Biochemical properties of extracellular α-mannosidases from the digestive fluid of Rhynchophorus palmarum larvae

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Abstract

Three extracellular α-mannosidases (EC 3.2.1.24) were purified from the digestive fluid of oil palm weevil larvae, Rhynchophorus palmarum (L.) and characterized in order to explore their potential for biotechnological application. Three steps procedure including size exclusion, anion exchange and hydrophobic interaction chromatography were used. The enzymes named RplM1, RplM2 and RplM3 had native molecular weights of approximately 125, 72 and 111 kDa, respectively and functioned as dimeric (RplM1) and monomeric (RplM2 and RplM3) structures. The three α-mannosidases displayed acidic and mesophilic activities by using para-nitrophenyl-α-D-Mannopyranoside as substrate. Apparent KM values were found to be 0.13, 0.22 and 0.16 mM for RplM1, RplM2 and RplM3, respectively. Ba²⁺, Ca²⁺, Mg²⁺, Zn²⁺ and ethylene diamine tetra acetic acid (EDTA) had no effect on the three enzyme activities whereas detergents and reducing agents were inhibitors. RplM1, RplM2 and RplM3 were more sensitive to swainsonine and cleaved α-1,2-α-1,3 and α-1,6 linked mannobiose. These characteristics prompted us to categorize them as catabolic α-mannosidases. Thus, they would help in the larval oil palm weevil nutrition allowing digestion by the removal of mannose residues in the beetle’s diet. Also, these enzymes could find potential application in biological, biochemical and pharmaceutical area.

Key words: extracellular α-mannosidases, characterization, oil palm weevil, Rhynchophorus palmarum, Coleoptera, Curculionidae.

Introduction

The α-mannosidases play an essential role in the processing of N-glycans to complex and hybrid oligosaccharides. The α-mannosidases involved in N-glycans processing are categorized as class I and class II, with different substrate specificities, intracellular locations, sizes, cations requirements, amino acid sequences and sensitivities to plant alkaloid inhibitors (Moremen et al., 1994; Herscovics, 1999; Moremen, 2000). According to the sensitivity of α-mannosidases to plant alkaloid inhibitors (Moremen et al., 1994; Herscovics, 1999; Moremen, 2000). According to the sensitivity of α-mannosidases to plant alkaloid inhibitors, the enzymes are categorized into two distinctive classes. The azapyanose analogues, such as kifunensine (Kif) and 1-deoxymannojirimycin (DMNJ), are potent inhibitors of class I α-mannosidases (Bischoff and Kornfeld, 1984; Elbein et al., 1990). In contrast, those sensitive to swainsonine (Sw) and 1,4-dideoxy-1,4-imino-D-mannitol (DIM) which are azafuranose analogues belong to class II α-mannosidases (Tulsiani et al., 1982a; Cenci di Bello et al., 1989). Based on the amino acid sequence alignments, class I and class II α-mannosidases belong to family 47 and family 38, respectively in Henrissat’s glycosidases classification (Moremen et al., 1994; Henrissat and Bairoch, 1996). On the other hand, microbial α-mannosidases have been widely studied (Keskar et al., 1996) and fallen into two groups. In group A, the enzymes hydrolyse non-reducing terminal α-mannosidic linkages regardless of the aglycon moiety. It includes those enzymes which act on para-nitrophenyl-α-D-Mannopyranoside (pNP-α-Man) and do not have strict linkage specificity. In group B, the enzymes hydrolyse specifically linked α-D-mannosidic bonds.

Apart from the vital role in glycoproteins synthesis and degradation, α-mannosidases are currently an important therapeutic target for the development of anticancer agents (Goss et al., 1995, 1997). The major potential application of α-mannosidases involves the strategies for treating the lysosomal storage disorder α-mannosidosis by enzyme replacement therapy. Indeed, α-mannosidosis results from deficient activity of α-mannosidase. The disease is characterized by massive intracellular accumulation of mannose-rich oligosaccharide, that is oligosaccharides carrying α-1,2-α-1,3 and α-1,6 mannosyl residues at the non-reducing termini (Saint-Pol et al., 1999; Sun et al., 1999; Hirsch et al., 2003). In this respect, the broad specific α-mannosidases become useful for application requiring removal of all α-mannosyl linkages. It is suggested that α-mannosidase and N-acetyl-β-hexosaminidase (isolated from latex) be used synergistically in the medical treatment of fungal diseases in the most severe cares to boost the known β-hexosaminidase antifungal action (Giordani et al., 1991).

α-mannosidases have been reported from various sources, e.g. plants (Woo et al., 2004; Ahi et al., 2007), fungi (Rey et al., 2000; Tatara et al., 2003), yeast (Kelly and Herscovics, 1998), animals (Weng and
Enzymes purification

All the purification procedure was carried out in cold room. To the crude extract was added solid ammonium sulphate to 80% saturation. The mixture was stirred for at least eight hours and centrifuged at 10,000 g for 15 min. The pellet was suspended in 20 mM sodium acetate buffer pH 5.0 and loaded onto a Sephacryl S-100 HR column (1.5 cm × 67 cm) equilibrated with the same buffer. Fractions of 1 ml were collected at a flow rate of 0.2 ml/min and, those containing the α-mannosidase activity were pooled.

The pooled fractions were applied to the following chromatography, a DEAE Sepharose Fast Flow column (2.5 cm × 4.5 cm) equilibrated with 20 mM sodium acetate buffer pH 5.0. The column was washed at a flow rate of 3 ml/min with two bed volumes of equilibration buffer to remove unbound proteins. Bound proteins were then eluted with a stepwise salt gradient (0.1, 0.2, 0.3 and 1 M) of NaCl in 20 mM sodium acetate buffer pH 5.0 and fractions of 1 ml were collected. Three peaks of α-mannosidase activity were obtained. To each pooled active fractions, solid ammonium sulphate was slowly added to give a final concentration of 1 M and the resulted enzyme solution was applied on a Phenyl Sepharose 6 Fast Flow column (1.5 cm × 3.2 cm) previously equilibrated with 20 mM sodium acetate buffer pH 5.0 containing 1 M ammonium sulphate. The column was washed with a reverse stepwise gradient of ammonium sulphate concentration (from 1 to 0 M) in the same sodium acetate buffer at a flow rate of 1 ml/min and fractions of 1 ml were collected.

The active fractions of each isoform were pooled, dialyzed overnight at 4 °C against 20 mM sodium acetate buffer pH 5.0 and constituted the purified enzymes.

Enzymes assay

All enzyme reactions were performed at 37 °C for 20 min in appropriate 100 mM sodium acetate buffer pHs containing 0.8 mM of pNP-α-Man. After prewarming the mixture at 37 °C for 5 min, the reactions were initiated by adding enzyme solution. The final volume was 150 µl and the reactions were stopped with 2 ml of sodium carbonate 2% (w/v). Enzymes activities towards 0.8 mM of pNP-α-Man were determined by measuring the released para-nitrophenol (pNP) at 410 nm using a spectrophotometer GENESIS 5. pNP was used as standard.

One unit of activity was defined as the amount of enzyme that hydrolyzes 1 µmol of substrate per min under the assay conditions. The specific activity was expressed as unit of activity per mg of protein.

Proteins estimation

Protein concentrations and elution profiles from chromatographic columns were determined by the Folin method (Lowry et al., 1951). Bovine serum albumin (BSA) was used as the standard protein.

Polyacrylamide gel electrophoresis (PAGE)

Electrophoresis was carried out by the method of Laemmli (1970) on 7.5% and 10% (w/v) acrylamide gels under denaturing and non-denaturing conditions,
respectively. In denaturing conditions, samples were incubated for 5 min at 100 °C with SDS-PAGE sample buffer containing 2-mercaptoethanol. Concerning non-denaturing conditions, samples were mixed just before running in sample buffer without 2-mercaptoethanol and SDS. Silver staining was used to localize protein bands (Blum et al., 1987). The standard molecular weights (Bio-Rad) comprising myosin (200 kDa), β-galactosidase (116.25 kDa), phosphorylase b (97.4 kDa), BSA (66.2 kDa) and ovalbumin (45.0 kDa) were used.

Native molecular weights determination

The purified enzymes were applied to gel filtration on Sephacryl S-200 HR column (0.8 cm × 35 cm) equilibrated with 20 mM sodium acetate buffer (pH 5.0) to estimate the native molecular weights. Elution was done at a flow rate of 0.2 ml/min and fractions of 0.5 ml were collected. Standard molecular weights (SIGMA) used for calibration were β-amylase from sweet potato (206 kDa), BSA (66 kDa), ovalbumin from egg white (45 kDa) and cellulase from Aspergillus niger (26 kDa).

pH and temperature optima

The effect of pH on the enzyme activities was determined by performing the hydrolysis of para-nitrophenyl-α-D-Mannopyranoside in a series of buffers (100 mM) at various pH values ranging from 3.0 to 8.0. The buffers used were sodium acetate buffer from pH 3.6 to 5.5; sodium phosphate buffer from pH 5.6 to 8.0 and sodium citrate buffer from pH 3.0 to 6.0. The pH values of each buffer were determined at 25 °C.

The effect of temperature on α-mannosidase activities was performed in 100 mM acetate buffer (appropriate pHs) over a temperature range of 30 to 80 °C using pNP-α-Man (2.5 mM) under the enzyme assay conditions.

pH and temperature stabilities

The pH stability of each α-mannosidase was studied in pH range of 3.0 to 8.0 with 100 mM buffers. The buffers were the same as in pH and temperature optima study (above). After 2 h preincubation at room temperature, residual α-mannosidase activities were measured at 37 °C for 20 min by adding substrate para-nitrophenyl-α-D-Mannopyranoside.

The thermal inactivation was determined at 37 °C and at each enzyme optimum temperature. Enzymes in appropriate buffers (pHs) were exposed to each temperature for 0 to 180 min. Aliquots were withdrawn at intervals (30 min) and immediately cooled.

Concerning thermal denaturation tests, the aliquots of each enzyme were preheated at different temperatures ranging from 30 to 80 °C for 25 min.

Residual activities, determined in the three cases at 37 °C under the enzyme assay conditions, were expressed as percentage activity of zero-time control of untreated enzymes.

Effect of some chemical agents

To determine the effect of various compounds (metal ions, detergents, reducing agents and α-mannosidase specific inhibitors) as possible activators or inhibitors of the purified α-mannosidases, each enzyme solution was preincubated at 37 °C for 30 min with the compounds and then the activity was assayed under the enzyme assay conditions. The residual activities were expressed as percentage refers to a control without chemical agents.

Substrate specificity and kinetic parameters determinations

The substrate specificity was determined by incubating each purified enzyme with substrates para-nitrophenyl-α-D-Mannopyranoside, para-nitrophenyl-α-D-Glucopyranoside, para-nitrophenyl-α-L-Fucopyranoside, para-nitrophenyl-α-D-Galactopyranoside, para-nitrophenyl-α-L-Arabinopyranoside, para-nitrophenyl-β-D-Fucopyranoside, para-nitrophenyl-β-D-Galactopyranoside, para-nitrophenyl-β-D-Xylopyranoside (2.5 mM) at 37 °C for 20 min in 100 mM sodium acetate buffer with appropriate pHs.

The kinetic parameters (K_M, ν_max and V_max/K_M) were determined at 37 °C in 100 mM sodium acetate buffer with appropriate pHs. The hydrolysis of synthetic aryl substrates was quantified on the basis of released pNP, as the standard enzymes assay. K_M and ν_max were determined from a Lineweaver-Burk plot using seven concentrations varying between 0 and 6 mM of the substrate pNP-α-Man.

Characterization of hydrolytic specificity

The hydrolytic specificity was determined at 37 °C by incubating 0.45 µg of each purified enzyme preparation with 1 mM of 2-O-α-D-mannopyranosyl-D-mannopyranoside, 3-O-α-D-mannopyranosyl-D-mannopyranoside or 6-O-α-D-mannopyranosyl-D-mannopyranoside, in 100 mM sodium acetate buffer (at appropriate pHs) for up to 24 h. Samples (5 µl) were removed at regular time intervals and applied to TLC plates to monitor the hydrolysis of different linked disaccharides. The TLC plates were run with butanol-acetic acid-water (9:3.75:2.25, v/v/v) and developed with naphtho-resorcinol in ethanol and H_2SO_4 20% (v/v). The sugar spots were visualized at 110 °C for 5 min.

Results

Enzymes purification

Purification results of digestive α-mannosidases from the oil palm weevil larvae are summarized in table 1. Three isoenzymes were purified from the crude extract and the purification procedure involved three chromatographic steps for each enzyme.

The peak of α-mannosidase activity resolved on gel filtration (Sephacryl S-100 HR) as first step was designated Rpl/M. Pooled fractions were collected and then applied onto DEAE-Sepharose Fast Flow, an anion exchange chromatography. Three peaks showing bounded α-mannosidase activities were eluted with 0.20, 0.25 and 0.30 M of NaCl, respectively. The three isoforms were termed Rpl/M1, Rpl/M2 and Rpl/M3 based on their elution order (data not shown).
Table 1. Purification procedure of α-mannosidases from the digestive fluid of R. palmarum larvae.

<table>
<thead>
<tr>
<th>Purification steps</th>
<th>Total protein (mg)</th>
<th>Total activity (UI)</th>
<th>Specific activity (UI/mg)</th>
<th>Purification fold</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>86.03</td>
<td>42.24</td>
<td>0.48</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ (80%)</td>
<td>72.35</td>
<td>36.64</td>
<td>0.51</td>
<td>1.06</td>
<td>88.80</td>
</tr>
<tr>
<td>Sephacryl S-100 HR Rpl/M</td>
<td>17.89</td>
<td>26.43</td>
<td>1.48</td>
<td>3.08</td>
<td>64.00</td>
</tr>
<tr>
<td>DEAE-Sepharose Fast Flow</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rpl/M1</td>
<td>0.93</td>
<td>1.25</td>
<td>1.34</td>
<td>2.79</td>
<td>3.00</td>
</tr>
<tr>
<td>Rpl/M2</td>
<td>0.33</td>
<td>0.64</td>
<td>1.94</td>
<td>4.04</td>
<td>1.60</td>
</tr>
<tr>
<td>Rpl/M3</td>
<td>1.02</td>
<td>0.69</td>
<td>0.68</td>
<td>1.42</td>
<td>1.70</td>
</tr>
<tr>
<td>Phenyl-Sepharose 6 Fast Flow</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rpl/M1</td>
<td>0.03</td>
<td>0.33</td>
<td>12.22</td>
<td>25.46</td>
<td>0.80</td>
</tr>
<tr>
<td>Rpl/M2</td>
<td>0.04</td>
<td>0.41</td>
<td>11.39</td>
<td>23.73</td>
<td>1.00</td>
</tr>
<tr>
<td>Rpl/M3</td>
<td>0.04</td>
<td>0.13</td>
<td>3.17</td>
<td>6.61</td>
<td>0.30</td>
</tr>
</tbody>
</table>

UI = 1µmol of pNP per min.

Figure 1. Native-PAGE analysis of purified α-mannosidases from the digestive fluid of R. palmarum larvae. The samples were loaded onto a 10% gel. Lane 1, Rpl/M1; Lane 2, Rpl/M2; Lane 3, Rpl/M3; Lane 4, crude extract.

Figure 2. SDS-PAGE analysis of purified α-mannosidases from the digestive fluid of R. palmarum larvae. The samples were loaded onto a 7.5% gel. Lane 1, Rpl/M1; Lane 2, Rpl/M2; Lane 3, Rpl/M3; Lane 4, Markers. Numbers on the right indicate the molecular weight (kDa) of the markers.

An ultimate hydrophobic interaction chromatography on a Phenyl-Sepharose 6 Fast Flow column as third step was necessary to each isofrom for purification. The purified enzymes named Rpl/M1, Rpl/M2 and Rpl/M3 were eluted with 0.61, 0.02 and 0.41 M of ammonium sulphate, and were enriched about 26, 24 and 7-fold, respectively (table 1).

Each isoenzyme showed a single protein band by silver staining on native polyacrylamide gel electrophoresis (figure 1).

Molecular weights estimation

After SDS-PAGE analysis under reducing conditions, each α-mannosidase (Rpl/M1, Rpl/M2 and Rpl/M3) showed a single protein band. Their relative molecular weights were estimated to be 62, 76 and 105 kDa, respectively (figure 2).

Molecular weights of the native enzymes, as determined by gel filtration, were approximately 125 kDa (Rpl/M1), 72 kDa (Rpl/M2) and 111 kDa (Rpl/M3) (table 2).
Effect of pH and temperature

The effect of pH and temperature on the three α-mannosidase activities is shown in table 2. The three isoenzymes were found to be acidic and mesophilic. At 37 °C, the purified enzymes showed best stability over pH values ranging from 4.3 to 5.2, conserving at least more than 90% of total activities. Their maxima activities were observed at 55, 50 and 60 °C for RplM1, RplM2 and RplM3, respectively. Values of temperature coefficients (Q10) calculated were found to be around 1.8 for the three α-mannosidases. From Arrhenius plot, values of 47, 42.9 and 50.4 kJ/mol were calculated for the activation energy of RplM1, RplM2 and RplM3, respectively (table 2).

The thermal stability was investigated by incubating each enzyme at various temperatures for 25 min. The three α-mannosidases retained 100% of their activities to temperatures up to their optima. Above, their activities declined rapidly as the temperature increased. Nevertheless, the enzymes were not completely inactivated even at 80 °C (figure 3).

A long-term stability study indicated that at 37 °C and in appropriate optimum pHs, the three α-mannosidases remained stable for 180 min. However, at their optimum temperatures, each enzyme is stable for 30 min and retained about 75% of its activity after 80 min preincubation. Above this preheating time, α-mannosidases were less stable showing half-life (50% of activity) nearby 150 min for RplM3 and 180 min for RplM1 and RplM2 (figure 4).

Effect of some metal ions, chelating and reducing agents and detergents

The effect of some chemical agents on α-mannosidases from oil palm weevil larvae digestive fluid using para-nitrophenyl-α-D-Mannopyranoside as substrate was examined.

Most of chemicals tested (Ba2+, Ca2+, Mg2+, Zn2+, EDTA, dithiothreitol, polyoxyethylene-9-lauryl ether and polyoxyethylten-10-oyl ether) were without effect on the enzymes activity. Nevertheless, Cu2+, 5,5′-dithio-2,2′-dinitro-dibenzoic acid (DTNB), L-cysteine and SDS were found to be the common inhibitors (60-100% inhibition observed on the three α-mannosidase activities), while para-chloromercuribenzoic acid (pCMB), tetradeyl trimethyl ammonium bromide and hexadecyl trimethyl ammonium bromide displayed 70 to 80% of inhibition on RplM2 and RplM3. Nonidet-P40, a non-ionic detergent, showed 34% of inhibition on RplM3 activity at the concentration of 1% (w/v). Only 2-mercaptoethanol displayed a particular stimulatory effect on RplM1 (137%) at the concentration of 0.1% (v/v).

Effect of α-mannosidase specific inhibitors

The influence of α-mannosidase specific inhibitors such as 1-deoxymannojirimycin (DMNJ), kifunsine (Kif), swainsone (Sw) and 1,4-dideoxy-1,4-iminomannitol (DIM) on the enzymes activities is presented in table 3.

Hydrolysis of pNP-α-Man by RplM1, RplM2 and RplM3 was strongly inhibited by the azafuranose ana-

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**Table 2.** Physicochemical properties of α-mannosidases from the digestive fluid of oil palm weevil larvae.

<table>
<thead>
<tr>
<th>Physicochemical properties</th>
<th>RplM1</th>
<th>RplM2</th>
<th>RplM3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Optimum temperature (°C)</td>
<td>55</td>
<td>50</td>
<td>60</td>
</tr>
<tr>
<td>Optimum pH</td>
<td>5.0</td>
<td>5.0</td>
<td>4.5</td>
</tr>
<tr>
<td>pH stability</td>
<td>4.4 - 5.2</td>
<td>4.6 - 5.2</td>
<td>4.3 - 4.8</td>
</tr>
<tr>
<td>Q10</td>
<td>1.8</td>
<td>1.7</td>
<td>1.8</td>
</tr>
<tr>
<td>Activation energy (kJ/mol)</td>
<td>47.0</td>
<td>42.9</td>
<td>50.4</td>
</tr>
<tr>
<td>Molecular weight (kDa)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

| SDS-PAGE                  | 62    | 76    | 105   |
| Gel filtration            | 125   | 72    | 111   |

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Figure 3. Thermal denaturation of α-mannosidases from the digestive fluid of *R. palmarum* larvae. The experiments were carried out at temperatures ranging from 30 to 80 °C. The enzymes were preincubated at each temperature for 25 min and the remaining activity measured at 37 °C under the enzyme assay conditions.

Figure 4. Thermal inactivation of α-mannosidases from the digestive fluid of *R. palmarum* larvae. Each enzyme was separately preincubated at 37 °C and its optimum temperature, in 100 mM sodium acetate buffers (pH 4.5 or 5.0). At the indicated times, aliquots were withdrawn and the residual activity measured at 37 °C under the enzyme assay conditions.
Table 3. Effect of specific inhibitors on the activity of the α-mannosidases from the digestive fluid of R. palmarum larvae.

<table>
<thead>
<tr>
<th>Specific inhibitors</th>
<th>Concentration (µM)</th>
<th>Relative activity (% of blank)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>RplM1</td>
</tr>
<tr>
<td>Blank</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Swainsonine</td>
<td>0.05</td>
<td>91</td>
</tr>
<tr>
<td>&quot;</td>
<td>0.5</td>
<td>29</td>
</tr>
<tr>
<td>&quot;</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>&quot;</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>1,4-dideoxy-1,4-iminomannitol</td>
<td>0.05</td>
<td>100</td>
</tr>
<tr>
<td>&quot;</td>
<td>0.5</td>
<td>91</td>
</tr>
<tr>
<td>&quot;</td>
<td>10</td>
<td>39</td>
</tr>
<tr>
<td>&quot;</td>
<td>20</td>
<td>23</td>
</tr>
<tr>
<td>Kifunensine</td>
<td>0.05</td>
<td>100</td>
</tr>
<tr>
<td>&quot;</td>
<td>0.5</td>
<td>97</td>
</tr>
<tr>
<td>&quot;</td>
<td>10</td>
<td>92</td>
</tr>
<tr>
<td>&quot;</td>
<td>20</td>
<td>88</td>
</tr>
<tr>
<td>1-deoxymannojirimycin</td>
<td>0.05</td>
<td>100</td>
</tr>
<tr>
<td>&quot;</td>
<td>0.5</td>
<td>100</td>
</tr>
<tr>
<td>&quot;</td>
<td>10</td>
<td>100</td>
</tr>
<tr>
<td>&quot;</td>
<td>20</td>
<td>80</td>
</tr>
</tbody>
</table>

Table 4. Kinetic parameters of α-mannosidases from the digestive fluid of R. palmarum larvae towards pNP-α-Man. The Michaelis-Menten constant (K_M) and the maximum velocity (V_max) are expressed as mM and units/mg of protein, respectively.

<table>
<thead>
<tr>
<th>α-mannosidases</th>
<th>K_M (mM)</th>
<th>V_max (µmol/min/mg)</th>
<th>V_max/K_M (ml/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RplM1</td>
<td>0.13</td>
<td>0.73</td>
<td>5.62</td>
</tr>
<tr>
<td>RplM2</td>
<td>0.22</td>
<td>2.46</td>
<td>11.18</td>
</tr>
<tr>
<td>RplM3</td>
<td>0.16</td>
<td>1.32</td>
<td>8.25</td>
</tr>
</tbody>
</table>

logues of mannose such as Sw and DIM when used in the reaction mixture at 20 µM. These inhibitions vary from 77 to 100%. In contrast, the azapyranose analogues as Kif and DMNJ had not such effect at the same concentration (0-20% of inhibition observed).

Substrate specificity and kinetic parameters

Purified α-mannosidases were assayed for hydrolytic activities towards a variety of synthetic and natural substrates. No detectable activities towards para-nitrophenyl glycopyranosides (para-nitrophenyl-α-D-Glucopyranoside, para-nitrophenyl-α-L-Fucopyranoside, para-nitrophenyl-α-D-Galactopyranoside, para-nitrophenyl-α-L-Arabinopyranoside, para-nitrophenyl-β-D-Fucopyranoside, para-nitrophenyl-β-D-Glucopyranoside, para-nitrophenyl-β-D-Galactopyranoside, para-nitrophenyl-β-D-Xylopyranoside) were observed except para-nitrophenyl-α-D-Manopyranoside (data not shown) on which Lineweaver-Burk-plot in range of concentrations 0 to 6 mM showed K_M and V_max values of the α-mannosidases (table 4). The K_M values were 0.13, 0.22 and 0.16 mM for isoforms RplM1, RplM2 and RplM3, respectively.

On the other hand, the linkage specificity was investigated using natural substrates such as disaccharides 2-O-α-D-mannopyranosyl-D-mannopyranoside (Man-α-1,2-Man), 3-O-α-D-mannopyranosyl-D-mannopyranoside (Man-α-1,3-Man) and 6-O-α-D-mannopyranosyl-D-mannopyranoside (Man-α-1,6-Man). Following incubation with RplM1, RplM2 and RplM3, released mannosone was separated by TLC (figure 5). All the α-mannosidases (RplM1, RplM2 and RplM3) showed broad specificity under acidic conditions by cleaving α-1,2; α-1,3 and α-1,6 Mannobioses at different rates. However, their hydrolytic on α-1,2 Mannobiose was greater than on α-1,3 or α-1,6 Mannobioses after 24 h (figure 5).

Discussion

α-mannosidases from insects have been largely studied physiologically with regard to their hydrolytic activities in crude extracts and many other properties (Ferreira et al., 1988; Silva and Terra, 1994; Francis et al., 2002; Erthal Jr. et al., 2007) to understand their importance in protein glycosylation, both in N-glycans elongation and in catabolism of mannosylated oligosaccharides (Moremen et al., 1994). However, few enzymatic studies have been realized and no work relative to oil palm weevil α-mannosidase has been so far reported. In this respect, three extracellular α-mannosidases were purified from the larvae of oil palm weevil and their properties were examined. The purification procedure of these enzymes involved three steps including size exclusion, anion exchange and hydrophobic interaction chromatography. The two latter chromatographies were crucial to purify the digestive α-mannosidases. Indeed, the anion-exchange chromatography on a DEAE-Sepharose Fast Flow column enabled the enzyme activity to be separated into three different isoforms (RplM1, RplM2 and RplM3), while hydrophobic interaction chromatography on Phenyl-Sepharose 6 Fast Flow led to separate each
Figure 5. TLC plates showing the hydrolytic activity of the purified α-mannosidases from the digestive fluid of *R. palmarum* larvae towards different linked disaccharides. Experiments were monitored with 45 µg of each enzyme, over 24 h incubation time at 37 °C in appropriate pHs. Separation of hydrolysed products was realized with butanol-acetic acid-water 9:3.75:2.25 (v/v/v). The plates were developed with naphtho-resorcinol in ethanol and H₂SO₄ 20% (v/v). The sugar spots were visualized at 110 °C for 5 min. (a), *RplM1* activity; (b), *RplM2* activity; (c), *RplM3* activity. 1,2 Mb, α-D-Manp(1,2)-D-Manp; 1,3 Mb, α-D-Manp(1,3)-D-Manp; 1,6 Mb, α-D-Manp(1,6)-D-Manp; M, mannose.
digestive α-mannosidase from the other proteins and impurities. Similar results concerning Phenyl-Sepharose gel have been reported for purification of the endopeptidase Thr-N from *Archachatina ventricosa* (Gould) digestive fluid (Niamké et al., 1999), endoxylanase and other glycosidases of *Macrotermes subhyalinus* (Ramburg) workers (Kouamé et al., 2005; Faul et al., 2006) and digestive β-galactosidase from *R. palmarum* larvae (Yapi et al., 2007).

Regarding molecular properties of the purified enzymes, α-mannosidases *Rpl*M2 and *Rpl*M3 have monomorphic structures. Except rat liver α-mannosidase I and jack bean α-mannosidase which are tetrameric polyepitides with different subunits (Tulsiani et al., 1982b; Kimura et al., 1999), most of α-mannosidases are monomeric (Bischoff and Kornfeld, 1986). As for α-mannosidase *Rpl*M1, it functions as a dimeric structure.

The purified α-mannosidase activities on *pNP*-α-Man were maximal in acidic region. This behaviour is in accordance with the majority of insect larvae α-mannosidases e.g. *Rhagium inquisitor* (L.) (Coleoptera) and *Rhyncoschiara americana* (Wiedemann) (Diptera) (Chipoulot and Charraras, 1985; Terra and Ferreira, 1994). In contrast, only a few insect α-mannosidases such as those from *D. melanogaster* and *S. frugiperda* which are both localized in Golgi apparatus have been reported to perform maximal activity near neutral pH region.

Since the enzymes behaved like lysosomal α-mannosidases and, also retained more than half of their hydrolytic activities near neutral pH region (data not shown), they would be advantageous for treating the lysosomal storage disorder α-mannosidosis by enzyme replacement. Indeed, the enzyme replacement therapy consists of a lysosomal α-mannosidase, which is formulated in an isotonic solution (Neufeld, 2004).

The optimum temperatures of these enzymes are in good agreement with those of the majority of α-mannosidases and other glycosidases purified from plants (Jagadeesh et al., 2004; Ahi et al., 2007), microbials (Yoshida et al., 1993; Gaikwad et al., 1995) and insects (Faul et al., 2006; Yapi et al., 2007) which are most of the time mesophilic enzymes. The digestive α-mannosidase activities from the oil palm weevil larvae were tested after a long time of pre-warming (150-180 min). Most published reports determined thermal stability over a very short incubation time, usually 10 min (Ichishima et al., 1981; Yoshida et al., 1993; Maruyama et al., 1994), but a long-term stability study gives more meaningful results, especially useful when lengthy concentration or purification steps (polyethylene glycol concentration or gel filtration) have to be employed.

The behaviour of the enzymes in presence of divalent cations tested and cation chelator (EDTA) is tempting to speculate that the concerning divalent cations are not necessary for enzyme catalytic activities. However, the sensitivity of the enzymes to pCMB and DTNB leads to assume that –SH groups participate probably in the enzymic reaction. This assumption is supported by the fact that L-cysteine, an amino acid containing –SH group, inhibited the concerning enzymes activity. In contrast, the particular enhanced effect of 2-mercaptoethanol on *Rpl*M1 activity suggests that, hydroxyl group of this molecule participate in this enzyme reaction compare to L-cystein which is also a small molecule as opposed to pCMB and DTNB.

Inhibitory effect is also noticed when detergents were tested on the three enzymes activities. These results contrast with those reported for rat liver α-mannosidases (Bonay et al., 1992). So, the concerning detergents should be avoided in these enzymes preparations. Nevertheless, the particular inhibitory effect (34%) that displays a non-ionic detergent (nonidet-P40) on *Rpl*M3 activity attracted our attention. This could be probably due to the enzyme’s structure. Indeed, it is possible that the active site of the enzyme contains sites for non-ionic ligands as in bovine serum albumin structure (Wasylewski and Kozik, 1979). Thus, nonidet-P40 may affect the activity of *Rpl*M3 by modifying its catalytic environment. Inhibition by this neutral detergent at extremely lower concentration (0.005%) had previously been reported on a rat calcium-activated ATPase (Batchelor and Stanworth, 1981).

The sensitivity of digestive α-mannosidases from *R. palmarum* larvae to *Sw* and *DIM* (specific inhibitors of α-mannosidases) largely matches the inhibition profile of lysosomal α-mannosidases previously characterized.

It is well known that pKa of *Sw* is 7.4 and thus, it would be fully ionized at pH 5.0 (Dorling et al., 1980). This may account for the apparent specificity of *Sw* for *R. palmarum* larvae α-mannosidases which show maximum activity at pH 4.5 and 5.0. Indeed, the ionization of *Sw* could improve its fixation in the catalytic centre of the enzymes. Using molecular models, it can be seen that the configuration of secondary hydroxyl groups of *Sw* is identical to that in DIM (Cenci di Bello et al., 1989). This observation supports the similar effect of DIM on the three digestive enzymes. However, the moderate effect of DIM may be explained by the accessibility of these compounds to the catalytic centre.

DMNJ and Kif, both pyranose analogues, are normally not known to inhibit class II α-mannosidases (Daniel et al., 1994; Moremen et al., 1994). Taken together, these results suggest that the three α-mannosidases from the digestive fluid of oil palm weevil larvae belong to the catabolic group of α-mannosidases with close resemblance to lysosomal α-mannosidases.

α-mannosidases *Rpl*M1, *Rpl*M2 and *Rpl*M3 are restricted towards the synthetic aryl-substrate (*pNP*-α-Man) normally acted upon by α-mannosidase, since they did not possess any other tested glycosidase activities. Based on their substrate specificity and their high activities in acidic regions (pH 4.5; 5.0), these enzymes behaved like lysosomal α-mannosidases. This hypothesis is supported by the fact that other extracellular enzymes are hydrolases and one finds glycosidase, phosphatase and protease activity. As for the lysosome hydrolases to which they bear a close resemblance, they are not very specific (Forestier, 1992). Indeed, these α-mannosidases display a broad natural substrate specificity by hydrolysing α-1,2; α-1,3 and α-1,6 mannosibiose linkages. Lysosomal α-mannosidases with these characteristics have previously been identified and purified from several sources as *Dictyostelium discoideum* (Schatzke et al., 1992) and a variety of mammalian tissues (Opheim and Touster, 1978; De Gasperi et al., 1991).

The three broad specific α-mannosidases are thought to
be very important and are presumably involved in cleaving the carbohydrate moieties of glycoproteins and in finishing the digestion of α-mannosylated compounds contained in R. palmarum larvae’s feeding habits and diets.

This study showed that the three α-mannosidases (RpmM1, RpmM2 and RpmM3) purified from the digestive fluid of oil palm weevil larvae behaved like lysosomal α-mannosidases by combination of a low pH optimum, broad natural substrate specificity, activity towards the synthetic aryl-substrate pNP-α-Man and a sensitivity to Sw. These enzymes due to their characteristics above could be useful for bioindustrial mannan removal by recycling glycoproteins and mannosylated compounds. They could also find application in pharmaceutical industries since they expressed lysosomal α-mannosidase activity. In addition, the inhibition profile of these enzymes should much contribute to the R. palmarum larva’s control.

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