Cobalt-ion chelate affinity chromatography for the purification of brain neutral α -D-mannosidase and its separation from acid α -D-mannosidase

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A method is described for the purification of neutral α -D-mannosidase and its separation from acid α -D-mannosidase from monkey brain by utilizing Co^{2+} chelate affinity chromatography. The neutral enzyme, which selectively bound to the metal-ion chelate column, was elutable by Tris at pH 7.5 and gave over 80-fold purification in a single step with 100% recovery.

The lysosomal acid α - and β -mannosidases are enzymes involved in the degradation of glycopolymers, and they have been implicated in the genetic disorders of α - and β -mannosidosis (Autio *et al.*, 1973; Jones & Dawson, 1981). The cytosolic neutral α - and β -D-mannosidases, which exhibit a pH optimum around neutrality, are suggested to be genetically, structurally and functionally distinct from the lysosomal acid mannosidases (Shoup & Touster, 1976; Dawson, 1982). Neutral α -D-mannosidases of liver, brain and other tissues, unlike the acid mannosidases, do not bind to concanavalin A-Sepharose and they are activated/stabilized by Co^{2+} ions (Phillips *et al.*, 1974, 1976; Shoup & Touster, 1976; Snaith, 1977; Hirani & Winchester, 1979; Mathur & Balasubramanian, 1981). Snaith (1977), who studied metal-ion activation of neutral α -D-mannosidase as a function of pH in tissue preparations, suggested the existence of multiple forms of this enzyme, although attempted separation of such forms proved unsuccessful. The neutral α -D-mannosidase, obtained in a 90%-pure form from rat liver (Shoup & Touster, 1976), has been shown to be a difficult enzyme to purify, with low yields, due mostly to its instability during several steps of purification. As part of a study on brain neutral α -D-mannosidase, we have attempted to purify it by metal-ion chelate chromatography. We now report here a substantial purification with 100% yield of monkey brain neutral mannosidase obtained in a single step of Co^{2+} chelate affinity chromatography.

Experimental procedures

Materials

p-Nitrophenyl α -D-mannopyranoside, butane-1,4-diol diglycidyl ether, iminodiacetic acid, Tris

and Sepharose 4B were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. All the metal salts and disodium EDTA were analytical-grade reagents. Concanavalin A-Sepharose 4B was prepared as described previously (Alam & Balasubramanian, 1978). Adult monkey (*Macaca radiata*) brain, removed under Nembutal anaesthesia, was stored frozen at -20°C until use.

Enzyme assays

Acid and neutral mannosidase activities were assayed at pH 4.8 and 6.3 respectively as described previously (Mathur & Balasubramanian, 1981). The assay mixture for neutral mannosidase consisted of 20 μmol of sodium citrate/phosphate buffer, pH 6.3, 0.4 μmol of *p*-nitrophenyl α -D-mannopyranoside and an appropriate amount of enzyme in a final volume of 0.2 ml. After an incubation for 30 min at 37°C , the reaction was stopped by the addition of 0.8 ml of 0.4 M-glycine/NaOH buffer, pH 10.5, and the liberated *p*-nitrophenol was read at 400 nm in a Carl Zeiss PMQ II spectrophotometer. One unit of the enzyme activity is defined as the amount of enzyme required to liberate 1 μmol of *p*-nitrophenol/h at 37°C under the standard conditions. Protein was determined by the method of Lowry *et al.* (1951), with crystalline bovine serum albumin as the standard. Polyacrylamide-gel electrophoresis was done on 7% gels by the procedure of Davis (1964) in glycine/Tris buffer, pH 8.3. Enzyme activity in the gel was assayed after slicing the gel.

Preparation of metal-ion chelate gels

Epoxy-activated Sepharose 4B was prepared by using butane-1,4-diol diglycidyl ether by the method of Porath (1974), and iminodiacetic acid was coupled to it as described by Porath *et al.* (1975).

The resulting biscarboxymethylamino-Sepharose 4B was packed into a column (1.5 cm $\times$ 5.7 cm) and saturated with Co^{2+} ions by passing 100 ml of CoCl_2 solution in water (3 mg/ml). Unbound Co^{2+} was washed off with 20 mM-potassium phosphate buffer, pH 7.5. Other metal-ion chelate gels of Ni^{2+} , Mn^{2+} , Cd^{2+} , Fe^{2+} , Cu^{2+} and Zn^{2+} were prepared in the same way by passing aqueous solutions of NiCl_2 , MnCl_2 , CdSO_4 , FeSO_4 , CuSO_4 and ZnSO_4 .

Purification of neutral α -D-mannosidase

All purification procedures were carried out at 4°C.

Preparation of post-concanavalin A-Sepharose fraction. Frozen monkey brain (100 g) was homogenized in 500 ml of 20 mM-potassium phosphate buffer, pH 7.0, in a Waring Blendor. The homogenate was centrifuged at 67 000 *g* for 30 min. The supernatant was adjusted to 60% saturation with $(\text{NH}_4)_2\text{SO}_4$, and the precipitate, recovered by centrifugation at 12 000 *g* for 30 min, was suspended and dialysed against 20 mM-potassium phosphate buffer, pH 6.0, containing 0.5 M-NaCl for 12 h. The contents of the dialysis bag were re-centrifuged to remove any sediment. The dialysed enzyme preparation, containing both acid and neutral α -D-mannosidases, was passed through a concanavalin A-Sepharose column (1.6 cm $\times$ 11 cm) pre-equilibrated with 20 mM-potassium phosphate buffer, pH 6.0, containing 0.5 M-NaCl. Acid mannosidase was selectively bound to the column (Mathur & Balasubramanian, 1981). The fractions containing neutral mannosidase, which did not bind to the column, were pooled.

Ammonium sulphate fractionation. The post-concanavalin A-Sepharose fraction was subjected to 30% saturation with $(\text{NH}_4)_2\text{SO}_4$. After being stirred for 3 h, the preparation was centrifuged at 12 000 *g* for 30 min. The precipitate was dissolved in 20 mM-potassium phosphate buffer, pH 7.5, and dialysed against 200 vol. of the same buffer for 12 h, with two changes.

Co^{2+} chelate chromatography. The enzyme prepared as above was applied to the Co^{2+} chelate column (1.5 cm $\times$ 5.7 cm) pre-equilibrated with 20 mM-potassium phosphate buffer, pH 7.5, at a flow rate of 5 ml/h, and 10 ml fractions were collected. The column was washed sequentially with 900 ml of 20 mM-potassium phosphate buffer, pH 7.5, and 150 ml of 1 M-NaCl in the same buffer. At the end of each washing it was made sure that the absorbance of the eluate at 280 nm and 218 nm fell almost to zero. After a final wash with 50 ml of equilibration buffer to remove the NaCl, the enzyme was eluted with 20 mM-Tris/acetate buffer, pH 7.5, at a flow rate of 2–3 ml/h, and 3 ml fractions

were collected. The active fractions were pooled and dialysed against 20 mM-potassium phosphate buffer, pH 7.5.

Results and discussion

In initial experiments we found that the Co^{2+} chelate gel selectively bound neutral mannosidase and separated it from acid mannosidase. When dialysed $(\text{NH}_4)_2\text{SO}_4$ fraction prepared from the 67 000 *g* supernatant of monkey brain homogenate which contained both acid and neutral mannosidases, was passed through the Co^{2+} chelate gel, all the acid mannosidase (assayed at pH 4.8) emerged unbound to the gel, while the neutral mannosidase (assayed at pH 6.3) bound to the column and was eluted by 20 mM-Tris/acetate buffer, pH 7.5 (Fig. 1). A small percentage of activity detectable when assayed at pH 6.3 in the unbound fractions is considered to be due to the acid mannosidase, which at pH 6.3 exhibits about 30% of its activity at pH 4.8 (Mathur & Balasubramanian, 1981). For routine purposes of the enzyme purification, a post-concanavalin A-Sepharose fraction as described in the Experimental procedures section was prepared and passed through the Co^{2+} chelate column to be doubly sure of the complete removal of the acid mannosidases. Table 1 gives the results of a typical purification procedure. An overall purification of 275-fold with 100% recovery over the post-concanavalin A-Sepharose fraction was achieved. The Co^{2+} chelate chromatography step alone gave an 80-fold purification over the previous step. These results were reproducible in several batches of purification, with an 80–85-fold purification and 90–105% recovery in the single step of Co^{2+} chelate chromatography. Gel electrophoresis of the purified enzyme followed by Coomassie Brilliant Blue staining showed three minor contaminating protein bands in addition to the major band corresponding to the neutral mannosidase activity. The enzyme was stable, with practically no loss in activity when kept at 0–4°C for 4 weeks in Tris/acetate buffer, pH 7.5.

It is believed that the selective pH-dependent affinity of transition-metal ions for histidine and possibly cysteine and the surface density of these groups are largely responsible for the chromatographic behaviour of a protein on metal-ion chelate gel (Porath *et al.*, 1975). The ability of Tris/acetate buffer, pH 7.5, to elute the enzyme is attributable to the chelating properties of Tris (Mahler, 1961), which could compete with the enzyme to complex with the Co^{2+} ion on the gel. Cysteine or histidine (5 mM concentration in 20 mM-phosphate buffer, pH 7.5), which are capable of binding to Co^{2+} , and a low-pH buffer, such as 20 mM-phosphate buffer,

Fig. 1. Co²⁺ chelate affinity chromatography showing the separation of acid and neutral α -D-mannosidases of monkey brain. A 67000g supernatant of monkey brain homogenate was prepared as described in the Experimental procedures section and subjected to 40% saturation with (NH₄)₂SO₄. The precipitate recovered by centrifugation at 12000g for 30 min was dissolved and dialysed against 20 mM-potassium phosphate buffer, pH 7.5. This preparation, which contained both acid and neutral mannosidases, was applied on the Co²⁺ chelate column and chromatographed as described in the Experimental procedures section. ○, A₂₈₀; ×, acid mannosidase activity (assayed at pH 4.8); ●, neutral mannosidase activity (assayed at pH 6.3).

Table 1. Purification of neutral α -D-mannosidase from monkey brain

These results were obtained by starting with 250g of brain and using a 2.3 cm × 6.2 cm Co²⁺ chelate column. Details of purification procedure, assay and unit of enzyme activity are given in the text.

Step	Protein (mg)	Activity (units)	Specific activity (units/mg of protein)	Purification (fold)	Recovery (%)
Post-concanavalin A-Sepharose 4B fraction	4400	44	0.01	—	100
0–30%-satn.-(NH ₄) ₂ SO ₄ fraction	1270	44	0.034	3.4	100
Co ²⁺ chelate affinity chromatography	16	44	2.75	275	100

pH 6.0, were also capable of eluting the enzyme from the gel. However, the purification achieved by using these eluting agents was much less when compared with Tris/acetate buffer, pH 7.5.

Ni²⁺, Cu²⁺, Zn²⁺, Mn²⁺, Cd²⁺ and Fe²⁺ chelate gels were tried for further purification of the enzyme. Ni²⁺, Cu²⁺ and Zn²⁺ chelate gels could bind the enzyme, but elution was achieved only by using 10 mM-EDTA. Although the recovery was 100% in these procedures, the purification was very low.

The Co²⁺ chelate chromatography described here may serve as a useful procedure to obtain a substantially purified neutral α -D-mannosidase preparation, free of acid α -D-mannosidase, in 100% yield for further purification or for isolating the postulated multiple forms of the enzyme (Snaith, 1977) or for checking its possible identity with a similar enzyme reported to be present in the rat liver endoplasmic reticulum (Bischoff & Kornfeld, 1983).

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