+	+
O-β-D-Galactosyl-(1→4)-*N*-acetyl-D-glucosamine	+	+
O-β-D-Galactosyl-(1→6)-*N*-acetyl-D-glucosamine	+	+
O-α-D-Galactosyl-(1→3)-D-galactose	−	+
O-α-D-Galactosyl-(1→6)-D-glucose	−	+
O-α-D-Galactosyl-(1→6)-*N*-acetyl-D-glucosamine	−	+

Table 5. *Action of β-galactosidase* 1 *on oligosaccharides from human milk*

The conditions of incubation were as in Table 4. The products of enzyme action were examined by paper chromatography in ethyl acetate–pyridine–water (2:1:2, by vol.) and detected with benzidine–trichloroacetic acid spray reagent (Bacon & Edelman, 1951).

Substrate	Structure	Products of enzyme action
2′-Fucosyl-lactose	β-D-Gal-(1→4)-D-Glc ↑ (1→2) α-L-Fuc	Nil
Lacto-difucotetraose	β-D-Gal-(1→4)-D-Glc ↑ (1→2) ↑ (1→3) α-L-Fuc α-L-Fuc	Nil
Lacto-*N*-tetraose	β-D-Gal-(1→3)-β-D-GlcNAc-(1→3)-β-D-Gal-(1→4)-D-Glc	Galactose and a trisaccharide $R_{lactose}$ 0.67
Lacto-*N*-neotetraose	β-D-Gal-(1→4)-β-D-GlcNAc-(1→3)-β-D-Gal-(1→4)-D-Glc	Galactose and a trisaccharide $R_{lactose}$ 0.67
Lacto-*N*-fucopentaose I	β-D-Gal-(1→3)-β-D-GlcNAc-(1→3)-β-D-Gal-(1→4)-D-Glc ↑ (1→2) α-L-Fuc	Nil
Lacto-*N*-fucopentaose II	β-D-Gal-(1→3)-β-D-GlcNAc-(1→3)-β-D-Gal-(1→4)-D-Glc ↑ (1→4) α-L-Fuc	Nil
Lacto-*N*-difucohexaose I	β-D-Gal-(1→3)-β-D-GlcNAc-(1→3)-β-D-Gal-(1→4)-D-Glc ↑ (1→2) ↑ (1→4) α-L-Fuc α-L-Fuc	Nil
Lacto-*N*-difucohexaose II	β-D-Gal-(1→3)-β-D-GlcNAc-(1→3)-β-D-Gal-(1→4)-D-Glc ↑ (1→4) ↑ (1→3) α-L-Fuc α-L-Fuc	Nil

spot running more slowly than lactose after 20min and three spots after 3h incubation.

Action of β-galactosidase 1 *on a blood-group-active glycoprotein.* A systematic examination of the action of the *T. foetus* β-galactosidases on blood-group active glycoproteins has not been made. To test the suitability of β-galactosidase 1 for such an examination tests were made with human Le^a substance (Annison & Morgan, 1952), selected because substances with this serological specificity are believed to have a proportion of their carbohydrate chains ending in unsubstituted β-D-galactosyl-(1 → 4)-*N*-acetyl-D-glucosaminyl residues (cf. Watkins, 1966*a*). Le^a substance (20mg) was incubated at 1% concentration in 0.01M-sodium phosphate buffer, pH6.5, with β-galactosidase 1 (1.2 units) for 18h at 37°C in the presence of toluene. The incubation mixture was heated for 5min at 100°C to stop enzyme action and then loaded on to a column (2g) of charcoal–Celite (1:1, w/w). The column was eluted with 5% (v/v) ethanol, the effluent evaporated to dryness, reconstituted in 0.15ml of water and examined by paper chromatography in 'amyl alcohol–dioxan–water–acetic acid (43:34:23:0.6, by vol.). The main product of enzyme action was chromatographically indistinguishable from galactose and only traces of other sugars were detected. The enzyme preparation therefore releases galactose from macromolecular glycoprotein.

DISCUSSION

Galactosidases in crude extracts of *T. foetus* hydrolyse a variety of low-molecular-weight α- and β-D-galactosides and release galactose from macromolecular blood-group active glycoproteins (Watkins, 1959). More than one form of α-galactosidase in these crude extracts was indicated by the different properties of the enzyme releasing terminal α-linked galactose from blood-group B substance and of that acting on low-molecular-weight α-galactosides. The results described in this paper demonstrate that the β-galactosidase in *T. foetus* also occurs in more than one form. Concentration of the β-galactosidase activity by ammonium sulphate precipitation, followed by fractionation on Sephadex G-200, separated the enzyme into two fractions. The first peak emerging from the Sephadex column, β-galactosidase 1, was, in terms of specific activity, 50 times as active as the crude extracts when *o*-nitrophenyl β-D-galactoside was used as substrate. The peak fractions are essentially free from enzymes that destroy the serological

activity of A, B, H and Le^a substances and from α-galactosidase and β-*N*-acetylglucosaminidase, but they contain a contaminating neuraminidase. Removal of this contaminant by fractionation on DEAE-cellulose leads to considerable loss of β-galactosidase activity. β-Galactosidase 2, the second peak of β-galactosidase activity, emerges with many other glycosidases; no attempt has been made to purify this enzyme further and it is possible that more than one isoenzyme of β-galactosidase occurs in this fraction.

Multiple forms of β-galactosidase from mammalian sources have been reported (cf. Doell & Kretchmer, 1962; Furth & Robinson, 1965). The most rigorously studied β-galactosidase of microbial origin, that from *Escherichia coli* ML 309 (cf. Wallenfels & Malhotra, 1960), occurs as a monomer and in an aggregated form that has a lower specific activity than the monomer (Sund & Weber, 1963; Rohlfing & Crawford, 1966). Aggregation does not, however, appear to be the explanation for the two forms of β-galactosidase from *T. foetus* because the enzymes differ in several properties in addition to the difference in size or shape that accounts for the elution pattern from the Sephadex G-200 column. β-Galactosidase 1 is activated by Mn^{2+} or Co^{2+} and inhibited by EDTA when *o*-nitrophenyl β-D-galactoside is used as substrate whereas β-galactosidase 2 is not activated by metal ions or inhibited by EDTA. In the presence of Mn^{2+} β-galactosidase 1 gives a sharp optimum at pH 5.8–6.0 whereas β-galactosidase 2 has a broad pH optimum of 4.5–6.0. β-Galactosidase 2 also has a lower K_m value than β-galactosidase 1 when *o*-nitrophenyl β-D-galactoside is used as substrate.

Of primary importance in the use of β-galactosidases as tools for degradative purposes is a knowledge of their specificity with respect to the aglycone. Both β-galactosidases released β-linked galactose joined to aliphatic and various carbohydrate aglycones (Table 4). Only qualitative tests for specificity were made with the di- and oligosaccharide substrates, but disaccharides with 1→3, 1→4 and 1→6 β-galactosyl linkages were all hydrolysed. The contaminating glycosidases in the β-galactosidase 2 preparation made it impossible to establish its specificity by tests with the fucose-containing oligosaccharides isolated from human milk, but several notable conclusions can be drawn about the specificity of β-galactosidase 1 (Table 5). The enzyme is an exoglycosidase; that is, it hydrolyses only terminal non-reducing galactosyl residues, as evidenced by the fact that galactose and a compound with the chromatographic mobility of a trisaccharide are the only products formed from lacto-*N*-tetraose and lacto-*N*-neotetraose. None of the fucose-containing oligosaccharides is hydrolysed by the β-galactosidase, although lacto-*N*-fucopentaose II and lacto-*N*-difucohexaose II both have terminal unsubstituted β-galactosyl residues. These two compounds differ from lacto-*N*-tetraose only by the attached fucose residues, and therefore it must be inferred that a fucose substituent on the subterminal *N*-acetylglucosamine unit renders the terminal β-galactosyl linkage resistant to hydrolysis. The presence of a fucose substituent on the terminal galactosyl unit itself, as in 2′-fucosyl-lactose and lacto-*N*-fucopentaose I, also prevents hydrolysis. Failure of the enzyme preparation to release galactose from an oligosaccharide or glycoprotein known to have terminal D-galactosyl residues therefore cannot necessarily be taken to indicate the absence of a β-linkage, since a substituent on the terminal galactose residue or on the subterminal sugar makes the molecule unacceptable to the enzyme. Neuraminic acid, which commonly occurs as a terminal non-reducing branching unit in glycoproteins, would presumably have the same effect as fucose. Another factor militating slightly against the use of either β-galactosidase 1 or 2 as hydrolytic catalyst is the fact that both have transferase activity in the presence of high concentrations of substrate. Nevertheless, if these facts are borne in mind, the broad specificity of the enzyme towards β-galactosyl residues joined to different sugars and to different positions in the aglycone moiety should make β-galactosidase 1, which is largely free of contaminating glycosidases, a useful tool for structural investigations of both low-molecular-weight carbohydrates and macromolecular glycoproteins.

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