Purification and immunological quantification of rat liver lysosomal glycosidases

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Although lysosomal enzyme activities are known to vary in response to numerous physiological and pharmacological stimuli, the relationship between lysosomal enzyme activity and enzyme concentration has not been systematically studied. Therefore we developed radioimmunoassays for two lysosomal glycosidases in order to determine lysosomal enzyme concentration. β -Galactosidase and β -glucuronidase were purified from rat liver 2780-fold and 1280-fold respectively, by using differential centrifugation, affinity chromatography, ion-exchange chromatography and molecular-sieve chromatography. Polyclonal antibodies to these enzymes were raised in rabbits, and two radioimmunoassays were established. Antibody specificity was shown by: (i) selective immunoprecipitation of enzyme activity; (ii) identical bands of purified enzyme on SDS/polyacrylamide-gel electrophoresis and immunoelectrophoresis; (iii) single immunoreactive peaks in molecular-sieve chromatography experiments. Sensitivities of the assays were such that 15 ng of β -galactosidase and 45 ng of β -glucuronidase decreased the ratio of bound to free radiolabel by 50%; minimal detectable amounts of immunoreactive enzymes were 2 ng and 10 ng respectively. The assays were initially used to assess the effects of physiological perturbations (i.e. fasting and age) on enzyme concentrations in rat liver; these experiments showed that changes in enzyme concentrations do not always correlate with changes in enzyme activities. This represents the first report of radioimmunoassays for lysosomal glycosidases. The results suggest that these radioimmunoassays provide useful technology for the study of regulatory control mechanisms of the concentrations of lysosomal glycosidases in mammalian tissues.

INTRODUCTION

Lysosomes are membrane-bound organelles containing hydrolytic enzymes that exhibit acid pH optima [1,2]. The major function of lysosomes is intracellular digestion, and lysosomal hydrolases are capable of degrading virtually all proteins, lipids and carbohydrates [2,3]. Lysosomal hydrolases can be categorized into groups depending on their substrate-specificities. One class of lysosomal enzymes, the glycosidases, hydrolyse the hemiacetal bonds of glycosides and polysaccharides [4]. Since glycosidases are also active against a variety of synthetic substrates, activity measurements can be accurately made by a variety of assays, including fluorimetric assays with methylumbelliferone substrates [5].

Although changes in assayable enzyme activity measured by fluorimetric assays may reflect true alterations in the mass of the enzyme protein, changes in activity may also reflect differences in the amounts of activators or inhibitors in the sample without a change in the mass of the enzyme protein [6]. In addition, an altered catalytic state of the same number of enzyme molecules, as may occur during aging or from covalent modification by phosphorylation, may cause changes in assayable enzyme activity [6,7]. Thus techniques that measure the actual mass or concentration of enzyme proteins may provide useful information. Moreover, simultaneous determinations of enzyme activity (i.e. units) by fluorimetric techniques and enzyme mass (i.e. nanograms) by an additional method would allow cal-

culation of the true specific activity of the enzyme protein (i.e. the units of enzyme activity per nanogram of enzyme protein) [8]. Such data can provide insight into the molecular mechanisms (e.g. number of enzyme molecules, alterations in catalytic state or changes in concentrations of inhibitors or activators) that account for alterations in enzyme activity observed under a variety of experimental conditions [8,9].

For these reasons, our aim was to develop radioimmunoassays for two lysosomal glycosidases: β -galactosidase (EC 3.2.1.23), which cleaves non-reducing terminal β -galactosyl residues from glycoproteins, glycosaminoglycans, oligosaccharides and glycolipids, and β -glucuronidase (EC 3.2.1.31), which cleaves non-reducing terminal β -glucuronosyl residues from glycosaminoglycans and conjugated steroids and drugs. We validated these assays by establishing their specificity, sensitivity and reproducibility, and applied them by assessing the effects of prolonged fasting and age on glycosidase activities and concentrations in rat liver.

METHODS

Analytical procedures

Assays for activities of β -galactosidase, β -glucuronidase and *N*-acetyl- β -glucosaminidase (EC 3.2.1.30) were performed in accordance with LaRusso & Fowler [5]. Malate dehydrogenase was determined from the rate of oxygenation of NADH, measured at 340 nm in the

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presence of acetic acid, under the conditions described by Dupourque & Kun [10]. Protein determinations were performed by the method of Bradford [11], with bovine serum albumin as standard. Polyacrylamide-gel electrophoresis was performed under both reducing and non-reducing conditions as described by Laemmli [12]. Amino acid composition of the purified proteins was determined, after acid hydrolysis of protein samples (10 µg) in 200 µl of 6 M-HCl containing 2.5 % (v/v) 2-mercaptoethanol for 24 h at 110 °C [13], on a Beckman 7300 automated amino acid analyser. Immunoblotting experiments were performed in accordance with Burnette [14]. For determination of the M_r values of the active enzymes, the purified glycosidases were chromatographed on a Bio-Sil TSK-250 (Bio-Rad Laboratories, Richmond, CA, U.S.A.) column (600 mm × 7.5 mm) by using h.p.l.c. with a Beckman 110B system (Beckman Instruments, Palo Alto, CA, U.S.A.) in a buffer system of 0.1 M-KH₂PO₄, pH 5, at a flow rate of 1.12 ml/min.

Purification of β -galactosidase and β -glucuronidase

(i) **Preparation of a fraction of rat liver enriched in lysosomes.** All procedures were carried out at 4 °C unless otherwise noted. Fifty-four male Sprague-Dawley rats (Harlan Sprague-Dawley, Madison, WI, U.S.A.) were killed by decapitation. The livers were removed, homogenized in iso-osmotic (5%, w/v) sucrose in a Potter-Elvehjem homogenizer (Kontes Glass Co., Vineland, NJ, U.S.A.), as previously described [5], and centrifuged at 700 *g* for 10 min. The supernatant was removed and centrifuged at 55000 *g* for 15 min; the resulting pellet, containing lysosomes and mitochondria, was suspended (0.5 ml/g of liver) in 5 mM-Tris/HCl buffer, pH 7.4, and dialysed overnight against 8 litres of 5 mM-Tris/HCl buffer, pH 7.4. Osmotic rupture of lysosomes was confirmed by determination of the latent activity [2] of β -galactosidase, which was only 2–3 % of total activity. The dialysis residue was centrifuged at 55000 *g* for 90 min, and the supernatant, containing soluble lysosomal enzymes, was 40 % saturated with (NH₄)₂SO₄ and, after 1 h, centrifuged at 14000 *g* for 20 min. The (NH₄)₂SO₄ saturation was increased to 60 %, and, after 1 h, the mixture was centrifuged at 14000 *g* for 20 min. The pellet was suspended in buffer A (50 mM-Tris/HCl buffer, pH 7.4, containing 0.5 M-NaCl, 0.1 mM-dithiothreitol and 0.02 % thimerosal) and dialysed overnight against 4 litres of the same buffer.

(ii) **Affinity chromatography with concanavalin A.** After dialysis, the dialysis residue was applied to a 10 cm × 2.5 cm column of Sepharose 4B-concanavalin A (Pharmacia, Piscataway, NJ, U.S.A.) that had been pre-equilibrated and washed with buffer A. β -Galactosidase and β -glucuronidase were eluted with buffer A containing 1 M-methyl α -glucopyranoside and dialysed overnight against buffer B (10 mM-potassium phosphate buffer, pH 7, containing 1 mM-Na₂EDTA, 2.5 % sucrose, 0.02 % thimerosal and 0.3 mM-calcium phosphate).

(iii) **Hydroxyapatite chromatography.** The sample from the previous step was applied to a high-performance hydroxyapatite (HPHT) system (Bio-Rad Laboratories) that had been pre-equilibrated with buffer B. Over 90 % of β -galactosidase did not bind to the hydroxyapatite column, whereas essentially 100 % of β -glucuronidase was bound. The proteins were eluted from the column

with a linear phosphate (0.01–0.3 mM) gradient at 1 ml/min. Fractions active in β -galactosidase and β -glucuronidase were suspended in buffer C (50 mM-Tris/HCl buffer, pH 7.4, containing 0.02 % thimerosal and 2.5 % sucrose).

(iv) **Molecular-sieve chromatography.** Soluble material from the previous step was applied to a 2.5 cm × 120 cm column of Sephadex G-200 (Pharmacia) and eluted with buffer C at 6 ml/h; 2 ml fractions were collected.

For the final purification step, the partially purified enzymes were chromatographed on two Bio-Sil TSK-250 columns connected in series by using h.p.l.c. (Beckman 110B system). The running buffer was buffer D (50 mM-KH₂PO₄ containing 2.5 % sucrose and 0.02 % thimerosal); the pH was adjusted to 7.4 or to 5.0, and the buffer was run at 1 ml/min. Fractions containing activities of the purified enzymes were concentrated by ultrafiltration and purified enzyme was stored in the running buffer at –80 °C.

Generation of antibodies and development of radioimmunoassays

(i) **Antibody preparation and characterization.** γ -Globulins against rat liver lysosomal β -galactosidase and β -glucuronidase were raised in New Zealand White rabbits. Initially, 200 µg of purified enzyme in complete Freund's adjuvant was injected in multiple places in the back; 4 weeks later, animals were boosted with the same amount of antigen and complete adjuvant; 5–9 weeks later, depending on the titres of antibodies, animals were bled 40–50 ml per animal by ear vein. Ouchterlony double-immunodiffusion studies were done to indicate the presence of antibodies.

For immunotitration experiments, increasing concentrations of antisera (100 µl), diluted in 50 mM-potassium phosphate buffer, pH 7.4, containing 0.1 % bovine serum albumin and 5 % sucrose, were added to a particle-free rat liver homogenate. After addition of normal rabbit serum (3 µl) and goat anti-(rabbit IgG) serum (10 µl) (Pel-Freez Biologicals, Rogers, AR, U.S.A.), the solutions were incubated at 4 °C for 24 h and then centrifuged at 2000 *g* for 20 min. The pellet was washed once with 1 ml of buffer and then resuspended in 100 µl of solution containing 250 mM-sucrose, 3 mM-imidazole and 0.1 % ethanol. Portions (5 µl) of supernatant were diluted in 95 µl of this same solution. Assays for activities of β -galactosidase and β -glucuronidase were performed on samples of both the pellet and supernatant.

(ii) **Radiolabelling of β -galactosidase and β -glucuronidase and characterization of labelled enzymes.** Enzymes were iodinated by the chloramine-T (Eastman Kodak Laboratories, Rochester, NY, U.S.A.) method [15]. Na¹²⁵I (500 µCi) (Amersham, Arlington Heights, IL, U.S.A.) in NaOH solution was added to 25 µg of β -galactosidase or β -glucuronidase in 10 µl of 0.5 M-KH₂PO₄, pH 7.5. Iodination by 25 µl of 0.1 % chloramine-T in the same buffer was allowed to proceed for 30 s. The reaction was stopped by addition of 100 µl of 0.1 % sodium metabisulphite. Approx. 3–10 ¹²⁵I atoms were introduced for every 10 enzyme molecules. The labelled enzymes were separated from free I₂ by chromatography through a 1 cm × 15 cm Bio-Gel P-2 column equilibrated with 50 mM-KH₂PO₄, pH 7.5. ¹²⁵I-

labelled enzyme was then repurified by chromatography on a Sephadex G-200 column equilibrated with buffer D, pH 7.4. Radiolabelled enzymes were stored in buffer D, pH 7.4, at -20°C at a concentration of 2×10^6 c.p.m./ml.

The amount of trichloroacetic acid-precipitable ^{125}I - β -galactosidase and ^{125}I - β -glucuronidase was determined [16]. Competitive binding of the different portions of the peak of ^{125}I radioactivity was assessed for both enzymes. Elution profiles of the activity in an enzyme-enriched fraction of rat liver and of the radioactivity in the ^{125}I -labelled proteins were compared after h.p.l.c. over two Bio-Sil TSK-250 columns. The extent of binding of the ^{125}I -labelled enzymes were studied for up to 24 weeks after iodination.

(iii) Radioimmunoassays: generation of competitive binding curves. Assays were conducted in a final volume of 0.4 ml in a buffer containing 50 mM- KH_2PO_4 , 1% bovine serum albumin and 0.02% thimerosal, pH 7.8. To generate competitive binding curves, increasing quantities (0–100 ng) of purified β -galactosidase or β -glucuronidase (100 μl), a constant amount of the respective antiserum (200 μl) and a constant amount of ^{125}I -labelled β -galactosidase or β -glucuronidase (100 μl) were added in that order to the assay tubes. The amount of antiserum was 200 μl of 1:5000 dilution for β -galactosidase and 1:1000 dilution for β -glucuronidase. These amounts of antisera were sufficient to precipitate 35–50% of the labelled enzyme. The amount of ^{125}I - β -galactosidase was 4 ng containing 10000 c.p.m. The amount of ^{125}I - β -glucuronidase was 40 ng containing 10000 c.p.m. All reaction mixtures were made in duplicate or triplicate and maintained at 4°C for 72 h. Bound label was separated from free by addition of a 1:25 dilution of normal rabbit serum (100 μl) and a 1:10 dilution of goat anti-(rabbit IgG) serum (100 μl) to each assay tube, and the tubes were incubated for 24 h at 4°C . After addition of 1 ml of buffer, centrifugation and aspiration of the supernatant, radioactivity in the pellet was counted for 5 min with a Packard Multi-Prias 4 γ -radiation counter (Packard Instruments, Downers Grove, IL, U.S.A.). Binding data were analysed by means of a computer program on the RDR 2002 instrument from ISO-Data, which was directly connected to the Packard Multi-Prias 4 counter. Results were expressed as $(B - \text{NSB}) / (B_0 - \text{NSB})$, where B is the total radioactivity (c.p.m.) in the presence of standards or unknown samples, NSB is the total radioactivity (c.p.m.) of the non-specific background, and B_0 is the total radioactivity (c.p.m.) in the absence of standard or unknown samples. A standard curve was generated by using ISO-Data's 'French' curve fit [17] and data were plotted on semi-logarithmic paper.

Liver samples were prepared for analyses as follows. Rats were killed by decapitation; the liver was excised and placed on ice in a buffer containing 250 mM-sucrose, 3 mM-imidazole and 0.1% ethanol, pH 7.4. The liver was minced and homogenized in the above buffer until a homogeneous 10-fold-diluted suspension was obtained.

The homogenates were frozen and thawed three times to disrupt all lysosomes, and centrifuged at 55000 g for 40 min to yield a particle-free supernatant. Competitive binding curves were generated by using 1:1 to 1:10000 dilutions of liver supernatants.

(iv) Evaluation and application of radioimmunoassays.

The following parameters were studied in an effort to optimize the assays: time of first incubation, final assay volume, buffer pH, incubation temperature, concentration of carrier (normal rabbit serum), concentration of second antibody [goat anti-(rabbit IgG) serum], effect of a detergent (Tween-20), effect of a proteinase inhibitor (aprotinin) and the use of an alternative to second antibody (*Staphylococcus* Protein A).

To determine the co-ordinancy of elution patterns for enzyme activity and immunoreactivity, a liver fraction enriched in soluble lysosomal enzymes was chromatographed on two 30 cm TSK-250 columns connected in series; 1 ml fractions were collected and analysed for enzyme activity and immunoreactivity.

For recovery experiments, 5, 10, 25 and 50 ng of purified enzymes were added to a buffer and to different dilutions of a fraction of rat liver enriched in soluble lysosomal enzymes. Sensitivities and reproducibilities of each radioimmunoassay were also determined.

To assess the effects of fasting on enzyme activities and immunoreactivities, rats were randomized to either Purina rat chow *ad libitum* and water ($n = 8$) or water only ($n = 8$) for 72 h. To assess the effects of age on enzyme activities and immunoreactivities, young ($n = 4$; approx. 1 month old) or mature ($n = 4$; approx. 4 months old) rats were studied. Homogenates of liver were prepared as described above.

RESULTS

Purification of β -galactosidase and β -glucuronidase

Results of the purification of β -galactosidase and β -glucuronidase are summarized in Table 1. Only 7% of the total activity of malate dehydrogenase, a mitochondrial marker, was found in the lysosome-enriched fraction after osmotic rupture. More than 99% of the activity of each glycosidase was bound to Sepharose 4B-concanavalin A at pH 7.4; addition of 0.5 M-NaCl diminished non-specific ionic binding. The final purification step, consisting of conventional chromatography and h.p.l.c., resulted in single peaks of activity and protein for both enzymes. Purified β -galactosidase had a calculated specific activity of 2.5 units/mg of protein, and β -glucuronidase had a calculated specific activity of 7.7 units/mg of protein. Enzyme activity for each enzyme was stable for 1 year when stored frozen at -80°C in buffer D and was unaffected by freezing and thawing. The pH optima for the activities of the purified enzymes were identical with the pH optima of the enzymes present in rat liver homogenates (β -glucuronidase, pH 4.4; β -galactosidase, pH 3.8), and activities of both purified enzymes were linear with mass and time (results not shown). Estimates of the M_r values of the purified enzymes by gel-filtration chromatography showed a major peak with an M_r of 122000 and a minor peak of approximately twice this size for β -galactosidase, and a single peak with an M_r of 204000 for β -glucuronidase. Under denaturing and reducing conditions, β -galactosidase consisted of one band with an M_r of approx. 62000 and β -glucuronidase of two bands with M_r values of approx. 66000 and 70000 (Fig. 1). Under non-denaturing and non-reducing conditions, β -glucuronidase exhibited only one band on 4% polyacrylamide-gel electrophoresis; enzyme activity co-

Table 1. Purification of lysosomal glycosidases from rat liver

Experimental details are given in the Methods section.

	β -Galactosidase				
	Enzyme (units)	Protein (mg)	Specific activity (units/mg of protein)	Purification (fold)	Enzyme activity yield (%)
Rat liver homogenate	139	154 200	0.0009	1	100
Lysosome-enriched fraction	45	3 600	0.0125	14	32
Concanavalin A chromatography	32	420	0.076	85	23
Hydroxyapatite chromatography	27	62	0.44	480	19
Molecular-sieve chromatography	19.5	7.8	2.5	2780	14

	β -Glucuronidase				
	Enzyme (units)	Protein (mg)	Specific activity (units/mg of protein)	Purification (fold)	Enzyme activity yield (%)
Rat liver homogenate	925	154 200	0.0060	1	100
Lysosome-enriched fraction	202	3 600	0.056	9.4	22
Concanavalin A chromatography	161	415	0.38	65	17
Hydroxyapatite chromatography	73	62	1.2	200	8
Molecular-sieve chromatography	37	4.8	7.7	1280	4

Fig. 1. Analytical SDS/polyacrylamide-gel electrophoresis of purified rat liver β -galactosidase and β -glucuronidase under reducing conditions

Standards (lane 1) and 20 μ g each of purified β -glucuronidase (lane 2) and β -galactosidase (lane 3) were treated and electrophoresed as described in the Methods section.

migrated exactly with protein staining (results not shown).

The amino acid composition of purified lysosomal β -glucuronidase (Table 2) was similar to that previously

Table 2. Amino acid compositions of β -galactosidase and β -glucuronidase from rat liver lysosomes

Amino acid composition was determined on 10 μ g each of purified β -galactosidase and β -glucuronidase after hydrolysis for 20 h as described in the Methods section. Data for each amino acid are expressed relative to lysine. For comparison, we have included previously published amino acid composition data for β -glucuronidase relative to lysine. —, Not detected.

Amino acid	Amino acid composition (mol of residue/mol of lysine)		
	β -Glucuronidase		
	β -Galactosidase	Present work	Tulsiani <i>et al.</i> [19]
Asp	1.92	2.09	2.13
Thr	1.03	1.15	1.27
Ser	1.82	1.97	1.16
Glu	2.08	2.21	2.20
Pro	1.11	1.19	1.14
Gly	3.74	2.77	1.75
Ala	2.48	1.28	1.45
Cys	—	—	—
Val	1.54	1.22	1.39
Met	0.12	0.04	0.30
Ile	0.83	0.88	0.82
Leu	1.89	1.71	1.78
Tyr	1.10	0.28	0.80
Phe	0.90	1.66	0.80
His	0.15	0.40	0.53
Lys	1.00	1.00	1.00
Arg	0.92	0.75	1.08
Trp	—	—	0.80

reported in rat liver by Himeno *et al.* [18], Tulsiani *et al.* [19] and Stahl & Touster [20]. We were unable to find reported information on the amino acid composition of rat liver β -galactosidase for comparison.

Generation of antibodies and development of radioimmunoassays

(i) **Antibody preparation and characterization.** Titres of antisera, defined as 200 μ l of a dilution of serum that bound 35% of 10000 c.p.m. of radiolabelled enzyme (see below), varied for β -galactosidase from 1/7500 to 1/60000 and for β -glucuronidase from 1/1250 to 1/5000. Antisera to β -galactosidase or β -glucuronidase specifically precipitated the activities of each enzyme from the supernatants of enzyme-enriched liver extracts (Fig. 2); activities in the pellet increased proportionately

Fig. 2. Immunotitration of β -galactosidase (a) and β -glucuronidase (b)

The experiment was done as described in the Methods section. Non-immune, anti- β -galactosidase and anti- β -glucuronidase sera were used in 2.5, 5, 10, 20, 40, 80, 160, 320, 640 and 1280 nl quantities or in dilutions from 1:40000 to 1:80. The enzyme activity remaining in the supernatant after immunoprecipitation is shown. (a) $\square$, β -Galactosidase activity with non-immune serum; $\diamond$, β -galactosidase activity with anti- β -galactosidase serum; $\triangle$, β -glucuronidase activity with anti- β -galactosidase serum. (b) $\square$, β -Glucuronidase activity with non-immune serum; $\triangle$, β -glucuronidase activity with anti- β -glucuronidase serum; $\diamond$, β -galactosidase activity with anti- β -glucuronidase serum.

Fig. 3. Immunoblot of purified glycosidases and a lysosome-enriched fraction of rat liver

Lanes 1 and 2 contain β -galactosidase (7 μ g/lane) incubated with non-immune rabbit serum (lane 1) or anti- β -galactosidase serum (lane 2). Lanes 3, 4 and 5 contain proteins (0.2 mg/lane) prepared from a fraction of rat liver enriched in lysosomes (see the Methods section for description of preparation) and incubated with anti- β -galactosidase serum (lane 3), non-immune rabbit serum (lane 4) and anti- β -glucuronidase serum (lane 5). Lanes 6 and 7 contain β -glucuronidase (2 μ g/lane) incubated with anti- β -glucuronidase serum (lane 6) or non-immune rabbit serum (lane 7). Proteins in each lane were exposed to 125 I-Protein A (2×10^5 c.p.m./ml); exposure time for autoradiograms was 17 h.

(results not shown). In high concentrations, the anti-serum to β -glucuronidase also slightly precipitated β -galactosidase activity. Immunoprecipitation curves for heart, spleen, kidney, brain and stomach were similar (results not shown). Western blots of the purified enzymes and of liver fractions enriched in solubilized lysosomal enzymes showed identical patterns and indicated that the antisera recognized specifically the proteins against which they were raised (Fig. 3).

(ii) **Characteristics of radiolabelled glycosidases.** Both 125 I- β -galactosidase and 125 I- β -glucuronidase were eluted as single peaks from a Sephadex G-200 column (results not shown). All components of the peak of radioactivity eluted from the column at the end of the iodination procedure showed identical binding curves with various concentrations of antisera. Binding characteristics of 125 I-labelled enzymes did not change over a period of 24 weeks. We routinely prepared fresh 125 I-labelled enzymes every 8–10 weeks, and used only the peak fraction from the Sephadex G-200 column for radioimmunoassays. On h.p.l.c., the peaks of radioactivity from 125 I-labelled enzymes and activities of unlabelled enzymes in rat liver were eluted in the same fractions (Fig. 4). Iodination did not alter the enzymic activity of β -glucuronidase, but it

Fig. 4. Co-elution of radioactivity and enzyme activity

Portions (10 μ l) of particle-free rat liver homogenate, which contained approx. 30000 c.p.m. of labelled β -galactosidase (a) or β -glucuronidase (b), were chromatographed over two Bio-Sil TSK-250 columns connected in series as described in the Methods section. Fractions (1 ml) were collected, and enzyme activity (●) and radioactivity (○) were measured.

completely inhibited the activity of β -galactosidase. With trichloroacetic acid, over 90 % of 125 I- β -galactosidase and 125 I- β -glucuronidase were precipitable.

(iii) **Development of radioimmunoassays for β -galactosidase and β -glucuronidase.** Purified enzymes or supernatants of tissue homogenates competed similarly with the radiolabelled enzymes, resulting in parallel displacement curves (Fig. 5). Three non-mammalian hydrolases (Fig. 5) and dog, cat, chicken and human liver homogenates (results not shown) did not compete for binding; also, β -galactosidase did not cross-react with antibody to β -galactosidase, and vice versa (Fig. 5).

Optimal conditions for this first incubation included a final incubation volume of 400 μ l, a pH of 7.8, a temperature of 4 °C and an incubation period of 72 h. For the second incubation, optimal conditions included 100 μ l of 1:25 buffer-diluted normal rabbit serum as carrier, 100 μ l of 1:10 buffer-diluted goat anti-(rabbit IgG) serum for separation of bound label from free, and an incubation time of 24 h at 4 °C. The addition of Tween-20 to the assay buffer did not influence the radioimmunoassay for β -glucuronidase. For β -

Fig. 5. Competitive binding curves for rat liver β -galactosidase (a) and β -glucuronidase (b)

Rabbit antisera dilutions were 1:6000 for anti- β -galactosidase serum and 1:40000 for anti- β -glucuronidase serum. (a) ■, Rat liver β -galactosidase; ○, rat liver β -glucuronidase; ◇, bacterial β -galactosidase; △, bacterial β -glucuronidase; □, almond β -glycosidase; ▲, particle-free rat liver homogenate. (b) ■, Rat liver β -glucuronidase; ○, rat liver β -galactosidase; △, bacterial β -glucuronidase; ◇, bacterial β -galactosidase; □, almond β -glycosidase; ▲, particle-free rat liver homogenate.

galactosidase, addition of 0.3 g of Tween-20/l caused a significant increase (30 %) in the antibody reactivity of the high-speed supernatant and a minor increase in immunoreactivity for the purified enzyme. Addition of 1000 kallikrein-inactivator units of aprotinin did not affect binding. Separation of bound label from free with *Staphylococcus* Protein A gave similar results to those with goat anti-(rabbit IgG) antibody. Enzyme activity and immunoreactivity were co-eluted in rat liver homogenates fractionated by h.p.l.c. (Fig. 6).

A 15 ng portion of β -galactosidase and 45 ng of β -glucuronidase decreased the ratio of bound label to free by 50 %. The minimal detectable amounts of immunoreactive β -galactosidase and β -glucuronidase were 2 ng and 10 ng respectively. Recovery of each of the purified enzymes added to an enzyme-enriched rat liver fraction was greater than 95 %. Intra- and inter-assay variation was less than 10 % for both assays.

(iv) **Application of radioimmunoassays.** The effects of fasting and age on lysosomal glycosidase activities and immunoassayable contents in rat liver are shown in Table 3. Fasting caused an increase (75%) in the enzyme activity of β -galactosidase and a quantitatively comparable increase (62%) in the immunoassayable content of β -galactosidase. In contrast, whereas fasting caused an increase (66%) in the enzyme activity of rat liver lysosomal β -glucuronidase, there was essentially no change in the immunoassayable content of this enzyme; as a consequence, the true specific activity of β -glucuronidase increased by approx. 50%. Young rats demonstrated significantly less enzyme activity (24%) and immunoassayable content (28%) for β -galactosidase than did mature rats; however, since these differences were proportionately the same, the true specific activities were virtually identical (Table 3). In contrast, whereas the enzyme activity for β -glucuronidase was significantly greater (59%) in young than in mature rats, the immunoassayable content was virtually identical. As a result, the true specific activity of β -glucuronidase was significantly greater (55%) in young rats.

Fig. 6. Co-elution of enzyme activity and immunoactivity

Portions (10 μ l) of particle-free rat liver homogenate were chromatographed over two Bio-Sil TSK-250 connected in series as described in the Methods section. Fractions (1 ml) were collected, and enzyme activity (○) and immunoactivity (●) were measured for β -galactosidase (a) and β -glucuronidase (b).

Table 3. Lysosomal glycosidase enzyme activity and immunoassayable content in rat liver

Data are means $\pm$ S.E.M. and were analysed by using an unpaired *t* test; N.S., not significant.

	Fed (<i>n</i> = 8)	Fasting (<i>n</i> = 8)	<i>P</i> value
Animal weight (g)	212 $\pm$ 5	152 $\pm$ 3	< 0.01
Liver weight (g)	9.7 $\pm$ 0.4	4.6 $\pm$ 0.2	< 0.01
β -Galactosidase (munits/g of liver)	142 $\pm$ 4	248 $\pm$ 10	< 0.01
(μ g/g of liver)	112 $\pm$ 3	182 $\pm$ 6	< 0.01
(munits/ μ g)	1.26 $\pm$ 0.02	1.36 $\pm$ 0.02	< 0.01
β -Glucuronidase (munits/g of liver)	345 $\pm$ 23	572 $\pm$ 65	< 0.01
(μ g/g of liver)	101 $\pm$ 3	112 $\pm$ 4	N.S.
(munits/ μ g)	3.4 $\pm$ 0.2	5.0 $\pm$ 0.4	< 0.01
	Young (<i>n</i> = 4)	Mature (<i>n</i> = 4)	<i>P</i> value
Animal weight (g)	108 $\pm$ 2.5	418 $\pm$ 2.5	< 0.05
Liver weight (g)	5.2 $\pm$ 0.18	12.1 $\pm$ 0.36	< 0.05
β -Galactosidase (munits/g of liver)	156 $\pm$ 7	194 $\pm$ 14	< 0.05
(μ g/g of liver)	125 $\pm$ 4	161 $\pm$ 13	< 0.05
(munits/ μ g)	1.25 $\pm$ 0.09	1.21 $\pm$ 0.06	N.S.
β -Glucuronidase (munits/g of liver)	400 $\pm$ 49	252 $\pm$ 41	< 0.05
(μ g/g of liver)	99.5 $\pm$ 5	95 $\pm$ 8	N.S.
(munits/ μ g)	4.05 $\pm$ 0.6	2.62 $\pm$ 0.2	< 0.05

DISCUSSION

The lysosomal enzymes β -galactosidase and β -glucuronidase have been previously purified by others: β -galactosidase from mouse macrophages and kidney, from human fibroblasts, from rat and human intestine, and from mouse, cat and human liver [21–28]; β -glucuronidase from hamster and mouse fibroblasts, from mouse kidney and urine, from rat preputial gland and intestine, and from mouse, rat, rabbit and bovine liver [18–20,29–35]. The method of purification presented here has certain differences from these published studies, including the use of affinity chromatography with concanavalin A, as well as the use of h.p.l.c. Our purification method is rapid and relatively simple, and yields results, based on degree of purification, gel electrophoresis and amino acid analysis, comparable with those of other investigators [19,20,25,32,33]. The specific activity of 7.7 units/mg of protein for β -glucuronidase compares reasonably well with the specific activities found by Lusis & Paigen (40 and 13 units/mg of protein) for β -glucuronidase isolated from urine of two strains of mice [31].

On polyacrylamide-gel electrophoresis under denaturing and reducing conditions, β -galactosidase consisted of one band with an apparent M_r of approx. 62000. This result compares closely with data previously published [21,23], in which β -galactosidases isolated from mouse macrophages and human fibroblasts consisted of single protein bands with M_r values of 63000 and 64000 respectively. During molecular-sieve chromatography, at pH 5, the active enzyme consisted of two peaks with apparent M_r values of approximately 2 and 4 times this size. These results are consistent with a previous report indicating that the active molecule of β -galactosidase probably consists of four identical subunits [26]. A small band with an M_r of 41000 co-purified with the active enzyme and was recognized by the antibody; it seems unlikely that this band represents a 'protective protein' as was described recently by Galjart *et al.*, since its M_r does not agree with the values reported for this 'protective protein' (i.e. a heterodimer of M_r 32000 and 20000) [23,36].

Polyacrylamide-gel electrophoresis of β -glucuronidase showed one band under non-denaturing and non-reducing conditions; enzyme activity co-migrated exactly with protein staining. In contrast, two bands of M_r 66000 and 70000 were observed on electrophoresis under denaturing and reducing conditions. Several groups have reported the existence of only one major subunit of β -glucuronidase with an M_r of approx. 75000 [20,21,32,33] or 71000 [31]. Himeno *et al.* also purified lysosomal β -glucuronidase from rat liver; their gels revealed two major bands of M_r 79000 and 74000 and one minor band of M_r 70000 [37]. This compares quite closely with our own results. During molecular-sieve chromatography at pH 5, the active protein had an apparent M_r of approx. 204000, suggesting that the active enzyme is also a tetramer. A fraction of rat liver enriched in lysosomes was prepared as the initial step of the purification, but the method did not otherwise separate between lysosomal and microsomal forms of β -glucuronidase. Therefore, on the basis of our methods, we cannot conclude whether the active molecule consists of four identical subunits or of two pairs of different subunits. Although unlikely, it is also possible that the band of M_r 66000 or the minor

band of M_r 61000 represents egasyn, a protein known to be complexed with microsomal, but not lysosomal, forms of β -glucuronidase in the mouse [38]. Heating a precipitate of β -glucuronidase and its antibody did not result in release of egasyn, as has been shown for the microsomal form of mouse β -glucuronidase (results not shown). Thus it seems unlikely that either one of these bands represents egasyn.

After radiolabelling of the purified enzymes by an oxidative technique [15], enzymic activity of β -glucuronidase was unchanged, whereas the activity of β -galactosidase was completely inhibited. This observation suggests that the catalytic site of β -galactosidase may be enriched in tyrosine residues. Iodination did not significantly alter the size of either enzyme protein, since the peaks of radioactivity of the radiolabelled enzymes and the peaks of activities of unlabelled enzymes were co-eluted on h.p.l.c.

Antisera to purified enzymes were raised in rabbits under standard conditions. Their specificity was established by: (i) selective precipitation of enzyme activities in liver homogenates and activity of antiserum–enzyme precipitate; (ii) identical bands of purified enzyme on polyacrylamide-gel electrophoresis and Western blots, corresponding to bands in liver homogenate; (iii) single immunoreactive peaks in molecular-sieve-chromatography experiments. Furthermore, immunocytochemical localization studies previously performed by us using electron microscopy confirmed the lysosomal origin of the purified enzymes [39]. Binding of antisera with enzymes did not interfere with the activity of either enzyme, suggesting that the catalytic sites (at least with the substrates employed) are not involved in immunological recognition. Moreover, the anti- β -galactosidase serum actually protected the enzyme from loss over time (results not shown). Both antisera also bound to enzymes in the heart, spleen, stomach, kidneys and brain of Sprague–Dawley rats and to rat liver enzymes of Wistar rats (results not shown).

The radioimmunoassays were reproducible over time. Specificity of the radioimmunoassays was shown by: (i) demonstrating parallelism between the curves of the purified enzymes and the dilutions of liver homogenate; (ii) co-elution of enzyme activity and radioimmunoactivity on gel filtration.

Initial application of the two radioimmunoassays showed that changes in immunoreactivity and in enzyme activity could occur independently. Our observations suggest that, although the hepatic activities of both lysosomal glycosidases increase in response to fasting, the mechanism of increased enzyme activity differs. For β -galactosidase, the actual amount of enzyme protein in liver increases; for β -glucuronidase, the catalytic activity alone is altered. Although other interpretations are possible, and although additional studies assessing transcriptional and translational regulatory events in response to perturbations will be necessary, our results raise the intriguing possibility that two related enzyme proteins (i.e. glycosidases) in one organelle (i.e. lysosomes) may respond to a single perturbation (i.e. fasting) in a similar fashion (i.e. increased enzymic activity) via different regulatory mechanisms.

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