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PURIFICATION OF A β-MANNANASE ENZYME FROM LUCERNE SEED BY SUBSTRATE AFFINITY CHROMATOGRAPHY

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Abstract—A technique which employs substrate affinity chromatography on glucomannan- or mannan-AH-Sepharose, has been developed for the purification of legume seed β-mannanases. Using this technique, lucerne seed β-mannanase B has been purified to near homogeneity with a final specific activity of 1080 nkat per mg protein. The preparation was completely devoid of α-galactosidase and β-mannosidase activities. β-Mannanases of microbial origin can also be purified using these materials.

INTRODUCTION

The usefulness of β-mannanase enzymes (EC 3.2.1.78) [1] in the analysis of the fine structures of galactomannans has been discussed by numerous workers [1–5]. However, in general, considerable difficulties have been experienced in obtaining this enzyme devoid of the interfering activities, β-mannosidase and α-galactosidase. In the few cases where this has been achieved for legume seed β-mannanases, tedious purification sequences were involved, resulting in considerable loss of enzyme activity [6, 7].

The aim of the present communication is to demonstrate the usefulness of an alternative procedure employing substrate affinity chromatography on mannan- or glucomannan-AH-Sepharose.

RESULTS AND DISCUSSION

β-Mannanase B of lucerne [6] has been obtained in an almost homogenous state by two simple chromatographic steps. Purification on DEAE-cellulose removed a large proportion of the protein as well as β-mannanases A and β-mannosidases A and B [6]. Chromatography of the active fraction in this eluate on mannan-AH-Sepharose gave a 64 fold purification with a recovery rate of 95% (Table 1). The resulting enzyme had a sp. act. of 1080 nkat/mg and appeared as a single major band (pI = 4.5) together with a much fainter band (pI = 4.4) on thin layer, polyacrylamide gel isoelectric focusing (Fig. 1). The preparation was completely devoid of α-galactosidase and β-mannosidase activities. Neither of these enzymes bind to the affinity column.

Details of the affinity chromatography procedure are shown in Fig. 2. At high salt concentrations and in the presence of soluble mannan, β-mannanase does not bind to mannan-AH-Sepharose (Fig. 2a); however in the absence of soluble mannan, other conditions being the same, the enzyme binds and subsequently can be eluted with soluble mannan (Fig. 2b). As shown in Fig. 2c, β-mannanase B binds only weakly to the immobilised mannan and can also be eluted by continued washing of the column with solution B. This reflects the relatively low affinity of the enzyme for the substrate i.e. with soluble mannan as substrate, a K_m of 1.9 mM anhydromannose (0.03% carbohydrate) was obtained. This elution of enzyme in the absence of soluble substrate does not reflect a degradation of the column, as these columns can be reused at least 4 times with only a slight differences in chromatographic patterns.

Since the purification of mannan for use in the preparation of these specific adsorbents is quite tedious, use has been made of readily extractable low viscosity glucomannans. Glucomannan-AH-Sepharose columns do bind β-mannanase but generally not as firmly as mannan-AH-Sepharose columns. However, using these

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Table 1. Purification of β-mannanase B of lucerne

Stage of purification	Total protein (mg)	Total activity (nkat)	Recovery % per Step	Recovery % Overall	Sp. act. (nkat/mg)	Purification
Crude extract	16100	5300	—	—	0.33	1
20–70% $(NH_4)_2SO_4$	4020	5300	100	100	1.32	4
DEAE cellulose (pH 5.5)	254	4300	81	81	16.9	51
Mannan-AH-Sepharose	3.8	4100	95	77	1080	3270

Fig. 1. Thin layer isoelectric focusing of β-mannanase B of lucerne using LKB,PAG plates. (1) Enzyme applied to affinity column (40 μl, 2%). (2) Enzyme applied to affinity column (20 μl, 2%). (3) Enzyme recovered from affinity column (20 μl, 1%).

columns highly purified β-mannanase preparations have been made. Thus, lucerne β-mannanase B has been purified to the same degree as on mannan-AH-Sepharose and the β-mannanases present in commercial Driselase* preparation (pIs 5.0 and 5.5) to a final sp. act. of 500 nkat/mg. β-1, 4-Glucanase present in commercial Driselase preparation does not bind to glucomannan-AH-Sepharose and β-glucosidase is only slightly retarded.

* A commercial product prepared from culture solutions of *Basidomycetes* sp. Kyowa Hakko Kogyo Co., Ltd., Tokyo, Japan.

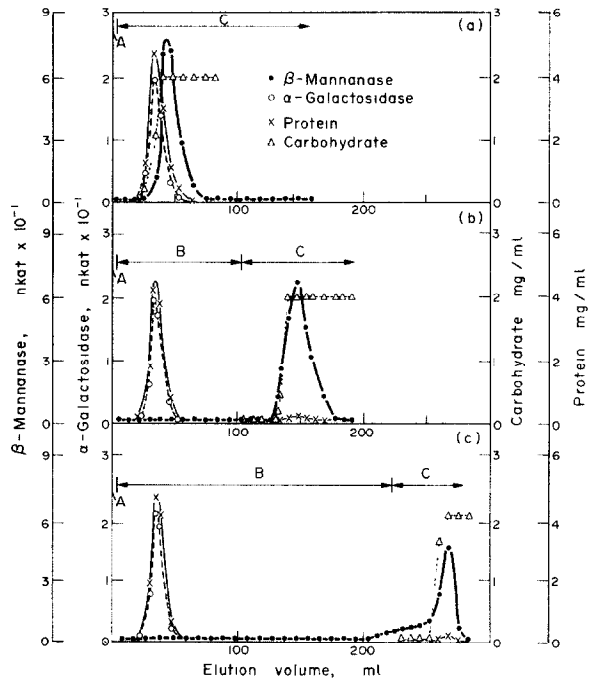

Fig. 2. Substrate affinity chromatography of β-mannanase B of lucerne on mannan-AH-Sepharose. Enzyme (4 ml, 1.7 μkat) was applied to a column (1.7 × 15 cm) pre-equilibrated with a soln of 0.5 M KCl plus 0.1 M NaOAc, pH 5. at 4°. Eluting solutions were (a) Enzyme preparation in 0.5 M KCl plus 0.1 M NaOAc, pH 5. (b) 0.5 M KCl plus 0 1 M NaOAc, pH 5 (c) 0.5 M KCl plus 0.1 M NaOAc plus 0.2% soluble mannan.

In summary, mannan-AH-Sepharose and glucomannan-AH-Sepharose columns are very useful in the preparation of β-mannanase completely devoid of other activities such as α-galactosidase, β-mannosidase, β-glucosidase and β-1,4-glucanase. This technique should be applicable to the purification of β-mannanase enzymes from most sources, allowing comparative studies of modes of action on galactomannans, glucomannans and galactoglucomannans. Enzymes purified by this technique are currently being used to study the fine structures of galactomannans.

EXPERIMENTAL

Mannans were extracted and purified from seeds of *Livistona australis* and *Archontophoenix cunninghamiana* (Bangalow palm) and low viscosity glucomannans from seeds of *Dracaena draco* and *Clivea miniata* by a procedure previously used to extract mannan from Bangalow palm seeds [6]. Seeds were dried at 50° before milling or extracting. Yields of mannan or glucomannan were: *L. australis*, 10%; Bangalow palm 5%; *D. draco*, 40%; and *C. miniata*, 14%. Glucose/mannose ratios determined by GLC of alditol acetates [4] were 49:51 for *D.* glucomannan and 43:57 for *C.* glucomannan. All products were white powders.

AH-Sepharose. This was prepared essentially according to the procedure of ref. [8].

Mannan-AH-Sepharose and Glucomman-AH-Sepharose Mannan or glucomannan (6 g) was dissolved in NaOH (10%, 60 ml). This was neutralised with HCl (M) and then $NaHCO_3$ (M, 200 ml) added immediately. CNBr (6 g), dissolved in MeCN (10 ml), was added with vigorous stirring and after 2 min at

room temp., AH-Sepharose (60 ml packed gel vol.) was added and stirring continued at 4° for 18 h. The gel was washed with $NaHCO_3$ (M, 500 ml), KCl (2 M, 500 ml) and NaOAc (2 M, 500 ml) and poured to give a column of dimensions of 1.7 × 15 cm which was washed with 10 bed vols of a soln of KCl (0.5 M) plus NaOAc (0.1 M, pH5) before use.

Soluble mannan. Reduced mannan (0.2 g) was dissolved in NaOH (10%, 2 ml), the soln diluted × 5, neutralised with HCl (1 M) and adjusted to 0.1% with NaOAc (0.1 M, pH5). At this concn the polymer remained in soln for at least a week at room temp. or 4°.

β-Mannanase assay Enzyme prepn (0.1 ml) was incubated with soluble mannan (1 ml, 0.1%) at 40° for 5 min. Reaction was stopped by adding *p*-hydroxybenzoic acid hydrazide reagent (5 ml) [9, 10] and colour developed by incubation at 100° for 6 min. β-Mannanase was also assayed using carob galactomannan dyed with Remazol-brilliant Blue as substrate, details of which will be presented separately. One unit (kat) is defined as the amount of enzyme that releases one mol of mannose reducing sugar equivalent/sec at 40° and pH 5 with soluble mannan as substrate. Sp. act. is expressed as nkat/mg of protein.

Assay of other glycosidase and glycanase activities. α-Galactosidase, β-mannosidase and β-glucosidase were assayed using the appropriate nitrophenyl sugar derivative as substrate [6]. β-Glucanase was assayed using a 1% suspension of cellulose powder as substrate and measuring reducing sugar increase by the PAHBAH method [9, 10].

Purification of β-mannanase B of lucerne. Germinated lucerne seeds (300 g) were extracted as previously described [6] and β-mannanase B purified and separated from β-mannanases A on DEAE-cellulose (pH 5.5). It was concentrated to 0.4 μkat/ml by dialysis against polyethylene glycol 4000 and then dialysed against soln B [KCl (0.5 M) + NaOAc (0.1 M, pH 5)]. A sample (4 ml, 1.7 μkat) was applied to an affinity column and eluted with soln B, followed by soln C [KCl (0.5 M) + NaOAc (0.1 M, pH 5) + soluble mannan (0.2%)].

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