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Preliminary communication

Separation and characterization of the β-D-glucan hydrolases from a species of *Cytophaga*

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Bacteria of the genus *Cytophaga* degrade cellulose readily[1], and their culture filtrates have been shown[2–6] to contain a mixture of enzymes capable of bringing about the *in vitro* hydrolysis of cellulose and the closely related polysaccharides laminarin and lichenin. However, the constitution of the enzyme complex has not been rigorously characterized nor the constituent enzymes purified. We now report on the constitution of this enzyme system and the significance of earlier work with the same crude preparation of *Cytophaga* enzyme.

The starting material was a commercially available, lytic enzyme preparation[7] from *Cytophaga* NCIB 9497 (available from British Drug Houses Ltd., Poole, Dorset; catalogue number 39072). Preliminary studies of the crude preparation showed the presence of activities towards laminarin, lichenin, and soluble cellulose derivatives such as carboxymethylcellulose (CM-cellulose). Recent techniques[8–10] developed for the purification of β-D-glucan hydrolases, using DEAE-cellulose, were used to fractionate the enzyme mixture into three protein fractions (FI–FIII) with β-D-glucanase activity (Fig. 1). FI, which was not adsorbed on to the ion-exchanger, acted only on laminarin to give (chromatography) higher oligosaccharides, the principal component having a d.p. of about seven or eight. FII, eluted with a high concentration of sodium chloride[9], degraded CM-cellulose and cellodextrins to give chromatographically mobile oligosaccharides, and lichenin to give a trisaccharide (4-*O*-β-laminaribiosyl-D-glucose) and a tetrasaccharide, as well as higher oligosaccharides. FIII, eluted by an acid wash as described earlier[10], acted on laminarin to give (paper chromatography) mainly glucose and laminaribiose, and on lichenin to give mainly glucose, laminaribiose, and a trisaccharide (3-*O*-β-cellobiosyl-D-glucose).

Chromatography of FI–FIII on Biogel P-60 did not result in further separation of enzymic activities, but did effect additional purification, particularly with FI where

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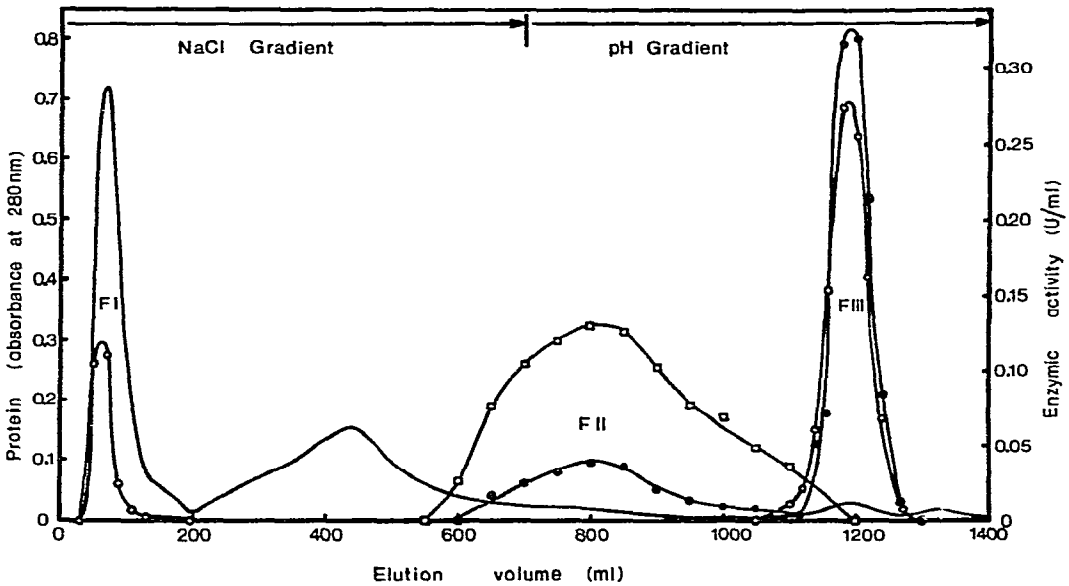

Fig. 1. Chromatographic separation of *Cytophaga* β-D-glucan hydrolases on DEAE-cellulose. Enzyme mixture (100 mg) was dissolved in 25mM citrate–phosphate buffer (pH 8.0) and applied to a column (2.8 × 9.0 cm) of DE-52 equilibrated with the same buffer. Protein was eluted with a gradient of salt [0 → 1.0M sodium chloride in 25mM citrate–phosphate buffer (pH 8.0) over 700 ml], followed by a gradient of pH (8.0 → 3.0 in M sodium chloride over 700 ml). Fractions (10 ml) were collected automatically and assayed for activity towards laminarin (○), lichenin (●), and CM-cellulose (□) by measurement of free reducing-groups produced by the action of the enzyme[29,30].

it served to remove the major contaminant, isoamylase[11,12]. After these procedures, the three enzymes appeared homogeneous by polyacrylamide gel-electrophoresis. Chromatography on Biogel is useful[13] for purification of glycoside hydrolases of low molecular-weight and estimation of their molecular weights. In the present work, the ratios of elution volume (V_e) to the void volume of the column (V_o) gave values of V_e/V_o for FI and FII of ~1.6 and for FIII of 2.2, suggesting molecular weights for FI and FII of ~18,000 and for FIII of ~8000. For the last enzyme, the unusually low molecular-weight is also indicated by the results[14] of polyacrylamide gel-electrophoresis in sodium dodecyl sulphate[15].

Enzymes of the three types present in this *Cytophaga* preparation have been reported previously, being produced by both plants and micro-organisms. Thus, β-(1 → 3)-D-glucan hydrolases (E.C. 3.2.1.6) of a type similar to FI, insofar as they are without action on mixed-linkage substrates, are present in extracts of barley malt[13], rye[16], and in the culture filtrate of a variety of yeast[17]. Endo-β-(1 → 4)-D-glucanase (E.C. 3.2.1.4) (the so-called C_x component of the cellulase complex[18]) is produced by many micro-organisms. The latter enzyme commonly also degrades lichenin[19], as we have found here for FII. Non-specific β-(1 → 3)-D-glucanases (E.C. 3.2.1.6*) of the FIII

*Although the specific and non-specific β-(1 → 3)-D-glucan hydrolases (FI and FIII, respectively) have the same systematic nomenclature and E.C. number, these are different enzymes. This anomaly should be resolved during the current revision of enzyme nomenclature.

type are the most-common type of β-(1 → 3)-D-glucanase and are produced by many micro-organisms[20, 21]. Their action on mixed-linkage D-glucans has been shown to give 3-*O*-β-cellobiosyl-D-glucose[19] as the major product. We have found no evidence for the presence of a specific 'lichenase'[22] in the enzyme complex.

Although many micro-organisms undoubtedly produce β-D-glucanases in the form of multicomponent enzyme complexes, the present work constitutes one of the few detailed characterizations of the nature of the components of such a complex, and is almost certainly the first occasion on which a number of closely related glucan hydrolases have been obtained in pure form from the same source.

The crude starting material used in the present study is a patented, commercially available, enzyme preparation which has been supplied in generous amounts by the patent holders to laboratories in several countries. Initially, laminarinase was believed to be the only carbohydrase present, and the crude material was described[5, 6] as 'a bacterial laminarinase preparation'. The enzyme preparation has come to be considered as a functionally pure β-(1 → 3)-D-glucanase suitable for use in structural studies of β-D-glucans[5, 6, 23–25], and for the degradation of yeast and fungal cell-walls[26,27]. It has also been used in studies of the mode of action of β-D-glucanases[28]. At an earlier date, the presence of isoamylase was demonstrated in the enzyme complex[11,12], and we have now shown the presence of three β-D-glucanases differing in specificity, rather than a single 'laminarinase' as hitherto believed. This means that structural studies, inferences about the 'laminarinase' specificity, and mechanistic studies carried out with the crude enzyme preparation are of little significance, and emphasizes the importance of using purified, characterized enzyme preparations for polysaccharide structural analysis. It remains to be determined which of the glucanases described is involved in lysis of living yeast cells[27].

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