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INTRODUCTION

The mechanism of the enzymatic breakdown of cellulose has not yet been unraveled. Like the hydrolysis of other polysaccharides (starch, dextran), two steps are generally recognized, the hydrolysis of the long chain to a disaccharide and the hydrolysis of the disaccharide to a simple sugar. The acceptance of this picture may be questioned on two points: (a) Is there a step preceding the hydrolysis of the 1,4- (or 1,6) linkages, by which the naturally occurring material is made available to the hydrolytic enzymes (1); and (b) is the hydrolysis of the long chain to the dimeric unit the work of one or of a complex of enzymes? That is, is Cx, the polysaccharase acting on the β -1,4-linkage of cellulose, a single enzyme or a group of similar enzymes? The present paper deals with the latter question as approached by the application of paper chromatography to the separation of cellulolytic enzymes.

Previous workers have chromatographed hydrolytic enzyme systems and found indications of multiple components: amylases (2); invertase (3); polygalacturonase (4). Our earlier attempts to obtain chromatograms of cellulolytic enzymes failed. The successful application of the paper strip method to use with the enzyme, Cx, stems from the observation of Whitaker (5) that protein stimulates the cellulolytic activity of a *Myrothecium verrucaria* filtrate. It is probable that the action of the added protein is to prevent the irreversible binding of some of the Cx. Application of protein to the paper strips (as described later) enhanced the recovery of Cx, and chromatograms were then possible.

METHODS

Cx Determination

Cx activity is measured by the reducing sugars (as glucose) produced by action of the enzyme on 0.5% carboxymethylcellulose (CMC) of low viscosity and

of low degree of substitution (D.S.) (Hercules CMC 50T, D.S. = 0.52), in 0.05 M citrate buffer at pH 5.4 and 50°C. A Cx unit is the amount of enzyme which under the above conditions gives a reducing value of 0.40 mg. reducing sugar/ml. reaction mixture/hr.

Organisms and Filtrate Production

The following organisms were used in this work: *Penicillium pusillum* QM 137g; *Myrothecium verrucaria* QM 460 (BPI 1334.2); *Streptomyces* sp. QM B814; *Trichoderma viride* QM 6a; *Pestalotia palmarum* QM 381.

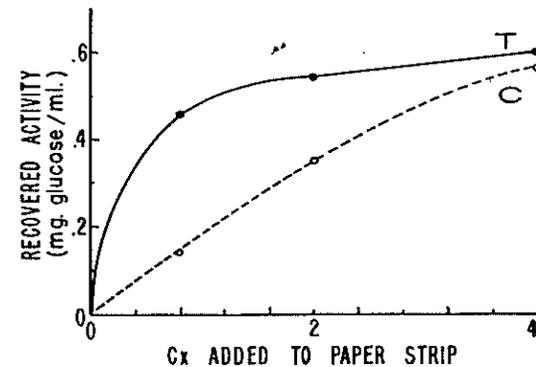


Fig. 1. Effect of the addition of gelatin to paper on the recovery of Cx from the paper.

C = Control strip (Whatman No. 1 paper)

T = Test strip sprayed with 1% gelatin

The Cx is that of *Penicillium pusillum* grown on duck. The abscissa is given in units of Cx.

Of these only *Penicillium pusillum* produces a filtrate of potency sufficient for direct analysis by the paper-strip method. Filtrates of the other organisms were concentrated 20-30-fold by precipitation with 2-3 vol. acetone in the cold. The precipitate was taken up in a minimal amount of 0.05 M citrate of pH 5.4. Solutions to be chromatographed should contain at least 50 Cx units/ml. The filtrates used are extracellular solutions obtained by techniques previously described (6).

Paper Chromatography

On the basis of results of preliminary experiments, the following method was developed for carrying out the paper chromatography. The method follows closely that of Cabib (3) and others in its general outline.

Large sheets (46 × 17 cm.) of Whatman No. 1 paper were immersed in a 4% zein solution in 75% ethyl alcohol at pH 4.0 (adjusted with 1 N HCl), removed, and air-dried. Strips (46 × 1 cm.) were cut from these sheets. The enzyme solution was spotted 6 cm. from the top of the paper, and the strip was placed in the chro-

matography chamber at 8°C. After irrigation by the descending method for 16–20 hr., the strips were removed, air-dried at room temperature, and cut into strips 2 cm. long. Each of these fragments was placed in a tube containing 1 ml. of CMC buffer mixture, and incubated at 50°C. for 2 hr. The amount of reducing sugars produced is a function of the Cx concentration. The irrigating solution was 0.3 M NaCl in 0.05 M citrate, pH 5.4. (Acetone–water 50:50 *v/v* was also used, but had no particular advantage.)

An example of the effect of coating the paper with protein on the recovery of Cx is shown by the curves in Fig. 1. In this particular test, gelatin was used as the coating protein, but zein gave identical results.

A plate-assay method, by which the developed strip is placed on an agar medium containing the substrate, also has been used in this work. Carboxymethyl-cellulose (CMC) was the substrate hydrolyzed. The undigested CMC was precipitated by addition of a 5% copper sulfate solution in 1 N sulfuric acid. Under the *M. verrucaria* strips, the clear zone extended the full length but the width of the zones was greatest at the peaks shown by the aliquot-assay method. With the other filtrates clear zones were found only at the position of the peaks. The method was discarded for routine purposes as less desirable than the aliquot-assay procedure.

The enzyme patterns obtained by the technique outlined have a high degree of reproducibility. The only deviation of any significance is in the last two aliquots of the strip. This is due to slight differences in the distance traveled by the solvent, and to the fact that the last fragment may not be 2 cm. in length, it being the remainder after all the rest has been divided into equal segments.

RESULTS

Comparison of Cellulolytic Filtrates by Paper-Strip Chromatography

About 0.02–0.04 ml. of several enzyme solutions were placed on zein-pretreated filter-paper strips (46 × 1 cm.) and developed with 0.3 M NaCl as described. Great differences in behavior are apparent (Fig. 2). The Cx of *Trichoderma viride* moves least under the test conditions, whereas most of the Cx of *Polyporus versicolor* appears to keep pace with the solvent front. Except for *T. viride*, all filtrates appear to have at least two Cx-type components, the slower-moving one remaining near the original spot in most cases.

To determine whether some foreign component of the solution is affecting the movement of Cx, the two filtrates showing the greatest difference in pattern were spotted on the same strip. The enzymes continued to move independently, the combined filtrates showing the same pattern as the combined patterns of the two filtrates.

In comparing chromatograms of several filtrates of *Myrothecium verrucaria*, we have noticed differences in the nature of the fast-moving

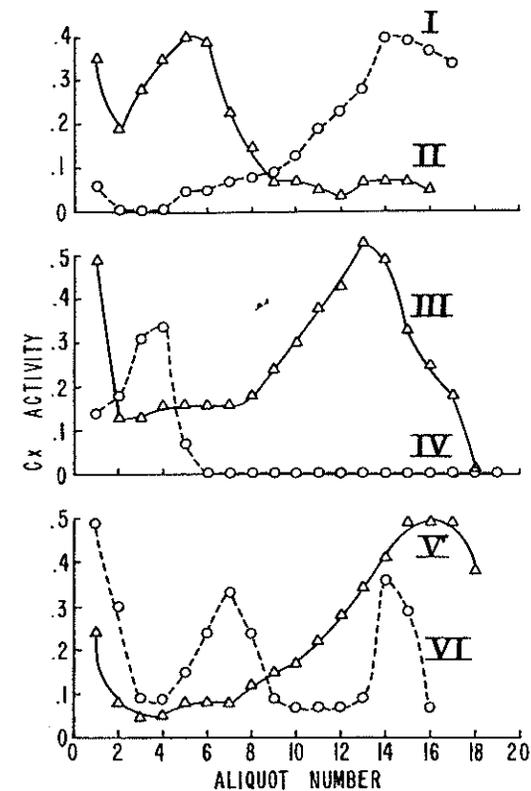


FIG. 2. Chromatographic patterns of the Cx of filtrates of various organisms. The last point on each curve is at the solvent front.

I *Torula* sp. QM 986 IV *Penicillium pusillum* QM 137g
 II *Rhizoctonia solani* QM 1039 V *Polyporus versicolor* QM 1013
 III *Chromosporium* sp. QM 806 VI *Aspergillus terreus* QM 72f

Position along the abscissa represents distance that the enzyme has moved down the paper strip.

component. There have been sharp peaks and broad peaks, and there have been differences in position relative to the solvent front. On the assumption that the moving component may be a complex between Cx and some other substance, various compounds were added to the enzyme solution. Glucose, maltose, cellobiose, and several proteins had

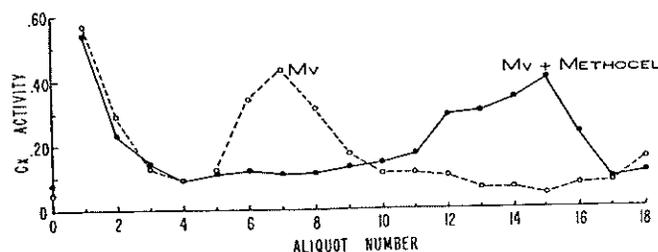


FIG. 3. Effect of Methocel on movement of Cx.
Mv = *Myrothecium verrucaria* filtrate
Mv + Methocel = *M. verrucaria* filtrate + 0.5% Methocel

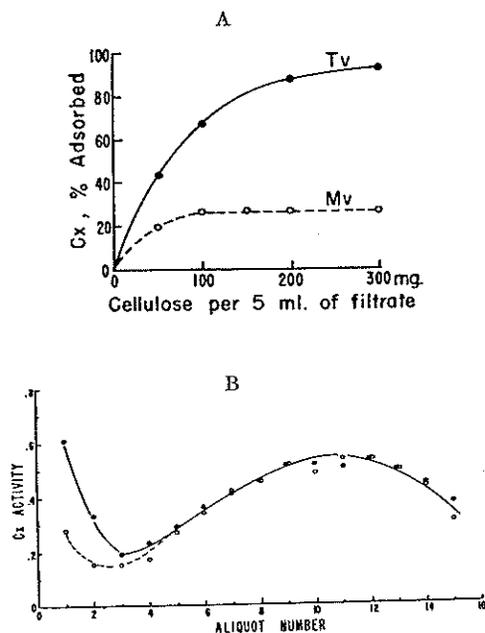


FIG. 4. Selective adsorption of Cx.
A. Adsorption of Cx on cellulose (Solka floc)
Tv = *Trichoderma viride* adsorbed at pH 4.8
Mv = *Myrothecium verrucaria* adsorbed at pH 5.7
B. Paper chromatogram of the above *M. verrucaria* filtrate before (●—●), and after (○—○), adsorption.

no effect. Methocel (methylcellulose) markedly increased the movement of the fast component, but had no effect on the slow component (Fig. 3). If there are substances present in some filtrates having an action similar to Methocel, this would account for part of the variation observed.

To determine whether acetone precipitation of the enzymes affected the patterns, comparison was made with lyophilized preparations. No differences in pattern were observed.

Separation of Components of the Cx Complex

An attempt was made to separate the components appearing on the chromatograms. The enzyme solutions were treated in various ways, and were chromatographed before and after treatment. Adsorption of the Cx of *T. viride* on cellulose (Solka Floc, Brown Co.) is great, and approaches 100%. On the other hand, adsorption of the Cx of *M. verrucaria* occurs to a much lower degree and approaches 30% (Fig. 4a). The Cx of the former thus appears to be a single adsorbable enzyme, while the Cx of the latter is composed of adsorbable and nonadsorbable components.

Chromatograms of these solutions before and after adsorption of part of the Cx (30% in *M. verrucaria*; 38% in *T. viride*) showed no change in pattern in *T. viride*. Since the Cx of this organism behaves as an entity, no change was expected. The part of the *M. verrucaria* filtrate removed by adsorption was the slow-moving component (Fig. 4b). This would indicate that the reason for the slow movement is adsorption of this Cx on the cellulose of the strip.

Removal of the fast-moving Cx component of *M. verrucaria* filtrates can be achieved by adsorption and elution from kaolin (Fig. 5b). After adsorption of 46% of the total Cx (pH 3.5), the kaolin was eluted twice with phosphate buffer (pH 7.0). The second eluate contained relatively little of the fast-moving component.

An alternate method was found for obtaining filtrates of *M. verrucaria* having a minimal amount of the fast-moving Cx component, namely, variation in the conditions under which the organism is grown. Filtrates were obtained by growth of this fungus in highly buffered solutions at pH 4.3, 5.3, and 7.0. At the high, and at the low, pH levels, production of the fast-moving component is suppressed (Fig. 5A). Attempts to get *T. viride* to form a fast-moving component by pH control were unsuccessful. In all cases, only the slow-moving Cx was produced.

Efforts to selectively inactivate one component by heat at different pH's were unsuccessful.

The question has arisen as to the presence and possible effect of cellobiase in experiments of the kind here described. We have shown

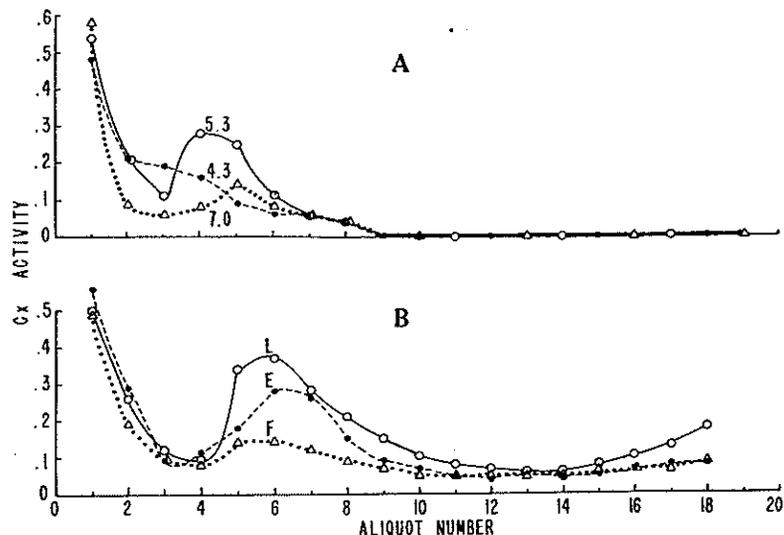


FIG. 5. Chromatographic patterns of *M. verrucaria* filtrates.

A. Effect of pH of growth medium: pH 4.3, pH 5.3, pH 7.0

B. Effect of treatment of filtrate with kaolin

E, First elution of adsorbed Cx

F, Second elution of adsorbed Cx

L, Cx unadsorbed on first batch of kaolin, was adsorbed on fresh kaolin and eluted. L is the eluate.

earlier (7) that the amount of cellobiase present in filtrates of the organisms used is negligible. Cellobiase does not hydrolyze CMC, and even the presence of amounts much larger than normally present would have little effect on the location of peaks in the curves.

DISCUSSION

The number of chromatographically separable Cx components in cellulolytic filtrates varies with the organism and with the conditions under which the organism is grown. These components hydrolyze car-

boxymethylcellulose, and are assumed to be attacking β -1,4-linkages in all cases, these being the only known linkages in cellulose whose hydrolysis leads to increased reducing values. In *T. viride* filtrates only one, a slow-moving, component has been found. In filtrates of *Streptomyces sp.* and of *M. verrucaria*, a slow-moving component is present, but a distinct fast-moving fraction is also found. In many cases, a third component appears to be present in *M. verrucaria* filtrates, appearing near the solvent front. In filtrates of *Penicillium pusillum* and of *Pestalotia palmarum*, the slow-moving component is relatively low in amount, and the fast-moving component relatively high. In *P. palmarum*, the bulk of the activity is found near the solvent front.

We cannot say whether the multiple components represent different enzymes, or whether but one Cx is present. Paramount to this decision is a consideration of what constitutes an enzyme and what differentiates one enzyme from another. While the components separated here have different motilities, it is not yet known whether they vary in substrate specificity, in pH and temperature optima, in effect of inhibitors (cellobiose), etc. We have shown that the addition of Methocel speeds up movement of the fast-moving component (in *M. verrucaria*), but no effect could be observed on the slow-moving fraction. It is likely that other materials may affect the rate of movement of Cx, either by the formation of complexes which resist adsorption on cellulose, or by an eluting action as it follows the Cx down the strip. The nonmoving component appears to be held back by adsorption on the paper. It could represent an uncomplexed Cx.

The similarity of the mechanisms of hydrolysis of different polysaccharides is again evident. To the list of hydrolytic systems having multiple components detectable by paper chromatography must now be added the cellulolytic system. It seems likely that the determination of whether the multiple components represent different factors or are due to complex formation may be more easily solved in one of the parallel systems (amylases, etc.), one in which well-characterized substrate fractions are procurable.

SUMMARY

It has been demonstrated by paper chromatography that filtrates of cellulolytic organisms contain up to three cellulolytic (Cx) components, depending on the species and on the conditions of growth.

The cellulolytic components may also be separated by differential adsorption on cellulose and kaolin.

The several components may represent multiple enzymes, or may be due to interactions of a single enzyme with other elements in the system.

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