LINKAGE-SPECIFIC α-D-GALACTOSIDASES FROM *TRICHOMONAS FOETUS*: CHARACTERISATION OF THE BLOOD-GROUP B-DESTROYING ENZYME AS A 1,3-α-GALACTOSIDASE AND THE BLOOD-GROUP P_1-DESTROYING ENZYME AS A 1,4-α-GALACTOSIDASE

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1. Introduction

Earlier investigations on glycosidases in extracts of the protozoan *Trichomonas foetus* indicated that there were at least two distinct α-D-galactosidases [1]; one acted on low-molecular-weight substrates and differed in heat stability and inhibitory properties from a second enzyme that released α-linked galactose from blood-group B-active structures in glycoproteins. Subsequently the *T. foetus* extract was found to contain an enzyme that destroyed the blood-group P_1 activity of a glycoprotein isolated from hydatid cyst fluids [2,3] and, as P_1 specificity is dependent on a terminal non-reducing α-galactosyl residue [3,4], it was inferred that the enzyme concerned was also an α-galactosidase.

The isolation of three linkage-specific α-D-galactosidases from *T. foetus* extracts is described in this paper. The enzyme that destroys blood-group B specificity is characterised as a 1,3-α-galactosidase, the enzyme that destroys blood-group P_1-specificity as a 1,4-α-galactosidase, and the third enzyme as a 1,6-α-galactosidase which also hydrolyses alkyl and aryl α-D-galactosides.

2. Materials and methods

T. foetus organisms were grown at the Microbiological Research Establishment, Porton and supplied as a frozen cell paste. Blood-group B substance was isolated from human ovarian cyst fluid [5] and blood-group P_1 glycoprotein from sheep hydatid cyst fluid [2]. Lactose, melibiose, *p*-nitrophenyl α- and β-D-galactosides and methyl α- and β-D-galactosides were purchased from Koch-Light Ltd., and raffinose (*O*-α-D-galactosyl-(1→6)-α-D-glucosyl β-D-fructoside) and stachyose (*O*-α-D-galactosyl-(1→6)-*O*-α-D-galactosyl-(1→6)-α-D-glucosyl β-D-fructoside) and *o*-nitrophenyl α-D-galactoside were purchased from Sigma Ltd. *O*-α-D-Galactosyl-(1→4)-D-galactose was isolated from okra pods as described [6]. *O*-α-D-Galactosyl-(1→4)-*O*-β-D-galactosyl-(1→4)-D-glucitol and *O*-α-D-galactosyl-(1→2)-*O*-α-D-glucosyl-(1→1)-glycerol were gifts from Dr J. Kawanami and Dr J. Baddiley, respectively. *O*-α-D-Galactosyl-(1→3)- galactose, isolated from λ-carrageenin was supplied by Dr K. Morgan, and synthetic crystalline *O*-α-D-galactosyl-(1→3)-*O*-α-D-galactosyl-(1→3)-galactose by Dr J. Koscielak. Radioactively labelled *O*-α-D[^{14}C]-galactosyl-(1→3)-*O*-[α-L-fucosyl-(1→2)] D-galactose and *O*-α-D-[^{14}C]galactosyl-(1→3)-*O*-[α-L-fucosyl-(1→2)]-*O*-β-D-galactosyl-(1→4)-D-glucose were prepared as described previously [7,8]. *O*-α-D-Galactosyl-(1→6)-*N*-acetyl-D-glucosamine was synthesised enzymically [9]. *O*-β-D-Galactosyl-(1→6)-*N*-acetyl-D-glucosamine and *O*-β-D-galactosyl-(1→3)-*N*-acetyl-D-glucosamine were gifts from the late Professor R. Kuhn and *O*-β-D-galactosyl-(1→4)-*N*-acetyl-D-glucosamine was supplied by Dr Zilliken. *O*-α-D-Galactosyl-(1→4)-*O*-β-D-galactosyl-(1→4)-*N*-acetyl-D-glucosamine was isolated from P_1 active glycoprotein [4]. L-Galactose was a gift from Professor T. Reichstein.

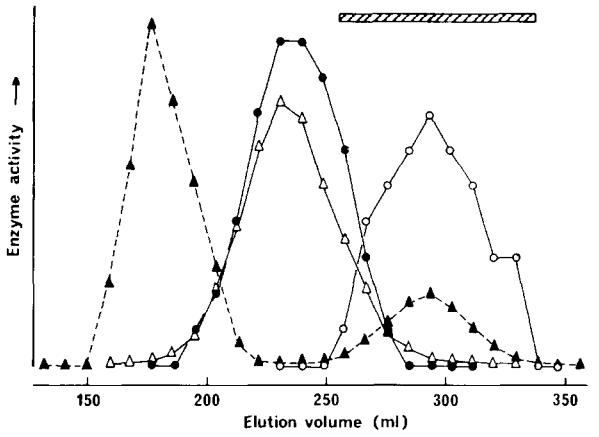

Fig.1. Separation of galactosidases in extracts of *T. foetus* by chromatography on Sephadex G-200. (○) Blood-group P_1-destroying enzyme (α-galactosidase *a*); (●) blood-group B-destroying enzyme (α-galactosidase *b*); (△), *p*-nitrophenyl α-D-galactoside hydrolysing enzyme (α-galactosidase *c*); (▲) *p*-nitrophenyl β-D-galactoside hydrolysing enzyme (β-galactosidase); ▨ Fractions pooled for isoelectric focusing.

Enzyme preparations. All procedures were carried out at 4°C. The packed, thawed *T. foetus* organisms (80 ml) were suspended in 120 ml 0.01 M phosphate buffer pH 7.0. The pH was adjusted to 6.0, the suspension was stirred for 2 h, and the cellular debris was then removed by centrifuging for 30 min at 10 000 *g*. The material that precipitated from the supernatant between 40–60% saturation with ammonium sulphate was dissolved in 5 ml 0.15 M NaCl. Insoluble material was removed by centrifuging for 1.5 h at 38 000 *g* and the supernatant was loaded onto a 90 × 2.5 cm column of Sephadex G-200 equilibrated with 0.15 M NaCl. The column was eluted by upward flow with 0.15 M NaCl and the fractions (3 ml) were tested for their action on *p*-nitrophenyl α- and β-D-galactosides and their capacity to destroy the serological activities of blood-group B and P_1 substances (fig.1). The fractions that contained the blood-group P_1-destroying enzyme were pooled and concentrated to 4 ml in an Amicon ultrafiltration cell. The sample was then subjected to isoelectric focusing [10] in an LKB Uniphor apparatus fitted with a 40 × 2.5 cm glass column. A linear sucrose gradient (0–40%) containing the sample and carrier ampholytes (LKB Ampholines, 2% pH range 3.5–5.0 and 0.2% pH range 3.5–10) was layered onto the anode solution. Focusing was carried out for 66 h with a maximum voltage of 800 V. The column was eluted by displacement with the cathode solution and the pH of each fraction (2.0 ml) was measured at 4°C. The fractions were tested for α- and β-galactosidase activity with *p*-nitrophenyl galactosides and for blood-group B- and P_1-destroying activities. The fractions containing P_1-destroying enzyme (α-galactosidase *a*; pI 4.43) were free from B-destroying enzyme and also from α- and β-galactosidase activity as measured by their action on the nitrophenyl substrates (fig.2). The fractions containing blood-group B-destroying enzyme (α-galactosidase *b*; pI 4.63) were free from blood-group P_1-destroying enzyme and α-galactosidase activity measured with *p*-nitrophenyl α-galactoside. This fraction contained β-galactosidase activity (fig.2) but α-galactosidase *b* could be freed from this contaminant by a second fractionation on Sephadex G-200 after isoelectric focusing. A third enzyme (α-galactosidase *c*; pI 5.39) that hydrolysed nitrophenyl α-galactoside was free from blood group B- or P_1-destroying enzymes (fig.2).

In order to obtain larger amounts of α-galactosidases *b* and *c* the fractions containing blood-group B-destroying enzyme from the Sephadex G-200 column (fig.1) may be subjected to isoelectric focusing. A preparation of B-destroying enzyme free from α-galactosidase *c* may also be prepared by heating the B-destroying enzyme from the Sephadex G-200 column at 50°C for 30 min.

Enzyme assays. Hydrolysis of nitrophenyl α- and β-galactosides and loss of blood-group serological activity were measured as described previously [1].

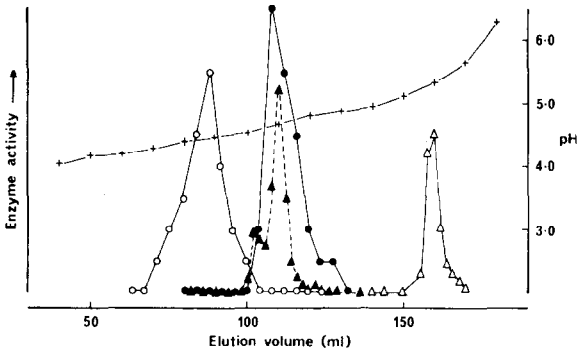

Fig.2. Separation by isoelectric focusing of galactosidases in pooled blood-group P_1-destroying enzyme fractions from Sephadex G-200 column (fig.1). (+) pH gradient; other symbols as in (fig.1).

Fractions from the isoelectric focusing column that were to be used for specificity studies were dialysed against several changes of 0.01 M phosphate buffer pH 6.2 to remove sucrose and ampholytes. To test for the release of galactose from the unlabelled oligosaccharides and glycoproteins, 10 μl of the dialysed enzyme solutions were incubated at 37°C for 4 h with 10 μl of 2% aqueous solutions of the oligosaccharides and for 18 h with 20 μl of 1% aqueous solutions of the glycoproteins. The reaction products were chromatographed in ethyl acetate–pyridine–water (10:4:3, by vol.) on Whatman No. 40 paper for 18 h and the sugars were detected with silver nitrate reagent [11]. The radioactive substrates (10 μl containing 10 000 cpm [^{14}C]galactose) were incubated with 10 μl of enzyme solution for 1 h at 37°C and examined by paper chromatography as described above. The labelled products were detected on a Packard Radiochromatogram Scanner (Model 3201) and counted in a Nuclear-Chicago Mark II Scintillation Counter.

Inhibition by simple sugars of the enzymes that destroy the serological activity of blood-group substances was carried out as described previously [12].

3. Results

Substrate specificity. The enzyme that destroyed blood-group P_1 specificity (α-galactosidase *a*) also destroyed the P^k activity of the hydatid cyst glycoprotein (see [13]) and the residual glycoprotein showed enhanced cross reactivity with horse anti-pneumococcal Type XIV serum (see [3]). These changes in serological activity were accompanied by the release of galactose; no other sugar was detected chromatographically. The destruction of the P_1 serological activity of the glycoprotein by α-galactosidase *a* was inhibited by D-galactose but not by L-galactose, indicating that the enzyme is a D-galactosidase. α-Galactosidase *a* did not induce any detectable chemical or serological change in blood-group B substance, and when tested on low molecular weight substrates of known structure hydrolysed only oligosaccharides containing terminal non-reducing α-D-galactosyl residues linked (1→4) to a subterminal sugar (table 1).

The enzyme that destroys blood-group B specificity, (α-galactosidase *b*) released galactose from blood-group B substance but had no action on the P_1 glycoprotein. As shown earlier [14] loss of B serological activity is accompanied by an increase in the blood-group H activity of the glycoprotein. With low-molecular-weight substrates this enzyme released galactose only from oligosaccharides containing terminal 1→3-linked α-galactosyl residues (table 1).

The enzyme that hydrolyses nitrophenyl α-D-galactoside (α-galactosidase *c*) hydrolyses methyl α-D-galactoside and disaccharides containing α-D-galactosyl residues joined in 1→6-linkage to another sugar (table 1). Occasionally preparations of this enzyme hydrolysed α-Gal 1→3 Gal, and the corresponding galactotriose, although they had no action on α-Gal 1→4 Gal, the B-active glycoprotein or the two radioactive compounds containing ^{14}C-labelled α-galactose linked (1→3) to a subterminal β-galactosyl residue substituted at C-2 position with L-fucose (table 1). The failure of some preparations to show this activity suggests the existence of a fourth α-galactosidase that hydrolyses the α-Gal 1→3 Gal bond only when there is no fucose substituent on the subterminal galactose. The pH gradient of the isoelectric focusing column is relatively steep in the region where α-galactosidase *c* is eluted (fig.2) and a small alteration in conditions might result in the overlap of two enzymes with isoelectric points that differed only slightly.

Heat stability and pH optima. With nitrophenyl α-galactoside as substrate the activity of α-galactosidase *c* is destroyed completely on heating the enzyme solution for 30 min at 40°C. With P_1-active glycoprotein as substrate no loss of α-galactosidase *a* activity is detectable after 30 min at 45°C but at 55°C almost all the activity is lost. α-Galactosidase *b* is the most stable to heat and with blood-group B substance as substrate only slight loss of activity is detectable after 30 min at 60°C. Activity is lost completely when this enzyme is heated at 70°C for 30 min. The pH optimum of all three α-galactosidases is pH 6.0–6.5.

4. Discussion

Few reports have appeared on α-galactosidases that are specific for positional linkages in the substrate molecules. The finding that the enzyme in *T. foetus* that destroys blood-group B specificity (α-galactosidase *b*) released galactose from low-molecular-weight sub-

Table 1
The release of galactose from glycoproteins and low molecular weight substrates by the α-galactosidases from *T. foetus*

Substrate	α-Galactosidase		
	a (P_1-destroying enzyme)	*b* (B-destroying enzyme)	*c*
P_1-active glycoprotein	+	–	–
B-active glycoprotein	–	+	–
p-Nitrophenyl α-D-galactoside	–	–	+
o-Nitrophenyl α-D-galactoside	–	–	+
Methyl α-D-galactoside	–	–	±[a]
α-Gal 1→2-α-Glc 1→1 glycerol	–	–	–
α-Gal 1→3 Gal	–	+	(+)–[b]
α-Gal 1→3-α-Gal 1→3 Gal	–	+	(+)–[b]
α-[^{14}C]Gal 1→3-[α-Fuc 1→2]-Gal	–	+[c]	–
α-[^{14}C]Gal 1→3-[α-Fuc 1→2]-β-Gal 1→4 Glc	–	+[c]	–
α-Gal 1→4 Gal	+	–	–
α-Gal 1→4-β-Gal 1→4 glucitol	+	–	–
α-Gal 1→4-β-Gal 1→4 GlcNAc	+	–	–
α-Gal 1→6 Glc	–	–	+
α-Gal 1→6 GlcNAc	–	–	+
α-Gal 1→6-α-Glc 1→2-β-Fru	–	–	+
α-Gal 1→6-α-Gal 1→6-α-Glc 1→2-β-Fru	–	–	+

p-Nitrophenyl β-D-galactoside, methyl β-D-galactoside, β-Gal 1→3 GlcNAc, β-Gal 1→4 Glc, β-Gal 1→4 GlcNAc and β-Gal 1→6 GlcNAc were not hydrolysed by α-galactosidases, *a*, *b* or *c*.
[a]α-Galactosidase *c* gave only partial hydrolysis of this compound.
[b]Hyrolysis observed only occasionally with preparations of α-galactosidase *c* (see text)
[c]100% of the [^{14}C]galactose was released by α-galactosidase b; none was released by α-galactosidases *a* or *c*.

+Galactose released
–No Galactose released

Abbreviations: Gal, D-galactose; Glc, D-glucose Fuc, L-fucose GlcNAc, *N*-acetyl-D-glucosamine; Fru, D-fructose

strates only when the terminal non-reducing α-galactosyl residue was linked (1→3) to the subterminal sugar is, however, in agreement with the known structure of the blood-group B determinant [15,16] which contains an α-D-galactosyl residue linked (1→3) to a β-D-galactosyl unit substituted at the 2-position with an α-L-fucosyl residue. A number of α-galactosidases of bacterial origin, and also the enzyme from coffee beans (see [17]), destroy blood-group B activity but the specificity of these enzymes has not been established in terms of their preferences for positional linkages. Coffee-bean α-galactosidase was without action on the P_1 substance under conditions which readily inactivated blood-group B substance [3], although Anstee and Pardoe [18] found some diminution of P_1 activity on more prolonged incubation with this enzyme. Naiki and Marcus [19] removed the terminal galactosyl residue of a P_1 glycolipid by treatment with fig α-galactosidase [20] but the detailed specificity requirements of the fig enzyme are not known. The finding that the *T. foetus* enzyme that destroys P_1 activity (α-galactosidase *a*) releases galactose from low-molecular-weight substrates only when the α-galactosyl residue is linked (1→4) to the subterminal sugar is in agreement with the structure, α-Gal 1→4-β-Gal 1→4 GlcNAc, established for the P_1

determinant in the glycoprotein from hydatid cyst fluid [4] and for the P_1 active glycolipid isolated from human red-cell membranes [21]. The enhanced reactivity of the residual glycoprotein with Type XIV pneumococcal serum after removal of the terminal α-galactosyl residue is to be expected from the exposure of the β-Gal 1→4 GlcNAc structure [22]. The destruction of the P^k activity of the hydatid cyst glycoprotein by the P_1 destroying enzyme is also reconcilable with the fact that both the P_1 and P^k determinants contain terminal α-Gal 1→4 Gal units [4,19,21].

Whether the *T. foetus* α-galactosidases are absolutely linkage specific can only be determined by more precise kinetic studies but the much greater facility with which each hydrolyses one positional linkage rather than others renders them very useful for establishing the structure either of oligosaccharides isolated from glycoproteins or of compounds formed in biosynthesis experiments.

Acknowledgements

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