Journal of Chromatography, 178 (1979) 515–523

CHROM. 12,119

ANALYSIS OF CELLULASE PROTEINS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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(Received June 14th, 1979)

SUMMARY

A new procedure using high-performance liquid chromatography for the rapid separation of cellulase proteins is described. The cellulase components of *Trichoderma reesei* are fractionated on a DEAE anion-exchange column using a phosphate buffer at pH 6.2. Activities of the individual components obtained from *T. reesei* QM6a, a wild strain, and several mutant strains have been determined. Each system examined contained β-glucosidase, at least two *exo*-β-1,4 glucanases and five *endo*-β-1,4 glucanases with the *endo*-β-1,4 glucanases accounting for 20–36% and the *exo*-β-1,4 glucanases for 64–80% of the soluble protein.

INTRODUCTION

The cellulases produced by microorganisms are complex mixtures of several types of enzymes wherein the major ones are *endo*-β-1,4 glucanase, *exo*-β-1,4 glucanase and cellobiase. These are frequently associated with other glycanases, glycosidases and proteases all of which act individually and/or synergistically to bring about the degradation and hydrolysis of various polymers often associated with cellulosic materials.

In order to evaluate these microorganisms for cellulase activity it is necessary to examine a large number of culture filtrates which have been produced under different conditions. This evaluation is usually done by the filter paper assay method[1]. This procedure gives useful information about the activity of the enzyme preparation but no information about the levels of the individual components and their role in saccharification. This type of information can best be obtained by fractionating the enzyme system.

Cellulase proteins have been fractionated on carbohydrate and polyacrylamide-gel columns[2–4]. The separations are achieved primarily by ion exchange, steric exclusion and affinity chromatography. Since these matrices are sensitive to changes in pH, ionic strength and pressure, the gradient elution of components must be carried out slowly. A typical run may require 18 to 24 h. One way to decrease this time is to increase the mechanical stability of the support material, by using inorganic

supports such as glass or silica beads. However, these materials must first be coated with an organic layer in order to prevent adsorption and/or denaturation of sensitive proteins by the silanol groups on the surface of the glass or silica. By covalently bonding a glycerolpropylsilyl layer to the beads these effects can be minimized[5]. Furthermore a variety of charged stationary phase groups can now be linked to the organic coating thus generating ion-exchange materials[6].

Proteins being amphoteric polyelectrolytes can be bound to both cation- and anion-exchange materials. The net charge of a protein in solution is a function of the pH of the buffer and the extent to which a protein is bound to an ion exchanger is dependent on the pH and ionic strength of the buffer and the isoelectric point of the protein. At pH values greater than the isoelectric point the protein can be attached to an anion exchanger since the protein carries a net negative charge. Below the isoelectric point a cation-exchange material would be used for binding.

This paper describes a high-performance liquid chromatography (HPLC) procedure utilizing a DEAE-glycophase anion-exchange column for the rapid separation, in 70 min or less, of protein components from cellulase preparations. The use of this procedure in protein separation was first described by Chang *et al.*[6]. A clear protein profile of an enzyme sample can be obtained from 1–2 mg of protein. When larger samples are used it is possible to collect and analyse individual components. The procedure is very gentle and no enzyme inactivation occurs on the column. We have used this procedure to evaluate cellulase preparations from *Trichoderma reesei* strains but the method is applicable to other enzyme systems.

EXPERIMENTAL

Materials

Cellulase preparations were obtained from *Trichoderma reesei* QM6a, a wild strain, and mutants QM9414 (ref. 7), MCG77 (ref. 8), Rutgers C-30 (ref. 9) and NG-14 (ref. 10) grown in 10-l fermentors on 6% two-roll milled cotton at 28° and pH > 3.0. Low- and medium-viscosity CM-cellulose (CMC), 7L2 and 7M, were obtained from Hercules Powder, Wilmington, Del., U.S.A. Phosphoric acid swollen cellulose (Walseth cellulose)[11], was prepared from BW200 cellulose (Brown, Berlin, N.H., U.S.A.). Cellobiose was obtained from Eastman, Rochester, N.Y., U.S.A.

DEAE-Glycophase-coated glass beads were prepared according to the procedure of Chang *et al.*[6] using 5–10-μm beads with 100-Å pore diameter. The column was slurry packed at 2000 p.s.i. using isopropanol.

General procedures

The protein concentration of cellulase solutions and fractions was determined using an ultraviolet absorption method[12].

A viscosimetric assay was used to determine *endo*-glucanase activity. A 1% solution of CMC (7L2) in phosphate buffer, pH 5.2, was used as substrate. The decrease in viscosity of the solution on incubation with enzyme at 40° was measured with an Ostwald-type Cannon 150 viscosimeter. The enzyme activity was determined from the slope of the linear line obtained by plotting specific fluidity *vs.* time. Specific activity was calculated from the enzyme activity and the amount of protein used.

The saccharifying cellulase activity of the enzyme fractions was determined by

assaying with Walseth cellulose (1%) at 40° and pH 5.2, for 60 min. Total reducing sugar was determined by the glucose–DNS method of Miller[13], and by HPLC on a Whatman Partisil PAC column (Whatman, Clifton, N.J., U.S.A.) using acetonitrile–water (80:20) as solvent. Glucose was also determined by a glucose analyzer (YSI Model 23A, Yellow Springs, Ohio, U.S.A.).

Liquid chromatography was carried out on a Micromeritics Model 7000 liquid chromatograph (Micromeritics, Norcross, Ga., U.S.A.) equipped with a Waters Assoc. U6k injector (Waters Assoc., Milford, Mass., U.S.A.). Protein was detected at 280 nm using a Varian Variscan, Model 635, UV-VIS spectrophotometer (Varian, Palo Alto, Calif., U.S.A.). The cellulase samples were separated on a DEAE-glycophase anion-exchange column using 20 m*M* potassium dihydrogen phosphate–3 m*M* NaN_3 buffer at pH 6.2. Components were eluted by using a 50-min linear salt gradient running from 0 to 0.15 *M* NaCl concentration. When necessary protein samples were concentrated using an Amicon ultrafiltration cell, Model 12, with a UM10 ultrafiltration membrane (Amicon, Lexington, Mass., U.S.A.).

RESULTS AND DISCUSSIONS

Studies have shown that most of the enzymes present in *T. reesei* cellulase have isoelectric points between 4.3 and 5.4 (ref. 14) and as such will bind to DEAE-glycophase if the solvent is buffered to a pH above the highest p*I*.

As noted earlier, inorganic supports may adsorb or inactivate proteins. In order to check this a cellulase sample of known protein concentration and activity was subjected to the liquid chromatograph and the eluted material collected and assayed. Ninety-six percent of the protein was recovered which still had 92% of the original CM-cellulase activity and 98% of the Avicelase activity. These results show that cellulase is not adversely affected by the DEAE-glycophase column or by the high pressure.

The protein profile obtained by HPLC from *T. reesei* 6a cellulase is shown in Fig. 1. The complete fractionation required only 70 min. Sufficient material was collected from each peak to determine the amount of protein present and to assay for CM-cellulase activity and Walseth cellulase activity (Table I).

It was known from previous work that *T. reesei* contained a β-glucosidase isoelectric at pH 8 (refs. 14, 15). Under the conditions used it was expected that this enzyme would elute with or near the solvent front. Assaying the various peaks for β-glucosidase activity showed that this was indeed the case as the only area to have activity was peak 1. The large fluidity change of a CMC solution with this peak would indicate the presence of an *endo*-β-1,4 glucanase (I) as β-glucosidase itself does not have an effect on CMC. Attempts to separate these components on an anion-exchange column were not successful. It seems likely that the p*I* of this *endo*-glucanase occurs at a pH greater than 7. By using a cation-exchange column the two components probably could be resolved. Recently Fagerstam[3] reported the isolation of an *endo*-glucanase (p*I* 7.5) from QM9414 using the cationic SP-Sephadex.

Based on their activities on CMC and Walseth cellulose, peaks 4 (II), 5 (III), 6 (IV) and 7 (V) are *endo*-β-1,4 glucanases (Table I). Liquid chromatographic (LC) analysis of the reducing sugars produced from Walseth cellulose showed only glucose (15–23%) and cellobiose to be present. With *endo*-glucanases II, III, IV and V this

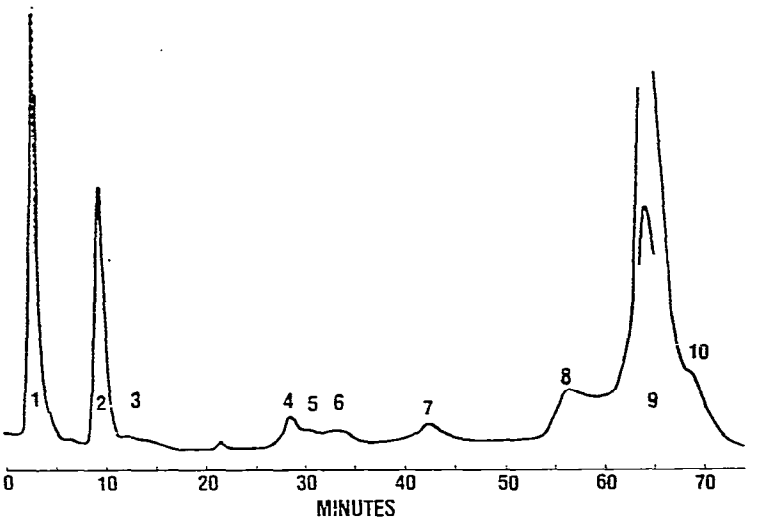

Fig. 1. HPLC protein profile of *Trichoderma reesei* QM6a. Conditions are as described in *General procedures*. See Table I for peak identification.

glucose did not arise from cellobiase activity as these fractions did not hydrolyse cellobiose. The change in viscosity of the CMC solution was more pronounced when a medium-viscosity (7M) CMC was used as compared to a low-viscosity (7L2) CMC (Table I).

exo-β-1,4 Glucanases have less action on Walseth cellulose than the *endo* enzymes and almost no action on CMC. They can be characterized by the low ratio of CM-cellulase to Walseth activities (Table I), and the fact that the hydrolysis product is predominantly cellobiose. On this basis peaks 2 (A), 9 (B) and 10 are *exo*-glucanases. The major reducing sugar produced from Walseth cellulose was cellobiose ($> 90\%$).

TABLE I

HPLC ANALYSIS OF *TRICHODERMA REESEI* QM6a (WILD STRAIN)

CMC specific fluidity = ΔF/mg protein; Walseth units = μmoles reducing sugar (DNS)/min/mg protein; R_1 = ratio ΔF (7L2)/Walseth; R_2 = ratio glucose–cellobiose from Walseth.

Fraction No.	*Total protein (%)*	*Specific enzyme activity*			R_1	R_2
		CMC		*Walseth units*		
		1% 7L2 ΔF	*0.4% 7M ΔF*			
1 *endo* I	13	11.2	24.6	2.3	4.9	0.18
2 *exo* A	11	0.3	0.4	0.85	0.4	0.03
3	2	0.3	2.3	1.04	0.3	0.09
4 *endo* II	3	3.0	12.5	1.2	2.5	0.26
5 *endo* III	1	8.7	26	1.8	4.8	0.28
6 *endo* IV	3	4.3	15	1.6	2.7	0.21
7 *endo* V	5	7.3	32	2.5	2.9	0.25
8	5	1.1	3.5	0.7	1.6	0.18
9 *exo* B	51	0.15	1.3	0.3	0.5	0.14
10	6	0.5	0.7	0.4	1.2	0.2

If indeed A and B are *exo*-glucanases then the products of hydrolysis should have inverted configurations[16]. Using cellohexaose as substrate and following the rotational change of the hydrolysis solutions (from A and B) with time, it was found that the rotations became more positive indicating an increase in the amount of α-anomer. When a drop of NH_4OH was added, the rotation immediately dropped to the α/β equilibrium level. Thus hydrolysis has taken place with inversion of configuration, and A and B are *exo*-glucanases. The amount of protein in the *exo*-glucanase fractions (peaks 2, 9 and 10) in *T. reesei* 6a cellulase is approximately 68% of the total protein. HPLC examination of QM9414 cellulase using a linear gradient gave results (not shown) similar to 6a, again with the *exo*-glucanases predominating (65%).

In Fig. 2 is shown the protein profile of the *T. reesei* mutant Rutgers C-30. Again the chromatogram is similar to that of 6a except that there are additional components in the area of *exo*-glucanase B. A summary of the properties of the various fractions is shown in Table II. From the CMC and Walseth assays it seems that the additional components are *exo*-glucanases. Thus in Rutgers C-30 *exo*-glucanases account for about 76% of the total protein.

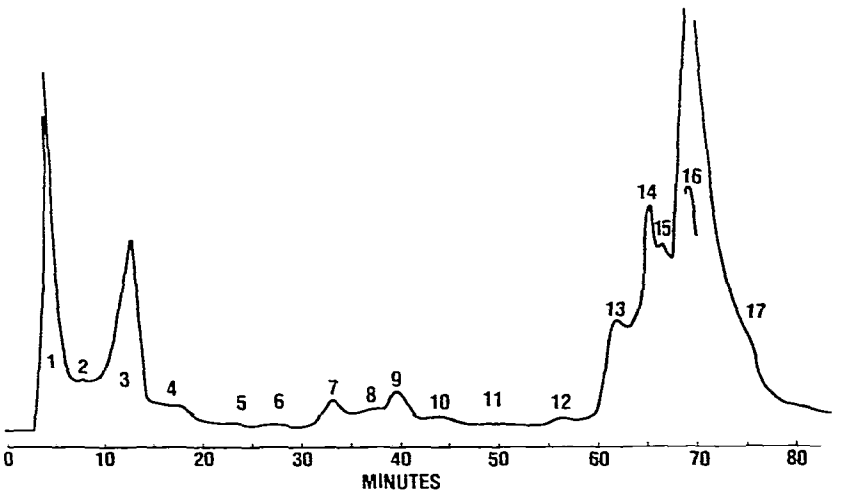

Fig. 2. HPLC protein profile of *Trichoderma reesei* Rutgers C-30 mutant. See Table II for peak identification.

A summary of the cellulase characteristics of various *T. reesei* strains is shown in Table III. Although these data show an increase in productivity for the mutant strains, the specific filter paper activities of the mutants are approximately the same as QM6a (0.68). HPLC analysis of these mutants gave patterns that were qualitatively similar (Fig. 3). Thus it would appear that these mutations have not significantly altered the nature of the cellulase enzymes.

The further utility of protein profiles from HPLC is shown in a study on the effects of biocides and temperature on the individual enzymes making up the cellulase system[17]. A standard biocide for cellulase has been 0.01% Merthiolate solution. Using this material cellulase samples could be stored for months in the cold without serious loss of CM-cellulase activity. Even when maintained at 50° for 90 h the preparations still retained at least 80% of the original CM-cellulase activity. However, when these

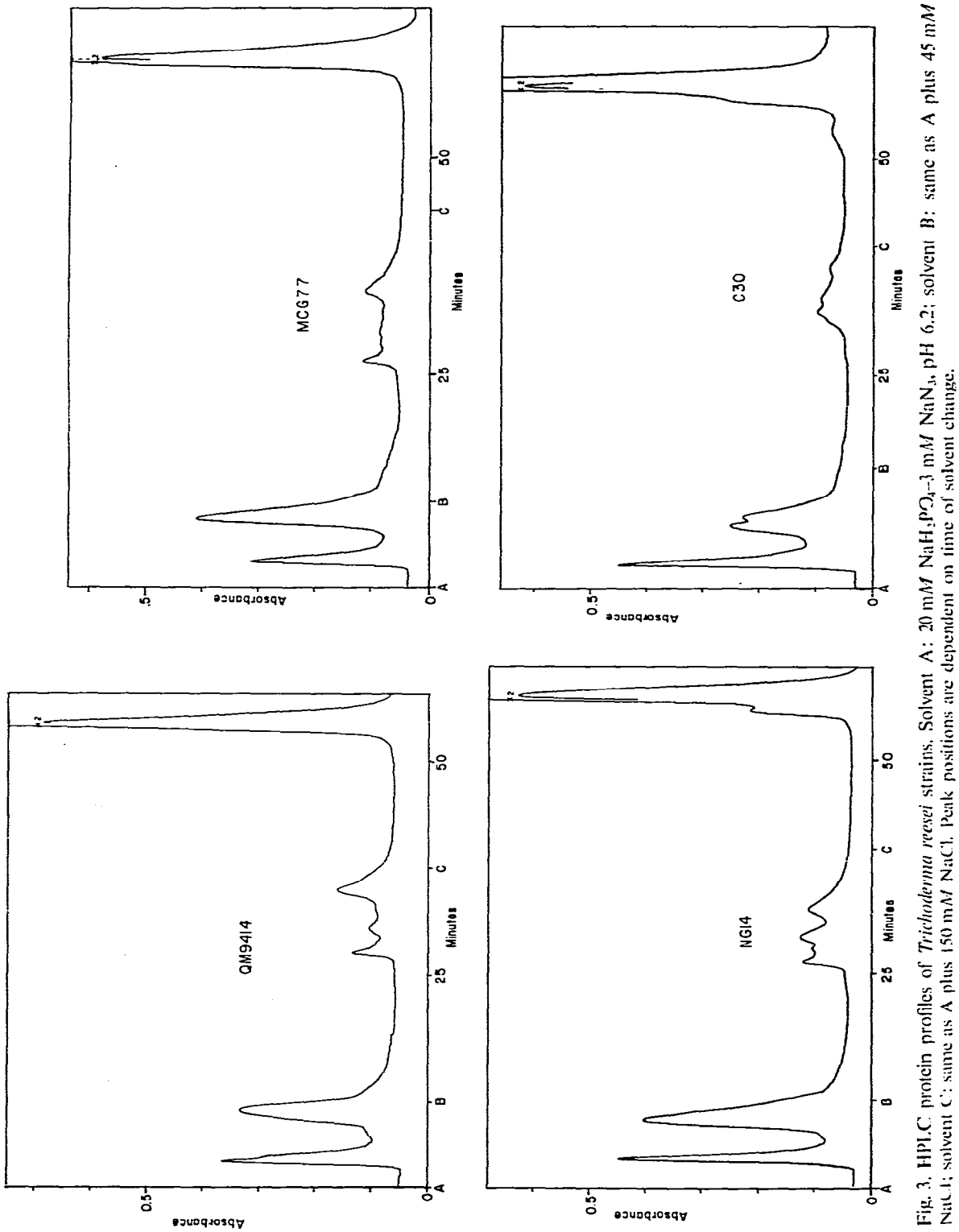

Fig. 3. HPLC protein profiles of *Trichoderma reesei* strains. Solvent A: 20 m*M* NaH_2PO_4–3 m*M* NaN_3, pH 6.2; solvent B: same as A plus 45 m*M* NaCl; solvent C: same as A plus 150 m*M* NaCl. Peak positions are dependent on time of solvent change.

TABLE II

HPLC ANALYSIS OF *TRICHODERMA REESEI* RUTGERS C-30 MUTANT

CMC specific fluidity = ΔF/mg protein; Walseth units = μmoles reducing sugar (DNS)/min/mg protein; R_1 = ratio ΔF CMC (7L2)/Walseth; R_2 = ratio glucose/cellobiose from Walseth.

Fraction No.	*Total protein (%)*	*Specific enzyme activity*		R_1	R_2
		CMC 7L2 ΔF	*Walseth units*		
1 *endo* I	10	8.3	1.6	5.2	0.20
2	2	1.2	1.1	1.1	0.05
3 *exo* A	12	0	1.1	0	0.05
4	3	2.4	1.2	2	0.19
5	0.5	8	4	2	0.19
6	0	—	—	—	—
7 *endo* II	3	6.5	1.5	4	0.32
8 *endo* III	1	7.8	2.2	3.5	0.35
9 *endo* IV	3	4.8	1.4	3.4	0.32
10 *endo* V	1	7	1.8	3.9	0.32
11	0.5	6.3	3	2.1	0.26
12	1	0	1.2	0	0.16
13	7	0	0.3	0	0.13
14	8	0	0.3	0	0.10
15	2	0	0.7	0	0.08
16 *exo* B	43	0	1.6	0	0.09
17	3	0	0.3	0	0.20

solutions were examined for Avicelase activity after 18 h at 50° only 45% of the original Avicelase activity remained. Examination by HPLC of these Merthiolate inactivated solutions showed that *exo*-glucanase A was no longer present and the amount of *exo*-glucanase B was reduced. The *endo*-glucanases were essentially unchanged (Fig. 4).

In the temperature studies it was found that when a solution of QM9414 not containing Merthiolate was placed in a 70° bath for 1 min and then quickly cooled, a precipitate formed resulting in a 50% reduction of soluble protein and a 65–70% reduction in Avicelase and CM-cellulase activity. A HPLC protein profile showed

TABLE III

CELLULASE FROM *TRICHODERMA REESEI* STRAINS

Cultures grown in 10-l fermentors on 6% two-roll milled cotton 20% mycelial inoculum, pH controlled >3.0, harvested at 14 days.

Strain	*Cellulase activity*			*Cotton activity*	*Soluble protein (mg/ml)*	*Total protein (%)***	
	CMC (units/ml)*	*FP (units*/ml)*	*Salicinase (units*/ml)*			*endo-Glucanase*	*exo-Glucanase*
QM6a	88	5	0.3	13.7	7.4	32	68
QM9414	109	10	0.6	16.7	13.6	36	64
MCG77	104	11	0.9	17.2	16.2	20	80
C-30	150	14	0.3	13.6	19	24	76
NG14	133	15	0.6	14.7	21	31	69

* Unit values in International Units/ml (ref. 1).

** Data from HPLC using a linear salt gradient as described in Experimental.

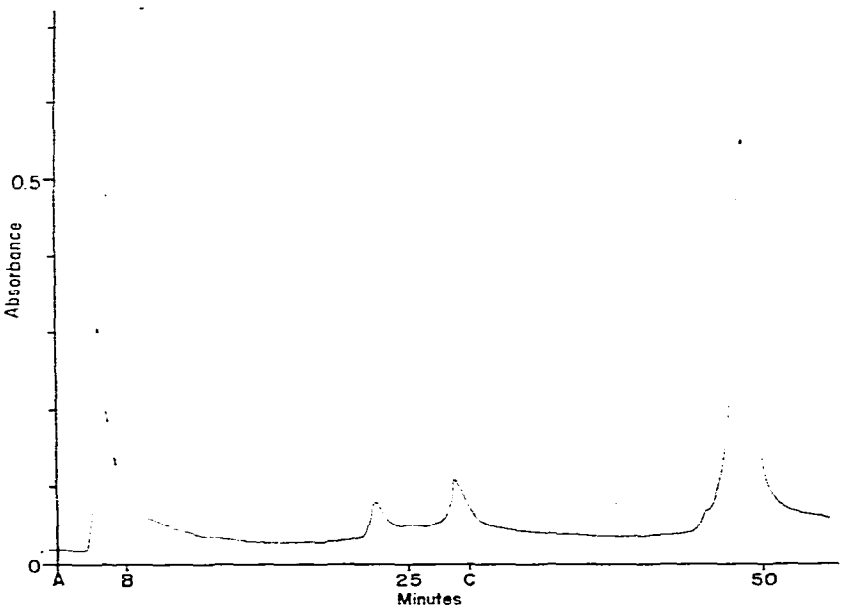

Fig. 4. Effect of a 0.01% Merthiolate on *Trichoderma reesei* QM9414 cellulase at 50°. Conditions are as described in Fig. 3.

that the amount of *exo*-glucanase B and *endo*-glucanase V had been greatly reduced, but that almost all of *exo*-glucanase A and the other *endo*-glucanases were still present. On heating for 5 min there was an additional 14% protein loss which corresponded to the loss of *exo*-glucanase A and an additional 22% loss of Avicelase activity with no further loss of CM-cellulase activity. These differences in the response of the two Avicelase fractions to Methiolate and to heat support the conclusions that they are structurally unrelated as noted by Fagerstam and Pettersson[18].

CONCLUSION

It has been demonstrated that HPLC can be used successfully to rapidly fractionate protein mixtures with no less of activity. By means of HPLC fractionation of *T. reesei*, it has been possible to demonstrate the presence of a β-glucosidase, at least five *endo*-β-1,4 glucanases and two *exo*-β-1,4 glucanases; to determine the approximate amount of the individual enzymes present and to assay these enzymes for CM-cellulase and Walseth activity, all within one day.

Larger amounts of individual components and sample purification can be obtained by repetitive injections and collections.

Protein profiles from HPLC will be useful in evaluating new mutant strains, studying effects of media and fermentation conditions on enzyme composition, analysis of residual or recovered enzyme from saccharification reactions, and evaluating the adverse effects of chemicals, heat or unfavorable pH.

ACKNOWLEDGEMENTS

The author thanks Dr. Mary Mandels and Dr. Elwyn T. Reese for their guidance and assistance, and Mr. R. E. Andreotti for the cellulase preparations.

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