J. Biosci., Vol. 4, Number 2, June 1982, pp. 133–138. © Printed in India.

Isolation and characterization of lysosomal alpha-mannosidase of placental tissue

FARHAT A. KHAN and DEBKUMAR BASU

Neurochemistry Division, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Trivandrum 695 011

MS received 17 December 1981; revised 2 March 1982

Abstract. The acidic α-mannosidase was purified 4400-fold by affinity chromatography on Concavalin A-Sepharose and heat treatment at 65°C in the presence of 1 mM zinc ion. The enzyme did not resolve into multiple forms as in the case of enzymes from human liver and human kidney. The pH optimum of the enzyme was 4.2 in citrate-phosphate buffer. The K_m value for p-nitrophenyl-α-D-mannose was 1.9 mM. The molecular weight of the enzyme determined by gel filtration was 300,000. The enzyme contained 10.6% neutral sugars.

Keywords. Placental tissue acid; α-mannosidase; Concanavalin A-Sepharose chromatography.

Introduction

Three types of α-D-mannosidase (EC 3.2.1.24) with different localizations were described in mammalian tissues. Acid α-mannosidase with pH optimum of 4.0-4.5, a typical lysosomal acid hydrolase, was widely distributed in mammalian tissues and plant seeds (Snaith and Levvy, 1973). The enzyme, with a pH optimum of 6.0-6.5 was first reported by Suzuki *et al.* (1969) and later purified to homogeneity by Shoup and Touster (1976) from the cytosol of rat liver. A third form of the enzyme with a pH optimum of 5.5,a component of Golgi-membrane of rat liver (Dewald and Touster, 1973), was solubilized by treatment with 0.5% deoxycholate. The enzyme was different from acid and neutral α-mannosidase not only on the basis of pH optimum but also on the basis of its kinetic properties as well as on its electrophoretic mobility. These enzymes did not require any lipid co-factor except for Zn^{2+} for the rat epididymis enzyme(Snaith and Levvy,1968).Forsee and Schutzbach (1981) partially purified after solubilization from rat liver microsomes, an α-mannosidase which required calcium ion as well as phosphatidyl choline or phosphatidyl ethanolamine for its activity. The enzyme was specific for α-l,2-mannosyl-mannose linkage and had a pH optimum between 5.0-5.5.

In the disease, mannosidosis, which occurs in both man (Ockermann, 1967; Kjellman *et al*., 1969) and cattle (Hocking *et al*., 1972), lysosomal acid α-D-mannosidase is absent but the neutral form is present in the normal amount (Carroll *et al*., 1972). In this paper we report the purification and properties of lysosomal acid α-D-mannosidase from human placental tissue an easily available human tissue.

Materials and methods

p-Nitrophenol, *p*-nitrophenyl-α-D-mannose, *p*-nitrophenyl-α-L-fucose, *p*-nitrophenyl-α-D-galactose, *p*-nitrophenyl-β-D-galactose, *p*-nitrophenyl-α-D-glucose, *p*-nitrophenyl-β-2-acetamido-2-deoxyglucose, crystalline bovine serum albumin, bovine serum albumin, acrylamide, N,N'-methylene-*bis*-acrylamide, N,N,N',N'-tetramethylene diamine, Coomassie Brilliant Blue R, α-methyl-D-glucose and cyanogen bromide were purchased from Sigma Chemical Co., St. Louis, Missouri, USA. Sephadex G-200, Sephadex G-50, blue-dextran 2000, DEAE-Sephadex A-50 and Sepharose 4B were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. The molecular weight marker proteins were purchased from Pierce Chemical Co., Rockford, Illinois, USA. All other chemicals used were of Analytical Reagent grade. *Canavalia gladiata* and *Canavalia ensiformis* were purchased locally. Concanavalin A-Sepharose 4B affinity column: Concanavalin A was isolated from *C. gladiata* or *C. ensiformis* and purified according to the method of Surolia *et al.* (1973). Concanavalin A-sepharose 4B column was prepared by cyanogen bromide activation of Sepharose 4B by the procedure of Bishayee and Bachhawat (1974). The concanavalin A content of the column was 6-8 mg/ml of Sepharose 4B.

Polyacrylamide gel electrophoresis

The purity of the enzyme protein was evaluated by electrophoresis on 7.5% (w/v) polyacrylamide gels as described by Davis (1964) at pH 4.3 in β-alanine-acetic acid buffer and at pH 8.3 in Tris-HCl buffer. Each tube contained 25-50 µg of protein. The protein was stained with Coomassie brilliant blue R and destained with methanol: acetic acid: distilled water (1:1.5:17.5, v/v) (Weber and Osborn, 1969). Protein was estimated with crystalline serum albumin as standard by the method of Lowry *et al.* (1951). The molecular weight of the enzyme was estimated by gel filtration on Sephadex G-200 column according to the procedure of Andrews (1964). The column was calibrated with the following standard proteins, cytochrome C (12,500), soybean trypsin inhibitor (21,500), ovalbumin (45,000), bovine serum albumin (67,000), aldolase (158,000) and catalase (240,000). The neutral sugar was estimated with galactose as standard by the phenolsulphuric acid method of Dubois *et al.* (1956).

Enzyme assay

The standard assay system for the acid α-D-mannosidase contained 200 µmol of citrate-phosphate buffer (pH 4.2), 0.5 µmol of *p* -nitrophenyl-α-D-mannose, 100 µg of bovine serum albumin (containing no enzyme activity), 1 µmol zinc sulphate and the enzyme in a final volume of 0.5 ml. After incubation at 37°C for 30 min, the reaction was stopped by heating the tubes at 100°C for 30 sec. The tubes after cooling were mixed with 2.5 ml of 0.4 M glycine/NaOH buffer (pH 10.5) and centrifuged for 5 min at 2000 *g*. The yellow colour was measured at 405 nm in Spectronic 20. One unit of enzyme was defined as the amount required to liberate 1 nmol of *p*-nitrophenol per min. Other glycosidases namely, α-L-fucosidase, β-D-galactosidase, α-D-galactosidase, α-D-glucosidase and β-D-hexosaminidase were assayed according to the method of Bossmann (1972).

Purification of the enzyme

The human placental tissues were collected in ice and freed from chord tissue and membrane. The tissue was thoroughly washed in cold distilled water and cut into small pieces and kept frozen at — 20°C until use. All operations were carried out at 0-4°C unless otherwise mentioned. The pH of the solution was always maintained at 7.0 with dilute ammonia solution during ammonium sulphate precipitation. Frozen tissue (500 *g*) were homogenized with 2500 ml of 0.02 M phosphate buffer, pH 7.0 containing 0.1 M NaCl in SORVALL Omnimixture for 3 min at full speed. The homogenate was stirred for 30 min and centrifuged at 20,000 *g* for 20 min in SORVALL RC-5B. The precipitate was discarded. Solid ammonium sulphate (490 g/litre) was added to the supernatant with constant stirring. After 30 min of stirring, the suspension was centrifuged as before and the precipitate was dissolved in 235 ml of 0.02 M phosphate buffer, pH 6.0 containing 0.1 M NaCl. The enzyme solution was then dialyzed against 3000 ml of 0.05 M phosphate buffer, pH 7.0 containing 0.1 M NaCl with 2 changes for 16 h. The precipitate formed during dialysis was discarded by centrifugation as before. The NaCl concentration of the enzyme solution was increased to 0.5 M by the addition of solid NaCl.

Concanavalin A-sepharose 4B column (1.4× 14 cm) was equilibrated with 0.05 M phosphate buffer, pH 7.0 containing 0.5 M NaCl and the enzyme solution was adsorbed on the column at a flow rate of 20 ml/h. The column was washed with 0.05 M phosphate buffer, pH 7.0 containing 1M NaCl at 25°C until the effluent had an absorbance of less than 0.05 at 280 nm. The enzyme was eluted at 25°C with 0.05 M phosphate buffer, pH 7.0 containing 1 M NaCl and 0.5 M α-methyl-D-glucoside and 10 ml fractions were collected. Active fractions (3 to 11) were pooled and dialyzed for 16 h against 100 vol of 0.02 M phosphate buffer pH 7.0 with 3 changes. The enzyme was precipitated from the dialyzed solution by the addition of solid ammonium sulphate (490 g/litre) with constant stirring. The enzyme was collected after 30 min stirring by centrifugation as before. The precipitate was dissolved in a minimum volume of 0.02 M phosphate buffer, pH 7.0. It was dialysed against 50 vol of the same buffer with 4 changes for 24 h. A DEAE-Sephadex A-50 column (1.6× 12 cm) was equilibrated with 0.02 M phosphate buffer pH 7.0 and 10 ml of the dialysed enzyme solution containing 100-110 mg of protein was subjected to ion-exchange chromatography on this column.The column, after the adsorption of the enzyme was washed with 50 ml of equilibrating buffer at a flow rate of 15 ml/h. The enzyme was eluted with the same buffer containing 0.05 M NaCl and 3 ml fractions were collected. Active fractions (4 to 16) were pooled and the protein was precipitated by the addition of solid ammonium sulphate (560 g/litre) with constant stirring. The enzyme was collected after 30 min stirring by centrifugation as before. The precipitate was dissolved in a minimum volume of 0.02 M phosphate buffer, pH 6.0 containing 0.1 M NaCl and was dialysed against 500 ml of 0.02 M phosphate buffer, pH 7.0 containing 0.1M NaCl with 4 changes for 10 h.

The enzyme solution containing 8-10 mg protein in 6-8 ml of total volume was passed through a Sephadex G-200 column (2×75 cm) which was equilibrated and

eluted with 0.02 M phosphate buffer, pH 7.0 containing 0.1 M NaCl at a flow rate of 10 ml/h and 3 ml fractions were collected. The active fractions (12-22) were pooled and concentrated by ultrafiltration through a PM-10 membrane. The enzyme solution was made 1 mM with respect to zinc ion by the addition of 0.01 M zinc sulphate solution and heated at 65°C for 1 h. The precipitated protein was discarded by centrifugation at 20,000 g for 30 min. The supernatant, containing the enzyme was concentrated by ultrafiltration through a PM-10 membrane. The enzyme when kept at —20°C was stable for at least 8 months.

Results and discussion

Purification of human placental tissue acidic α-mannosidase

The acidic α-mannosidase from placental tissue was purified 4400-fold (table 1).

Table 1. Purification of α-mannosidase.

Steps	Total units	Total protein (mg)	Sp. act.	% Recovery
Homogenate	27,520	33,560	0.8	100
Supernatant	24,348	23,411	1.5	89
Ammonium sulphate	21,320	14.026	1.5	78
ConA-Sepharose eluate	18,667	438	42.5	68
DEAE Sephadex	12,806	124	103.3	47
Sephadex G-200	9,540	8.9	1077.0	35
Heat treatment at 65°C	7,747	2.2	3603.0	28

Since the chromatography on concanavalin A-Sepharose was utilised at an early stage there was no neutral α-mannosidase activity. The ion-exchange chromatography was done at pH 7.0 on DEAE-Sephadex A-50. The enzyme did not resolve into multiple forms as was the case with human liver (Phillips *et al.*, 1975) and human kidney (Marinkovic and Marinkovic, 1976) α-mannosidases. The only difference was that in earlier cases the ion-exchange chromatography was done at pH 6.0. In the present case, we found that the enzyme was not retained at pH 6.0, but that it was retained at pH 7.0. The enzyme was free from other acidic glycosidase activity after Sephadex G-200 gel filtration except that for β-hexosaminidase activity. Hexosaminidase activity was completely removed by heat treatment at 65°C for 1 h at pH 6.0 in the presence of zinc ions.

pH optimum and kinetic constants

The enzyme from the human placental tissue had optimum activity at pH 4.2 under standard assay conditions.

The K_m value obtained with *p*-nitrophenyl α-D-mannoside as substrate by the plot S/v·against S was 1.9 mM and V_{max} was 105.5 nmol/min/mg protein. This K_m value was of the same order as obtained with human kidney and human liver enzyme (Phillip *et al.,* 1976; Marinkovic and Marinkovic, 1976).

Molecular weight

The molecular weight of placental α-mannosidase determined by Sephadex G-200 gel filtration was found to be 300,000 while that of the kidney enzyme was 180,000; that of the liver A form was 220,000 and of the B-form was 300,000 (figure 1).

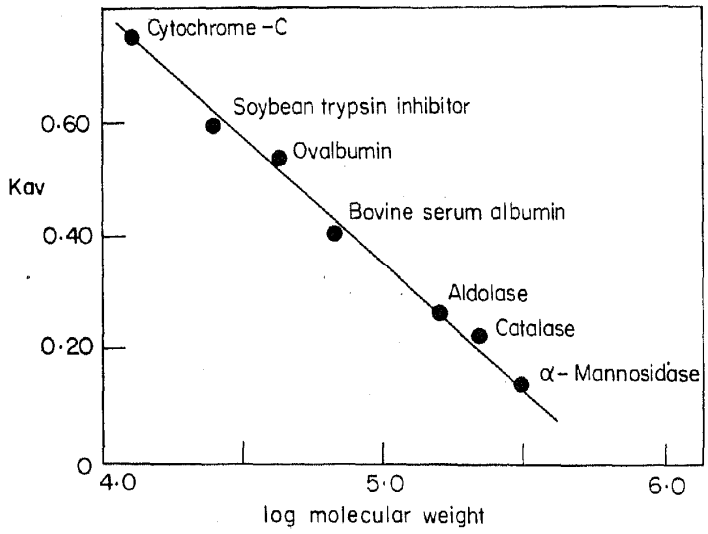

Figure 1. Determination of molecular weight of α-mannosidase by gel filtration on Sephadex G-200.

Effect of zinc and other metal ions

Snaith and Levvy (1968) showed that zinc ions were required for the maximal activity of α-mannosidase from rat epididymis. Snaith (1975) characterised the same enzyme from Jack bean meal as a metalloprotein containing 2 atoms of zinc/ mol of enzyme of molecular weight 230,000. In the present case the enzymic activity of the placental homogenate was completely dependent on zinc ion as was shown by Phillips *et al.* (1976) with human liver enzyme. However, the purified enzyme was only marginally activated (20 to 30%) by zinc ions in the concentration range 20 to 600 mM. At present we are unable to offer any explanation for this behaviour of the enzyme.

In a separate set of experiments the purified enzyme was preincubated for 30 min at 25°C in pH 4.0 buffer in the presence of EDTA (4-250 mM), the enzymic activity was found to be 72 to 85% of the original. Several other metals like Hg^{2+}, Mn2+, Cu $^{2+}$, Co^{2+} had no effect at 1 mM concentration, but Ag^{1+} ion at a similar concentration inhibited upto 60% of the activity in the absence of EDTA. The enzyme was protected by Zn^{2+} against inactivation on heating at 65°G for 1 h. Sodium chloride and sodium acetate (1 to 5 mM) had no effect on the enzyme activity.

Electrophoresis

The enzyme on electrophoresis at pH 4.3 on 7.5% and 5% acrylamide gel showed a single band when stained for protein. But the same protein on electrophoresis between pH 7.00 and 8.3 on 5%, 7.5% and 10% acrylamide did not move from the top of the gel, except for a slight diffusion.

Carbohydrate content

It is well established that lysosomal acid glycohydrolases are glycoprotein. The glycoprotein nature of α-mannosidase of placental tissue was indicated by its binding to concanavalin A-Sepharose column and subsequent elution with α-methylglucoside. The neutral sugar content of the purified protein was 10.6%.

Acknowledgements

This work is financed by a grant from the Council of Scientific and Industrial Research, New Delhi. We thank Ms. Annama for excellent technical assistance.

References

Andrews, P. (1964) *Biochem. J.,* **91,** 222.
Bishayee, S. and Bachhawat, B. K. (1974) *Neurobiology,* **4,** 48.
Bossmann, H. B. (1972) *Biochim, Biophys. Acta,* **258,** 265.
Carroll, M., Dance, N., Masson, P. K., Robinson, D., and Winchester, B. G. (1972) *Biochem. Biophys, Res. Commun.,* **49,** 579.
Davis, B. J. (1964) *Ann. N. Y. Acad. Sci.,* **121,**404.
Dewald, B. and Touster, O. (1973) *J. Biol. Chem.,* **248,**7223.
Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. and Smith, F. (1956) *Anal. Chem.,* **28,** 350.
Forsee, W. T. and Schutzbach, J. S. (1981) *J. Biol. Chem.,* **256,** 6577.
Hocking, J. D., Jolly, R. D. and Batt, R. D. (1972) *Biochem., J.,* **128,** 69.
Kjellman, B., Gamstrop, I., Brun, A., Ockermann, P. A. and Palmgren, B. (1969) *J. Pediat.,* **76,** 366.
Lowry, O. H., Rosenbrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.,* **193,** 265.
Marinkovic, D. V. and Marinkovic, J. N. (1976) *Biochem. J.,* **155,**217.
Ockermann, P. A. (1967) *Lancet,* **2,** 239.
Phillips, N. C, Robinson, D. and Winchester, B. G. (1976) *Biochem. J.,* **153,** 579.
Shoup, V. A. and Touster, O. (1976) *J. Biol. Chem.,* **251,** 3845.
Snaith, S. M. and Levvy, G. A. (1968) *Nature,* **218,** 91.
Snaith, S. M. and Levvy, G. A. (1973) *Adv. Carbhyd. Chem. Biochem.,* **28,** 401.
Snaith, S. M. (1975) *Biochem. J.,* **147,** 83.
Surolia, A., Prakash, N., Bishyaee,S. and Bachhawat, B.K.(1973*)Indian J. Biochem. Biophys.,* **10,**145.
Suzuki, I.,Kushida, H. and Shida, H. (1969) *J. Biochem. (Tokyo),* **41,**334.
Tulsiani, D. R. P., Opheim, D. J. and Touster, O. (1977) *J. Biol. Chem.,* **252,** 3227.
Weber, K. and Osborn, M. (1969) *J. Biol. Chem.,* **244,** 4406.