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Note

Purification of glucan hydrolases from a commercial preparation of *Trichoderma viride* by chromatofocusing

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Estimation of polysaccharides in barley and in malted barley requires the use of specific enzymes. Analysis of the β-D-glucan content requires amylase-free β-D-glucanases, and the estimation of starch and starch-degradation products generally utilises amyloglucosidase preparations. Pure commercial enzymes are not available at present and previous attempts to purify crude enzyme preparations have involved either heat or acid treatment[1,3], or several stages of enzyme purification[4,6]. We now report that chromatofocusing[7] has provided a means of separating the α- and β-D-glucanase activities of a commercial preparation of *Trichoderma viride* by a single-stage procedure. Previous studies using CM- and DEAE-Sephadex chromatography[6] indicated that this fungus contains a number of enzymes capable of hydrolysing β-D-glucans.

Fig. 1 shows the fractionation of a crude preparation of *T. viride* by chromatography and confirms earlier results obtained in separations on one-tenth of this scale. Enzyme B1, consisting of eluate from fractions 49–59, and enzyme B2 (fractions 65–71) had isoelectric points of 5.25 and 4.95, respectively. Each of these enzymes cleaved barley β-D-glucan, as indicated by the positive response to the Nelson–Somogyi reagent. However, the D-glucose oxidase method showed that D-glucose was absent and it was therefore concluded that these enzymes were acting in an endo or random fashion. In contrast, enzyme B3 (fractions 97–110) hydrolysed barley β-D-glucan with the rapid production of D-glucose, indicating the presence of an exo-acting enzyme. Enzyme B3 had an isoelectric point of 4.20. In addition, fractions B1, B2, and B3 each released reducing sugars from CM-pachyman, but were completely devoid of any activity towards starch or pullulan.

Enzyme A (fractions 76–95) did not attack barley β-D-glucan or CM-pachyman, but it caused a rapid release of D-glucose from starch as indicated by both the

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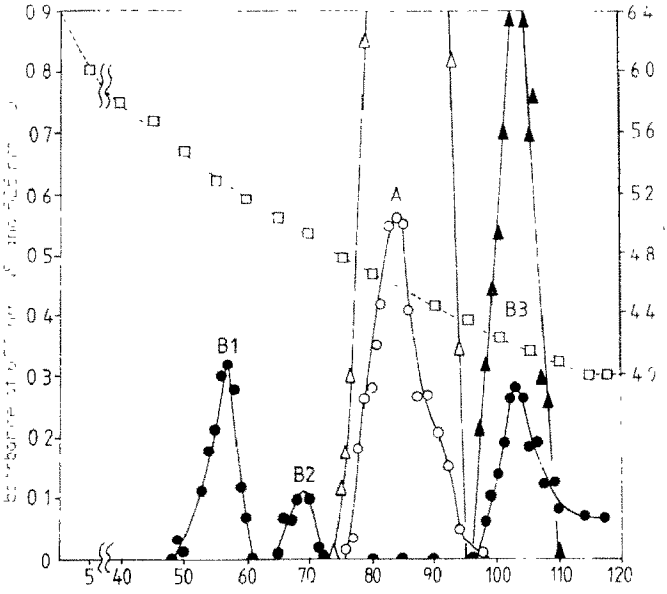

Fig. 1. Fractionation of a crude preparation of *Trichoderma viride* by chromatography. Fractions were assayed against β-D-glucan with the Nelson–Somogyi reagent (NS) (–●–) and D-glucose oxidase (GO) (–▲–). Hydrolysis of starch was monitored by using the Nelson–Somogyi reagent (– –) and D-glucose oxidase (· · ·). The pH values are also shown (– – –).

reducing-power and D-glucose oxidase methods. This fraction (isoelectric point 4.6) therefore contained an amyloglucosidase which showed only very slight activity towards pullulan after extended incubation. Thus, the method provides, in one step, β-D-glucanase-free amyloglucosidase as well as amyloglucosidase-free β-D-glucanases.

In preliminary studies, lowering of the pH has been used to inactivate the β-D-glucanase contaminant in amyloglucosidase preparations. However, the conditions of inactivation have to be carefully controlled and there is always some doubt that traces of the β-D-glucanase contaminant may remain. The same is true of selective heat-treatment to inactivate the amyloglucosidase contaminant in β-D-glucanase preparations[2]. The other approach to the separation of these enzymes has generally involved several stages[4, 5] and there was always a limitation on the quantities that could be applied, particularly to gel-filtration columns. Therefore, it is clear that the present single-stage separation of gram quantities of this crude commercial preparation represents a considerable improvement on previous methods.

The foregoing, isolated enzymes provide a useful means of estimating the levels of γ- and β-D-glucans in cereal samples and of following the degradation of starch and barley β-D-glucan throughout the malting and brewing processes.

EXPERIMENTAL

Materials. — The enzyme preparation used was a crude, lyophilised powder

from *Trichoderma viride*, supplied by Sigma and marketed as Cellulase Type IV. Starch was a Lintner soluble-starch from the American Society of Brewing Chemists, and barley β-D-glucan was obtained from Biocon U.K. Ltd. The sodium salt of CM-pachyman was prepared by Dr. J. W. Spouge by the method of Clarke and Stone[8]. Pullulan was prepared from *Aureobasidium pullulans*[9]. Reducing sugars were determined by a modification of the Somogyi–Nelson method[10], and the specific assay of D-glucose was by a D-glucose oxidase method[11].

Enzyme fractionation by chromatofocusing. — A solution of the crude enzyme powder (2 g) in water (50 mL) was dialysed overnight in running tap-water, and then applied to a column (23 × 40 mm) containing a slurry (150 mL) of Polybuffer Exchanger 94 (Pharmacia) which had been equilibrated with 25mM imidazole–HCl buffer (pH 6.5). The column was then eluted with polybuffer 74–HCl (pH 4.0) at 50 mL/h. Fractions (10 mL) were collected, and analysed for enzyme activity against starch and against β-D-glucan. The pH of each fraction was measured.

Enzyme assays. — D-Glucanase activities were measured in digests containing 1% of substrate (barley β-D-glucan or starch) (0.5 mL), column fraction (0.5 mL), and 0.1M acetate buffer (pH 4.8, 4.0 mL). Digests were incubated at 37° for 1 h for the analysis of reducing sugars and 3 h for the analysis of D-glucose.

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