The binding of human liver acid β -galactosidase to wheat-germ lectin is influenced by aggregation state of the enzyme

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At low ionic strength and pH 6.0 human liver acid β -galactosidase exists in two aggregation states, monomeric and multimeric. These species can be separated on wheat-germ lectin–Sephadex, Cellogel electrophoresis and gel filtration on Sephadex G-200, and are not normally interconvertible. On re-application of either form to wheat-germ lectin–Sephadex the equilibrium is re-established and the two forms are interconverted.

Lectins are able to interact with specific sugars either free or glycosidically linked, and this property has been used extensively in the purification of lysosomal hydrolases, many of which are glycoproteins (see Dulaney, 1979a). In most cases the lectin concanavalin A, linked covalently to Sepharose 4B, has been used. This lectin binds mainly to oligosaccharide moieties containing α -D-mannose either as the non-reducing terminal sugar or internally glycosidically linked, but interactions with α -D-glucose and *N*-acetyl- α -D-glucosamine residues have been reported (Goldstein, 1976). Another lectin that has been used to study lysosomal hydrolases is wheat-germ lectin, which interacts specifically with *N*-acetyl-D-glucosamine (Nagata & Burger, 1974), *N*-acetylneuraminic acid and *N*-acetyl-D-galactosamine (Peters *et al.*, 1979; Monsigny *et al.*, 1980).

Acid β -galactosidase purified from human liver has been shown to contain approx. 75 μ g of carbohydrate/mg of protein, the predominant sugars being D-mannose, *N*-acetyl-D-glucosamine, D-galactose and *N*-acetyl-D-neuraminic acid (Frost *et al.*, 1978). This enzyme binds strongly to concanavalin A, and may be eluted with α -methyl D-mannoside (Norden & O'Brien, 1974; Fiddler *et al.*, 1979). However, the binding to wheat-germ lectin is complex and suggests an intrinsic heterogeneity in the enzyme. Norden & O'Brien (1974) reported that only 60% of the β -galactosidase bound specifically to this lectin, and that on re-application of this bound enzyme 95% of the activity was again bound. In contrast, Fiddler *et al.* (1979) found that over 90% of the enzyme was bound to lectin, but that only 65% of this activity could be eluted with a competitive sugar. The resolution of two components with β -galactosidase activity on gel electro-

phoresis (O'Brien, 1975; de Wit *et al.*, 1977) is further evidence of possible microheterogeneity.

We have recently reported that the activity, stability and aggregation state of β -galactosidase is highly dependent on pH and ionic strength (Heyworth *et al.*, 1981a). The present study was undertaken to re-assess the heterogeneity of β -galactosidase, as evidenced by lectin binding and electrophoresis, and to determine the aggregation states of the resolvable components.

Experimental

Materials

Concanavalin A linked to Sepharose 4B and Sephadex G-200 were from Pharmacia, Hounslow, Middlesex, U.K. Wheat-germ lectin linked to Sepharose 6MB, α -methyl D-glucoside, α -methyl D-mannoside, *N*-acetyl-D-glucosamine and disodium β -glycerophosphate (grade II) were from Sigma Chemical Co., Poole, Dorset, U.K. Cellogel sheets (17 cm $\times$ 10 cm) were from Whatman Lab-sales, Maidstone, Kent, U.K. 4-Methylumbelliferyl β -D-galactoside was from Koch–Light Laboratories, Colnbrook, Bucks., U.K.

Human livers, which appeared normal on gross inspection, were obtained at autopsy and used fresh or after storage at -20°C for less than 6 months.

Preparation of enzyme

For most experiments, a supernatant from a crude liver homogenate was used. All stages were performed at 4°C . A liver homogenate was prepared by suspending human liver (1 g) in 10 mM-sodium phosphate buffer, pH 6.0 (10 ml), in a Potter–Elvehjem homogenizer. The homogenate was soni-

cated three times for 30 s with a Kerry ultrasonicator (Kerrys Ultrasonics, Basildon, Essex, U.K.) tuned to maximum frequency, and then centrifuged at 48 000 g for 30 min to obtain a clear supernatant.

In some experiments partially and highly purified β -galactosidase prepared as previously described (Heyworth *et al.*, 1981a) were used.

Assay of β -galactosidase activity

A stopped assay method with 0.5 mM-4-methylumbelliferyl galactoside in 50 mM-sodium acetate buffer, pH 4.5, containing 100 mM-NaCl was used (Heyworth *et al.*, 1981a).

Lectin binding

The method of Fiddler *et al.* (1979) was used after modification. A column of Sepharose linked to either concanavalin A or wheat-germ lectin (1 ml) was equilibrated with 10 mM-sodium phosphate buffer, pH 6.0, before use. A 1 ml portion of enzyme solution was applied and washed through with 2 ml of equilibration buffer. The column was further washed with 5 ml of equilibration buffer supplemented with 500 mM-NaCl and allowed to warm to room temperature (about 20°C).

Specific elution of the adsorbed enzyme activities was achieved with 1 M-monosaccharide competitor in 10 mM-Tris/HCl buffer, pH 7.0, containing 500 mM-NaCl. This competitor was α -methyl D-mannoside or α -methyl D-glucoside for concanavalin A-Sepharose columns and N-acetyl-D-glucosamine for wheat-germ lectin-Sepharose columns. Fractions of volume 1 ml were collected throughout the procedure. If partially or highly purified enzyme preparations were used, they were dialysed exhaustively at 4°C against 10 mM-sodium phosphate buffer, pH 6.0, before application.

Cellogel electrophoresis

A modification of the method described by de Wit *et al.* (1977) was used. The running buffer was 50 mM-disodium β -glycerophosphate adjusted to pH 6.2 with orthophosphoric acid, and the cellulose acetate strips were maintained at a constant amper-

age with an initial voltage of 200 V for 3 h at 4°C. Location of the enzyme activity was achieved by incubating the Cellogel with buffered substrate and examination under an u.v. light.

When quantitative results were required, the gel was cut into strips (10 mm $\times$ 2 mm) after electrophoresis and assayed for β -galactosidase as indicated above.

Gel filtration

Calibrated columns of Sephadex G-200 were prepared as described previously (Heyworth *et al.*, 1981a) and eluted with 20 mM-Pipes (1,4-piperazinediethanesulphonic acid)/NaOH buffer, pH 6.0, containing 100 mM-NaCl. All samples were dialysed exhaustively against this buffer before application.

Results

Lectin binding

Acid β -galactosidase binds strongly to concanavalin A-Sepharose at pH 6.0, and over 95% of the activity can be specifically eluted with 1 M- α -methyl D-mannoside at pH 7.0. Elution with 1 M- α -methyl D-glucoside under the same conditions gives a broader elution profile with only 60% recovery. The small amount of neutral β -galactosidase activity assayed under these conditions does not bind to the lectin.

Separation of β -galactosidase into two components is observed on application to a wheat-germ lectin-Sepharose column. At low flow rates (0.2 ml/min) over 60% of the applied activity is bound, whereas at higher flow rates (2 ml/min) only approx. 20% is bound. For this reason, samples were allowed to equilibrate with the lectin for 15 min before low-salt washing. The ratio bound/unbound enzyme is independent of the state of purity of the applied sample (Table 1). Unbound enzyme increases markedly if the procedure is performed in the presence of 500 mM-NaCl or at 22°C, but is independent of the buffer used to maintain pH 6.0.

The K_m of both the bound and the unbound enzyme against 4-methylumbelliferyl galactoside

Table 1. *Binding of β -galactosidase to wheat-germ lectin-Sepharose*
For experimental details see the text. Percentage recovery is defined with respect to the activity applied.

Stage of purification	Recovery unbound (%)		Recovery bound (%) Eluate
	Application and low-salt washing	High-salt washing	
(1) Crude liver homogenate	27	6	63
(2) Fraction from batch separation on DEAE-cellulose	26	8	63
(3) Fraction from separation on concanavalin A-Sepharose	24	7	68
(4) Fraction from affinity chromatography on <i>p</i> -amino-phenyl thiogalactoside	25	8	66

was found to be 0.25 mM, a similar value to that for the unfractionated enzyme.

The stability of the fractionated enzyme to preincubation for 2 h at 37°C in 20 mM-Pipes buffer, pH 6.0, containing 100 mM-NaCl is markedly less than that of unfractionated enzyme. The unfractionated enzyme is relatively stable under these conditions, losing only 10% of its activity over a 2 h preincubation (Heyworth *et al.*, 1981a), whereas both bound and unbound enzyme lose over 90% of their activity over this period.

Cellogel electrophoresis

The normal pattern showing resolution into two components moving to the anode is observed on electrophoresis of crude enzyme. These components have been separated by wheat-germ lectin binding, as shown by the presence of only the more-anodal component in the unbound enzyme and only the

Fig. 1. Cellogel electrophoresis of β -galactosidase fractions from wheat-germ lectin-Sepharose chromatography

For further details see the text. (a) Unfractionated enzyme; (b) unbound enzyme; (c) bound enzyme.

less-anodal component in the bound enzyme (Fig. 1).

Gel filtration

On Sephadex G-200, the enzyme that did not bind to wheat-germ lectin was resolved mainly into low-molecular-weight forms, with a 27% recovery. In contrast, higher-molecular-weight forms, with a 41% recovery, are apparent in the bound enzyme (Fig. 2). Both fractions appear to contain some dimeric enzyme (A_2). Unfractionated enzyme has been shown previously to be mainly dimeric, with an 84% recovery. Re-equilibration to give the dimer as the major species does not occur when isolated monomeric or multimeric fractions are re-applied to gel-filtration columns under the same conditions.

Rechromatography of unbound and bound enzyme on wheat-germ lectin-Sepharose

Unbound enzyme was re-applied immediately after the first chromatography, and the bound enzyme was exhaustively dialysed against a low-ionic-strength buffer, pH 6.0, before re-application. The results are summarized in Table 2. Both the unbound and the bound enzyme separate on

Fig. 2. Gel filtration on Sephadex G-200 of β -galactosidase fractions from wheat-germ lectin-Sepharose chromatography

Elution was at 4°C with 20 mM-Pipes buffer containing 100 mM-NaCl. For further details see the text. Δ , Unbound fraction; $\blacktriangle$, bound fraction. A_3 , A_2 and A_1 represent the calculated elution positions of multimer, dimer and monomer respectively.

Table 2. *Re-chromatography of unbound and bound enzyme on wheat-germ lectin-Sephadex*
For experimental details see the text.

Run no.	Sample	Recovery unbound (%)	Recovery bound (%)
1	Unfractionated enzyme	32	67
2	Unbound enzyme from run no. 1	34	65
3	Bound enzyme from run no. 1	29	64
4	Unbound enzyme from run no. 2	31	61
5	Bound enzyme from run no. 2	30	63
6	Unbound enzyme from run no. 3	28	59

re-application into two fractions, with the same ratio of activities observed in the original separation, suggesting the re-establishment of an equilibrium between the two forms of the enzyme, which exist at low ionic strength and pH 6.0, on binding to wheat-germ lectin.

Discussion

Our results on the binding of β -galactosidase to wheat-germ lectin are very similar to those obtained by Norden & O'Brien (1974) but in marked contrast with those reported by Fiddler *et al.* (1979), who observed 90% binding of the enzyme but only a 60% specific elution. We have shown that this discrepancy is not a function of the purity of the enzyme used, since even highly purified enzyme resolves into two components with the same ratio as for crude liver supernatants. Neutral β -galactosidase has been ruled out as one of the components, since this enzyme does not bind to concanavalin A and would be absent from purified enzyme preparations.

The gel filtration of the isolated fractions from the wheat-germ lectin-Sephadex chromatography suggests that these fractions are different aggregation species of the enzyme and are not interconvertible. Our previous suggestions on the relative stability and activity of the monomeric, dimeric and multimeric aggregation states of β -galactosidase (Heyworth *et al.*, 1981a) find support in the present study. Both the unbound enzyme, which is mainly monomeric, and the bound enzyme, which is mainly multimeric, are far less stable than the unfractionated enzyme, which is dimeric in high salt concentrations at pH 6.0. The recoveries of bound and unbound enzyme on gel filtration support the idea that at 4°C the enzyme exists in a stable equilibrium of aggregation states, and that it is only if the temperature is increased or these aggregation forms are separated during gel filtration that the equilibration is shifted and losses of enzymic activity are observed.

The finding that the bound and the unbound enzyme fractions have different electrophoretic mobilities on Cellogel provides direct evidence that

this mobility is correlated with the aggregation state of the enzyme, in agreement with Cheetham & Dance (1976). It seems probable that, during aggregation, some masking of negative charge has taken place, giving a lowered electrophoretic mobility of the multimeric species.

The nature of the specific interaction of β -galactosidase with wheat-germ lectin is less clear. The enzyme is known to contain *N*-acetyl-D-glucosamine and *N*-acetyl-D-neuraminic acid residues (Frost *et al.*, 1978), both of which could interact with the lectin. Treatment of β -galactosidase with neuraminidase has been shown to convert the more-anodal electrophoretic component to one that is slower-moving (Cheetham & Dance, 1976; Hoeksema *et al.*, 1980). Since the enzyme bound to wheat-germ lectin is a slower-moving component, it would appear that it is the *N*-acetyl-D-glucosamine residues that are primarily responsible for the interaction of β -galactosidase with wheat-germ lectin.

The re-establishment of the equilibrium between the monomeric and multimeric forms of β -galactosidase on re-application of either the bound or the unbound fraction to wheat-germ lectin-Sephadex is most unexpected, and was not observed by Norden & O'Brien (1974). A similar phenomenon was observed by Dulaney (1979b) when caeruloplasmin was re-applied to concanavalin A-Sephadex. A possible explanation is that, on binding of β -galactosidase to wheat-germ lectin by the specific sugar residues, these are no longer available to influence the aggregation state of the enzyme, and inter-conversion of monomer and multimer is allowed. The re-equilibration invalidates the suggestion of microheterogeneity in the carbohydrate content put forward by Norden & O'Brien (1974).

Although the results reported in the present paper are with human liver acid β -galactosidase, essentially similar results have been obtained with human fibroblasts grown in culture (Heyworth *et al.*, 1981b).

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References

- Cheetham, P. S. J. & Dance, N. E. (1976) *Biochem. J.* **157**, 189–195
- de Wit, J., Hoeksema, H. L., Halley, D., Hagemeijer, A., Bootsma, D. & Westerveld, A. (1977) *Somat. Cell Genet.* **3**, 351–363
- Dulaney, J. T. (1979a) *Mol. Cell. Biochem.* **21**, 43–63
- Dulaney, J. T. (1979b) *Anal. Biochem.* **99**, 254–267
- Fiddler, M. B., Ben-Yoseph, Y. & Nadler, H. L. (1979) *Biochem. J.* **177**, 175–180
- Frost, R. G., Holmes, E. W., Norden, A. G. W. & O'Brien, J. S. (1978) *Biochem. J.* **175**, 181–188
- Goldstein, I. J. (1976) in *Concanavalin A as a Tool* (Bittiger, H. & Schnebli, H. P., eds.), pp. 55–65, John Wiley and Sons, London
- Heyworth, C. M., Neumann, E. F. & Wynn, C. H. (1981a) *Biochem. J.* **193**, 773–779
- Heyworth, C. M., Kirkham, S. & Wynn, C. H. (1981b) *Biochem. Soc. Trans.* **9**, 397–398
- Hoeksema, H. L., de Wit, J. & Westerveld, A. (1980) *Hum. Genet.* **53**, 241–248
- Monsigny, M., Roche, A. C., Sene, C., Maget-Dana, R. & Delmotte, F. (1980) *Eur. J. Biochem.* **104**, 147–153
- Nagata, Y. & Burger, M. M. (1974) *J. Biol. Chem.* **249**, 3116–3122
- Norden, A. G. W. & O'Brien, J. S. (1974) *Biochem. Biophys. Res. Commun.* **56**, 193–198
- O'Brien, J. S. (1975) *Clin. Genet.* **8**, 303–313
- Peters, B. P., Ebisu, S., Goldstein, I. J. & Flashner, M. (1979) *Biochemistry* **18**, 5505–5511