The Fractionation of *Myrothecium verrucaria* Cellulase by Gel Filtration

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(Received 13 August 1964)

1. Culture filtrates from *Myrothecium verrucaria* have been fractionated by gel filtration on Sephadex G-75 to give three major cellulolytic components with molecular weights of about 55 000, 30 000 and 5300. 2. The middle component has the bulk (90%) of the total carboxymethylcellulase activity and is little affected by exposure to cotton. The other two, which are mainly responsible for the activity of the filtrate towards cotton, are removed or deactivated by exposure to it. These observations accord with the previously reported behaviour of the whole culture filtrate. There is no evidence for interconversion of, or synergism between, these components. 3. Temperature control during gel filtration is necessary for reproducible results at high resolution. The effect of a change in temperature has been explained in terms of changes in the degree of swelling of the gel particles.

In the previous paper of this series (Selby, Maitland & Thompson, 1963) it was concluded that the cellulase system from *Myrothecium verrucaria* contained at least two enzyme types that differed in the rate and extent of their attack on fibrous cellulose. It was suggested that the enzyme (A-enzyme) necessary for extensive degradation of cotton was in short supply in the cell-free culture filtrate as normally prepared, and that it was inactivated by exposure to cotton yarn; another cellulase (B-enzyme) was not so deactivated but was able to weaken cotton to a limited extent only.

Gel filtration offers a means of separating enzymes with little risk of deactivation. A successful separation of the active components from the cellulase of *Polyporus versicolor* on Sephadex G-75 has been reported (Pettersson, Cowling & Porath, 1963; Pettersson & Porath, 1963), although Whitaker, Hanson & Datta (1963), using the same method with *M. verrucaria* cellulase, found no evidence of heterogeneity, a view that our work did not support (Selby *et al.* 1963). The cellulase system of *M. verrucaria* has now been fractionated on Sephadex G-75, and it is the purpose of the present paper to explain, in terms of the properties of the separated components, some features of the behaviour of the whole culture filtrate.

MATERIALS

Myrothecium verrucaria was strain 45541 from the Commonwealth Mycological Institute, Kew, Surrey. The cotton flock on which it was grown and the cotton yarn that was used for measurements of cellulase activity were as

described by Selby *et al.* (1963). Sephadex G-25 and G-75 were supplied by A.-B. Pharmacia, Uppsala, Sweden; they were medium-grade 'block-polymerized' (fragmented) dextrans. The eluting buffer contained Na_2HPO_4 (3.3 mM), NaH_2PO_4 (6.7 mM) and NaCl (0.1 M) (pH approx. 6.5); in the '10× buffer' these concentrations were multiplied by ten.

Thyroglobulin was prepared by the method of Edelhoch (1960). Crystalline bovine-plasma albumin (serum albumin) was supplied by Armour Laboratories, London, and β -lactoglobulin (bovine) by Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. α -Lactalbumin was prepared by the method of Aschaffenburg & Drewry (1957). Cytochrome *c* (horse heart) was obtained from L. Light and Co. Ltd., Colnbrook, Bucks. Glucagon hydrochloride was obtained from Eli Lilly and Co., Indianapolis, Ind., U.S.A. 2,4-Dinitrophenylalanine was provided by our colleague Mr J. T. B. Shaw.

METHODS

The preparation of the culture filtrate and measurements of activity towards enzyme-pretreated cotton yarn and towards CM-cellulose were as described by Selby *et al.* (1963). For measurement of CM-cellulase in a series of fractions a more rapid, but less accurate, method was devised. A simple capillary-outflow viscometer shaped like a pipette was used (bulb volume, 1.3 ml.; flow time for water at 30°, 8.4 sec.). It was enclosed, except for the bottom 2 cm. of the capillary, in a constant-temperature water jacket. At the top it was connected to a vacuum line by a fine plastic tube and provided with an opening that was closed with the finger to draw the solution into the viscometer. A sample (5 ml.) of the CM-cellulose solution was measured into each of 20 test tubes held in a rack in a water bath at 30°, and the enzyme samples (0.5 ml.) were then added with stirring at 90 sec. intervals. The viscosity of each solution was

Fig. 1. Calibration of method for rapid measurement of CM-cellulase activity. Experimental details are given in the text.

measured after incubation for 30 min. by filling the viscometer, then raising its outlet clear of the liquid surface and timing the outflow. The method was calibrated against known dilutions of a culture filtrate whose activity had been accurately measured by the original method (Selby *et al.* 1963). Over a range of activities (10×10^{-2} to 0.5×10^{-2} units) a plot of $\log(\text{activity})$ against flow time was nearly linear (Fig. 1), indicating that the proportional sensitivity was constant, an error in timing of 0.1 sec. corresponding to a 3% error in activity; outside this range the sensitivity was less but still useful.

Preparation of Sephadex G-75 for column chromatography. Medium-grade (100–250 mesh) Sephadex G-75 was fractionated by sieving dry. The fraction (A; 41% by weight of the original) that passed through a 200-mesh sieve (hole size 75μ) was judged fine enough for use and was suspended in distilled water, which caused the effective diameter of the particles to be doubled. The coarse fraction, after similar swelling, was cut, suspended in water in a rotary homogenizer (MSE Ato-Mix used at half speed) and sieved wet on a 100-mesh sieve (hole size 150μ); the coarse particles were returned to the homogenizer for further cutting. All the particles that passed the 100-mesh sieve were added to fraction A. The extreme fines were removed by repeated suspension of the gel in distilled water, sedimentation and decantation; the fines were decanted when microscopic examination of a sample drawn from just above the sedimented layer showed few particles larger than 80μ . Thus the particles used to form the column were between 80μ and 150μ when wet, equivalent to 40–75 μ (375–200 mesh) dry. These particles were further swollen by suspension for 14 days in a culture filtrate from *M. verrucaria* diluted fivefold with chloroform-saturated eluting buffer. They were finally washed with, and resuspended in, eluting buffer. A vertical glass tube (2.5 cm. $\times$ 100 cm.) with a sintered disk (porosity no. 3) 1 cm. from its lower end was fitted with a constant-temperature (22°) water jacket. A rubber bung, drilled to take a fine-bore vinyl tube (internal diam. 1.1 mm.), was pushed into the bottom of the glass tube to

leave a uniform gap of 0.5 mm. between the bung and the sinter. The suspension of Sephadex G-75 was poured into the glass tube and allowed to settle under gravity. The eluting buffer was allowed to percolate through the column of gel as it settled; after a further 2 days the length had stabilized (88.5 cm.) but the flow of buffer (12 ml./hr. under a 30 cm. head) was continued, even when the column was not in use. The eluting buffer was deaerated to prevent the formation of air bubbles among the gel particles, and was kept saturated with chloroform to prevent bacterial contamination. Some trouble was experienced initially because of loss of chloroform through the walls of the plastic tubing used to lead the eluting buffer to the head of the column. It was overcome by resaturating the buffer with chloroform in a small trap so built in to the top of the column that it was kept at constant temperature. The eluate was led through the fine-bore vinyl tube to a volume-actuated fraction collector.

Calibration of the Sephadex G-75 column with substances of known molecular weight. The materials chosen for calibration were thyroglobulin (mol.wt. 670 000), crystalline bovine-plasma albumin (mol.wt. 67 000), β -lactoglobulin (mol.wt. approx. 35 000), α -lactalbumin (mol.wt. 15 500), cytochrome *c* (mol.wt. 12 400), glucagon (mol.wt. 3500), and 2,4-dinitrophenylalanine (mol.wt. 255). The molecular weights given are the currently accepted values. The method was similar to those described by Whitaker (1963) and by Andrews (1964). The materials were used as supplied except for glucagon, which was prepared by dissolving the hydrochloride (2.5 mg.) in the minimum volume of 0.02 N-NaOH and immediately making up to 5 ml. with eluting buffer. The other materials were dissolved separately in eluting buffer (approx. 0.2 mg./ml.) and the solutions diluted to give an extinction of about 0.5 (1 cm. cuvette) at 360 $m\mu$ for 2,4-dinitrophenylalanine, 412 $m\mu$ for cytochrome *c* and 280 $m\mu$ for the remainder. The density of each solution (5 ml.) was increased by adding 10 $\times$ buffer (0.1 ml.).

The solutions were filtered through glass paper (Whatman GF/B) and applied, one at a time, to the column by siphoning through a plastic tube attached to a fine glass capillary that ended within 3 mm. of the top of the gel. The flow of buffer was interrupted until the solution had spread over the surface of the gel and covered the end of the capillary; it was then resumed and the application of the sample completed at such a rate that the layer of denser solution continued to cover the end of the capillary. In this way interruption of the flow and mixing of the sample with the eluting buffer were minimized. When the sample had sunk into the gel a small amount of buffer solution was allowed to flow back through the capillary, which was then sealed off. The eluate was collected automatically in 4.55 ± 0.02 ml. fractions; collection was started when half of the solution under examination had entered the top surface of the gel. The extinction of each fraction was measured and the calibration completed by plotting for each material the fraction number at which the peak of extinction was observed against the logarithm of its molecular weight. During experiments involving 2,4-dinitrophenylalanine the column was shielded from light to prevent decomposition (Russell, 1963).

Fractionation of culture filtrates by gel filtration. Culture filtrates, both as prepared and after concentrating, were fractionated by gel filtration. When a filtrate was used

without concentration, its density was first increased by the addition of 0.02 ml. of the $10\times$ buffer/ml. Otherwise a tenfold concentrate of the filtrate was prepared in a rotary evaporator at 30° and no further addition of salt was necessary. To each cellulase solution 2,4-dinitrophenylalanine (approx. 0.2 mg.) was added as a marker to confirm that the column was correctly loaded and was running without distortion, and that the fractions were of constant size. The column was operated exactly as in the calibration experiment. The collected fractions were examined by measuring extinctions at 280 and $360\text{ m}\mu$, CM-cellulase activity and activity towards enzyme-pretreated cotton yarn (A-activity).

RESULTS

Calibration of the column of Sephadex G-75. The relation between elution volume for each material and the logarithm of its molecular weight is shown in Fig. 2. The positions of the peaks of extinction of each material were reproducible to within half a fraction, provided that the temperature of the column was not allowed to change. In previous experiments in which this requirement was not met the positions were less reproducible, particularly when the temperature in the laboratory fell during weekends. The importance of this requirement is discussed below.

Fractionation of a culture filtrate from *M. verrucaria* on Sephadex G-75. The properties of the separated fractions from a filtrate of *M. verrucaria* (10 ml., unconcentrated) are shown in Fig. 3. The dilution of any solute caused by passage through this column is about $2\frac{1}{2}$ -fold. A corresponding dilution of the culture filtrate before fractionation had an extinction (1 cm. cuvette) at $280\text{ m}\mu$ of 0.35

and a CM-cellulase activity of 1.84×10^{-2} units, and caused a 7.7% loss of strength of pretreated cotton yarn during three further treatments. There are evident in Fig. 3 at least three cellulolytic components differing in molecular size and with different relative activities towards CM-cellulose and towards cotton yarn. Component II has the greater part of the CM-cellulase activity of the original filtrate, whereas components I and III are slightly more active towards yarn but much less active towards CM-cellulose. From the positions of the peaks of CM-cellulase activity the approximate molecular weights of the three components are estimated by reference to Fig. 2 to be 55 000, 30 000 and 5300. The material of still lower molecular weight, evident in Fig. 3 as peaks of extinction, has no cellulolytic activity but accounts for most of the yellow colour of the filtrate.

Fractionation of a concentrate of a culture filtrate from *M. verrucaria* on Sephadex G-75. The three components were obtained in greater quantity from a tenfold concentrate (10 ml.) of a culture filtrate from *M. verrucaria* by fractionation on the same column of Sephadex G-75. About one-third of each of the main components was re-run separately to establish homogeneity and reproducibility of behaviour (● in Fig. 4). Another third of each was exposed at 30° to three fresh samples of cotton yarn (each for a period of 24 hr. and with a liquor: cotton ratio of 300:1, v/w) and re-run (△ in Fig. 4). Fractions were pooled as shown and CM-cellulase activities and activities towards pretreated cotton

Fig. 2. Relation between elution volume and log (molecular weight) for reference materials on a Sephadex G-75 column [2.5 cm. $\times$ 88.5 cm.; 0.01 M-phosphate buffer, pH 6.5 containing NaCl (0.1 M)]. A, Thyroglobulin; B, serum albumin; C, β -lactoglobulin; D, α -lactalbumin; E, cytochrome c; F, glucagon; G, 2,4-dinitrophenylalanine.

Fig. 3. Fractionation on a Sephadex G-75 column (details as given in Fig. 2) of a filtrate from a culture of *M. verrucaria*.

Fig. 4. Effect of exposure to cotton on the components from the fractionation of *M. verrucaria*. Details of the column were as given in Fig. 2. (a) Component I re-run before (●) and after (Δ) exposure to cotton; (b) component II re-run before (●) and after (Δ) exposure to cotton; (c) component III re-run before (●) and after (Δ) exposure to cotton. On each graph the shading shows which fractions were united and the arrow shows the position of the peak of extinction of the 2,4-dinitrophenylalanine marker.

were measured (Table 1). Clearly components I and III were decreased in activity towards both pretreated cotton and towards CM-cellulose by exposure to cotton, whereas component II was little affected by this treatment. There was no evidence for interconversion of components I, II and III after exposure to cotton.

Systematic recombination of components I, II and III, as originally separated, did not lead to any significant enhancement of activity either towards cotton or towards CM-cellulose, showing that synergism between these components is not a major factor in the action of the culture filtrate.

DISCUSSION

Factors affecting the efficiency of the gel filtration. Flodin (1961, 1962) showed that the resolution obtainable on Sephadex gels could be improved by using small particles, low flow rates and fractions with a narrower distribution of particle size than are commercially available. In the present work an adequate flow (3 cm./hr.) was obtained with only a small hydrostatic pressure (approx. 30 cm.) with columns up to 100 cm. long and particles as small as 40–75 μ (dry), provided that certain precautions were taken in the preparation of the gel and of the column. It was of utmost importance to allow the gel particles to swell to their maximum size in the buffer system to be used. Further, it was observed that, although exposure of Sephadex G-75 to a culture filtrate from *M. verrucaria* caused no solubilization, a slight swelling occurred, which was demonstrated as an increase in water retention when the gel was centrifuged under standard conditions. Therefore as a routine procedure the gel was swollen in a mixture of culture filtrate and eluting buffer for 2 weeks before being used to pack the column. It was also important to form the column at the temperature at which it was to be used. It was suspected that the gel particles swelled further on cooling because the effect of temperature on the rate of flow of eluting buffer through the column was greater than could be

explained solely in terms of viscosity changes. Apart from causing inconveniently low rates of flow, an increase in particle size must also affect the behaviour of molecules passing through the column. The change in size of the irregularly shaped particles of Sephadex prepared by block-polymerization is difficult to measure, but the change in diameter of the spherical 'bead-form' particles now available can be measured under the microscope and is about 1% per 10°. A diametral change of 1% corresponds to a volume change of 3%. Since there is about twice as much buffer solution in the particles as in the voids, this increase will cause an approx. 6% decrease in void volume, and hence approx. 6% less buffer solution will be required to elute a molecule large enough to be excluded from the gel. By contrast, a small molecule, to which most of the buffer solution present both in and between the gel particles is accessible, will be little affected since the total volume of the column remains unchanged. In confirmation it was found that thyroglobulin was eluted from Sephadex G-75 about two fractions (7%) earlier at 8° than at 22°; serum albumin behaved similarly, although not completely excluded from the gel. Both Whitaker (1963) and Andrews (1964) have noticed a temperature effect; Whitaker gives findings that agree closely with our observations, although the agreement is obscured by his presentation of elution volumes as ratios relative to γ_2 -globulin so that it appears that only the smaller molecules are affected. It is obviously necessary to control the temperature during gel filtration if the otherwise excellent reproducibility of this technique is to be utilized.

Behaviour of the separated components of M. verrucaria cellulase. Previous work with the culture filtrate from *M. verrucaria* suggested that it contained at least one exhaustible and one persistent enzyme, and that the CM-cellulase activity was largely associated with the latter (Selby *et al.* 1963). These deductions are supported by the present results, which show that the enzyme contained in component II from the fractionation on Sephadex

Table 1. *Effect of exposure to cotton on the activities of the separated components from a culture filtrate of M. verrucaria*

Experimental details are given in the text.			
Component	Treatment	Further loss of strength (%) by pretreated cotton after incubation for 24 hr. with three fresh quantities of fractions pooled as in Fig. 4	10 ² × CM-cellulase activity of pooled fractions (units)
I	None	12.2	0.42
	Exposed to cotton	2.0	0.13
II	None	2.5	4.9
	Exposed to cotton	3.0	3.8
III	None	5.5	0.24
	Exposed to cotton	2.0	0.04

G-75 exhibits the bulk (about 90%) of the CM-cellulase activity of the whole filtrate, and that its activities are unaffected by exposure to cotton (Table 1 and Fig. 4b). It seems that this component contains the B-enzyme and that its molecular weight is about 30000. The other two active components (I and III) separated by gel filtration have molecular weights estimated to be 55000 and 5300. They both lose activity on exposure to cotton, and in this respect behave as A-enzyme. It is not possible at this stage of the work to say what, if any, is the connexion between components I and III. The estimates of molecular weight do not take account of differences of molecular shape, or density, or of adsorption effects, and must therefore be treated with reserve. It was considered possible, for example, that adsorption could have contributed to the retardation of component III on Sephadex G-75, so leading to a false estimate of the molecular weight. Such adsorption would be more obvious on Sephadex G-25, from which proteins with molecular weights over 5000 are completely excluded. In fact the volumes of buffer solution required to elute component III, serum albumin and 2,4-dinitrophenylalanine from a column (1.8cm. $\times$ 40cm.) of Sephadex G-25 were 38, 37 and 124ml. respectively, showing that adsorption of component III was negligible. It is therefore extremely likely that component III was retarded on Sephadex G-75 solely on account of its small molecular size (cf. Pettersson *et al.* 1963).

Whitaker *et al.* (1963), working with a filtrate from *M. verrucaria*, found only one cellulolytic component in the eluate from a column (100cm. $\times$ 3.2cm.) of Sephadex G-75 under conditions apparently similar to ours. By scaling the elution volumes in proportion to the column volumes it is possible to deduce that, on Whitaker's column, the separation between components I and II would have been 55ml., and between II and III 264ml. If it is assumed that the peak of CM-cellulase activity (380ml.; Fig. 4A in Whitaker *et al.* 1963) represents the emergence of component II, components I and III would have emerged at 325 and 644ml. respectively. It is therefore impossible to tell whether component III was present or not, as no results are given beyond 470ml. This is also true for Fig. 4B in Whitaker *et al.* (1963), which ends at 525ml., in a region where there is still CM-cellulase activity, since component III would have emerged at 570ml. The trace of extinction at 260m μ (Fig. 4A in Whitaker *et al.* 1963) shows no resolution of components I and II, but the observed displacement (about 30ml.) of the peak of CM-cellulase activity relative to that of extinction suggests that both components were present, though incompletely resolved. The CM-cellulase activity would be almost entirely due to com-

ponent II, and the extinction at 260m μ to the contributions of both components, giving a peak about midway between the positions of components I and II, i.e. about 28ml. before component II, as observed.

The peaks of ultraviolet absorption, of CM-cellulase activity and of activity towards cotton still did not coincide in the present work, in which the resolution was higher (Fig. 3); a similar effect was noticed by Pettersson *et al.* (1963) in their separations of *P. versicolor* cellulase. It seems that each peak of activity may still contain several components, not necessarily all cellulolytic, and that these skew effects may be due to their incomplete separation.

Because it had been thought that B-enzyme might be formed by complex-formation between A-enzyme and carbohydrate (Selby *et al.* 1963), evidence for the formation of component II by exposure of components I and III to cotton was sought. No evidence of such interconversion was found (Fig. 4). Thus if A-activity is lost by the formation of a soluble enzyme-carbohydrate complex the change in molecular weight must be too small to be detectable by gel filtration on Sephadex G-75.

It is concluded that the fractionation by gel filtration of culture filtrates from *M. verrucaria* into several active components has confirmed the heterogeneity of this cellulase system. The separated components differ in molecular size and in their relative activities towards different cellulosic substrates. Each is stable under the conditions of separation employed and, if passed again through the same column of gel, emerges in the same position as before.

The authors are grateful to their colleague Mrs K. V. A. Thompson, who supplied cultures of *M. verrucaria*, and to Dr P. Andrews for advice on the choice of proteins for calibration of the Sephadex column. A part of this research was conducted under a project authorized by U.S. Public Law no. 480, 85th Congress.

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