CHROM. 6400

Note

Nature of the binding of a β-1,4-glucan hydrolase to ion exchangers

In an earlier paper[1] the abnormally strong binding of an endo-β-1,4-glucanase (β-1,4-glucan 4-glucanohydrolase, EC 3.2.1.4.) to microgranular DEAE-cellulose was reported. During chromatography on this medium at pH 8.0 the enzyme could only be recovered by prolonged elution with a high concentration (1.0 M) of sodium chloride. It was difficult to rationalize this behaviour, which suggested the enzyme was exceptionally acidic, with the fact that most enzymes of this type are, like lysozyme, rather basic in nature[2]. Examination of the behaviour of the same enzyme on other ion-exchange media has now confirmed that binding to anion-exchange cellulose is due mainly to the charge properties of the enzyme rather than being of a substrate affinity type as has been observed in the case of some related enzymes[3-5]. However, the possibility that the binding is enhanced by affinity interactions cannot be ruled out. The present work has also suggested an alternative, possibly superior, procedure for purification of the β-glucan hydrolase, namely by chromatography on microgranular CM-cellulose. Interaction with this medium is solely of an affinity type as is evident by comparison with the behaviour of the enzyme on CM-Sephadex where little or no binding takes place.

Experimental

The β-1,4-glucanase preparation had been partly purified from the extracellular culture filtrate of a species of Cytophaga (BDH Ltd., Poole, Great Britain, catalog No. 39072), and was free from the other glucan hydrolases[6], and most of the carbohydrate present in the crude starting material. Chromatography was performed at room temperature on columns (9 × 2.8 cm I.D.) of DEAE-Sephadex A-50, CM-Sephadex C-25 (products of Pharmacia Fine Chemicals) and microgranular CM-cellulose CM-52 (a product of H. Reeve Angel). Chromatography on the anion-exchanger was
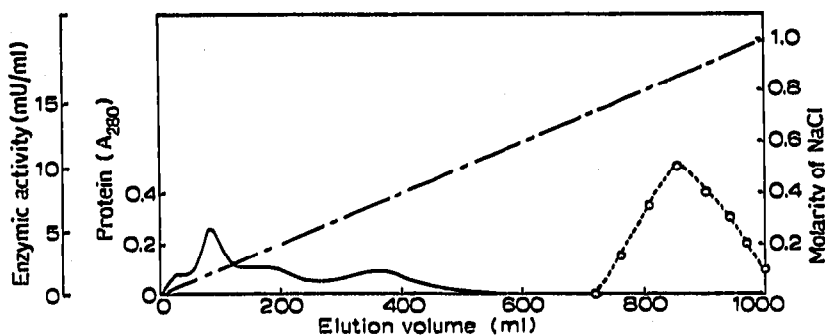

Fig. 1. Chromatography of Cytophaga endo-β-1,4-glucanase preparation on DEAE-Sephadex. Elution was performed with a gradient of sodium chloride (— – – —) 0–1.0 M over 1 l in 25 mM citrate–phosphate buffer, pH 8.0. Fractions of 9.0 ml were collected automatically. Protein in the fractions (—) was detected by measurement of ultraviolet absorption at 280 nm (A_{280}). β-1,4-glucanase activity (○ – – – ○) was measured using CM-cellulose as substrate.
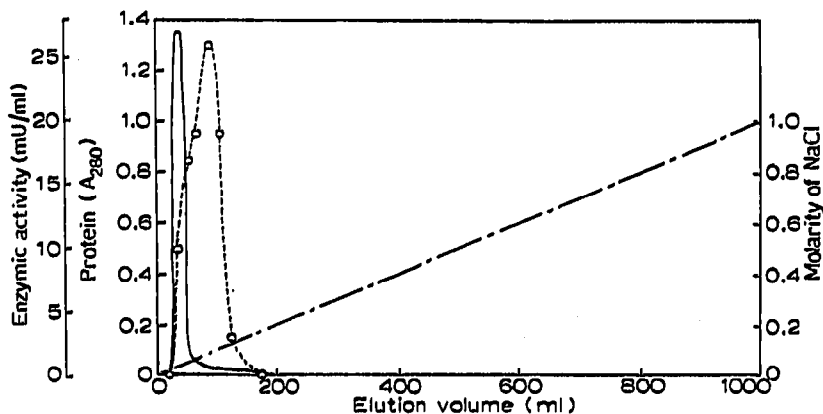
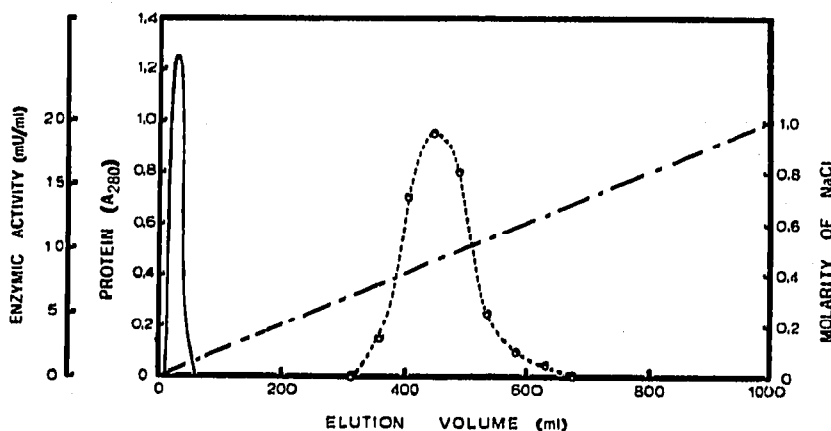Fig. 2. As for Fig. 1, but chromatography performed on CM-Sephadex in 10 mM acetate buffer pH 5.0.

Fig. 3. As for Fig. 1, but chromatography performed on CM-cellulose in 10 mM acetate buffer pH 5.0.

performed in 25 mM citrate–phosphate buffer, pH 8.0; in the case of the cation exchangers the column buffer was 10 mM acetate, pH 5.0. In all cases elution was carried out after application of the sample, using a salt gradient (0 → 1.0 M sodium chloride over 1 l) and fractions of 9 ml were collected automatically. Figs. 1–3 show the distribution of protein and activity towards soluble CM-cellulose (Cellofas B, medium viscosity, Imperial Chemical Industries Ltd.), measured by increase in reducing power[7,8], in the effluents from the three columns.

Chromatography on DEAE-Sephadex gave a peak of enzymic activity eluted as a fairly sharp peak (cf. elution from DEAE-cellulose[1]) at high salt concentration (ca. 0.8 M sodium chloride), and associated with only very small amounts of protein (Fig. 1). The enzyme showed no significant adsorption to CM-Sephadex and was recovered in the fractions containing unbound protein (Fig. 2.) When the enzyme preparation was chromatographed on CM-cellulose the protein elution profile was closely similar to that from CM-Sephadex but in this case the enzyme was only eluted after the concentration of sodium chloride in the gradient applied to the column reached about 0.5 M (Fig. 3). In all cases the fractions containing the enzymic activity

were combined, concentrated using an ultrafiltration cell fitted with a UM-10 Diaflo membrane (Amicon Corp., Lexington, Mass., U.S.A.), assayed for protein and β-1,4-glucanase activity, and the specific activities calculated. The results are shown in Table I.

TABLE I

PURIFICATION OF CYTOPHAGA β-1,4-GLUCANASE BY CHROMATOGRAPHY ON ION EXCHANGERS

Ion exchanger	Nature of binding	Specific activity
DEAE-Cellulose	ionic (+ affinity ?)	4.0[a]
DEAE-Sephadex	ionic	2.8
CM-Sephadex	no binding	0.37
CM-Cellulose	affinity	5.9

[a] From ref. 1.

Discussion

While ion-exchange chromatography is one of the most useful techniques for protein purification[9–11], it has the limitation that it results, more often than not, in only partial separation of the components of mixtures. There are, however, cases where specific single-step purifications to homogeneity may be effected by chromatography on cellulosic anion exchangers as the result of the matrix of the ion exchanger acting as an affinity adsorbent. Advantage has been taken of this phenomenon to purify endo-β-1,3- and endo-β-1,4-glucanases from several sources[3–5]. Generally the strength of the enzyme–matrix interaction in such cases is strong enough to resist displacement of the protein by a salt gradient with the result that specific elution can be achieved by an acid wash after removal of unwanted protein by elution with a high concentration of salt.

In the case of Cytophaga endo-β-1,4-glucanase, a rather abnormally strong binding to DEAE-cellulose has been reported previously[1]. Since the binding to DEAE-cellulose could be reversed by prolonged elution with a high salt concentration it was not, in this case, entirely clear what factors were involved in binding. These could be either (a) ionic interactions, (b) affinity interactions, or (c) a combination of both types. The behaviour of the enzyme on DEAE-Sephadex (Fig. 1), where the enzyme is again eluted at the end of the salt gradient suggests that the binding is due to ionic forces. The rather sharper nature of the peak from DEAE-Sephadex as compared with that from DEAE-cellulose might suggest the additional involvement of affinity interactions with the latter medium. However, this cannot, by itself, be taken as definitive evidence that the interaction is ionic since cases are known where anomalous binding, but by an interaction less specific than enzyme–substrate binding, to both modified cellulose and modified Sephadex ion exchangers (but not to the corresponding polyacrylamide ion exchanger) takes place[12]. For this reason the behaviour on the cation exchangers was investigated.

The inability of the enzyme to bind significantly to CM-Sephadex at pH 5.0 is indicative that the protein is still negatively charged at this pH, *i.e.* is an acidic protein. Chromatography on this medium does not lead to any significant purification and, as is apparent from Table I, the enzyme recovered is far from pure.

To test for the involvement of any affinity interactions with the modified

celluloses the behaviour on CM-cellulose was investigated. Since the negatively-charged carboxymethyl groups cannot bind the enzyme, ionic and affinity binding can be clearly differentiated by comparison of the behaviour on cation-exchange cellulose and Sephadex. The relatively strong binding to the cellulosic cation exchanger, in contrast to the Sephadex cation exchanger, only overcome by 0.5 M sodium chloride, is a clear indication of affinity binding. Thus it cannot be ruled out that affinity binding also plays a part during chromatography on DEAE-cellulose as mentioned above.

The conclusion is, therefore, that the Cytophaga endo-β-1,4-glucanase is unusual for this type of enzyme, in being acidic, this accounting for its behaviour on anion exchangers. The enzyme shows what appears to be affinity interaction with CM-cellulose and this may also be involved in the binding to DEAE-cellulose.

This enzyme may therefore be purified specifically in any of three ways, *viz.* by salt-gradient chromatography on DEAE-cellulose, DEAE-Sephadex or on CM-cellulose. The preparations obtained by each method are of comparable specific activity (Table I) and all are considered to be homogeneous. The highest specific activity is obtained by chromatography on CM-cellulose and this probably reflects a lower extent of enzyme inactivation on this cation exchanger than on the two anion exchangers. This is now the preferred procedure for purification of this enzyme.

With the elucidation of the nature of the interactions of this enzyme with ion-exchange media we are one step nearer our aim of establishing systematic methods for purification of the glycoside hydrolases. This will greatly aid the preparation of quantities of these enzymes for our work on their structures and mechanisms of action.

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1 J. J. MARSHALL, *J. Chromatogr.*, 71 (1972) 367.
2 J. J. MARSHALL, unpublished work.
3 J. J. MARSHALL, *Trans. Biochem. Soc.*, 1 (1973) 143.
4 J. J. MARSHALL, *Anal. Biochem.*, (1973) in press.
5 J. J. MARSHALL, *Comp. Biochem. Physiol.*, (1973) in press.
6 J. J. MARSHALL, *Carbohydrate Res.*, (1973) in press.
7 N. NELSON, *J. Biol. Chem.*, 153 (1944) 375.
8 M. SOMOGYI, *J. Biol. Chem.*, 195 (1952) 19.
9 E. A. PETERSON, *Cellulosic Ion Exchangers*, North-Holland, Amsterdam, 1970.
10 S. R. HIMMELHOCH, *Methods Enzymol.*, 22 (1971) 273.
11 J. J. MARSHALL, *An Introduction to Protein Purification*, Koch-Light Laboratories, Colnbrook 1972.
12 E. A. PETERSON, personal communication (1972).

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