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EVIDENCE OF MULTIPLE CELLULASE FORMS IN *TRICHODERMA HARZIANUM* E58 AND THEIR SIGNIFICANCE IN CELLULOSE HYDROLYSIS

by

Suzanne Porter

Supervised by Dr. J. N. Saddler

A thesis submitted in partial fulfillment of
the requirements for the degree of
Master of Science

School of Graduate Studies
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ABSTRACT:

The occurrence of multiple cellulase components of Trichoderma harzianum E58 and the implications of their existence on the hydrolysis of cellulose were examined. The combined action of these three cellulase enzymes is generally measured by their ability to hydrolyze insoluble cellulosic substrates, such as filter paper, over a defined period of time and conditions. A single commercial enzyme preparation, Novo-Celluclast, showed different extents of hydrolysis of several cellulosic substrates over time, illustrating the influence of substrate characteristics on hydrolysis.

The filter paper activities of six batches of T. harzianum E58 showed poor correlation with the ability of these enzymes to hydrolyze other cellulosic substrates over extended periods of time. Hydrolysis of a single substrate by a single enzyme preparation resulted in similar slopes in reducing sugar production with enzyme concentration, between one-hour and twenty-four-hour hydrolyses, indicating that hydrolysis is dependent on the initially adsorbing set of cellulase components. In attempts to understand the substrate effects on the individual cellulase components, these components must first be isolated and characterized.

The multiplicity of the cellulase components of T. harzianum E58 was examined, and the number of endoglucanase components and their specificities towards β-1,4-linkages

were studied. Separation of the enzyme components by chromatofocusing indicated that several types of endoglucanases were produced by the fungus. Their specificities towards β-1,4-glucosidic linkages and β-1,4-xylosidic linkages were characterized using a zymogram technique, using carboxymethylcellulose (CMC) and xylan, respectively, as the substrates. Although many of the protein bands exhibited specificity towards either carboxymethylcellulose or xylan, there were also components that appeared to be capable of hydrolyzing both substrates. The existence of these non-specific endoglucanases (or endoglycanases) may be due to either complex formation of both specific endoglucanases and xylanases, or the result of broad specificity towards both substrates. The necessity of a non-specific endoglycanase may be due to regions in the natural substrate where cellulose and hemicellulose (xylan) meet.

As enzymes exhibiting exoglucanase activity could not be readily differentiated by zymogram technique, attempts were made to purify and characterize a component with this activity. A protein with an isoelectric point (pI) of 4.8 and molecular weight of 63 kDa by SDS-PAGE was isolated. This enzyme hydrolyzed filter paper and Avicel, producing cellobiose as the main product. The exoglucanase, or cellobiohydrolase, did not hydrolyze carboxymethylcellulose, salicin, or cellobiose.

The role of the exoglucanase, as an enzyme which cleaves cellobiose from the non-reducing chain ends of the oligosaccharides produced by the endoglucanase, was examined using sub-saturation concentrations of T. harzianum E58 cellulase. No significant increase in hydrolysis was observed when purified exoglucanase was added to the cellulase mixture. Since sufficient binding sites should be present, it is probable that the exoglucanase must be present in a certain ratio with the other components, either for proper adsorption or for enhanced hydrolytic efficiency. The high proportion of non-specific endoglucanases and the need for an efficient endoglucanase-to-exoglucanase ratio are discussed in terms of a modified model for cellulose hydrolysis.

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LIST OF ABBREVIATIONS

CBH	Cellobiohydrolase (I, II, etc.)
CI	Crystallinity index
CMC	Carboxymethylcellulose
DNS	Dinitrosalicylic acid
DP	Degree of Polymerization
EG	Endoglucanase (I, II, etc.)
FPU	Filter Paper Unit
IEA	International Energy Association
IEF	Isoelectricfocusing
IUPAC	International Union of Pure and Applied Chemistry
MCC	Microcrystalline cellulose
MUC	Methylumbelliferylcellobioside
PAGE	Polyacrylamide Gel Electrophoresis
PIPES	Piperazine buffer
SDS	Sodium dodecyl sulphate
TRIS	Tris (Hydroxymethyl) Aminomethane buffer

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INTRODUCTION:

The ability of certain microorganisms to degrade cellulose to soluble sugars was dramatically obvious to troops in the South Pacific during the 1940's, whose cotton uniforms were susceptible to this process. This promoted research into the mechanisms of cellulose hydrolysis, and after decades of study the action of the cellulase enzymes is still the subject of great debate.

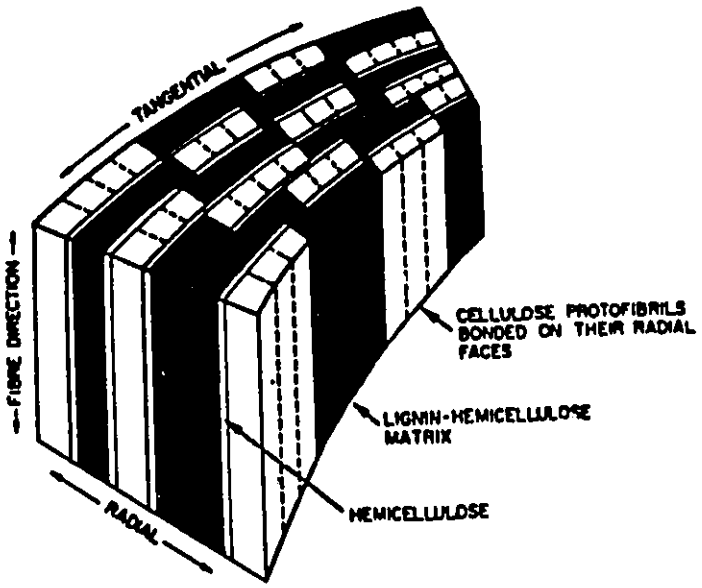
A resurgence of interest in cellulose hydrolysis was sparked during the 1970's, during the energy crisis. The production of ethanol from lignocellulosics has been attempted on numerous occasions, but previous processes have involved the use of acid hydrolysis of the substrate. This method has its disadvantages; acid hydrolysis is not specific, and glucose is not the only product. Costly neutralization steps are required after hydrolysis to remove the acids. An alternative route for the production of sugars from lignocellulosics is the use of enzymatic hydrolysis.

Many fungi and bacteria produce and secrete enzymes which hydrolyze cellulose to oligomers, cellobiose, and glucose. However, native cellulose is rarely found in the pure state. It is generally associated with other polymers in plant cell walls, forming a three-dimensional matrix

(Fig. 1) with lignin, a high molecular weight phenolic compound, and hemicellulose, a polymer of pentoses, predominantly xylan. While this association between cellulose and the other cell wall components gives wood its strength and durability, it also hinders its accessibility to enzymes. Evidence that lignin may also decrease hydrolysis of cellulose by binding cellulase enzymes, rather than by merely acting as a physical barrier, has also been reported (Sutcliffe and Saddler, 1986). Therefore, the lignocellulosic substrate must be pretreated to decrease the association between cellulose and the other polymers before efficient hydrolysis can be performed.

Various pretreatment processes have been assessed for their ability to fractionate the lignocellulosic components, as well as to enhance the accessibility of the cellulose to hydrolytic enzymes. These different physical and chemical methods include options such as steam treatment, milling, and the use of acids and alkalis (Ryu et al., 1982; Knappert et al., 1980; Gharpury et al., 1983; Brownell et al., 1984). However, these processes also affect structural features of the cellulose itself, such as degree of polymerization (DP), crystallinity, surface area, and pore volume. These features also affect the accessibility of the substrate to cellulases, therefore, it is difficult to determine if increased hydrolysis is mostly due to the removal of lignin or xylan, or due to changes in the cellulose itself (Bertran

FIGURE 1: Model Showing Arrangement of Carbohydrates and Lignin in Cell Wall



(Salmén, 1985)

(Courtesy of John Wiley and Sons)

and Dale, 1985; Enriquez et al., 1981; Ford, 1983; Weimer and Weston, 1985; Stone et al., 1969; Fan et al., 1980; Knappert et al., 1980; Gharpury et al., 1983; Wong et al., 1988).

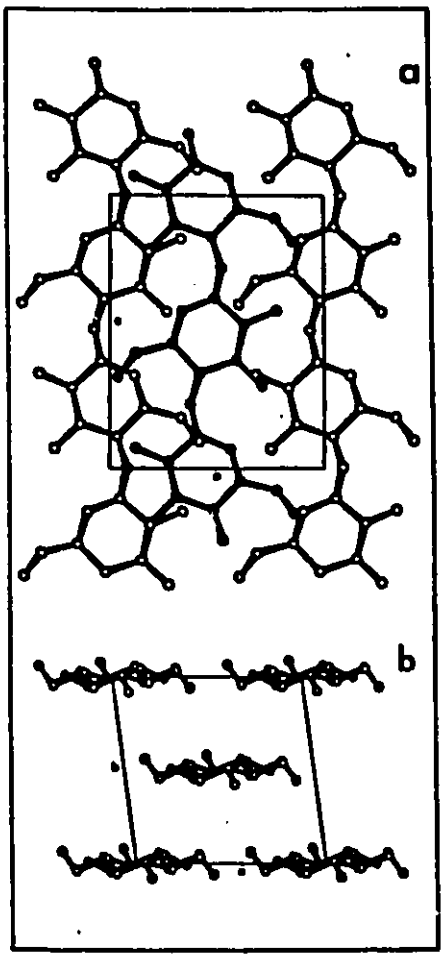
Some naturally occurring celluloses are found in a relatively pure state. Cotton, algal-derived microcrystalline cellulose from organisms such as Valonia, and the cellulose produced by the bacterium Acetobacter xylinum have been used to study hydrolysis of pure cellulose without altering the cellulose structure by pretreatment steps.

(I) CELLULOSE STRUCTURE:

Native cellulose is composed of β-D-glucopyranose residues linked by (1,4)-glucosidic bonds, forming long linear macromolecules. Each residue is rotated 180° on its axis, therefore, cellobiose is the recurring unit. Although cellulose appears to have a simple structure, the insolubility of the substrate allows for a great diversity of cellulosic substrates.

Native cellulose, or cellulose I, is found in chains of up to 10,000 glucose units in length (Fig. 2). These cellulose molecules align lengthwise, forming distinct units called microfibrils. Each plant microfibril contains about 40 cellulose chains with a total diameter of about 5 nm (Preston, 1986). Algal microfibrils have been observed to

FIGURE 2: Projections of the Parallel Chain Model for Cellulose I



1. Projections of the parallel chain model for cellulose I. (a) Projection perpendicular to the ac plane. The center chain (black) is staggered with respect to that at the origin. (b) Projection perpendicular to the ab plane, looking along the fiber axis.

(Blackwell et al., 1978)

be about 20 x 20 nm^2, containing 1200-1400 cellulose chains (Sugiyama et al., 1985).

The cellulose chains in the microfibrils are thought to be aligned in a parallel manner, allowing for significant internal hydrogen bonding. These parallel, ordered areas of the microfibrils are termed "crystalline regions". The hydrogen bonding in the crystalline regions makes the cellulose very compact and relatively inaccessible to hydrolytic agents. When crystalline cellulose is treated with weak acids, the DP, or average number of glucose units, of the chains is rapidly reduced. This shortened chain length is persistent and suggests that the crystalline regions are of constant length separated by regions that are more accessible to protons. These hydrolyzable portions are thought to be non-parallel and are called "amorphous regions" (Young, 1986). The reduction in extensive hydrogen bonding in these regions makes them a more open structure than the crystalline regions, and therefore, more accessible to catalysts.

Enzymatic hydrolysis of cellulose seems to occur by a different mechanism than that described for acid hydrolysis. Comparison of average chain length showed that for a similar weight loss, the DP of cellulose was much less after acid hydrolysis than after enzymatic hydrolysis (Walseth, 1952b). This suggests that the interior of the substrate is relatively inaccessible to cellulase enzymes; a surface

peeling of the outer chains occurs and the average DP remains high (Chang and Tsao, 1981; Lee et al., 1983). Evidence that the proportion of crystalline material in a sample increases during the initial stages of hydrolysis indicates that the amorphous regions are attacked first, as observed with acid hydrolysis (Enriquez et al., 1981; Lee et al., 1983).

The importance of the duality of the substrate, with respect to crystallinity, is observed by the fact that the ratio of crystalline to amorphous cellulose in a cellulosic substrate is a major determinant of hydrolysis. The amount of crystalline material in a substrate can be determined by X-ray diffraction. The relative proportion of crystalline to amorphous cellulose is called the "Crystallinity Index" (CI).

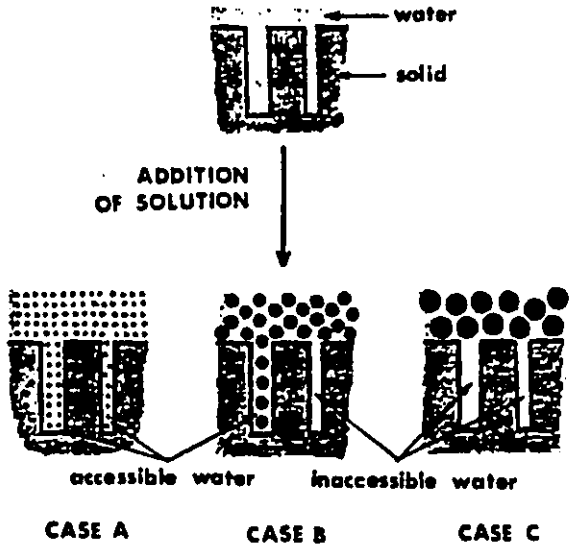
The Crystallinity Index has been shown to exhibit an inversely proportional relationship to both the initial rate and to the extent of hydrolysis of cellulosic substrates (Bertran and Dale, 1985; Fan et al., 1980). However, pretreatments that decrease crystallinity are usually milling processes that also increase the specific surface area of the substrate. This would be expected to produce a larger adsorptive surface for the cellulase enzymes. Improved hydrolysis with decreased particle size has been observed (Ford, 1983), but other workers have stated that surface area had no effect on hydrolysis (Fan et al., 1980).

However, the latter study measured surface area by nitrogen adsorption, which would not reflect the surface area accessible to larger particles, such as the cellulase enzymes.

The surface area of cellulosic substrates that is accessible to cellulase enzymes can be estimated by the solute exclusion technique. The fibrillar nature of the substrate results in pores, or spaces between lamella, into which cellulases can migrate. On the premise that a more open structure will reveal more surface area to the enzymes, this technique measures the water that is extruded from the substrate by a series of carbohydrate probes (Fig. 3). Smaller probes will be capable of entering more pores, resulting in a greater dilution of the solvent than by the larger probes. The accessible pore volume of several substrates is determined for each size probe, and plotted against initial reaction rates of hydrolysis of the same celluloses. The size probe whose line passes through the origin is the best size estimate of the cellulase enzyme, since no hydrolysis would occur if the substrate were inaccessible (Fig. 4). Similar estimates of cellulase diameters have been obtained by various groups, 4.3 nm (Weimer and Weston, 1985), 4.0 nm (Stone et al., 1969), and 5.1 nm (Grethlein, 1985).

The estimates for cellulase diameters are averages for the different cellulase components found in a cellulase

FIGURE 3: Illustration of Accessibility of Internal Regions of a Solid Substrate to Particles of Various Sizes by the Solute Exclusion Technique



(Stone and Scallan, 1969)

FIGURE 4: Estimation of Molecular Diameter of Rate-Limiting Cellulase Components

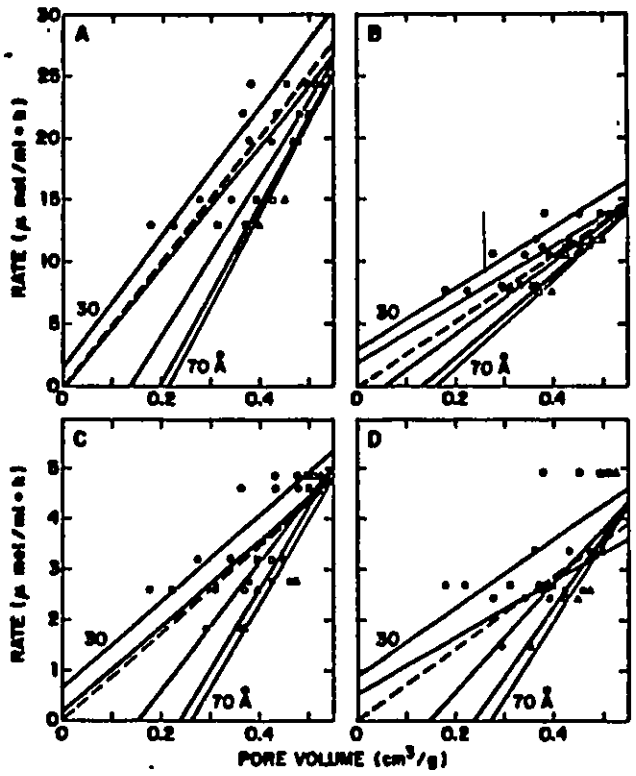


Figure Estimation of molecular diameter of rate-limiting component of cellulase complexes. (A) *T. reesei*, expt 1; (B) *T. reesei*, expt 2; (C) *C. thermocellum*, expt 1; (D) *C. thermocellum*, expt 2. Each solid line represents a line of best fit between reaction rate and pore volume at given maximum pore diameter for all six celluloses. Pore diameters: (●) 30 Å; (○) 40 Å; (■) 50 Å; (□) 60 Å; (▲) 70 Å. Broken lines represent lines of best fit, derived by interpolation, which pass through their respective origins. These lines correspond to the following pore diameters: (A) 39.0 Å; (B) 46.0 Å; (C) 41.2 Å (D) 43.8 Å.

(Weimer and Weston, 1985)

mixture; purifications of the cellulase enzymes of bacteria and fungi have shown that the cellulase enzyme is actually a group of several types of enzymes. The size and shape of one cellulase component of T. reesei has been estimated by small-angle X-ray scattering. The enzyme had a tadpole-like shape with a head and long tail. The length was 18 nm, and the width was 4.4 nm (Schmuck et al., 1986).

(II) CELLULOLYTIC ENZYMES:

Many species of bacteria and fungi produce and secrete extracellular enzymes which are required for the efficient breakdown of cellulose (Walseth, 1952a; Mandels and Reese, 1964; Saddler et al., 1985; Almin et al., 1975). Since the initial research on hydrolysis of cellulose began, it has been known that several types of enzymes, collectively called the "cellulase enzyme", are required for efficient hydrolysis of crystalline cellulose.

Separation of cellulase components has led to several theories describing their collective and individual actions during hydrolysis of native cellulose. Mandels and Reese (1964) proposed one of the first and most widely accepted theories of cellulose hydrolysis which incorporated the action of more than one enzyme component. They introduced the (C_1-C_x) theory for the mode of action of cellulases during hydrolysis of native cellulose. During purification of Trichoderma viride cellulases, they isolated the

β-glucosidase enzyme and two other components. One component showed activity toward the soluble substrate, carboxymethylcellulose, and the other showed slight activity on cotton. The activity towards cotton was increased when the two components were combined. The component active against cotton was reported to be a non-hydrolytic, solubilizing factor. This "C_1" component was thought to convert native cellulose to a form that would be receptive to further enzymatic hydrolysis, because little solubilization of cotton occurred by mixtures that had the C_1 component removed. The cellulose would then be acted upon by an unspecified number of hydrolytic "C_x" enzymes, which would cleave the glucosidic bonds. The action of the C_x enzymes was detected by the production of the soluble reducing sugars that were released.

The C_x enzymes can act alone on the soluble substrate, carboxymethylcellulose, but both C_1 and C_x are required for the efficient hydrolysis of ordered cellulose. The combined action of C_1 and C_x components on crystalline cellulosic substrates was shown to be synergistic. When the cellulase components of T. koningii (Wood and McCrae, 1979; Wood, 1975), T. viride (Selby, 1967), or Fusarium solani (Wood and McCrae, 1979) were separated, their activity towards cotton greatly decreased. When the components were recombined, cellulolytic activity was restored to close to that of the crude filtrate (Table 1).

TABLE 1: Evidence of Synergism between Various Fungal Cellulase Components

ENZYME	COTTON SOLUBILIZATION (%) F. solani	T. koningii
C_1 (cellobiohydrolase)	2	1
C_x (endoglucanases)	1	1
β-glucosidase	1	nil
$C_1 + C_x$	58	53
$C_1 + \beta$-glucosidase	18	20
$C_1 + C_x + \beta$-glucosidase	71	72
Original culture filtrate	71	71

(Wood and McCrae, 1979)

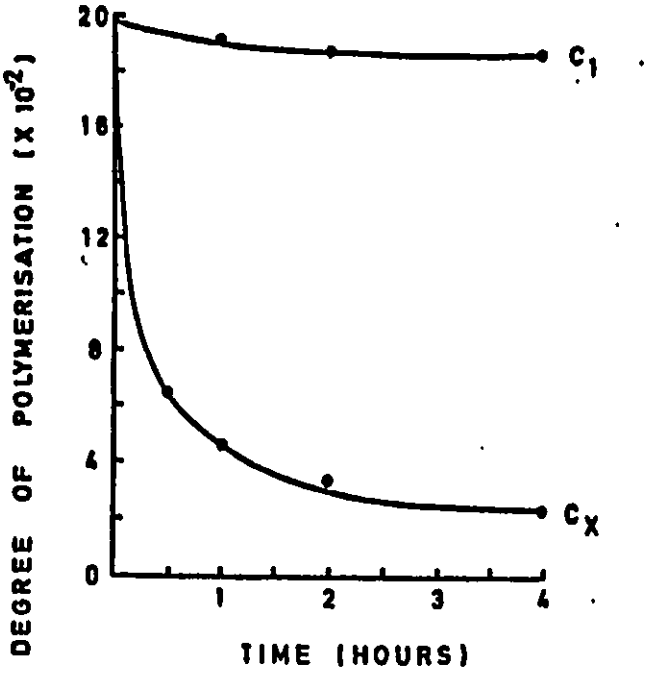
Subsequently, the C_1 component was observed to possess hydrolytic activity. Researchers have shown that the C_1 component of fungi produces cellobiose as the major product from substrates such as the commercial cellulose Avicel, phosphoric acid-treated Avicel, cotton, cellohexaose, and cellotetraose (Berghem and Petterson, 1973; Wood and McCrae, 1979).

Removal of cellobiose units from cellulose must occur from the chain ends because simultaneous cleavage of two different internal glucosidic bonds by one enzyme is unlikely. This suggests that C_1 is an exo-enzyme. This was supported by the slow decrease in DP of the cellulose, with increasing production of reducing sugars (Fig. 5). The exoglucanase has subsequently been defined as a cellobiohydrolase (1,4-β-D-glucan cellobiohydrolase, EC 3.2.1.91).

Conversely, the C_x component causes a rapid decrease in chain length with little reducing sugar production, suggesting that it hydrolyzes internal bonds in the cellulose chains, and is therefore an endoglucanase. This enzyme is referred to as 1,4-β-D-glucan 4-glucanohydrolase, EC 3.2.1.4.

The presence of both an exoglucanase and an endoglucanase gave rise to a possible mechanism for synergistic action of the two components during hydrolysis. According to the most widely accepted theory of cellulose hydrolysis,

FIGURE 5: Comparison of Change in Degree of Polymerization of Phosphoric Acid Swollen Cellulose by C_1 and C_x Enzymes of T. koningii



(Wood and McCrae, 1979)

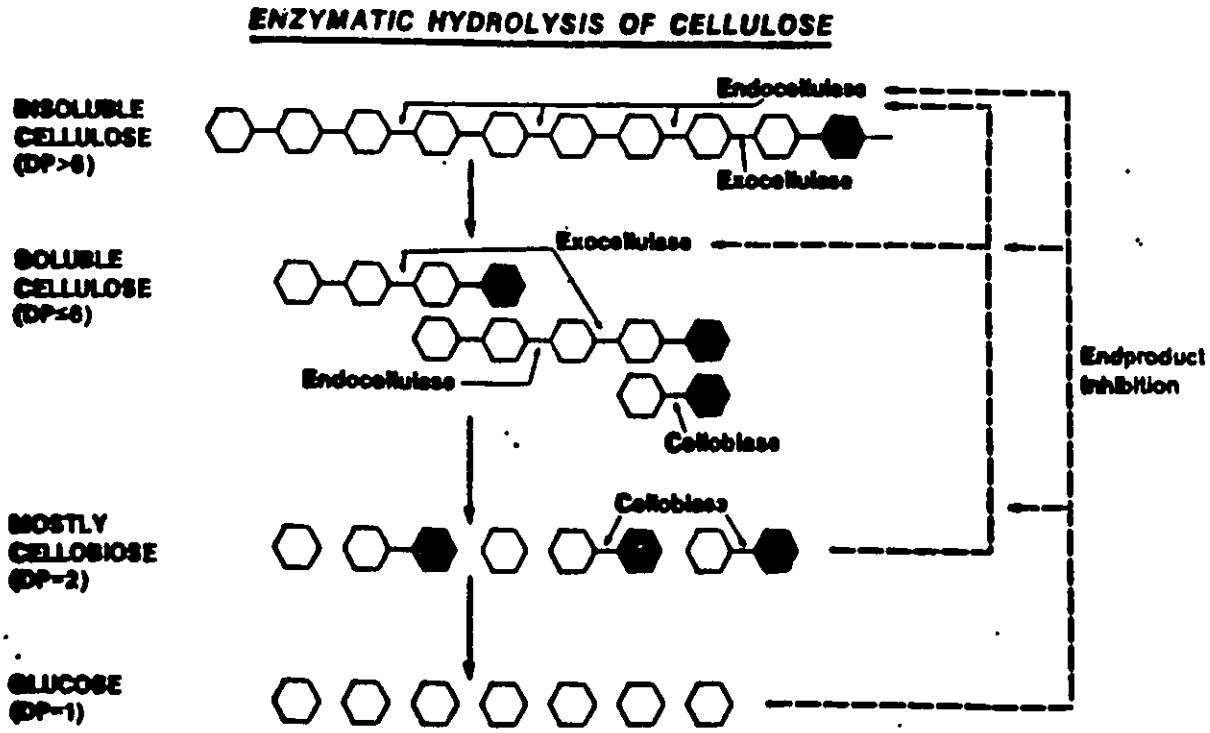
the endoglucanase is the first to attack, randomly cleaving the cellulose chains. The exoglucanase would then attack the newly formed non-reducing ends, producing cellobiose. Cellobiose is finally cleaved by the β-glucosidase (cellobiase), producing glucose as the final product (Fig. 6).

Evidence that the endoglucanase (formerly C_x) initiates the attack on the cellulose substrate, and that the exoglucanase (formerly C_1) possesses hydrolytic activity, confuses the old nomenclature such that the terms C_x and C_1 are rarely used.

Visual observations of the cellulase components on cellulose support this synergistic mechanism of hydrolysis by endoglucanases and exoglucanases. Electron microscopy studies have provided evidence which supports this synergistic breakdown of algal or bacterial microfibrils by crude cellulases. Although individual endoglucanase or exoglucanase components were shown to have little effect, dissolution of the fibres was observed when the components were recombined (Chanzy et al., 1983; White and Brown, 1982; Chanzy and Henrissat, 1983; Chanzy et al., 1984; Chanzy and Henrissat, 1985).

Some researchers still propose the necessity of a factor that activates the cellulose, before the hydrolytic enzymes can act upon it (Coughlan, 1985). Swelling factors (Mandels and Reese, 1964) and short-fibre-forming factors

FIGURE 6: Diagrammatic Representation of the Synergistic Hydrolysis of Cellulose by the Various Cellulase Components



(Saddler, 1986)

(Halliwell and Riaz, 1970; Griffin et al., 1984; Krull et al., 1988) have been suggested as non-hydrolytic components that make the cellulose susceptible to hydrolysis. These functions were formerly attributed to the original C_1 component. The exact number of cellulase activities required for efficient hydrolysis is still unknown. The similarity in the enzyme activities of the individual cellulase components and in the substrate structures makes the interpretation of the assaying of these components difficult.

(III) ENZYME ASSAYS:

Cellulase activities in an enzyme preparation are quantified by several assays. However, similarities in substrate specificities and the synergistic action of the components make quantitation of the activities of individual cellulase components difficult in a mixed sample.

Endoglucanase (EG) activity is traditionally determined by its activity on substituted cellulose, such as carboxymethylcellulose. Hydrolysis of the substrate is calculated by glucose production or by a decrease in viscosity of the substrate. However, exoglucanase can also hydrolyze this substrate slightly, until its action is hindered by the presence of the substituent groups.

Exoglucanase or cellobiohydrolase (CBH) activity is not readily detected in a mixture of cellulase activities, due

to interference by the activities of the other cellulase components. On crystalline substrates, the synergistic action of the endoglucanase will elevate the apparent exoglucanase activity. Avicel is often used to measure exoglucanase activity, as it is a very crystalline substrate with low DP, and therefore, is more resistant to endoglucanase action. Soluble substrates such as methylumbelliferylcellobioside (MUC) are becoming popular for the detection of exoglucanase activity. However, there is evidence that some endoglucanases (van Tilbeurgh and Claeyssens, 1985) and xylanases (Gilbert et al., 1988) can also cleave these substrates.

The β-glucosidase activity, purified or in mixtures, does not adsorb to the polymer cellulose. This enzyme cleaves the soluble cellobiose, thus producing the final product, glucose. The activity is assayed by measuring the glucose produced from cellobiose, salicin, or p-nitrophenyl-β-glucoside. It has been shown that the activities determined using these substrates do not always correspond to each other. These discrepancies may be due to different enzyme specificities towards the different substrates, or they may be the result of the presence of multiple enzyme forms with different specificities (Khan et al., 1985).

Filter paper has become the substrate of choice by most laboratories for measurement of the combined cellulase activities. It is apparent, however, that the β-glucosidase

content of the cellulase mixture has a great influence on the filter paper activity which is obtained. This activity must be present in excess to eliminate the feedback inhibition on the adsorbable endoglucanase and exoglucanase components (Khan et al., 1985; Bailey, 1981).

One drawback to the assay substrates used to assess the cellulolytic activity of fungal culture filtrates is that they possess structural differences from natural celluloses. Carboxymethylcellulose contains substituent groups to render it soluble. Filter paper is prepared from milled cotton, and as an insoluble cellulosic substrate, its structural features will determine the rate and extent of its hydrolysis by an enzyme preparation. These substrate factors may differ from those of an alternate substrate, and therefore, the action of the same enzyme preparation may differ on other forms of cellulose. It is questionable whether hydrolysis of these assay substrates reflects hydrolysis of native celluloses.

Another drawback with the current assay methods involves the recent studies examining the presence of multiple forms of cellulase components. Assay methods measure a combined action of many cellulase components without detecting differences in activity or specificity. It is now apparent from purification studies that most fungi produce more than one of each cellulase type. It has not yet been resolved from those enzymes isolated whether they

are true isozymes, which are formed from two or more common sub-unit types, or are actually the products of post-translational modifications. The purpose of this complexity in the cellulase system is unknown, but suggests that the current hypothesis of endoglucanase, exoglucanase, and β-glucosidase activities may need refining.

(IV) MULTIPLICITY OF CELLULASES:

The current theory of cellulose hydrolysis explains the presence of exoglucanase and endoglucanase activities, but does not explain the heterogeneity of cellulase components observed in many cellulase systems. Purification of various fungal cellulases has shown the presence of several components exhibiting endoglucanase and exoglucanase activity. For example, five endoglucanases were obtained from both Fusarium lini (Rao et al., 1986) and Sporotrichum pulverulentum (Almin et al., 1975). The complete cellulase system of T. viride was also found to contain at least six endoglucanases, three exoglucanases, and one β-glucosidase (Beldman et al., 1985).

It has been suggested that the occurrence of multiple cellulase forms is the result of proteolytic cleavage and/or differential glycosylation. However, two different mRNA's of each cellulase component of Schizophyllum commune were observed before the onset of protease secretion (Willick and Seligy, 1985). Additional evidence of multiple enzyme forms

arising before extracellular secretion of proteases has been reported for the cellulase system of T. reesei (Labudova and Farkas, 1983). Another study involving T. reesei showed the presence of two distinct endoglucanase precursors by SDS-PAGE of cell-free extracts (Messner and Kubicek, 1988).

The endoglucanases of the thermophilic actinomycete Thermonospora curvata were characterized in different phases of the growth cycle. Those present in the early exponential phase differed from those in the stationary phase in terms of molecular weight, and K_m for carboxymethylcellulose (Lupo and Stutzenberger, 1988).

Exoglucanase activities have also been observed to occur in multiple forms in the same organism. Two immunologically unrelated cellobiohydrolases of T. reesei have been isolated (Fagerstam and Petterson, 1979). They showed no obvious sequence homology in the first 20 amino acids (Fagerstam and Petterson, 1980).

Purification and characterization of some T. harzianum E58 cellulase components indicate that more than one type of each enzyme may be present. Two enzyme complexes with β-glucosidase activity have been observed (Tan et al, 1987) and three xylanases have been obtained (Tan et al., 1985a; Tan et al., 1985b; Wong et al., 1986). Ion-exchange chromatography of T. harzianum E58 cellulases produced a peak that showed activity towards Avicel, but little activity towards carboxymethylcellulose or salicin. This

suggested the presence of an exoglucanase. This peak was homogeneous on SDS-PAGE, but other peaks also showed a component of the same molecular weight, suggesting that more than one exoglucanase of similar weights may be present (Tan et al., 1986). Two peaks that were eluted from the ion-exchange column showed activity towards carboxymethyl-cellulose, and may indicate the presence of multiple forms of endoglucanase components (Tan et al., 1986).

The necessity of multiple forms of cellulases for efficient hydrolysis is not completely understood. The production of several enzymes with the same function would be costly to an organism, unless the enzymes had different optima, with respect to physical conditions, or had different specificities dependent on variations in the substrate. Most cellulases secreted by a given organism have similar temperature and pH optima, therefore, different substrate specificities are likely.

(V) SPECIFICITY OF CELLULASES:

The current definition of exoglucanase, endoglucanase, and cellobiase involves activity towards β-1,4-glucosidic linkages. The differences in the specificity of these enzymes is dependent on the chain length, the distance from the purported adsorption site to the non-reducing end of the chain, and the degree of substitution of the substrate.

Isolation of a single cellulase activity has proven difficult because of similarities in physical characteristics of cellulases, inadequate assay substrates for indicating each specific type of cellulase enzyme, or formation of complexes between cellulase components. Alternatively, cellulases may contain more than one substrate bond-type specificity per enzyme.

Endoglucanases have been observed to show variations in linkage specificity, complicating the definition of their role in cellulose hydrolysis. Many supposed endoglucanase enzymes have also been observed to possess activity towards β-1,4-xylosidic linkages. Several workers have attempted to purify and characterize all cellulase components produced by a microorganism, and to determine which are required for efficient cellulose hydrolysis (Wood, 1985; Beldman et al., 1988a).

Differences in the action of various cellobiohydrolases indicate that the definition of exoglucanase activity may be an over simplification. Exoglucanases were classified as cellobiohydrolases based on the production of cellobiose as the major hydrolysis product from cellulose (Berghem and Petterson, 1973). However, CBHII of T. viride has been observed to be capable of hydrolyzing carboxymethylcellulose to completion, thereby showing endoglucanase activity. Cellobiohydrolase II did not act upon MUC, which CBHI of T. viride was capable of hydrolyzing (Niku-Paavola et al.,

1986). Cellobiohydrolase II also facilitated the production of small fibres during breakdown of Solka Floc, as detected by phase contrast microscopy (Kyriacou et al., 1987). This action is characteristic of an endoglucanase, and did not resemble that of CBHI. Discrepancies such as these have raised suggestions that CBHII is actually an endoglucanase.

The CBHI of T. reesei also seems to have some endo-wise activity. High pressure liquid chromatography (HPLC) analysis of the hydrolysis products of CBHI on substituted cellooligosaccharides showed that CBHI lacks specificity for the terminal cellobiosyl group that is characteristic of cellobiohydrolases (van Tilbeurgh et al., 1982). The authors suggested CBHI may be an endoglucanase whose lack of activity against carboxymethylcellulose may be due to its specificity towards unsubstituted cellobiosyl groups.

The adsorption of CBHI to Valonia microcrystals was not limited to the chain ends, as might be expected of an exo-enzyme. Colloidal gold-labelling of CBHI showed that it adsorbed all along the edges of the microcrystals, corresponding to one face which is more readily digested during synergistic hydrolysis than the other faces (Chanzy et al., 1984).

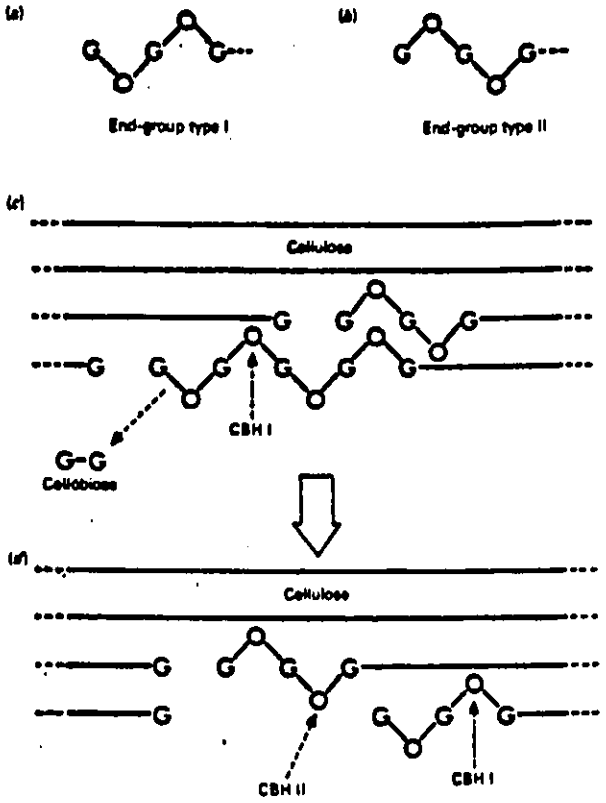
Additional evidence for endo-type action by CBHI is the observed sequence homology between CBHI and endoglucanase II (EGII) (Bhikhabhai and Petterson, 1984). Also, the two CBH's act synergistically together, as determined by

reducing sugar production from Avicel. The sum of the activities of the individual CBH's was exceeded by the activity of the two enzymes incubated together. Wood (1985) has shown the presence of synergistic action by the CBHI and CBHII components of Penicillium funiculosum during filter paper hydrolysis. He postulated that an asymmetry in the substrate may exist to necessitate the presence of two enzymes which cleave cellobiose units, and showed two possible chain ends that may exist (Fig. 7).

Alternatively, the two cellobiohydrolases may combine to form a complex. Evidence for complex formation by cellulase components comes from the cross-synergism seen in exoglucanases and endoglucanases from different fungi. Both CBHI and CBHII of P. funiculosum interact synergistically with the endoglucanases from the same fungus to hydrolyze cotton, but only CBHI will cross-synergize with endoglucanases from T. koningii and Fusarium solani (Wood, 1985). Only certain combinations form a functional complex, and cross-synergistic combinations may sometimes be more active than combining components from the same species (Wood and McCrae, 1979). Alternatively, cleavage by the endoglucanase will likely produce one of the two possible chain ends recognized by the exoglucanases.

The endoglucanase components of various cellulolytic fungi have also been observed to have diverse substrate specificities. It is not certain that these enzymes are

FIGURE 7: Two Possible Chain End Conformations in Cellulose



Possible explanation of the synergism between cellobiohydrolases (CBH) I and II of P. funiculosum in solubilizing crystalline cellulose

Because cellobiose is the repeating unit in cellulose, theoretically there will be two types of non-reducing end groups (*a* and *b*) in the cellulose crystallite. These end groups will be held in position by non-covalent bonds and will require two different stereospecific enzymes for hydrolysis. (*c*) shows only cellobiohydrolase I attacking; (*d*) shows cellobiohydrolase II attacking the new chain end exposed by cellobiohydrolase I action.

(Wood, 1985)

homogeneous and are not complexed with other enzymes. A component of P. funiculosum has been purified to homogeneity as detected by analytical polyacrilamide gel electrophoresis (with or without SDS) and by isoelectricfocusing. It was observed, however, that the purified enzyme still showed activity towards substrates with β-1,4-; β-1,3-; β-1,6-; and α-1,6-glycosidic as well as β-1,4-xylosidic linkages (Sahasrabudhe et al., 1987). Similarly, a cellulolytic component of T. reesei, which was homogeneous by isoelectricfocusing, was also observed to be active against both β-1,4-glucosidic and β-1,4-xylosidic linkages (Sprey and Lambert, 1983). This component was subsequently separated into several fractions using a titration-curve technique in the presence of the detergent ureaoctylglucoside. Separation of the different enzyme activities was verified by zymograms. As the complex was not dissociated by treatment with SDS during an SDS-PAGE fractionation, these workers proposed the existence of a cellulolytic complex associated by the carbohydrate portions of the proteins.

Cloned cellulases of mixed specificities have been obtained. An endoglucanase from Clostridium thermocellum, cloned in Escherichia coli, also cleaved an assay substrate of exoglucanases, p-nitrophenyl-β-cellobioside, at the agluconic bond (Pétré et al., 1986). An endoglucanase from Cellulomonas fimi, cloned in E. coli, was capable of cleaving xylan (Gilkes et al., 1984).

The ability to hydrolyze xylan, as well as cellulose, is not common to all β-1,4-glucan glucanohydrolases. Several fungi have been observed to produce both specific endoglucanases, as well as non-specific endoglycanases that hydrolyze other glycosidic bonds in addition to β-1,4-glucosidic linkages. Many such enzymes have been observed to hydrolyze β-1,4-xylosidic linkages (Kyriacou et al., 1987; Beldman et al, 1985). If the lack of specificity were due solely to random complexing via the carbohydrate portion of the proteins, it seems unlikely that both specific and non-specific complexes would occur. The presence of endoglucanase and xylanase activities together may indicate that regions of the substrate may require enzymes with broad specificities.

(VI) ADSORPTION:

The differences in substrate specificity of cellulase components is generally determined by action on soluble substrates, such as carboxymethylcellulose and MUC. It has become apparent that differences in substrate specificity detected on soluble assay substrates may be exaggerated on insoluble celluloses due to differences in the adsorption of the individual cellulase components. Therefore, structural features of the cellulose that affect adsorption of the enzymes may alter the synergistic effect of the components.

This could determine the extent of the hydrolysis of the substrate.

It is difficult to speak of exoglucanases or endoglucanases as a collective term when discussing adsorption, because it appears that the individual components may possess different adsorption capacities towards the substrate. The adsorption of various endoglucanase activities from several cellulase systems to affinity chromatography columns has been compared, and it was suggested that there are weakly and strongly adsorbing components (Rabinovitch et al., 1982). Another study determined adsorption of purified T. viride cellulase components by protein adsorbed, rather than by loss of enzyme activity. These researchers also showed that some components adsorbed strongly while others had weaker associations with the substrate (Beldman et al., 1987).

Interactions between the adsorbed activities have been suggested (Ryu et al., 1984). Competitive adsorption between sequentially added T. reesei cellulase components supports the concept of complex formation by the endoglucanases and exoglucanases. These workers postulated that this competition is an important factor in the synergistic behaviour of the enzymes. It is possible that the exoglucanase and endoglucanase require the other enzyme to be adsorbed to the substrate surface so they can properly cleave the cellulose chain.

The effect of substrate factors on synergism between cellulase components has also been studied using combinations of purified T. reesei cellulase components on various substrates. It was reported that synergism was not dependent solely on the combination of enzyme components, but was also affected by the properties of the substrate (Henrissat et al., 1985).

If the endoglucanase or exoglucanase components require the presence of the other enzyme on the substrate surface, then the extent of saturation of the surface by the enzymes would affect this interaction. Comparison of hydrolysis of Avicel by saturating and non-saturating concentrations of purified T. reesei cellulases showed that synergism was greatest when non-saturating conditions were used (Woodward et al., 1988). If the various cellulase components have to compete for binding sites, as proposed by Ryu et al. (1984), and some components are adsorbed to the surface with non-productive binding (Rabinovitch et al., 1982), saturation of the cellulose surface may prevent efficient interaction between exoglucanases and endoglucanases. They may also block the subsequent site of a component after a hydrolysis step is completed.

Several studies have shown that various substrate factors can affect the adsorption of the different cellulase components. These include particle size (Goel and Ramachandran, 1983), amorphous and crystalline regions

(Klyosov et al., 1986), and the lignin and xylan content (Chernoglazov et al., 1988). These results indicate that the various cellulase components may adsorb differently on a different substrate.

Because adsorption is necessary for degradation to occur, hydrolysis of a substrate is dependent on the ratio of components which adsorb to it and on the efficiency of the adsorption in terms of binding site and complex formation with other components. Therefore, the enzyme components which adsorb to and hydrolyze a substrate such as filter paper, which is used to determine the apparent activity of cellulase preparations, will not necessarily reflect the enzyme mixture's ability to bind to and hydrolyze another cellulosic substrate.

(VII) RESEARCH OBJECTIVES:

The diversity of cellulase enzymes and the complexity of the cellulosic substrate have become more apparent as purification processes and analytical equipment have improved. The need for multiple cellulase forms varying in substrate specificity has yet to be resolved. Differences between substrates used for assaying cellulolytic activity and substrates used for actual bioconversion raise questions about the usefulness of the various assays.

Filter paper activity has traditionally been used as a measure of total cellulase activity of an enzyme

preparation. This assay is advocated as the best compromise in determining whether the three major enzyme components (endoglucanase, exoglucanase and β-glucosidase) are present in a mixture, as well as in assessing the probable hydrolytic potential towards other cellulosic substrates. However, the filter paper assay is not standardized, and most laboratories use their own modification of Mandels' method (Mandels et al., 1976).

The reproducibility of a standard filter paper assay was examined. The filter paper activity of Novo-Celluclast was determined using the same conditions as groups from several other laboratories world-wide and the results were compared. The relative activity, as determined by the standard assay, was also compared with the activity obtained from the method commonly used at Forintek.

Observations that the substrate structure of a cellulose affects the adsorption and hydrolysis by the cellulase components makes it seem unlikely that a short-term hydrolysis of one substrate will reflect the long-term hydrolysis of another substrate. The usefulness of the filter paper assay in the prediction of the extent of hydrolysis, over extended periods of time, was examined.

The presence of a multiplicity of cellulase components, and the evidence that these components may possess differences in substrate specificities, reveal that filter

paper activity values do not differentiate between the individual components.

In addition, the quantity and activity of other components, such as pectinases and xylanases, will vary between preparations. A seemingly inactive enzyme mixture, based on the filter paper assay, may actually contain highly active cellulases present in low concentrations. Initial screening based solely on the filter paper assay may bypass fungi containing highly active enzymes.

This thesis examines the existence of multiple cellulase components in T. harzianum E58. Several of the cellulases of T. harzianum E58 had been fractionated previously. Three distinct xylanases were purified and characterized (Tan et al., 1985a; Tan et al., 1985b; Wong et al., 1986), and two complexes with β-glucosidase activity were partially resolved (Tan et al., 1987).

Purification and characterization studies of cellulases from other fungi have shown that more components with endoglucanase activity are generally produced by a species than those with exoglucanase activity (Beldman et al., 1985). In addition, the substrate specificities of these endoglucanase components are known to differ. This is probably due to the greater likelihood of substrate variations in the internal regions of the cellulose encountered by the endoglucanase. The chain ends cleaved by exoglucanases are unlikely to show significant diversity.

Many endoglucanase components have been observed to hydrolyze xylans in addition to glucans, and are called "non-specific endoglycanases". The role of these components in hydrolysis has yet to be resolved. The fact that microorganisms produce both specific and non-specific endoglucanases raises questions as to whether both are required in a complete cellulase, and whether they have different functions in hydrolysis. The number and activities of endoglucanase components produced by a typical batch of T. harzianum E58 were determined and their specificities towards xylans and glucans were examined by a zymogram overlay technique.

Inadequate zymogram techniques for exoglucanase activity necessitated the purification of one of these cellulase components. This enzyme was characterized and compared to the current definition of exoglucanase.

Use of zymograms, in addition to the filter paper assay, is recommended for future assessment of cellulase mixtures. This would give an indication of the number of cellulase components and of their relative activities and specificities.

MATERIALS AND METHODS:

(I) ENZYME SOURCES:

The fungus Trichoderma harzianum E58 was obtained from the Forintek Culture Collection. T. harzianum E58 was grown in a New Brunswick Scientific Co. 30 L fermentor at 28°C, using 20 L Vogel's medium, and 1% cellulose, usually Solka Floc, a commercial cellulose (Saddler et al., 1982). T. harzianum E58 was harvested after three days growth, when highest cellulase activities were obtained. The culture was filtered through a Whatman 934AH glass fiber filter and concentrated on a Pellicon ultrafiltration unit using a membrane with a 10 kDalton cut-off. The cellulases remained in the retentate which was precipitated by two volumes of cold acetone on ice, then centrifuged at 3000xg for 30 minutes. The pellet was redissolved in distilled water and freeze-dried. Lyophilized enzyme was weighed and made to volume in buffer (0.05 M sodium citrate buffer, pH 4.8, for hydrolysis experiments and enzyme assays, or the corresponding start buffer for chromatofocusing).

Commercial cellulase enzymes from Novo (Denmark), termed Novo-Celluclast and Novozym, were also used.

(II) ENZYME ASSAYS:

All enzyme activities were determined as units per millilitre of enzyme preparation (IU/mL) based on micromoles of glucose per minute of reaction time.

(a) Filter Paper Assay: In the Forintek method, a modification of Mandels' method (Mandels et al., 1976), 1.0 mL enzyme was serially diluted in citrate buffer. One mL citrate buffer was added. The tubes were mixed and incubated in a 50°C water bath for five minutes. A 50 mg strip of Whatman No. 1 chromatography paper was added to each tube and the tubes were incubated for one hour at 50°C. After incubation, 3 mL of the colorimetric dinitrosalicylic acid reagent (DNS) were added to each tube, to quantify glucose equivalents. The tubes were boiled for 5 minutes. After cooling, the absorbance was determined spectrometrically at 575 nm with respect to a blank containing buffer.

The method suggested by the International Union of Pure and Applied Chemistry (IUPAC) is also a modification of the Mandels' method, but differed from the Forintek method in that 0.5 mL enzyme was used instead of 1 mL, and that after boiling with DNS, 20 mL distilled water is added to each tube (Ghose, 1987). Absorbance was read at 540 nm.

(b) Endoglucanase Assay: The enzyme was serially diluted in 1 mL volumes, and pre-incubated in a 50°C water bath for five minutes. One ml of pre-incubated 1% carboxymethyl-cellulose was added to each tube; the tubes were mixed and incubated at 50°C for 30 minutes. Three mL DNS were added and the tubes were treated as in the Forintek method for filter paper.

(c) Exoglucanase ("Avicelase") Assay: Exoglucanase activity is determined by reducing sugar production from hydrolysis of Avicel. The enzyme was serially diluted in citrate buffer to 1 mL and pre-incubated at 50°C. One mL of 1% Avicel was added; tubes were mixed and incubated at 50°C for one hour, then treated as in the Forintek method.

(d) β-Glucosidase Assay: One mL of 1% cellobiose or 1% salicin was added to one mL of serially diluted enzyme preparation after pre-incubation. Tubes were mixed and incubated at 50°C for 30 minutes. Samples containing salicin were treated as in the Forintek method to determine reducing sugar production. Cellobiose reacts with DNS and gives about 85% of the absorbance of glucose on an equimolar ratio. Cellobiose hydrolysis can be determined from glucose production measured by the Glucostat assay, by YSI analyzer, which measures gluconolactone produced from glucose by

immobilized glucose oxidase, or by high pressure liquid chromatography (HPLC).

(e) Xylanase Assay: The same procedure used for the endoglucanase assay was followed, with 1% xylan substituted for 1% carboxymethylcellulose.

(III) MEASUREMENT OF HYDROLYTIC POTENTIAL:

Hydrolyses were performed in 60 mL Wheaton serum vials. Enzyme was added to 0.05 M citrate buffer, pH 4.8, to a volume of 10 mL. Enzyme and substrate blanks were prepared similarly. Vials were incubated in a shaker at 50°C, 150 rpm., unless otherwise specified. Following the incubation period the non-digested substrate was removed from the filtrate by vacuum filtration using Whatman 934AH glass fibre filters. All experiments were carried out in duplicate.

When Novo-Celluclast was adjusted to 20 FPU/gram cellulose, this was equivalent to an enzyme activity of 0.1 FPU/mL and a substrate concentration of 0.5%. Ten μL of Novozym was added per vial to supplement the β-glucosidase activity of the enzyme.

Glucose and cellobiose concentrations were determined by the DNS method, or HPLC. The DNS method was performed as detailed in the enzyme assays. Three mL DNS were added to 1 mL hydrolyzate, boiled for 5 minutes, cooled and absorbance

was read at 575 nm. This method gives total reducing sugars, and does not distinguish between glucose and cellobiose. A Varian HPLC equipped with a Biorad H column was used for HPLC analysis, with a flow rate of 0.8 mL/minute. The eluent was 0.05 M sulfuric acid. Column temperature was 70°C.

Samples were de-ionized by passage through columns packed with ion-exchange resins, IR45(OH) and IR50(H), to reduce the citrate peak between the cellobiose and glucose peaks. Erythritol was used as an internal standard to quantitate dilution and loss of material during the de-ionizing step. Samples were prepared in duplicate and HPLC analyses were performed twice.

The substrates used for the hydrolyses were filter paper, Sigmacell, Avicel, Solka Floc BW300 and α-floc. The filter paper was Whatman No. 1 chromatography paper (Whatman Laboratories Inc., Clifton, N. J.). Sigmacell Type 50 (Sigma Chemical Company, St. Louis, Mo.) and Avicel PH-101 (FMC Co., Philadelphia, Penn.) are microcrystalline celluloses with an average particle size of 50μ. Solka Floc BW300 (Lee Chemicals Ltd., Toronto, Ont.) and α-floc (James River Corporation, Berlin, N. H.) are ball-milled celluloses.

(IV) PURIFICATION AND CHARACTERIZATION OF THE ENDOGLUCANASE COMPONENTS OF TRICHODERMA HARZIANUM E58:

(a) Chromatofocusing: The cellulase components of T. harzianum E58 were separated by isoelectric point (pI), using a Pharmacia Phastsystem, for fast protein liquid chromatography (FPLC), with a MonoP column 5/20. Proteins were detected by their absorbance at 280 nm using an ultraviolet (UV) spectrometer. Pharmacia Polybuffer PB74 or PB96 was used as the eluent buffer in all separations and the pH was adjusted according to the separation gradient used. The starting buffers used were: PIPES HCl pH 5.5; Bis-Tris HCl ph 6.5; or Tris HCl pH 8.5.

Buffers contained 0.02% sodium azide and were de-gassed before use. After each run, residual protein was removed from the MonoP column with 2 M NaCl before re-equilibration with start buffer.

(b) Isoelectricfocusing: Fractions corresponding to chromatofocusing peaks were separated on the Pharmacia Phastsystem, using Pharmacia IEF gels with pH gradients of 4-6.5, or 3-9. Bands were visualized using the silver-staining kit from Pharmacia.

(c) Preparation of Zymograms: The zymogram technique of Teather and Wood (1982) was adapted for use with gels from the Pharmacia Phastsystem. Substrate gels contained 2%

Difco Bacto-agar in 0.05 M sodium citrate buffer, pH 4.8. Carboxymethylcellulose gels contained 0.1% substrate (w/v); xylan gels contained 0.1% oat spelts xylan (w/v). Autoclaved solutions were left to cool to about 50°C, de-gassed, then poured on the hydrophilic side of GelBond sheets. When cooled, the sheets were cut and stored at 4°C.

Protein samples were separated on Native-PAGE gels on the Pharmacia Phastsystem. Duplicate gels were run simultaneously. One gel was stained by the silver-staining method to visualize the protein bands. The second gel was used in activity staining by an overlay method on the substrate gel.

Carboxymethylcellulose gels were pre-incubated with the unstained Native-PAGE gels for one minute. This released sufficient protein to act upon the substrate, without loss of resolution due to excessive hydrolysis. The substrate gel alone was then incubated for 5-10 minutes at 50°C. Xylan gels were incubated at 50°C with the same Native-PAGE gel for 10-15 minutes. The enzyme activity in the carboxymethylcellulose and xylan gels was visualized by washing the gel surface with 1 M NaCl, then pouring Congo red stain on the agar surface (Teather and Wood, 1982). Gels were placed on a shaker for 30 minutes. The Congo red was poured off, and replaced with 1 M NaCl. The sodium chloride was replaced until clear bands were observed in the gels.

(d) Localization of Active Components on Protein-Stained Gels: Bands evident on the gels were measured from the origin in millimetres, and compared to the bands on the protein-stained gels. Alternatively, gels can be placed directly on top of the protein-stained gels.

(V) PURIFICATION OF THE EXOGLUCANASE FROM T. HARZIANUM E58:

(a) Ion-Exchange Chromatography: The culture filtrate of the fungus T. harzianum E58, grown on 1% Solka Floc, was concentrated by ultrafiltration using a Pellicon cassette system with a 10 kDa molecular weight cut-off (Millipore Ltd.). The retentate was passed through a DEAE-Sephadex A50 (Pharmacia) ion-exchange column, equilibrated with 15 mM potassium phosphate buffer, pH 6.5 (Tan et al., 1986). The column was developed with a linear gradient of 15 mM and 300 mM potassium phosphate buffer, pH 6.5. Four main peaks were eluted. The last peak, Peak D, contained predominantly Avicelase activity, which suggested the presence of an exoglucanase. This protein was shown to be homogeneous on SDS-PAGE and had a molecular weight of 67 kDa.

(b) Molecular Weight Determination of Cellulases:
Molecular weight estimations were performed on the Pharmacia Phastsystem. Polyacrilamide gel electrophoresis (PAGE) was carried out in the presence of sodium dodecyl sulphate (SDS)

as the denaturing agent on gradient gels (10-15). Bands were visualized by silver staining.

(c) Chromatofocusing: The combined ion-exchange fractions corresponding to Peak D (gift from P. Mayers) were buffer-exchanged on a Biogel P6G desalting column. The protein was eluted with PIPES HCl, pH 5.5. Samples were concentrated in an Amicon 10 mL ultrafiltration unit before application to FPLC columns. Fractions eluting with pI 4.8 were collected and combined for further purification by gel filtration.

(d) Affinity Chromatography: Ion-exchange fractions from the DEAE-Sephadex column (Peak D), containing the exoglucanase with pI 4.8, were passed through a Pasteur pipet containing 1 mL packed cellulose, or about 0.25 g Solka Floc BW300. Unbound protein was eluted with 0.05 M citrate buffer pH 4.8. The eluent was collected and concentrated using an Amicon 10 mL filter unit with a PM10 membrane (10 kDalton molecular weight cut-off). The bound protein was eluted with 10 mM NaOH. The eluent was collected and concentrated with an Amicon filter unit, to a volume of about 2 mL. The bound and unbound fractions were further concentrated in the Centricon-10 UF units. Final volume was approximately 0.5 mL per UF unit. The profiles of the bound and unbound protein components were compared,

by Native-PAGE, to the enzyme control of Peak D that was not passed through the column.

(e) Gel Filtration: The combined fractions from the chromatofocusing step corresponding to the peak at pI 4.8 were run on the Superose-12 gel filtration column using the Pharmacia FPLC system. The eluent buffer was 0.05 M sodium citrate buffer pH 4.8, at a flow rate of 0.1 or 0.05 mL/min.

(VI) CHARACTERIZATION OF THE EXOGLUCANASE:

(a) Effect of pH on Peak D: Peak D protein was buffer-exchanged on a Biogel P6G column with 0.05 M citrate buffer, pH 4.8 as the eluent. Final protein concentration was about 2.2 mg/mL as determined by the Lowry method (Lowry et al., 1951). Triplicate tubes were prepared containing 1.9 mL 0.05 M citrate buffer adjusted to various pH's. Enzyme was added to each sample to obtain a total volume of 2.0 mL, and an enzyme concentration of approximately 0.1 mg/mL. Tubes were heated in a 50°C water bath, a 50 mg strip of filter paper was added to each tube, and the filter paper activity of each sample was determined by the Forintek method. Percentage activity relative to the controls at pH 4.8 were determined.

(b) Substrate Specificity: Activities towards Avicel, carboxymethylcellulose, salicin, cellobiose and filter paper were performed as described in the Assays section.

(c) Assessment of Synergism between the Exoglucanase and Endoglucanase Components: Ten μL of the purified exoglucanase (approximately 1 mg/mL, estimated from the purification steps) and ten μL of the endoglucanase-rich Fraction 26 (from chromatofocusing run in the pH gradient 6.5-4.0) were assayed, either individually or combined, in an extended filter paper assay. The procedure for the Forintek method was followed, but, because the amounts of reducing sugars detected after a one-hour incubation were negligible, the tubes were incubated for 24 hours. Glucose and cellobiose production was determined by HPLC analysis.

(d) Effect of Added Cellobiohydrolase on Filter Paper Hydrolysis by T. harzianum E58 Cellulases: Tubes containing 40 or 200 μg (10 or 50 μL; 4mg/mL) of total T. harzianum E58 cellulases, in a final volume of 2.0 mL of 0.05 M citrate buffer pH 4.8, were prepared. At these concentrations, cellulases were below the purported saturation level of 20 FPU/g cellulose. Ten or twenty μL of the purified cellobiohydrolase were added to duplicate tubes of T. harzianum E58 cellulases, and the tubes were incubated

with filter paper for 25 hours. Glucose and cellobiose production was determined by HPLC.

RESULTS AND DISCUSSION:

The first part of this thesis examined the filter paper assay, the assay most commonly used to assess the cellulolytic potential of fungi, bacteria, or their enzymes. The activity obtained for Novo-Celluclast, by a proposed standard assay, was compared with the results from several other laboratories, and also with the activity obtained by the Forintek method used at this laboratory. The effectiveness of the filter paper assay as a means of measuring long-term hydrolysis of other substrates was also examined.

The occurrence of multiple forms of cellulases produced by T. harzianum E58 and the differences in their substrate specificities were investigated. Cellulase components possessing endoglucanase activity produced by T. harzianum E58 grown on the commercial cellulose, Solka Floc, were isolated to verify the presence of multiple endoglucanase components as observed with many other fungal systems. These enzymes were assayed for their specificities towards both β-D-glucans and β-D-xylans by a zymogram technique, to see if a variation in specificities of these endoglucanase components exists.

Endoglucanase forms are generally more numerous than exoglucanase forms in a cellulase mixture, however, it has

been suggested that the latter enzymes make up a large percentage of the total protein (Hayn and Esterbauer, 1985). Exoglucanase components are not readily detected by zymogram technique due to interfering activity by some endoglucanases (van Tilbeurgh and Claeyssens, 1985) and xylanases (Gilbert et al., 1988) on the fluorogenic substrate used. Therefore, an exoglucanase was purified and characterized. The action of this enzyme with the other cellulase components during the hydrolysis of cellulose was examined and discussed in the context of the current theory of hydrolysis.

(I) HYDROLYSIS:

(a) Standard Filter Paper Assay:

The purpose of any enzyme assay is to determine the amount of enzyme required to produce a given amount of product under defined conditions. However, the influence of structural differences between celluloses, such as Solka Floc, filter paper, and the probable bioconversion celluloses, such as steam-treated wood, makes it difficult to extrapolate the hydrolytic potential of a cellulase from the filter paper activity.

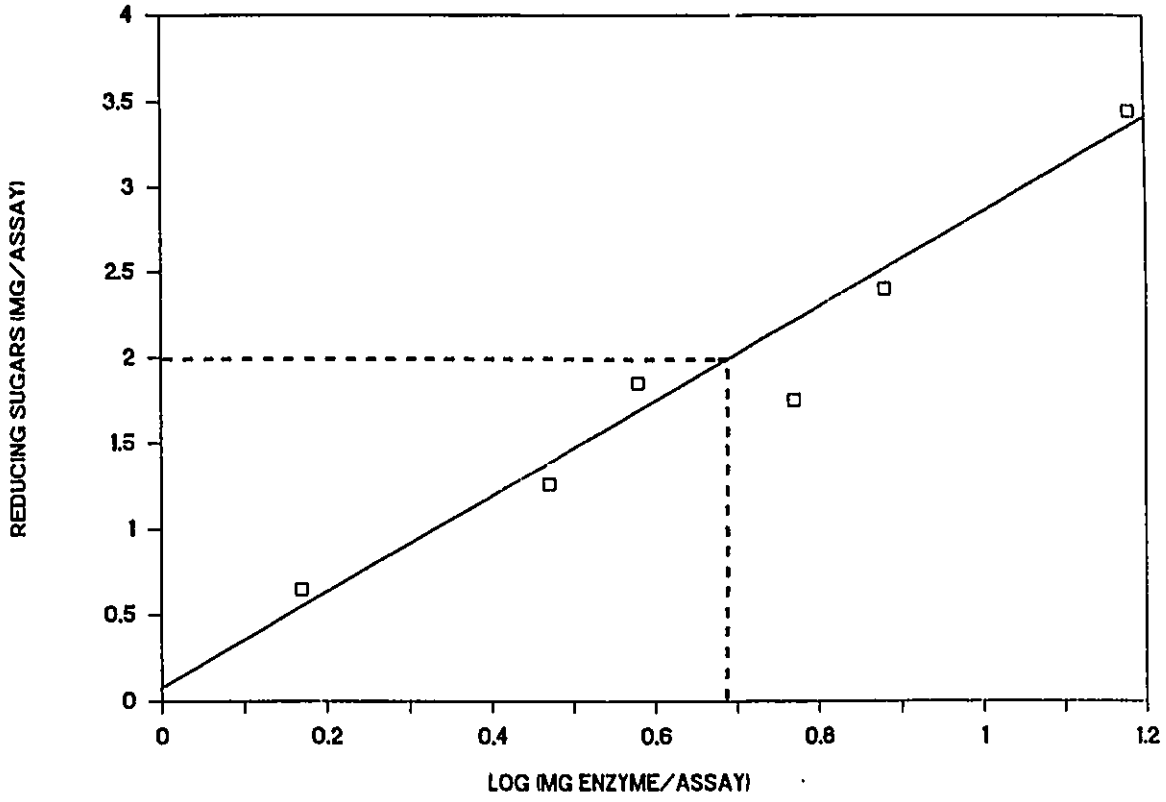
Most laboratories use their own version of the filter paper assay to determine cellulolytic activity, generally modifications of the Mandels' method (Mandels et al., 1976), making it extremely difficult to compare results. Recently,

Forintek participated in an International Energy Agency (IEA) discussion where it was agreed by several international laboratories to perform the filter paper assay under identical conditions using the same batch of enzyme.

Samples from a common batch of a commercial enzyme preparation, Novo-Celluclast, were sent to several laboratories to test the reproducibility of the filter paper assay. The method specified by the International Union of Pure and Applied Chemistry (IUPAC) determines the amount of enzyme required to produce 2.0 mg reducing sugars from a 50 mg strip (1 x 6 cm) of Whatman No. 1 filter paper in one hour at 50°C (Ghose, 1987). A straight line is obtained when reducing sugar production is plotted versus the logarithm of the amount of enzyme preparation that is added. Two milligrams of sugar corresponds to a 4% conversion of the filter paper strip to reducing sugars. This value is used because it allows for greater conversion than can be expected from the readily hydrolyzed amorphous component alone, which could result in values that are erroneously high. A 4% conversion is also not sufficiently high for end-product inhibition to become a factor.

The amount of Novo-Celluclast enzyme preparation required to produce 2.0 mg reducing sugars from filter paper by the IUPAC method was the inverse logarithm of 0.69 mg, which corresponds to 4.9 mg of enzyme preparation (Fig. 8).

FIGURE 8: Effect of Enzyme Loading of Novo-Celluclast on Reducing Sugar Production in IUPAC Filter Paper Assay (Filter Paper activity is based on the amount of enzyme preparation required to produce 2.0 mg of glucose equivalents in a one hour assay. One filter paper unit corresponds to micromoles of glucose equivalents produced per minute.)



When filter paper units are expressed as micromoles of glucose equivalents produced per minute, the release of 2.0 mg glucose in one hour gives a value of 0.185 μmole min^{-1}, and consequently, 4.9 mg enzyme corresponds to an activity of 38 FPU/gram of the enzyme preparation.

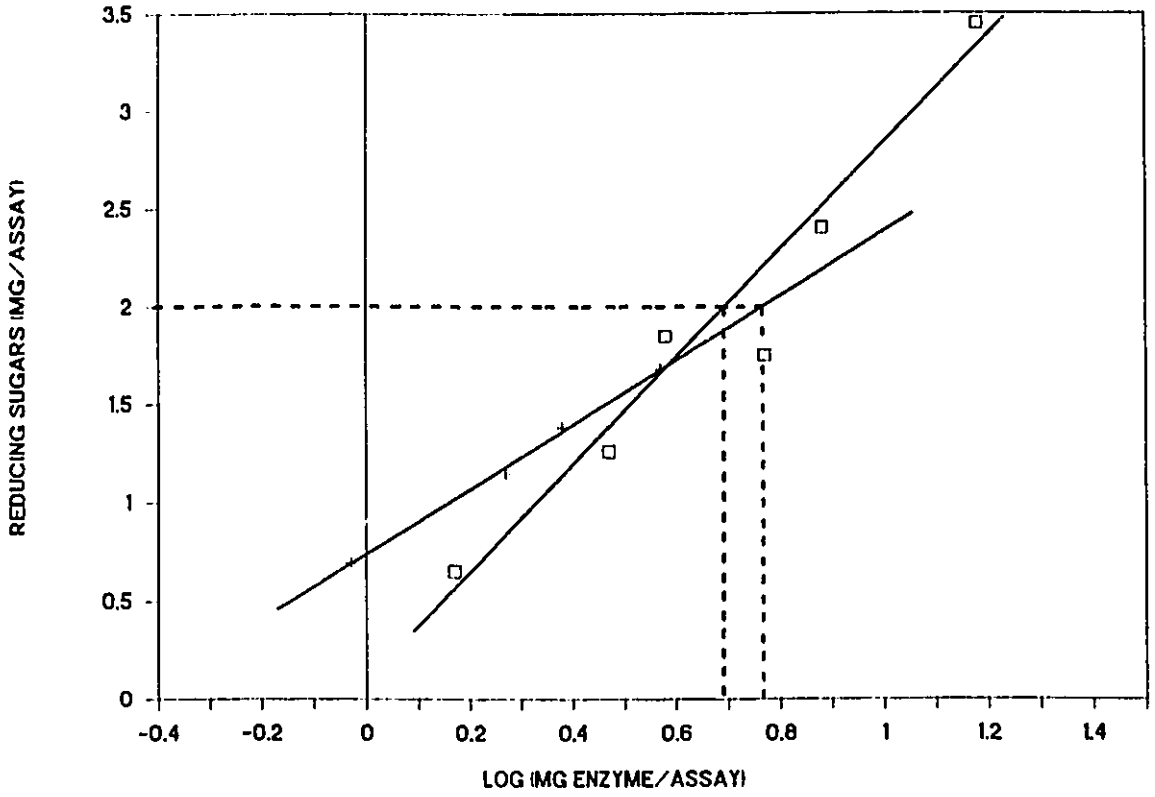
When laboratories in Austria, France, and New Zealand carried out a round robin comparison as part of the IEA network, values of 45, 48, and 57 filter paper units per gram of Novo-Celluclast enzyme preparation were obtained, respectively.

As the samples from the Novo-Celluclast batch were identical, it is unlikely that enzyme variations were responsible for the discrepancies in the reported filter paper activities. It is probable that slight differences in incubation temperature or pipet volume accounted for the variations in results reported by the laboratories. The uniformity and equivalency of the filter paper which was used as the substrate may have also been a factor. Whatman No. 1 filter paper is made from a milled cotton cellulose, and it is possible that slight variations may occur from batch to batch as a result of the milling process. Inter-laboratory comparison of results was possible, but actual values are not necessarily reproducible.

(b) Comparison of Two Filter Paper Assay Methods: The cellulase activity of the Novo-Celluclast was compared by the IUPAC and Forintek methods. The Forintek method eliminates the addition of 20 ml wa' r prior to reading the absorbance, therefore, less dilute enzyme preparations can be assayed than can be determined by the IUPAC method. This allows the monitoring of cellulase production in culture filtrates during the early stages of growth of the fungal culture. During this phase, reducing sugar production from filter paper may be lower than that specified by the IUPAC method.

The reducing sugars released from filter paper by the action of Novo-Celluclast differed between the two filter paper assay methods (Fig. 9). A range of amounts of Novo-Celluclast enzyme preparation were used which would result in values which could be assayed directly by both methods. The amount of enzyme required to produce 2 mg of reducing sugar was determined. The values obtained for the logarithm of the enzyme concentration, in milligrams per assay, were 0.77 and 0.69 for the Forintek and IUPAC methods, respectively. These values are equivalent to 5.9 mg enzyme required to produce 2 mg reducing sugars by the Forintek method, or 31 FPU/g, and 4.9 enzyme, or 38 FPU/g by the IUPAC method. However, the amount of enzyme required and the amount of reducing sugars are based on a "per assay"

FIGURE 9: Comparison of Reducing Sugar Production from Filter Paper by IUPAC and Forintek Methods in Assaying Filter Paper Activity by Equivalent Amounts of Novo-Celluclast Enzyme Preparation
IUPAC method (□ - □), Forintek method (+ - +)
(Filter Paper activity is based on the amount of enzyme preparation required to produce 2.0 mg of glucose equivalents in a one hour assay. One filter paper unit corresponds to micromoles of glucose equivalents produced per minute.)

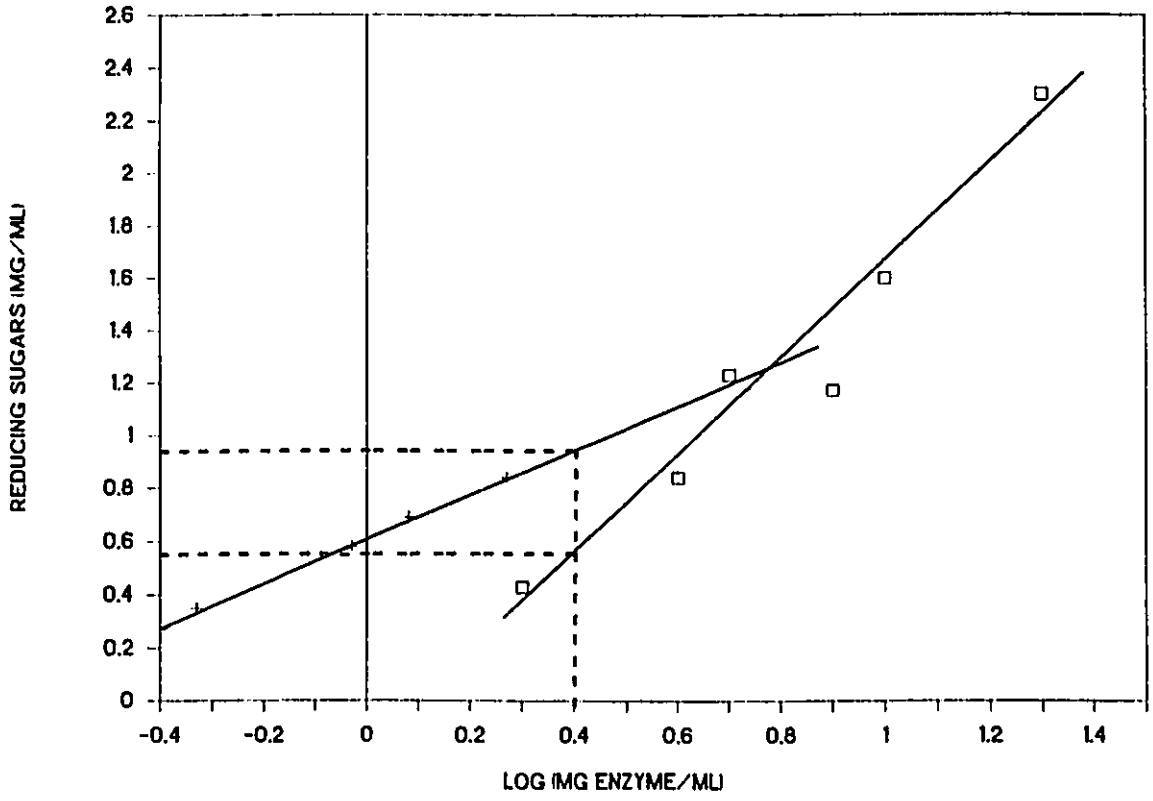


basis. This may be misleading since the reaction volumes differ with the two assays, and as a result, the ratio of enzyme/product concentrations are not equal.

Reducing sugar production was also compared at equivalent enzyme concentrations (Fig. 10). At a value of 0.4 on the X-axis, corresponding to 2.5 mg enzyme preparation per milliliter, a reducing sugar concentration of about 0.58 mg/ml was obtained by the IUPAC assay, while a value of 0.95 mg/ml was obtained with the Forintek method. This corresponded to a difference in hydrolysis of about 40%. The filter paper activity values obtained on a "per assay" basis were more similar for the two methods than the values obtained at equivalent concentrations.

The filter paper strip was used at a constant size and weight per assay tube but the reaction volume differed for the two assays. Therefore, it was apparent that the Forintek samples actually contain 33% more enzyme per unit cellulose than the IUPAC samples at equal concentrations of enzyme. This means that more enzyme was available for adsorption to the substrate in the Forintek samples. Unlike reactions with soluble substrates, the interaction between the adsorbed enzyme and the substrate result in a concentrating effect of the enzyme around and on the substrate. The initial enzyme concentrations of cellulase in the two assay methods were equal, but, as adsorption

FIGURE 10: Comparison of Reducing Sugar Production from Filter Paper by IUPAC and Forintek Methods in Assaying Filter Paper Activity at Equivalent Concentrations of Novo-Celluclast Enzyme Preparation
IUPAC method (□ - □), Forintek method (+ - +)
(Filter Paper activity is based on the amount of enzyme preparation required to produce 2.0 mg of glucose equivalents in a one hour assay. One filter paper unit corresponds to micromoles of glucose equivalents produced per minute.)



proceeded, the surface concentration of the Forintek method's samples would have exceeded that of the IUPAC samples. Increased surface concentration of cellulases could have a significant effect, as some of the enzymes are thought to be ineffectively bound to the substrate (Rabinovitch et al., 1982; Chernoglazov et al., 1984). The formation of cellulase complexes on the surface of the cellulose is supported by some researchers (Fagerstam and Petterson, 1980; Wood and McCrae, 1979). The probability of formation of functional complexes would increase with increased surface concentration. In addition, the presence of more enzyme implies less inhibition by the product.

In the same manner, it is apparent that the substrate concentration is greater in the IUPAC method, due to the smaller volume. Increased substrate concentrations in the filter paper assay have been shown to result in an increase in apparent activity (Mandels et al., 1976). With soluble substrates, reactions are performed such that the substrate concentration is not limiting, however, this is not as easily arranged when insoluble substrates are used. The effective concentration of an insoluble substrate as viewed by the cellulase enzymes is a function of the available surface area. Increasing the available surface area causes an increase in mass which results in mass-transfer problems.

For this reason, filter paper strips of standard size are used in the assays.

The extent of hydrolysis measured by the IUPAC method during the assay also had a significant effect on the calculated filter paper activity of the Novo-Celluclast. The linear plot demonstrated that the amount of enzyme required to produce 1.0 mg reducing sugars was less than half the amount required to produce 2.0 mg reducing sugars from filter paper, particularly by Mandel's method. An enzyme concentration of 4.5 mg/ml was required to produce 1 mg/ml of reducing sugars, but more than three times this amount of enzyme (15.5 mg/ml) was required to produce twice the concentration of reducing sugars. When the end-point product concentration is lowered, more emphasis is placed on the hydrolysis of the more readily hydrolyzed amorphous components of the substrate (Mandels et al., 1976).

It is apparent that the amount of enzyme required to hydrolyze crystalline cellulose is high. In this one-hour hydrolysis, the amount of enzyme used is in the same order of magnitude as the product concentration. This reflects the requirement that cellulases must first be adsorbed to the substrate surface, unlike reactions involving soluble substrates, before hydrolysis can take place. Also, the amount of non-cellulase components present in the enzyme mixtures is unknown and low activities may be obtained for

highly active cellulases that are contaminated with high concentrations of other proteins. An assay method that can distinguish between cellulase protein and non-cellulase protein would give a better indication of the true cellulolytic activity of an enzyme mixture than the traditional filter paper assay.

It is apparent from these results that the IUPAC method does not result in more reproducible values for inter-laboratory comparison than other established procedures. However, a standard procedure must be established. The temperature and length of the assay should remain at 50°C and one hour. The extent of conversion of the original substrate to reducing sugars should be around 2-4% in order to limit the effects of enzyme inhibition and amorphous cellulose content on the assay. Since the IUPAC method necessitates a dilution step, with mixing and settling time adding approximately one hour to the assay, the Forintek method was used throughout the rest of this research program. Since filter paper activities by the two methods were more comparable when enzyme and reducing sugars were compared by their amounts in the assay, rather than by concentrations, researchers must agree upon a standard reaction volume.

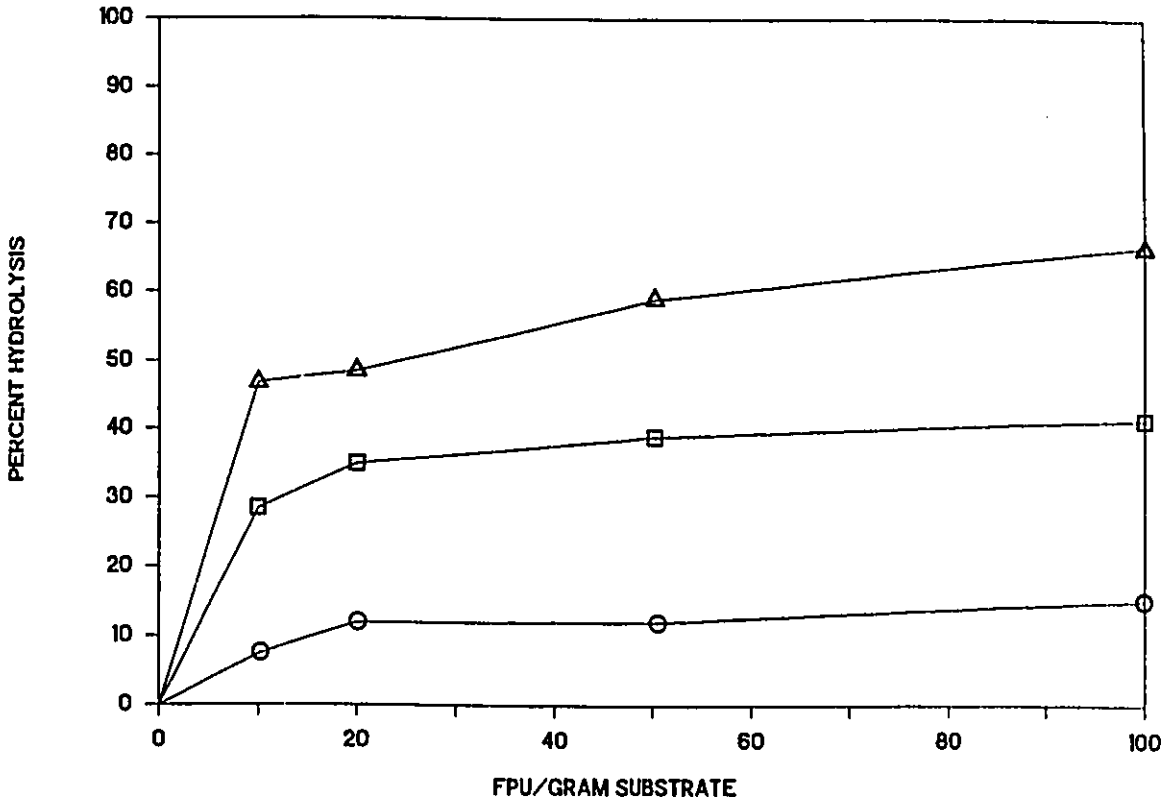
(c) The Ability of the Filter Paper Assay to Predict the Hydrolytic Potential of Cellulase Preparations on Various Cellulosic Substrates: The activity of a cellulase preparation should be of use in determining the ability of the enzymes to hydrolyze cellulose over extended periods of hydrolysis. The amount of reducing sugars released after incubation of 2% Sigmacell with increasing concentrations of Novo-Celluclast was examined. The shapes of the curves for 4-, 24- and 72-hour hydrolyses were similar.

The extent of hydrolysis increased with enzyme loading until about 20 filter paper units per gram of cellulosic substrate (FPU/gram), at which point increased addition of enzyme had little effect on the degree of hydrolysis (Fig. 11).

The levelling-off of the extent of hydrolysis, with increased enzyme loading, above 20 FPU/gram suggested that the available cellulose surface had become saturated with cellulase enzymes. As the surface area available to the cellulase enzymes has a finite value, it would limit the extent of hydrolysis by the enzymes.

Correlations between the available surface area measured by the solute exclusion method and the initial reaction rate of cellulose hydrolysis have been calculated by other workers (Weimer and Weston, 1985; Stone et al., 1969; Grethlein, 1985). The diameters of the cellulase

FIGURE 11: The Percent Hydrolysis of 2% Sigmacell after Incubation with Increasing Concentrations of Novo-Celluclast for 4 (○ - ○), 24 (□ - □) and 72 hours (△ - △) (Duplicate Samples)

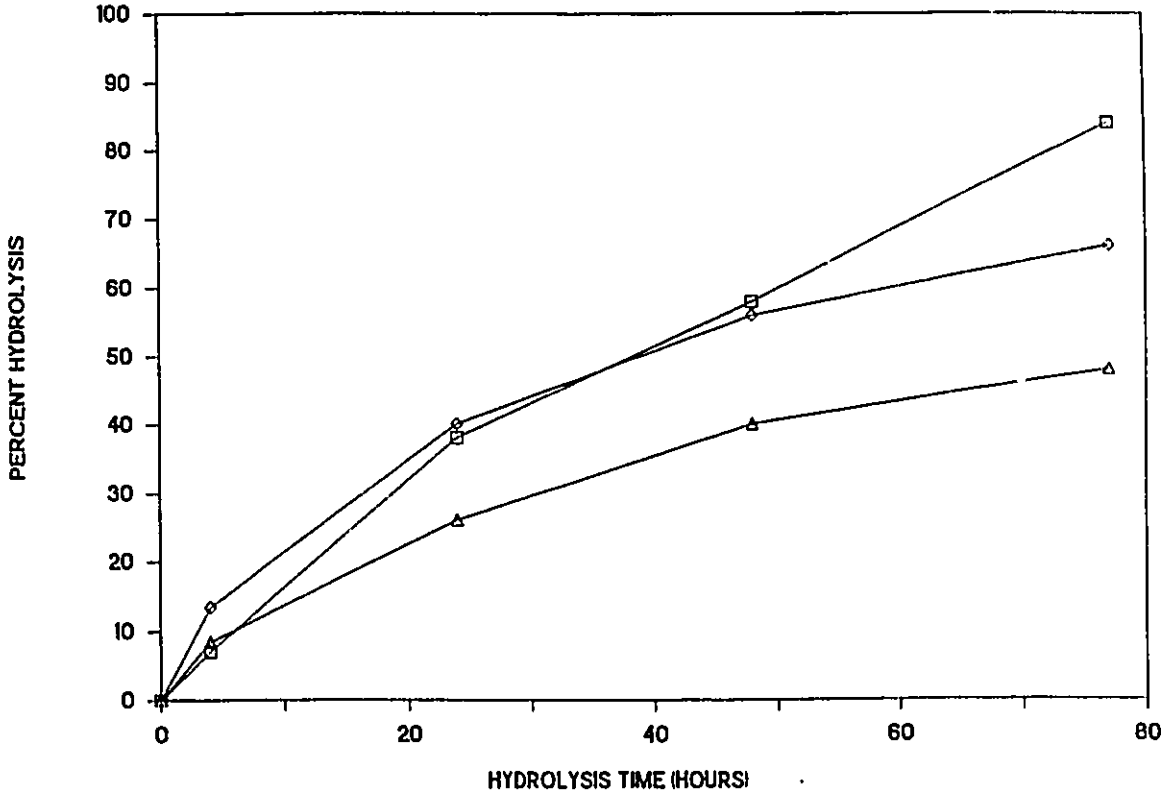


enzymes can be estimated by this correlation, and therefore, the physical space that the enzymes occupy on the substrate can also be calculated. As the adsorption of cellulases on cellulose follows the Langmuir isotherm of a monomolecular layer (Goel and Ramachandran, 1983), an increase in the amount of adsorbed enzyme should correspond to greater hydrolysis. This has been observed on pretreated poplar, where a six-fold increase in the amount of enzyme adsorbed to the pretreated cellulose resulted in a significant increase in hydrolysis (Grethlein, 1985).

A fixed concentration of Novo-Celluclast was then added to several cellulosic substrates to detect differences in their hydrolysis profiles. The extents of hydrolysis of filter paper, Solka Floc and Sigmacell, by 20 FPU of Novo-Celluclast per gram of cellulose, were compared over time (Fig. 12). The rates of reducing sugar production slowed after about twenty-four hours for each substrate. This could be the result of various factors, such as enzyme inactivation due to denaturation, end-product inhibition, increased recalcitrancy of the substrate as the more readily hydrolyzed portions are removed, or site blockage by inactivated enzymes.

Although an identical concentration of Novo-Celluclast was added to each substrate, differences in the reducing sugars released from filter paper, Sigmacell and Solka Floc

FIGURE 12: Reducing Sugars Released from Various Cellulosic Substrates (0.5% w/v) after Extended Hydrolysis with 20 FPU/G Novo-Celluclast with Added Novozym: Filter Paper (□ - □), Solka Floc BW 300 (◇ - ◇) and Sigmacell (△ - △) (Duplicate Samples)

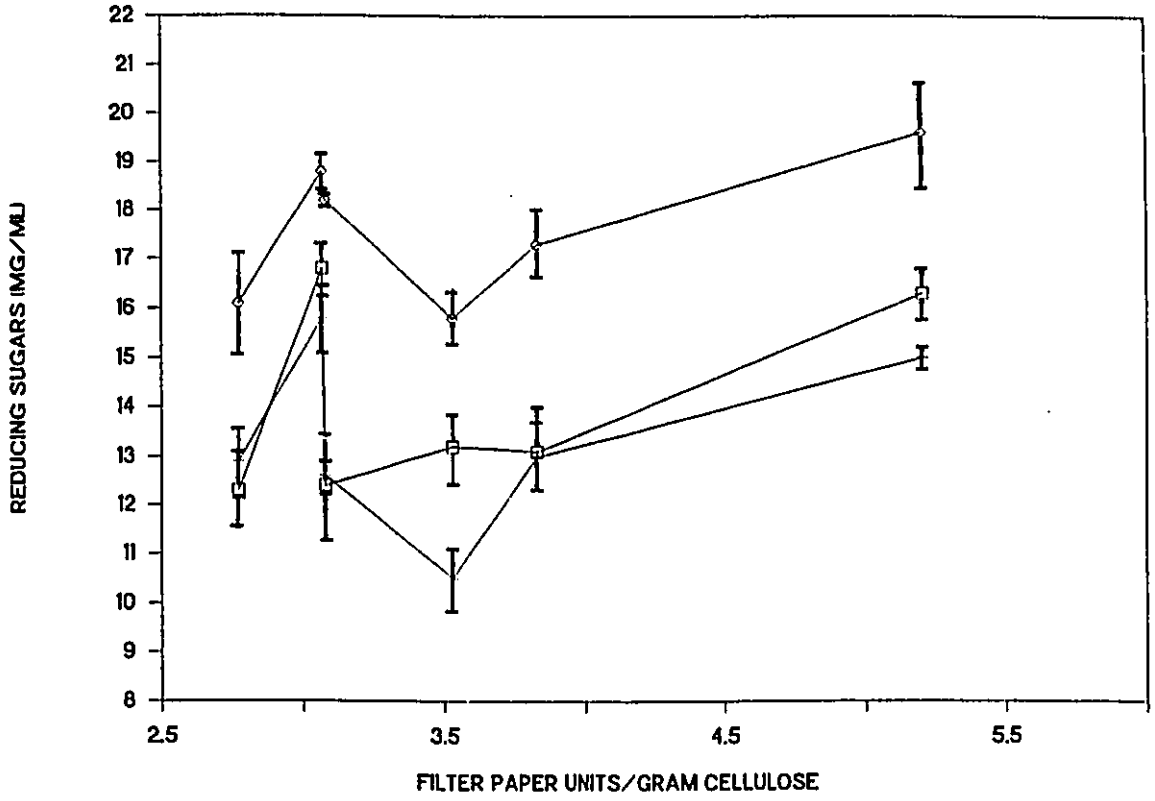


BW300 were observed. Filter paper and Solka Floc showed a similar extent of hydrolysis over the first two days, however, Solka Floc hydrolysis greatly decreased in the third day. Sigmacell was the least susceptible to enzymatic breakdown. Since the curves differed for the three substrates, there was no reason to assume that hydrolysis of one cellulosic substrate could be used to estimate hydrolysis of another.

The ability of the one-hour filter paper assay to reflect the hydrolysis of cellulosic substrates in twenty-four hours when using more than one batch of enzyme was next examined. The cellulase activities of the culture filtrates from six batches of T. harzianum E58, grown on different cellulosic substrates, were determined by the Forintek filter paper assay. Filter paper activities of less than 10 FPU/g were chosen because the concentrations at the lower part of the curve may be linear with percent hydrolysis (Fig. 11).

There was no correlation between the extent of hydrolysis after twenty-four hours and the filter paper activity of these enzymes over a range of 2-6 FPU/g (Fig. 13). Filter paper after one-hour hydrolysis will likely differ structurally from filter paper that has undergone extended hydrolysis. The physical nature of the cellulosic substrate is known to change during hydrolysis,

FIGURE 13: Relationship between Filter Paper Activity, by the Forintek Method, of Six Different Batches of Trichoderma harzianum Cellulases and Long-Term Reducing Sugar Production from Filter Paper (□ - □), Solka Floc BW 300 (◇ - ◇) and Avicel (+ - +)
(Duplicate Samples)



therefore the short-term and long-term hydrolyses may not show any correlation, even on the same substrate.

Differences in the extent of hydrolysis of the three substrates were obvious. For example, two enzyme preparations had similar activities of about 3 FPU/g and therefore, produced equivalent amounts of reducing sugars over the one-hour filter paper assay. However, after a twenty-four-hour hydrolysis of filter paper, one enzyme preparation released 12 mg/ml reducing sugars from filter paper, while the other one released 17 mg/ml reducing sugars, corresponding to a difference of about 30% hydrolysis. Solka Floc was more readily hydrolyzed than filter paper or Avicel, by most of the enzyme preparations used (Fig. 13).

Differences in β-glucosidase activity in the batches of enzymes may result in differences in filter paper activity, and in the extent of hydrolysis (Bailey, 1981). The reducing sugar production from filter paper, by the six batches of T. harzianum E58 cellulases, was plotted against cellobiase activity to detect if this activity were the limiting factor affecting hydrolysis (results not shown). There was no correlation between the two, therefore differences in hydrolysis are dependent on the interaction between the substrate and the adsorbable cellulase components.

The DNS assay, which is usually used to determine the filter paper activity of an enzyme preparation, does not distinguish between the different reducing sugars released from the substrate. Measurements of glucose equivalents by the DNS assay may mask differences in the profile of sugars produced. Although it would be desirable to measure the specific hydrolysis products, standards of oligosaccharides are not readily available.

(e) Differential Analysis of Hydrolysis Products: The differential hydrolysis of filter paper, α-floc, Avicel, and Solka Floc by the endoglucanase and exoglucanase components was determined by monitoring glucose and cellobiose production by high pressure liquid chromatography (HPLC). At each concentration, the β-glucosidase activity was constant, therefore, differences in glucose and cellobiose production from each substrate was dependent on the ability of the endoglucanase and exoglucanase components to adsorb to and hydrolyze each of the substrates.

The glucose (Fig. 14) and cellobiose (Fig. 15) production from these four different substrates, with increasing T. harzianum E58 enzyme loadings up to 20 FPU/g cellulose, was measured. Glucose production (Fig. 14) and the total reducing sugar production (Fig. 16) were approximately linear for all substrates, over the range of

enzyme concentrations assayed. However, cellobiose production quickly levelled off at about 15 μL of enzyme preparation, or 7 FPU/g cellulose. It could be concluded that β-glucosidase activity was not limiting, otherwise the cellobiose would accumulate and its concentration would increase, rather than level off.

Although the patterns of glucose and cellobiose production from filter paper, α-floc, Avicel, and Solka Floc were similar, the rates of hydrolysis were different. For this particular enzyme preparation, filter paper was the most readily-hydrolyzed substrate. (It was seen in Fig. 13 that most preparations hydrolyzed Solka Floc more easily than filter paper, but this would depend on the characteristics of the cellulase forms present in each batch.) The slopes of glucose production and the levels of cellobiose attained varied with the different substrates, even though the composition of the added enzyme mixture remained constant. Structural factors of the substrates determined the extent of hydrolysis by the T. harzianum E58 cellulases.

It appears that the factors that affect the hydrolysis of the substrates by the various cellulase components remain relatively constant throughout hydrolysis, as evidenced by the similarity between the slope of glucose production from filter paper after twenty-five hours and the slope of

FIGURE 14: Influence of Increased Enzyme Loadings of T. harzianum on Glucose Released from Filter Paper (□ - □), Avicel (+ - +), Solka Floc BW 300 (◇ - ◇) and α-floc (▽ - ▽) after 25 Hours Hydrolysis at 50°C
(Triplicate Samples, Analyzed Twice)

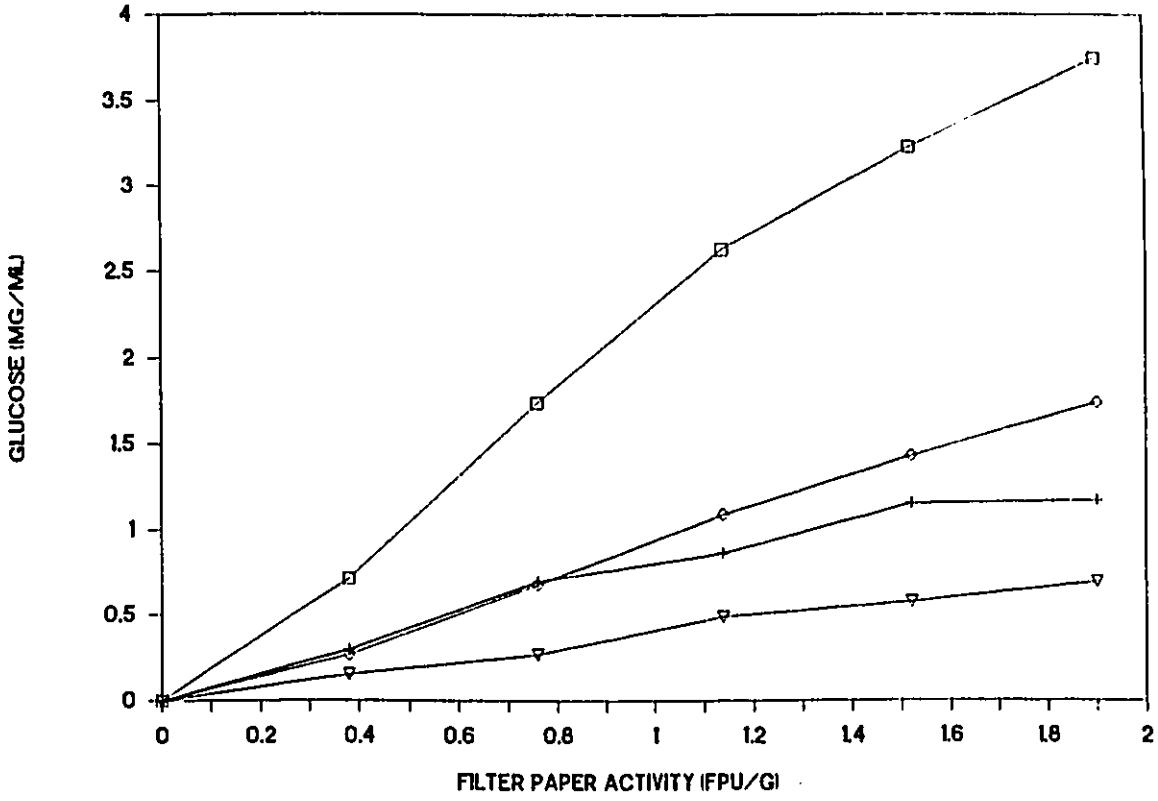


FIGURE 15: Influence of Increased Enzyme Loadings of T. harzianum on Cellobiose Released from Filter Paper (□ - □), Avicel (+ - +), Solka Floc BW 300 (◇ - ◇) and α-floc (▽ - ▽) after 25 Hours Hydrolysis at 50°C
(Triplicate Samples, Analyzed Twice)

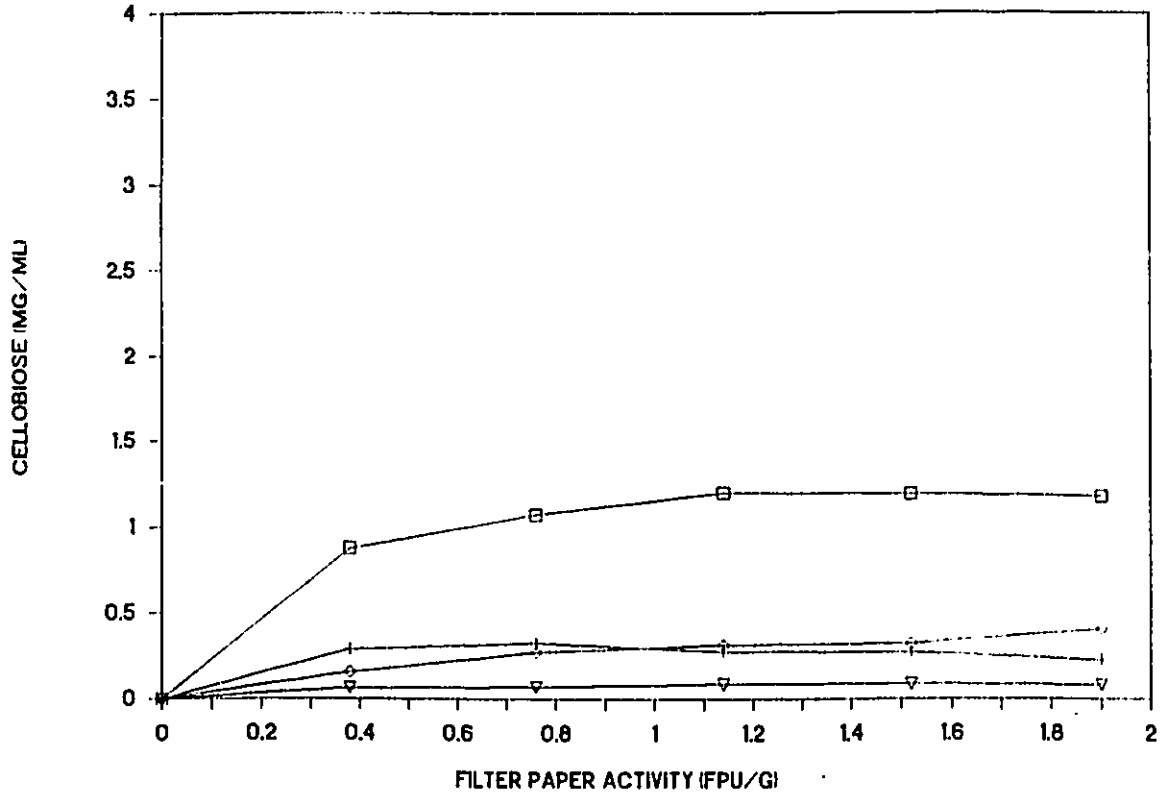
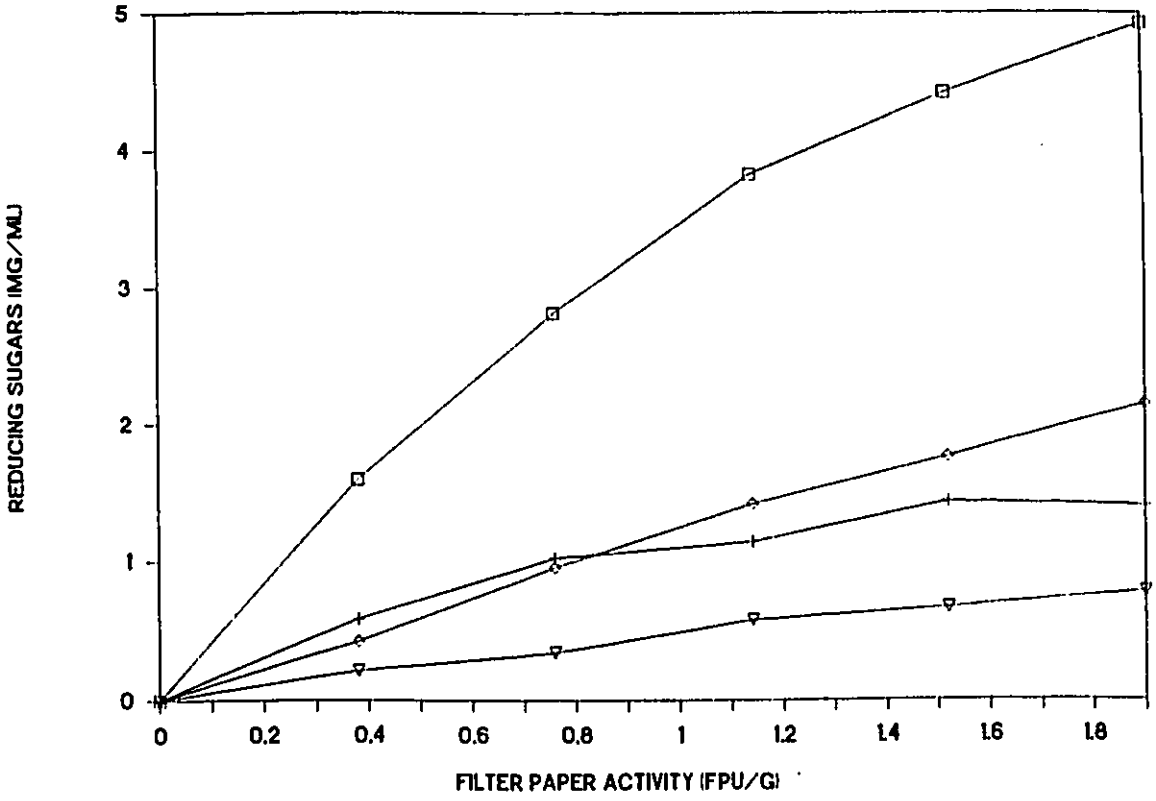


FIGURE 16: Influence of Increased Enzyme Loadings of T. harzianum on the Reducing Sugars Released from Filter Paper (□ - □), Avicel (+ - +), Solka Floc BW 300 (◇ - ◇) and α-floc (▽ - ▽) after 25 Hours Hydrolysis at 50°C (Triplicate Samples, Analyzed Twice)



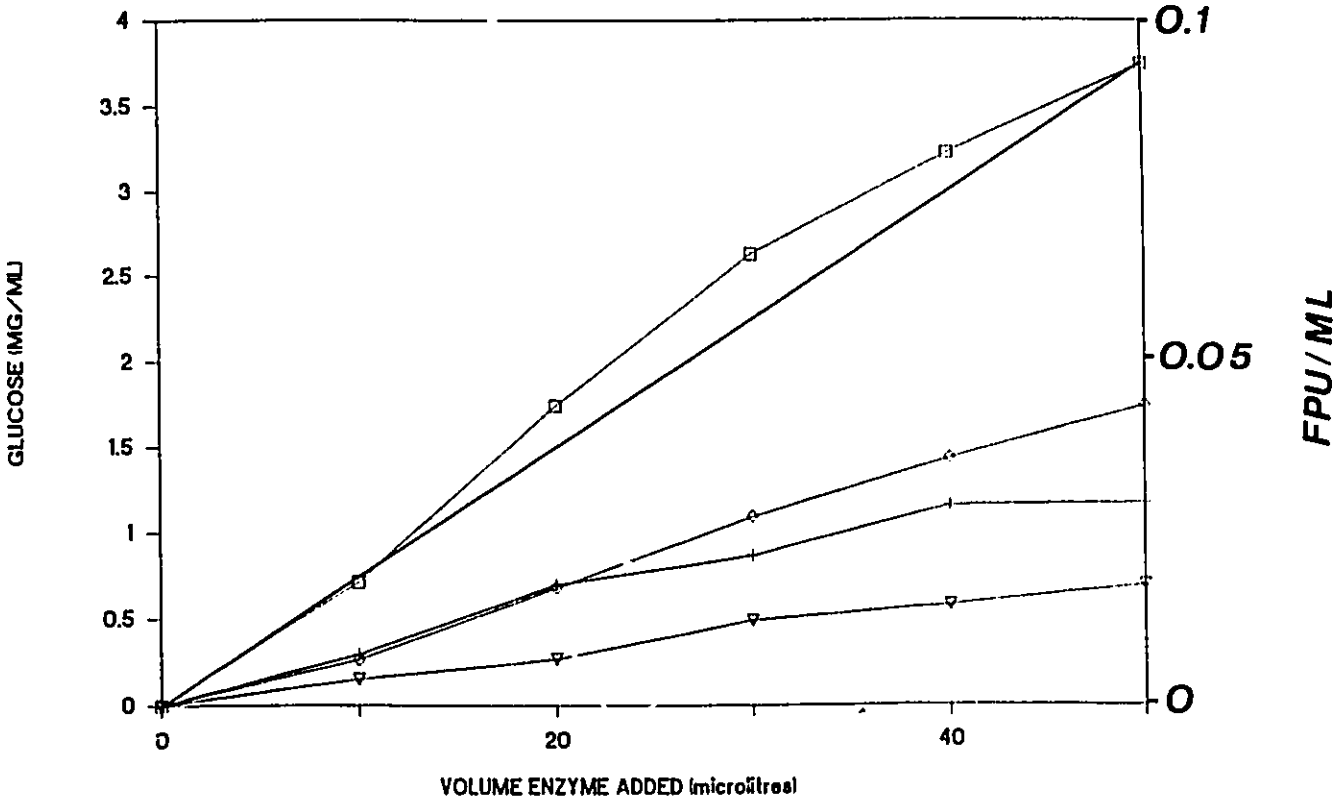
(Reducing sugars were calculated from the sum of glucose and cellobiose produced during hydrolysis as measured by HPLC. Other saccharides were negligible.)

reducing sugars released, described as IU/mL, in the one-hour filter paper assay (Fig. 17).

For a given substrate, there appears to be a relationship between its susceptibility to a given enzyme preparation and the concentration of the enzyme preparation. This seems to be more important than other factors such as heat inactivation and end-product inhibition which would have a significant impact on long-term hydrolysis. The slopes for long-term glucose production from the other substrates were dissimilar to the slope for filter paper activity indicating that this relationship is inherent in each substrate. These results are supported by those of Lee and Fan (1982), who showed that the enzymes that were initially adsorbed onto the substrate were responsible for the extent of hydrolysis. Therefore, the array of enzyme components responsible for the hydrolysis of filter paper in one hour would be identical to that hydrolyzing filter paper over twenty-four hours. The composition of the adsorbed cellulase components, or the ability of these enzymes to hydrolyze the substrate once adsorbed, will differ on other cellulosic substrates, due to the effect of substrate factors on their adsorption (Beldman et al., 1986; Chernoglazov et al., 1987; Rabinovitch et al., 1982).

It seems that the predictability of long-term hydrolysis by a one-hour assay on the same sample is only

FIGURE 17: Comparison of the Calculated Filter Paper Activity (solid line) and the Glucose Released During a 25-Hour Hydrolysis when Increasing Amounts of A Trichoderma harzianum Enzyme Preparation were added to Filter Paper (□-□), Avicel (+-+), Solka Floc (◇-◇) and α-floc (▽-▽)
(The enzyme preparation contained 4 mg protein per mL buffer.)



Filter Paper activity was determined by the DNS colorimetric analysis of hydrolysis products released during the one-hour Forintek filter paper assay.

feasible when dilutions of the same batch of enzyme are used. Different batches of enzymes possess different ratios of cellulase components and each of the various cellulase components produced may differ in substrate specificity.

In order to gain a better understanding of enzyme-substrate interactions during enzymatic hydrolysis of cellulose, it would be desirable if one could isolate or purify the various cellulase components and differentiate them with respect to their individual adsorption and substrate specificities.

(II) PURIFICATION AND CHARACTERIZATION OF THE ENDOGLUCANASE COMPONENTS OF T. HARZIANUM E58:

Several groups have tried to elucidate the synergistic interaction of the various enzyme components required for efficient cellulose hydrolysis by purifying the individual enzymes and adding them back in varying proportions (Wood and McCrae, 1979; Beldman et al., 1988a). This work has shown that the synergistic interaction between endoglucanases, exoglucanases and β-glucosidases is necessary for efficient degradation of cellulosic substrates. The necessity for a multiplicity of these enzymes exhibiting similar activities has yet to be fully understood.

Evidence that cellulases possess the capability to hydrolyze both β-1,4-glucans and β-1,4-xylans was obtained through competition experiments (Toda, Suzuki and Nisizawa, 1971). Using purified endoglucanases from T. viride they showed that the endoglucanase and xylanase activities occurred at the same active site. This indicates the possibility of a third group of cellulase enzymes, that are non-specific enzymes with activity towards both β-D-glucans and β-D-xylans. Evidence of these non-specific endoglycanases has been observed in other microorganisms. Three endoglucanases of Streptomyces viridosporus were observed to possess activity towards xylan (Ramachandra et al., 1987). A xylanase purified from Clostridium thermocellum showed significant activity towards carboxymethylcellulose and acid-swollen cellulose (Lee et al., 1987). Three of the six endoglucanases and one of the three exoglucanases isolated from T. viride were shown to be capable of hydrolyzing xylan in addition to cellulose, with varying specificities (Beldman et al., 1988b). Evidence of cloned cellulases that possess activity towards both β-D-glucans and β-D-xylans has been reported which indicates that specificity to both substrates can be inherent in a protein and not solely from complex formation.

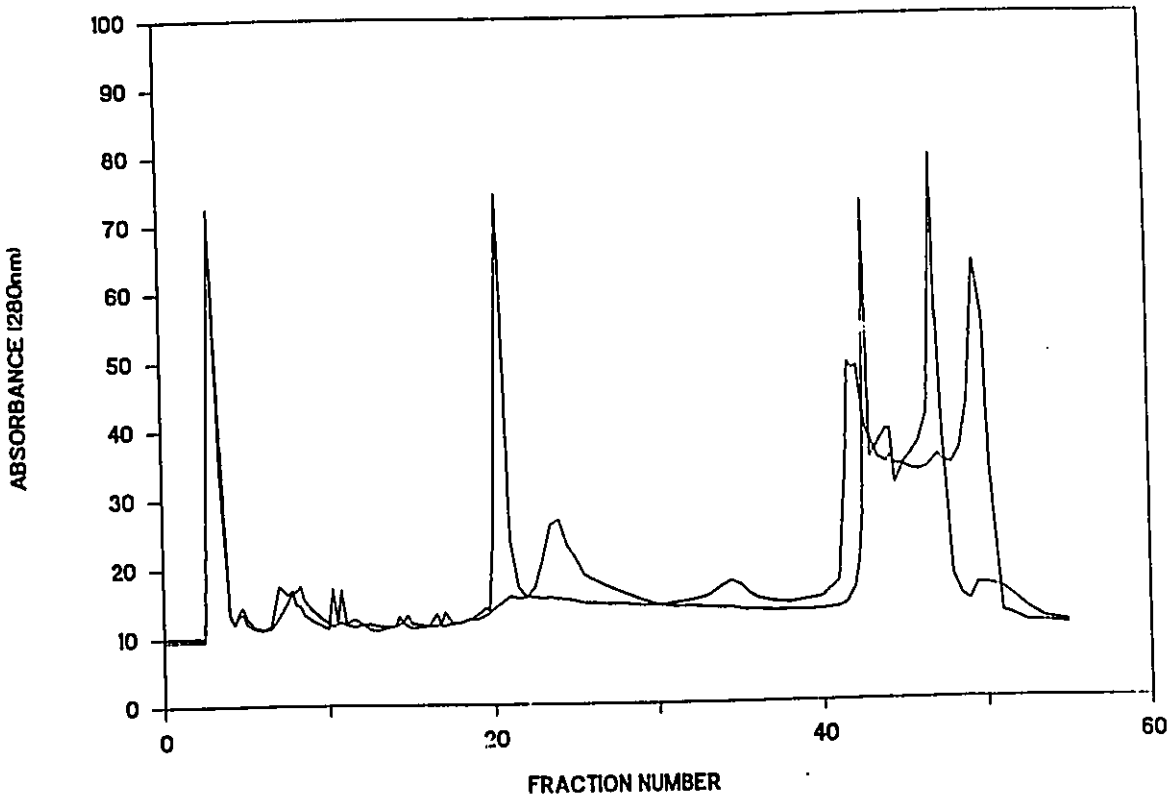
To try to determine the key components of the T. harzianum E58 cellulase system, a purification regime aimed

at characterizing some of these main cellulase components was followed. The cellulase components of a T. harzianum E58 preparation grown on Solka Floc were characterized and the multiplicity of the cellulases and their substrate specificities were examined.

Chromatofocusing (Sluyterman and Elgersma, 1978) was chosen as a preliminary separation step in the isolation of the cellulase components of T. harzianum E58 due to the ease of the method and minimal sample preparation. It has previously been shown to be effective in the separation of fungal extracellular enzymes (Thomas et al., 1983; Hayn and Esterbauer, 1985; Ridout et al., 1988).

A cellulase enzyme preparation from T. harzianum E58 grown on Solka Floc for three days was separated by FPLC using a pH gradient of 6.5-4.0 (Fig. 18). This technique was used in attempts to detect differences in adsorption of individual cellulase components on several cellulosic substrates, in response to substrate factors. Control runs were performed to test the reproducibility of the separation process and peak areas. Two identical runs were performed using the same enzyme. The two chromatographs were very similar at the beginning of the chromatofocusing run, but by fraction 20 large variations in peak shape and position of elution were obvious. Proteins were eluted at their pI, therefore, it appears that some factor(s) were affecting the

FIGURE 18: Assessment of Suitability of Chromatofocusing for Detection of Changes in Peak Areas as a Reflection of Enzyme Adsorption.



Two consecutive runs using the same enzyme were performed under the same experimental conditions. Flow Rate: 0.25 ml/min for the first 100 minutes, then 0.1 ml/min.

pH gradient generated by the column so that the chromatographs were not reproducible. Experimental conditions were kept constant between the two runs, but possibly the temperature of the column may have varied slightly between runs. It was apparent that the peaks were subject to change, and minor variations in peak area that might result from differential adsorption on cellulosic substrates would not be detected. The separation method was well-suited, however, to partial purification and characterization of cellulase components.

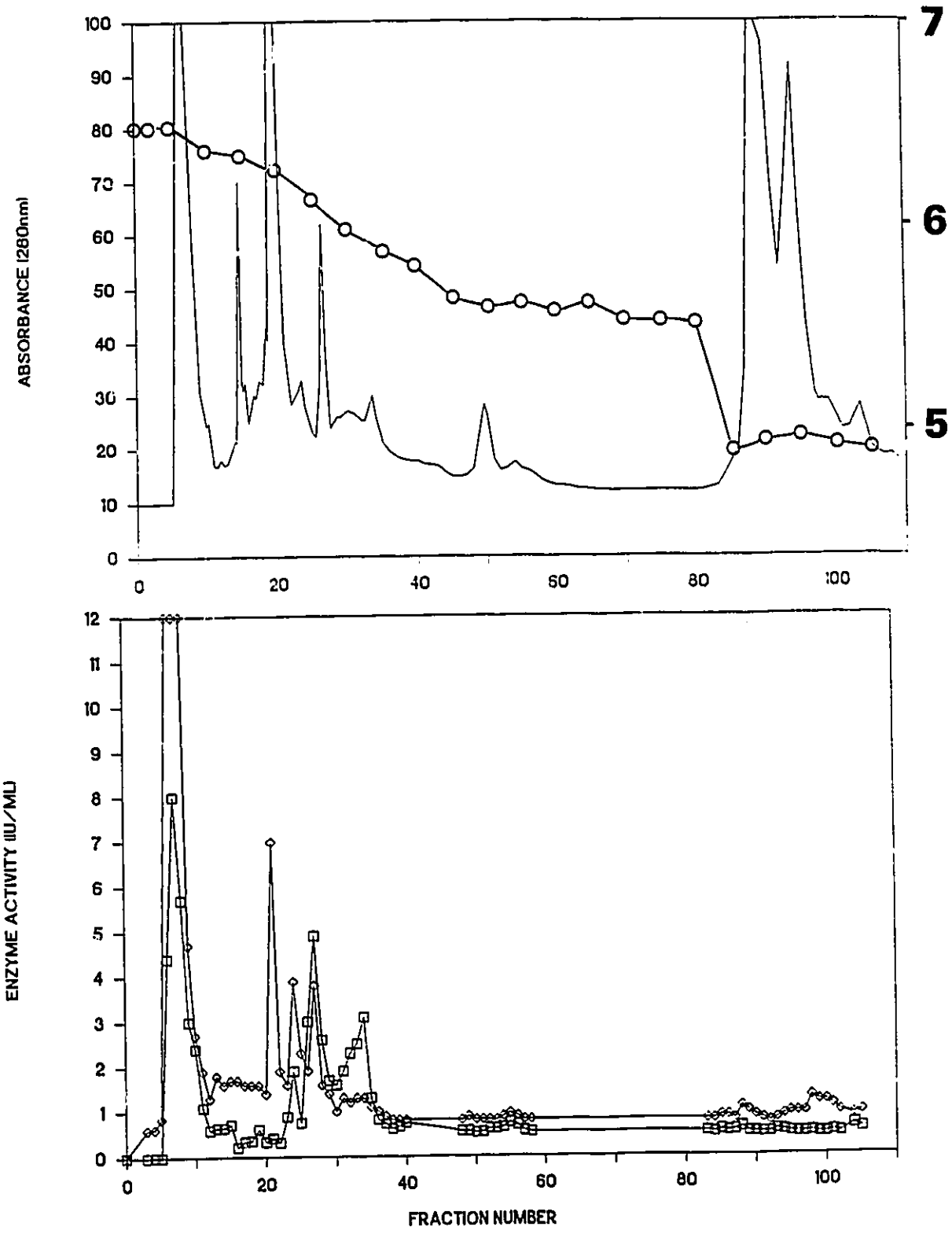
The presence of cellulase components was determined by performing enzyme assays on the fractions eluted from the chromatofocusing column. Fractions eluted from a chromatofocusing run were assayed for endoglucanase and xylanase activities. The endoglucanase assay was performed instead of the filter paper assay because neither the endoglucanase nor the exoglucanase produces significant reducing sugar release from filter paper when acting alone. Many cellulosic substrates also contain small amounts of residual hemicellulose, therefore, xylanase activity was also assayed to ensure that reducing sugar production was the result of only cellulases acting on cellulose. Oat spelts xylan also contains contaminating glucans, so minimal activity on xylan was not classified as xylanase activity. The presence of endoglucanase and xylanase activities in the

same peak may indicate the presence of a non-specific endoglycanase.

Protein peaks that were suspected of containing exoglucanase, based on the presence of a large amount of protein containing negligible endoglucanase activity, were subsequently assayed for their ability to interact synergistically with the endoglucanase components to hydrolyze filter paper. The proteins were eluted from a chromatofocusing run by decreasing pI in a pH gradient of 6.5-4.0 (Fig. 19A). Fourteen protein peaks were detected and were assayed for endoglucanase and xylanase activities (Fig. 19B). It was apparent that most peaks contained both endoglucanase and xylanase activities, although the protein peak at fraction 21 appeared to contain only xylanase activity.

Other studies of extracellular fungal enzymes separated by chromatofocusing assessed each peak for particular enzyme activities by enzyme assays, but did not examine each peak to determine whether only one protein was actually being eluted per peak (Thomas et al., 1983; Hayn and Esterhauer, 1985; Ridout et al., 1988). It is possible that proteins of similar pI are being eluted together, which would account for apparent multiple linkage specificities of cellulase components. It was necessary to determine whether each peak eluted contained a pure protein with activity on both

FIGURE 19: Protein Profile of E58 Culture Filtrate by Chromatofocusing; pH Gradient 6.5-4.0 (a) U.V. absorbance 280nm and pH (○ - ○) (b) Enzyme Activities: carboxymethylcellulose (□ - □) and xylan (◇ - ◇)



carboxymethylcellulose and xylan, or whether more than one component was present per peak.

Fractions that were eluted from the chromatofocusing column were run on isoelectricfocusing gels and Native-PAGE gels using the Pharmacia Phastsystem to determine the number of proteins per peak. Isoelectricfocusing and chromatofocusing methods both separate proteins on the basis of pI, however, the IEF procedure gives better resolution of proteins of similar pI. Proteins of similar pI would be resolved more readily as separate bands on a gel than would be detected as separate components by ultraviolet (U.V.) detector. Consecutive peaks showed an average decreasing pI as expected, therefore, the IEF gels are not shown here. Each peak contained several proteins which migrated on the IEF gels to similar R_f values, and in no case did one peak correspond to a pure protein.

Eluted fractions were run on Native-PAGE gels and the proteins were stained by silver staining. Fractions corresponding to the first seven peaks from the chromatofocusing run (in the pH gradient 6.5-4.0 shown in Figure 19) were shown to contain several components per peak (Fig. 20). This supported the IEF results that none of the peaks contained a pure protein. Although enzyme assays of eluted fractions showed that most peaks contained both endoglucanase and xylanase activities, it is unknown at this

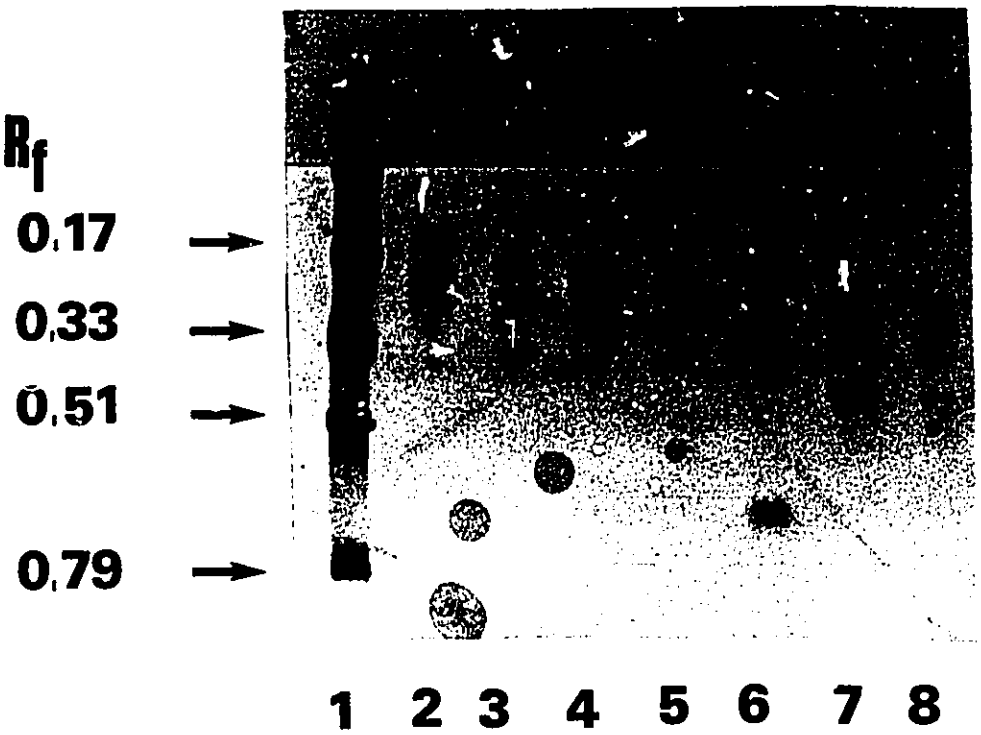
stage whether both activities are in one protein or in separate proteins.

Previous studies of cellulase enzymes have detected activity towards various substrates by overlay techniques (Considine and Coughlan, 1982; Farkas et al, 1985). These methods detected the presence of cellulase activity, but did not differentiate substrate specificities of individual cellulase components.

Separation of protein by electrophoretic techniques, prior to performing the overlay technique, allows differentiation between the cellulase enzymes. Zymograms were performed to distinguish which enzyme activities could be attributed to which protein band. This method was adapted for use with the gels of the Pharmacia Phastsystem. Substrate gels containing carboxymethylcellulose for detection of endoglucanase activity and oat spelts xylan for detection of xylanase activity were incubated with the electrophoresced gels. Intact polysaccharides interact with the Congo red dye, but clearing was observed where hydrolysis has occurred (Teather and Wood, 1982).

Isoelectricfocusing and Native-PAGE gels have the advantage of being non-denaturing gels, therefore, enzymes retain their activities. Native-PAGE gels were used for the overlay method, because a stronger difference between the bands of clearing and background was observed with these

FIGURE 20: Native-PAGE Gel of Fractions from Chromatofocusing (pH 6.5-4.0) corresponding to first seven major Protein Peaks
Lane 1: Standards; Lane 2: Fractions 6-8; Lane 3: Fraction 15; Lane 4: Fraction 20; Lane 5: Fraction 21; Lane 6: Fraction 24; Lane 7: Fraction 27; Lane 8: Fraction 34
(Arrows mark the positions of molecular weight standards used as a measure of consistency between runs, and not in determination of molecular weights.)



gels than with the IEF gels. The pH gradient of the IEF gels appeared to affect the interaction between the unhydrolyzed substrate and the Congo red dye, making hydrolyzed regions less distinct than on the Native-PAGE zymograms, particularly in the acidic portions of the gels. This has been reported previously (MacKenzie and Williams, 1984), however, most researchers continue to perform separation of the cellulase components by isoelectric-focusing.

Molecular weight standards are used for SDS-PAGE rather than Native-PAGE. The shapes of the cellulase enzymes may be significantly different from those of the standards, such that their migration is not linear with the reference proteins under the conditions of Native-PAGE. Schmuck et al. (1986) showed that a cellobiohydrolase of T. reesei had an elongated shape with dimensions of 44 nm x 180 nm. Average dimensions for a variety of purified cellulase components are 33 nm x 200 nm (Lee and Fan, 1980). Standard proteins would be chosen on the basis of a more uniform, spherical shape and these differences in shape between sample and standards would be instrumental in affecting migration through the acrylamide gel. Therefore, standards were used as an indication of consistency between runs, and not used in determining the molecular weights of the cellulases.

The first chromatofocusing peak eluted with the column's void volume, and therefore, contained the proteins with pI greater than or equal to pH 6.5. By comparing the hydrolysis of the two substrate gels, different patterns of enzymatic activity were apparent for the protein components.

The four protein bands in lane 2, that did not enter the separation gel but remained in the stacking gel, were detected by silver staining of the Native-PAGE gel (Fig. 20). These components did not show activity on the carboxymethylcellulose gel (Fig. 21), but produced clearing on the xylan gel (Fig. 22). It is unlikely that their molecular weights are greater than the capabilities of the gel (750,000 for native, globular proteins); they probably have pI's greater than the pH 8.3 of the buffer. These bands likely contain several xylanases of high pI, purified previously (Tan et al., 1985a; Wong et al., 1986).

Also apparent were specific endoglucanases with R_f values of 0.29, 0.51 and 0.53 (Fig. 20, lane 2), which hydrolyzed carboxymethylcellulose, but not xylan. The last two components were detected by their activity on the substrate gels and their R_f values were estimated from them. The literature on the Pharmacia Phastsystem states that protein quantities as low as 5 ng are detected by this method. The sensitivity of the zymogram technique appears to be better than this, but would depend on the enzyme.

FIGURE 21: Carboxymethylcellulose Zymogram performed by Overlay Method using Native-PAGE Gel of Fractions from Chromatofocusing (pH 6.5-4.0) corresponding to first seven major Protein Peaks
Lane 1: Standards; Lane 2: Fractions 6-8; Lane 3: Fraction 15; Lane 4: Fraction 20; Lane 5: Fraction 21; Lane 6: Fraction 24; Lane 7: Fraction 27; Lane 8: Fraction 34
(Arrows mark the positions of molecular weight standards used as a measure of consistency between runs, and not in determination of molecular weights.)

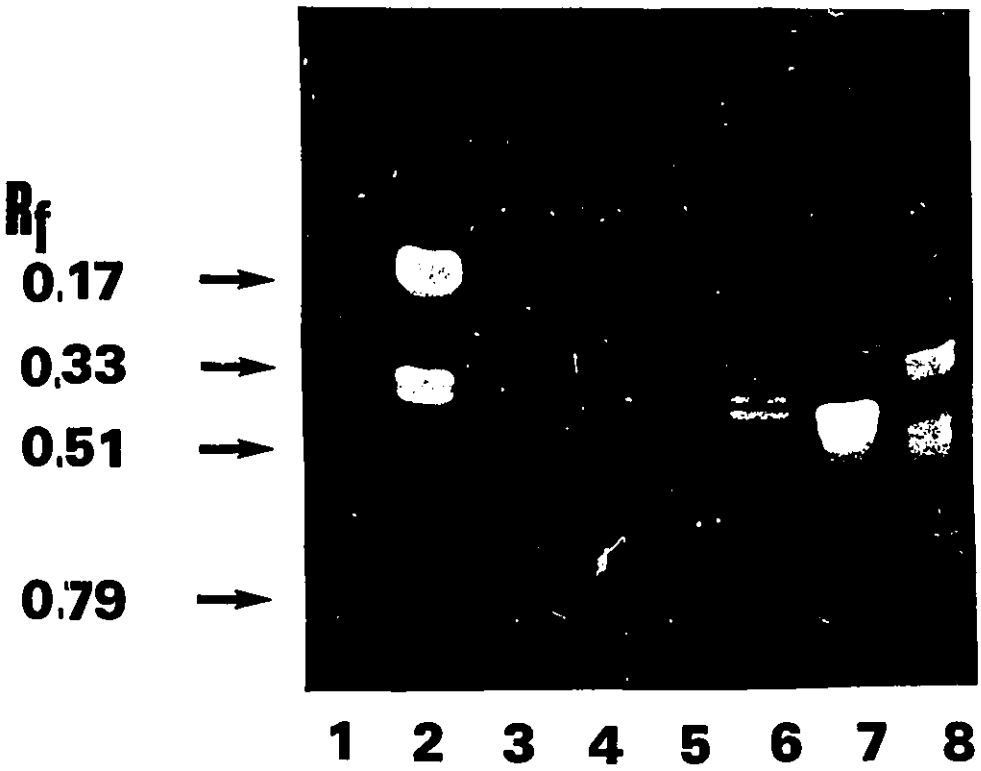
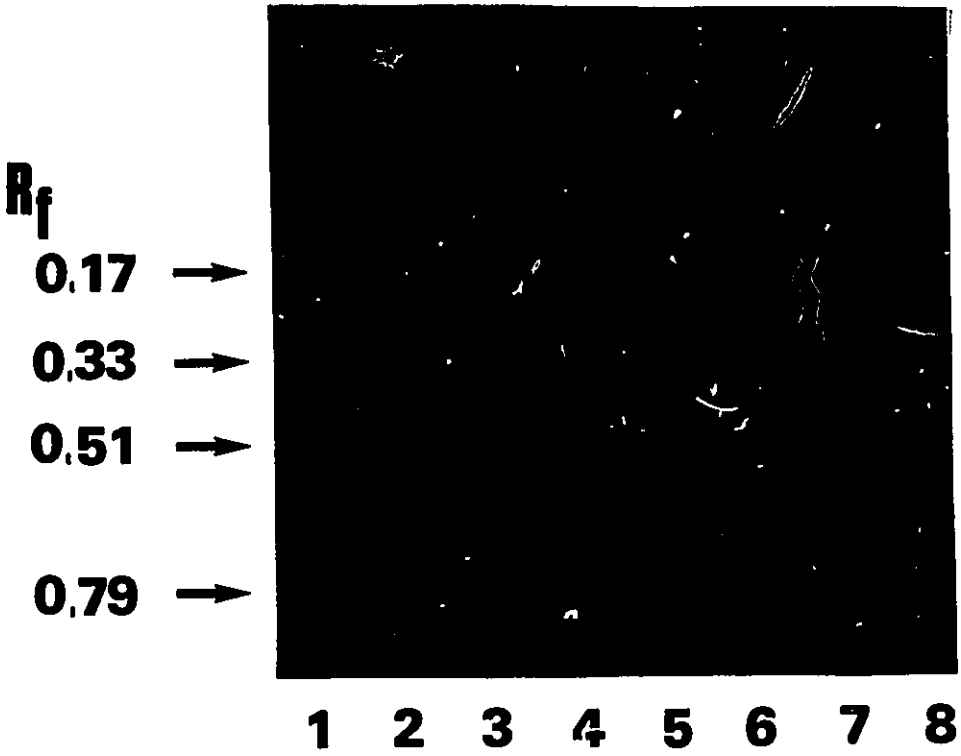


FIGURE 22: Xylan Zymogram performed by Overlay Method using Native-PAGE Gel of Fractions from Chromatofocusing (pH 6.5-4.0) corresponding to first seven major Protein Peaks
Lane 1: Standards; Lane 2: Fractions 6-8; Lane 3: Fraction 15; Lane 4: Fraction 20; Lane 5: Fraction 21; Lane 6: Fraction 24; Lane 7: Fraction 27; Lane 8: Fraction 34
(Arrows mark the positions of molecular weight standards used as a measure of consistency between runs, and not in determination of molecular weights.)



Detectable clearing on the substrate gel is dependent on the specific activity of each enzyme component, in addition to its concentration.

Bands corresponding to proteins with R_f values of 0.19, 0.23, 0.27, 0.37 and 0.40 (Fig. 20, lane 2) were observed to possess activity towards both carboxymethylcellulose and xylan.

Fraction 15 contained two major protein bands, visible on the silver-stained Native-PAGE gel, that were not active towards either substrate (Fig. 20, lane 3). Several other components were present in concentrations too low for detection by the silver-staining method, but showed activity on either carboxymethylcellulose or xylan. There was no evidence of components active on both substrates in this peak.

The IEF gels showed that each peak contained numerous proteins, covering a range of pI's. Many protein components were present in more than one chromatofocusing peak, or were eluted over a wide range of pH. For example, a protein component with an R_f value of 0.27, which possessed activity towards both carboxymethylcellulose and xylan, was present in the first four peaks of the chromatofocusing run. Some enzyme components produced a dramatic change in the xylan substrate gel without producing much clearing. Those bands with R_f values of 0.21 and 0.25 (Fig. 22, lanes 4 and 5)

produced regions on the xylan substrate gel that were significantly darker than the background with Congo red, indicating an acidic pH. These components may be esterases that function in the breakdown of xylan. The hydrolysis of an ester bond may result in acidification of the medium and a darkening of the Congo red.

The fractions corresponding to the last seven peaks on the chromatofocusing run in the pH gradient seen (Fig. 19A) were separated on IEF and Native-PAGE gels and stained by the silver-staining method. The Native-PAGE gel is shown in Figure 23. Carboxymethylcellulose and xylan zymograms (Figs. 24 and 25) showed similar results to those seen in Figures 21 and 22 for the first seven chromatofocusing peaks. Both specific endoglucanases and endoxylanases were present, as well as non-specific endoglycanases which could cleave both carboxymethylcellulose and oat spelts xylan.

One band of protein in fraction 50 with pI about 5.6 (Fig. 23, lane 2) was detectable by the silver-staining method on Native-PAGE, but this component did not possess activity towards either substrate. A second component in this fraction, with an R_f value of 0.51 was detected. It was not concentrated enough to be detected by silver staining, but was active towards carboxymethylcellulose on the substrate gel.

FIGURE 23: Native-PAGE Gel of Fractions from Chromatofocusing (pH 6.5-4.0) corresponding to last seven major Protein Peaks
Lane 1: Standards; Lane 2: Fraction 50; Lane 3: Fraction 54; Lane 4: Fraction 88; lane 5: Fraction 89; Lane 6: Fraction 94; Lane 7: Fraction 99; Lane 8: Fraction 104
(Arrow marks the position of exoglucanase)

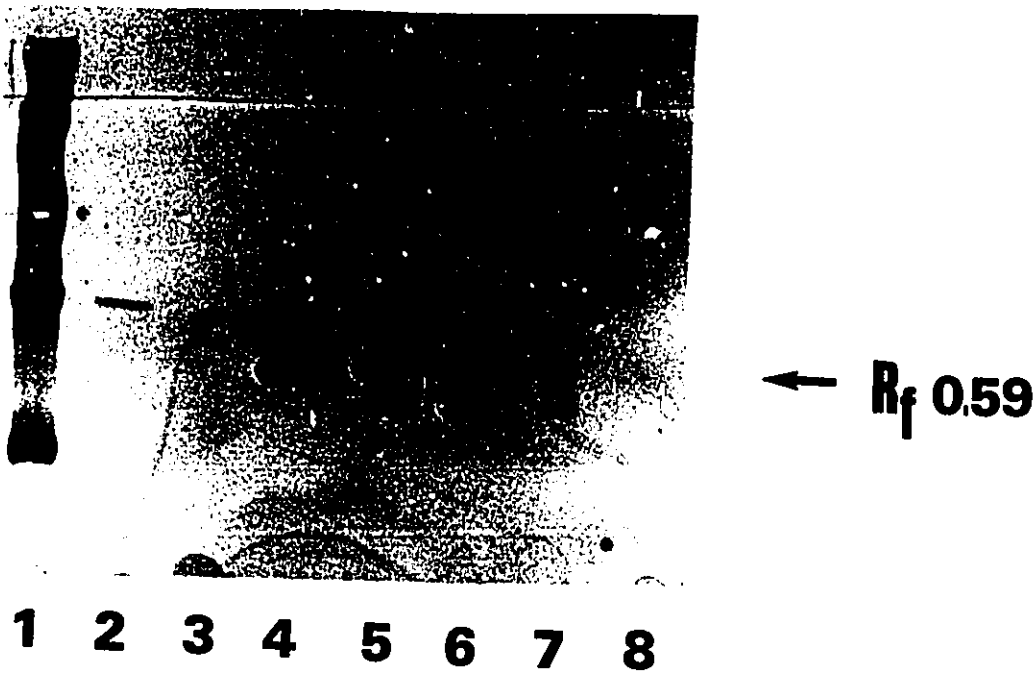
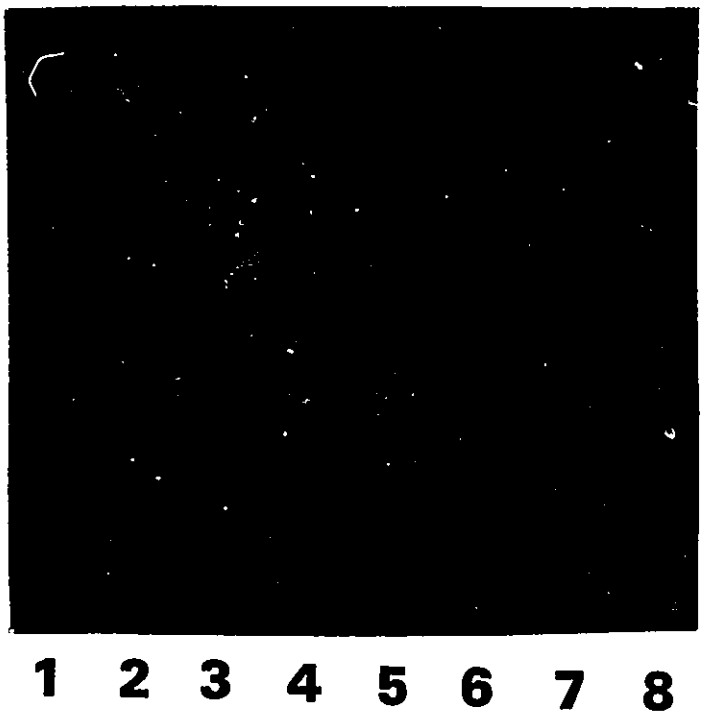


FIGURE 24: Carboxymethylcellulose Zymogram performed by Overlay Method using Native-PAGE Gel of Fractions from Chromatofocusing (pH 6.5-4.0) corresponding to last seven major Protein Peaks
Lane 1: Standards; Lane 2: Fraction 50; Lane 3: Fraction 54; Lane 4: Fraction 88; Lane 5: Fraction 89; Lane 6: Fraction 94; Lane 7: Fraction 99; Lane 8: Fraction 104
(Arrow marks the position of exoglucanase)

FIGURE 25: Xylan Zymogram performed by Overlay Method using Native-PAGE Gel of Fractions from Chromatofocusing (pH 6.5-4.0) corresponding to last seven major Protein Peaks
Lane 1: Standards; Lane 2: Fraction 50; Lane 3: Fraction 54; Lane 4: Fraction 88; Lane 5: Fraction 89; Lane 6: Fraction 94; Lane 7: Fraction 99; Lane 8: Fraction 104
(Arrow marks the position of exoglucanase)

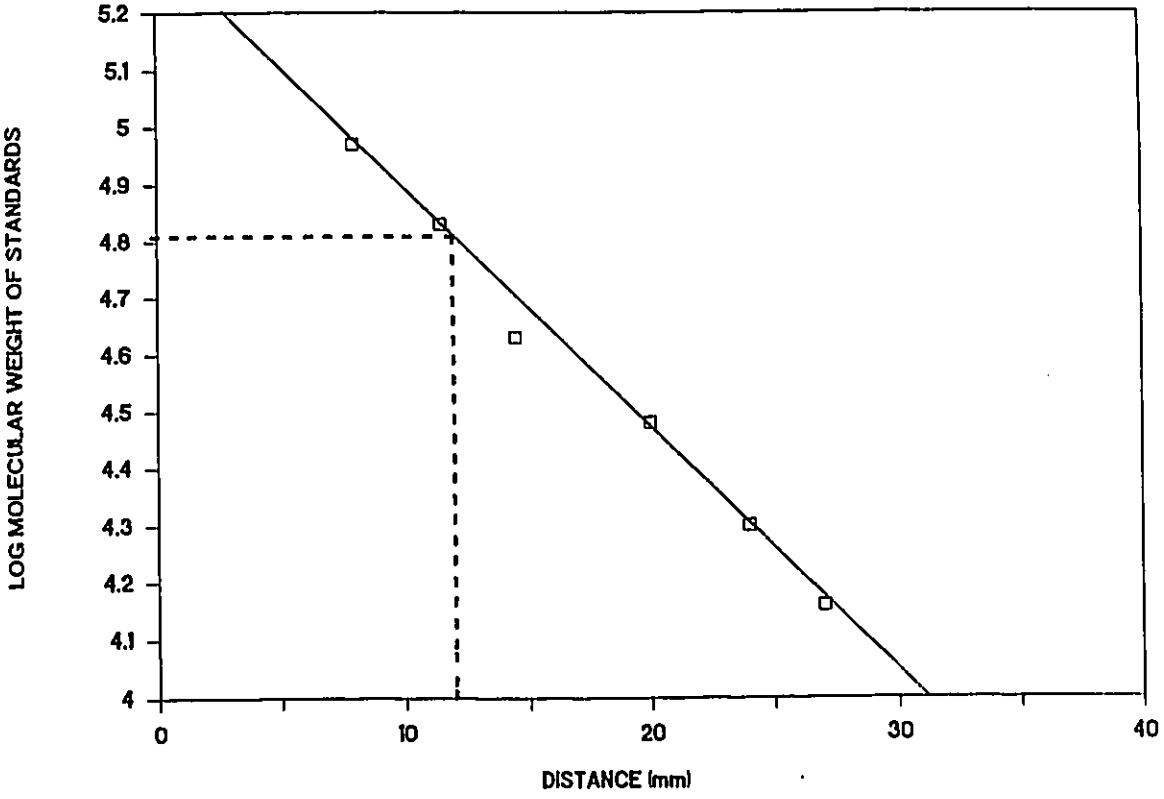


Fractions 54 and 104 (Fig. 23, lanes 3 and 8, respectively) did not contain enough protein to be detected by the silver-staining method. It can be seen in Figure 19 that the protein peaks which correspond to these fraction numbers are significantly smaller than the other peaks. All components were examined in their original proportion respective to the other enzyme components, therefore, these peaks were not concentrated to give a measurable quantity on the Native-PAGE gel. However, the carboxymethylcellulose zymogram for fraction 104 shows the presence of specific endoglucanase activity. Three distinct bands of clearing were apparent (Fig. 24, lane 8).

A major protein component found in fractions 88 and 89 (Fig. 23, lanes 4 and 5) had an R_f value of 0.59 on Native-PAGE. This component showed very little activity on the carboxymethylcellulose (Fig. 24, lanes 4 and 5) and xylan (Fig. 25, lanes 4 and 5) zymograms. Only faint clearing was observed on each. The concentration of this protein and its relative absence of endo-type activity suggested that it may be an exoglucanase.

Purified exoglucanases show minimal activity towards filter paper, and activity towards MUC has also been observed with some endoglucanases (van Tilbeurgh and Claeyssens, 1985) and xylanases (Gilbert et al., 1988). Verification of exoglucanase activity was performed by the

FIGURE 26: Estimation of Molecular Weight of Exoglucanase Component by Comparison of Migration of Standards by SDS-PAGE



action of this protein with an endoglucanase. It showed synergism towards filter paper with an endoglucanase component obtained from the chromatofocusing run in the pH gradient 6.5-4.0 (fraction 26). This enzyme had a molecular weight of about 63 kDa by SDS-PAGE (Fig. 26), which corresponded to an Avicelase component partially isolated previously by ion-exchange chromatography (Tan et al., 1986).

Proteins with pI greater than 6.5 were further separated by chromatofocusing over the pH gradient 8.5-6.0 (Fig. 27A). Most activity in the chromatofocusing peaks, as detected by enzyme assays, was xylanase (Fig. 27B). The Native-PAGE gel for the pH gradient 8.5-6.0 was not shown, due to insufficient protein for detection by the silver-staining method. Protein bands were evident on the IEF gel, but adequate separation of components could not be achieved because the IEF gel gradient was in the range 3-9, and the bands were closely packed at the cathode end of the gel. The minimum detectable concentration of protein differs for the IEF and Native-PAGE gels, because identical protein concentrations that were not detected on the silver-stained Native-PAGE gel are evident on the IEF gel.

Although the protein bands were not apparent, several components were active on zymograms. A component that was active towards carboxymethylcellulose, with an R_f value of

FIGURE 27: Protein Profile of E58 Culture Filtrate by Chromatofocusing; pH Gradient 8.5-6.0 (a) U.V. absorbance 280nm and pH (O-O) (b) Enzyme Activities: carboxymethylcellulose (□-□) and xylan (◇-◇)

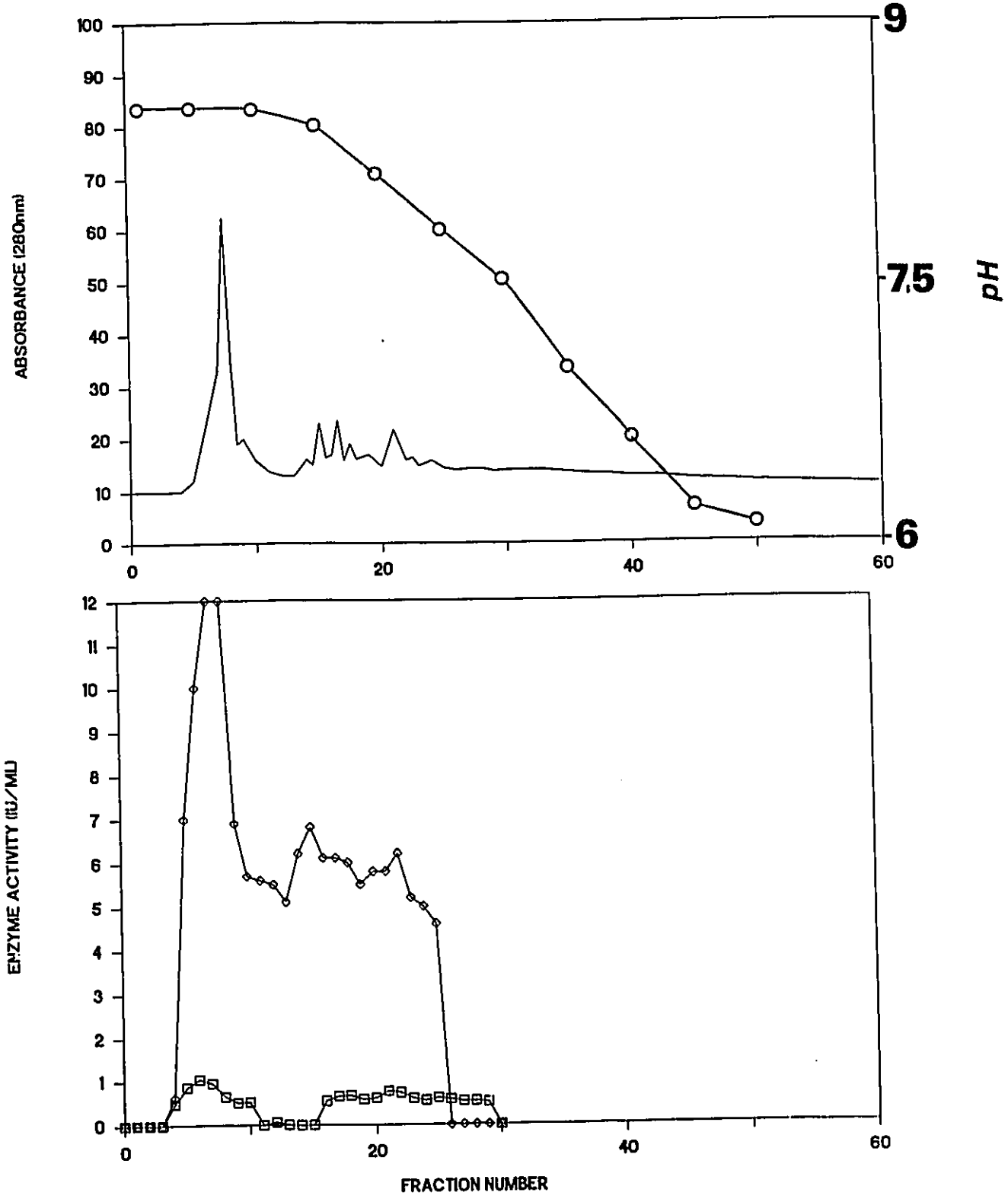


FIGURE 28: Carboxymethylcellulose Zymogram performed by Overlay Method using Native-PAGE Gel of Fractions from Chromatofocusing (pH 8.5-6.0) corresponding to major Protein Peaks
Lane 2: total extracellular protein of T. harzianum; Lane 3: Fraction 23; Lane 4: Fraction 20; Lane 5: Fraction 15; Lane 6: Fraction 9; Lane 7: Fraction 7; Lane 8: Fraction 6
(Arrows mark the positions of molecular weight standards used as a measure of consistency between runs, and not in determination of molecular weights.)

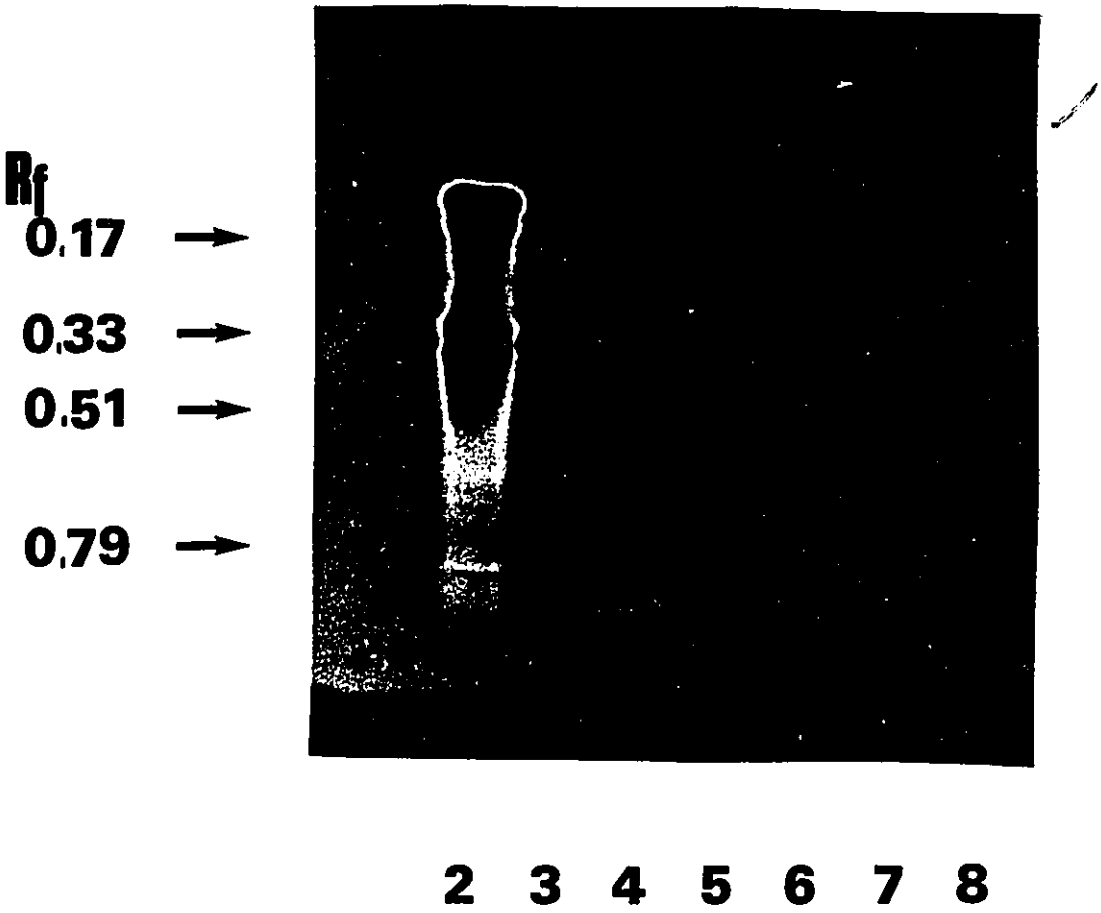
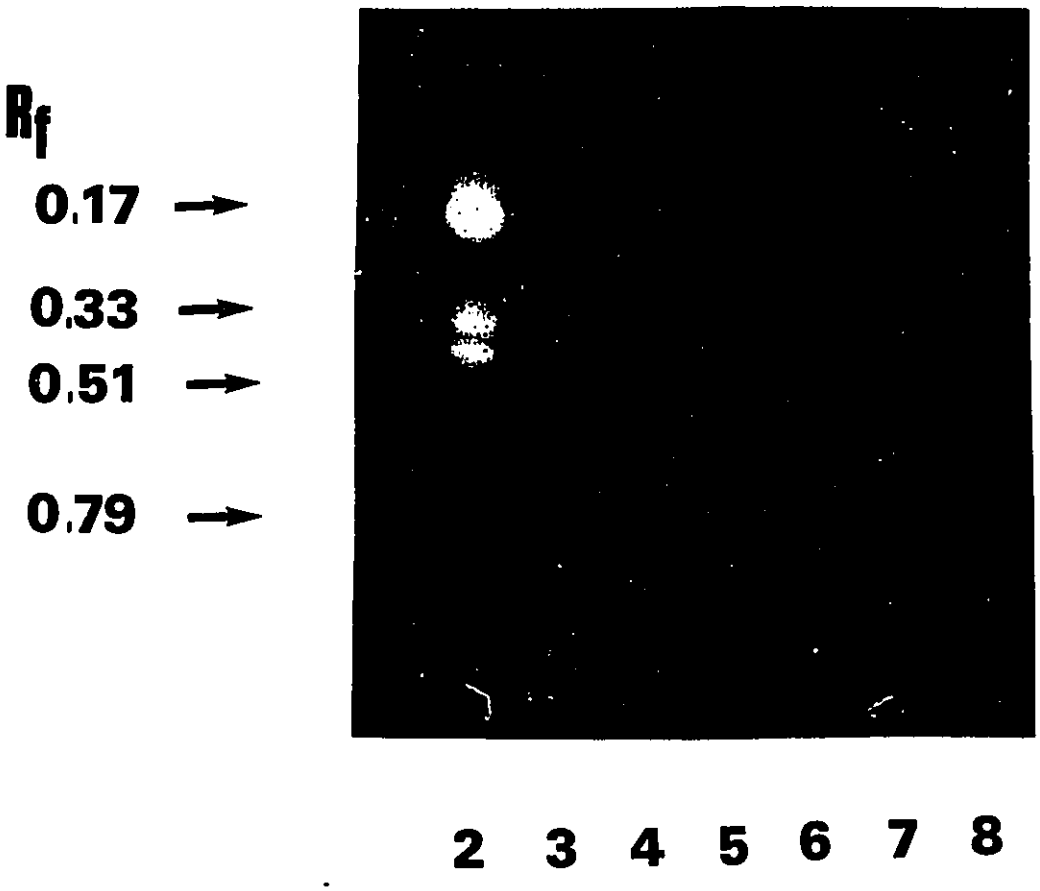


FIGURE 29: Xylan Zymogram performed by Overlay Method using Native-PAGE Gel of Fractions from Chromatofocusing (pH 8.5-6.0) corresponding to major Protein Peaks
Lane 2: total extracellular protein of T. harzianum; Lane 3: Fraction 23; Lane 4: Fraction 20; Lane 5: Fraction 15; Lane 6: Fraction 9; Lane 7: Fraction 7; Lane 8: Fraction 6
(Arrows mark the positions of molecular weight standards used as a measure of consistency between runs, and not in determination of molecular weights.)



0.13, was observed in fractions 6 and 7 (Fig. 28, lanes 8 and 7). A xylanase component with an R_f value of 0.20 was detected in fractions 6, 7 and 9 (Fig. 29, lanes 8, 7 and 6, respectively).

Evidence of clearing in zymogram gels, by enzymes that were not concentrated enough to be detected by silver staining of the Native-PAGE gels, illustrates the sensitivity of the zymogram method with carboxymethylcellulose or xylan as the substrates. Detection of Streptomyces flavogriseus endoglucanase activity after isoelectricfocusing, from 1 ng protein in a five-minute assay, has been reported (MacKenzie and Williams, 1984). This corresponded to 2.5×10^{-4} units of activity towards carboxymethylcellulose. Detection of 6.2×10^{-4} units of Clostridium thermocellum endoglucanase activity has been reported, from a 60-minute assay, following separation of proteins by SDS-PAGE (Béguin, 1983). Spectrophotometric detection of endoglucanase activity can be reproducible to about 2×10^{-2} units, based on glucose production from a 30-minute assay and a limit of detection of about 0.1 mg glucose, by the DNS method. In addition to greater sensitivity, the zymogram method also has the benefit of distinguishing the respective enzyme components, whereas the colorimetric DNS method measures an average of activities.

Comparison of clearing on the carboxymethylcellulose and xylan gels gives an indication of the substrate specificities of the cellulase components. The zymogram technique for determining substrate specificity of cellulase components differentiates between cellulase components that were specific to either glucans or xylans and those capable of hydrolyzing both substrates. It also gave an indication of the ratio of cellulase to non-cellulase protein.

Due to the extremes in R_f values of the protein components detected by the zymogram technique, the protein bands observed may not be representative of individual proteins, but instead they may actually be enzyme-enzyme complexes. These components have been separated by both pI and molecular weight, therefore it is likely that both activities are present in the same protein "unit" and have not merely co-migrated under both separation processes. Whether each band contains only one enzyme sub-unit type is not certain. Individual components would have to be purified to examine this, but that is beyond the scope of this study.

Evidence of formation of dimers by microbial glucosidases has been described in other systems. The α-amylase of Bacillus subtilis was shown to exist as a dimer that dissociated under high dilutions (Isemura and Kakiuchi, 1962; Kakiuchi et al., 1964).

It is possible that these T. harzianum E58 enzyme components are in the form of complexes of high molecular weight, as suggested by research done by Sprey and Lambert (1983). These workers isolated a component of T. reesei that was homogeneous by isoelectricfocusing and showed β-glucosidase activity towards p-nitrophenol-β-D-glucoside and cellobiose; xylanase activity; and slight activities on filter paper, carboxymethylcellulose, and cotton. When urea was added to the gels, a second band was observed which contained the β-glucosidase activity. Addition of urea-octylglucoside to the gels further split the complex, separating the cellulase and xylanase activities. They did not give the molecular weight of the complex.

Complex formation has been detected in bacterial cellulase systems. A large component of C. thermocellum, homogeneous by several purification techniques, had a molecular weight of 2.1 million Daltons. When this "cellulosome" was electrophoresced in the presence of sodium dodecyl sulfate, fourteen different polypeptide types were apparent (Lamed et al., 1983). Other researchers have estimated that the size of the C. thermocellum cellulosome may range from between 4 and 102 million Daltons, resulting from small sub-units (Coughlan et al., 1985).

This was not the case with T. harzianum E58. Two chromatofocusing fractions (fractions 26 and 88, pH 6.5-

4.0) that showed the presence of many protein components by IEF or Native-PAGE appeared as single bands when electrophoresced in the presence of sodium dodecyl sulfate (results not shown).

Evidence that complex formation may have occurred in this T. harzianum E58 system lies in the fact that certain components eluted in one peak, then were eluted several peaks later, but not in the intervening peaks. For example, an endoglucanase component with an R_f value of 0.29 and a xylanase component with an R_f value of 0.32 both eluted in the first peak, corresponding to proteins with pI's greater than 6.5. They did not elute from the column again until the sixth and seventh peaks. Assuming that the subsequent proteins with the same molecular weight were the same components, this suggests that these enzymes complexed with various other components, forming combinations differing in pI's. However, this also suggests that these complexes are dissociated by the Native-PAGE step, which seems unlikely. It is more likely that the proteins being eluted in different peaks were not identical, but may represent proteins that underwent slight differences in post-translational modifications.

In addition, the xylanase components with pI's greater than 8.3 (Fig. 20, lane 2) only showed activity on zymograms after separation by chromatofocusing (Fig. 22, top of lane

2) and not when present in the total extracellular protein (Fig. 29, lane 2). It is possible that the enzymes are complexing with some component(s) in the total protein that is being removed during the chromatofocusing step, thereby activating the xylanases.

The diversity of the endoglucanase system hints that the exoglucanases may also be more numerous or complex than previously considered. During preliminary chromatofocusing experiments, two protein peaks demonstrated synergism with an endoglucanase component (results not shown). This suggested that at least two exoglucanase components may be present in T. harzianum E58.

Due to the nature of the substrates attacked by exoglucanases, zymogram detection of multiple forms of exoglucanase is limited. Methylumbelliferylcellobioside hydrolysis in gels is detected by fluorescence, but its hydrolysis is not specific to exoglucanase, according to recent studies. Evidence that endoglucanases can also hydrolyze the substrate lessens its use in differentiating exoglucanases from other cellulase components (van Tilbeurgh and Claeyssens, 1985). Therefore, an exoglucanase was purified and characterized.

(III) PURIFICATION AND CHARACTERIZATION OF AN EXOGLUCANASE OF TRICHODERMA HARZIANUM E58:

In attempts to amend the current theory of cellulose hydrolysis to account for the presence of multiple cellulase forms, the endoglucanase and exoglucanase components must be separated and characterized with respect to their substrate specificities. This can be done for endoglucanases, in part, by the use of zymogram overlay methods. However, the assay substrates of exoglucanase are not conducive to these methods.

Ion-exchange of T. harzianum E58 cellulases on DEAE-Sephadex (Tan et al., 1986) resulted in the elution of four main peaks (Fig. 30). The fourth peak, Peak D, possessed little activity towards carboxymethylcellulose or cellobiose, but was active on Avicel. These data suggested that it was neither an endoglucanase nor β-glucosidase, but might be an exoglucanase. The peak was homogeneous on SDS-PAGE with a molecular weight of 67 kDa, as detected by brilliant Coomassie Blue staining.

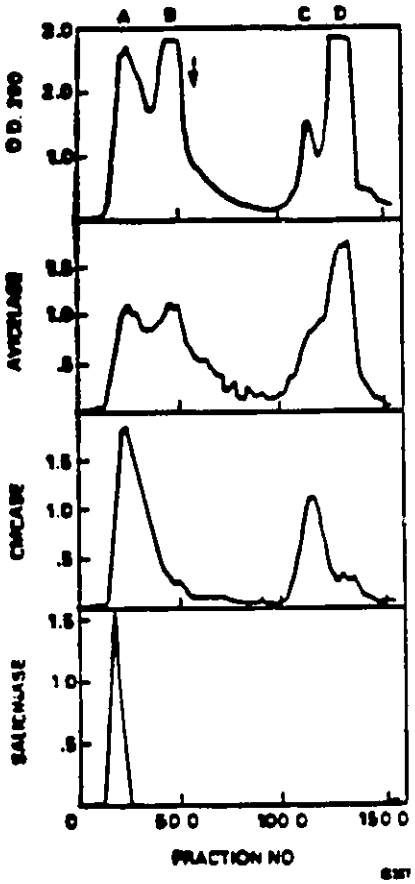
The pH optimum of Peak D was determined by its activity on filter paper at various pH's. Its pH optimum was observed to be 4.8 (Fig. 31). This is similar to those of many other fungal cellulases, and is therefore the pH at which cellulase assays are generally performed.

The ion-exchange fractions of Peak D were subjected to isoelectricfocusing to verify the homogeneity observed on SDS-PAGE. An IEF gel of the ion-exchange fractions containing Peak D showed that a major protein had a pI of 4.8 (Fig. 32) and was not homogeneous, but was contaminated by several components (Fig. 35, lane 7).

In attempts to separate the exoglucanase from these contaminating proteins, Peak D was run on the chromatofocusing column in the pH gradient 5.5-4.0 (Fig. 33). Fractions containing the major peak with pI 4.8 were collected, but isoelectricfocusing (Fig. 35, lane 6) and Native-PAGE (Fig. 34, lanes 1-6) indicated that contaminants were still present.

Based on the strong interaction between exoglucanase and cellulose, affinity chromatography was attempted. Assessment of enzyme leaching from a column of Solka Floc showed that the exoglucanase remained strongly adsorbed while the endoglucanase activity was eluted out with the reducing sugar stream (Tan et al., 1986). On this premise, it was thought that the contaminating proteins would have less affinity for the cellulose than the exoglucanase would, and would be more readily eluted from the column. The exoglucanase would remain adsorbed to its substrate, and would require more stringent conditions of elution. Although the homogeneous band for Peak D on SDS-PAGE was

FIGURE 30: Separation of Protein Components of T. harzianum by Ion-Exchange and Determination of Cellulase Activities of Protein Peaks



Chromatogram of *Trichoderma harzianum* E58 cellulase concentrated by ultrafiltration on DEAE-Sephadex A50. O.D. 280 represents protein profile, avicelase denotes exoglucanase activity, CMCase corresponds to endoglucanase activity, while salicinase represents β-glucosidase activity. The arrow marks the application of a linear gradient composed of 15 mM to 300 mM phosphate buffer, pH 6.5

(Tan et al., 1986)

FIGURE 31: Effect of pH on Filter Paper Activity of Exoglucanase-containing Peak D

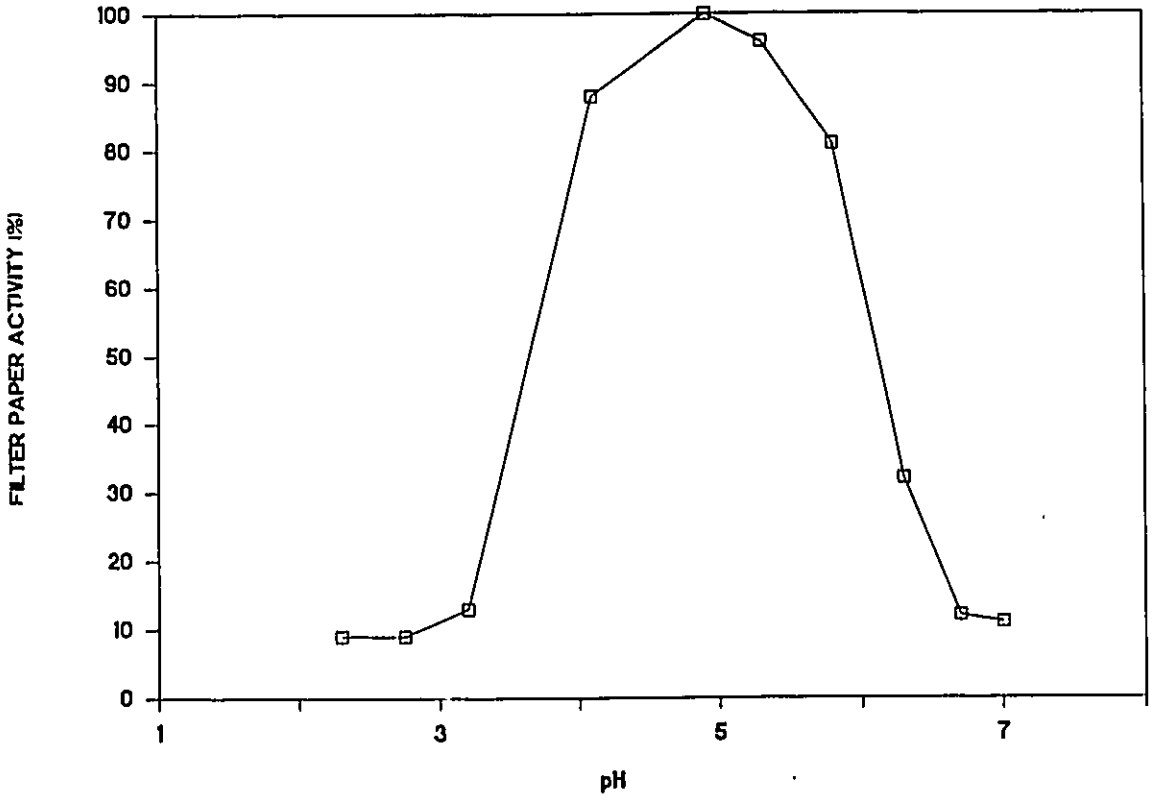


FIGURE 32: Estimation of Isoelectric Point of Exoglucanase by Comparison of Migration of Standards by Isoelectric-focusing

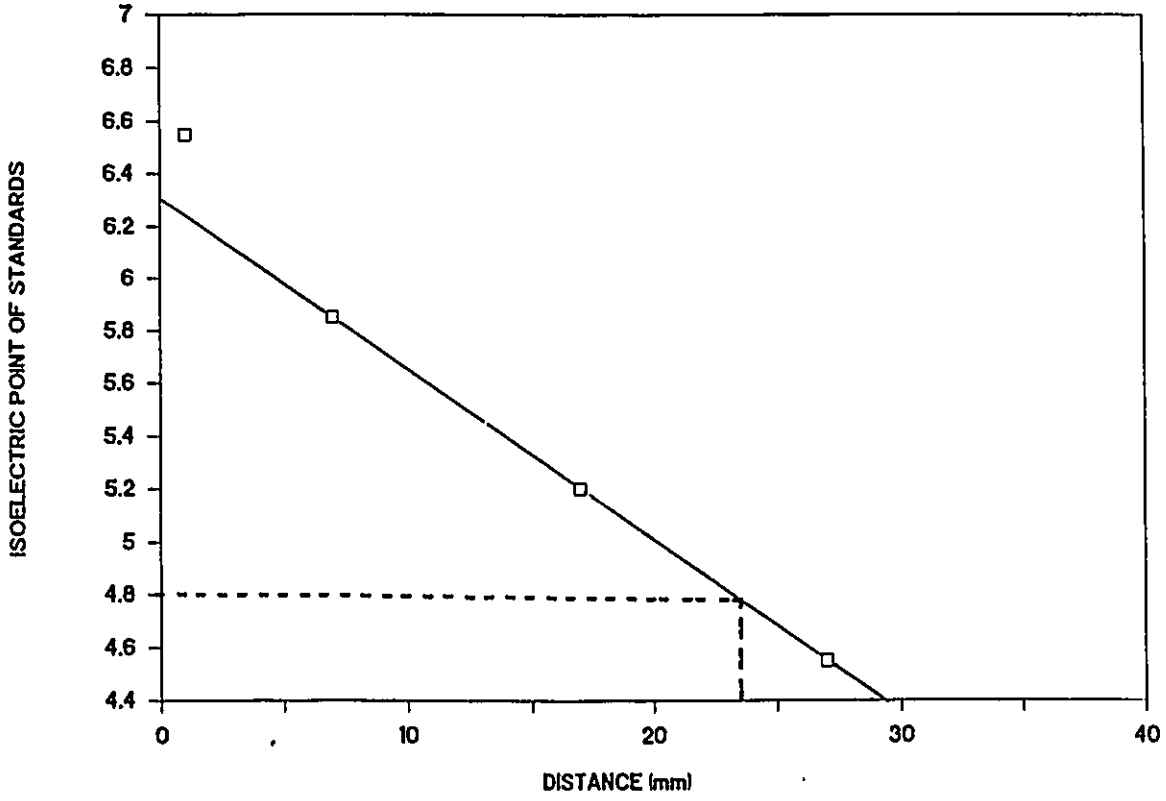
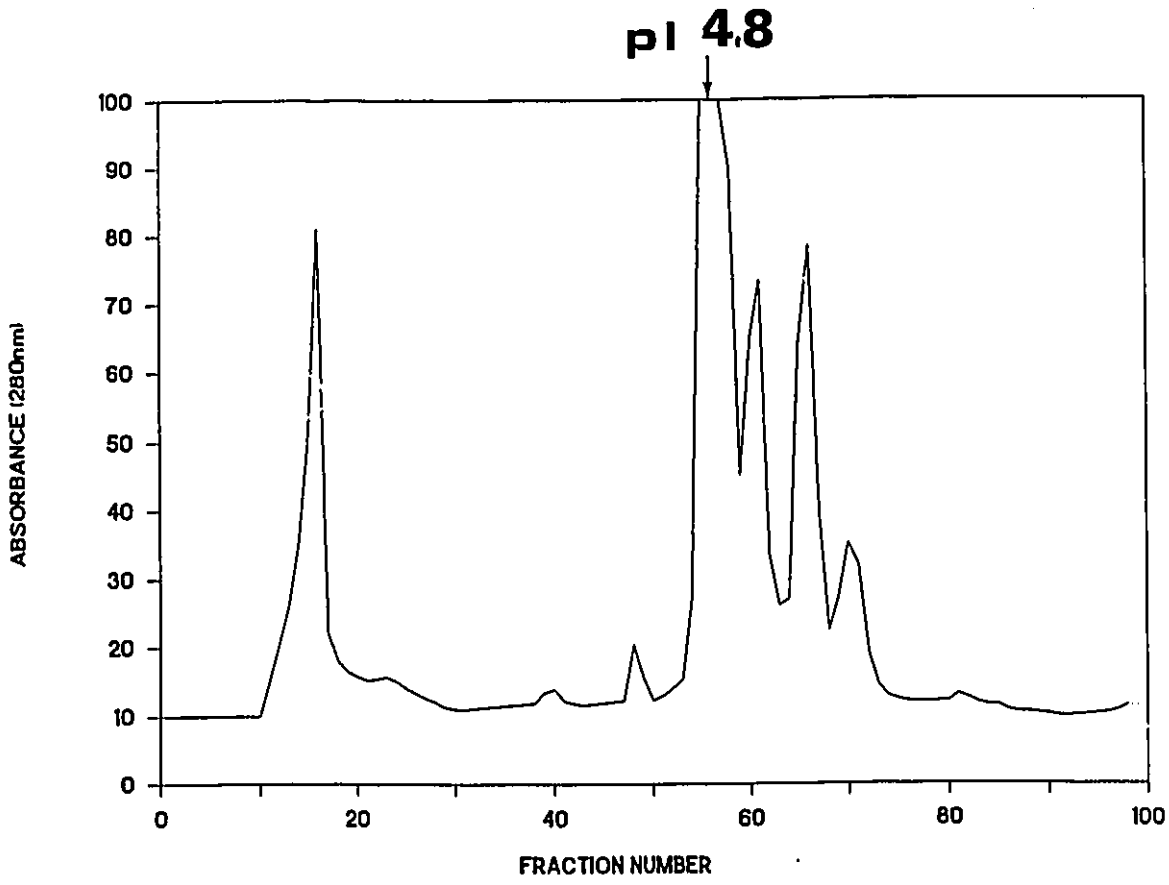


FIGURE 33: Protein Profile of Ion-Exchange Fractions Corresponding to Peak D by Chromatofocusing (pH Gradient 5.5-4.0)



seen to become adsorbed to Solka Floc when monitored by brilliant Coomassie Blue (Tan et al., 1986), it was thought that perhaps some of the contaminating proteins would have been unbound but undetected by the less sensitive staining method. The silver stain is much more sensitive and would detect unbound contaminating proteins in the fresh eluent. However, the silver-stained IEF gel of the unbound and bound proteins also showed that all components were strongly adsorbed to the substrate, therefore, the exoglucanase cannot be purified by affinity chromatography under these conditions (Fig. 35, lanes 3 and 4). Non-cellulase components do not adsorb to cellulose, therefore, most of the extracellular protein appears to be cellulase. These results corresponded to those obtained by the zymogram technique; most protein bands represented cellulases.

Separation of the contaminating proteins from the exoglucanase was attempted by gel filtration, taking advantage of the differences in molecular weights observed on the Native-PAGE gel (Fig. 34, lanes 1-6). The chromatofocusing fractions corresponding to the peak that eluted with pI 4.8 from the pH gradient 5.5-4.0 (Fig. 33) were collected and run on a Superose-12 gel filtration column at a flow rate of 0.1 ml/min (Fig. 36). Native-PAGE gel of the peak fractions showed that the exoglucanase eluted with two higher molecular weight components (Fig. 37,

FIGURE 34: Native-PAGE Gel of Chromatofocusing Fractions containing Exoglucanase with pI 4.8
Lanes 1-6: Fractions 54-59 inclusive; Lane 8: Standards

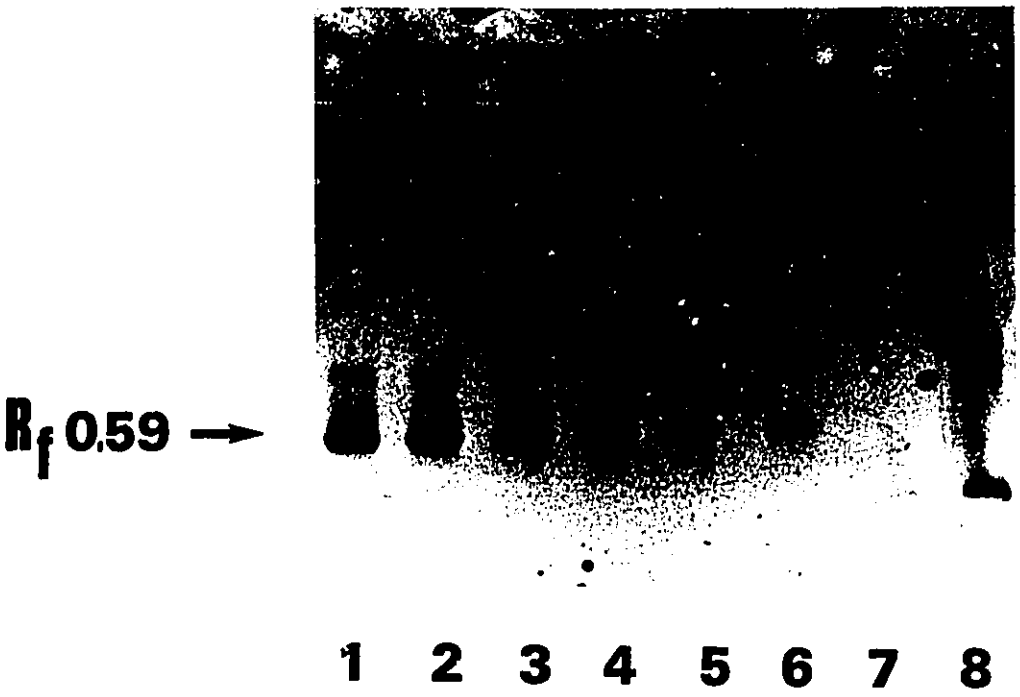
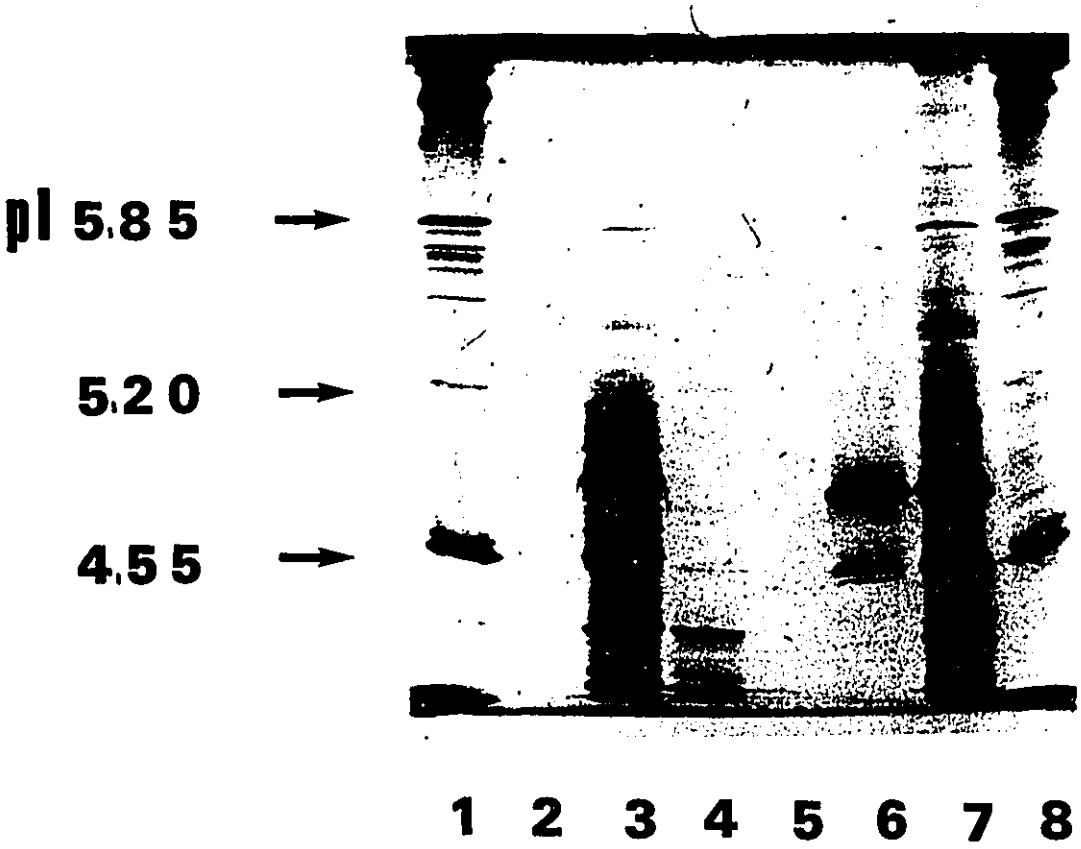


FIGURE 35: Affinity Chromatography of Peak D (IEF Gel; pH 4.0-6.5)
Lanes 1,8: Standards; Lane 3: bound protein; Lane 4: unbound protein; Lane 6: chromatofocusing peak pI 4.8; Lane 7: desalted Peak D



lanes 1-6), but a minor component eluted first (Fig. 37, lane 7). The contaminating proteins could not be removed by selective adsorption onto cellulose. The exoglucanase was the lowest molecular weight protein in the fractions, therefore, gel filtration was reattempted, but at a slower flow rate of 0.05 ml/min. Isoelectricfocusing (Fig. 38) and Native-PAGE (Fig. 39, lane 2) gels of eluted fractions indicated that pure exoglucanase eluted from the trailing edge of the peak. The purification profile of the exoglucanase is shown in Figure 39.

The purified exoglucanase had no activity towards carboxymethylcellulose, xylan, salicin or cellobiose. It produced only cellobiose from filter paper and Avicel, as detected by HPLC, therefore, the purified exoglucanase is a β-1,4-D-glucan cellobiohydrolase.

In the initial stages of characterizing this enzyme, it was observed that Peak D showed synergistic action with an endoglucanase-rich fraction from the chromatofocusing run in the pH gradient 6.5-4.0 (fraction 26). Initial IEF and Native-PAGE gels showed the presence of contaminating proteins, therefore, it was not certain if the synergistic response was due to the exoglucanase or the contaminants. The purified cellobiohydrolase was examined for the same capability to verify that this was the component from Peak D that acted with the endoglucanase. The two components still

showed synergistic hydrolysis of filter paper (Table 2). This synergistic interaction was then examined with the complete cellulase of T. harzianum E58.

The role of the purified cellobiohydrolase in synergistic hydrolysis was examined by adding purified enzyme to T. harzianum E58 cellulase and measuring glucose and cellobiose production. Addition of 10 or 20 μg of purified exoglucanase to 40 or 200 μg of T. harzianum E58 cellulases had no significant effect on production of either glucose or cellobiose (Table 3). In sub-saturation concentrations, additional exoglucanase did not increase cleavage of cellobiose units from the cellulose chains.

The substrate surface was not saturated, therefore, the lack of increased reducing sugar production could not be due to a lack of substrate binding sites. This lack may be related to the competitive adsorption observed by Ryu et al. (1984). These researchers suggested that continued hydrolysis by the exoglucanase is dependent on the presence of adsorbing endoglucanase, and vice versa. This competition for adsorption sites is thought to promote successive hydrolytic steps by the enzymes. Alternatively, the hydrolysis is dependent on the proper ratio of endoglucanase to exoglucanase, allowing formation of functional complexes on the substrate surface.

FIGURE 36: Gel Filtration on Superose-12 Column of Combined Chromatofocusing Fractions Containing Peak with Exoglucanase with pI 4.8 (flow rate: 0.1 ml/min)

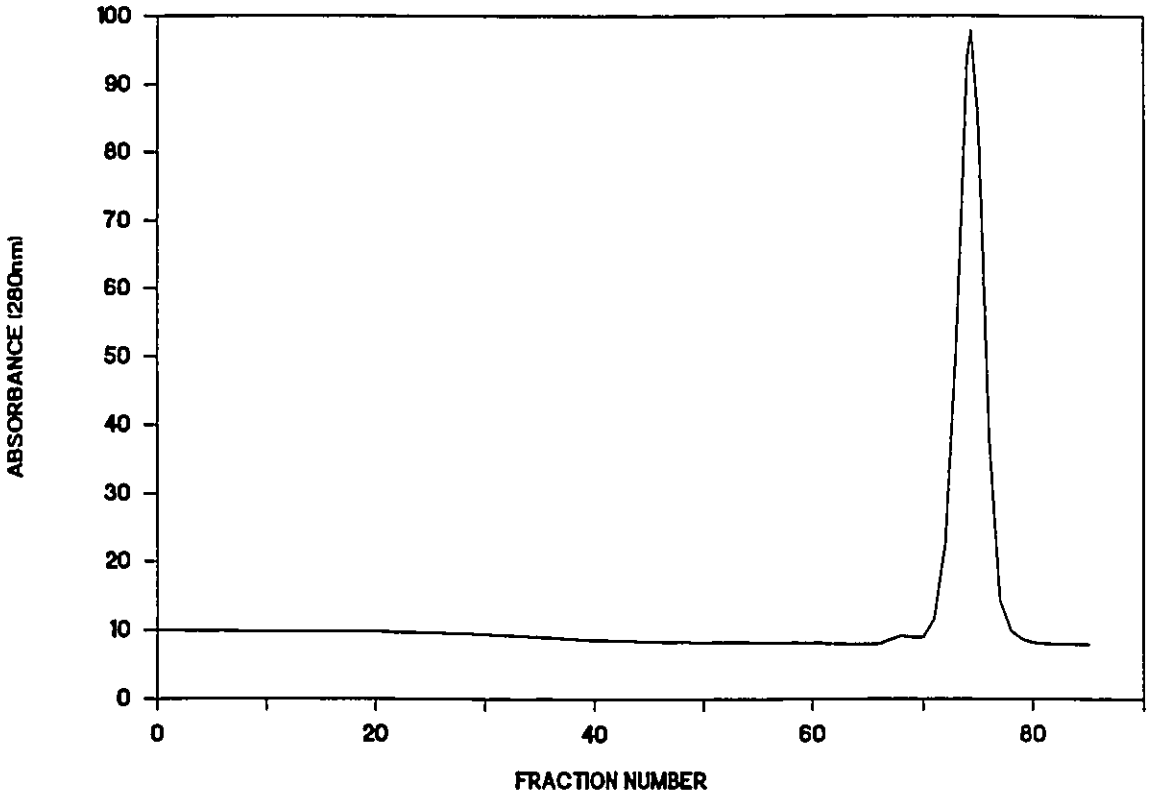


FIGURE 37: Native-PAGE Gel of Fractions from Gel Filtration Run containing Exoglucanase with pI 4.8
(flow rate: 0.1 ml/min.)
Lanes 1-6: Fractions 77-72 inclusive; Lane 7: Fraction 69;
Lane 8: Standards
(Arrow marks the position of exoglucanase)

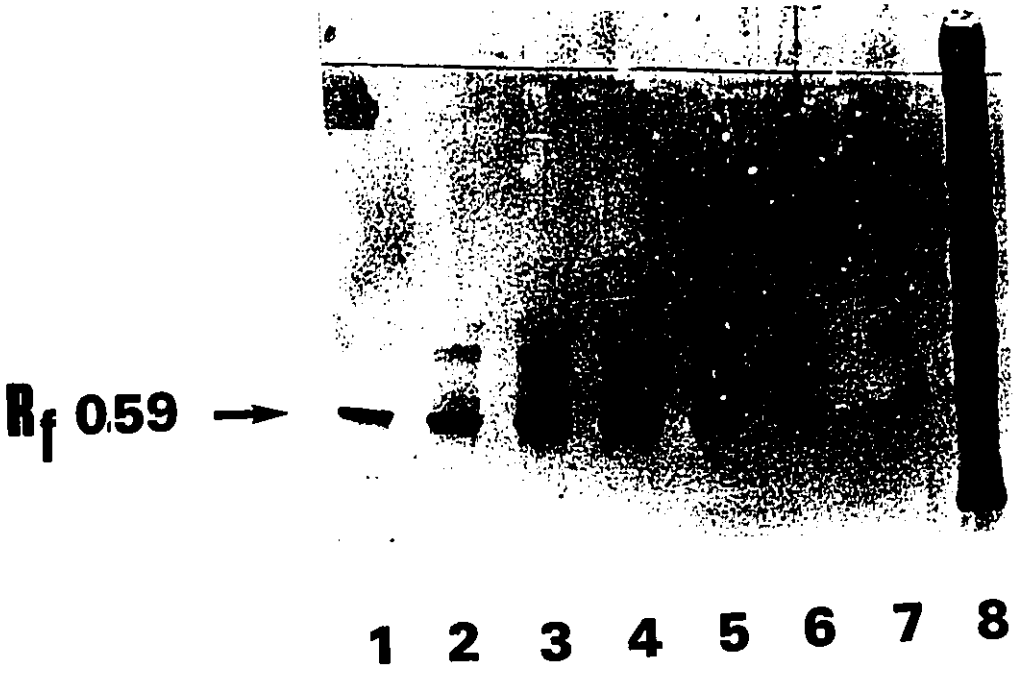


FIGURE 38: IEF Gel (pH 4-6.5) of Fractions from Gel Filtration Run containing Exoglucanase with pI 4.8 (flow rate: 0.05 ml/min.)
Lane 1: Standards; Lanes 2-8: Fractions 152-146 inclusive

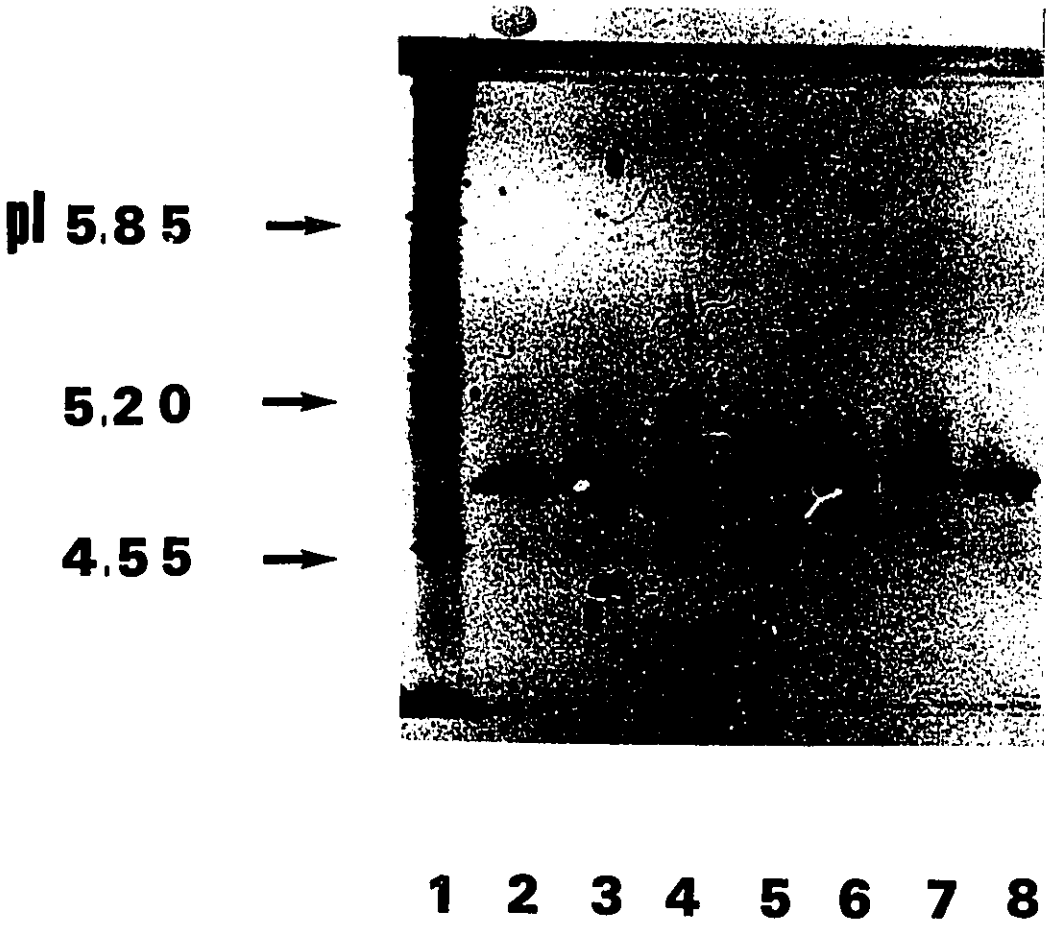


FIGURE 39: Purification Profile of T. harzianum Exoglucanase with pI of 4.8 on Native-PAGE Gel
Lanes 1 and 6: Standards; Lane 2: purified exoglucanase; Lane 3: fractions containing protein from ion-exchange fractions with pI 4.8 from chromatofocusing run in pH gradient 5.5-4.0; Lane 4: ion-exchange fractions corresponding to Peak D; Lane 5: total extracellular protein of T. harzianum
(Arrow marks the position of exoglucanase)

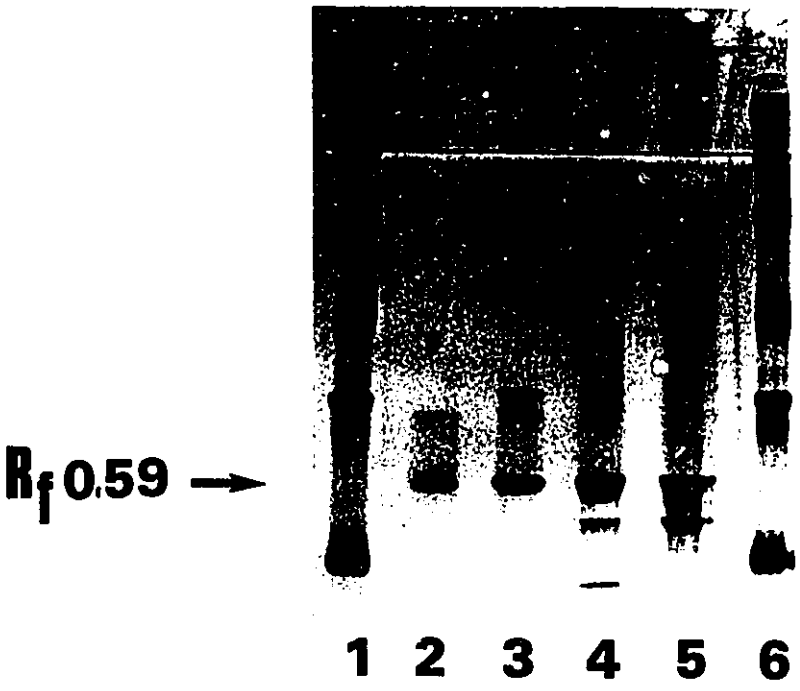


TABLE 2: Evidence of Synergism, between Purified Exoglucanase and FPLC Fraction with Carboxymethylcellulase Activity, on Filter Paper (Units are in mg/mL, Produced During a Twenty-four Hour Hydrolysis, as Measured by HPLC. Results are the average values for duplicate samples, analyzed twice.)

SAMPLE	GLUCOSE	CELLOBIOSE
EXO	0	ND
ENDO	0	0.009
EXO + ENDO	0	0.056

ND: not detected; HPLC tracings indicated very small peaks, but these were not quantified by the detector system.

If the action of a cellobiohydrolase is merely the cleavage of a cellobiose unit from the non-reducing end of a cellulose chain, then additional enzyme added to sub-saturating conditions of a cellulase mixture should result in an increase in hydrolysis. This was not the case here and other workers have also observed contradicting data to this hypothesis. Beldman et al. (1988a) observed a negative synergistic (or antagonistic) effect with certain combinations of cellobiohydrolases and endoglucanases of T. viride, which would not be expected according to current hypotheses involving co-operative action of an exo-type and an endo-type enzyme. In addition, Wood et al. (1989) reported that efficient synergistic action by combinations of Penicillum pinophyllum cellulases occurred only when both cellobiohydrolases, CBHI and CBHII, were present and only with certain endoglucanases. Therefore, when one cellobiohydrolase of T. harzianum E58 was added in excess to the complete cellulase on filter paper, no significant increase in hydrolysis could occur without addition of the other cellobiohydrolase which cleaves the alternate cellulose chain end conformation.

TABLE 3: Effect of Added Purified Exoglucanase on Glucose and Cellobiose Production from Filter Paper by T. harzianum E58 Cellulases (Units are in MG/ML, Produced During a Twenty-five Hour Hydrolysis, as Measured by HPLC. Results are the average values for duplicate samples, analyzed twice.)

Amount Enzyme	Amount Exo Added	Glucose (mg/ml)	Cellobiose (mg/ml)	Total Reducing Sugars (mg/ml)
40 µg	0 µg	0.38	0.77	1.15
40 µg	10 µg	0.37	0.77	1.14
40 µg	20 µg	0.58	0.84	1.42
200 µg	0 µg	2.95	1.14	4.10
200 µg	10 µg	3.02	1.16	4.18
200 µg	20 µg	3.26	1.22	4.49

CONCLUSIONS:

By comparing the extended hydrolyses of several substrates by various enzyme preparations, it was observed that the filter paper assay was not accurate in predicting the long-term hydrolysis of any cellulosic substrate other than filter paper itself. Even then, a single batch of enzymes must be used, so that the specificity and ratio of cellulase components remains constant.

It is apparent that the filter paper assay measures an average of combined cellulase activities, but cannot give a specific activity for individual cellulase components or group of cellulase types. In addition, the filter paper activity per gram enzyme preparation does not account for the non-cellulase protein in the preparation, which is usually unknown. However, the use of zymograms with representative substrates was seen to be an effective way to partially quantify some of the cellulase components and determine their specificity. It was apparent that the filter paper assay is more suitable as a screening method for the detection of cellulase activity in enzyme preparations, while the zymogram technique is more effective in determining the activity of individual enzyme components.

Preliminary separation of extracellular proteins of T. harzianum E58 by chromatofocusing revealed the presence of approximately twenty peaks with different isoelectric points. Partial characterization of the peak components by various enzyme assays indicated that most peaks contained activity towards both cellulose and xylan. It could not be determined whether the activities were found in separate enzymes with similar pI, or in enzymes with broad specificity, without further purification steps. Through the use of electrophoresis and zymograms, the presence of several endoglucanases specific to β-1,4-glucosidic linkages and xylanases specific to β-1,4-xylosidic linkages were detected. However, components that appeared to be active towards both substrates were also observed.

Attempts have been made to correlate endoglucanase specificity to adsorption affinity for crystalline substrates or to the degree of randomness of action, however, no correlations have been observed (Beldman et al., 1987). The requirement for these non-specific endoglycanase components has yet to be determined. Their use may be inherent in the substrate, involving the hydrolysis of the linking regions between cellulose and hemicellulose (xylan). Alternatively, the high molecular weights of the enzymes, as determined by Native-PAGE, may indicate the existence of enzyme complexes and not of pure cellulase components.

The nature of the substrates used to characterize the exoglucanase makes this enzyme less suited to characterization by the zymogram technique than the endo-type enzymes, therefore, the enzyme was purified for further study. The purified exoglucanase was found to have a pI of 4.8, and a molecular weight of 63 kDa by SDS-PAGE. It did not hydrolyze salicin, cellobiose, or carboxymethylcellulose. It produced cellobiose as the major hydrolysis product from its action on filter paper and Avicel and was, therefore, classified as a cellobiohydrolase.

The cellobiohydrolase showed synergistic action with a chromatofocusing fraction containing endoglucanase activity. Exoglucanases are thought to be active in the cleavage of cellobiose units from the non-reducing chain ends created by the endoglucanase. In which case, as long as the exoglucanase is not in excess, its addition to a cellulase mixture should increase the extent of cellulose hydrolysis over a given length of time. However, this was not observed with the T. harzianum E58 system. Using a range of subsaturation concentrations of enzyme, the addition of purified exoglucanase did not have a significant effect on the hydrolysis of filter paper during a twenty-five-hour hydrolysis.

Recent results from Wood et al. (1989) indicated that significant synergism by P. pinophyllum cellulases only

occurred when both cellobiohydrolases were present with certain endoglucanases. This evidence reflects the two possible conformations of chain ends which may be produced during hydrolysis. Therefore, the results obtained with T. harzianum E58 were not totally unexpected, due to the addition of only one cellobiohydrolase. The second cellobiohydrolase would then likely be limiting.

The negative synergistic effect that was observed between certain combinations of exoglucanases and endoglucanases indicated that not only are some combinations incapable of increasing cellulose hydrolysis, others may actually decrease the enzymes' effectiveness on the substrate. This supports the theory of complex formation by the cellulase components on the substrate surface, such that some components may interfere with the adsorption or hydrolysis step of another cellulase component, if they do not act synergistically. A correlation between the binding affinity of endoglucanases and their synergistic action with the exoglucanase components (Beldman et al., 1988a) suggests that synergism is dependent on an exoglucanase/endoglucanase complex obtaining a "tight hold" on the substrate surface. Alternatively, the less tightly binding endoglucanases function on the soluble oligosaccharides and synergism is not as pronounced on these substrates.

The proposed modified mechanism of cellulose hydrolysis involves the hydrolysis of the crystalline regions of the cellulose chain by randomly acting endoglucanases of high binding affinity. These components could form a complex on the cellulose surface, with either of the tightly binding exoglucanases, which would increase hydrolysis, or the hydrolytic action of one enzyme would promote the action of the other group of enzymes. As short chain oligosaccharides are released from the substrate surface, the enzyme components with less affinity for crystalline cellulose hydrolyze these to cellobiose and glucose. The non-specific endoglucanases would be capable of hydrolyzing either cellulose or hemicellulose regions and the possible linking-regions between the two, exposing more substrate for the specific enzymes to hydrolyze.

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