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ENZYME THERAPY II
PURIFIED HUMAN α-GALACTOSIDASE A

STABILIZATION TO HEAT AND PROTEASE DEGRADATION BY COMPLEXING WITH ANTIBODY AND BY CHEMICAL MODIFICATION

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SUMMARY

Methods were investigated for the stabilization of human splenic α-galactosidase A (α-D-galactoside galactohydrolase, EC 3.2.1.22). Anti-α-galactosidase A antiserum was produced in a goat by repeated immunization with highly purified α-galactosidase A. When this antiserum was incubated at 37 °C with α-galactosidase A in varying enzyme: antiserum ratios, a significant increase in the thermal (50 °C) stability and in the resistance to protease digestion of these mixtures was observed compared to appropriate controls. The enzyme was also treated with the bifunctional cross-linking reagent, hexamethylene diisocyanate, and the thermal stability and protease resistance of the cross-linked derivative were increased compared to the native enzyme treated with the monofunctional reagent, butyl isocyanate.

INTRODUCTION

Recent interest in enzyme therapy for inherited lysosomal enzymatic deficiencies has stimulated the exploration of various approaches to modify purified enzymes. The goal of such modification is the prolongation of the half-life of the enzymatic activity with respect to thermal inactivation and degradation by circulating proteases, two enzymatic properties desirable for therapeutically more effective in vivo administration.

Interactions of enzymes with specific antibodies have been demonstrated to alter various physical characteristics of native or mutant proteins, including stabilization toward heat [1, 2] or alkaline [3] denaturation, reversal of heat denaturation and restoration of enzymatic activity [4], restoration of activity of a mutant enzyme [5], and enhancement of enzymatic activities [10, 11]. In addition, various enzymes

Abbreviations: 4-MUgal, 4-methylumbelliferyl-α-D-galactopyranoside; CTH, galactosyl-α-(1→4)-galactosyl-β-(1→4)-glucosyl-β-(1→1)-ceramide; HMDC, hexamethylene diisocyanate.

have been altered by inter- and intramolecular cross-linking by bifunctional organic reagents [12]; such treatment has been shown to significantly increase thermal stability and protease resistance [13]. This communication reports preliminary data on the successful in vitro stabilization of α-galactosidase A by immunologic and chemical methods.

MATERIALS AND METHODS

Fresh-frozen human spleens obtained at splenectomy were the source of α-galactosidase A, which is rapidly heat inactivated at 50 °C [14]. The enzyme was isolated by successive methanol–butanol extraction, $(NH_4)_2SO_4$ precipitation, Sephadex G-200, diethylaminoethyl (DEAE)-cellulose, and carboxymethyl (CM)-cellulose column chromatography. Enzymatic activity was monitored with the artificial fluorogenic substrate, 4-methylumbelliferyl-α-D-galactopyranoside (4-MUgal) [14] and with the natural substrate, galactosyl-α-(1→4)-galactosyl-β-(1→4)-glucosyl-β-(1→1)-ceramide (CTH; Bernlohr, R. W., et al. unpublished). In addition, α-galactosidase has previously been shown to hydrolyze the terminal α-linked galactosyl moiety from digalactosyl ceramide which accumulates in Fabry kidney [15] and the terminal α-linked galactosyl moiety from rabbit blood group B-active glycolipid [16]. Approximately 99% of the purified splenic α-galactosidase activity was the heat labile A form. The specific activity of the CM-cellulose enzyme preparations used to innoculate the goat averaged 835 nmoles 4-MUgal hydrolyzed per min per mg protein and 125 nmoles CTH hydrolyzed per min per mg protein. Immunization was carried out over a six-month period (3 mg total protein given) and antiserum was obtained seven days after each antigen challenge; antisera used in this report were collected following the second and third challenges. Normal goat serum and the antiserum were freed of major lipids and fibrinogen by silica gel treatment (Condie, R. M., et al., unpublished) and both sera were decomplemented at 56 °C for 30 min which totally inactivated endogenous α-galactosidase A activity. Protein concentration of these sera averaged 75 mg protein per ml.

RESULTS

Antiserum treatment

α-Galactosidase A fractions purified through the DEAE-cellulose step (specific activity averaging 250 nmoles 4-MUgal hydrolyzed per min per mg protein and 40 nmoles CTH hydrolyzed per min per mg protein) were incubated with the undiluted goat sera. An enzyme stock solution at 0.5 mg protein per ml was used for all the stability tests. Microliter quantities of this stock solution were incubated for 90 min at 37 °C with microliter quantities of antiserum to give enzyme: antiserum ratios ranging from 1:10 to 200:1 (μl:μl) in a constant volume of 0.4 ml in 0.1 M phosphate buffer, pH 7.5. Normal goat serum or phosphate buffer controls were incubated with enzyme at the same ratios in a constant volume of 0.4 ml. Enzyme assay of the enzyme: antiserum and control mixtures prior to the 37 °C incubation revealed no loss of enzymatic activity due to the addition of antiserum and normal goat serum. In some cases, however, an increase of up to 15% of the initial enzymatic activity was observed after treatment with antiserum and normal goat serum. Following the

90-min incubation, thermal stability studies were carried out with 0.4 ml of the enzyme: antiserum mixtures at 50 °C and 0.02-ml aliquots were taken for enzyme assay over 1.5 h. An enzyme:antiserum ratio of 10:1 or 20:1 (μl:μl) conferred maximum thermal stability to the α-galactosidase A. Normal goat serum or 0.1 M phosphate buffer, pH 7.5, at the same ratios to enzyme concentration did not thermally stabilize the enzyme. Protease degradation studies were carried out by the addition of 300 μg of trypsin (EC 3.4.4.19; Worthington Biochemical Corp.) following 90 min incubation of enzyme–antiserum mixtures (0.4 ml). The mixtures were then incubated at 37 °C and 0.02-ml aliquots were withdrawn for enzyme assay at intervals over 2.5 h. Again, an enzyme:antiserum ratio of 10:1 or 20:1 (μl:μl) maximized the trypsin resistance of the enzyme, whereas the normal goat serum or buffer did not stabilize the enzymatic

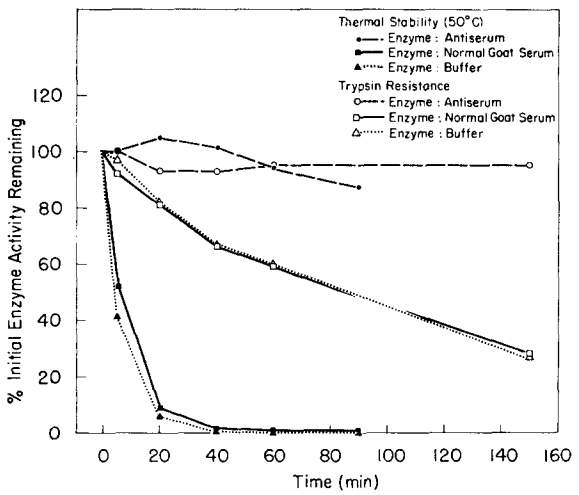

Fig. 1. Thermal stability and resistance to trypsin digestion of enzyme–antiserum, enzyme–normal goat serum and enzyme–buffer mixtures at a 20:1 ratio (μl:μl). Enzyme concentration was 0.5 mg protein per ml and total incubation volume was 0.4 ml in 0.1 M phosphate, pH 7.5.

activity against protease digestion. These observations are summarized in Fig. 1 for an enzyme:antiserum ratio of 20:1.

Solubility of the enzyme–antibody complex

In all thermal stability studies and in several cold (4 °C) precipitation studies carried through 72 h, no precipitating material was observed, indicating that the enzyme–antibody complex was soluble.

Chemical modification

To test the effectiveness of enzyme stabilization using a bifunctional cross-linking reagent, partially purified (DEAE-cellulose fractions) α-galactosidase A was again used. The bifunctional reagent, hexamethylene diisocyanate (HMDC, Eastman Kodak Co.) was dissolved in acetone (100 μmoles HMDC per 1.0 ml acetone) and 0.01 ml of this solution was added per 1.0 ml of enzyme (0.5 mg protein per ml) in 0.02 M phosphate buffer, pH 6.5. The mixture was mixed vigorously and then allowed

to stand at room temperature for 15 min. For controls, 0.01 ml of the monofunctional reagent, butyl isocyanate (Eastman Kodak Co.; 200 μmoles per 1.0 ml acetone) or 0.01 ml of acetone were added to 1.0-ml aliquots of α-galactosidase A. Enzyme assay of the cross-linked and control mixtures exhibited activity identical to that of the untreated enzyme. Cross-linked and control mixtures were precipitated with saturated $(NH_4)_2SO_4$ and resuspended in 0.1 M phosphate, pH 7.5. The resuspended enzyme (0.3 ml, 450 μg protein) was incubated at 50 °C and aliquots were withdrawn at intervals over 2 h for enzyme assay. The cross-linked enzyme exhibited markedly increased thermal stability compared to the native enzyme (Fig. 2). The cross-linked and native

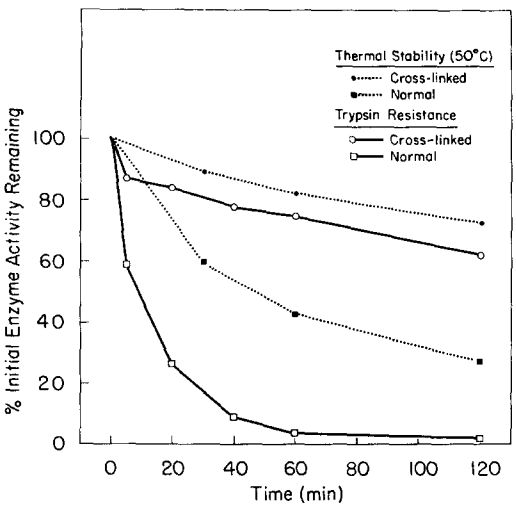

Fig. 2. Thermal stability and resistance to trypsin digestion of HMDC cross-linked and native α-galactosidase A. Enzyme concentration was 0.5 mg protein per ml and total incubation volume was 0.3 ml in 0.1 M phosphate, pH 7.5. The data for enzyme treated with monofunctional reagent have not been included; extensive inactivation of the enzyme was observed, but the remaining activity was indistinguishable from that of native enzyme.

α-galactosidase A (0.3 ml) were incubated at 37 °C after the addition of 250 μg of trypsin and aliquots were withdrawn at regular intervals for assay. Similarly, the resistance to trypsin digestion of the cross-linked enzyme was markedly increased compared to that of the native enzyme, as shown in Fig. 2. In addition, resistance to protease digestion was observed when the cross-linked enzyme was treated with either pronase (Calbiochem Inc.) or chymotrypsin (EC 3.4.4.5; Worthington Biochemical Corp.) under comparable conditions of pH and concentration. Enzyme treated with the monofunctional reagent, butyl isocyanate, was inactivated. It was also observed that excessive cross-linking with HMDC (three or more sequential 0.01-ml additions) increased the thermolability of α-galactosidase A.

DISCUSSION

These results may have therapeutic significance for specific enzymatic deficiency diseases. Previous attempts have been made to replace the deficient α-galacto-

sidase A activity in hemizygous patients with Fabry's disease [17–19]. However, effective replacement was limited by the short half-life of the infused native enzyme. Administration of antibody-stabilized or cross-linked enzyme with a greatly increased half-life and stability may provide a more successful method of therapy. Further studies on the antigenicity and toxicity of a stabilized α-galactosidase A preparation as well as the design of a suitable delivery system must be accomplished, however, before in vivo therapeutic trials are undertaken.

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