Journal of Industrial Microbiology, 8 (1991) 229–236
Elsevier

SIM 00368

Studies on the galactomannan-degrading enzymes produced by *Sporotrichum cellulophilum*

Alberto Araujo* and Owen P. Ward

Department of Biology, University of Waterloo, Waterloo, Ontario, Canada

(Received 14 March 1991; revision received 3 June 1991; accepted 17 June 1991)

Key words: Mannanase; *Sporotrichum cellulophilum*; Galactomannan; Hemicellulase

SUMMARY

Extracellular mannanase activity produced by *Sporotrichum cellulophilum* was purified into two components using acetone precipitation, SP-Sephadex C50 ion exchange chromatography and preparative polyacrylamide gel electrophoresis. The purified mannanse components, M1 and M2, had molecular weights of 108 000–112 000 and 32 200–36 000 respectively. Component M1 was shown to contain 2 subunits having molecular weights of 62 000 and 50 000. M1 and M2 had similar pH-activity profiles with pH optima of 5.5 and 6.0 respectively. M1 was more thermostable than M2: half lives of the enzymes at 70 °C were 30 and 9 min for M1 and M2 respectively.

INTRODUCTION

Hemicellulose is the second largest renewable resource after cellulose, with some hardwood plant species containing up to 35% of hemicellulosic substances [47]. The major hemicellulose components of wood are galactomannans, glucomannans, arabinogalactans and xylans. Galactomannan is the main storage carbohydrate in leguminous seeds and constitutes 18–20% of the dry weight of guar and locust beans [20]. Many palm seeds contain mannans as a major component, causing thickening of the endosperm wall and conferring characteristic hardness on the palm kernel [36]. Mannans are present in substantial quantity in the endosperms of copra and in the roots of konjak [1]. Mannanases may be used to hydrolyse mannans of coffee bean in coffee manufacture [46]. These enzymes have potential applications for processing of other plant materials, including their use as supplementary enzymes to cellulase in the conversion of wood polysaccharides to hexoses [21,24]. A variety of mesophylic fungi and bacteria produce mannan-degrading enzymes. Fungal β-1,4-mannanases or β-mannosidases are produced by *Aspergillus* species [7,9,16,40,42], *Crysosporium lignorum* [18], *Polyporus* species [44], *Rhizopus niveus* [24], *Sclerotium rolfsii* [43], *Tyromyces* [25] and *Trichoderma* species [41]. α-Galactosidase also participates in the hydrolysis of galactomannan by removal of galactosyl side chain residues [13]. When beet sugar is produced by the Steffen process, the presence of traces of raffinose interferes with sucrose crystallization. While treatment with α-galactosidase, which converts raffinose to galactose and sucrose improves process efficiency, industrial sources of this enzyme are rare and the low heat tolerance of available enzymes makes them unsuitable for effective use in this application [22].

The advantages in carrying out enzyme reactions at higher temperatures include the increased reaction rates attained, the ability to markedly reduce microbial infection of the material being processed (above 60–65 °C), and the fact that higher temperatures increase the solubility of carbohydrate polymers rendering them more amenable to enzymatic attack [8,45]. These advantages have motivated screening programmes to identify more thermostable industrial enzymes, and a number of thermophilic fungi producing more thermostable cellulases have been isolated [10,15,26,31,33]. We have previously screened a variety of cellulase-producing thermophilic fungi, including species of *Talaromyces*, *Thielavia*, *Thermoascus* and *Sporotrichum*, as possible future commercial sources of mannanase activity since, in many of the potential applications, these latter enzymes would be used in combination with cellulase [5]. *Sporotrichum cellulophilum* ATCC 20493 produces a significant level of galactomannan-degrading activity. Enzyme activity was shown to be induced by locust bean gum with no detectable enzyme being observed in media containing glucose as carbon source. We have also shown that this

Correspondence: O.P. Ward, Department of Biology, University of Waterloo, Waterloo, Ontario, Canada N2L 3G1.
* Present address: Allelix Biopharmaceuticals Inc., 6850 Goreway Drive, Mississauga, Ontario, Canada, L4V 1P1.

0169-4146/91/$03.50 © 1991 Society for Industrial Microbiology

organism is a good producer of arabinase [27]. Studies have now been carried out on production of galactomannan-degrading activity by *S. cellulophilum* ATCC 20493 in laboratory fermenters and the crude enzyme has been purified into two components which have been characterised.

MATERIALS AND METHODS

Microorganism

Sporotrichum cellulophilum ATCC 20493 was maintained on potato-dextrose agar medium. Inoculated slants were incubated at 45 °C for 4 days and then stored at 5 °C with monthly subculturing.

Medium and culture conditions

The basal medium for enzyme production contained: $NaNO_3$, 2 g/l; KH_2PO_4, 2 g/l; $CaCl_2 \cdot 2H_2O$, 0.3 g/l; $MgSO_4 \cdot 7H_2O$, 0.3 g/l and 1 ml/l of trace metal solution [2]. The basal medium was supplemented with locust bean gum (Sigma, St. Louis, MO), corn steep solids, yeast extract (Difco, Detroit, MI) and potato extract as indicated in the results section. Potato extract was prepared as previously described [6]. The pH of the culture medium was adjusted to 5.5.

Inoculum was prepared in 25-ml Erlenmeyer flasks containing 65 ml medium for preliminary shake-flask optimisation studies and in 2-l Erlenmeyer flasks containing 500 ml of medium for studies in fermenters. These flasks were inoculated with spores of *S. cellulophilum*, taken from slants of the maintenance medium, and incubated at 45 °C on an orbital shaker set at 200 rpm for 24 h. A 5% (v/v) actively growing culture from these flasks was employed as mycelial inoculum for enzyme production studies. Shake flask enzyme optimisation studies were carried out in 250-ml Erlenmeyer flasks containing 65 ml medium, inoculated as described above and incubated at 45 °C on an orbital shaker at 200 rpm for 48 h. Fermenter cultures were carried out in 20-l Chemap fermenters, model 2000, containing 15 l of medium, and sterilised in situ. Fermenter settings were: agitation, 400 rpm; aeration, 0.5 vvm; and temperature, 45 °C, with and without pH control. At the end of 36 h, mycelia were separated by straining through a cheese cloth and further centrifuged at 1000 × *g* for 10 min. Media used in inoculum cultures were the same as those used in the subsequent enzyme production culture.

Estimation of biomass

Biomass determinations were carried out by drying the mycelium on preweighed Whatman glass microfibre filters (GF/D) to constant weight at 80 °C. Filtered mycelia were washed with water prior to drying to remove soluble medium constituents.

Measurement of enzyme activity

Mannanase was assayed using locust bean galactomannan as substrate, purified from locust bean gum (Sigma, St. Louis, MO) by the method of Halmer et al. [23]. The assay mixture contained 0.5 ml of 1% (w/v) galactomannan, prepared in 0.1 M sodium acetate buffer, pH 5.6 and 0.5 ml of enzyme, appropriately diluted in the same buffer. The reaction mixture was incubated at 50 °C for 30 min. Reducing sugars produced due to enzyme activity were determined as mannose-reducing equivalents using the dinitrosalicylic acid (DNS) method of Miller [38]. β-Mannosidase activity was determined using *p*-nitrophenyl β-D-mannopyranoside as previously described [4]. α-Galactosidase activity was determined by incubation of 0.1 ml of enzyme with 1 ml of substrate, containing 1 mM/l *p*-nitrophenyl α-D-galactopyranoside (Sigma) in 0.05 M sodium acetate, pH 5.6, for 10 min at 50 °C. The reaction was terminated by addition of 2 ml of 2% w/v Na_2CO_3. The yellow colour was measured at 410 nm and related to absorbance values obtained with standard *p*-nitrophenol solutions. One unit of β-mannosidase and α-galactosidase activity is defined as the amount producing 1 μmol of product in 1 min.

Determination of D-galactose

D-Galactose was determined using the lactose/D-galactose test-combination kit of Boehringer (Mannheim, GmbH). In this kit, galactose is oxidized by α-galactose dehydrogenase with production of NADH which is determined spectrophotometrically.

Determination of protein

Protein was estimated according to the method of Lowry et al. [30] with bovine serum albumin as the standard. Protein eluted during column chromatography was monitored by absorbance at 280 nm and estimated by assuming that one absorbance unit corresponded to 0.5 mg/ml.

Enzyme purification

One volume of the cell-free supernatant at − 4 °C was precipitated with two volumes of chilled acetone and allowed to stand for 2 h. The precipitated enzyme was separated by centrifugation at 10 000 × *g* for 20 min, redissolved in 0.02 M sodium acetate buffer, pH 5.6 and passed through a Sephadex G-25 column (2.5 × 20 cm). The active fractions were pooled and lyophilised. Lyophilised enzyme (180 mg) was dissolved in 5 ml of 50 mM sodium acetate buffer at pH 4.3 and applied to a SP-Sephadex C50 column (2.5 × 24 cm). The bound enzyme was eluted using a linear NaCl gradient (0–0.7 M) in 50 mM sodium acetate buffer at pH 4.3, and 5-ml fractions were collected.

Gel electrophoresis

Polyacrylamide gel electrophoresis (PAGE) was carried out using 7.5% gel concentration according to the method of Ornstein [39] and Davis [11]. The gels were stained using the silver-staining procedure described by Merril et al. [37]. Preparative PAGE was carried out in a modified mini-protean II (Biorad, Richmond, VA) electrophoresis apparatus. The thickness of the gel was 2.5 mm. Desalted enzyme (2 mg) was charged onto the gel and electrophoresis was carried out using Tris/glycine buffer at pH 8.5 with constant current of 14 mA at 5 °C for 6 h. The gel was then sliced into 2.5-mm sections and broken by syringe passage. Enzyme was eluted by using 4 ml of sodium acetate buffer, 0.1 M, pH 5.0.

Enzyme characterisation

Enzyme pH optima were determined at various pH values in 0.05 M sodium acetate/0.1 M sodium phosphate buffer. Carbohydrate contents of the purified components were determined using the phenol sulphuric acid method of Dubois et al. [14]. Molecular masses were determined by SDS-PAGE according to the previously described method [35]. Bovine serum albumen monomer, 66 kDa, and dimer, 132 kDa; ovalbumin, 42.7 kDa; carbonic anhydrase, 31 kDa; and soya bean trypsin inhibitor, 21 kDa, were also used as standards to determine the molecular mass of the enzymes in undissociated form by gel filtration. Enzyme fractions and standards were applied to a 1.5 × 90 cm column, packed with Toyopearl TSK HW55 and eluted with 0.05 M sodium acetate, pH 5.6.

RESULTS

Preliminary studies on optimisation of enzyme production were carried out in shake flask cultures containing the basal medium supplemented with varying amounts of locust bean gum, potato extract, corn steep solids and yeast extract. Mannanase activities ranging from 1.3 to 5.2 U/ml were observed in the cell-free supernatant after 48 h. A medium containing locust bean gum, 5 g/l; potato extract, 5 ml/l; corn steep solids, 0.1 g/l; and yeast extract, 1.0 g/l, which resulted in production of 5.1 U/ml mannanase, was selected for use in fermentation and enzyme purification studies. The production of mannanase activity and mycelial biomass in this medium was investigated in three laboratory fermenters with pH uncontrolled and controlled at 4.5 and 5.5, respectively. Highest enzyme production was observed in the fermentation run with uncontrolled pH (Fig. 1).

The cell-free supernatant recovered after 36 h under these fermentation conditions was used for enzyme purification. The clarified supernatant contained 6 units per ml

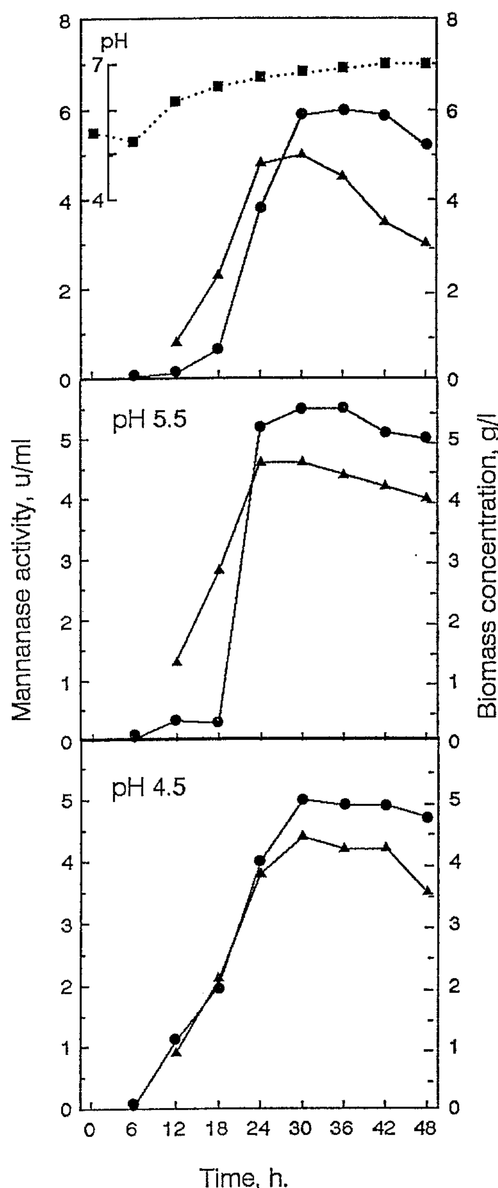


Fig. 1. Production of enzyme and biomass in 20-l laboratory fermenters without pH control and with pH controlled at 4.5 and 5.5. ■, pH; ●, mannanase activity; ▲, biomass concentration.

mannanase activity with a specific activity of 32 U/mg The enzyme was purified from the cell-free supernatant by precipitation with two volumes of acetone, resuspension and passage through a Sephadex G-25 column, lyophilisation and then separation using SP-Sephadex C50 ion exchange chromatography. Only one enzyme peak was observed following elution of the SP-Sephadex column using a sodium chloride linear gradient (Fig. 2). On checking the homogeneity of the protein in this peak,

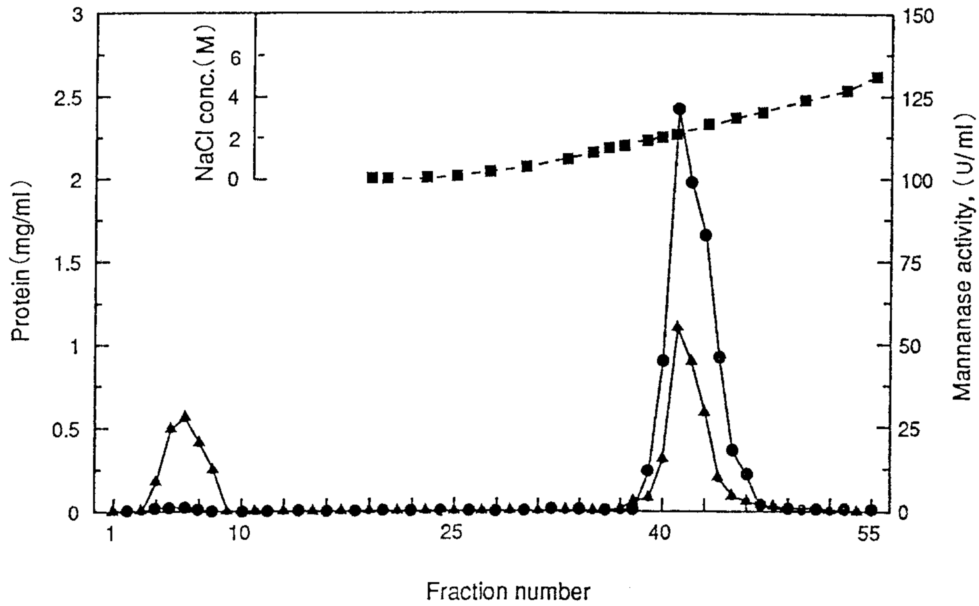


Fig. 2. Enzyme elution profile with NaCl gradient from SP-Sephadex C 50 column. ●, Mannanase; ▲, protein; ■, NaCl concentration.

at least four bands were observed on PAGE electrophoresis. The four electrophoretic bands were not successfully separated using gel filtration through a TSK HW55 or Sephadex G-100 gel filtration columns. The enzyme peak recovered from the ion exchange column was successfully fractionated into two active mannanase components using preparative PAGE. The patterns of enzyme elution from preparative PAGE are presented in Fig. 3 and the electrophoretic homogeneity of the isolated components is illustrated in Fig. 4. A summary of the purification scheme is presented in Table 1. Final specific activities of M1 and M2 were 198 and 153 U/mg respectively.

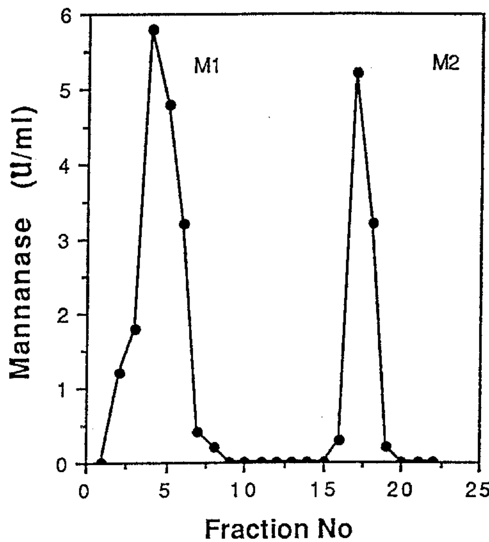


Fig. 3. Purification of mannanases by preparative PAGE of the SP-Sephadex C 50 peak.

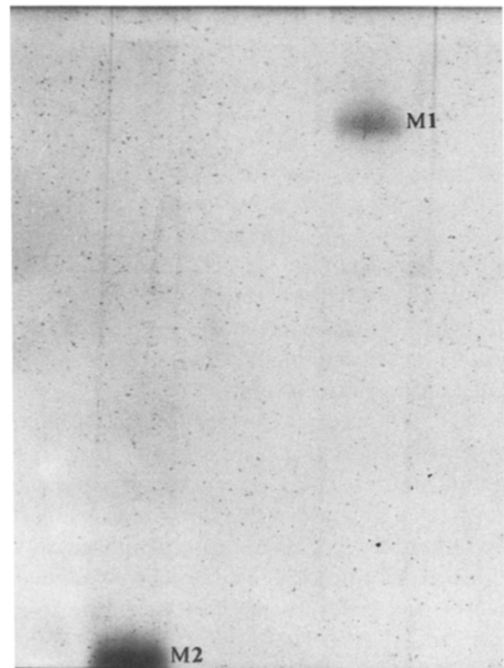


Fig. 4. Polyacrylamide gel electrophoresis of purified mannanases M1 and M2.

TABLE 1

Summary of enzyme purification

	Total protein		Total mannanase activity recovered (U)	Specific activity (U/mg)	Recovery (%)
	Used in purification step (mg)	Recovered in purification step (mg)			
Culture filtrate	–	748	23936	32	100.0
Acteone precipitate	748	452	19888	44	83.1
Sephadex G25	452	399	19568	49	81.8
SP-Sephadex C50	180	16	1920	120	20.1
Preparative electrophoresis	2				
M1		0.38	75	198	13.1
M2		0.53	81	153	

The pH activity profiles of the enzyme components, M1 and M2, are presented in Fig. 5. Similar pH-activity patterns were observed with M1 and M2 having pH optima of 5.5 and 6.0 respectively. The comparative thermostabilities of the enzymes were determined by incubation at a range of temperatures (50–70 °C) for up to 3 h. Residual activities (%) are presented in Fig. 6. Both enzymes retained full activity for 2 h at 50 °C. Component M1 was considerably more thermostable than M2 at higher temperatures, retaining 80% activity after 2 h at 65 °C, compared to a corresponding value of 4% for M2. The half lives of the enzymes at 70 °C were 30 min and 9 min for M1 and M2, respectively. Molecular masses of components M1 and M2, determined by gel filtration, were 108 kDa and 32.2 kDa, respectively. The molecular masses of components M1 and M2, determined from SDS PAGE, were 112 kDa and 35 kDa, respectively. Mannanase component M1 was shown to contain two subunits having molecular masses of 62 kDa and 50 kDa. Carbohydrate content was 24% for M1 and 11% for M2.

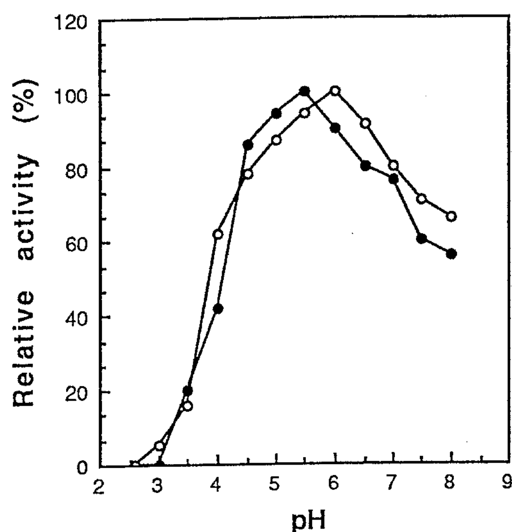
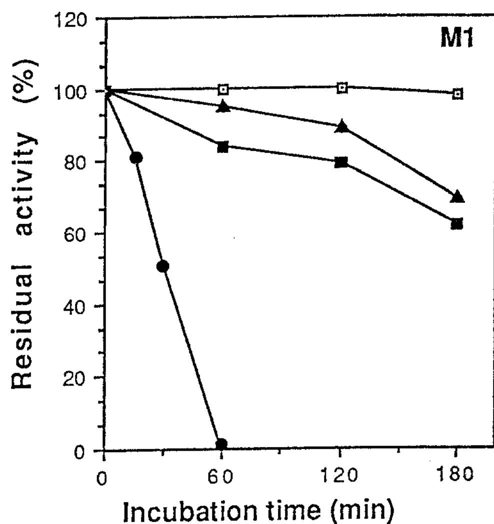


Fig. 5. Effect of pH on mannanase activity. ●, Mannanase M1; ○, mannanase M2.

Production of reducing sugars on enzymatic hydrolysis of galactomannan could be due to random cleavage of the main mannan chain by endo-β-1,4-mannanase, cleavage of single D-mannosyl residues by β-mannosidase from the non-reducing end of the mannan chair, or removal of α-D-galactosyl side-chain residues by α-galactosidase. M1 manifested no β-mannosidase or α-galactosidase activity, confirming that enzyme activity was due to endo-β-1,4-mannanase.

When electrophoretically homogeneous component M2 was incubated with galactomannan substrate, and the digest analysed for reducing sugars and galactose, 95% of the reducing equivalents produced on the digestion of galactomannan was attributed to mannanase activity, with the remaining 5% due of production of galactose. Comparative activities of M2 towards galactomannan and *p*-nitrophenyl-α-galactopyranoside of 3.8 (100%) and 0.14 (3.7%) U/ml, respectively, confirmed the presence of low galactose cleaving ability. When the pH activity and thermostability profiles for α-galactosidase activities (Figs. 7, 8) were compared with the corresponding mannanase profiles for M2, the data suggested that α-galactosidase activity was due to a distinct enzyme. However, all attempts to separate this α-galactosidase activity from the mannanase activity were unsuccessful. Nevertheless, by heat treatment of M2 at 60 °C for 1 h, the thermostability differences between mannanase and α-galactosidase activities of M2 were exploited to reduce α-galactosidase activity to 0.6% of mannanase activity. M2 manifested no activity towards *p*-nitrophenyl-β-D-mannopyranoside.



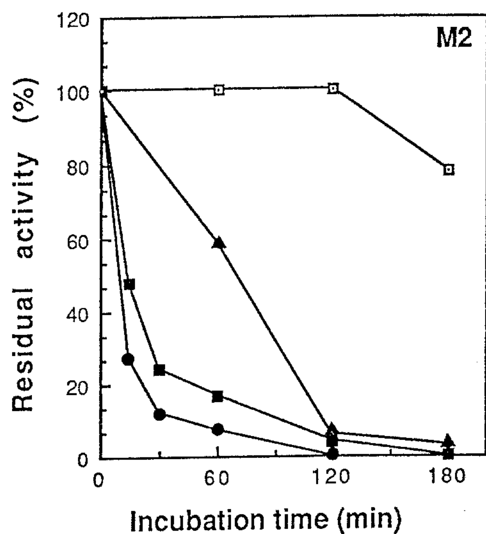


Fig. 6. Thermal stability of mannanase M1 and mannanase M2. Each enzyme was incubated at various time intervals and residual activity was determined. □, 50 °C; ▲, 60 °C; ■, 65 °C; and ●, 70 °C.

DISCUSSION

Mannanase activity, 5–6 U/ml, was produced with *Sporotrichum cellulophilum* in 36–48 h in shake flasks and pH uncontrolled fermenters in a basal salt medium, supplemented with (g/l) locust bean gum, 5; yeast extract, 1; corn steep solids, 0.1 and 5 ml/l potato extract. The clarified supernatant was purified into active mannanase components using acetone precipitation, SP-Sephadex C50 ion exchange chromatography and preparative polyacrylamide gel electrophoresis. Properties of purified

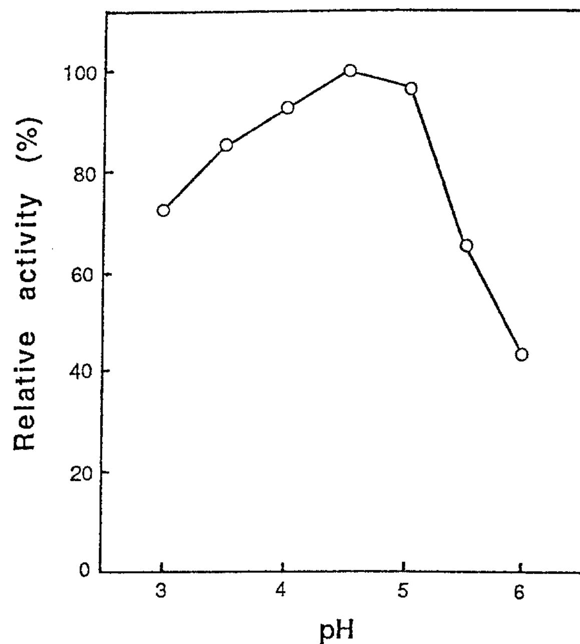


Fig. 7. Effect of pH on α-galactosidase activity.

mannanase component M1 were: molecular mass, 108–112 kDa; carbohydrate content, 24%; pH optimum 5.5 and half-life at 70 °C, 30 min. Corresponding prop-

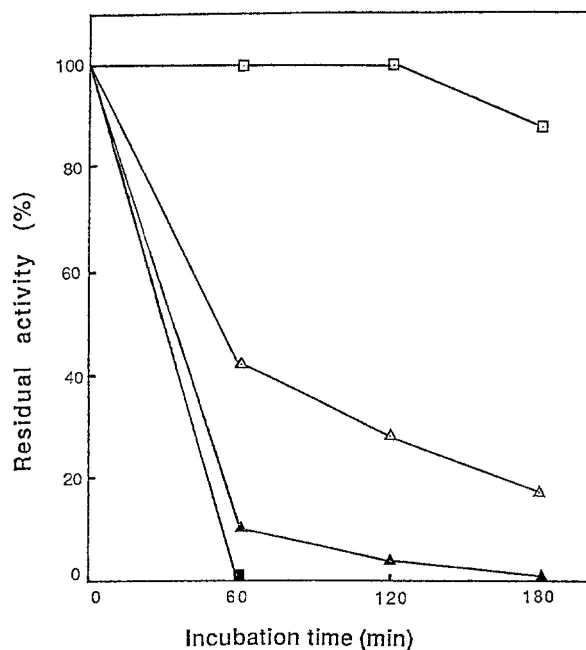


Fig. 8. Thermal stability of α-galactosidase activity present in fraction M2. Each enzyme was incubated for various time intervals and residual activity was determined. □, 50 °C; △, 55 °C; ▲, 60 °C; and ■, 65 °C.

erties for M2 were: molecular mass, 32.2–36 kDa; carbohydrate content, 11%; pH optimum 6.0 and half-life at 70 °C, 9 min. Component M1 was shown to contain two subunits having molecular masses of 62 kDa and 50 kDa, respectively. Specific activities of purified M1 and M2 were 198 and 153 U/mg, respectively. A low level of α-galactosidase activity, which co-purified with M2, was almost completely denatured by heat treatment for 1 h at 60 °C.

Molecular mass values obtained by gel filtration were 108 and 32.3 kDa for M1 and M2, respectively. Mannanase produced by *Tyromyces palustris* contained at least two mannanase components of different molecular mass [25] while a β-mannosidase enzyme and four mannanase components were isolated from culture filtrates of *T. terrestris* [3]. A single mannanase component is produced by *A. niger* [19,42], *Irpex lacteus* and *Bacillus subtilis* [17,32]. In contrast, mannanases from cell-free supernatants of *Bacillus pumilus* [3] and *Streptomyces* SP.17 [28] were separated into two and four active enzyme components, respectively. The molecular mass of component M1 from *S. cellulophilum* (112 kDa) was considerably higher than any of the components from *T. terrestris* (30–89 kDa), *A. niger* (45 kDa), *Irpex lacteus* (53 kDa), *B. pumilus* (38 kDa; 55 kDa) or *B. subtilis* (37 kDa). M1 and M2 had carbohydrate contents of 24% and 11%, respectively. Mannanase components of *T. terrestris* also contained varying amounts of carbohydrate ranging from 6 to 36%. pH optima for *S. cellulophilum* components M1 and M2 at 5.5 and 6.0, respectively, were relatively high for fungal mannanases. pH optima for other fungal mannanases ranged from 3 to 4.0 in the case of *Aspergillus* enzymes to 5.5 for the mannanase from *R. niveus* [12]. Mannanases from *Bacillus* species have pH optima around 6.0 [4]. Component M1 was relatively thermostable, having a half-life of 30 min at 70 °C. Mannanase components from *Bacillus pumilus* also manifested food stability with half-lives of 21 min and 60 min, respectively, at 70 °C [3]. *A. niger* mannanase was totally inactivated after 1 h at 70 °C [5]. Arabinan-degrading enzymes from *S. cellulophilum* were less thermostable than mannanase components M1 and M2 [27]. *S. cellulophilum* also produces thermostable cellulases and xylanases [15].

α-Galactosidase has been found by many researchers to exist in a variety of interconvertable multimolecular forms which has caused difficulties in protein purification procedures [13,29]. Co-purification of some α-galactosidase with mannanase may be related to the high tendency of α-galactosidase to form protein–protein interactions. The purified mannanase components of *T. terrestris* were shown to degrade mannan substrate in a synergistic manner [6]. Substrate specificity and patterns of action of the purified mannanase components from *S. cellulophilum* will now be investigated in more detail and compared with other microbial mannanase components.

ACKNOWLEDGEMENT

Support for this research by the Natural Sciences and Engineering Research Council of Canada is gratefully acknowledged. O.P. Ward is holder of an NSERC Industrial Research Chair, co-sponsored by Allelix, Canada.

REFERENCES

1 Akino, T., C. Kato and K. Horikoshi. 1989. Two *Bacillus* β-mannanases having different COOH termini are produced in *Escherichia coli* carrying pMAH5. Appl. Environ. Microbiol. 55: 3178–3183.

2 Araujo, A. and J. D'Souza. 1980. Production of biomass from enzymatic hydrolysate of agricultural waste. J. Ferment. Technol. 58: 399–401.

3 Araujo, J. and O.P. Ward. 1990. Production, purification and properties of the mannanase components of *Bacillus pumilus*. Appl. Environ. Microbiol. 56: 1954–1956.

4 Araujo, J. and O.P. Ward. 1990. Hemicellulases of *Bacillus* species: preliminary comparative studies on production and properties of mannanases and galactanases. J. Appl. Bacteriol. 68: 253–261.

5 Araujo, J. and O.P. Ward. 1990. Extracellular mannanases and galactanases from selected fungi. J. Industr. Microbiol. 6: 171–178.

6 Araujo, A. and O.P. Ward. 1990. Purification and some properties of the mannanases from *Thielavia terrestris*. J. Industr. Microbiol. 6: 269–274.

7 Bailey, P.J., W. Liese, R. Roesch, G. Keilich and E.G. Afting. 1969. Cellulase (β-1,4-glucan 4-glucanohydrolase) from the wood-degrading fungus, *Polyporus schweinitzii* Fr. Biochim. Biophys. Acta 185: 381–391.

8 Brock, T.D. 1986. Introduction, an overview of the thermophiles, general molecular and applied microbiology (Brock, T.D., ed.), pp. 1–16, John Wiley & Sons, New York, NY.

9 Civas, A., R. Eberhard, P. Le Dizet and F. Petek. 1984. Glucosidases induced in *Aspergillus tamarii*: α-D-galactosidase and β-D-mannanase. Biochem. J. 219: 857.

10 Coughlan, M.P. 1985. The properties of fungal and bacterial cellulases with comment on their production and application. In: Biotechnology and Genetic Engineering Reviews (Russel, G.E., Ed.), Vol. 3, pp. 39–109, Intercept, Newcastle.

11 Davis, B.J. 1964. Disc electrophoresis – II Method and application to human serum protein. Ann. New York Acad. Sci. 121: 404–427.

12 Dekker, R.F.H. and G.N. Richards. 1976. Hemicellulases: their occurrence, purification, properties and mode of action. Adv. Carbohydr. Chem. 32: 277–352.

13 Dey, P.M. and J.B. Pridham. 1972. Biochemistry of α-galactosidases. Adv. Enzymol. 36: 91–130.

14 Dubois, M., K.A. Gilles, J.K. Hamilton, P.A. Rebers and F.

Smith. 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. 28: 350–356.

15 Durand, H., P. Soucaille and G. Tiraby. 1984. Comparative study of cellulases and hemicellulases from four fungi: mesophiles *Trichoderma reesei* and *Penicillium* sp. and thermophiles *Thielavia terrestris* and *Sporotrichum cellulophilum*. Enzyme Microb. Technol. 6: 175–180.

16 Elbein, A.D., S. Adya and Y. Chuan Lee. 1977. Purification and properties of a β-mannosidase from *A. niger*. J. Biol. Chem. 252: 2026–2031.

17 Emi, S., J. Fukumoto and T. Yamamoto. 1972. Crystallization and some properties of mannanase. Agric. Biol. Chem. 36: 991–1001.

18 Eriksson, K.-E. and W. Rzedowski. 1969. Extracellular enzyme system utilized by the fungus *Chrysosporium lignorum* for breakdown of cellulose. 1. Studies on the enzyme production. Arch. Biochem. Biophys. 129: 683–688.

19 Eriksson, K.-E. and M. Winell. 1968. Purification and characterization of a fungal β-mannanase. Acta. Chem. Scand. 22: 1924–1934.

20 Fincher, G.B. and B.A. Stone. 1981. Metabolism of noncellulosic polysaccharides. In: Encyclopedia of Plant Physiology, New Series, Vol. 13 (Tanner, W. and F.A. Loewus, eds.), pp. 69–132, Springer-Verlag, Berlin.

21 Godfrey, T. 1983. Edible oils. In: Industrial Enzymology (Godfrey, T. and J. Reichelt, eds.), pp. 424–427, Nature Press, New York, NY.

22 Godfrey, T. 1983. Dextranase and sugar. In: Industrial Enzymology (Godfrey, T. and J. Reichelt, eds.), pp. 422–423, Nature Press, New York, NY.

23 Halmer, P., J.D. Bewley and T.A. Thorpe. 1975. An enzyme to degrade the lettuce endosperm cell wall during gibberellin and light-induced germination. Nature 258: 716–718.

24 Hashimoto, Y. and J. Fukumoto. 1969. Studies on the enzyme treatment of coffee beans. Purification of mannanase of *Rhizopus niveus* and its action on coffee mannan. Nippon Nogei Kagaku Kaishi 43: 317–322.

25 Ishihara, M. and K. Shimuzu. 1980. Hemicellulases of brown rotting fungus, *Tyromyces palustris*. Purification and some properties of an extracellular mannanase. Mokuzai Gakkaishi 26: 811–818.

26 Johnson, E.A., M. Sakajah, G. Halliwell, G. Media and A.L. Demain. 1982. Saccharification of complex cellulosic substrates by the cellulase system from *Clostridium thermocellum*. Appl. Environ. Microbiol. 43: 1125–1132.

27 Karimi, S. and O.P. Ward. 1989. Comparative study of some microbial arabinan degrading enzymes. J. Industr. Microbiol. 4: 173–180.

28 Kusakabe, I. and R. Takahashi. 1988. β-Mannanase of *Streptomyces*. Methods Enzymol. 160: 611–614.

29 Lee, Y.C. and V. Wacek. 1970. Galactosidase from *Aspergillus niger*. Arch. Biochem. Biophys. 138: 264–271.

30 Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall. 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193: 265–275.

31 Maloney, A.P., S.I. McCrae, T.M. Wood and M.P. Coughlan. 1985. Isolation and characterization of the 1,4-β-D-glucan glucanohydrolases of *Talaromyces emersonii*. Biochem. J. 2250: 365–374.

32 McCleary, B.V. 1988. β-D-Mannanases. Methods Enzymol. 160: 596–610.

33 McHale, A. and M.P. Coughlan. 1981. The cellulolytic system of *Talaromyces emersonii*. Purification and characterization of the extracellular and intracellular glucosidases. Biochim. Biophys. Acta, 662: 152–159.

34 McHale, A. and M.P. Coughlan. 1982. Properties of the β-glucosidases and *Talaromyces emersonii*. J. Gen. Microbiol. 128: 2327–2331.

35 Meghji, K., O.P. Ward and A. Araujo. 1990. Production, purification and properties of extracellular carboxyl esterases from *Bacillus subtilis* NRRL 365. Appl. Environ. Microbiol. 56: 3735–3740.

36 Meier, H. and J.S.G. Reid. 1981. Reserve polysaccharides other than starch in higher plants. Encyclopedia of plant physiology, New Series (Pirson, A. and M.H. Zimmermann, eds.), Vol. 13A, pp. 418–471, Springer-Verlag, Berlin.

37 Merril, C.R., D. Goldman, S.A. Sedman and M.H. Ebert. 1981. Ultrasensitive stain for proteins in polyacrylamide gels shows regional variation in cerebrospinal fluid proteins. Science 211: 1438–1438.

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

39 Ornstein, L. 1964. Disc electrophoresis – I Background and Theory. Ann. New York Acad. Sci. 121: 321–349.

40 Ratto, M. and K. Poutanen. 1988. Production of mannan-degrading enzymes. Biotechnol. Lett. 10: 661–664.

41 Reese, E.T. and Y. Shibata. 1965. β-Mannanases of fungi. Can. J. Microbiol. 11: 167–193.

42 Tsujisaki, Y., K. Hujama, S. Takenishi and J. Fukumoto. 1972. Studies on the hemicellulases. III. Purification and some properties of mannanases from *Aspergillus niger* van Tieghem sp. Nippon Nogei Kagaku Kaishi 46: 155–161.

43 Van Etten, H.D. and D.F. Bateman. 1969. Enzymatic degradation of galactan, galactomannan and xylan by *Sclerotium rolfsii*. Phytopathology 59: 968–972.

44 Wan, C.C., J.E. Muldrey, S.C. Li and Y.-T. Li. 1976. β-Mannosidase is also produced by the mushroom *Polyporus sulfureus*. J. Biol. Chem. 251: 4384–4388.

45 Ward, O.P. and M. Moo-Young. 1988. Thermostable enzymes. Biotechnol. Adv. 6, 39–69.

46 Ward, O.P. and M. Moo-Young. 1989. Enzymatic degradation of cell wall and related plant polysaccharides. In: Critical Reviews in Biotechnology (Stewart, G.G. and I. Russell, eds.), Vol. 8, pp. 237–274, CRC Press, Boca Raton, FL.

47 Yalpani, M. 1988. Polysaccharides. Studies in Organic Chemistry. 36. Elsevier, Amsterdam.