Affinity Purification of α-Galactosidase A from Human Spleen, Placenta, and Plasma with Elimination of Pyrogen Contamination

PROPERTIES OF THE PURIFIED SPLENIC ENZYME COMPARED TO OTHER FORMS

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The substrate analog α-D-galactosylamine was synthesized, linked to 6-aminohexanoic acid, and coupled to carboxyhexyl-Sepharose. This affinity support permitted the purification of human α-galactosidase A (α-D-galactoside galactohydrolase, EC 3.2.1.22) from spleen, placenta, and plasma. When used in conjunction with conventional procedures, affinity chromatography enabled the rapid and specific purification of α-galactosidase A from epidermal source. Significantly, pyrogenic endotoxins were eliminated from enzyme preparations by the use of the affinity column. Splenic α-galactosidase A was purified in high yield (38%) with a specific activity of 1.9 × 10^6 units/mg. The purified enzyme was a homodimer with a native molecular weight of 101,000 and a subunit weight of 49,800. The UV absorption coefficient was ε²³⁰ = 18 and the λmax was 282 nm. The plasma form was purified with a markedly improved yield to a specific activity (229,000 units/mg) which was 3 times greater than that achieved previously. The enzymes from plasma, spleen, and placenta were immunologically identical. The physical and kinetic properties of the purified enzymes were consistent with and confirmed previous findings.

The evaluation of replacement therapy for inherited enzyme deficiency diseases has been stymied by the limited availability of sufficient amounts of purified, pyrogen-free enzyme for long term clinical trials (1, 2). Single or multiple doses of partially purified α-galactosidase A, isolated by conventional chromatographic methods from human plasma (3), spleen (4), and placenta (5), have been administered to patients with Fabry disease. Markedly different kinetics of the plasma clearance of enzyme and substrate were observed for the plasma and tissue forms (6, 7). The plasma form had an 8-fold longer half-life and depleted 25 times more substrate than the splenic enzyme. These findings suggested differences in the post-translational modification of the forms and emphasized the need to further characterize their carbohydrate structures as well as to perform additional replacement trials.

Several purification schemes have been reported for α-galactosidase A from human placenta (8, 9), plasma (10), liver (11), spleen (4), and urine (12). The reported specific activities varied over a 20-fold range (5 × 10^2 to 1 × 10^6 units/mg) with yields of 3 to 18% (including α-N-acetylgalactosaminidase in the crude extract). In this paper, we report 1) the rapid, specific, and high yield purification of α-galactosidase A from human spleen, placenta, and plasma using conventional chromatographic techniques and the α-D-galactosylamine affinity ligand of Harpaz et al. (13-15), 2) the characterization of physical and kinetic properties of the purified splenic enzyme and comparison of selected properties with α-galactosidase A from other sources, 3) the correlation of pyrogenic acid content with the charge heterogeneity of the different α-galactosidase A forms, and 4) the elimination of the previously troublesome problem of pyrogen contamination.

EXPERIMENTAL PROCEDURES

Materials
6-Aminohexanoic acid, benzylisoxycarbonyl chloride, dimethylformamide, 10% p-bromamine on activated carbon, triethylamine, and melibiose were purchased from Aldrich. Cynogen bromide, D-galactose, (5-methyl resorcinol), silica gel H (10-40 μ), imidazole (fluorimetric grade), neumaminidase (Clostridium perfringens, type IX), pyruvate kinase (rabbit muscle), fumarase (pig heart), α-methylglucoside, myo-inositol, and stachyose were obtained from Sigma. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide HCl was purchased from Bio-Rad. Silica gel G thin layer plates (250 μ) were from Analtech, Inc., Newark, DE. Isobutyl chloroformate was purchased from Eastman Kodak, Rochester, NY; anhydrus ammonia was obtained from the Matheson Co., Inc. East Rutherford, NJ. Fluorescamine was purchased from Pierce Chemical Co., Rockford, IL. Goat anti-rabbit IgG, 4-methylumbelliferyl α-D-galactopyranoside, α-nitrophenyl-2-acetamido-2-deoxy-α-D-galactopyranoside, and the other synthetic glycoside substrates were purchased from Research Products International Corp., Elk Grove Village, IL. Plasmamate was from Cutter Biological, Berkeley, CA. Sepharose 4B, octyl-Sepharose, and α-Sepharose 1-4B, 1octyl-Sepharose, Con A-Sepharose, 1-2 Sephadex G-200, Sephacryl S-200, and the SDS molecular weight marker kit were obtained from Pharmacia. Amphotere were obtained from LKB Products, Stockholm, Sweden. Butyl-agarose was from P-L Biochemicals, Sodium taurocholate, raffinose, and 1-(2-hydroxyethyl)-1-piperazineethanesulfonic acid were purchased from Calbiochem-Behring Corp., La Jolla, CA. Microgranular CM and DEAE-cellulose were obtained from Whatman Laboratory Products, Clifton, NJ. The Pyrostat Limulus Amebocyte Lysate Kit was purchased from Millipore Corp., Bedford, MA. GB-Cer was purified from Fabry kidney (16) and [1]H]Gb-Cer was a gift from Dr. C. C. Sweeney, Michigan State University. Highly polymerized bovine serum albumin (Lot Y4033) was obtained from Schwarz/Mann. Aldolase (rabbit muscle) and cytochrome c (horse heart) were from Boehringer Mannheim. Human Cohn Fraction IV-1 was obtained from the Michigan Department of Public Health, Lansing, MI.

Methods
α-Galactosidase A Assays—The method of Desnick et al. (17) was used.

1 The abbreviations used are: Con A, concanavalin A; Gb-Gal, globotrihexosylceramide; 4-MU-α-Gal, 4-methylumbelliferyl-α-D-galactopyranoside; SDS, sodium dodecyl sulfate; α-GalNH₂-C₆-Sephrose, N-6-aminoxy-anxyl-α-D-galactopyranosylamine-Sepharose.
Affinity Purification of Human α-Galactosidase A

used for artificial substrate assays with the following modifications. A stock solution of 5.0 mM 4-MU-α-Gal was prepared in citrate-phosphate buffer, pH 4.6 (18), with gentle solubilization in an ultrasonic bath. The reaction mixture, containing 150 μl of substrate solution and 25 μl of enzyme solution, was incubated at 37°C for 10 to 60 min. The reaction was terminated with 2.3 ml of 0.1 M ethylenediamine.

The reaction mixture contained 50 μl of 0.1 M sodium acetate, pH 3.8, 350 μg of sodium taurocholate, 5 nmol of 4-MU-α-Gal substrate, the activity was 44% of that obtained following modifications. The reaction mixture contained 50 μl of 0.1 M sodium acetate, pH 3.8, 350 μg of sodium taurocholate, 5 nmol of 4-MU-α-Gal substrate, and 50 μg of enzyme in a final volume of 100 μl. Incubation was at 37°C for 15 min.

For both assays, one unit of activity is that amount of enzyme which hydrolyzes 1 nmol of substrate/h under the conditions of the assay.

Preparation of α-Galactosidase A—Methods of Lowry et al. (21). The determination of the native molecular weight of α-galactosidase A was determined by gel filtration on Sephadex G-200. The protein was equilibrated and eluted with 0.15 M NaCl, 50 mM sodium phosphate, pH 6.0, and 0.02% sodium azide. The column size was 1.5 × 100 cm and the flow rate was 5 ml/h. The sample consisted of 10,000 units of α-galactosidase A, and 500 μg each of cytochrome c, ovalbumin, and aldolase in 0.2 ml of column buffer. The enzyme was detected by 4-MU-α-Gal activity and the protein markers were detected by the fluorescent assay.

The subunit molecular weight was determined in 5% acrylamide, 0.1% SDS-phosphate gels according to the method of Weber and Osborn (22). The sample solutions contained 5 μg of each protein standard or 6.2 μg of placent al α-galactosidase A or 13 μg of α-galactosidase A (Lowry assays). The samples containing SDS, β-mercaptoethanol, and protein were heated at 100°C for 15 min prior to addition of bromphenol blue, sucrose, and additional β-mercaptoethanol. The final sample volume was 50 μl. Electrophoresis was conducted for 6 h at 8 mA/tube constant current.

Synthesis of N-(N-Benzoxycarbonyl-6-aminohexyl)-α-α-galactopyranosylamine—The procedure of Harpaz et al. (13) was used as described below. The mixed anhydride of N-benzoxycarbonyl-6-aminohexanoic acid and isobutyl chloroformate was prepared exactly as described (13) and the precipitate (13) was redissolved in 6 N HCl and stored overnight at 4°C. The complex was used immediately since it was reported that mutarotation (to predominantly the β-form at equilibrium) occurred rapidly in solutions of this type.

The reaction mixture contained 52 (±0.5°C) warm enzyme solution, was incubated at 37°C for 10 to 60 min. The reaction was terminated with 2.3 ml of 0.1 M ethylenediamine.

The reaction mixture contained 50 μl of 0.1 M sodium acetate, pH 3.8, 350 μg of sodium taurocholate, 5 nmol of 4-MU-α-Gal, 1,000 ng of α-β-galactosidase A, 160 μg of whole rabbit serum and 25 mM sodium phosphate buffer, pH 6.0, containing 1 mg/ml of human serum albumin to a total volume of 90 μl in 1.0-ml conical polycarbonate centrifuge tubes. After 30 min at 37°C, 10 μl of goat anti-rabbit IgG (250 μg of protein) was added and incubated for an additional 20 min. After storage overnight at 4°C, the tubes were centrifuged for 20 min at 25,000 × g. Aliquots (25 μl) of the supernatant were assayed for 4-MU-α-Gal activity at 37°C for 1 h.

Preparation of the α-D-Galactopyranosylamine—Ammonia Complex—A modification of the method of Frish and Isbell was used (26). α-Galactosylboric acid (0.22 mol) and NHCl (0.019 mol) were partially dissolved in 100 ml of anhydrous methanol. Ammonia was bubbled into this stirred suspension through a fritted glass sparger for 7 h. The mixture was stored at 4°C for 2 days under ammonia and the precipitate was then filtered and washed at 4°C with dry methanol. This precipitate was recrystallized at 4°C from 200 ml of 3% NH4OH by the addition of 400 ml of dry methanol saturated with ammonia. The precipitate was recovered by centrifugation and was dissolved in 250 ml of 6 N HCl, and the supernatant was assayed for 4-MU-α-Gal activity at 37°C for 1 h. The final precipitate was washed with an additional 50 ml of methanol/ammonia and was desiccated overnight and analyzed as described (26). After these recrystallizations with proportionately smaller volumes, the melting point was 95-96°C and the specific rotation ([α]84°) (c, 5, in water) approximated the reported value of 138° (26). The complex was used immediately since it was reported that mutarotation (to predominantly the β-form at equilibrium) occurred rapidly in solutions of this type.

The reaction mixture consisted of 50 μl of 0.1 M sodium acetate, pH 3.8, 350 μg of sodium taurocholate, 5 nmol of 4-MU-α-Gal, 1,000 ng of α-β-galactosidase A, 160 μg of whole rabbit serum and 25 mM sodium phosphate buffer, pH 6.0, containing 1 mg/ml of human serum albumin to a total volume of 90 μl in 1.0-ml conical polycarbonate centrifuge tubes. After 30 min at 37°C, 10 μl of goat anti-rabbit IgG (250 μg of protein) was added and incubated for an additional 20 min. After storage overnight at 4°C, the tubes were centrifuged for 20 min at 25,000 × g. The supernatant (25 μl) was assayed for 4-MU-α-Gal activity at 37°C for 1 h.
The supernatant was diluted 1:4 with buffer B and gently stirred overnight with 35 ml (settled volume) of Con A-Sepharose. The slurry was stored in an ice bath such that the last 2 to 5 h of stirring was at room temperature. The subsequent washing and elution steps also were done at 23°C. The slurry was filtered on a 60-ml Buchner sintered glass (coarse) filter and washed with 10 bed volumes of buffer B. The Con-A-Sepharose was packed in a column (diameter 3.1 cm) to a height of 5.2 cm and eluted with buffer B containing 1 M methylglucoside at a flow rate of 1.0 ml/min. The enzyme eluted in approximately 300 ml as a skewed peak with reasonably constant specific activity. The sample was concentrated 10-fold and desalted against buffer A.

DEAE-cellulose was precycled according to the manufacturer's instructions, equilibrated with buffer A and packed into a column (2.5 × 70 cm). The Con-A-Sepharose concentrate was applied and washed with 500 ml of buffer A at a 1.0-ml/min flow rate until the effluent protein was approximately 0.01 mg/ml. The α-galactosidase A was eluted with a 400-ml linear gradient of 10 to 200 mM NaCl in buffer A at pH 6.5.

In preparation for affinity chromatography, the pooled α-galactosidase A activity from the DEAE-cellulose step was diluted with 0.5 volume of buffer C [0.15 M citrate-phosphate (18), 0.15 M NaCl, pH 4.7] and adjusted to pH 4.7 with 1.0 N HCl. Occasionally a precipitate formed in the reaction mixture. The slurry was removed by centrifugation. The supernatant was applied to a column (0.76 × 10.0 cm) of α-GalNH₂-C₅⁺-Sepharose. The column was washed with a 40-ml linear gradient from buffer C to 0.15 M citrate-phosphate, 0.5 M NaCl, pH 6.0 (buffer D). The enzyme was eluted with 20 ml of buffer D containing 0.4 M α-galactoside. The flow rate was 0.5 ml/min for all steps. The pooled activity was concentrated to a single peak by ultrafiltration with an Amicon model 3 stirred cell and was desalted on Sephadex G-50.

Purification of Plasma α-Galactosidase A—The plasma enzyme was partially purified from human Cohn Fraction IV-1 by a modification (4) of our previous method (10). This enzyme preparation (27 ml, 390,000 units, sterile) was diluted with an equal volume of sterile buffer C containing 1 mg/ml of human serum albumin (jellid Plasmanate) and adjusted to pH 4.7 with 0.5 N HCl. It was applied to a column (2.5 × 10 cm) of α-GalNH₂-C₅⁺-Sepharose which had been washed prior to use with 300 ml of sterile buffer C containing 1 mg/ml of human serum albumin. After application, the column was washed with an additional 500 ml of this buffer and eluted with 200 ml of sterile 0.15 M citrate-phosphate buffer, pH 6.0, containing 0.15 M NaCl, 0.4 M galactose and 1 mg/ml of human serum albumin. The flow rate was 1.5 ml/min. The pooled activity was dialyzed against three 1,500-ml changes of sterile 120 mM NaCl, 25 mM sodium phosphate, and 2 mg/ml of human serum albumin over a 48-h period. The enzyme solution was sterile-filtered and packaged in sterile 20-ml serum vials in a laminar-flow hood.

RESULTS

Binding Capacity of the Affinity Resin—The binding capacity of α-GalNH₂-C₅⁺-Sepharose for α-galactosidase A was a function of pH, temperature, and column height. The enzyme was absorbed more effectively by the affinity resin at the pH optimum of the artificial substrate assay (pH 4.6) than at the pH of maximal enzyme stability (pH 6.0). When approximately 3,500 units of partially purified splenic α-galactosidase A activity in 2.0 ml of either citrate-phosphate buffer, pH 4.6, or sodium phosphate buffer, pH 6.0, were applied to an affinity column (0.75 × 1.8), 92% of the activity bound to the column at pH 4.6 while only 25% bound at pH 6.0. Since the enzyme lost activity below pH 4.6, the standard affinity buffer was fixed at pH 4.7 for optimal binding and recovery. Enzyme binding was also a function of temperature; at 4°C, pH 4.7, 94% of the activity bound while only 65% bound at room temperature. For a constant flow rate of about 0.2 ml/min/cm², capacity was also dependent on column height. For a column (0.45 × 1.0 cm), only 70% bound for a capacity of 45,000 units/ml packed beads. In contrast, for a column (0.76 × 10 cm) the capacity was greater than 100,000 units/ml packed beads with 97% of the applied activity bound to the column.

The Behavior of α-N-Acetylgalactosaminidase on Affinity Chromatography—The binding and elution of α-N-acetylga-
lactosaminidase was similar to that of a-galactosidase A. When 691,000 units of the former enzyme was applied to a column (0.76 × 10 cm), about 70,000 units were recovered in the flow-through and wash, while the galactose eluate contained 540,000 units with a 20-fold purification. Therefore, the DEAE-cellulose step was required in order to exclude this activity prior to affinity chromatography of a-galactosidase A.

Affinity Purification of Plasma a-Galactosidase A—The plasma enzyme was purified from 77 kg of Cohn Fraction IV-1 by conventional procedures as described under “Methods” and summarized in Table Ia. The final affinity step resulted in a 300-fold purification and 92% recovery as compared to the flow-through and wash, while the galactose eluate contained 540,000 units with a 20-fold purification. Therefore, the column (0.76 × 10 cm) and washed with a 30-ml gradient of buffer C to buffer D. The activity was eluted with 15 ml of 0.4 M galactose in buffer D. The shift to pH 6.0 resulted in improved removal of contaminating protein without significant leakage of activity during the wash and afforded a more efficient elution of enzymatic activity by 0.4 M galactose. Assays were performed on diluted aliquots (e.g. 1:100), which minimized competitive inhibition by galactose.

Removal of Pyrogens from a-Galactosidase A—Prior to affinity chromatography, the preparation described in Table Ia (Sephadex G-200 step) contained high levels of pyrogen, making it unsuitable for human trials of enzyme replacement. Fig. 2 shows that the pyrogens did not bind to the affinity resin and were eluted in the wash buffer while the a-galactosidase A activity remained bound. The highest level of pyrogen measured in the column flow-through was 6.25 ng/ml; after extensive washing, the level dropped 300-fold to the low background level in the buffers, 0.02 ng/ml. After pooling the fractions containing a-galactosidase A activity, dialyzing, sterile filtering, and packaging in sterile vials, the endotoxin concentration in the final preparation was 0.15 ng/ml.

Affinity Purification of Splenic and Placental a-Galactosidase A—The purification of splenic and placental a-galactosidase A was similar and is summarized in Table II. Typically, spleen had 4 to 5 times more total 4-MU-a-Gal activity than an equal weight of placenta. Furthermore, while the ratio of a-galactosidase A to a-N-acetylgalactosaminidase in spleen was 5.7 to 1, it was reversed in placenta, with a ratio of 0.4 to 1 for the respective activities. Thus, the average yields of a-galactosidase A per 1 kg of spleen and placenta were approximately 2,000,000 and 200,000 units, respectively.

Through the use of affinity chromatography, the splenic enzyme was purified to a final specific activity of 4,070,000 units/mg (fluorescamine) and 1,880,000 units/mg (Lowry) with 31% recovery. A single affinity step gave a specific activity of 100,000 to 500,000 units/mg for various preparations of the placental enzyme. With rechromatography of the pooled placental preparations on the affinity column, the specific activity was increased to 2,060,000 units/mg (fluorescamine) and 980,000 units/mg (Lowry).

Removal of a-N-acetylgalactosaminidase activity was accomplished by DEAE-cellulose chromatography. The a-galactosidase A activity eluted at 70 mM NaCl followed by β-hexosaminidase A at 150 mM NaCl and a-N-acetylgalactosaminidase at 175 mM NaCl. Fractions containing 75% of the a-galactosidase A activity were pooled so as to eliminate over 99% of the contaminating a-N-acetylgalactosaminidase activity. However, this cut retained 4,000,000 units of β-hexosaminidase.

![Figure 2](image_url)

**FIG. 2. Separation of pyrogens from plasma a-galactosidase A by affinity chromatography.** The pyrogen content of solutions of purified a-galactosidase A was estimated relative to Gram-negative endotoxin by the Limulus Amebocyte Lysate Pyrostat kit. Due to a manufacturer’s change in the reference endotoxin, the Pyrostat assay reported here resulted in pyrogen values that were 75% lower than before. Thus, the preparation that gave 1.0 ng/ml in a previous report (3) and a 0.5°C temperature rise in rabbits) would give 0.25 ng/ml in the current test. X, pyrogen concentration in the pooled enzyme fractions after packaging in sterile vials.

<table>
<thead>
<tr>
<th>Table I</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Purification of plasma a-galactosidase A</strong></td>
</tr>
<tr>
<td><strong>Step</strong></td>
</tr>
<tr>
<td>ml</td>
</tr>
<tr>
<td>a. Conventional purification</td>
</tr>
<tr>
<td>Crude extract</td>
</tr>
<tr>
<td>First DEAE-cellulose</td>
</tr>
<tr>
<td>Second DEAE-cellulose</td>
</tr>
<tr>
<td>CM-Cellulose</td>
</tr>
<tr>
<td>Octyl-Sepharose</td>
</tr>
<tr>
<td>Sephadex G-200</td>
</tr>
<tr>
<td>a-GalNHA-C12-Sepharose</td>
</tr>
<tr>
<td>a. Rapid purification</td>
</tr>
<tr>
<td>Crude extract</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
</tr>
<tr>
<td>a-GalNHA-C12-Sepharose</td>
</tr>
</tbody>
</table>

* Sum of 12 separate preparations; the starting material was 6.4 kg of Cohn Fraction IV-1 per preparation.
* Sum of six preparations (two first DEAE-cellulose batches pooled for each second DEAE-cellulose).
* All six octyl-Sepharose batches pooled and run as one batch in these two steps.
* Based on a separate 1/5 scale experiment. Due to human serum albumin in the elution buffer, the actual protein was 70 mg for a specific activity of 5,140 units/mg.
idase A activity (16% of the total), 920,000 units of P-galactosidase A activity, and 99.3% of the protein while the purified splenic enzyme was eluted with 67% recovery.

The specific activity of the purified splenic enzyme depended on the method used for determining the protein concentration. For the purified splenic and placentary enzymes the fluorosamine assay yielded protein concentrations of 0.27 and 0.58 mg/ml, respectively. Using the Lowry assay, however, the protein concentrations were 0.58 and 0.97 mg/ml, respectively. Since both assays were standardized against bovine serum albumin, the disparate values suggested that the enzyme had fewer primary amines than other proteins (perhaps due to carbohydrate attachment points) or more than the average number of tyrosine and/or tryptophan residues. Indeed, the 280 nm absorbances of the splenic and placentary enzymes suggested apparent protein concentrations of 1.2 and 2.1 mg/ml of protein, respectively, when calculated by the method of Warburg and Christian (32). The spectrum of the splenic enzyme itself (Fig. 4) was quite similar to the spectrum for tryptophan and showed no other characteristic absorbances in the region of 300 to 700 nm (360 to 700 nm not shown, but was flat or decreasing). The extinction coefficient (ε 280) for splenic α-galactosidase A was 18 based on the Lowry protein determination. The A 280/A 260 ratio of the splenic enzyme was 1.80.

Enzyme Purity—The final splenic α-galactosidase A preparation was nearly homogeneous, containing only 0.32% α-N-acetylgalactosaminidase, 0.09% β-hexosaminidase A and less than 1% of all other lysosomal hydrolases assayed (Table III). Apparently, none of these activities represented multiple specificities of α-galactosidase A since they were absent in the plasma preparation. In the case of β-hexosaminidase A, the activity eluted after α-galactosidase A on butyl-agarose chromatography (4) and is thus a separate enzyme. The splenic enzyme was homogeneous by the criterion of single bands in the 280 nm absorbances of the splenic and placentary enzymes suggested apparent protein concentrations of 1.2 and 2.1 mg/ml of protein, respectively, when calculated by the method of Warburg and Christian (32). The spectrum of the

### Table II

**Affinity Purification of Human α-Galactosidase A**

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume (ml)</th>
<th>Activity (units/mg)</th>
<th>Specific Activity (units/mg)</th>
<th>Purification Fold</th>
<th>Yield %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude splenic extract</td>
<td>1,000</td>
<td>1,640,000</td>
<td>164</td>
<td>1.0</td>
<td>100</td>
</tr>
<tr>
<td>(NH4)2SO4, ppt.</td>
<td>750</td>
<td>1,790,000</td>
<td>556</td>
<td>3.4</td>
<td>107</td>
</tr>
<tr>
<td>Con A-Sepharose</td>
<td>75</td>
<td>1,250,000</td>
<td>13,900</td>
<td>85</td>
<td>75</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>120</td>
<td>750,000</td>
<td>4,070,000</td>
<td>24,800</td>
<td>31</td>
</tr>
<tr>
<td>α-GalNH₄⁺-C₁₂-Sepharose</td>
<td>0.46</td>
<td>503,000</td>
<td>4,730</td>
<td>156</td>
<td>82</td>
</tr>
<tr>
<td>Crude placentary extract</td>
<td>5,000</td>
<td>930,000</td>
<td>30.4</td>
<td>1.0</td>
<td>100</td>
</tr>
<tr>
<td>(NH₄)₂SO₄, ppt.</td>
<td>725</td>
<td>961,000</td>
<td>169</td>
<td>5.6</td>
<td>103</td>
</tr>
<tr>
<td>Con A-Sepharose</td>
<td>260</td>
<td>767,000</td>
<td>136,000</td>
<td>4,480</td>
<td>32</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>96</td>
<td>374,000</td>
<td>4,420</td>
<td>145</td>
<td>40</td>
</tr>
<tr>
<td>First α-GalNH₄⁺-C₁₂-Sepharose</td>
<td>58.5</td>
<td>311,000</td>
<td>136,000</td>
<td>4,480</td>
<td>32</td>
</tr>
<tr>
<td>Second</td>
<td>0.50</td>
<td>480,000</td>
<td>2,060,000</td>
<td>67,800</td>
<td>---</td>
</tr>
</tbody>
</table>

* From 220 g of human spleen.
* These assays were done on desalted samples.
* 1,880,000 units/mg by Lowry.
* From 1,000 g of human placenta.
* From 510,000 units of placentary enzyme pooled and concentrated from the first affinity step (specific activity = 528,000 units/mg).
* 990,000 units/mg by Lowry.

![Fig. 3. Affinity purification of splenic α-galactosidase A. See "Experimental Procedures" for details.](image)

**Fig. 4.** UV-absorbance spectra of purified splenic α-galactosidase A. The specific activity was 1.9×10⁶ units/mg (Lowry) in 25 mm phosphate buffer, pH 6.0, and the protein concentration was 0.58 mg/ml. The spectra was determined in a Gifford model 2000 spectrophotometer.

### Table III

**Per cent glycosidase relative to α-galactosidase A**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate conc.</th>
<th>Buffer</th>
<th>pH</th>
<th>Per cent of α-Galactosidase A activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-d-Galactosidase A</td>
<td>4.3</td>
<td>0.1 M Cit</td>
<td>4.6</td>
<td>100</td>
</tr>
<tr>
<td>α-d-Glucosidase</td>
<td>1.0</td>
<td>0.1 M Ac</td>
<td>4.0</td>
<td>0</td>
</tr>
<tr>
<td>β-d-Glucosidase</td>
<td>1.0</td>
<td>0.1 M Cac</td>
<td>6.0</td>
<td>0</td>
</tr>
<tr>
<td>α-d-Mannosidase</td>
<td>6.0</td>
<td>0.3 M C-P</td>
<td>4.2</td>
<td>0.01</td>
</tr>
<tr>
<td>α-d-Mannosidase</td>
<td>4.0</td>
<td>0.1 M C-P</td>
<td>6.0</td>
<td>0.01</td>
</tr>
<tr>
<td>α-d-N-Acetylgalactosamidase</td>
<td>10.0</td>
<td>0.1 M Cit</td>
<td>4.3</td>
<td>0.32</td>
</tr>
<tr>
<td>α-d-N-Acetylgalactosamine</td>
<td>10.0</td>
<td>0.1 M Cit</td>
<td>4.4</td>
<td>0.0</td>
</tr>
<tr>
<td>α-L-Arabinosidase</td>
<td>0.5</td>
<td>1.0 M Cit</td>
<td>5.0</td>
<td>0.05</td>
</tr>
<tr>
<td>α-L-Fucosidase</td>
<td>0.78</td>
<td>0.1 M C-P</td>
<td>5.7</td>
<td>0.0</td>
</tr>
<tr>
<td>β-d-Galactosidase</td>
<td>1.0</td>
<td>0.1 M C-P</td>
<td>4.6</td>
<td>0.78</td>
</tr>
<tr>
<td>β-d-Glucosidase</td>
<td>5.0</td>
<td>0.2 M C-P</td>
<td>6.0</td>
<td>0.0</td>
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<tr>
<td>β-d-Glucuronidase</td>
<td>10.0</td>
<td>0.1 M Ac</td>
<td>4.8</td>
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<tr>
<td>β-Hexosaminidase A</td>
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<td>0.06 M C-P</td>
<td>4.4</td>
<td>0.09</td>
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<tr>
<td>β-d-Xylosidase</td>
<td>2.0</td>
<td>0.1 M Ac</td>
<td>5.5</td>
<td>0.0</td>
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</table>

* 4-Methylumbelliferone glycosides except as noted.
* The abbreviations for buffers used are: C-P, citrate-phosphate; Ac, acetate; Cac, cacodylate; Cit, citrate.
* p-Nitrophenyl glycosides.
polyacrylamide gel electrophoresis using two different gel concentrations and SDS-gel electrophoresis (Fig. 5).

The placental enzyme also showed a single band in non-denaturing and denaturing polyacrylamide gel electrophoresis but contained 11% α-N-acetylgalactosaminidase (as 4-MU-α-Gal activity). Both splenic and placental enzymes migrated as wide bands compared to the sharp bands for the polymerized albumin multimers. The plasma form contained 0.25% β-hexosaminidase A as the only measured lysosomal enzyme consistent with its known activity. Both splenic and placental enzymes were in native polyacrylamide gels at pH 7.0. To each gel, 25 μg of protein was applied (Lowry assay). Gels 1 and 4 contain bovine serum albumin. The purified splenic and placental enzymes (final step, Table III) were applied to Gels 2 and 4 and to Gels 7 and 8, respectively. B, SDS-polyacrylamide gel electrophoresis. The standards in Gel 7 were bovine serum albumin, fumarase, ovalbumin, glyceraldehyde-3-phosphate dehydrogenase, and chymotrypsinogen. The splenic and placental enzymes were in Gels 8 and 9, respectively. See “Experimental Procedures” for details.

**Enzyme Stability**—It was essential to keep the final protein concentration of the purified enzyme above 0.1 to 0.2 mg/ml to minimize activity loss. Dilute solutions could be stabilized with 1 mg/ml of human serum albumin or 5% polyvinylpyrrolidone. With storage under these conditions at 4°C and pH 6.0 (25 mM sodium phosphate) the activity loss was less than 3%/month. Repeated freezing and thawing increased enzyme inactivation. Splenic α-galactosidase A, stored at 23°C for 4 h with 1 mg/ml of human serum albumin, was stable from pH 4.5 to 7.0 but was rapidly inactivated at lower and higher pH values. The splenic and plasma enzymes were thermolabile with a half-life of 7 min at 55°C in 25 mM sodium phosphate buffer, pH 6.0, with 1 mg/ml of human serum albumin.

**Molecular Weight of Splenic α-Galactosidase A**—The pH optimum of splenic α-galactosidase A was 3.8 for the natural substrate, GbCer, and 4.6 for the artificial substrate, 4-MU-α-Gal. While the latter optimum was broad, the natural substrate optimum was sharp, with no detectable activity above pH 5.

The K_m for GbCer was 0.078 ± 0.03 mM while that for 4-MU-α-Gal was 2.03 ± 0.3 mM (standard deviations for five determinations). With 4-MU-α-Gal, the enzyme exhibited normal Michaelis-Menten kinetics with a V_max of 2.8 × 10^5 units/mg (Lowry) for the purified enzyme. The turnover number (catalytic center activity) for 4-MU-α-Gal was 2.3 × 10^4 min^{-1}, assuming two active sites/molecule. With the natural substrate however, the Lineweaver-Burk plots were nonlinear, curving upwards at lower substrate concentrations. The K_m reported above was obtained by plotting 1/v versus 1/S^1/2 which resulted in a linear plot. Using the V_max obtained in this way, the Hill equation was plotted and it confirmed the apparent n value of 1.9 which is consistent with positive cooperativity between at least two active sites. The ratio of artificial to natural substrate activities was 7.3, measured at substrate concentrations of 4.3 mM and 0.15 mM, respectively. With both substrates at 1.0 mM, the ratio was 3.1. At V_max the calculated ratio was 9.4.

The enzyme rate with the natural substrate was a complex function of protein, sodium taurocholate, and substrate concentrations as previously reported (11, 32-36). Substrate stabilized the activity at low concentrations of added human serum albumin but was unable to significantly reverse the inhibition by high protein concentrations (Table IV).

The inhibition of α-galactosidase A by various substances is summarized in Table V. For the substrate analogs, the inhibition was competitive and decreased with increasing
chain length. Myo-inositol, an activator of α-N-acetylgalactosaminidase (37), was a very weak noncompetitive inhibitor. N-6-Aminohexanoyl-α-D-galactosylamine, prepared as described under "Methods," was the best competitive inhibitor, with a $K_i$ of 2.3 mM.

**Immunological Identity of Plasma and Tissue Forms of α-Galactosidase A**—Antibody raised in rabbits against purified human spleen α-galactosidase A (see "Experimental Procedures") was used to evaluate the immunological relationship between the plasma and tissue enzymes. Ouchterlony double immunodiffusion showed a line of identity between the antibody and the splenic, plasmonic, and plasma forms as detected by 4-MU-α-gal activity (Fig. 7, inset). The amounts of rabbit anti-human splenic α-galactosidase A (IgG fraction) required to precipitate 50% of the splenic and plasma forms were similar at 325 and 255 ng, respectively (Fig. 7). The slight difference in titer for these two forms may have been due to the presence of inactive enzyme or to the variation in sialic acid content.

**Differences in Lectin and Ion-Exchange Binding of α-Galactosidase A Forms**—While both forms bound to Con A-Sepharose, the amount of plasma form bound was less than that of splenic α-galactosidase A. Equal amounts of splenic and plasma enzyme (4,400 units) were individually mixed with 0.5-ml aliquots (packed volume) of Con A-Sepharose in buffer B (10 ml) at 23°C for 4 h. The resulting filtrates from the splenic and plasma experiments contained 0 and 11% of the respective initial activities. The filtrate of a mixture of both enzymes (8,800 total units) plus 0.5 ml of Con A-Sepharose also contained 12% of the initial plasma activity. Isoelectric focusing (see below) gave a single pH 4.2 form, showing that all 12% was the plasma form. Thus, a property of the plasma form itself inhibited binding. It is possible that the reduced binding of the plasma form was due to more extensive sialylation of the enzyme or to the presence of a nonbinding subform. The earlier elution of the splenic enzyme from DEAE-cellulose is consistent with its lower sialic acid content as compared to that of the plasma form. Using a DEAE-cellulose column (0.75×12 cm), 1,000 units of enzyme, and a 50-ml 10 to 300 mM NaCl gradient in 25 mM sodium phosphate, pH 6.0, the NaCl elution molarities of the splenic and plasma forms were 65 and 73 mM, respectively.

**Isoelectric Focusing of α-Galactosidase A**—The nature of the charge difference between these two forms was best demonstrated by isoelectric focusing experiments. The plasma form (Fig. 8, Lane 1) always migrated as a single, broad band with a pI of 4.2. In contrast, splenic enzyme always migrated as a set of closely spaced forms clustered about a pI of 4.7. Although the number and relative positions of the bands varied from one preparation to another, the bands in adjacent lanes always appeared at identical pI values. Furthermore, when the same sample was focused in ampholytes from a different manufacturer (Pharmalyte, Pharmacia), the same number of bands appeared but at slightly different positions. This suggested that each subform of the enzyme focused with the specific ampholyte(s) of pH nearest to that form. The

<table>
<thead>
<tr>
<th>Human Serum Albumin</th>
<th>Enzyme activity*</th>
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<tbody>
<tr>
<td>µg</td>
<td>0.1 mm Gb.Cer</td>
</tr>
<tr>
<td>0</td>
<td>538</td>
</tr>
<tr>
<td>2</td>
<td>1440</td>
</tr>
<tr>
<td>10</td>
<td>1650</td>
</tr>
<tr>
<td>100</td>
<td>538</td>
</tr>
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</table>

* Assayed as described under "Methods."

**TABLE IV**

<table>
<thead>
<tr>
<th>Sugar</th>
<th>Enzyme activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-6-aminohexanoyl-α-D-galactosylamine</td>
<td>2.3 Competitive</td>
</tr>
<tr>
<td>d-Galactose</td>
<td>21 Competitive</td>
</tr>
<tr>
<td>Melibiose</td>
<td>35 Competitive</td>
</tr>
<tr>
<td>(Gala1→6Glc)</td>
<td>170 Competitive</td>
</tr>
<tr>
<td>Raffinose</td>
<td>0 No inhibition</td>
</tr>
<tr>
<td>(Gala1→6Glc1→2Fru)</td>
<td></td>
</tr>
<tr>
<td>Stachyoside</td>
<td>750 Noncompetitive</td>
</tr>
<tr>
<td>Myo-Inositol</td>
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</tr>
</tbody>
</table>

* Determined by plots of 1/v versus I.

**FIG. 7. Immunological identity of splenic and plasma α-galactosidase A.** The titration procedure is described under "Methods." The inset is an Ouchterlony plate stained for 4-MU-α-Gal using the overlay method described for isoelectric focusing. The center well contained 10 µg of rabbit anti-human spleen α-galactosidase A. The outer wells contained 60 µg each of purified α-galactosidase A from human spleen (1 and 4), placenta (2 and 5), and plasma (3 and 6).

**FIG. 8. Isoelectric focusing of purified α-galactosidase.** Lane 1 contained 11 units of the plasma form; Lane 2 contained 16 units of the splenic form, purified by conventional methods; Lanes 3 and 4 contained 23 units of the splenic form and 30 units of the placental form, respectively, purified by affinity chromatography (final step, Table II); and Lane 5 contained 3 units of the placental α-N-acetyl-galactosaminidase. The method is described under "Experimental Procedures."
placental form (Lane 4), on the other hand, focused into only two major bands at higher pl values of 5.0 and 5.1. The minor band at pl 4.6 coincided with that of α-N-acetylgalactosaminidase (Lane 5), a known contaminant of this preparation (Table III).

Neuraminidase Studies—That the pl differences were due mainly to different sialic acid content was suggested by the results of progressive hydrolysis by highly purified neuraminidase from C. perfringens. Fig. 9A shows that both the plasma (Lanes 1 to 3) and splenic forms (Lanes 4 to 6) were converted to similar higher pl forms by treatment with neuraminidase while α-N-acetylgalactosaminidase activity was unaffected. Again, there was a striking correspondence between the various pl values of the two preparations. For example, in Lanes 3 and 5, the patterns of the neuraminidase-treated plasma and spleen enzymes were very similar. With prolonged treatment, both enzymes focused to a single major band with a pl of 5.0 which was presumably characteristic of the completely desialylated enzyme (Fig. 9B). However, since the rates of hydrolysis by neuraminidase were different for the two preparations and since there was a minor pl 4.9 band in the neuraminidase-treated splenic preparation (Fig. 9B, Lane 3), the sialic acid content may not constitute the only difference between the plasma and splenic forms.

Although no protease activity had been detected in the neuraminidase used, it was important to confirm that the pl changes were not due to any contaminating enzyme. This was accomplished with highly purified neuraminidase from Vibrio cholera. This enzyme was strongly inhibited by low concentrations of EDTA. Thus, α-galactosidase A forms were incubated with V. cholera neuraminidase for 40 min with the latter enzyme being specifically inhibited by EDTA added at earlier times. Though not shown, the pattern was quite similar to Figure 9A. The observed change in isoelectric points could thus be ascribed to lack of sialic acid rather than to protease activity or to activity by some other enzyme in the neuraminidase preparation.

DISCUSSION

The methods described here for the purification of α-galactosidase A from human spleen, placenta, and plasma have resulted in preparations with higher specific activities and greater yields than those previously reported (4, 8–12). The enhanced purification was primarily due to the use of α-galactosylamine as an immobilized affinity ligand, a procedure previously described by Harpaz et al. (13) for the purification of plant α-galactosidases. Their procedure was modified by coupling the second aminohexyl group directly to Sepharose 4B rather than synthesizing N-6-aminohexanoyl-N-6-aminohexanoyl-α-D-galactopyranoside directly. High ligand concentrations were obtained since an excess of the readily available 6-aminohexanoic acid could be used to make carboxyethyl-Sepharose via cyanogen bromide activation. This was then linked to the 6-aminohexanoyl-α-galactosyamine ligand by the efficient dehydration with carbodiimide to generate the identical C₂ ligand of Harpaz et al. (13). With this procedure, the unreacted affinity ligand also was recovered and reused.

The affinity step permitted the rapid and high yield purification of large quantities of α-galactosidase A for subsequent use in structural studies and clinical trials. The final specific activity (229,000 units/mg) of the plasma enzyme was more than 3 times greater than that previously reported using conventional techniques (10). The use of the affinity step for the splenic enzyme resulted in a 31% yield of enzyme with a specific activity of 4,070,000 units/mg; in contrast, our previous procedure (4) gave enzyme of 1,460,000 units/mg in 8% yield.

The affinity ligand was highly specific for α-galactosidase A. Indeed, the affinity step resulted in almost total removal of β-hexosaminidase A, the major contaminant in α-galactosidase A preparations purified by conventional chromatographic methods (4). The presence of trace amounts of β-hexosaminidase activity may be due to the enzyme's recognition of the structurally related ligand. The binding affinity of the ligand for α-N-acetylgalactosaminidase was similar to that for α-galactosidase A. Thus, the ligand can be used for the purification of either or both activities. α-Galactosidase A and α-N-acetyl-galactosaminidase can be separated by DEAE-cellulose (8) or hydroxyapatite chromatography (9, 11). When care was taken to eliminate all α-N-acetylgalactosaminidase at the DEAE-cellulose step, nearly homogeneous α-galactosidase A was obtained in the subsequent affinity step. For removal of the remaining minor contaminants, more extensive washing or rechromatography on the affinity resin may be required.

A major advantage of the affinity step was the removal of pyrogens in the buffer wash (Fig. 2). Even with aseptic technique, enzyme preparations often contained pyrogens of varying molecular weights, most larger than 1 × 10⁴. Gel filtration on Sephadex G-200 was used to reduce the content of high molecular weight pyrogens, but lower molecular weight (50,000 to 150,000) contaminants were not removed by this procedure (4). However, a single affinity chromatographic step, with adequate washing of the affinity-bound enzyme, resulted in the removal of pyrogenic contaminants to levels lower than that required for clinical use. Thus, these studies demonstrate that affinity chromatography provides an effective procedure for the removal of pyrogens and, presumably, other toxic contaminants, from this and other purified protein preparations prior to clinical evaluation.

The final specific activity of the affinity-purified splenic enzyme was 4.07 × 10⁶ units/mg (fluorescamine assay) or 1.88 × 10⁶ units/mg (Lowry assay), the latter being almost twice that reported by Dean and Sweeley (11) for α-galactosidase from human liver, which was the highest previously reported from any human source. The placental preparation was less pure, with a specific activity of 0.99 × 10⁶ units/mg (Lowry). However, it should be noted that the placental preparation contained 25% α-N-acetylgalactosaminidase (Table III). Resolution of the α-galactosidase A and α-N-acetylgalactosaminidase activities in the purified placental preparation was not accomplished by polyacylamide gel electrophoresis, even in the presence of SDS, because the enzymes have similar native (39) and, presumably, subunit molecular weights. Further-
more, the pl of placental α-N-acetylgalactosaminidase and that of at least one tissue α-galactosidase A form were nearly identical (see Fig. 8). Thus, estimation of α-N-acetylgalacto-
saminidase activity in preparations of purified α-galactosidase A is most reliably determined by direct assay using the synthetic substrate, α-nitrophenyl-α-N-acetylgalactosaminide and, for confirmation, by isoelectric focusing with and without neuraminidase pretreatment, since α-N-acetylgalactosaminidase is unaffected by neuraminidase (Fig. 9A).

In contrast to previous findings concerning the subunit molecular weight of highly purified α-galactosidase A preparations (8, 9), analytical SDS-polyacrylamide gel electrophoresis of the affinity-purified splenic preparation revealed only a single protein band (Fig. 5B) corresponding to a molecular weight of 49,800 (Fig. 6F). These data were consistent with the enzyme being a homodimer with the observed native molecular weight of 101,000 (Fig. 6A).

The kinetic properties of purified splenic α-galactosidase A were similar to those previously reported for the tissue forms (9, 11). The cooperative kinetics observed with the natural glycoprotein substrate confirmed previous findings (11, 34). Although there are several possible explanations for these observations (39, 40), the apparent Hill number of 1.9 was consistent with cooperativity between the monomers of the homodimeric enzyme, each with an interacting catalytic site. This finding suggests that enzymatic activity and stability may be dependent on dimerization.

Previous studies in this (4, 6) and other laboratories (10, 41-47) suggested that the plasma and tissue forms of α-galacto-
sidase A are dimeric glycoproteins, the plasma form being the most highly sialylated (6, 10). The possibility that they rep-
resent α-galactosidase A isoforms resulting from the expres-
sion of different genes is ruled out since all forms are absent in Fabry disease (47-49) and since the normal forms are immunologically identical (Fig. 7). Thus, the multiple forms observed in the isoelectric focusing patterns of various tissue samples (Figs. 8 and 9) almost certainly represent varying degrees of sialylation. This finding is in agreement with a recent report (47). By using V. cholerae neuraminidase in conjunction with its specific inhibitor, EDTA, we have dem-
strated more conclusively that the neuraminidase activity in the preparation was, in fact, responsible for the observed increase in pl values.

Isoelectric focusing before and after neuraminidase treat-
ment of α-galactosidase A from the various sources revealed at least 10 distinct and reproducible species. It has been suggested that these bands are due to the number of sialic acid residues on each form (50). It might therefore be esti-
mated that the plasma form (pl 4.2) contains 10 to 12 moieties while the placental form (pl 5.9) only 1 or 2 residues. This difference in sialylation may partly explain the differences in subunit molecular weights observed for the splenic and pla-
cental forms (Fig. 5B). Indeed, the heterogeneity in carbohy-
drate structure may be responsible for the wide bands ob-
served on gel electrophoresis.

The precise carbohydrate content and interrelationships among the various forms of α-galactosidase A requires detailed structural studies. With the ability to isolate milligram quantities of purified enzyme by affinity chromatography, these studies, as well as others, can be accomplished.

Finally, the purification methods reported here should permit the availability of large quantities of highly purified, pyrogen-free enzyme for evaluation of replacement therapy in Fabry disease.

Acknowledgments—We wish to express our appreciation to Jeffrey A. Leavy for carrying out the preparation of the affinity ligand and for his assistance with the affinity purification of the enzymes. In addition, we thank Lauri McBride, Raman Reddy, and Cecilia Kovac for their expert technical assistance in enzyme purification and character-
ization. Drs. Gregory A. Grabowski and Evelyn A. Devine for review of the manuscript, and Linda Lugo and Anna DiGianantonio for clerical assistance. We thank Dr. Jerome Schuman for suggesting the use of EDTA to inhibit V. cholerae neuraminidase.

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