IN VITRO CORRECTION OF DEFICIENT HUMAN FIBROBLASTS BY β-GLUCURONIDASE FROM DIFFERENT HUMAN SOURCES

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SUMMARY

Human β-glucuronidase has been isolated from four sources by affinity chromatography on a heterologous antibody resin. Partial purification of 30-180X has been achieved in one step. β-Glucuronidase from these sources were taken up by β-glucuronidase deficient fibroblasts in culture and produced correction of the abnormal acid mucopolysaccharide accumulation. Enzyme preparations from blood platelets had far greater uptake and corrective activity than enzymes from liver, placenta, or urine.

INTRODUCTION

β-Glucuronidase (EC 3.2.1.31) is a hydrolytic enzyme for which a dual localization in the lysosome and microsomes[1-3] and multiple intracellular forms are reported[4,5]. The deficiency of this enzyme in man[6,7] has recently joined the list of lysosomal hydrolase deficiencies which produce a mucopolysaccharide storage disease. Hall, et al[8] demonstrated that fibroblasts from a β-glucuronidase deficient patient accumulate excessive amounts of ^{35}S-acid mucopolysaccharides in culture, and that this biochemical abnormality is corrected by the addition of partially purified β-glucuronidase from bovine liver[8]. β-Glucuronidase from human fibroblasts also shows corrective activity[9].

We report here a general method of isolating β-glucuronidase from a variety of human sources using an immunoadsorbent gel and studies which compare the uptake properties and the corrective activity of these enzyme preparations.

MATERIALS AND METHODS

Preparation of antibody-Sepharose gel. β-Glucuronidase, purified from rat preputial glands[10] and homogeneous by polyacrylamide gel electrophoresis, was injected subcutaneously into goats in Freund's adjuvant over six weeks. Immunoglobulins (IgG) from goat sera were isolated by ammonium sulfate precipitation and DEAE-cellulose chromatography[11] and lyophilized. Prior to coupling, the IgG fraction was dissolved and dialyzed against 0.2 M sodium citrate, pH 6.5. Five grams of IgG were coupled to 400 g of cyanogen bromide activated Sepharose 2B (0.3g CNBr/ml packed gel) by mixing for 16 hr at 4^{o} in a total volume of 730 ml of the citrate buffer[12]. The antibody-Sepharose was washed extensively with 0.15 M NaCl before use.

Lysosomal hydrolase activity assays measured release of p-nitrophenol or 4-methylumbelliferone from synthetic substrates (Sigma) after 100 µl substrate and 20 µl enzyme were incubated for 30 min at 37^{o}. The reaction was stopped by addition of 1.90 ml of 0.32 M glycine, 0.20 M sodium carbonate, pH 10.4 buffer. Substrate solutions were p-nitrophenyl phosphate disodium (10 mM), and 4-methylumbelliferyl-β-D-glucuronide (1 mM), -N-acetyl-β-D-glucosaminide (1 mM), -β-D-galactoside (1 mM), -α-D-mannoside (0.8 mM) in the buffers previously described[13].

Preparation of liver, placenta and platelet extracts. Frozen liver (100 g) or placenta (20 g) was homogenized in a Waring blendor in five volumes of TRIS-saline (0.01 M TRIS (hydroxymethyl) aminomethane. HCl, 0.15 M NaCl, pH 7.0). Ammonium sulfate was added to the 12,000 x g supernatant. The 30-45% (liver) precipitate or 0-40% (placenta) was dissolved in and dialyzed against TRIS-saline. Platelets were isolated, washed[14] and ruptured by sonication or lysed by 1% DOC treatment in 0.05 M TRIS, pH 8.0 and frozen for storage. Prior to use DOC was preciptated from lysates with 0.01 M $CaCl_2$.

Isolation of β-glucuronidase by affinity chromatography. The tissue extract was passed over a column of antibody-Sepharose (0.9 cm x 2-10 cm). After washing the column with 10 bed volumes of TRIS-saline or until the $O.D._{280}>0.01$, the β-glucuronidase activity was eluted with 6 M urea in 0.01 M TRIS, pH 7.0. The eluate was dialyzed against TRIS-saline and concentrated by ultrafiltration.

Table I

Specific activities[a] of β-glucuronidase and accompanying lysosomal enzymes from human sources before[b] and after [c]purification by affinity chromatography

	Placenta	Urine	Liver	Platelet (DOC)	Platelet Sonic
β-Glucuronidase					
before	379	288	207	413	37
after	68,180	19,500	6,880	12,511	4,600
β-N-acetylglucosaminidase					
before	1,158	882	1,000	1,362	425
after	n.d.[c]	345	n.d.	n.d.	735
α-Mannosidase					
before	15.8	59	42	54	13.9
after	n.d.	n.d.	26	n.d.	n.d.
β-Galactosidase					
before	31.6	512	400	5	13.5
after	n.d.	n.d.	n.d.	n.d.	n.d.
Acid Phosphatase					
before	5,800	48,300	1,360	342	2,000
after	n.d.	n.d.	n.d.	n.d.	n.d.

a) One unit is nmole/hr. p-nitrophenol (for acid phosphatase activity) or 4-methylumbelliferone (for other hydrolase activities). Protein was determined by the method of Lowry[17].

b) Crude enzyme extracts were prepared for adsorption on affinity resin as described in Materials and Methods. Prior to use all extracts were centrifuged, extracts of liver, placenta and platelet (sonic) at 12,000 x g, platelet (DOC) $CaCl_2$ precipitation mixture at 5,000 x g, and freshly collected, cooled urine at 1,000 x g.

c) Not detectable; enzymatic activity was considered real if greater than two times the blank value.

β-Glucuronidase uptake and biochemical correction. Primary fibroblasts from one normal patient and one patient with β-glucuronidase deficiency mucopolysaccharidosis were maintained in Eagle's Minimal Essential Medium with 10% heat inactivated fetal calf serum. Enzymes to be incubated with fibroblasts were mixed with diluent (saline-10% fetal calf serum) prior to sterilization through Millipore filters. Uptake and correction of ^{35}S-acid MPS accumulation were measured simultaneously using cultured fibroblasts from the same passage. Enzyme uptake was measured by determining the percentage of added enzyme taken up by fibroblasts in 35 mm dishes during 48 hours. Correction of MPS accumulation was measured by the reduction in the

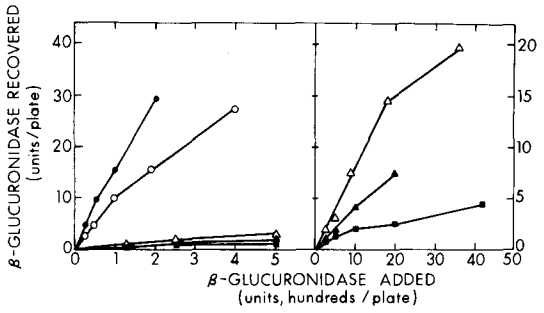

Figure 1. Uptake of human β-glucuronidase from urine (-▲), placenta (-Δ), liver (-■-), platelet sonicate (-o-), Platelet-DOC (-●-) in fibroblasts defective in β-glucuronidase activity. Cells were cultured in 35 mm plastic petri dishes for 48 hr in medium containing the β-glucuronidase. The dishes of cells were then washed three times with 0.15 M NaCl and fixed by treatment in -20° acetone for 20 sec. The plates were assayed with 0.5 ml of 1 mM 4-methylumbelliferyl-β-D-glucuronide at 37° for 1 hr.

$^{35}SO_4$ accumulation by deficient fibroblasts in 100 mm plates over 48 hours in response to added β-glucuronidase according to the method of Barton and Neufeld[15,16]

RESULTS

The selective retention of human β-glucuronidase on goat anti-rat-β-glucuronidase-Sepharose is shown in Table I. A 30-180 fold increase in specific activity of β-glucuronidase was obtained by adsorption to and elution from the gel. By contrast, the other lysosomal hydrolases measured were not detectable or greatly reduced in the gel purified β-glucuronidase preparations. The gel retained 95-99% of applied β-glucuronidase from platelet or placenta extracts, but at least 80% of the other activities measured were recovered in the effluent of crude extract. Recovery of adsorbed β-glucuronidase in the 6 M urea eluate varied from 60-100%, with recovery improving on repeated use of the gel. We suggest that the highest affinity sites for β-glucuronidase do not release the enzyme in the presence of 6 M urea and that one approaches quantitative recovery of adsorbed enzyme when the high affinity sites become saturated.

Fig. 1 shows the uptake of β-glucuronidase from five enzyme preparations by β-glucuronidase deficient fibroblasts. Although each preparation of β-glucuronidase is taken up, there is a striking difference between the platelet enzyme and enzyme

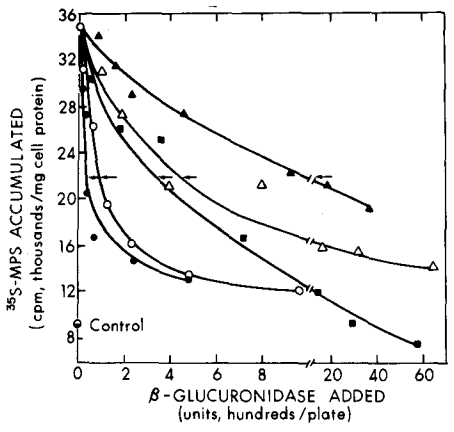

Figure 2. Response of ^{35}S-MPS accumulation to added human β-glucuronidase from urine (-▲-), placenta (-△-), liver (-■-), platelet sonicate (-o-), platelet-DOC (-●-) fibroblasts defective in β-glucuronidase activity. Cells were cultured in 100 mm plastic petri dishes for 48 hr in medium containing β-glucuronidase and ^{35}S-SO_4, and analyzed according to the procedure of Barton and Neufeld[15,16]. The ^{35}S-MPS accumulation of a normal line of fibroblasts (●) without added enzyme, was used to establish a value for 100% correction. Arrows denote point of half-maximal correction.

derived from liver, placenta and urine in the amount taken up per unit of catalytic activity added to the culture medium. In separate experiments a range of 8-25% uptake has been obtained for various platelet preparations. The presence of deoxycholate (DOC) or Triton X-100 during platelet extraction releases 50-100% more enzyme into the supernatant than is released in the absence of detergent, but does not affect uptake.

Figure 2 demonstrates the reduction in ^{35}S-MPS accumulation by added β-glucuronidase from the five preparations. There is a general correlation in the order of uptake properties of a given enzyme preparation and the corrective activity. The platelet β-glucuronidase shows both the highest uptake and the greatest corrective activity per unit of catalytic activity. The exception is the liver preparation which shows poorer uptake than any other preparation, but better corrective activity than the urinary enzyme. The linear uptake of enzyme far exceeds the amount required for half-maximal correction (see arrows in Figure 2). A similar uptake phenomenon has been reported for urinary α-L-iduronidase[18] and placenta N-acetyl-α-gluco-

saminidase[19] by fibroblasts deficient in those enzyme activities respectively. To compare Fig. 1 and Fig. 2 we calculate that 100 units per plate in an uptake experiment (Fig. 1) is equivalent to about 1400 units per plate in a correction experiment (Fig. 2) by accounting for the difference in diameter of the plates in which the former and latter studies were conducted (35 mm vs 100 mm, respectively) and the difference in volume of medium in which the enzyme was supplied to the growing cells in each study (2 ml vs 10 ml, respectively).

DISCUSSION

The discovery and isolation of specific corrective factors for a number of mucopolysaccharide storage disorders has stimulated great interest in the possibility of enzyme replacement in lysosomal storage diseases, first suggested by Hers[20]. Mixed results with fresh frozen plasma infusions[21,22] in these disorders have emphasized the need to focus attention on purified enzyme preparations for such investigations. The finding by Hickman and Neufeld[9] that I-cell enzyme has little ability to be taken up by and correct deficient human fibroblasts suggested that enzyme in the "proper recognition form" is important to in vitro correction. The studies reported here demonstrate large differences of both the uptake properties and corrective activity among β-glucuronidases from various human sources. These differences may have an important bearing on the choice of tissue for purification of human enzymes for enzyme replacement.

As earlier suggested[9] the variation in uptake ability of the same enzyme activity from different sources may be due to subtle structural differences such as type and number of sugar residues. The in vivo uptake ability of certain plasma proteins has been shown to increase significantly when the terminal sialic acid is removed to expose a galactose residue[23,24].

The platelet β-glucuronidase used in these experiments demonstrate three electrophoretic forms on acrylamide gel electrophoresis whereas placenta enzyme shows two unresolved bands. If one of the platelet enzyme forms prove to be the "high uptake form" analysis of the structural differences compared to the "low uptake form"

found in platelets, placenta, urine or liver could account for its recognition and uptake and shed light on the mechanism of lysosomal recognition for uptake. Such information should be valuable to understanding normal lysosomal enzyme maturation and localization as well as to investigation of lysosomal enzyme replacement.

CONCLUSION

β-Glucuronidase from several human sources was isolated by an antibody-Sepharose immunoadsorbent column. The enzyme preparations from platelets shows very high uptake by deficient fibroblasts and biochemical correction compared to enzyme preparations from urine, liver or placenta. β-Glucuronidase deficient human fibroblasts provide a useful model for defining the requirements for in vitro biochemical correction.

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REFERENCES

1. De Duve, C., Pressman, B. C., Gianetto, R., Wattiaux, R., and Appelmans, F. (1955) Biochem. J. 60, 604-617.
2. Paigen, K. (1961) Expt. Cell Res. 25, 286-301.
3. Fishman, W. H., Ide, H., and Rufo, R. (1969) Histochemie 20, 287-299.
4. Swank, R. T. and Paigen, K. (1973) J. Mol. Biol. 77, 371-389.
5. Plapp, B. V. and Cole, R. D. (1967) Biochemistry 6, 3676-3681.
6. Sly, W. S., Quinton, B. A., McAlister, W. H., and Rimoin, D. L. (1973) J. Pediatrics 82, 249-257.
7. McKusick, V. A. (1972) Heritable Orders of Connective Tissue, pp. 521-686, 4th Ed. C. V. Mosby Co., St. Louis.
8. Hall, C. W., Cantz, M., and Neufeld, E. F. (1973) Arch. Biochem. Biophys. 155, 32-38.
9. Hickman, S. and Neufeld, E. F. (1972) Biochem. and Biophys. Res. Comm. 49, 992-999.
10. Ohtsuka, K. and Wakabayashi, M. (1970) Enzymologia 39, 109-124.
11. Weir, D. M. (1973) Handbook of Experimental immunology, pp. 5-35, F. A. Davis, Philadelphia.
12. Cuatrecasas, P. (1971) Methods in Enzymology, Vol. 22 pp. 345-378, Academic Press, New York.
13. Glaser, J. H. and Sly, W. S. (1973) J. Lab. Clin. Invest. 82, 969-977.
14. Baenziger, N. L. and Majerus, P. W. Methods in Enzymology "Biomembranes", Academic Press, New York. (in press)

15. Fratatoni, J. C., Hall, C. W., and Neufeld, E. F. (1969) Proc. Nat. Acad. Sci., U.S. 64, 360-366.
16. Barton, R. W. and Neufeld, E. F. (1971) J. Biol. Chem. 246, 7773-7779.
17. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265-275.
18. Bach, G., Friedman, R., Weissman, B., and Neufeld, E. F. (1972) Proc. Nat. Acad. Sci., U.S. 69, 2048-2051.
19. O'Brien, J. S., Miller, A. L., Loverde, A. W., and Veath, M. L. (1973) Science 181, 753-755.
20. Hers, H. G. (1965) Gastroenterology 48, 625-633.
21. Di Ferrante, N., Nichols, B. L., Donelly, P. V., Neri, B., Hrgovcic, R., and Berglund, R. K. (1971) Proc. Nat. Acad. Sci., U.S. 68, 303-307.
22. Dekaban, A. S., Holden, K. R., and Constantinopoulos, G. (1972) Pediatrics 50, 688-692.
23. Morell, A. G., Gregoriadis, G., Scheinberg, I. H., Hickman, J., and Ashwell, G. (1971) J. Biol. Chem. 246, 1461-1467.
24. Gregoriadis, G., Morell, A. G., Sternlieb, I., and Scheinberg, I. H. (1970) J. Biol. Chem. 245, 5833-5837.