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SEPARATION OF HYDROLYTICALLY ACTIVE COMPONENTS OF CELLULASE FROM MYROTHECIUM VERRUCARIA BY STARCH GEL ELECTROPHORESIS

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Summary

Using starch gel electrophoresis according to Smithies, desalted crude cellulase from *Myrothecium verrucaria* was separated into at least 12 protein zones. These were tested on their activity towards p-nitrophenyl-β-D-glucoside, sodium carboxymethylcellulose and α-cellulose. They were all hydrolytically active.

When the separate proteins were subjected to re-electrophoresis, they appeared at the same position as that, at which they appeared upon electrophoresis of the crude preparation. In some of the protein zones reducing sugars were found. The results obtained are indicative of a multiple nature of the cellulase from *M. verrucaria,* but are not contradictory to the conception that one, or a limited number of proteins, would be associated with other substances, thus yielding conjugated proteins with different physical and enzymatic properties.

INTRODUCTION

Several investigators, working with a number of different cellulolytically active micro-organisms, including *M. verrucaria,* have in the past obtained cellulase preparations. These mostly seemed to contain a number of different cellulases (1 - 15). Sometimes, however, they were found to contain only one cellulolytic component (16 - 22).

The separation techniques applied, very often included column chromatography and gel filtration (1, 3, 5, 9, 10, 12, 15), but also electrophoresis on starch blocks (6, 7, 8), paper (22, 23), Pevikon powder (2) or starch gel (18) and, recently, also iso-electric focusing (24, 25).

The starch gel electrophoresis of cellulase from *M. verrucaria,* described by Whitaker et al. (18), was simultaneously and independently applied

in our laboratory. Our results, however, indicate a multiple nature of this cellulase, whereas Whitaker found no major indications of heterogeneity. Two main differences between our and Whitaker's experimental conditions are the amounts of crude enzyme applied in the electrophoresis (about ten times as much in our experiments) and the character of the buffers used. Moreover, Whitaker applied a number of other purification methods before carrying out the final electrophoresis.

In the following description of our experiments it will be shown that starch gel electrophoresis of crude cellulase from *M. verrucaria* under our experimental conditions gives a great number of cellulolytically active protein zones. Moreover, upon electrophoresis of cellulase fractions, obtained after chromatography of the crude cellulase over Sephadex columns, all protein zones are found again.

DESCRIPTION OF ELECTROPHORETIC TECHNIQUE

The experimental arrangement was essentially the same as the vertical starch gel electrophoresis developed by Smithies (26). The gel, prepared from hydrolysed starch (Connaught) was 255 mm long, 120 mm wide and 6 mm thick, with on each end a thicker part, where the connections with the electrode vessels were made. At a distance of 75 mm from the upper side, 7 slots of each 12 x 1 x 5 mm were present. In general 0.05 ml of a 10 % cellulase solution was applied to these slots. Albumin, when used for comparison, was used as a 2 % solution (0.05 ml). The gel surface was coated with paraffin. The lower side was placed on some layers of Whatman 3 MM paper, in one compartment of a divided electrode vessel, a platinum electrode being placed in the other compartment. Connections were made by cotton wool. On the upper side, the connection between gel and electrode compartment was obtained by a triple layer of Whatman 3 MM paper.

An electric field was applied of 2.5 to 5 Volts/cm, depending on the buffer used. The time of electrophoresis was 16 - 18 hours at 4° C. After termination, the paraffin layer was removed and the gel was cut overlength in two parts, each 3 mm thick, using a strained steel thread. One part was coloured with Amido-black to determine the position of the protein zones. During this procedure some shrinkage occurs. To establish the position of the protein zones in the uncoloured part, the position of the zones on the coloured part was marked onto elastic band, which was then stretched to the size of the untreated part, and the zones were cut out according to the positions indicated on the band.

PRODUCTION OF CELLULASE AND ASSAYS OF ENZYMATIC ACTIVITY

Details on the methods employed for the production of cellulase and on the enzymatic determinations are published elsewhere (27). The filtrates of the submerged cultures, obtained after 7 days at 26° C on a rotatory shaker, were concentrated about 10-fold. They were desalted over a column of Sephadex G-25 and freeze-dried.

Enzymatic activities were determined towards :

(a) *sodium carboxymethyl cellulose* (NaCMC) ; by definition (10) an enzyme solution contains 1 unit of cellulase activity per ml, when 1 ml of it, incubated with 5 ml NaCMC (0.7 %) produces 0.4 mg glucose equivalent per ml, the reducing sugars being determined by dinitrosalicylic acid ; culture filtrates usually contained about 10 units of cellulase activity per ml.

(b) *α-cellulose ;* the mixture of 1 ml enzyme solution and 5 ml of a 1 % suspension of α-cellulose is, after incubation, centrifuged and the

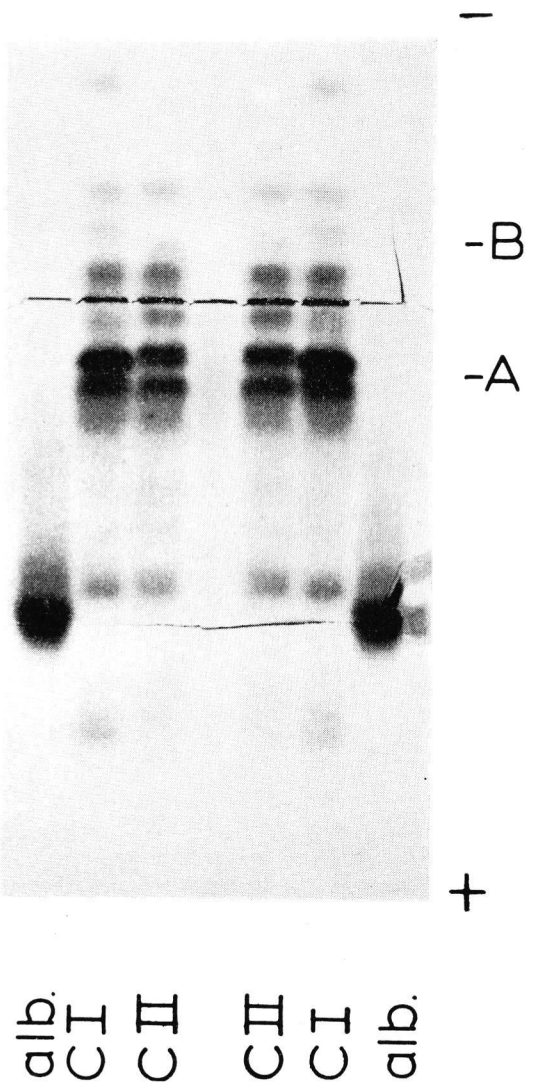

Fig. 1. — Starch gel electrophoresis of various cellulase preparations in Tris-citric acid buffer pH 7.

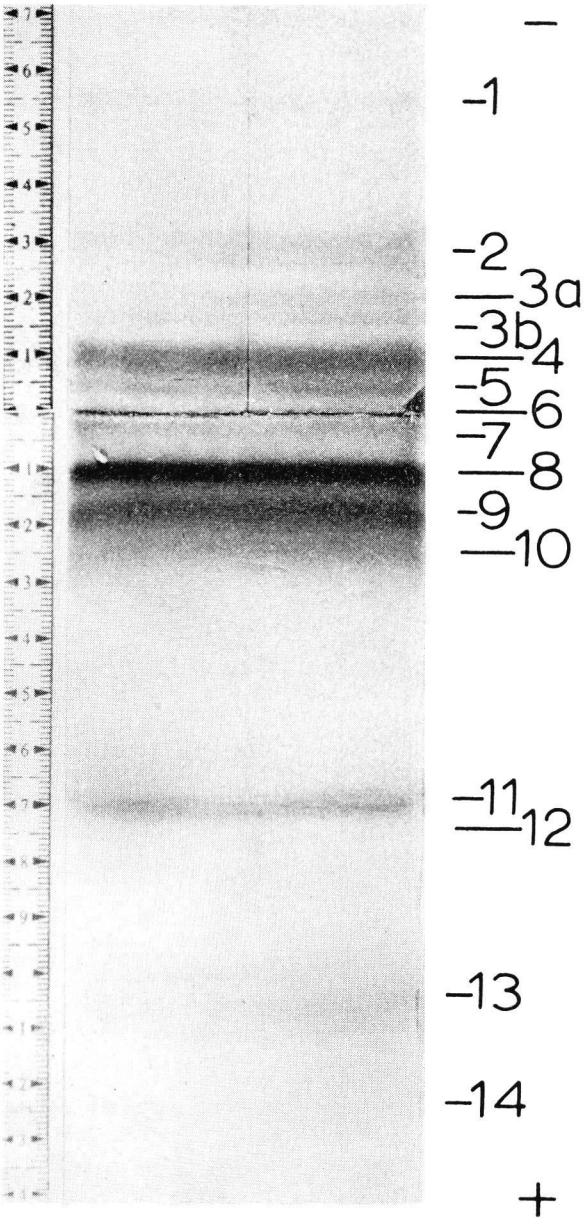

Fig. 2. — Starch gel electrophoresis of 30 mg cellulase in Tris-citric acid buffer pH 7.0.

reducing sugars are determined according to Somogyi ; unfortunately this very sensitive method cannot be applied to NaCMC, due to formation of a precipitate.

(c) *nipheglucc (p-nitrophenyl-β-D-glucoside);* the mixture of 1 ml enzyme solution and 0.5 ml nipheglucc is incubated and the amount of p-nitrophenol formed is read from a calibration curve.

RESULTS

The best separations were obtained when Tris-citric acid buffers, having a pH between 6.9 and 7.2 and a molarity of 0.02 (in the gel) were used. The buffer around the electrodes was of the same pH, the molarity, however, was 0.05.

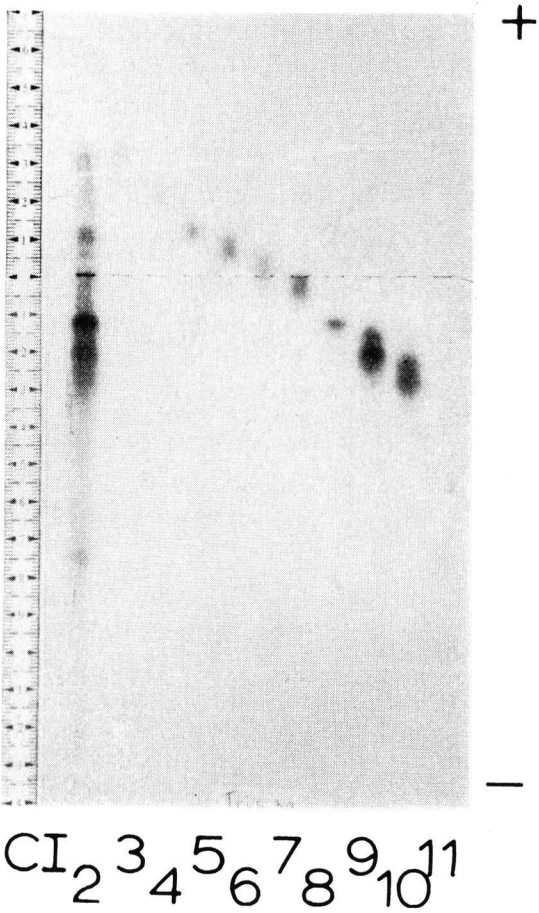

Fig. 3. — Re-electrophoresis in Tris-citric acid buffer pH 7.0 of proteins eluted from zones cut out of a semi-preparative electrophoresis of cellulase in the same Tris-citric acid buffer.

Several cellulase preparations were tested. They showed a similar overall picture but were not completely identical. Figure 1 shows the results for the batches C-I and C-II. Zone A, the first strong band at the (+) side, for example, was much weaker in C-II and the same is true for zone B the second weak zone at the (—) side. The zone of C-I moving fastest to the (—) side and that moving fastest to the (+) side, were absent or much weaker in C-II. A third batch of cellulase, not shown in the figure, gave a pattern which was almost identical to that of C-II.

To investigate the possibility that some of the proteins in the zones were artefacts, originating from proteins in other zones, and to check the reproducibility of the electrophoretic behaviour of these proteins, the proteins were subjected to re-electrophoresis. Therefore, a larger amount of C-I (30 mg in 0.3 ml) was pipetted into a slot of 70 x 1 x 5 mm. The electrophoresis in Tris-citric acid buffer and the establishment of the positions of the protein zones was carried out as described before. The result is shown in figure 2. The zones were cut out, eluted by repeated freezing and defrosting according to Smithies (26) and centrifuged. The gel was washed out three times with buffer and the fractions were concentrated by means of the apparatus of the Membranfilter Gesellschaft in collodion hulls, to a volume of 0.05 to 0.1 ml. Of each fraction 0.02 ml was used for gel electrophoresis. Eleven very small slots (5 x 0.75 x 5 mm) were now used simultaneously in one experiment.

In the Tris-citric acid buffer the proteins were found at their original position (Fig. 3) ; only some contamination with proteins from adjacent zones was observed.

Upon electrophoresis in borate buffer (pH 8.5), the same order of positions from negative to positive pole was found, but several of the fractions were found to split up into two or three zones. In acetic acid-acetate buffer (pH 4), however, the relative positions were completely different ; e.g. fraction 8 ran faster than fraction 4 (Fig. 4).

It was, of course, of interest to find out which of the protein zones contained cellulolytic enzymes. Therefore the preparative electrophoresis of 30 mg cellulase C-I in Tris-citric acid buffer was repeated twice. The zones were again cut out and eluted as described before, but, in order not to dilute the fractions, the gel was not washed. In the combined eluates of each pair of corresponding zones the enzymatic activities towards NaCMC, α-cellulose and nipheglue were determined as well as the amount of protein according to the Folin procedure. The possibility had to be taken into account that some of the extracts contained sugars or other reducing groups, even before the incubation with the enzyme (which would influence the determinations of the hydrolytic activity towards NaCMC and α-cellulose) and also, that they could contain light-absorbing components (this would influence the determination of the activity towards nipheglue). Therefore in each enzymatic determination, when a certain amount of extract was mixed with the substrate, the same amount was also mixed with distilled water and treated identically to the incubated samples. The results are shown in Tables IA-ID. The number of the zones correspond with those of figure 2.

The extinctions of the blank $E_{bl.}$ were substracted from those of the incubated samples $E_{inc.}$ to give $E_{corr.}$.

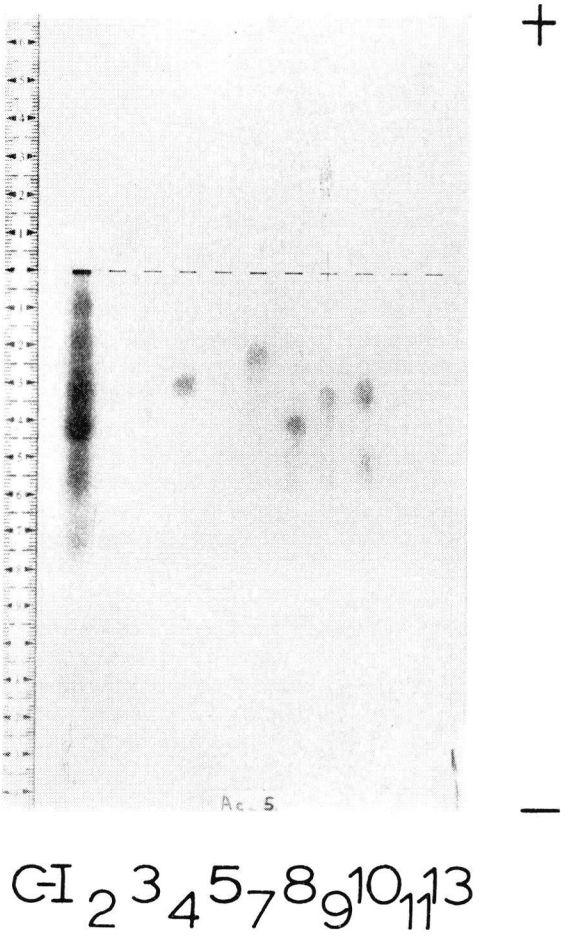

Fig. 4. — Re-electrophoresis in acetic acid-acetate buffer pH 4.0 of proteins eluted from zones cut out of a semi preparative electrophoresis of cellulase in Tris-citric acid buffer pH 7.0.

The blanks of Tables IB and IC are within the experimental error, but in the determinations of the hydrolytic activity towards α-cellulose (Table ID) appreciable $E_{bl.}$ values were found. In this series the reducing sugars were determined by the Somogyi method, which, as mentioned before, is much more sensitive than the DNS method, which was applied for the activity determinations towards NaCMC.

In fractions 6 and 7 the highest amount of reducing sugars were found. In fraction 6 the amount was equivalent to 0.24 mg in 0.05 ml. In the determination of the activity towards NaCMC only 0.01 ml of this fraction

TABLE I A

Protein determination (Folin)

Zone	*ml*	*E*
1	0.2	0.148
2	0.2	0.223
3a	0.2	0.244
3b	0.2	0.274
4	0.2	0.519
5	0.1	0.272
6	0.1	0.375
7	0.2	0.594
8	0.2	0.794
9	0.2	0.575
10	0.2	0.332
11	0.2	0.172
12	0.2	0.177
13	0.2	0.157
14 is blank	0.19	0.164

TABLE I B

Hydrolytic activity towards Na-CMC

Zone	*ml*	$E_{inc.}$	$E_{bl.}$	$E_{corr.}$	*mg equ. glucose in reaction mixture (1.1 ml)*
1	0.1	0.019	—0.010	0.029	0.09
2	0.1	0.715	—0.010	0.725	0.47[5]
3a	0.05	0.349	—0.016	0.365	0.27
3b	0.1	1.550	—0.006	1.556	0.90
4	0.01	0.998	—0.005	1.003	0.63
5	0.01	0.580	—0.006	0.586	0.40
6	0.01	0.685	0.000	0.685	0.45
7	0.01	0.870	0.000	0.870	0.55[5]
8	0.01	0.248	—0.012	0.260	0.21
9	0.02	0.118	—0.015	0.133	0.14
10	0.1	0.695	—0.005	0.700	0.46
11	0.1	0.000	—0.010	0.010	$<$0.08
12	0.1	—0.012	0.015	0.003	$<$0.08
13	0.1	0.065	—0.005	0.070	0.11
14 is blank	0.1	0.007	0.000	0.007	$<$0.08

TABLE I C

Hydrolytic activity towards nipheglucc

Zone	*ml*	$E_{inc.}$	$E_{bl.}$	$E_{corr.}$	*m. mol. p. nitrophenol formed (5 ml)*
1	0.2	0.004	0.001	0.003	—
2	0.2	0.000	0.000	0.000	—
3a	0.2	0.010	—	—	—
3b	0.2	0.028	0.000	0.028	0.005
4	0.2	0.500	0.000	0.500	0.110
5	0.2	0.714	0.000	0.714	0.157[5]
6	0.2	0.650	0.003	0.647	0.142[5]
7	0.2	0.345	—0.001	0.346	0.075
8	0.2	0.055	0.002	0.053	0.012[5]
9	0.2	0.010	0.000	0.010	—
10	0.2	0.012	0.000	0.012	—
11	0.2	0.008	—0.005	0.013	—
12	0.2	0.010	—0.005	0.015	—
13	0.2	0.005	0.000	0.005	—
14 is blank	0.2	0.000	0.000	0.000	—

TABLE I D

Hydrolytic activity towards α-cellulose

Zone	*ml*	$E_{inc.}$	$E_{bl.}$	$E_{corr.}$	*mg equ. glucose in reaction mixture (6 ml)*
1	0.5	0.192	0.142	0.050	0.018
2	0.5	0.350	0.138	0.212	0.078
3a	0.3	0.385	0.216	0.169	0.060
3b	0.3	0.661	0.280	0.381	0.141
4	0.05	0.799	0.159	0.640	0.234
5	0.05	0.829	0.313	0.516	0.189
6	0.05	1.18	0.662	0.518	0.189
7	0.05	1.09	0.595	0.495	0.180
8	0.05	0.830	0.047	0.783	0.285
9	0.05	0.476	0.036	0.440	0.162
10	0.3	0.901	0.188	0.713	0.261
11	0.5	0.425	0.127	0.298	0.108
12	0.45	0.278	0.134	0.144	0.054
13	0.5	0.238	0.184	0.054	0.021
14 is blank	0.3	0.164	0.122	0.042	0.015

was available, which would contain about 0.24/5 mg glucose, which is not detectable by the DNS method.

It would be quite conceivable that some reducing sugars can be eluted from starch gel after several times of freezing and thawing. This was tested with a blank gel, subjected to the usual electrophoresis. The amount of reducing sugars present in the protein zones 4, 5, 6 and 7 was, however, far higher than that found in any of the eluates of the blank gel. This suggests, that in these zones, reducing sugars are associated with the proteins. The low $E_{bl.}$ values of zones 8 and 9 in Table ID, can, however, easily be attributed to sugars eluted from the gel. These two most heavy bands, therefore, appear not to contain any reducing sugars.

Fractions 6 and 7 were hydrolysed with dilute sulphuric acid and were analysed chromatographically with respect to the possible presence of specific reducing sugars. The results were compared with those obtained for comparable eluates of blank gel (Fig. 5).

The hydrolysate of the eluate from the blank gel (g) showed a number of spots giving the normal sugar reaction with anisaldehyde. The most prominent of them was probably glucose. These spots were also present in

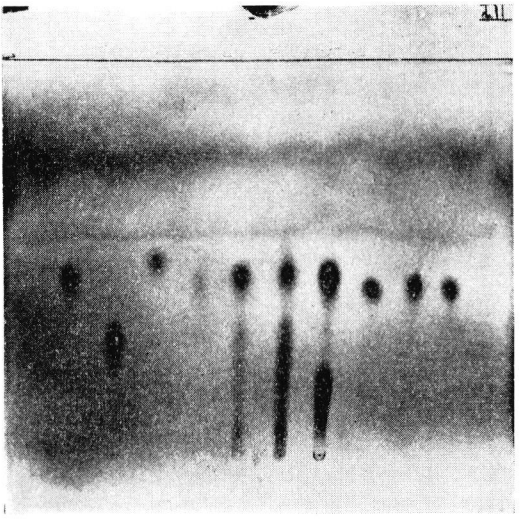

Fig. 5. — Thin-layer chromatogram of hydrolysates of eluates from zones 6 and 7 and from a blank gel in comparison with some sugars.
Adsorbent : Silicagel H.
Solvent : Butanol-acetone-water (4+5+4).
Detection : Anisaldehyde reagent.
a = glucose, b = fructose, c = rhamnose, d = xylose, e = hydrolysate zone 6, f = hydrolysate zone 7, g = hydrolysate blank gel, h = galactose, i = mannose, j = maltose.

the hydrolysates (e and f) of the zones 6 and 7, but these contained at least two spots which were not observed in the blank. The identity of these spots could not be established with certainty, but they were evidently not glucose. Therefore, zones 6 and 7 may indeed contain characteristic glycoproteins, that are responsible for the high blank values in the determination of the activity towards α-cellulose.

From the $E_{corr.}$ values of the Tables IA - ID it appears that a hydrolytic activity is associated with most of the zones. Only for zone 1 the activity is doubtful.

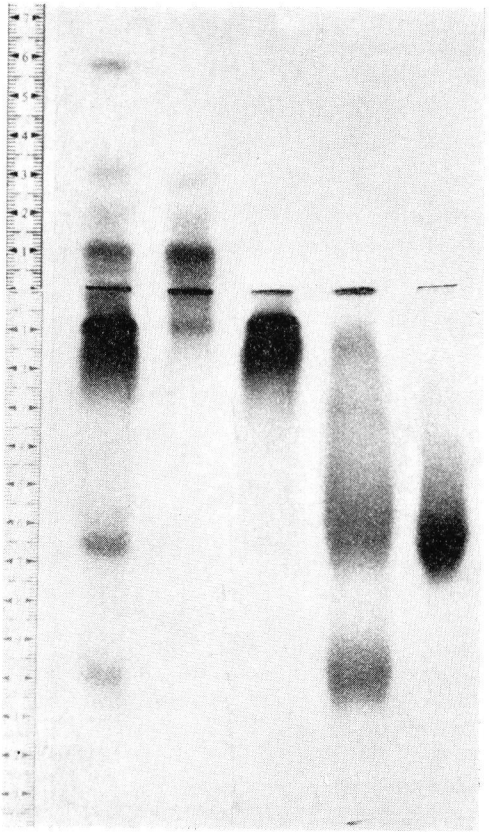

Fig. 6. — Starch gel electrophoresis in Tris-citric acid buffer pH 7.0 of protein fractions obtained on chromatography of cellulase over a column of DEAE-Sephadex A 50.

It appears that the ratio of the activities towards the three substrates is different for different zones. This is most obvious when comparing fractions 4 to 8, where the same amounts of the fractions are taken for the measurements of the activities. According to Table ID for example, fraction 8 has the highest activity towards α-cellulose, whereas, according to tables IB and IC, it has a much smaller activity towards niphegluc and NaCMC than fraction 4. This fraction has the highest activity towards NaCMC, whereas fraction 5 is most active towards niphegluc. This suggests that enzymes with different enzymatic characteristics are present in the various zones.

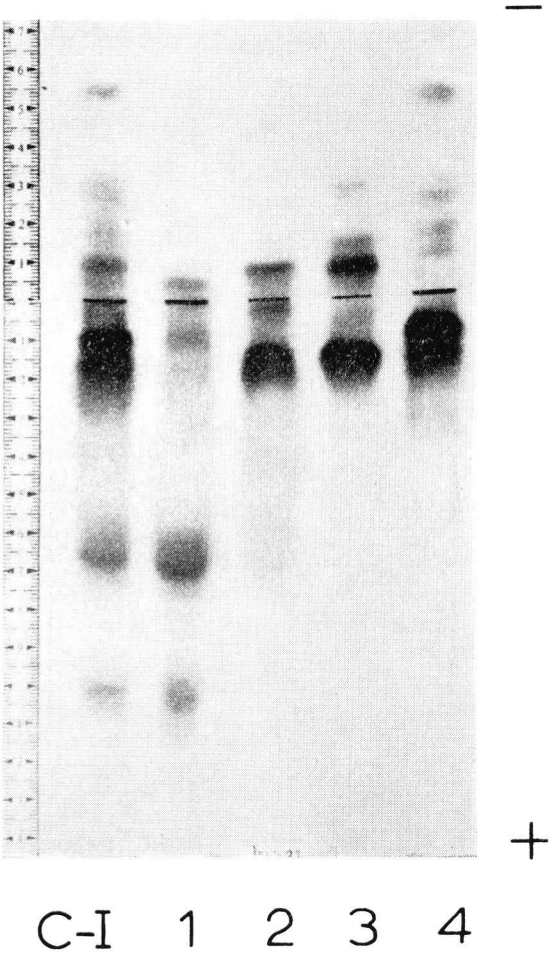

Fig. 7. — Starch gel electrophoresis in Tris-citric acid buffer pH 7.0 of protein fractions obtained on chromatography of cellulase over a column of CM-Sephadex.

The cellulase batch C-I has also been chromatographed in our laboratory over a column of DEAE-Sephadex, using a phosphate buffer pH 8 and 0.01 M. Part of the enzyme was not absorbed (fraction 1). The absorbed enzyme was eluted, using a linear gradient from 0 up to 0.5 M NaCl in the same buffer. The eluate was collected into the fractions 2 and 3. All three fractions contained enzymatic activity towards the three substrates ; only in fraction 3 very little activity towards nipheglucwas present. These fractions were subjected to the usual starch gel electrophoresis in Tris-citric acid buffer.

The same cellulase batch was also chromatographed over CM-Sephadex C-50 medium, in phosphate-citrate buffer pH 5.0 and 0.01 M. The absorbed enzyme was eluted with a linear gradient from 0 up to 0.3 M NaCl in the same buffer. Four fractions were obtained, the first one representing unabsorbed enzyme. These fractions too were subjected to the same kind of electrophoresis. The results of the two separations are shown in figure 6 and 7. It appears that all the fractions found in the original cellulase preparations are found again in the fractions obtained from the columns. Some proteins which were not absorbed on CM-Sephadex (Fig. 7, fraction 1), were strongest absorbed on DEAE-Sephadex (Fig. 6, fraction 3) and vice versa. Fraction 3 of figure 6 showed little activity towards nipheglucThis corresponds with the data of table IC, showing that the proteins of the electrophoretic zones 8-13 showed hardly any activity towards nipheglucc.

DISCUSSION

When crude cellulase from *M. verrucaria* is subjected to starch gel electrophoresis, either as such or after fractionation on substituted Sephadex columns, a number of separate zones are found, which all contain hydrolytic activity. Some are more active towards α-cellulose, others towards NaCMC or nipheglucc. It would be conceivable that some of the enzymes in these zones contain the same protein moiety, but are associated with various other substances (e.g. sugars). Especially the recent communications of Eriksson, Pettersson and Björndal (28, 29) would suggest such a possibility. They found for the fungus Stereum sanguinolentum two chromatographically separated cellulase peaks. After dialysing to zero carbohydrate content, however, the mixed enzymes gave only one homogeneous cellulase peak, upon rechromatography as well as on electrophoresis. Their results suggest, that combination of a cellulase with a carbohydrate can change the properties of the enzyme considerably. The data we obtained in the determination of the reducing groups in some of the eluted electrophoretic zones would be in agreement with such a conception and the results of thin-layer chromatography of hydrolysates of two fractions support the idea that some of the proteins are associated with sugars. Whether the protein moieties in some or all of the hydrolytically active zones are identical or not, is a question that cannot be answered definitely from our data.

There seems to be no doubt, however, that upon electrophoresis of crude cellulase from *M. verrucaria* a large number of hydrolytically active substances can be separated from each other. Some of them may be simple proteins and others are probably conjugated with sugars.

These conclusions may seem to be contradictory to those of Whitaker (18), who also applied gel electrophoresis. However, he carried out a number of purification methods before the electrophoresis and also worked under slightly different conditions.

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