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CHARACTERIZATION OF NEUTRAL ISOZYMES OF HUMAN α-GLUCOSIDASE

DIFFERENCES IN SUBSTRATE SPECIFICITY, MOLECULAR WEIGHT AND ELECTROPHORETIC MOBILITY

FRANK MARTINIUK and ROCHELLE HIRSCHHORN

Department of Medicine and Department of Biology, New York University School of Medicine, New York University Medical Centre, 550 First Avenue, New York, NY 10016 (U S A)

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Summary

We have previously defined two isozymes of neutral α-glucosidase (α-D-glucoside glucohydrolase, EC 3 2.1 20) on the basis of differences in electrophoretic mobility and designated these neutral α-glucosidase AB and α-glucosidase C (Swallow, D M , Corney, G , Harris, H and Hirschhorn, R (1975) Ann. Hum Gen. 38, 391—406) We now describe differences between the two isozymes with respect to molecular weight, solubility in $(NH_4)_2SO_4$, glycosylation, isoelectric point and substrate specificities Neutral α-glucosidase C is precipitable in 40—60% $(NH_4)_2SO_4$, has a molecular weight of 92 000, an isoelectric point of 5 5 and releases glucose from glycogen as well as from low molecular weight artificial and natural substrates containing α1-4 glucosidic linkages Neutral α-glucosidase AB precipitates at 0—40% $(NH_4)_2SO_4$, binds to concanavalin A, has a molecular weight of greater than 150 000, and does not utilize α1-4 linked glucose substrates larger than a disaccharide Neutral α-glucosidase AB migrates more rapidly to the anode than α-glucosidase C when agarose, Cellogel, acrylamide or starch are used as support media. Both isozymes are equally inhibited by Zn^{2+}

Introduction

The glucosidases are a group of enzymes which catalyze the cleavage of glucosidic linkages and which can be subdivided according to their pH optima

into acid and neutral forms Acid α-glucosidase (α-D-glucoside glucohydrolase, EC 3.2 1 20) is a lysosomal hydrolase which primarily splits α1-4 glucosidic linkages, with glycogen as its substrate, and has been extensively studied and well characterized in mammals, including man [1—4]. Inherited deficiency of this enzyme results in glycogen storage disease Type II or Pompe' disease [4] Neutral α-glucosidase activity has not been well characterized in the past. Attempts have been made to define the neutral α-glucosidases [5—11] but often without a clear realization that there appear to be several different isozymes.

We have previously reported that α-glucosidase activity can be subdivided into two forms on the basis of differences in mobility following electrophoresis in starch gel We have called these two isozymes 'neutral α-glucosidase AB' and 'neutral α-glucosidase C' [12]. Dreyfus and Alexandre [13] have similarly described two isozymes of neutral α-glucosidase detected by electrophoresis of tissue extracts in Cellogel and staining for enzyme activity. Our recent studies have also shown that human neutral α-glucosidase C is genetically polymorphic, with four alleles, including a 'null' allele and is coded for by a genetic locus provisionally, assigned to chromosome 15 [14,15] * and separate from that for neutral α-glucosidase AB An additional neutral enzyme has also been reported in renal tissue [13,16—19].

The presence of these neutral isozymes of α-glucosidase with broad pH optima has complicated prenatal diagnosis, heterozygote detection and quantitation of residual enzyme activity in infantile and adult onset acid α-glucosidase deficiency disease [16,20—22]. Various maneuvers, including the use of anti-acid α-glucosidase antibodies and differential inhibition by turanose, Zn^{2+}, K^{+}, and ratios of activities at different pH, etc., have been used in an attempt to distinguish between glucosidase activity of the acid as opposed to the neutral isozyme(s) in whole tissue extracts [16,23—29]. We have attempted to directly separate and characterize the neutral isozymes of α-glucosidase which we described earlier, and report here the results of these studies

Materials

4-Methylumbelliferone, 4-methylumbelliferyl-α-D-glucopyranoside (4-methylumbelliferyl-α-glucoside), β-naphthyl-α-glucoside, bovine serum albumin, ovalbumin, catalase, aldolase, transferrin, apoferritin chymotrypsinogen, α-methyl-D-mannoside, maltose and a glucose determination kit (Cat. No 510-A) were obtained from Sigma Chemical Co; Sephadex G-100, G-200, Sephacryl S-200 (Superfine), Sepharose 4B and Con A-Sepharose from Pharmacia Fine Chemicals, Bio-gel P-300 (100—200 mesh), agarose, acrylamide, bisacrylamide, Temed and $(NH_4)_2S_2O_8$ from Bio-Rad Labs; starch hydrolyzed from Connaught Labs Ltd., Canada, $(NH_4)_2SO_4$ (Ultra-pure) from Schwarz-Mann; rabbit liver glycogen from Boehringer-Mannheim, Statzyme-Glucose 16 from Worthington, $MnCl_2$, $ZnCl_2$ and $HgCl_2$ from J T. Baker Chemical Co.;

* Neutral α-glucosidase C has been designated GANC by the International System for Human Gene Nomenclature (ISGN 1979) In Human Gene Mapping 5 (1979), Fifth International Workshop on Human Gene Mapping Birth Defects Original Artical Series, Vol 15, No 11 (The National Foundation, New York 1979), also in Cytogenet Cell Genet (1979) Vol 24

Cellogel from Kalex Science Co (Manhasset, NY), $MgCl_2$ from Fisher Scientific Co , RPMI 1640, fetal calf serum, glutamine and penicillin-streptomycin from Grand Island Biological Co , Lymphoprep from Accurate Chemical and Scientific Co., PM-10 membranes from Amicon Corp , maltotriose from Aldrich Co and ampholines from LKB.

Methods

Sample preparation Tissues (obtained within 24—48 h post mortem and frozen at —60°C) were suspended in an equal volume of H_2O/g weight, homogenized in a Waring Blendor for 5 min at 4°C, sonicated briefly (Heat systems—Ultrasonics; Sonifier-Cell disruptor) and centrifuged for 15 min at 15 000 × *g* at 4°C For preparation of α-glucosidase AB from placenta, the placenta was washed extensively with 0 1 M sodium phosphate pH 7.5, for 3—4 days at 4°C, any connective tissue was removed and the placenta frozen at —60°C Peripheral blood lymphocytes were isolated on Hypaque Ficoll gradients [30]. Lymphoid line cells were grown in RPMI 1640 with 16% heat-inactivated fetal calf serum, washed, suspended at 10^8 cell/ml H_2O freeze-thawed five times and sonicated.

Enzyme assays The 4-methylumbelliferyl-α-D-glucopyranosidase activity was determined essentially as described previously [12,16] Bovine serum albumin (1 mg/ml) was included for assay of partially purified enzyme The fluorescence of the free 4-methylumbelliferone produced was measured in a Turner fluorimeter (Model 110) with a 7—60 excitation filter and 2A/2ND emission filters. 1 unit enzyme activity was defined as the production of 1 μmol 4-methylumbelliferone/min at 37°C For determination of glucoamylase activity, 40 μl 100 mg/ml glycogen in either 0.1 M sodium phosphate, pH 7.5 or 0.5 M CH_3CO_2Na pH 4 (containing 3 mg/ml bovine serum albumin) and a 20 μl sample were incubated for 30—360 min at 37°C and the reaction terminated by boiling for 2 min Glucose was determined with Sigma's glucose determining kit No. 510, modified according to Dr N Beratis by diluting the *O*-dianisidine to 50 mg/20 ml H_2O. 1 unit was defined as the production of 1 μmol glucose/min at 37°C For determination of maltase and maltotriase activity, 40 μl 10 mM maltose or maltotriose in 0.5 M sodium phosphate, pH 7 5, and a 20 μl sample were incubated at 37°C, for 30—180 min and the reaction terminated by boiling for 2 min. Glucose was determined using either the Sigma glucose kit No. 510 or the Worthington Statzyme-glucose 16 kit according to the manufacturer's direction Protein was determined by the method of Lowry et al. [31] with human albumin as a standard.

Column chromatography Sephadex G-100, G-200, Sephacryl S-200 and Bio-Gel P-300 column were eluted with 0.05 M sodium phosphate, pH 7.5. The Bio-Gel P-300 column was run at 4 ml/h for 60 days [32] prior to use Columns were calibrated with standard markers and also with endogenous enzyme markers (purine nucleoside phosphorylase, M_r 89 000, peptidase B, 54 000, adenosine deaminase, 35 000 and adenylate kinase, 24 000) when whole tissue extracts were applied.

Electrophoresis Electrophoresis in starch gel (10 1% hydrolysed starch in 0.01 M sodium phosphate pH 6.5) was performed as previously described [14]

Horizontal slab agarose (1%) or slab polyacrylamide (5%) electrophoresis was carried out in 0 04 M sodium phosphate, pH 7.5, 10 V/cm for 3.5—4 h for the agarose gels and 0.05 M sodium phosphate, pH 6.5 or 7.5, 9 V/cm for 4 h at 4°C for the acrylamide gels using the LKB Multiphor apparatus. Cellogel was presoaked and prerun in 0.04 M sodium phosphate, pH 6 5, samples applied and electrophoresed at 15 V/cm, 6 mA for 3 h Isoelectric focusing in horizontal slab gels was performed as previously described [14]. Enzyme activity was detected as described for starch gel electrophoresis, except that Cellogel was also used to incorporate the stain following electrophoresis in Cellogel. Vertical slab polyacrylamide gel electrophoresis (4% stacking, 6% running gels) for the kidney isozyme was performed in a Pharmacia Apparatus GE-4 using 0.05 M sodium phosphate, pH 7.0, with constant current of 45 mA per slab for 18 h at 4°C. Enzyme activity was detected by staining with β-naphthyl-α-D-glucoside and fast blue B at pH 5.6, as previously described [12].

Con A-Sepharose binding and elution. Samples were applied to a 5 ml Con-A-Sepharose column previously equilibrated in 0.1 M glycine/1 mM $MgCl_2$/1 mM $MnCl_2$, pH 7.0 The column was then washed with 0.1 M sodium phosphate/1 M NaCl, pH 7 0 (100 ml at 60 ml/h) and the bound glycoprotein eluted by addition of 1 M α-methyl-D-mannoside to the wash buffer.

Production of rabbit anti-placental acid α-glucosidase Purified human placental acid α-glucosidase was prepared by a modification of the method of de Barsy et al [1]. After homogenization and centrifugation, excess hemoglobin was removed by adding CM-Sephadex C-50 to the sample (previously adjusted to pH 5.0) and stirring for 30 min at 25°C, maintaining the pH at 5.0. The mixture was filtered on a Buchner funnel and the filtrate acidified to pH 4 with slow addition of 2 N HCl, while stirring Completion of the purification was identical to that previously described [1].

Rabbits were injected intradermally with purified placental acid α-glucosidase (0.5 mg protein/ml in complete Freund's adjuvant), boosted with 0.25 mg protein in complete Freund's adjuvant 4 and 6 weeks later and then subcutaneously at 8 weeks with 0.125 mg antigen in complete Freund's adjuvant. All rabbit serum, following the first booster, contained antibody to acid α-glucosidase as detected by Ouchterlony double-immunodiffusion and staining of precipitin lines for enzyme activity with β-naphthyl-α-D-glucoside (1 mg/ml) and fast blue B (2 mg/ml) in 0.1 sodium citrate, pH 4.6 [12]. The rabbit antibody to human placental acid α-glucosidase (obtained at 8 weeks) was attached to CNBr-activated Sepharose 4B. The Sepharose antibody column (1 × 10 cm) was equilibrated with phosphate-buffered saline at a flow rate of 10 ml/h Samples (0.5 ml) were applied, 1 ml fractions collected and fractions containing neutral α-glucosidase activity pooled, concentrated and reassayed for both neutral and acid α-glucosidase activity

Results

Electrophoresis in starch and polyacrylamide gels and detection of isozymes of neutral α-glucosidase Electrophoresis of extracts of tonsil, liver, lymphoid line cells and peripheral blood lymphocytes in starch gels reveals two major areas of α-glucosidase activity when the gels are incubated with 4-methyl-

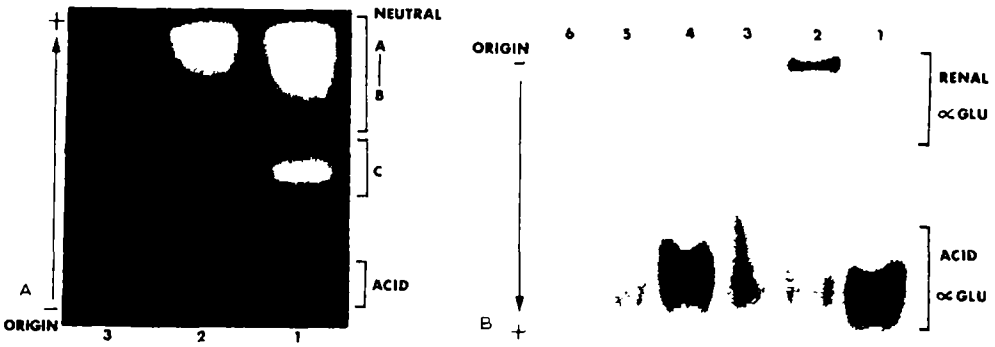

Fig 1 A Electrophoresis is starch gel of neutral α-glucosidases Tissue extracts were electrophoresed in starch gels and the gels incubated with 4-methylumbelliferyl-α-glucoside at neutral or acid pH in order to visualize α-glucosidase activity. Channels 1 and 3 contain an extract of human tonsil and channel 2 an extract of human placenta Channels 1 and 2 have been stained at neutral pH The most anodally migrating area is usually seen to consist of two bands of enzyme activity, but in this photograph they have been blurred into a single area (channel 1) although a suggestion of a second band can be seen in 2 These two bands have been previously called α-glucosidase A and B A second major area of enzyme activity is seen migrating midway between the origin and the A-B isozyme and has previously been called α-glucosidase C This isozyme is usually of weaker intensity than α-glucosidase AB in most tissues or cells other than tonsil, including liver, kidney and fibroblasts In placenta (channel 2), the C isozyme is barely detectable When the same extracts are stained at acid pH (channel 3), a single area of enzyme activity is seen which remains close to the origin (acid α-glucosidase) B. Electrophoresis in polyacrylamide of various tissues stained at pH 5.6 for β-napthyl-α-glucosidase activity and demonstration of the renal isozyme. Channel 1 contains purified human placental α-glucosidase (αGLU), channel 2 = human kidney, channel 3 = human liver, channel 4 = human placenta, channel 5 = human tonsil and channel 6 = human lymphoid line Kidney demonstrates a very prominent glucosidase activity which is clearly separated from the acid α-glucosidase activity (Neutral α-glucosidase AB and C are not visualized with this stain)

umbelliferyl-α-D-glucoside at neutral pH (7 5) (Fig. 1A). The more anodally-migrating double-banded isozyme has been designated α-glucosidase AB, while the usually weaker-staining, more slowly migrating isozyme has been designated α-glucosidase C [12]. Placental extracts do not exhibit easily detectable α-glucosidase C activities under these conditions. The acid α-glucosidase isozyme (detected by staining at acid pH) migrated minimally to the anode in all tissues used in these studies (Fig 1A) and a genetically polymorphic rare allozyme of acid α-glucosidase [12] was not present in any of the tissues utilized in these studies

In contrast to the above tissues, extracts of kidney exhibit an additional isozyme This isozyme migrates in starch gel to a point either between the origin and the common allozyme of acid α-glucosidase or to a position very slightly anodal to that of acid α-glucosidase and is often difficult to visualize separately. The kidney isozyme can be most clearly differentiated from the acid α-glucosidase by electrophoresis in 6% vertical slab polyacrylamide gels, pH 7 and staining with β-naphthyl-α-D-glucoside at pH 5 6. Kidney extracts exhibit a double band of enzyme activity migrating near the origin (Fig 1B) and this isozyme is not observed in any of the other tissues examined. Under these conditions of electrophoresis and staining, the acid α-glucosidase is clearly separate from the kidney isozyme and migrates more rapidly to the anode while the neutral α-glucosidase AB and C are not visualized.

Finally, platelet-rich buffy coat preparations exhibit an additional 4-methylumbelliferyl-α-glucosidase activity visible, in starch gel following electrophoresis, as a less clearly defined area of activity extending from the α-glucosidase C region through the α-glucosidase AB region, with greatest intensity between the two isozymes. The following studies deal only with the α-glucosidase AB and C isozymes.

Neutral α-glucosidase activity The total neutral α-glucosidase activity at pH 7 5, as determined with 4-methylumbelliferyl-α-glucoside as substrate, was very similar in homogenates of the various human tissues or cultured cells tested Thus, long-term lymphoid line cell extracts had a specific activity of 0 71 ± 0 06 U/g (n = 5), tonsil of 1.5 ± 0 5 (n = 9), liver of 0.6 (n = 2) and placenta of 0.54 (n = 2).

Separation of isozymes on Sephadex G-100 and determination of relative electrophoretic mobilities in agarose, acrylamide and Cellogel When extracts of human liver, tonsil or lymphoid lines were chromatographed on Sephadex G-100 columns, the neutral α-glucosidase activity eluted as two major peaks (Fig. 2). The first peak eluted over a broad range with an apparent molecular weight of 152 000 ± 9800 (n = 4), while a second peak eluted with an apparent molecular weight of 92 250 ± 9800 (n = 4).

Fractions containing each of the two peaks separated by chromatography on Sephadex G-100 were concentrated and electrophoresed in starch gels. The 150 000 molecular weight peak migrated rapidly to the anode as two bands of enzyme activity corresponding to neutral α-glucosidase AB isozyme found in whole homogenates. The 92 000 molecular weight peak migrated less rapidly to the anode and migrated to the same position as the α-glucosidase C isozyme in whole homogenates. There was some cross contamination in each of the frac-

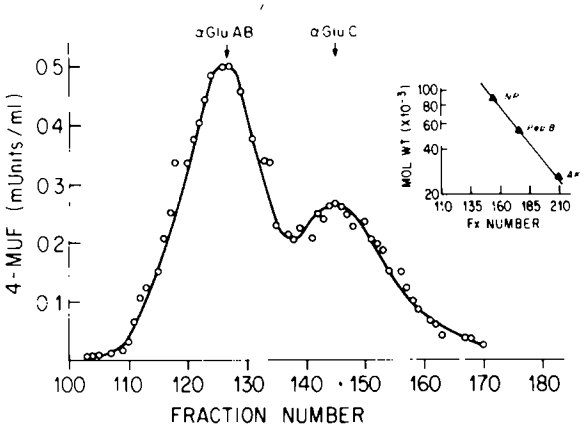

Fig. 2. Sephadex G-100 (2 5 X 100 cm) chromatography of human lymphoid line extract Two major peaks of neutral α-glucosidase activity 4-methylumbelliferyl-α-glucoside (4-MUF) at pH 7 5) were observed with apparent molecular weights of 152 000 and 92 000, respectively. Fractions 115—131 and 136—155 (1.5 ml/fraction) were pooled, concentrated and electrophoresed in starch gel The 152 000 peak co-migrated with the most rapidly migrating anodal enzyme bands (α-glucosidase AB, αGluAB), while the 92 000 peak co-migrated with the least anodal migrating enzyme band (α-glucosidase C, αGlu) Identical results were obtained using extracts from human liver and tonsil

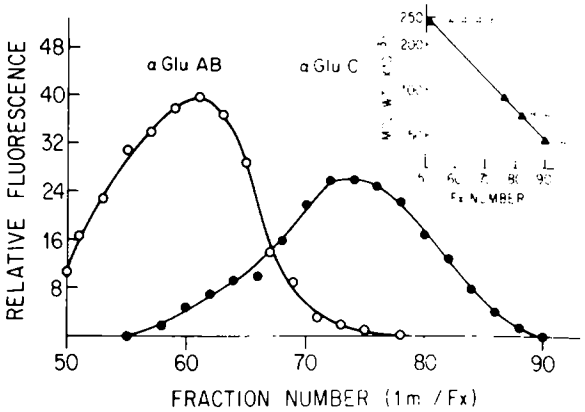

Fig 3 Sephadex G-200 chromatography (1 5 X 100 cm) of partially purified human neutral α-glucosidase Each isozyme was partially purified from human liver The α-glucosidase AB (α-Glu AB) peak (as determined by starch gel electrophoresis) eluted with an apparent molecular weight of 152 000 and α-glucosidase C (αClu C) (as determined by starch gel electrophoresis) eluted with an apparent molecular weight of 95 600 Identical results were obtained with partially purified material from human tonsil, lymphoid lines and placenta (α-glucosidase AB only)

tions. The higher molecular weight peak (152 000) will now be referred to as 'neutral α-glucosidase AB' while the lower molecular weight peak (92 000) will now be referred to as 'neutral α-glucosidase C'.

Since two isozymes of neutral α-glucosidase have been previously defined by electrophoresis in Cellogel [13,29], we also determined the relative mobility of the neutral α-glucosidase AB and neutral α-glucosidase C isozymes on non-starch-containing support media Electrophoresis of whole homogenates in agarose or polyacrylamide gels as well as in Cellogel revealed two sharp bands of neutral α-glucosidase activity When the two isozymes were separated by chromatography on Sephadex G-100 and then electrophoresed, the neutral α-glucosidase AB migrated most rapidly to the anode on all three support media, as it did on starch gels, but only a single, rather than a double band could be distinguished Neutral α-glucosidase C migrated less anodally on all four electrophoretic supports The relative mobilities of the two isozymes was further confirmed by electrophoresis of an extract of a long-term lymphoid line from an individual genetically deficient in neutral α-glucosidase C [14]. Electrophoresis in starch, agarose and Cellogel of this extract revealed only the most anodally migrating isozyme, or neutral α-glucosidase AB.

Isoelectric point Isoelectric focusing of crude extracts of liver, tonsil, peripheral blood lymphocytes or lymphoid lines consistently revealed one area of enzyme activity with a p*I* value of approx 5 5. On occasion a second area of enzyme activity with a p*I* value of approx. 6.1 was seen.

The isoelectric point of neutral α-glucosidase C was determined using the Sephadex G-100 lower molecular weight peak obtained from human peripheral blood lymphocytes, lymphoid line cells, tonsil and liver. The p*I* value for neutral α-glucosidase C from all these sources was 5.5 ± 0.2 (n = 7) on a narrow (5—7) or a wide (3—9) pH gradient range The p*I* for neutral α-glucosidase AB initially could not be clearly determined using either homogenates or partially

purified material (Sephadex G-100 fractionated or Con A-Sepharose bound and eluted material) from any tissues studied. However, a 1000-fold purified preparation of the placental AB isozyme had a p*I* of 6.1, corresponding to the p*I* occasionally observed in whole homogenates. These p*I* values are clearly different from the isoelectric point of 4.7 which we determined for acid α-glucosidase under these conditions

Separation of isozymes by binding to Concanavalin A and $(NH_4)_2SO_4$ precipitation The two neutral α-glucosidases could also be separated by chromatography on Con A-Sepharose columns [34] or by $(NH_4)_2SO_4$ precipitation. Con A-Sepharose bound approx. 0 64 units enzyme activity/ml packed beads. When lower total amounts of enzyme activity were applied, approx. 75% (75.5 ± 0 7) of the total neutral α-glucosidase activity was bound and subsequently eluted with α-methylmannoside, while 25% (25.5 ± 0.7) was not bound The bound enzyme was entirely neutral α-glucosidase AB, as defined by molecular weight and by electrophoretic mobility in starch gel The unbound material contained mostly neutral α-glucosidase C with some contaminating neutral α-glucosidase AB

Starch gel electrophoresis of material precipitated at 0—40% and at 40—60% $(NH_4)_2SO_4$ saturation indicated that a separation of the two isozymes had occurred. Neutral α-glucosidase AB was precipitated at 40% saturation and represented 62.5 ± 6.4% of total neutral α-glucosidase activity while neutral α-glucosidase C was precipitated at 60% saturation and represented 37.5 ± 6 4% of total neutral α-glucosidase activity The relative contribution of each of the isozymes to the total neutral α-glucosidase activity as determined by $(NH_4)_2SO_4$ precipitation, agreed with that obtained by fractionation on Con A-Sepharose.

Determination of molecular weights by column chromatography on support media containing different glucosidic linkages Whole homogenates and partially purified isozymes of neutral α-glucosidase were chromatographed on various types of support media (Sephadex G-100, G-200, Bio-Gel P-300 and Sephacryl S-200). Following column chromatography, fractions containing neutral α-glucosidase activity were pooled, concentrated and electrophoresed in starch gels to determine which isozyme was present.

Sephadex G-100 and G-200 are dextran polymers and contain α1-4 (as well as α1-6 and α1-3) glucosidic linkages Sephacryl is predominantly acrylamide but also contains dextran while Bio-Gel P-300 contains only acrylamide and thus no glucosidic linkages. These enzymes might be expected to show affinity for and bind to the α-glucosidic linkages present in some of the support media. The isozymes could then elute with an apparently lower molecular weight from Sephadex G-100 or G-200 as compared to Sephacryl S-200 or Bio-Gel P-300.

Neutral α-glucosidase AB activity of either whole tissue homogenates or of a 0—40% $(NH_4)_2SO_4$ fraction eluted with a molecular weight of approx. 160 000 following chromatography on either Sephadex G-100 or G-200 (Figs. 2 and 3). However, a higher molecular weight of 350 000 was observed following chromatography on Bio-Gel P-300 (polyacrylamide) or Sephacryl S-200 (Fig. 4)

The molecular weight of neutral α-glucosidase C in either whole homogenate or of a 40—60% $(NH_4)_2SO_4$ fraction was also determined using the several different support media. Neutral α-glucosidase C activity eluted with an average molecular weight of 95 600 ± 6700 (n = 7) on Sephadex G-100, G-200, Bio-Gel

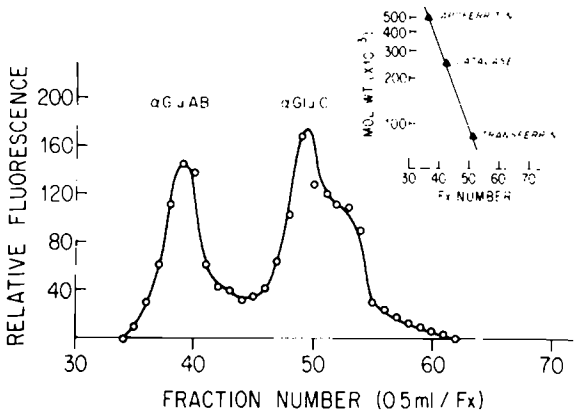

Fig 4 Bio-Gel P-300 (1 5 X 30 cm) chromatography of human tonsil neutral α-glucosidases. Isozymes were separated by Con A-Sepharose before chromatography. α-Glucosidase AB and C eluted with apparent molecular weights of 350 000 and 92 000, respectively. Fractions 37—42 and 46—55 were pooled, concentrated and electrophoresed in starch gel The 350 000 molecular weight peak correspond to α-glucosidase AB (αGlu AB) and the 92 000 molecular weight peak corresponded to α-glucosidase C (αGlu C) in starch gel electrophoresis.

P-300 and Sephacryl S-200. No retardation was found with any of the support media (Figs. 2, 3 and 4)

Substrate specificities. In order to determine substrate specificities, the neutral α-glucosidase AB and α-glucosidase C were each partially purified, using the differences found in precipitation by $(NH_4)_2SO_4$, binding to Con A-Sepharose and molecular weight. An approx. 500—1000-fold purified α-glucosidase AB was obtained from placenta by sequential $(NH_4)_2SO_4$ precipitation, Sephacryl S-200 column chromatography and binding to, and elution from, Con A-Sepharose columns This preparation contained no detectable acid α-glucosidase activity (as determined by assay with 4-methylumbelliferyl-α-glucoside at

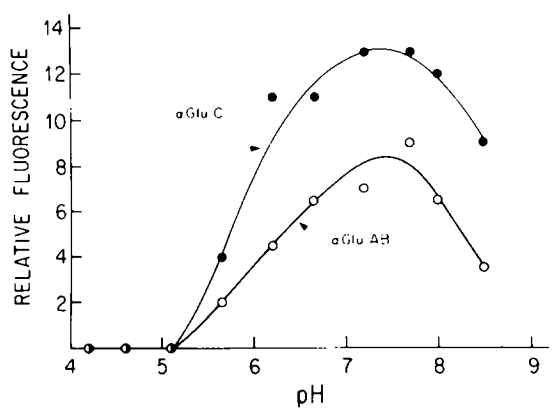

Fig 5. pH optima (4-methylumbelliferyl-α-glucoside) of neutral α-glucosidases Placental α-glucosidase AB (αGlu AB) was purified 500—1000-fold by 40% $(NH_4)_2SO_4$ precipitation, Sephacryl S-200 and Con A-Sepharose (bound and eluted) chromatography Liver α-glucosidase C (α-Glu C) was purified 100-fold by 40—60% $(NH_4)_2SO_4$ precipitation, Sephacryl S-200 and Con A-Sepharose chromatography

pH 4 as well as starch or polyacrylamide gel electrophoresis and staining for enzyme activity). The final specific activity of the material used was 598 μmol/g protein per min and the yield was 10%. The α-glucosidase C isozyme was purified approx. 100—200-fold from liver by sequential $(NH_4)_2SO_4$ precipitation (40—60%), Sephacryl S-200 chromatography and passage through a Con A-Sepharose column This preparation was still contaminated with acid α-glucosidase activity and was therefore passed through a Sepharose-anti-acid α-glucosidase antibody column. This resulted in removal of any detectable acid α-glucosidase activity, as determined by assay with both 4-methylumbelliferyl-α-glucoside and glycogen at pH 4 The final specific activity of the material used was 49 μmol/g protein per min with an approx. 3% yield.

The α-glucosidase AB had slightly lower activity for maltose as compared with activity for 4-methylumbelliferyl-α-glucoside (if μmol substrate hydrolysed rather than μmol glucose produced are compared). (Table I). The AB isozyme had no detectable activity when glycogen or maltotriose were used as substrates

In contrast, the α-glucosidase C isozyme had substantial activity for maltotriose and glycogen. This activity was not due to contamination by acid α-glucosidase, since assay at pH 4 did not reveal any glycogenolytic activity (despite substantial activity at pH 7.5). Similar to α-glucosidase AB, the α-glucosidase C isozyme degraded maltose and 4-methylumbelliferyl-α-glucoside at approximately equal rates.

pH optima and K_m Both isozymes demonstrated a broad pH optimum for 4-methylumbelliferyl-α-glucoside between pH 5.7 and 8.5, with a broad peak at approx. pH 7.5 (Fig. 5) Both isozymes also had essentially the same K_m for 4-methylumbelliferyl-α-glucoside (3.7 and 3.5 10^{-5} M).

Inhibitors. In preliminary experiments, various divalent cations were added to the staining mixture following electrophoresis in starch gels in order to determine their effect upon relative staining intensities of the neutral α-glucosidases. These initial experiments suggested that divalent cations, maltose and turanose might be potential inhibitors for the isozymes.

Partially purified placental neutral α-glucosidase AB and liver neutral α-glucosidase C were assayed in the presence of various concentrations of the

TABLE I

ACTIVITY OF NEUTRAL α-GLUCOSIDASES FOR VARIOUS SUBSTRATES

Activity is expressed relative to the activity for 4-methylumbelliferyl-α-glucoside Activity is calculated as μmol glucose or 4-methylumbelliferone produced/min at 37°C, rather than μmol substrate degraded in order to allow for comparison with glycogen and maltotriose The activity of the α-glucosidase AB preparation used was 0 044 μmol/min (spec act = 598 μmol/g protein per min) The activity of the α-glucosidase C preparation used was 0 008 μmol/ml/min (spec act = 49 μmol/g protein per min)

Substrate	α-Glucosidase AB	α-Glucosidase C
4-Methylumbelliferyl-α-glucoside	1 0	1 0
Maltose	1 49	1 66
Maltotriose	<0 02 *	0 72
Glycogen	<0 05 *	0 45

* Lower limits of detection

TABLE II

INHIBITION OF HUMAN NEUTRAL α-GLUCOSIDASES BY DIVALENT CATIONS, MALTOSE AND TURANOSE

Human placental α-glucosidase AB and liver α-glucosidase C were the same as the partially purified material in Table I 4-Methylumbelliferyl-α-glucoside at pH 7 5

Inhibitor	Concentration	% Inhibition	
		α-Glucosidase AB	α-Glucosidase C
1 Divalent cations	(mM)		
Hg^{2+}	1 00	100	100
	0 10	100	90
	0 01	100	9
Zn^{2+}	10 00	100	100
	1 00	91	87
	0 10	78	58
Mn^{2+}	10 00	48	17
	1 00	0	0
	0 10	0	0
2 Turanose and maltose	(M)		
Turanose	0 90	33	33
	0 45	12	47
	0 10	0	10
Maltose	1 00	40	85
	0 50	0	72
	0 10	0	30

divalent cations (Table II). The two isozymes were differently inhibited by Hg^{2+}. Neutral α-glucosidase AB was totally inhibited at 0 01 mM Hg^{2+}, while neutral α-glucosidase C activity was minimally affected at this concentration. Zn^{2+} inhibited both isozymes essentially equally, with total inhibition at 10 mM. Mn^{2+} only partially inhibited both enzymes, even at 10 mM concentration, with neutral α-glucosidase AB being slightly more inhibited than α-glucosidase C (48 vs. 17%) Mg^{2+} (10 mM) did not affect either isozyme (data not shown).

Interestingly, turanose (often used as a specific inhibitor of the acid isozyme) at 0 45 M concentration inhibited both isozymes in a fashion similar to that observed with Mn^{2+} approx. 50% of the neutral α-glucosidase C activity and 12% of the α-glucosidase AB activity were inhibited (Table II). However, lower concentrations of turanose (0 1 M) capable of inhibiting 90% of purified acid α-glucosidase activity (data not shown) had no effect on the AB activity and only inhibited α-glucosidase C activity by 10% Maltose (0 5 M) substantially inhibited α-glucosidase C activity for 4-methylumbelliferyl-α-glucoside but had no effect on activity of α-glucosidase AB (Table II).

Discussion

Neutral α-glucosidase activity has been studied in several different tissues of different species [5—11,13,17—19,22,35], yet no definitive characterization of this enzyme activity has been forthcoming It has recently been demonstrated that in most tissues at least two different isozymes of neutral α-glucosidase can

be clearly identified by differences in mobility following electrophoresis of extracts and staining for enzyme activity with the artificial substrate 4-methylumbelliferyl-α-glucoside [12,13,29].

We have utilized electrophoresis in starch gel, Cellogel, agarose and polyacrylamide gel, as well as enzymatic assay to identify the two neutral α-glucosidase isozymes following various separative procedures and have compared several different properties of the neutral isozymes. We have found that the neutral α-glucosidase C isozyme precipitated at 40—60% $(NH_4)_2SO_4$, had a molecular weight of approx 92 000 and was not retarded on chromatographic supports containing α1-6 or α1-4 glucosidic linkages. This isozyme had a p*I* value of approx. 5.5, migrated less anodally on all support media at pH 6 5, and did not bind to Con A-Sepharose columns The α-glucosidase AB isozyme migrated more anodally on all support media, precipitated at 40% $(NH_4)_2SO_4$, bound to Con A-Sepharose as previously reported [34] and had a higher molecular weight of 150 000 on Sephadex G-100 or G-200 This molecular weight could reflect retardation on these supports, containing α1-6 and some α1-4 and α1-3 glucosidic linkages, since the molecular weight was considerable higher when determined on Bio-Gel P-300 and Sephacryl S-200 (350 000). However, we do not know if these differences in elution volume reflect enzyme-substrate interaction or some other alteration in the molecule on the different support media Further studies would be required to determine the significance of the differences we have observed Additionally, determination of the isoelectric point of the α-glucosidase AB was not straightforward. No clearcut isoelectric point could be determined consistently by isoelectric focussing of whole tissue extracts in slab gels, although an enzyme activity with a p*I* value of 6 1 was occasionally seen Since the α-glucosidase AB appears to contain sialic acid [12], we suspect that the inability to detect a single isoelectric point might be due to the existence of multiple sialated forms spread out over a range of isoelectric points. The isoelectric point of the 1000-fold purified α-glucosidase AB could be determined by slab gel isoelectric focusing and was found to be 6.1. The electrophoretic mobility of this preparation on starch gel electrophoresis was like that of the B (desialated) isozyme. The AB isozyme migrated rapidly to the anode on all support media, at pH 6.5, a finding inconsistent with this isoelectric point.

Using the difference in properties of the two isozymes, we were able to partially purify both isozymes α-glucosidase AB 1000-fold and α-glucosidase C approc 100-fold (but devoid of acid α-glucosidase). Using these partially purified preparations we found that the two isozymes were markedly different in their substrate specificities. Surprisingly, α-glucosidase AB had no detectable activity on maltotriose as well as no detectable activity on glycogen The isozyme would not appear to be a general disaccharidase since preliminary investigations did not reveal activity on sucrose or lactose. However, this isozyme accounts for approx. 75% of neutral α-glucosidase activity in the tissues tested other than kidney, when maltose or 4-methylumbelliferyl-α-glucoside are used as substrated. The α-glucosidase C isozyme appears to be a glucoamylase since it releases glucose from glycogen as well as from maltotriose and maltose and the artificial 4-methylumbelliferyl-α-glucoside substrate. The specificities of the α-glucosidase C are, therefore, similar to the specificities of the acid isozyme.

However, until α-glucosidase C is purified more extensively we cannot be certain that we may not be co-purifying an α-amylase which degrades glycogen to smaller polysaccharides which can then be further degraded by the neutral α-glucosidase C. The K_m value of neutral α-glucosidase C for low molecular weight substrates is markedly lower than that of the acid isozyme (0.05 vs. 5 mM). If the K_m values for glycogen bear a similar relationship, the neutral α-glucosidase C may play a significant role in glycogenolysis during conditions of low glycogen content

The observation that α-glucosidase C appears to degrade glycogen is of potential biologic significance. We have previously found that approx. 3% of an apparently normal young population are homozygous deficient for neutral α-glucosidase C [14] As a result of this high frequency of deficiency for α-glucosidase C, it can be expected that some individuals who are homozygous for an acid α-glucosidase partial deficiency gene (phenotype of adult onset acid maltase deficiency disease) would by chance also be homozygous deficient for neutral α-glucosidase C If different levels of α-glucosidase C can affect the rate of overall hydrolytic glycogen catabolism, the interaction of deficiency alleles at the two separate genetic loci could explain the reported occurrence of adult onset and infantile phenotype of acid maltase deficiency within the same family [36].

On a practical level, our studies with turanose, demonstrating inhibition of the neutral α-glucosidase C isozyme, point out the need to carefully control the concentration of turanose if this inhibitor is to be used to specifically quantitate the acid isozyme. Our studies with inhibitors also confirm previous reports of inhibition of neutral α-glucosidase by Zn^{2+} [28].

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