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# ANALYTICAL MEDIUM-PRESSURE LIQUID CHROMATOGRAPHY OF CELLULOLYTIC ENZYMES ON SPHERON ION EXCHANGERS*

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## SUMMARY

Cellulolytic enzymes from the cultivation liquid of *Trichoderma viride-reesei* were submitted to a rapid medium-pressure liquid chromatography, analytical and semipreparative, on weakly, medium and strongly acidic cation exchangers and on medium and strongly basic anion exchangers Spheron 1000. The course of gradient elution was monitored by measurement of effluent absorbance at 285 nm and 254 nm, conductivity, pH and enzymatic activity assays (cleavage of crystalline cellulose, filter-paper, carboxymethylcellulose and *p*-nitrophenyl-β-D-glucopyranoside). On all ion exchangers tested, optimum conditions for separation were found. The chromatographic profiles and separated enzymatic activities of our preparation were compared with those of commercial technical cellulolytic preparations from the cultivation liquids of *Trichoderma viride* and *Aspergillus niger*, using Spheron DEAE-1000. The results are discussed from the point of view of the possible use of rapid column chromatography in the diagnosis of enzymatic preparations and as a modern analytical method for biotechnology.

## INTRODUCTION

Cellulolytic enzymes belong to the group of enzymes which take part in the gradual degradation of cellulose to glucose[1]. They are produced by various types of organisms, *e.g.*, bacteria, fungi or actinomycetes. In the majority of cellulolytic organisms the cellulase complex is composed of several enzymes, which hydrolyse cellulose substrates synergically. The cellulase system contains *exo*-β-1,4-β-glucanases which hydrolyse the cellulose chain from the non-reducing end and split off either glucose or cellobiose, *endo*-β-1,4-glucanases which randomly cleave internal bonds of the cellulose fibres and β-glucosidase which hydrolyses cellobiose and lower cellodextrins to glucose[2]. The accessibility of highly active cellulases is a prerequisite for the successful application of enzymatic conversion of cellulose in industry.

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A number of screening methods has been developed for the selection of highly productive mutant strains[3]. The evaluation of the samples is usually carried out from the point of view of cellulase activity, the total activity of the non-fractionated systems being determined by the method of cleavage of filter-paper[4], known as filter-paper activity (FPA). Different preparations of cellulases differ in the proportions of individual components, depending on the source, cultivation and treatment of the sample. The FPA method provides no information on the levels of individual enzymes of the cellulolytic system or on their rôle in the hydrolysis of cellulose substrates. Such information is best obtained by fractionation of the cellulase complex and by purification of the sample. For the preparation of technical enzymes, modern biotechnology requires rapid, accurate and reproducible analytical methods.

The recent development of the modern high-performance liquid chromatography (HPLC) of biopolymers[5–8] has permitted the application of this method to the separation of cellulolytic enzymes of various origins[9–11]. In earlier papers we demonstrated the possibility of using the hydrophilic polymer Spheron for the preparation of macroporous ion exchangers[12,13]. These ion exchangers proved suitable for rapid chromatography of technical enzymes[14–16]. The aim of this work was to extend these studies to rapid separations of the cellulolytic enzyme complex on an analytical and semipreparative scale.

## EXPERIMENTAL

### *Materials*

*Enzymes.* The desalted lyophilized cellulase preparation from the *Trichoderma viride-reesei* mutant was prepared from a cultivation liquid obtained from the Research Institute of the Food Industry in Prague. The cultivation took place at 30°C for 6 days in a 5-l tank at 300 rpm and 1:1 aeration, on a medium composed of 1.5 % of cellulose and 1 % of wheat bran, at pH 6.2. The cellulase preparations Onozuka R-10 and *Aspergillus niger* were commercial samples supplied by Serva (Heidelberg, F.R.G.).

*Chromatographic materials.* For the separation of cellulases, commercially accessible (Lachema, Brno, Czechoslovakia) ion-exchange derivatives of Spheron 1000 were used, with a particle size 20–40 μm: weakly acidic cation exchanger Spheron C-1000, nominal capacity 1.85 mequiv./g; medium acidic cation exchanger Spheron Phosphate-1000, 3.1 mequiv./g; strongly acidic cation exchanger Spheron S-1000, 1.72 mequiv./g; medium basic anion exchanger Spheron DEAE-1000, 1.5 mequiv./g; strongly basic Spheron TEAE-1000, 1.4 mequiv./g.

*Substrates.* For the determination of cellulolytic activity, carboxymethyl (CM)-cellulose from Serva was used, having a substitution degree of 0.7 %, a polymerization degree of 500–520 and molecular weight about 100,000. Avicel pH 105, a microcrystalline cellulose, was supplied by FMC Corporation (Philadelphia, PA, U.S.A.), Whatman No. 1 paper from Whatman (Springfield Mill, Maidstone, U.K.) and *p*-nitrophenyl-β-D-glucopyranoside from Lachema.

### *Methods*

*Chromatography.* The preparation of ion-exchange derivatives of Spheron and the chromatographic apparatus were described earlier[12,13]. For the separation of

cellulases a combination of isocratic elution and elution with a linear gradient of the ionic strength of the buffer were used. The concentration of the buffers is always given in relation to the counter-ion. The chromatographic columns were packed by the slurry method, using the stepwise pulse-packing method[17], at 2.0–2.5 MPa. The effluent from the column was monitored by a tandem system of two flow-through photocells, recording absorbance at 254 and 285 nm. Conductivity, pH and enzymatic activity were measured in each fraction.

*Enzymatic activity assays.* After treatment with enzymes, the newly formed reducing ends were determined with the arsenomolybdate reagent of Somogyi[18] and Nelson[19]. The reaction mixture contained 2 ml of a 1% solution of CM-cellulose in 0.05 *M* sodium citrate of pH 4.9 and 100 μl of the enzyme solution. The incubation lasted for 30 min at 40°C. The unit of enzymatic activity was defined as the amount of enzyme which sets free within 1 min an amount of reducing sugars corresponding to 1 μmol of glucose.

Filter-paper (1 × 1 cm) was incubated in 1 ml of 0.05 *M* citrate buffer pH 4.7 with 100 μl of sample for 3–14 h at 40°C. After the end of the incubation 200 μl of supernatant were withdrawn and the content of liberated sugars was determined using the method of Somogyi[18] and Nelson[19].

β-Glucosidase activity was determined with *p*-nitrophenyl-β-D-glucopyranoside as substrate. The reaction mixture contained 1 ml of a 1 m*M* solution of the substrate in 0.05 *M* acetate buffer pH 5 and 100 μl of the enzyme solution. Since the β-glucosidase activity was relatively low the incubation was carried out for 3–14 h at 40°C. The reaction was stopped by addition of 2 ml of 1 *M* $Na_2CO_3$. The unit of enzyme activity was defined as the amount of enzyme which sets free 1 nmol of *p*-nitrophenol within 1 min.

Avicelase activity was determined by suspending 16 mg of Avicel pH 105 in 1 ml of 0.05 *M* citrate buffer of pH 4.7 and incubating with 100 μl of an enzyme sample for 3 h under slow constant rotation of the test-tube at 37°C. [The equipment described earlier (ref. 20, Fig. 1) was used for this purpose.] The reducing sugars were determined by the Somogyi[18] and Nelson[19] method using 200 μl of the supernatant.

## RESULTS

We carried out a systematic investigation of the possibilities of rapid medium-pressure ion-exchange chromatography of the cellulolytic complex of the imperfect fungus *Trichoderma viride-reesei*, known to be one of the most suitable microbial producers of technical cellulolytic enzymes. The experiments were carried out on all commercially available Spheron ion exchangers. Experiments with isoelectric focusing of the cellulolytic complex, which will be described in a subsequent paper[21], served as a partial guide in the selection of chromatographic conditions. Optimum conditions found for chromatography on Spheron DEAE-1000 were then used for the comparison of our cellulolytic preparation with commercially available cellulolytic preparations Onozuka R-10 (*T. viride*) and preparations from *Aspergillus niger*.

### *Chromatography on cation exchangers*

The cellulases from *T. viride* were separated on an analytical scale on Spheron carboxyl-1000 at pH 3, 4 and 5, and with the same ionic strength gradient. The best

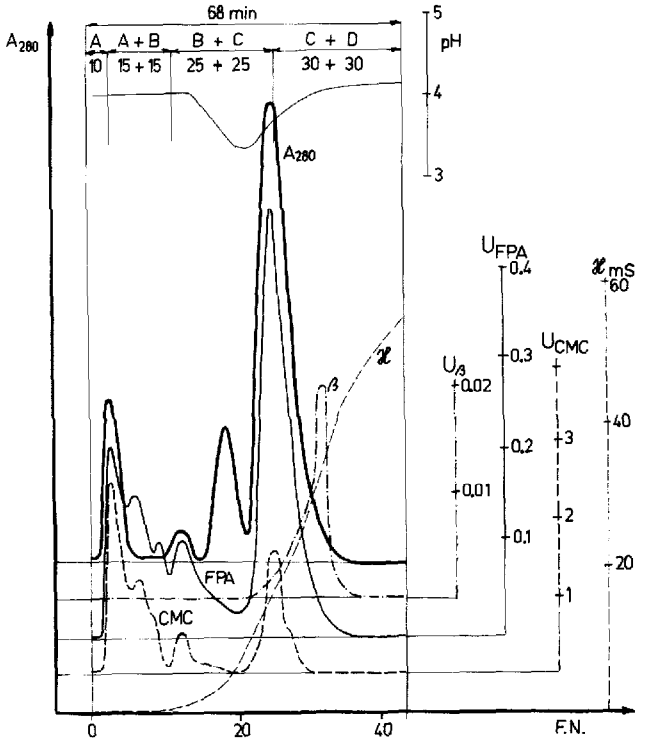


Fig. 1. Chromatography of 20 mg of a desalted lyophilized preparation of the cellulolytic system from *T. viride* on Spheron C-1000. Conditions: column, 20 × 0.8 cm; fractions, 3.3 ml at 90-sec intervals; pressure 0.3 MPa; flow-rate 2.2 ml/min; room temperature. Buffers (all of pH 4) for linear gradients: A, 0.025 *M* NaOH + citric acid; B, 0.05 *M* + citric acid; C, 0.25 *M* NaOH + citric acid + 1 *M* NaCl; D, 0.25 *M* NaOH + citric acid + 2 *M* NaCl. $A_{280}$ = Detector response; $\kappa$ = conductivity in mS; $U_{CMC}$ = units of carboxymethylcellulase activity; $U_{FPA}$ = units of filter-paper activity; $U_\beta$ = units of $\beta$-glucosidase activity. The activities were determined in 0.8-ml aliquots of the effluent (see Experimental); F.N. = fraction number.

separation was achieved —contrary to the original assumption— in the proximity of the isoelectric point of the main fraction (p*I* = 4.2) at pH 4. The concentration of 0.025 *M* of the starting buffer was optimal. The chromatographic course under these conditions is illustrated in Fig. 1. The main fraction of the carboxymethylcellulase (CM-cellulase) activity and a part of the filter-paper cleaving activity (FPA) or the Avicel cleaving activity (not shown) were eluted isocratically. The gradient of increasing citrate molarity indicates at least three minor components, characterized both by CM-cellulase activity and by FPA. Using a linear NaCl gradient an apparently non-protein peak was obtained, which displayed a higher absorbance at 254 nm than at 280 nm but none of the investigated activities. Toward the end of this gradient, separate maxima of FPA and $\beta$-glucosidase activity were detected. In all the chromatograms (at various pH values and ionic strengths), a part of the protein material was eluted at approximately the hold-up volume of the column. It is possible that this is due to the presence of non-ionogenic admixtures which interact with the enzymes and transport them.

On medium acidic Spheron Phosphate-1000, chromatograms were obtained at

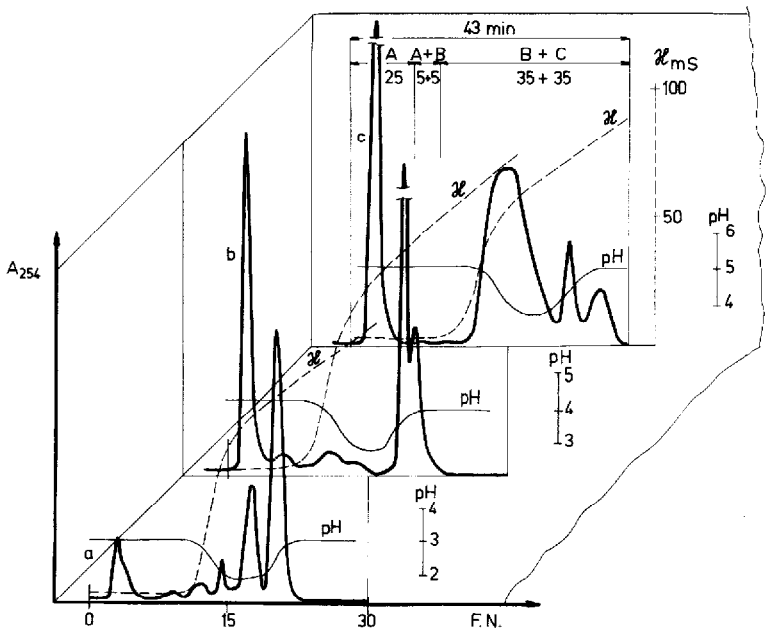


Fig. 2. Determination of the optimum pH for the separation of the cellulolytic system of *T. viride* on Spheron phosphate-1000. Conditions: 7.5 mg of lyophilized preparation; column, 20 × 0.8 cm; fractions, 3.5 ml at 85-sec intervals; pressure 0.2 MPa; flow-rate 2.4 ml/min; room temperature. Buffers: A, 0.05 *M* NaOH + citric acid; B, 0.05 *M* NaOH + citric acid + 1 *M* NaCl; C, 0.05 *M* NaOH + citric acid + 3 *M* NaCl. Citric acid was always added until the required pH value was achieved, *i.e.*, 3, 4 and 5 in a, b and c. $A_{254}$ = Detector response.

pH 3–5, using similar combinations of the gradients (see Fig. 2). At a pH value one unit lower than the isoelectric point of the main fraction of cellulases, the mixture was strongly retained on the column (pH 3, see curve a). Experiments at still lower pH values were not carried out owing to the instability of the enzymes. As is evident from Fig. 2, the quality of separation decreased with increasing pH, and an increasing proportion of the mixture was not retained on the column. It is interesting that the absorbance of individual components increased with pH. Chromatography of the cellulolytic complex on Spheron Phosphate-1000 at pH 3 proved most suitable as the first step of fractionation of the complex on a preparative scale and therefore it was subsequently investigated in greater detail, including the evaluation of enzymatic activities[21].

Fig. 3 shows a chromatogram on the cation-exchange derivative Spheron sulfate-1000 at pH 4, with the mentioned gradient systems. Three types of activity were determined in the effluent, in accord with the detector record at 280 nm. The increased acidity of the sulphonic groups, in comparison with the carboxyl and phospho derivative, was most striking in the complete separation of the $\beta$-glucosidase activity, which was eluted towards the end of the chromatogram in the form of a single peak. At least four forms of the CM-cellulase activity were detected, of which the main fraction was eluted with the hold-up volume. The FPA and Avicelase activities (not shown) were determined in two forms; that in smaller amount was practically not bound to the column, and the main fraction was obtained on elution with buffers B + C.

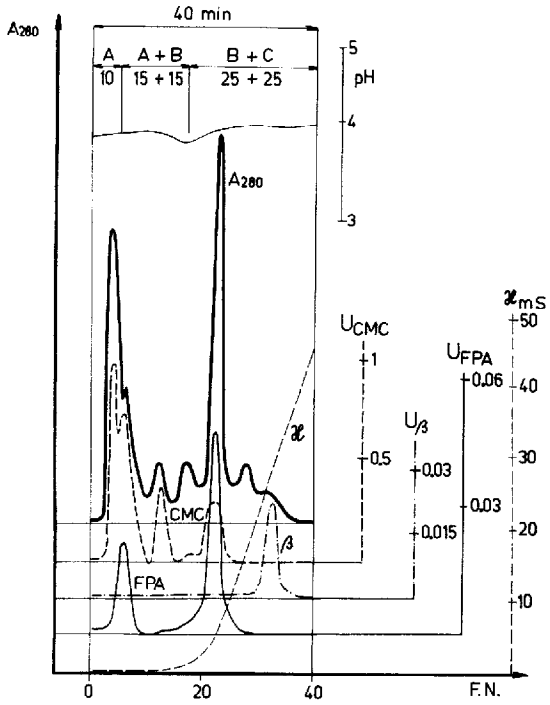


Fig. 3. Chromatography of 8 mg of the cellulolytic system from *T. viride* on Spheron S-1000. Conditions: column, 20 × 0.8 cm; fractions, 2.2 ml taken at 60-sec intervals; pressure 0.5 MPa; flow-rate 2.2 ml/min; room temperature. All buffers were of pH 4: A, 0.01 *M* NaOH + citric acid; B, 0.05 *M* NaOH + citric acid; C, 0.25 *M* NaOH + citric acid + 1 *M* NaCl. For enzymatic activities see Fig. 1.

*Chromatography on anion exchangers*

Cellulolytic enzymes are acidic proteins, and as such they are usually chromatographed on anion-exchange resins. We investigated the separation of the cellulolytic system of *T. viride* on a medium basic exchanger, Spheron DEAE-1000, at pH 4–7. The best separation was achieved at pH 5 and with the gradient systems given in Fig. 4. Similarly to cation exchangers, this ion-exchange derivative also did not afford a complete separation of any of the investigated activities for high-molecular-weight substrate; the protein peaks were characterized by the presence of several enzymatic activities. However, it is possible that this imperfect separation of the CM-cellulase activity and FPA is not a result of an incomplete chromatographic separation, but that it is generally caused by insufficient specification of individual activities, following from the choice of the substrate, and by the fact that in both cases the activity is determined via the reducing ends formed (for details see Discussion). The β-glucosidase activity, which was obtained in the form of a single peak on cation-exchange derivatives, was separated into four forms on the DEAE derivatives. CM-cellulase was obtained in four forms and FPA in two forms. The last three peaks in the chromatographic profile had no cellulase activity.

The series of chromatographic separations was completed by experiments on a strongly basic Spheron TEAE-1000 packing. Fig. 5 illustrates the chromatographic profile at two wavelengths; the proportions of the absorbances indicate that

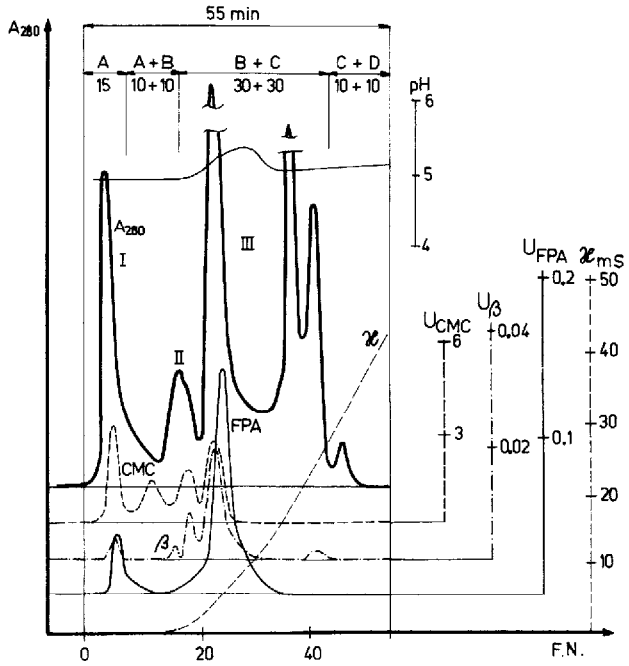


Fig. 4. Chromatography of the cellulolytic system of *T. viride* on Spheron DEAE-1000. Conditions: 20 mg of the preparation; column, 20 × 0.8 cm; fractions, 2.3 ml in 66-sec intervals; flow-rate 2 ml/min; pressure 0.5 MPa; room temperature. Buffers of pH 5: A, 0.005 *M* citric acid + NaOH; B, 0.1 *M* citric acid + NaOH; C, 0.25 *M* citric acid + NaOH + 1 *M* NaCl; D, buffer C + 3 *M* NaCl. For the enzymatic activities see Fig. 1.

the last two peaks are probably not proteins. Contrary to expectation, we could not obtain a better chromatographic profile than in the case of the DEAE derivative, despite the fact that the elution sequence of the peaks was similar. Therefore we did not evaluate the obtained fractions by activity assays.

*Comparison of the chromatographic profiles of cellulases from T. viride-reesei and from two commercial preparations of technical cellulase*

A comparison of our cellulolytic preparation from the mutant of *T. viride* with some commercial preparations was of great interest. Such a comparison is not only useful from the point of view of the evaluation of the enzymatic composition of the preparations themselves, but also as an evaluation of the efficiency of the conditions found for the distinction of the cellulolytic preparations according to their origins. All the chromatograms of the three different preparations were obtained on the medium basic anion exchanger Spheron DEAE-1000 in citrate buffers of pH 5 and with similar gradients of increasing molarity of the buffers.

Chromatography of the technical preparation Onozuka R-10 is shown in Fig. 6. Isocratic elution with the starting buffer gave the main protein fraction I which was little bound to the DEAE packing under these conditions. It contained a double peak of high β-glucosidase activity, another of CM-cellulase activity and the main fraction of the FPA. Using a linear gradient of citrate ions the minor peak of the CM-cellulase activity, Ia, was eluted. A further peak, II, was again characterized by the presence of

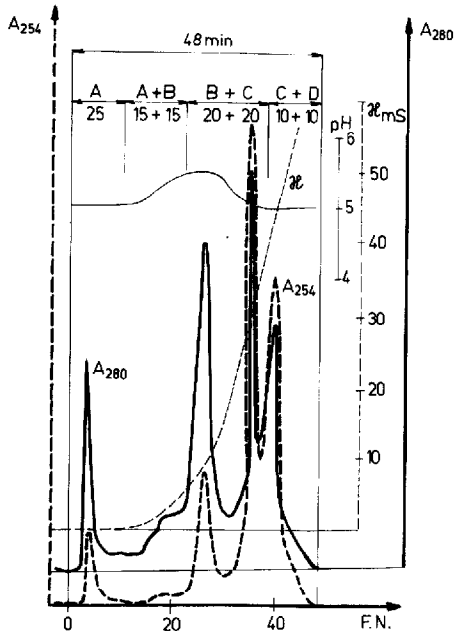


Fig. 5. Chromatography of the cellulolytic system of *T. viride* on Spheron TEAE-1000. Conditions: column, 20 × 0.8 cm; 7 mg of the preparation; flow-rate 2.4 ml/min; fractions collected in 60-sec intervals; pressure 0.4 MPa; room temperature. Buffers of pH 5: A, 0.005 *M* citric acid + NaOH; B, 0.1 *M* citric acid + NaOH; C, 0.25 *M* citric acid + NaOH + 1 *M* NaCl; D, 0.25 *M* citric acid + NaOH + 3 *M* NaCl. $A_{254}$, $A_{280}$ = Detector responses.

two β-glucosides with one third of the activity of the first peak, CM-cellulase activity and FPA. Elution with an NaCl gradient gave peak III in which only FPA and a minor fraction of β-glucosidase activity could be detected. The last two peaks of the chromatographic profile did not display any cellulolytic activity.

Fig. 7 illustrates the chromatography of the commercial preparation from *Aspergillus niger*. The protein profile recorded as absorbance at 280 nm was different from that in Figs. 4 and 6. The determination of the enzymatic activities confirmed that this preparation was different from those originating from *T. viride*. The starting buffer eluted the main fraction of the CM-cellulase activity and FPA. No β-glucosidase activity could be found. Gradients of increasing ionic strength gave the asymmetric peak II in which a high β-glucosidase activity was found in the form of a simple peak as well as a double peak of low CM-cellulase activity and a simple peak of FPA.

In Figs. 4, 6 and 7 records of the absorbance at 280 nm are shown, from which basic information on chromatographed samples follows. In the case of the preparations from *T. viride* (Figs. 4 and 6) the mutual relationship is evident from the profiles of measured absorbance at 280 nm alone but different levels of individual enzymes are also evident without activity assays. In Figs. 4 and 6 the opposite ratio of the heights of the two main protein peaks I and III is recorded. When these records were completed by the determination of three different activities, a more detailed knowledge of these materials was obtained. The preparation Onozuka R-10 displays a 28 times higher total β-glucosidase activity (compared to our enzyme),which is practi-

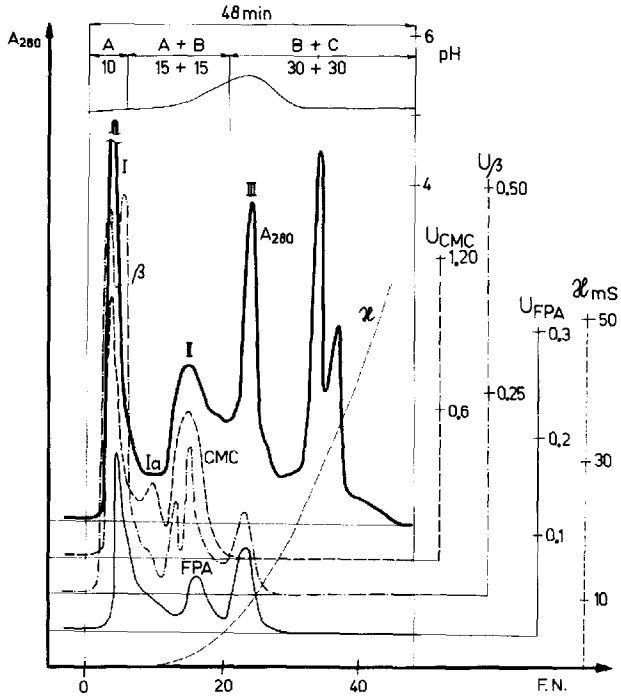


Fig. 6. Chromatography of 20 mg of the preparation Onozuka R-10 on Spheron DEAE-1000. Conditions: column, 20 × 0.8 cm; fractions at 60-sec intervals; flow-rate 2.1 ml/min; pressure 0.5 MPa; room temperature. Buffers of pH 5: A, 0.005 *M* citric acid + NaOH; B, 0.1 *M* citric acid + NaOH; C, 0.25 *M* citric acid + NaOH + 1 *M* NaCl. 0.5 ml of the effluent were withdrawn for the determination of enzymatic activities. For the enzymatic activities see Fig. 1.

cally all eluted with the hold-up volume. This form of the enzyme is almost absent in our preparation from *T. viride*. Further peaks of the β-glucosidase activity were eluted at the same values of buffer molarity, but they were present in different relative proportions. In the case of Onozuka R-10 three chromatographically different forms of filter-paper cleaving enzymes were found, while in the case of our preparation only two were found in opposite quantitative ratio in comparison with Onozuka R-10. The component which cleaved filter-paper and Avicel, which was eluted in both cases at the beginning of the gradient B + C, had the ability to liberate individual fibres from the filter-paper.

The determination of the activities in the preparation *A. niger* (Fig. 7) provided further information on this material. The preparation had an 83 times higher β-glucosidase activity than our material. This activity was eluted with the gradient B + C (see peak II in Fig. 7), in a position where, in the case of the preparation from *T. viride* (Fig. 4), an enriched FPA with the ability to liberate filter-paper fibres was found. The latter enzyme property was not found in the preparation from *A. niger*, where FPA indicates only the ability to liberate reducing sugars.

## DISCUSSION

We examined the chromatography of cellulolytic enzymes on cation-exchange

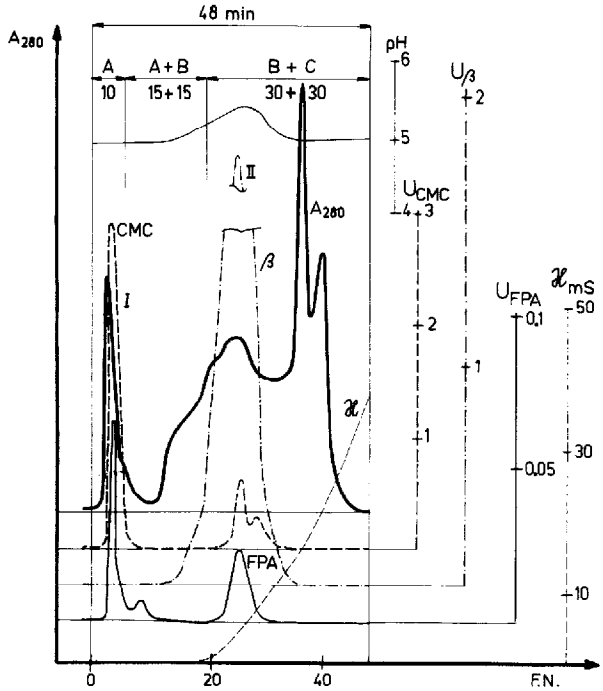


Fig. 7. Chromatography of 20 mg of the cellulase preparation from *Aspergillus niger* on Spheron DEAE-1000. Conditions as in Fig. 6.

and anion-exchange derivatives of Spheron at various pH values, using gradients of sodium or citrate ions and sodium chloride ionic strength gradients or pH gradients. The results of the pH-gradient separations are not shown because they did not contribute in any experiment to a better separation than that illustrated in preceding sections. Chromatography took 40–70 min. A good separation depended on the complexity of the gradient systems and the choice of the flow-rate. From the point of view of flow-rate the possibilities are not yet exhausted and the separation could be carried out at even higher rates, *i.e.*, in a shorter time.

The separations carried out with the preparation of the cellulolytic system from *T. viride* demonstrated the presence of five forms of CM-cellulase and at least two forms of the enzymes which cleaved filter-paper. It remains to be determined whether genetically different isoenzymes are involved, or whether these differences are cases of polymorphism of the same types of enzymes, due, for example, to subsequent changes in the polysaccharide composition, or to changes caused by a limited proteolysis.

In this study we could not completely separate the CM-cellulase activity from FPA. Both activities were always found in the fractions, but at different ratios. This fact could be ascribed to an imperfection of the separation process, probably due, for example, to the close proximity of the isoelectric points of individual components of the mixture, although this is improbable considering the choice of such a broad range of conditions. The quality of the chromatography is demonstrated by the complete separation of the β-glucosidase activity on the cation-exchange derivatives of Spheron (see Fig. 3).

The explanation for the observed multiple specificity of the separated fractions may lie in an imperfection in the determination of enzymatic activities, *i.e.*, in the fact that individual enzymes cannot be distinguished in principle when CM-cellulase and filter-paper are used as substrates. These macromolecular substrates cannot be defined as unambiguously as the relatively simple low-molecular-weight substrate for β-glucosidase activity —*p*-nitrophenyl-β-D-glucopyranoside. In addition, the activity was estimated in both cases by the determination of the reducing ends formed.

The problem may be considered from another point of view. The essence of the catalytic effect of all three types of the cellulolytic enzymes is the cleavage of the β-glucosidic bond in cellulose. The amounts of the individual detectable activities may reflect the polymorphism of the substrate, an expression of the adaptability of the microorganisms to the degradation of most types of arrangement of cellulose fibres in natural materials. From this it would follow that the tests used differentiate only quantitative differences in these activities, and that the whole spectrum of the cellulolytic enzymes does not display strictly qualitative differences in their specificities. If this hypothesis is correct, than any one enzyme of the cellulolytic system would be able, in principle, to cleave any cellulose, although with different efficiencies. This problem requires further study and HPLC separations represent a good approach.

Medium-pressure liquid chromatography of cellulolytic enzymes on ion-exchange derivatives of Spheron generally permits a comparison of preparations from various sources and from various mutant strains. The method elaborated consists of the initial comparison of elution profiles, which may be completed by the determination of enzymatic activities in the effluent, in order to increase our knowledge of the enzymatic properties of the preparations under investigation. This procedure may also be used to study the effect of various parameters during fermentation (temperature, pH, aeration, medium composition) on the individual activities, by the withdrawal of samples during fermentation and their rapid analysis, as well as the effect of storage.

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