Short Communications

Purification of α -Mannosidase from Jack-Bean Meal Suitable for use as Reagent

By CLEMENT Y. T. CHU and MICHAEL D. TURNER

*The Isaac Gordon Laboratory for Gastrointestinal Research, University of Rochester
School of Medicine and Dentistry, Rochester, N. Y. 14642, U.S.A.*

(Received 13 February 1974)

A simple method of purification of α -mannosidase from jack-bean meal is described which yields a product free of β -*N*-acetylglucosaminidase activity.

Exoglycosidases are useful tools for the structural analysis of glycoproteins. If an enzyme is to be used for this purpose, its purity is of critical importance.

α -Mannosidase (EC 3.2.1.24) has been used for analysis of glycoproteins and has been shown to liberate the terminal mannose from ovalbumin, ovalbumin-glycopeptide, ovomucoid or glycopeptide from rat liver microsomal fractions and from the O antigen of *Salmonella typhimurium* (Li, 1966, 1967). It is known to be present in considerable amounts in jack-bean meal (Li, 1966) and has been isolated and characterized by Li (1966, 1967).

The method of isolation of α -mannosidase originally proposed by Li (1967) and its more recent modification (Li, 1972) both involve many steps including water extraction, salt fractionation, alcohol fractionation, gel filtration on Sephadex G-200 and ion-exchange chromatography on DEAE-Sephadex. After these procedures, the mannosidase was often contaminated with β -*N*-acetylglucosaminidase. Li (1972) found that this contamination could be eliminated by treatment with pyridine. Preliminary results in this laboratory, however, indicated that even after pyridine treatment many preparations still showed β -*N*-acetylglucosaminidase activity. Experiments were therefore conducted to develop a more satisfactory procedure for producing α -mannosidase free of β -*N*-acetylglucosaminidase activity.

Materials and methods

Jack-bean meal and Sephadex G-200 (micro-granules) were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A. The following enzyme substrates were obtained from Pierce Co., Rockford, Ill., U.S.A.: *p*-nitrophenyl α -*N*-acetylglucosaminide, *p*-nitrophenyl α -D-mannopyranoside, *p*-nitrophenyl β -D-galactopyranoside, *p*-nitrophenyl α -D-glucopyranoside, *p*-nitrophenyl β -D-glucopyranoside. $(\text{NH}_4)_2\text{SO}_4$ (special enzyme grade) was purchased from Schwarz-Mann, Orangeburg, N.Y., U.S.A. DEAE-cellulose (Whatman DE 32) was purchased from W. and R. Balston, Maidstone, Kent, U.K.

A 5 μmol portion of each of the above substrates was added to 0.9 ml of 0.05 M-sodium citrate buffer at pH 3.5 for *p*-nitrophenyl α -D-galactopyranoside and *p*-nitrophenyl β -D-galactopyranoside, at pH 4.5 for *p*-nitrophenyl α -D-mannopyranoside and at pH 5.0 for *p*-nitrophenyl β -*N*-acetylglucosaminide, *p*-nitrophenyl α -D-glucopyranoside and *p*-nitrophenyl β -D-glucopyranoside. Then 0.1 ml of the appropriately diluted enzyme solution, equivalent to 1–100 μl of undiluted enzyme solution, was added to the substrate solution and the final volume made to 1 ml with the appropriate buffer. The mixture was incubated at 25°C for 5 min and the reaction was terminated by adding 3 ml of 0.2 M-sodium borate buffer, pH 9.8. The E_{430} was measured in a Hitachi-Perkin-Elmer model 139 spectrophotometer. Protein was measured by the method of Lowry *et al.* (1951).

One enzyme unit was defined as the amount of enzyme that hydrolysed 1 μmol of substrate/min at 25°C. The specific activity of the enzyme preparation was expressed as units/mg of protein.

Enzyme isolation procedure

Jack-bean meal (20 g) was suspended in 6 vol. of water with continuous stirring at 25°C for 1 h. The suspension was centrifuged at 3000g for 10 min at 4°C in an angle-head centrifuge and the slightly turbid supernatant collected.

Solid $(\text{NH}_4)_2\text{SO}_4$ was added to obtain 30% saturation according to Dixon's (1953) nomogram. The suspension was left at 4°C for 2 h and then centrifuged at 5000g for 15 min. The sediment was discarded and the supernatant was adjusted to 60% saturation with $(\text{NH}_4)_2\text{SO}_4$ and left at 4°C overnight. After centrifugation at 5000g for 15 min, the precipitate was dissolved in 15 ml of 0.1 M-Sørensen's phosphate buffer, pH 7.0 (prepared by mixing 100 ml of 0.1 M- KH_2PO_4 and appropriate amounts of 0.1 M- Na_2HPO_4 to give the desired pH). The solution was dialysed against the same buffer overnight at 4°C. The enzyme solution was then centrifuged at 3000g for 10 min to remove any insoluble material.

Then 16ml of the enzyme extract, containing 383mg of protein, was applied to a column (2.7cm×90cm) of Sephadex G-200 and eluted with 0.1M-Sörensen phosphate buffer, pH7.0, at a flow rate of 26ml/h. Fractions (5ml) were collected. α -Mannosidase was eluted first, followed successively by β -N-acetylglucosaminidase and β -galactosidase. The three glycosidases were not, however, completely separated by this step.

Whatman DEAE-cellulose (DE 32) was prepared according to the manufacturer's recommendation and thereafter equilibrated with 0.01M-Sörensen phosphate buffer, pH8.0. It was packed into a 2.5cm×30cm column. The crude α -mannosidase obtained from the previous gel filtration was dialysed against 0.01M-Sörensen phosphate buffer, pH8.0, and applied to the column. The column was eluted with

0.01M-Sörensen phosphate buffer, pH8.0, at a flow rate of 33ml/h. Fractions (3ml) were collected. β -Galactosidase was eluted from the column first and α -mannosidase appeared when the eluting buffer was changed to 0.01M-Sörensen buffer, pH8.0, containing 0.1M-NaCl. At this salt concentration, β -N-acetylglucosaminidase was still retained on the column.

The separation in the DEAE-cellulose column can also be achieved by applying a continuous NaCl gradient. By this method, a gradual NaCl gradient was generated from a six-chamber gradient generator, the chambers containing respectively 0.05M-, 0.10M-, 0.15M-, 0.20M-, 0.25M- and 0.30M-NaCl in 0.01M-Sörensen phosphate buffer, pH8.0. The column was eluted with the starting buffer for 60 fractions before the NaCl gradient was started. α -Mannosidase appeared at 0.012M-NaCl and β -N-acetylglucos-

Fig. 1. Elution profile of ion-exchange chromatography on Whatman DE32 DEAE-cellulose

Crude enzyme solution (20ml) from gel filtration containing 123 mg of protein was applied to a Whatman DE 32 DEAE-cellulose column (2.5cm×3.0cm) previously equilibrated with 0.01M-Sörensen phosphate buffer, pH8.0. The column was eluted with the same buffer for 60 fractions and then a gradual NaCl gradient (●) was started (shown by the arrow). The column was eluted at a flow rate of 33ml/h. Fractions (3ml) were collected. Fractions 90–130 were pooled. Δ , E_{280} ; $\circ$, α -mannosidase; $\square$, β -galactosidase; $\blacktriangle$, β -N-acetylglucosaminidase.

Table 1. Purification of α -mannosidase from jack-bean meal

For details see the text.

Step	Total activity (units)	Yield (%)	Specific activity (units/mg)		Purification factor
			α -Mannosidase	β -N-Acetylglucosaminidase	
(1) Water extraction	382	100	0.13	0.02	1
(2) $(\text{NH}_4)_2\text{SO}_4$ fractionation	360	94	0.94	0.15	7
(3) Gel filtration	216	56	1.7	0.3	13
(4) DEAE-cellulose (DE 32) ion-exchange chromatography	206	54	15.6	0	120

aminidase was eluted at 0.11M-NaCl and reached its peak at 0.125M-NaCl (Fig. 1).

Undiluted enzyme pooled from fractions 90–130 was assayed for β -N-acetylglucosaminidase activity by using the assay method described above. Even after an incubation time of 30min, no β -N-acetylglucosaminidase activity could be detected. The results of the purification procedure are summarized in Table 1.

Li (1967) stated that jack-bean meal α -mannosidase is very vulnerable to freeze-drying. We found that when the purified enzyme from the eluate was exhaustively dialysed against water and then freeze-dried, the enzyme activity was almost lost; however, when the enzyme was dialysed against 0.01M-Sørensen phosphate buffer, pH8.0, before freeze-drying, over 90% of the enzyme activity could be recovered. The stability of the enzyme in this condition may be explained on the basis of the stability of its enzyme-metal-ion complex formed with Zn^{2+} in this pH as observed by Snaith & Levvy (1973).

This procedure appears to be a simple reproducible method for the isolation and purification of α -mannosidase from jack-bean meal which yields a product free of contamination from the other glycosidases, i.e. β -N-acetylglucosaminidase, α -galactosidase, β -galactosidase, α -glucosidase and β -glucosidase.

This work was supported by Grants CA-13453 and Center Grant CA-11198 from the National Cancer Institute, and by Grant 5-TO-1-AM-05177 from the National Institute of Arthritis, Metabolic and Digestive Diseases, U.S. Public Health Service.

- Dixon, M. (1953) *Biochem. J.* **54**, 457–458
Li, Y. T. (1966) *J. Biol. Chem.* **241**, 1010–1012
Li, Y. T. (1967) *J. Biol. Chem.* **242**, 5474–5480
Li, Y. T. (1972) *Methods Enzymol.* **288**, 703–713
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
Snaith, S. M. & Levvy, G. A. (1973) *Advan. Carbohydr. Chem. Biochem.* **28**, 401–445