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ALPHA-GALACTOSIDASE A FROM HUMAN PLACENTA

STABILITY AND SUBUNIT SIZE

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Summary

α-Galactosidase A (α-D-galactoside galactohydrolase, EC 3.2.1.22) was purified from human placenta. The purified enzyme showed one major band on polyacrylamide gel electrophoresis and a single precipitin line on double immunodiffusion. Electrophoresis of the purified, *S*-carboxymethylated enzyme on sodium dodecyl sulfate polyacrylamide gel showed one component with a molecular weight of about 65 000, but electrophoresis of the non-*S*-carboxymethylated enzyme showed two components, a major band with a molecular weight of 67 500 and a diffuse band with a molecular weight of 47 000. We suggest that the smaller diffuse component is a degradation product and that the enzyme is a dimer with a molecular weight of approximately 150 000 and a subunit of molecular weight of about 67 500. Antibody raised against the purified enzyme quantitatively precipitated α-galactosidase A, but not α-galactosidase in Fabry's disease fibroblasts.

The α-galactosidase A is very heat labile and pH sensitive. It is most stable in concentrated solution at low temperature and at a pH of 5.0 to 6.0. When added to plasma at 37°C, it has a half-life of only 17 min. This imposes a serious obstacle to its use in the treatment of Fabry's disease.

Introduction

Fabry's disease is a lysosomal sphingolipid storage disease characterized by the accumulation of ceramide trihexoside [1,2]. It is caused by an X-linked mutation that results in a deficiency of ceramide trihexosidase [3], an α-galactosidase which can be measured with artificial substrates [4].

Human tissues contain two acid α-galactosidases (α-D-galactoside galactohydrolase, EC 3.2.1.22), α-galactosidase A and α-galactosidase B [5—7]. α-Galactosidase A is thermolabile while α-galactosidase B is thermostable. These isozymes also differ in electrophoretic mobility, substrate specificity, kinetic properties, and isoelectric points [8]. There is no immunological cross reactivity between

the two enzymes [9,10] and, despite early suggestions to the contrary [11] they cannot be interconverted by treatment with neuraminidase [9,12]. Some investigators have indicated that only α-galactosidase A has ceramide trihexosidase activity [13,14], but more recent reports suggests that both the A and B enzyme have ceramide trihexosidase activity [15,16]. It is α-galactosidase A activity which is absent or reduced in patients with Fabry's disease [11,17,18] whereas α-galactosidase B activity is normal or increased.

In previous studies [8] the two α-galactosidases from human placenta were separated. The B enzyme was purified to homogeneity and the A enzyme was partially purified. We now report the purification of the placental A enzyme to near-homogeneity with further characterization of the enzyme.

Materials and Methods

Human placentas were obtained from local hospitals, stripped of the outer membranes, and frozen at −20°C until ready for use. α-Galactosidase was assayed as previously described [8]. Protein determinations were carried out according to the method of Lowry et al. [19] with bovine serum albumin as standard.

Polyacrylamide gel electrophoresis was carried out in 7% polyacrylamide gels at pH 8.7 by the method of Davis [20]. Samples were applied to the stacking gel in 25% sucrose. Protein bands were detected by staining overnight with 0.025% Coomassie Blue R-250 in water/methanol/acetic acid (5 : 5 : 1) and destaining in 10% acetic acid. Activity bands were detected by incubating the gels in 0.5 M citrate buffer, pH 4.4, containing 4 mM 4-methylumgelliferyl α-D-galactoside at 37°C for 30 min, decanting, adding 1 M glycine buffer, pH 10.7, and observing under long wave ultraviolet light.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed by the method of Weber and Osborn [21]. Protein samples were denatured in 1% sodium dodecyl sulfate and 1% 2-mercaptoethanol at 100°C for 2 min and then for 2 h at 37°C. The electrophoresis of the denatured proteins was carried out in sodium dodecyl sulfate polyacrylamide gels at 22°C with 6 mA/tube. Bovine serum albumin, ovalbumin, and cytochrome *c* were used as standards for the determination of the molecular weights of the protein subunits. Sodium dodecyl sulfate polyacrylamide gel electrophoresis was also carried out in 8 M urea and with enzyme that had been *S*-carboxymethylated according to the procedure of Crestfield et al. [22].

Antibody against the enzyme was raised by injecting a mixture of 20 μg of the final preparation with an equal volume of complete Freund's adjuvant into the hind foot pads of rabbits twice, at 4-week intervals. The rabbits were bled at least 7 days after the last injections. The antibody was characterized by the standard double immunodiffusion technique and by precipitation of α-galactosidase activity.

The materials were obtained as follows: Concanavalin A-Sepharose from Pharmacia, ECTEOLA-cellulose from Sigma Chemical Co., CM-52 and DE-52 celluloses from Whatman, Inc., hydroxyapatite powder from Bio-Rad Laboratories, and 4-methylumbelliferyl α-galactoside from Koch-Light Laboratories.

Results

Purification. All the purification steps were performed at 0—4° C except the concanavalin A-Sepharose chromatography, which was carried out at room temperature. 1200 g of placenta were thawed, ground in a meat grinder and homogenized with 3000 ml of cold water in a Waring blendor for 5 min. The crude homogenate was centrifuged at 16 000 × *g* for 30 min. For each 9 parts of supernatant, 1 part of 0.125 M succinate buffer, pH 6.0 containing 5 mM each of magnesium chloride, manganese chloride, and calcium chloride and 1% sodium azide was added. The mixture was allowed to reach room temperature, and was applied to 80 ml concanavalin A-Sepharose column. The column was then washed with 1000 ml of 0.0125 M succinate buffer, pH 6.0 containing 0.5 M sodium chloride. Activity was eluted with 0.5 M α-D-methylmannoside in 0.01 M phosphate buffer, pH 6.5 [23]. The eluate was added directly to a DEAE-cellulose column (2.54 × 38 cm). After washing the column with 300 ml of 0.01 M phosphate buffer, pH 6.5, the α-galactosidases were separated with a gradient of sodium chloride in the phosphate buffer. α-Galactosidase A (first peak) was eluted with a 700 ml linear gradient of sodium chloride from 0 to 0.1 M. α-Galactosidase B was eluted with a 700 ml linear gradient of sodium chloride from 0.1 to 0.25 M. The fractions from the first peak were combined and the activity precipitated by adding 334 mg ammonium sulfate per ml. The precipitate was dissolved in cold water and dialyzed against three changes of 1 l each of 0.01 M phosphate buffer, pH 6.5 for 2 h. The pH of the dialysate was adjusted to 5.2 with 1 M citric acid and the solution added to a CM-cellulose column (2.54 × 12 cm) that had been equilibrated with 0.01 M citrate buffer, pH 5.2. Activity was washed through the column with the same buffer. After combining the tubes containing the activity, the solution was dialyzed against 3 changes of 1 l of 0.005 M phosphate buffer, pH 6.8 for 2 h each and added to a hydroxyapatite column (1 ml of bed volume per mg of protein). The column was washed with 100 ml of 0.005 M phosphate buffer, pH 6.8 and the activity eluted with an 800 ml linear gradient of this buffer at a concentration from 0.005 to 0.1 M. Tubes containing the activity were pooled and the solution concentrated with an Amicon PM-30 diaflow membrane. The concentrate was dialyzed against 3 changes of 1 l each of 0.01 M phosphate buffer, pH 7.5 for 2 h each and added to a ECTEOLA-cellulose column (1.5 × 23 cm) that had been equilibrated with 0.01 M phosphate buffer, pH 7.5. After washing the column with the buffer, the activity was eluted with a 500 ml linear gradient of 0—0.1 M sodium chloride in 0.01 M phosphate buffer, pH 7.5. Tubes containing the activity were combined, pH adjusted to 6.2 with 0.1 M KH_2PO_4, and the solution concentrated with an Amicon PM-30 diaflow membrane.

An overall purification of about 36 000-fold with a yield of 18% was achieved. A summary of the purification procedure is given in Table I. The procedure has been repeated many times with similar results.

Purity. Polyacrylamide disc gel electrophoresis patterns of the purified enzyme are shown in Fig. 1A and B. At lower concentrations, only one band of protein could be observed, but at higher concentrations several minor bands were visible. The stain for activity coincided with the major protein band with both having an R_F of 0.31 (Fig. 1C and D).

TABLE I

PURIFICATION OF α-GALACTOSIDASE A FROM HUMAN PLACENTA

Step	Total activity * (units)	Yield (%)	Total protein (mg)	Specific activity (μunits/mg)	Fold purification
Crude homogenate	18.9	—	80 640	234	—
Supernatant	15.5	82	25 900	600	2.5
Concanavalin A-Sepharose	13.6	72	336	40 500	173
DEAE-celluose	11.6	61	100	116 000	496
Ammonium sulfate	10.9	58	72	151 400	647
CM-cellulose	8.9	47	51	174 500	746
Hydroxyapatite	6.3	33	10	630 000	2 692
ECTEOLA-cellulose	3.4	18	0.4	8500 000	36 325

* α-Galactosidase A only.

When anti α-galactosidase A (anti A) serum was tested against the purified enzyme by double immunodiffusion, only one precipitin line was observed and this line showed α-galactosidase activity. Only one precipitin line was also observed with concentrated crude placental extract. The anti-A serum also quantitatively precipitated α-galactosidase A from solution, but did not precipitate partially purified α-galactosidase B. The anti-serum precipitated about 70% of the α-galactosidase activity of normal cells but did not precipitate

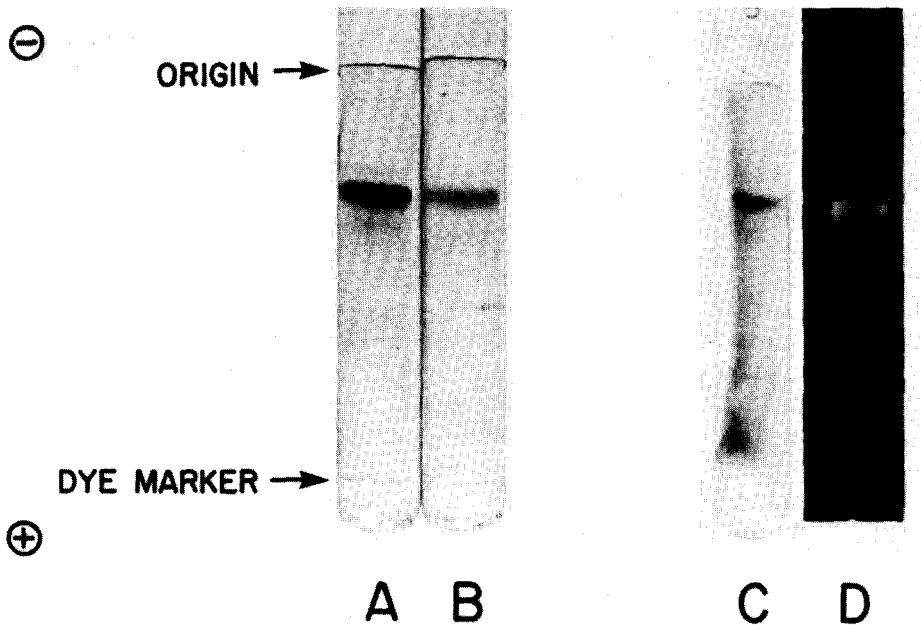

Fig. 1. Polyacrylamide gel electrophoresis of the purified α-galactosidase A. Gel in photograph A contains 8.4 μg of purified enzyme and was stained for protein. Gel in photograph B contains 2.1 μg of enzyme and was stained for protein. Gel in photographs C and D contains 2.1 μg of enzyme. It was first stained for α-galactosidase activity (photograph D) and then stained for protein (photograph C).

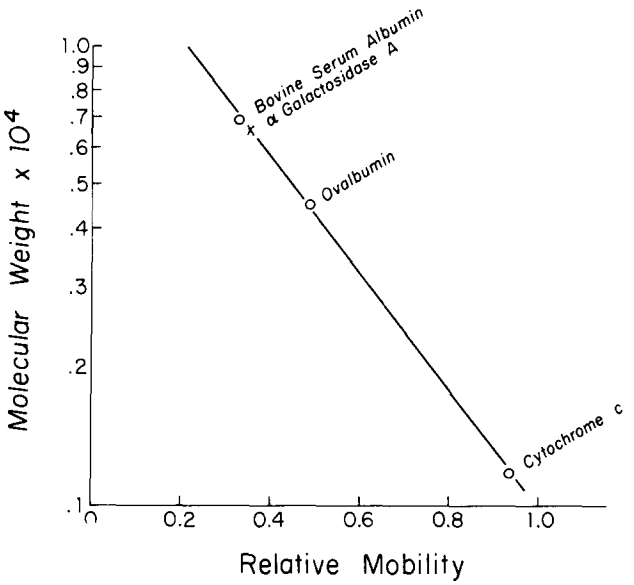

Fig. 2. Molecular weight of the subunits of *S*-carboxymethylated α-galactosidase A as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis.

any of the activity of Fabry's cells. This confirms that the antibody is specific for the A isoenzyme and does not react with the B isozyme.

Subunit molecular weight. Electrophoresis of *S*-carboxymethylated α-galactosidase A in sodium dodecyl sulfate polyacrylamide gel showed one component with a molecular weight of 65 000 (Fig. 2). However, electrophoresis of

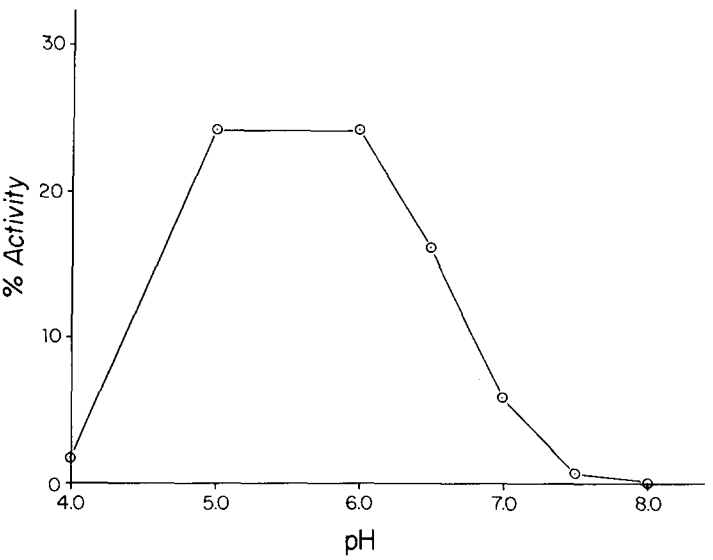

Fig. 3. Heat stability of α-galactosidase A at various pH values. Solutions of enzyme in universal buffer (6.01 g of citric acid, 3.89 g of dihydrogen potassium phosphate, 1.77 g of boric acid, and 5.27 g of diethylbarbituric acid per 1. pH adjusted with o.2 M sodium hydroxide) at various pH values were heated at 50° C for 30 min, cooled and assayed for α-galactosidase activity.

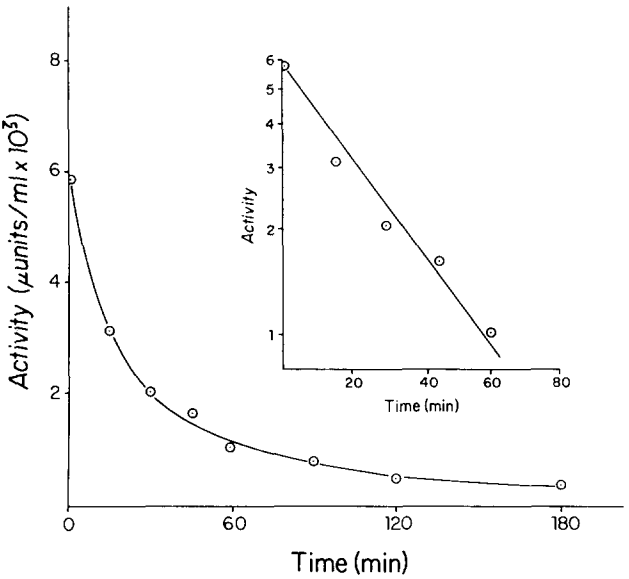

Fig. 4. Stability of α-galactosidase A in plasma. Purified enzyme (0.029 units) was added to 5 ml of plasma and incubated at 37°C. pH was maintained at 7.4 with a CO_2/air atmosphere. Samples were taken at various times and assayed for α-galactosidase.

sodium dodecyl sulfate denatured, non-*S*-carboxymethylated enzyme showed two components, a major band with a molecular weight of 67 500 and a diffuse band with a molecular weight of 47 000. In order to rule out reassociation of the enzyme, sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed in 8 M urea. The pattern on electrophoresis in urea was similar to that without urea, a major band with a molecular weight of 69 000 and a diffuse band with a molecular weight of 47 000.

Stability. The effect of pH on heat stability is shown in Fig. 3. The enzyme was most stable at a slightly acid pH with maximum stability between pH 5 and 6. Stability of the enzyme in plasma maintained at a pH 7.40 at 37°C is shown in Fig. 4. Activity was lost very rapidly with a half life of 17 min. However, the enzyme was very stable at 4°C at pH 6.2 in a concentrated solution with little or no loss of activity in over 20 days.

Discussion

In earlier study from this laboratory, α-galactosidase B was purified to homogeneity from human placenta, but attempts at complete purification of α-galactosidase A, the enzyme which is lacking in Fabry's disease, were unsuccessful. This was the case because of the instability of the enzyme at later purification stages. Investigation of the relationship between enzyme stability and pH has now made possible, for the first time, purification of this important enzyme to near-homogeneity. In the first step of the purification, advantage was taken of the fact that most or all of the lysosomal glycosyl hydrolase are reversibly bound by concanavalin A-Sepharose [23]. This resulted in high degree of purification with a high yield. The enzyme was then further purified

by conventional methods. After the last purification step, it is important to immediately adjust the pH to 6.2 in order to prevent loss of activity. The purified protein formed one major band on polyacrylamide gel electrophoresis and one precipitin line on double immunodiffusion with antibody against the purified enzyme. The specific activity of the purified enzyme is about 10 times higher than the partially purified enzyme previously reported from this laboratory [8] and 230 times as great as that infused into patients with Fabry's disease by Brady and co-workers [24] as experimental enzyme replacement therapy.

Antibody against purified α-galactosidase A precipitated the purified enzyme and about 70% of the α-galactosidase activity in normal fibroblast, but it could not precipitate α-galactosidase B or the α-galactosidase activity remaining in Fabry's fibroblasts. This confirms our earlier observation that the activity remaining in Fabry's fibroblast (α-galactosidase B) is immunologically distinct from α-galactosidase A [9].

Electrophoresis of the purified enzyme in sodium dodecyl sulfate showed two protein bands, a major band with a molecular weight of approximately 67 500 and a diffuse minor band with a molecular weight of 47 000. After *S*-carboxymethylation only the major, larger molecular band was observed. A number of factors may cause the appearance of multiple bands. Although contamination by another protein is possible, this seems unlikely, since no major contamination was observed on polyacrylamide gel electrophoresis or on double immunodiffusion. One band could be a multimer of the other, but the molecular weight determinations and the failure of *S*-carboxymethylation to produce further dissociation do not agree with this concept. It seems most likely that degradation of the enzyme occurred in the dissociation procedure or in sodium dodecyl sulfate electrophoresis. Hybridization studies [25,26] in man-Chinese hamster somatic cell hybrids indicate that the enzyme is a dimer with similar subunits. The molecular weight of the enzyme was previously determined by Sephadex gel filtration to be 150 000 [8]. Considering the molecular weight determinations of glycoproteins by Sephadex gel filtration is an estimation, the most reasonable structure for the enzyme is a dimer with a native molecular weight of approximately 150 000 and a subunit molecular weight of about 67 500 with the diffuse, smaller molecular weight band representing a degradation product.

Fabry's disease is one of the inborn errors of metabolism that might be amenable to enzyme therapy. There are, of course, some formidable obstacles to the success of such treatment. The cells of several patients with Fabry's disease have been studied for the presence of antigenically cross-reacting material, and α-galactosidase A antigen has not been detected [27,28]. If, in fact, patients with Fabry's disease have never had immunologic experience with an α-galactosidase A-like protein, they may easily become sensitized to exogenous α-galactosidase A, even from human sources. It is necessary, too, for exogenously administered α-galactosidase A to persist for a sufficiently long time at sites where it has an opportunity to hydrolyze significant amounts of ceramide trihexoside. Preliminary attempts at replacement therapy have been reported. Brady et al. [24] injected partially purified α-galactosidase intravenously into two patients with Fabry's disease. A decrease in plasma ceramide

trihexoside was observed in both patients, but levels returned to preinfusion values within 48 h of enzyme injection. However, on the basis of our studies one of the major obstacles to enzyme replacement with α-galactosidase is the instability of the enzyme. We have observed that α-galactosidase A is fairly stable at slightly acid pH and it can be stored for several weeks in the cold at pH 6.2 without loss of activity. This was very helpful in studying the properties of the enzyme, but the instability of the enzyme at pH 7.4 severely limits its use in enzyme replacement. As expected from our pH-stability data the activity is lost very rapidly in plasma at 37°C. The half life of the enzyme in plasma at 37°C could account for most of the reported disappearance of activity from the circulation after infusion of the enzyme into patients with Fabry's disease [24].

The instability of α-galactosidase A in plasma has recently been observed by other investigators. Rietra et al. [29] have shown that α-galactosidase activity in plasma and serum decreased very rapidly when incubated at 37°C, but if the pH of the samples was adjusted to 6.1, the activity was very stable. Desnick et al. [30] have demonstrated that the half life of α-galactosidase A incubated in plasma from a hemizygote with Fabry's disease at pH 7.3 and 37°C was about 9 min. This half life is shorter than the one (17 min) we observed. The details of their experiment are not given so it is difficult to compare results directly.

Snyder et al. [31] have reported the stabilization of α-galactosidase A to heat and protease degradation by complexing with antibody and by chemical cross linking of the enzyme. This is a potentially useful approach, although such treatment might enhance the antigenicity of the enzyme.

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