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USE OF IMMOBILIZED ANTIBODIES IN INVESTIGATING ACID α-GLUCOSIDASE IN URINE IN RELATION TO POMPE'S DISEASE

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Summary

(1) A simple method is described for the isolation of the lysosomal enzyme, acid α-glucosidase (α-D-glucoside glucohydrolase, EC 3.2.1.20) from normal human liver. Antibodies raised against the purified enzyme were immobilized by covalent coupling to Sepharose 4B.

(2) Acid α-glucosidase can be quantitatively removed from normal urine by incubating with an excess of immobilized antibody. With *p*-nitrophenyl-α-glucoside as substrate, acid α-glucosidase accounts for 91 ± 3% of the total α-glucosidase activity at pH 4.0 in normal urine.

(3) In urine from a patient with the infantile form of Pompe's disease ('acid maltase deficiency'), no α-glucosidase activity could be removed by the immobilized antibody, in agreement with the fact that acid α-glucosidase is absent in these patients.

(4) In urine from patients with the late-onset form of Pompe's disease, 46 ± 11% of the α-glucosidase activity at pH 4.0 can be removed by incubation with immobilized antibodies, indicating that residual acid α-glucosidase activity is present in urine of these patients. The residual acid α-glucosidase activity amounts to about 5% of that in the urine of control persons.

(5) If acid α-glucosidase is adsorbed to immobilized antibodies, the activity can still be measured with *p*-nitrophenyl-α-glucoside as substrate. The K_m for *p*-nitrophenyl-α-glucoside is not significantly changed by adsorbing purified acid α-glucosidase to immobilized antibodies.

(6) The properties of acid α-glucosidase from urine of patients with late-

onset Pompe's disease were compared with those of acid α-glucosidase from normal urine, both adsorbed to immobilized antiserum. The pH-activity profile of the enzyme from urine of patients with late-onset Pompe's disease can not be distinguished from that of the normal urinary enzyme. The K_m for *p*-nitrophenyl-α-glucoside of the two enzymes is identical, both at pH 4 and 3. The titration curves of the two enzymes with immobilized antibodies are identical.

Introduction

Glycogen storage disease Type II (Pompe's disease [1]) is characterized by a deficient activity of acid α-glucosidase [2], a lysosomal enzyme [2—4], causing accumulation of glycogen in the lysosomes [2]. In the infantile form of the disease, death usually occurs in the first year of life [1]. Acid α-glucosidase hydrolyses both α(1 → 4) and α(1 → 6) glucosidic linkages [5,6], thus bringing about the complete breakdown of glycogen to glucose. Deficient activity of the enzyme has been demonstrated in many different types of tissues and body fluids in Pompe's disease (see for example Refs. 2—9).

The biochemical diagnosis of Pompe's disease is complicated by the occurrence of at least two isoenzymes of α-glucosidase in human tissues and body fluids. The lysosomal enzyme has a pH optimum at about 4.5, and the microsomal enzyme is a neutral α-glucosidase with a pH optimum at 6.5 [3,7]. In addition, a distinct isoenzyme of α-glucosidase may be present in kidney [10]. The microsomal enzyme hydrolyses maltose and the artificial substrates *p*-nitrophenyl-α-glucoside and 4-methylumbelliferyl-α-glucoside, but has little if any activity towards glycogen [11,12]. Only the lysosomal enzyme is deficient in Pompe's disease [7]. Since neutral α-glucosidase has considerable activity at acidic pH, particularly with maltose as the substrate, this isoenzyme may interfere with the determination of acid α-glucosidase activity [8,13]. For this reason, the ratio between the activities at pH 4.5 (or 4.0) and pH 6.5 has frequently been used as a diagnostic indication for Pompe's disease [13,14].

Pompe's disease is clinically heterogeneous (for a review, see Ref. 15), and besides the infantile form of the disease, late-onset forms (juvenile and adult) have been described. Symptoms like glycogen storage are usually restricted to skeletal muscle [15—17]. In cells and tissues of cases with the infantile form of Pompe's disease there is a virtually absolute deficiency of acid α-glucosidase and absence of cross-reactive immunological material [18,19]. De Barsy et al. [20] and others [17,21,22] have shown that residual acid α-glucosidase activity (7—22% or normal) is present in patients with the late-onset form of the disease.

The diagnosis of the infantile and the late-onset forms of Pompe's disease can be facilitated by the use of antibodies against acid α-glucosidase [17—19, 22]. Furthermore, the complex between acid α-glucosidase and antibodies against the enzyme still exhibits activity towards low molecular weight substrates [18,19]. These facts prompted us to use immobilized antibodies against acid α-glucosidase in a study of Pompe's disease. This method has two advantages. First, quantitation is facilitated (cf. Ref. 23), and second, the kinetic parameters of the adsorbed α-glucosidase can be studied (cf. Ref. 24)

without interference from neutral α-glucosidase. This paper reports a study of acid α-glucosidase in urine from normal persons, from a patient with the infantile form of Pompe's disease and from six patients with the late-onset form of the disease.

Materials and Methods

Purification of acid α-glucosidase from human liver

Liver tissue was obtained at autopsy within 6 h after death from a control subject, frozen immediately, and stored at −20°C until use. The control liver showed no manifest pathological changes on morphological examination.

60 g control liver were thoroughly homogenized in ice-cold distilled water in an Ultra-Turrax at 0—3°C. The homogenate (concentration about 3% w/v) was centrifuged for 30 min at 50 000 × g and 0°C. The pH of the supernatant was adjusted to pH 7.0 by adding 1 M potassium phosphate buffer (pH 7.0) to a final concentration of 50 mM, and 4 M NaCl solution was added to bring the final NaCl concentration to 500 mM.

The next step involved adsorption of glycoprotein by Concanavalin A and selective elution with α-methyl-D-glucoside. 20 ml packed Concanavalin A-Sepharose 4B (Pharmacia), which had been exhaustively washed with distilled water, were added to the supernatant. After incubation with stirring at 0°C for 30 min, the mixture was poured onto a column (25 × 3.3 cm inner diameter). The Concanavalin A-Sepharose 4B was washed with 200 ml 50 mM potassium phosphate buffer (pH 7.0)/500 mM NaCl. Glycoproteins were eluted from the column with 100 ml washing buffer to which α-methyl-D-glucoside (Koch-Light) had been added to a final concentration of 1 M. The eluate, which contained about 0.7% of the protein and 35% of the α-glucosidase activity initially present in the homogenate was concentrated by ultrafiltration (Amicon PM 30 filter) to about 3 ml and dialysed for 16 h against 2000 ml 10 mM potassium phosphate buffer (pH 6.5). The dialysed solution was layered onto a Sephacryl-S 200 column (Pharmacia; 100 × 1.2 cm) and eluted with 10 mM phosphate buffer (pH 6.5)/100 mM NaCl. 4-ml fractions were collected; the flow rate was 5 ml/h. The fractions were measured at 280 nm on a Zeiss PMQ_2 spectrophotometer and α-glucosidase activities were determined using *p*-nitrophenyl-α-glucoside and glycogen as substrates (see below). The α-glucosidase fractions were pooled and dialysed for 16 h against 10 mM phosphate buffer (pH 6.5) and concentrated by ultrafiltration (Amicon PM 30 filter). Protein was measured according to Lowry et al. [25], as modified by Eggstein and Kreuz [26], using crystallized egg albumin as standard.

Enzyme assay conditions

To determine *p*-nitrophenyl-α-glucosidase activity, the reaction mixture contained 100 mM sodium acetate buffer (pH 4.0), 6.6 mM *p*-nitrophenyl-α-D-glucoside (Koch-Light), enzyme preparation (0.025—0.25 ml) and water to 0.5 ml. In control incubations, either enzyme or substrate was omitted. After incubation at 37°C for 10—60 min, the reaction was stopped by adding 1 ml 0.3 M glycine/NaOH buffer (pH 10.6). The liberated *p*-nitrophenol was estimated spectrophotometrically at 405 nm, using a molar extinction coeffi-

cient of $18.5 \cdot 10^6$ $M^{-1} \cdot cm^{-1}$ [27]. One unit of enzyme activity is defined as 1 μmol *p*-nitrophenyl-α-D-glucopyranoside hydrolysed/min at 37°C. To determine *N*-acetyl-β-glucosaminidase, the reaction mixture contained 50 mM sodium acetate buffer (pH 5.0), 3 mM *p*-nitrophenyl-*N*-acetyl-β-glucosaminide, enzyme preparation and water to 0.5 ml. The reaction was stopped as described for α-glucosidase.

When α-glucosidase attached to the immobilized antiserum was used as an enzyme source the reaction mixture was the same as that used with the free enzyme, except that the volume of the reaction mixture was 1 ml, and that the reaction was started by adding 0.05—0.5 ml of the immobilized antiserum-α-glucosidase complex. During incubation the mixture was well shaken. The reaction was stopped by adding 2 ml glycine/NaOH buffer (pH 10.6) after which the Sepharose was centrifuged and the *p*-nitrophenol liberated was measured in the supernatant. Lineweaver-Burk plots from which the corresponding K_m values were calculated, were obtained by varying the *p*-nitrophenyl-α-glucoside concentration from 1.6 to 13.2 mM.

To determine glycogen-α-glucosidase activity, the reaction mixture contained 100 mM sodium acetate (pH 4.0), 10 mg/ml glycogen, enzyme preparation (0.025—0.25 ml) and water to 0.5 ml. In control incubations either enzyme or substrate was omitted. After incubation at 37°C for 10—60 min, the reaction was stopped by heating the mixture at 100°C for 2 min. The liberated glucose was estimated using hexokinase and glucose-6-phosphate dehydrogenase [28].

Immunological methods: preparation of antiserum and immobilization of antiserum on Sepharose 4B

α-Glucosidase was purified from normal human liver as described above. A mixture of about 200 μg purified enzyme, 0.5 ml phosphate-buffered saline and 1 ml complete Freund's adjuvant was injected intramuscularly into a rabbit in four different places. Three booster injections of 150 μg of the same preparation with incomplete Freunds' adjuvant were given 3, 5 and 7 weeks later. After a further 2 weeks, plasma was withdrawn and complement was inactivated by incubation of the antiserum at 56°C for 30 min. A crude immunoglobulin fraction was prepared by 50% $(NH_4)_2SO_4$ precipitin followed by dialysis against phosphate-buffered saline (pH 7.4), and was used as a source of anti-α-glucosidase antibodies (anti-α-glucosidase IgG).

A control immunoglobulin preparation was obtained in a similar way from rabbits injected with complete Freund's adjuvant only (control IgG). Incubations of different dilutions of antiserum with α-glucosidase preparations were carried out for 30 min at 37°C and then for 2 h at 0°C. 20 mM potassium phosphate (pH 7.0) was present. Subsequently, the incubation mixture was centrifuged at 10 000 × *g* for 4 min at room temperature. The resulting supernatant was assayed for α-glucosidase activities as described above.

Antibodies were immobilized by covalent coupling to Sepharose 4B using CNBr-activated Sepharose (Pharmacia) according to the instructions of the manufacturer, the immunoglobulins being added at a concentration of 5 mg protein/ml packed gel (recovery of bound protein 80%). The Sepharose 4B was carefully washed on a glass filter with 500 ml 500 mM NaCl and suspended and stored in phosphate-buffered saline (same volume of buffer as volume packed

Sepharose 4B) to which 0.01% NaN_3 was added to prevent bacterial growth.

Incubations of α-glucosidase preparations with immobilized anti-α-glucosidase were carried out at 25°C for 2 h with continuous shaking. The suspension of antiserum-Sepharose 4B was well stirred before use. The incubation mixture was subsequently centrifuged at 2000 × *g* for 2 min at room temperature. The supernatant was assayed for α-glucosidase activity as described above. The immobilized anti-α-glucosidase was resuspended in 0.5 ml distilled water to measure the adsorbed α-glucosidase activities as described above. The enzyme is not bound to normal rabbit serum coupled to Sepharose.

Sepharose suspensions were dispersed with a Finn pipette, using a tip with a wide opening and keeping the suspension well stirred.

Collection and processing of urine

Fresh urine (30—100 ml) was collected from controls and from patients with Pompe's disease and processed within 6 h after voiding. (Preliminary observations indicated that rapid processing is essential, since prolonged storage (>24 h) either at 0—4% or at −20°C might significantly affect acid α-glucosidase activity). Urine was usually collected in the morning.

Urine was cooled to 0°C and solid $(NH_4)_2SO_4$ was added to 80% saturation. After stirring for 20 min, the precipitate was centrifuged down (50 000 × *g* for 30 min), taken up in distilled water and dialysed against distilled water for 2 h. Any precipitate formed was removed by centrifugation and the dialysed urinary protein preparation was stored at 0—4°C and used within 48 h.

Description of control group and patients with infantile and late-onset form of Pompe's disease

The control group consisted of 27 healthy persons ranging in age from 3—53 years. The patients with the late-onset form of Pompe's disease ranged in age from 12—62 years. The diagnosis of the disease was established at the age of 10—59 years on the basis of a deficiency of acid α-glucosidase in muscle (Koster, J.F. and Slee, R., Personal communication). All patients showed muscular weakness. In muscle biopsies, vacuolar myopathy was evident, the vacuoles being filled with periodic acid-Schiff reactive material (Busch, H.F.M., personal communication). The infantile form of Pompe's disease was diagnosed in one patient by Tegelaers, W.F. and Schutgens, R.B.H. (personal communication).

Results

Purification of α-glucosidase from human liver

The procedure developed by us to purify acid α-glucosidase from normal human liver consists of only two steps: (i) batch-wise Concanavalin A adsorption of glycoproteins (cf. Ref. 29), and (ii) Sephacryl S-200 chromatography of the eluted glycoproteins. The elution profile after Sephacryl S-200 chromatography is shown in Fig. 1. With glycogen as substrate, a single activity peak is obtained. The column was calibrated with proteins of known molecular weight. The effluent volume containing the enzyme corresponds to an apparent molec-

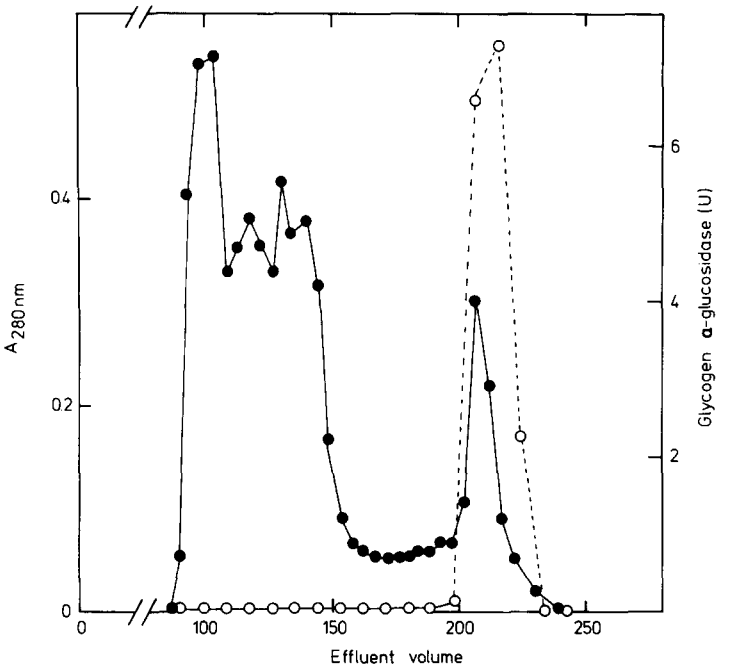

Fig. 1. Chromatography of glycogen α-glucosidase from liver on Sephacryl S-200. Elution pattern of protein (●———●) and glycogen α-glucosidase (○———○) after chromatography of the fraction obtained from the Sepharose 4B-Concanavalin A column.

ular weight of <10 000, indicating that the enzyme is retarded on the column; polysaccharide Sephacryl S-200, like Sephadex, acts as a substrate analogue for acid α-glucosidase [30].

Results of a typical isolation are shown in Table I. Acid α-glucosidase (assayed with *p*-nitrophenyl-α-glucoside) is obtained in a yield of approx. 10%, with a specific activity with glycogen as subtrate at least equivalent to that of preparations isolated by other more time-consuming procedures [13,19,30]. It is unlikely that the preparation contains neutral α-glucosidase, since it is known that the neutral α-glucosidase is not retarded during chromatography in polysaccharides like Sephadex [30].

TABLE I

PURIFICATION OF ACID α-GLUCOSIDASE FROM HUMAN LIVER

The initial homogenate was prepared from 60 g liver.

Step	Total activity with *p*-nitrophenyl α-glucoside (units)	Total protein (mg)	Specific activity (units/mg protein)	Recovery (%)
Homogenate	12	—		100
Concanavalin-A-Sepharose 4B	4.3	31	0.14	35.8
Sephacryl S-200	1.25 *	2.7	0.46	10.4

* Total activity with glycogen as substrate, 6 units.

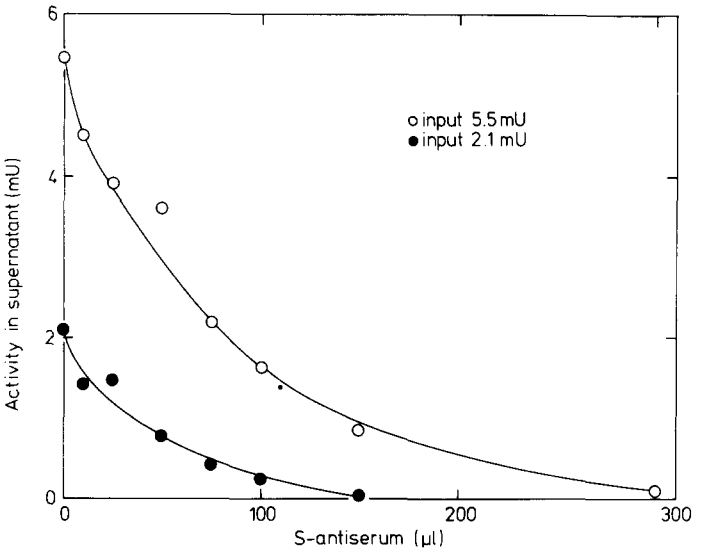

Fig. 2. Effects of preincubation with Sepharose-4B-anti-α-glucosidase IgG on the *p*-nitrophenyl-α-glucosidase activity of two concentrations of purified acid α-glucosidase. Activity was measured in the supernatant after centrifugation of the enzyme-immobilized antiserum mixture. No activity was removed after incubation with immobilized normal serum. S-antiserum, antiserum coupled to Sepharose 4B.

Characterization and immobilization of antibodies against acid α-glucosidase

The antibodies against purified α-glucosidase precipitated α-glucosidase activity depending on the relative amount of antigen and antibodies present in the system. In contrast, there was no effect of control IgG on the α-glucosidase activity. With both glycogen and *p*-nitrophenyl-α-glucoside, similar precipitation curves were obtained with the same titre.

The effects of immobilized antiserum on two concentrations of purified acid α-glucosidase are shown in Fig. 2. Upon increasing the amount of immobilized antiserum the activity remaining in the supernatant gradually decreased and reached zero at higher amounts of antiserum-Sepharose 4B. The amount of immobilized antiserum required to remove α-glucosidase activity was a function of the input activity. Control serum coupled to Sepharose 4B had no effect on the acid α-glucosidase activity.

Use of immobilized antibodies in studying α-glucosidase in urine

In a dialysed urinary protein preparation obtained by 80% $(NH_4)_2SO_4$ saturation of urine, hydrolysis of substrates containing α(1 → 4) glucosidic linkages is brought about not only by the lysosomal acid α-glucosidase, but also by the other glycosidases present. Thus glycogen is hydrolysed by amylase, and *p*-nitrophenyl-α-glucoside by neutral α-glucosidase [11,12,31,32] and by the specific form of α-glucosidase present in kidney [33]. It is therefore difficult to detect a deficiency of acid α-glucosidase in urine from a patient with Pompe's disease [8]. The pH activity profiles with glucagon as substrate are very similar, whereas those with *p*-nitrophenyl-α-glucoside as substrate differ only slightly (Fig. 3A and B).

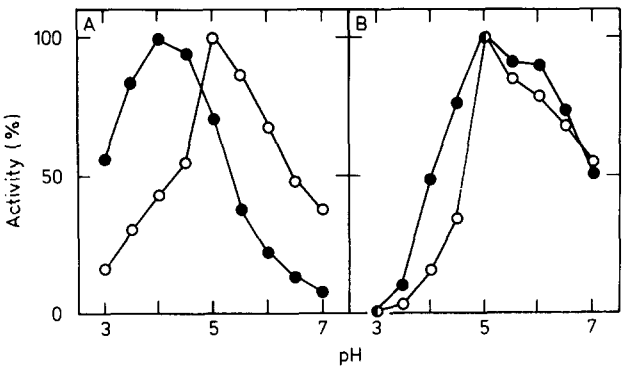

Fig. 3. Effect of pH on α-glucosidase activity in urine from a normal control (A), and urine from a patient with the infantile form of Pompe's disease (B). The substrate was *p*-nitrophenyl-α-glucoside (●———●) or glycogen (○———○).

By using immobilized specific antibodies, the acid α-glucosidase activity in urine can easily be distinguished from other enzymes hydrolysing α-glucosidic linkages. Thus in normal urine about 90% of the *p*-nitrophenyl-α-glucosidase activity at pH 4 can be removed by incubation with the immobilized antibodies (Fig. 4A). Only 35% of the activity at pH 6.5 is removed by this treatment (Fig. 4A). The nonprecipitable activity must be due to neutral α-glucosidase. In urine from a patient with the infantile form of Pompe's disease, preincubation with immobilized antibody has no effect whatsoever on the activity either at pH 4 or 6.5 (Fig. 4B). This finding indicates that the antiserum does not contain antibodies against α-glucosidases other than the lysosomal enzyme. This result confirms that acid α-glucosidase activity is absent in urine from patients with the infantile form of Pompe's disease.

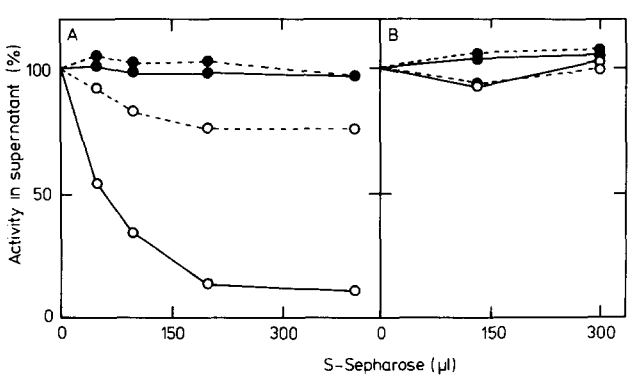

Fig. 4. Effect of preincubation with immobilized anti-α-glucosidase IgG or immobilized control IgG on the *p*-nitrophenyl-α-glucosidase activity in urine from a normal person and from a patient with the infantile form of Pompe's disease. The activity was measured at pH 4 (———) and at pH 6.5 (- - - - -) in the supernatant obtained after centrifugation. A, normal urine. B, urine from patient with Pompe's disease. The input of *p*-nitrophenyl-α-glucosidase activity, measured at pH 4, was 4.3 mU in the normal urine and 1.0 mU in the Pompe's disease urine, whereas the ratio activity at pH 4/activity at pH 6.5 was 7.0 in the normal urine and 0.7 in the urine of the patient with Pompe's disease. ○———○, immobilized anti-α-glucosidase IgG; ●———●, immobilized control IgG. S-Sepharose, antiserum coupled to Sepharose 4B.

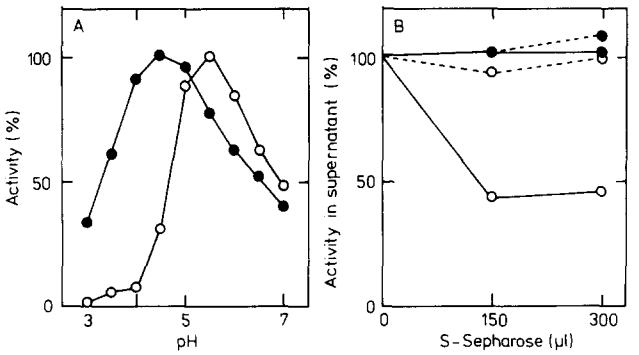

Fig. 5. α-Glucosidase activity in urine from a patient with the late-onset form of Pompe's disease. A, effect of pH. The substrate was *p*-nitrophenyl-α-glucoside (●———●) or glycogen (○———○). B, effect of preincubation of urine preparation with immobilized anti-α-glucosidase IgG (○———○) or control IgG (●———●) on *p*-nitrophenyl-α-glucosidase activity. The activity was measured at pH 4.0 (———) or pH 6.5 (- - - - -) in the supernatant obtained after centrifugation. The *p*-nitrophenyl-α-glucosidase activity at pH 4 before preincubation was 1.9 mU, whereas the ratio activity at pH 4.0/acitivity at pH 6.5 was 1.7. S-Sepharose, antiserum coupled to Sepharose 4B.

The immobilized antibodies can also be used in the study of α-glucosidase activity in urine from patients with late-onset Pompe's disease. In this case the pH activity profile (Fig. 5A) is very similar to that in normal urine (Fig. 3A). After incubation of the preparations from late-onset patients with immobilized antibodies, about 55% of the *p*-nitrophenyl-α-glucosidase activity at pH 4 is removed (Fig. 5B). This result clearly shows that residual acid α-glucosidase activity is present in the urine of late-onset patients, as has also been shown for liver and other material [17,20] and cultured skin fibroblasts [22].

Quantitative assay of the acid α-glucosidase activity in urine

The amount of acid α-glucosidase activity present in urine can be calculated from the difference in α-glucosidase activity before and after treatment with immobilized antibodies against acid α-glucosidase. By relating the activity to that of another lysosomal enzyme activity, for instance total hexosaminidase, a correction can be made for the degree of diuresis, assuming that the excretion of both enzymes is similarly affected by diuresis [34].

Data on total α-glucosidase at pH 4, hexosaminidase and acid α-glucosidase activities in urine from 27 normal controls and 6 patients with late-onset Pompe's disease are presented in Table II. In normal controls, the amount of total α-glucosidase at pH 4, hexosaminidase, and acid α-glucosidase per ml urine varies over a very wide range due to differences in diuresis. Acid α-glucosidase comprises about 90% of the total α-glucosidase activity at pH 4. The mean value for the ratio of acid α-glucosidase/hexosaminidase is 1.36 ± 0.74.

In the urine from patients with late-onset Pompe's disease the total α-glucosidase activity at pH 4 is reduced; only about 45% is due to acid α-glucosidase, with a mean value for the ratio acid α-glucosidase/hexosaminidase of 0.064 ± 0.013. The amount of acid α-glucosidase activity in the patients' urine is thus about 5% of that in the urine of normal controls.

TABLE II

TOTAL α-GLUCOSIDASE ACTIVITY AT pH 4, HEXOSAMINIDASE AND ACID α-GLUCOSIDASE IN NORMAL URINE AND IN URINE FROM PATIENTS WITH LATE-ONSET POMPE'S DISEASE

The amount of acid α-glucosidase is that removed by incubating the urinary protein preparation with immobilized antibodies.

Parameter	Normal controls	Patients with late-onset Pompe's disease
Total α-glucosidase at pH 4 (mU/ml urine)		
Mean ± SD	1.49 ± 0.69	0.22 ± 0.11
Range	0.11—3.00	0.13—0.44
n	27	6
Hexosaminidase (mU/ml urine)		
Mean ± SD	1.25 ± 0.73	1.51 ± 0.55
Range	0.18—3.64	0.95—2.46
n	27	6
Acid α-glucosidase (mU/ml urine)		
Mean ± SD	1.45 ± 0.52	0.095 ± 0.02
Range	0.79—2.73	0.06—0.13
n	22	6
Acid α-glucosidase (% of total α-glucosidase at pH 4.0)		
Mean ± SD	90.6 ± 3.2	45.8 ± 11.2
Range	84—95	30—58
n	22	6
Acid α-glucosidase/hexosaminidase		
Mean ± SD	1.36 ± 0.74	0.064 ± 0.013
Range	0.56 —3.93	0.05—0.08
n	22	6

Kinetic studies of acid α-glucosidase from urine as measured with enzyme adsorbed to immobilized antibodies

De Groot et al. [24] have shown that when the lysosomal enzymes α-galactosidase A and *N*-acetyl-α-galactosaminidase are adsorbed to immobilized antisera, they still exhibit activity towards low molecular weight substrates. Similarly, Koster and Slee [19] have shown that the complex between acid α-glucosidase and anti-α-glucosidase is able to hydrolyse maltose and methylumbelliferyl-α-glucoside, but not glycogen. We have made use of this phenomenon in order to compare the kinetic properties of urinary acid α-glucosidase from normal persons and from patients with Pompe's disease.

Fig. 6A shows pH activity profiles of total *p*-nitrophenyl-α-glucosidase, acid *p*-nitrophenyl-α-glucosidase (as measured with the enzyme adsorbed to immobilized antibodies) and the residual acid α-glucosidase activity remaining in the supernatant after removal of the Sepharose. It is clear that the pH activity profile of total α-glucosidase is made up of at least two components. It is of interest to note that the sum of the activities at pH 3 on the Sepharose-antiserum and in the supernatant is greater than the activity at this pH in untreated urine.

The mean pH activity profiles of acid α-glucosidase (measured with adsorbed enzyme) from normal urine and from urine of patients with late-onset Pompe's disease are compared in Fig. 6B. It is clear that there is no significant difference

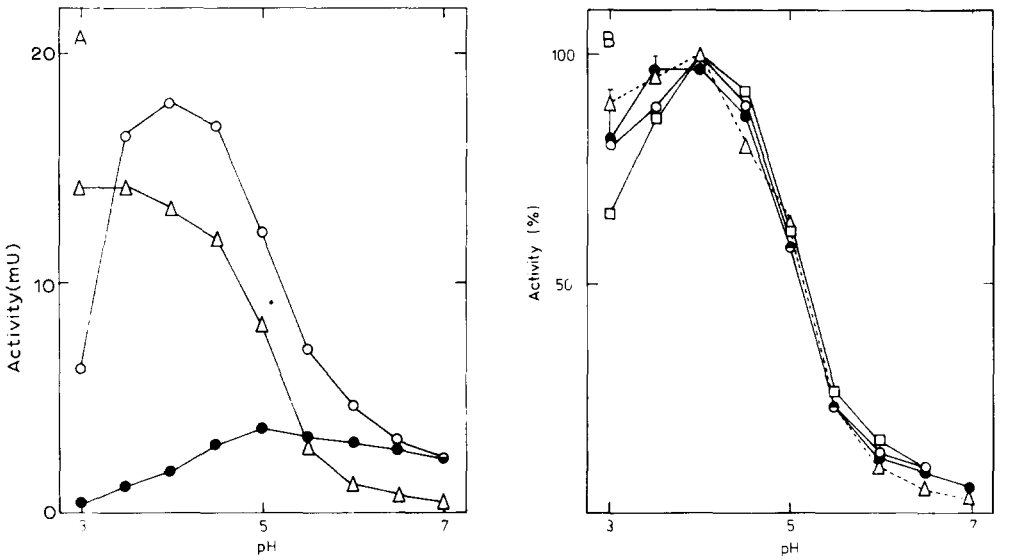

Fig. 6. A. Effect of pH on *p*-nitrophenyl-α-glucosidase activity in normal urine before and after incubation with immobilized anti-α-glucosidase and on acid α-glucosidase adsorbed to the immobilized antiserum. ○———○, activity before immunoadsorption. ●———●, activity after immunoadsorption. △———△, activity of α-glucosidase adsorbed to immobilized antiserum. The latter two curves were obtained after preincubation of 2.2 ml urinary protein preparation (17.8 mU *p*-nitrophenyl-α-glucosidase activity at pH 4.0) with 1.2 ml Sepharose-antiserum suspension. The concentration of *p*-nitrophenyl-α-glucoside was 6.6 mM. B. Effect of pH on the *p*-nitrophenyl-α-glucosidase activity of acid α-glucosidase in urine from normal persons and in patients with the late-onset form of Pompe's disease after adsorption of the enzyme to immobilized anti-α-glucosidase IgG. ●———●, normal urine, the bars show the S.D. (n = 5). ○———○ and □———□, urine from late-onset Pompe's disease; △———△, acid α-glucosidase purified from normal liver.

between the two enzymes with regard to this effect of pH on the activity; purified acid α-glucosidase from normal liver shows a similar pH curve (Fig. 6B).

Table III shows that the K_m for *p*-nitrophenyl-α-glucoside at pH 4 of the

TABLE III

EFFECT OF ATTACHMENT OF α-GLUCOSIDASE TO IMMOBILIZED ANTIBODIES ON THE K_m FOR *p*-NITROPHENYL-α-GLUCOSIDE AT pH 4 AND 3

The enzyme assay was carried out at pH 4.0 or 3.0; the *p*-nitrophenyl-α-glucoside concentration was changed from 1.6 to 13.2 mM.

Source of α-glucosidase	K_m (mM) for *p*-nitrophenyl-α-glucoside		
	Activity of enzyme in solution	Enzyme absorbed to immobilized antibodies	
		pH 4.0	pH 3.0
Purified enzyme from liver	6.7	3.5	—
Urine from controls (n = 8)	3.3 ± 0.28	3.2 ± 0.8	6.2 ± 1.5
Urine from patients with late-onset Pompe's disease (n = 3)	5.6 ± 2.0	3.2 ± 0.4	5.3 ± 1.3

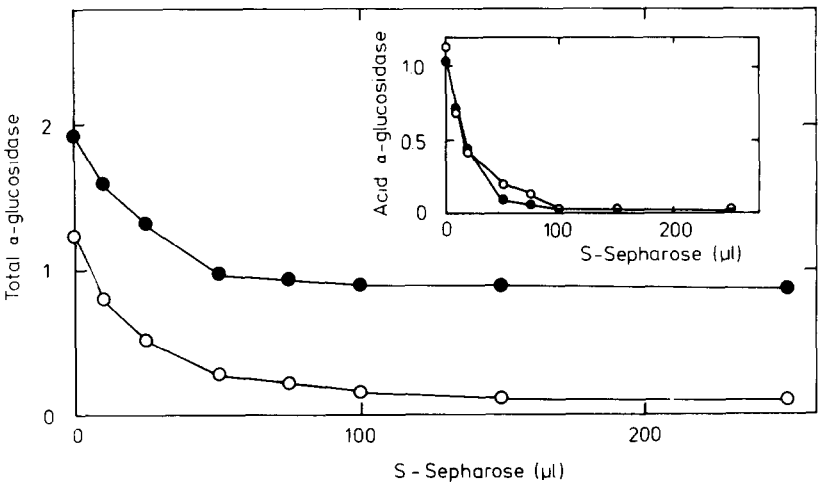

Fig. 7. Comparison of titration curves with immobilized antiserum of total α-glucosidase activity at pH 4.0 in normal urine and in urine from patients with late-onset Pompe's disease. The input was 1.23 and 1.93 mU total *p*-nitrophenyl-α-glucosidase in the normal urine and in the urine from the patients, respectively, corresponding to 1.12 and 1.04 mU of acid *p*-nitrophenyl-α-glucosidase, respectively. The inset shows the titration curves for acid α-glucosidase, which were obtained by subtracting the values for non-acid-α-glucosidase (i.e., nonprecipitable activity) from those for total α-glucosidase at pH 4.0. ○———○, normal urine. ●———●, urine from patients with late-onset Pompe's disease.

purified enzyme from liver is of the same order of magnitude whether measured with the free enzyme or with enzyme adsorbed to immobilized antiserum. Furthermore, the K_m for *p*-nitrophenyl-α-glucoside of the urinary enzyme from patients with late-onset Pompe's disease is the same as that of the enzyme from normal urine, both at pH 4 and 3 (Table III).

Comparison of immunological characteristics of acid α-glucosidase from normal urine and from urine of patients with late-onset Pompe's disease

When protein preparations from normal urine and from urine of patients with late-onset Pompe's disease containing approximately the same amount of acid α-glucosidase (1.12 and 1.04 mU, respectively) were titrated with different amounts of immobilized antiserum, the precipitation curves shown in Fig. 7 were obtained. With excess immobilized antiserum, about 90% of the α-glucosidase activity was removed in the normal urine and 55% in the patients' urine (Fig. 7; cf. Figs. 4A and 5B). When these curves are corrected by subtracting the nonacid α-glucosidase activity, the curves shown in the inset of Fig. 7 are obtained.

The corrected titration curves for the normal controls and patients with late-onset Pompe's disease are almost identical, indicating that the immunological characteristics of the α-glucosidase in the two preparations are very similar. Furthermore, these results indicate that no enzymologically unreactive material which crossreacts with acid α-glucosidase is present in urine of patients with late-onset Pompe's disease, in agreement with the findings of Reuser et al. [22] in cultured fibroblasts.

Discussion

Previously, three methods have been used to measure acid α-glucosidase activity in urine. First, the ratio of the activity at pH 4 to that at pH 6.5 has been used as a measure of the relative amounts of acid α-glucosidase and non-acid α-glucosidase activity [8,32,35]. Second, the sensitivity to turanose, which inhibits acid α-glucosidase but not nonacid α-glucosidase has been used as a diagnostic tool [8]. Third, a method has been described in which the assay is carried out at pH 4 in the presence of a high KCl concentration, which inhibits nonacid α-glucosidase activity [35].

The immunological technique described in this paper provides a simple, reliable and sensitive means of quantitatively measuring the amount of acid α-glucosidase in urine. It is preferable to the three methods described above because it allows exact quantitative measurement of the amount of acid α-glucosidase present. By relating the amount of acid α-glucosidase (i.e., the amount removable by immobilized antiserum) to the activity of another lysosomal enzyme, a correction is made for the degree of diuresis. Thus the ratio acid α-glucosidase/hexoaminidase may be used as a measure of the specific activity of acid α-glucosidase in the urine.

With this method, it is possible to distinguish not only between patients with Pompe's disease and normal controls using easily available biological fluids, but also between the infantile and late-onset forms of the disease (cf. Ref. 32). Whereas acid α-glucosidase was absent from the urine of a patient with the infantile form of the disease, the urine of late-onset patients was found to contain residual acid α-glucosidase activity, the level of which was about 5% of that in control urine. The presence of residual α-glucosidase activity in the urine of late-onset patients confirms the results of de Barsy [20], DiMauro et al. [17,21] and Reuser et al. [22] obtained with other materials. It is, of course, desirable to confirm the diagnosis by measurements of enzyme activity in cells (e.g., leukocytes, cultured fibroblasts) or in tissues (e.g., muscle).

The question arises of the nature of the mutation in the late-onset form of Pompe's. The present study shows that acid α-glucosidase in urine from late-onset patients is indistinguishable from that in normal urine with regard to its kinetic characteristics (see also Refs. 21 and 22), or reactivity with antibodies raised against acid α-glucosidase from normal liver (see also Ref. 22). Reuser et al. [22] suggested that the mutation in the late-onset form of the disease may lead either to a defect in a regulatory mechanism resulting in a decreased rate of synthesis of the enzyme or, alternatively, to a structural alteration resulting in an increased rate of degradation of the enzyme. Studies are in progress to detect possible structural differences in acid α-glucosidase in urine from normal persons and from patients with late-onset Pompe's disease.

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