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Short communication

Problems and prospects of using Sephacryl S-200 for separation of cellulase and xylanase complexes of *Coprinus cinereus*

(Masalah dan prospek penggunaan Sephacryl S-200 untuk pemisahan kompleks selulase dan xilanase daripada *Coprinus cinereus*)

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Key words: ultrafiltration, cellulases, xylanases, gel filtration chromatography, SDS-polyacrylamide gel electrophoresis

Abstrak

Hasil turasan kasar yang mengandungi kompleks selulase dan xilanase yang diperoleh daripada *Coprinus cinereus* yang dikulturkan di atas Solka Floc, telah dipekatkan melalui membran pengultraturasan (Diaflo PM10, berat molekul tertapis 10 000) dan kemudiannya dipisahkan menerusi kromatografi penurasan gel (Sephacryl S-200). Hasil daripada kromatografi penurasan gel ini di luar dugaan kerana berat molekul protein dari setiap puncak aktiviti enzim kurang daripada jangkaan. Jelaslah bahawa proses jerapan atau tindak balas elektrostatik antara enzim dan Sephacryl S-200 telah berlaku menandakan gel tidak berfungsi hanya sebagai penyaring molekul yang pasif. Andaian ini terbukti apabila nilai berat molekul protein yang ditentukan dengan gel elektroforesis (SDS-PAGE) didapati lebih bermakna.

Abstract

The crude culture filtrates obtained from the growth of *Coprinus cinereus* on Solka Floc which contained cellulase and xylanase complexes, were concentrated through ultrafiltration membrane (Diaflo PM 10, molecular weight cut-off of 10 000) and later fractionated through gel filtration chromatography (Sephacryl S-200). Results from gel filtration chromatography showed that the apparent molecular weight of protein from each activity peak was very much less than expected. It appeared that the gel did not act solely as a passive molecular sieve suggesting that an adsorption or electrostatic interaction might have occurred between the enzyme and the matrix of the column. Molecular weight determination from SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gave a higher and more realistic molecular weight thus supporting the idea.

Introduction

Coprinus cinereus is mesophilic Basidiomycetes which are known as alkaline tolerant fungi (Burrows 1980). In solid substrate fermentation, it utilises cellulose and hemicellulose best under alkaline conditions (pH 8–9) (McShane 1976; Burrows 1980). Under liquid culture

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conditions, Long (1991) found that *C. cinereus* produced cellulase complexes that had activities towards avicel, carboxymethylcellulose and p-nitrophenyl ß-D-glucopyranoside. In addition, it also produced xylanase complexes which were found to be constitutive enzymes. The profile of pH optima (pH 6–6.5) and pH stability [nearly 100% stable at pH 7–9 for 24 h at 37 °C (Long 1991)] of cellulase under alkaline conditions indicate the potential use of the fungus or its enzymes as the agent in on-farm biodegradation processes. It may also be useful for biotechnological processes such as the production of laundry detergents which require the use of cellulase at alkaline pH (Shikata et al. 1990). The stability of its cellulases under alkaline conditions further suggests that an investigation on the characteristics and mechanisms of action of each individual enzyme is necessary. Moreover, the purification of these enzymes has not been reported previously.

Enzyme separation in this study involves the use of gel filtration chromatography that is well documented for the determination of the molecular weight and size of protein. The technique is based on the well established ability of gel filtration media such as Sephadex, Sepharose and Sephacryl (Pharmacia Inc., Sweden) to separate molecules according to their sizes. In this study, Sephacryl S-200 which has separation range nominally equivalent to that of Sephadex G-200, was used. The gel is a hybrid prepared by covalently cross-linking allyl dextran with N, N'-methylenebisacrylamide, forming a rigid matrix that can withstand high flow rates. This paper reports on the problems and prospects of using Sephacryl S-200 for separating cellulase and xylanase complexes from *C. cinereus.*

Materials and methods

Organism and cultural conditions

Stock cultures of *C. cinereus* IMI 140506 (monokaryon) was obtained from the Commonwealth Mycological Institute (Kew, London) and were maintained on slopes of malt extract agar (MEA) (Oxoid Ltd, Basingstoke, Hants, U.K.)

All experiments were conducted using 500 mL Erlenmeyer flasks containing 200 mL of sterile mineral salts medium and 1% w/v Solka Floc inoculated with 5 mL of spore suspension. The inoculums and the growing media were prepared as described by Long and Knapp (1991). Cultures were incubated at 37 °C on an orbital shaker at 210 rev/min (25 mm displacement).

Estimation of protein

Protein from the gel filtration fractions was estimated using the Bradford method (Bradford 1976). The samples were thoroughly mixed and 100 µL was added to a test tube containing 5 mL of Coomassie Brilliant Blue G-250 reagent. The content of the tube was then vigorously mixed and allowed to stand for at least 10 min but less than an hour. The absorbance was then measured using spectrophotometer at 595 nm. Bovine serum albumin in 1 *M* NaOH was used as the protein standard.

Enzymes assays

All enzyme activities were assayed by the method of Long and Knapp (1991), modified from Wood and Bhat (1988). Activity of avicelase and carboxymethylcellulase was quoted as µmol reducing sugar released, expressed as glucose which was measured by the Somogyi-Nelson methods (Wood and Bhat 1988). In the case of xylanase, its reducing sugar released was measured by the dinitrosalicylic acid method (Miller 1959). While the activity of ß-glucosidase and ß-xylosidase was determined by measuring the release of p-nitrophenol from p-nitrophenyl ß-D-glucopyranoside and p-nitrophenyl ß-D-xylopyranoside respectively. The names of the enzymes involved in the activity were referred to in terms of the substrate attacked.

Ultrafiltration of protein

The hydrolytic enzyme was harvested from 7-day-old cultures of *C. cinereus* grown on a medium containing Solka Floc as a major carbon source. The enzyme preparation (250 mL) was concentrated to 12.5 mL (one-twenty of the original volume) by ultrafiltration with Diaflo PM 10 membrane filter (43 mm diameter) (Amicon, Ltd) using 60 mL Amicon stirred pressure cell. This membrane had a molecular weight cut-off of 10 000. A 10 mL sample of concentrated enzyme was then freeze-dried (–20 °C) and later dissolved in 5 mL distilled water to give a solution of 40 times concentration factor.

Gel filtration chromatography

The concentrated enzyme preparation was fractionated by gel filtration chromatography using Sephacryl S-200 (Pharmacia LKB Biotechnology, Uppsala, Sweden). The samples (volume of 1–2 mL of total bed volume) were loaded into a Sephacryl S-200 gel filtration column which was pre-equilibrated with 0.1 *M* phosphate buffer, pH 7.0 and 0.01 % sodium azide. The total bed volume of the column was 190 mL (diameter of 2.2 cm and height of 50 cm). Elution of the samples was carried out at 4 °C using the similar buffer at a constant flow rate of 10 mL/h and a fraction volume of 2.4 mL. The effluent was continuously monitored for protein by measuring UV light absorption at 280 nm.

Four molecular weight (mw) standards (Pharmacia, Low Molecular Weight Gel Filtration Calibration Kit) were used to calibrate the column. These were ribonuclease A (mw 13 700), chymotrypsinogen A (mw 25 000), ovalbumin (mw 43 000) and albumin (mw 43 000). The similar buffer and elution rate were used in calibrating the column. A molecular weight calibration curve to define the relationship between the elution volumes (defined by the parameter K_{av} of a set of standard proteins) and the logarithm of their respective molecular weight was plotted.

The K_{av} values for each protein were calculated using the equation:

$$K_{av} = V_e - V_o / V_t - V_o$$

where V_e = elution volume for the protein
V_o = column void volume (equal to the elution volume for Blue Dextran 2 000)
V_t = total bed volume

SDS-polyacrylamide gel electrophoresis

In the gel electrophoresis experiments, a dissociating gel and discontinuous buffer (discontinuities of buffer composition and pH) system were employed. Sodium dodecyl sulphate (SDS), an anionic detergent, was used as dissociating agent. The method used was based on the method described by Laemmli (1970).

Sample preparation

Samples (0.5 mL) from the fractions obtained during gel filtration chromatography were placed in minifuge tubes and the proteins were precipitated with 0.5 mL of 50 % (w/v) trichloroacetic acid (TCA). The samples were then incubated on ice for 15 min followed by centrifugation for another 10 min. The supernatant were removed and the pellets were washed twice with acetone. Samples were then dried at room temperature before 50 µL of protein solubiliser was added. The mixtures were boiled for about 4 min and the denatured protein samples were loaded onto SDS-polyacrylamide gel electrophoresis (PAGE).

Molecular weight estimation

Similar procedures of sample preparation were employed in preparing protein markers. The marker polypeptides used were phosphorylase b (mw 97 000), bovine serum albumin (mw 68 000), ovalbumin (mw 43 000), carbonic anhydrase (mw 30 000) and soybean trypsin (mw 20 500). The calibration line of $\log_{10}$ polypeptide of molecular weight was plotted versus relative mobility (Rf) value or distance (mm) migrated by the SDS-PAGE.

Results and discussion

Ultrafiltration and freeze-drying process

In the ultrafiltration process, nearly 40% of the protein in the culture supernatant was lost *(Table 1)*. The loss of a large amount of protein is not uncommon as similar result has also been reported by Marui et al. (1985) in the ultrafiltration process of xylanase from *Streptomyces* spp. No. 3137. There was an increase in specific activities of all enzymes tested except xylanase *(Table 1)*. The loss of large amount of xylanase activity (76%) during this process was perhaps due to the removal of a divalent cation which has been shown to enhance xylanase activity as well as its stability (Long 1991). Results also showed that the freeze-drying process did not significantly decrease the activity of CMCase, ß-glucosidase and ß-xylosidase *(Table 1)*. The nearly complete recovery of all enzyme activities after freeze-drying was very good as it has been noted that some enzymes lose activity during the freeze-drying process; an example being the exoglucanase of *Aspergillus fumigatus* which lost about 73% of its activity after freeze-drying (Parry et al. 1983).

Elution profile of protein on Sephacryl S-200

The elution profile of the protein was measured by two methods: continuous monitoring of UV light absorption at 280 nm and analysis of fractions using the Coomassie Brilliant Blue (Bradford 1976). As expected, both methods were responsive to different protein profiles or structures *(Figure 1)*. The absorption of light in the region of 280 nm is almost entirely due to the tyrosine, phenylalanine and tryptophan (Herbert et al. 1971). While the analysis using Coomassie Brilliant Blue involved the binding of a dye to the protein.

The elution profile of the enzyme activities showed that xylanase had 3 peaks of activity, CMCase 2 peaks , avicelase 1 peak, ß-glucosidase 2 peaks and ß-xylosidase 3 peaks *(Figure 2)*. Some fractions had peaks comprising more than one enzyme activities. An example was ß-glucosidase that had two peaks which coincided with the peaks for ß-xylosidase activity *(Figure 2)*. The sharing of similar activity peak by some enzymes was probably due to the presence of several enzymes in a fraction leading to several

Table 1. Purification of cellulase and xylanase from *Coprinus cinereus*

Step	Hydrolytic enzyme	Total protein* (mg)	Total activity (µmol)	Specific activity (µmol/mg)	Purification (fold)
Original	Avicelase		1 650	40.00	
filtrate	CMCase		6 450	156.70	
	ß-glucosidase	41.3	6 225	150.90	1.0
	Xylanase**		12 435	301.50	
	ß-xylosidase		431	10.50	
Ultrafiltration	Avicelase		1 538	61.50	1.5
PM 10	CMCase		4 786	191.40	1.2
	ß-glucosidase	25.0	5 794	231.70	1.5
	Xylanase**		2 983	119.32	0.4
	ß-xylosidase		293	11.72	1.1
Freeze-drying	Avicelase		1 545	61.80	1.6
	CMCase		4 742	189.68	1.2
	ß-glucosidase	25.0	5 771	230.84	1.5
	Xylanase**		2 983	119.32	0.4
	ß-xylosidase		291	11.64	1.1

*measured using Coomassie Brilliant Blue
**µmol reducing sugar x 10^2

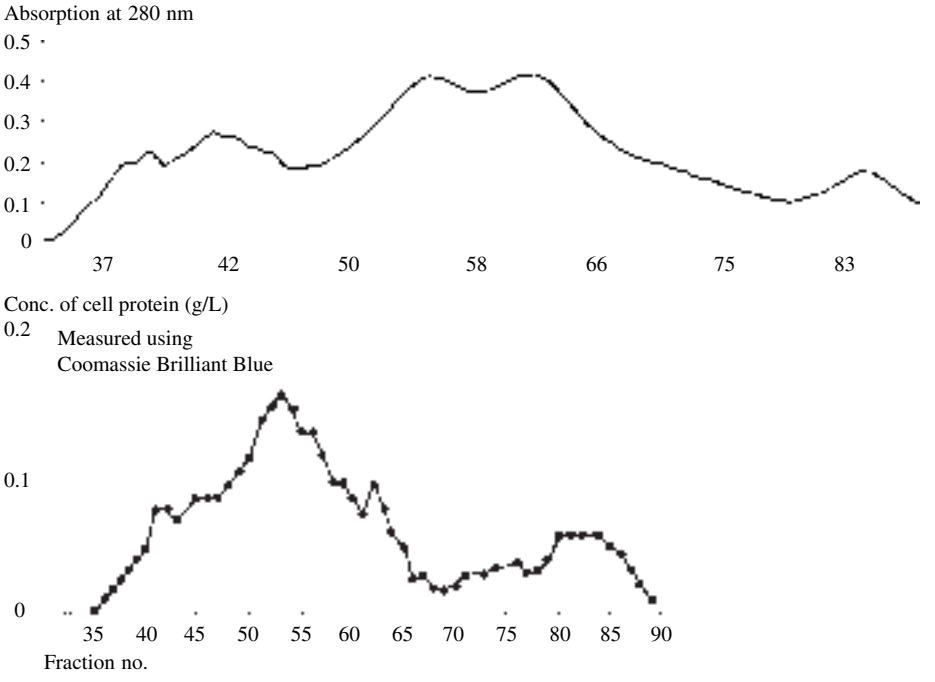

Figure 1. Elution profile of protein applied to Sephacryl S-200 gel filtration column

enzyme activities, or the presence of a single enzyme in a fraction that had multiple substrate specificity (Fournier et al. 1985). *Table 2* shows the specific activity of enzyme from the gel filtration fractions. Fraction 71 had a specific activity of CMCase four times higher than in fraction 57. While fraction 84 gave specific activity of ß-xylanase six and nine times higher than in fractions 48 and 56 respectively.

The K_{av} and the apparent molecular weight of each protein peak are presented in *Table 3* (due to a very high K_{av}, some values are extrapolated to demonstrate the possible range of values). The apparent molecular weights were very much less than expected. Fraction 48 which had activity more towards ß-glucosidase and ß-xylosidase, recorded the highest molecular weight of 30 000 while fraction 84 had the lowest molecular weight of less than 1 000 which was too low and was clearly improbable. Overall, it appeared that the gel did not act solely as a passive molecular sieve suggesting that there was some kind of interaction between the enzymes and the matrix. Generally, there are two ways in which a solute can depart from its 'true' elution volume, viz. either by appearing earlier or appearing later than expected. Substances that appear later are retarded either by adsorption or by electrostatic interaction. The adsorption of solute to the gel matrix is based on the structure of the substance and may be due to hydrophobic interactions. To prevent this adsorption, ethylene glycol was added (on the advice of Pharmacia, the manufacturer of Sephacryl S-200) to the eluent buffer. Unfortunately, the addition of 5% ethylene glycol produced a very similar (almost identical) pattern of results to that obtained without it.

Determination of molecular weight by SDS-PAGE

Results from SDS-PAGE showed that fraction 71 gave three protein bands which had molecular weight ranging from 34 000 to 47 000 *(Plate 1).* The apparent molecular weight of fraction 71 calculated from this method was far higher than that of gel filtration chromatography technique (mw of

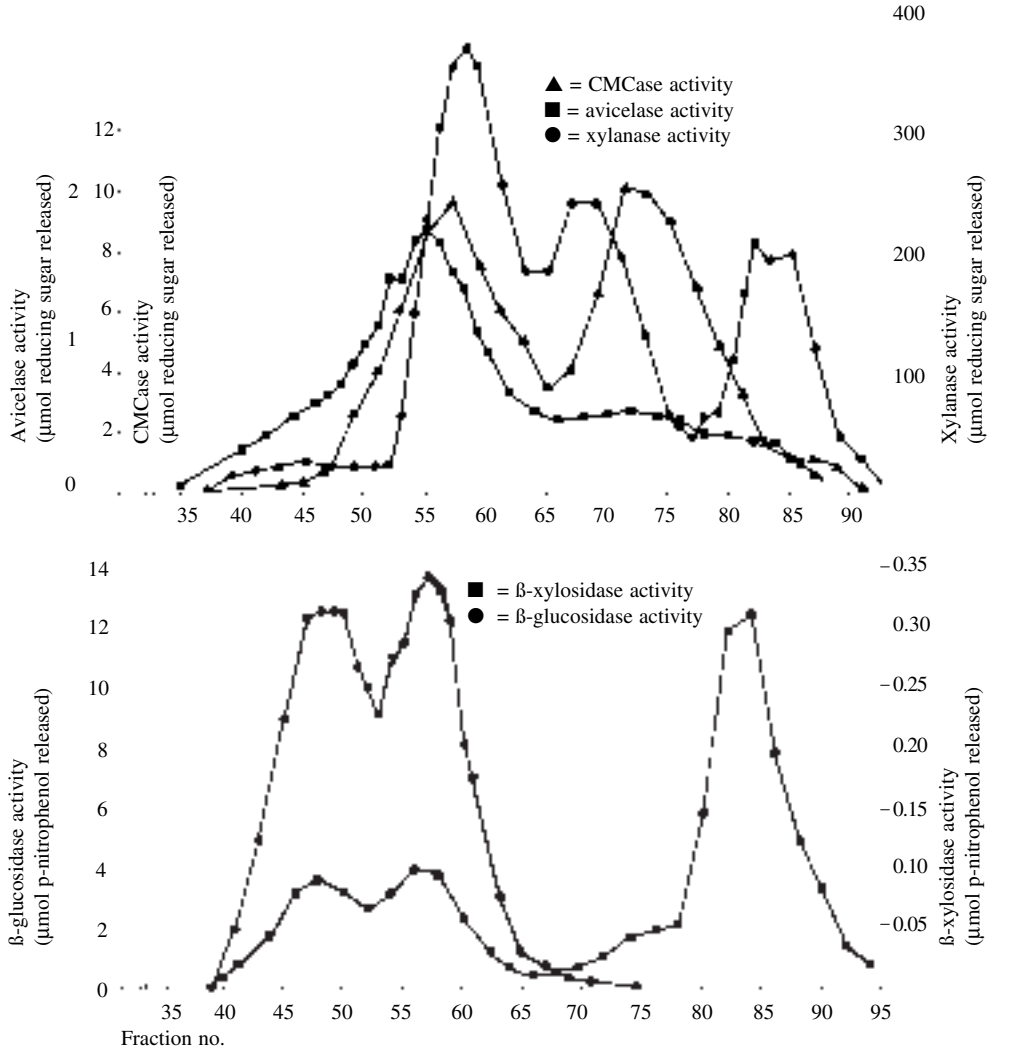

Figure 2. Elution profile of hydrolytic enzyme activities on Sephacryl S-200 gel filtration chromatography

2 600). This conspiracy was also noticed when fraction 84 (most likely contained xylanase and/or ß-xylosidase) was subjected to SDS-PAGE. Results from gel filtration chromatography showed that this fraction had molecular weight of less than 1 000. However, with SDS-PAGE the molecular weight was about 31 000. The molecular weight determination by SDS-PAGE supported the idea that an adsorption or electrostatic interaction had occurred between the complex enzymes of *C. cinereus* and the matrix of Sephacryl S-200.

The adsorption phenomena on Sephacryl S-200 have also been reported by Belew et al. (1978) where they found that the absorption depended upon the pH of the eluent buffer employed. At pH 3.5, the gel could adsorb acidic, neutral and basic protein and at pH 8 the gel began to behave as a cation exchanger and strongly adsorb any protein that was positively charged. Although the eluent buffer pH used in this experiment was 7, the result did not conform to the result by Belew et al. (1978) which

Table 2. Enzyme activities after fractionated through gel filtration chromatography Sephacryl S-200

Hydrolytic enzyme	Fraction no.	Total protein* (mg)	Total activity (µmol)	Specific activity (µmol/mg)
Avicelase	55	0.34	10.50	31.3
CMCase	57	0.30	58.20	194.0
	71	0.07	60.60	841.7
ß-glucosidase	49	0.26	75.00	284.0
	57	0.30	82.20	274.0
Xylanase**	58	0.24	17.76	74.0
	57	0.05	11.52	240.0
	82	0.14	10.08	70.0
ß-xylosidase	48	0.22	0.43	2.0
	56	0.34	0.48	1.4
	84	0.14	1.86	12.9

*measured using Coomassie Brilliant Blue
**µmol reducing sugar x 10^2

Table 3. The apparent K_{av} and molecular weight of enzyme activities obtained (calculated from peak activity of each enzyme) from gel filtration chromatography Sephacryl S-200 fractions

Hydrolytic enzyme	Fraction no.	K_{av}	Apparent molecular wt.
Avicelase	55	0.54	10 500
CMcase	57	0.52	11 200
	71	0.82	2 600*
ß-glucosidase	48	0.33	30 000
	57	0.52	11 500
Xylanase	58	0.54	10 500
	68	0.76	3 500*
	82	1.06	<1 000*
ß-xylosidase	48	0.33	30 000
	56	0.50	12 700
	84	1.10	<1 000*

*extrapolated values

showed that solute gel interaction was minimal at or near pH neutral.

Obviously, the separation of protein observed from this study was not strictly size-dependent, indicating that the gel does not act solely as a passive molecular sieve. Further studies need to be carried out to elaborate the possible way of interaction between matrix of the column and the enzymes of *C. cinereus.*

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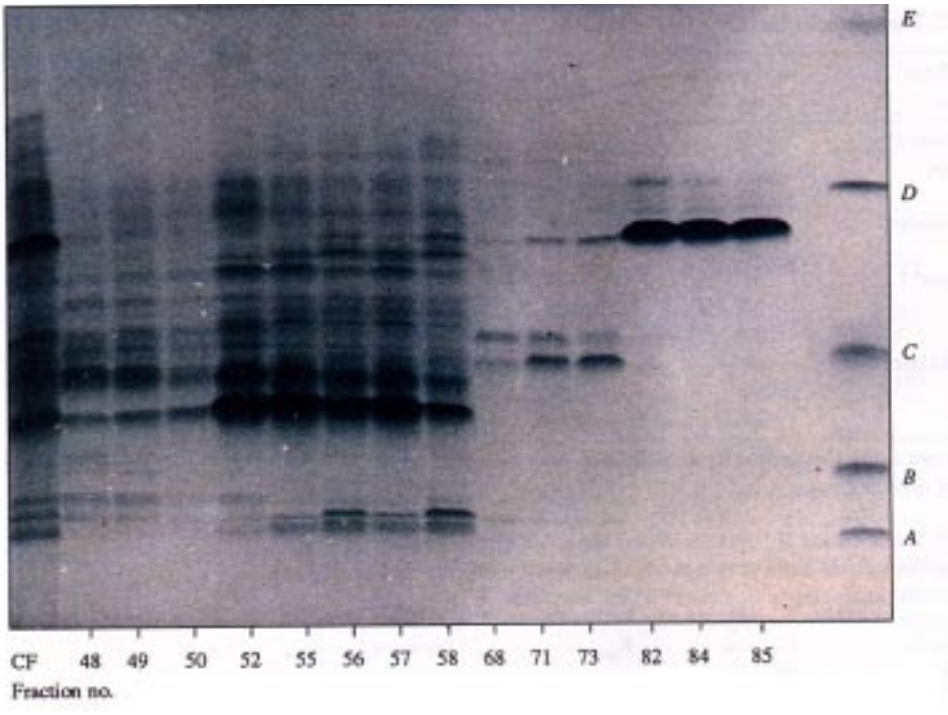

Plate 1. Molecular weight determination by SDS-PAGE of protein contained in fraction obtained from gel filtration chromatography (A to E are marker proteins, CF is crude culture filtrate)

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