Antonie van Leeuwenhoek **51** (1985) 139–150

Production, purification and partial characterization of an endo-polygalacturonase from *Cryptococcus albidus* var. *albidus*

FEDERICO FEDERICI

Istituto di Biologia Vegetale, Sezione di Microbiologia, University of Perugia, 06100 Perugia, Italy

FEDERICI, F. 1985. Production, purification and partial characterization of an endo-polygalacturonase from *Cryptococcus albidus* var. *albidus*. Antonie van Leeuwenhoek **51**: 139–150.

Cryptococcus albidus var. *albidus* produced an extracellular endo-polygalacturonase (poly (1,4-α-D-galacturonide) glycanohydrolase EC 3.2.1.15) when grown in a synthetic medium containing one of a variety of pectic substances or galacturonic acid. The highest level of enzyme activity (15.5 VU·ml^{-1}) was obtained after 72 h of growth on 1.0% low-methoxyl pectin. The enzyme, purified by gel filtration (Sephadex G-100) after repeated ammonium sulphate precipitation and dialysis, showed only one band by polyacrylamide gel electrophoresis and had the following properties: mol wt (MW_r) 41 000 dal; isoelectric point (pI) = 8.10 ± 0.10; optimum temperature and pH for activity around 37 °C and pH 3.75, respectively; pH stability in the pH range 4.0 to 8.0; complete heat inactivation after 10 min at 55 °C; K_m and V_{max} values $5.7 \cdot 10^{-1}$ mg·ml^{-1} and $5.1 \cdot 10^{-1}$ mmoles·min^{-1}, respectively.

INTRODUCTION

The pectolytic ability of yeasts and yeast-like fungi has long been known (Luh and Phaff, 1951; Bell and Etchells, 1956; Vaughn et al., 1969). Very few yeast species, however, have been thoroughly studied with regard to this capacity. So far, most research has been carried out with *Kluyveromyces fragilis* (Luh and Phaff, 1954; Wimborne and Rickard, 1978; Lim et al., 1980) which, among these microorganisms, is regarded as the most potent producer of pectinase.

In a previous study (Federici, 1983*a*) strong pectolytic activity was found in strains of *Cryptococcus albidus*, a yeast of particular interest since it was found to be involved, with some filamentous fungi, in the breakdown of preserved fruit (Dennis and Harris, 1979; Harris and Dennis, 1979).

This paper deals with the production, purification and partial characterization of the extracellular endo-polygalacturonase from strain IMAT-4735 of *Cr. albidus* var. *albidus* which, in a preliminary screening, was selected for its high pectin-depolymerizing (viscosity-diminishing) activity (Federici, 1983*b*).

Materials and Methods

Organism, medium and culture conditions

Cryptococcus albidus var. *albidus* IMAT-4735, obtained from the culture collection of the Laboratory of Agricultural Microbiology of the Istituto di Biologia Vegetale, University of Perugia, Italy, was maintained routinely on Bacto Malt Agar (Difco).

The growth medium employed consisted of ($g \cdot l^{-1}$ of distilled water): $(NH_4)_2SO_4$ 1.0; KH_2PO_4 2.0; $Na_2HPO_4 \cdot 2H_2O$ 0.9; $MgSO_4 \cdot 7H_2O$ 1.0; yeast extract (Difco) 1.0. Unless indicated otherwise, pectin (Sigma) was added as the sole carbon source, to a final concentration of 10 $g \cdot l^{-1}$, and the pH of the medium was adjusted to 5.0.

Cultures were grown in 100 ml of the above medium in 500-ml Erlenmeyer flasks in shake culture (150 rpm) for 96 h at 25 °C. Growth was followed by viable counts on malt agar. Culture broth samples were collected every 6–8 h, centrifuged (3000 × *g* for 10 min) and the supernatants tested for enzyme activity.

Preparation of cell extract

The cells were washed twice in 10 mM citrate-phosphate buffer (pH 7.0), resuspended in sterile distilled water and then broken by five 1-min periods of sonication in a 20 KHz 150 W MSE Mullard Ultrasonic Disintegrator operating at maximum output. The supernatant fraction was recovered after centrifugation (6500 × *g* for 20 min) of the sonicated suspension.

Enzyme assays

Polygalacturonase activity was quantified by measuring the reduction in viscosity of buffered pectin solutions, according to the procedure of Tuttobello and Mill (1961). The incubation temperature was 37 °C. One viscometric unit of activity (VU) was taken as the amount of enzyme which reduced the initial viscosity to 50% in 20 min.

Polygalacturonase was also determined by measuring the release of reducing end-groups from either pectin or Na-polypectate by the dinitrosalicylic acid (DNS) method of Miller (1959), using galacturonic acid as a standard.

Presence of pectin and/or pectate lyase activities was determined colorimetrically by the thiobarbituric acid (TBA) method of Neukom (1960).

Recovery of crude enzyme

Cell-free culture broth (200 ml with specific enzyme activity of 15.5 VU · ml^{-1}) was brought to 50% saturation with ammonium sulphate and centrifuged (5000 × *g* for 10 min). The supernatant was then brought to 90% saturation with the same salt and left overnight at 4 °C. The precipitate formed was collected by centrifugation (8000 × *g* for 15 min), dissolved in a minimum amount of distilled water and dialysed overnight against several volumes of water. Precipitation with ammonium sulphate and dialysis were repeated twice. The final enzyme solution, which contained almost 90% of the initial pectinase activity, was concentrated by lyophilization. The lyophilized material had an activity of 70.0 VU per mg of dry crude preparation.

Analytical methods

Protein was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

Gel filtration chromatography of the crude enzyme preparation was performed at 6 °C by using a Sephadex G-100 column (1.5 × 125 cm) equilibrated and eluted with 20 mM phosphate buffer, pH 7.0. The flow rate was 6.0 ml · h^{-1}. 1.5-ml fractions were collected and their enzyme activity was determined viscometrically.

The apparent molecular weight (MW) of the enzyme was estimated by gel filtration on Sephadex G-100 (as above) according to the procedure of Andrews (1965). Lysozyme (MW_r 14000 dal), myoglobin (MW_r 18000 dal), trypsin (MW_r 23500 dal), ovalbumin (MW_r 43000 dal) and bovine serum albumin (MW_r 67000 dal) were the reference proteins. The void volume V_o was determined using a 0.4% Blue Dextran 2000000 solution.

In order to determine its MW the enzyme was also subjected to sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie blue staining (Weber and Osborn, 1969). The MW of the enzyme was determined by comparing its migration rate with that of standard proteins with known MW.

Polyacrylamide gel electrophoresis under non-denaturing conditions was carried out according to Davis (1964). After electrophoresis, the protein was detected in one gel by staining with 0.25% Coomassie blue while a duplicate gel was stained for glycoproteins by the periodic acid-Schiff technique of Zacharius et al. (1969).

Isoelectric point (pI) was determined by analytical isoelectric focusing in polyacrylamide gel (Drysdale et al., 1971). The ampholine carrier ampholytes used had a pH range of 3.5–10.0.

In all electrophoresis experiments the protein concentration of the enzyme was made up to 1.0 mg · ml^{-1}.

pH and temperature relations

Effects of pH and temperature on enzyme activity were determined under standard assay conditions.

Effect of pH on enzyme stability was measured by incubating the enzyme solution at various pH values for 12 h at 6 °C. 100 mM citrate – phosphate, 200 mM boric acid – 50 mM borax and 50 mM borax – 200 mM NaOH buffers were used for the pH ranges 2.5–7.0, 7.5–9.2 and 9.3–10.0, respectively.

Thermal stability of the enzyme preparation was studied by incubating enzyme solutions which were heated for 10 min at various temperatures between 30 °C and 60 °C.

Chemicals

Citrus pectin (galacturonic acid content approximately 88.9% and methoxy content approximately 9.7%) and Na-polypectate were obtained from Sigma (London) Chemical Co. Ltd, Kingston-upon-Thames, Surrey, England. The galacturonic acid was obtained from Fluka AG, Buchs, Switzerland. Low methoxyl pectin LM-380 and high methoxyl pectin HM-Rapid Set were a gift from Cesalpinia S.p.A. (Pectin Division), Bergamo, Italy.

Results and Discussion

The effect of the carbon source on polygalacturonase production by *Cr. albidus* var. *albidus* IMAT-4735 is reported in Table 1.

Enzyme production was obtained in all cultures containing pectic substances

Table 1. Effect of the carbon source on growth and pectinase activity of *Cryptococcus albidus* var. *albidus* IMAT-4735[1]

Carbon source (%)	Biomass (cells·ml^{-1}, × 10^7)	Enzyme activity (VU·ml^{-1})
Glucose 0.2	5.0	1.8
Sucrose 0.2	5.3	2.2
Galacturonic acid 0.2	4.8	8.0
Glucose 1.0	21.8	none
Sucrose 1.0	21.3	none
Galacturonic acid 1.0	18.0	13.5
Na-polypectate 1.0	18.5	13.0
Pectin 1.0	18.0	13.8
Pectin LM-380 1.0	19.9	15.5
Pectin HM-RS 1.0	19.7	14.0
Glucose 0.2 + pectin 0.8	20.6	7.2
Galacturonic acid 0.2 + pectin 0.8	20.8	14.0

[1] Biomass and pectinase activity at the time of maximal enzyme production.

as well as galacturonic acid. This implies that the enzyme was not induced by the substrate but produced constitutively. The enzyme activity, however, was reduced to less than 15% when the microorganism was grown on 0.2% glucose. Enzyme synthesis was completely repressed by an initial glucose concentration in the culture medium of 1.0%. In this respect, the polygalacturonase of *Cr. albidus* appears to be rather different from both the inducible enzyme of *Aspergillus niger* (Tuttobello and Mill, 1961) and the constitutive ones of other yeasts such as *K. fragilis* (Phaff, 1966; Wimborne and Rickard, 1978) and *Rhodotorula rubra* and *Rh. minuta* (Vaughn et al., 1969).

Pectin LM-380 was the carbon source that supported the highest level of enzyme activity and, therefore, was chosen as substrate for enzyme induction in the study of the kinetics of polygalacturonase production by the microorganism.

Contrary to what has been reported for other yeasts whose ability to produce polygalacturonase is completely repressed by high oxygen tensions (Phaff, 1966; Wimborne and Rickard, 1978), *Cr. albidus* var. *albidus* showed high enzyme production when grown aerobically in shake culture (Fig. 1). Growth occurred with an average doubling time of approximately 4–5 h. Polygalacturonase activity increased progressively during exponential growth and early stationary phase, reaching a maximum (15.5 VU $\cdot$ ml^{-1}) after 72 h. Almost all the enzymatic activity was found in the fermentation medium. The polygalacturonase was thus a true extracellular enzyme and its appearance in the culture supernatant was not due to cell lysis.

Enzyme was also produced with galacturonic acid as the sole carbon source (Fig. 2). This feature seems to be of particular interest being rather uncommon among yeasts (Luh and Phaff, 1951).

The crude enzyme rapidly reduced the viscosity of both sodium polypectate and pectin solutions (Fig. 3); the rate of reduction in viscosity was, however, twice as high on the first substrate as on pectin. At 50% viscosity reduction only c. 4% of the available glycosidic bonds of both substrates had been hydrolysed. This mechanism is characteristic of polygalacturonases which act in an 'endo' fashion (endo-PG), as confirmed by the linearity of the relationship between enzyme concentration and the reciprocal of the 50% reduction time (Phaff, 1966; Finkelman and Zajic, 1978) (Fig. 4). No lyase was present in the crude enzyme preparation as shown by the negative TBA test (Neukom, 1960).

Purification of *Cr. albidus* endo-PG activity was achieved by gel filtration of the crude enzyme preparation obtained after repeated ammonium sulphate precipitation and dialysis.

The lyophilized material (c. 65 mg) was dissolved in 3.0 ml of distilled water and applied onto a Sephadex G-100 column. The enzyme activity eluted as a single symmetric peak and the active fractions were pooled, dialysed overnight against water and concentrated by lyophilization. Total recovery of activity was c. 75%. Attempts to further purify the enzyme did not lead to any increase in specific activity.

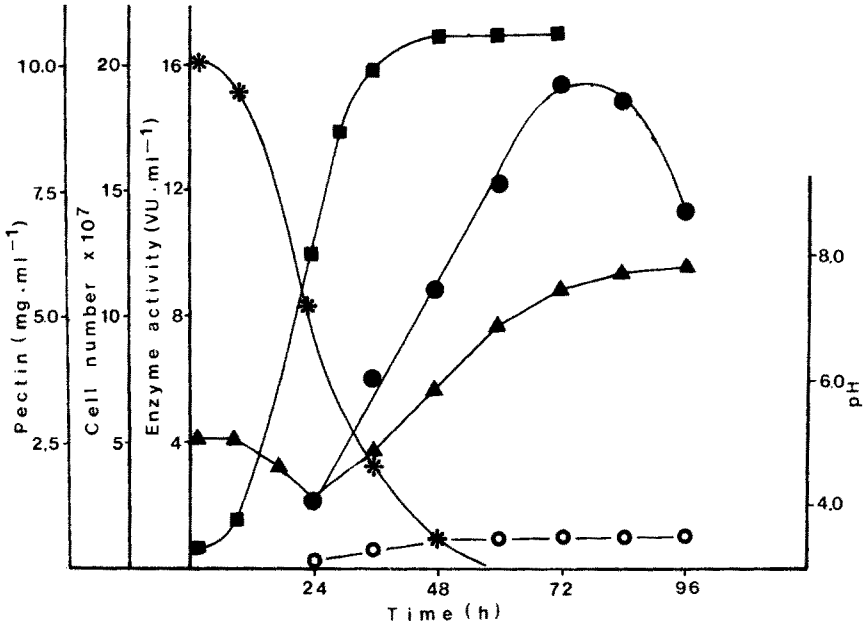

Fig. 1. Growth and pectinase activity of *Cryptococcus albidus* var. *albidus* IMAT-4735 in shaken culture on pectin LM-380. Growth (■); enzyme activity of culture supernatant (●) and cell extract (○); pectin consumption (*); pH (▲).

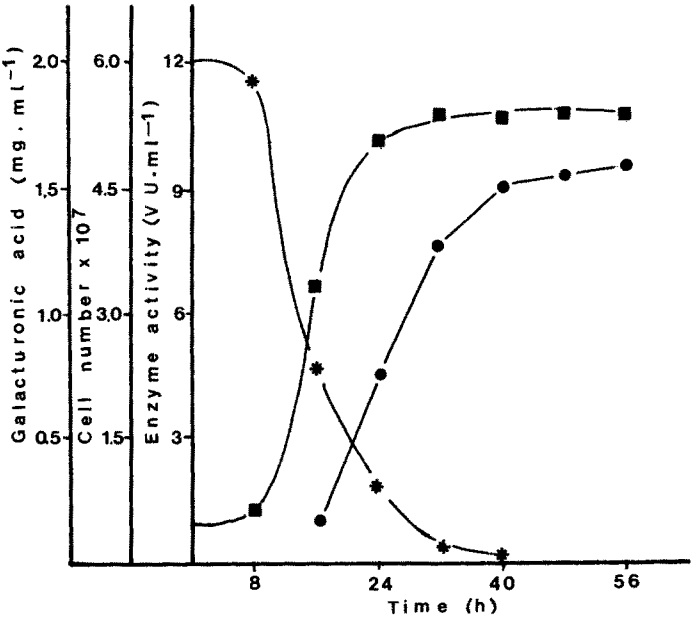

Fig. 2. Growth (■) and pectinase activity (●) of *Cryptococcus albidus* var. *albidus* IMAT-4735 in shaken culture on 0.2% galacturonic acid (*).

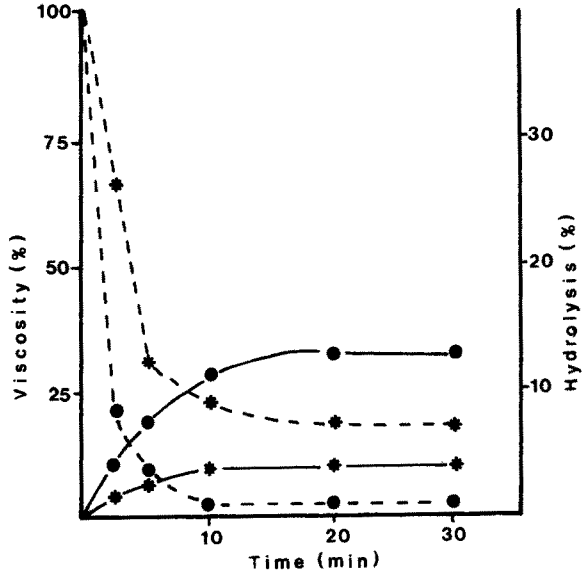

Fig. 3. Reduction in viscosity (----) and hydrolysis (——) of Na-polypectate (●) and pectin (*) solutions by the crude pectinase of *Cryptococcus albidus* var. *albidus* IMAT-4735.

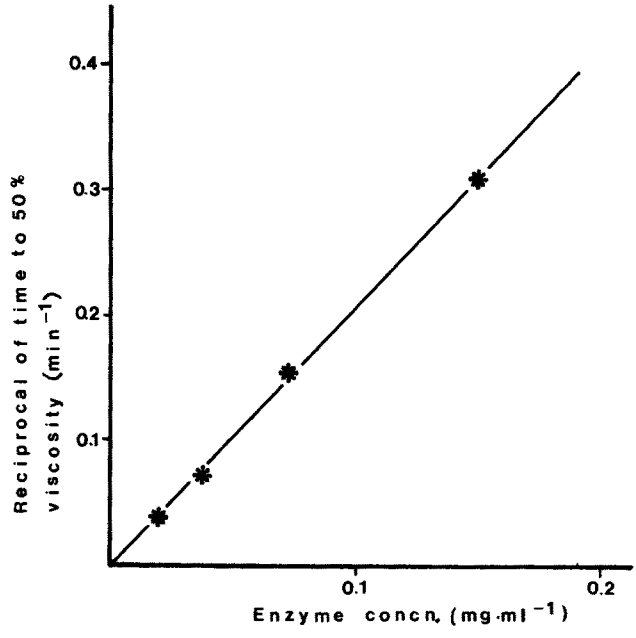

Fig. 4. Effect of the concentration of the crude pectinase of *Cryptococcus albidus* var. *albidus* IMAT-4735 on the reciprocal of the 50% viscosity reduction time.

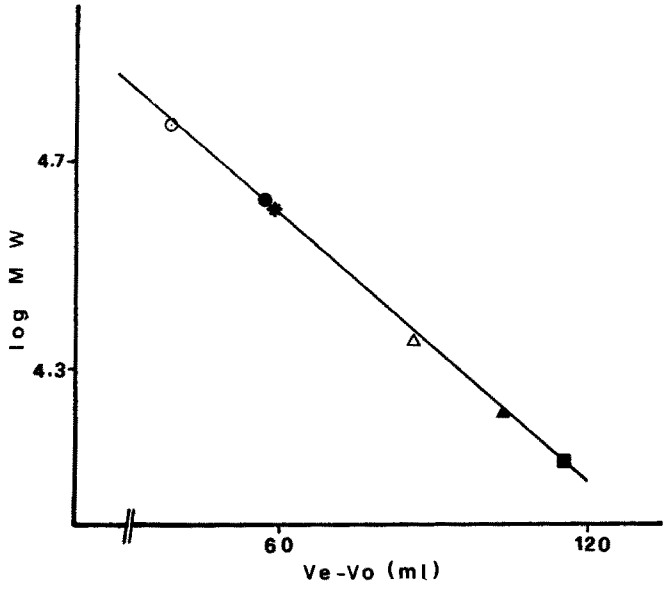

Fig. 5. Estimation of molecular weight by gel filtration. Lysozyme (■); myoglobin (▲); trypsin (△); *Cryptococcus albidus* endo-PG (*); ovalbumin (●); bovine serum albumin (○).

Enzyme purity was demonstrated by electrophoresis experiments under both denaturing and non-denaturing conditions.

Isoelectric focusing gave a pI of 8.10 ± 0.10. This value is higher than those reported for the endo-PGs I, II and III of *K. fragilis* (Lim et al., 1980), but similar to those of the pectic enzymes of some phytopathogenic fungi (Cervone et al., 1977).

The enzyme had an apparent MW_r of 41 000 dal, as estimated by gel filtration on Sephadex G-100 (Fig. 5) and as confirmed by SDS-PAGE experiments (MW_r c. 39 500 dal).

The endo-PG was most active at pH 3.75 and 37 °C (Figs 6–7). It appeared to be fairly stable in the pH range 4.0–8.0 (Fig. 6). Heat inactivation was not significant up to 40 °C; at higher temperatures, however, enzyme activity decreased sharply and complete inactivation occurred at 55 °C (Fig. 7).

Lineweaver-Burk plots obtained from initial velocities at varying substrate concentrations, under standard assay conditions, gave a K_m value of $5.7 \cdot 10^{-1}$ mg·ml^{-1} for activity on Na-polypectate (Fig. 8), which is very close to that observed for endo-PG III of *K. fragilis* (Lim et al., 1980).

The enzyme was completely inhibited by Hg^{2+} (Table 2). Partial inhibition was also obtained with Pb^{2+} and Cd^{2+} at a concentration of $5 \cdot 10^{-3}$ M. However, this inhibition was almost nil at an ion concentration of $5 \cdot 10^{-4}$ M.

The positive reaction of the pure enzyme with the periodic acid-Schiff reagent,

Table 2. Effect of metal ions and enzyme inhibitors on the activity of *Cryptococcus albidus* endo-PG

Metal ion or inhibitor	Residual activity (%) at concentrations:	
	$5 \cdot 10^{-3}$M	$5 \cdot 10^{-4}$M
None	100	100
Ca^{2+} (chloride)	100	100
Cd^{2+} (sulphate)	35	80
Fe^{3+} (sulphate)	100	100
Hg^{2+} (acetate)	0	0
K^{+} (chloride)	100	100
Mn^{2+} (sulphate)	100	100
Mg^{2+} (sulphate)	86	100
Na^{+} (chloride)	100	100
Pb^{2+} (acetate)	50	84
EDTA	100	100
Tris	100	100

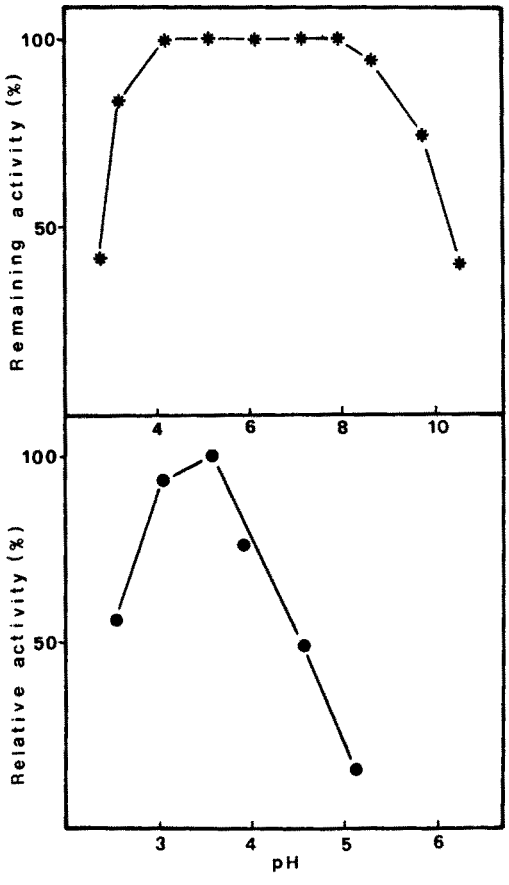

Fig. 6. Effect of pH on activity (●) and stability (*) of *Cryptococcus albidus* endo-PG.

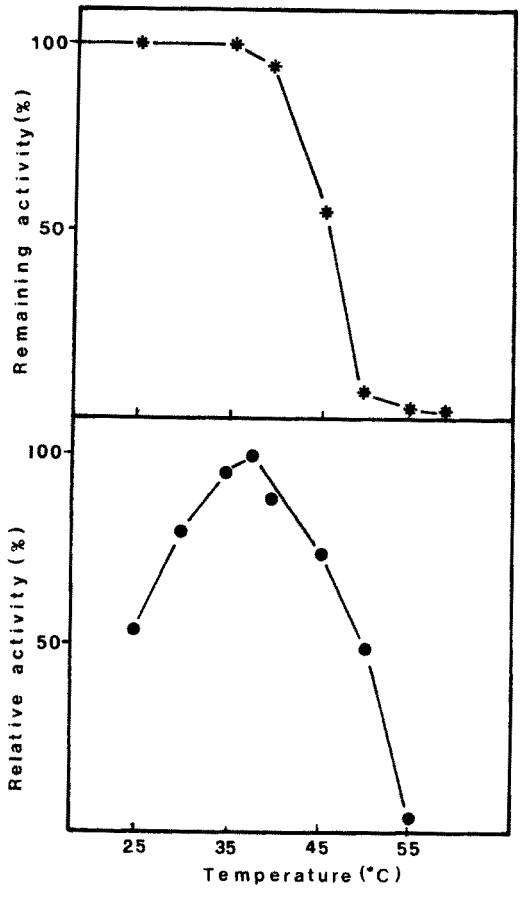

Fig. 7. Effect of temperature on activity (●) and stability (*) of *Cryptococcus albidus* endo-PG.

with which the polyacrylamide gel was stained after electrophoresis under non-denaturing conditions (Zacharius et al., 1969), suggested that the endo-PG was a glycoprotein.

The author thanks Mrs Rita Gallo for her skilful technical assistance. The research was supported by a grant from the Italian Ministero della Pubblica Istruzione, Rome, Italy.

Accepted 24 April 1985

References

Andrews, P. 1965. The gel-filtration behaviour of proteins related to their molecular weights over a wide range. — Biochem. J. **96**: 595–606.

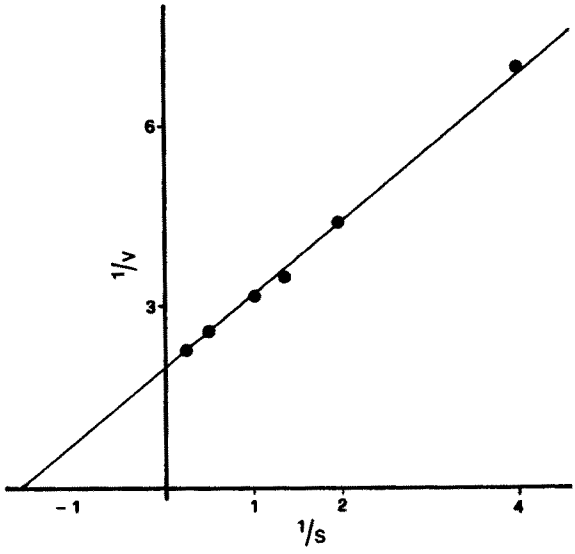

Fig. 8. Effect of Na-polypectate concentration on *Cryptococcus albidus* endo-PG activity. Velocity (V) is expressed as mmoles of galacturonic acid · ml^{-1} of the reaction mixture · min^{-1} and substrate concentration (S) as mg of Na-polypectate · ml^{-1} of the reaction mixture. Incubation time was 3 min.

BELL, T.A. and ETCHELLS, J.L. 1956. Pectin hydrolysis by certain salt-tolerant yeasts. — Appl. Microbiol. **4**: 196–210.

CERVONE, F., SCALA, A., FORESTI, M., CACACE, M. G. and NOVIELLO, C. 1977. Endopolygalacturonase from *Rhizoctonia fragariae*. Purification and characterization of two isoenzymes. — Biochim. Biophys. Acta **482**: 379–385.

DAVIS, B. J. 1964. Disc electrophoresis – II. Method and application to human serum proteins. — Ann. N. Y. Acad. Sci. **121**: 404–427.

DENNIS, C. and HARRIS, J. E. 1979. The involvement of fungi in the breakdown of sulphited strawberries. — J. Sci. Food Agric. **30**: 687–691.

DRYSDALE, J. W., RIGHETTI, P. and BUNN, H. F. 1971. The separation of human and animal hemoglobins by isoelectric focusing in polyacrylamide gel. — Biochim. Biophys. Acta **229**: 42–50.

FEDERICI, F. 1983*a*. Extracellular enzymes production in yeasts. — Ann. Microbiol. (Milan) **33**: 15–20.

FEDERICI, F. 1983*b*. Preliminary study of the pectolytic activity of *Cryptococcus albidus* var. *albidus*. — Boll. Soc. Ital. Biol. Sper. **59**: 1509–1512.

FINKELMAN, M. J. and ZAJIC, J. E. 1978. Pectinase from *Aureobasidium pullulans*. p. 459–464. *In* L. A. Underkofler (ed.), Developments in Industrial Microbiology, Vol. 19. — Society for Industrial Microbiology, Washington D.C.

HARRIS, J. E. and DENNIS, C. 1979. The stability of pectolytic enzymes in sulphite liquor in relation to breakdown of sulphited strawberries. — J. Sci. Food Agric. **30**: 704–710.

LIM, J., YAMASAKI, Y., SUZUKI, Y. and OZAWA, J. 1980. Multiple forms of endo-polygalacturonase from *Saccharomyces fragilis*. — Agric. Biol. Chem. **44**: 473–480.

LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. and RANDALL, R. J. 1951. Protein measurement with the Folin phenol reagent. — J. Biol. Chem. **193**: 265–275.

LUH, B. S. and PHAFF, H. J. 1951. Studies on polygalacturonase of certain yeasts. — Arch. Biochem. Biophys. **33**: 212–227.

LUH, B. S. and PHAFF, H. J. 1954. Properties of yeast polygalacturonase. — Arch. Biochem. Biophys. **48**: 23–37.

MILLER, G. L. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. — Anal. Chem. **31**: 426–428.

NEUKOM, H. 1960. Uber Farbreaktionen von Uronsauren mit Thiobarbitursaure. — Chimia **14**: 165–167.

PHAFF, H. J. 1966. α-1,4-Polygalacturonide glycanohydrolase (endopolygalacturonase) from *Saccharomyces fragilis*. p. 636–641. *In* S. P. Colowick and N. O. Kaplan (eds), Methods in Enzymology, Vol. 8. — Academic Press, New York.

TUTTOBELLO, R. and MILL, P. J. 1961. The pectic enzymes of *Aspergillus niger* 1. The production of active mixtures of pectic enzymes. — Biochem. J. **79**: 51–57.

VAUGHN, R. H., JAKUBCZYK, T., MACMILLAN, J. D., HIGGINS, T. E., DAVÉ, B. A. and CRAMPTON, V. M. 1969. Some pink yeasts associated with softening of olives. — Appl. Microbiol. **18**: 771–775.

WEBER, K. and OSBORN, M. 1969. The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. — J. Biol. Chem. **244**: 4406–4412.

WIMBORNE, M. P. and RICKARD, P. A. D. 1978. Pectinolytic activity of *Saccharomyces fragilis* cultured in controlled environments. — Biotechnol. Bioeng. **20**: 231–242.

ZACHARIUS, R. M., ZELL, T. E., MORRISON, J. H. and WOODLOCK, J. J. 1969. Glycoprotein staining following electrophoresis on acrylamide gels. — Anal. Biochem. **30**: 148–152.