Properties of Selected Hemicellulases of a Multi-Enzymatic System from Penicillium funiculosum

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A multi-enzymatic system from Penicillium funiculosum displayed α-L-arabinofuranosidase, endo-1,4-β-D-xylanase, β-D-xylosidase and endo-1,3-1,4-β-D-glucanase activities at high levels over a wide acidic pH range of 2.0 to 5.5. Moreover, the pH stability was particularly extended over the wide range of pH of 2.0 to 8.0 with endo-1,3-1,4-β-D-glucanase and endo-1,4-β-D-xylanase; however, α-L-arabinofuranosidase and β-D-xylosidase exhibited higher stability in the pH range of 2.0 to 5.5. The results indicate that the optimal temperature of α-L-arabinofuranosidase (65°C) and β-D-xylosidase (70°C) as well as their thermal stability were higher than those of endo-1,3-1,4-β-D-glucanase (60°C) and endo-1,4-β-D-xylanase (50°C). Although $V_{max}$ of β-D-xylosidase and endo-1,4-β-D-xylanase was higher than that of α-L-arabinofuranosidase and endo-1,3-1,4-β-D-glucanase, respectively, their catalytic efficiency was lower. High levels of feruloyl esterase, α-D-galactosidase, β-D-mannosidase and endo-1,4-β-D-mannanase activities were also detected in the multi-enzymatic system. The overall features of the multi-enzymatic system from P. funiculosum reveal its potential for degrading and modifying plant cell walls from a variety of food and feedstuffs.

Key words: multi-hemicellulolytic system; Penicillium funiculosum; endo-1,4-β-D-xylanase; β-D-xylosidase; endo-1,3-1,4-β-D-glucanase

The plant cell wall contains a variety of complex polysaccharide polymer, which are cross-linked and form compact structures. The major components of the plant cell wall consist of celluloses, hemicelluloses and lignins, with celluloses being the most abundant component, followed by hemicelluloses. Unlike celluloses, hemicelluloses are more complex and have a heterogeneous composition of various sugar units; indeed, they include xylan consisting of β-1,4-linked xylose units, glucuronomannans composed of β-1,4-linked glucose and mannose units, and arabinans and galactans in which the respective main chain sugars include arabinose and galactose. Due to the heterogeneous nature of hemicelluloses, they require for their degradation and modification a complex mixture of enzymes, collectively called hemicellulases, such as endo-xylanases, β-xylosidases, endoglucanases, endomannanases, β-mannosidases, α-arabinofuranosidases and α-galactosidases. The efficiency of the synergistic or combined action of hemicellulases has been reported to be higher than that of their individual actions.

Some cellulolytic bacteria and filamentous fungi produce a set of cellulases and hemicellulases which synergistically hydrolyze crystalline celluloses and hemicelluloses to smaller oligosaccharides and subsequently to monomeric sugars upon the cleavage action of exo-enzymes. However, most filamentous fungi such as Trichoderma and Aspergillus strains are more efficient producers of cellulases than hemicellulases. Penicillium species have shown the ability to produce high cellulase and hemicellulase sets, but much less is known about their properties than those from other fungi. Endoxylanases are the most characterized hemicellulases because their substrates, xylans, constitute the largest proportion of hemicelluloses in plants. In particular, Aspergillus and Trichoderma have been the most extensively studied as xylanase-producing fungi.

In our previous study, selected cellulases that were needed for the complete hydrolysis of the native cellulose to glucose were identified in the multi-enzymatic system produced by P. funiculosum, and their catalytic properties were characterized. The overall objective of the present study is the characterization, in terms of pH, temperature, thermal stability and other kinetic properties, of selected hemicellulolytic activities expressed in the multi-enzymatic system from P. funiculosum, including endo-1,3-1,4-β-D-glucanase, endo-1,4-β-D-xylanase, α-L-arabinofuranosidase and β-D-xylosidase activities. The catalytic efficiency of feruloyl esterase and other selected mannanolytic activities is also investigated. The overall study is useful in understanding the mode of action of the hemicellulolytic system produced by P. funiculosum and in assessing its practical value.

Materials and Methods

Materials. Birchwood xylan, p-nitrophenyl-β-D-xylloside, p-nitrophenyl-α-L-arabinofuranoside, p-nitrophenyl-β-D-mannoside, p-nitrophenyl-α-D-galactoside, methyl ferulate, glucose, xylose, mannose and...
Enzymatic assays. Endo-1,4-β-D-xylanase, endo-1,3,1,4-β-D-glucanase and endo-1,4-β-D-mannanase activities were respectively assayed by using birchwood xylan (1%, w/v), barley β-glucan (1% w/v) and guar galactomannan (1%, w/v) as substrates, according to a modification of the method described by Bailey et al.(13) Poly saccharide substrate solution was preheated at 70 °C for 20 min before being hydrolyzed by the enzymatic product. The reaction mixture (1 ml), consisting of 0.5 ml of the substrate solution and 0.5 ml of an appropriately diluted enzyme solution, was incubated at 50 °C with continuous agitation of 150 rpm for 60 min. At regular temperature intervals (5 min), the hydrolysis reaction was stopped by adding 1.5 ml of a 1% (w/v) dinitrosalicylate reagent, which was prepared in 1.6% NaOH. The reaction mixture was then placed in a boiling-water bath for 5 min to develop the reducing sugar color. Prior to cooling at room temperature, 0.5 ml of a potassium sodium tartrate solution (50%) was added to the mixture. The absorbance of the resulting mixture was measured spectrophotometrically at 540 nm, against the reagent blank, by using a DU-65 spectrophotometer (Beckman Instruments, San Ramon, CA, USA). Two blank assays, without the substrate or without the enzyme, were conducted in parallel. The amount of the released reducing sugar was determined from a standard curve, which had been constructed with standard solutions of glucose, xylose and mannose. One unit of specific activity is defined as the amount of enzymatic protein that liberates one μmol of reducing sugar per min of the reaction under the assay conditions.

The β-D-xylanase, α-L-arabinofuranosidase, α-D-galactosidase and β-D-glucosidase activities were assayed by using p-nitrophenyl-β-D-xyloside, p-nitropheny-α-L-arabinofuranoside, p-nitropheny-α-D-galactoside and p-nitropheny-β-D-mannoside, respectively, according to a modification of the method previously described by Deshpande et al.(14) An assay was performed by mixing 0.2 ml of the 4 mM substrate and 0.2 ml of the appropriately diluted enzyme solution in a suitable buffer. After 15 min of incubation at 40 °C, the reaction was stopped by adding 1.2 ml of 1% sodium carbonate. The absorbance of the reaction mixture was measured at 400 nm with the Beckman spectrophotometer. Two blank assays, without the enzyme or without the substrate, were prepared in tandem for the experiments. Both enzymatic reactions and blanks were run in triplicate. One unit of specific activity is defined as the amount of enzymatic protein that liberates one μmol of p-nitrophenol per min of the reaction under the assay conditions. The molar extinction coefficient of p-nitrophenol was estimated to be 21.6 μmol/mg/cm by using a standard solution of p-nitrophenol.

The feruloyl esterase assay was performed by using methyl ferulate as a substrate, according to a modification of the procedure described by Donaghy and McKay.15) The reaction mixture consisted of 0.25 ml of methyl ferulate in an ethanolic solution, 0.20 ml of a sodium acetate buffer (0.2 M, pH 5.0) and 0.05 ml of an appropriately diluted enzyme solution. The mixture was subjected to continuous shaking for 30 min at 37 °C. The feruloyl esterase assay was then halted by adding to the reaction homogenate 20 μl of 12N HCl. A control trial, without the enzyme, was carried out in tandem with the enzymatic assay. The concentration of the released ferulic acid was measured by an HPLC analysis, according to a modification of the method described by Kermasha et al.,(16) using a 126 reversed-phase column (5 μm, 250 × 4.6 mm, Agilent Technologies, Wilmington, DE, USA), using a linear gradient of acetonitrile from 15 to 50% with an aqueous TFA solution (0.2%, v/v). Elution was performed at room temperature at a flow rate of 1.0 ml/min. The reaction components were monitored at 280 nm with continuous scanning in the region of 190–500 nm at 1-s intervals. One unit of enzyme specific activity was defined as the amount of enzymatic protein which released one μmol of ferulic acid per min of the reaction.

Production and preparation of the multi-enzymatic extract. The P. funiculosum strain (IMI 378536) was cultivated in a modified medium of Steiner et al.,(7) containing cellulose and corn steep liquor as the main carbon and nitrogen sources, respectively. The preparation of the multi-enzymatic extract was carried out according to the procedure described by Karboune et al.(8)

Properties of a Hemicellulolytic System from Penicillium funiculosum 1287

Effect of pH on selected hemicellulolytic activities

The effect of pH on different hemicellulolytic activities is shown in Fig. 1. Endo-1,4-β-D-xylanase (Fig. 1A) and β-D-xylodisidase (Fig. 1B) showed their maximum specific activities at pH 3.5 and 4.0, respectively, and retained more than 60% of these activities in the pH ranges of 2.0 to 4.5 and 2.5 to 5.0. The results (Fig. 1C) also indicate that the endo-1,3,1,4-β-D-glucanase exhibited its maximal specific activity in the pH range of 3.5 to 5.0 and maintained more than 60% of its maximum activity in a wide pH range of 2.0 to 5.5. Regarding the α-L-arabinofuranosidase specific activity (Fig. 1D), this showed an optimum pH of 2.5 with more than 90% of its maximum specific activity obtained in the pH range of 2.0 to 3.0; however, no α-L-arabinofuranosidase specific activity could be detected in the alkaline pH region (above pH 6.0). The overall results reveal that the multi-hemicellulolytic system from P. funiculosum displayed endo-1,4-β-D-xylanase, β-D-xylodisidase, endo-1,3,1,4-β-D-glucanase and α-L-arabinofuranosidase specific activities at high levels in the acidic pH region (2.0 to 5.5).

The pH stability profiles of selected hemicellulolytic activities are shown in Fig. 2. Endo-1,4-β-D-xylanase (Fig. 2A) and endo-1,3,1,4-β-D-glucanase (Fig. 2C) showed high stability over a broad range of pH 2.0 to 8.0, where they maintained more than 70% of their maximum specific activity after 2 h of incubation at 25 °C; at pH 9.0, these respective enzymes still showed 63 and 61% of their maximum specific activity. Figure 2B indicates that the β-D-xylodisidase specific activity was stable in the pH range of 2.5 to 5.5, retaining more than 74% of its maximum value;
however, after incubating at pH 2.0 and above 6.0, this enzyme showed only 47% and less than 60% of its initial specific activity. Although the specific activity of β-D-Xylosidase (Fig. 2D) was stable in the pH range of 2.0 to 5.5, its pH stability decreased thereafter in the alkaline region. The overall experimental findings indicate that the specific activities of endo-1,4-β-D-xylanase and endo-1,3-1,4-β-D-glucanase showed higher stability in the alkaline pH range (7.0 to 9.0) than those of β-D-xylosidase and α-L-arabinofuranosidase.

The pH activity and stability profiles of the multimultihemicellulolytic system from P. funiculosum showed different features from those of other Penicillium hemicellulases. In fact, most endo-1,4-β-D-xylanases from Penicillium spp. have been reported to have an optimal pH in the range of 4.5 to 6.0, with the exception of those from Penicillium purpurogenum and Penicillium sp., which were optimally active at pH 7.0 and 2.0, respectively. Moreover, most endo-1,4-β-D-xylanases from Aspergillus spp. and Fusarium oxysporum f. spp. have displayed optimal activity in the pH range of 4.0 to 5.5. Regarding the β-D-xylosidase, its optimal pH of 4.0 (Fig. 1B) is similar to that reported for the xylanolytic extract from P. funiculosum and for the purified enzyme from Penicillium wortmanni, Penicillium herquei, and Trichoderma reesei. However, a higher pH optimum of 5.0 to 6.5 has been reported for the β-D-xylosidase from Aspergillus spp.

Hemicellulases classified as endo-1,3,1,4-β-D-glucanase are mainly produced by plants and bacteria; as far as the authors are aware, only two endo-1,3,1,4-β-D-glucanases have been isolated from a fungal source, including anaerobic Orpinomyces sp. and thermophilic aerobic Talaromyces emersonii. The optimum pH for the endo-1,3,1,4-β-D-glucanase from P. funiculosum (Fig. 1C) is close to that of the same enzyme from

Fig. 1. Effect of pH on Selected Hemicellulolytic Activities of the Multi-Enzymatic System from P. funiculosum: (A) Endo-1,4-β-D-xylanase; (B) β-D-Xylosidase; (C) Endo-1,3,1,4-β-D-glucanase; (D) α-L-Arabinofuranosidase.

Fig. 2. pH Stability of Selected Hemicellulolytic Specific Activities of the Multi-Enzymatic System from P. funiculosum: (A) Endo-1,4-β-D-xylanase; (B) β-D-Xylosidase; (C) Endo-1,3,1,4-β-D-glucanase; (D) α-L-Arabinofuranosidase.
T. emersonii;\textsuperscript{27} however, a higher optimum pH value of 6.0 has been reported for the endo-1,3-1,4-β-D-glucanase from *Orpinomyces* sp.\textsuperscript{26} On the other hand, α-L-arabinofuranosidase was most active at a remarkably acidic pH value of 2.5 and was inactive above pH 6.0 (Fig. 1D). A similar optimum pH of 2.5 has been reported for α-L-arabinofuranosidase from *Corticum rolfsii*.\textsuperscript{28} However, α-L-arabinofuranosidases from *Penicillium capsulatum*\textsuperscript{29} and *P. purpurigenum*\textsuperscript{30} have displayed optimum activity at pH 4.0, whereas that from *Penicillium canescens*\textsuperscript{31} has shown an optimal pH in the range of 5.0 to 6.5. As far as the authors are aware, no α-L-arabinofuranosidase activity from *P. funiculosum* has been characterized with regards to its optimum pH. α-L-arabinofuranosidase has been mainly produced by *Aspergillus* spp. and has shown optimum activity in the pH range of 4.0 to 5.5.\textsuperscript{32}

**Effect of temperature on selected hemicellulolytic activities**

The effect of temperature on selected hemicellulolytic activities of the multi-enzymatic system from *P. funiculosum* is shown in Fig. 3. The endo-1,4-β-D-xylanase specific activity increased 2.5 times when the reaction temperature was increased from 25 to 50°C, then remained unchanged at 55°C and subsequently decreased at higher temperatures (Fig. 3A). The results (Fig. 3C) also show an increase in the specific activity of endo-1,3,1,4-β-D-glucanase by a factor of 3.2 to its maximum value in the temperature range of 25 to 60°C, before remaining constant at 65°C and subsequently decreased at higher temperatures (Fig. 3A). The results (Fig. 3C) also show an increase in the specific activity of endo-1,3,1,4-β-D-glucanase by a factor of 3.2 to its maximum value in the temperature range of 25 to 60°C, before remaining constant at 65°C and subsequently decreased at higher temperatures (Fig. 3A). The results (Fig. 3C) also show an increase in the specific activity of endo-1,3,1,4-β-D-glucanase by a factor of 3.2 to its maximum value in the temperature range of 25 to 60°C, before remaining constant at 65°C and subsequently decreased at higher temperatures (Fig. 3A). The results (Fig. 3C) also show an increase in the specific activity of endo-1,3,1,4-β-D-glucanase by a factor of 3.2 to its maximum value in the temperature range of 25 to 60°C, before remaining constant at 65°C and subsequently decreased at higher temperatures (Fig. 3A). The results (Fig. 3C) also show an increase in the specific activity of endo-1,3,1,4-β-D-glucanase by a factor of 3.2 to its maximum value in the temperature range of 25 to 60°C, before remaining constant at 65°C and subsequently decreased at higher temperatures (Fig. 3A). The results (Fig. 3C) also show an increase in the specific activity of endo-1,3,1,4-β-D-glucanase by a factor of 3.2 to its maximum value in the temperature range of 25 to 60°C, before remaining constant at 65°C and subsequently decreased at higher temperatures (Fig. 3A).

\[\text{Specific activity (mol/min.mg of protein)}\]

**Fig. 3.** Effect of Temperature on Selected Hemicellulolytic Activities of the Multi-Enzymatic System from *P. funiculosum*: (A) Endo-1,4-β-D-xylanase; (B) β-D-Xylosidase; (C) Endo-1,3-1,4-β-D-glucanase; (D) α-L-Arabinofuranosidase.
xylosidase, whose specific activity still showed 50% of its initial value after incubating at 70 °C. On the other hand, there was no loss in the specific activity of α-L-arabinofuranosidase after its incubation at temperatures up to 50 °C; however, above this temperature, the enzyme was less stable and retained only 33% and 19% of its initial specific activity after incubating at 60 and 70 °C, respectively (Fig. 4D). The overall results indicate that the endo-1,3-1,4-β-D-glucanase specific activity exhibited higher thermal stability than that of endo-1,4-β-D-xylanase. Similarly, Sahasrabudhe et al. have reported that the thermal stability of endo-glucanase from P. funiculosum was greater than that of the endo-1,4-β-D-xylanase. The experimental findings (Fig. 4) also indicate that the specific activities of both endo-1,4-β-D-xylanase and endo-1,3-1,4-β-D-glucanase were less thermostable than those of β-D-xylosidase and α-L-arabinofuranosidase at temperatures above 60 °C. Similarly, β-D-xylosidase from Aspergillus awamori has shown higher thermal stability at 70 °C than endo-1,4-β-D-xylanases from the same strain which rapidly lost their activity above 55 °C. As far as the authors are aware, there is no information in the literature on the kinetic parameters of β-D-xylosidase from P. funiculosum. However, β-D-xylosidase from P. janthinellum has been reported to have a $K_m$ value for p-nitrophenyl-β-D-xyloside of 1.07 mM, which is higher than that estimated for β-D-xylosidase present in the multi-enzyme system from P. funiculosum (0.51 mM); however this $K_{app}$ value is lower than those reported for β-D-xylosidases from P. wortmanni (from 0.6 to 4.2 mm) for different xylooligosaccharides. On the other hand, the $K_{app}$ value for endo-1,4-β-D-xylanase (9.2 mg/ml) is close to those reported for two isoenzymes of endo-1,4-β-D-xylanase from P. capsulatum using wheat straw xylan as substrate (7.0 and 9.8 mg/ml), but lower than those obtained using oat spelt xylan as substrate (46.0 and 33.7 mg/l). However, lower $K_m$ values of 3.4, 4.1 and 4.7 mg/ml have been obtained for other endo-1,4-β-D-xylanases from P. chrysogenum, recombinant P. funiculosum and P. canescens, respectively. The apparent $K_{app}$ value (9.2 mg/ml) of endo-1,3-1,4-β-D-

**Kinetic parameters**

In spite of the complexity of the multi-enzyme system produced by P. funiculosum, it was still considered important to determine the apparent kinetic parameters of the selected hemicellulolytic enzymes. The apparent Michaelis-Menten constants, $K_{app}$ and $V_{max}$, estimated by non-linear regression are given in Table 1. Although the $V_{max}$ value of β-D-xylosidase was higher than that of α-L-arabinofuranosidase, its catalytic efficiency was 1.4 times lower. The results also indicate that the respective $K_{app}$ and $V_{max}$ values of endo-1,4-β-D-xylanase were 4.6 and 1.2 times higher than those of endo-1,3-1,4-β-D-glucanase; as a result, the catalytic efficiency of endo-1,3,1,4-β-D-glucanase was substantially greater.

As far as the authors are aware, there is no information in the literature on the kinetic parameters of β-D-xylosidase from P. funiculosum. However, β-D-xylosidase from P. janthinellum has been reported to have a $K_m$ value for p-nitrophenyl-β-D-xyloside of 1.07 mM, which is higher than that estimated for β-D-xylosidase present in the multi-enzyme system from P. funiculosum (0.51 mM); however this $K_{app}$ value is lower than those reported for β-D-xylosidases from P. wortmanni (from 0.6 to 4.2 mm) for different xylooligosaccharides. On the other hand, the $K_{app}$ value for endo-1,4-β-D-xylanase (9.2 mg/ml) is close to those reported for two isoenzymes of endo-1,4-β-D-

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**Table 1. Kinetic Parameters of Selected Hemicellulases Present in the Multi-Enzyme System Produced by P. funiculosum**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$K_{app}$ b</th>
<th>$V_{max}$ b</th>
<th>Catalytic efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endo-1,4-β-D-xylanase</td>
<td>9.24</td>
<td>72,992.7</td>
<td>197,491.1</td>
</tr>
<tr>
<td>Endo-1,3,1,4-β-D-glucanase</td>
<td>2.01</td>
<td>58,562.3</td>
<td>7,575,222.8</td>
</tr>
<tr>
<td>β-D-Xylosidase</td>
<td>0.51</td>
<td>83.6</td>
<td>163.9</td>
</tr>
<tr>
<td>α-L-Arabinofuranosidase</td>
<td>0.26</td>
<td>59.1</td>
<td>227.3</td>
</tr>
</tbody>
</table>

*aThe michaelis constant is defined as mg/ml of birchwood xylan and barley β-glucan for endo-1,4-β-D-xylanase and endo-1,3,1,4-β-D-glucanase, respectively, as well as nm of p-nitrophenyl-β-D-xyloside and p-nitrophenyl-α-L-arabinofuranoside for β-D-xylosidase and α-L-arabinofuranosidase, respectively.

*bThe maximum velocity is defined as μmol of released reduced sugar or p-nitrophenol per mg of protein per min of reaction.

The catalytic efficiency is defined as the ratio of $V_{max}/K_{app}$.**
glucanase for β-glucan was higher than that reported for the same enzyme from *Orpinomyces* sp. (0.91 mg/ml), but lower than that from *T. emersonii* (13.4 mg/ml). The $K_{m}$ value of α-L-arabinofuranosidase falls within the range of those obtained for α-L-arabinofuranosidases from *P. capsulatum* of 0.18 mM and 1.3 mM. α-L-arabinofuranosidases from *P. purpureogenum* have shown a $K_{m}$ value of 1.23 mM for the same substrate, p-nitrophenyl-α-L-arabinofuranosidase.

**Investigation of feruloyl esterase and selected mannanolytic enzymes**

To ensure the complete hydrolysis of plant cell wall polysaccharides, the side-chain substituent removal may be needed. Indeed, it has been reported that the presence of side-chain ferulic acid can limit further enzymatic hydrolysis of arabinoxylans and xyloglucans by endo-1,4-β-D-xylanase and β-D-xylosidase. The experimental findings also show that the endo-1,4-β-D-mannanase activities, and as m M of galactomannan is comparable to those reported (from 0.18 to 0.51 mM), the mannase. Although the presence of side-chain ferulic acid can limit further enzymatic hydrolysis of arabinoxylans and xyloglucans by endo-1,4-β-D-xylanase and β-D-xylosidase. The maximum velocity is defined as µmol of released reduced sugar or p-nitrophenol or fufural acid per mg of protein per min of reaction. The catalytic efficiency is defined as the ratio of $V_{max}/K_{m}$. The range reported for other fungal α-D-galactosidase showed comparable characteristics in terms of temperature optimum, pH optimum and stability. Conversely, α-L-arabinofuranosidase was mostly active and stable in the extreme acidic region as compared to β-D-xylosidase. The catalytic properties of selected hemicellulases, in terms of the optimum pH, reaction temperature and kinetic parameters, were similar to those obtained from other sources. The overall features of the hemicellulolytic system from *P. funiculosum* reveal its promising potential for degrading the cell wall in a biotechnological process.

**Conclusion**

Selected hemicellulolytic activities were detected at high levels in the multi-enzymatic system from *P. funiculosum*. Endo-1,3,1,4-β-D-glucanase and endo-1,4-β-D-xylanase showed comparable characteristics in terms of temperature optimum, pH optimum and stability.

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**References**