THE ROLE OF β-GLUCANASES AND ASSOCIATED ENZYMES FROM *CLAVICEPS PURPUREA* IN THE INFECTION OF CEREALS

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by

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ABSTRACT

The locations of three enzymes, β-1,3-glucanase, β-glucosidase and β-fructofuranosidase from the Ascomycete fungus Claviceps purpurea (ergot), during its parasitic development on rye, were investigated by immunofluorescent staining techniques.

An infective strain of C. purpurea was selected for extracellular production of B-glucanase in axenic culture; culture conditions were optimised for good recovery of activity. The β-glucanase and β-glucosidase were extracted from culture media, but the β-fructofuranosidase was obtained from disrupted mycelium. The enzymes were purified by ion-exchange and gel chromatography and characterised. Antisera to the purified enzymes were prepared in rabbits. Indirect antibody staining, using fluorescein-conjugated anti-rabbit immunoglobulin serum, was employed to locate the enzymes, or very closely related enzymes, in longitudinal sections of parasitic tissue at various stages of ergot development. Visualisation by fluorescence microscopy necessitated the development of a mild non-denaturing quenching treatment for the bright autofluorescence of plant tissue sections, which would have masked fluorescence arising from the staining of the pathogen.

Fluorescence was largely absent in the early stages of infection. After ten days, the β-glucanase and β-glucosidase could be identified, to varying degrees, in areas of the fungus where there was active growth; particularly at sporulating hyphal tips in the sphacelium and at the host/parasite interface. As the ergot matured, fluorescence for both enzymes persisted only at the interface and in the residual sphacelium that had been displaced acropetally. The B-fructofuranosidase was identified initially in sphacelial tissue, but did not persist there after differentiation. An increased level of fluorescence due to β-fructofuranosidase was found in sclerotial tissue, which contrasts with that observed for

β-glucanase and β-glucosidase.

The roles of the β-glucenase in hyphal wall growth, the β-glucanase and β-glucosidase in the possible dissolution of plant saccharides, and of the β-fructofuranosidase in carbohydrate assimilation are discussed in relation to their observed locations.

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1. INTRODUCTION

1.1 Historical significance of ergot

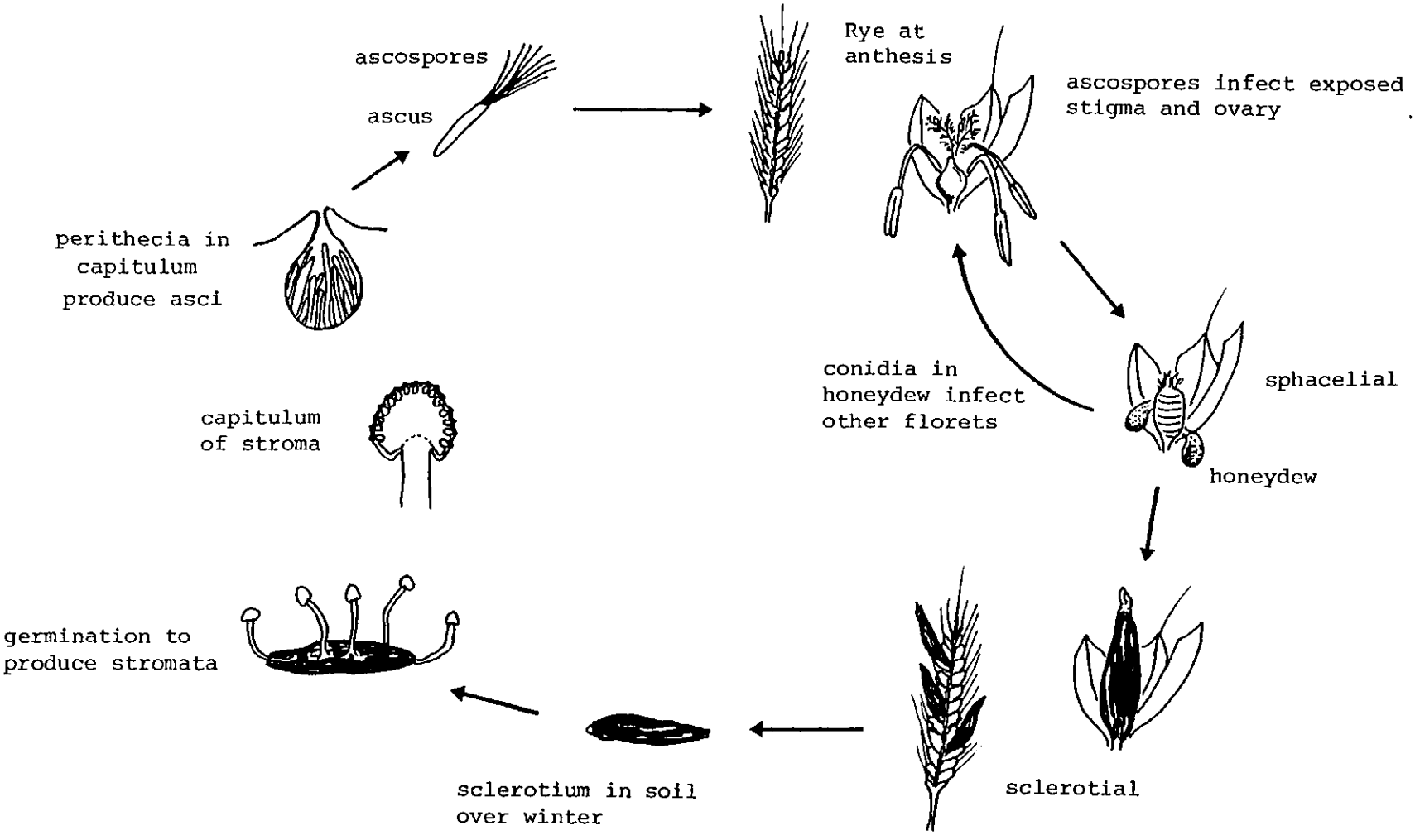
Claviceps purpurea (Fr.) Tul. was first identified as a parasitic fungus on graminaceous grasses by Tulasne in 1853. However, ergot had been known, in the guise of an aberrant seed, for several centuries previously and noted by Loncier in his Krauturbuch (1582) for its use in midwifery. A comprehensive review of historical reports on Claviceps has been provided by Bove (1970).

The importance of Claviceps lies in the production of alkaloids in its characteristic sclerotium or 'ergot'. Some of these alkaloids are pharmacologically active and useful as drugs, e.g. ergotamine, used in the treatment of migraine and ergometrine which is used as an oxytocic and to control post-partum haemorrhage. Other physiological effects of natural and semi-synthetic ergot alkaloids are vasoconstriction, promotion of smooth muscle contraction, antagonism to adrenalin and serotonin and stimulation of the central nervous system (Bové, 1970). In contrast, accidental human and animal consumption of alkaloids in ergot contaminated grain has led to widespread and often fatal outbreaks of ergotism (St. Anthony's Fire) recorded over several centuries (Barger, 1931). A detailed report of an outbreak as recent as 1951 in France has been given by Fuller (1969) and suspected occurrences have been noted since.

1.2 The life cycle of ergot (Figure 1).

Claviceps in an Ascomycete fungus belonging to the class Pyrenomycetes (Webster, 1970). In spring and early summer ergot sclerotia in the soil germinate to produce stromata. The capitulum of the stroma contains peritheca which discharge filamentous, binucleate ascospores to be disseminated by air movement. The ascospores may infect a host inflorescence if they land on a stigma

Figure 1: The life cycle of ergot



or ovary exposed during anthesis. Germinating ascospores produce hyphae which grow down the style and penetrate the ovary. The ovary tissue is destroyed and replaced within 10 days by a loosely packed, white hyphal mycelium, the 'sphacelium', which does not penetrate beyond the base of the original ovary. Sphacelial tissue sporulates profusely and conidia can be found in a sugary honeydew that is a characteristic of this stage of infection; the honeydew exudes from the edges and tip of the infected spikelet often forming large droplets. The conidia act as a stage of secondary infection and can be transferred to infect other florets by vectors such as insects, rainsplash and wind.

About two weeks after infection, conidiation and honeydew exudation subside, and the mycelium differentiates into sclerotial hyphae. Further growth of sclerotial tissue generates the characteristic spur-shaped ergot sclerotium four weeks after infection, while the sphacelial tissue diminishes. The sclerotium comprises densely packed, thick walled, plectenchymatic storage hyphae full of lipid; with a purple-pigmented outer layer. It continues to grow while the host inflorescence matures, and is usually much larger than seeds in the same ear which have developed from uninfected ovaries. The ergots fall or are knocked from the ear, before or during harvest, and lie dormant in the soil over winter. After a period of cold (Mitchell and Cooke, 1968; Cunfer & Marshall, 1977) the ergots will germinate more or less synchronously with cereal seeds to infect a new crop in the spring.

1.3 The pathology of ergot

The agricultural problem posed to cereals by the many species of ergot fungus found in temperate areas has been countered by cultural controls such as the use of sclerotia-free seed, crop rotation, deep tillage and weed control. The possibility of biological

control of ergot using other fungi parasitic on ergot mycelia has also been investigated (Mantle, 1965; Puranik & Mathre, 1971; Mower, Snyder & Hancock, 1975) as well as host resistance in cereal varieties (Bernier, 1976, 1978).

The chemotheraputic use of ergot alkaloids has stimulated interest in the production of alkaloids in saprophytic culture instead of their inefficient and costly isolation from field cultivated sclerotia on cereals (Bové, 1970). Since alkaloid production is associated with sclerotial tissue, much research has centred on the diverse metabolic factors affecting the establishment of sclerotial over sphacelial cultures (Stoll et al., 1957; Taber & Vining, 1958; Arcamone et al, 1962; Tonolo, 1966; Mantle & Tonolo, 1968; Mantle, Morris & Hall, 1969; Arcamone et al., 1970; Mantle, 1975; Mantle & Nisbet, 1976). The chemical and biosynthetic aspects of ergot alkaloids have also been extensively reviewed (Abe & Yamotodani, 1964; Groger, 1972; Thomas & Bassett, 1972; Stadler & Stutz, 1975).

Concurrently studies of the metabolism of ergot in the in vivo parasitic condition have investigated amino-acid, lipid and carbohydrate metabolites in sclerotial germination after winter (Cooke & Mitchell, 1970; Corbett, Dickerson & Mantle, 1975) and in sphacelial and sclerotial tissue during development on rye florets (Corbett, Dickerson & Mantle, 1974). The technique of supplying radioactive CO_2 as photosynthetic carbon source to infected plants, previously used for rusts and mildews (Lunderstadt, 1966; Edwards & Allen, 1966), was employed to trace the path of carbon into the parasite's amino-acid pool (Baker, 1979) and carbohydrate metabolism (Dickerson, Mantle & Nisbet, 1976; Nisbet, Dickerson & Mantle, 1977).

The progress of Claviceps infections, particularly at the cellular or ultrastructural level, have received less attention than the biosynthesis of alkaloids by this organism, and published accounts of different stages of the life cycle have not all been in agreement

(Engelke, 1902; Kirchoff, 1929; Dickson, 1947; Milovidov, 1954,1956; Ramstad & Gjerstad, 1955; Campbell, 1958; Kybal, 1964). This has been rectified to some extent by the recent comprehensive electron microscopical studies on the parasitic development of Claviceps paspali infecting Dallisgrass (Luttrell, 1977) and the Claviceps purpurea infection of rye and wheat by Luttrell (1980) and Shaw & Mantle (1980a, 1980b).

Agreement exists, however, that Claviceps purpurea does not penetrate the host spikelet further than a discrete interface equivalent in depth to 2 or 3 plant cells across the base of the ovarian tissue distal to the rachilla. The rachilla contains xylem and phloem vascular elements which are not continuous in the region where glumes, palea and lemma are attached to the rachilla. The discontinuity is filled by a central core of thick-walled transfer cells, which would therefore be responsible for the transfer of solutes from the plant stem to a developing seed or ergot (Zee & O'Brien, 1970; Shaw, 1971, 1979). Claviceps relies on the host phloem exudate completely for its nutrients; primarily in the form of sucrose which the fungus converts into more readily assimilable glucose by means of a β-fructofuranosidase (Dickerson, 1972). The heterotrophic fungus is considered to act as 'sink' and the autotrophic plant, the 'source' of nutrients in the parasitic relationship (Smith, Muscatine & Lewis, 1969); the developing ergot lies in the floret 'bathed' in phloem exudate (Ramstad & Gjerstad, 1955). It has been suggested that the exudation and evaporation of honeydew, which is acted upon by the fungal β-fructofuranosidase, from the floret, draws host phloem exudate into the floret by osmotic pressure (Mower & Hancock, 1975 a & b - see below) but this has not been substantiated. Since Claviceps relies solely on the plant's nutrient supply, the establishment of the host/parasite interface and its relationship to the vascular elements of the rachilla is of vital

importance to the infection process and development of the ergot.

The developing ergot could be considered to be an 'honourary seed', i.e. mimicing a healthy seed and thereby not disturbing the plant's normal translocation pattern. Yet the superior metabolic activity of the parasite over that of developing healthy seeds enables it to divert preferentially host nutrients to the detriment of adjacent uninfected florets (Corbett et al,1974; Swan, 1980). In response to the discrete fungal invasion, in effect a 'wound', and consequent nutrient drain, the host might be expected to deposit callose, a β-1,3-glucan which forms an impermeable lattice (Currier, 1957; Aist, 1976), to seal the wound and vascular tissue stopping the nutrient loss. However, ergot infections have the potential to overcome this (Ramstad & Gjerstad, 1955). It was proposed by Dickerson et al, (1978) that, as Claviceps spp. produce an extracellular β-1,3-glucanase (Dickerson, Mantle & Szczyrbak, 1970), this enzyme could possibly degrade any callose produced by the host adjacent to the areas of infection, ensuring a continued supply of nutrients. A β-glucosidase, produced in conjunction with the β-glucanase, may also be involved in this process to hydrolyse various glycosides, some of which could arise from the action of the β-glucanase (Dickerson et al, 1978).

Accordingly, the emphasis of the present work has been to locate the β-glucanase, β-glucosidase and β-fructofuranosidase in the parasitic tissue of Claviceps purpurea growing on rye. Immunofluorescence was used as a technique that would accurately identify the situation of these enzymes in tissue, and thereby clarify their possibly important roles in the establishment and maintenance of infection and nutrient supply in the host/parasite system.

Subsequent sections of this introduction will describe each of the three enzymes in greater detail as well as β-glucans which are substrates for the β-1,3-glucanase, and lastly the applications of immunofluorescence to studies on fungi and plants.

1.4 β-glucans

Glucans with different linkages : β-1,2, β- 1,3, β-1,4, β-1,6 and combinations of these, occur widely in micro-organisms and plants, notably cellulose - a β-1,4-glucan (Whistler & Smart, 1955, Aspinall, 1980). However, in the present context this discussion will mainly be confined to β-1,3-glucans, since they represent the substrates for β-1,3-glucanase.

1.4.1 β-1,3-glucans

These homopolysaccharides of glucose, containing predominantly β-1,3 glycosidic linkages are found in fungi, algae, lichens and higher plants (Clarke & Stone, 1963; Gorin & Spencer, 1968; Aspinall, 1970). In fungi, β-1,3-glucan occurs as a structural polymer of the cell wall in conjunction with chitin, mannan or cellulose. A classification of fungi on the basis of major wall constituents has been made by Bartnicki-Garcia (1968); the most common type being group V (which includes Claviceps spp.) having a cell wall of mainly chitin and β-glucan. In the outer layer of the wall chitin microfibrils are embedded in an amorphous matrix of β-1,3-glucan and proteins (Hunsley & Burnett, 1970; Wessels et al., 1972; Schaeffer, 1977). Degrading the wall with glucanase and chitinase, Seitsma & Wessels (1979, 1981) demonstrated that, in Schizophyllum commune, the glucan and chitin are in the form of a covalently linked matrix involving lysine and citrulline. In yeasts, β-1,3-glucan is also an important component of the cell wall, where it is present in varying quantities in conjunction with mannans, chitin and branched β-1,6-glucans (Phaff,

1963; Manners, Masson & Patterson, 1973; Fleet & Manners, 1977).

Many fungi produce extracellular β-1,3-glucans when grown in culture, notably Plectania occidentales (Davis, Rhodes & Schulke, 1967); Schizophyllum commune (Wessels et al, 1972); Monilia fructigena (Archer, Clamp & Migliore, 1977; Santamaria, Reyes & Lahoz, 1978); Sclerotium rolfsii (Kritzman, Chet & Henis, 1979) and Claviceps purpurea and fusiformis (Perlin & Taber, 1963; Buck et al, 1968). In some cases the glucan was partially remetabolized late in the growth cycle suggesting that the glucan can act as a storage polysaccharide (Willets, 1971). The glucans from these organisms had similar structures, a β-1,3 linked backbone with short β-1,6 linked side chains branching with varying frequency from the main chain; Buck et al (1968) and Wessels et al (1972) also found that the extracellularly produced polysaccharide had a very similar structure to that of the corresponding cell wall glucan. Differing structures were found by the two authors for the glucan produced by Monilia fructigena, this was probably due to the use of different culture media (Archer et al, 1977; Santamaria et al, 1978).

In cultures of Claviceps spp. glucan was produced concurrently with the appearance of sclerotial cells (Perlin & Taber, 1963; Buck et al, 1968), and is thought to provide a reserve carbon source along with triglyceride oils for use in sclerotial germination (Dickerson et al, 1970). Moreover, in the ergot fungi Claviceps purpurea and Sphacelia sorghi, enzymes such as the β-fructofuranosidase, β-glucanase and β-glucosidase are bound to both the extracellular and cell wall glucan (Dickerson & Baker, 1979). The polymer is thought to provide a stabilizing matrix for the bound enzyme, enhancing its stability.

Mycolaminarins, similar in structure to laminarin, the storage β-1,3 glucan of brown algae (Wang & Bartnicki-Garcia, 1974), occur in

Oomycetes such as Phytophthora spp., where they serve as food storage polysaccharides and may also be present in the cell wall with cellulose (Wang & Bartnicki-Garcia, 1974 , 1980). Inoculation of plants with extracted mycolaminarins produces phytotoxic symptoms in the plant similar to those observed in a true fungal infection (Keen et al, 1975) Such phytotoxicity has been observed with other fungal β-glucans, frequently resulting in the 'elicitation' of phytoalexin synthesis by the plant. Phytoalexins are a group of compounds, toxic to fungi, which are produced non-specifically in hypersensitive and resistant reactions, stopping the spread of a fungal infection (Kiraly, Barna & Ersek, 1972; Yoshikawa, Yamaguchi & Masago, 1978; Albersheim & Valent, 1978). Currently considerable interest is directed toward investigating phytoalexin elicitation, and the role β-1,3 glucans from fungal cell walls might have in the recognition systems for disease resistance in plants (Keen, 1975; Albersheim & Anderson-Prouty, 1975; Ayers et al, 1976; Steckoll & West, 1978; Cline & Albersheim, 1981). The antitumour activity, demonstrated in some animals, of β-glucans from fungi and yeasts, especially β-1,3 glucans containing some mannose residues (Whistler et al, 1976), could be another aspect of the role of β-glucans in cell recognition systems.

1.4.2 Callose

β-1,3-glucans also occur in higher plants. Amongst such glucans is callose which is of particular importance and wide occurrence. This is an unbranched β-1,3 linked glucan (Aspinall, 1980), whose conformation is thought to be a stable triple helix (Rees, 1973). The importance of callose to the plant is as a plugging and sealing agent (Currier & Strugger, 1956). This relies upon the relative insolubility and strength of callose, the impermeability of callose plugs to the passage of small molecules (Heslop-Harrison, 1966) and the fact that

it can be very rapidly deposited in most plant cells (Fulcher et al, 1976). The major locations of callose in plants are as cylinders around the connecting strands of sieveplate pores and in lateral pit areas of phloem sieve tubes (Currier, 1957; Crafts & Currier, 1963), as a constituent of pollen tube walls (Rosen, 1968; Cresti & Van Went, 1976; Reynolds & Dashek, 1976) and in depositions as a response to cell injury or 'wounding' (Aist, 1976).

Cell injury may result from mechanical damage (Currier, 1957; Lipetz, 1970; Nims, Halliwell & Rosberg, 1967), fungal infections (McKeen & Rimmer, 1973) or viral invasions (Shimomura & Dijkstra, 1975). The cellular response to damage involves the deposition of material between the cell wall and plasmalemma to seal the 'wound', and prevent loss of cytoplasm or entry of substances which might be toxic to the cell (Nims et al, 1967; Bushnell & Bergquist, 1975; Skipp, Harder & Samborski, 1974). Callose is a major component of this deposited material (Aist & Williams, 1971), and occurs in conjunction with lignins, cellulose, chitin, pectins or proteins (Aist, 1976). In fungal infections, the wall depositions - termed papillae - may be produced as a result of pressure exerted by intercellularly growing hyphae, in addition to chemical stimuli such as degradation of the plant cell wall (Heath & Heath,1971; Bushnell, 1972), and the papillae are capable of preventing penetration of the cell by the fungus (Bushnell & Bergquist, 1975; Aist, 1977). The callose that occurs in the sieve plates and lateral pit areas of functional phloem sieve tubes is thought to act as a valve; increases or decreases in the depositions of callose controlling translocation across the sieve plates and between cells (Webster & Currier, 1968). Hence, the greater deposits of callose that are found in injured vessels would bring about the blockage of the sieve plates and lateral pits, preventing loss of translocates from healthy to injured cells (Crafts & Currier, 1963; Eschrich & Currier, 1964).

Apart from callose, β-1,3 glucan occurs in higher plants as hemicellulose - a structural component of some cell walls. Hemicelluloses are linear β-glucans possessing mixed linkages, usually β-1,3 and β-1,4 in a ratio of approximately 1:2 to 1:3 (Whistler & Richards, 1970); they occur in the cell wall of many cereals and related grasses (Smith & Montgomery, 1959; Nevins, Yamamoto & Huber, 1978; Aspinall, 1980) and in cotton fibre (Huwyler, Franz & Meier, 1978).

1.5 β-1,3- Glucanases (E.C. 3.2.1.6)

1.5.1 β-1,3 - glucanases of fungi and plants

Glucans with β-1,3 linkages are common in the environment and the enzymes which hydrolyse them, β-1,3 - glucanases or laminarinases, are ubiquitous. These enzymes have been found in bacteria, fungi, algae, plants and some invertebrates (Moscatelli, Ham & Rickes, 1961; Reese & Mandels, 1959a; Duncan, Manners & Ross, 1956; Clarke & Stone, 1962; Mandels, Parrish & Reese, 1967; Cutter & Yellowlees, 1979). In fungi β-1,3-glucanases are usually constitutive and were found to occur in 90% of the organisms tested by Reese & Mandels (1959a). β-1,6-glucanases are also constitutive in fungi, although β-1,6 - glucans are less common; whereas β-1,2 - glucanases are found to be adaptive or inducible, their substrates being rare in nature (Reese, Parrish & Mandels, 1962; Villanueva et al, 1976).

The activities demonstrated by β-1,3 - glucanases may be either endo- or exo-hydrolytic. Endo-glucanases attack β-1,3-glucans at random points in the chain producing oligosaccharides, e.g. laminaritriose and laminaribiose, which are eventually cleaved to glucose units. Exo-glucanases hydrolyse the polymer sequentially from a chain end, the sole product being glucose (Reese & Mandels, 1959a; Chesters & Bull, 1963a). A β-glucosidase, which would hydrolyse the oligosaccharides generated by the action of endo-glucanases,

or by exo-glucanases on β-1,3 -glucans with β-1,6 linked branches, is often produced by fungi in conjunction with the β-1,3-glucanase (Dickerson et al, 1970; Villanueva et al, 1976). Separation of the glucanase from glucosidase activity, where present, is therefore necessary in determining the precise hydrolytic pattern of a particular glucanase. An extracellular β-1,3-glucanase from cultures of Basidiomycete QM806 was selected by Reese & Mandels (1959a) as the best source of an exohydrolytic β-1,3-glucanase, and this enzyme has been widely used in studies of glucan structure and for comparison with the activities of other glucanases (Reese & Mandels, 1959b; Chesters & Bull, 1963b; Buck et al, 1968).

Many fungi produce β-1,3-glucanases extracellularly when grown in axenic culture (Reese & Mandels, 1959a; Chesters & Bull, 1963a; Dickerson et al, 1970; Jones, Gordon & Bacon, 1974; Miyazaki et al, 1977; Jirku, Kraznerova & Krumphanzl , 1980). In this context the function of the fungal glucanases is the hydrolysis of β-1,3-glucans in the local environment of the fungus, either as a competitive function or for the purposes of assimilation and growth. Moreover, since many fungi produce both extracellular (section 1.4.1) and intracellular glucans (Reese & Mandels, 1959a), the glucanase would be responsible for the reutilization of these carbohydrates as a reserve when required (Villanueva et al, 1976).

The most widely accepted role of β-1,3-glucanases in fungi (as well as of β-1,6 - glucanases (Wessels, 1969)), is their involvement in hyphal wall morphogenesis (Burnett, 1968; Gooday, 1978). It was proposed by Bartnicki-Garcia (1973) that hyphal wall growth was brought about by a delicate balance between cell wall polymer lytic and synthesizing enzymes. The wall is softened or 'plasticized' by partial digestion of polymers in the cell wall layers by enzymes such as glucanase, chitinase and mannanase, followed by the

intussusception of new wall material by polymer synthesizing enzymes. Consistent with this model, Mahadevan & Mahadkar (1970) have found β-1,3-glucanase activity associated with the cell walls of Neurospora crassa, and a correlation between the degree of branching and apical growth and the level of this enzyme's activity. Subsequently, Sukumaran & Mahadevan (1975) demonstrated the presence of β-1,3-glucanase and protease in the branching tips and cross-septa of N. crassa hyphae using immunofluorescent tracing techniques. Kritzman et al (1978) have also used immunofluorescent staining to show the location of β-1,3-glucanase in the hyphal tips, new septa and lateral branching points of Sclerotium rolfsii. Santos et al, (1979) suggested that β-1,3-glucanases in Penicillium italicum are associated with the cell wall in the periplasmic space, and cellulase and β-1,3-glucanase activity have been found in the apical vesicles of Saprolegnia monoica by Fevre (1977, 1979) using sub-cellular fractionation. In conjunction with the lytic enzymes, β-glucan synthetases have been found to be bound to the cell walls of Phytophthora, Neurospora and Saprolegnia by Wang & Bartnicki-Garcia (1966), Mishra & Tatum (1972) and Fevre & Dumas (1977) respectively.

Both β-1,3- and β-1,6- glucanases occur in yeasts (Abd-el-Al & Phaff, 1968; Barras, 1972) and their main function is also thought to be in the process of budding and cell wall growth (Villanueva et al, 1976). Fleet & Phaff (1974) have shown the association of β-1,3-glucanases with the cell walls of Schizosaccharomyces. Using synchronous cultures, Maddox & Hough (1971) found that β-1,3 glucanases are produced maximally during the budding process, and Matile et al. (1971) have isolated vesicles containing β-glucanase which were secreted into the cell wall at the sites of new buds. In some yeasts, different forms of β-1,3-glucanase, both exo- and endo-hydrolytic, can occur in the cell walls and culture media of the same

organism (Villa, Notario & Villanueva, 1975; Villanueva et al, 1976), although a less specific glucanase, active against both β-1,3 and β-1,6 linkages has also been reported (Farkas, Biely & Bauer, 1973).

The occurrence of cell wall autolysis in some organisms (Polacheck & Rosenberger, 1975) suggests the unchecked action of β-1,3-glucanase and other lytic enzymes (Reese & Mandels, 1959a; Maddox & Hough, 1971; Barras, 1972). In fact the lytic activity of β-1,3-glucanases has been useful in investigations of cell wall structure by enzymic digestion (Hunsley & Burnett, 1970; Fleet & Manners, 1977; Seitsma & Wessels, 1979), in the structural analysis of polysaccharides (Marshall, 1974; Huwyler et al, 1978; Miyazaki et al, 1978), in the preparation of protoplasts from fungal and yeast cells (Peberdy, 1976, 1978) and in immobilized enzyme systems for the treatment of cereal worts (Linko & Linko, 1979).

The presence of native β-1,3-glucanase in plants was inferred from the very rapid deposition and disappearance of callose in plant cells (Currier, 1957; Heslop-Harrison, 1966; Fulcher et al, 1976). Clarke & Stone (1962) found that β-1,3-glucanase and β-glucosidases were present in many plants, especially in the phloem and xylem vessels. They suggested that β-1,3-glucanase functions in the removal of callose after dormancy from sieve tubes in the grapevine Vitis vinifera. The enzyme, therefore, may be involved in the regulation of cell to cell translocation, by control of the callose deposits in sieve plates and lateral pits of vascular cells (Abeles & Forrence, 1970), although this has not been conclusively demonstrated. Cereal grains, both ungerminated and germinating have been found to possess β-1,3-glucanase activity (Clarke & Stone, 1962; Manners & Marshall, 1969, 1973), which in this context are thought to be involved in the mobilization of hemicelluloses during germination (Ballance & Manners, 1978). In addition, levels of

native β-1,3-glucanase in some plants have been found to increase upon infection by fungi (Abeles et al, 1971; Wargo, 1975; Pegg, 1976). This has led these authors to propose that β-1,3-glucanase could have a further function in plant defence mechanisms by attacking the glucan in the hyphal walls of invading fungi.

1.5.2 β-1,3-glucanase from ergot

The first reported occurrence of β-1,3-glucanase activity in Claviceps spp. was by Dickerson et al (1970). Cultures of Claviceps fusiformis, intended for the production of alkaloids, elaborated a glucan which, after seven days growth, had become a stable viscous 'froth' preventing adequate aeration and adversely affecting alkaloid production and extraction (Buck et al, 1968). A variant strain of C. fusiformis, which arose spontaneously, was found to be capable of complete autohydrolysis of the glucan, allowing some renewed growth and successful alkaloid extraction (Dickerson et al, 1970). The autohydrolysis was due to an extracellularly produced β-1,3-glucanase having an exohydrolytic action the same as that observed for the Basidiomycete QM806 glucanase (Reese & Mandels, 1959a; Nelson et al,1963). Dickerson et al,(1970) also reported that the same enzyme activity was found in media from cultures of Claviceps purpurea, even though these cultures produced little or no glucan.

β-1,3-glucanase activity has been shown to be present in exuded honeydew, and in parasitic tissue of C. purpurea throughout its development on host cereals; the level of activity being higher in sclerotial than in sphacelial tissue (Dickerson et al, 1978). The glucanase activity, along with β-glucosidase and β-fructofuranosidase, was shown to occur bound to both the extracellular glucan produced by Sphaelia sorghi in culture and to the glucan in cell wall preparations from cultured mycelium of C. purpurea (Dickerson & Baker, 1979). This is consistent with the proposed roles of β-1,3-glucanase in hyphal

wall growth (Bartnicki-Garcia, 1973) and in the mobilization, during germination, of β-1,3-glucan reserves that occur in ergot sclerotia (Buck et al, 1968; Dickerson et al, 1970).

Another possible role for β-1,3-glucanase from Claviceps has, however, been proposed by Dickerson et al,(1978). The plant host might be expected to deposit callose in sieve tubes and cells adjacent to damaged cells in response to the fungal invasion (See above, section 1.4.2). But in ergot-infected ovaries no callose was detectable by aniline blue fluorescence microscopy. It was proposed that the extra cellularly produced fungal β-1,3-glucanase could hydrolyse any callose deposited in host cells, or across the sieve plates of the vascular tissue at the base of the ovary. This would allow the fungal colonization to become established and ensure the maintenance of a supply of nutrients to the parasite from the host. In support of this proposal, Dickerson et al (1978) have shown that the Claviceps purpurea β-1,3-glucanase is active against plant callose. Callose deposits, induced in healthy wheat ovaries by the insertion of needles and detected in sections of the ovaries by aniline blue fluorescence microscopy, were removed i.e. hydrolysed by the application of either extracted β-1,3-glucanase or honeydew from C. purpurea.

A complementary approach to the above studies would be to determine the location of β-glucanases in tissues from the in vivo host/parasite system, in order to ascertain whether they were suitably placed to function in the roles that have been proposed for them. The present work was undertaken with this object in view.

1.6 β-glucosidases (E.C. 3.2.1.21)

The β-glucosidases are active against β-linked glucose containing dissacharides and oligosaccharides of low degree of polymerisation, but do not attack glucans; in contrast, the glucanases attack

oligosaccharides only very slowly or not at all (Reese, Maguire & Parrish, 1968). The β-glucosidases have a wide distribution in animals, plants and micro-organisms (Nisizawa & Hashimoto, 1970); they are commonly produced extracellularly into culture media by many fungi (Jermyn, 1959; Sternberg, Vijayakumar & Reese, 1977).

Since β-glucosidases are able to hydrolyse β-1,4 linked glucosides, these enzymes are important in the dissolution of cellulose - a β-1,4-glucan - by micro-organisms, where they appear to operate in conjunction with cellulases. Hence, the involvement of β-glucosidases from several cellulolytic micro-organisms in the process of cellulose hydrolysis has been studied in relation to, for example, the breakdown of plant cellulose material in the soil (Han & Srinivasan, 1969; Bucht & Eriksson, 1969; Herr, Baumer & Dellweg, 1978) and the possible use of these enzymes in the industrial production of glucose syrups from waste cellulose (Sternberg, 1976; Maguire, 1977; Sternberg *et al*, 1977). Carbohydrases which attack β-1,4 glycosidic linkages are thought to be primarily inducible enzymes in fungi (Villanueva *et al*, 1976), although the presence of constitutive β-glucosidases in yeasts and *Neurospora* has also been reported (Hu *et al*, 1960; Eberhart & Beck, 1973). The induction of β-glucosidase activity by various glycosides has thus provided a useful system to study the control of enzyme induction and production in these organisms (Herman & Halvorson, 1963; Wilson & Niederpruem, 1967; Urey, 1971; Borgia & Sypherd, 1977). Most β-glucosidases studied were induced by cellulose or cellobiose and repressed, or the enzyme activity inhibited, by glucose (Wilson & Niederpruem, 1967; Desphande, Eriksson & Pettersson 1978; Smith & Gold, 1979).

Fungal β-glucosidases, in common with other carbohydrate hydrolysing enzymes (Dedonder, 1961; Nisizawa & Hashimoto, 1970), exhibit both direct hydrolytic splitting of disaccharides and glycosyl

transferase activity with the production of glucose and trisaccharides from cellobiose (Berger & Eberhart, 1961; Krishna-Murti & Stone, 1961; Mahadevan & Eberhart, 1964). In addition, two main types of substrate specificity have been found to occur in fungal β-glucosidases. First, a specific action against the β-1,4 linked diglucoside cellobiose called cellobiase, and secondly a more general activity often termed aryl-β-glucosidase (Mahadevan & Eberhart, 1964; Bucht & Eriksson, 1969; Lusis & Becker, 1973; Maguire, 1977). The latter enzymes are active in varying degrees against the glucosyl-β-glucosides of different linkages, i.e. gentiobiose, cellobiose, laminaribiose, sophorose and β,β-trehalose, and aryl-β-glucosides such as esculin, arbutin, salicin, amygdalin and p-nitrophenol-β-glucoside, but much less active against alkyl-β-glucosides (Reese et al, 1968; Sternberg et al, 1977). An extensive study of the mechanism of action of the aryl-β-glucosidase from Stachybotris atra has been made by Jermyn (1966, 1969).

The aryl-β-glucosidase and cellobiase activities are not mutually exclusive and both activities may be exhibited by the same enzyme or iso-enzymes; alternatively, the two activities may be represented by two distinct β-glucosidases from the same organism (Bucht & Eriksson, 1969; Lusis & Becker, 1973; Gong, Ladisch & Tsao, 1977; Herr et al, 1978; Canevascini & Meyer, 1979). The production of two or more types of β-glucosidase by a fungus is a common occurrence, and it has been found that these enzymes may be located in different parts of the fungal cell, i.e. intracellularly and cell wall bound in addition to being produced extracellularly (Horikoshi & Ikeda, 1965; Mahadevan & Menon, 1968; Eberhart & Beck, 1970; Smith & Gold, 1979; Dekker, 1981).

In ergot fungi, β-glucosidase is produced extracellularly in culture, and, in conjunction with the β-1,3-glucanase, it was

'jointly' responsible for the autolysis of glucan produced by Claviceps fusiformis in culture (Dickerson et al, 1970; see section 1.5.2). The Claviceps β-1,3-glucan contains single glucose unit branches which are β-1,6 linked to the main chain (Buck et al, 1968). The branches are released as gentiobiose by the action of the β-1,3-glucanase and are rapidly hydrolysed by the β-glucosidase to yield glucose as the sole product of glucan hydrolysis. The β-glucosidase activity was separated from the β-1,3-glucanase by ion-exchange chromatography and was shown also to be active against cellobiose and p-nitrophenol-β-D-glucopyranoside (Dickerson et al, 1970). These authors also found that β-glucosidase activity was associated with the purified extracellular glucan. It was shown that the glucan provided a matrix to which the enzyme bound strongly and that the stability of the bound β-glucosidase over that for the unbound enzyme was greatly enhanced. This effect has been demonstrated for β-glucosidases bound to the extracellular glucan from other ergot fungi and to the cell wall glucan from Claviceps purpurea (Dickerson & Baker, 1979).

In addition to the β-glucanase discussed above (section 1.5.2), β-glucosidase activity was also found in exuded honeydew and in extracts from C. purpurea growing on rye (Dickerson et al, 1978). It was suggested by these authors that, since cereal grasses contain glucans of mixed linkage (see section 1.4.2), the β-glucosidase could act to limit the accumulation of glucosides that might arise from the action of β-glucanase on these glucans. In the hydrolysis of extracellular glucan in culture the β-glucosidase had acted upon the products of β-glucanase activity; it was therefore of interest to investigate whether this could occur in the parasitic tissue and thereby fulfil the role proposed above. The present studies were accordingly directed towards identifying the precise location of the

β-glucosidase, to determine whether there was any correlation between its distribution and that observed for the β-glucanase.

1.7 β-fructofuranosidases (E.C.3.2.1.26)

The enzymes which hydrolyse sucrose, invertases, occur widely in micro-organisms, plants and animals (Myrback, 1960). Hydrolysis of sucrose by different types of invertase may involve either its direct cleavage giving glucose and fructose, or the initial transfer of the fructosyl moiety to other sucrose molecules which releases glucose and produces several different oligosaccharides. Further hydrolysis subsequently releases the fructose from these oligosaccharides (Bealing & Bacon, 1953; Dedonder, 1961; Nisizawa & Hashimoto, 1970). It is usually this latter type of invertase or β-fructofuranosidase which occurs in fungi, and the β-fructofuranosidases from yeasts and Neurospora have been intensively studied (Metzenberg, 1963a, 1964; Neumann & Lampen, 1967; Gascon, Neumann & Lampen, 1968; Lampen, 1971; Guthrie et al, 1979).

Ergot fungi growing on cereal grasses rely mainly on the phloem exudate, which contains primarily sucrose, for carbohydrate nutrients. Little sucrose is assimilated directly by the fungus (Dickerson, Mantle & Nisbet, 1976), so that a means for converting the disaccharide into more easily assimilable monosaccharide units, such as a β-fructofuranosidase, is important to the fungus (Long et al, 1975). The honeydew exuded from florets during ergot parasitism contains up to 30% (w/v) sucrose in its initial stages, but subsequently this is mostly replaced by glucose, fructose and oligosaccharides (Fuchs & Pohm, 1953). In the case of C. purpurea, these products were shown to be the result of the action of a β-fructofuranosidase from the fungus on host sucrose (Dickerson, 1972). The pattern of sucrose hydrolysis by the ergot enzyme is such that glucose is released to be assimilated by the fungus

whilst a number of glucose-fructose and fructosyl oligosaccharides are generated. It has also been reported that the types of oligosaccharides produced by ergot fungi is species dependant; this being due to a species specific β-transfructofuranosidase being produced by a variety of ergot fungi (Mower, Gray & Ballou, 1973; Mower & Hancock, 1975 a & b).

Numerous proposals have been put forward in an attempt to account for the elaboration of oligosaccharides by β-fructofuranosidase from Claviceps spp. . First, the heterotrophic fungus acts as a 'sink' for nutrients from the autotrophic plant host (Smith, Muscatine & Lewis, 1969; Lewis, 1974); the generation of oligosaccharides releases glucose which is rapidly converted to the polyol mannitol and trehalose by the fungus (Cooke & Mitchell, 1970; Dickerson et al, 1976) and, unless mannitol and trehalose are normally present as intermediates in a plant's carbohydrate metabolism, these saccharides are only poorly metabolised by higher plants (Lewis & Smith, 1967). Therefore, it is possible that hydrolysis of sucrose and rapid transfer of liberated glucose to polyol creates a decreasing concentration gradient of sucrose from host tissues to fungal tissues which draws more host sugar into the floret. The concentration gradient would also prevent reciprocal movement of fungal carbohydrates into the host (Smith et al, 1969). Secondly, it has been proposed that the production of oligosaccharides by the fungus and the evaporation of exuded honeydew raises the osmotic pressure in the floral cavity drawing more phloem exudate into the floret (Mower et al, 1973; Mower & Hancock, 1975b). However, the process by which this net change in osmotic pressure is achieved has not been made clear. Thirdly, the oligosaccharides may be generated to regulate the level of free fructose; in axenic culture, fructose is not metabolised in the presence of glucose and at high concentrations (30%, w/v) inhibits growth and sporulation (Dickerson, 1972). Last,

oligosaccharides might serve as carbon reserves for use in later growth stages (Arcamone et al, 1970, Dickerson et al, 1970).

The β-fructofuranosidase of C.purpurea has been extracted, purified and characterized from both parasitic tissue grown on the cereal host and from axenic culture (Dickerson, 1972, Baker, 1979). Since sucrose is not assimilated directly by the fungal hyphae, this enzyme might be expected to be produced extracellularly in order to hydrolyse sucrose in the phloem exudate yielding glucose which could then be taken up by the hyphae. However, little activity was found in the culture medium by the above authors, most of the enzyme activity being cell-associated. The β-fructofuranosidases of Neurospora and yeasts have also been shown to be cell-associated (Metzenberg, 1963b; Gascon & Lampen, 1968). Dickerson & Baker (1979) demonstrated that the β-fructofuranosidase of C.purpurea was tightly bound to the cell wall glucan and to a glucan produced extracellularly in copious amounts by other ergot fungi such as C.fusiformis and Sphacelia sorghi. Binding to the glucan greatly enhances the stability of the bound enzyme over that of the unbound enzyme; this enhancement has also been observed for yeast β-fructofuranosidases bound to yeast cell wall mannan (Arnold, 1969; Woodward & Wiseman, 1976).

In order to study the distribution β-fructofuranosidase in C.purpurea sclerotia of different ages, Baker (1979) cut developing sclerotia into transverse sections, each comprising approximately 1/5 of the length of the sclerotium, and assayed these sections for the presence of β-fructofuranosidase activity. Although all of the ergot tissue demonstrated activity, the maximum level of activity was found 28 days after infection sited in sclerotial tissue close to the host/parasite interface. The specific activity had decreased 50% by 56 days after infection, by which time the major enzyme activity was

found to be sited distal from the interface near the top of the mature sclerotium. The present studies have sought to extend this work by identifying the distribution of the β-fructofuranosidase precisely, and thereby to define as accurately as possible this enzyme's role in carbon assimilation throughout the period of parasitic development.

1.8 Immunofluorescence

The techniques of immunofluorescent detection of proteins, employing immune serum globulin labelled with a fluorescent dye to locate the corresponding antigen in tissues, were introduced by Coons et al, (1942) and are now well established. These methods have been used extensively in the medical field for the detection of microbial pathogens and in studies of antigenic substances in animal cells. Several comprehensive reviews of the principles, experimental methods and applications of immunofluorescence in the medical and zoological fields have been written (Beutner, 1961; Goldman, 1968; Johnson, Holborow & Dorling, 1978; Nairn, 1976).

In the study of fungi, immunofluorescence is of importance in the detection of pathogenic species in infections of human and animal tissue (Nairn, 1976), although other applications have been found. Antisera raised against crude preparations of fungal hyphae have enabled the serological typing of species within a generic group for taxonomic purposes (Madhosingh, 1964; Burrell et al, 1966; Amos & Burrell, 1967; Halsall, 1976). Coupled with fluorescent dyes, these sera can distinguish between species (White, 1976) and they provide a simple method for identifying the presence of hyphae from a particular fungus in soil samples (Beno & Allen, 1964; Schmidt & Bankole, 1965; Kumar & Patton, 1964; Eren & Pramer, 1966). Fluorescent antisera directed against antigenic preparations of fungal cell walls

have been used as a means of following visually the areas of cell wall extension during hyphal morphogenesis (May, 1962; Goos & Summers, 1964; Marchant & Smith, 1968) and changes in the distribution of surface antigens on the aggregating cells of the cellular slime mould Dictyostelium (Takeuchi, 1963). More recently, Hunsley & Kay (1976) have used antisera against the different polysaccharide fractions of Neurospora hyphal walls, i.e. glucan, chitin and glucan-peptide-galactosamine, to follow, by fluorescence, the deposition of these components in apical growth.

Purified fungal enzymes have successfully been used to prepare antibodies, leading to the precise serological characterization of equivalent enzymes from related species, which can be useful in resolving taxonomic problems (Marchin & Duerkson, 1969; Cook & Cain, 1977; Lachance & Phaff, 1979; Baker & Dickerson, 1980). Specific fungal enzyme antisera have also facilitated the cellular localization,by immunofluorescence, of enzymes. In Aspergillus, α-amylase was found to be bound to the hyphal cell wall by Tonomura (1964) and the siting of invertases has been demonstrated on hyphal and conidial cell walls of Neurospora (Chung & Trevithick, 1970; Chang & Trevithick, 1972) and on the cell surface of the yeast Saccharomyces (Tkacz and Lampen, 1973). β-1,3-glucanases, thought to be involved in hyphal morphogenesis (see section 1.5.1), have been located in association with hyphal apices and branch points by Sukumaran & Mahadevan (1975) and Kritzman et al (1978).

The use of immunofluorescence is currently being extended into the areas of plant physiology and pathology. As regards native plant antigens, Knox (1971) and Howlett (1973) have studied the location of proteins in the cell wall of pollen grains and their fate during germination, and the distribution of water soluble proteins in wheat

grains was examined by Barlow, Simmonds & Kenrick (1973). The cellular localization of plant enzymes; in particular, a β-fructofuranosidase from tomatoes (Iki et al, 1977) and ribulose 1,5 biphosphate carboxylase from wheat leaves (Hattersley, Watson & Osmond, 1977) has also been achieved.

The first reports of immunofluorescent detection of plant pathogens were the identification of pseudomonads in plant cells by Paton (1960,1964) and of Polyporus hyphae on the roots of pine trees infected by this fungus by Kumar & Patton (1964). Preece (1966) suggested that immunofluorescence, using whole cells or cell wall fractions as antigens, would be a useful technique in identifying plant pathogenic fungi in plant tissues. Subsequently there have been reports on the detection of bacteria in the cells of leaf-nodulated plants of the Rubiaceae family (Bettelheim, Gordon & Taylor, 1968), the hyphae of Alternaria, Aspergillus and Penecillium on barley grains (Warnock, 1971, 1973) and studies following the colonization of wheat and oat roots by hyphae of Ophiobolus (Choo & Holland, 1970) and of dahlia roots by Verticillium (Fitzell, Evans & Fahy, 1980). Choo & Holland (1970) and Warnock (1973) also point to the value of this technique in identifying particular fungi in mixed populations on the surfaces of plant tissues. Whole cells and cell walls represent rather crude antigens for the preparation of antisera, but the technique has now been applied to more specific antigens from plant pathogens. Hislop et al, (1974) located the extracellular pectolytic enzymes from Sclerotinia fructigena along hyphae growing through apple tissue, and Paynter & Alconero (1979) detected the phytotoxin syringomycin produced by a pseudomonad pathogen in infected peach tree cells.

The investigations described above demonstrate that immunofluorescence is established as a means of detection and localization

of enzymes in fungi and the plants on which those fungi are pathogenic. Consequently, in the present studies on the roles of enzymes, this technique was chosen to locate precisely the β-1,3-glucanase, β-glucosidase and β-fructofuranosidase from Claviceps purpurea associated with parasitic tissue growing on rye.

A major limiting problem in the application of immunofluorescence to plant tissues is autofluorescence. This was noted by Paton (1964), Preece (1966), Choo & Holland (1970) and several of the other authors mentioned above; it was also encountered in this work. Autofluorescence is exhibited to a certain extent by most animal, plant and microbial cells (Van Duijn, 1954; Sonea & De Repentigny, 1960), but the slight pale blue autofluorescence of animal tissue does not constitute a great problem in investigations using fluorescent staining (Nairn, 1976). Plant tissues, however, demonstrate very bright autofluorescence across the spectrum of visible light wavelengths. The autofluorescence may be due to the large number of cyclic and phenolic compounds in plant cells which are naturally fluorescent e.g. chlorophyll, NAD, FAD and growth hormones, or to the production of scopoletin in damaged or sectioned tissue (Udenfriend, 1964), and Fitzell et al, (1980) also found that the intensity of autofluorescence was dependent on the thickness of sections used.

In some cases (Knox, 1971; Hislop et al, 1974, Iki et al,1977), the colour of the autofluorescence contrasted with that of the antibody-coupled fluorochrome used, hence no quenching was required; but usually some form of autofluorescence quenching is required in order that antibody-coupled fluorescence is not confused with autofluorescence (Preece & Cooper, 1969). Quenching is usually achieved by the use of a fluorescent counterstain of a different colour (Paton, 1964; Choo & Holland, 1970; Nairn, 1976; Fitzell et al, 1980). Paton (1964) also suggests that treatment of plant tissues with dimethylformamide or mercuric chloride followed by washes of EDTA is necessary

to remove autofluorescent material, e.g. chlorophyll. The present work on the siting of native enzymes required the development of a less harsh quenching treatment than that previously described, which would not denature proteins.

2. MATERIALS AND METHODS

2.1 Organisms and Media

2.1.1 Organisms

Strains of Claviceps purpurea were obtained from the collection of Dr. P.G. Mantle, Biochemistry Dept., Imperial College, London, stored as conidial suspensions in 10% (^{v}/v) glycerol in sealed ampoules under liquid nitrogen. Claviceps purpurea strain 6, which was used for further experiments, was originally derived from ergots isolated from Cocksfoot grass (Dactylis glomerata (L.)). Svalofs Fourex Rye (Swedish Seed Association, Svalof, Sweden) was grown in outside plots and in pots under greenhouse conditions at Chelsea Physic Garden.

2.1.2 Media and culture conditions

Medium T (modified after Stoll et al., 1954) was used for the saprophytic growth of Claviceps spp. in culture. It contained the following constituents per litre of distilled water: Sucrose, 100 g; L-asparagine, 10 g; $Ca(NO_3)_2,4H_2O$, 1 g; KH_2PO_4, 0.25 g; $MgSO_4.7H_2O$, 0.25 g; KCl, 0.125 g; $FeSO_4.7H_2O$, 0.033 g; $ZnSO_4.7H_2O$, 0.027 g; Yeast extract, 0.1 g; Cysteine (HCl), 0.01 g. The pH was adjusted to 5.2 with 2.5 M NaOH.

Modified medium T was developed as an amino-nitrogen medium (Mantle and Nisbet, 1976) and was used for Claviceps strain selection. It contained the above constituents per litre of distilled water without the $Ca(NO_3)_2.4H_2O$, Yeast extract or Cysteine (HCl).

Liquid media (100 ml) were dispensed into 500 ml Erlenmeyer flasks closed with styrofoam bungs, prior to autoclaving for 30 min. at 5 p.s.i. For solid media, 1.5% (w/v) Oxoid agar number 3 was added before autoclaving.

Antibiotic media plates. A 0.25% (w/v) solution of tetracycline hydrochloride, sterilized by passage through a Millipore filter (0.45 µm), was added to agar media as a bacteriostatic agent as plates were being poured, to give a final concentration of 50 µg antibiotic ml^{-1} media.

Preparation of mycelial cultures. Conidial suspensions were thawed slowly from liquid nitrogen, heated to 37°C for 10 min. in a waterbath (Shaw, 1975), and diluted 100 fold in sterile distilled water. The diluted suspension was streaked out on medium T agar plates containing antibiotic and incubated for 3 days at 24°C. Uncontaminated mycelium was subcultured on to medium T agar slopes in 20 ml McCartney bottles from which, after 14 days incubation at 24°C, a spore suspension of 5×10^7 spores ml^{-1} in sterile distilled water was prepared. Flasks containing 100 ml liquid medium were inoculated with 1 ml spore suspension per flask and incubated at 24°C on a rotary shaker (240 r.p.m).

2.2 General analytical techniques

2.2.1 Glucose determination

Glucose as a product of enzyme reactions was determined using the glucose oxidase method of Fleming & Pegler (1963). The method was adapted in the present study for the use of ABTS (2,2'-azino-di-[3-ethyl-benzthiazoline-sulphonate (6)], Boehringer), a chromogen replacing O-dianisidine, and considered to be less carcinogenic than the latter. The accuracy of Fleming & Pegler's method for determining glucose in the presence of maltose was maintained. The commercial glucose oxidase had no detectable activity against the disaccharides, referred to in section 2.3.2 and table 6, within the time limit of the assay below. Glucoseoxidase reagent, 2 ml, containing glucose oxidase, 6 mg; peroxidase, 1 mg; ABTS, 10 mg; per 40 ml 0.5 m Tris-HCl buffer, pH 7.0, was added to a 1 ml volume containing the glucose

sample and the mixture incubated at room temperature for 15 min. A green colour was produced in the presence of glucose which was stable for a further 25 min. The absorbance was read at 420 nm, and the concentration of glucose calculated from a standard curve of 0 to 50 μg ml^{-1} glucose in distilled water.

2.2.2 p-Nitrophenol determination

Addition of 2 ml 0.1M NaOH to a reaction sample contained in 1 ml distilled water was used to stop the reaction and develop the yellow nitrophenate ion. The absorbance was determined at 410 nm, and the concentration determined from a standard curve of 0 to 25 μg ml^{-1} NaOH treated nitrophenol.

2.2.3 Determination of reducing sugar

Reducing sugars (i.e. monosaccharides) were detected using the Formazan method (Baker, 1979). An equal volume of 0.1% (w/v) triphenyl tetrazolium chloride (TTC) in 1M NaOH was added to a sample and the mixture incubated for 15 min in the dark at 25°C. The TTC reacts with reducing sugar under alkaline conditions to give a red formazan complex. The formazan is unstable and has no specific absorption peak in the visible spectrum; hence this serves only as a qualitative test.

2.2.4 Protein concentrations

Protein in solution was estimated by a spectrophotometric method based on that of Warburg & Christian (1942). The concentration was calculated from the extinction coefficients at 280 and 260 nm using the method of Layne (1957).

2.2.5 Paper chromatography

Carbohydrate samples were analysed by descending paper chromatography on Whatman No.1 or 3 mm paper, in a solvent comprising propan-1-ol, ethyl acetate, water; 7:1:2 parts by volume, for 16 to 40 h (Buck et al, 1968). To develop, chromatograms were dipped in an aniline-phthalate reagent modified from Partridge (1949) (O-phthallic acid, 1.7 g; aniline, 1 ml; 40% (w/v) trichloro-acetic acid, 5 ml; glacial acetic acid, 5 ml; ethanol, 90 ml), dried and heated to 100°C for 5 min. Carbohydrate spots appeared dark brown against a pale background.

Chromatograms containing U-^{14}C radioactive samples were autoradiographed using Fuji RX-3 film to locate spots. The spots were cut out, cut into 5 mm squares, placed in 5 ml scintillation fluid (N-butyl-PBD, 6 g; napthalene, 50 g; toluene, 1 L) and counted in a Beckman LS-240 scintillation counter.

2.2.6 Gel electrophoresis

Polyacrylamide tube gels were run in a disc electrophoresis unit according to the methods of Weber, Pringle & Osborn (1972). For electrophoresis of native proteins, 6% (w/v) polyacrylamide gels with 1% crosslinking were prepared in 0.04 M tris-acetate buffer, pH 8.0, and polymerized with 1.5% (w/v) ammonium persulphate and 1% (w/v) tetramethyldiaminomethane (TEMED). The gels were pre-run for 2 h to remove impurities before protein samples (10-50 µg) were applied in 20 µl 50% (w/v) sorbitol saturated with bromophenol blue and the gels run anodically at a current of 7mA/gel for 2 to 3 h at 4°C. For denatured protein, 8% (w/v) polyacryamide gels with 1% cross-linking were prepared in 0.1 M sodium phosphate buffer, pH 7.2, containing 0.01 M EDTA and 0.1% (w/v) sodium dodecylsulphate (SDS), and polymerized and pre run as above. Protein samples (5-50 µg) were denatured in a solution (60 µl) containing 1% (w/v) SDS and

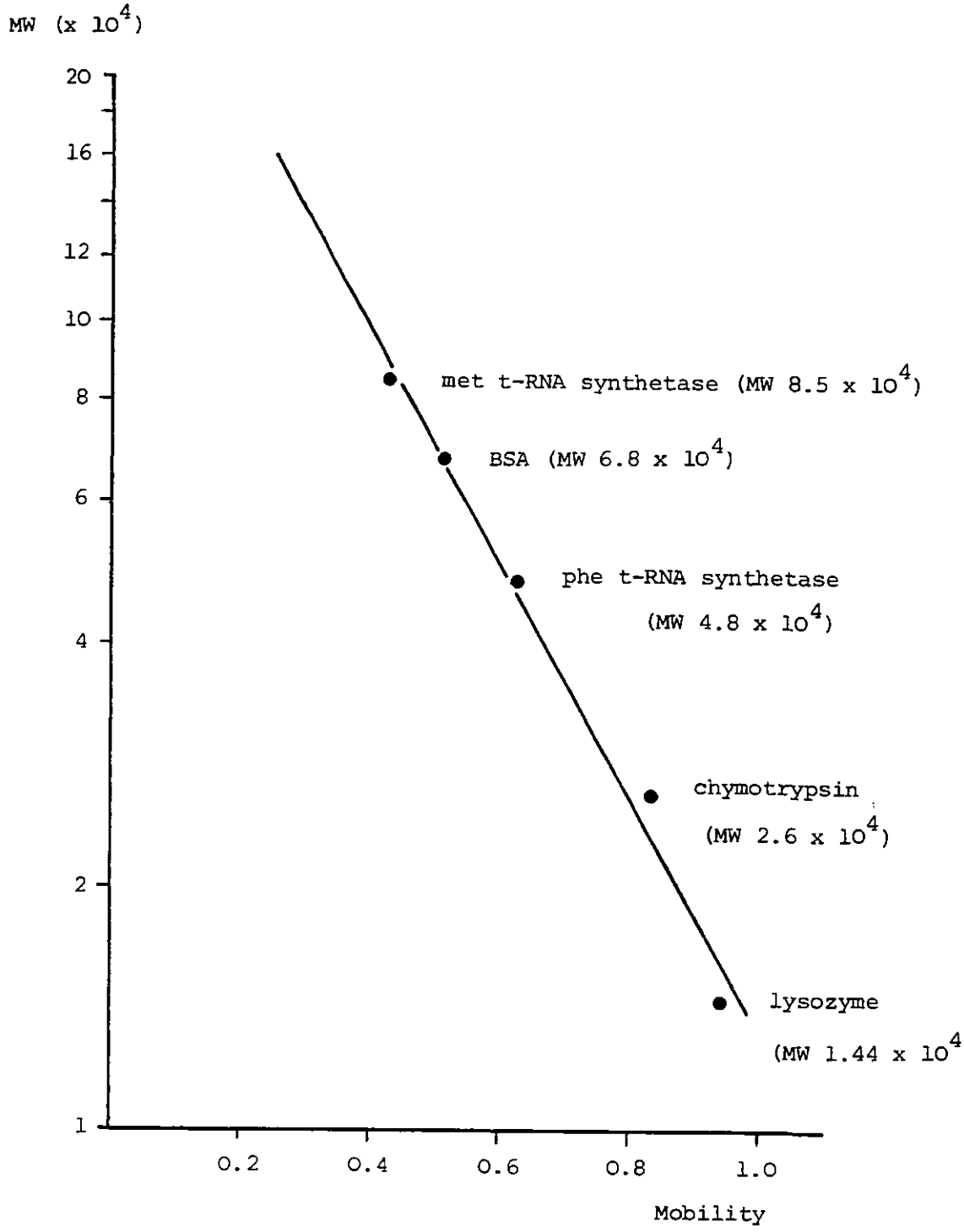
1% (v/v) β-mercaptoethanol at 100°C for 3 min, to which was added 30 µl of the sorbitol-bromophenol blue solution prior to application to the gels. The gels were run anodically at 6 mA/gel for 4 h at room temperature.

Gels were stained for protein with Coomassie blue after Chrambach et al (1967) (Coomassie brilliant blue R, 0.25% (w/v); methanol, 50% (v/v); glacial acetic acid, 10% (v/v)) for 3 h. They were destained in several changes of 7.5% (v/v) glacial acetic acid in 25% (v/v) methanol.

Detection of β-fructofuranosidase activity in gels was carried out using a modification of the 'post-incubation capture' technique of Gabriel & Wang (1969). Native protein gels were incubated in 10 mM sodium phosphate buffer, pH 6.0, containing 1.5% (w/v) sucrose for 1 h at 24°C. They were then washed thoroughly in distilled water, treated with 0.1% (w/v) TTC in 1 M NaOH in the dark for 15 min. and fixed in 10% (v/v) glacial acetic acid to fix the red formazan product (see above section 2.2.3).

Estimation of the molecular weights of proteins was carried out using denatured protein gels according to the methods described by Weber, Pringle & Osborn (1972). The following protein standards, lysozyme (MW 1.44 x 10^4), chymotrypsin (MW 2.6 x 10^4), phenylalanine tRNA synthetase (MW 4.8 x 10^4), bovine serum albumin (MW 6.8 x 10^4) and methionine tRNA synthetase (MW 8.5 x 10^4), were used to construct a standard curve of log molecular weight against mobility relative to the dye front (Figure 2). The molecular weight of an unknown was estimated from this curve by its migration distance relative to the dye front.

Figure 2: Standard curve for protein molecular weight estimation on SDS polyacrylamide gels



Mobility was relative to the dye front which was given a value of 1.0.

For details see Methods section 2.2.6.

2.3 Enzyme assays

2.3.1 Exo-β-1,3-glucanase

The activity of β-1,3-glucanase was assayed by following the production of glucose from the hydrolysis of a fungal β-1,3-D-glucan (Dickerson et al, 1970). Enzyme samples, 10 µg to 1 mg, and substrate at concentrations of 0.2 to 0.6% (w/v) were incubated in 0.1 M sodium acetate buffer, pH 5.0, at 30°C in a volume of 1 ml. Samples taken at intervals were heated to 100°C for 3 min. in a boiling water-bath to stop the reaction. Glucose was determined as described in section 2.2.1.

The fungal glucan substrate, prepared from Claviceps fusiformis cultures (see below, section 2.4.6), is a β-1,3 linked glucan possessing single glucose residue side chains joined to the main chain every third residue by β-1,6 linkages (Buck et al, 1968). Hydrolysis by this glucanase yields glucose and gentiobiose in a molar ratio of approximately 2:1. In assaying the β-glucanase activity of the crude enzyme extracts (see below), 'contaminating' β-glucosidase hydrolysed completely the gentiobiose produced by the action of the β-glucanase on the β-glucan. This effectively doubled the yield of glucose and hence, apparently, the rate of hydrolysis by the β-glucanase. The true activity of the β-glucanase was obtained as described previously (Dickerson et al, 1970) by adjusting, in this case halving, the rate of glucose production.

2.3.2 β-glucosidase

The activity of β-glucosidase was assayed using p-nitrophenol -β-D-glucopyranoside (Koch-Light) as substrate at a concentration of 1 mM. The reaction was carried out in 0.1M sodium acetate buffer, pH 5.0, at 30°C in a volume of 1 ml with 2 $\mu g ml^{-1}$ to 1 mg ml^{-1} of enzyme depending on purity. Samples were taken at intervals and

p-nitrophenol determined as described in section 2.2.2. Assays of the glucosidase against 'natural' disaccharide substrates were carried out under the same conditions. Samples taken at intervals were heated to 100°C for 3 min. to stop the reaction and glucose in the sample was determined as described in section 2.2.1.

2.3.3 β-fructofuranosidase

The activity of β-fructofuranosidase on sucrose was determined on a qualitative basis using the formazan method of Baker (1979) to detect the liberation of reducing sugar, i.e. monosaccharides. Enzyme samples, 10 μg ml^{-1} to 1 mg ml^{-1}, were incubated with an equal volume of 3% (w/v) sucrose in 0.1M sodium phosphate buffer, pH 6.0, at 30°C for 30 min. The reaction mixture was then tested for reducing sugar as described in section 2.2.3.

Quantitative determination of β-fructofuranosidase activity was obtained using radioactive U -^{14}C-sucrose as substrate (Dickerson, 1972). Enzyme, 3μg to 1 mg, was incubated with 2% (w/v) sucrose containing 2 μCi U-^{14}C-sucrose (394 m Ci $mmole^{-1}$) in 0.1M sodium phosphate buffer, pH 6.0, in a volume of 0.1 ml at 30°C. Samples, taken at intervals, were spotted on to paper chromatograms over mercuric chloride and developed as described in section 2.2.5.

2.4 Extraction procedures

2.4.1 Strain testing

Mycelial cultures of various strains of C. purpurea (see Results) were prepared in duplicate flasks of modified medium T, as described in section 2.1.2, and incubated at 24°C for 8 to 16 days. Flasks were harvested by filtering off mycelium under vacuum on a Buchner funnel, and the resulting culture filtrate centrifuged on a small bench centrifuge to remove spores. A sample of 40 ml of culture medium was tested for the presence of glucan by the addition of 2

volumes of cold absolute ethanol (Buck et al, 1968). Any glucan that precipitated was collected on a glass rod, resuspended in distilled water and reprecipitated to purify further. Finally it was dialysed against 5 L distilled water at 4°C before freeze drying. The remainder of the culture medium was dialysed against 3 changes of 5 L 1 mM sodium acetate buffer, pH 5.2, containing 0.1 mM EDTA at 4°C and freeze dried. This crude extract was assayed for protein content (section 2.2.4) and β-glucanase activity (Section 2.3.1).

2.4.2 Media modifications

Cultures of C. purpurea strain 6 were prepared, incubated, harvested and assayed as described above (2.4.1). However, various changes (see Results) were made in the constituents of the medium and their concentrations, prior to the flasks being inoculated and incubated.

2.4.3 Growth of C. purpurea 6 in modified medium T.

Mycelial cultures were prepared as described in section 2.1.2 and duplicate flasks were harvested at intervals up to 13 days after inoculation. Mycelium filtered off from the culture medium was washed in distilled water, dried on filter paper in an oven at 70°C to constant weight and weighed. The culture medium was assayed for glucan content, protein content and β-1,3 glucanase activity as described in section 2.4.1.

2.4.4 Isolation of protein from batch culture medium after growth of C. purpurea 6.

Mycelial cultures prepared in modified medium T (2.1.2) were harvested after 13 days culture by filtration under vacuum and the culture medium centrifuged at 4°C for 45 min. at 15,000 g to remove spores and cell debris. Subsequent steps were all carried out at 4°C. The resultant clear yellow liquid (2.5 litres) was dialysed against

2 changes of 10 litres 1 mM sodium acetate buffer, pH 5.2, containing 0.1 mM EDTA for 48 h. The non-dialysable residue was rapidly frozen over a mixture of acetone and solid CO_2 and freeze-dried. The freeze-dried solid was immediately resuspended in 80 ml 20 mM sodium acetate buffer, pH 5.2, and the resulting very viscous suspension further dialysed against 2 changes of 10 litres 1 mM sodium acetate buffer, pH 5.2, containing 0.1 mM EDTA for 72 h. The residue was centrifuged for 45 min. at 26,000 g to remove debris; minor lipid deposits were removed with lead free glass wool and the clear supernatant was freeze-dried. The protein content and enzyme activity of the resultant crude extract were determined, and the remainder was stored in a vacuum desiccator over anhydrous $CaCl_2$ at $-20^{\circ}C$.

Freeze drying was only employed for large quantities of crude or partially purified protein. Purer preparations were stored, after ultrafiltration, as concentrated buffered solutions in the presence of 0.02% (w/v) sodium azide at $4^{\circ}C$.

2.4.5 Isolation of protein from mycelium of C. purpurea 6 (For β-fructofuranosidase)

Mycelial cultures prepared in modified medium T (2.1.2) were harvested after 7 days culture by filtration as above. The mycelium was washed with 10 mM sodium phosphate buffer, pH 6.0, and disrupted by 2 passages through an Edebo X-press (A.B. Biox, Sweden) at $-20^{\circ}C$ using a pressure of 10 tons. To extract the β-fructofuranosidase, the disrupted fungal material was suspended and gently stirred for 2 h at $24^{\circ}C$ in 5 ml g^{-1} freshweight 10 mM sodium phosphate buffer, pH 6.0, containing 5% (w/v) sucrose. Subsequent steps were all carried out at $4^{\circ}C$. The suspension was centrifuged for 30 min. at 12,000 g to remove cell debris and filtered through lead free glass

wool to remove floating lipid. The solution was re-centrifuged for a total of 4 h at 35,000g to clear. The clear yellow supernatant was dialysed against 3 changes of 10 litres 1 mM sodium phosphate buffer, pH 6.0, containing 0.1 mM EDTA for 72 h. The non-dialysable residue was freeze-dried, immediately resuspended in 20 ml 1 mM sodium phosphate buffer, pH 6.0, containing 0.1 mM EDTA and further dialysed against 2 changes of 10 litres of the same buffer for 48 h. This final dialysis residue was re-centrifuged for 1 h at 35,000 g to clear and freeze-dried. The protein content and β-fructofuranosidase activity of the resultant crude intracellular extract were determined, and the remainder was stored in a vacuum desiccator over anhydrous $CaCl_2$ at -20°C.

2.4.6 Isolation of β-glucan from cultures of C. fusiformis

Mycelial cultures of Claviceps fusiformis strain 139/2/1 which did not autolyse glucan were prepared as described by Buck et al (1968). The viscous cultures were diluted with an equal volume of distilled water prior to harvesting by filtration under vacuum. The harvested culture medium was heated to 100°C over a boiling water bath for 30 min. to destroy any enzyme activity present in the glucan and cooled. The glucan was precipitated with an equal volume of cold absolute ethanol, collected on a glass rod and re-suspended in distilled water by stirring at room temperature for 20 h. This suspension was then centrifuged at 35,000 g for 1 h to remove debris, dialysed at 4°C against 3 changes of 10 litres distilled water for 48 h and freeze dried.

2.5 Enzyme purification procedures

The following ion exchange and gel chromatographic procedures were employed in attempts to purify the particular enzymes being studied from crude extracts:

i) β-1,3-glucanase: Sephadex G-200, diethylaminoethyl cellulose DE-52, carboxymethyl cellulose CM-52, Sulphopropyl Sephadex C-50 and Sephacryl S-200 superfine.

ii) β-glucosidase: Sulphopropyl Sephadex C-50, Sephacryl S-200 superfine and hydroxyapatite.

iii) β-fructofuranosidase: diethylaminoethyl cellulose DE-52, Sephacryl S-200 superfine and hydroxyapatite.

Of these chromatographic techniques, the methods described in detail below gave consistently successful results in the purification of these enzymes. All columns were run at 4°C and all buffers used were degassed.

2.5.1 Sulphopropyl Sephadex

Sulphopropyl Sephadex C-50 (Pharmacia), 5 g, was swollen in 500 ml of the running buffer (0.1M sodium acetate buffer, pH 5.0) for 3 days. A column of the gel, dimensions 18.5 x 2 cm, was prepared and equilibrated at 4°C in running buffer at a flow rate of 20 ml h^{-1} using a peristaltic pump; 6 column volumes of buffer were used. Crude extracellular extract (2.4.4) was suspended in the running buffer to a maximum concentration of 3 mg protein ml^{-1} and dialysed against running buffer for 24 h. The dialysed suspension was applied to the column using a flow rate of 20 ml h^{-1} and washed with 2.5 column volumes of running buffer.

A linear gradient, prepared according to the method of Bock & Ling (1954), of 0 to 0.15M NaCl in a total of 8 column volumes of running buffer was applied at the same flow rate as before. This was followed by a second linear gradient of 0.15 to 0.5M in a total of 5 column volumes of running buffer. Fractions, 5 ml, were collected, their protein content estimated, and fractions containing protein

were assayed for enzyme activity. Active fractions were bulked, their volume reduced by ultrafiltration (Amicon 52, 10000 mol.wt. exclusion limit filter, 30 p.s.i. at 4°C), dialysed against 2 changes of 5 litres 1mM sodium acetate buffer, pH 5.0, containing 0.1 mM EDTA and freeze dried.

2.5.2 Sephacryl

Sephacryl S-200 superfine (Pharmacia) was equilibrated in several changes of degassed column running buffer (0.1M sodium acetate buffer, pH 5.2, containing 0.5M NaCl), poured into a glass column using an extension tube, and packed using a peristaltic pump and descending buffer with successive flow rates of 40 ml h^{-1} for 2 h and 6 ml h^{-1} for 18 h. The flow was reversed and equilibration completed at a rate of 4 ml h^{-1} for 30 h. The final column dimensions were 85 x 1 cm. Enzyme samples, 2 to 10 mg, were applied in a maximum volume of 0.75 ml running buffer to the base of the column and eluted by ascending flow at a rate of 3 ml h^{-1}. Fractions, 1 ml, were collected, their protein content determined, and fractions containing protein assayed for enzyme activity. Active fractions were bulked, reduced in volume by ultrafiltration (Amicon 12, 10,000 mol.wt. exclusion limit filter, 30 p.s.i. at 4°C) and stored at 4°C in the presence of 0.02% (w/v) sodium azide.

2.5.3 Diethyaminoethyl cellulose

This was used specifically for the purification of β-fructofuranosidase. Diethylaminoethyl (DEAE) cellulose DE-52 (Whatman) was degassed in 0.15M H_3PO_4, pH 2.0, for 30 min, titrated to pH 6.0 with 0.5M NaOH and washed several times in the running buffer, 50 mM sodium phosphate, pH 6.0, to remove fines. A column of the gel, dimensions 18.5 x 2 cm, was poured and packed at a flow rate of 45 ml h^{-1} for 1 h using a peristaltic pump. Equilibration was completed at the rate of

20 ml h^{-1} using a further 6 column volumes of buffer.

Crude intracellular extract (2.4.5), containing 100 mg protein was suspended in running buffer (4 mg ml^{-1}) and dialysed against the same buffer for 24 h prior to application. The column was washed after application with 2.5 column volumes of running buffer before applying a linear gradient of 0 to 0.15M NaCl in 8 column volumes of the same buffer. Fractions, 5 ml, were collected, their protein content determined, and fractions containing protein assayed for β-fructofuranosidase activity by the Formazan method (2.3.3). Active fractions were bulked, the volume reduced by ultrafiltration (Amicon 52, as above), dialysed against 2 changes of 5 litres 1mM sodium phosphate buffer, pH 6.0, containing 0.1 mM EDTA and freeze-dried.

2.5.4. Hydroxyapatite

Hydroxyapatite was equilibrated in several changes of 10mM sodium /potassium phosphate buffer, pH 6.8, using 20 volumes on each occasion. A column, dimensions 4 x 1.2 cm and volume 5 ml, was made up, which was further equilibrated with 15 ml of the same buffer. Protein samples, 2 to 10 mg, were dialysed against the same buffer for 24 h and applied in a volume of 1 to 2 ml directly to the top of the column followed by a wash of 10 ml buffer.

The elution of β-glucosidase samples was carried out using successive washes at concentrations of 0.25 and 0.5M sodium/potassium phosphate buffer, pH 6.8, and 0.8M sodium/potassium phosphate buffer, pH 7.5, each in 3 column volumes. For β-fructofuranosidase samples, elution was carried out using a linear gradient of increasing buffer concentration from 10 to 250 mM sodium/potassium phosphate buffer, pH 6.8. In both types of elution a flow rate of 1.5 ml h^{-1} was obtained by hydrostatic pressure, and 1 ml fractions were collected. The protein content of fractions was determined, and fractions contain-

ing protein assayed for enzyme activity. Active fractions were bulked, the volume reduced by ultrafiltration (Amicon 12, see above) and stored at 4°C in the presence of 0.02% (w/v) sodium azide.

2.6 Immunological methods

2.6.1 Preparation of antisera

Antisera were prepared in Old English rabbits and, prior to immunization, a sample of blood was taken as control non-immune serum. Purified enzyme, 75 µg, was suspended in 0.5 ml phosphate buffered saline (PBS; 0.03M sodium phosphate buffer, pH 7.2, containing 0.85% (w/v) NaCl) and emulsified with 0.5 ml Freund's complete adjuvant. Of this emulsion 0.25 ml was inoculated intramuscularly into each of 2 sites in the rabbit's leg, and 0.15 ml inoculated subcutaneously at each of 3 sites. After 14 days this inoculation procedure was repeated. A final inoculation using 75 µg purified enzyme in 0.5 ml PBS emulsified with 0.5 ml Freund's incomplete adjuvant was carried out after a further 14 days, again at 2 intramuscular and 3 subcutaneous sites. Prior to the second and final inoculations, a small sample of 1.5 ml blood was removed from the rabbit and the serum tested for the appearance of antibodies by immunodiffusion plates (2.6.3).

The rabbit was bled from the ear 12 days after the final injection; 30 ml of blood was collected and clotted by incubation at 37°C for 2 h and stored at 4°C overnight. Clotted blood cells were removed by centrifugation at 25,000 g for 45 min. at 4°C. The remaining clear serum was divided into 2 ml aliquots, frozen rapidly over a mixture of acetone and solid CO_2 and stored at -20°C.

2.6.2 Purification of immunoglobulin G (IgG) fraction from serum

A modification of the methods of Levy & Sober (1960) and Brandtzaeg (1973) was followed. DEAE - cellulose was suspended in a 20 fold excess of 5% (w/v) KH_2PO_4, pH 4.7, and degassed. It was then extensively equilibrated by washing in running buffer (10mM potassium phosphate buffer, pH 7.8) until the pH remained constant. A 5 ml column, dimensions 4 x 1.2 cm, was poured and washed with 2 column volumes of running buffer to complete equilibration at 4°C. Frozen serum, 2 ml, was thawed slowly, dialysed against 3 changes of running buffer for 36 h at 4°C, and applied directly to the top of the column. The column was eluted with 2 column volumes of running buffer, followed by the same volume of buffer containing 50 mM NaCl. Fractions, 1 ml, were collected and the protein content determined (2.2.4). The 2 elution steps produced 2 separate protein containing fractions, both of which were substantially pure IgG. The samples comprising these fractions were bulked separately, the NaCl concentration in each made up to 0.15M (0.85%, w/v), and sodium azide added to a concentration of 0.01% (w/v) before storage at 4°C.

2.6.3 Immunodiffusion plates.

Immunodiffusion was carried out in agar plates according to the methods of Ouchterlony & Nilsson (1978). A solution of 0.5% (w/v) Ionagar was prepared in PBS, poured on to glass microscope slides and allowed to set. Wells, 2.5 mm in diameter, were cut in a hexagonal array using a template and cutter, and sealed with 0.1% Ionagar in PBS. Serial doubling dilutions of 1:1 to 1:64 of antiserum and enzyme were prepared in PBS. Antiserum dilutions (10 µl) were pipetted into the outer circle of wells and an enzyme dilution (10 µl containing 0.6 - 1.5 µg of enzyme) pipetted into the centre well. The slides were placed in a sealed humid atmosphere in the dark and developed for 20 h.

They were then washed in 3 changes of PBS for 24 h, stained with Coomassie blue for 30 min. and destained as described in section 2.2.6.

2.6.4 Preparation of infected plant material

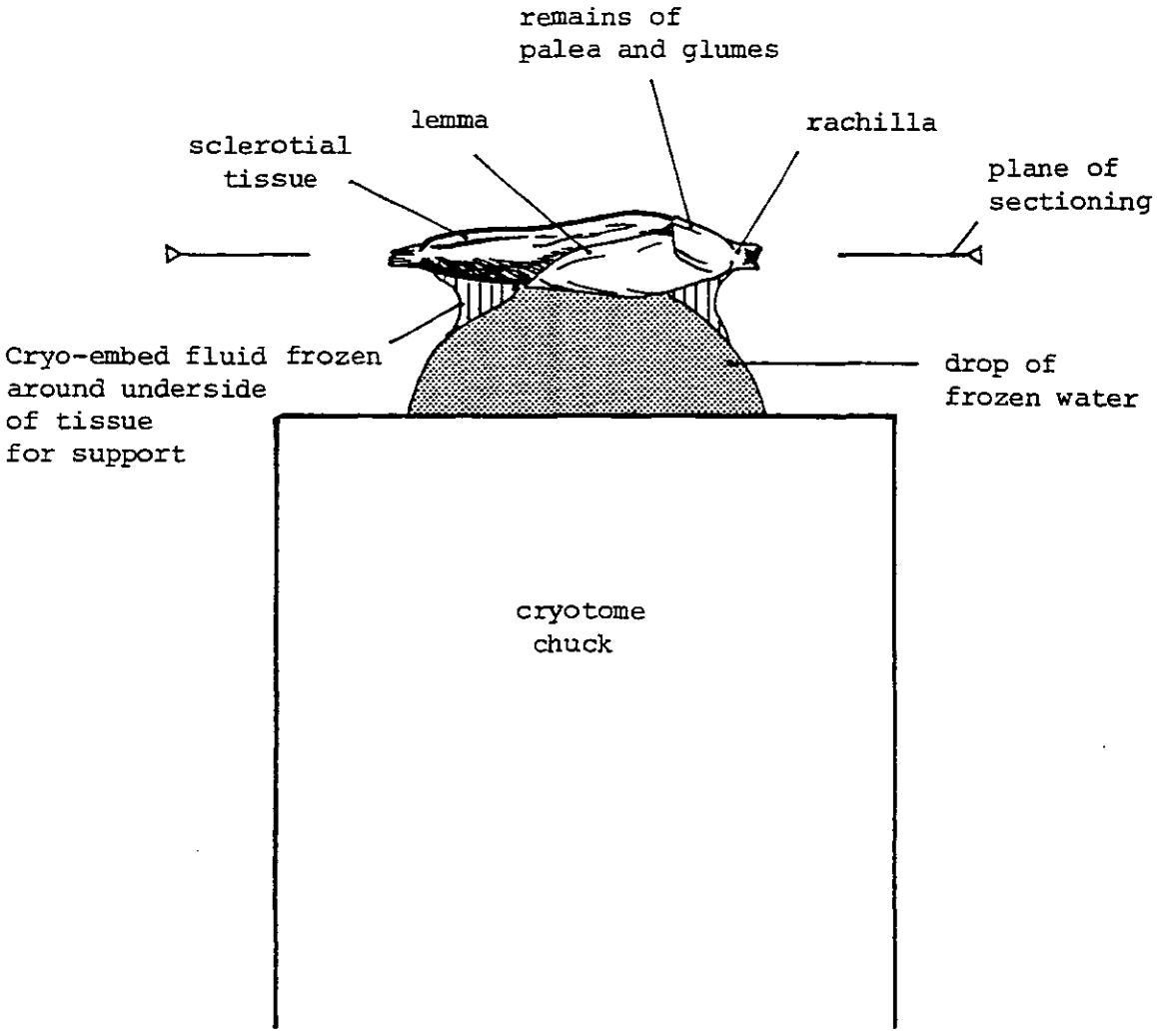
Ears of rye, spring sown, were inoculated just prior to anthesis. Infection was achieved by injection of a suspension of Claviceps purpurea conidia (10^5 spores ml^{-1}), into the prefloral cavity with a small hypodermic syringe, allowing the conidia direct access to the tip of the stigma and ovary. At intervals after infection (4,7,10,12, 14,18,21,28 and 35 days) both infected and uninfected florets were removed from the ears and the glumes and palea were carefully dissected away from the floret. The remaining infected ovary, or developing ergot, attached to the rachilla was then 'snapfrozen' by plunging into freon cooled to -180°C over liquid nitrogen. Frozen tissue samples were stored at -70°C.

2.6.5 Tissue Sectioning

Because of the differing structure and hydration of plant and fungal tissue, it proved difficult to maintain the original structure during cryostat sectioning of infected plant tissues. The following protocol was therefore developed (see Results).

Frozen florets were mounted horizontally on a cryotome chuck on top of a drop of water which was just freezing. A minimal amount of Bright Cryoembedding liquid (50 - 100 µl) was frozen around the underside of the floret to support it (Figure 3).

Figure 3: The mounting of frozen tissue for cryostat sectioning



Ergot tissue at 28 days old is shown approximately 5x life size

For details see Methods section 2.6.5.

The material was sectioned longitudinally in a cryostatic chamber (Bright Instrument Company, model OTF/AS/M/V) using a steel microtome knife. The temperature of both chamber and knife were maintained at ~25°C. Two consecutive ribbons of 3 to 5 sections, 10 μm thick, were transferred to each end of a microscope slide at room temperature and air dried.

2.6.6 Preparation of antibody stained sections

The antibody staining protocol employed was a modification of standard indirect staining methods (Goldman, 1968; Nairn, 1976) (See Results). All steps were carried out at 25°C.

i) Sections dried onto slides were fixed in 90% (v/v) ethanol for 30 min.

ii) Slides were placed flat in a humid chamber, and one ribbon of sections was covered with a few drops of a 1:4 dilution of purified enzyme antiserum in PBS. The other ribbon on the slide was covered with the same dilution of control non-immune serum and the slides were incubated for 45 min.

(iii) Slides were washed in 4 changes of PBS for 20 min, rinsed in distilled water, air dried and returned to the humid chamber. Both ribbons were covered with a 1:40 dilution (manufacturer's recommendations) of fluorescein isothiocyanate (FITC) conjugated IgG fraction of goat anti-rabbit IgG (Miles) in PBS, and incubated for a further 45 min.

(iv) Slides were again washed in 4 changes of PBS for 20 min, rinsed in distilled water and air dried.

(v) They were counterstained with 0.03% (w/v) methylene blue (C.I.52015, B.D.H.) in 0.1M sodium borate buffer, pH 8.0, for 3 min, washed in distilled water for 5 min, air dried, and mounted

in 25 µl 90% (v/v) glycerol in PBS.

2.6.7 Microscopy

Specimens were studied using epi-illumination from a HBO 50 watt mercury vapour lamp in a Zeiss WL microscope. A band-pass filter (450-490 nm) and 510 nm interference mirror gave a narrow band width of illumination from 450 nm to 500 nm. A 520 nm barrier filter in conjunction with large aperture objectives (x 16 and x 40), were used for viewing. Photomicrographs were taken with a Zeiss MC 63 camera attachment using Ektachrome high speed daylight professional reversal film (DIN 24), uprated onestop to DIN 25 for exposure.

3. RESULTS

3.1 Growth of Claviceps purpurea in axenic culture

3.1.1 Strain selection

In order to select a strain giving high yields of β-1,3-glucanase, strains of Claviceps purpurea were cultured, harvested and assayed as described in Methods section 2.4.1. The strains, namely 5/1, 5/8I, 6, 12/1, 17, 17/2, 25B, 29/4, 44, 88 and 330 were known to be infective on rye (Swan, 1980). Dickerson et al, (1970) had shown that β-1,3-glucanase activity in the culture medium of C. fusiformis was at a maximum by the 13th day of culture; preliminary experiments with C. purpurea strains 88 and 330 agreed with this and also indicated that β-glucanase activity in the medium was declining by the 16th day. Consequently cultures were routinely harvested after 12 days incubation.

Many of the strains produced glucan in the culture medium during incubation. For example, on day 6, strain 6 contained 0.6 mg glucan ml^{-1} medium and strain 88 contained 2.3 mg glucan ml^{-1} medium. However, no glucan was detectable in the culture medium after 10 days in all except 2 strains, 17 and 25B, both of which still contained glucan at day 12 (0.36 mg ml^{-1} and 0.94 mg ml^{-1} respectively), and had, as expected, correspondingly very low levels of β-glucanase activity. The protein content and β-1,3 glucanase activity of the culture medium obtained for the strains tested (with the exception of 17 and 25B) are given in table 1. On the basis of these results strain 6 gave the highest yield of β-glucanase activity and was selected for further experiments.

3.1.2 Growth of C. purpurea 6 following various changes to the medium

In an attempt to increase the production of β-glucanase by C. purpurea 6 during growth, changes were made in the carbon source,

Table 1: Protein content and β-1,3-glucanase activity from the culture media of Claviceps purpurea strains

Strain	β-1,3-glucanase activity*, μg glucose liberated $min^{-1}mg^{-1}$	Protein content of medium, mg ml^{-1}	Total activity, units 100 ml $medium^{-1}$ (i.e.per flask)
5/1	2.5	0.19	48.0
5/8I	2.6	0.16	43.7
6	6.3	0.10	67.7
12/1	0.2	0.31	6.4
17/2	3.2	0.10	34.6
29/4	2.8	0.18	51.2
44	2.1	0.09	20.8
88	2.6	0.08	22.6
330	1.4	0.22	31.4

For experimental procedures see sections 2.4.1 and 3.1.1.

* Activities are adjusted for the presence of β-glucosidase - see methods section 2.3.1.

nitrogen source and pH of the culture medium. Details of these alterations and the resultant protein yields and enzyme activities are given in table 2. The yield of glucanase from C. purpurea 6 grown in the original medium T (2.1.2) was also compared to that obtained with modified medium T. Cultures were harvested and assayed after 12 days incubation as described in methods sections 2.4.1 and 2.4.2.

The results represent experiments performed on different occasions for the changes in pH, carbon source, nitrogen source and comparison with medium T. The growth of the fungus was not always comparable between experiments, and this is reflected in the variation in yield of activity obtained in the control modified medium T samples. Despite this fact, no significant improvement in the yield of β-glucanase (total activity) was observed with any of the changes in media that were tested. Therefore the initial composition of modified medium T (Methods 2.1.2) was used for succeeding cultures of C. purpurea 6.

The course of C. purpurea 6 growth in modified medium T over 12 days is shown in Figure 4. Flasks were harvested and assayed as described in section 2.4.3. every 2 days.

3.1.3 Isolation of protein from the culture medium after growth of C. purpurea 6

Extracellular protein was prepared as described in Methods section 2.4.4. An average yield of 400 mg freeze-dried solid L^{-1} culture medium (± 10%) was obtained; the protein content of the solid was 51%. The specific activity of β-1,3-glucanase in the extract was 4.7 µg glucose liberated min^{-1} mg^{-1} protein with the assay procedure used, the value being adjusted for the presence of β-glucosidase (2.3.1). The β-glucosidase also present in the extract was assayed as described in section 2.3.2 and had a specific activity of 31.1 µg p-nitrophenol liberated min^{-1} mg $protein^{-1}$.

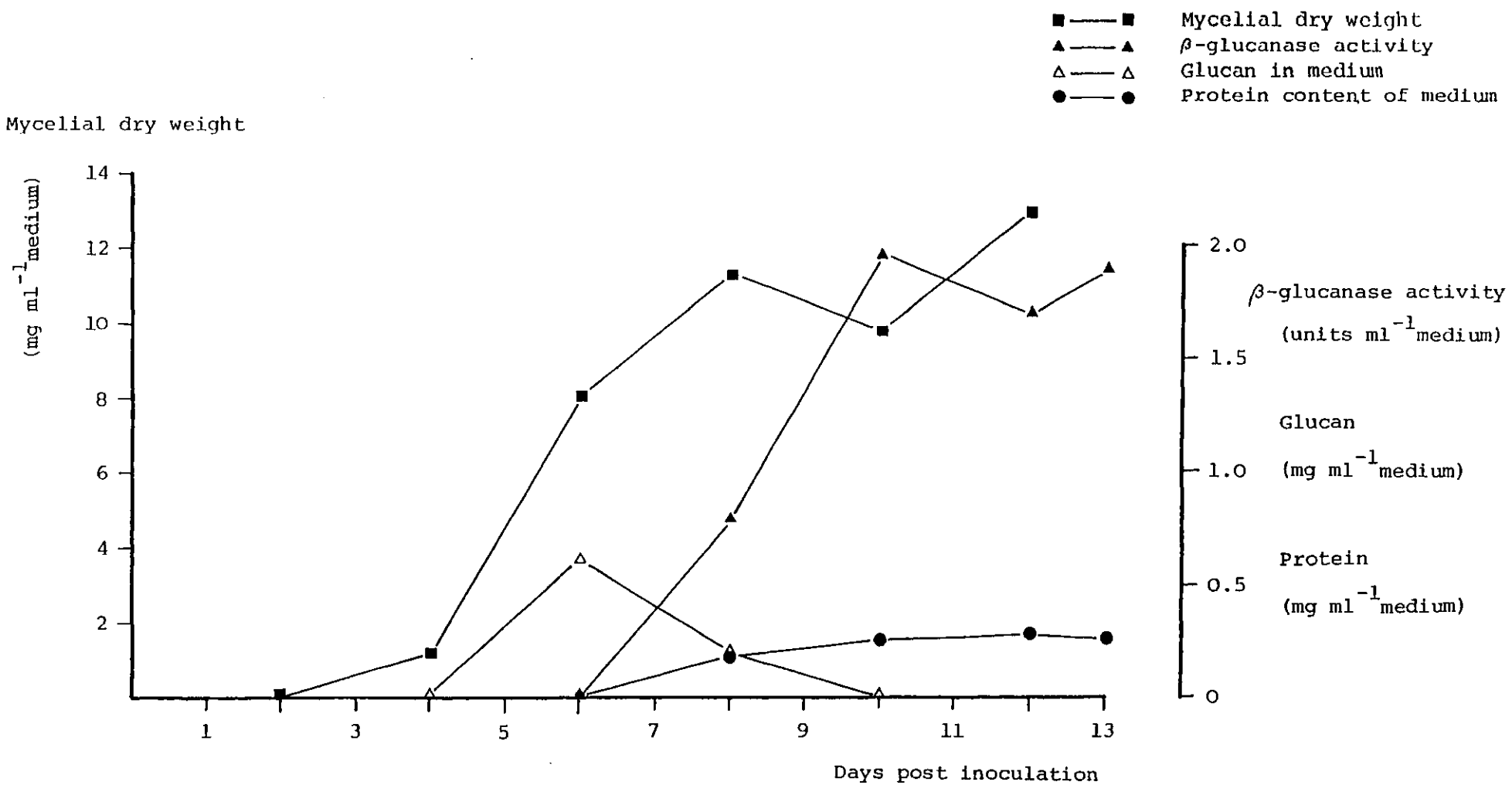
Table 2: Protein content and β-1,3-glucanase activity from the culture media of Claviceps purpurea strain 6 grown in altered media

		β-1,3-glucanase activity,* ug glucose liberated $min^{-1} mg^{-1}$	Protein content of medium, mg ml^{-1}	Total activity, units 100 ml medium $^{-1}$
Increased pH				
(control)	5.2	4.0	0.18	73.2
	5.6	3.3	0.14	48.5
	6.0	3.0	0.10	30.0
	6.4	2.2	0.10	23.1
Carbon source				
(control)	10% (w/v) sucrose	6.6	0.06	44.8
	5% (w/v) sucrose	4.4	0.05	23.8
	20% (w/v) sucrose	1.3	0.12	16.4
	10% (w/v) mannitol	2.5	0.01	.4.0
Nitrogen source				
(control)	0.5%(w/v) asparagine	4.2	0.22	95.8
	1.0%(w/v) asparagine	3.3	0.27	90.7
	1.0%(w/v) aspartic acid	3.6	0.26	93.6
Modified medium T		4.0	0.11	46.8
Medium T		1.9	0.18	34.4

For experimental procedures, see Methods section 2.4.2 and Results section 3.1.2. In each case, control represents the composition of modified medium T given in section 2.1.2.

* Activities are adjusted for the presence of β-glucosidase - see section 2.3.1.

Figure 4: Growth of C.purpurea 6 in modified medium T.



For experimental details see section 3.1.2.

3.1.4 Isolation of protein from the mycelium of C. purpurea 6

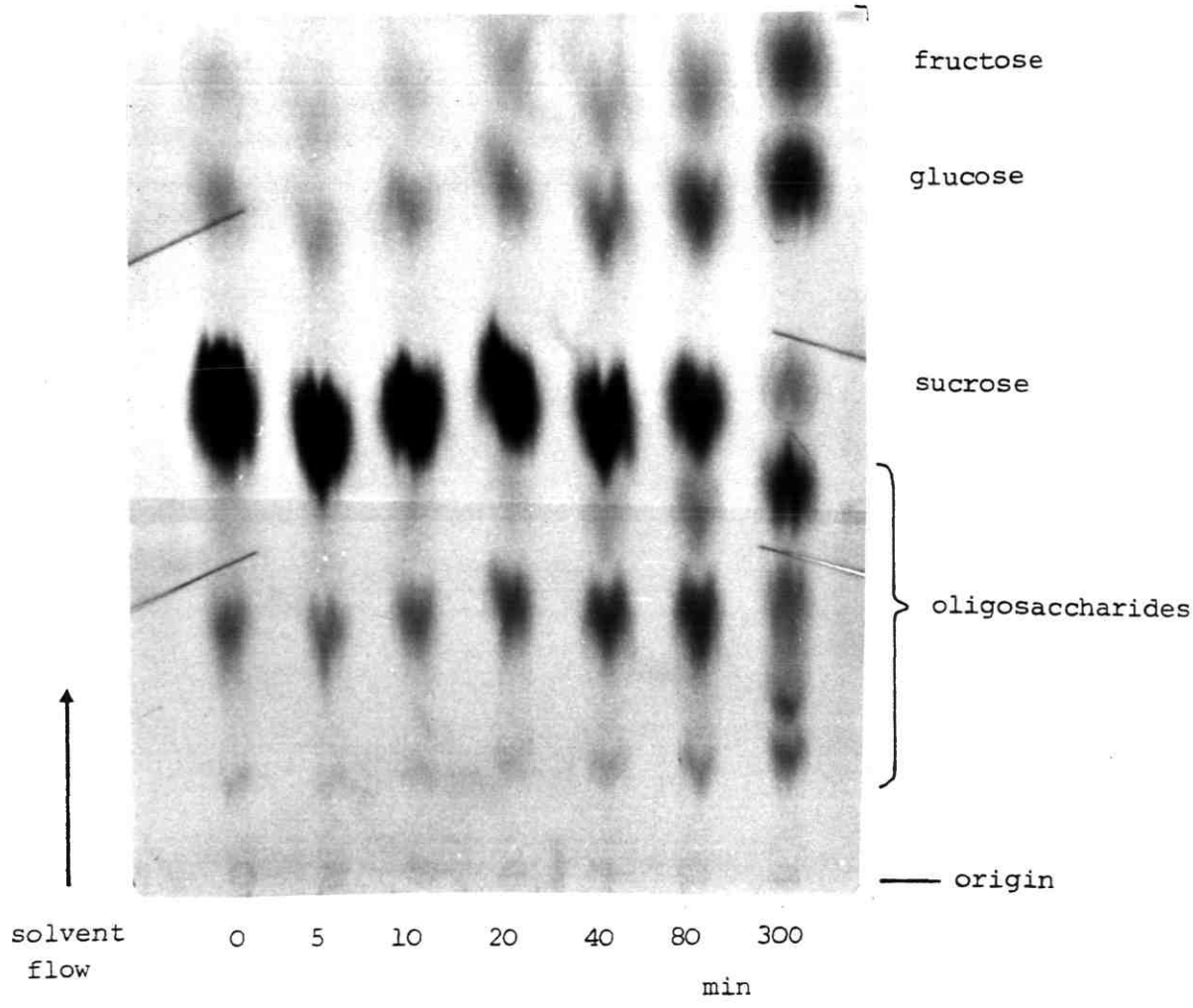
Intracellular protein was extracted as described in Methods section 2.4.5. The fresh weight of tissue upon harvesting after 7 days incubation was 66 g litre $^{-1}$ culture. The final freeze dried solid yield after extraction was equivalent to 2.21 mg g^{-1} fresh weight of original tissue. The protein content of this freeze dried extract was 71%. The presence of β-fructofuranosidase activity was detected using the Formazan test and assayed by hydrolysis of radioactively labelled U-^{14}C-sucrose (Methods section 2.3.3). It had a specific activity of 0.2 μmole sucrose hydrolysed min^{-1} mg^{-1} protein. The action of the β-fructofuranosidase in crude extract on sucrose gave rise to a number of oligosaccharides (Figure 5). This type of transfer activity, where glucose is released while the liberation of fructose is limited by the production of oligofructosides has been described previously for C. purpurea β-fructofuranosidase by Dickerson (1972).

3.2 Purification and properties of β-1,3-glucanase from C. purpurea 6

3.2.1 Purification of β-glucanase

Some difficulty was encountered in initial attempts to purify the β-glucanase, in particular because of the presence of β-glucosidase, which tended to co-migrate with the β-glucanase on various gels. Table 3 lists some of the gel chromatographic methods which were tried with varying success. The β-glucanase and β-glucosidase were found to co-purify in elutions from Sephadex G-200, DEAE-cellulose DE-52 and CM-cellulose CM-52. A much higher salt concentration than that used for elution from CM-52 was required to elute the enzymes from the strong cation exchanger Sulphopropyl Sephadex C-50, and some separation was achieved. The gradient for elution on this gel was adjusted until an ionic strength was obtained that gave clear separation;

Figure 5: Activity of β-fructofuranosidase from crude intracellular extract on U-^{14}C-sucrose - autoradiograph of products



For experimental details see section 3.1.4 and Methods sections 2.3.3 and 2.2.5.

Table 3: Column chromatography techniques employed in attempts to separate β-1,3-glucanase and β-glucosidase activities from crude extracellular extracts

Column gel	Chromatography conditions	Reference	Result
Sephadex G-200	Column dimensions 45 x 2.5 cm, void volume 65 ml. Buffer, 10 mM sodium acetate, pH 5.2, Flow rate, 0.3 ml min^{-1}, 160 x 2.5 ml fractions collected	Baker, 1979	Broad heterogeneous range of protein species which were not separated. β-glucanase and β-glucosidase activity eluted in the same range from fractions 55 to 80.
DEAE-cellulose De-52	Dimensions 19 x 2 cm, buffer 0.1 mM sodium acetate, pH 5.2. Elution gradient 0.5 to 125 mM concentration of the same buffer. Flow rate, 0.5 ml min^{-1}, 70 x 5 ml fractions collected.	Dickerson et al, 1970	β-glucanase and β-glucosidase activities did not bind to the column and were eluted in the void volume.

Table 3: Continued

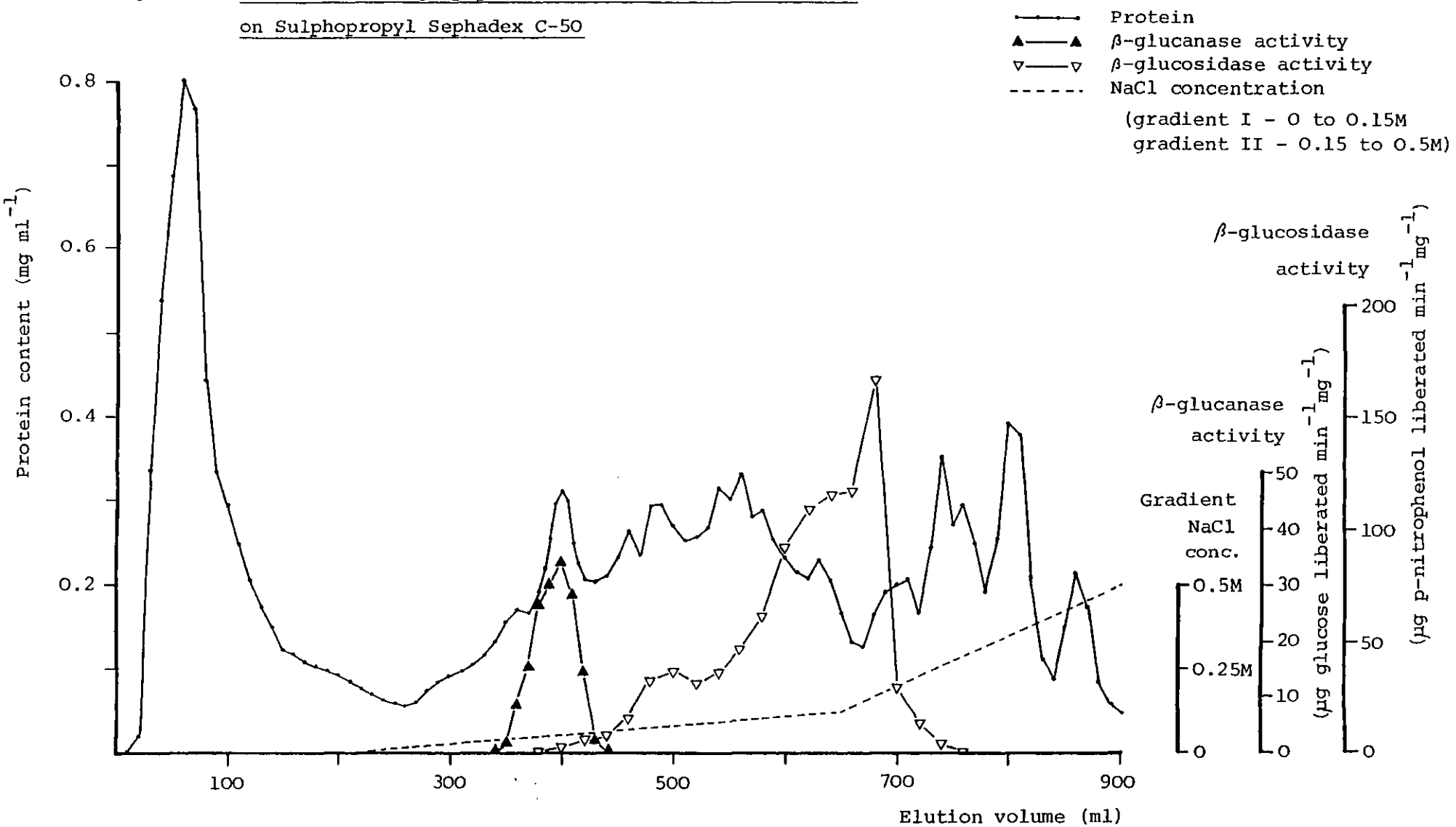
CM-cellulose CM-52	Dimensions 11 x 1.5 cm, buffer 5 mM sodium phosphate, pH 7.5. Elution gradient 5 to 75 mM concentration of the same buffer. Flow rate 0.5 ml min^{-1}, 70 x 5 ml fractions collected.	Both enzyme activities bound to column. β-glucanase eluted in fractions 31-38 and β-glucosidase in fractions 15 - 50, and hence were not separated.
Sulphopropyl Sephadex C-50	Dimensions 13 x 1.5 cm, buffer 0.1M sodium acetate, pH 5.0. Elution gradient 0 to 0.2 M NaCl in the same buffer. Flow rate 0.5 ml min^{-1}, 45 x 5 ml fractions collected.	Both activities bound to the column and some separation was achieved. β-glucanase eluted in fractions 23 - 33 and β-glucosidase in fractions 27 - 45.

this was found to be a very 'shallow' gradient. Sulphopropyl Sephadex was therefore employed as the first stage in the fractionation of crude extracellular extract and purification of β-1,3 glucanase.

Extracellular extract containing 175 mg protein was applied to a column of Sulphopropyl Sephadex C-50 prepared and eluted as described in Methods section 2.5.1, and 180 x 5 ml fractions were collected. The protein content and enzyme activity of the fractions are shown in Figure 6. The activity of β-glucanase eluted as a single peak between 0.04M and 0.07M NaCl on the gradient and was almost completely separated from the β-glucosidase activity (see below for further purification), which eluted over a broad range of 0.07 to 0.26M NaCl on the gradient. Fractions containing β-glucanase activity (71-84) were bulked (2.5.1) and gave a total protein yield of 15.3 mg. Fractions containing β-glucosidase activity (105-140) having a total protein content of 55.8 mg were bulked and retained for further purification (see section 3.3.1). The activity of β-fructofuranosidase was detectable by the Formazan test in the protein which did not bind to the column; this material (fractions 4 to 30 containing 51 mg protein) was also retained (see section 3.4.3).

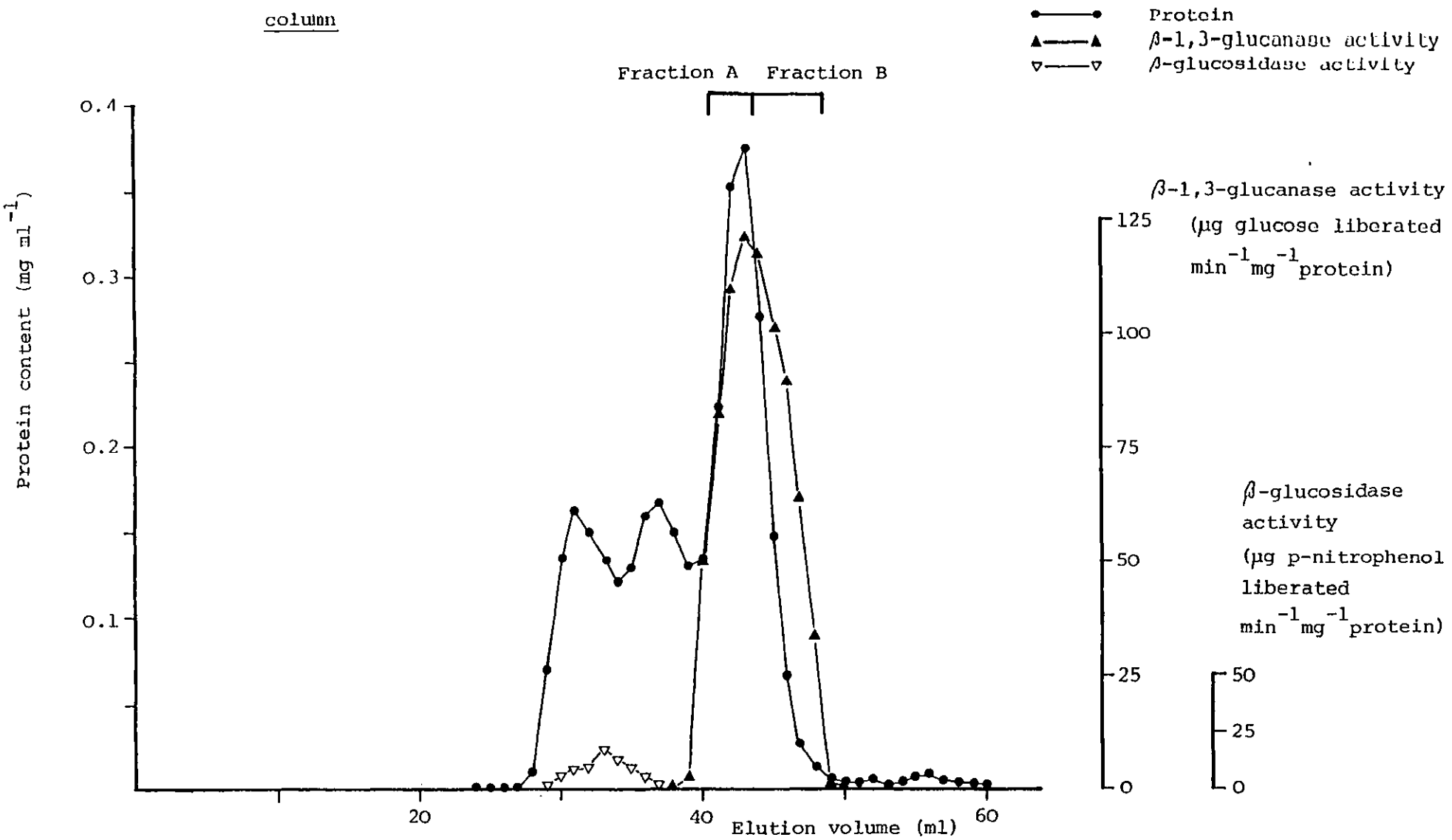
The β-glucanase was further purified using Sephacryl S-200 superfine. Samples containing β-glucanase activity (5 mg protein) were applied to the column and eluted as described in Methods section 2.5.2. The protein content and enzyme activity of fractions (80 x 1 ml) eluted from this column are given in figure 7. The activity of β-glucanase was eluted as a single peak and was free from any contaminating β-glucosidase activity. The fractions comprising the peak of β-glucanase activity were bulked as two separate samples (A, fractions 41 to 43 and B, fractions 44 to 47), because it was deemed likely, from the elution profile, that the latter half of the peak (sample B) would be free from traces of the protein which eluted

Figure 6: Column chromatography of crude extracellular extract on Sulphopropyl Sephadex C-50



For experimental details see section 3.2.1.

Figure 7: Elution of β-1,3-glucanase activity from a Sephacryl S-200 column



For experimental details see section 3.2.1.

in the peak immediately preceding the peak containing β-glucanase activity. The total protein yield from this column was 2.8 mg.

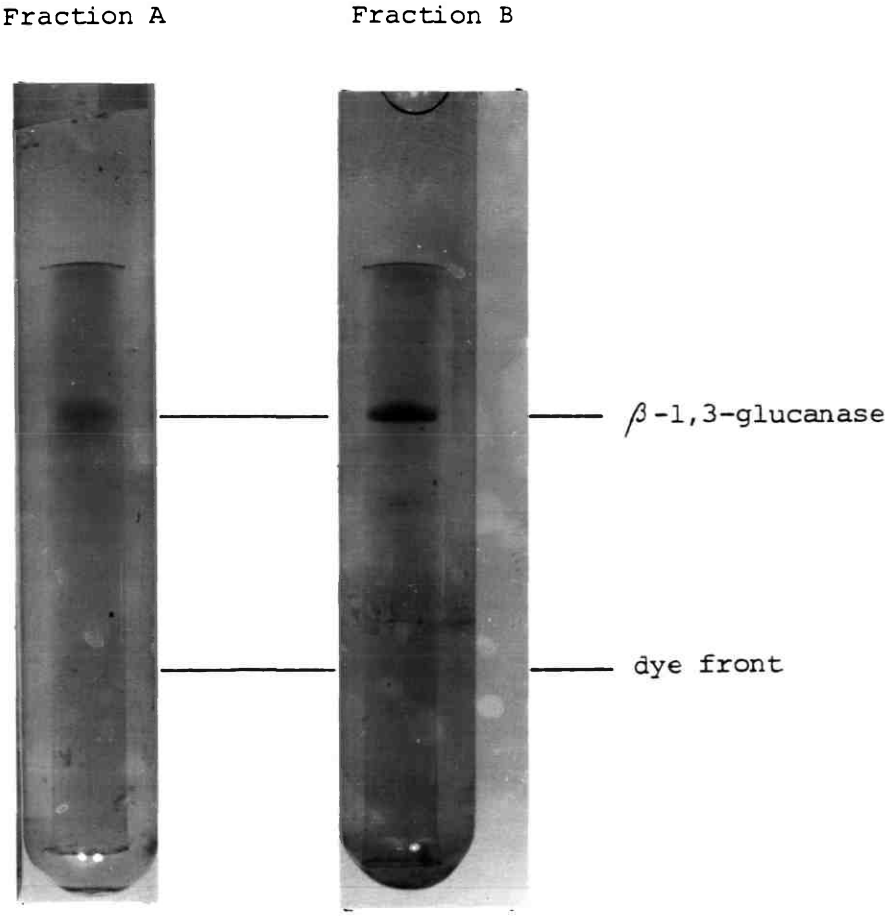
3.2.2 Analysis of purified β-glucanase

The samples from the column described above were analysed by native and SDS gel electrophoresis (Methods section 2.2.6). Analysis by native protein gels was not pursued further, since, under the electrophoresis conditions employed, the β-glucanase samples migrated no more than 1-2 mm into the gel. On denatured protein SDS gels, sample B from the above column was seen to contain only a single protein species (Figure 8). The purity of the β-glucanase in this sample was therefore confirmed.

3.2.3 Specific activity of purified β-glucanase

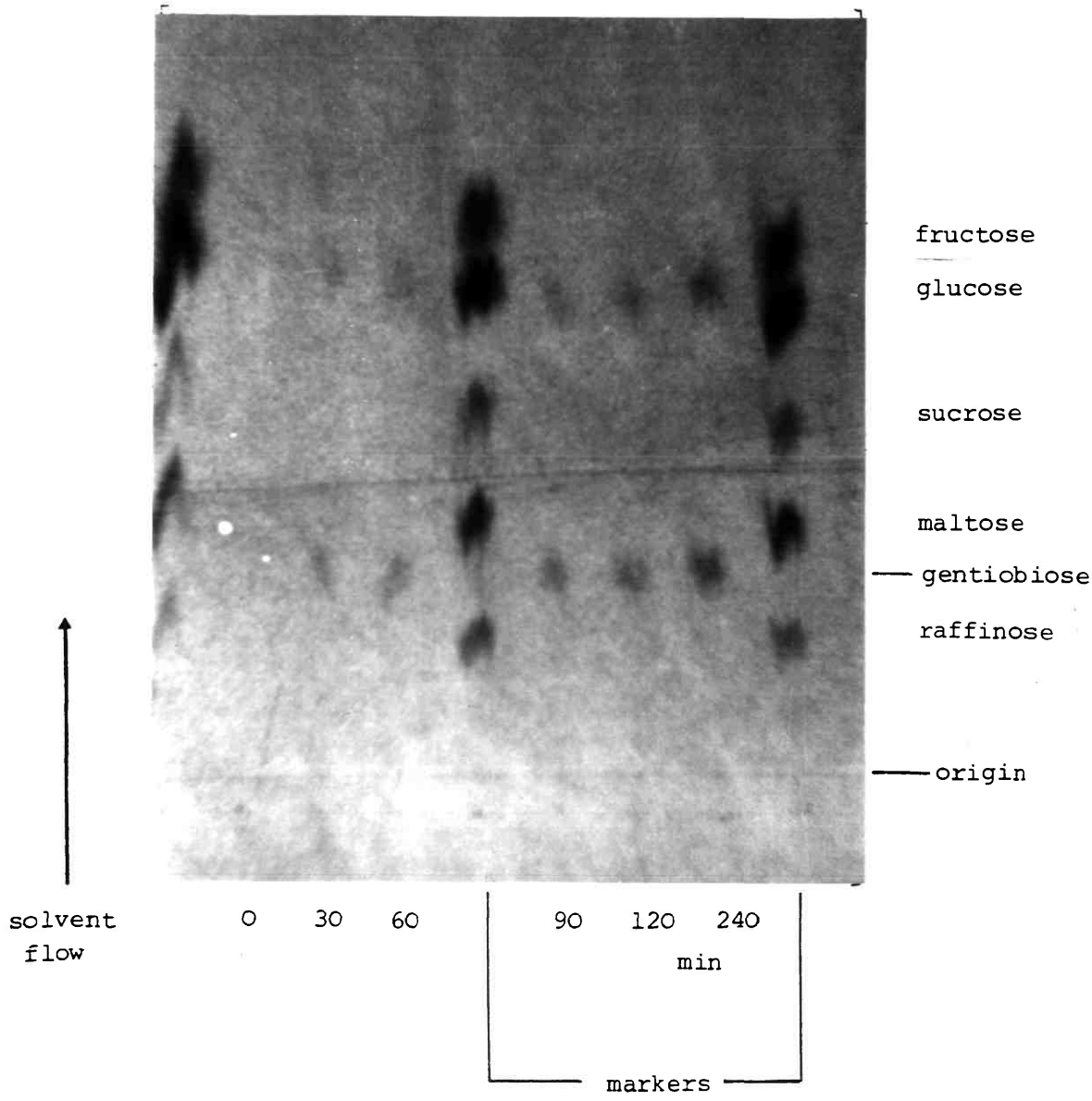
Purified enzyme, 50 μg ml^{-1}, was incubated at 30°C with 0.5% (w/v) glucan in 0.1M sodium acetate buffer, pH 5.0. The reaction products were determined as in Methods sections 2.2.1 and 2.3.1, and also on paper chromatograms as described in Methods section 2.2.5. The specific activity was 209 μg glucose liberated min^{-1} mg^{-1} protein. Chromatograms demonstrated that the only detectable reaction products from the hydrolysis of the glucan were glucose and gentiobiose (Figure 9). The ratio of glucose to gentiobiose was compared on chromatograms with that observed for the hydrolysis of this glucan by the QM806 exo-β-1,3-glucanase (Buck et al, 1968; Dickerson et al, 1970; Baker, 1979). On a qualitative basis, the ratios were the same, and it was concluded that no hydrolysis of gentiobiose was occurring during the period of the assay, and that all contaminating β-glucosidase activity had been removed. The activities, protein yields and purification factors for the steps in the purification of the C. purpurea β-1,3-glucanase are given in Table 4.

Figure 8: Analysis of protein containing β-1,3-glucanase activity from the Sephacryl column on SDS polyacrylamide gels



For experimental details see section 3.2.2 and Methods section 2.2.6.

Figure 9: Activity of the purified β-1,3-glucanase - chromatograph of products



For experimental details see section 3.2.3.

Table 4: Purification of the exo-β-1-3-glucanase from C. purpurea 6.

	Protein mg	Specific Activity*	Total Activity	Fold Purification	Yield %
Crude extract	175	4.7	823	1	100
Sulphopropyl sephadex C-50	15.3	43	658	9.1	80
Sephacryl S-200 superfine	2.8	209	585	44.5	71

For experimental procedures, see Methods section 2.5 and Results Section 3.2.1.

* µg glucose liberated min^{-1} mg $protein^{-1}$.

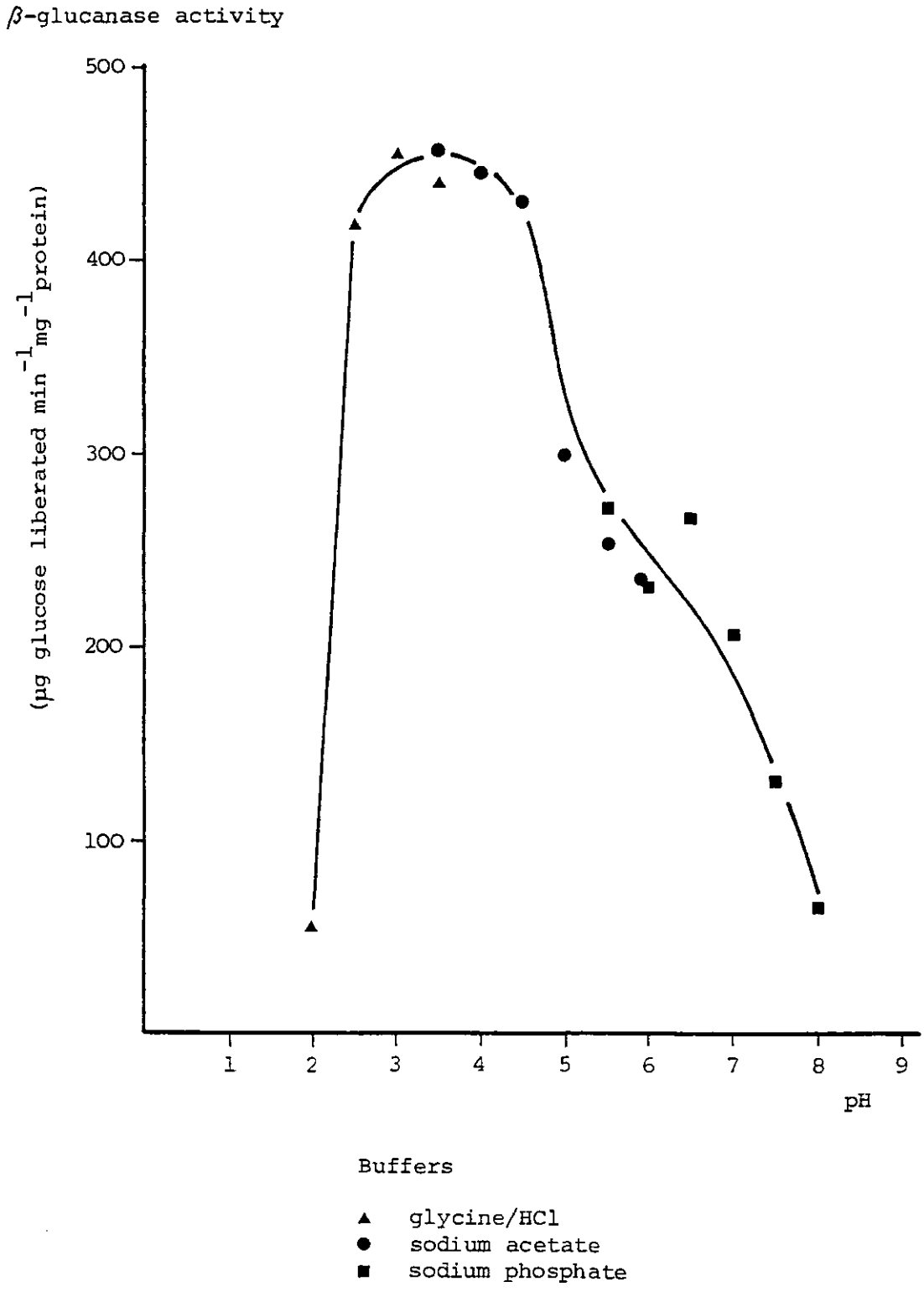
3.2.4 The pH optimum of β-glucanase

Purified enzyme, 12.5 μg ml^{-1}, was incubated at 30°C with 0.2% (w/v) glucan in each of the following buffer ranges; glycine/HCl, pH 2.0 to 3.5; sodium acetate, pH 3.5 to 6.0; sodium phosphate, pH 5.5 to 8.0. The buffers were used at a concentration of 0.2M to ensure adequate buffering close to the limits of particular buffer ranges. The substrate concentration was lower than previously used, in order to minimise inaccuracies arising from possibly inadequate mixing of the small amount of enzyme with the viscous glucan substrate. Preliminary experiments had determined that the reaction rate was still 1^{st} order after 10 min. with these concentrations of enzyme and substrate. The results (Figure 10) indicate that the β-glucanase had a pH optimum of 3.5 to 4.0, and retained 75% of its maximum activity between pH 2.4 and 5.5.

3.2.5 The molecular weight of β-glucanase

The molecular weight was estimated from SDS polyacrylamide gels as described in Methods section 2.2.6. The β-glucanase had an average mobility of 0.39 (3 replicates) relative to the dye front, with a consequent molecular weight of 99,000 ± 6,000. The rather large margin of error arises from the fact that no suitable protein standards with molecular weight over 85,000 were available at the time of experimentation.

Figure 10: The pH optimum of β-1,3-glucanase



For experimental details see section 3.2.4.

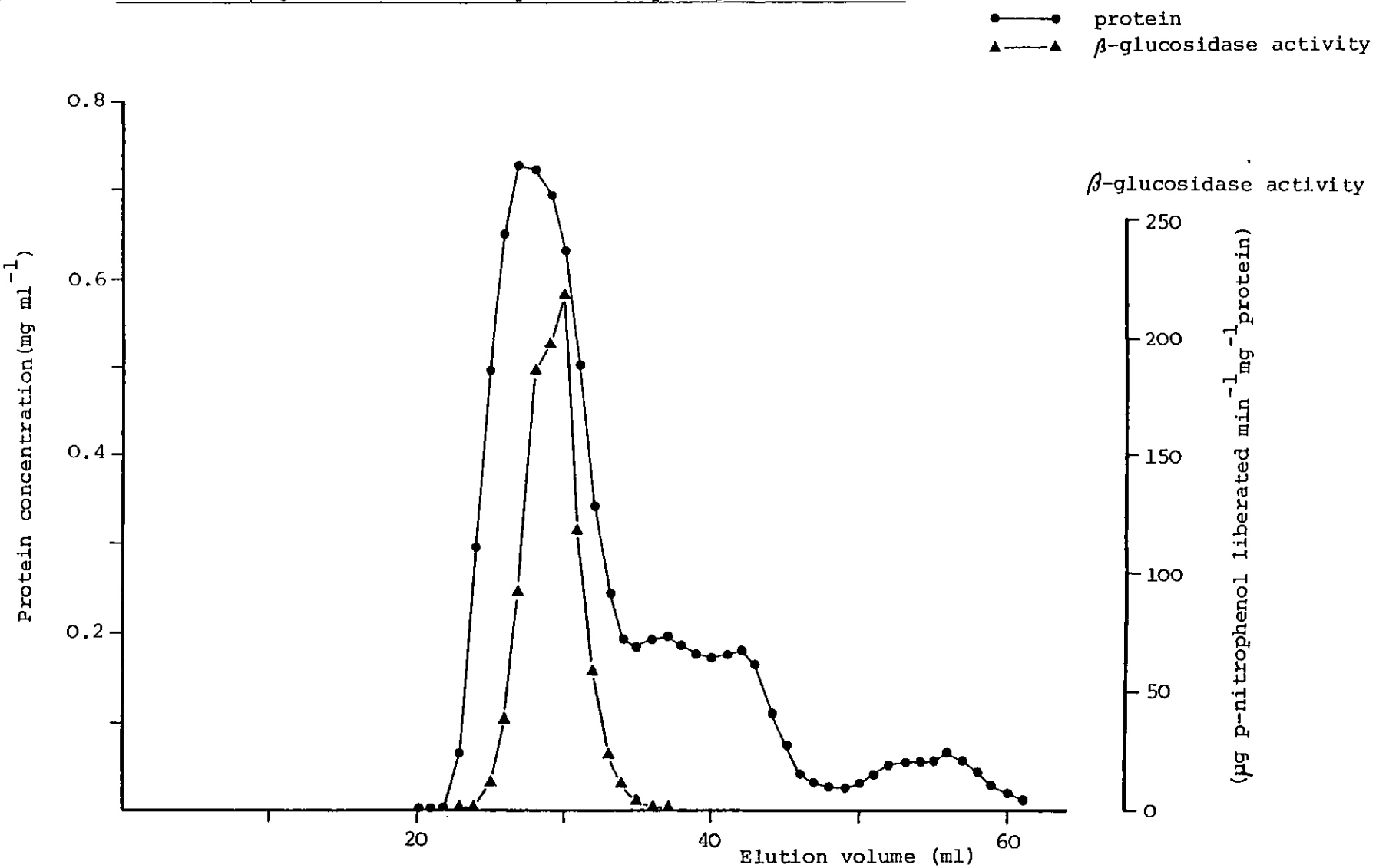
3.3 Purification and properties of β-glucosidase from C.purpurea 6

3.3.1 Purification of β-glucosidase from extracellular extracts

Combined fractions containing β-glucosidase activity from the Sulphopropyl Sephadex C-50 column (3.2.1) containing 10 mg protein were applied to a Sephacryl S-200 column as described in Methods section 2.5.2. Fractions, 80 x 1 ml, were collected and assayed for protein content and β-glucosidase activity (Methods sections 2.2.4 and 2.3.2). The β-glucosidase activity was eluted in a single peak, fractions 26 to 33, which contained 80% of the protein applied to the column (Figure 11). Active fractions were analysed by SDS gel electrophoresis (2.2.6) and the gels showed that five different protein species were still present in fractions containing β-glucosidase activity (Figure 13).

In an attempt to purify further the β-glucosidase, active fractions from the Sephacryl column, described above, were applied to a second Sulphopropyl Sephadex C-50 column. The column was prepared as described in Methods section 2.5.1, with the exceptions that 0.1M sodium acetate buffer, pH 4.5, containing 0.1M NaCl was used, and the final column dimensions were 5.5 x 1.5 cm. A linear gradient of 100 ml, increasing in ionic strength from 0.1M to 0.4M NaCl in the same buffer, was employed for elution. Fractions, 110 x 1 ml, were collected and assayed for protein content and β-glucosidase activity as above. The elution of β-glucosidase activity resembled that obtained in the previous Sulphopropyl Sephadex column (3.2.1), in that the enzyme activity was eluted in a broad peak between 0.14M and 0.26M NaCl on the gradient. Analysis of the fractions containing β-glucosidase activity by SDS gel electrophoresis (2.2.6) revealed that there were four different protein species still present in the fractions (Figure 13) of which, presumably, not all were β-glucosidase. Analysis of β-glucosidase samples by native

Figure 11: Elution of β-glucosidase activity from a Sephacryl S-200 column



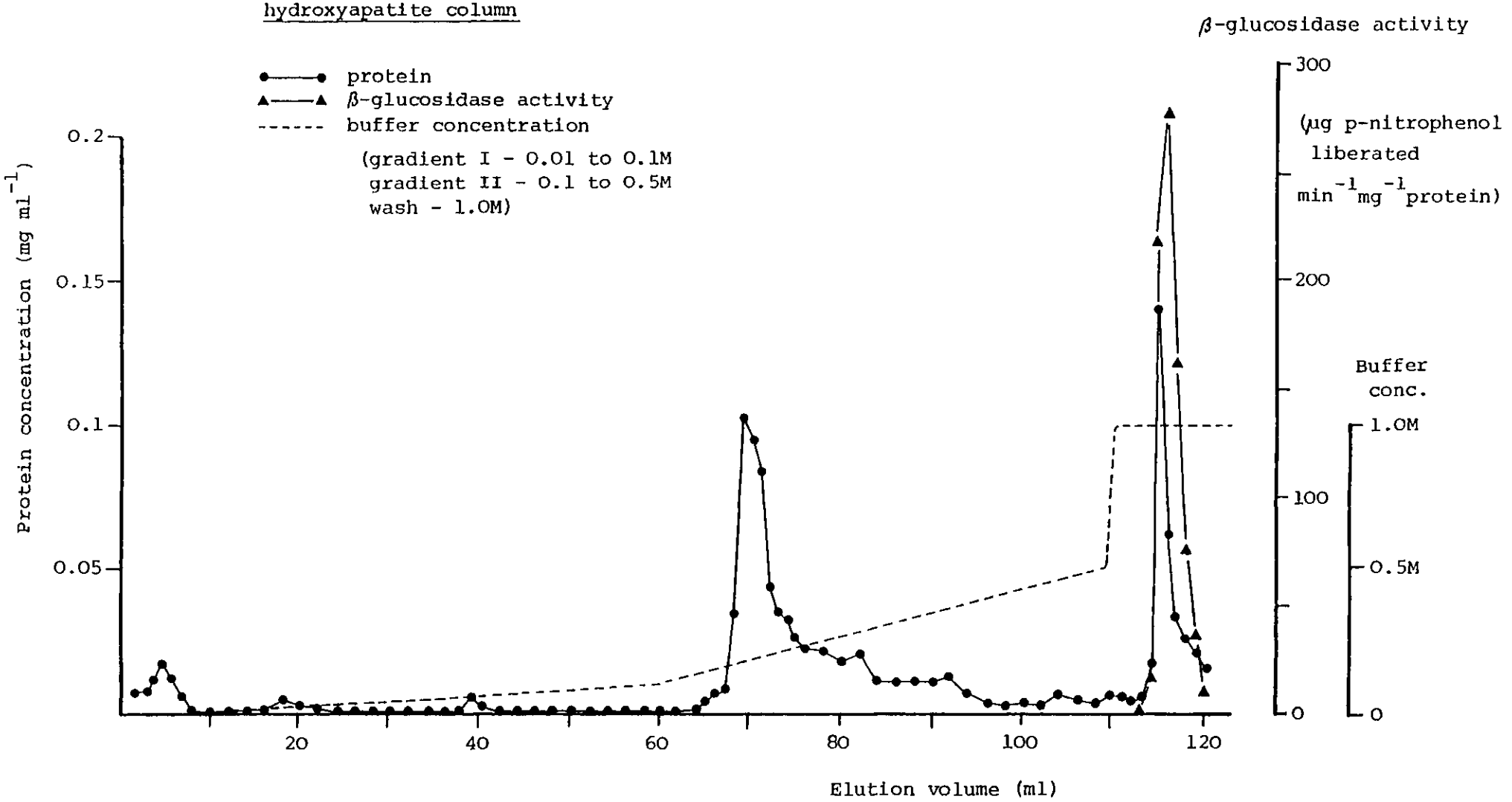
For experimental details see section 3.3.1.

protein gels (2.2.6) was not pursued, since, as had been seen for the β-glucanase, β-glucosidase migrated only 1 to 2 mm into the gel under the electrophoresis conditions used.

Hydroxyapatite was employed in an attempt to achieve a better purification of the β-glucosidase. Active fractions, eluted from the Sephacryl column described above and containing 5 mg protein, were applied to a hydroxyapatite column prepared as described in Methods section 2.5.4, followed by a wash of 10 ml. A linear gradient increasing in strength from 0.01M to 0.1M of sodium/potassium phosphate buffer, pH 6.8 (total volume 50 ml), was applied, followed by a second gradient increasing in concentration from 0.1M to 0.5M of the same buffer and in the same volume. The eluted fractions, 110 x 1 ml, were assayed for protein content and β-glucosidase activity as described above. The results indicated several peaks of protein (Figure 12), but no β-glucosidase activity was detectable in any of the eluted fractions. Therefore, to elute the protein which was still bound to the column, a wash of 15 ml 1.0M sodium/potassium phosphate buffer, pH 7.5, was applied, and the eluted fractions assayed as above. High levels of β-glucosidase activity were detected confined to a single peak of protein eluted by this wash, which peak contained approximately 30% of the protein originally applied to the column (Figure 12). The fractions containing β-glucosidase activity were analysed by SDS gel electrophoresis (2.2.6). The gels (Figure 13) showed a single protein species, confirming the purification of β-glucosidase by this method.

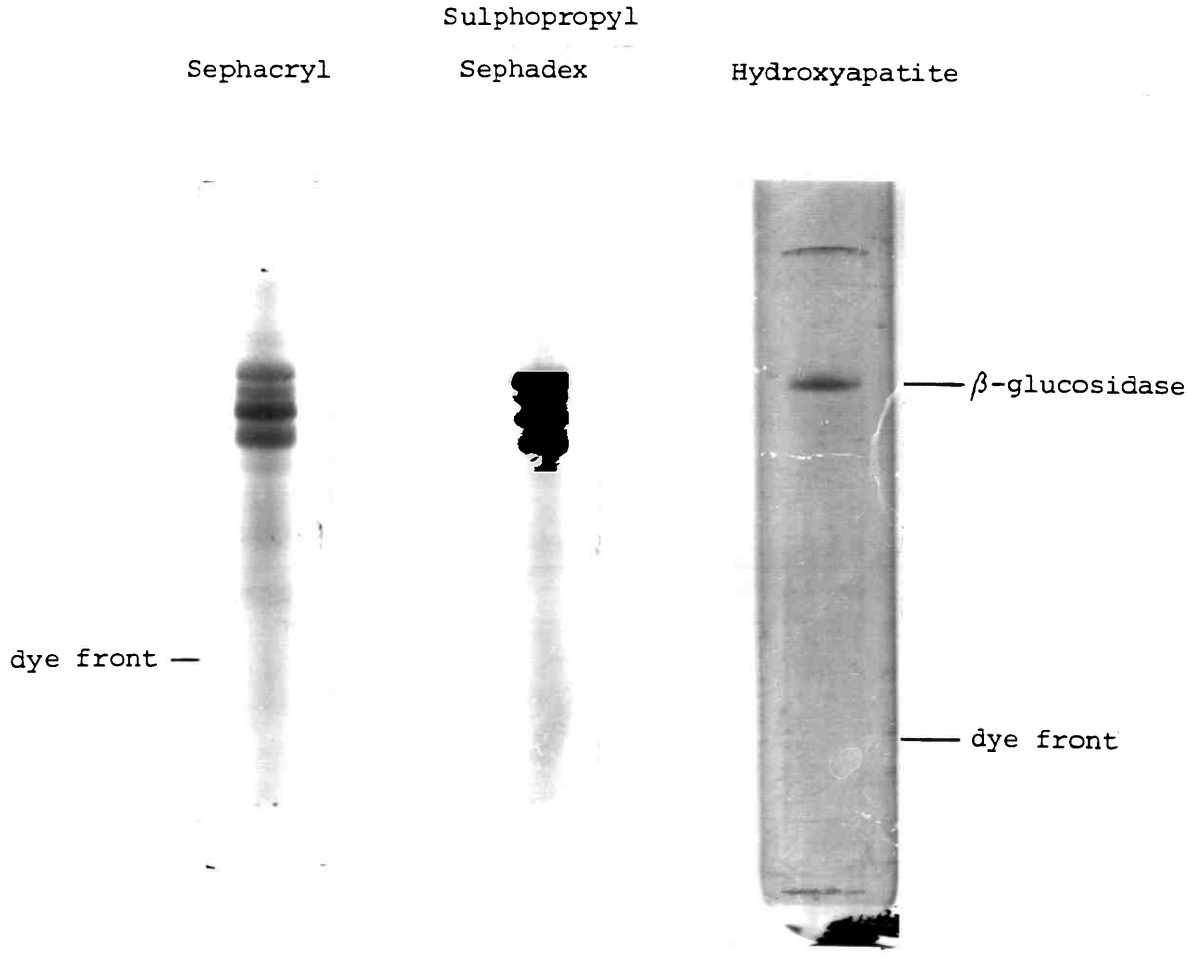
In subsequent purifications of β-glucosidase using hydroxyapatite columns a stepwise method of elution was adopted. Protein was applied and the column washed as described above. Then washes, each in 3 column volumes, at the buffer concentrations detailed in Methods section 2.5.4, were applied successively to the column. Fractions,

Figure 12: Gradient elution of β-glucosidase activity from a hydroxyapatite column



For experimental details see section 3.3.1.

Figure 13: Analysis of protein containing β-glucosidase activity from columns on SDS polyacrylamide gels



For experimental details see section 3.3.1 and Methods section 2.2.6.

60 x 1 ml, were assayed for protein content and β-glucosidase activity as above. The elution profile (Figure 14) demonstrated that the β-glucosidase was eluted as a single peak of pure enzyme by the 0.8M buffer wash. The activities, protein yields and purification factors for the steps in the purification of the C. purpurea β-glucosidase are given in Table 5.

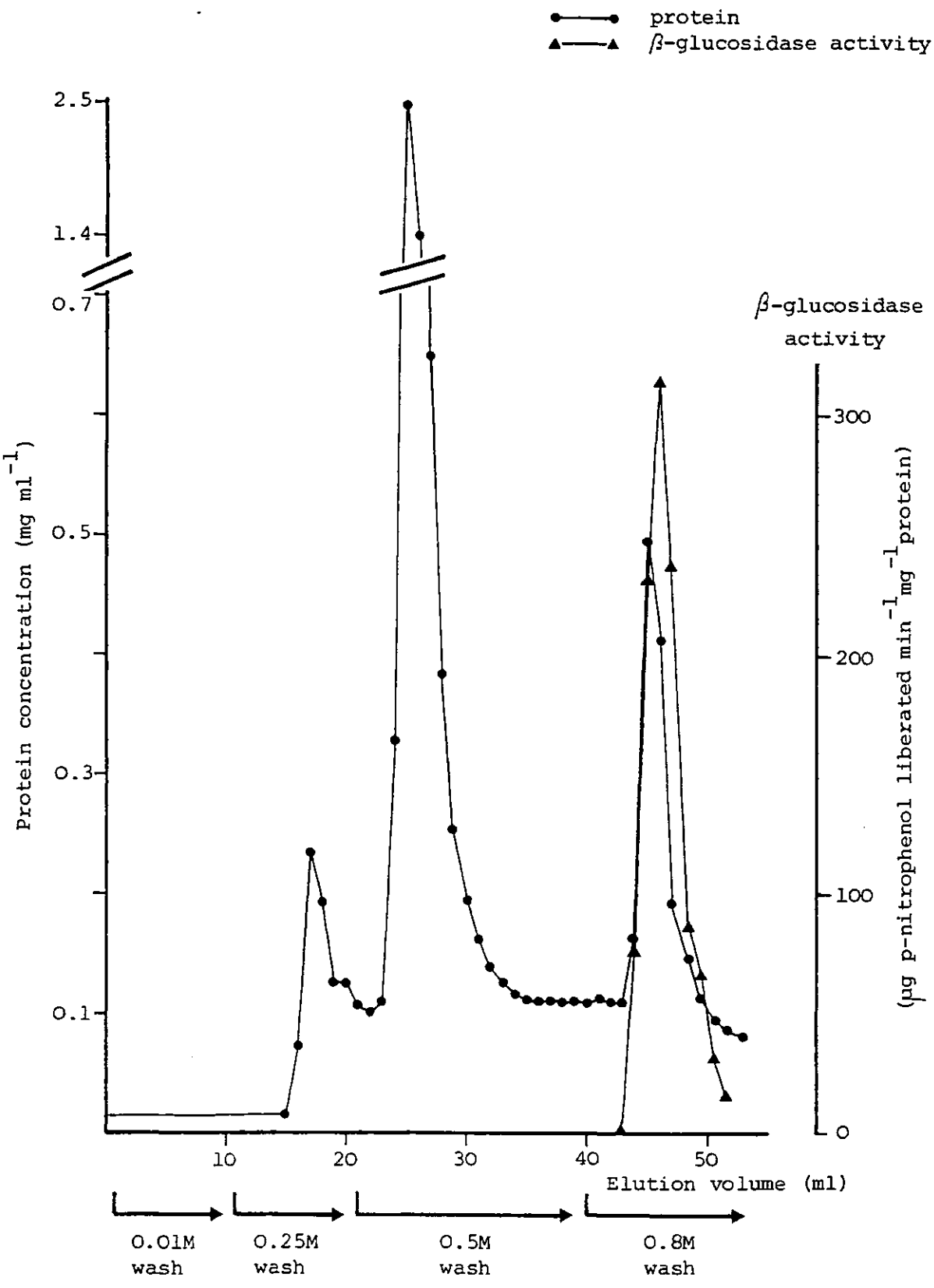
3.3.2 Specific activity of purified β-glucosidase

Purified enzyme, 25 μg ml^{-1} and 5 μg ml^{-1}, was incubated at 30°C with 1 mg ml^{-1} p-nitrophenol-β-D-glucopyranoside in 0.1M sodium acetate buffer, pH 5.0. Samples were taken at intervals and products determined as described in Methods section 2.2.2. Purified enzyme showed high activity against this substrate and a rate of 3.99 μmol p-nitrophenol liberated min^{-1} mg^{-1} protein was obtained with the 5 μg ml^{-1} concentration of enzyme (Figure 15). Due to the high reaction rate of the purified enzyme, subsequent reactions were stopped after only 3 minutes, to ensure first order reaction conditions were being observed.

3.3.3 The Michaelis constant for β-glucosidase

Purified enzyme, 2 μg ml^{-1}, was incubated at 30°C with p-nitrophenol-β-D-glucopyranoside in 0.1M sodium acetate buffer, pH 5.0, for 3 min. After preliminary experiments, substrate concentrations in the range 0.05 mM to 3.0 mM were utilised. The results are presented as a double reciprocal plot of reaction rate against substrate concentration (Figure 16). The K_m for β-glucosidase was found to be 2.4 x 10^{-4}M and it had a V_{max} of 8.3 μmol p-nitrophenol liberated min^{-1} mg^{-1} protein. The results also showed that substrate inhibition occurred at concentrations above 1 mM.

Figure 14: Step wise elution of β-glucosidase activity from a hydroxyapatite column



For experimental details see section 3.3.1.

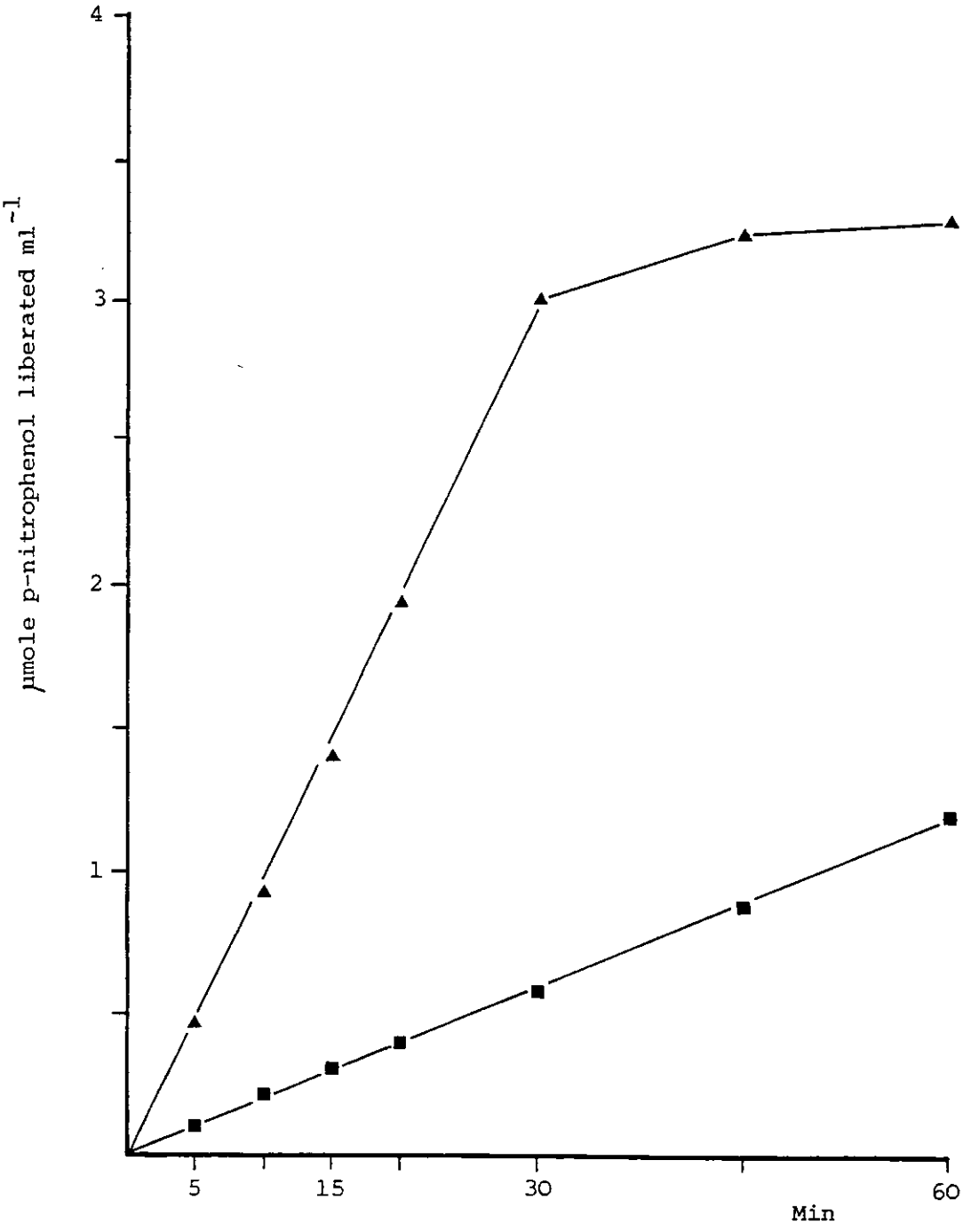
Table 5; Purification of the β-glucosidase from C. purpurea 6.

	Protein mg	Specific Activity*	Total Activity	Fold Purification	Yield %
Crude extract	175	0.21	36.8	1	100
Sulphopropyl sephadex C-50	55.8	0.48	26.8	2.3	73
Sephacryl S-200 superfine	23.5	0.89	20.9	4.2	56
Hydroxyapatite	2.6	5.99	15.6	28.5	42

For experimental procedures, see Methods section 2.5 and Results section 3.3.1.

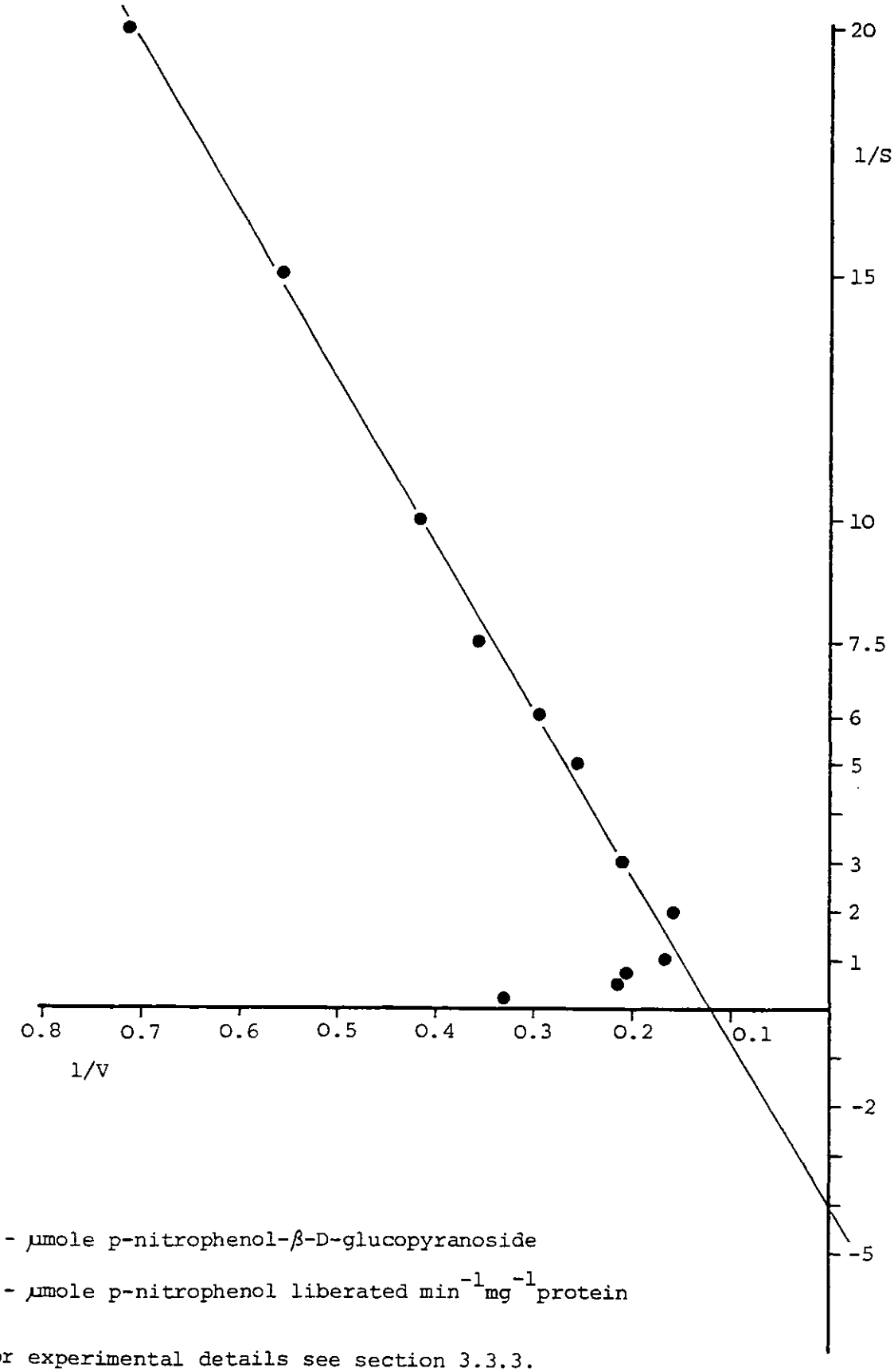
*μmoles p-nitrophenol liberated min^{-1} $mg\ protein^{-1}$.

Figure 15: Activity of the β-glucosidase



For experimental details see section 3.3.2.

Figure 16: Double reciprocal plot to show the effect of substrate concentration on β-glucosidase activity



S - μmole p-nitrophenol-β-D-glucopyranoside

V - μmole p-nitrophenol liberated $min^{-1}mg^{-1}$protein

For experimental details see section 3.3.3.

3.3.4 The pH optimum of β-glucosidase

Purified enzyme, 2 μg ml^{-1}, was incubated at 30°C with 1mM p-nitrophenol-β-D-glucopyranoside in each of the following buffers at 0.2M: glycine/HCl, pH 2.0 to 3.5; sodium acetate, pH 3.5 to 6.0; sodium phosphate, pH 5.5 to 8.0. Buffer concentrations were higher than those used previously to ensure adequate buffering close to the limits of particular buffer ranges. The results (Figure 17) indicated that the β-glucosidase had a pH optimum of 4.5, and that it retained 75% of its maximum activity between pH 3.5 and pH 6.5.

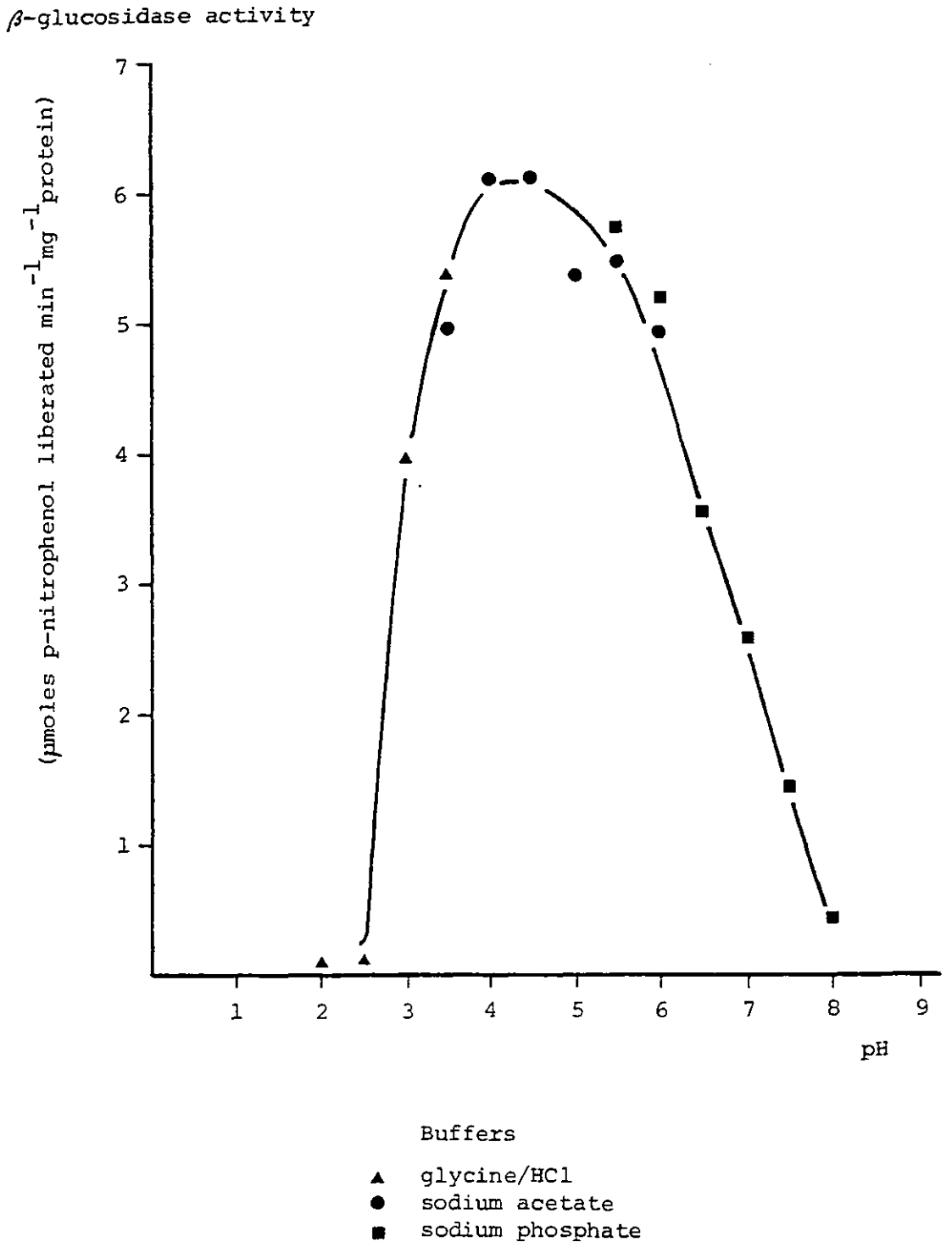
3.3.5 Activity of β-glucosidase against different glucosides

Purified enzyme, 2 μg ml^{-1}, was incubated at 30°C with saccharides of differing glycosidic linkages in 0.1M sodium acetate buffer, pH 5.0, for 3 min. The substrates used were the disaccharides laminaribiose, cellobiose, gentiobiose, maltose, sucrose, p-nitrophenol-β-D-glucopyranoside, and the trisaccharide laminaritriose, all at concentrations of 1 mM; and the fungal β-D-1,3-glucan (2.4.6) at a concentration of 0.6% (w/v). In the case of p-nitrophenol-β-D-glucopyranoside the reaction was stopped and products determined as described in Methods section 2.2.2. For the other 'natural' substrates, the reaction was stopped by heating to 100°C in a boiling water bath for 3 min, which was sufficient to inactivate the enzyme, and reaction products determined by the glucose oxidase method (Methods section 2.2.1). The results are given in table 6. The β-glucosidase was active to varying degrees against β-linked di- and tri-saccharides but not against α-linked substrates. The β-glucosidase showed no activity against the fungal β-glucan, even when the reaction was allowed to continue for 12 h.

3.3.6 The molecular weight of β-glucosidase

The molecular weight was estimated by SDS polyacrylamide gels

Figure 17: The pH optimum of β-glucosidase



For experimental details see section 3.3.4.

Table 6: Substrate specificity of the β-glucosidase

Substrate	Linkage	Specific Activity*
Laminaribiose	β-1-3	5.5
Cellobiose	β-1-4	2.9
Gentiobiose	β-1-6	7.3
p-Nitrophenol-β-D-glucopyranoside	β-1-6	2.7
Maltose	α-1-4	0
Sucrose	α1 - β2	0
Laminaritriose	β-1-3	0.7
β-Glucan	β-1-3 and β-1-6	0

For experimental procedures see Results section 3.3.5.

* μmoles substrate hydrolysed min^{-1} $mg\ protein^{-1}$.

as described in Methods section 2.2.6. The β-glucosidase had an average mobility of 0.28 (3 replicates) relative to the dye front, giving a molecular weight 145,000 ± 12,000. The margin of error takes into account the fact that no suitable protein standards with a molecular weight over 85,000 were available at the time of experiments.

3.4 Purification of β-fructofuranosidase from C. purpurea 6.

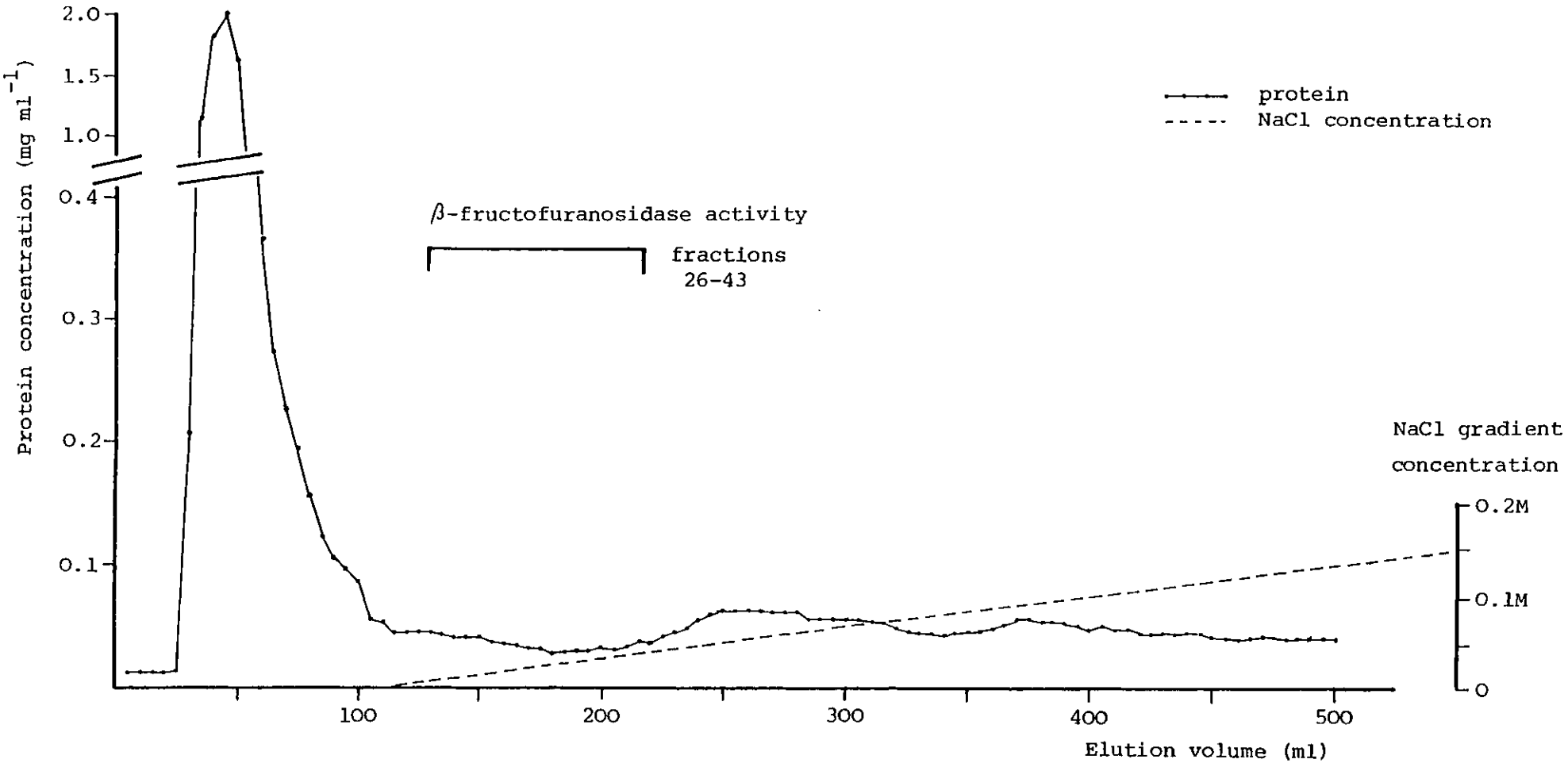
In Claviceps spp., β-fructofuranosidase is expressed both extracellularly and as a cell bound or intracellular activity, but there is no clear evidence whether these are the same enzyme or not (Dickerson, 1972; Baker & Dickerson, 1980). Therefore both cell bound and extracellular protein preparations were purified.

3.4.1 Purification of cell bound β-fructofuranosidase

The quantity of protein containing β-fructofuranosidase activity that could be extracted per unit weight of mycelium was low (1.6 mg protein gm^{-1} fresh weight). Therefore, having established that β-fructofuranosidase was present by assaying the crude extract against radioactive U-^{14}C-sucrose (2.3.3), all subsequent tests relied on the Formazan method (2.2.3), using sucrose as substrate, to indicate the presence of β-fructofuranosidase in fractions from columns and in protein bands on gels.

Crude intracellular extract (3.1.5), 150 mg containing 100 mg protein, was partially purified on a DEAE-cellulose column as described in Methods section 2.5.3. The elution profile is given in Figure 18. The fractions from the column, 110 x 5 ml, were assayed for protein content and β-fructofuranosidase activity as described in Methods sections 2.2.4 and 2.3.3. Of the total protein applied to the column, 50% did not bind, but this fraction contained no β-fructofuranosidase activity. The β-fructofuranosidase activity eluted at the very beginning of the gradient (between 10 mM and 35 mM

Figure 18: Column chromatography of crude intracellular extract on DEAE-cellulose



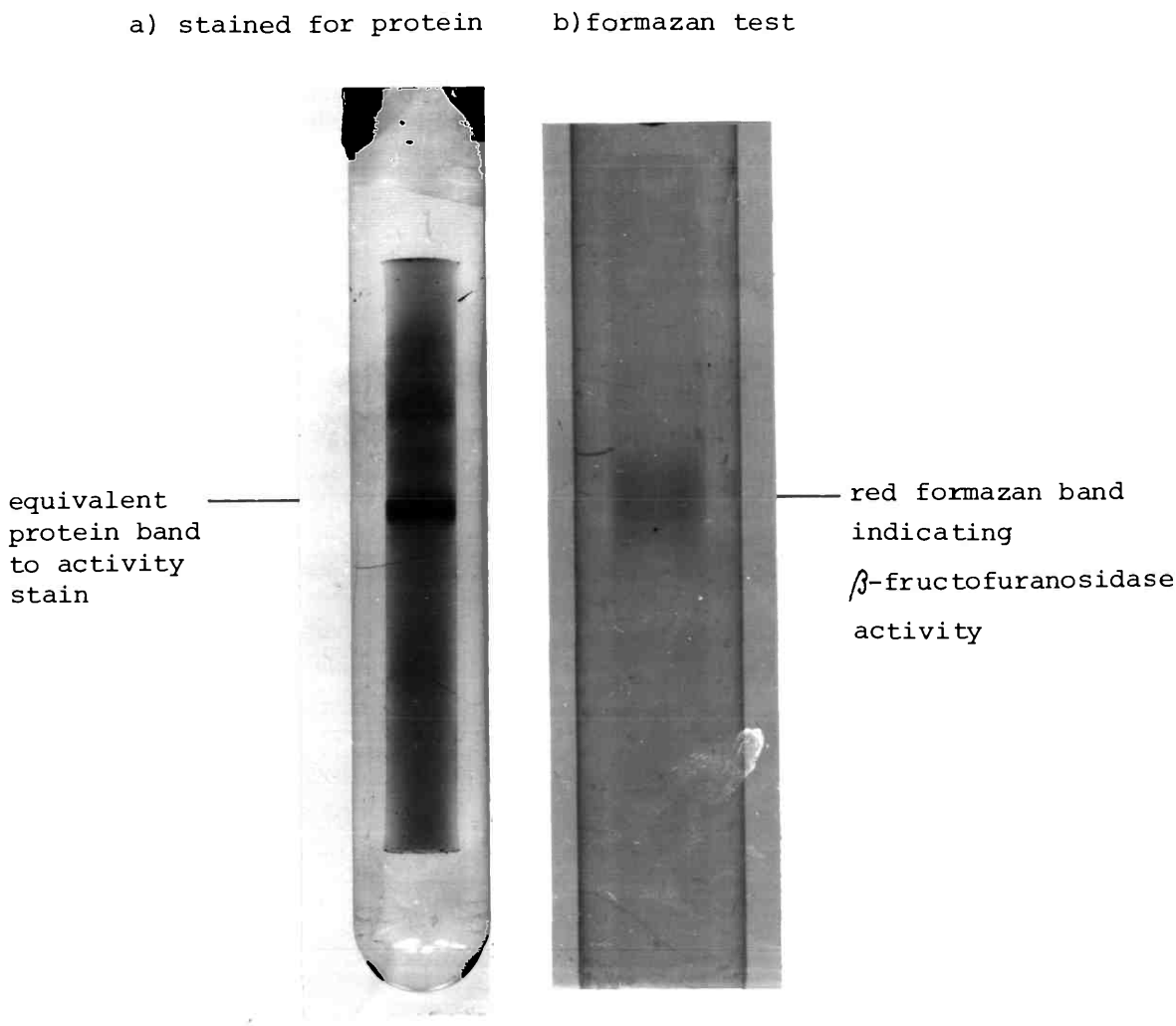
For experimental details see section 3.4.1.
β-fructofuranosidase activity was detected by the formazan test (Methods sections 2.2.2 and 2.3.3)

NaCl on the gradient), but not as a single protein peak. Active fractions were bulked, dialysed and freeze dried as described in Methods section 2.5.3. A yield of 2.8 mg protein, containing β-fructofuranosidase, was obtained. The active fractions were analysed by native protein gel electrophoresis (2.2.6). Gels stained for protein demonstrated at least 5 separate protein species present in the fraction. Identical gels were assayed specifically for β-fructofuranosidase activity as described in Methods section 2.2.6. A band of the red formazan product was deposited in the gel; indicating the point where sucrose had been converted to reducing sugar by the β-fructofuranosidase. This formazan band corresponded with a major band on the identical gels stained for protein (Figure 19).

The protein fraction containing β-fructofuranosidase activity, derived from the DEAE-cellulose column described above, was applied to a Sephacryl S-200 column and eluted as described in Methods section 2.5.2. Fractions, 80 x 1 ml, were collected and assayed for protein content and β-fructofuranosidase activity as described previously. The elution profile (Figure 20) showed that the enzyme activity eluted between fractions 31 and 36, and active fractions were bulked, dialysed against 1 mM sodium phosphate buffer, pH 6.0, containing 0.1 mM EDTA and freeze dried. The yield of enzymically active protein from this column was 0.36 mg.

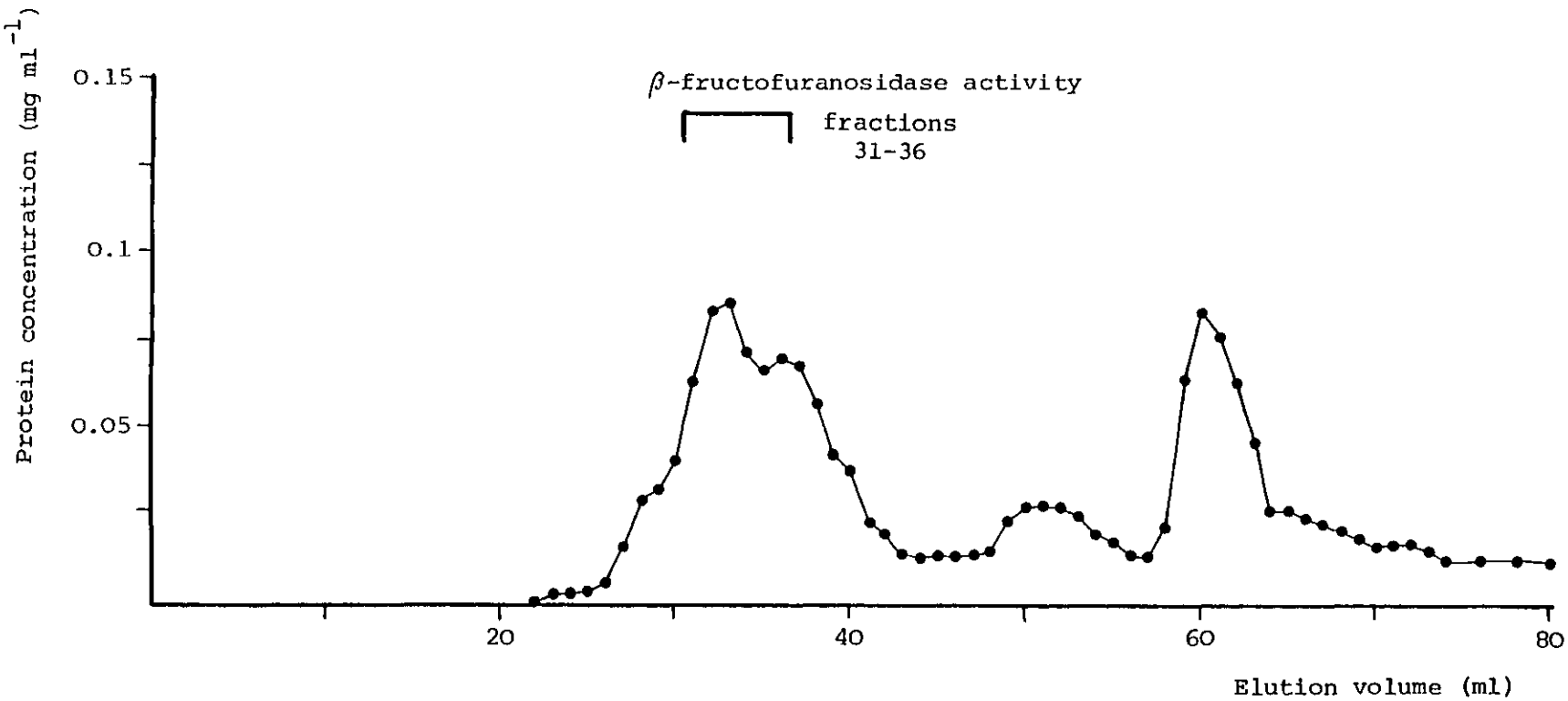
In order to purify further the β-fructofuranosidase, protein, 1.4 mg, obtained from the columns described above, was applied to a hydroxyapatite column prepared as described in Methods section 2.5.4. Elution was carried out using a linear gradient of increasing buffer concentration from 10 mM to 250 mM sodium/potassium phosphate buffer, pH 6.8, in 10 column volumes, followed by a wash of the same buffer at 0.5M concentration. Fractions, 70 x 1 ml, were assayed for protein content and β-fructofuranosidase activity as described above. The

Figure 19: Analysis of cell bound β-fructofuranosidase activity by native polyacrylamide gels



For experimental details see section 3.4.1 and Methods section 2.2.6.

Figure 20: Elution of cell bound β-fructofuranosidase activity from a Sephacryl S-200 column



For experimental details see section 3.4.1.
β-fructofuranosidase activity was detected by the formazan test (Methods sections 2.2.2 and 2.3.3)

elution profile (Figure 21) shows that the β-fructofuranosidase activity was eluted as a single peak of protein between the concentrations 75 mM to 115 mM on the gradient. Active fractions were bulked, reduced in volume by ultrafiltration and stored as described in Methods section 2.5.4; the yield of enzymically active protein was 0.22 mg.

The fraction containing β-fructofuranosidase activity from the hydroxyapatite column was analysed by SDS polyacrylamide gels (2.2.6). The gels showed a single protein species present in the fraction (Figure 22), confirming the purification of the cell-associated β-fructofuranosidase by this method. Calculation of the protein yields for each stage of the purification (Table 7), indicated that the β-fructofuranosidase represented 0.11% of the protein in the original crude intracellular extract (3.1.5).

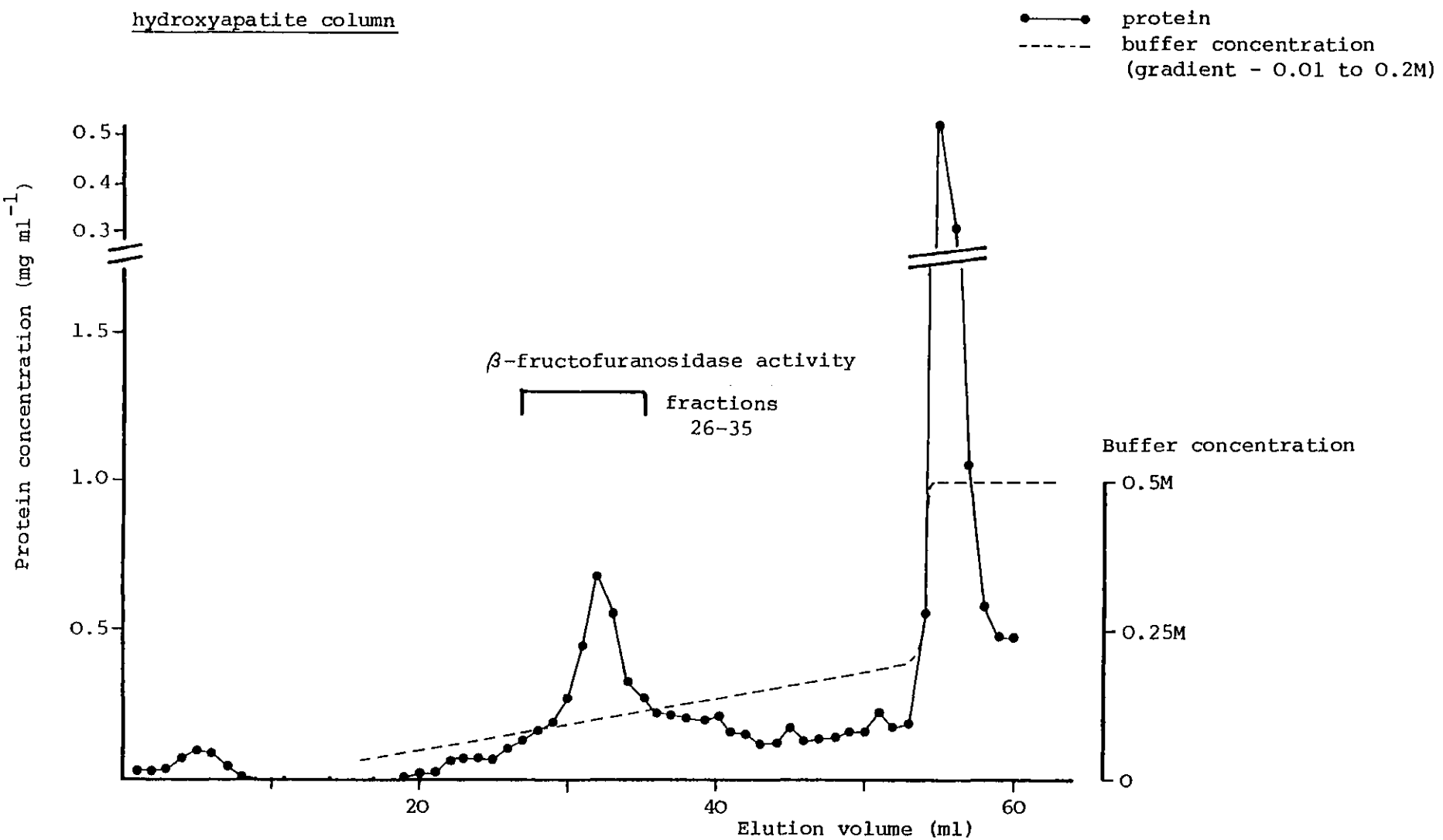
3.4.2 The molecular weight of β-fructofuranosidase

The molecular weight of the cell-associated β-fructofuranosidase was estimated by SDS gel electrophoresis as described previously (2.2.6). As average mobility of 0.31 (3 replicates) was obtained, giving a molecular weight of 134,000 ± 12,000 for the β-fructofuranosidase. As noted for the β-glucanase and β-glucosidase, the margin of error again takes into account the fact that suitable protein standards with molecular weights over 85,000 were not available at the time of experimentation.

3.4.3 Purification of the β-fructofuranosidase from extracellular extracts

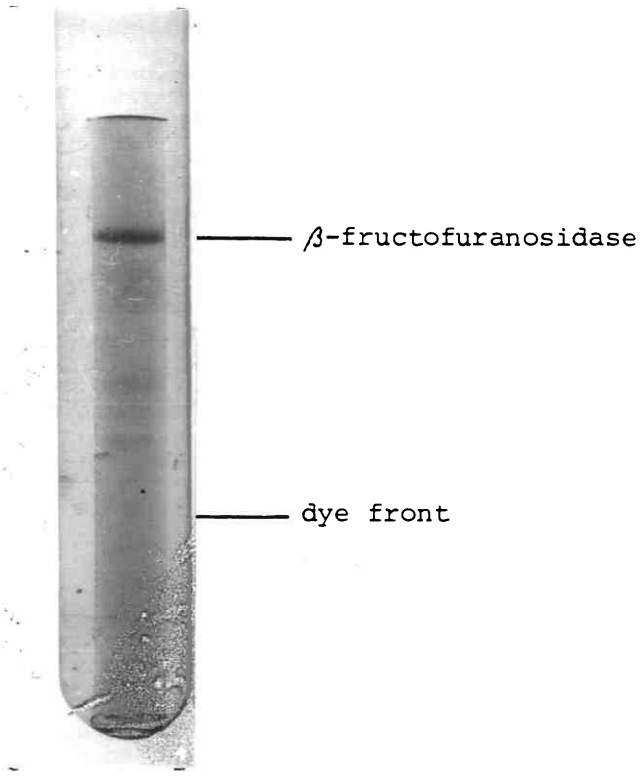
As far more protein having β-fructofuranosidase activity was available in this case, compared with the intracellularly located activity, it was possible to follow the purification by both the formazan test and assaying the enzyme activity against radioactive

Figure 21: Elution of cell bound β-fructofuranosidase activity from a hydroxyapatite column



For experimental details see section 3.4.1.
β-fructofuranosidase activity was detected by the formazan test (Methods sections 2.2.2 and 2.3.3)

Figure 22: Analysis of cell bound β-fructofuranosidase activity from the hydroxyapatite column on SDS polyacrylamide gels



For experimental details see section 3.4.1 and Methods section 2.2.6.

Table 7: Purification of the cell-associated β-fructofuranosidase from C. purpurea 6

	Protein mg	Specific Activity*	Total Activity	Fold Purification	Yield %
Crude extract	206	0.2	41.2	1	100
DEAE-cellulose	7.2	4.0	28.8	20	70
Sephacryl S-200 superfine	1.4	15.7	22.0	78	53
Hydroxyapatite	0.22	84.3	18.5	421	45

For experimental procedures, see Methods section 2.5 and Results section 3.4.1.

*μmol sucrose hydrolysed min^{-1} mg $protein^{-1}$

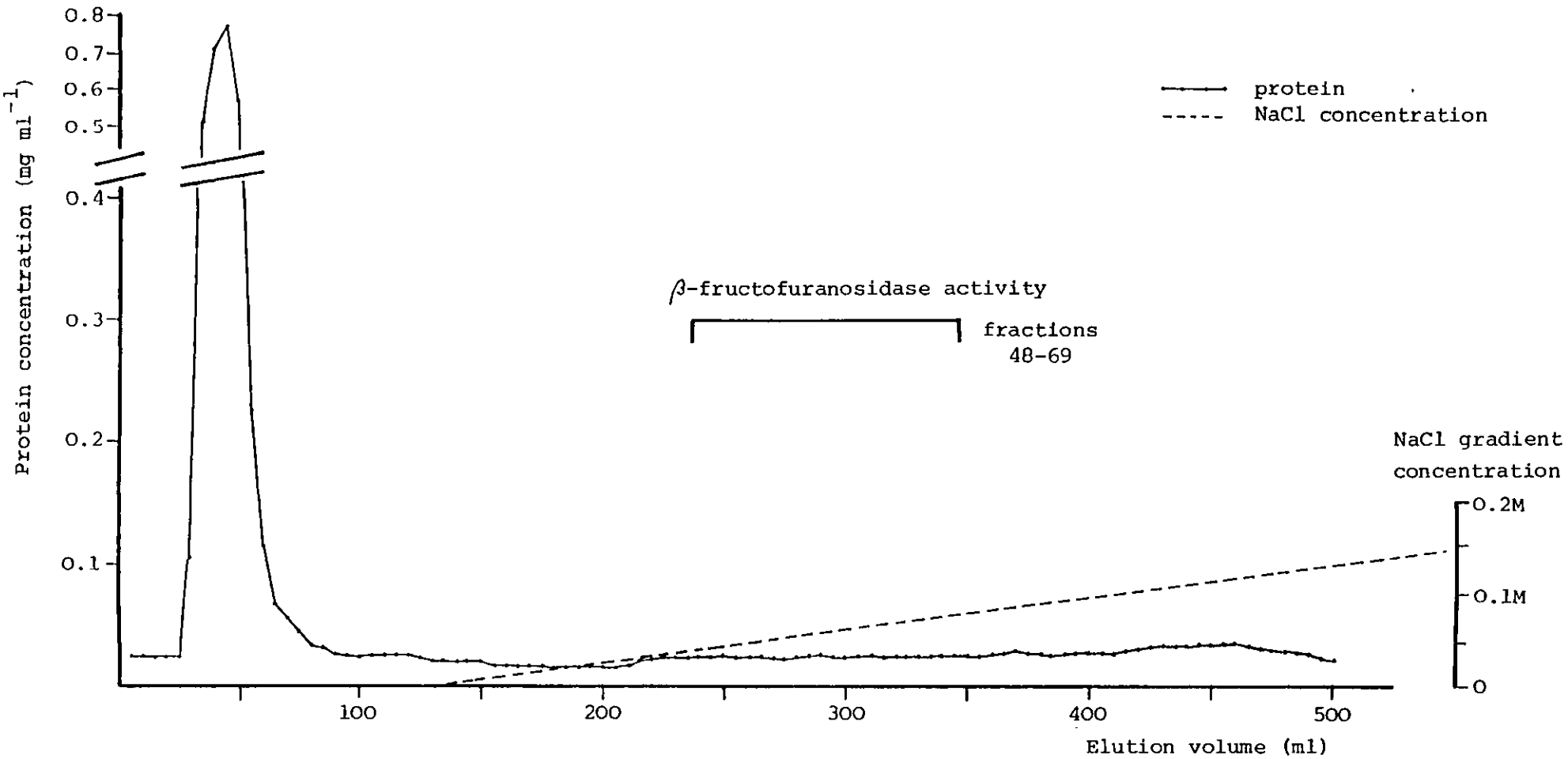
U-^{14}C-sucrose. The same procedure as that described above for the intracellular enzyme was used for the purification of β-fructofuranosidase activity present in the culture medium.

The β-fructofuranosidase activity in extracellular extracts did not bind to the Sulphopropyl Sephadex column described previously (3.2.1). This protein, 19 mg, was applied to a column of DEAE-cellulose prepared and eluted as described in Methods section 2.5.3, and fractions, 110 x 5 ml, were assayed for protein content and β-fructofuranosidase activity as above. The activity of β-fructofuranosidase was eluted over the range of buffer concentrations from 35 mM to 75 mM on the gradient, which is a higher molarity than that observed for the intracellular enzyme (Figure 23). Active fractions were bulked, dialysed and freeze-dried as before, and the yield of enzymically active protein was 0.5 mg; which represented less than 3% of the protein applied to the column. Analysis of the active fraction by native gel electrophoresis (2.2.6) demonstrated at least 5 different protein species present in the fraction.

The active fraction from the above column was applied to a Sephacryl S-200 column and eluted as described in Methods section 2.5.2. Fractions, 80 x 1 ml were assayed for protein content and β-fructofuranosidase activity as before; the elution profile is given in figure 24. The activity of β-fructofuranosidase was eluted as a single peak between fractions 31 and 38, and active fractions were bulked and reduced in volume by ultrafiltration as described previously for the intracellular enzyme. The yield of active protein from this column was 0.17 mg, analysis of which by SDS gel electrophoresis (2.2.6) showed that 3 protein species were present in the fraction, only one of which represented β-fructofuranosidase.

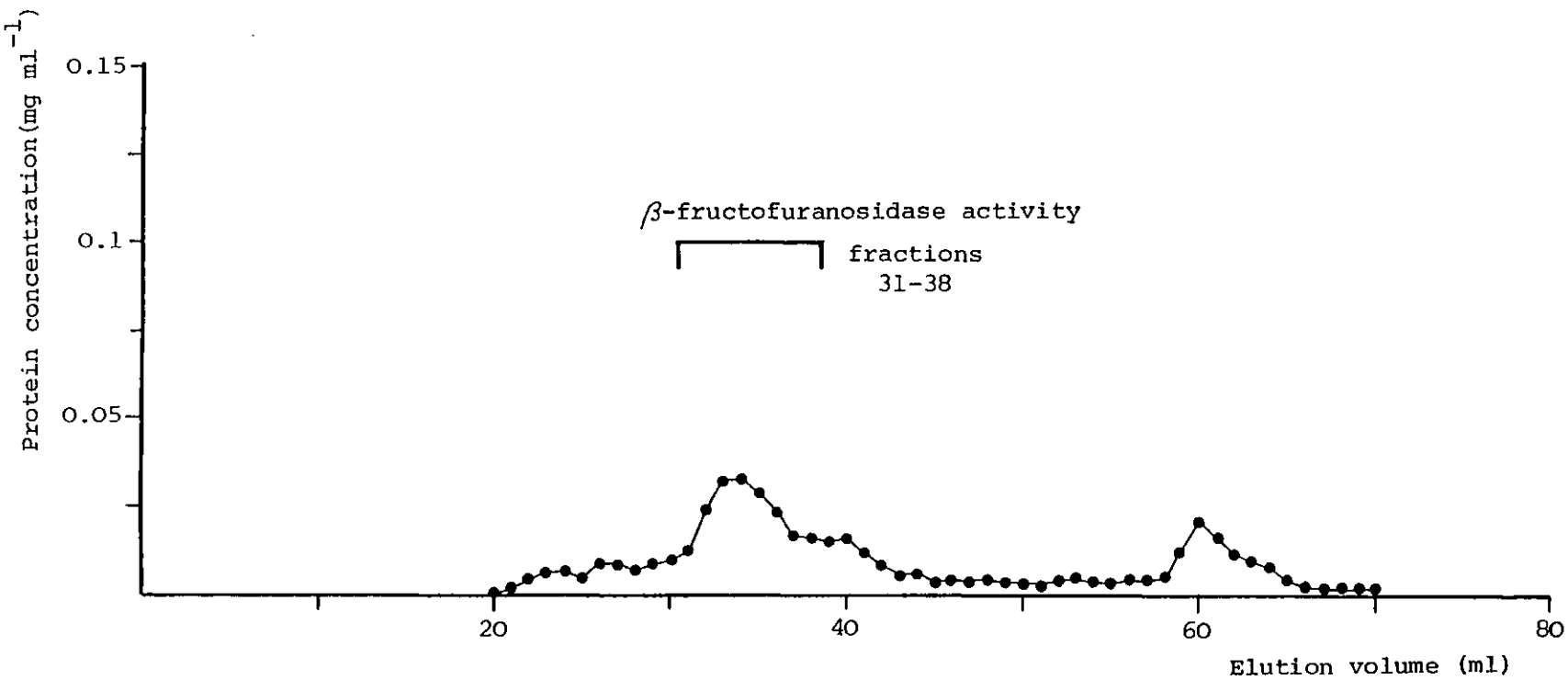
For further purification, active fractions from the DEAE-cellulose column, containing 0.9 mg protein, were applied to a hydroxyapatite column, prepared and eluted as described for the

Figure 23: Elution of extracellular β-fructofuranosidase activity from a DEAE-cellulose column



For experimental details see section 3.4.3.
β-fructofuranosidase activity was detected by the formazan test (Methods sections 2.2.2 and 2.3.3)

Figure 24: Elution of extracellular β-fructofuranosidase activity from a Sephacryl S-200 column



For experimental details see section 3.4.3.
β-fructofuranosidase activity was detected by the formazan test (Methods sections 2.2.2 and 2.3.3)

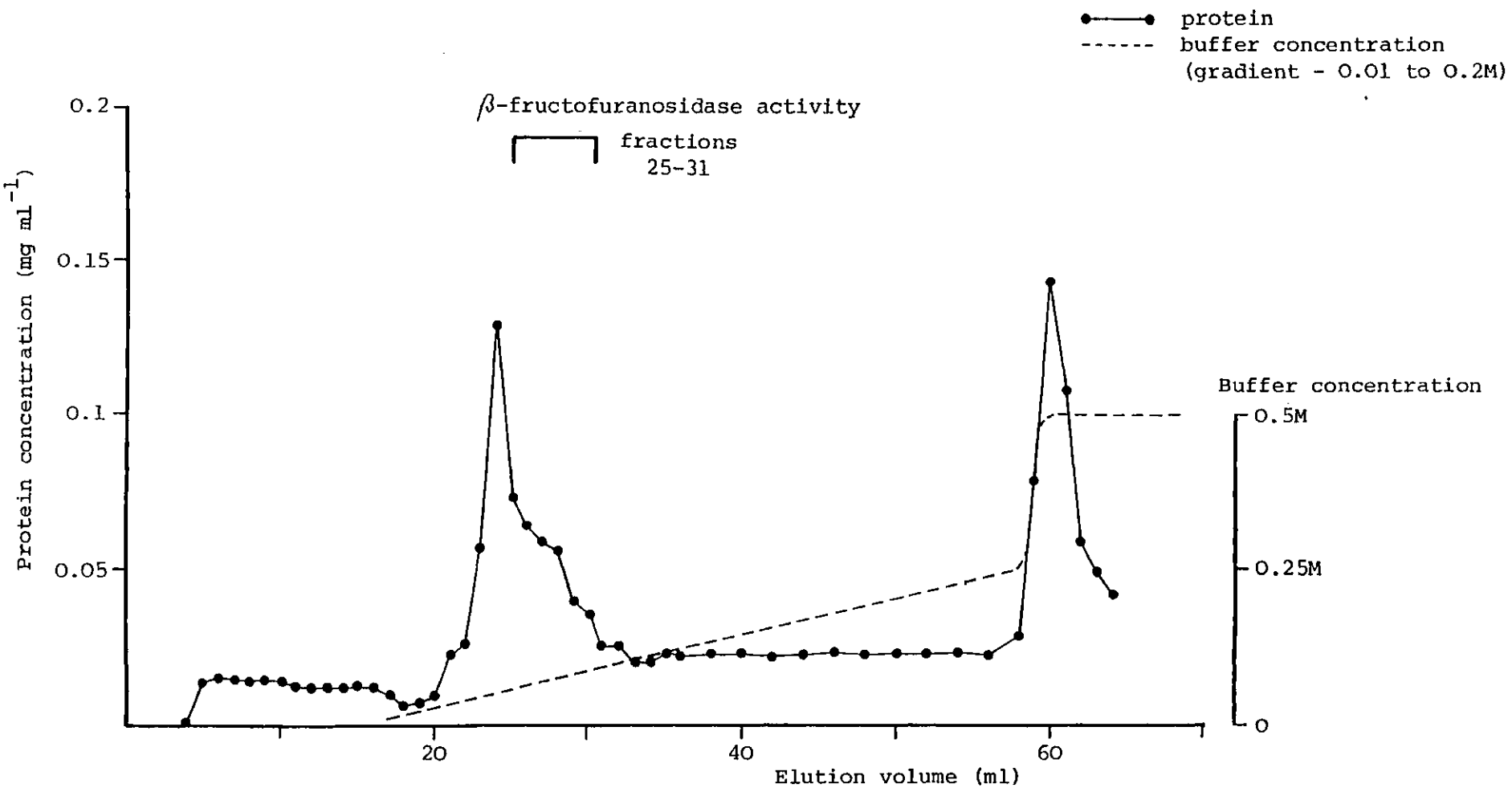
intracellular enzyme in section 3.4.1. Fractions, 70 x 1 ml, were assayed for protein content and β-fructofuranosidase activity as before, and the elution profile (Figure 25) showed that the extracellular β-fructofuranosidase activity was eluted at the same buffer molarity as had been observed for the intracellular enzyme. However, in this case it was contaminated by a more abundant protein species which was eluted just in front of it on the gradient. Active fractions were bulked, reduced in volume by ultrafiltration as before and analysed by SDS gel electrophoresis (2.2.6). Three distinct protein species were found to be present in the fraction. The yield of protein from the active fractions was 0.19 mg, which was equivalent to 0.11% of the protein present in the crude extracellular extract. However, since complete purification was not achieved, the extracellular β-fructofuranosidase activity would have represented an even smaller proportion of the total extracellular protein than this.

As the cell-associated β-fructofuranosidase was purified to homogeneity and as this enzyme's activity would most probably be expressed in the parasitic tissue rather than that of a possibly secreted extracellular enzyme, it was chosen as the first preference for antibody production.

3.4.4 Specific activity of β-fructofuranosidase from the culture medium

Partially purified β-fructofuranosidase (3-4 mg protein) from both the Sephacryl and hydroxyapatite columns described above (3.4.3) was assayed using radioactive U-^{14}C-sucrose as described in Methods section 2.3.3. The β-fructofuranosidase fraction from the Sephacryl column had an activity of 108 μmole sucrose hydrolysed min^{-1} mg^{-1} protein, and that from the hydroxyapatite column had an activity of 383 μmole sucrose hydrolysed $min^{-1}mg^{-1}$ protein. The progress of the

Figure 25: Elution of extracellular β-fructofuranosidase activity from a hydroxyapatite column



For experimental details see section 3.4.3.
β-fructofuranosidase activity was detected by the formazan test (Methods sections 2.2.2 and 2.3.3)

hydrolysis of sucrose by the extracellular β-fructofuranosidase was similar in each case and is shown in an autoradiograph of a chromatogram of the reaction products (Figure 26). The enzyme exhibited a more directly hydrolytic action against sucrose, producing fewer oligosaccharides, than that shown by the β-fructofuranosidase in crude intracellular extract (Section 3.1.5, Figure 5).

3.5 Immunological studies

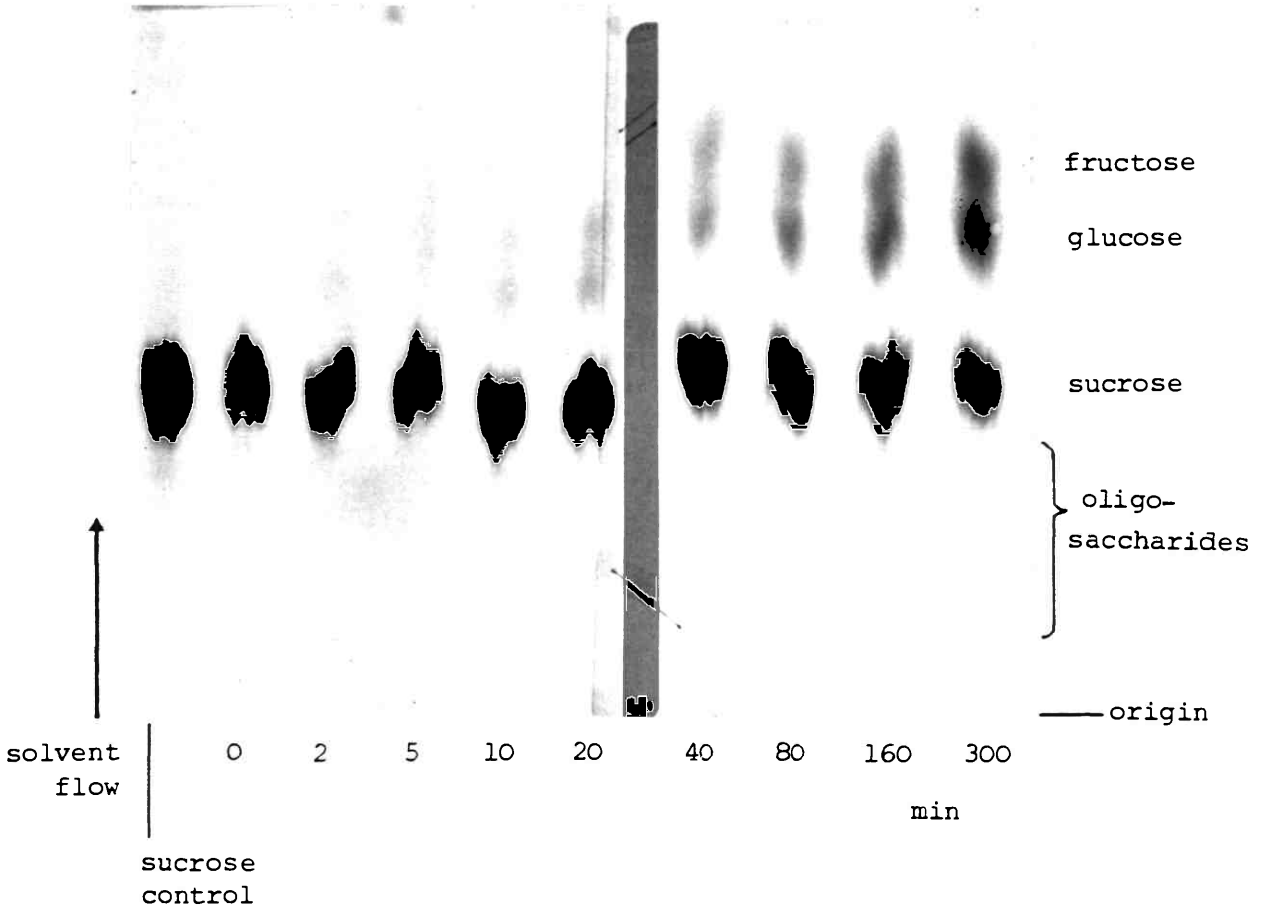
3.5.1 Preparation of antisera

Antisera to the three enzymes, β-1,3-glucanase, β-glucosidase and cell-associated β-fructofuranosidase were prepared in rabbits as described in Methods section 2.6.1. The 1.5 ml samples of blood, taken during the course of immunization and tested for the presence of antibodies by immunodiffusion (2.6.3), indicated that, for each enzyme, antibodies became detectable in the serum of samples taken prior to the final inoculation. In each case a volume of approximately 14 ml of clear serum was obtained after clotting and centrifugation of the final bleed. Of this, 0.5 ml was mixed with an equal volume of glycerol (as a bacteriostatic agent) for use in immunodiffusion assays; the remainder was frozen.

3.5.2 Purification of the IgG fraction from serum

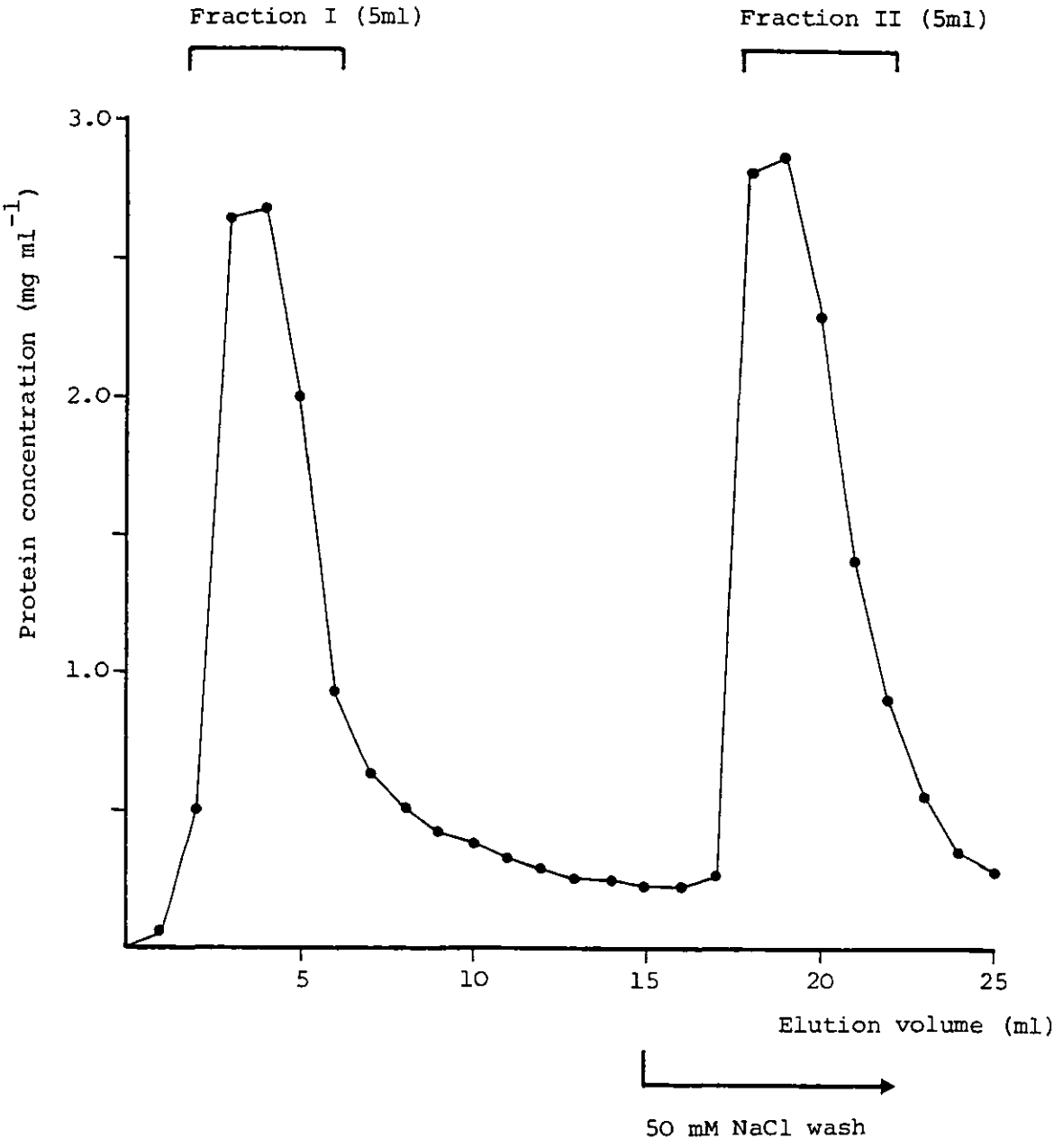
This was carried out for each enzyme antiserum as described in Methods section 2.6.2. The elution of the IgG fractions of β-glucanase serum by this method is given in figure 27 as an example. The two IgG components eluted from the column, which were bulked separately, were each contained in a volume of 5 ml. Therefore, in effect, the concentration of IgG in the purified samples represented a 1:5 dilution of its concentration in the original unpurified antiserum sample. Both IgG fractions, from each enzyme antiserum, contained antibody

Figure 26: Activity of partially purified extracellular β-fructofuranosidase on U-^{14}C-sucrose - autoradiograph of products



For experimental details see section 3.4.4 and Methods sections 2.3.3 and 2.2.5.

Figure 27: Elution of IgG fractions of β-1,3-glucanase antiserum from a DEAE-cellulose column



For experimental details see section 3.5.2 and Methods section 2.6.2.

activity against the respective enzyme used as antigen, as demonstrated by immunodiffusion plate tests (3.5.3). However, for reasons given below (3.5.3 iv) only the first fraction was employed for staining sections.

3.5.3 Immunodiffusion plate studies

i) β-glucanase. Serial doubling dilutions, 1:2 to 1:64, of β-glucanase antiserum (serum: glycerol, 1:1 (v/v), 3.5.1) were prepared in PBS. Aliquots, 10 µl, of 3 consecutive dilutions were pipetted into the outer six wells of a plate prepared as described in methods section 2.6.3, opposite wells being duplicates (see figure 28). The centre well contained 10 µl of purified β-glucanase at a concentration of 150 µg ml^{-1} in PBS. The plates were washed and stained as described in Methods section 2.6.3. Precipitin bands (indicating enzyme/antibody binding) were visible between β-glucanase and antiserum dilutions of 1:2 to 1:16 (Figure 29). This was equivalent to a titre of 1:32 of the original antiserum. No reaction was observed between β-glucanase and control non-immune serum.

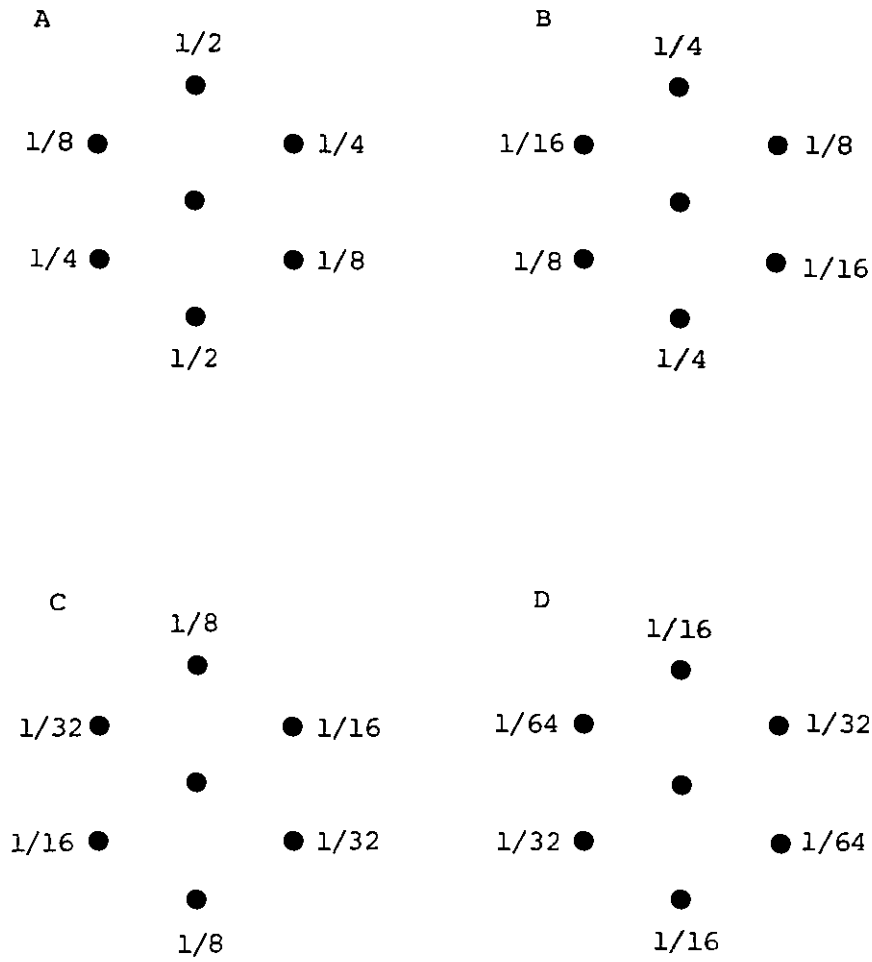
ii) β-glucosidase. Doubling dilutions of β-glucosidase antiserum were prepared and plates, containing 10 µl of 75 µg ml^{-1} purified β-glucosidase in the centre well, were prepared and developed as described above. The developed plates are shown in figure 30, and precipitin bands were visible between β-glucosidase and antiserum dilutions of 1:2 to 1:32, equivalent to a titre of 1:64 of the original antiserum. Again, no reaction was observed with the control non-immune serum.

iii) β-fructofuranosidase. Immunodiffusion plates for β-fructofuranosidase antiserum dilutions, with the centre well containing 10 µl of 60 µg ml^{-1} purified β-fructofuranosidase, were prepared and developed as described above for β-glucanase. In this case the developed plates (Figure 31) demonstrated precipitin bands at antiserum dilutions

Figure 28: Layout of samples applied to wells in immunodiffusion plates

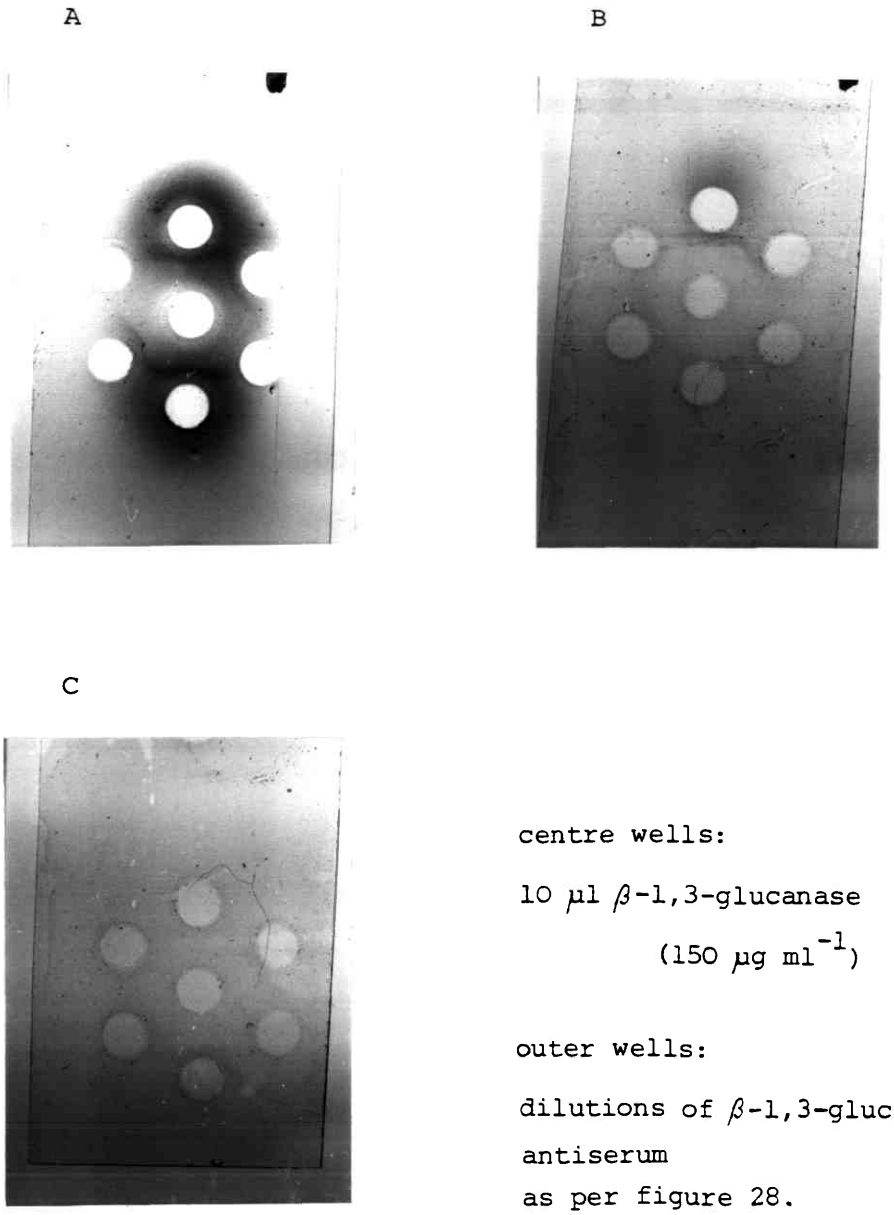
Central well: enzyme (i.e. antigen) 10 µl

Outer wells: serial dilutions of antiserum in duplicate 10µl



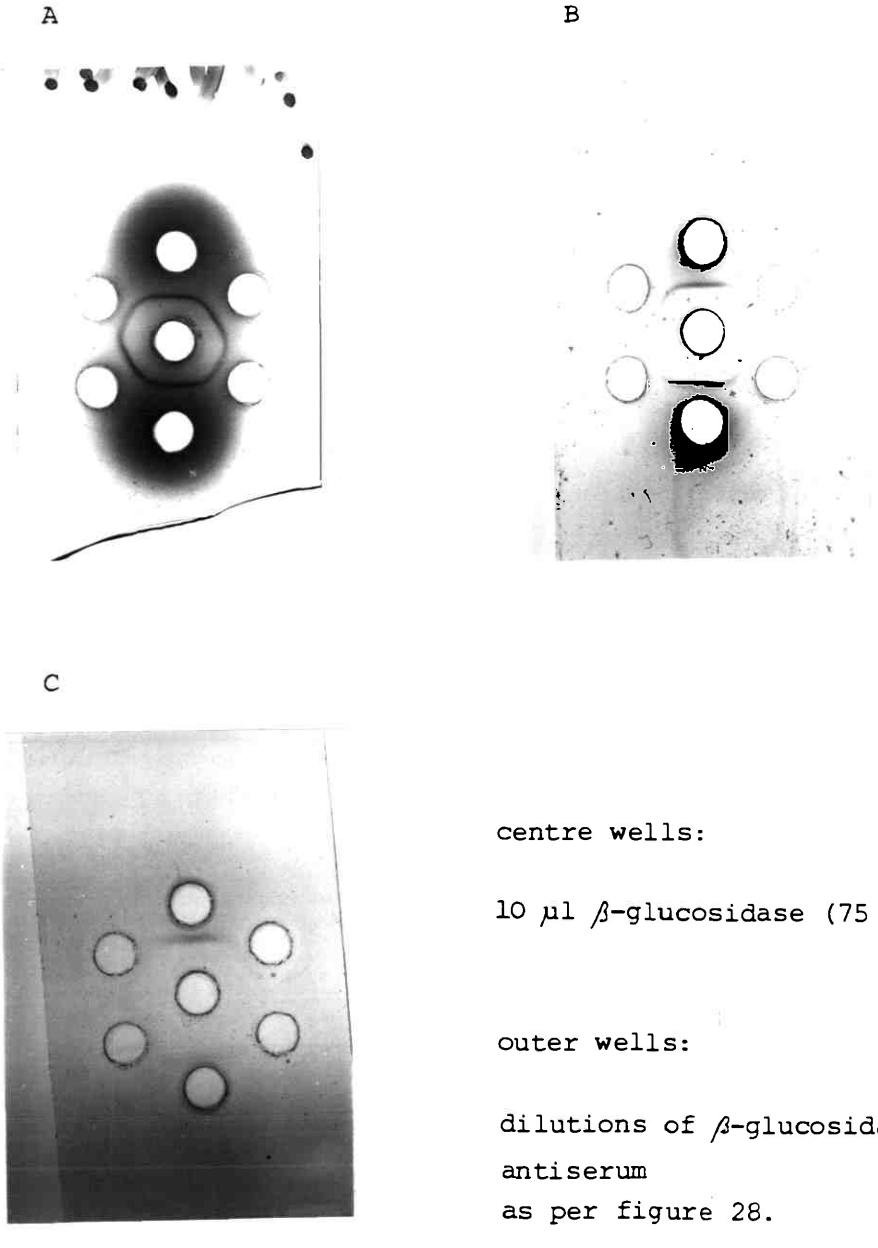
For details see Methods section 2.6.3.

Figure 29: Immunodiffusion of β-1,3-glucanase antiserum against the purified β-1,3-glucanase



For details see section 3.5.3 (i).

Figure 30: Immunodiffusion of β-glucosidase antiserum against the purified β-glucosidase



centre wells:

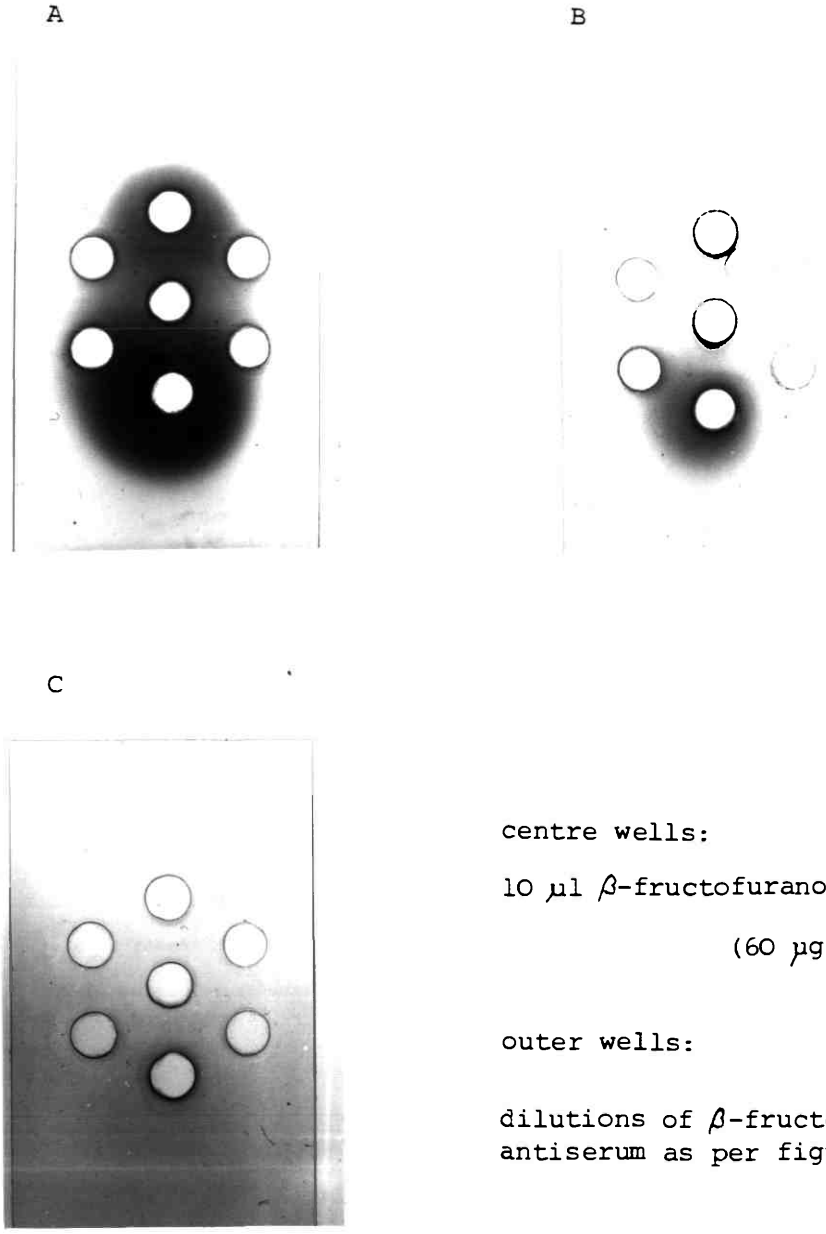
10 µl β-glucosidase (75 µg ml^{-1})

outer wells:

dilutions of β-glucosidase antiserum
as per figure 28.

For details see section 3.5.3 (ii).

Figure 31: Immunodiffusion of β-fructofuranosidase antiserum against the purified cell bound β-fructofuranosidase



centre wells:

10 µl β-fructofuranosidase

(60 µg ml^{-1})

outer wells:

dilutions of β-fructofuranosidase antiserum as per figure 28.

For details see section 3.5.3 (iii).

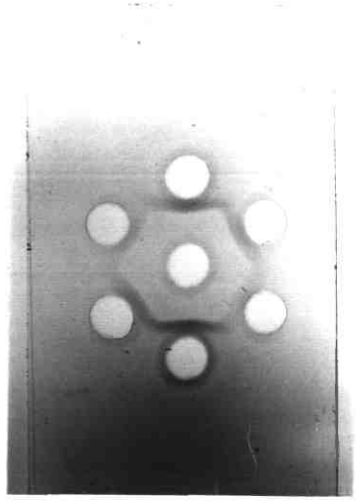
of 1:2 to 1:8, equivalent to a titre of 1:16 of the original antiserum. No reaction was observed with control non-immune serum, or between the partially purified extracellular β-fructofuranosidase and the antiserum.

iv) IgG fractions of antisera. Dilutions, 1:1 to 1:4 in PBS, of the two purified IgG fractions from each antiserum were tested against their respective enzyme antigens by the methods described in section 2.6.3 and above. For each enzyme, both IgG fractions (3.5.2) demonstrated antibody activity; the precipitin bands between the enzyme antigen and IgG fraction I were stronger than those between enzyme and fraction II in each case. As an example, the developed immunodiffusion plates for β-glucanase against its antiserum IgG fractions are shown in figure 32. Since, for each enzyme, IgG fraction I showed the greater affinity for antigen, this fraction was used in antibody staining experiments.

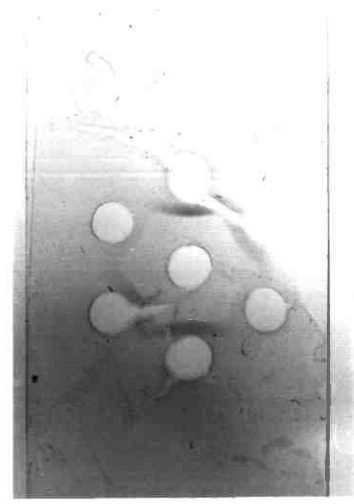
v) Commercial FITC-conjugated goat anti-rabbit IgG. In order to confirm the specificity of the commercially prepared fluorescein-conjugated antiserum, an immunodiffusion plate was prepared as described in Methods section 2.6.3. A 1:4 dilution of purified anti-β-glucanase IgG, 10 µl in PBS, was pipetted into the centre well, 10 µl of a 1:8 dilution in PBS of the FITC-conjugated IgG fraction of goat anti-rabbit lgG pipetted into each of two outer wells, and the plate developed as described in Methods. The developed plate showed strong precipitin bands between the two antisera, confirming the specificity of the commercial antiserum for the experimentally prepared rabbit antisera (Figure 33).

vi) Cross reactivity. Three immunodiffusion plates were prepared as described previously in duplicate. Aliquots, 10 µl, of 1:1 and 1:2 dilutions in PBS of each of the three experimentally prepared enzyme antisera (purified IgG fraction) were pipetted into the six outer wells

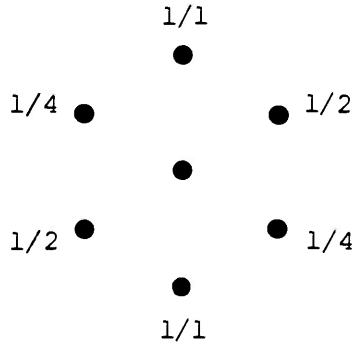
Figure 32: Immunodiffusion of the IgG fractions of β-glucanase antiserum against the purified β-glucanase

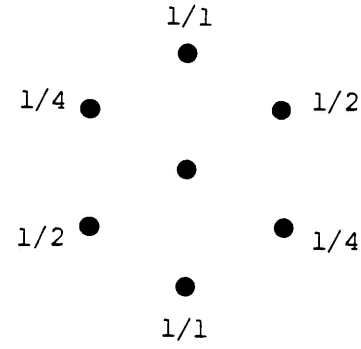


Fraction I



Fraction II



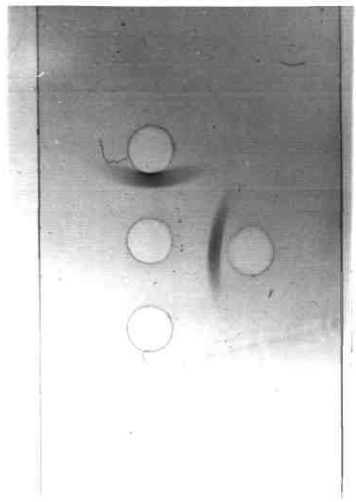


outer wells: dilutions of IgG fraction of serum

centre well: β-1,3-glucanase 10 µl

For details see section 3.5.3 (iv)

Figure 33: Immunodiffusion of the commercial fluorescein-conjugated antiserum against experimentally prepared antiserum



FITC-goat anti-rabbit IgG serum

1/8 dilution

β-1,3-glucanase antiserum

1/4 dilution

For details see section 3.5.3 (v).

of each plate as shown in the diagram (Figure 34). Aliquots, 10 µl, of the three purified enzymes (β-glucanase, β-glucosidase and β-fructofuranosidase) were pipetted into the centre wells of three separate plates. The developed plates demonstrated that no cross-reactivity was detectable between any enzyme and the undiluted antisera raised against the other two enzymes (Figure 34).

3.5.4 Activity of β-glucanase in the presence of specific antiserum.

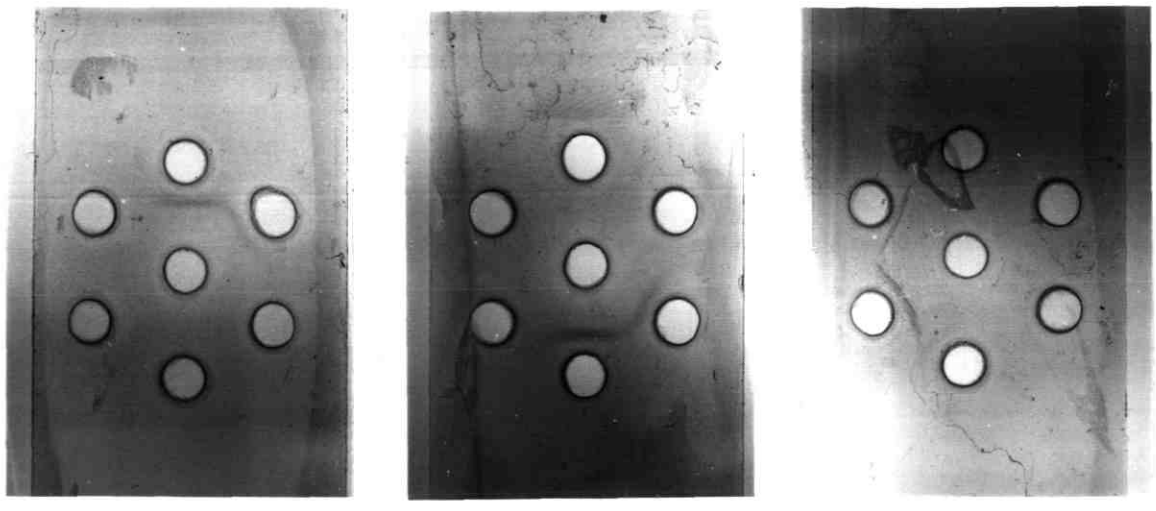
Purified β-glucanase, 25 µg ml^{-1}, was assayed against 0.5% (w/v) glucan substrate in PBS, in the presence of the IgG fraction of β-glucanase antiserum (3.5.2). The ratios of enzyme to antibody protein used were 1:0, 1:10, 1:25, 1:50 and 0:50. Samples were taken at intervals and the reaction products determined as described in Methods sections 2.2.1 and 2.3.1. The results demonstrated that the presence of antibody had no significant effect upon the activity of the β-glucanase (Figure 35).

3.5.5 Activity of β-glucosidase in the presence of specific antiserum.

Purified β-glucosidase, 6 µg ml^{-1}, was assayed against 0.1% (w/v) P-nitrophenol-β-D-glucopyranoside in PBS, in the presence of the IgG fraction of β-glucosidase antiserum (3.5.2). The ratios of enzyme to antibody protein used in this case were 1:0, 1:25, 1:100 and 0:100. Samples were taken at intervals and reaction products determined as described in Methods sections 2.2.2 and 2.3.2. The results are presented in figure 36 and show an increase in activity at the highest ratio of antibody to enzyme. Since this experiment was carried out on only two occasions which gave somewhat different results for the 1:100 enzyme:antibody sample (reaction rates of 2.1 and 2.5 µg P-nitrophenol liberated min^{-1}), the apparent rate increase observed may not be significant.

Figure 34: Immunodiffusion of the three enzyme antisera against each of the three purified enzymes

(to test for cross-reactivity between antisera)



a)

centre well: 10 µl

β-1,3-glucanase

b)

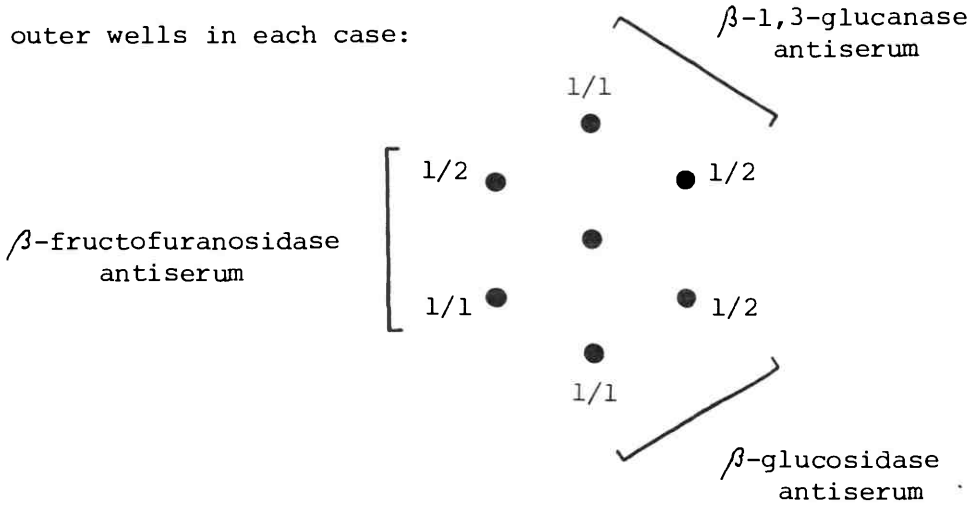
centre well: 10 µl

β-glucosidase

c)

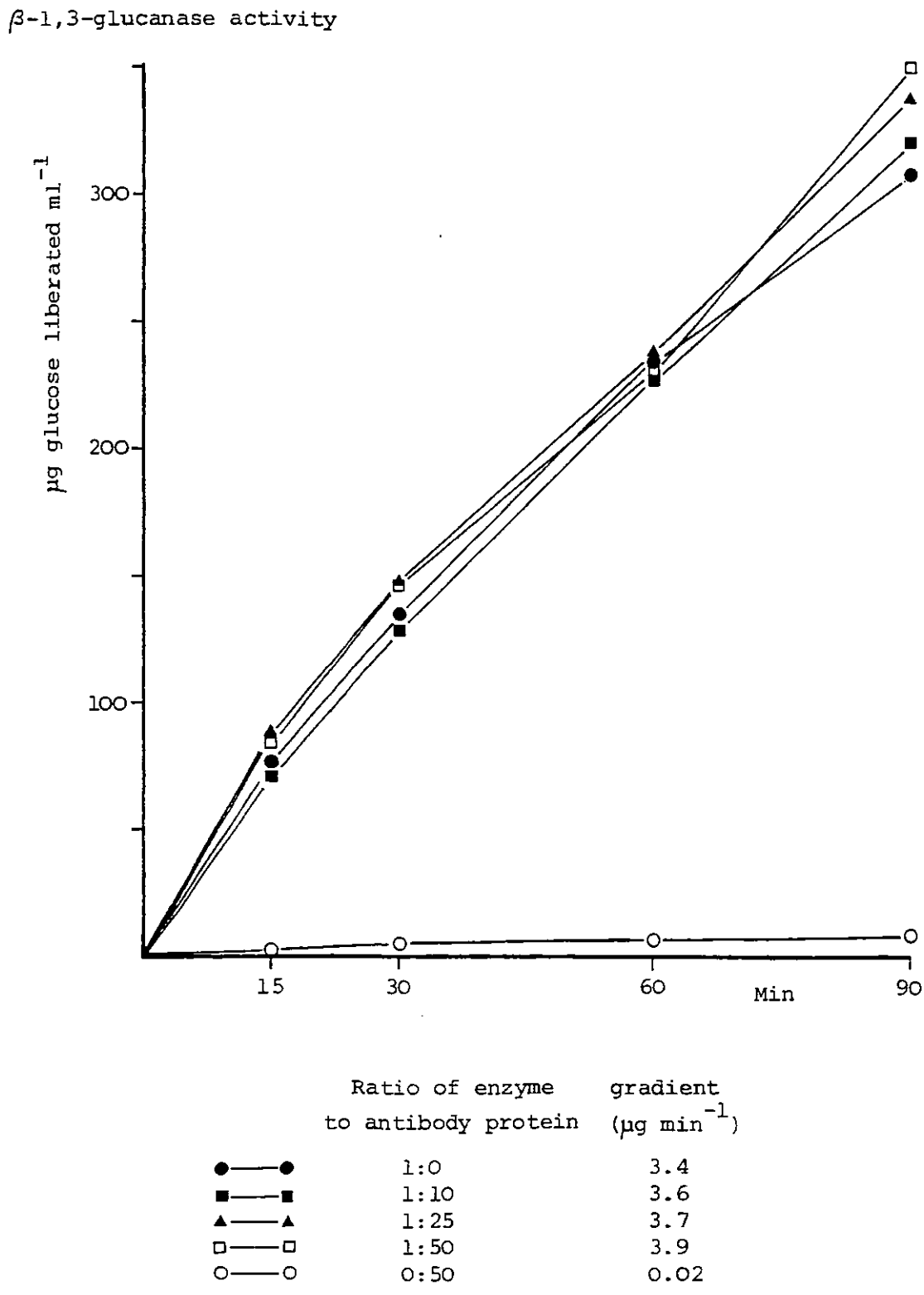
centre well: 10 µl

β-fructofuranosidase



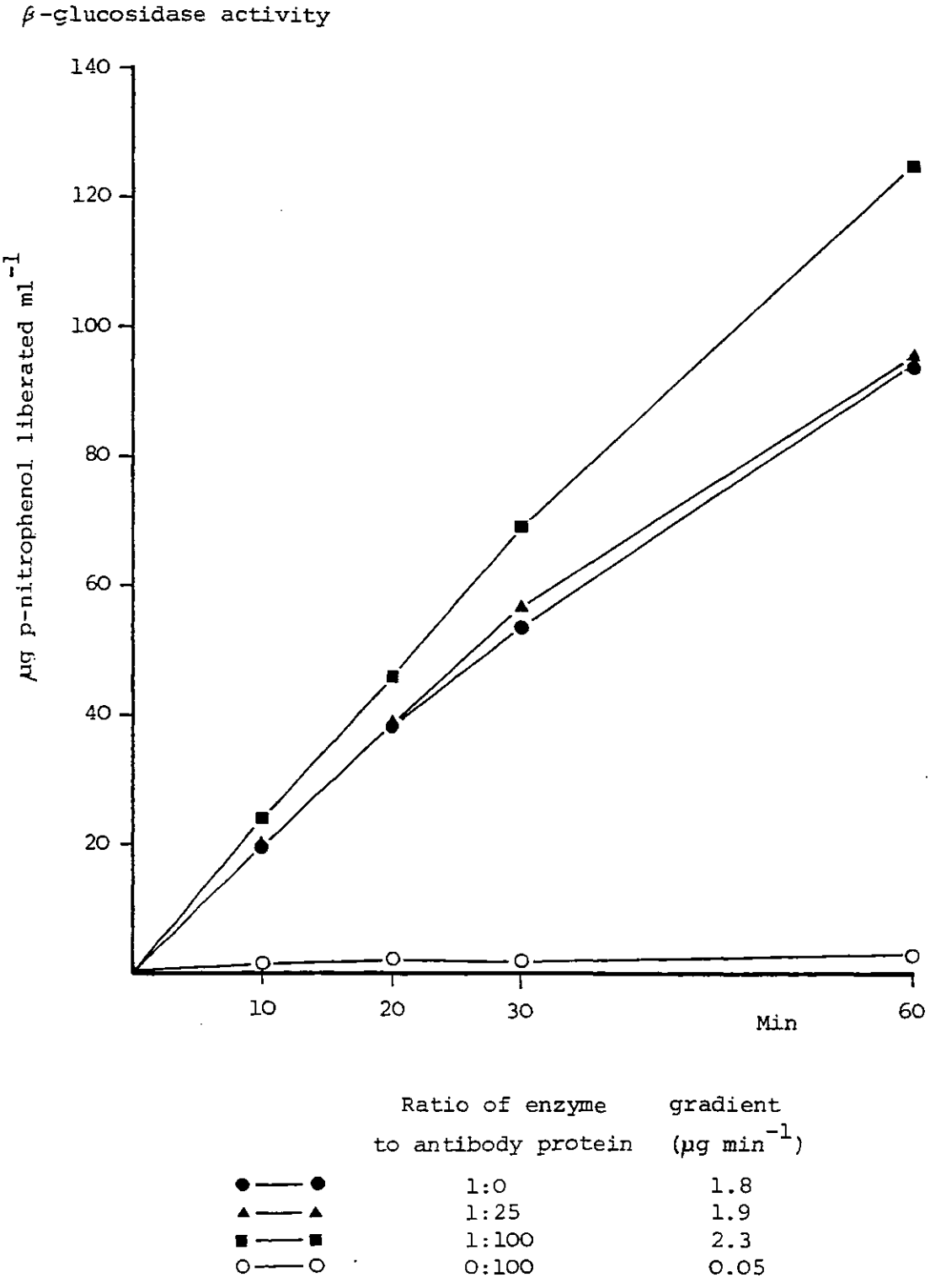
For details see section 3.5.3 (vi).

Figure 35: Activity of the β-1,3-glucanase in the presence of specific antiserum



For experimental details see section 3.5.4.

Figure 36: Activity of the β-glucosidase in the presence of specific antiserum



For experimental details see section 3.5.5.

3.6 Production of antibody stained sections

3.6.1 Sectioning of infected plant tissues

Approximately half of the florets on over 30 rye inflorescences were inoculated simultaneously with C. purpurea 6 spores as described previously (Corbett et al, 1974). For each of the subsequent samples, the florets from at least 3 inflorescences were removed and frozen as described in Methods section 2.6.4.

The technique of tissue sectioning (Methods section 2.6.5) was developed after initial experimentation varying the temperature of sectioning and the orientation of the tissue, in order to prevent disintegration of the section and maintain the close association of the fungal and plant tissue at the interface. The developing ergot in the floret is approximately cylindrical in shape and can be considered more or less symmetrical about its central (long) axis. Therefore, it was considered that longitudinal sections through the ergot and rachilla of the rye floret would supply the most information about the location of enzymes within the tissue.

Sections of 10 μm thickness were routinely produced as these provided the most convenient quantity of tissue for histochemical studies (Pearse, 1968). Consecutive ribbons of sections were placed at each end of the same microscope slide; one ribbon was stained with enzyme antiserum and the other stained with control non-immune serum (Methods section 2.6.6). This provided control tissue sections which were as identical as was practically possible to the tissue sections which were stained for enzyme.

3.6.2 Immunofluorescent staining and quenching of autofluorescence

Sectioned plant tissue, and to a lesser extent fungal tissue, demonstrated bright autofluorescence when illuminated under a fluorescence microscope across a broad spectrum of light from 280 to

500 nm (Plate 1); this masked the fluorescence due to the antibody-coupled fluoresceinwhich was being used to identify the fungal enzymes *in situ*.

Attempts to quench the autofluorescence in tissue sections, by washing with mild oxidising and reducing reagents (iodine in KI and 1% (w/v) sodium borohydride in PBS), prior to antibody treatment, were ineffective. Counterstaining, also prior to antibody treatment, with 1% (w/v) toluidine blue in sodium borate buffer, pH 10, was also ineffective as the stain was washed out by the subsequent steps of the immunofluorescent staining method. Similarly, counterstaining the sections after antibody treatment with Evans blue (Nichols & McComb, 1962), neutral red, aniline blue or crystal violet in aqueous solution, and with Evans blue or crystal violet in sodium borate buffer, pH 8.0, gave insufficient quenching of autofluorescence although the tissue was stained (Table 8).

It was noted that methylene blue or toluidine blue at a concentration of 1% (w/v) in 0.1M sodium borate buffer, pH 10.0, had been shown to stain plant and ergot tissues effectively (Shaw & Mantle, 1980a). Counterstaining tissue sections, after antibody treatment, with these dyes in borate buffer at the relatively lower pH value of 8.0, to prevent dissociation of antibody-antigen complexes and denaturation, completely quenched the autofluorescence. Quenching was less effective if these dyes were used in water instead of borate buffer. Since sclerotial tissue of the ergot, stained with toluidine blue under the above conditions, demonstrated a low level of fluorescence upon illumination at 495 nm (the optimum incident light wavelength for fluorescein) and that stained with methylene blue did not; methylene blue was routinely used for autofluorescence quenching. The concentration of methylene blue employed for counterstaining was reduced to the minimum commensurate with adequate quenching of plant

Table 8: Quenching of plant autofluorescence.

Unless otherwise stated, tissue sections were irrigated after antibody treatment with 1% w/v of the following in 0.1M sodium borate buffer, pH 8.0.

Treatment of sectioned material	Degree of quenching*
Sodium borohydride (1% w/v in PBS)**	None
Iodine in KI (aqueous)**	None
Borate buffer	None
Aniline blue (aqueous)	+
Neutral red (aqueous)	+
Crystal violet (aqueous)	+
Crystal violet	++
Evans blue (aqueous)	+
Evans blue	++
Methylene blue (aqueous)	++
Methylene blue	++++
Toluidine blue (aqueous)	++
Toluidine Blue	++++
Toluidine Blue**	None

* visual estimate of increase in quenching from a trace (+) to complete (++++)

** before antibody treatment.

tissue autofluorescence, to prevent its significantly masking the intensity of any fluorescence visible due to the presence of antibody-coupled fluorescein bound to antigens in the tissue section. This concentration was found to be 0.03% (w/v).

The antibody staining protocol developed (Methods section 2.6.6) was therefore a modification of the standard indirect or sandwich staining techniques (Goldman, 1968; Nairn, 1976). Acetone was not used for fixation as it was found to remove tissue sections from the slides. A 1:4 dilution of the purified IgG fraction of antiserum (equivalent to a 1:20 dilution of original serum) was used for each enzyme antiserum in order to reduce non-specific antibody staining to a minimum (Nairn, 1976), while providing adequate detection of the enzyme antigens in the tissue. Sections from at least three different developing ergots of the same age were stained and photographed under the microscope (Methods section 2.6.7), for each of the three enzyme antisera - β-glucanase, β-glucosidase and β-fructofuranosidase.

3.7 Observations on immunofluorescent stained sections

The approximate areas of sections which are illustrated in the plates are shown in figures 37 to 40.

3.7.1 Development of the ergot within the floret

The development of *C. purpurea* 6 within infected florets was studied over a period of 35 days and was similar to that described previously for this organism (Bove, 1970; Luttrell, 1980, Shaw & Mantle, 1980a, 1980b). During the first 12 to 14 days of infection the host ovary tissue was degraded and replaced by the fungal sphacelium. Fungal invasion did not extend deeper than a discrete interface across the base of the ovary tissue during the complete development of the ergot (Plate 2), except at its centre where it

penetrated to the top of the xylem vessels within the rachilla (Plates 18 and 26). Honeydew exudation was visible from infected florets between 9 and 16 days post-inoculation, and sporulation was evident around the periphery and on the highly convoluted invaginations of sphacelial tissue in sections from 10, 12, 14 and 18 day samples (Plates 7, 9, 23 and 32).

In the period from 14 to 21 days after infection, sporulation and honeydew exudation ceased and differentiation to form sclerotial hyphae took place. Differentiation commenced near the base of the sphacelium, and further differentiation was accompanied by the acropetal displacement of the diminishing sphacelial tissue which could be found primarily at the top of the developing sclerotium (Plates 12, 24, 25 and 35). The ergot was fully differentiated by 28 days after infection, and only a small fragment of sphacelial tissue remained at the apex of the sclerotium (Plate 16). Further development after this stage involved a thickening and maturation of the sclerotium.

Florets not infected with C. purpurea, but which were from the same inflorescence as infected florets, developed seeds as has been described elsewhere (Luttrell, 1980), although these seeds have often been found to be less viable than healthy seeds from an uninfected inflorescence (Corbett et al, 1974; Swan, 1980). No fluorescence was observed when sections of the uninfected seeds were stained with fluorescein - conjugated antibody to detect the fungal enzymes.

3.7.2 Sections stained with β-glucanase antiserum

The early stages of host colonization were not accompanied by any particular localization of fluorescence attributable to the presence of β-glucanase; however, a general fluorescence was present which persisted throughout the sphacelial stage (Plate 3). In this plate, hyphae which have been exposed or cut in the sectioning

process can be detected outlined by a slightly brighter fluorescence within the general fluorescence. At 10 days post-infection a faint band of fluorescence across the base of the sphacelial tissue was visible (Plate 4), indicating the presence of β-glucanase at the interface which had become established between host and parasite. This band of fluorescence had become more pronounced by 14 days, and was brighter around the periphery of the interface than at its centre (Plates 5 and 6).

In addition to the fluorescence at the host/parasite interface, very bright fluorescence was observed 10 days post-infection around the peripheral areas, both superficial and invaginated, of the developing sphacelium. This appears as a series of highly convoluted fluorescent bands in sections (Plates 7 and 9). This part of the sphacelium corresponds to the area where conidiation is occurring; a pocket of conidia connecting with the outer surface of the sphacelium and divided from the sphacelial tissue by a band of bright fluorescence is shown in plate 7. Bright fluorescence at the boundaries of the conidiating tissue was visible throughout the period between 10 and 18 days post-infection, whilst the sphacelial form of hyphae predominated in the developing ergot (Plates 9 and 10).

Sclerotial tissue demonstrated a much lower overall fluorescence than sphacelial tissue when stained with β-glucanase antiserum. So, as differentiation progressed between 14 and 21 days, the more brightly fluorescent sphacelial tissue was displaced and can be observed above the darker sclerotial tissue in plate 11. Furthermore, after 21 days a distinct border was detected between the contrastingly stained tissues (Plate 12). This border corresponded exactly to the layer of purple pigmented cells which form the outer cortex and rind of the mature sclerotium. By 21 days the fluorescence that had been associated with the peripheral sporulating areas of the

sphacelial tissue was greatly diminished (Plate 12). However, the band of fluorescence at the interface did not diminish, indicating the continued presence of β-glucanase (Plate 13), which had, by this stage become more diffuse across the base of the ergot (Plate 14). For comparison, plate 15 shows a control section of the same age stained with control non-immune serum and demonstrating the absence of fluorescence across the interface.

At 28 days post-infection differentiation of the ergot was complete, but fluorescence persisted in the fragment of sphacelial tissue remaining outside the purple pigmented boundary of the sclerotial apex (Plate 16). Fluorescence was absent from the tissues within the mature sclerotium, except across the base (i.e. the host/parasite interface) where it retained the band of fluorescence due to β-glucanase (Plates 17 and 18); the lower intensity of the fluorescence is probably ascribable to the fact that it was more diffuse than had been seen during differentiation.

3.7.3 Sections stained with β-glucosidase antiserum

Throughout the development of the ergot, fluorescence due to β-glucosidase was observed in similar locations in the fungal tissue to that seen with β-glucanase (Plates 3 to 18).

During the early stages of establishment of the sphacelium from 4 to 10 days post-infection, a general fluorescence was present without showing particular localization (Plates 19 and 20). Fungal hyphae, exposed by sectioning and delineated by brighter fluorescence than the general background along their cell walls are visible in plate 20. The 'loose' appearance of the tissue in this plate possibly demonstrates that the hyphae were partially conforming to the cell structure of the original host ovary tissue, which was still in the process of being degraded and replaced by sphacelial tissue. As with β-glucanase, a faint band of fluorescence due to β-glucosidase

became visible across the base of the sphacelium after 10 days (Plate 21), and by 14 days this band was quite distinct (Plate 22). Fluorescence was also observed around the peripheral and invaginated conidiating surfaces of the sphacelium with β-glucosidase antiserum; however, it was less intense than that observed with β-glucanase antiserum (Plate 23).

Sclerotial tissue showed much lower overall fluorescence than sphacelial tissue, again as noted for β-glucanase. Plates 24 and 25 demonstrate the brightly fluorescent sphacelial tissue that was displaced acropetally during differentiation by the darker sclerotial tissue. The small sphacelial cap remaining on the apex of the mature ergot continued to show β-glucosidase fluorescence similar to that seen in sections stained with β-glucanase antiserum (Plate 16).

During differentiation (14 to 21 days after infection) the band of fluorescence due to β-glucosidase at the interface across the base of the ergot became much brighter (Plate 26), especially above the region where the fungal hyphae had penetrated the rachilla more deeply close to the xylem vessels. At 21 days the fluorescence was more intense than that observed for β-glucanase; and, unlike the β-glucanase at this stage, the β-glucosidase was confined to a much more discrete area across the base of the sclerotium (Plates 27 and 28). Moreover, this very bright narrow band of fluorescence due to the β-glucosidase at the interface was maintained in mature sclerotial tissue, and was observed in sections from 28 and 35 days after infection (Plates 29 and 30).

3.7.4 Sections stained with β-fructofuranosidase antiserum

The overall level of fluorescence ascribable to β-fructofuranosidase was much less than that seen for β-glucanase and β-glucosidase. That fluorescence was present can be seen by comparison of plate 37 with the control, plate 38; the latter micrograph

had an exposure time of 4 min, twice that required for the micrograph in plate 37.

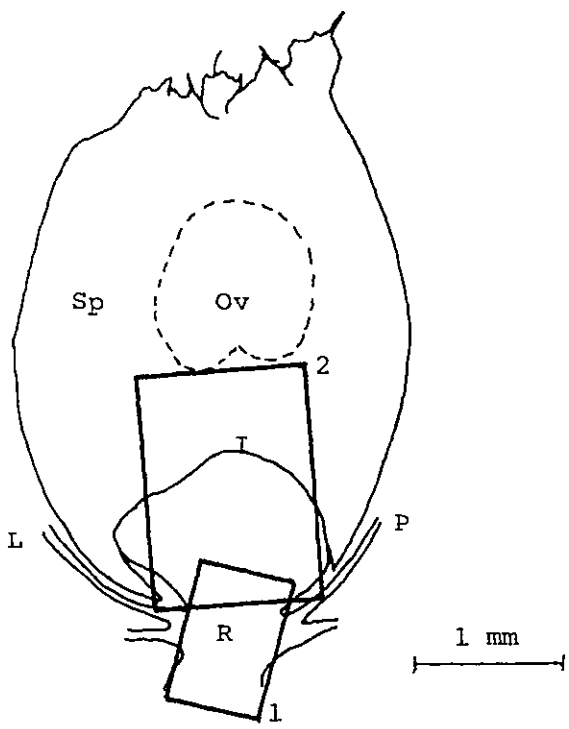
Sphacelial tissue stained for β-fructofuranosidase demonstrated a general fluorescence indicating the presence of the enzyme throughout the tissue, without any particular localization, similar to that observed for β-glucanase and β-glucosidase (Plate 31). However, in contrast to the other two enzymes, the later sphacelial stages were not characterized by a band of fluorescence, due to the enzyme, across the base of the ergot at the interface. Fluorescence due to β-fructofuranosidase was apparent at the peripheral sporulating tissues of the sphacelium, but it was significantly less intense than that seen for β-glucanase and only slightly brighter than the surrounding sphacelial mass (Plate 32).

During differentiation (14 to 21 days post infection) a band of fluorescence developed across the interface extending in places into the host cells of the rachilla (Plate 33). This effect was not found to occur in sections at other stages of ergot development. Differentiated sclerotial tissue demonstrated an increased intensity of fluorescence over that found for sphacelial tissue in sections stained for β-fructofuranosidase (Plate 34). It is evident from this micrograph that the fluorescence exhibited by sectioned hyphae is confined to a discrete cell boundary and is not confluent between cells. Sphacelial tissue became less fluorescent during differentiation of the ergot, and thus, in plate 35, the sphacelial cells displaced at the top are dark compared to the brightly fluorescent sclerotial tissue below the cortical layer of purple-pigmented hyphae. This represents the opposite case to that observed for β-glucanase and β-glucosidase.

The differentiated ergot (28 days) showed only traces of fluorescence at the base of the sclerotium (Plate 36); moreover, none

was visible in the remaining sphacelial cap once differentiation was complete. However, mature sclerotial hyphae in the apical half of the ergot continued to demonstrate fluorescence due to the presence of β-fructofuranosidase (Plate 37). A control section at the same age stained with non-immune serum provides comparison (Plate 38). The area of fluorescence was confined to tissues inside the pigmented cortical rind of the ergot (Plate 39). Sections of tissues 35 days after infection showed that fluorescence due to β-fructofuranosidase persisted in the medulla of mature ergots (Plates 40 and 41), whereas the β-glucanase and β-glucosidase had disappeared from this area upon maturation.

Figure 37: Diagram representing a section of developing ergot at 10 days and showing the areas of the section illustrated in plates 1 and 2.



Sp - sphacelial tissue; Ov - ovule; I - interface; L - lemma; P - palea; R - rachilla. Numbers refer to the corresponding plates. which are both at 160x magnification.

Since individual ergot samples varied in actual size, the sizes of the plates indicated in the diagram are not strictly to scale.

Plate 1: Autofluorescence of plant tissue sections

Section of plant tissue from the rachilla of the floret showing yellow-green autofluorescence that was visible upon illumination at 495 nm.

Plate 2: The host-parasite interface

Section at 10 days photographed by white light (tungsten filament) showing fungal and plant tissue stained blue by the methylene blue-borate reagent used to suppress autofluorescence. The discrete interface between the fungal sphacelium and uninvaded plant cells at the base of the ovary is indicated.

Sp - sphacelial tissue; I - interface; R - part of rachilla

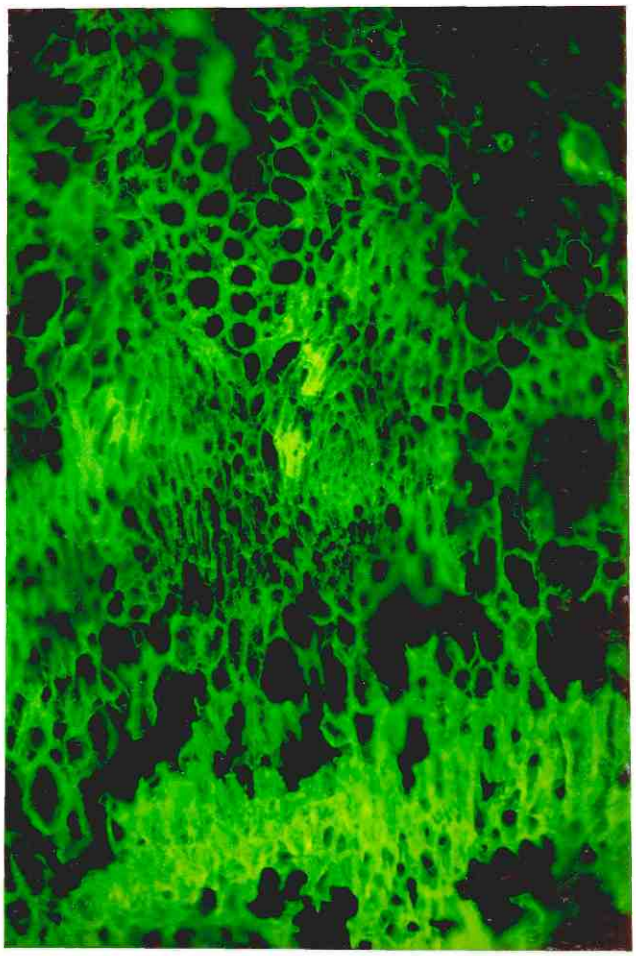


Plate 1

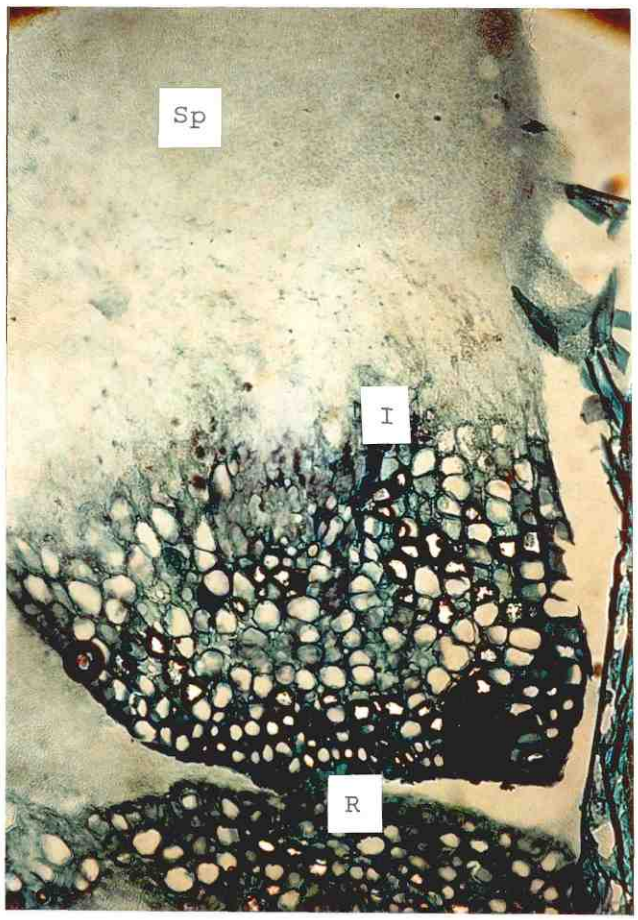
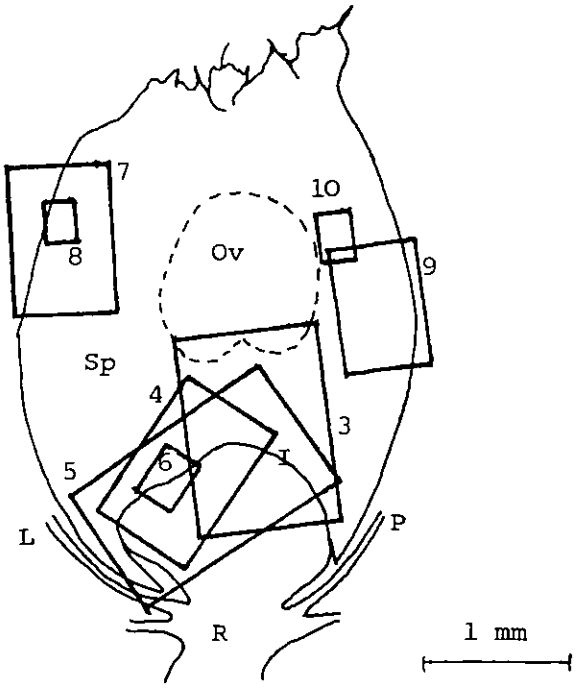


Plate 2

Figure 38: Diagrams representing sections of developing ergot stained with /3-1,3-glucanase antiserum showing the areas of sections illustrated in plates 3 to 18.

a) Sections from 7 to 14 days.

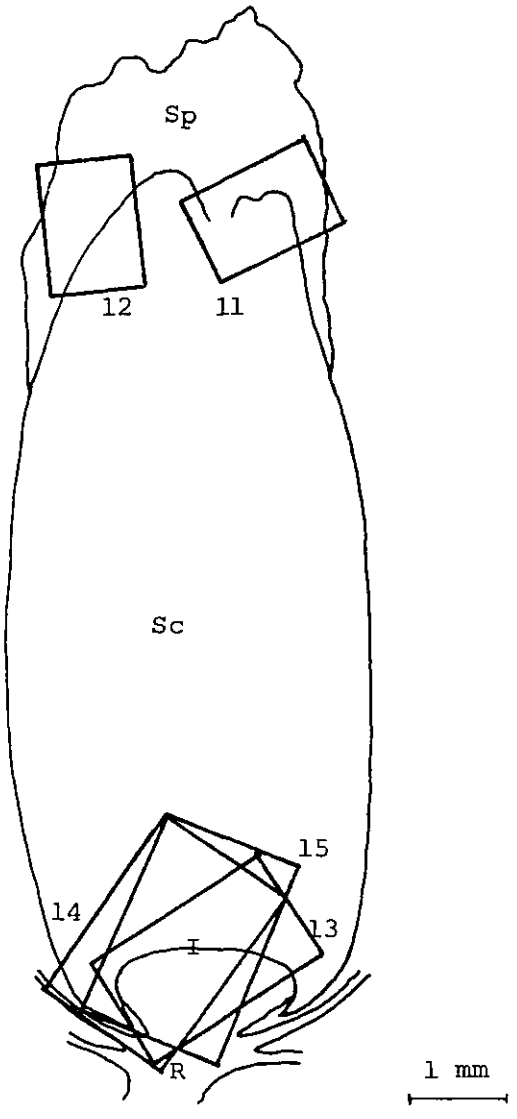


Sp - sphacelial tissue; Ov - ovule; I - interface; L - lemma; P - palea; R - rachilla. Numbers refer to the corresponding plates. Plates 3,4,5, 7 and 9 are at 160x magnification, plates 6,8 and 10 are at 400x.

Since individual ergot samples varied in actual size, the sizes of the plates indicated in the diagram are not strictly to scale.

Figure 38: (continued)

b) Sections from 14 to 21 days.

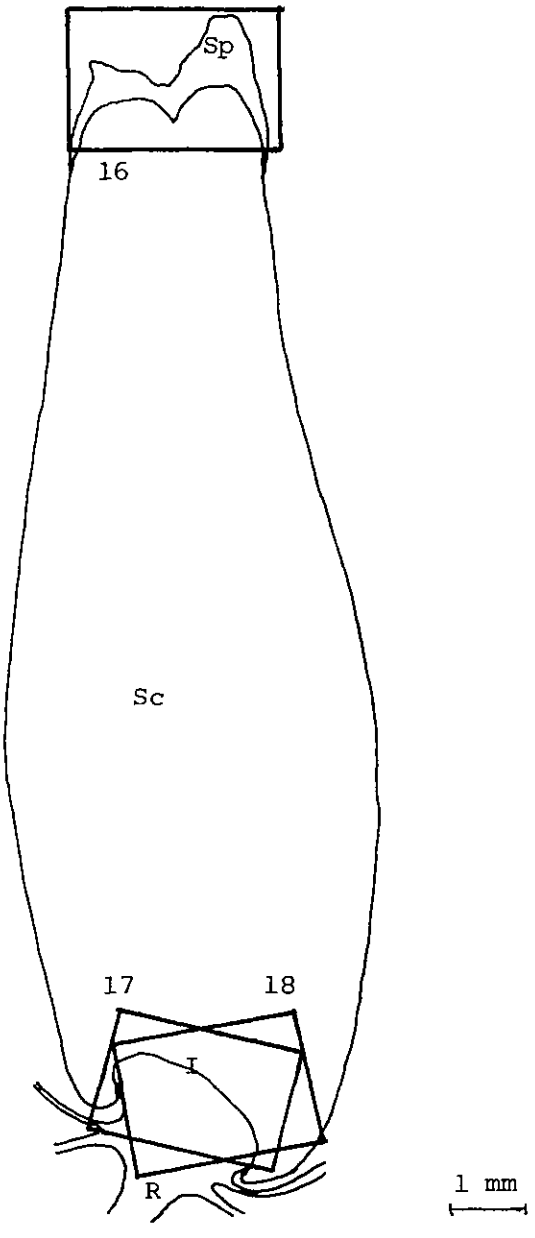


Sp - sphacelial tissue; Sc - sclerotial tissue; I - interface;
R - rachilla. Numbers refer to the corresponding plates.
Plates 11 to 15 are all at 160x manification.

Since individual ergot samples varied in actual size, the sizes of the plates indicated in the diagram are not strictly to scale.

Figure 38: (continued)

c) Sections from 28 to 35 days.



Sp - sphacelial tissue; Sc - sclerotial tissue; I - interface;
R - rachilla. Numbers refer to the corresponding plates.
Plates 16,17 and 18 are at 160x magnification.
Since individual ergot samples varied in actual size, the sizes of the plates indicated in the diagram are not strictly to scale.

Plate 3: Early fungal mycelium at day 7 stained for β-1,3-glucanase

Fungal mycelium was evident between the remains of the ovule at the top of the plate and uninvaded host cells at the base of the ovary. (The fungus colonises the parenchyma of the ovary wall before the ovule tissues are degraded (Shaw, 1979)). Some fungal hyphae appear outlined by slightly brighter fluorescence.

Ov - ovule; HC - host cells; FM - fungal mycelium;
FH - fungal hyphae.

Plate 4: Host-parasite interface at day 10 stained for β-1,3-glucanase

Fluorescence above the general level of fluorescence was visible as a faint band across the interface.

Sp - sphacelial mycelium; HC - host cells at base of ovary;
I - interface.

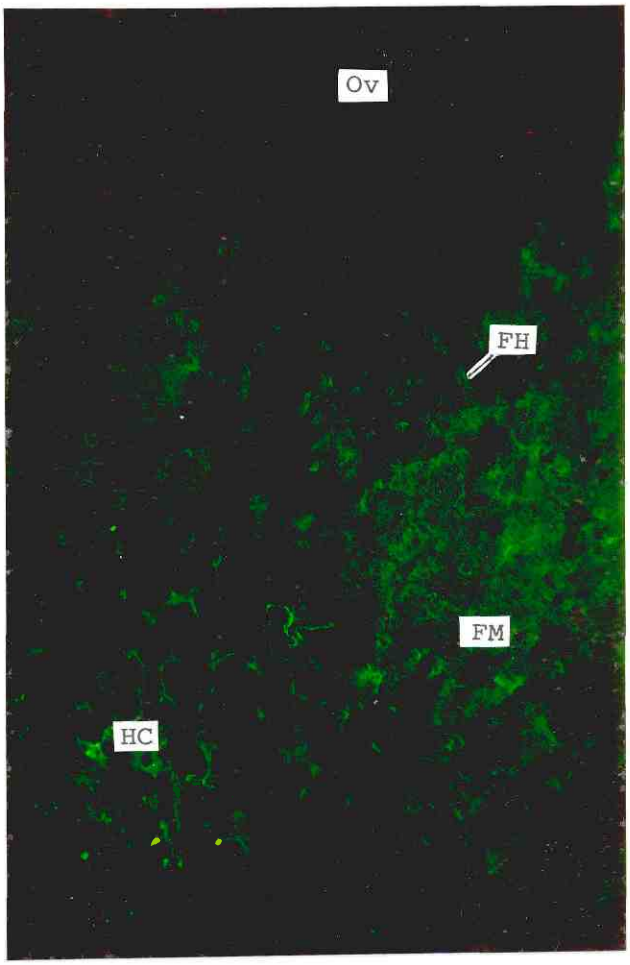


Plate 3

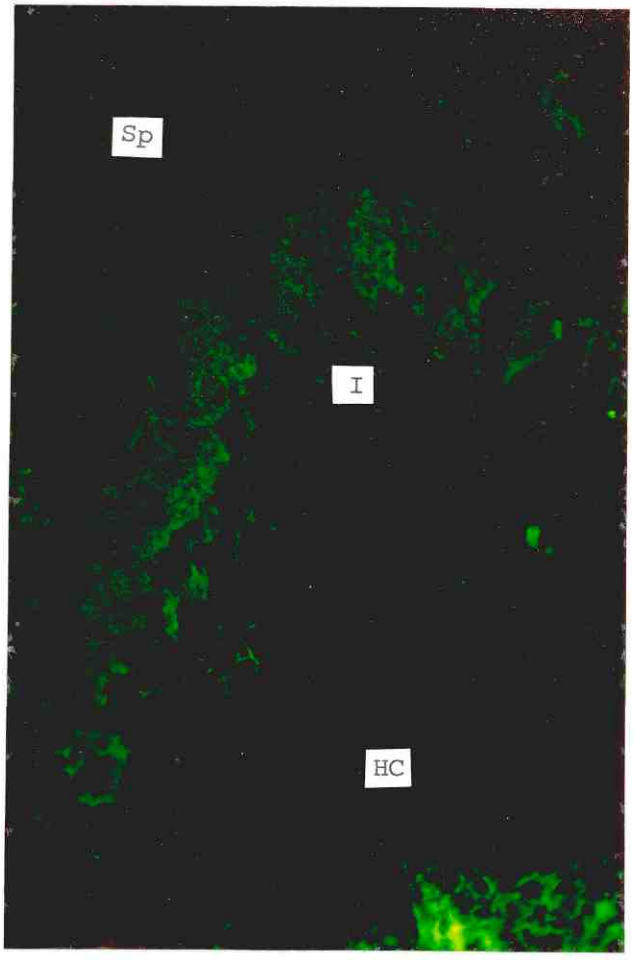


Plate 4

Plate 5: Host-parasite interface at 14 days stained for β-1,3-glucanase

A band of fluorescence, brighter towards its edges than at its centre, was located at the base of the fungal sphacelium where the mycelium was in contact with plant cells.

Sp - sphacelial mycelium; HC - host plant cells; I - interface; AF - area of fluorescence.

Plate 6: Detail of host-parasite interface at 14 days stained for β-1,3-glucanase

Enlarged section of plate 5 showing highly fluorescent fungal hyphae next to uninvaded host cells.

Sp - sphacelial mycelium; HC - host plant cells; I - interface; AF - area of fluorescent hyphae.

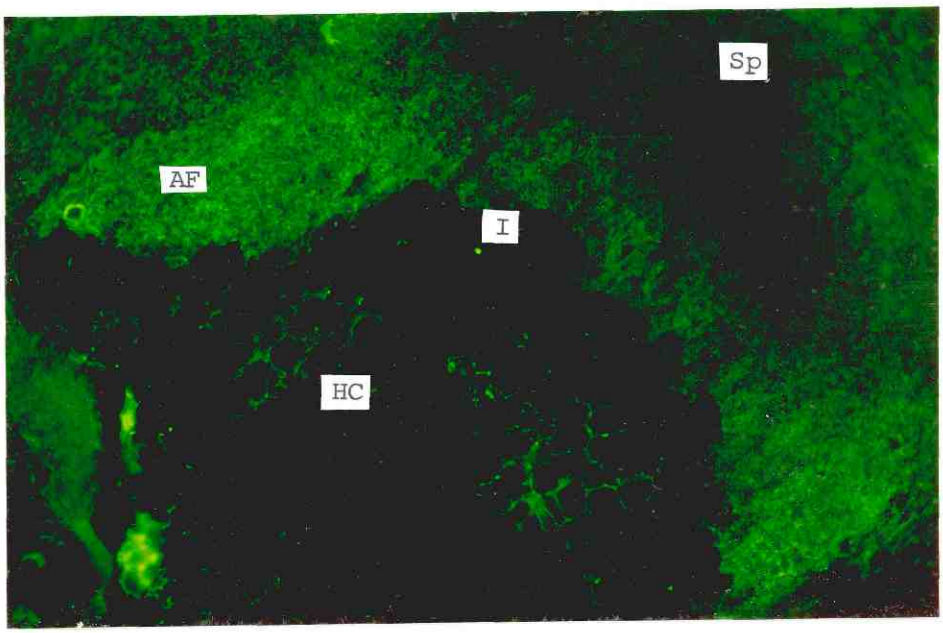


Plate 5

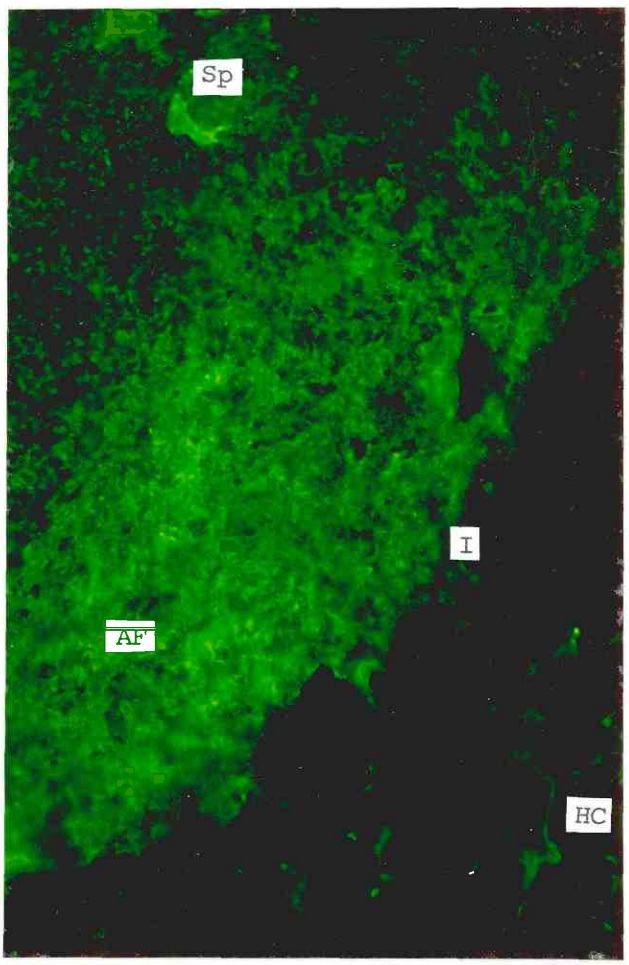


Plate 6

Plate 7: Invaginated pocket of conidiation in sphacelial tissue at 10 days stained for β-1,3-glucanase

The pocket of conidiating hyphae opens to the surface of the sphacelium. Fluorescence was visible along the convoluted periphery of the pocket. The pocket would be filled with honeydew in which the conidia are exuded from the floret.

Sp - sphacelial tissue; PC - pocket of conidiation; AF - area of fluorescence; Op - opening to outer surface of spacelium.

Plate 8: Detail of conidiophores at 10 days stained for β-1,3-glucanase

Enlarged section of plate 7 showing highly fluorescent conidiating hyphae on the borders of a pocket of conidiation. Conidia appear as dark silhouettes against the fluorescence.

PC - pocket of conidiation; C - conidia.

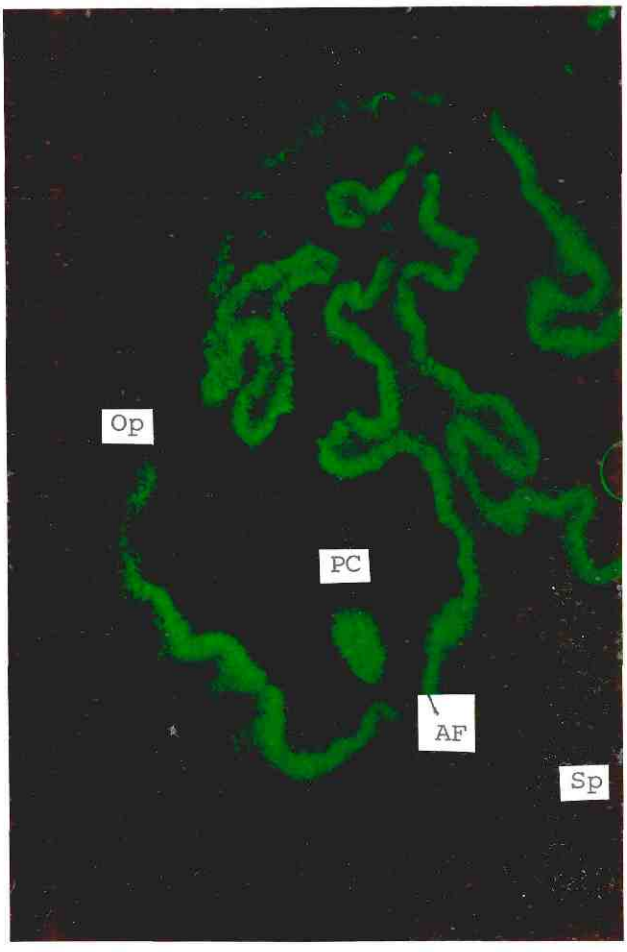


Plate 7

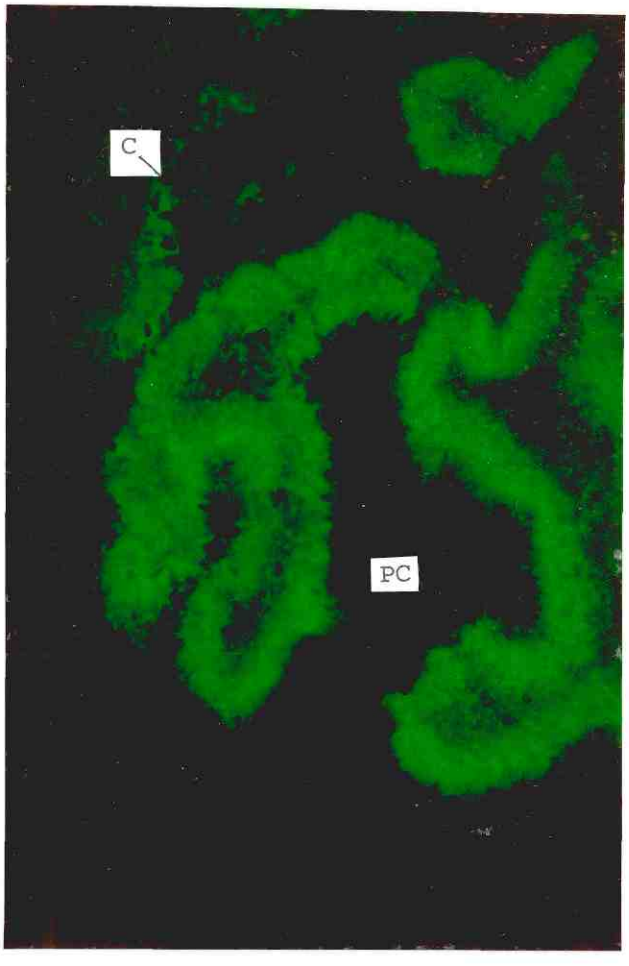


Plate 8

Plate 9: Sphacelial tissue at day 14 stained for β-1,3-glucanase

Conidiating hyphae extend throughout the sphacelial mycelium forming highly convoluted pockets connected to the outer surface of the sphacelium.

Sp -sphacelial tissue; PC - pocket of conidiation; OSp - outer surface of sphacelium.

Plate 10: Detail of conidiophores within sphacelial tissue at 14 days stained for β-1,3-glucanase

Conidia are generated by the highly fluorescent hyphae bordering the pocket of conidiation.

Sp - sphacelial tissue; C - conidia; PC - pocket of conidiation; AF - area of fluorescence.

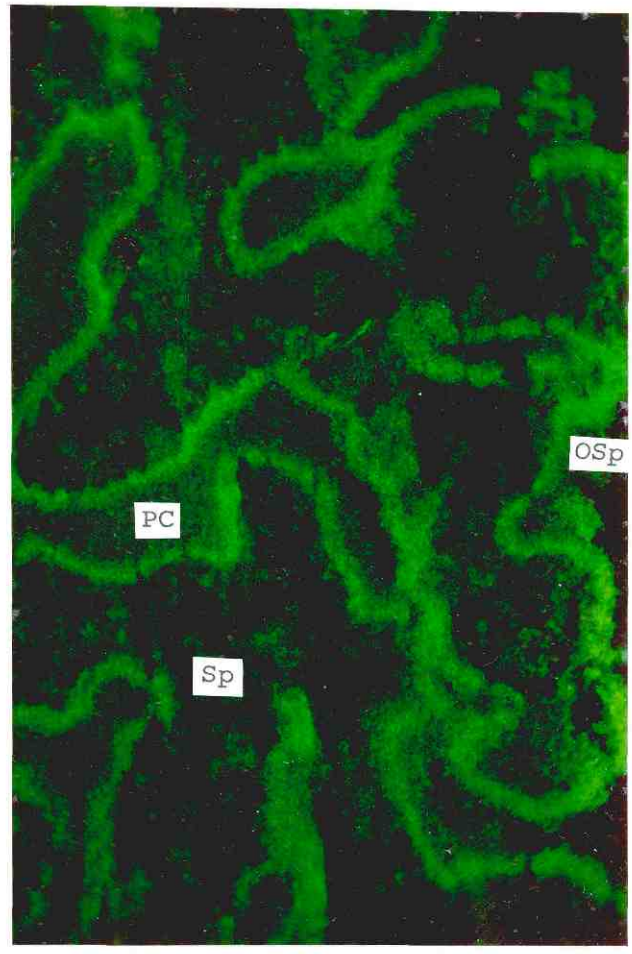


Plate 9

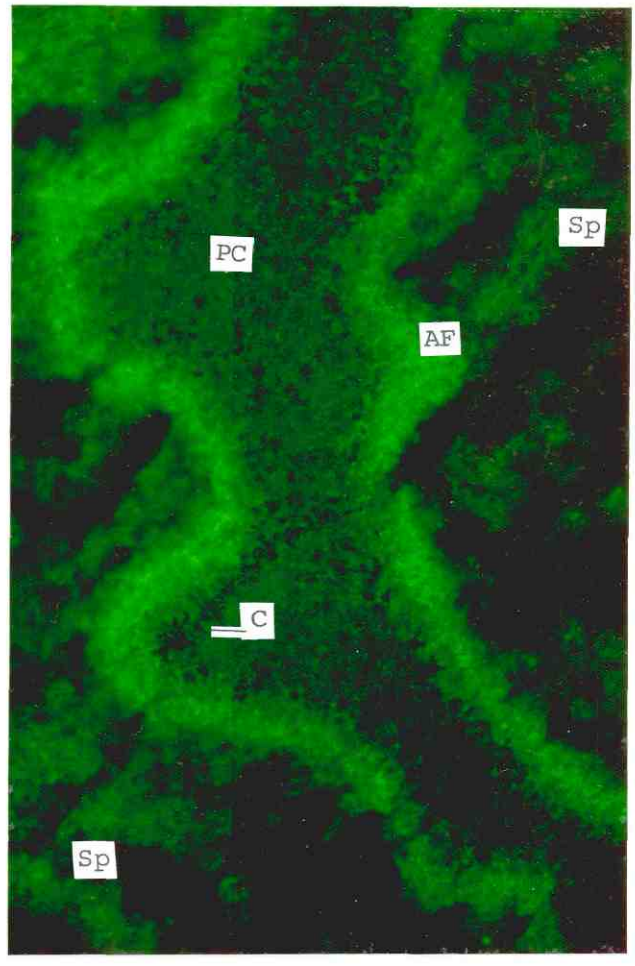


Plate 10

Plate 11: Apex of developing ergot at 18 days stained for β-1,3-glucanase

Sclerotial tissue appears darker below the more brightly fluorescent sphacelial tissue.

Sc - sclerotial tissue; Sp - sphacelial tissue.

Plate 12: Apex of developing ergot at 21 days stained for β-1,3-glucanase

Fluorescent sphacelial tissue was separated from the non-fluorescent sclerotial tissue by a dark band (PL) which corresponded to the purple pigmented outer cortex of the ergot. The borders of pockets of conidiation in the remaining sphacelial tissue had ceased to show fluorescence at this stage.

Sc - sclerotial tissue; Sp - sphacelial tissue; PL - pigmented layer; PC - pocket of conidiation.

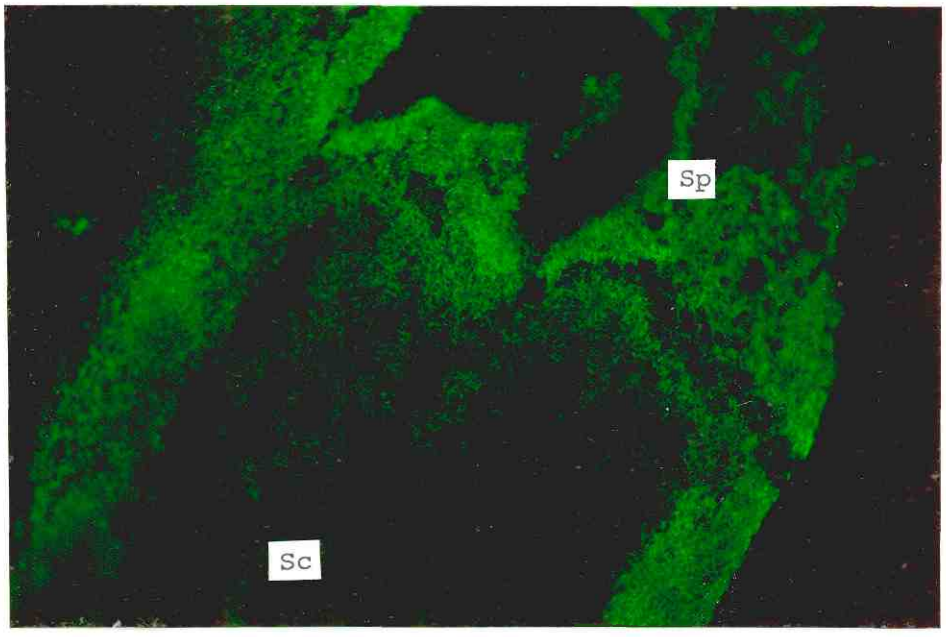


Plate 11

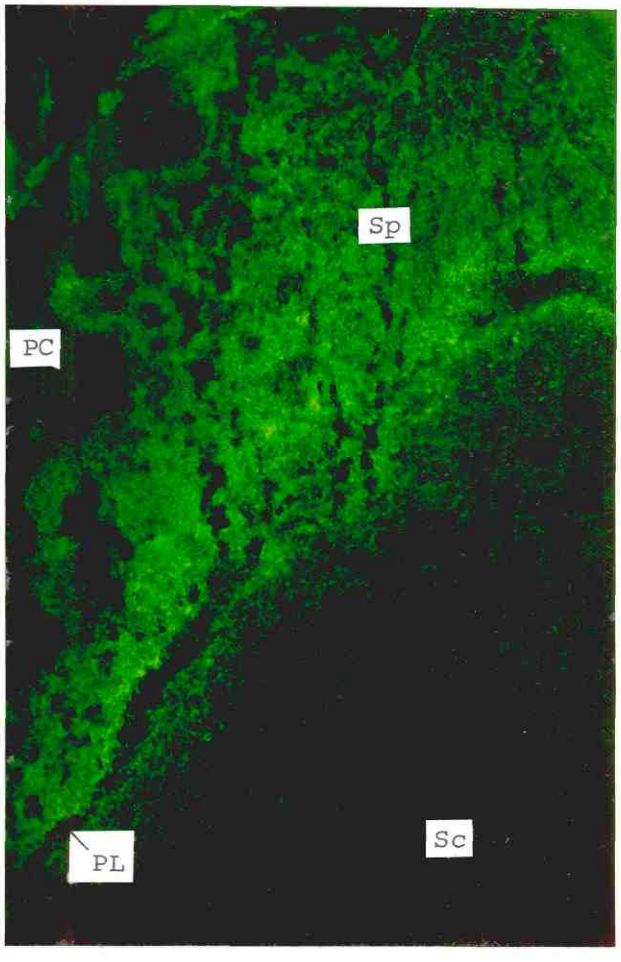


Plate 12

Plate 13: Host-parasite interface at 18 days stained for β-1,3-glucanase

Fungal mycelium (FM) can be seen to grow around underneath the uninvaded plant cells at the base of the ovary - this serves to anchor the ergot inthe floret.

Sc - sclerotial tissue; I - interface; HC - host plant cells; FM - fungal mycelium.

Plate 14: Host-parasite interface at 21 days stained for β-1,3-glucanase

Fluorescence across the base of the sclerotium was more diffuse at this stage. At the centre of the interface (Ic) the fluorescence was brighter near the area at which fungal hyphae extend downwards to the vascular tissue in the rachilla.

Sc - sclerotial tissue; I - interface; Ic - centre of interface; HC - host plant cells; L - lemma.

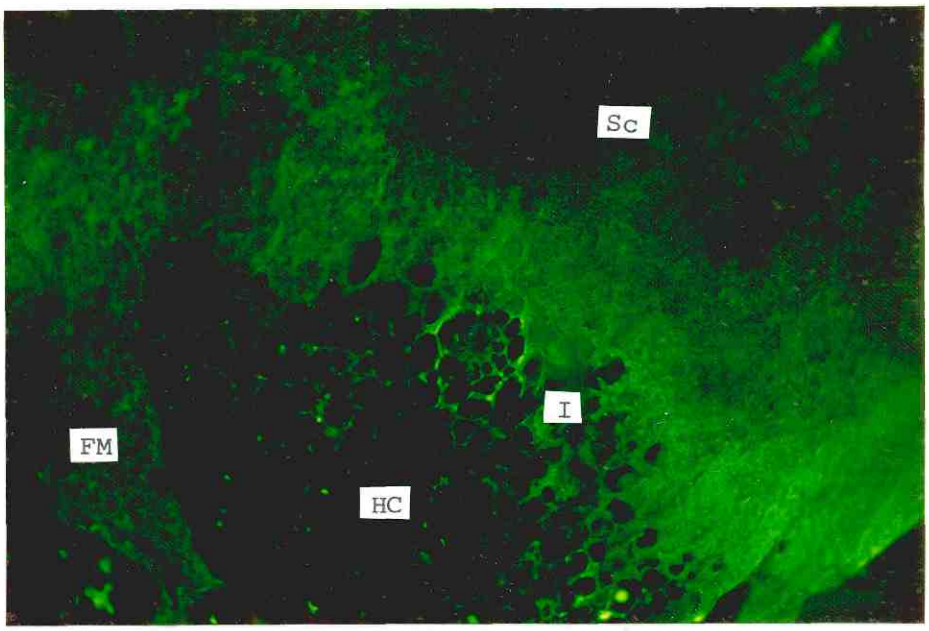


Plate 13

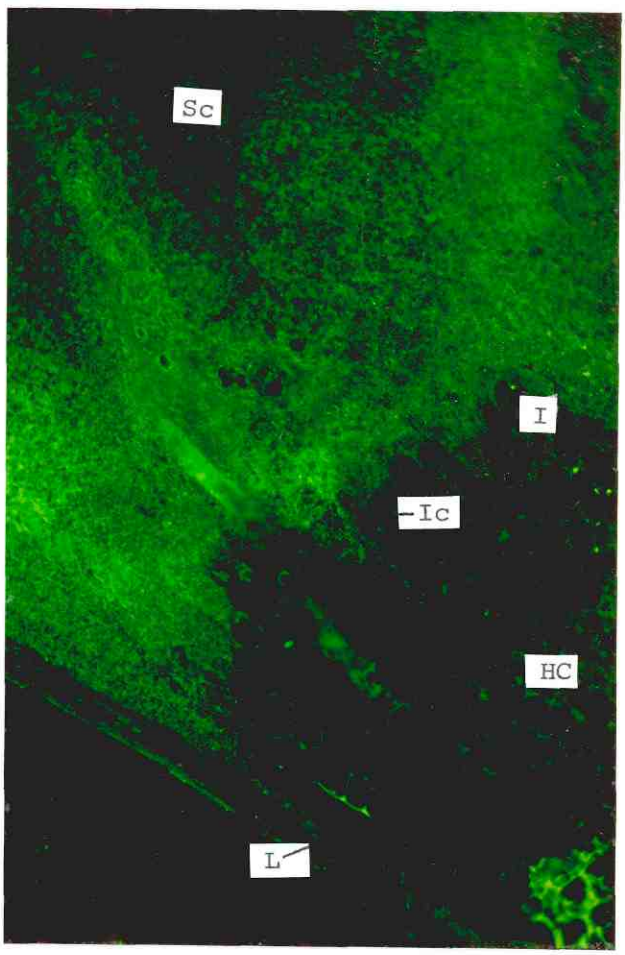


Plate 14

Plate 15: Host-parasite interface at 21 days stained with control non-immune serum

Control section showing no fluorescence presented for comparison with plate 14.

Sc - sclerotial tissue; I - interface; HC - host plant cells.

Plate 16: Apex of developing ergot at 28 days stained for β-1,3-glucanase

A remnant of fluorescent sphacelial tissue remained outside the pigmented rind at the tip of the sclerotium.

Sp - sphacelial tissue; Sc - sclerotial tissue; PL - pigmented layer.

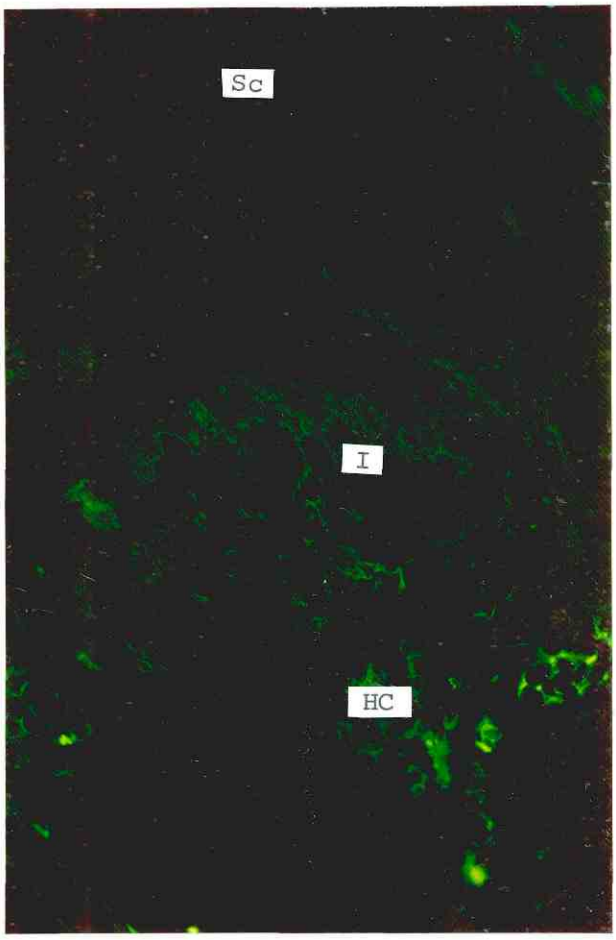


Plate 15

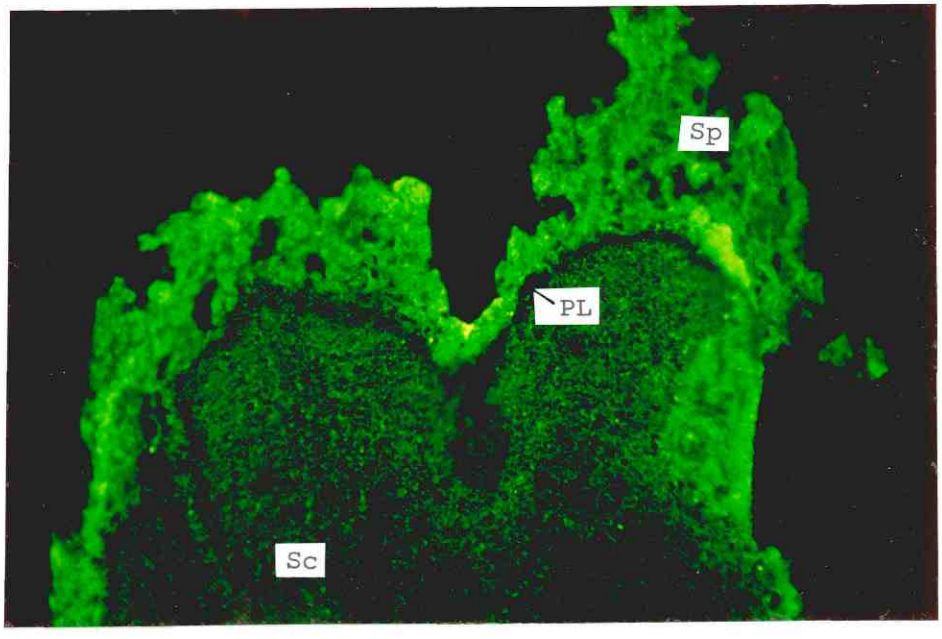


Plate 16

Plate 17: Host-parasite interface at 28 days stained for β-1,3-glucanase

Diffuse fluorescence was present across the base of the sclerotium and above vascular tissue at this stage.

Sc - sclerotial tissue; I - interface; HC - host plant cells; V - vascular elements from the rachilla.

Plate 18: Host-parasite interface at 35 days stained for β-1,3-glucanase

A band of fluorescence persisted in the mature sclerotium.

Sc - sclerotial tissue; I - interface; HC - host plant cells; V - vascular elements from the rachilla.

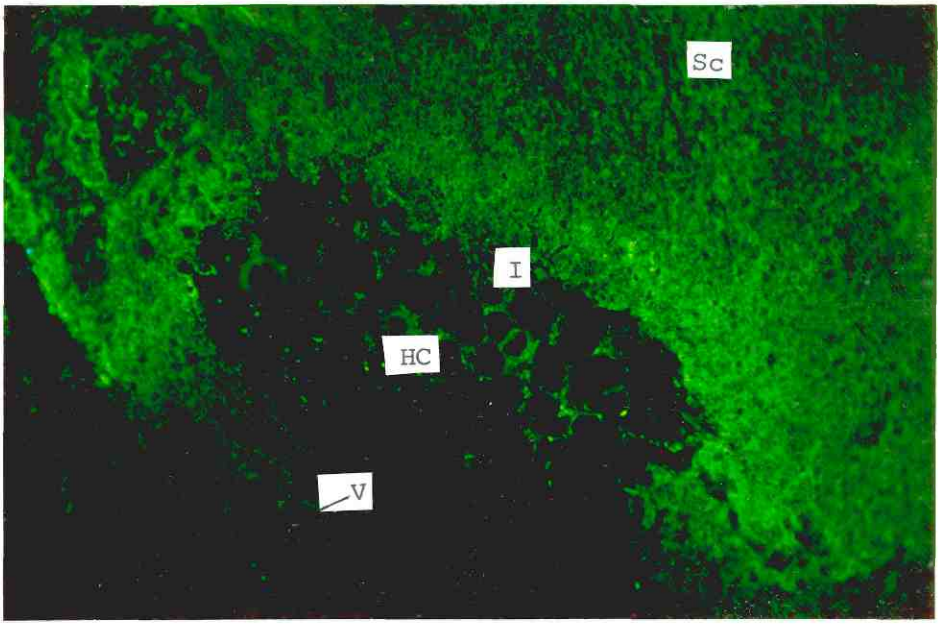


Plate 17

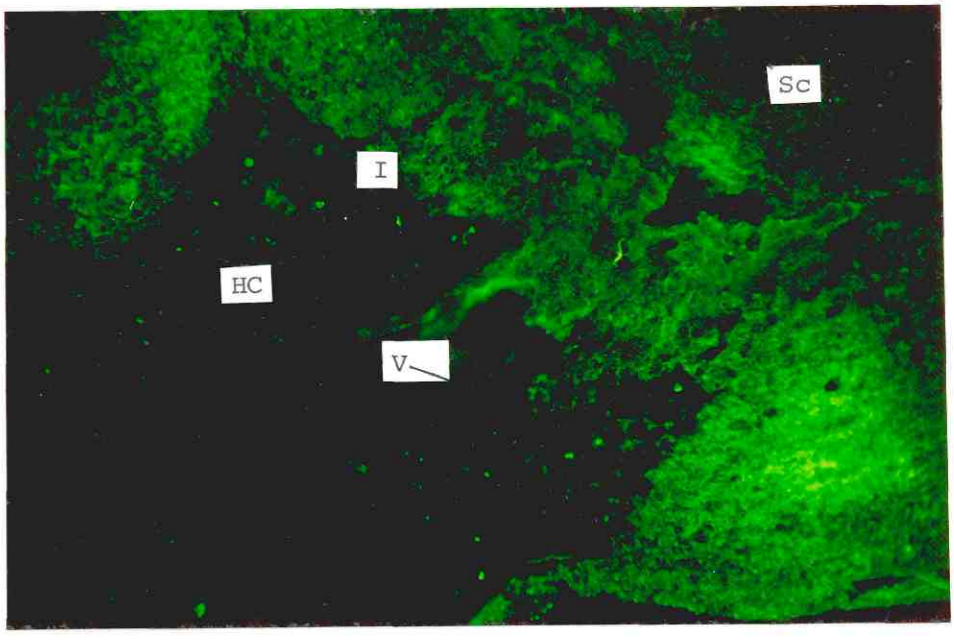
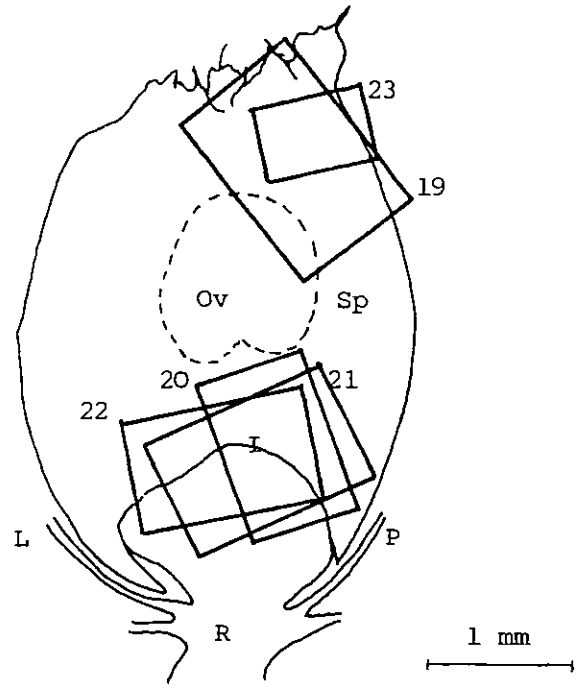


Plate 18

Figure 39: Diagrams representing sections of developing ergot stained with β-glucosidase antiserum showing the areas of sections illustrated in plates 19 to 30.

a) Sections from 4 to 14 days.

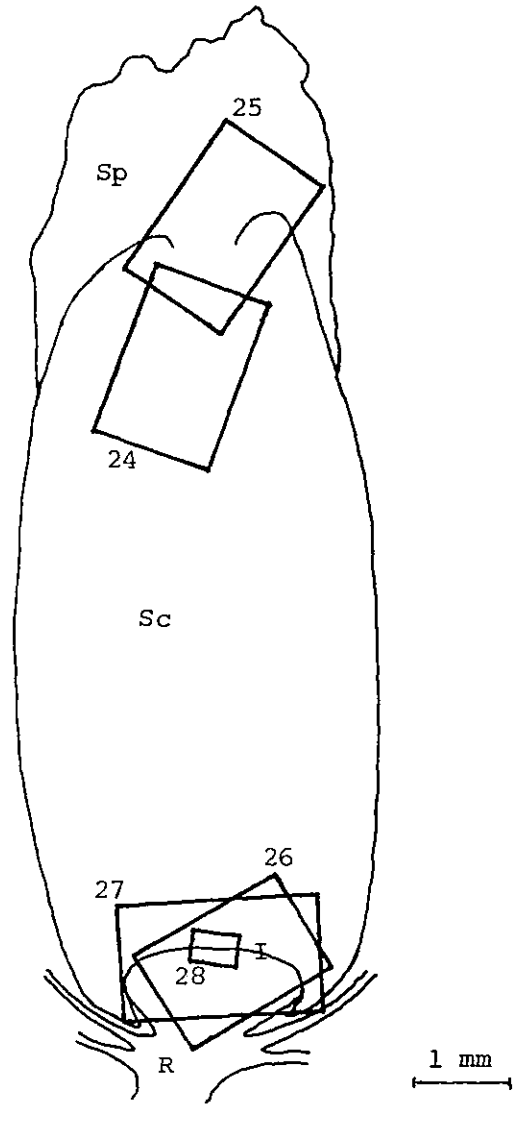


Sp - sphacelial tissue; Ov - ovule; I - interface; L - lemma; P - palea; R - rachilla. Numbers refer to the corresponding plates.
Plates 19 to 23 are all at 160x magnification.

Since individual ergot samples varied in actual size, the sizes of the plates indicated in the diagram are not strictly to scale.

Figure 39: (continued)

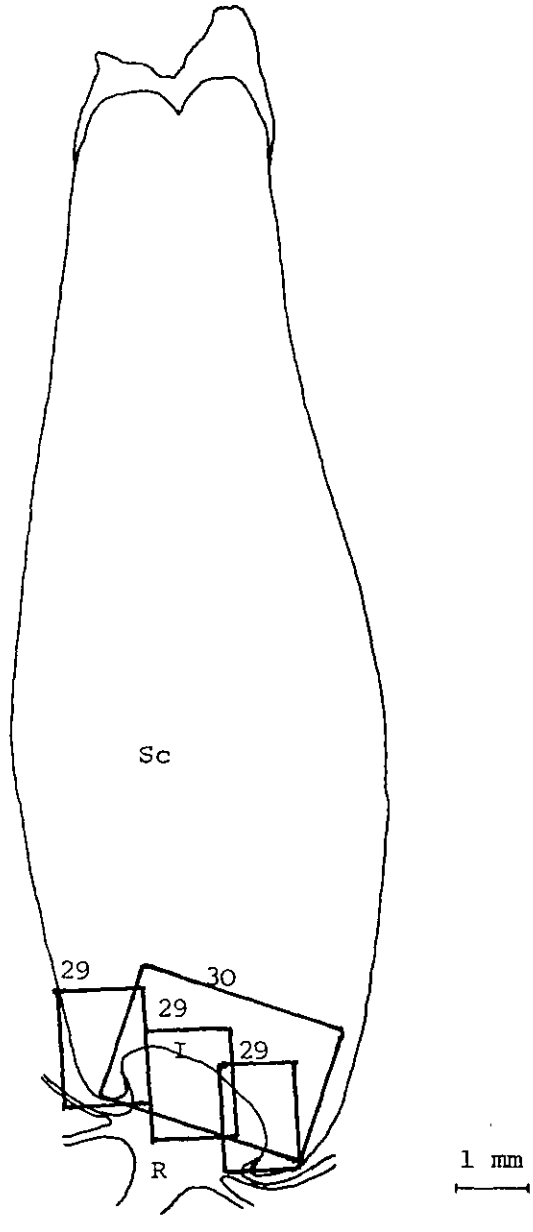
b) Sections from 14 to 21 days.



Sp - sphacelial tissue; Sc - sclerotial tissue; I - interface; R - rachilla. Numbers refer to the corresponding plates. Plates 24 to 27 are at 160x and plate 28 is at 400x magnification.
Since individual ergot samples varied in actual size, the sizes of the plates indicated in the diagram are not strictly to scale.

Figure 39: (continued)

c) Sections from 28 to 35 days.



Sc - sclerotial tissue; I - interface; R - rachilla. Numbers refer to the corresponding plates, both of which are at 160x magnification.

Since individual ergot samples varied in actual sizes, the sizes of the plates indicated in the diagram are not strictly to scale.

Plate 19: Infected ovary at day 4 stained for β-glucosidase

Disruption of the ovary tissue was apparent although the fungal mycelium had not become established.

Ov - ovule; L - lemma; DHC - disrupted ovary parenchyma.

Plate 20: Early fungal mycelium at day 7 stained for β-glucosidase

Fluorescence is visible on fungal hyphae at the right of the section. The morphology of the mycelium at this stage still resembles the original structure of plant cell walls in the ovary.

FH - fungal hyphae; FM - fungal mycelium; HC - host plant cells.

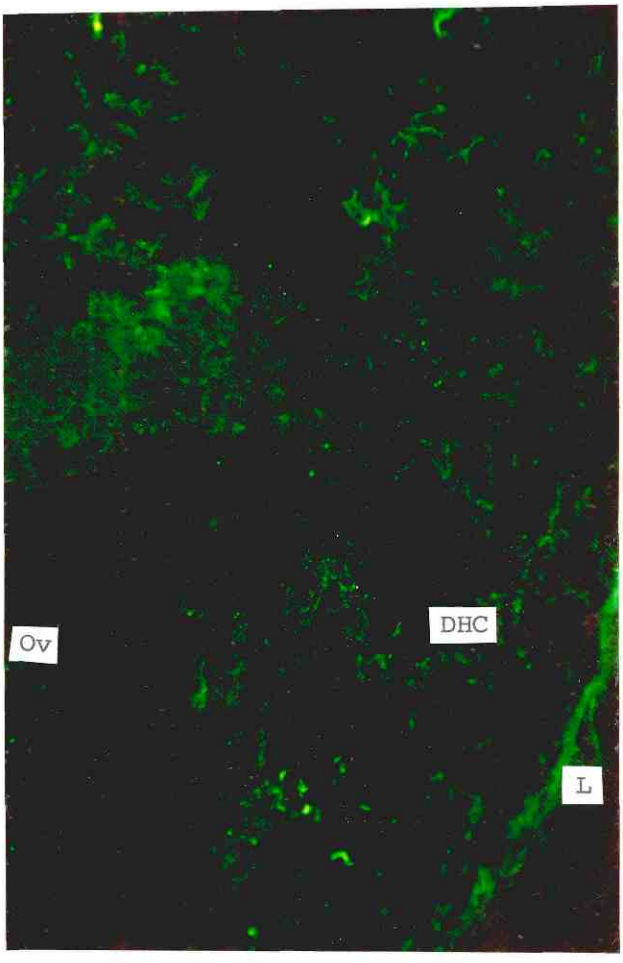


Plate 19

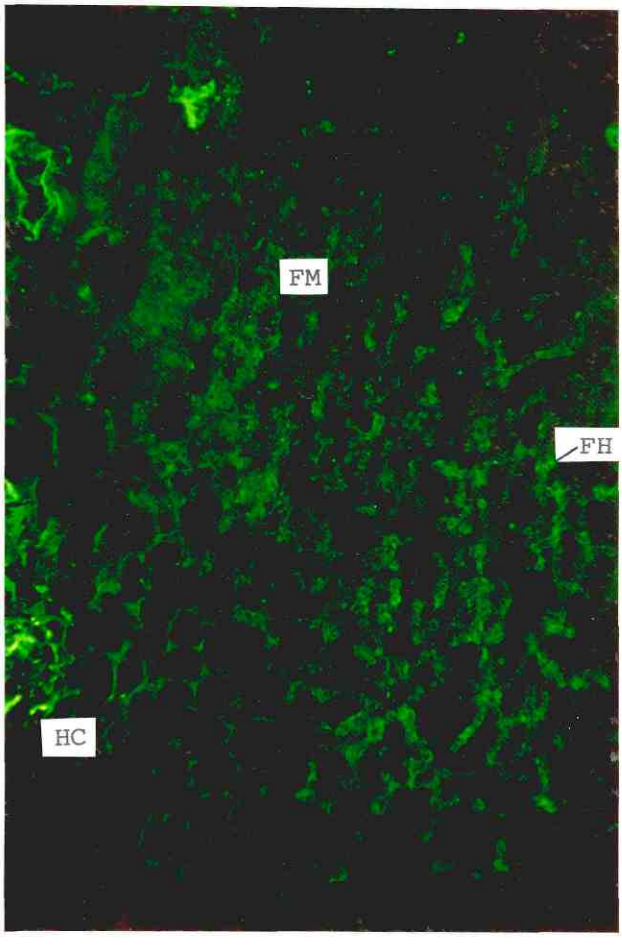


Plate 20

Plate 21: Host-parasite interface at 10 days stained for β-glucosidase

As was observed for β-1,3-glucanase, fluorescence was visible across the base of the sphacelium at this stage.

Sp - sphacelial tissue; I - interface; HC - host plant cells; AF - area of fluorescence.

Plate 22: Host-parasite interface at 14 days stained for β-glucosidase

Sp - sphacelial tissue; I - interface; V - vascular tissue.

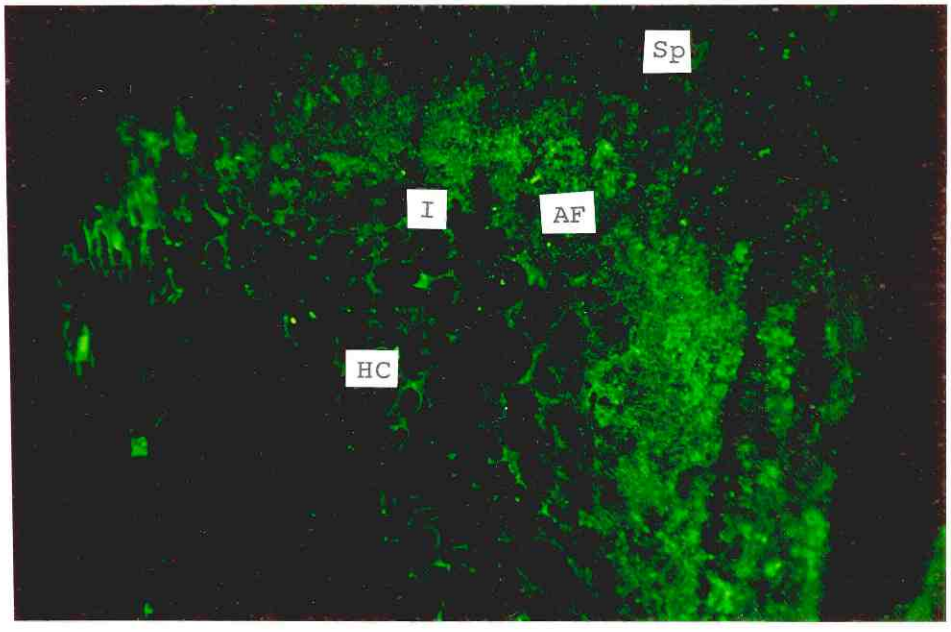


Plate 21

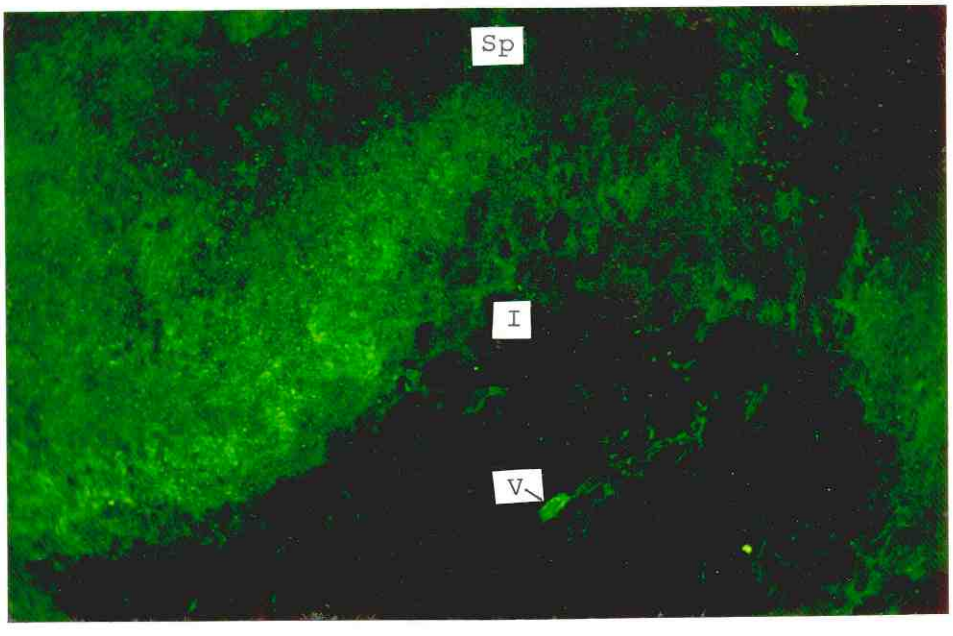


Plate 22

Plate 23: Sporulating sphacelial tissue at 14 days stained for β-glucosidase

Invaginated pockets of conidiation showed fluorescence at a lower level than that seen for the β-1,3-glucanase.

Sp - sphacelial tissue; PC - pocket of conidiation; OSp - outer surface of the sphacelium.

Plate 24: Central region of developing ergot at 14 days stained for β-glucosidase

In this sample differentiation had commenced at this stage. The more fluorescent sphacelial tissue was displaced by growth of the darker less fluorescent sclerotial tissue which can be seen in the lower half of the plate.

Sp - sphacelial tissue; Sc - sclerotial tissue.

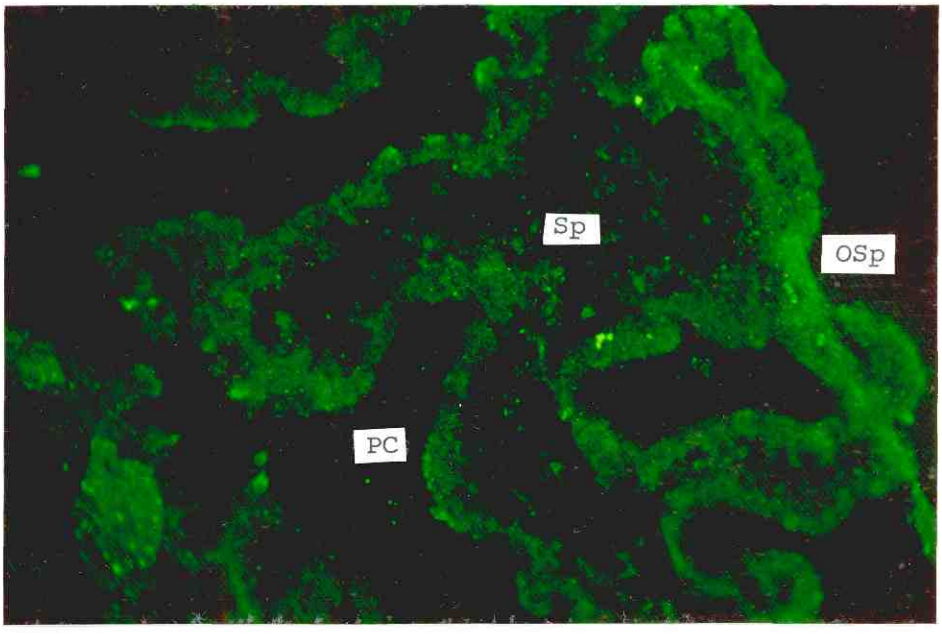


Plate 23

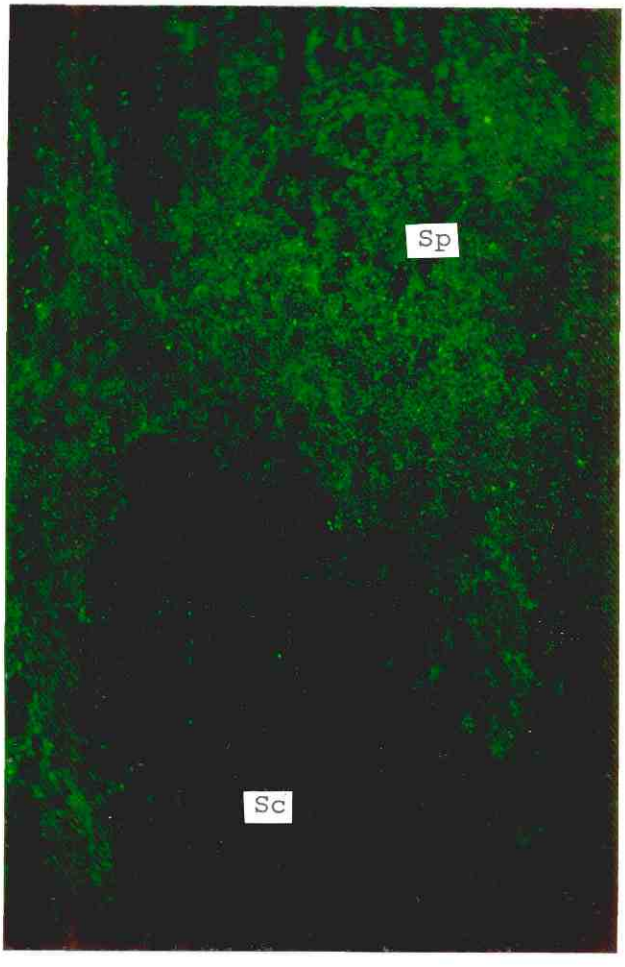


Plate 24

Plate 25: Apex of the ergot at 18 days stained for β-glucosidase

This section shows a still fluorescent pocket of conidiation in sphacelial tissue above dark non-fluorescent sclerotial tissue.

Sp - sphacelial tissue; PC - pocket of conidiation; Sc - sclerotial tissue.

Plate 26: Host-parasite interface at 18 days stained for β-glucosidase

Fluorescence was apparent at the interface, particularly at its centre, where fungal mycelium extended downwards into the host tissue to reach the vascular elements.

Sc - sclerotial tissue; I - interface; HC - host plant cells; V - vascular elements.

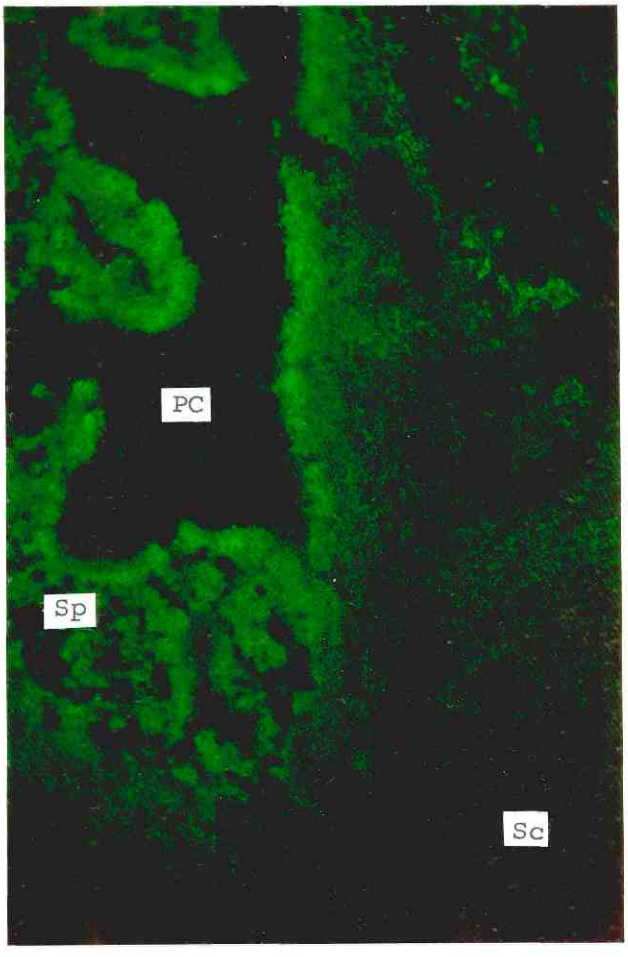


Plate 25

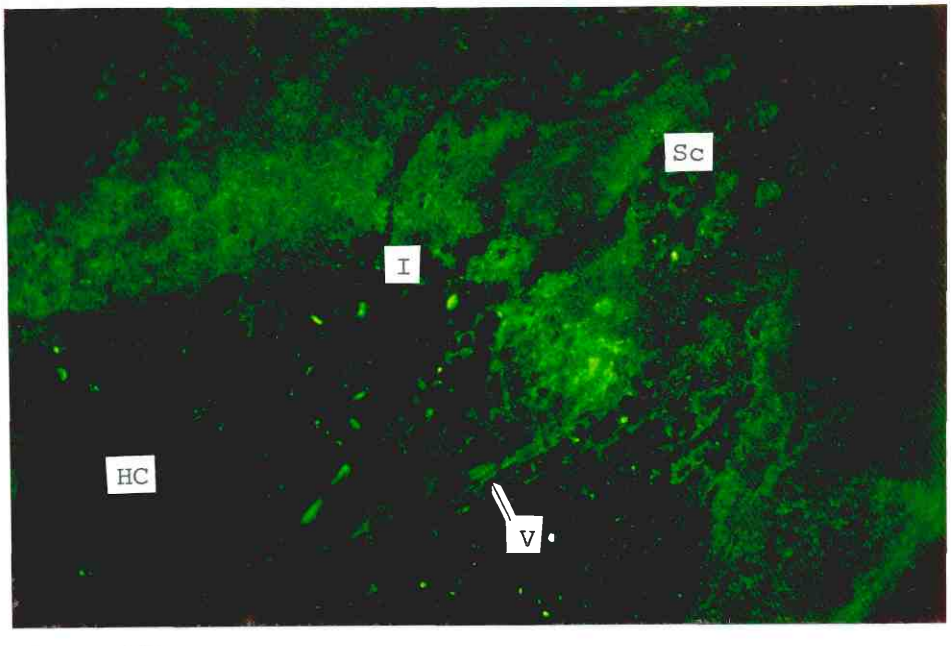


Plate 26

Plate 27: Host-parasite interface at 21 days stained for β-glucosidase

At this stage the band of fluorescence due to β-glucosidase was much brighter than at earlier stages in the infection, and was confined to the fungal tissue immediately distal to the interface.

Sc - sclerotial tissue; I - interface; HC - host plant cells.

Plate 28: Detail of host-parasite interface at 21 days stained for β-glucosidase

Fluorescence was present in hyphae closely associated with the uninvaded plant cells below the interface.

Sc - sclerotial hyphae; I - interface; HC - uninfected host cells; AF - area of fluorescence.

Plate 30: Host-parasite interface at 35 days stained for β-glucosidase

A band of fluorescence remained at the base of mature sclerotia.

Sc - sclerotial tissue; I - interface; AF - area of fluorescence.

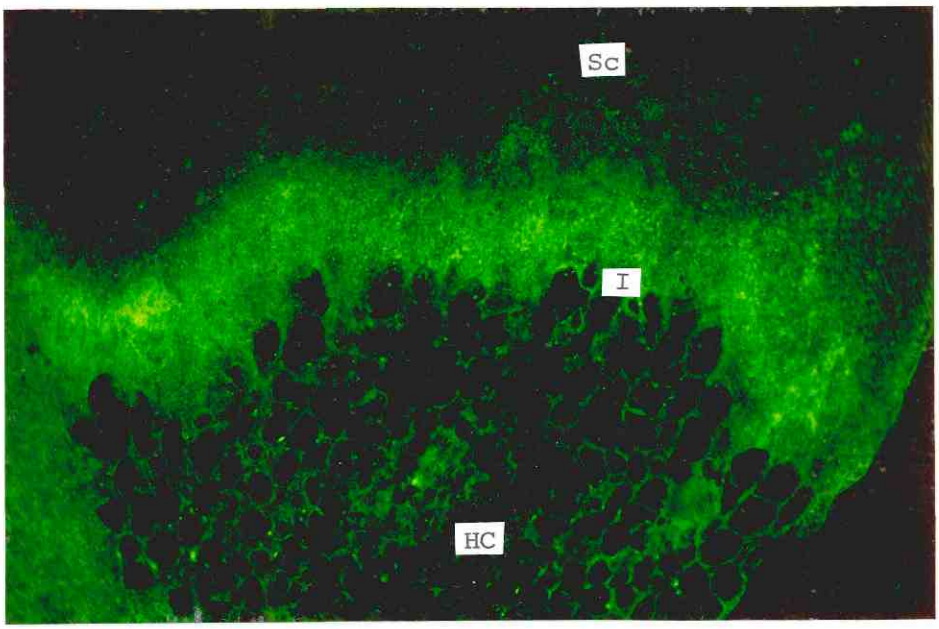


Plate 27

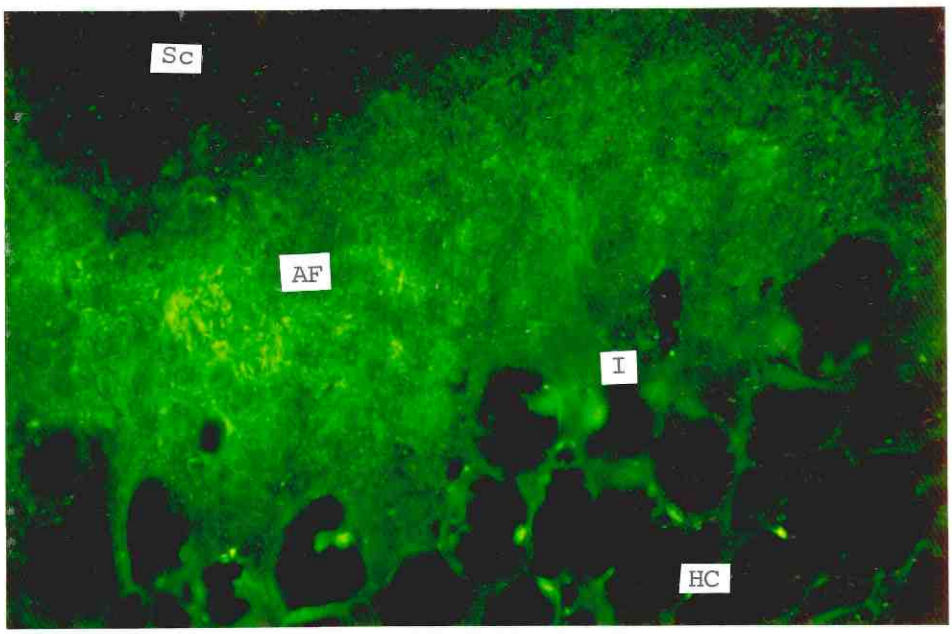


Plate 28

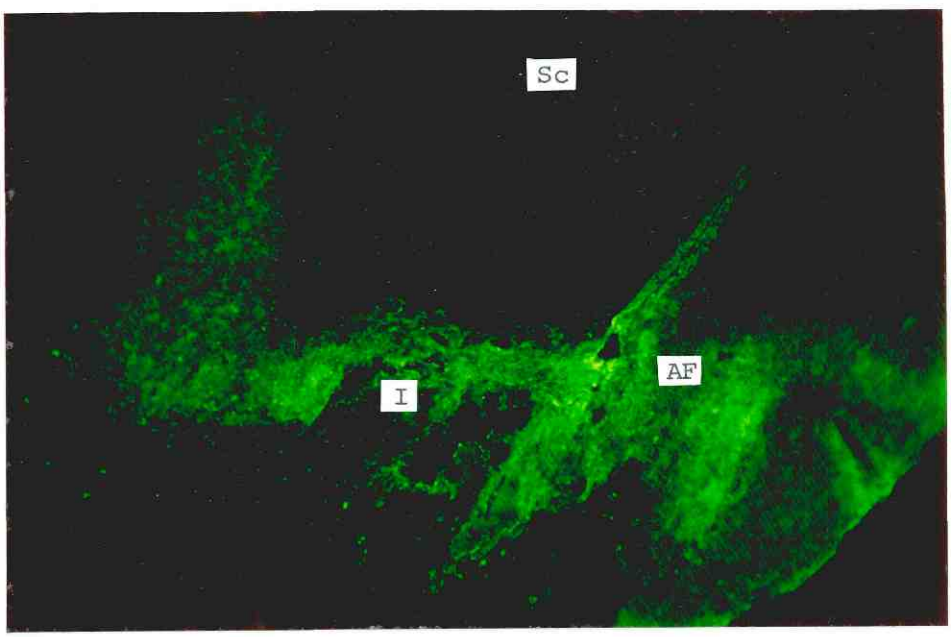


Plate 30

Plate 29: Host-parasite interface at 28 days stained for β-glucosidase

Section from large ergot sample showing the clearly defined bright band of fluorescence above the interface.

Sc - sclerotial tissue; I - interface; HC - host plant cells.

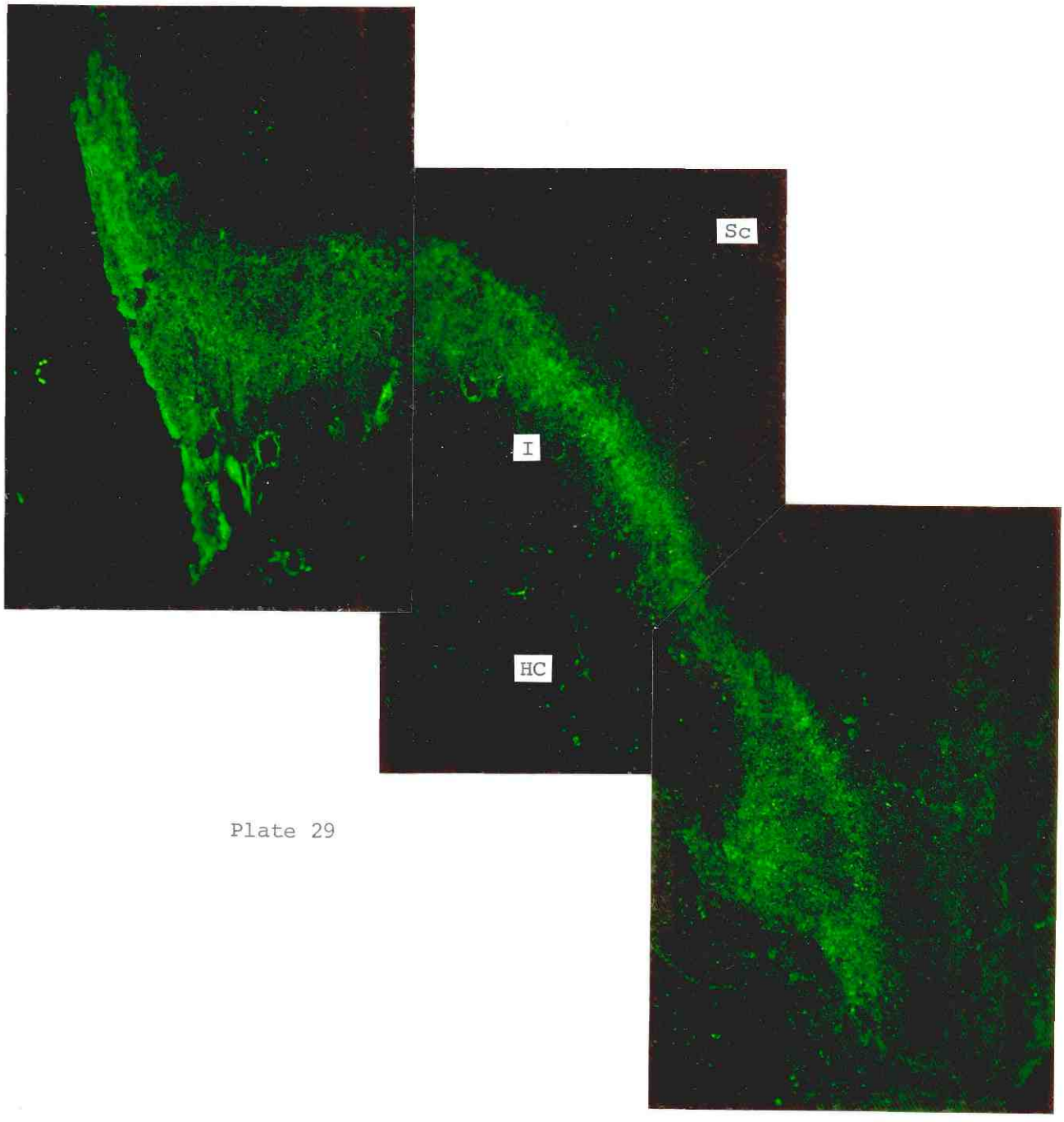
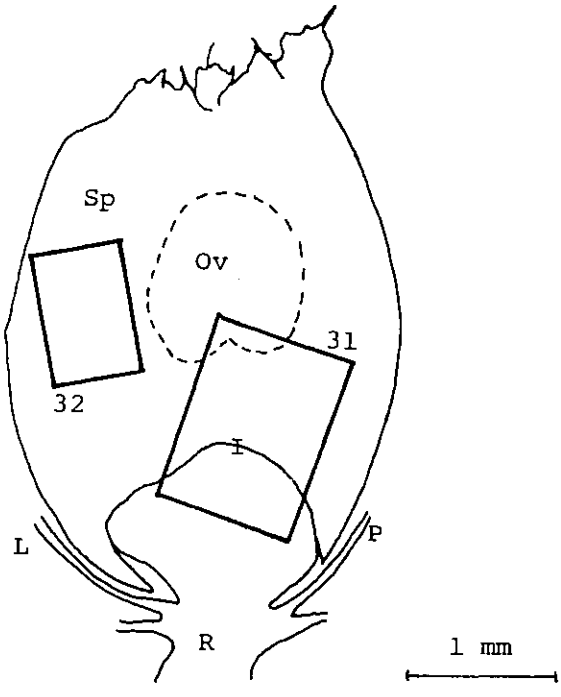


Plate 29

Figure 40: Diagrams representing sections of developing ergot stained with β-fructofuranosiadse antiserum showing the areas of sections illustrated in plates 31 to 41.

a) Sections from 7 to 14 days.

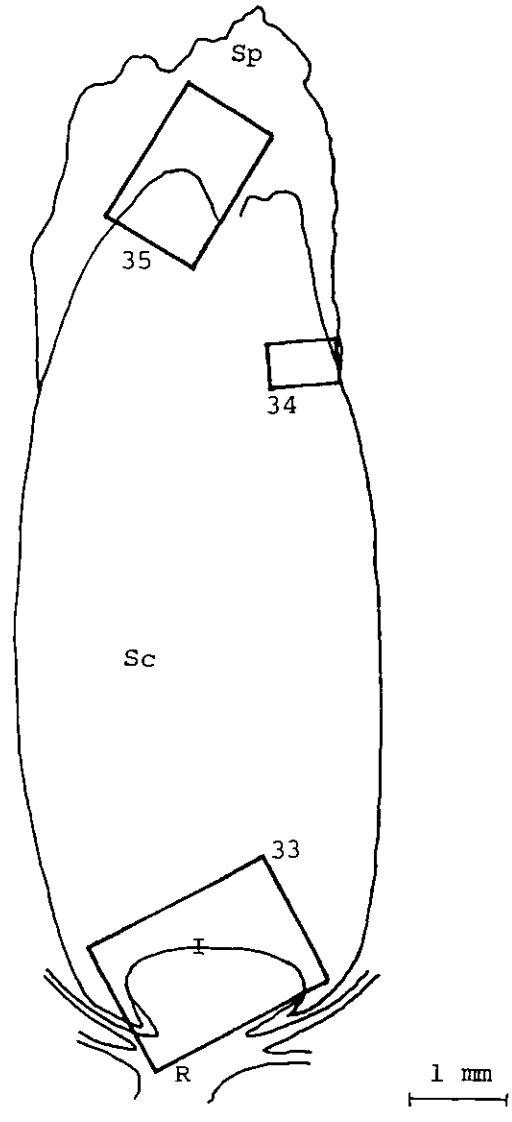


Sp - sphacelial tissue; Ov - ovule; I - interface; L - lemma; P - palea; R - rachilla. Numbers refer to the corresponding plates, both of which are at 160x magnification.

Since individual ergot samples varied in actual size, the sizes of the plates indicated in the diagram are not strictly to scale.

Figure 40: (continued)

b) Sections from 14 to 21 days.

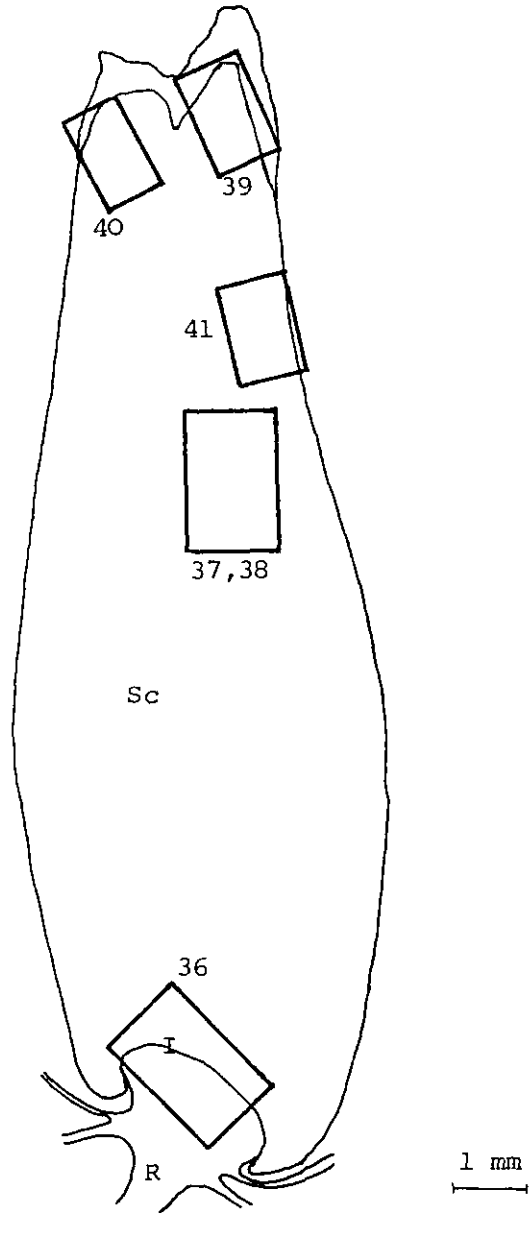


Sp - sphacelial tissue; Sc - sclerotial tissue; I - interface; R - rachilla. Numbers refer to the corresponding plates. Plates 33 and 35 are 160x, plate 34 is 400x magnification.

Since individual ergot samples varied in actual size, the sizes of the plates indicated in the diagram are not strictly to scale.

Figure 40: (continued)

c) Sections from 28 to 35 days.



Sc - sclerotial tissue; I - interface; R - rachilla. Numbers refer to the corresponding plates. Plates 36 to 41 are all at 160x magnification.

Since individual ergot samples varied in actual size, the sizes of the plates indicated in the diagram are not strictly to scale.

Plate 31: Early fungal mycelium at 7 days stained for β-fructofuranosidase

The fungal mycelium was not well established at this stage and only a very low level of fluorescence was evident.

Ov - ovule; DHC - disrupted ovary parenchyma cells; FM - fungal mycelium.

Plate 32: Sporulating sphacelial tissue at 12 days stained for β-fructofuranosidase

Hyphae producing conidia at the edges of pockets of conidiation were only marginally more fluorescent than the surrounding sphacelial tissue.

Sp - sphacelial tissue; PC - pocket of conidiation.

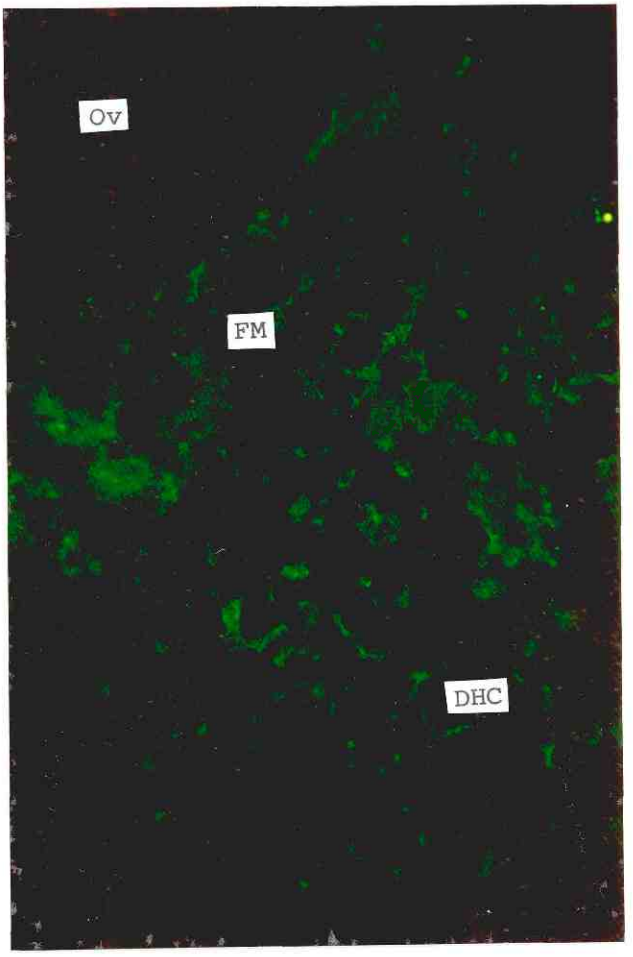


Plate 31

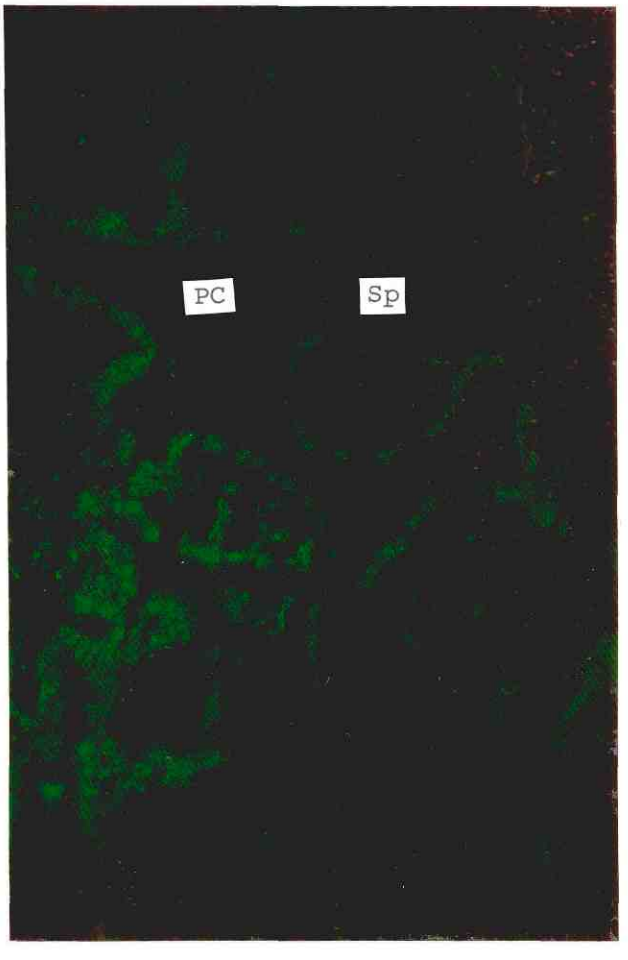


Plate 32

Plate 33: Host-parasite interface at 18 days stained for β-fructofuranosidase

The fluorescence across the interface shown in this section was only seen to occur in a sample at this stage of infection.

Sc - sclerotial tissue; I - interface; HC - host plant cells.

Plate 34: Detail of sclerotial tissue at 21 days stained for β-fructofuranosidase

Individual sclerotial hyphae are visible in this section delineated by a ring of fluorescence around the cell boundary.

SC - sclerotial cell.

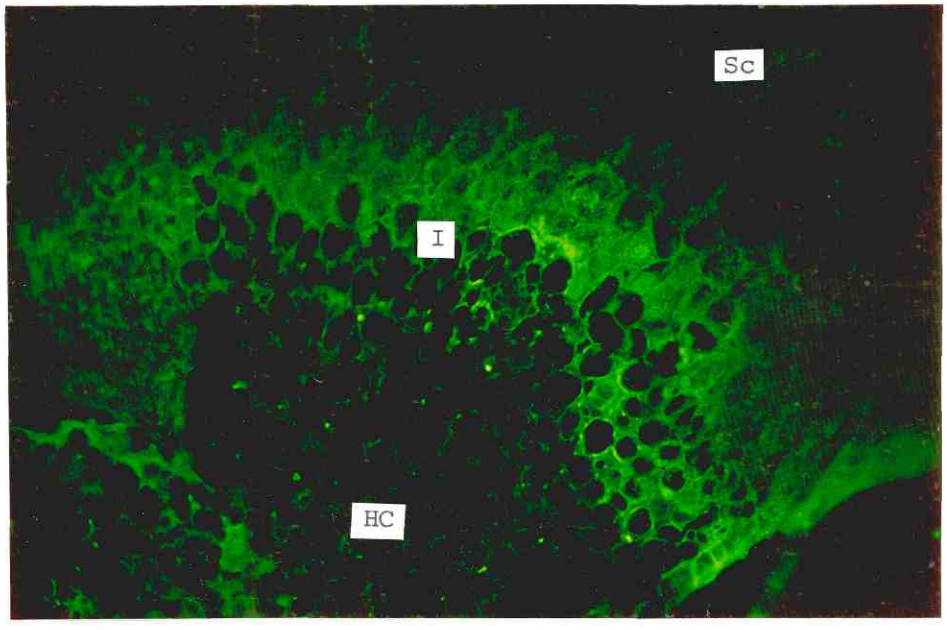


Plate 33

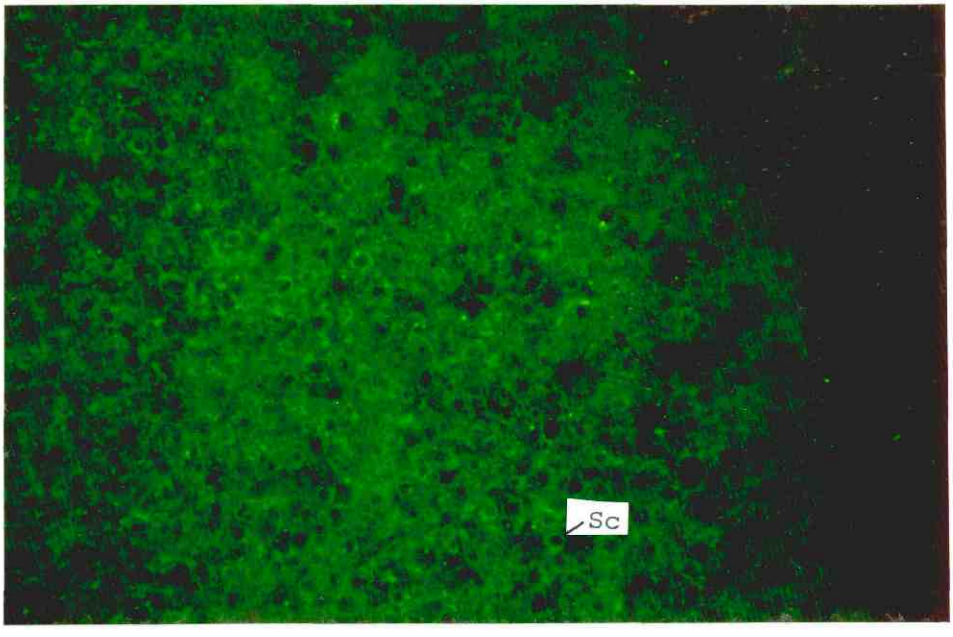


Plate 34

Plate 35: Apex of sclerotium at 21 days stained for β-fructofuranosidase

The sclerotial tissue at the bottom of this section showed a higher level of fluorescence than the diminishing sphacelial tissue. The dark boundary between the two (PL) corresponded to the pigmented cortex which formed the outer rind of the sclerotium.

Sc - sclerotial tissue; Sp - sphacelial tissue; PL - pigmented layer.

Plate 36: Host-parasite interface at 28 days stained for β-fructofuranosidase

No fluorescence was observed at the base of the developed sclerotium incontrast to that observed in sections stained for β-1,3-glucanase and β-glucosidase.

Sc - sclerotial tissue; I - interface; HC - host plant cells.

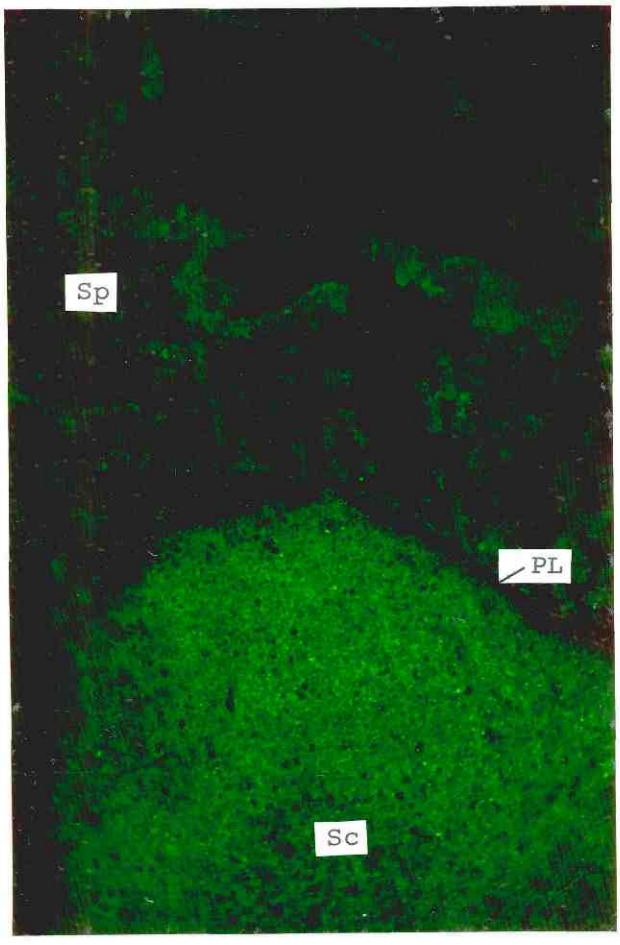


Plate 35

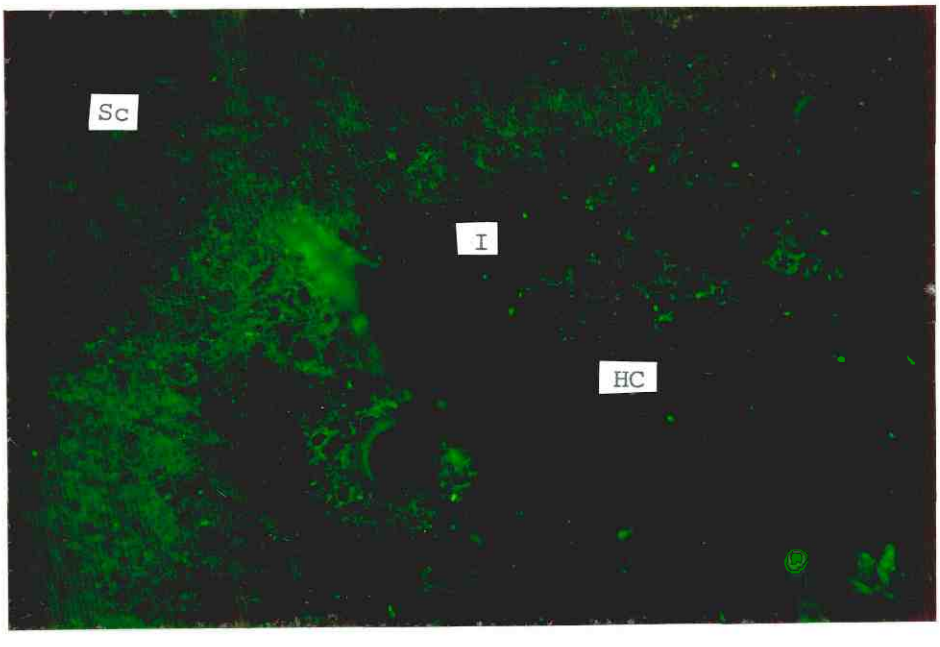


Plate 36

Plate 37: Sclerotial tissue at 28 days stained for β-fructofuranosidase

Plate 38: Sclerotial tissue at 28 days stained with control non-immune serum

The level of fluorescence due to β-fructofuranosidase in sclerotial tissue is indicated by comparison with identical tissue stained with control non-immune serum.

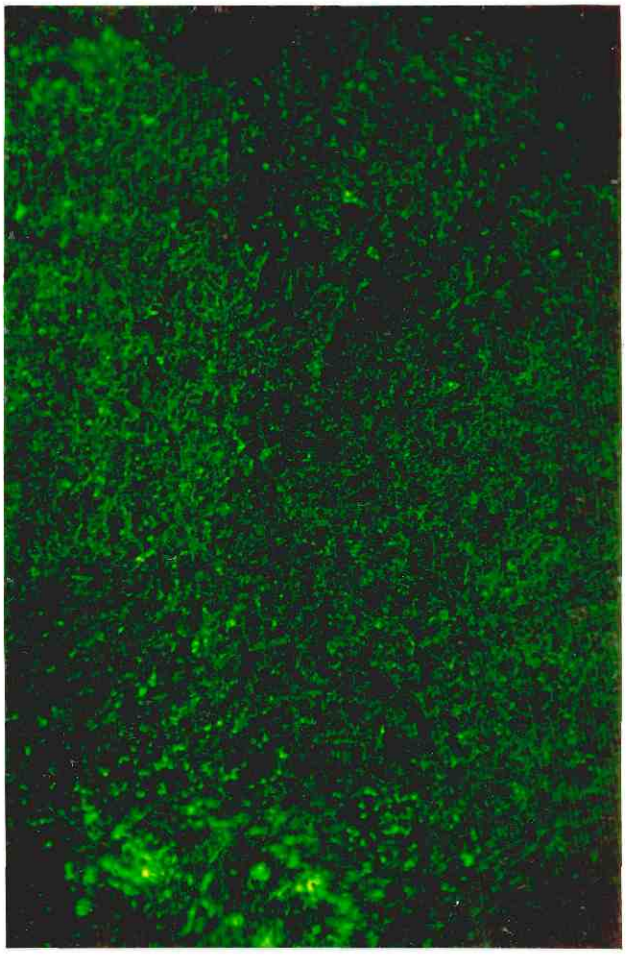


Plate 37

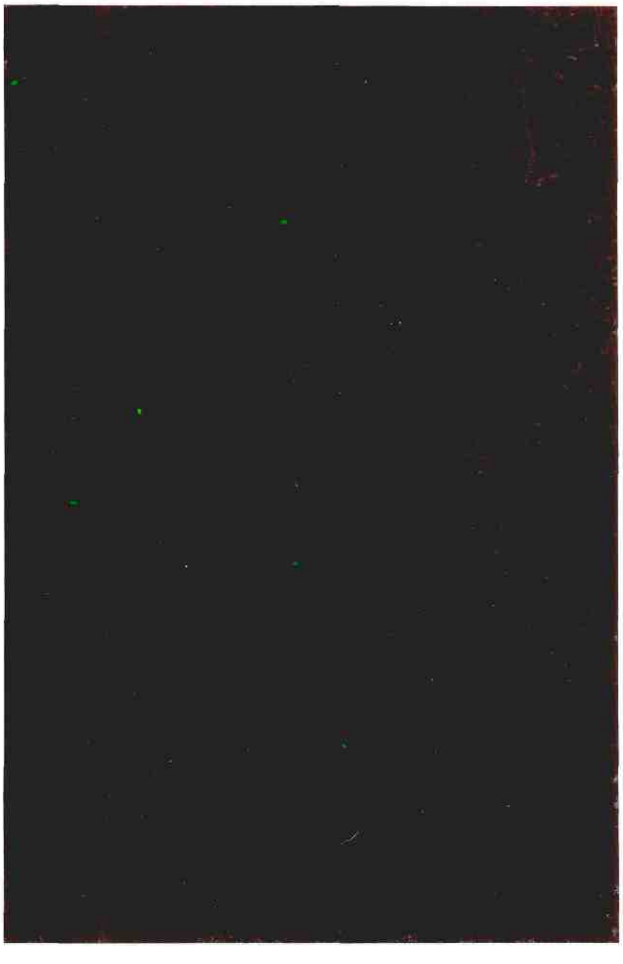


Plate 38

Plate 39: Apex of sclerotium at 28 days stained for β-fructofuranosidase

Fluorescence observed in sclerotial tissue was confined by the outer pigmented rind of the sclerotium. At this stage the remaining sphacelial fragment demonstrated very little or no fluorescence.

Sc - sclerotial tissue; PL - pigmented layer; Sp - sphacelial tissue.

Plates 40 & 41: Sclerotial tissue at 35 days stained for β-fructofuranosidase

The medulla of the mature sclerotium continued to show fluorescence due to the presence of β-fructofuranosidase.

MSc - medulla of sclerotium; CSc - outer cortical layer of sclerotium.

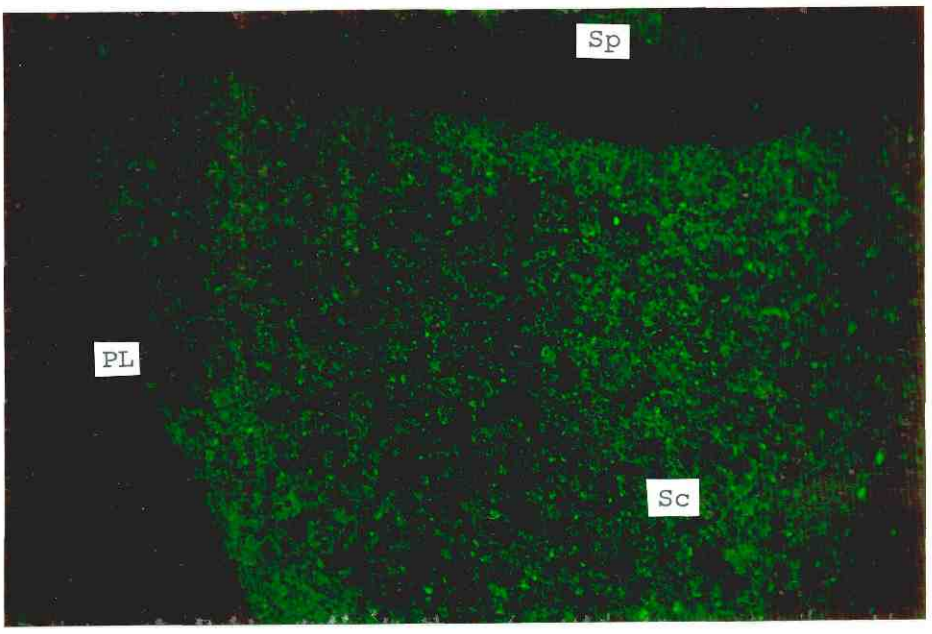


Plate 39

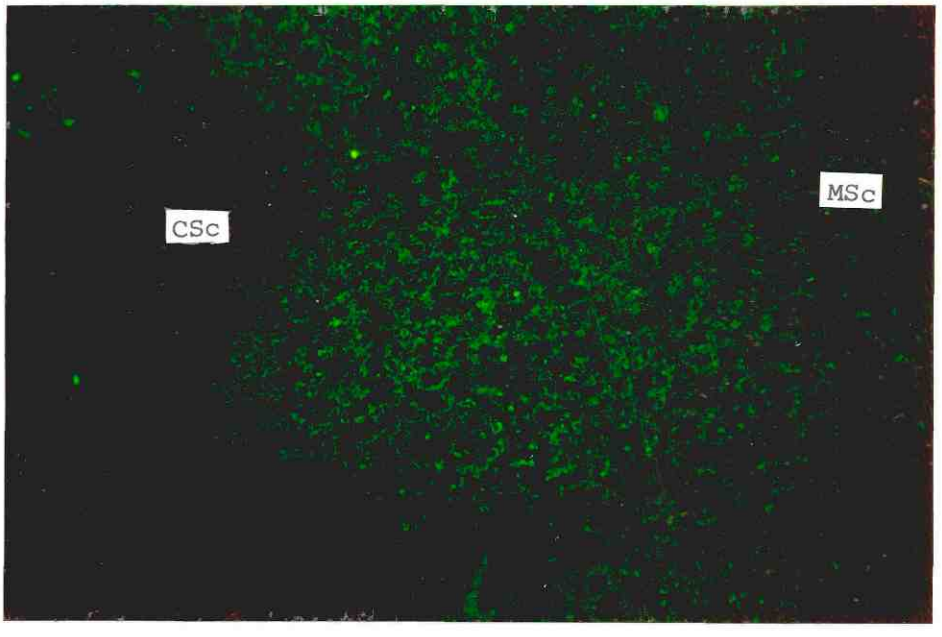


Plate 40

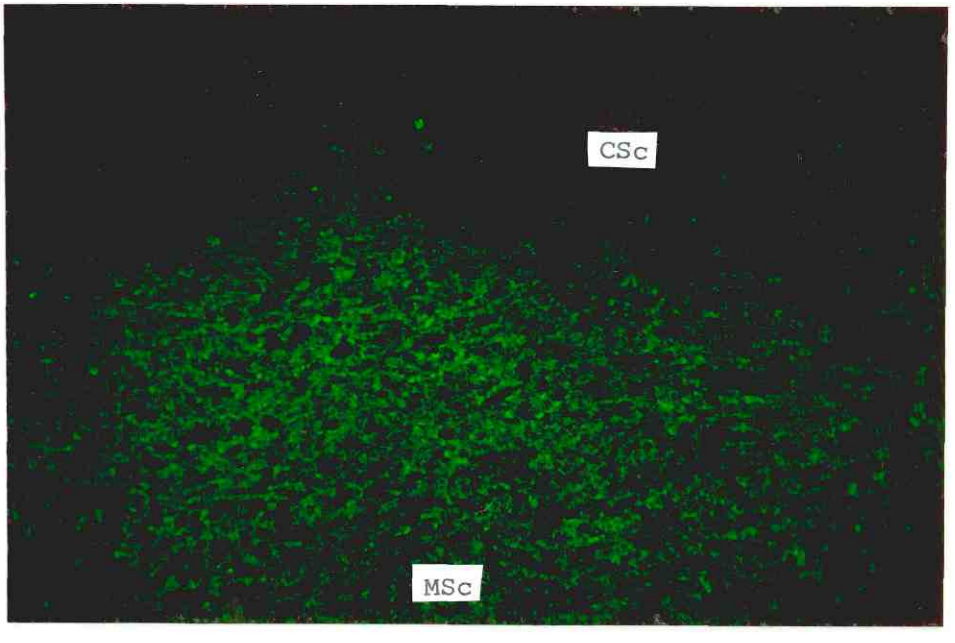


Plate 41

4. DISCUSSION

The present studies have centred on the examination of the locations of β-glucanase, β-glucosidase and β-fructofuranosidase from Claviceps purpurea, during its development on rye, as a means of determining the roles of these enzymes in the parasitic system. Discussion of this work is presented in four sections. First, the purification and characterization of the enzymes is considered since, of the three enzymes, only the β-fructofuranosidase from C. purpurea had been completely purified previously. Secondly, aspects of the application of immunofluorescent techniques, in particular the suppression of autofluorescence, are discussed, as this technique has not been previously applied to investigations of ergot enzymes. Thirdly, the functions of β-glucanase, β-glucosidase and β-fructofuranosidase, at the stages of ergot development studied, are discussed in detail on the basis of the locations observed in the micrographs. Last, more general consideration is given to the cellular location and roles of these enzymes in cereal parasitism by C. purpurea and to the use of immunofluorescence in other investigations of a similar nature.

4.1 Purification of enzymes

The use of immunofluorescence as a means of locating enzymes in tissues required the preparation of purified enzymes from axenic cultures as immunogens. Whereas β-fructofuranosidase had been purified from mycelial cultures of C. purpurea and Sphacelia sorghi by Dickerson (1972) and Baker & Dickerson (1980), the β-glucanase and β-glucosidase from C. purpurea had not been completely purified previously, since only crude culture filtrate extracts were used in earlier investigations of these enzymes by Dickerson et al, (1970) and Shaw (1979). Of the C. purpurea strains used by the above authors,

only strain 29/4 remained; hence, the β-glucanase activity of several other strains besides 29/4 was tested, to determine whether higher yields of enzyme activity than reported previously could be obtained.

The proposal of Dickerson et al, (1978), concerning the importance of β-glucanase to the establishment and maintenance of nutrient flow from host to fungus, implies that there might be a correlation between the infectivity of a strain in vivo and the levels of glucanase produced by it in culture. Although there was considerable variation in specific and total activity of glucanase from the different strains (Table 1), all the strains tested were known to be infective on rye (Swan, 1980). The exception was strain 12/1 which was not a particularly infective strain, and this did produce much lower levels of glucanase than the others. This suggests that there may be a threshold level of glucanase activity, below which infectivity is reduced, but, above which, increased activity does not noticeably improve the success of the strain as a pathogen.

C. purpurea strain 6, which gave the highest yield of glucanase, produced enzyme in the culture medium at the same stage of growth as had been observed for C. fusiformis (Dickerson et al,1970), although far less glucan had been elaborated by the former species. This is the case for most C. purpurea strains, in cultures of which often no glucan is detected β-glucanase being produced in sufficient quantities, presumably, degrading any glucan before it accumulates (Shaw, 1979). Production of β-glucanase by C. purpurea 29/4 in culture was associated with the appearance of sclerotial hyphae in the mycelium (Shaw, 1979) and this correlation was again observed with C. purpurea 6. The modified medium T used in the present work had been developed as an amino-nitrogen medium which promoted the development of sclerotial

hyphae in culture (Mantle & Nisbet, 1976). Thus, although various changes in the composition and pH of the medium were tested for their effect on enzyme production, growth of C. purpurea 6 in the modified medium T still proved to give the highest yield of β-glucanase activity.

Separation of the β-glucanase and β-glucosidase activities from media extracts using the anion exchanger DEAE-cellulose, as reported for the C. fusiformis enzymes by Dickerson et al (1970), proved impossible. Under the same experimental conditions the C. purpurea enzymes did not bind to DEAE - cellulose but to the cation exchanger CM-cellulose. It is possible that different methods of crude enzyme extract preparation - acetone precipitation in the case of C. fusiformis and freeze-drying in the present case of C. purpurea - caused the differences in binding properties, i.e. different charges on the enzymes. More probably, it arises because of species or strain specificity; particularly, the differences could arise here because the two ergot species are usually parasitic on different cereal hosts, the enzymes consequently function under different conditions within the host. The fact that a strong cation exchanger and 'shallow' gradient were necessary to separate the C. purpurea β-glucanase and β-glucosidase, and that neither enzyme migrated into the gel when electrophoresed on polyacrylamide gels at pH 8.0, means that both enzymes were basic enzymes carrying predominantly negative charges under the purification conditions used, and they might be expected to demonstrate similar isoelectric points. Kritzman et al, (1978) have demonstrated that a β-1,3-glucanase from Sclerotium rolfsii also did not bind to DEAE-cellulose and was a basic enzyme with an isoelectric point at pH 8.5.

Claviceps glucan was used in assays of β-glucanase activity in preference to laminarin, an algal β-1,3-glucan commonly used as a

substrate in β-1,3-glucanase assays (Nelson et al, 1963), since it was more closely related to substrates that the C. purpurea β-glucanase would encounter in vivo. Also, the structure and branching pattern of the glucan were determined by Buck et al, (1968) and this permits an indication of the degree of β-glucosidase contamination in a β-glucanase sample to be obtained by estimation of the ratio of glucose to gentiobiose in reaction products (Dickerson & Pollard, 1982). The purified β-1,3-glucanase from C. purpurea 6 possessed the same type of activity against β-1,3-glucan, i.e. exohydrolytic, as had previously been observed for the C.fusiformis and Basidiomycete QM806 enzymes (Dickerson et al, 1970; Huotari et al, 1968). Glucose and gentiobiose were the only products of complete hydrolysis, with no detectable trace of intermediate oligosaccharides that would indicate endohydrolytic activity.

The β-glucanase and β-glucosidase both possessed similar broad pH optima, 2.5 to 5.5 and 3.5 to 6.5 respectively, reflecting the fact that both must tolerate more diverse conditions functioning outside the cell than within. The values of the pH optima for the Claviceps enzymes are very similar to those observed for extracellular β-1,3-glucanases and β-glucosidases from other fungi (Villanueva et al, 1976; Eberhart & Beck, 1970; Sternberg et al, 1977; Dekker, 1981).

The β-glucosidase was active against disaccharides of different glycosidic linkages indicating that it was an aryl β-glucosidase, rather than the more specific cellobiase type which also occurs in other fungi (Mahadevan & Eberhart, 1964; Bucht & Eriksson, 1969). It demonstrated higher activity against gentiobiose and laminaribiose, which it would encounter in vivo as products of β-1,3-glucanase hydrolysis of Claviceps glucan, than against cellobiose (Table 6). With the artificial substrate p-nitrophenol-β-D-glucopyranoside,

the β-glucosidase had a K_m of 2.4 x 10^{-4}M which agreed with the value obtained by Dickerson et al, (1970) for the β-glucosidase from C. fusiformis, although for the C. purpurea enzyme, substrate inhibition was found to occur at substrate concentrations above 1mM.

Purification of the intracellular or cell-associated β-fructofuranosidase from C. purpurea 6 was achieved under approximately similar conditions to those described previously for ergot β-fructofuranosidase (Dickerson, 1972; Baker & Dickerson, 1980). The pattern of oligosaccharides elaborated by this enzyme in the hydrolysis of sucrose was the same as had been reported by the above authors. Also, the molecular weight of 134,000 ± 12,000, estimated by gel electrophoresis, for the cell-associated β-fructofuranosidase was close to the value that had been obtained by Baker (1979) on gel chromatography columns for the same enzyme from C. purpurea strain 29/4 and Sphacelia sorghi.

The quantity of protein containing β-fructofuranosidase activity that could be purified from both intra- and extra-cellular extracts was very low, although the yield obtained for the intracellular enzyme was slightly the greater. Apart from requiring a slightly higher molarity of salt in its elution from DEAE-cellulose (Section 3.4.3), the extracellular enzyme was purified by the same steps as the cell-associated enzyme. Since β-fructofuranosidase occurs bound to cell wall glucan and extracellularly produced glucan in ergot fungi (Dickerson & Baker, 1979), the extracellular activity may represent cell-associated activity which had become dissociated from the cell wall during growth or had been released as the extra-cellular glucan, to which it was bound, was hydrolysed by the β-1,3-glucanase in the culture medium. The difference in elution volume from DEAE-cellulose therefore, may be due to a change in overall charge or conformation resulting from its separation from glucan. This might also explain the more directly hydrolytic action on sucrose observed for the extra-

cellular β-fructofuranosidase, which generated fewer oligosaccharides and more free fructose than the cell-associated enzyme. Thus, although the two enzymes did not possess identical characteristics, the β-fructofuranosidase activity in the culture medium extracts probably represents the same enzyme as the cell-associated β-fructofuranosidase.

4.2 Immunofluorescence

The antiserum titres for the three enzymes, estimated from immunodiffusion plates, were not as high as had been obtained previously for ergot β-fructofuranosidase by Baker & Dickerson (1980). On that occasion the enzyme preparation used as immunogen was bound to a β-glucan which would have prolonged enzyme stability and enhanced presentation of the antigen, permitting a higher titre to be obtained. On the other hand, use of the indirect method of immunofluorescent staining increases the sensitivity of the technique (Nairn, 1976), and has the added advantage that the same fluorescein-conjugated anti-rabbit antiserum could be used for each of the three enzyme antisera.

Fluorescein has the disadvantage of fading rapidly or photobleaching under higher magnifications on the microscope (Nairn, 1976). Rhodamine does not fade, but its red fluorescence is less intense than the green of fluorescein (Johnson et al, 1978) and a filter system for use with rhodamine was not available at the time of experimentation. The use of lower magnifications, which were necessary to visualize the overall locations of the enzymes within Claviceps tissue, helped to reduce the consequences of fluorescence fading in the photography. Moreover, higher magnifications would have required thinner sections than the 10 μm sections used, in order to distinguish fluorescence at the level of single cells or at the subcellular level. Information on the freeze-sectioning of unfixed, frozen fungal and

plant tissues for immuno-or histochemical purposes is not as extensive as that for animal tissues (Pearse, 1968; Hall, 1978). Fungal and plant tissues are more hydrated than animal tissue, on which previous immunochemical work has mostly concentrated, rendering the cellular structure of plant and fungal tissues less dense and more 'fragile' under freeze-sectioning. Also, fixing prior to sectioning is to be avoided as this might adversely affect the antigenicity of enzymes in tissues. Further investigation of techniques for obtaining thinner sections of fungal and plant tissue than were possible in the present work would therefore be of value. One possible solution to this problem, suggested by Tokuyasu (1973), involved the infusion of a concentrated sucrose solution (30%, w/v) into tissues or cell suspensions prior to freezing, which would help to support frozen material being sectioned. However, this particular approach would not be very effective in the case of Claviceps tissues, since the fungus contains a β-fructofuranosidase which would hydrolyse the sucrose. In the case of ergot and plant tissue, the maintenance of the morphology of the host/parasite interface was of prime importance. Thus, the sectioning conditions developed (Methods section 2.6.5) might not have been optimal for either plant or fungal tissues when sectioned alone, but provided that the best results where these tissues were in discrete but close association.

The antibody staining procedure involved considerable periods of immersion and repeated washing of sections in buffers. This would be expected to remove much of the freely soluble enzymes from tissues, which enzymes, despite having been fixed in alcohol, were not bound or associated to cellular components such as membranes, cell walls or glucan. In particular, a large proportion of the honeydew, which would have been present in sections from 10 to 18 days, would have been lost from sections and any enzymes it might have contained would

not be detected by fluorescence. The resultant fluorescence observed in sections may therefore be ascribed largely to cell-associated enzymes, which, since they react with the corresponding antisera, must be considered the same or very closely related to those produced in and extracted from *in vitro* culture (Dickerson & Pollard, 1982).

In the event that no translocation occurred, for which there was no evidence in the present work (i.e. gradual change in the position and level of fluorescence between subsequent sections was not observed), the localization of fluorescence demonstrated the presence of cell-associated enzymes at their sites of action, and, to a certain extent, demonstrated areas of synthesis of these enzymes where hyphae may have been disrupted by sectioning exposing the cytoplasm. It should be noted that intracellular enzymes, in the strict sense of the word, would not be detected in whole tissue, because antibodies could not cross the cell membrane without some prior treatment rendering the membrane permeable (Rennie, 1976).

Autofluorescence is not normally found where fungal hyphae alone are being studied (Hunsley & Kay, 1976; Kritzman *et al*, 1978), although slight autofluorescence was observed in sections of sclerotial tissue from *Claviceps purpurea* - presumably due to the presence of alkaloids, which exhibit fluorescence, in these cells (Mantle & Nisbet, 1976). Where plant tissue is present in samples, autofluorescence can cause difficulties in the application of immunofluorescence as it might confuse the interpretation of fluorescence localization. There have, however, been relatively few attempts reported to overcome this problem. Knox (1971) found that the autofluorescence in pollen grain walls was primarily green in colour, and consequently changed from using the green-emitting fluorochrome fluorescein to the red-emitting fluorochrome rhodamine in order to

obtain contrast between the auto- and antibody coupled fluorescence. Hislop et al, (1974) were fortunate, when using fluorescein to trace fungal pectinases in apples, that the apple tissue autofluorescence was red, again providing contrast with the fluorochrome. Autofluorescence was evident in the photomicrographs presented in other reports (Barlow et al, 1973; Iki et al, 1977; Paynter & Alconero, 1979; Fitzell et al, 1980), although suppression of the autofluorescence was considered unnecessary or had not been attempted by these authors.

The use of mercuric chloride or dimethyl-formamide followed by EDTA washes, as proposed by Paton (1964), to remove autofluorescent material from plant cells was suitable where the antigen to be located was the whole bacterial cell and therefore the denaturation, which would occur with this treatment, would not be a very significant problem. In the present case, where native enzymes were being studied, the attendant denaturation of protein with such a treatment would seriously impair binding of antibody to enzyme. The most effective solution to the problem of autofluorescence, advocated by several authors (Paton, 1964; Choo & Holland, 1970; Nairn, 1976), is counterstaining with a fluorochrome of a different colour to that of the antibody coupled fluorochrome to provide a background of a different colour. Counterstaining tissue sections prior to antibody staining has the drawback that antigenic sites may be masked or the counterstain leached out by the numerous washes involved in antibody staining. Counterstaining after antibody staining is preferable, but additionally should be carried out at a pH between 6.5 and 8.5, as a higher pH (i.e. above pH 9.0) may interfere with the immunological reactivity or fine tissue morphology of sections (Nairn, 1976).

The use of methylene blue in borate buffer as counterstain, developed in the present study, met the above criterion and was successfully used to quench the bright yellow-green autofluorescence of

sections. An added advantage was that the stain itself was non-fluorescent and hence provided a dark background against which the antibody-coupled fluorescence could be visualized. Since borate might be expected to complex with carbohydrate residues in the plant cell walls (Pittet, 1965), and as the methylene blue is also involved in the suppression of autofluorescence, it is possible that a methylene blue-borate-wall complex is formed. The quenching could therefore arise directly due to the derivatisation of the wall altering any fluorescent properties it might possess, or the ternary complex could filter out fluorescence where it arises from illumination of other residues in the plant tissue.

4.3 Location and role of the enzymes

Examination of the tissue sections revealed that the development of C. purpurea 6 in the rye floret followed closely the pattern and morphology of growth described previously by Luttrell (1980) and Shaw & Mantle (1980, a & b).

The destruction of the host ovary tissue within the floret by invading fungal hyphae is brought about by polysaccharases such as pectinases and cellulases acting on the plant cell walls (Bateman, 1976; Shaw & Mantle, 1980a; Mankarios & Friend, 1980). Extracellularly produced β-1,3-glucanase from the fungal hyphae could also be involved in this process since cereal grass cell walls contain hemicelluloses - mixed linkage glucans - which would be susceptible to β-glucanase action. This has been demonstrated by Tanimoto & Masuda (1968) and Masuda (1968) who observed softening of the cell walls of barley and oat coleoptiles in the presence of a fungal β-1,3-glucanase. The extracellular β-glucosidase could also have a role in this context, since it would hydrolyse oligosaccharides of low D.P. (2-5) arising from the dissolution of cell wall polymers by cellulase and glucanase. The β-glucosidase provides the final

step in the breakdown of carbohydrates to glucose which can be utilized by the fungus. These functions were reflected in the presence of fluorescence due to β-1,3-glucanase and β-glucosidase without specific localization in the early stages of ovary colonization. In particular, in plate 20, the pattern of fluorescence corresponding to β-glucosidase and the 'loose' appearance of the tissue appeared to conform to the shape of ovary cell walls. This probably represented the detection of extracellularly produced β-glucosidase located along the outer surfaces of fungal hyphae that were growing intercellularly; which points to the involvement of this enzyme in the degradation of plant cell walls. A similar pattern of fluorescence outlining the shape of plant cells was seen in sections of the same age stained for β-fructofuranosidase, although the level of fluorescence was lower. There would not be an especial requirement for the β-fructofuranosidase at this stage as the fungus could utilize glucose resulting from the degradation of host cells, but the enzyme was obviously already active against host sucrose present in the degenerating ovary cells.

After 10 days infection degeneration of the host ovary was almost complete and the sphacelial stage of infection was established. This was characterized by a general presence of fluorescence in sphacelial tissue sections attributable to all three enzymes studied. The presence of β-1,3-glucanase will be due to its proposed role in hyphal morphogenesis, i.e. partial hydrolysis of the cell wall glucan matrix prior to intussusception of new material (Bartnicki-Garcia, 1973). Once the invading hyphae have reached the base of the ovule, proliferation of the hyphae to generate the sphacelium and consume the host tissue takes place in an amorphous manner throughout the ovary rather than growth extending from one location (Shaw & Mantle, 1980a; Luttrell, 1980). Hence, β-glucanase involved in hyphal wall growth would be expected to be detectable throughout the sphacelial tissue,

as was observed.

As regards the β-glucosidase, until host cells were totally degraded it would still be necessary for the final conversion of plant saccharides to glucose. It is also possible that the β-glucosidase has a role to play in Claviceps hyphal wall growth. The β-1,3-glucan in Claviceps hyphal walls possesses β-1,6 linked glucose side-chains (Buck et al, 1968); therefore the gentiobiose which would be liberated by the action of the β-1,3-glucanase on this polysaccharide might be converted to glucose by the β-glucosidase for re-utilization by the fungus. Confirmation of this, however, would require further study of whether the enzyme occurs bound to hyphal walls at growing apices or branching points, and what effect, if any, on growth could be observed in mutants lacking this enzyme. In this context Rudick, Fitzgerald & Rudick (1979), who used peroxidase coupled antibodies to localize intracellular and cell wall bound α-glucosidases in Aspergillus hyphae, have proposed a function for this enzyme in the cell wall degradation associated with hyphal growth. Also, Urey (1971) found an increase in the levels of β-glucosidase during hyphal growth and conidiation in Neurospora, but this enzyme was not essential to the conidiation process as mutants lacking the enzyme produced conidia in the same quantities as the wild type.

The presence of β-fructofuranosidase throughout the sphacelium indicates that sucrose from the host phloem exudate was utilized as carbon source where required by the fungal tissues, rather than being hydrolysed as it entered the base of the sphacelium from the host vascular tissue and translocated to more distal sphacelial hyphae as glucose, which would have required localization of the β-fructofuranosidase near the interface. The phloem exudate flows through and around the sphacelial tissues and excess sucrose not utilized by the fungus passes out of the floret in the early stages of honeydew exudation (Bassett

et al, 1972). The action of the β-fructofuranosidase on host sucrose generates the oligosaccharides characteristic of honeydew (Dickerson, 1972; Mower & Hancock, 1975a) which predominate over sucrose in 'late' honeydew as greater amounts of the sucrose are utilized for growth by the fungus (Fuchs & Pohm, 1953; Dickerson, Mantle & Nisbet, 1976).

Exuded honeydew contains large numbers of conidia generated from vegetative conidiophores which develop all over the surfaces of the sphacelium and in deeply convoluted invaginations. All three enzymes were located in the sporulating hyphae, particularly in the case of β-1,3-glucanase where fluorescence was very bright (Plates 7 to 10). Conidiogenesis would require rapid cell growth and septation at the hyphal tips from which conidia are released, and the detection of high concentrations of β-glucanase was therefore due to this enzyme's lytic role in hyphal wall extension and the formation of cross-septa in order to release the conidia. The levels of β-glucosidase in conidiating hyphae were much lower than those for the β-glucanase, but its presence at this location might be accounted for by the possibly complementary role of the β-glucosidase to the β-glucanase in hyphal wall extension which was discussed above. β-fructofuranosidase has been found to be present in conidiophores of Neurospora by Chang & Trevithick (1972) using immunofluorescent and histochemical techniques, which suggests that a consequence of rapid conidiogenesis would be a high metabolic rate in these hyphae. The β-fructofuranosidase detected in Claviceps conidiophores would thus be producing glucose from host sucrose to support the higher metabolic rate at this location. Enzyme activities of β-glucanase, β-glucosidase and β-fructofuranosidase are all detectable in exuded Claviceps honeydew (Dickerson et al, 1976; Shaw, 1979), and it is possible that synthesis and export into honeydew of these enzymes occurs in conidiating hyphae. This might contribute to the fluorescence which was

observed for each enzyme in this region.

Mahadevan & Menon (1968) found that β-glucanase and β-glucosidase were bound tightly to the coats of Neurospora crassa conidia, and proposed that the β-glucanase would have a role in conidial germination, hydrolysing conidial wall glucans. In conidia from Aspergillus oryzae, β-glucosidase occurs strongly bound to the spore coat and its activity was found to increase upon germination of the conidia (Horikoshi & Ikeda, 1965). Also, O'Day and Paterno (1979) proposed that β-glucosidase, along with cellulase, was responsible for the hydrolysis of glucan and cellulose in the cell walls of slime mould microcysts to facilitate germination. It is likely, therefore, that β-glucanase and β-glucosidase will be present in the walls of Claviceps conidia, and both enzymes may be necessary in the germination of these conidia. Claviceps conidia, isolated from agar slope cultures and stained for β-1,3-glucanase by immunofluorescence, exhibited fluorescence in the spore coat, whereas, in conidia isolated from honeydew, the coat was much less evident (J. Willingale, P.G., Imperial College, personal communication). Also, conidia from honeydew germinate more rapidly than those from slope cultures. The reduction in spore coat, permitting the more rapid germination, was probably due to the hydrolysis of conidial wall glucans by the β-glucanase activity present in honeydew.

The sectioning procedure used maintained the integrity of plant cells, fungal mycelium and the interface between the two, which was established and clearly observable 10 days after infection. The interface appeared as a discrete junction across the base of the ovary between fungal mycelium and uninvaded plant cells (Plate 2). It was observed, however, that where sections were median across the host/parasite interface (Plates 18 and 25), the fungal tissue extended downwards at the centre of interface to make contact with the vascular

elements from the rachilla. This confirmed the observations of Luttrell (1980) as to the shape of the interface, which had not been previously described. Fungal mycelium grows downwards around the edges of the remaining undegraded plant cells at the base of the ovary anchoring the ergot in the floret (Plates 13 and 17), and the extension of fungal hyphae at the centre of the interface into the rachilla provides direct access for the parasite to vascular elements and hence phloem exudate. In the established sphacelium β-glucanase and β-glucosidase were observed to be localized in a band across the base of the fungal tissue just above the interface, and were found to be present at this location for the period of development studied.

The presence of β-1,3-glucanase activity at this location is consistent with the proposals of Dickerson et al (1978). Extracellularly produced β-glucanase from this region of the sphacelium would be in contact with plant cells across the interface, especially where fungal hyphae penetrate to the vascular traces. Therefore any callose which might be deposited adjacent to damaged plant cells in the interface or in vessels in the rachilla could be removed by the β-glucanase. This would ensure that the translocation of nutrients in phloem exudate from the host into the infected floret was not restricted or curtailed by the plant's wound response. Alternatively the band of β-glucanase could also represent the onset of differentiation. Differentiation usually commences about 12 to 14 days after infection near the base of the sphacelium, and the sclerotial hyphae generated are displaced distally by further hyphal growth across the base of the ergot (Luttrell, 1980; Swan, 1980). This region would hence contain a high concentration of actively growing hyphal tips, in which case the β-glucanase detected would be involved in the process of hyphal wall extension as discussed above.

The role of β-glucosidase in the breakdown of plant poly-

saccharides to glucose would not be required after colonization of the ovary/ since further degradation of plant cells does not take place. The band of β-glucosidase across the base of the fungal mycelium, particularly where it was located distal to the junction between hyphae and host vascular tissue (Plate 26), could then serve to hydrolyse oligosaccharides of D.P. 2-3 other than sucrose in the phloem exudate, since this enzyme was shown to be active against glycosides having various linkages. The β-glucosidase, apart from its role in polysaccharide breakdown discussed above, would therefore also function to degrade plant glycosides, some of which might be toxic to the fungus.

The β-fructofuranosidase was not found to be localized across the base of the ergot, except in one sample at 18 days after infection, at which stage generation of sclerotial hyphae was in progress. Baker (1979) suggested that β-fructofuranosidase was produced in the actively growing basal cells of the sclerotium and remained in sclerotial hyphae stabilized by glucan in the cell wall (Dickerson & Baker, 1979), as they matured and were displaced acropetally by further growth (i.e. there was no de novo synthesis of the enzyme in the mature ergot). The fluorescence in this particular section could represent the detection of β-fructofuranosidase in the basal cells. However, since the band of fluorescence was detected across the interface and in one sample only, the above conclusion needs further confirmation.

The sphacelium was displaced acropetally, after the onset of differentiation, by growth of sclerotial hyphae, and was observed to degenerate into a small fragment remaining above the maturing sclerotium. Degeneration of the tissue would result from the breakdown of cell walls and cell lysis, in which the β-glucanase, still detectable in the declining sphacelium, would act by hydrolysing cell wall glucans. The additional continued presence of β-glucosidase in the

sphacelial cap would enable carbohydrates to be completely broken down. This might allow for some reutilization of the breakdown products by sclerotial hyphae. The degenerating tissue, in which conidiation and honeydew exudation had ceased, would have no requirement for sucrose because its metabolism was primarily catabolic, and the β-fructofuranosidase was not found to persist in the sphacelial cap. The sphacelial cap would have little or no access to nutrients, since the maturing sclerotium, which becomes much larger than the sphacelium and distends the floret, could utilize all the available nutrients from the phloem exudate. Furthermore, the purple-pigmented outer cortical rind of the sclerotium, which was seen to develop after 18 to 21 days infection, would tend to seal off the sphacelial cap from the sclerotial hyphae. This would prevent sucrose reaching the sphacelial remnant, but also, might possibly prevent complete reutilization of the degraded mycelium, hence a fragment remains.

Sclerotial hyphae are characterised by an active secondary metabolism involved in the production of alkaloids and carbon reserve materials such as triglyceride oils and glucan (Corbett et al, 1974; Mantle & Nisbet, 1976). The tissues of the sclerotium, which are mostly given over to storage, are supplied with host sucrose via veins of undifferentiated hyphae which run up the centre of the ergot and radiate into the surrounding tissues (Luttrell, 1980). In the sclerotial hyphae the β-fructofuranosidase continues to function in the conversion of host sucrose to glucose to fuel the secondary metabolism until maturation is completed. The β-fructofuranosidase was detected throughout the medulla of the sclerotium in sections from 21 days after infection onwards, and was more concentrated nearer the apex of the sclerotium than at its base. This location agrees with the levels of β-fructofuranosidase activity observed by Baker (1979) in sections of ergot sclerotia.

In contrast, the β-glucanase and β-glucosidase did not persist in sclerotial tissue except at its base. Since sclerotial tissue is generated and displaced upwards from actively growing hyphae in a meristematic region near the interface (Luttrell, 1980), the band of fluorescence due to β-glucanase across the base of the sclerotium, which became more diffuse as maturation progressed (Plate 18), would correspond to this enzyme's function in growing hyphal apices. Sclerotial hyphae more distal to this region would be undergoing secondary metabolism rather than hyphal extension, in which maturation process the β-glucanase, unlike the β-fructofuranosidase, would not be required and was not detected in sections. Similarly, the very discrete band of β-glucosidase persistent above the interface during maturation (Plate 29), would continue to be active against plant saccharides entering the fungal tissue in phloem exudate, but the β-glucosidase did not remain in sclerotial hyphae undergoing secondary metabolism. As both enzymes were present in the growing basal hyphae, but lost from mature sclerotial hyphae during distal displacement from the interface, it is possible that proteins were turned over in maturing cells to initiate the secondary metabolism.

4.4 General Considerations

The action of β-1,3-glucanases in wall plasticising in hyphal morphogenesis has not been conclusively proved, although the evidence in favour of this role is quite substantial (Gooday, 1978). In Claviceps the β-1,3-glucanase was located in regions of active hyphal growth where the major substrate available to the enzyme in this context must be presumed to be cell wall glucan; this strongly implies that a β-1,3-glucanase, thought to be wall bound, is involved to some degree in hyphal morphogenesis. In the wall polymer matrix, glucan chain ends against which an exohydrolytic β-glucanase could act would probably be infrequent; hence a β-glucanase possessing endohydrolytic

activity might be more suited to a role in hyphal wall plasticising than the exo-β-1,3-glucanase found in Claviceps. In fact, this role is usually ascribed to endohydrolytic β-glucanases in other fungi (Villanueva et al,1976), although these authors quote that "in a variety of yeasts, only those β-glucanases have been found which act after an exo-splitting step has occurred".

Endohydrolytic activity was not detected in assays of the purified Claviceps β-glucanase, but this does not exclude the possibility that this type of activity can be expressed in parasitic tissue. It is possible that the enzyme detected in sections associated with growing hyphae could be a homologous endo-β-1,3-glucanase, which was therefore detected by the antiserum raised against the purified exo-β-glucanase. More probably, the Claviceps β-glucanase might possess a low level of endohydrolytic activity not detectable in the assay used (i.e. oligosaccharides of a low enough degree of polymerization to be distinguishable from the substrate polysaccharide on chromatograms were not generated). The endohydrolytic activity would be expressed initially in the hydrolysis of glucan, but the resultant rapid increase in the effective molarity, i.e. concentration of "chain ends", would permit the exohydrolytic activity to supercede and predominate the former. Chesters & Bull (1963a) and Villanueva et al, (1976) have reported that fungal β-glucanases which can follow a complex mechanism that is neither purely exo- nor purely endo-hydrolytic do occur in some fungi, and this also may be the case for the Claviceps purpurea β-1,3-glucanase.

Dissolution of plant polysaccharides such as callose and hemicelluloses is another role that can be ascribed to the Claviceps β-1,3-glucanase where it was present in the initial colonization of the ovary and at the host/parasite interface. This can be accounted for by the action of an extracellular β-glucanase in addition to the wall

bound enzyme, since the former would have greater access to plant polysaccharides than the latter. In particular, the hyphae that extend downwards from the centre of the interface, and which grow intracellularly in the vascular tissues of the rachilla (Luttrell, 1980), could secrete an extracellular β-glucanase into the vascular elements to prevent the accumulation of callose, as was proposed by Dickerson et al, (1978). Immunofluorescent detection of the fungal β-glucanase actually within invaded cells of the vascular bundle was prevented, however, because of residual autofluorescence in the xylem vessels and the possibility that the enzyme might have been washed from ovary cells by the antibody staining process.

In other plant pathogen studies, β-1,3-glucanases from fungi are also thought to be active against host polysaccharides. Anderson & Powelson (1979) found an increase in fungal laminarinase among other plant cell wall degrading enzymes in infections of pea plants by Phoma medicaginis, and a similar increase in β-1,3-glucanase activity has been noted in the infection of Brassicas by the downy mildew Peronospora parasitica (J.A. Lucas, University of Nottingham, personal communication). More specifically, Hohl & Stossel (1976) proposed that, in potato cultivars susceptible to infection by Phytophthora infestans, β-1,3-glucanases present in the fungal hyphae (Holten & Bartnicki-Garcia, 1972) enabled the fungal haustoria to degrade the callose in wall appositions produced by invaded plant cells, giving the haustoria access to the plant cell cytoplasm. These authors also proposed that in a resistant potato cultivar, phytoalexins are produced by the plant which can inhibit the production of β-glucanase by the fungus, preventing haustoria from penetrating cells and even reducing hyphal growth (Stossel & Hohl, 1981). Mechanisms such as this may perhaps be crucial to the ability of plants to resist pathogenic fungi possessing a range of hydrolytic enzymes such as pectinases, cellulases and glucan-

ases (Kuc, 1979).

The same functions as those proposed for the Claviceps β-1,3-glucanase in ergot parasitism are carried out by the plant's own β-1,3-glucanases. In uninfected plants, β-glucanases are thought to be involved in the regulation of callose deposits in vascular elements, thus controlling cell to cell translocation (Clarke & Stone, 1962; Abeles & Forrence, 1970; Wong & Maclachlan, 1980). Native β-1,3-glucanases have been implicated in the growth of cereal plant cell walls containing hemicelluloses, since levels of β-glucanase were seen to increase in conjunction with auxin induced cell elongation in barley, oat and corn coleoptiles (Masuda, 1968; Tanimoto & Masuda, 1968; Goldberg, 1977; Huber & Nevins, 1979, 1981; Yamamoto & Nevins, 1981). Also, β-1,3-glucanases occur in ungerminated, germinating and malted cereal grains, notably barley, rye and wheat (Clarke & Stone, 1962; Anderson et al, 1964; Manners & Marshall, 1969, 1973) and Ballance & Manners (1978) suggested that the β-glucanase may be involved in cell wall mobilization during germination. Moreover, it has been proposed that plant β-1,3-glucanases could act as a defence mechanism by attacking the glucan component of fungal cell walls, causing cell lysis and restricting the spread of an infection (Abeles et al, 1971; Wargo, 1975; Pegg, 1976). These authors found increases in the levels of constitutive plant β-1,3-glucanases following fungal invasion and Cline & Albersheim (1981a, 1981b) have reported on the ability of a β-glucan hydrolase from beans to hydrolyse fungal β-1,3-glucans; the products of this reaction may possibly act as phytoalexin elicitors. Further studies are needed to confirm this proposal; however, it is unlikely that the proposed mechanism would play a part in Claviceps infections since the fungus does not cause extensive damage to the plant tissues or invade beyond the rachilla.

The β-glucosidase was located, to varying degrees, in the same areas of the fungus as the β-glucanase. It is possible, therefore, that the two enzymes are complementary in the metabolism of C.purpurea, and that the presence of β-glucosidase ensures the hydrolysis of disaccharides, i.e. gentiobiose, laminaribiose and cellobiose, produced by the action of the β-1,3-glucanase on both fungal cell wall glucan and plant glucans. Increases in the levels of fungal β-glucosidase activity have also been observed in the infections of Brassicas by Peronospora parasitica (Kluczeswki & Lucas, 1982) and alfalfa grass by Aschochyta imperfecta (Olah & Sherwood, 1973). In the latter case, the authors noted a parallel increase in aglycone derivatives of the flavonoid glycosides which are normal constituents of alfalfa leaves, and they suggested that the fungal β-glucosidase was responsible for the hydrolysis of these glycosides. Similarly, Cohen (1975) found enhanced levels of β-glucosidase and scopoletin in sunflower plants infected by Plasmopora halstedii, and proposed that the fungal β-glucosidase hydrolysed scopolin, which is present in host cells, to yield the scopoletin. It is interesting to note that scopoletin is implicated in the autofluorescence of damaged plant cells (Udenfriend, 1964), and Cohen observed that infected host tissue exhibited greater primary or auto-fluorescence than uninfected healthy plant cells. A similar reaction might possibly be caused by the β-glucosidase from Claviceps purpurea in the host cells surrounding the developing ergot, and this would have contributed to the autofluorescence of plant cells that was observed in tissue sections in the present work.

In Neurospora and Monilia β-glucosidases have been detected intra- and extracellularly as well as bound to the cell wall (Eberhart & Beck, 1970; Dekker, 1981). It is probable that, for Claviceps, extracellular and wall bound β-glucosidase were being detected by immunofluorescence, since these locations would be the most suitable for

enzymes involved in the breakdown of glycosides which the fungal hyphae might encounter. However, the possibility that intracellular β-glucosidase was also being detected cannot be ruled out on the basis of the present results. Clarification of this point would result from a more detailed study of the cellular location of β-glucosidase in Claviceps hyphae by immunofluorescence.

The location of β-1,3-glucanase in hyphae from axenic cultures of Claviceps purpurea has since been successfully studied by immunofluorescence, using the antisera prepared for the present investigation (J. Willingale, P.G., Imperial College, personal communication). Fluorescence was observed as a cap on growing hyphal apices, especially conidiating hyphal tips, and as rings around the hyphae which would correspond to points where septa were being formed within the hyphae. These locations for β-1,3-glucanase agree closely with those observed in other fungi by immunofluorescence (Sukumaran & Mahadevan, 1975; Kritzman et al, 1978) and provide further evidence in favour of a role for β-glucanase in hyphal morphogenesis.

In the case of β-fructofuranosidase, some indication of the cellular location could be gained from sections of sclerotial hyphae photographed at higher magnification (Plate 34). The fluorescence followed the contour of the sclerotial cell boundary, but fluorescence in adjacent cells was discrete, i.e. did not meet at the boundary between the cells. This suggests a location where the enzyme is either bound to polysaccharide in the inner layer of the cell wall or in the mural space outside the cell membrane. This is the same location as has been observed for the β-fructofuranosidase in Neurospora by chemical and cell fractionation techniques (Metzenberg, 1963b). A cell wall bound location for all three enzymes studied; β-1,3-glucanase, β-glucosidase and β-fructofuranosidase, is also supported by the demonstration of the activity of each enzyme in cytoplasm-free prepara-

tions of C. purpurea cell walls by Dickerson & Baker (1979).

The immunofluorescent technique has proved valuable in the detection of fungal enzymes from Claviceps purpurea in vivo. Previously, localization studies in Claviceps purpurea had only been performed for enzymes or polysaccharide using cytochemical techniques under the electron microscope (Vorisek, Ludvik & Rehacek, 1974; Vorisek, 1977; Vorisek & Rehacek, 1978; Vorisek & Lojda, 1979). Fluorescence studies have stressed that the β-1,3-glucanase has important functions in ergot parasitism as discussed above; additionally, immunofluorescence could be applied to other stages in the Claviceps purpurea life cycle where a role for β-glucanase might occur. For example, glucan is thought to be present as a storage material in dormant, overwintering sclerotia (Dickerson et al, 1970) and Streiblova, Ryc & Kybal (1978) have reported that cytoplasmic vesicles transport hydrolases, including β-1,3-glucanase, into the cell wall upon activation following absorption of water after winter. The latter authors proposed that the β-glucanase could remove plug material (possibly glucan) from the pores between cortical cells to re-establish cell to cell communication after dormancy, and that glucose liberated from cell wall glucan by this enzyme may act as an additional carbon source in the activated sclerotium. Immunofluorescent localization of the β-glucanase in germinating sclerotia might help to test the validity of this proposal. Also, it has been shown that Claviceps conidia do not readily infect fertilized ovaries (Shaw, 1975); yet conidia germinating at the same time as pollen grains on host stigmata can compete successfully to reach the ovary, although the pollen germ tube has a faster growth rate than fungal hyphae (Shaw, 1979). Shaw (1979) proposed that an extracellular β-1,3-glucanase produced by the fungus could hydrolyse the callose present in pollen tube walls, which

would disrupt the growth of the pollen tube (Roggen & Stanley, 1969) and enable the fungus to colonise the ovary before fertilization took place. Detection of the fungal β-glucanase around pollen tubes in the stigma by immunofluorescence would give support to this hypothesis.

Certain improvements in the immunofluorescent technique presented in this study are possible, such as the use of rhodamine as fluorochrome, in certain cases, to overcome problems arising from the fading observed with fluorescein. An investigation of the levels of autofluorescence suppression that can be obtained with the borate-methylene blue reagent in sections of different thicknesses and of different types of plant tissue would also be useful for general application of the technique. Nevertheless, the technique in its present form is still capable of being applied more widely to explore, for example, the levels, locations and roles of specific enzymes, or other immunogenic metabolites, in the interactions between fungal pathogens and their hosts.

REFERENCES

ABD-EL-AL, A.T.H. & PHAFF, H.J. (1968) Exo-β-glucanases of yeast. Biochemical Journal 109, 347.

ABE, M. & YAMATODANI, S. (1964) Preparation of alkaliods by saprophytic culture of Ergot fungi. Progress in Industrial Microbiology 5, 205.

ABELES, F.B. & FORRENCE, L.E. (1970) Temporal and hormonal control of β-1,3-glucanase in Phaseolus vulgaris L.. Plant Physiology 45, 395.

ABELES, F.B., BOSSHART, R.P., FORRENCE, L.E. & HABIG, W.H. (1971). Preparation and purification of Glucanase and Chitinase from Bean leaves. Plant Physiology 47, 129.

AIST, J.R. (1976). Papillae and related wound plugs of plant cells. Annual Review of Phytopathology 14, 145.

AIST, J.R. (1977). Mechanically induced wall appositions of plant cells can prevent penetration by a parasitic fungus. Science 197,568.

AIST, J.R. & WILLIAMS, P.H. (1971). The cytology and kinetics of cabbage root hair penetration by Plasmodiophora brassicae. Canadian Journal of Botany 49, 2023.

ALBERSHEIM, P. & ANDERSON-PROUTY, A.J. (1975). Carbohydrates, proteins, cell surfaces and the biochemistry of pathogenesis. Annual Review of Plant Physiology 26, 31.

ALBERSHEIM, P. & VALENT, B.S. (1978). Host-pathogen interactions in plants. Journal of Cell Biology 78, 627.

AMOS, R.E. & BURRELL R.G. (1967). Serological differentiation in Ceratocystis. Phytopathology 57, 32.

ANDERSON, A.J. & POWELSON, M.L. (1979). Production of plant cell wall degrading enzymes by Phoma medicaginis f. sp. pinodella. Phytopathology 69, 372.

ANDERSON, F.B., CUNNINGHAM, W.L. & MANNERS, D.J. (1964). Studies on carbohydrate metabblising enzymes. 10:Barley β-glucosidases. Biochemical Journal 90, 30.

ARCAMONE, F., CHAIN, E.B., FERRETTI, A., MINGHETTI, A., PENELLA, P. & TONOLO, A. (1962). The effect of tryptophan and methyltryptophan on submerged lysergic acid fermentation in Claviceps paspali (Stevens & Hall). Biochimica et Biophysica Acta 57, 174.

ARCAMONE, F., CASSINELLI, G., FERNI, G., PENCO S., PENELLA, P & POL,C. (1970). Ergotamine production and metabolism of Claviceps purpurea strain 275 F1 in stirred fermenters. Canadian Journal of Microbiology 16, 923.

ARCHER, S.A., CLAMP, J.R. & MIGLIORE, D. (1977). Isolation and partial characterization of an extracellular branched D-glucan from Monilia fructigena. Journal of General Microbiology 102, 157.

ARNOLD, W.N. (1969). Heat inactivation kineties of yeast β-fructofuranosidase. A polydisperse system. Biochimica et Biophysica Acta 178, 347.

ASPINALL, G.O. (1970). Polysaccharides. Commonwealth and International Library - A course in Organic Chemistry. Ed. R. Robinson. Pergamon Press, Oxford.

ASPINALL, G.O. (1980). Chemistry of cell wall polysaccharides. In "Biochemistry of Plants". Eds. P.K. Stumpf and E.E. Conn. Vol.III. Ed. J. Preiss. pp. 473-500. Academic Press, London.

AYERS, A.R., EBEL, J., VALENT, B. & ALBERSHEIM, P. (1976). Host-pathogen interactions. X: Fractionation and biological activity of an elicitor isolated from the mycelial cell walls of Phytophthora megasperma sojae. Plant Physiology 57, 760.

BAKER, R.C.F.B. (1979). Metabolic and enzymic studies on ergot fungi in axenic culture and during parasitic development. Ph.D. Thesis. University of London.

BAKER, R.C.F.B. & DICKERSON, A.G. (1980). A serological study of a β-D-fructofuranosidase from Sphacelia sorghi McRae. Journal of General Microbiology 121, 267.

BALLANCE, G.M. & MANNERS, D.J. (1978). Partial purification and properties of an endo-1,3-β-D-glucanase from germinated rye. Phytochemistry 17, 1539.

BARGER, G. (1931). Ergot and Ergotism. Gurney & Jackson, London.

BARLOW, K.K., SIMMONDS, D.H. & KENRICK, K.G. (1973). The localization of water soluble proteins in wheat endosperm as revealed by fluorescent antibody techniques. Experientia 29, 229.

BARRAS, D.R. (1972). A β-glucan endo-hydrolase from Schizosaccharomyces pombe and its role in cell wall growth. Antonie V. Leeuwenhoek 38, 65.

BARTNICKI-GARCIA, S. (1968). Cell wall chemistry, morphogenesis and taxonomy of fungi. Annual Review of Microbiology 22, 87.

BARTNICKI-GARCIA, S. (1973). Fundamental aspects of hyphal morphogenesis. Symposia of the Society of General Microbiology 22, 245.

BASSETT, R.A., CHAIN, E.B., CORBETT, K., DICKERSON, A.G. & MANTLE, P.G. (1972). Comparative metabolism of Claviceps purpurea in vivo and in vitro. Proceedings of the Biochemical Society 127, 3P.

BATEMAN, D.F. (1976). Plant cell wall hydrolysis by pathogens. In "Biochemical aspects of plant-parasite relationships". Phytochemical Society Symposia Series. No.13. Eds. J. Friend and D.R.Threllfall. pp. 79-103. Academic Press, London.

BEALING, F.I. & BACON, J.S.D. (1953). The action of mould enzymes on sucrose. Biochemical Journal 53, 277.

BENO, D.W. & ALLEN, O.N. (1964). Immunofluorescent staining for the identification of Puccinia sorgi germinated urediospores. Phytopathology 54, 872.

BERGER, L.S. & EBERHART, B.M. (1961). Extracellular β -transglucosidase activity from conidia of Neurospora crassa. Biochemistry and Biophysics Research Communications 6, 62.

BERNIER, C.C. (1976). Resistance to ergot in the genus Triticum. Proceedings of the Canadian Phytopathology Society. No.43. 42nd session.

BERNIER, C.C. (1978). Evaluation of resistance to Claviceps purpurea in the genus Triticum. Abs. 3rd International Congress of Plant Pathology, Munich, 1978.

BETTELHEIM, K.A., GORDON, J.F. & TAYLOR, J. (1968). The detection of a strain of Chromobacterium lividum in the tissues of certain leaf-nodulated plants by immunofluorescent techniques. Journal of General Microbiology 54, 177.

BEUTNER, E.H. (1961). Immunofluorescent staining - the fluorescent antibody method. Bacteriological Reviews 25,49.

BOCK, R.M. & LING, N.-S. (1954). Devices for gradient elution in chromatography. Analytical Chemistry 26, 1543.

BORGIA, P. & SYPHERD, P.S. (1977). Control of β-glucosidase synthesis in Mucor racemosus. Journal of Bacteriology 130, 812.

BOVÉ, F.J. (1970). The story of ergot. S. Karger, Basel.

BRANDTZAEG, P. (1973). Conjugates of IgG with different fluorochromes I. Characterization by anion exchange chromatography. Scandinavian Journal of Immunology 2, 273.

BUCHT, B. & ERIKSSON, K.E. (1969). Extracellular enzyme system utilized by the rot fungus Stereum sanguinolentum for the breakdown of cellulose IV. Separation of the cellobiase and aryl β-glucosidase activities. Achives of Biochemistry and Biophysics. 129, 416.

BUCK, K.W., CHEN, A.W., DICKERSON, A.G. & CHAIN, E.B. (1968). Formation and structure of extracellular glucans produced by a Claviceps species. Journal of General Microbiology 51, 337.

BURNETT, J.H. (1968). Fundamentals of Mycology. San Martin's Press, New York.

BURRELL, R.G., CLAYTON, C.W., GALLEGLY, M.E. & LILLY, V.G. (1966). Factors affecting the antigenicity of the mycelium of three species of Phytophthora. Phytopathology 56, 422.

BUSHNELL, W.R. (1972). Physiology of fungal haustoria. Annual Review of Phytopathology 10, 151.

BUSHNELL, W.R. & BERGQUIST, S.E. (1975). Aggregation of cytoplasm and the formation of papillae and haustoria in powdery mildew of barley. Phytopathology 65, 310.

CAMPBELL, W.P. (1958). Infection of barley by Claviceps purpurea. Canadian Journal of Botany 36, 615.

CANEVASCINI, G. & MEYER, H.P. (1979). β-glucosidase in the cellulolytic fungus Sporotrichium thermophile Apinis. Experimental Mycology 3,203.

CHANG, P.L.Y. & TREVITHICK, J.R. (1972). Distribution of wall bound invertase during the asexual life-cycle of Neurospora crassa. Journal of General Microbiology 70,23.

CHESTERS, C.G.C. & BULL, A.T. (1963a). Enzymic degradation of Laminarin 1. Microbial laminarinases. Biochemical Journal 86, 28.

CHESTERS, C.G.C. & BULL, A.T. (1963b). Enzymic degradation of laminarin 2. Multi-component nature of fungal laminarinases. Biochemical Journal 86, 31.

CHOO, Y.S. & HOLLAND, A.A. (1970). Direct and indirect fluorescent antibody staining of Ophiobolus graminis Sacc. in culture and in the rhizosphere of cereal plants. Antonie v. Leeuwenhoek 36, 549.

CHRAMBACH, A., REISFELD, R.A., WYCKOFF, M. & ZACCARI, J. (1967). A procedure for the rapid and sensitive staining of protein fractionated by polyacrylamide gel electrophoresis. Analytical Biochemistry 20, 150.

CHUNG, P.L.Y. & TREVITHICK, J.R. (1970). Biochemical and histochemical localization of invertase in Neurospora crassa during conidial germination and hyphal growth. Journal of Bacteriology 102, 423.

CLARKE, A.E. & STONE, B.A. (1962). β-1,3-glucan hydrolases from the grape vine (Vitis vinifera) and other plants. Phytochemistry 1, 175.

CLARKE, A.E. & STONE, B.A. (1963). Chemistry and biochemistry of β-1,3-glucans. Reviews of Pure and Applied Chemistry 13, 134.

CLINE, K. & ALBERSHEIM, P. (1981a). Host pathogen interactions XVI. Purification and characterization of a β-glucosyl hydrolase/transferase present in the walls of soybean cells. Plant Physiology 68, 207.

CLINE, K. & ALBERSHEIM, P. (1980b). Host pathogen interactions XVII. Hydrolysis of biologically active fungal glucans by enzymes isolated from soybean cells. Plant Physiology 68, 221.

COHEN, Y. (1975). Fluorescence microscopy and related enzyme activity of Sunflower plants infected by Plasmopora halstedii. Physiological Plant Pathology 7, 9.

COOK, K.A. & CAIN, R.B. (1977). Enzymes of the β-oxoadipate pathway in fungi; a serological study of 3-carboxymuconate cyclase(lactonizing enzyme) in fungi and its possible taxonomic significance. Journal of General Microbiology 102, 81.

COOKE, R.C. & MITCHELL, D.T. (1970). Carbohydrate physiology of sclerotia of Claviceps purpurea during dormancy and germination. Transactions of the British Mycological Society 54, 93.

COONS, A.H. CREECH, H.J., JONES, R.N. & BERLINER, E. (1942). The demonstration of pneumococcal antigen in tissues by the use of fluorescent antibody. Journal of Immunology 45, 159.

CORBETT, K., DICKERSON, A.G. & MANTLE, P.G. (1974). Metabolic studies on Claviceps purpurea during parasitic development on rye. Journal of General Microbiology 84, 39.

CORBETT, K., DICKERSON, A.G. & MANTLE, P.G. (1975). Metabolism of the germinating sclerotium of Claviceps purpurea. Journal of General Microbiology 90, 55.

CRAFTS, A.S. & CURRIER, H.B. (1963). On sieve tube function Protoplasma 57, 188.

CRESTI, M. & VAN WENT, J.L. (1976). Callose deposition and plug formation in Petunia pollen tubes "in situ". Planta 133, 35.

CUNFER B.M. & MARSHALL, D. (1977). Germination requirements of Claviceps paspali sclerotia. Mycologia 69, 1137.

CURRIER, H.B. (1957). Callose substance in plant cells. American Journal of Botany 44, 478.

CURRIER, H.B. & STRUGGER, S. (1956). Aniline blue and fluorescence microscopy of callose in bulb scales of Allium cepea L. Protoplasma 45, 552.

CUTTER, R.L. & YELLOWLEES, D. (1979). Purification and characterization of two endo-(1→3)-β-D-glucanases from Telescopium telescopium. Carbohydrate Research 75, 221.

DAVIS, E.N., RHODES, R.A. & SCHULKE, H.R. (1967). Fermentative production of exocellular glucans by fleshy fungi. Applied Microbiology 13, 267.

DEDONDER, R.A. (1961). Glycosidases and Transglycosidation. Annual Review of Biochemistry 30, 347.

DEKKER, R.F. (1981). Induction, localization and characterization of β-D-glucosidases produced by a species of Monilia. Journal of General Microbiology 127, 177.

DESPHANDE, V., ERIKSSON, K.E. & PETTERSSON, B. (1978). Production, purification and partial characterization of 1,4-β-glucosidase enzymes from Sporotrichium pulverulentum. European Journal of Biochemistry 90, 191.

DICKERSON A.G. (1972). A β-D-fructofuranosidase from Claviceps Purpurea. Biochemical Journal 129, 263.

DICKERSON, A.G., MANTLE, P.G. & SZCZYRBAK, C.A. (1970). Autolysis of extracellular glucans produced in vitro by a strain of Claviceps fusiformis. Journal of General Microbiology 60, 403.

DICKERSON, A.G., MANTLE, P.G. & NISBET, L.J. (1976). Carbon assimilation by Claviceps purpurea growing as a parasite. Journal of General Microbiology, 97, 267.

DICKERSON, A.G., MANTLE, P.G., NISBET, L.J. & SHAW B.I.(1978). A role for β-glucanases in the parasitism of cereals by Claviceps purpurea. Physiological Plant Pathology 12, 55.

DICKERSON, A.G. & BAKER, R.C.F. (1979). The binding of enzymes to fungal β-glucans. Journal of General Microbiology 112, 67.

DICKERSON, A.G. & POLLARD, C.M.D. (1982). Observations on the location of a β-glucanase and an associated β-glucosidase in Claviceps purpurea during its development on rye. Physiological Plant Pathology 21, 175.

DICKSON, J.G. (1947). In "Diseases of field crops". p.p. 152-158. McGraw-Hill.

DUNCAN, W.A.M., MANNERS, D.J., & ROSS, A.G. (1956). Enzyme systems in marine algae. Carbohydrase activities. Biochemical Journal 63, 44.

EBERHART, B.M. & BECK, R.S. (1970). Location of β-glucosidases in Neurospora crassa. Journal of Bacteriology 101, 408.

EBERHART, B.M. & BECK, R.S. (1973). Induction of β-glucosidases in Neurospora crassa. Journal of Bacteriology 116, 295.

EDWARDS, H.H. & ALLEN, P.J. (1966). Distribution of products of photosynthesis between powdery mildew and barley. Plant Physiology 41, 683.

ENGELKE, C. (1902). Neue Beobachtugen über die vegetations - Formen des Mutterkorn pilzes Claviceps purpurea Tulasne. Hedwigia 41, 221.

EREN, J. & PRAMER, D. (1966). Application of immunofluorescent staining to studies of the ecology of soil microorganisms. Soil Science 101, 39.

ESCHRICH, W. & CURRIER, H.B. (1964). Identification of callose by its diachrome and fluorochrome reactions. Stain Techniques 39,303.

FARKAS, V., BIELY, P. & BAUER, S. (1973). Extracellular β-glucanase from the yeast Saccharomyces cerevisiae. Biochimica et Biophysica Acta 321, 246.

FÈVRE, M. (1977). Subcellular localization of glucanase and cellulase in Saprolegnia monoica (Pringsheim). Journal of General Microbiology 103, 287.

FÈVRE, M. (1979). Intracellular and cell wall associated 1-3-β-glucanases of Saprolegnia. Mycopathologia 67, 89.

FÈVRE, M. & DUMAS, C. (1977). β-glucan synthetases from Saprolegnia monoica. Journal of General Microbiology 103, 297.

FITZELL, R., EVANS, G. & FAHY, P.C. (1980). Studies on the colonization of plant roots by Verticillium dahliae Klebahn with use of immunofluorescent staining. Australian Journal of Botany 28, 357.

FLEET, G.H. & MANNERS, D.J. (1977). Enzymatic degradation of alkali soluble glucan from cell walls of Saccharomyces cerevisiae.Journal of General Microbiology 98, 315.

FLEET, G.H. & PHAFF, H.J. (1974). Glucanases in Schizosaccharomyces: Isolation and properties of the cell wall associated β-(1-3)-glucanases. Journal of Biological Chemistry 249, 1717.

FLEMING, I.D. & PEGLER, H.F. (1963). The determination of glucose in the presence of maltose and isomaltose by a stable, specific enzyme reagent. Analyst 88, 967.

FUCHS, L. & POHM, M. (1953). Zur Kenntuis des Honigtaues. Scientia Pharmaceutica 21, 239.

FULCHER, R.G., McCULLY, M.E., SETTERFIELD, G. & SUTHERLAND, J. (1976). β-1,3-glucans may be associated with cell plate formation during cytokinesis. Canadian Journal of Botany 54, 539.

FULLER, J. (1969). The day of St. Anthony's Fire. Hutchison, London.

GABRIEL, O. & WANG, S.F. (1969). Determination of enzyme activity in polyacrylamide gels. Analytical Biochemistry 27, 545.

GASCON, S. & LAMPEN, J.O. (1968). Purification of the internal invertase of yeast. Journal of Biological Chemistry 243, 1567.

GASCON, S., NEUMANN, P. & LAMPEN, J.O. (1968). Comparative study of the properties of purified internal and external invertases of yeast. Journal of Biological Chemistry 243, 1573.

GOLDBERG, R. (1977). On possible connections between auxin induced growth and cell wall glucanase activities. Plant science Letters 8,233.

GOLDMAN, M. (1968). Fluorescent Antibody Methods. Academic Press, London.

GONG, C.S., LADISCH, M.R. & TSAO, G.T. (1977). Cellobiase from Trichoderma viride: Purification, properties, kinetics and mechanism. Biotechnology and Bioengineering 19, 959.

GOODAY, G.W. (1978). The enzymology of hyphal growth. In "The Filamentous Fungi". Vol. III Developmental Mycology. Eds. J.E. Smith and D.R. Berry. pp 51-77. E. Arnold, London.

GOOS, R.D. & SUMMERS, D.F. (1964). Use of fluorescent antibody technique in observations on the morphogenesis of fungi. Mycologia 56, 701.

GORIN, P.A.J. & SPENCER, J.F.T. (1968). Structural chemistry of fungal polysaccharides. Advances in Carbohydrate Chemistry 23,367.

GROGER, D. (1972). Ergot. In "Microbial Toxins". Vol. VIII. Fungal Toxins. Eds. S. Kadis, A. Ciegler & S.J. Ajl. p.321. Academic Press London.

GUTHRIE, R.D., JENKINS, I.D., ROGERS, P.J., WING, F.S., WATTERS, J.J. & YAMASAKI, R. (1979). Studies of invertase (β-D -fructofuranosidase). Carbohydrate Research 75, c1.

HALL, J.L. (1978). Electron microscopy and cytochemistry of plant cells. Elsevier, N. Holland.

HALSALL, D.M. (1976). Specificity of cytoplasmic and cell wall antigens from four species of Phytophthora. Journal of General Microbiology 94, 149.

HAN, Y.W. & SRINIVASAN, V.R. (1969). Purification and characterization of β-glucosidase of Alcaligenes faecalis. Journal of Bacteriology 100, 1355.

HATTERSLEY, P.W., WATSON, L. & OSMOND, C.B. (1977). In situ immunofluorescent labelling of ribulose 1,5 biphosphate carboxylase in leaves of C_3 and C_4 plants. Australian Journal of Plant Physiology 4, 523.

HEATH, M.C. & HEATH, I.C. (1971). Ultrastructure of an immune and a susceptible reaction of cowpea leaves to rust infection. Physiological Plant Pathology 1, 277.

HERMAN, A. & HALVORSON, H. (1963). Genetic control of β-glucosidase synthesis in Saccharomyces lactis. Journal of Bacteriology 85, 901.

HERR, D., BAUMER, F. & DELLWEG, H. (1978). Purification and properties of an extracellular β-glucosidase from Lenzites trabea. European Journal of Applied Microbiology and Biotechnology 5, 29.

HESLOP- HARRISON, J. (1966). Cytoplasmic continuities during spore formation in flowering plants. Endeavour 25, 65.

HISLOP, E.C., SHELLIS, C., FIELDING, A.H., BOURNE, F.J. & CHILDLOW, J.W. (1974). Antisera produced to purified extracellular pectolytic enzymes from Sclerotinia fructigena. Journal of General Microbiology 83, 135.

HOHL, H.R. & STOSSEL, P. (1976). Host parasite interfaces in a resistant and susceptible cultivar of Solanum tuberosum inoculated with Phytophthora infestans: tuber tissue. Canadian Journal of Botany 54, 900.

HOLTEN, V.Z. & BARTNICKI-GARCIA, S. (1972). Intracellular β-glucanase activity of Phytophthora palmivora. Biochemica et Biophysica Acta 276, 221.

HORIKOSHI, K. & IKEDA, Y. (1965). Studies on the spore coats of Aspergillus oryzae II. Conidia coat bound β-glucosidase. Biochemica et Biophysica Acta 101, 352.

HOWLETT, B.J., KNOX, R.B. & HESLOP-HARRISON, J. (1973). Pollen wall proteins: Release of the allergen antigen E from intine and exine sites in pollen grains of Ragweed and Cosmos. Journal of Cell Science 13, 603.

HU, A.S.L., EPSTEIN, R., HALVORSEN, H.O. & BOCK, R.M. (1960). Yeast β-glucosidase - Comparison of the physical-chemical properties of purified constitutive and inducible enzymes. Archives of Biochemistry and Biophysics 91, 210.

HUBER, D.J. & NEVINS, D.J. (1979). Autolysis of the cell wall β-D- glucan in corn coleoptiles. Plant and Cell Physiology 20, 201.

HUBER, D.J. & NEVINS, D.J. (1981). Partial purification of endo-β-D-glucanase and exo-β-D-glucanase from Zea mays seedlings and their involvement in cell wall autohydrolysis. Planta 151, 206.

HUNSLEY, D. & BURNETT, J.H. (1970). The ultrastructural architecture of the walls of some hyphal fungi. Journal of General Microbiology 62, 203.

HUNSLEY, D. & KAY, D. (1976). Wall structure of the Neurospora byphal apex: Immunofluorescent localization of wall surface antigens. Journal of General Microbiology 95, 233.

HUOTARI, P.I., NELSON, T.E., SMITH, F. & KIRKWOOD, S. (1968). Purification of an exo-β-D(1-3) glucanase from Basidiomycete QM 806. Journal of Biological Chemistry 243, 952.

HUWYLER, H.R., FRANZ, G. & MEIER, H. (1978). β-1,3-glucan in the cell walls of cotton fibres (Gossypium arboretum L.). Plant Science Letters 12, 55.

IKI, K., NAKAGAWA, H., OGURA, N. & TAKEHANA, H. (1977). Intracellular localization of β-fructofuranosidase in tomato fruit tissue. Agricultural and Biological Chemistry 41, 1311.

JERMYN, M.A. (1959). Some comparative properties of β-glucosidases secreted by fungi. Australian Journal of Biological Science 12,213.

JERMYN, M.A. (1966). Fungal cellulases XV. Acceptor specificity of the aryl β-glucosidase of Stachybotrys atra. Australian Journa of Biological Science 19, 903.

JERMYN M.A. (1969). Interaction of linear analogues of D-glucose with the β-glucosidase of Stachybotrys atra. Australian Journal of Biological Science 22, 1039.

JIRKU, V., KRAZNEROVA, B. & KRUMPHANZL, V. (1980). The extracellular system of β-1,3-glucanases of Alternaria tennissima and Aspergillus vesicolor. Folia Microbiologia 25, 24.

JOHNSON, G.D., HOLBOROW, E.J. & DORLING, J. (1978). 'Immunofluorescence'. In 'Handbook of Experimental Immunology'. Ed. D.M. Weir. 3rd Edn. Vol I. pp 15.0-15.30. Blackwell, Oxford.

JONES, D., GORDON, A.H. & BACON, J.S.D. (1974). Co-operative action by endo and exo β-1,3-glucanases from parasitic fungi in the degradation of cell wall glucan of Sclerotinia sclerotiorum (Lib.) de Bary. Biochemical Journal 140, 47.

KEEN, N.T. (1975). Specific elicitors of plant phytoalexin production: determinants of race specificity in pathogens? Science 187, 74.

KEEN, N.T., WANG, M.C., BARTNICKI-GARCIA, S. & ZENTMYER, G.A. (1975). Phytotoxicity of mycolaminarins- β-1,3-glucans from Phytophthora spp. Physiological Plant Pathology 7, 91.

KIRALY, Z., BARNA, B. & ERSEK, T. (1972). Hypersensitivity as a consequence, not the cause of plant resistance to infection. Nature 239, 456.

KIRCHOPF, H. von. (1929). Beitrage zur biologie und physiologie des Mutterkornpilzes. Centr. Bakteriol. Parasitenk.Abt. II 77, 310.

KLUCZESWKI, S.M. & LUCAS, J.A. (1982). Development and physiology of infection by the downy mildew fungus Peronospora parasitica (Pers. ex Fr.) Fr. in susceptible and resistant Brassica species. Physiological Plant Pathology (in press).

KNOX, R.B. (1971). Pollen wall proteins: localization, enzymic and antigenic activity during development in Gladiolus (Iridacae) Journal of Cell Science 9, 209.

KRISHNA-MURTI, C.R. & STONE, B.A. (1961). Fractionation of the β-glucosidases from Aspergillus niger. Biochemical Journal 78, 715.

KRITZMAN, G. CHET, I. & HENIS, Y. (1978). Localization of β-1,3-glucanase in mycelium of Sclerotium rolfsii. Journal of Bacteriology 134, 470.

KRITZMAN, G., CHET, I. & HENIS, Y. (1979). Isolation of extracellular polysaccharides from Sclerotium rolfsii. Canadian Journal of Botany 57, 1855.

KUC, J. (1979). Specificity between plants and their parasites: a molecular confrontation. In "Host parasite interfaces". Ed.B.B. Nickol. pp. 87-101. Academic Press, London.

KUMAR, D. & PATTON, R.F. (1964). Fluorescent antibody technique for detection of Polyporus tormentosus. Phytopathology 54, 898.

KYBAL, J. (1964). Neue beobachtungen uber die bildung der sclerotien von Claviceps purpurea (Fr.)Tul. Planta Medica 12, 166.

LACHANCE, M.A. & PHAFF, H.J. (1979). Comparative study of molecular size and structure of exo-β-glucanases from Kluyveromyces and other yeast genera: evolutionary and taxonomic implications. International Journal of Systematic Bacteriology 29, 70.

LAMPEN, J.O. (1971). Yeast and Neurospora Invertases. In 'The Enzymes'. 3rd. Edn. Ed. P.D. Boyer. Vol. V, pp 291-305. Academic Press, London.

LAYNE, E. (1957). Spectrophotometric and turbidimetric methods for measuring protein. In "Methods in Enzymology". Vol. III. Eds. S.P. Colowick & N.O. Caplan. Academic Press, London.

LEVY, H.B. & SOBER, H.A. (1960). A simple chromatographic method for preparing γ-globulin. Proceedings of the Society for Experimental Biology and Medicine 103, 250.

LEWIS, D.H. (1974). Microorganisms and plants - evolution of parasitism and mutualism. Symposia of the Society for General Microbiology 24, 367.

LEWIS, D.H. & SMITH, D.C. (1967). Sugar alcohols (polyols) in fungi and green plants I. Distribution, physiology and metabolism. New Phytologist 66, 143.

LINKO, Y.Y. and LINKO, P. (1979). Immobilized β-glucanases and their applications. Biotechnology Letters 1,489.

LIPETZ, J. (1970). Wound healing in higher plants. International Review of Cytology 27, 1.

LONG, D.E., FUNG, A.K., McGEE, E.E.M., COOKE, R.C. & LEWIS, D.H.(1975). The Activity of invertase and its relevance to the accumulation of storage polysaccharides in leaves infected by biotrophic fungi. New Phytologist. 74, 173.

LUNDERSTADT, J. (1966). Effect of rust infection on hexokinase activity and carbohydrate dissimilation in primary leaves of wheat. Canadian Journal of Botany 44, 1345.

LUSIS, A.J. & BECKER, R.R. (1973). The β-glucosidase system of the thermophilic fungus Chaetomium thermophile var. coprophile N.var.*

LUTTRELL, E.S. (1977). The disease cycle and fungus host relationships in Dallisgrass ergot. Phytopathology 67, 1641.

LUTTRELL, E.S. (1980). Host parasite relationships and the development of the ergot sclerotium in Claviceps purpurea. Canadian Journal of Botany 58, 942.

MADDOX, I.S. & HOUGH, J.S. (1971). Yeast glucanase and mannanase. Journal of the Institute of Brewing 77, 44.

MADHOSINGH, C. (1964). A serological comparison of three Fusarium species. Canadian Journal of Botany 42, 1143.

MAGUIRE, J.R. (1977). Kinetics of the hydrolysis of cellobiose and p-nitrophenol-β-D-glucopyranoside by the cellobiase of Trichoderma viride. Canadian Journal of Biochemistry 55, 19.

MAHADEVAN, P.R. & EBERHART, B.M. (1964). The β-glucosidase system of Neurospora crassa II. Purification and characterization of the aryl β-glucosidase. Archives of Biochemistry and Biophysics 108, 22.

MAHADEVAN, P.R. & MENON, C.P.S. (1968). Laminarase of Neurospora crassa I. Enzyme activity associated with conidia and conidial wall. Indian Journal of Biochemistry 5, 6.

MAHADEVAN, P.R. & MAHADKAR, V.R. (1970). Role of enzymes in growth and morphology of Neurospora crassa: cell wall bound enzymes and their possible role in branching. Journal of Bacteriology 101, 941.

MANDELS, M., PARRISH, R.W. & REESE, E.T. (1967). β-1,3-glucanases from plant callus cultures. Phytochemistry 6,1097.

* Biochimica et Biophysica Acta 329, 5.

MANKARIOS, A.T. & FRIEND, J. (1980). Polysaccharide degrading enzymes of Botrytis allii and Sclerotium ceviporum. Enzyme production in culture and effect of enzymes on isolated cell walls. Physiological Plant Pathology 17, 93.

MANNERS, D.J. & MARSHALL, J.J. (1969). Studies on carbohydrate metabolizing enzymes. Part XXII: the β-glucanase system of malted barley. Journal of the Institute of Brewing 75, 550.

MANNERS, D.J. & MARSHALL, J.J. (1973). Some properties of a β-1,3-glucanase from rye. Phytochemistry 12, 547.

MANNERS, D.J. MASSON, A.T. & PATTERSON, J.C. (1973). The structure of a β-1,3-D-glucan from yeast cell walls. Biochemical Journal 135, 19.

MANTLE, P.G. (1965). Hypercapsulated growth of Leuconostoc mesenteroides in the sphacelial stage of Claviceps purpurea. Journal of Microbiology and Serology 31, 414.

MANTLE, P.G. (1975). In "The Filamentous Fungi - Industrial Mycology" Vol. I. Eds. J.E. Smith & D.R. Berry. E. Arnold, London.

MANTLE, P.G. & TONOLO, A. (1968). Relationship between the morphology of Claviceps purpurea and the production of alkaloids. Transactions of the British Mycological Society 51, 499.

MANTLE, P.G., MORRIS, L.J. & HALL, S.W. (1969). Fatty acid composition of sphacelial and sclerotial growth forms of Claviceps in relation to the production of ergoline alkaloids in culture. Transactions of the British Mycological Society 53, 441.

MANTLE, P.G. & NISBET, L.J. (1976). Differentiation of Claviceps purpurea in axenic culture. Journal of General Microbiology 93, 321.

MARCHANT, R. & SMITH, D.G. (1968). A serological investigation of hyphal growth in Fusarium culmorum. Archiv fur Mikrobiologie 63, 85.

MARCHIN, D.L. & DUERKSON, J.D. (1969). Comparison of the catalytic and immunological properties of β-glucosidases from three strains of Saccharomyces lactis. Journal of Bacteriology 97, 237.

MARSHALL, J.J. (1974). Application of enzymic methods to the structural analysis of polysaccharides (Part 1). Advances in Carbohydrate Chemistry and Biochemistry 30, 257.

MASUDA, Y. (1968). Role of cell-wall-degrading enzymes in cell wall loosening in oat colesptiles. Planta 83, 171.

MATILE, P. CORTAT, M., WIEMKEN, A. & FREY-WYSSLING, A. (1971). Isolation of glucanase containing particles from budding Saccharomyces cerevisiae. Proceedings of the National Academy of Science (U.S.A) 68, 636.

MAY, J.W. (1962). Sites of cell wall extension demonstrated by the use of fluorescent antibody. Experimental Cell Research 27, 170.

McKEEN, W.E. & RIMMER, S.R. (1973). Initial penetration process in powdery mildew infection of susceptible barley leaves. Phytopathology 63, 1049.

METZENBERG, R.L. (1963a). Purification and properties of invertase from Neurospora crassa. Archives of Biochemistry and Biophysics. 100, 503.

METZENBERG, R.L. (1963b). Localization of β-fructofuranosidase in Neurospora. Biochimica et Biophysica Acta 77, 455.

METZENBERG, R.L. (1964). Enzymically active subunits of Neurospora invertase. Biochimica et Biophysica Acta 89, 291.

MILOVIDOV, P. (1954). Prisperek k mikroskopicko-morfologickeniu studiu vyvoje namele Claviceps purpurea (Fr.) Tul. I. Prestia 26, 415.

MILOVIDOV, P. (1956). Prisperek k mikroskopicko-morfologickeniu studiu vyvoje namele Claviceps purpurea (Fr.) Tul. II. Prestia 28, 380.

MISHRA, N.C. & TATUM, E.L. (1972). Effect of L-sorbose on polysaccharide synthetases of Neurospora crassa. Proceedings of the National Academy of Science (USA) 69, 313.

MITCHELL, D.T. & COOKE, R.C. (1968). Some effects of temperature on germination and longevity of sclerotia in Claviceps purpurea. Transactions of the British Mycological Society 51, 721.

MIYAZAKI, T., YADOMAE, T., YAMADA, H. & OIKAWA, N. (1977). An endo (1-3)-β-D-glucanase from Mucor hiemalis. Carbohydrate Research 55, 65.

MIYAZAKI, T., OIKAWA, N., YAMADA, H. & YADOMAE, T. (1978). Structural examination of antitumour water soluble glucans from Grifora umbellata by use of four types of glucanase. Carbohydrate Research 65, 235.

MOSCATELLI, E.A. HAM, E.A. & RICKES, E.L (1961). Enzymatic properties of a β-glucanase from Bacillus subtilis. Journal of Biological Chemistry 236, 2858.

MOWER, R.L., GRAY, G.R. & BALLOU, C.E. (1973). Sugars from Sphacelia sorghi honeydew. Carbohydrate Research 27, 119.

MOWER, R.L., SNYDER, W.C. & HANCOCK, J.G. (1975). Biological control of ergot by Fusarium. Phytopathology 65, 5.

MOWER, R.L. & HANCOCK, J.G. (1975a). Sugar composition of ergot honeydews. Canadian Journal of Botany 53, 2813.

MOWER, R.L. & HANCOCK, J.G. (1975b). Mechanism of honeydew formation by Claviceps. Canadian Journal of Botany 53, 2826.

MYRBACK, K. (1960). Invertases. In "The Enzymes" 2nd. ed. Eds. PD. Boyer, H. Lardy & K. Myrback. Vol 4, p. 374. Academic Press, London.

NAIRN, R.C. (1976). Fluorescent Protein Tracing. 4th edn. E. & S. Livingston, London.

NELSON, T.E., SCALETTI, J.V., SMITH, F. & KIRKWOOD, S. (1963). The use of enzymes in structural studies on polysaccharides 1. The mode of attack of a β-D-1,3-glucanase on laminarin. Canadian Journal of Chemistry 41, 1671.

NEUMANN, N.P. & LAMPEN, J.O. (1967). Purification and properties of yeast invertase. Biochemistry 6, 468.

NEVINS, D.J., YAMAMOTO, R. & HUBER, D.J. (1978). Cell wall β-D-glucans of five grass species. Phytochemistry 17, 1503.

NICHOLS, R.L. & McCOMB, D. (1962). Immunofluorescent studies with Trachmoma and related antigens. Journal of Immunology 89, 545.

NIMS, R.C., HALLIWELL, R.S. & ROSBERG, D.W. (1967). Wound healing in cultured tobacco cells following microinjection. Protoplasma 64, 21.

NISBET, L.J. DICKERSON, A.G. & MANTLE, P.G. (1977). Changes in respiration patterns of Claviceps purpurea during parasitic development. Transactions of the British Mycological Society 69, 11.

NISIZAWA, K. & HASHIMOTO, Y. (1970). Glycoside hydrolases and glycosyl transferases. In 'Carbohydrates - Chemistry and Biochemistry'. 2nd edn. Eds. W. Pigman and D. Horton. Vol. IIA, pp 241-300. Academic Press, London.

O'DAY, H. & PATERNO, G.F. (1979). Intracellular and extracellular C.M. cellulase and β-glucosidase activity during germination of Polysphondylium pallidum microcysts. Archives of Microhiology 121, 231.

OLAH, A.F. & SHERWOOD, R.T. (1973). Glycosidase activity and flavonoid accumulation in alfalfa infected by Aschochyta imperfecta. Phytopathology 63, 739.

OUCHTERLONY, O. & NILSSON, L.-A. (1978). Immunodiffusion and immuno-electrophoresis. In 'Handbook of Experimental immunology'. 3rd edn. Ed. D.M. Weir. Vol. I, chap. 19. Blackwell, Oxford.

PARTRIDGE, S.M. (1949). Aniline hydrogen phthalate as a spraying agent for chromatography of sugars. Nature (London) 164, 443.

PATON, A.M. (1960). Role of Pseudomonas in plant disease. Journal of Applied Bacteriology 23, 526.

PATON, A.M. (1964). The adaptation of the immunofluorescence technique for use in bacteriological investigations of plant tissue. Journal of Applied Bacteriology 27, 237.

PAYNTER, V.A. & ALCONERO, R. (1979). A specific fluorescent antibody for detection of syringomycin in infected peach tree tissues. Phytopathology 69, 493.

PEARSE, A.G.E. (1968). Histochemistry - theoretical and applied. 3rd edn. Churchill, London.

PEBERDY, J.F. (1976). Isolation and properties of protoplasts from filamentous fungi. In 'Microbial and Plant Protoplasts'. Eds. J.F. Peberdy, A.H. Rose, H.J. Rogers & E.C. Cocking. Academic Press, London.

PEBERDY, J.F. (1978). Protoplasts and their development. In "The Filamentous Fungi". Vol. III. Developmental Mycology. Eds. J.E. Smith and D.R. Berry. pp 119-131. E. Arnold, London.

PEGG, G.F. (1976). The occurrence of 1,3-β-glucanase in healthy and Verticillium infected resistant and susceptible tomato plants. Journal of Experimental Botany 27, 1093.

PERLIN, A.S. & TABER, W.A. (1963). A glucan produced by Claviceps purpurea. Canadian Journal of Chemistry 41, 2278.

PHAFF, H.J. (1963). The cell wall of yeasts. Annual Review of Microbiology 17, 15.

PITTET, A.O. (1965). In 'Methods in Carbohydrate Chemistry'. Vol.5. General polysaccharides. Ed. R.L. Whistler. p 4. Academic Press, London.

POLACHECK, Y. & ROSENBERGER, R.F. (1975). Autolytic enzymes in hyphae of Aspergillus nidulans: their action on old and newly formed walls. Journal of Bacteriology 121, 332.

PREECE, T.F. (1966). Application of the fluorescent antibody technique to the identification of plant pathogenic fungi. Journal of General Microbiology 42, v.

PREECE, T.F. & COOPER, D.J. (1969). The preparation and use of a fluorescent antibody reagent for Botrytis cinerea grown on glass slides. Transactions of the British Mycological Society 52, 99.

PURANIK, S.B. & MATHRE, D.E. (1971). Biology and control of ergot on male sterile wheat and barley. Phytophathology 61, 1075.

RAMSTAD, E. & GJERSTAD, G. (1955). The parasitic growth of Claviceps purpurea (Fries) Tulasne on rye and its relation to alkaloid formation. Journal of the American Pharmaceutical Association 44, 741.

REES, D.A. (1973). Polysaccharide conformation. In MTP International Review of Science: Organic Chemistry, Series 1, vol.7. (Carbohydrates). Ed. G.O. Aspinall, pp 251-283. Butterworth, London.

REESE, E.T. & MANDELS, M. (1959a). β-D-1,3-glucanases in fungi. Canadian Journal of Microbiology 5, 173.

REESE, E.T. & MANDELS, M. (1959b). Use of enzymes in isolation and analysis of polysaccharides. Applied Microbiology 7,378.

REESE, E.T., PARRISH, F.W. & MANDELS, M. (1962). β-D-1,6-glucanases in fungi. Canadian Journal of Microbiology 8, 327.

REESE, E.T., MAGUIRE, A.H. & PARRISH, F.W. (1968). Glucosidases and exo-glucanases. Canadian Journal of Biochemistry 46, 25.

RENNIE, R.J. (1976). Immunological detection of nitrogenase proteins in whole cells. Journal of General Microbiology 97, 289.

REYNOLDS, J.D. & DASHEK, W.V. (1976). Cytochemical analysis of callose localization in Lilium longiflorum pollen tubes. Annals of Botany, 40, 409.

ROGGEN, H.P.J.R. & STANLEY, R.G. (1969). Cell wall hydrolysing enzymes in wall formation as measured by pollen tube extension. Planta 84,295.

ROSEN, W.G. (1968). Ultrastructure and physiology of pollen. Annual Review of Plant Rhysiology 19, 435.

RUDICK, M.J., FITZGERALD, Z.E. & RUDICK, V.L. (1979). Intra- and extracellular forms of α-glucosidase from Aspergillus niger. Archives of Biochemistry and Biophysics 193, 509.

SANTAMARIA, F., REYES, F. & LAHOZ, R. (1978). Extracellular glucan containing β-1,3 and β-1,6 linkages isolated from Monilia fructigena. Journal of General Microbiology 109, 287.

SANTOS, T., SANCHEZ, M., VILLANUEVA, J.R. & NOMBELA, C. (1979). Derepression of β-1,3-glucanases in Penecillium italicum:localization of various enzymes and correlation with cell wall glucan mobilization and autolysis. Journal of Bacteriology 137, 6.

SCHAEFFER, H.P. (1977). An alkali soluble polysaccharide from the cell walls of Coprinus lagopus. Archives of Microbiology 113, 79.

SCHMIDT, E.L. & BANKOLE, R.O. (1965). Specificity of immunofluorescent staining for study of Aspergillus flavus in soil. Applied and Environmental Microbiology 13, 673.

SEITSMA,J.H. & WESSELS, J.G.H. (1979). Evidence for covalent linkages between chitin and β-glucan in a fungal wall. Journal of General Microbiology 114, 99.

SEITSMA, J.H. & WESSELS, J.G.H. (1981). Solubility of (1-3)-β-D/(1-6)-β-D-glucan in fungal walls: importance of presumed linkage between glucan and chitin. Journal of General Microbiology 125, 209.

SHAW, B.I. (1979). Infection of cereals by Claviceps purpurea and the role of some enzymes in parasitism. Ph.D. thesis, University of London.

SHAW, B.I. & MANTLE, P.G. (1980a). Host infection by Claviceps purpurea; Transactions of the British Mycological Society 75, 77.

SHAW, B.I. & MANTLE, P.G. (1980b). Parasitic differentiation of Claviceps purpurea. Transactions of the British Mycological Society 75, 117.

SHAW, S. (1975). Aetiology of ergot disease of male sterile wheat. Ph.D. thesis. University of London.

SHIMOMURA, T. & DIJKSTRA, J. (1975). The occurrence of callose during the process of local lesion formation. Netherlands Journal of Plant Pathology 81, 107.

SKIPP, R.A. HARDER, D.E. & SAMBORSKI, D.J. (1974). Electron microscopy studies of resistant (Sr 6 gene) and susceptible near isogenic wheat lines by Puccinia graminis fsp. tritici. Canadian Journal of Botany 52, 2615.

SMITH, D. MUSCATINE, L. & LEWIS, D. (1969). Carbohydrate movement from autotrophs to heterotrophs in parasitic and mutualistic symbiosis. Biological Reviews 44, 17.

SMITH, F. & MONTGOMERY, R. (1959). Cereal Gums. In 'Chemistry of Plant Gums and Mucilages', Monograph Series 141, pp 373-380. Rheinhold Publishing Corp., New York.

SMITH, M.H. & GOLD, M.H. (1979). Phanerochaete chrysosporium β-glucosidases: induction, cellular localization and physical characterisation. Applied and Environmental Microhiology 37, 938.

SONEA, S. & DEREPENTIGNY, J. (1960). Observations sur les couleurs de la fluorescence primarie des microorganisms. Canadian Journal of Microbiology 6, 519.

STADLER, P.A. & STUTZ, P. (1975). The ergot alkaloids. In 'The alkaloids'. Vol. XV, Ed. R.H.F. Manske. Academic Press, London.

STECKOLL, M. & WEST, C.A. (1978). Purification and properties of an elicitor of castor bean phytoalexin from culture filtrates of the fungus Rhizopus stolonifer. Plant Physiology 61, 38.

STERNBERG, D. (1976). β-glucosidase of Trichoderma: its biosynthesis and the role in the saccharification of cellulose. Applied and Environmental Microbiology 31, 648.

STERNBERG, D., VIJAYAKUMAR, P. & REESE, E.T. (1977). β-glucosidase; microbial production and effect on enzymatic hydrolysis of cellulose. Canadian Journal of Microbiology 23, 139.

STOLL, A., BRACK, A., KOBEL, H., HOFFMANN, A. & BRUNNER, R. (1954). Die Alkaloide eine Mutterkornpilzes von Pennisetum typhoideum, Rich und deren Bildung in Saprophytischer kultur. Helvetica Chimica Acta 37, 1815.

STOLL, A., BRACK, A., HOFFMANN, A. & KOBEL, H. (1957). Process for the preparation of ergotamine, ergotaminine and ergometrine by saprophytic culture of ergot (Claviceps purpurea (Fr.) Tul). in vitro and isolation of alkaloids thus produced. U.S. patent No.2809920.

STOSSEL, P. & HOHL, H.R. (1981). Effect of phytoalexins on hyphal growth and β-glucanases of Phytophthora infestans. Mycopathologia 73, 153.

STREIBLOVA, E., RYC, M. & KYBAL, J. (1978). Fine structure of imbibed sclerotial cells of Claviceps purpurea (Fr.) Tul. revealed by freeze etching. Zeitschrift fur Allgemeine Mikrobiologie 18, 123.

SUKUMARAN, C.P. & MAHADEVAN, P.R. (1975). Localization of enzymes in the cell walls of Neurospora crassa. Indian Journal of Experimental Biology 13, 127.

SWAN, J. (1980). Competitive interactions in the parasitism of cereals by Claviceps purpurea. Ph.D. thesis, University of London.

TABER, W.A. & VINING, L.C. (1958). The influence of certain factors in the in vitro production of ergot alkaloids by Claviceps purpurea Canadian Journal of Microbiology 4, 611.

TAKEUCHI, I. (1963). Immunochemical and immunohistochemical studies on the development of the cellular slime mould Dictyostelium mucoroides. Developmental Biology 8, 1.

TANIMOTO, E. & MASUDA, Y. (1968). Effect of auxin on cell wall degrading enzymes. Physiologia Plantarum 21, 820.

THOMAS, R. & BASSETT, R.A. (1972). The biosynthesis of ergot alkaloids. Progress in Phytochemistry 3, 47.

TKACZ, J.S. & LAMPEN , J.O. (1973). Surface distribution of invertase on growing Saccharomyces cells. Journal of Bacteriology 113, 1073.

TOKUYASU, K.T. (1973). A technique for ultracryotomy of cell suspensions and tissues. Journal of Cell Biology 57, 551.

TONOLO, A. (1966). Production of peptide alkaloids in submerged culture by a strain of Claviceps purpurea (Fr.) Tul. Nature (London) 209, 1134.

TONOMURA, K. (1964). Localization of cell bound α-amylase in Aspergillus oryzae demonstrated by fluorescent antibody technique. Journal of Bacteriology 87, 226.

TULASNE, L.R. (1853). Mémoire sur l'ergot des glumacées. Annales des Sciences Naturelles Botanique 20, 5.

UDENFRIENDS, S. (1964). In 'Fluorescence Assays in Biology and Medicine.' p.383. Academic Press, New York.

UREY, J.C. (1971). Enzyme patterns and protein synthesis during synchronous conidiation in Neurospora crassa. Developmental Biology 26, 17.

VAN DUIJN, C. (1954). Fluorescence microscopy - primary fluorescence. The Microscope 10, 1.

VILLA, T.G., NOTARIO, V. & VILLANUEVA, J.R. (1975). β-glucanases of the yeast Pichia polymorpha. Archives of Microbiology 104, 201.

VILLANUEVA, J.R., NOTARIO, V., SANTOS, T. & VILLA, T.G. (1976). β-glucanases in nature. In 'Microbial and Plant Protoplasts'. Eds. J.F. Peberdy, A.H. Rose, H.J. Rogers, & E.C. Cocking, pp 323- 355. Academic Press, London.

VORISEK, J. (1977). Electron cytochemical demonstration of acid phosphatase in saprophytic Claviceps purpurea. Archives of Microbiology 111, 289.

VORISEK, J. LUDVIK, J. & REHACEK, Z. (1974). Cytochemical localization of polysaccharides in Claviceps paspali ultrastructure during submerged fermentation of alkaloids. Journal of Bacteriology 118, 285.

VORISEK, J. & REHACEK, Z. (1978). Fine structural localization of alkaloid synthesis in the endoplasmic reticulum of submerged Claviceps purpurea. Archives of Microbiology 117, 279.

VORISEK, J. & LOJDA, Z. (1979). Electron microscopic localization of acetyl coenzyme A carboxylase in cells of Claviceps purpurea. Histochemistry 65, 59.

WANG, M.C. & BARTNICKI-GARCIA, S. (1966). Biosynthesis of β-1,3 and β-1,4 linked glucan by Phytophthora cinnamomi hyphal walls. Biochemistry and Biophysics Research Communications 24, 832.

WANG, M.C. & BARTNICKI-GARCIA, S. (1974). Mycolaminarins: storage (1→3)-β-D-glucans from the cytoplasm of the fungus Phytophthora palmivora. Carbohydrate Research 37, 331.

WANG, M.C. & BARTNICKI-GARCIA, S. (1980). Distribution of mycolaminarins and cell wall β-glucans in the life cycle of Phytophthora. Experimental Mycology 4, 269.

WARBURG, O. & CHRISTIAN, W. (1942). Isolierung und kristallisation des garungsferments enolase. Biochemische Zeitschrift 310, 384.

WARGO, P.M. (1975). Lysis of the cell wall of Armillaria mellea by enzymes from forest trees. Physiological Plant Pathology 5, 99.

WARNOCK, D.W. (1971). Assay of fungal mycelium in grains of barley, including the use of the fluorescent antibody technique for individual fungal species. Journal of General Microbiology 67, 197.

WARNOCK, D.W. (1973). Use of immunofluorescence to detect mycelium of Alternaria, Aspergillus and Penecillium in barley grains. Transactions of the British Mycological Society 61, 547.

WEBER, K., PRINGLE, J.R. & OSBORN, M. (1972). Measurement of molecular weights by electrophoresis on SDS acrylamide gels. Methods in Enzymology 26C, 3.

WEBSTER, D.H. & CURRIER, H.B. (1968). Heat induced callose and lateral movement of assimilates from phloem. Canadian Journal of Botany 46, 1215.

WEBSTER, J. (1970). Introduction to fungi. Cambridge University Press.

WESSELS, J.G.H. (1969). A β-1,6-glucan glucanohydrolase involved in hydrolysis of cell wall glucan in Schizophyllum commune. Biochimica et Biophysica Acta 178, 191.

WESSELS, J.G.H., KREGER, D.R., MARCHANT, R., REGENSBURG, B.A., & DE VRIES, O.M.H., (1972). Chemical and morphological characterization of the hyphal wall surface of the Basidiomycete Schizophyllum commune. Biochimica et Biophysica Acta 273, 346.

WHISTLER, R.L. & SMART, C.L. (1953). Polysaccharide chemistry. Academic Press, London.

WHISTLER, R.L. & RICHARDS, E.L. (1970). Hemicelluloses. In 'The Carbohydrates - Chemistry and Biochemistry'. 2nd edn. Eds. W. Pigman & D. Horton Vol. IIA, pp 447-469. Academic Press, London.

WHISTLER, R.L., BUSHWAY, A.A., SINGH, P.P., NAKAHARA, W., & TOKUZEN, R. (1976). Non-cytotoxic, anti-tumour polysaccharides. Advances in Carbohydrate Chemistry and Biochemistry 32, 235.

WHITE, D.G. (1976). Preparation and use of a fluorescent antibody reagent for the detection of Pythium graminicola. Phytopathology 66, 523.

WILLETTS, H.J. (1971). The survival of fungal sclerotia under adverse environmental conditions. Biological Reviews 46, 387.

WILSON, R.W. & NIEDERPRUEM, D.J. (1967). Control of β-glucosidases in Schizophyllum commune. Canadian Journal of Microbiology 13, 1009.

WONG, Y.S. & MACLACHLAN, G.A. (1980). 1,3-β-D-glucanases from Pisum sativum seedlings III. Development and distribution of endogenous substrate. Plant Physiology 65, 222.

WOODWARD, J. & WISEMAN, A. (1976). Folding pathway selection or cross-linking as the cause of thermal stabilization of yeast invertase conformation by its mannan. Biochemical Society Transactions 4, 80.

YAMAMOTO, R. & NEVINS, D.J. (1981). Coleoptile growth inducing capabilities of exo -β-(1,3)- glucanases from fungi. Physiologia Plantarum 51, 118.

YOSHIKAWA, M., YAMAGUCHI, K. & MASAGO, H. (1978). Glyceollin: its role in restricting fungal growth in resistant soybean hypocotyls infected with Phytophthora megasperma var. sojae. Physiological Plant Pathology 12, 73.

ZEE, S.Y. & O'BRIEN, T.P. (1970). A special type of tracheary element associated with xylem discontinuity in the floral axis of wheat. Australian Journal of Biological Science 23, 783.

ZEE, S.Y. & O'BRIEN, T.P. (1971). Vascular transfer cells in the wheat spikelet. Australian Journal of Biological Science 24, 35.

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Observations on the location of a β-glucanase and an associated β-glucosidase in *Claviceps purpurea* during its development on rye

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The location of two fungal enzymes, a β-glucanase and a β-glucosidase, was studied during the growth and differentiation of *Claviceps purpurea* on rye. The enzymes, purified from axenic cultures of the fungus where they are produced extracellularly, were used as antigens. The antibodies to them, attached to fluorescent markers, permitted the identification of the enzymes, or very closely related enzymes, in tissue sections cut longitudinally from ergots at various stages of development. Fluorescence was largely absent in the early stage of infection but, after 10 days, both enzymes could be identified, to varying degrees, in areas of the fungus where active growth occurs; particularly, at sporulating hyphal tips in the sphacelium and at the host-parasite interface. As the ergot matured, fluorescence, for both enzymes, persisted only at the interface and in the residual sphacelium that had been displaced acropetally. The role of the enzymes in fungal wall growth and in the possible dissolution of plant saccharides is discussed.

INTRODUCTION

Both β-1,3 and β-1,6-D-glucanases are widespread in fungi where they have, in some cases, been found bound tightly to both cell walls and extracellular glucans and are responsible for the hydrolysis of the matrices to which they are bound; in the case of cell walls this lytic or "plasticizing" action is considered to be linked to hyphal growth [*14*, *17*, *40*].

With respect to the parasitism of cereals by *Claviceps purpurea* (Fr.) Tul., it has been proposed that β-glucanases have a role in establishing the sphacelial mycelium of the fungus and sustaining the subsequent development of its sclerotium by ensuring an abundant supply of nutrients—particularly a carbon supply in the form of sucrose which can be mobilized by a β-fructofuranosidase produced by the fungus [*11*, *12*, *13*, *25*, *34*]. Such a rôle for glucanases could require the dissolution of callose [*13*]. Callose is a β-D-1,3-linked glucan with a conformation that is probably a triple helix [*30*]; it is deposited widely in plants, especially in response to wounding. Deposition of callose between host plasmalemma and cell wall, in structures known as papillae, has been observed in response to infection of plants by fungi and viruses [*1*, *33*]. Dickerson *et al.* [*13*] have produced evidence that the absence of callose at the interface between the parasite, *C. purpurea*, and its host cereal and in host sieve tubes is consistent with the action of a fungal β-1,3-D-glucanase, and an associated β-glucosidase present in the infected region. Further investigation has sought to establish more precisely the location of these fungal enzymes in order to determine

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whether or not their siting is such that they could play the effective rôle that has been proposed for them in the nutrition and development of the parasite, *C. purpurea*.

METHODS

Organisms

C. purpurea strain 6, originally derived from ergots isolated from cocksfoot grass [*Dactylis glomerata* (L.)] [*38*], was cultured from a conidial suspension stored in 10% (v/v) glycerol in sealed ampoules under liquid nitrogen. Svalofs Fourex Rye (Swedish Seed Association, Svalof, Sweden) was grown in outside plots at Chelsea Physic Garden.

Media and culture conditions

Modified Medium T, an amino-nitrogen medium modified after Stoll *et al.* [*24*, *36*], was used for the saprophytic growth of *C. purpurea* in culture and contained the following constituents per litre of distilled water: Sucrose, 100 g; L-asparagine, 10 g; KH_2PO_4, 0·25 g; $MgSO_4 . 7H_2O$, 0·25 g; KCl, 0·125 g; $FeSO_4 . 7H_2O$, 0·033 g; $ZnSO_4 . 7H_2O$, 0·027 g. The pH was adjusted to 5·2 with 2·5 M NaOH. The liquid medium (100 ml) was dispensed into 500 ml Erlenmeyer flasks closed with styrofoam bungs, prior to autoclaving for 30 min at 5 p.s.i. For solid media, 1·5% (w/v) Oxoid agar number 3 was added before autoclaving. A 0·25% (w/v) solution of tetracycline hydrochloride, sterilized by passage through a Millipore filter (0·45 μm), was added to media as a bacteriostatic agent as plates were being poured to give a final concentration of 50 μg antibiotic ml^{-1} media.

Mycelial cultures were prepared from conidial suspensions on modified medium T agar plates containing antibiotic. Uncontaminated mycelium was subcultured on to modified medium T agar slopes from which, after 14 days incubation at 24 °C, a spore suspension of 5×10^7 spores ml^{-1} in sterile distilled water was prepared. Flasks containing 100 ml modified medium T were inoculated with 1 ml spore suspension per flask and incubated at 24 °C on a rotary shaker (240 r min^{-1}) for 13 days.

Enzyme assays

The activity of exo-β-1,3-glucanase was assayed by following the production of glucose from the hydrolysis of a fungal β-1,3-D-glucan [*10*]. Enzyme samples, 10 μg to 1 mg, and substrate at a concentration of 0·6% (w/v) were incubated in 0·1 M sodium acetate buffer, pH 5·0, at 30 °C in a volume of 1 ml. Samples, taken at intervals, were heated to 100 °C for 3 min in a boiling water bath to stop the reaction. Glucose was determined as described below. Hydolysis by this glucanase yields glucose and gentiobiose in a molar ratio of approximately 2 : 1. In assaying the β-glucanase activity of the crude enzyme extracts (see below), "contaminating" β-glucosidase hydrolysed completely the gentiobiose produced by the action of the β-glucanase on the β-glucan. This effectively doubled the yield of glucose and hence, apparently, the rate of hydrolysis by the β-glucanase. The true activity of the β-glucanase was obtained as described previously [*10*] by adjusting, in this case halving, the rate of glucose production.

The activity of β-glucosidase was assayed using *p*-nitrophenol-β-D-glucopyranoside (Koch-Light) as substrate at a concentration of 1 mM. The reaction was carried out in 0·1 M sodium acetate buffer, pH 5·0, at 30 °C in a volume of 1 ml with 2 μg ml^{-1} to 1 mg ml^{-1} of enzyme depending on purity. Samples were taken at intervals and *p*-nitrophenol determined as described below.

Assays of the glucosidase against "natural" disaccharide substrates were carried out under the same conditions. Samples taken at intervals were heated to 100 °C for 3 min to stop the reaction and glucose in the sample was determined as described below.

Glucose determination

Glucose was determined using the glucose oxidase method of Fleming & Pegler [*18*]. The method was adapted by the authors for the use of ABTS (2,2′-azino-di-[3-ethyl-benzthiazoline-sulphonate(6)], Boehringer), a chromogen replacing *O*-dianisidine. The accuracy of Fleming & Pegler's method for determining glucose in the presence of maltose was maintained. The commercial glucose oxidase had no detectable activity on the disaccharides, referred to above, within the time limit of the assay below. Glucose oxidase reagent, 2 ml, containing glucose oxidase, 6 mg; peroxidase, 1 mg; ABTS, 10 mg; per 40 ml 0·5 M Tris–HCl buffer, pH 7·0, was added to a 1 ml volume containing the glucose sample and the mixture was incubated at room temperature for 15 min. A green colour was produced in the presence of glucose which was stable for a further 25 min. The absorbance was read at 420 nm, and the concentration of glucose calculated from a standard curve of 0 to 50 μg ml^{-1} glucose in distilled water.

p-*Nitrophenol determination*

Addition of 2 ml 0·1 M NaOH to a reaction sample contained in 1 ml distilled water was used to stop the reaction and develop the yellow nitrophenate ion. The absorbance was determined at 410 nm, and the concentration determined from a standard curve of 0 to 25 μg ml^{-1} NaOH treated nitrophenol.

Protein concentrations

Protein in solution was estimated by a spectrophotometric method based on that of Warburg & Christian [*41*]. The concentration was calculated from the extinction coefficients at 280 and 260 nm using the method of Layne [*22*].

Gel electrophoresis

This was carried out according to the method of Weber, Pringle & Osborn [*43*]. Polyacrylamide (8%, w/v) gels with 1% cross-linking were prepared. Protein samples (5 to 50 μg) were denatured in a solution (60 μl) containing 1%(w/v), sodium dodecyl sulphate (SDS) and 1% (v/v) β-mercaptoethanol at 100 °C for 3 min. Sorbitol, 50% (w/v) saturated with bromophenol blue (30 μl), was added to the sample prior to application to the gel. The gels were run anodically at a current of 6 mA per gel for 4 h at room temperature. Gels were stained with Coomassie blue by the method of Chrambach *et al.* [*8*].

Isolation of protein from culture medium after growth of C. purpurea *strain 6*

Mycelium was harvested after 13 days culture by filtration under vacuum, and the culture medium centrifuged at 4 °C for 45 min at 15 000 ***g*** to remove spores and cell

debris. Subsequent steps were all carried out at 4 °C. The resultant clear yellow liquid (2·5 l) was dialysed against 2 changes of 10 l 1 mM sodium acetate buffer, pH 5·2, containing 0·1 mM EDTA for 48 h. The non-dialysable residue was freeze-dried, immediately resuspended in 80 ml 20 mM sodium acetate buffer, pH 5·2, and further dialysed against 2 changes of 10 l 1 mM sodium acetate buffer, pH 5·2 containing 0·1 mM EDTA for 72 h.

The residue was centrifuged for 45 min at 26 000 ***g*** to remove debris, minor lipid deposits were removed with lead-free glass wool and the clear supernatant was freeze-dried. The protein concentration and enzyme activity of the resultant crude extract were determined; the protein yield was 0·22 g protein l^{-1} culture filtrate. The extract was stored in a vacuum desiccator over anhydrous $CaCl_2$ at −20 °C.

Purification of exo-β-1,3-glucanase

Crude extract (175 mg), suspended in 0·1 M sodium acetate buffer, pH 5·0 (3 mg protein ml^{-1}), was applied to a column of Sulphopropyl Sephadex C-50 (Pharmacia), dimensions 18·5 × 2 cm, equilibrated according to the manufacturer's recommendations at 4 °C. Elution was by 2 successive linear gradients [*4*] of 0 to 0·15 M NaCl in 8 column volumes of buffer followed by 0·15 to 0·5 M NaCl in 5 column volumes of buffer both at a flow rate of 20 ml h^{-1}. Fractions, 180 × 5 ml, were assayed for protein content, β-glucanase and β-glucosidase activity. The activity of β-glucanase eluted as a single peak between 0·04 and 0·07 M on the salt gradient, and was separated from the β-glucosidase which eluted between 0·08 and 0·25 M. Active fractions were bulked, reduced by ultrafiltration (Amicon 52, 10 000 mol. wt exclusion limit filter, 30 p.s.i. at 4 °C), dialysed against 2 changes of 1 mM sodium acetate buffer, pH 5·0, containing 0·1 mM EDTA and freeze dried.

Samples (*c.* 5 mg) of β-glucanase from above were applied in 0·75 ml 0·1 M sodium acetate buffer, pH 5·2, containing 0·5 M NaCl to the base of a column 85 × 1 cm of Sephacryl S-200 Superfine (Pharmacia) equilibrated in the same buffer and eluted by ascending flow at a rate of 3 ml h^{-1}. Fractions, 80 × 1 ml, were assayed for protein content and enzyme activity. The activity of β-glucanase eluted as a single peak, free from β-glucosidase activity. The elution volume corresponding to the position of the peak (fractions 41 to 48) indicated a molecular weight of approximately 100 000 for this enzyme. Active fractions were bulked, reduced by ultrafiltration and stored at 4 °C in the presence of 0·02% (w/v) NaN_3.

Purification of β-glucosidase

Freeze dried protein samples (*c.* 10 mg) from the Sulphopropyl Sephadex column described above containing β-glucosidase activity were applied to the Sephacryl column and eluted as described above. Fractions, 80 × 1 ml, were assayed for protein content and β-glucosidase activity. The enzyme eluted at a position (fractions 26 to 33) which indicated an approximate molecular weight of 150 000. Active fractions were bulked and reduced in volume by ultrafiltration as described previously.

Further purification of β-glucosidase was obtained using a column of hydroxyapatite, dimensions 4 × 1·2 cm and volume 5 ml, equilibrated in 10 mM sodium/potassium phosphate buffer, pH 6·8. Samples (*c.* 5 mg), containing β-glucosidase activity, from the above column were dialysed against the same buffer for 24 h and

applied (in approximately 1 ml) directly to the top of the column followed by a wash of 10 ml buffer. Washes at concentrations of 0·25 and 0·5 M sodium/potassium phosphate buffer, pH 6·8, and 0·8 M sodium/potassium phosphate buffer, pH 7·5, each in 3 column volumes were applied successively to the column. Fractions, 1 ml, were collected, their protein content determined, and then assayed for β-glucosidase activity. The activity of β-glucosidase was eluted as a single peak by the 0·8 M buffer wash, and active fractions were bulked, reduced by ultrafiltration as described above and stored at 4 °C in the presence of 0·02% (w/v) NaN_3.

Preparation of antisera

Antisera were prepared in Old English rabbits and, prior to immunization, a sample of blood was taken as control, non-immune serum. Purified enzyme, 75 μg, was suspended in 0·5 ml phosphate buffered saline (PBS) (0·03 M sodium phosphate buffer, pH 7·2, containing 0·85% w/v NaCl) and emulsified with 0·5 ml Freund's complete adjuvant. Of this emulsion 0·25 ml was inoculated intramuscularly into each of 2 sites in the rabbit's leg, and 0·15 ml inoculated subcutaneously at each of 3 sites. After 14 days this inoculation procedure was repeated. A final inoculation using 75 μg purified enzyme suspended in 0·5 ml PBS and emulsified with 0·5 ml Freund's incomplete adjuvant was carried out after a further 14 days, again at 2 intramuscular and 3 subcutaneous sites.

The rabbit was bled from the ear 12 days after the final injection; 30 ml of blood was collected and clotted by incubation at 37 °C for 2 h and stored at 4 °C overnight. Clotted blood cells were removed by centrifugation at 25 000 *g* for 45 min at 4 °C. The remaining clear serum was divided into 2 ml aliquots, frozen rapidly over a mixture of acetone and solid CO_2 and stored at −20 °C.

Purification of immunoglobulin G (IgG) fraction from serum

A modification of the method of Brandtzaeg was followed [*6*]. A 5 ml column, 4 × 1·2 cm, of DEAE-cellulose was prepared and equilibrated with 10 mM potassium phosphate buffer, pH 7·8, at 4 °C. Frozen serum (2 ml) was thawed slowly, dialysed against 3 changes of running buffer for 36 h at 4 °C, and applied directly to the top of the column. The column was eluted with 2 column volumes of running buffer, followed by the same volume of buffer containing 50 mM NaCl. Fractions, 1 ml, were collected and the protein content determined as above. The 2 elution steps produced 2 separate protein containing fractions, both of which were substantially pure IgG. The samples comprising the fractions were bulked separately, the concentration of NaCl in each made up to 0·15 M (0·85%, w/v), and NaN_3 added to a concentration of 0·01% (w/v) before storage at 4 °C.

Immunodiffusion plates

A solution of 0·5% (w/v) Ionagar was prepared in PBS, poured on to glass microscope slides and allowed to set. Wells, 2·5 mm in diameter, were cut in a hexagonal array using a template and cutter, and sealed with 0·1% Ionagar in PBS. Serial doubling dilutions of 1 : 1 to 1 : 64 of antiserum and enzyme were prepared in PBS. Antiserum dilutions (10 μl) were pipetted into the outer circle of the wells, and an enzyme dilution (10 μl containing 1·5 μg of enzyme) pipetted into the centre well.

The slides were placed in a sealed humid atmosphere in the dark and developed for 20 h. They were then washed in 3 changes of PBS for 24 h, stained with Coomassie blue for 30 min and destained.

Preparation of infected plant material

Ears of rye, spring sown, were inoculated just prior to anthesis by injecting a suspension of *C. purpurea* conidia (10^5 spores ml^{-1}) into the prefloral cavity with a small hypodermic syringe, allowing the conidia direct ascess to the tip of the stigma and ovary. At intervals after infection (4, 7, 10, 12, 14, 18, 21, 28 and 35 days) both infected and uninfected florets were removed from the ears, and the glumes and palea were carefully dissected away from the floret. The remaining infected ovary, or developing ergot, attached to the rachilla was then "snapfrozen" by plunging into freon cooled to −180 °C over liquid nitrogen. Frozen tissue samples were stored at −70 °C.

Tissue sectioning

Owing to the differing structure and hydration of plant and fungal tissue, it proved difficult to maintain the original structure during cryostat sectioning of infected plant tissues. The method described below was developed after initial experiments varying the temperature of sectioning and the orientation of the tissue. The developing ergot in the floret is approximately cylindrical and can be considered more or less symmetrical about its central axis. Therefore, longitudinal sections through the ergot and rachilla of the plant tissue were used to obtain information about the location of enzymes within the tissue.

Frozen florets were mounted horizontally on a cryotome chuck on top of a drop of water which was just freezing. A minimal quantity of Bright Cryoembedding liquid (50 to 100 µl) was frozen around the underside of the floret to support it. The material was sectioned longitudinally in a cryostatic chamber (Bright Instrument Company, model OTF/AS/M/V) using a steel microtome knife. The temperature of both chamber and knife were maintained at −25 °C. Two consecutive ribbons of 3 to 5 sections, 10 µm thick, were transferred to each end of a microscope slide at room temperature and air dried.

Preparation of antibody stained sections

The sectioned plant tissues demonstrated bright yellow autofluorescence, when illuminated across a broad spectrum from 280 to 500 nm, that masked any fluorescence due to fluorescein (see below).

Methylene blue and toluidine blue in borate buffer stain plant and ergot tissues effectively [*31*]. Counterstaining after antibody treatment with these dyes in borate buffer at relatively low pH (see below), to prevent dissociation of antibody antigen complexes, completely quenched the plant tissue autofluorescence. The antibody staining protocol employed was therefore a modification of the standard sandwich technique [*19*, *26*]. All steps were carried out at 25 °C.

(i) Sections dried on to slides were fixed in 90% (v/v) ethanol for 30 min (acetone was not employed for fixation as it removed sections from slides).

(ii) Slides were placed flat in a humid chamber, and one ribbon of sections was covered with a few drops of a 1 : 4 dilution of purified enzyme antiserum in PBS (equivalent to a 1 : 20 dilution of original antiserum). The other ribbon on the slide was covered with the same dilution of non-immune control serum and the slides were incubated for 45 min.

(iii) Slides were washed in 4 changes of PBS for 20 min, rinsed in distilled water, air dried and returned to the humid chamber. Both ribbons were covered with a 1 : 40 dilution (manufacturer's recommendations) of fluorescein isothiocyanate (FITC) conjugated IgG fraction of goat anti-rabbit IgG (Miles) in PBS, and incubated for a further 45 min.

(iv) Slides were again washed in 4 changes of PBS for 20 min, rinsed in distilled water and air dried.

(v) The sections were counterstained with 0·03% (w/v) methylene blue in 0·1 M sodium borate buffer, pH 8·0, for 3 min, washed in distilled water for 5 min, air dried and mounted in 25 µl 90% (v/v) glycerol in PBS.

Microscopy

Specimens were studied using epi-illumination from a HBO 50 W mercury vapour lamp in a Zeiss WL microscope. A band-pass filter (450 to 490 nm) and 510 nm interference mirror gave a narrow band width of illumination from 450 to 500 nm. A 520 nm barrier filter in conjunction with large aperture objectives, was used for viewing. Photomicrographs were taken with a Zeiss MC 63 camera attachment using Ektachrome high speed daylight professional reversal film (DIN 24), uprated one stop to DIN 25 for exposure.

RESULTS

Enzyme purification

The β-glucanase and β-glucosidase were purified from extracellular extracts as described in Methods (Table 1). Purification was monitored by denaturing SDS polyacrylamide gel electrophoresis. Active fractions from the Sephacryl column for

TABLE 1

Purification of exo-β-1,3-glucanase and β-glucosidase[a]

	Protein (mg)	Specific activity	Total activity	Fold purification	Yield (%)
	(a) exo β-1-3-glucanase				
Crude extract	175	4·7[b]	823	1	100
Sulphopropyl Sephadex C-50	15·3	43	658	9·1	80
Sephacryl S-200	2·8	209	585	44·5	71
	(b) β-glucosidase				
Crude extract	175	0·21[c]	36·8	1	100
Sulphopropyl Sephadex C-50	55·8	0·48	26·8	2·3	73
Sephacryl S-200	23·5	0·89	20·9	4·2	56
Hydroxyapatite	2·6	5·99	15·6	28·5	42

[a] For experimental procedures, see Materials and Methods.
[b] µg glucose liberated min^{-1} mg^{-1} protein.
[c] µmol *p*-nitrophenol liberated min^{-1} mg^{-1} protein.

β-glucanase and from hydroxyapatite for β-glucosidase demonstrated single protein bands when applied to polyacrylamide gels.

The purified β-glucanase had a specific activity of 209 μg glucose liberated min^{-1} mg^{-1} protein against the β-1,3-D-glucan substrate and the same specificity as observed previously [*10*]. The purified β-glucosidase demonstrated activity against various di- and trisaccharide substrates having β-D-1,6, β-D-1,4 and β-D-1,3-glucosidic linkages (Table 2). With *p*-nitrophenol-β-D-glucopyranoside as substrate the purified β-glucosidase had a K_m of $2{\cdot}4 \times 10^{-4}$ M and a V_{max} of 8·3 μmol *p*-nitrophenol liberated min^{-1} mg^{-1} protein.

TABLE 2

Substrate specificity of β-glucosidase[a]

Substrate	Linkage	Specific activity[b]
Laminaribiose	β-1,3	5·5
Cellobiose	β-1,4	2·9
Gentiobiose	β-1,6	7·3
p-Nitrophenol-β-D-glucopyranoside	β-1,6	2·7
Maltose	α-1,4	0
Sucrose	α1,β2	0
Laminaritriose	β-1,3	0·7
β-Glucan	β-1,3 & β-1,6	0

[a] Substrates (1 mM) in 0·1 M sodium acetate buffer, pH 5·0, were incubated at 30 °C with 2 μg ml^{-1} of enzyme; in the case of the β-glucan 0·6% (w/v) was used with 10 μg ml^{-1} of enzyme.
Samples were taken at intervals during the period when the reaction was first order and assayed either for glucose, by the glucose oxidase method or for *p*-nitrophenol.

[b] μmol substrate hydrolysed min^{-1} mg^{-1} protein.

Specificity of antisera

Immunodiffusion plates showed visible precipitin lines after staining with Coomassie blue at dilutions down to 1 : 32 of the original antiserum. No reaction was observed with control non-immune serum. No cross reaction was observed between β-glucanase and undiluted anti-β-glucosidase serum or β-glucosidase and undiluted anti-β-glucanase serum. The diffusion plates also confirmed the strong binding of the commercially prepared fluorescein conjugated IgG fraction of goat anti-rabbit IgG to the experimentally prepared antisera.

Preparation of antibody stained sections

The development of *C. purpurea* within the host floret was studied over a period of 28 days. The approximate areas of the sections taken during this period, illustrated in the plates, are shown in Fig. 1.

During the first 12 to 14 days of infection, the ovary tissue was degraded and was replaced by the fungal sphacelium. The early stage of this colonization was not accompanied by any particular localization of fluorescence attributable to the presence of β-glucanase; however, a general fluorescence was present which persisted throughout the sphacelial stage (Plate 1). This effect was also observed with the

Abbreviations used in plates and figures: I, host/parasite interface; Sp, sphacelial tissue; Sc, sclerotial tissue; P, palea; L, lemma; R, rachilla; O, base of ovary; Ov, ovule; H, host plant cell; C, conidia; PC, pocket of conidiation; PL, purple pigmented layer; AF, area of fluorescence; V, vascular elements from rachilla.

PLATE 1. Overall fluorescence due to β-glucanase in young sphacelial tissue at 7 days. Scale bar is 100 μm.

PLATE 2. Fluorescence due to β-glucanase at the interface of sphacelial and plant tissue at 10 days. Scale bar is 100 μm.

PLATE 3. The interface of sphacelial and host tissue at 14 days, showing fluorescence due to β-glucanase. Scale bar is 100 μm.

PLATE 4. Enlarged section of Plate 3, showing fluorescence due to β-glucanase closely associated with the host/parasite interface. Scale bar is 50 μm.

PLATE 5. Invaginated pocket of conidiation of sphacelial tissue at 10 days showing fluorescence due to β-glucanase around the convoluted periphery. Scale bar is 100 μm.

PLATE 6. Fluorescence due to β-glucanase around an invaginated pocket of conidiation in the sphacelium at 14 days. Scale bar is 50 μm.

PLATE 7. Fluorescence due to β-glucosidase at the periphery of sphacelial tissue at 14 days. Scale bar is 100 μm.

PLATE 8. Apex of developing sclerotium with fluorescence due to β-glucanase present in the cap of residual sphacelial tissue at 18 days. Scale bar is 100 μm.

PLATE 9. Diminishing sphacelial tissue with fluorescence due to β-glucanase separated from the apex of the developing sclerotium by a layer of purple pigment. 21 days. Scale bar is 100 μm.

PLATE 10. Host/parasite interface at 18 days, showing fluorescence due to β-glucanase. Scale bar is 100 μm.

PLATE 11. Fluorescence due to β-glucanase at the interface at 21 days. Scale bar 100 μm.

PLATE 12. Control section showing the host/parasite interface at 21 days, stained with non-immune control serum. Scale bar is 100 μm.

PLATE 13. Fluorescence due to β-glucosidase at the interface at 18 days. Scale bar is 100 μm.

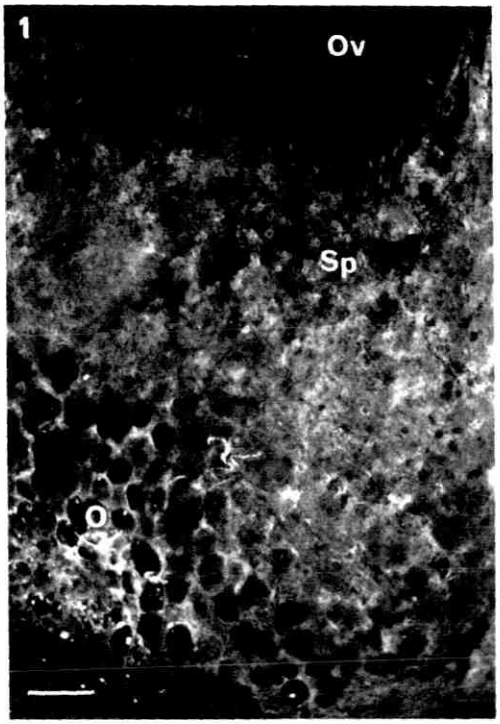
PLATE 14. Host/parasite interface at 21 days demonstrating fluorescence due to β-glucosidase. Scale bar is 100 μm.

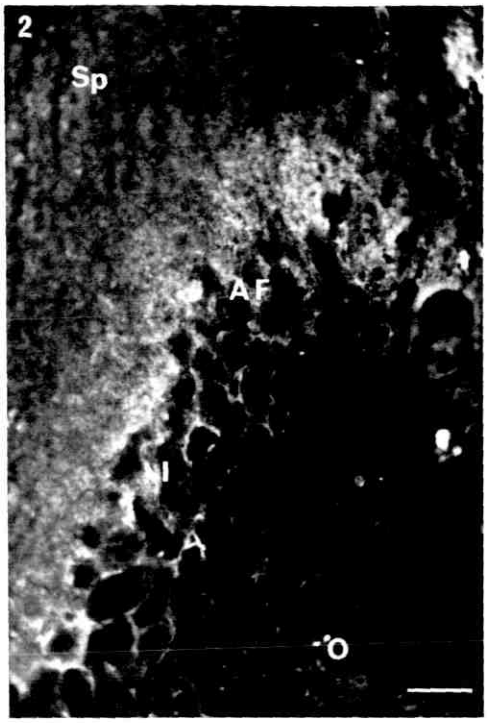
PLATE 15. Apex of maturing sclerotium at 28 days with cap of residual sphacelial tissue showing fluorescence due to β-glucanase. Scale bar is 100 μm.

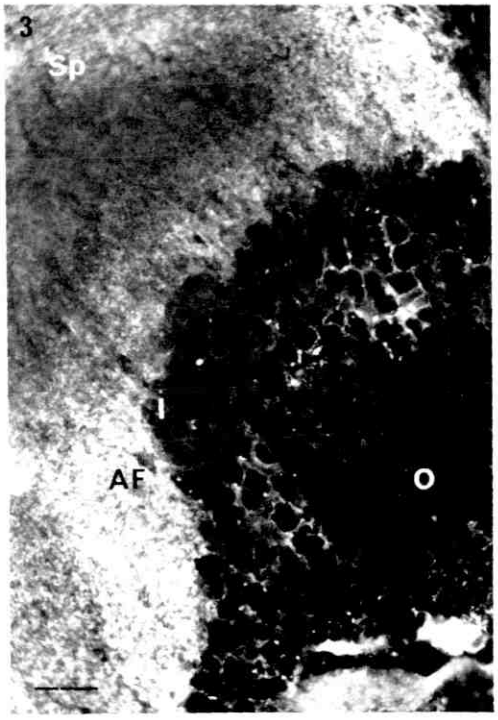
PLATE 16. Host/parasite interface of maturing ergot at 28 days showing fluorescence due to β-glucanase. Scale bar is 100 μm.

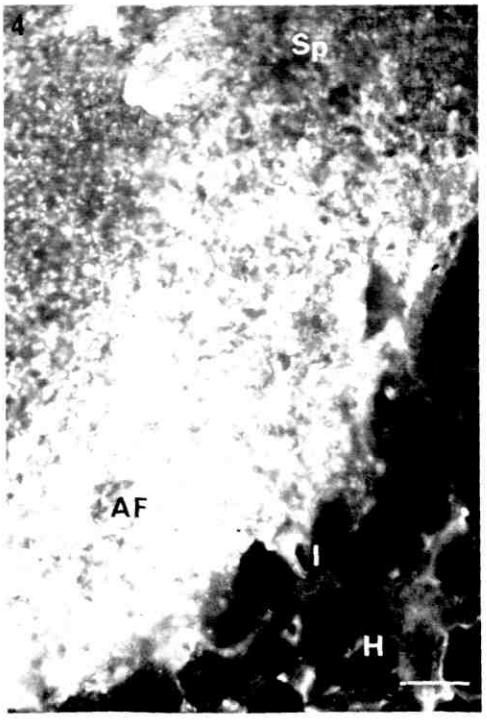
PLATE 17. Band of fluorescence due to β-glucosidase at the interface at 28 days. Scale bar is 100 μm.

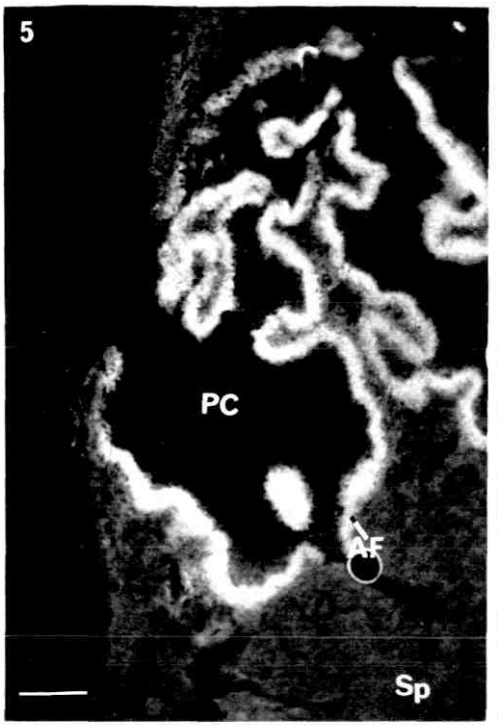
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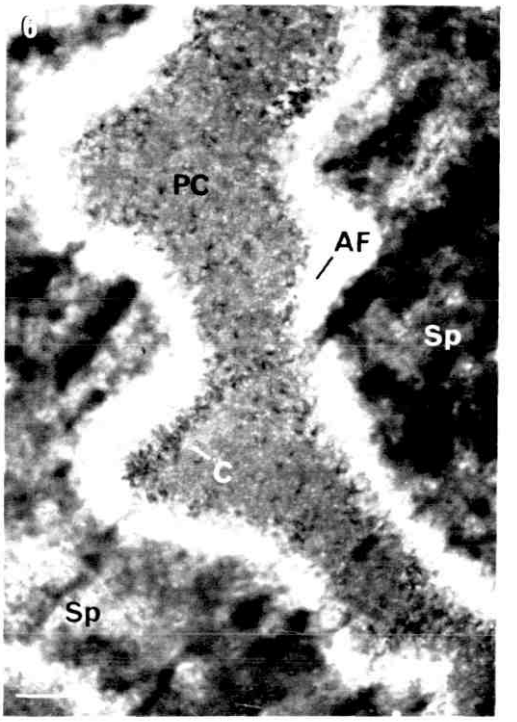

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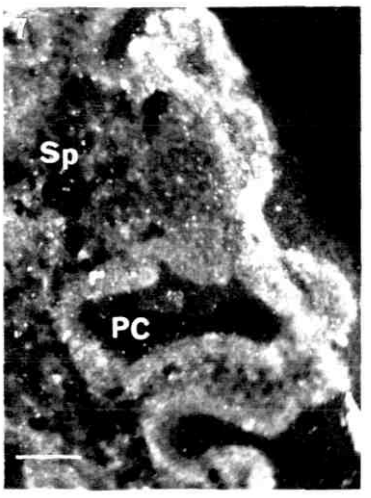

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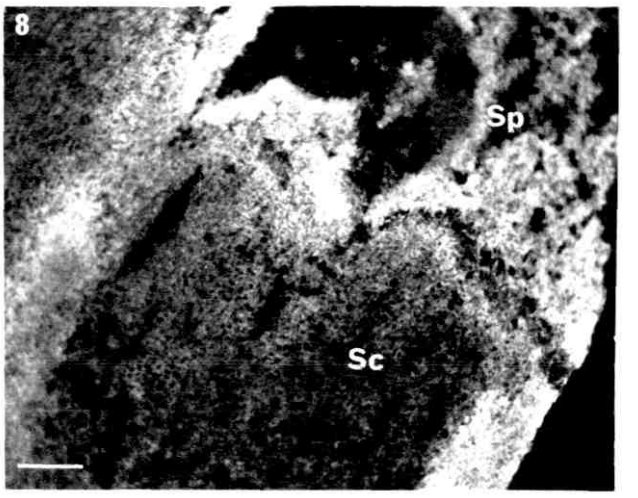

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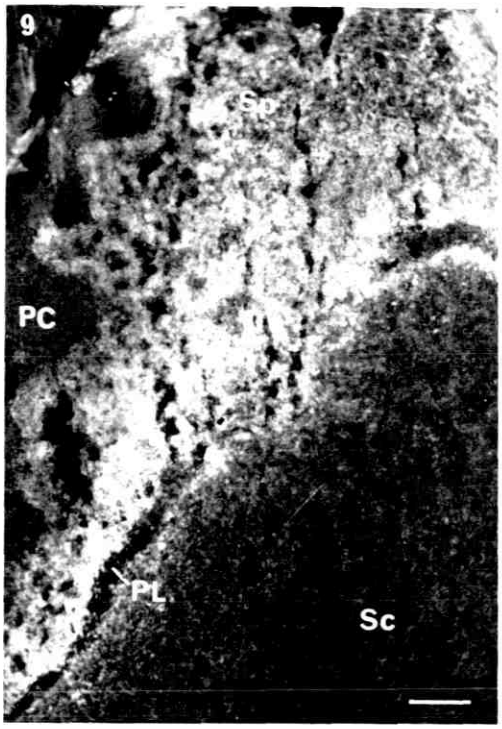

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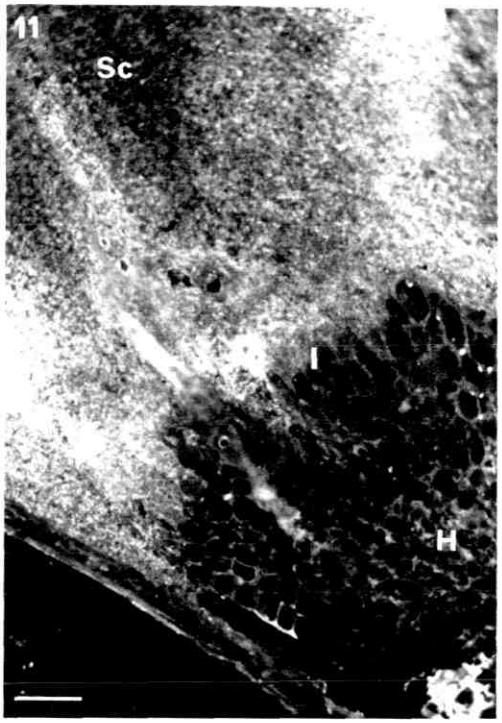

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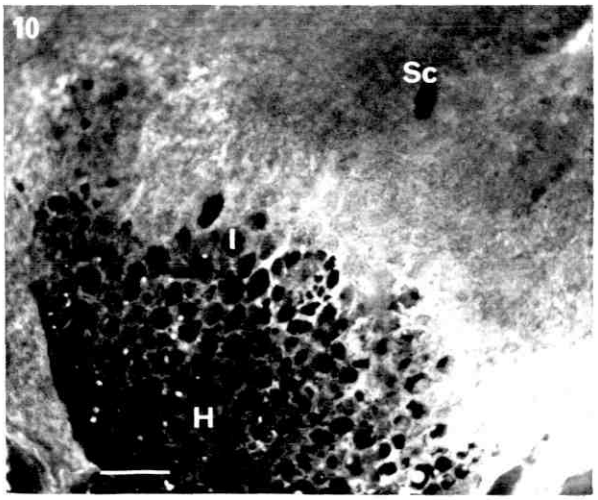

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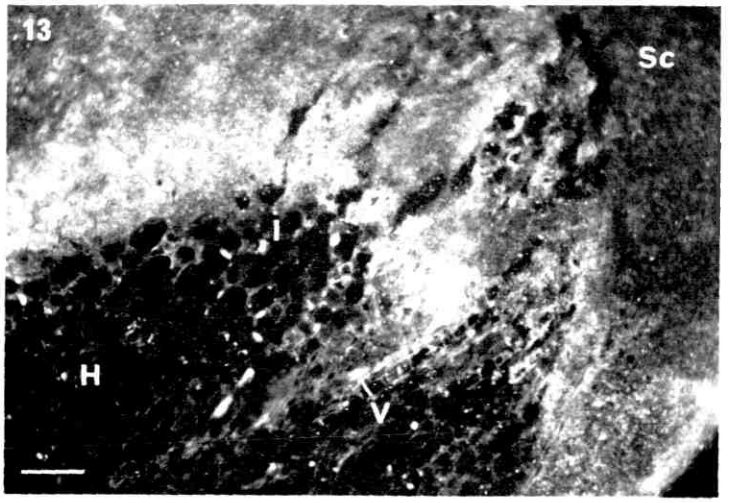

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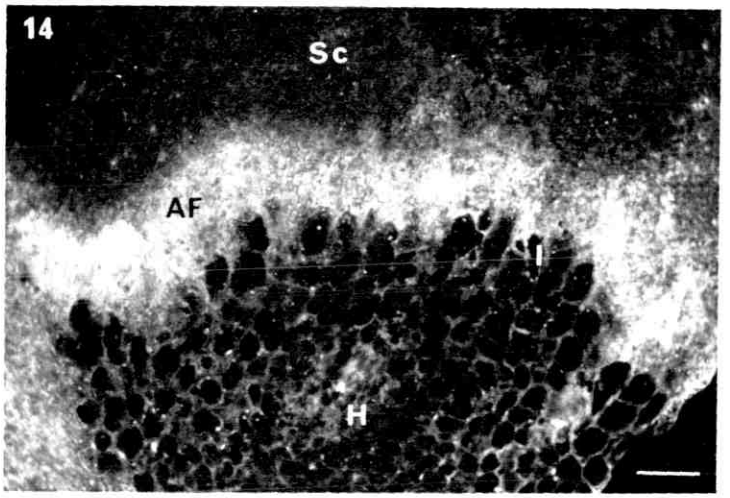

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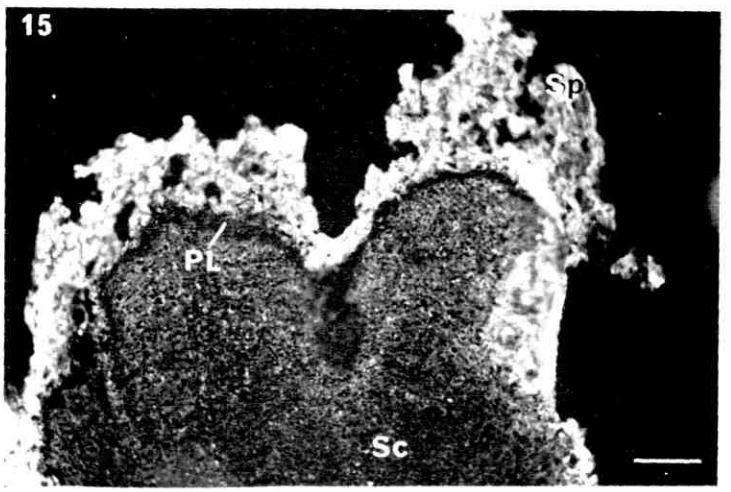

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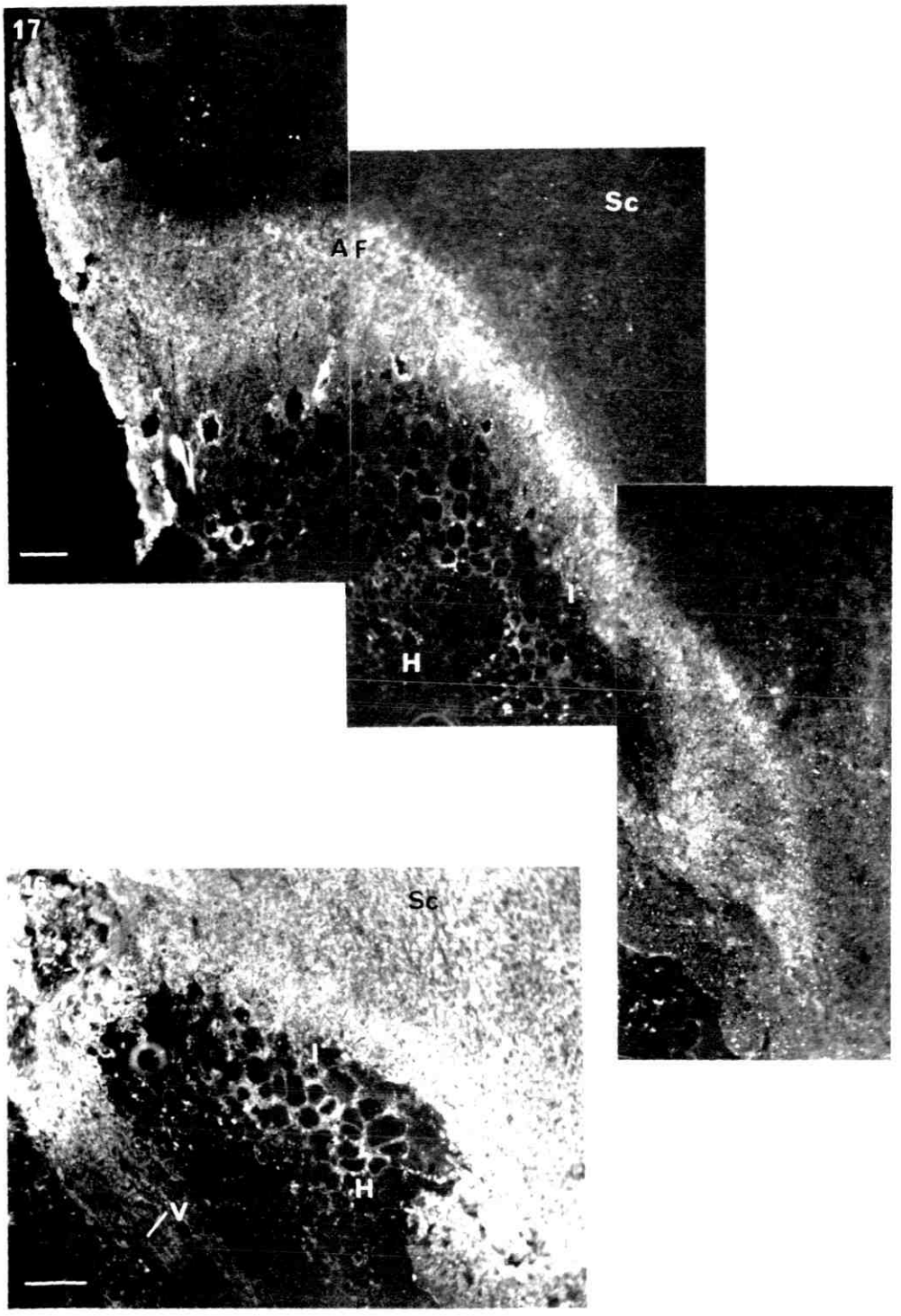

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β-glucosidase. At 10 days after infection, a faint band of fluorescence across the base of the sphacelial tissue was visible (Plate 2), indicating the presence of β-glucanase at the interface of host and parasite. This band of fluorescence was more pronounced by 14 days and was brighter around the periphery of the interface than at its centre (Plates 3 and 4). In addition to the fluorescence at the host/parasite interface, very bright fluorescence was observed at 10 days after infection around peripheral areas,

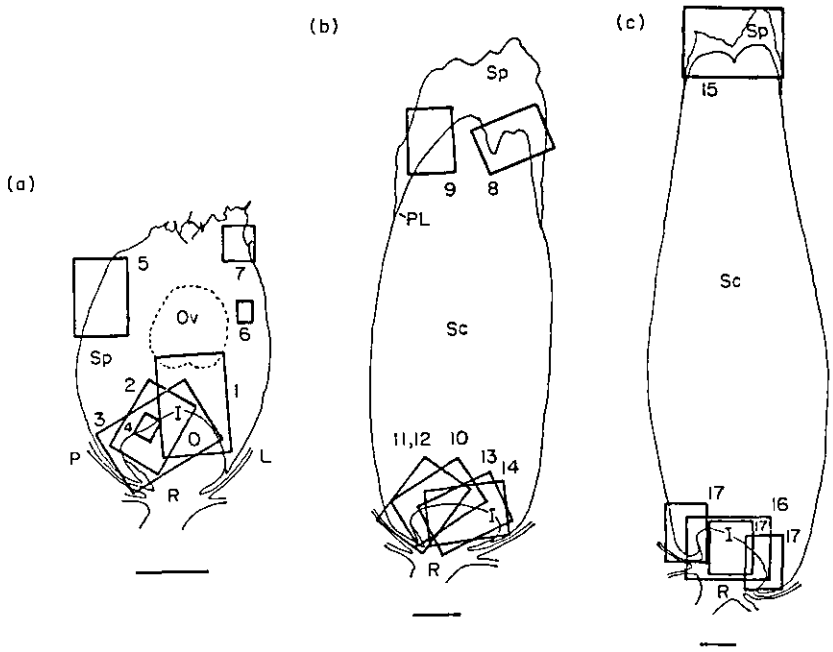


FIG. 1. Diagrams representing sections of developing and maturing ergots showing areas of sections presented in plates. (a) Sections from 7 to 14 days shown in Plates 1 to 7; (b) Sections from 18 to 21 days shown in plates 8 to 14; (c) Sections from 28 days shown in plates 15 to 17. For abbreviations see legend for plates. Bar represents 1 mm.

both superficial and invaginated, of the developing sphacelium. This appears as a series of highly convoluted bands in the sections (Plates 5 and 6). This part of the sphacelium corresponds to the area where sporulation is occurring; a pocket of spores connecting with the outer surface of the sphacelium and divided from the sphacelial tissue by the band of bright fluorescence can be seen in Plate 5. Fluorescence at the boundaries of sporulating tissue was observed with both antisera, but that for β-glucanase was more intense (cf. Plates 6 and 7).

During the period from 14 to 21 days after infection, sporulation ceased and differentiation to form sclerotial tissue took place. This was accompanied by the acropetal displacement of sphacelial tissue which could be found at the top of the developing sclerotium (Plates 8 and 9). Differentiated sclerotial tissue demonstrated a lower overall fluorescence than sphacelial tissue when stained with either β-glucanase or β-glucosidase antiserum (Plate 8). Furthermore, a distinct border was detected 21 days after infection between the dark barely fluorescent sclerotial tissue and the

remaining brightly fluorescent sphacelial tissue (Plate 9). This border corresponded exactly to the layer of purple pigmented tissue which covers the outer surface of the mature sclerotium.

By 21 days, the fluorescence that had been associated with the periphery of the sphacelial tissue was greatly diminished (Plate 9). However, the band of fluorescence at the interface did not diminish, indicating the continued presence of β-glucanase (Plate 10) which by this stage had become more diffuse across the base of the ergot (Plate 11) although still distinct when compared with sections treated with non-immune control serum (Plate 12). Similarly, a band of fluorescence across the interface was visible from 10 days after infection onwards in sections stained with β-glucosidase antiserum. Moreover, during differentiation, this band of fluorescence became very much brighter than that observed for β-glucanase at the same stage of development (cf. Plates 13 and 14 with Plate 10—note, particularly, the contrasts in Plate 14 and Plate 10). Also, unlike the β-glucanase, the β-glucosidase was confined to a very discrete area across the base of the developing ergot (Plate 14).

The ergot was fully differentiated 28 days after infection. At this time, only a small, still fluorescent fragment of sphacelial tissue remained at the top of the sclerotium outside the pigmented boundary (Plate 15). The mature ergot retained the band of β-glucanase across its base, i.e. the interface (Plate 16), but the lower intensity of fluorescence showed that it was more diffuse than had been seen during differentiation. In contrast, the very bright, narrow band of fluorescence associated with the presence of β-glucosidase at the interface was still present in the sclerotial tissue at 28 days (Plate 17).

DISCUSSION

Immunofluorescent techniques have been employed for the detection of bacterial [*3, 28*] and fungal [*7, 16, 42*] pathogens of plants, as well as for the localization of proteins within plant tissues [*20*]. In the present study, proteins of a fungal phytopathogen were identified in the parasite growing on the host by complexing with antisera prepared to them and visualizing using fluorescein conjugated IgG fraction of goat anti-rabbit IgG. To achieve this, the bright autofluorescence of the plant tissue [*39*] had to be quenched by a method that was not destructive to the fungal enzymes under investigation. The main quenching agent was borate buffer which presumably complexes with carbohydrate residues [*29*] in the plant cell walls. The use of immunofluorescence microscopy to trace the occurrence of the enzymes under investigation during development of the ergot required the purified enzymes themselves to be used as the antigens. The procedure for fixing and developing sections necessitated successive irrigations of the tissue which would be expected to remove most of the soluble enzymes. The resultant fluorescence, therefore, may be ascribed largely to cell-associated β-glucanase and β-glucosidase, which, since they react with the corresponding antisera, must be considered the same or very closely related to those produced extracellularly during *in vitro* culture.

The progress of development of the ergot was similar to that described previously [*5, 23, 31, 32*]. Fluorescence due to the β-glucanase was first observed at the interface and around the peripheral (invaginated in section) areas of the sphacelium 10 days

after infection; it persisted at the interface throughout the developmental period, but, although initially very bright, had disappeared from the sphacelial periphery by 18 days.

The β-glucanase was found to be localized within the parasitic system in areas of the fungus which would normally be those identifiable as having the most rapid growth rates, i.e. at the base of the ergot in acropetal development [*38*] and at the hyphal tip during sporulation. The surface of the sphacelium is where sporulation takes place [*23*] and this would be expected to be accompanied by active wall growth. It has been noted that wall growth is probably preceded by partial degradation of polymers in the growing areas prior to intussusception of new material and proposed that β-glucanases represent causal agents for this degradation [*2, 40*]. The presence of β-glucanases in situations similar to that described here have also been observed by other workers [*21, 37*] who used immunofluorescent methods to detect this enzyme in the hyphal walls, apices and branching points of *Sclerotium rolfsii* and *Neurospora crassa*. Fevre [*15*] has also demonstrated β-glucanase activity in the apical vesicles of *Saprolegnia monoica* by techniques of subcellular fractionation.

The β-glucosidase was identifiable during the development of the ergot in much the same regions as the β-glucanase. The areas fluorescing owing to the presence of the former enzyme were more discrete than those observed from the latter, especially with respect to the interface. The β-glucanase could hydrolyse any callose that the host would normally deposit in response to wounding as proposed by Dickerson *et al.* [*13*] and also other polysaccharides, primarily glucans of more mixed linkage, present in the plant cell wall [*27, 35*] which would, on hydrolysis, give rise to a variety of glucosides that, unlike the laminarin type of oligosaccharides, cannot be hydrolysed by the β-glucanase itself [*10*]. The similar location of the two enzymes might occur because such products of the action of the β-glucanase could be acted upon by the β-glucosidase.

A lytic function may account for the presence of both of the enzymes in the residual sphacelial tissue to be found at the apex of the maturing ergot. The fact that the enzymes do not persist in the mature sclerotial tissue reflects the differences in metabolism between this and sphacelial tissue. Sclerotial cells are characterized by an active secondary metabolism [*9*]; it is possible that proteins are turned over during maturation to initiate this.

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REFERENCES

1. Aist, J. R. (1976). Papillae and related wound plugs of plant cells. *Annual Review of Phytopathology* **14**, 145–163.
2. Bartnicki-Garcia, S. (1973). Fundamental aspects of hyphal morphogenesis. In *Microbial Differentiation*, 23rd Symposium of the Society for General Microbiology, Ed. by J. M. Ashworth & J. E. Smith, pp. 245–267. Cambridge University Press, Cambridge.
3. Bettelheim, K. A., Gordon, J. F. & Taylor, J. (1968). The detection of a strain of *Chromobacterium lividium* in the tissues of certain leaf nodulated plants by immunofluorescent techniques. *Journal of General Microbiology* **54**, 177–184.
4. Bock, R. M. & Ling, N. S. (1954). Devices for gradient elution in chromatography. *Analytical Chemistry* **26**, 1543–1546.
5. Bove, F. J. (1970). *The Story of Ergot*. S. Karger, Basel, Switzerland.

6. Brandtzaeg, P. (1973). Conjugates of IgG with different fluorochromes I. Characterisation by anion exchange chromatography. *Scandinavian Journal of Immunology* **2,** 273–290.
7. Choo, Y. S. & Holland, A. A. (1970). Direct and indirect fluorescent antibody staining of *Ophiobotus graminis* Sacc. in culture and in the rhizosphere of cereal plants. *Antonie Van Leeuwenhoek* **36,** 549–554.
8. Chrambach, A., Reisfeld, R. A., Wyckoff, M. & Zaccari, J. (1967). A procedure for rapid and sensitive staining of protein fractionated by polyacrylamide gel electrophoresis. *Analytical Biochemistry* **20,** 150–154.
9. Corbett, K., Dickerson, A. G. & Mantle, P. G. (1974). Metabolic studies on *Claviceps purpurea* during parasitic development on rye. *Journal of General Microbiology* **84,** 39–58.
10. Dickerson, A. G., Mantle, P. G. & Szczyrbak, C. A. (1970). Autolysis of extra-cellular glucans produced *in vitro* by a strain of *Claviceps purpurea*. *Journal of General Microbiology* **60,** 403–415.
11. Dickerson, A. G. (1972). A β-D-fructofuranosidase from *Claviceps purpurea*. *Biochemical Journal* **129,** 263–273.
12. Dickerson, A. G., Mantle, P. G. & Nisbet, L. J. (1976). Carbon assimilation by *Claviceps purpurea* growing as a parasite. *Journal of General Microbiology* **97,** 267–276.
13. Dickerson, A. G., Mantle, P. G., Nisbet, L. J. & Shaw, B. I. (1978). A role for β-glucanases in the parasitism of cereals by *Claviceps purpurea*. *Physiological Plant Pathology* **12,** 55–62.
14. Dickerson, A. G. & Baker, R. C. F. (1979). The binding of enzymes to fungal β-glucans. *Journal of General Microbiology* **112,** 67–75.
15. Fevre, M. (1977). Subcellular localization of glucanase and cellulase in *Saprolegnia monoica* Pringsheim. *Journal of General Microbiology* **103,** 287–295.
16. Fitzell, R., Evans, G. & Fahy, P. C. (1980). Studies on the colonization of plant roots by *Verticillium dahliae* Klebahn with use of immunofluorescent staining. *Australian Journal of Botany* **28,** 357–368.
17. Fleet, G. H. & Phaff, H. J. (1974). Glucanases in Schizosaccharomyces. Isolation and properties of the cell wall associated β-(1-3)-glucanases. *Journal of Biological Chemistry* **249,** 1717–1728.
18. Fleming, I. D. & Pegler, H. F. (1963). The determination of glucose in the presence of maltose and isomaltose by a stable, specific enzymic reagent. *Analyst* **88,** 967–968.
19. Goldman, M. (1968). *Fluorescent Antibody Methods.* Academic Press, London & New York.
20. Knox, R. B. (1971). Pollen wall proteins: localization, enzymic and antigenic activity during development in *Gladiolus* (Iridaceae). *Journal of Cell Science* **9,** 209–237.
21. Kritzman, G., Chet, I. & Henis, Y. (1978). Localization of β-(1-3)-glucanase in the mycelium of *Sclerotium rolfsii*. *Journal of Bacteriology* **134,** 470–475.
22. Layne, E. (1957). Spectrophotometric and turbidimetric methods for measuring protein. In *Methods in Enzymology,* Ed. by S. P. Colowick & N. O. Kaplan, vol. III, pp. 447–454. Academic Press, London & New York.
23. Luttrell, E. S. (1980). Host–parasite relationships and development of the ergot sclerotium in *Claviceps purpurea*. *Canadian Journal of Botany* **58,** 942–958.
24. Mantle, P. G. & Nisbet, L. J. (1976). Differentiation of *Claviceps purpurea* in axenic culture. *Journal of General Microbiology* **93,** 321–334.
25. Mower, R. L. & Hancock, J. G. (1975). Mechanism of honeydew formation by *Claviceps* species. *Canadian Journal of Botany* **53,** 2826–2834.
26. Nairn, R. C. (1976). *Fluorescent Protein Tracing,* 4th edn. E. and S. Livingstone, London.
27. Nevins, D. J., Yamamoto, R. & Huber, D. J. (1978). Cell wall β-D-glucans of five grass species. *Phytochemistry* **17,** 1503–1505.
28. Paton, A. M. (1964). The adaptation of immunofluorescence techniques for use in bacteriological investigations of plant tissue. *Journal of Applied Bacteriology* **27,** 237–243.
29. Pittet, A. O. (1965). In *Methods in Carbohydrate Chemistry,* Ed. by R. L. Whistler, Vol. 5, p. 4. Academic Press. London & New York.
30. Rees, D. A. (1973). Polysaccharide conformation. In *MTP International Review of Science: Organic Chemistry,* Series One, vol. 7 (*Carbohydrates*). Ed. by G. O. Aspinall, pp. 251–283. Butterworth, London.
31. Shaw, B. I. & Mantle, P. G. (1980*a*). Host infection by *Claviceps purpurea*. Transactions of the British Mycological Society **75,** 77–90.
32. Shaw, B. I. & Mantle, P. G. (1980*b*). Parasitic differentiation of *Claviceps purpurea*. *Transactions of the British Mycological Society* **75,** 117–121.
33. Shimomura, T. & Dijkstra, J. (1975). The occurrence of callose during the process of local lesion formation. *Netherlands Journal of Plant Pathology* **81,** 107–121.
34. Smith, D., Muscatine, L. & Lewis, D. (1969). Carbohydrate movement from autotrophs to heterotrophs in parasitic and mutualistic symbiosis. *Biological Reviews* **44,** 17–90.

35. SMITH, F. & MONTGOMERY, R. (1959). Cereal Gums. In *Chemistry of Plant Gums and Mucilages*, Monograph Series 141, pp. 373–380. Rheinhold Publishing Corporation, New York.
36. STOLL, A., BRACK, A., KOBEL, H., HOFFMANN, A. & BRUNNER, R. (1954). Die Alkaloide eine Mutterkornpilzes von *Pennisetum typhoideum*, Rich und deren Bildung in saprophytischer kultur. *Helvetica Chimica Acta* **37,** 1815–1825.
37. SUKUMARAN, C. P. & MAHADEVAN, P. R. (1975). Localization of enzymes in the cell walls of *Neurospora crassa*. *Indian Journal of Experimental Biology* **13,** 127–130.
38. SWAN, J. (1980). Competitive interactions in the parasitism of cereals by *Claviceps purpurea*. Ph.D. Thesis, University of London.
39. VAN DUIJN, C. (1954). Fluorescence microscopy—Primary fluorescence. *The Microscope* **10,** 1–6.
40. VILLANUEVA, J. R., NOTARIO, V., SANTOS, T. & VILLA, T. G. (1976). β-Glucanases in nature: biochemistry and function of β-glucanases in yeast. In *Microbial and Plant Protoplasts*, Ed. by J. F. Peberdy, A. H. Rose, H. J. Rogers & E. C. Cocking, pp. 323–355. Academic Press, London & New York.
41. WARBURG, O. & CHRISTIAN, W. (1942). Isolierung und Kristallisation des Garungasferments enolase. *Biochemische Zeitschrift* **310,** 384–421.
42. WARNOCK, D. W. (1973). Use of immunofluorescence to detect mycelium of *Alternaria*, *Aspergillus* and *Penicillium* in barley grains. *Transactions of the British Mycological Society* **61,** 547–552.
43. WEBER, K., PRINGLE, J. R. & OSBORN, M. (1972). Measurements of molecular weights by electrophoresis on acrylamide gels. *Methods in Enzymology* **26C,** 3–27.

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