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Purification and some properties of the mannanases from *Thielavia terrestris*

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SUMMARY

Thielavia terrestris NRRL 8126 cell free supernatants contained mannanase and β-mannosidase when cultured on a complex media containing locust bean gum. Using acetone precipitation, SP-Sephadex C50 ion exchange chromatography and preparative gel electrophoresis, the crude enzyme was resolved into one β-D-mannosidase and four β-D-mannanase components. β-D-mannosidase had a specific activity of 0.02 (U/mg) on *p*-nitrophenyl-β-D-mannopyranoside substrate. Mannanase components M1, M2, M3 and M4 had specific activities of 28.2, 38.7, 52.8 and 4.17 (U/mg) respectively on purified locust bean galactomannan substrate. pH optima for the enzymes were in the range 4.5–5.5. Mannanase component M4 manifested the greatest thermostability, retaining full activity for 3 h at 60 °C. Molecular weights determined by SDS–PAGE were 72000 for β-mannosidase and 52000, 30000, 55000 and 89000 for M1, M2, M3 and M4 respectively. Carbohydrate contents of the enzymes ranged from 6–36%. Preliminary studies indicate that enzyme components hydrolyse the mannan substrate in a synergistic manner.

INTRODUCTION

The hemicelluloses of plant materials rank second only to cellulose in abundance as naturally occurring compounds [5]. They include a large group of galactomannans present in seeds of leguminous plants and the gluco- and galactogluco-mannans of coniferous woods [25,28]. Ivory nut and green coffee beans contain substantial levels of mannans [17]. Mannanases may be used in the production of coffee and have potential applications in the processing of other plant materials [10,23]. The most important microbial enzymes involved in the degradation of plant mannans are endo-1,4-D-mannanase (EC 3.2.1.78) and β-mannosidase (EC 3.2.1.25).

Microbial species known to produce mannanases include *Aeromonas hydrophile* [23], *Bacillus subtilis* [8], *Cellumonas* sp. [13], *Streptomyces olivochromogenes* [22], *Aspergillus* sp. [3,9,33], *Botrytis cinerea* [26], *Tyromyces palustris* [14], *Trichoderma* sp. [24] and *Rhizopus niveus* [12]. β-Mannosidase is produced by *A. awamori*, *A. niger* [32], *B. cinnerea* [26], *T. palustris* [14] and *Cellumonas* sp. [13]. A limited number of microbial mannanases have been purified and the mannanases from *T. palustris* [14] and *A. hydrophile* have each been separated into components.

In using hemicellulases for the processing of plant materials, operation at increased reaction temperatures is advantageous in reducing microbial infection of the material being processed, in increasing the solubility of the carbohydrate substrate and in accelerating the reaction rate [2,27,29]. Consequently, there is substantial interest in identifying hemicellulase enzymes having increased thermostability. The mould *Thielavia terrestris* has been shown to produce thermostable cellulases, xylanases and arabanases [7,15]. In this paper we report the purification and initial characterisation of five mannan degrading enzymes produced by *T. terrestris*.

MATERIALS AND METHODS

Microorganism. Thielavia terrestris NRRL 8126 was maintained on potato-dextrose agar. The culture was incubated at 45 °C for 4 days, stored at 5 °C and periodically subcultured.

Culture medium. Medium for enzyme production contained (g/l): locust bean gum (Sigma, St. Louis), 5; corn steep solids, 1; yeast extract, 0.1; $NaNO_3$, 2; KH_2PO_4, 2; $CaCl_2 \cdot 2H_2O$, 0.3; $MgSO_4 \cdot 7H_2O$, 0.3. In addition, the medium contained (ml/l) potato extract, 10 and trace metal solution [1], 1. pH of the medium was adjusted to 5.0. Potato extract was prepared by boiling 150 g diced potato in 250 ml water for 1 h. The extract was recovered by straining through cheesecloth and made up to 500 ml.

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Inoculum and culture conditions. For the inoculum culture development, 250-ml Erlenmeyer flasks containing 80 ml of the medium were used whereas 2-l Erlenmeyer flasks containing 300 ml of medium were used in the second culture stage. A two stage shake flask culture procedure was used to produce extracellular mannanase from *Thielavia terrestris* NRRL 8126. The inoculum development culture was loop inoculated and incubated for 36 h at 45 °C on an orbital incubator shaker set at 200 rpm. A 1% (v/v) vegetative inoculum from this 36 h culture was used to inoculate the second culture stage which was incubated under the same conditions for 48 h. Clarified cell free supernatants were recovered initially by removal of the mycelium using cheesecloth followed by centrifugation at 10000 × *g* for 10 min.

Enzyme assays. Mannanase was assayed using locust bean galactomannan as substrate. This was prepared by ethanol precipitation of an aqueous solution of locust bean gum followed by redissolving and freeze drying according to the method of Halmer et al. [11]. 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 solution. The reaction mixture was incubated at 50 °C for 30 min. Reducing sugars produced due to enzyme activity were determined by the dinitrosalicylic acid method of Miller [19]. 1 unit (U) of activity is the amount of enzyme producing 1 μmol of mannose reducing equivalents per min.

The assay mixture for β-mannosidase determination contained 0.5 ml of a 2 mM solution of *p*-nitrophenyl-β-D-nitrophenyl-β-D-mannopyranoside (Sigma) and 0.5 ml of enzyme solution. The reaction mixture was incubated at 50 °C for 30 min and was then stopped by addition of 2 ml of 2% (w/v) sodium carbonate solution. Released *p*-nitrophenol was determined by absorbance at 420 nm. 1 unit (U) of β-mannosidase is defined as the amount of enzyme producing 1 μmol of *p*-nitrophenol per min.

To determine the effect of pH on enzyme activity, assays were carried out in 0.05 M sodium citrate–0.1 M sodium phosphate buffer with pH varied.

In temperature optima determinations, the assay temperature was varied in the range 40–90 °C.

Protein and carbohydrate determination. Protein content of culture filtrates was determined using the method of Lowry et al. [16] with crystalline bovine serum albumin (Sigma, St. Louis) as a reference 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. Carbohydrate content of the purified enzymes was determined using the phenol sulphuric acid method.

Enzyme purification. Buffers: Buffer 1 contained 0.02 M sodium acetate, pH 5.6. Buffer 2 contained 0.05 M sodium acetate, pH 4.0.

Acetone precipitation: 1 volume of clarified culture filtrate at 4 °C was precipitated with 2 volumes of chilled acetone and allowed to stand for 2 h. The precipitated enzyme was separated by centrifugation at 10000 × *g* for 20 min. The enzyme was redissolved in Buffer 1 and concentrated using an Amicon cell containing a YM-10 membrane having a molecular weight cut off of 10000. The concentrated enzyme was then lyophilized.

Ion exchange chromatography: 140 mg of the lyophilized enzyme was dissolved in 4 ml of Buffer 2 and applied to a 2 × 27 cm SP-Sephadex C50 column. The enzyme was eluted using a linear NaCl gradient, 0–0.75 M in Buffer 2.

Electrophoresis: polyacrylamide gel electrophoresis (PAGE) was carried out at a 7.5% gel concentration according to the method of Ornstein [21] and Davis [4]. The gels were stained using the silver staining procedure described by Merril et al. [18]. Preparative PAGE was carried out in a modified Miniprotean II (BioRad) electrophoresis apparatus. The thickness of the gel was 2.4 mm. Four mg of desalted enzyme was charged and electrophoresis was carried out using Tris/glycine buffer at pH 8.3 with constant current of 15 mA, at 5 °C for about 4 h. The gel was then sliced to a thickness of 2.5 mm and broken by syringe passage.

Mannan hydrolysis. Coffee bean mannan was produced by first preparing holocellulose by the method of Whistler et al. [30] and then by isolation of mannan from the purified holocellulose by the method of Wolfrom et al. [31]. The digestion mixture contained mannan, 50 mg; enzyme solution, 1 ml; 0.1 M sodium acetate buffer, pH 5.6, 1 ml; H_2O, 1 ml. Digestion mixtures were incubated for 1 h at 50 °C and the reducing sugars were determined using the Nelson-Somogyi method [20]. Each enzyme solution contained a protein content of 26 μg/ml. The unfractionated enzyme consisted of the acetone precipitate, resuspended in 0.1 M sodium acetate buffer, pH 5.6 and passed through a Biogel-P10 column, 2 cm × 27 cm. The active enzyme fractions were pooled and used in the digestion procedure.

RESULTS

The culture supernatant contained 0.314 mg/ml protein and mannanase and β-mannosidase activities of 6.7 U/ml and 0.001 U/ml respectively. The initial purification steps involved precipitation with chilled acetone, resuspension, dialysis and lyophilisation. The precipitate was redissolved in Buffer 2 and fractionated using a SP-Sephadex C50 column. The elution profile is illustrated in Fig. 1. The void volume peak (fractions 6–12) and the major enzyme peak (fractions 56–62) recovered by

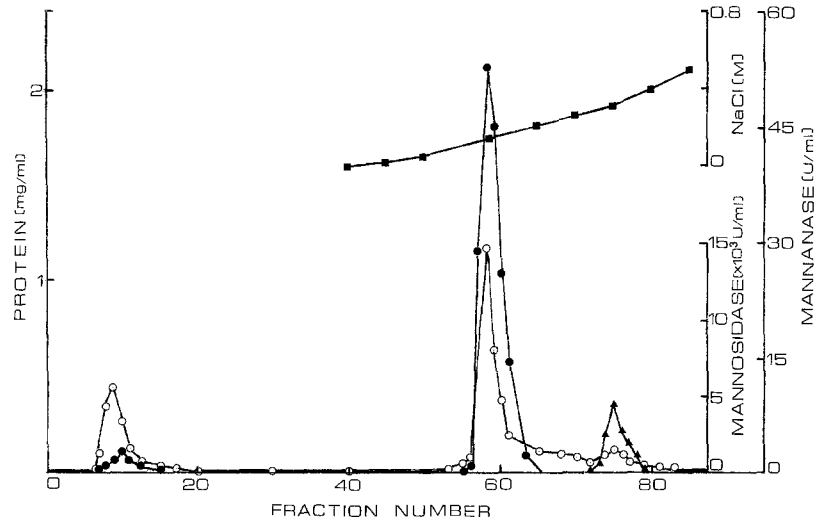

Fig. 1. Enzyme elution profile with NaCl gradient from SP-Sephadex C50 column. ●, Mannanase; ▲, β-Mannosidase; ○, Protein; ■, NaCl.

gradient elution contained mannanase activity with no associated β-mannosidase. The second peak (fractions 72–78) eluted from the SP-Sephadex column contained β-mannosidase activity. The β-mannosidase fraction exhibited a single protein band on polyacrylamide gel electrophoresis (PAGE). The minor and major mannanase peaks contained two and three protein bands when separated on PAGE. Both peaks were subsequently separated by preparative gel electrophoresis. Electrophoretic separation resulted in production of one mannanase activity band from the minor peak, purified to homogeneity. Electrophoresis of the major peak resulted in isolation of three mannanase components as illustrated in Fig. 2. The purity of the β-mannosidase fraction and the four mannanase components, designated M1–M4, are illustrated in Fig. 3. A summary of the purification scheme is presented in Table 1.

The pH activity profiles of the β-mannosidase and four mannanase components are illustrated in Fig. 4. pH optima values for β-mannosidase and mannanase components M1, M2, M3 and M4 were 5.0, 4.5, 5.5, 5.0 and 5.5 respectively. In order to determine temperature optima for activity, the enzyme assay was carried out at a range of temperatures between 40 and 90 °C. Temperature optima for β-mannosidase, M1, M2, M3 and M4 were 65, 70, 75 and 75 °C, respectively. The comparative thermostabilities of the enzymes were determined by incubation at a range of temperatures (50–80 °C) for up to 3 h. Percentage residual activities of mannanases and β-mannosidase are indicated in Figs. 5 and 6. Mannanase component M4 manifested the greatest thermostability, retaining full activity for 3 h at 60 °C. All the other

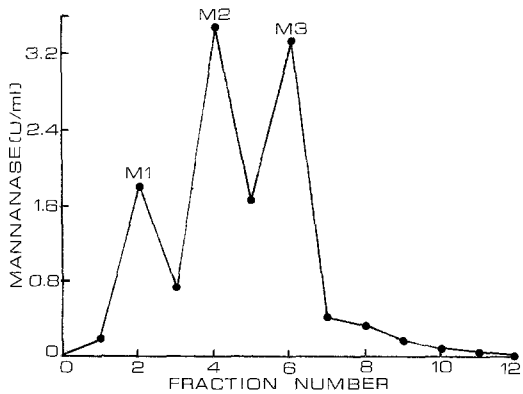

Fig. 2. Preparative electrophoresis of the major SP-Sephadex mannanase peak. ●, Mannanase.

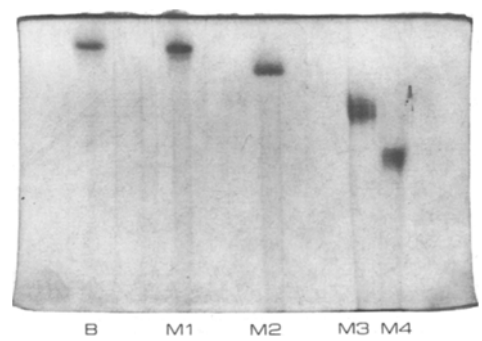

Fig. 3. Demonstration of homogeneity of the five enzyme components by PAGE. B, β-Mannosidase; M1–M4, Mannanase components.

TABLE 1

Summary of enzyme purification

Fraction	Mannanase			β-Mannosidase	
	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Total activity (U)	Specific activity (U/mg)
Clarified culture filtrate	390.0	8308.0	21.3	1.30	0.002
Acetone precipitate	163.8	6861.6	41.8	1.20	0.007
SP-Sephadex C50					
peak A	9.3	35.3	3.8		
peak B	10.6	597.7	56.6		
peak C	1.3	0.3	0.3	0.03	0.026
Preparative electrophoresis					
from peak B M1	1.8	50.8	28.2		
from peak B M2	2.3	89.0	38.7		
from peak B M3	1.7	87.1	52.8		
from peak A M4	4.3	17.9	4.2		

enzymes retained 100% residual activity at 50 °C. Each of the enzyme components was fully or almost fully denatured after 1 h at 80 °C.

Molecular weights were determined by SDS–PAGE electrophoresis using a series of molecular weight markers (phosphorylase *b*, 97400; bovine serum albumin, 66.000; ovalbumin, 42700; carbonic anhydrase; 31000; soybean trypsin inhibitor, 21500; lysozyme, 14400; all from BioRad). Carbohydrate content of the purified enzymes was also determined. The molecular weight and carbohydrate compositions of the five enzymes are presented in Table 2.

The five purified enzymes were tested individually and

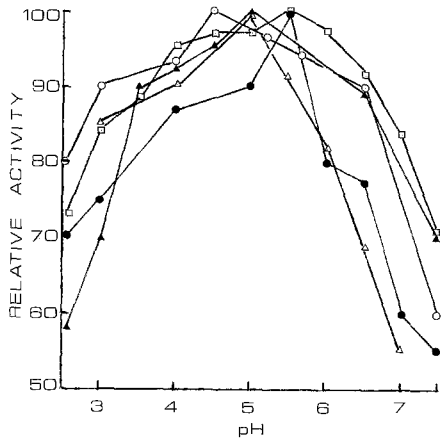

Fig. 4. Effect of pH on activity of the five enzyme components. ▲, β-Mannosidase; ○, M1; ●, M2; △, M3; □, M4.

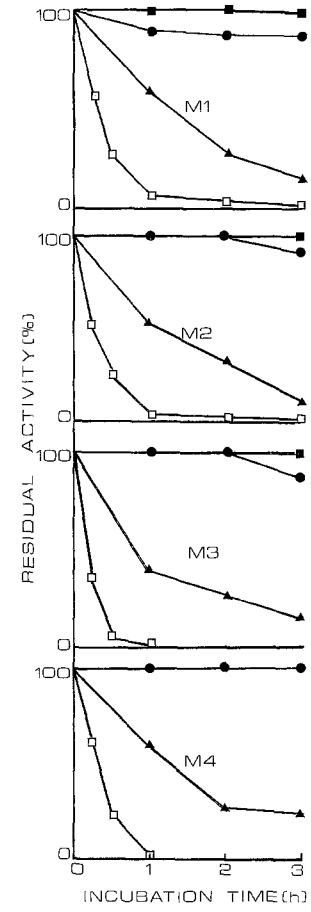

Fig. 5. Effect of temperature on stabilities of the mannanase components. ■, 50 °C; ●, 60 °C; ▲, 70 °C; □, 80 °C.

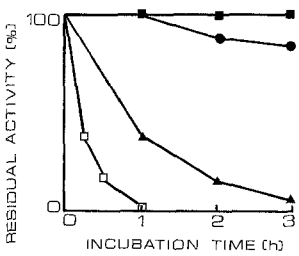

Fig. 6. Effect of temperature on stability of β-mannosidase. ■, 50 °C; ●, 60 °C; ▲, 70 °C; □, 80 °C.

in a wide range of combinations for effectiveness in hydrolysing mannan from coffee beans and compared with the unfractionated enzyme preparation. Each enzyme reaction contained the same total added enzyme protein content. The data for the unfractionated enzyme preparation, the individual components and the most effective combination of components are presented in Table 3. This preliminary study illustrates the synergistic effect of the individual components in hydrolysis of the mannan substrate.

TABLE 2

Molecular weights and carbohydrate contents of the enzyme components

Enzyme component	Molecular weight	Carbohydrate content (%)
β-Mannosidase	72000	9
Mannanase		
M1	52000	18
M2	30000	12
M3	55000	6
M4	89000	37

DISCUSSION

A distinctive feature of microbial cellulases and hemicellulases is the number of enzyme components isolated which degrade the substrate. The crude mannanase produced by the brown rotting fungus, *T. palustris*, contained at least two mannanase components of different molecular weights [14] while, as observed above, *T. terrestris* produces at least five mannan degrading enzymes.

pH optima for the β-mannosidase and the four mannanase components were in the range 4.5–5.5. pH optima for other fungal mannanases ranged from 3–4.0 in the case of *Aspergillus* enzymes to 5.5 for the mannanase from *Rhizopus niveus* [20]. β-D-mannanase from *B. subtilis* had a higher pH optimum at 6.0. The β-mannosidase produced by *A. niger* had a pH optimum of 4.5 [32].

Temperature optima of most mannanases are quoted to be in the range 40–65 °C. Purified D-mannanase from *B. subtilis* [8] and β-mannosidase from *A. niger* had temperature optima at 60 °C [32]. The β-mannosidase of *T. terrestris* had a temperature optimum of 65 °C whereas temperature optima of the mannanase components M1 and M2–M4 were 70 and 75 °C respectively. One of the mannanase components of *T. palustris* was also characterised as having a temperature optimum of 75 °C.

It was noted that the molecular weights of the mannanase components of *T. terrestris* ranged from 30000 to 89000 and that carbohydrate contents ranged from 6.4 to 36.7%. The component with highest thermostability, M4, had the highest carbohydrate content. Molecular weights of some other fungal mannanases ranged from 37000 to 61000 [6].

Synergism was observed in the preliminary study of

TABLE 3

Hydrolysis of mannan by enzyme components

Enzyme components (% based on protein content)						Mannose reducing equivalents produced μmol
Unfractionated enzyme	Manannases				β-Mannosidase	
	M1	M2	M3	M4		
100	–	–	–	–	–	14.3
–	100	–	–	–	–	7.7
–	–	100	–	–	–	9.0
–	–	–	100	–	–	9.5
–	–	–	–	100	–	2.1
–	–	–	–	–	100	3.4
–	18	26	36	10	10	14.0

the effectiveness of the purified components to degrade mannan. The patterns of action of the five component enzymes in the degradation of mannan will now be more fully characterised.

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