Heterogeneity of pig lysosomal acid α -glucosidase

Affinity to Sephacryl S-200 gel and tissue distribution

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Acid α -glucosidase purified from pig liver showed heterogeneity in its affinity to Sephacryl S-200 gel. Acid α -glucosidase was separated into two fractions (S1 and S2) by Sephacryl S-200 affinity chromatography. Each fraction contained components at apparent 76 kDa and 67 kDa on SDS/PAGE. The amount of S1 fraction was about 1.3 times that of the S2 fraction. In the kidney the ratio of S1 to S2 fraction was similar to that in the liver. However, the heart contained 1.3 times as much S2 fraction as S1 fraction. The spleen acid α -glucosidase consisted mainly of S1 fraction, containing only a 76 kDa component. Immunohistochemically, acid α -glucosidase was demonstrated in the macrophages of the spleen. Thus the 76 kDa component in the spleen must come mainly from the macrophages. Lectin-binding analysis was carried out on the components present in the S1 and S2 fractions after electrophoresis and transfer to nitrocellulose sheets. Slight differences in binding observed suggest differences in the structure of the sugar side chains.

INTRODUCTION

The importance of the role of acid α -glucosidase in the lysosomal degradation of glycogen has been emphasized because the deficiency of this enzyme in glycogenosis type II causes glycogen accumulation in lysosomes (Hers, 1963; Baudhuin *et al.*, 1964). Many biochemical reports of acid α -glucosidase have been published (Auricchio *et al.*, 1968; Bruni *et al.*, 1969; Jeffery *et al.*, 1970; Murray *et al.*, 1978; Hasilik & Neufeld, 1980a,b; Hilkens *et al.*, 1981; Oude Elferink *et al.*, 1985; Mutsaers *et al.*, 1987), and Hoefsloot *et al.* (1988, 1990a,b) and Martiniuk *et al.* (1990) reported the cDNA and amino acid sequence of human acid α -glucosidase.

Acid α -glucosidase can be purified on the basis of its affinity to the dextran type of gel-filtration media such as Sephadex (Auricchio *et al.*, 1968; Bruni *et al.*, 1969), which may act as a substrate analogue of the natural substrates, such as glycogen, containing α -linked glucose units. Employing this principle, Hasilik & Neufeld (1980a,b) and Hilkens *et al.* (1981) purified this enzyme from human placenta. They found that the enzyme contained two components, with molecular masses of 76 kDa and 70 kDa. Tashiro *et al.* (1986) also reported that acid α -glucosidase purified from pig liver with Sephadex G-100 as an affinity medium contained two components. It was possible to separate these two components from each other by DEAE-5 pw ion-exchange column chromatography. Furthermore, Henkel *et al.* (1985) reported the charge heterogeneity in human acid α -glucosidase, and obtained four active forms with different charges by chromatofocusing. Two contained 76 kDa and two contained 70 kDa polypeptides, which also contained a small amount of different low-molecular-mass peptides. They reported that the oligosaccharide chains in the hepatic enzyme contained 13.1 mol of mannose and 8.3 mol of *N*-acetylglucosamine/mol of enzyme. Mannose 6-phosphate and sialic acid were not detected. Thus they reported that the charge heterogeneity in the hepatic enzyme could not be attributed to the presence of phosphate or sialic acid residues in the carbohydrate structure and that charge heterogeneity resulted from proteolytic modification of the enzyme.

The present study demonstrates that pig acid α -glucosidase shows heterogeneity in its affinity to Sephacryl S-200 gel.

MATERIALS AND METHODS

Materials

Materials used in this work included PBE 94, Polybuffer 74, Sephacryl S-200, Sephadex G-100 and G-25, from Pharmacia Fine Chemicals (Uppsala, Sweden); TSK gel Toyopearl HW-55 from Tosho Mfg. Co. (Tokyo, Japan); 4-methylumbelliferone, 4-methylumbelliferyl α -D-glucoside (4-MU- α -glucoside), glycogen (from rabbit liver), turanose and phenylmethanesulphonyl fluoride from Sigma Chemical Co. (St. Louis, MO, U.S.A.); leupeptin and pepstatin A from Nakarai Chemicals (Kyoto, Japan); maltose and glucose assay kit (Glucose B-Test Wako) from Wako Pure Chemical Industries (Osaka, Japan); and isomaltose and concanavalin A (Con A) from Seikagaku Kogyo Co. (Tokyo, Japan); 250 mg of Con A was used for coupling with 30 ml of CNBr-activated Sepharose 4B according to the instructions given by Pharmacia, and of this 230 mg was coupled. Peroxidase-Con A, peroxidase-*Ricinus communis* agglutinin (RCA), peroxidase-wheat-germ agglutinin (WGA), peroxidase-*Lens culinaris* agglutinin (LCA), peroxidase-peanut agglutinin (PNA) and peroxidase-phytohaemagglutinin-E4 (PHA-E4) were obtained from Seikagaku Kogyo Co. Endo- β -N-acetylglucosaminidase H (Endo H) was purchased from Genzyme Co. (Boston, MA, U.S.A.); endo- β -N-acetylglucosaminidase (Endo F) and peptide *N*-glycosidase F (PNGase) were from Boehringer (Mannheim, Germany); and neuraminidase was from Sigma. As controls for lectin reactivity of acid α -glucosidase, ovalbumin (Seikagaku Kogyo), transferrin (Wako Pure Chemical Industries), and fetuin (Canadian Bioclinical, Scarborough, Ont., Canada) were used. Nitrocellulose membrane was purchased from Bio-Rad Laboratories (Richmond, CA, U.S.A.).

Purification of acid α -glucosidase from pig liver

Purification was performed as reported previously (Iwamasa *et al.*, 1982, 1986; Tashiro *et al.*, 1986) by using Sephacryl S-200,

Abbreviations used: Con A, concanavalin A; 4-MU-, 4-methylumbelliferyl; RCA, *Ricinus communis* agglutinin; WGA, wheat-germ agglutinin; LCA, *Lens culinaris* agglutinin; PNA, peanut agglutinin; PHA-E4, phytohaemagglutinin E4; Endo H and F, endo- β -N-acetylglucosaminidase H and F; PNGase, peptide *N*-glycosidase F.

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Table 1. Summary of the purification of the acid α -glucosidase from pig liver

Details of the purification procedures were previously reported (Iwamasa *et al.*, 1982, 1986; Tashiro *et al.*, 1986). A m-unit of enzyme activity is 1 nmol of 4-methylumbelliferone/min at pH 4.5.

Purification step	Enzyme activity		Recovery (%)	Purification factor (fold)
	(m-units/g of tissue)	(m-units/mg of protein)		
Homogenate	33.8	0.34	100	1
Crude extract	25.0	0.45	74.0	1.3
(NH ₄) ₂ SO ₄	24.6	3.79	72.8	11.1
Sephacryl S-200	10.9	224	32.2	650
Con A-Sephadex 4B	7.80	338	23.1	990
Sephadex G-100	6.32	377	18.8	1100

Con A-Sephadex 4B and Sephadex G-100 affinity-chromatography procedures, in the presence of proteinase inhibitors (5 μ g of leupeptin/ml, 0.1 μ g of pepstatin A/ml and 0.1 mM-phenylmethanesulphonyl fluoride). Bound α -glucosidase was eluted by 0.2 M-methyl mannoside (Con A-Sephadex B) and continuous elution with equilibration buffer (10 mM-sodium phosphate buffer, pH 6.0) of the gel (Sephadex G-100). Auricchio *et al.* (1968) and Bruni *et al.* (1969) reported the affinity of acid α -glucosidase to a dextran-type gel, containing α -linked glucose units, which is known to act as a substrate analogue. A summary of the purification steps is shown in Table 1. The resulting enzyme solution was used in subsequent studies.

Analytical Sephacryl S-200 gel affinity chromatography

The purified enzyme solutions (1 mg/ml) from pig liver and other enzyme solutions from kidney, spleen and heart, each of which were from the Con A-Sephadex 4B step, were applied to a Sephacryl S-200 column (1.6 cm $\times$ 90 cm) which had been equilibrated with 10 mM-sodium phosphate buffer, pH 6.0, containing 0.5 M-NaCl and 0.01 % NaN₃. The purification factors of the other enzyme solutions, from kidney, spleen and heart, were 990-, 970- and 900-fold respectively. With continued elution by the same buffer under the same conditions, the acid α -glucosidase activity was noted shortly after the bed-volume elution. The eluate with acid α -glucosidase activity was collected.

Analytical procedures

Acid α -glucosidase activity was assayed with 4-MU- α -glucoside, glycogen, maltose, isomaltose and turanose as substrates. Each assay mixture (50 μ l) contained 0.1 M-sodium acetate buffer (pH 4.0–5.0), 0.2 mg of BSA/ml, 10 μ l of enzyme source and substrate. The concentrations of each of the substrates were as follows: 0.2 mM-4-MU- α -glucoside, 20 mg of glycogen/ml, 20 mM-maltose, 20 mM-isomaltose and 20 mM-turanose. The 4-MU- α -glucoside-hydrolysing activity was stopped by addition of 3 ml of 0.1 M-glycine/NaOH buffer, pH 10.5, and the liberated 4-methylumbelliferone was assayed fluorimetrically as described previously (Ohshita *et al.*, 1989). The reactions that hydrolyse glycogen, maltose and isomaltose were terminated by heating the mixture at 100 °C for 1 min. The turanose-hydro-

lysing activity was stopped by direct addition of glucose assay reagent, because the turanose is hydrolysed non-enzymically by heating. The liberated glucose was determined with a glucose assay kit (glucose oxidase-peroxidase and phenol-4-aminoantipyrine system) prepared by Wako Chemical Industries. The activity of acid α -glucosidase in the tissue homogenate was measured separately from neutral α -glucosidase, since the neutral α -glucosidase is effectively inactivated when the homogenate is preincubated in the presence of 0.1 M-sodium acetate buffer, pH 4.5, for 10 min at 37 °C. One unit was defined as the amount of enzyme which catalysed the formation of 1 μ mol of glucose/min from glycogen or the hydrolysis of 1 μ mol of one of the other substrates/min.

Protein was determined by the method of Lowry *et al.* (1951), with BSA as a standard.

Polyacrylamide-disc-gel electrophoresis was performed in 5 mm $\times$ 75 mm glass tubes in a cold-room (4 °C) in 2.5 % acrylamide stacking gel (pH 6.8) and 7.3 % acrylamide separating gel (pH 8.9) and a non-dissociating buffer system (0.0624 M-Tris/HCl, pH 6.8, in stacking gel; 0.025 M-Tris/0.192 M-glycine, pH 8.3, as electrode buffer). Samples (20 μ g) were mixed with equal volumes of 50 % (w/v) sucrose containing Bromophenol Blue and applied to the stacking gel. After electrophoresis, gels were stained with Coomassie Brilliant Blue R-250 (0.04 % in 3.5 % HClO₄). Other unstained gels were cut into 2 mm sections and were used for measurement of the enzyme activity after extraction with 100 mM-sodium acetate (pH 4.5).

SDS/PAGE was performed by the method of Laemmli (1970) in 7 % or 10 % acrylamide gel. Protein bands were detected after staining with Coomassie Brilliant Blue.

Chromatofocusing

Chromatofocusing was performed essentially according to the instructions given by Pharmacia. All procedures were carried out in a cold-room (4 °C). The buffers were degassed before use. A Polybuffer-exchanger PBE 94 column (0.9 cm $\times$ 27.0 cm) and a Sephadex G-25 column (1.9 cm $\times$ 22.0 cm) were equilibrated before the experiment with 25 mM-piperazine/HCl buffer, pH 5.2, containing 10 % sucrose. The sample was desalted by passage through the Sephadex G-25 column. The desalted sample was then applied to the PBE 94 column. The enzyme was subsequently eluted from the column with diluted Polybuffer 74 (1:10, v/v) containing 10 % sucrose, pH 4.0.

Preparation of antiserum

Antiserum against acid α -glucosidase purified from pig liver was prepared. A 0.15 mg portion of the purified enzyme (S1 76 kDa component isolated by chromatofocusing; Fig. 1) was emulsified with an equal volume of Freund's complete adjuvant. This emulsion (containing 200 μ g of the enzyme) was then injected subcutaneously into rabbits three times at 2-week intervals. Serum was collected 7 days after the third injection. Anti-(acid α -glucosidase) IgG was isolated by using a Sephacryl S-200 column after precipitation with (NH₄)₂SO₄ (50 % saturation). It was then applied to the acid α -glucosidase (76 kDa component used for antigen)-conjugated Sepharose 4B affinity column. The anti-(acid α -glucosidase) IgG was eluted with 0.5 M-NaCl/0.1 M-glycine/HCl, pH 2.6. The pH of the eluate was immediately raised by its collection into tubes containing 0.5 M-sodium phosphate buffer, pH 7.0. Anti-(acid α -glucosidase) Fab' fragment conjugated with peroxidase was prepared as described previously (Ohshita *et al.*, 1989).

Western-blot analysis

SDS/polyacrylamide-slab-gel electrophoresis was performed by the method of Laemmli (1970) in a 7 % or 10 % gel.

Fig. 1. Separation of 76 kDa component from liver S1 by chromatofocusing

About 3 mg of S1 purified from liver was applied to a PBE 94 column (0.9 cm $\times$ 27 cm). The enzyme was eluted from the column with Polybuffer 74 as described in the Materials and methods section; 3 ml fractions were collected and their 4-MU- α -glucosidase activity (○), pH (●●●) and A_{280} (□) were measured.

The purified enzyme (2 μ g; from the analytical Sephacryl S-200 chromatography step) was directly dissolved in sample buffer, consisting of 1% SDS/0.0625 M-Tris/HCl (pH 6.8)/5% mercaptoethanol/3 M-urea/0.002% Bromophenol Blue. The material was then heated for 5 min in a boiling bath. After electrophoresis, the sample was transferred on to a nitrocellulose sheet (0.1 A/30 V, 12 h), followed by treatment with anti-(acid α -glucosidase) Fab'-peroxidase for 4 h at 8 °C. The bound peroxidase activity was detected by means of the colour reaction with 0.05% diaminobenzidine and 0.01% H_2O_2 in 50 mM-Tris/HCl buffer, pH 7.2, containing 150 mM-NaCl.

Immunohistochemical procedures

Pig spleen was fixed in ice-cold 4% (v/v) formaldehyde in 0.2 M-sodium phosphate buffer (pH 7.2) for 3 h. Deparaffinized sections were then prepared by routine histochemical methods. Fresh-frozen sections were also prepared. The sections were treated with methanol containing 0.1% H_2O_2 for 30 min to block the endogenous peroxidase activity. Subsequently, they were washed with PBS (10 mM-sodium phosphate buffer, pH 7.4, 0.15 M-NaCl) and were treated with blocking buffer (3.5% skim milk containing PBS). After washing, the sections were incubated with anti-(acid α -glucosidase) Fab'-peroxidase (10 μ g/10 ml of blocking buffer) at room temperature in a moist chamber for 30 min. The sections were then stained with 0.05% diaminobenzidine in 0.05 M-Tris/HCl buffer, pH 7.4, containing 0.01% H_2O_2 at room temperature. After staining with Methyl Green, the sections were dehydrated and mounted.

Lectin-binding analysis of acid α -glucosidase by use of the lectin-nitrocellulose method

Lectin reactivity of the S1 and S2 fractions. This was performed by the method of Kijimoto-Ochiai *et al.* (1989). Liver enzyme from the Con A-Sepharose 4B step was separated into two fractions (S1 and S2, each 2 μ g) and used for this experiment. After SDS/PAGE, S1 and S2 fractions were transferred on to a nitrocellulose sheet (60 V, 3 h). Peroxidase-coupled lectins were diluted appropriately (Kijimoto-Ochiaia *et al.*, 1985) with 10 mM-Tris/HCl buffer, pH 7.4, containing 0.05% Tween 20

and 0.15 M-NaCl. In a clean flat plastic dish, S1 and S2 fractions on a nitrocellulose sheet were stained with a lectin solution (1 ml for a 10 cm $\times$ 10 cm sheet) at 4 °C for 1 h. After washing the nitrocellulose sheet with 10 mM-Tris/HCl buffer, pH 7.4, containing 0.05% Tween 20 and 0.15 M-NaCl for 4 $\times$ 15 min, the peroxidase staining was carried out by the colour reaction with 0.05% diaminobenzidine and 0.01% H_2O_2 in 50 mM-Tris/HCl buffer, pH 7.4, containing 0.15 M-NaCl.

Glycosidase digestion and weak-acid treatment of S1 and S2 fractions on a nitrocellulose sheet. These were performed as follows. Endo H digestion was carried out with 35 m-units of Endo H (1 unit = 1 nmol/min) in 700 μ l of 0.05 M-citrate/0.1 M-sodium phosphate buffer, pH 5.1, at 37 °C for 4 h. Endo F digestion was performed at 37 °C for 46 h with 1 unit of Endo F (1 unit = 1 μ mol/h) in 800 μ l of 0.1 M-sodium acetate buffer, pH 4.0, containing 10 mM-EDTA, 0.8% 2-mercaptoethanol and 0.15 M-NaCl. For PNGase digestion, 1 unit each of PNGase (1 unit = 1 μ mol/min) and Endo F in 200 μ l of 0.1 M-Tris/HCl buffer, pH 8.5, containing 40 mM-EDTA were used. The incubation was carried out at 37 °C for 20 h.

Weak-acid treatment was performed with 400 ml of 0.025 M- H_2SO_4 in a glass dish on a water bath at 80 °C for 1 h to remove the sialic acid.

Neuraminidase treatment. S1 and S2 fractions were incubated with 60 m-units of neuraminidase (1 unit = 1 μ mol/min) in 100 μ l of 0.1 M-acetate buffer, pH 4.8, at 37 °C for 1 h in the presence of proteinase inhibitors. After neuraminidase treatment, the samples were analysed by Western blotting.

RESULTS

Analytical Sephacryl S-200 gel affinity chromatography

Enzyme purified 1100-fold from the liver was obtained at the final step from the Sephadex G-100 affinity column. It was then applied to a gel-filtration column (Toyopearl HW-55) which had previously been equilibrated with 10 mM-sodium phosphate buffer, pH 6.0, containing 0.5 M-NaCl and 0.01% NaN_3 . The enzyme was eluted as a single peak with a molecular mass of 70 kDa. The purification factor after Toyopearl HW 55 gel chromatography was similar to that of Sephadex G-100 gel chromatography. The resulting solution was loaded on to a Sephacryl S-200 affinity column which had previously been equilibrated with 10 mM-sodium phosphate buffer, pH 6.0, containing 0.5 M-NaCl and 0.01% NaN_3 . The enzyme was separated into two fractions (S1 and S2), which were eluted after the bed volume with the same buffer (Fig. 2a). The separation patterns on the Sephacryl S-200 column of rechromatography of S1 and S2 fractions were the same as those of the previous chromatography, and were reproducible. Acid α -glucosidase from the kidney, spleen and heart was also analysed on the same Sephacryl S-200 column; Con A-Sepharose 4B-step enzymes were used after Toyopearl HW-55 gel filtration. The kidney acid α -glucosidase showed a similar elution pattern (Fig. 2b). However, the spleen acid α -glucosidase was separated into large amounts of S1 and very small amounts of S2 (Fig. 2c). Heart enzyme separated into large amounts of S2 and small amounts of S1 (Fig. 2d). The ratio of S1 to S2 fraction was 1.3 in the liver and kidney, and 0.77 in the heart.

Characterization of the purified enzyme

The purified enzyme, obtained after the final Toyopearl HW 55 gel filtration, and S1 and S2 fractions after Sephacryl S-200 affinity chromatography, were loaded on to the polyacrylamide disc gel (7.3%). After electrophoresis, all of them showed a single band which coincided with the position of the activity

Fig. 2. Sephacryl S-200 affinity chromatography of the liver (a), kidney (b), spleen (c) and heart (d) acid α -glucosidase

Each tissue enzyme (3 mg) was applied to a Sephacryl S-200 column (1.6 cm $\times$ 90 cm) which had been equilibrated with 10 mM-sodium phosphate buffer (pH 6.0) containing 0.5 M-NaCl. The enzyme was eluted from the column with the same buffer; 2.3 ml fractions were collected, and their acid α -glucosidase activity was measured at pH 4.5 with 4-MU- α -glucoside (●) as substrate. The enzyme activity appeared after the total volume of the column on continued elution with the same buffer. A m-unit of enzyme activity is 1 nmol of 4-methylumbelliferone produced/min at pH 4.5. Applied total activity of each sample was 566.6 m-units.

located in the slices of unstained gel (Fig. 3), and this activity increased with the amount of the enzyme protein.

The purified enzyme was also analysed by use of SDS slab-gel electrophoresis. The S1 and S2 fractions from the liver, kidney and heart each contain components of apparent 76 kDa and 67 kDa (Fig. 4), whereas in the spleen, the S1 fraction and also the very small S2 fraction consisted of only one 76 kDa component (Fig. 4). The 76 kDa and 67 kDa components from the liver S1 and S2 which were separated by chromatofocusing were all able to hydrolyse glycogen, maltose, isomaltose and turanose. The liver S1 76 kDa-component activities for the hydrolysis of glycogen were all maximal at pH 4.0–4.5 in the presence of a carrier protein such as BSA. The pH values at which maltose and isomaltose were maximally hydrolysed were 4.0–5.0 and 5.0 respectively; that for turanose was 3.5–5.0, and the three other components also revealed similar pH profiles for the hydrolysis of these substrates. The maximum velocity and K_m values of the four components for the hydrolysis of the various substrates were also examined. The four components had similar enzymic properties. For the S1 76 kDa component, K_m values were 17.0 mg/ml for glycogen, 9.0 mM for maltose, 67 mM for isomaltose and 2.3 mM for turanose. The S2 76 kDa component has 18.3 mg/ml as the K_m value for glycogen, 7.0 mM for maltose, 71 mM for isomaltose and 2.5 mM for turanose.

The inhibitory effects of isomaltose, maltose, glycogen and turanose on the initial rate of hydrolysis of 4-MU- α -glucoside

were examined, and the data were analysed by Dixon plots. The K_i values for the S1 76 kDa component were 16.0 mg/ml for glycogen, 4.5 mM for maltose, 50.0 mM for isomaltose and 0.8 mM for turanose. The results indicated that all these substrate compounds competitively inhibited the 4-MU- α -glucosidase activity of the four components of the liver acid α -glucosidase. These four components may therefore be considered to have a single substrate-binding site which is common to isomaltose, maltose, glycogen, turanose and 4-MU- α -glucoside.

Western-blot analysis

Antibody against acid α -glucosidase obtained from a rabbit (1500 g) which had been immunized with an S1 76 kDa component of the liver mixed with an equal volume of Freund's complete adjuvant reacted with the three other components (Fig. 5).

Immunohistochemical observations

In the spleen, the reaction was found only in the macrophages in the sinus and splenic corpuscles (lymphatic nodules) (results not shown). Macrophages were identified morphologically and by immunohistochemistry of α -L-iduronidase, which was detected in the macrophages in the spleen (Sakuda *et al.*, 1990). In the lymphocytes, the reaction was not demonstrated. Immunohistochemical reactions in the frozen sections were slightly stronger than those in the deparaffinized sections.

Fig. 3. Polyacrylamide-disc-gel electrophoresis of the purified liver enzyme

Purified enzyme (Toyopearl HW 55 step) (20 μ g) was applied to the gel. Electrophoresis was carried out as described in the Materials and methods section. The gel was stained with Coomassie Brilliant Blue: $\blacktriangle$, band of the enzyme; *, Bromophenol Blue dye. Other unstained gels were cut into 2.0 mm sections, which were used for measurement of the enzyme activity ($\circ$) after extraction with 100 mM-sodium acetate (pH 4.5). A m-unit of enzyme activity is nmol of 4-methylumbelliferone produced/min at pH 4.5.

Fig. 4. SDS/PAGE of S1 and S2 fractions

Electrophoresis was performed as described in the Materials and methods section. Lane L, liver (2 μ g); lane K, kidney (2 μ g); lane H, heart (2 μ g); lane S, spleen (2 μ g); lane M, molecular-mass markers (2 μ g each), including phosphorylase *b* (94 kDa), BSA (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa) and trypsin inhibitor (20 kDa). After electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250 (0.04% in 3.5% HClO_4).

Lectin-binding analysis of acid α -glucosidase by use of the lectin-nitrocellulose method

Both 76 kDa and 67 kDa components of the S1 and S2 were heavily stained with Con A. Another nitrocellulose sheet treated with Endo F was also stained with Con A. The staining patterns of both 76 kDa and 67 kDa components of S1 and S2 became very weak. The 76 kDa and 67 kDa components of S1 showed

Fig. 5. Western-blot analysis of S1 and S2 fractions of the liver and spleen

Western-blot analysis was performed as described in the Materials and methods section: 2 μ g of each sample was used for SDS/PAGE. After SDS/PAGE, Western blotting was performed. Lane A, S1 fraction of the liver; lane B, S2 fraction of the liver; lane C, S1 fraction of the spleen; lane D, S2 fraction of the spleen; lane M, molecular-mass markers (2 μ g each), as in Fig. 4.

weaker stains than those of S2 (Fig. 6a). Con A staining of all the bands was also decreased by PNGase treatment (Fig. 6b). However, no changes were observed after digestion with Endo H (Fig. 6c). The 76 kDa and 67 kDa components of both S1 and S2 showed only slight staining by WGA. Even after treatment with neuraminidase the bands showed similar stains (Fig. 6d). With RCA, only the 76 kDa component of S1 and S2 showed faint stains (results not shown). However, after treatment with neuraminidase or H_2SO_4 , the 67 kDa band of both S1 and S2 became weakly stained (results not shown). The 76 kDa and 67 kDa components of S1 and S2 showed faint stains with PHA-E4 (results not shown), and moderate stains with LCA (results not shown). PNA did not react with the either 76 or 67 kDa components of S1 or S2 (results not shown).

DISCUSSION

Acid α -glucosidase from pig liver was purified and separated into two fractions (S1 and S2) by use of Sephacryl S-200 gel affinity chromatography. The amount of the S1 fraction was 1.3 times that of the S2 fraction. In the kidney, the amount of S1 fraction was also 1.3 times that of the S2 fraction, whereas the enzyme from the heart showed 1.3 times as much S2 as S1. In the spleen, acid α -glucosidase consisted mainly of the S1 fraction. The S1 and S2 fractions were reproducible on rechromatography. When two fractions were analysed by SDS/PAGE, S1 and also S2 from the liver, kidney and heart had a 76 kDa and a 67 kDa component. However, spleen enzyme consisted of only a 76 kDa component. Immunohistochemically, acid α -glucosidase was demonstrated only in the macrophages in the spleen (results not shown). The 76 kDa component in the spleen is likely to come mainly from the macrophages. This was also confirmed by use of human leukaemia cell line THP-1 (obtained from American Type Culture Collection). The monocyte cell line THP-1 consisted of only a 76 kDa component (Nakasone *et al.*, 1989). In the liver,

Fig. 6. Lectin-binding analysis by the nitrocellulose method

Portions (2 μ g each) of S1 and S2 were used in each experiment. As controls, 2 μ g of ovalbumin (O) and 1 μ g each of fetuin (F) and transferrin (T) were used. S1 and S2 fractions from Sephacryl S-200 affinity chromatography were used. On another nitrocellulose sheet, bands of S1 and S2 fractions were confirmed by Western-blot analysis. Procedures are described in the Materials and methods section. The right-hand panels are stained after enzymic treatment. The left-hand panels are stained without enzymic treatment; (—) indicates mock incubation. (a) Con A staining after Endo F treatment. The 76 and 67 kDa components of S1 and S2 showed weaker stains than those in the 'mock' incubation (—), without Endo F. The 76 and 67 kDa components of S1 showed weaker stains than those of S2. W, Western-blot analysis of S1; M, molecular-mass markers (2 μ g each) including phosphorylase *b* (94 kDa), BSA (67 kDa) and ovalbumin (43 kDa). (b) Con A staining after PNGase treatment. The staining patterns of both 76 and 67 kDa components of S1 and S2 became weak after treatment with PNGase. (—) 'Mock' incubation with PNGase. (c) Con A staining after Endo H treatment. No changes were observed after digestion with Endo H. (—) 'Mock' incubation without Endo H. (d) WGA staining of the 76 and 67 kDa components of S1 and S2, which showed only slight staining. No changes were observed after treatment with neuraminidase. (—) 'Mock' incubation without neuraminidase.

Kupffer cells are known to be cells of the mononuclear phagocyte system, similar to macrophages. It seems likely that the Kupffer-cell enzyme consisted of only a 76 kDa component, and the hepatocyte enzyme of both 76 kDa and 67 kDa components. Murray *et al.* (1978) and Henkel *et al.* (1985) have also reported heterogeneity of the acid α -glucosidase from human liver in terms of its pI and molecular size. Furthermore, Henkel *et al.* (1985) obtained four enzymically active forms by chromato-

focusing, and reported that sialic acid and mannose 6-phosphate were not detected in the carbohydrate structure. Thus the charge heterogeneity resulted from differences in the protein structure of the charge forms. Tsuji *et al.* (1988) found that acid α -glucosidase is transported to lysosomes by a phosphomannosyl-receptor-independent system in I-cell-disease fibroblasts. Independently Hasilik & Neufeld (1980b), Reuser *et al.* (1985) and Iwamasa *et al.* (1986) reported the incorporation of phosphate into human

acid α -glucosidase in cultured fibroblasts. The heterogeneity in the pI of pig liver enzyme molecule was considered in part to reflect differences in post-translational modification. However, the question arises as to whether our 67 kDa molecule represents a normal mature form of the enzyme. Hasilik & Neufeld (1980a) found that acid α -glucosidase purified from human placenta contained two components with molecular masses of 76 kDa and 70 kDa, and that of the enzyme in normal fibroblasts labelled with [3 H]leucine was processed to a 76 kDa component, and a small amount to a 70 kDa component. Oude Elferink *et al.* (1985) reported more recently that a 76 kDa component constituted a mature form in cultured human skin fibroblasts, and that it had been processed from a 95 kDa intermediate form. From our results of amino acid sequence analysis of the 76 kDa and 67 kDa components of S1 and S2 (Yagami *et al.*, 1989), our 67 kDa molecule was considered to be produced from the 76 kDa mature form. In the macrophages which contained only a 76 kDa component, the processing or degradation of acid α -glucosidase is now understood to be different from that of other cells. An interesting finding obtained in the present study was that acid α -glucosidase was heterogeneous in terms of its affinity to Sephacryl S-200 gel. If Sephacryl S-200 gel were to act as a substrate analogue of the natural substrate, such as glycogen (Schram *et al.*, 1979), the components of the enzyme that could be separated by this gel would be expected to differ in their affinity for that substrate. However, their catalytic properties for the hydrolysis of glycogen and other substrates differ slightly. Separately, CRM (immunologically cross-reactive material from Pompe's-disease fibroblasts without enzyme activity) also showed affinity to Sephacryl S-200 gel (results not shown). It may be considered that the affinity to the gel did not completely depend on the active-site structure of the enzyme. Jeffery *et al.* (1970) and Koster & Slee (1977) postulated that acid α -glucosidase from rat liver and human liver had multiple substrate-binding sites. Our results, however, indicate that acid α -glucosidase from pig liver had a single substrate-binding site, since the 4-MU- α -glucosidase activities of the four components of this enzyme were all competitively inhibited by isomaltose, maltose, glycogen and turanose. Onodera *et al.* (1989) also reported that rabbit liver acid α -glucosidase has a single active site for hydrolysis of maltose and glycogen. The reason why acid α -glucosidase shows heterogeneity on Sephacryl S-200 gel is obscure. However, the elution pattern of the acid α -glucosidase in Sephacryl S-200 affinity chromatography was reproducible, and was the same at any time. The macrophage enzyme consisted of mainly the 76 kDa component of S1, even after rechromatography. It was considered that the heterogeneity reported in the present paper was not due to artifact or random degradation of the enzyme. The possibility that differences in their carbohydrate structure cause their heterogeneous affinity to Sephacryl S-200 gel was examined in the present paper. Slight differences between the S1 and S2 were demonstrated after treatment with Endo F and PNGase. After treatment with Endo F or PNGase, the affinity to Sephacryl S-200 gel and also Con A-Sepharose 4B gel changed (results not shown). However, it still showed affinity to Sephacryl S-200 gel even after glucosidase digestion. The heterogeneity of affinity might partly depend on the protein structure. Con A reacted strongly with both S1 and S2. However, after Endo F treatment, the S1 showed weaker stains than the S2, and the 67 kDa band of S1 was stained the weakest. Con A staining of all the bands was decreased by PNGase treatment. Use of WGA, LCA, RCA and PHA-E4 produced similar staining in both S1 and S2. Mutsaers *et al.* (1987) reported that human acid α -glucosidase (76 and 70 kDa components) contained four *N*-glycosidic carbohydrate chains per molecule, and reported the presence of mannose, *N*-acetylglucosamine and fucose. In addition, trace

amounts of galactose and *N*-acetylneuraminic acid was also detected. In our present studies, a mannose-rich *N*-linked sugar chain is contained in both S1 and S2. Con A-staining patterns before and after digestion with Endo H, Endo F and PNGase indicated that there may be small amounts of other sugar chains with different structure. The sugar components reported by Mutsaers *et al.* (1987) were also confirmed, but slight differences between S1 and S2 were found. Thus the difference in affinity to Sephacryl S-200 between S1 and S2 may in part depend on the differences in their sugar chains. In the present report, it was demonstrated that the amounts of S1 and S2 fractions after Sephacryl S-200 gel chromatography in various tissues was different, and the processing or degradation of acid α -glucosidase in macrophages was different from that in other cells. Separately, it was reported that, for β -glucuronidase from mouse macrophage P388D1 cells, small amounts of complex-type sugar chains were also found (Goldberg & Kornfeld, 1981). However, further studies are required to determine whether or not such heterogeneity has a relationship to the manifestation of glycogenosis type II.

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