Purification and Properties of Human α-Galactosidases*

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SUMMARY

The thermostable α-galactosidase (α-galactosidase A) and thermostable α-galactosidase (α-galactosidase B) were separated and purified from human placenta. A homogeneous α-galactosidase B preparation was obtained, but the α-galactosidase A preparation contained small amounts of contaminating protein and various other acid hydrolase activities. Each preparation had a molecular weight of approximately 150,000, as estimated by Sephadex filtration. α-Galactosidase A had a $K_m$ of 3.4 mM for the artificial substrate, 4-methylumbelliferyl-α-D-galactopyranoside, and of 40.6 mM for melibiose. α-Galactosidase B hydrolyzed 4-methylumbelliferyl-α-D-galactopyranoside with first order kinetics and appeared to have no activity with melibiose. Both enzymes had maximal enzyme activity at pH 4.5, but α-galactosidase A had a broad pH-activity curve, while that of the B enzyme was sharply peaked. α-Galactosidase A was inhibited by myo-inositol; α-galactosidase B was not. The isoelectric point of α-galactosidase A was 4.70 ± 0.07; the isoelectric point of α-galactosidase B was 4.42 ± 0.04.

Antibodies were produced against both the α-galactosidase A and α-galactosidase B preparations. No cross reactivity between the two enzyme preparations was found on double immunodiffusion. Neither antiserum neutralized enzyme activity, but the anti-α-galactosidase A serum precipitated α-galactosidase A activity from solution and the anti-α-galactosidase B serum precipitated α-galactosidase B activity from solution. Treatment of α-galactosidase A with neuraminidase does not change its immune reactivity or kinetic properties.

These studies lend no support to the concept that α-galactosidase A is the neuraminyl derivative of galactosidase B or that the two enzymes are closely structurally related.

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These studies lend no support to the concept that α-galactosidase A is the neuraminyl derivative of galactosidase B or that the two enzymes are closely structurally related.

Fabry's disease is an inborn error of metabolism in which the glycolipid, ceramide trihexoside, accumulates in various tissues (1). Brady et al. (2) showed that normal human small intestinal mucosa had the capacity to enzymatically hydrolyze ceramide trihexoside, and that this enzyme activity was lacking in specimens from two patients with this disorder. Subsequently, a decrease in the capacity of leukocytes and fibroblasts to hydrolyze artificial α-galactosidases was demonstrated in patients with Fabry's disease (3). Investigating the biochemical genetics of Fabry's disease, we found that human fibroblasts and human leukocytes contained two α-galactosidases (4, 5). The major component, designated α-galactosidase A, was thermostable and was absent from fibroblasts and lymphocytes of patients with Fabry's disease. The other component, α-galactosidase B, was somewhat increased in activity in Fabry's disease, was thermostable, and could, in addition, be distinguished from α-galactosidase A on the basis of its higher $K_m$ for the artificial substrate and different electrophoretic mobility (4, 5). Similar findings have now also been reported by others (6, 7).

The relationship between the two α-galactosidases, which are found also in other human tissue (8), is of importance in furthering our understanding of the genetic basis of this disease. It has been proposed that α-galactosidase B is a precursor of α-galactosidase A (6, 7). We have now undertaken purification of human α-galactosidase A and α-galactosidase B to clarify the relationship between these enzymes and to provide pure enzyme for possible replacement therapy of patients with Fabry's disease.

MATERIAL AND METHODS

Refrigerated human placentas, no more than 48 hours old, served as starting material. They were stripped of their outer membranes, cut into 2- to 3-inch pieces, washed with cold 0.9% sodium chloride, blotted on filter paper, and ground in a meat grinder. Ammonium sulfate fractionation and column chromatography were performed at 4°C using standard techniques.

α-Galactosidase activity was measured in a 100-μl system containing 0.1 M sodium citrate, pH 4.0; 0.1% bovine serum albumin; and 4.0 mM 4-methylumbelliferyl-α-D-galactopyranoside (Koch Light Laboratories, England). A 15- or 30-min incubation at 37°C was terminated by addition of 4.0 ml of 0.2 M glycine buffer, pH 10.7. Fluorescence was measured using a Turner 110 or 111 fluorometer with a Corning 7-37 and 0-51 primary filter and a 3-73 secondary filter. A standard containing 0.1325 μM 4-methylumbelliferone in the glycine buffer was read with each assay. Protein determinations were carried out by the method of Lowry et al. (9) when determining specific activity of enzyme in the crude placental extract and by the ratio of absorbance at 280 nm to that at 260 nm (10) in monitoring column elutions. Activity was expressed as micromoles per mg of protein (picomoles of substrate hydrolyzed per min per mg of protein).

Other acid hydrolases were tested in the same assay system substituting the following substrates for 4-methylumbelliferyl-α-D-galactoside: β-glucosidase, 0.5 mM 4-methylumbelliferyl-β-D-glucopyranoside; β-galactosidase, 0.5 mM 4-methylumbelliferyl-
**RESULTS**

**Preliminary Concentration of α-Galactosidase from Human Placenta**—Ground placenta was homogenized at a concentration of approximately 25% (w/v) in 0.15 M potassium chloride using a Sorvall Omni-Mixer at high speed for 5 to 10 min. In order to determine the amount of the two isozymes present in the original starting material, thermal stability assays were done on the crude homogenate. The amount of stable enzyme, extrapolated to zero time, represented the initial activity of α-galactosidase A and the difference of this from the total initial activity was then the α-galactosidase A activity. The homogenate was centrifuged at 5,000 × g for 30 min. A 25 to 50% ammonium sulfate cut was taken on the supernatant, dissolving the protein cake in 0.15 M potassium phosphate buffer at pH 6.5. The supernatant was dialyzed for 20 to 24 hours against the same buffer and the precipitate removed by centrifuging at 75,000 × g for 45 min. At this point there is a 6 to 7 fold purification of total α-galactosidase activity with a 25 to 30% yield from crude, ground placenta.

**Separation of α-Galactosidase A and α-Galactosidase B—DEAE-cellulose (DE52, Whatman)** was equilibrated with 0.01 M potassium phosphate buffer, pH 6.5, in a column 2.5 × 45 cm. The supernatant from the dialyzed enzyme was applied, and the enzyme was then eluted with a 700-ml sodium chloride gradient. The sodium chloride was found to be thermolabile. This peak represented α-galactosidase A activity. The second peak, eluted at approximately 0.08 M sodium chloride, was found to be thermostable. This peak represented α-galactosidase A activity. The second peak, eluted at approximately 0.15 M sodium chloride, was thermostable and represented α-galactosidase B activity.

**Further Purification of α-Galactosidase A (Table 1)**—The fractions comprising the first peak were pooled and dialyzed against

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**Fig. 1.** DEAE-cellulose chromatography of placental α-galactosidase in 0.01 M potassium phosphate buffer, pH 6.5, with a 0 to 0.5 M sodium chloride gradient. Conditions for chromatography and thermal stability measurement are described in the text. The sodium chloride gradient was measured by conductivity and proteins determined by 280:260 nm ratio.
Purification of α-galactosidase A from placenta

| Fraction               | Enzyme units | Protein | Specific activity | Yield | Purification |
|------------------------|--------------|---------|-------------------|-------|-------------|
| Crude homogenate*      | 15.9         | 78,600  | 202 ml/mg         | 100   | *           |
| DEAE-cellulose......... | 3.9          | 453     | 8,510 mg/mg       | 24.2  | 42          |
| CM-cellulose fraction  | 4.45         | 346     | 12,900 mg/mg      | 27.9  | 64          |
| 25-50% (NH₄)₂SO₄      | 2.93         | 146     | 20,100 mg/mg      | 18.2  | 99          |
| G-200 Sephadex        | 2.36         | 53      | 44,500 mg/mg      | 14.8  | 220         |
| ECTEOLA-cellulose..... | 0.98         | 4.5    | 318,000 mg        | 6.1   | 1,580b      |

* Representing 1026 g, wet weight, of fresh human placenta.

5 mM potassium phosphate buffer, pH 6.0. The dialysate was allowed to pass slowly through a bed volume of 100 ml of CM-cellulose (CM52, Whatman) which was previously equilibrated with the pH 6.0 buffer in a 500-ml Buchner funnel. The CM-cellulose was washed with an equal volume of the buffer, and the washing and sample eluate containing the enzyme were combined.

The CM-cellulose-treated fraction was then concentrated by collecting the protein precipitated between 30 and 50% (NH₄)₂SO₄ saturation. Ascending chromatography of the redissolved precipitate was carried out on G-200 Sephadex in a column, 2.5 x 36.5 cm, with a flow adaptor (Pharmacia). The flow rate was maintained at 20 ml per hour with 10 mM potassium phosphate, pH 7.5, as the eluting buffer. The column was calibrated for molecular weight determinations using as markers: catalase (240,000), hemoglobin (60,000), egg albumin (45,000), trypsin (23,700), and cytochrome c (12,400). The enzyme was eluted at a position corresponding to a molecular weight of 150,000.

The peak enzyme activity was pooled and applied to a column of ECTEOLA-cellulose (Sigma Chemical Company) which had been equilibrated with the pH 7.5 buffer. Chromatography was carried out in a column 1.5 x 30 cm, with enzyme elution with 400 ml of a 0 to 0.2 M sodium chloride gradient: the first chamber of the gradient maker contained 100 ml of buffer followed by 100 ml each of 0.05 M, 0.1 M, and 0.2 M sodium chloride in buffer. The peak of enzyme activity was concentrated by ultrafiltration for use in immunological studies and determining properties of the enzyme.

Further Purification of α-Galactosidase B (Table II)—The fractions comprising the second peak of the DEAE-cellulose chromatography were pooled and dialyzed against 5 mM potassium phosphate buffer, pH 6.0. A 35 to 50% saturated ammonium sulfate fraction was subjected to G-200 Sephadex ascending chromatography, as described in the purification of the A enzyme except that the pH 6.0 buffer was used. The enzyme was eluted at a position corresponding to a molecular weight of 150,000. The fractions comprising the peak of enzyme activity were then pooled and subjected to isoelectric focusing. After dialysis against 0.01 M potassium phosphate buffer, pH 7.5, ECTEOLA-cellulose chromatography was performed as described in the purification of the A enzyme. The α-galactosidase B was concentrated by ultrafiltration and used for immunological studies and determining properties of the enzyme.

Purity of Enzyme Preparation

Purity of the final enzyme preparations was tested with polyacrylamide disc electrophoresis. The α-galactosidase B enzyme showed a single band when staining both for enzymatic activity and for protein. The α-galactosidase A preparation showed one major and two to four minor bands when stained for protein. The single band of enzyme activity corresponded to the major protein band.

Although protein determinations on the crude extract gave essentially identical values whether measured by the 280:260 nm method or Lowry’s method, protein estimation based on the 280:260 nm method on either of the purified preparations gave values approximately 3- to 3.5-fold higher than when measured by the Lowry technique.

The B isoenzyme preparations manifested no β-glucosidase, β-galactosidase, β-hexosaminidase, acid phosphatase, and α-mannosidase activity. The A isozyme preparation had no β-glucosidase activity; however, β-galactosidase, β-hexosaminidase, acid phosphatase, and α-mannosidase, representing 0.06%, 1.07%, 0.3%, and 0.17%, respectively, of the α-galactosidase activity were detected.

Study of distribution of these acid hydrolases in the fractions of the final (ECTEOLA-cellulose) chromatographic step demonstrated that the position of elution of the hydrolases was not identical with that of α-galactosidase A, indicating that these represented small amounts of contaminating enzymes rather than activities intrinsic to the α-galactosidase A preparation.

Enzymatic Properties of α-Galactosidase A and B

Enzyme kinetics were studied with both enzymes using 4-methylumbelliferyl-α-D-galactopyranoside or melibose (6-(α-D-galactoside)-D-glucose) as substrate (Fig. 2). α-Galactosidase A has a Kₘ of 3.4 mM and 40.6 mM, respectively, for these two substrates. α-Galactosidase B appeared to have first order kinetics with 4-methylumbelliferyl-α-D-galactopyranoside and was unable to hydrolyze melibose even at concentrations up to 500 mM. The apparent first order relationship between 4-methylumbelliferyl-α-D-galactopyranoside concentration and the velocity of the α-galactosidase B reaction could well be more apparent than real. Such apparent first order kinetics are observed if the Kₘ of the enzyme for substrate is high and all levels tested are well under the half-saturating concentration. Assay of the enzyme activity at higher substrate concentrations was

| Fraction     | Enzyme units | Protein | Specific activity | Yield | Purification |
|--------------|--------------|---------|-------------------|-------|-------------|
| Crude homogenate* | 6.65         | 78,600  | 202 ml/mg         | 100   | *           |
| DEAE-cellulose | 1.91         | 646     | 2,960 mg/mg       | 27.5  | 34          |
| 35-50% (NH₄)₂SO₄ fraction | 1.35     | 254     | 5,310 mg/mg       | 19.4  | 60          |
| G-200 Sephadex | 1.72         | 77      | 21,100 mg/mg      | 23.3  | 239         |
| Ectrofocusing | 1.08         | 11.8    | 91,100 mg/mg      | 15.5  | 1,006       |
| ECTEOLA-cellulose | 0.98         | 1.29    | 351,000 mg        | 6.3   | 3,900b      |

* Representing 1026 g, wet weight, of fresh human placenta.

b 0.394 mg of protein, 1,124,000 micromoles per mg and 12,500-fold purification when based on Lowry’s method.
FIG. 2. Lineweaver-Burk plot for placental α-galactosidase A and B with 4-methylumbelliferyl-α-D-galactopyranoside as substrate. The assay system contained 0.1 M citrate buffer, pH 4.0, 0.7% bovine serum albumin, and substrate concentrations from 0.5 to 28.0 mM. Activity was measured as described in the text.

FIG. 3. pH activity curves for placental α-galactosidase A and B. Citrate buffer (0.02 M) from pH 3.0 to 6.5 was used in the assay system as described in the text. Activity is expressed as milliunits per ml (nanomoles of substrate hydrolyzed per min per ml of enzyme solution).

Not possible, however, because of the relative insolubility of the umbelliferone derivative.

Ho studied the ability of the purified enzymes to hydrolyze the terminal galactose of ceramide trihexoside and found that α-galactosidase A was able to cleave galactose from this glycolipid, but that α-galactosidase B was not. Ho found the ratio of activity of α-galactosidase A with 4-methylumbelliferyl-α-
galactopyranoside as substrate to that with ceramide trihexoside as substrate to be approximately 6.4.

pH-activity curves (Fig. 3) were determined using 0.02 M citrate buffer at pH 3.0 to 6.5. Both enzymes manifest maximal activity at pH 4.5; however, while α-galactosidase A shows a broad curve, the B enzyme gives a rather sharp peak of activity.

Fig. 4 presents the effect of temperature on α-galactosidase A and α-galactosidase B as an Arrhenius plot. Assays were done as described in the text at temperatures of 5, 10, 15, 25, 30, 37, 40, and 45°.

The energy of activation of α-galactosidase A was approximately 15,700 cal per mole, while α-galactosidase B produced a very unusual plot manifesting optimal activity at 25°. The low activities observed at higher temperatures were obviously not due to instability of the enzyme, since α-galactosidase B is quite stable even at 50°, and were probably due to a conformational change in the enzyme.

M. W. Ho, personal communication.
The separated enzymes after DEAE-cellulose chromatography were tested for inhibition by myo-inositol (13). \( \alpha \)-Galactosidase A was found to be 43% inhibited when concentrations up to 750 mM were used. \( \alpha \)-Galactosidase B was not inhibited by this concentration of myo-inositol but showed a slight increase in activity. The purified preparations of both enzymes were found to require albumin in order to maintain activity in assay.

**Isoelectric Points**

Isoelectric focusing revealed the isoelectric points to be 4.70 ± 0.07 (mean ± 1 S.E.) for \( \alpha \)-galactosidase A and 4.42 ± 0.04 for \( \alpha \)-galactosidase B. This was not used as a purification step for the A enzyme because of an excessive loss of enzyme activity in this procedure.

**Immunological Studies**

The final enzyme preparations were mixed with equal parts of complete Freund’s adjuvant to obtain an emulsion and injected into the hind foot pads of rabbits. Protein injected ranged from 15 to 50 μg. Injections were given approximately every 2 weeks, and the rabbits were bled from an ear vein at least 7 days following the last injection. The serum was heated at 50° for 30 min and then filtered through 0.2-μ Millipore filters. Serum from rabbits injected with the purified \( \alpha \)-galactosidase A preparation was designated as “anti-A serum”; serum from the rabbits injected with purified \( \alpha \)-galactosidase B was designated as “anti-B serum”; and serum from normal rabbits or rabbits injected only with Freund’s adjuvant was designated as “control serum.” When tested with standard double immunodiffusion techniques, anti-A serum reacted with \( \alpha \)-galactosidase A, but not with \( \alpha \)-galactosidase B preparations. Conversely, anti-B serum reacted with \( \alpha \)-galactosidase B and not with \( \alpha \)-galactosidase A.

Mixing antiserum with enzyme had no appreciable effect on the activity of either \( \alpha \)-galactosidase A or B, but when the mixture of enzyme and antiserum was diluted 1:10 in 10 mM phosphate buffer, pH 7, and centrifuged at 40,000 × g for 1 hour, anti-B enzyme removed \( \alpha \)-galactosidase B activity and anti-A serum removed \( \alpha \)-galactosidase A activity. A 1:4 dilution of anti-B serum removed 80% of \( \alpha \)-galactosidase B activity and no \( \alpha \)-galactosidase A activity; a 1:4 dilution of anti-A serum removed 75% of \( \alpha \)-galactosidase A activity and no \( \alpha \)-galactosidase B activity from solution. Undiluted anti-B serum did appear to remove approximately 10% of A enzyme from solution, but this capacity was lost even by 1:4 dilution. The \( \alpha \)-galactosidase activity of fibroblasts from a patient with Fabry’s disease was almost completely removed by treatment with anti-B serum, while only a small proportion of enzyme from normal fibroblasts was removed by this antibody.

**Effect of Neuraminidase Treatment on Properties of \( \alpha \)-Galactosidase A**

Since it has been suggested that \( \alpha \)-galactosidase B might be the neuraminyl derivative of \( \alpha \)-galactosidase A, \( \alpha \)-galactosidase A preparations were treated with *C. perfringens* neuraminidase, and the properties of the converted enzyme were studied. Earlier investigations had shown that such treatment alters the electrophoretic mobility of \( \alpha \)-galactosidase A (7, 8) and alters its isoelectric point to resemble that of \( \alpha \)-galactosidase B (7). The thermal stability of \( \alpha \)-galactosidase A was unaltered by neuraminidase treatment. The \( K_m \) of the treated enzyme for 4-methylumbelliferyl-\( \alpha \)-d-galactopyranoside was 2.3 mM, and that for melibiose was 37.1 mM, in close agreement with that of the untreated enzyme. Neuraminidase-treated \( \alpha \)-galactosidase A did not react with anti-B serum in the double immunodiffusion system, and enzyme activity was not precipitated from solution by the antisera. Its reaction with anti-A serum was unaltered.

**DISCUSSION**

Fibroblasts from patients with Fabry’s disease were shown to contain residual \( \alpha \)-galactosidase activity which was different from those of normal cells (4–7, 14). This was originally interpreted as indicating that Fabry’s disease represented a structural mutation of the \( \alpha \)-galactosidase locus, and that an abnormal enzyme was produced (14). Subsequently, however, we were able to show that the residual enzyme in Fabry’s disease fibroblasts seemed to be identical to a minor \( \alpha \)-galactosidase component found also in normal cells. We named the normal thermolabile \( \alpha \)-galactosidase which was missing in Fabry’s disease \( \alpha \)-galactosidase A and designated the other, thermostable component \( \alpha \)-galactosidase B (4, 5). Wood and Nadler (6) and Ho et al. (7) suggested, however, that \( \alpha \)-galactosidase B was also abnormal and that there might be a close genetic relationship between \( \alpha \)-galactosidase A and B. It was proposed that \( \alpha \)-galactosidase B might be the precursor of \( \alpha \)-galactosidase A, the latter enzyme representing the neuraminyl derivative of \( \alpha \)-galactosidase B.

Separation and purification of these two forms of \( \alpha \)-galactosidase have now made it possible to study separately their properties and to produce an antibody against each of these forms. These investigations showed that the biochemical properties of \( \alpha \)-galactosidase A and B correspond fairly closely to the properties deduced from studies on crude extracts of normal fibroblasts and fibroblasts from patients with Fabry’s disease (5). \( \alpha \)-Galactosidase A is, indeed, thermolabile and is found to have a \( K_m \) of 3.4 mM for 4-methylumbelliferyl-\( \alpha \)-galactopyranoside, a \( K_m \) of 4.9 mM having been estimated previously (9). \( \alpha \)-Galactosidase B, previously thought to have a \( K_m \) of about 20 mM for 4-methylumbelliferyl \( \alpha \)-galactopyranoside (5), apparently reacts with this substrate with first order kinetics. Since both enzymes appear to have the same molecular weight on Sephadex filtration, the possibility that one isozyme represents a polymer of the other is ruled out. It was previously predicted (5) that \( \alpha \)-galactosidase B, which is active in Fabry’s disease, would have no activity with respect to the glycolipid which accumulates in this disease. Indeed, although \( \alpha \)-galactosidase A was found to be active with ceramide trihexoside, no activity was found with \( \alpha \)-galactosidase B. It is of interest, in this respect, that \( \alpha \)-galactosidase A was also found to be active against melibiose, the \( \alpha \)-galactoside of glucose, while \( \alpha \)-galactosidase B manifested no activity against this compound.

In the case of another glycolipid storage disorder, Tay-Sachs disease, there is reason to believe that the fundamental defect may be failure to transform the B isozyme of hexosaminidase to the A isozyme (15, 16). Recently, it has been found that antibodies produced against either isozyme of hexosaminidase react also with the other isozyme (17, 18). This was not found to be the case with \( \alpha \)-galactosidase: antibody against \( \alpha \)-galactosidase A showed no reaction against \( \alpha \)-galactosidase B and antisera to the B enzyme gave only minimal reaction with \( \alpha \)-galactosidase A. Furthermore, removing neuraminic acid from \( \alpha \)-galactosidase A did not convert it to a form which made it active against the anti-\( \alpha \)-galactosidase B antibody. Neither did removal of neuraminic acid from \( \alpha \)-galactosidase A change its kinetic properties with respect to 4-methylumbelliferyl-\( \alpha \)-d-galacto-
pyranoside or melibiose nor alter its sensitivity to heat inactivation. Thus, it is clear that $\alpha$-galactosidase B is not merely the neuraminyl derivative of $\alpha$-galactosidase A. Presumably, this enzyme does have a natural function as an $\alpha$-galactosidase, but as yet its natural substrate remains unknown. We have previously presented indirect evidence that $\alpha$-galactosidase B is not merely an associated hydrolytic activity of one of the other acid hydrolases (5), and the present studies show that the homogeneous preparation is free of $\beta$-glucosidase, $\beta$-galactosidase, $\beta$-hexosaminidase, acid phosphatase, and $\alpha$-mannosidase activities.

Efforts to treat Fabry's disease by enzyme replacement have consisted of plasma infusion (19) and of kidney transplantation (20, 21). The $\alpha$-galactosidase A activity of normal human plasma is approximately 145 microunits per ml. Thus, 2.2 liters of plasma or approximately 8 pints of whole blood would have to be infused to provide the amount of enzyme activity in 1 mg of our final placental $\alpha$-galactosidase A preparation. Since the infusion of purified placental enzyme is a much simpler procedure than kidney transplantation, and normal human placentas are freely available, it is possible that purified $\alpha$-galactosidase may prove to be of some value in the treatment of this serious disorder. It must be emphasized, however, that even infusions of massive amounts of $\alpha$-galactosidase A may prove to be of no benefit to patients with Fabry's disease. For exogenous enzyme to be of help to these individuals, it would be necessary for the infused enzyme to reach the sites of ceramide trihexoside accumulation and to survive degradation for a sufficiently long period of time to remove accumulated glycolipid. Further studies are needed to determine whether such replacement therapy is feasible.

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