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ORIGINAL PAPER

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Evaluation of an endo-β-mannanase produced by *Streptomyces ipomoea* CECT 3341 for the biobleaching of pine kraft pulps

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Abstract An endo-β-mannanase (EC 3.2.1.78) from *Streptomyces ipomoea* CECT 3341 was purified and applied to the biobleaching of pine kraft pulps. The maximum level of endo-β-mannanase activity (0.6 units ml^{-1}) was achieved after 4 days of growth in a medium containing locust bean gum and yeast extract. Zymograms revealed mannanase bands (Man) with high and low electrophoretic mobility on the second and seventh days of incubation (Man1, Man3) and three bands of high, medium and low mobility from the third to sixth days of growth (Man1, Man2, Man3). Although these exhibited different molecular masses, their amino-terminal sequences were identical. The action of proteases detected in the culture supernatant could be responsible for such events, suggesting that only one endo-β-mannanase is produced by *S. ipomoea*. The purified Man3 exhibited a molecular mass of 40 kDa, an isoelectric point of 4.0 and an optimal temperature and pH reaction of 55 °C and 7.5, respectively. It was strongly inhibited by Ag^{+}, Hg^{2+}, Al^{3+} and Fe^{3+}, and was strongly activated by Mn^{2+}. The ability of the purified endo-β-mannanase to improve the bleachability of pine kraft pulp, when applied with alkaline extraction, was demonstrated by an increase in the pulp brightness (1.7%, using the International Standards Organisation's test) and an absence of variations in the viscosity values. A relationship between the increase in pulp brightness and the presence of manganese in the pulps could be established.

Introduction

Kraft pulping of wood is the most widespread industrial process used for paper production (Fengel and Wegener 1984). Through this process, most of the lignin of the substrate is removed. Prior to obtaining paper, multistage bleaching is used to remove the residual lignin. During the past few years, more environmentally friendly chemical and biological methods have replaced the traditional use of elemental chlorine in the bleaching of pulps (Malinen and Fuhrmann 1995; McDonough 1995). Enzyme-aided pretreatments increase the final paper brightness, while reducing chemical costs and adsorbable organic halogen levels (Skerker et al. 1992). One of the most feasible alternatives is the pretreatment of pulps with hemicellulases, such as xylanases and mannanases, prior to the chemical bleaching. This enzymatic step partially removes the re-precipitated xylan and/or mannan from the surfaces of kraft fibres, which facilitate subsequent removal of lignin (Suurnäkki et al. 1997). Although most attention has been towards the use of xylanases in biobleaching, mannanases were recently shown to exceed the xylanase effect in enhancing the bleachability of modified continuous-cooking pulps (Suurnäkki et al. 1996).

Glucomannan is one of the major hemicelluloses of softwood pulps. The main enzyme needed for depolymerisation of this polymer is endo-1,4-β-D-mannanase (EC 3.2.1.78), which attacks the backbone of the polysaccharide, resulting in short-chain oligosaccharides. Endo-1,4-D-β-mannanases are widely distributed in micro-organisms, plants and animals (Flari et al. 1995; Hossain et al. 1996; Nonogaki et al. 1995; Stälbrand et al. 1993).

We have previously reported the selection of the strain *Streptomyces ipomoea* CECT 3341 as a producer of high levels of β-mannanase activity in a medium containing locust bean gum as inducer (Montiel et al. 1999). We also described the potential effectiveness of the crude extract produced by this micro-organism on the biobleaching of pine kraft pulp. The aim of this work is to determine the contribution of the purified β-mannanase produced by *S. ipomoea* to the bleaching of such kraft pulp. Consequently, a full study including production, purification and characterisation of the extracellular activity has been performed.

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Materials and methods

Micro-organism and growth conditions

S. ipomoea CECT 3341 was maintained as a suspension of spores and hyphal fragments in 20% glycerol at –20 °C. The strain was routinely grown on GAE agar medium plates containing (per litre): 10 g glucose, 1 g L-asparagine, 0.5 g yeast extract, 0.5 g K_2HPO_4, 0.5 g $MgSO_4 \cdot H_2O$ and 0.01 g $FeSO_4 \cdot H_2O$ (Hernández et al. 1994) at 28 °C until sporulation occurred (4–6 days). For enzyme production, mineral basal (MBS) medium (Crawford 1978) supplemented with galactomannan 1% (w/v) and yeast extract 0.2% (w/v) was used. Erlenmeyer flasks (100 ml) containing 20 ml of this medium were inoculated with 1 ml of a standard spore suspension (10^7 colony-forming units ml^{-1}) harvested from sporulated plates with 0.01% Tween 80. Cultures were incubated for 7 days at 28 °C under shaking conditions at 200 rpm. Flasks were removed daily during growth and filtered through Whatman No. 1 filter paper. Cell-free supernatants were used as crude extracellular enzyme preparation for enzyme assay, protein determination and zymogram analysis.

Mannanase assays and protein determination

Mannanase activity was assayed by estimation of released reducing sugars using 0.5% (w/v) locust bean gum as substrate in 70 mM phosphate buffer, pH 7.5. The reaction mixture (25 µl culture supernatant, 25 µl substrate solution) was maintained at 55 °C for 10 min and the reducing sugars were determined by the bicinchoninate/Cu^{2+} method (Copa-Patiño et al. 1993). One unit (U) of mannanase activity was defined as the amount of enzyme producing 1 µmol of mannose min^{-1}. Solubilisation of galactomannan by the purified enzyme was monitored using a turbidimetric procedure (Wang and Broda 1992). One unit of mannan solubilisation activity was defined as the amount of enzyme that solubilised 1 mg of locust bean gum suspension min^{-1}.

Extracellular protein concentration was estimated by the method of Bradford (1976). Bovine serum albumin was used to prepare the standard curve.

Electrophoretic analysis

Culture supernatants were obtained daily and native PAGE was used to separate the extracellular proteins. Gels were developed as zymograms for mannanase and protease activities. After electrophoresis, gels were overlaid on a 1.5% (w/v) agarose gel sheet (10 mm thick) containing 0.1% (w/v) galactomannan or 1% (w/v) gelatine, respectively, in 70 mM phosphate buffer (pH 7.5). In both cases, PAGE and agarose gel sheets were placed on a glass plate and incubated for 15–30 min at 45 °C. The PAGE sheets were torn from the agarose gel sheets and proteins remaining in the gels were stained with Coomassie brilliant blue R-250 (Sigma). To stain mannanase, the agarose gel sheet was dipped into 0.05% (w/v) Congo red (Sigma) solution and stained for 15 min. The Congo red was poured off and the gel sheet was washed with 1 M NaCl, until excess stain was removed. To stain protease, the agarose gel sheet was dipped into Frazier solution (15 g $HgCl_2$, 20 ml HCl, 80 ml distilled water) for 15 min and then stained with Coomassie brilliant blue R-250.

Alongside the purification process, SDS-PAGE analysis was also carried out. Protein bands were stained with Coomassie brilliant blue R-250 or silver stain (Bio-Rad). A low-molecular-weight calibration kit (Pharmacia) was used as standard.

Isoelectric focusing (IEF) was carried out in a Bio-Rad mini-IEF cell system, according to the manufacturer's instructions, at pH 3–9. Marker proteins in this pH range (pH 3.5–9.3: Pharmacia, pH 4.5–9.6: Bio-Rad, pH 3.6–6.9: Bio-Rad) were used as standards.

Purification of β-mannanase

S. ipomoea CECT 3341 was cultivated in 2-l Erlenmeyer flasks containing 400 ml of MBS medium. Flasks were incubated at 28 °C under shaking conditions (200 rpm) for 4 days. Cultures were centrifuged (4,000 *g*, 30 min) and supernatants were concentrated in a Christ Alpha 1-4 freeze-dryer (B. Braun-Biotech) and then ten-fold concentrated by re-dissolution in 70 mM phosphate buffer, pH 7.0.

Liquid chromatography was carried out in a GP-250 plus fast performance liquid chromatographic system (Pharmacia). Aliquots (2 ml) of concentrated enzyme preparation were first applied to an anion exchange column (Econo-Pac Q cartridge, Bio-Rad). Mannanase-active fractions were pooled, concentrated and applied to a Mono Q HR 5/5 column (0.5×5 cm; Pharmacia). In all cases, columns were equilibrated with 70 mM phosphate buffer (pH 7.0) and the adsorbed proteins were eluted with the same buffer, using a discontinuous NaCl gradient (0–1 M) at a flow rate of 1 ml min^{-1}. Chromatograms were obtained by monitoring the absorbance at 280 nm. β-Mannanase activity and proteins were assayed in all collected fractions (1 ml).

Throughout the purification process, fractions were analysed by native and denaturing PAGE, as described above.

Enzyme characterisation

Estimates, both of optimal temperature and pH, and of thermal and pH stability of purified mannanase (4.8 µg ml^{-1}) were performed over a temperature range of 30–90 °C and a pH range of 3.0–9.0. Michaelis–Menten kinetic parameters, K_m and V_{max}, for the purified mannanase were determined at 55 °C in 70 mM phosphate buffer (pH 7.5), using galactomannan as substrate, in a range from 0.025% to 1.25% (w/v). The effect of metal ions (Na^+, K^+, Ag^+, Mg^{2+}, Ca^{2+}, Mn^{2+}, Cu^{2+}, Zn^{2+}, Hg^{2+}, Ba^{2+}, Fe^{3+}, Al^{3+}), EDTA and phenylmethylsulfonyl fluoride, all at a concentration of 1 mM, was tested. All assays were performed at optimal pH and temperature under standard enzyme assay conditions.

To determine the β-mannosidase activity of the purified enzyme, 20 mM *p*-nitrophenyl-β-D-mannopyranoside in 70 mM phosphate buffer (pH 7.5) was used as substrate, as described by Rättö and Poutanen (1988).

N-Terminal sequence determination

After SDS-PAGE electrophoresis, proteins on denaturing PAGE sheets of purified mannanase were transferred to a polyvinylidene difluoride (PVDF) membrane (PVDF-Immobilon-P, 0.45 µm; Millipore), using a Trans-Blot electrophoretic transfer cell system (Bio-Rad). The band, after staining with Coomassie brilliant blue R-250, was excised and subjected to N-terminal amino acid sequence analysis, using the degradation method (Edman 1950) in a Procise protein sequencer (Applied Biosystems), according to the manufacturer's instructions.

Processing experiments

Aliquots (100 µl) of the supernatant from the seventh day of incubation were mixed with aliquots (100 µl) from either the second or fourth day. The mixtures were incubated at 37 °C for 1 h and 24 h. The samples were freeze-dried and re-suspended in the electrophoresis buffer and zymogram analysis for mannanase activity was carried out.

Application of purified mannanase to the biobleaching of pine kraft pulps

The effect of the purified mannanase and the effect of enzyme treatment followed by alkaline extraction on the biobleaching of a pine kraft pulp (kindly supplied by Prof. Liisa Viikari, VTT, Finland) were evaluated. Enzyme treatment of pine kraft pulp

and further alkaline extraction of control and treated pulps were carried out as described by Montiel and co-workers (1999). Kappa number [International Standards Organisation test (ISO) 302-1981 E], viscosity (ISO 5351/1-1981 E) and brightness (ISO 2469) of pulps were determined by ENCE Industry (Pontevedra, Spain). All incubations and assays were performed in triplicate. Results are the means of three replicates, with standard errors where appropriate.

Determination of manganese in the pine kraft pulp

Manganese determination was carried out with a model 2380 atomic absorption spectrophotometer (Perkin Elmer) equipped with a model 561 register and multi-element hollow cathode lamps. The working conditions were as described by González et al. (1988).

Results

Production of mannanases and zymogram analysis

The time course of the production of β-mannanases from *S. ipomoea* in a medium containing galactomannan as inducer of activity was studied. Maximum mannanase activity (0.6 U ml^{-1}) was achieved after 4 days of incubation. Through a zymogram technique, different patterns of bands were detected along the incubation time-line. Three bands of mannanase activity, named Man1, Man2, and Man3 were identified by their different electrophoretic mobility. Whereas single bands of Man1 or Man3 were detected at the second and seventh days of incubation, respectively, Man1, Man2 and Man3 were visualised from the third to the sixth day (Fig. 1).

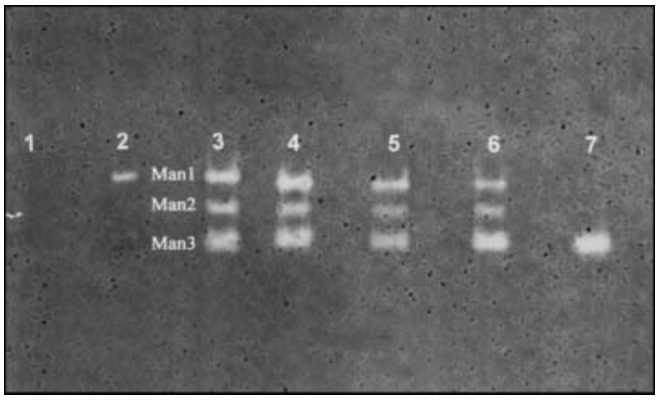

Fig. 1 Zymographic analysis of mannanase (*Man1–3*) activities produced by *Streptomyces ipomoea* after different days of incubation. *Lanes 1–7* correspond to the total days of incubation

Purified mannanase properties and N-terminal amino acid sequencing

Mannanase activities from the concentrated supernatants were purified by anion-exchange chromatography, as described in Materials and methods.

At the end of the purification process, an enzyme preparation of purified Man3 (Fig. 2) was obtained with a specific activity of 56 U mg^{-1} protein, which corresponds to a final yield of 13%. Also, Man1 and Man2 were partially purified (data not shown).

The molecular mass of the purified enzyme (Man3) was 40 kDa and its isoelectric point was 4.0. The optimum pH for this enzyme was 7.5 and it was stable at pH 7.5–8.0. The optimum temperature for the purified enzyme was 55 °C. The enzyme retained up to 60% of its activity after 120 min of incubation and more than 50% of activity after 30 min at 80 °C in the pH range 7.5–8.0. Mannanase activity was inhibited by Ag^{+}, Al^{3+}, Fe^{3+} and Hg^{2+}. In contrast, the addition of Mn^{2+} resulted in a significant activation of the enzyme, up to 200% compared with the initial activity.

The kinetic constants of the purified β-mannanase were determined under the optimal assay conditions. The K_m value of the purified enzyme (Man3) was 3.4 mg ml^{-1} and the V_{max} was 55.7 U mg^{-1} of protein. The physiological efficiency (V_{max}/K_m) was 14.8. The purified enzyme demonstrated its ability to solubilise galactomannan (35% in 10 min). β-Mannosidase activity was not detected in the purified mannanase, Man3.

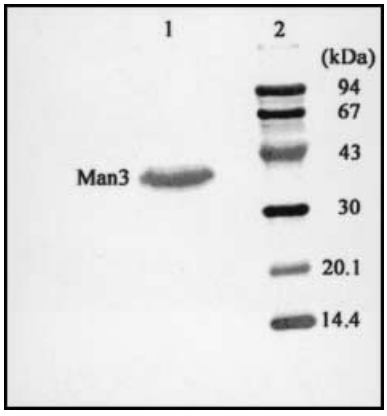

Fig. 2 Protein bands visualised in the polyvinylidene difluoride Immobilon membrane after SDS-PAGE. *Lane 1* Mannanase (*Man3*; 4.8 μg ml^{-1}) purified by an Econo Pac-Q anionic cartridge, followed by Mono Q HR5/5 column, both coupled to a fast performance liquid chromatography system. *Lane 2* Molecular mass markers

10
A G G I H V S D G R V V E G N *S. ipomoea* CECT 3341
A A G G I H V S N G R V V E G N *S. lividans* 66
10

Fig. 3 Comparison between N-terminal amino acid sequence of β-mannanases from *S. ipomoea* CECT 3341 and *S. lividans* 66

The purified enzyme (Man3) and both partially purified Man1 and Man2 mannanases were subjected to N-terminal amino acid sequence analysis. The N-terminal sequences of the three mannanases displayed 100% homology. The deduced sequence was compared with that corresponding to mannanase from *S. lividans* 66 (Arcand et al. 1993) and showed a 93% homology (Fig. 3).

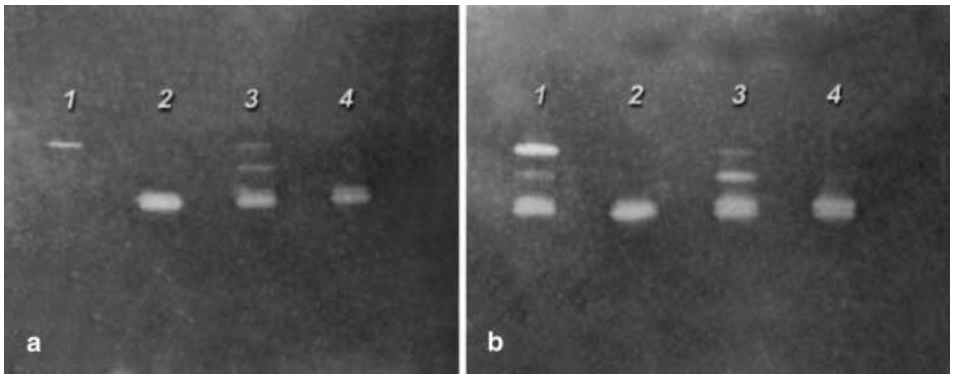

Fig. 4a, b Zymographic analysis of the extracellular processing of Man1 mannanase activity obtained after mixing active supernatants from the second and seventh days (**a**) and from the fourth and seventh days of incubation (**b**). *Lanes 1 and 2* Controls, *lane 3* reaction mixture after 1 h, *lane 4* reaction mixture after 24 h. See text for details

Table 1 Kappa number, brightness and viscosity of pine kraft pulps after enzymatic treatment (*ET*) and after enzymatic treatment followed by alkaline extraction (*ET+AE*). All values are the means of three determinations ± standard error. *ISO* International Standards Organisation test

	Control	ET	Control +AE	ET+AE
Kappa number	23.3±0.15	23.2±0.34	21.0±0.2	20.7±0.4
Brightness (%, by ISO)	31.8±0.26	31.9±0.35	32.4±0.6	34.1±0.2
Viscosity (mg ml^{-1})	1029±45	1036±51	1034±36	1044±48

Processing of mannanase Man1

In order to know whether Man2 and Man3 mannanases are derived from Man1, aliquots of the supernatant from the seventh day of incubation were mixed with aliquots from either the second or fourth day. After 1 h of incubation of the supernatants of the second day (Fig. 4a, lane 1) and seventh day (Fig. 4a, lane 2), an activity band corresponding to Man2 was observed, concomitant with a decrease in the intensity of Man1 (Fig. 4a, lane 3). In the same way, an increase in the activity band corresponding to Man2 was detected in parallel with a remarkable decrease in the intensity of the Man1 band (Fig. 4b, lane 3) when aliquots from the fourth day (Fig. 4b, lane 1) and seventh day (Fig. 4b, lane 2) supernatants were mixed. In both experiments, after 24 h of incubation, only a single band of mannanase activity, corresponding to Man3, was visualised (Fig. 4a, b, lane 4). In addition, at least three bands of protease activity could be detected when zymogram analysis for this activity was performed (data not shown).

Application of the purified mannanase to biobleaching of a pine kraft pulp

The softwood kraft pulp, containing 113 µg Mn g^{-1} pulp, was treated with the purified mannanase from *S. ipomoea*. In addition, enzyme treatment followed by alkaline extraction of kraft pulp was also undertaken. Following the enzymatic treatment, the physical properties of the pine kraft pulps, both with and without alkaline extraction, were examined and the results are shown in Table 1. A slight reduction in kappa number (from 21.0 units to 20.7 units) and an important increase in brightness (1.7 ISO units) were obtained after enzyme treatment plus alkaline extraction. No appreciable differences in viscosity between control and treated pulps were detected.

Discussion

The present work was initiated with the study of the production kinetics of β-mannanase activity from *S. ipomoea* and its analysis using zymograms. Three different bands of β-mannanase activity (Man1, Man2, Man3) were visualised along the time course of growth. Although this fact is not uncommon among micro-organisms (Araujo and Ward 1990; Gübitz et al. 1996), it is important to note that the different patterns of bands detected in the culture supernatants of *S. ipomoea* differed along the incubation time-line.

The purification procedure for Man3 resulted in a tenfold purification and a yield of 13%, which are comparable with data reported for the mannanase from *S. lividans* 66 (Arcand et al. 1993). The molecular mass (40 kDa) and the isoelectric point (4.0) were similar to those of other bacterial and fungal mannanases (Ademark et al. 1998; Arcand et al. 1993; Mendoza et al. 1994; Stälbrand et al. 1993; Tamaru et al. 1995).

Results obtained about the stability of Man3 to high temperature can be considered industrially advantageous, when compared with mannanases produced by other streptomycetes such as *S. lividans* 66 (Arcand et al. 1993). In fact, high stability of mannanases at high temperature and alkaline conditions is an important requirement for their application in the bleaching of kraft pulps (Suurnäkki et al. 1997).

The ability to solubilise galactomannan, as showed by the purified mannanase, joined with the absence of β-mannosidase activity would suggest the *endo*- character of this enzyme.

The enzyme activity was strongly inhibited by Ag^{+}, Al^{3+}, Fe^{3+} and Hg^{2+}, as described for mannanases from different origin (Tamaru et al. 1995). Moreover, the enzyme activity was strongly activated by Mn^{2+}. To our

knowledge, there are no reports about the activation of mannanases by this ion. The N-terminal amino-acid sequence of the enzyme (Man3) shared 93% identity with that from *S. lividans* 66 (Arcand et al. 1993). Because of this high homology and its similarity in physico-chemical characteristics, the mannanase produced by *S. ipomoea* could be included in the glycosyl hydrolase family 5. In order to confirm this hypothesis, genetic and crystallographic studies are in progress.

As deduced from the N-terminal amino-acid sequence, the first 15 amino acids of all three mannanases (Man1, Man2, Man3) are identical, although their molecular masses are different. The heterogeneity in the molecular mass of these proteins could be the result either of a different degree of glycosylation or a proteolytic cleavage (Hilge et al. 1996; Stoll et al. 1999). From our results, it could be inferred that Man2 and Man3 are the products of a proteolytic processing of Man1. In fact, a unique single band corresponding to Man3 was visualised after mixing aliquots of the supernatants from the second and seventh days, and from the fourth and seventh days of incubation, probably due to the extracellular proteases secreted by the micro-organism. A similar behaviour was recently described for the mannan-degrading system of *Cellulomonas fimi*. In this system, the multiple bands detected on the zymogram were the result of proteolytic cleavage of a mature protein (Stoll et al. 1999).

Preliminary results of the bleaching of pine kraft pulp with the crude extract produced by this micro-organism showed the suitability of this crude material to improve the quality of pulp (Montiel et al. 1999). Taking into account that, in this extract, both mannanase and xylanase activities (ratio 4:1) were present (Montiel et al. 1999), the increase in the bleachability of the pulps could not be strictly attributed to mannanase. Thus, in this work, the effect was evaluated of the purified Man3, followed by an alkaline extraction. According to the results, the enzymatic treatment by itself did not show any effect on the bleaching of pulp. Moreover, no significant decrease in kappa number was observed after enzymatic treatment followed by alkaline extraction, in contrast with the result obtained when crude extract was used. From this result, it can be concluded that most of the decrease in kappa number is due to the xylanase activity in the crude extract. However, Man3 produced a remarkable effect on the pulp brightness, with an increase of 1.7% ISO units. This was probably due to the profound influence of the multivalent counter-ions of carboxylic groups in the effectivity of the xylanases in pulp treatment (Buchert and Viikari 1995). Although this effect was not demonstrated with mannanases, the enhancement of brightness obtained with Man3 could be a consequence of the presence of multivalent ions in pulps. According to this, the enhancement of the brightness observed after the enzymatic treatment of the pine kraft pulp could be due to the presence of manganese detected in the pulps (113 μg Mn g^{-1}). In fact, Mn^{2+} was demonstrated to increase the mannanase activity produced by *S. ipomoea*, by approximately 200%.

Finally, another factor that makes Man3 suitable for bleaching is the lack of appreciable change in the pulp viscosity.

The positive qualities showed by the mannanase produced by *S. ipomoea* CECT 3341 open new possibilities for the application of streptomycete enzymes in the pulp and paper industry.

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