

α -Mannosidases from the Digestive Fluid of *Rhynchophorus Palmarum* Larvae as Novel Biocatalysts for Transmannosylation Reactions

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Abstract Two biological fluids, namely hemolymph and digestive fluid from the larval stage of *Rhynchophorus palmarum* Linnaeus, a serious pest in agroecosystem exploiting oil palm, were screened for hydrolytic activities, by the use of synthetic and natural glycoside substrates. Several exo and endoglycosidase activities were observed but, the interesting α -mannosidase activity (0.41 ± 0.04 UI) had attracted our attention. So, we have previously demonstrated that this activity harbours four distinctive α -mannosidase isoforms named *RplM*, *RplM1*, *RplM2* and *RplM3*. We have extended this work to determine the ability of these enzymes to catalyze synthesis reactions. Finally, we have revealed that, α -mannosidases from the digestive fluid of *R. palmarum* larvae catalyze transmannosylation reactions. The stability of the enzymes and the optimization of the transfer product yield were studied as functions of pH, enzyme unit, starting concentration of donor or acceptor and time. It was shown that, in experimental optimum conditions, average yields of 12.34 ± 0.75 , 12.15 ± 0.79 , 5.59 ± 0.35 and $8.43 \pm 0.50\%$ were obtained for the α -mannosidases *RplM*, *RplM1*, *RplM2* and *RplM3*, respectively. On the basis of this work, α -mannosidases from the digestive fluid of *Rhynchophorus palmarum* larvae appear to be a valuable tool for the preparation of neoglycoconjugates.

Keywords α -Mannosidases · Transmannosylation reactions · Oil palm weevil larvae · *Rhynchophorus palmarum* · Coleoptera

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Introduction

The recent increase in papers dealing with all aspects of insect digestive enzymes seems to have two major causes. One is the finding that insects provide excellent model for studying digestive enzymes, particularly because there are species adapted to almost all kinds of habitats and feeding habits. The second is the realization that the digestive tract is the major interface between the insect and its environment. In this respect, insect enzymes involved in digestion of lipids (lipases), proteins (proteases) and carbohydrates (carbohydrases) have been extensively reviewed [1, 2]. Since that time, there has been significant progress in insect digestive enzymology.

Carbohydrates digestive enzymes are glycosidases and although their originated function is the cleavage of glycosidic linkages, glycosidases can also be induced to serve as glycosidic-bond forming catalysts [3]. The pivotal roles of glycostructures in biological processes have become increasingly evident [4, 5]. So, efforts to understand and control the bioprocessing of carbohydrates are contributing much to glycobiology and for the development of new therapeutic strategies [6, 7]. In this respect, enzyme-based strategies for the synthesis of oligosaccharides represent emerging technologies that have the potential to greatly simplify glycan assembly to the detriment of chemical methods. Furthermore, the recent dynamic development of glycosciences has brought about the increasing need for new glycostructures, and glycosidases are widely used for this aim [8]. These enzymes are advantageous because they tolerate environmental stress and thanks to their broad substrate specificity, they are able to accept a wide range of cheap donors with different aglycon moieties and acceptors [9, 10]. These attractive characteristics have aroused a tendency to search cheap glycosidases, quite robust to handle with interesting transglycosylation capabilities. Thus, although they often exhibit a poor regioselectivity and sometimes low yields, they are readily available from different sources. Thus, glycosidases from several sources have been largely explored in transglycosylation reactions [11–13]. Concerning α -mannosidases, they have already served in oligomannosides synthesis reactions. However, the major sources originated from plants, usually Jack bean and almond α -mannosidases [14–17].

Our contribution in pursuit of new glycosidases (easy to obtain and purify, robust to handle and able to catalyze transglycosylation reactions) comes from the observation of a particular behaviour in nature. Indeed, *Rhynchophorus palmarum* larvae are known to feed exclusively on live vegetative tissue within the trunk of palms, often destroying the apical growth area and causing eventual death of the palm [18]. Therefore, it was suggested that this bug borer would certainly have enzymatic system that enabled it to hydrolyze polysaccharides as celluloses and hemicelluloses, which are the major constituents of plant cell walls [19]. In this respect, we have screened the hemolymph and the digestive fluid from the larval stage of *R. palmarum* for several glycosidase activities in order to explore the best one for biotechnological application. α -mannosidase activity from the digestive fluid was interesting than most of the other tested glycosidases. So, in previous studies, we have purified and characterized four isoforms of α -mannosidases namely *RplM*, *RplM1*, *RplM2* and *RplM3* [20, 21]. Through extensive biochemical characterizations, these α -mannosidases were distinguished on the basis of their size, physicochemical and kinetic properties. In the current study, data on screening are discussed, and the purified α -mannosidases are explored for their potential to catalyze transmannosylation reactions. This is the first report on an insect larva α -mannosidases with transmannosylation activities.

Materials and Methods

Enzymatic Source and Biological Fluids Extraction

Oil palm weevil *R. palmarum* larvae were collected locally in Côte d'Ivoire from their host trees (oil palm *Elaeis guineensis*). The biological fluids (hemolymph and digestive fluid) were extracted in an ice bath after clipping the head of the larva. Hemolymph (H) and digestive fluid (DF) were obtained by drawing fluids into syringes. The two solutions were filtered through cotton wool and then centrifuged for 30 min at $10,000\times g$ ($4\text{ }^{\circ}\text{C}$) to remove particulates. The resulted supernatants constituted the enzymatic sources (H or DF).

Chemicals

para-Nitrophenyl- (*p*NP-) glycopyranoside substrates (*p*NP- α -D-mannopyranoside, *p*NP- α -D-glucopyranoside, *p*NP- β -D-glucopyranoside, *p*NP- α -D-galactopyranoside, *p*NP- β -D-galactopyranoside, *p*NP- α -L-fucopyranoside, *p*NP- α -L-arabinopyranoside, *p*NP- β -D-xylopyranoside), *para*-nitrophenol (*p*NP), sucrose, starch, carboxymethylcellulose, Inulin, xylan and glucose were purchased from Sigma Aldrich. Bovine serum albumin (BSA) was from Fluka Biochemika. The α -mannosidases *Rpl*M, *Rpl*M1, *Rpl*M2 and *Rpl*M3 (EC 3.2.1.24) used for transmannosylation reactions originated from the purified enzymes collection of "Laboratoire de Biotechnologies, UFR Biosciences, Université de Cocody (Abidjan, Côte d'Ivoire)". The α -mannosidases were purified as described previously [20, 21]. Their purification procedures involved at least three steps including ammonium sulphate saturation, size exclusion, anion exchange and hydrophobic interaction chromatography. All other chemicals and reagents are commercially available and of analytical grade.

Hydrolytic Screening of Synthetic and Natural Substrates by Biological Fluids

For synthetic aryl-glycoside hydrolytic activities, each enzymatic source (H, DF or hemolymph and digestive fluid (H+DF) in a ratio of one to one (v/v)) was mixed in a total volume of 150 μl composed of *para*-nitrophenyl-glycoside substrate (2.5 mM, final concentration), in 100 mM starting concentration of sodium acetate buffer pH 5.0. The reaction mixture was incubated with shaking at $37\text{ }^{\circ}\text{C}$ for 10 min. The liberated *p*NP was quantified spectrophotometrically at 410 nm under alkaline conditions (2% w/v, Na_2CO_3) referred to a standard *p*NP (absorbance as a function of concentration) curve obtained in similar conditions.

For the natural substrates hydrolysis, the total volume was 250 μl , composed of 50 μl of the enzymatic source (H, DF or H+DF), 100 μl of substrate (0.25% w/v, final concentration) in 100 mM starting concentration of sodium acetate buffer pH 5.0. The reaction mixture was incubated with shaking at $37\text{ }^{\circ}\text{C}$ for 30 min and, the reactions were stopped by adding 150 μl of dinitro salicylic acid (DNS) and heating the resulted solution at $100\text{ }^{\circ}\text{C}$ for 5 min [22]. The liberated reducing sugars were quantified spectrophotometrically at 540 nm referred to a standard glucose (absorbance as a function of concentration) curve obtained in similar conditions.

All values were determined in triplicate and corrected for auto-hydrolysis of the substrate. One unit of enzymatic activities in the two cases (synthetic and natural substrates) released 1 μmol of liberated product (*p*NP or reducing sugar) per min under the above conditions, respectively. The activity was expressed as micromole per min per mg of protein (UI).

Estimation of Proteins Concentration

The concentration of the proteins was measured using the Folin ciocalteus method [23]. BSA was used as the standard protein.

Transmannosylation Reactions

The ability of α -mannosidases *RplIM*, *RplIM1*, *RplIM2* and *RplIM3* from the digestive fluid of oil palm weevil *R. palmarum* larvae [20, 21] to catalyze transmannosylation reactions was tested with *pNP*- α -D-mannopyranoside (*pNP*- α -Man) used either as the mannosyl donor and acceptor.

In typical experiment, the transmannosylation reactions were carried out at 37 °C in a total reaction mixture of 60 μ l containing 400 mM of citrate/phosphate buffer pH 4.0. The reactions were stopped by immersion in boiling water for 3 min followed by cooling in ice bath. Prior to each HPLC analysis, the reaction mixtures were filtered using Ultrafree-MC filter (0.45 μ m) devices (Millipore). Phenol was used as the internal standard to correct chromatographic product areas. Ten microliters (10 μ l) aliquots of each reaction mixture always containing the internal standard (10 mM final concentration) were analyzed quantitatively by HPLC at room temperature. The column used was SPHERECLONE 5 μ ODS (2) (250 mm \times 4.60 mm; Phenomenex) and phenolic compounds were detected at 254 nm with a SPECTRA SYSTEM UV 1000 detector. The elution was done with a BECKMAN 114 M solvent delivery module pump, at a flow rate of 1 ml/min using degassed methanol/water in the ratio 35:65 (v/v) as eluent. The chromatograms were obtained with a SHIMADZU C-R8A CHROMATOPAC V1.04 integrator.

The detailed experimental conditions for studying parameters likely to affect the transmannosylation reactions (pH, enzyme unit, donor or acceptor concentration and time) are given below.

Determination of Optimum pH The pH values were determined at 25 °C. For determination of optimum pH, the transmannosylation reactions were performed by incubating at 37 °C for 1 h each α -mannosidase (20 μ l) in a pH range of 3.5 to 7.0 (citrate/phosphate buffer, 400 mM), with 10.5 mM final concentration of *pNP*- α -Man. The reactions were stopped by heating (3 min in boiling water), and the mixed products analyzed by HPLC as described in the typical transmannosylation reaction above.

Determination of Enzyme Unit For this study, the optimum pH determined for each enzyme was fixed. A amount of each α -mannosidase (0 to 11 μ g) was mixed with 10.5 mM of *pNP*- α -Man, in 400 mM citrate/phosphate buffer at appropriate optimum pH. The transmannosylation reactions were performed at 37 °C for 1 h. The reactions were stopped by immersion in boiling water for 3 min and the products quantified by HPLC as described in the typical transmannosylation reaction above.

Determination of Mannosyl Donor or Acceptor Concentrations The influence of mannosyl donor or acceptor concentrations (0 to 21 mM) on the transmannosylation reactions was determined by using *pNP*- α -Man either as donor and acceptor of the mannosyl residue. Under the optimum conditions of pH and enzyme unit, α -mannosidases *RplIM*, *RplIM1*, *RplIM2* and *RplIM3* were separately incubated at 37 °C for 1 h with different concentrations of the aryl substrate (*pNP*- α -Man). The reactions were stopped by immersion in boiling

water for 3 min and the products quantified by HPLC as described in the typical transmannosylation reaction above.

Determination of Optimum Time To determine the optimum time of transmannosylation, the optima conditions of pH, enzyme unit and donor or acceptor concentrations were kept. Only the times of transmannosylation reactions varied from 0 to 5 h. At regular time interval, aliquots of the reaction mixture were withdrawn, heated in boiling water for 3 min to stop the reaction and the resulted products quantified by HPLC as above.

Transmannosylation in Optimum Conditions Ultimately, the optimum conditions of pH, enzyme unit, donor or acceptor concentrations and time reaction were fulfilled to perform a unique transmannosylation reaction with α -mannosidases *Rp1tM*, *Rp1M1*, *Rp1M2* and *Rp1M3* purified from the digestive fluid of oil palm weevil *R. palmarum* larvae. These reactions were conducted in triplicate with regard to the typical conditions described in the other experiments, and the products were quantified as described previously (HPLC).

Estimation of the Yield of Transmannosylation

The hydrolysis of 1 mol of *pNP*- α -Man liberates 1 mol of *pNP* and 1 mol of mannose. Consequently, the area of the released *pNP* correspond with that of the mannosyl residue. After adjusting areas with the internal standard (phenol), the yield of transmannosylation was calculated as follow: area of transfer product/area of released *para*-nitrophenol \times 100 (%).

Results

Screening for Glycosidases Hydrolytic Activities

The potential of synthetic and natural glycoside derivatives to act as substrates for the biological fluids (H, DF and H+DF) of *R. palmarum* larvae was assayed and the results are shown in Table 1. We noticed that the hemolymph and the digestive fluid of the larval stage of the insect are able to hydrolyze all the substrates with varying degrees. However, the hydrolysis of each substrate observed from the usage of the digestive fluid is better than that showed in the presence of either the hemolymph or the mixture (*v/v*) of the two biological fluids (H+DF), suggesting that digestive fluid is the best enzymatic source (Table 1).

As regards *pNP*-glycoside substrates hydrolysis by the digestive fluid, the α -mannosidase activity was the highest (0.41 ± 0.04 UI). Apart from this activity, β -glucosidase, β galactosidase, α -glucosidase and α -fucosidase activities are also interesting with specific activity values of 0.36 ± 0.03 , 0.29 ± 0.02 , 0.24 ± 0.02 and 0.10 ± 0.01 UI, respectively. The other glycoside (α -galactoside, α -arabinoside and β -xyloside) activities are less interesting (activity values inferior to 0.02 UI; Table 1).

Concerning natural substrates hydrolysis, we noticed as in synthetic substrates (*pNP*-glycosides) the tendency of the digestive fluid to hydrolyze these substrates efficiently. The α -amylase activity in this biological fluid was extremely higher (17.10 ± 2.81 UI). Compared with it, there are virtually no sucrose, carboxymethylcellulose, inulin and xylan hydrolyzing activity. These activities were less than 1 UI (Table 1).

Table 1 Screening of biological fluids from the larval stage of the oil palm weevil *Rhynchophorus palmarum* for glycosidase activities over synthetic and natural substrates.

Substrates	Specific activity ($\mu\text{mol}/\text{min}/\text{mg}$)		
	DF	H	H+DF (v/v)
Synthetic			
<i>p</i> NP- α -D-mannopyranoside	0.41 \pm 0.04	0.07 \pm 0.02	0.17 \pm 0.02
<i>p</i> NP- β -D-glucopyranoside	0.36 \pm 0.03	0.03 \pm 0.01	0.15 \pm 0.03
<i>p</i> NP- β -D-galactopyranoside	0.29 \pm 0.02	0.04 \pm 0.01	0.13 \pm 0.01
<i>p</i> NP- α -D-glucopyranoside	0.24 \pm 0.02	0.03 \pm 0.01	0.11 \pm 0.02
<i>p</i> NP- α -L-fucopyranoside	0.10 \pm 0.01	0.03 \pm 0.01	0.05 \pm 0.01
<i>p</i> NP- α -D-galactopyranoside	0.01 \pm 0.01	\leq 0.01	\leq 0.01
<i>p</i> NP- α -L-arabinopyranoside	0.01 \pm 0.01	\leq 0.01	\leq 0.01
<i>p</i> NP- β -D-xylopyranoside	0.01 \pm 0.01	\leq 0.01	\leq 0.01
Natural			
Starch	17.10 \pm 2.81	6.06 \pm 0.94	5.78 \pm 1.01
Sucrose	0.59 \pm 0.05	0.12 \pm 0.02	0.16 \pm 0.02
Carboxymethyl cellulose	0.46 \pm 0.03	0.10 \pm 0.02	0.24 \pm 0.03
Inulin	0.24 \pm 0.04	0.04 \pm 0.01	0.21 \pm 0.03
Xylan	0.21 \pm 0.02	0.07 \pm 0.01	0.11 \pm 0.01

DF digestive fluid, H hemolymph, H+DF (v/v) mixture of digestive fluid and hemolymph (volume per volume)

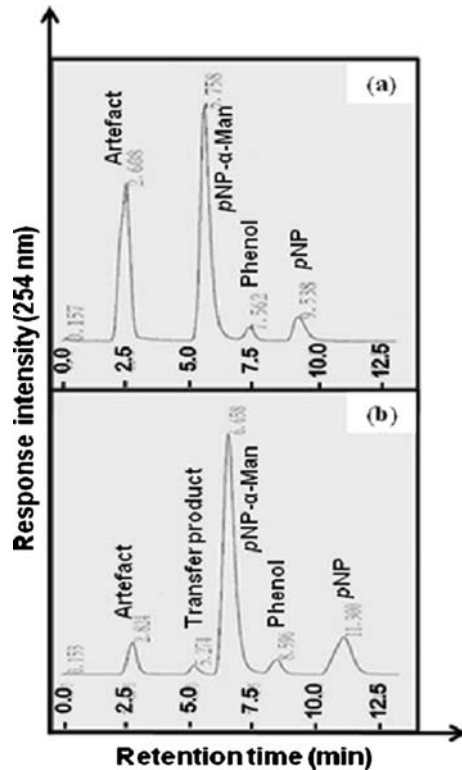
Potentiality of the α -mannosidases to Catalyze Transmannosylation

α -mannosidases *RplM*, *RplM1*, *RplM2* and *RplM3* from the digestive fluid of *R. palmarum* larvae were assayed for their ability to catalyze transmannosylation by increasing *p*NP- α -Man concentration (10.5 mM, final concentration). Analysis of the reaction mixture by high-performance liquid chromatography (Fig. 1) has confirmed this ability. The chromatogram (Fig. 1b) shows a fifth pic of newly synthesized product (transfer product) with a retention time of 5.274 min, sited between these of the artefact (2.824 min) and the *p*NP- α -Man substrate (6.658 min). In this context, some physicochemical parameters usually determined to optimize the experimental conditions of transglycosylation reactions were studied.

Influence of pH

Different pHs varying from 3.5 to 7.0 of a citrate/phosphate buffer (400 mM) were tested for their susceptibility to influence transmannosylation reaction catalyzed by α -mannosidases *RplM*, *RplM1*, *RplM2* and *RplM3*. Maximum transfer yields of 5.87%, 6.15%, 4.18% and 4.99% were obtained at pH 4.0 for α -mannosidases *RplM*, *RplM1*, *RplM2* and *RplM3*, respectively (Table 2). Calculation of the yield of transmannosylation was possible only at pH 3.5 and 4.0. From pH 4.0 to 6.0, although the synthesized product was observed, it was impossible to calculate the yields because of missing area of liberated *para*-nitrophenol useful for doing calculation (Table 2, Fig. 2b). However, above pH 6.0, the transmannosylation product was not observed (Table 2).

Fig. 1 HPLC chromatograms of the typical transmannosylation assay catalyzed by α -mannosidases *RplM*, *RplM1*, *RplM2* and *RplM3* from the digestive fluid of *Rhynchophorus palmarum* larvae. The experiments were monitored at 37 °C for 1 h by incubating appropriate amount of each α -mannosidase with pNP- α -Man (10.5 mM) substrate used either as the donor or the acceptor of the mannosyl residue, in 400 mM citrate/phosphate buffer pH 4.0. Ten microliters (10 μ l) aliquots of the reaction mixture were quantified. Phenol was used as the internal standard. **a** control, **b** assay



Influence of Enzyme Unit

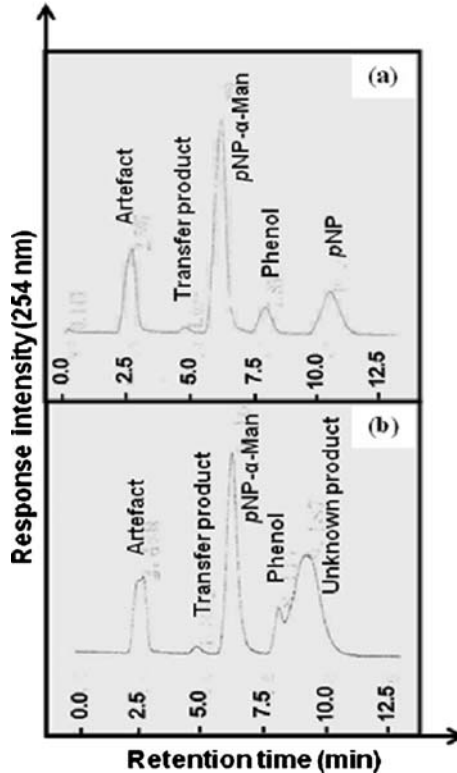
The efficiency of the α -mannosidases from *R. palmarum* larvae in catalysis of transmannosylation reactions was also largely dependent on the respective enzyme units (Fig. 3). The best yields (6.06%, 6.32%, 4.61% and 5.21%) were obtained with enzyme

Table 2 Influence of pH on the yields of transmannosylation reaction catalyzed by α -mannosidases *RplM*, *RplM1*, *RplM2* and *RplM3* from the digestive fluid of *Rhynchophorus palmarum* larvae.

pH values	Transfer product area	pNP area	Yield of transmannosylation (%)			
			<i>RplM</i>	<i>RplM1</i>	<i>RplM2</i>	<i>RplM3</i>
3.5	+ or –	+	3.81	5.92	0	0
4.0	+	+	5.87	6.15	4.18	4.99
4.5	+	–	nd	nd	nd	nd
5.0	+	–	nd	nd	nd	nd
5.5	+	–	nd	nd	nd	nd
6.0	+	–	nd	nd	nd	nd
6.5	–	–	0	0	0	0
7.0	–	–	0	0	0	0

– Absent, + present; nd not determined

Fig. 2 HPLC chromatograms of the effect of pH on the liberated *para*-nitrophenol in transmannosylation assay catalyzed by α -mannosidases *Rpl1M*, *Rpl1M1*, *Rpl1M2* and *Rpl1M3* from the digestive fluid of *Rhynchophorus palmarum* larvae. The experiments were performed at 37 °C for 1 h by incubating appropriate amount of each α -mannosidase with 10.5 mM of *pNP*- α -Man in 400 mM citrate/phosphate buffer ranging from pH 3.5 to 7.0. The *pNP*- α -Man substrate was used either as the donor or the acceptor of the mannosyl residue. Ten microliters (10 μ l) aliquots of the reaction mixture were quantified. Phenol was used as the internal standard. **a** pH 4.0; **b** pH 4.5



units of around 3.63 μ g (*Rpl1M*), 4.40 μ g (*Rpl1M1*), 9.08 μ g (*Rpl1M2*) and 7.04 μ g (*Rpl1M3*), respectively. On both sides of these enzyme unit values, the ability to catalyze the transmannosylation reactions was affected.

Donor or Acceptor Concentrations Dependence

Transmannosylation yields were considerably influenced by variation in donor or acceptor (*pNP*- α -Man) concentrations (Fig. 4). Maximum transmannosylation was observed for each α -mannosidase in the presence of 21 mM of donor or acceptor. At this concentration, optimum yields of 9.80%, 10.09%, 5.64% and 6.57% were obtained for α -mannosidases *Rpl1M*, *Rpl1M1*, *Rpl1M2* and *Rpl1M3*, respectively. Above this concentration (21 mM) the substrate *pNP*- α -Man was not soluble in the citrate/phosphate buffer (400 mM).

Kinetically-Controlled Reactions

Transmannosylation kinetics were also studied as a function of incubation time. The yields (%) of the transfer products obtained at various times (min) are shown in Fig. 5. In the initial stage of the reaction, much oligomannosides were synthesized by the transmannosylation activity of the enzymes. However, as the reaction proceeded, the transmannosylation product was gradually reduced (Fig. 5). The optimum times of transmannosylation were 45 min with a yield of 5.93% for *Rpl1M2* and 2 h for *Rpl1M*, *Rpl1M1* and *Rpl1M3*. At 2 h, yields of 13.08%, 12.94% and 8.92% were obtained, respectively for the latter three.

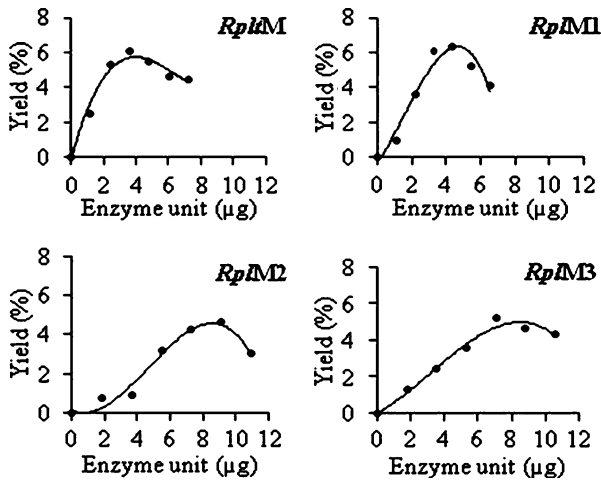


Fig. 3 Effect of enzyme unit on transmannosylation activity of α -mannosidases *RplM*, *RplM1*, *RplM2* and *RplM3* from the digestive fluid of *Rhynchophorus palmarum* larvae. The optimum enzyme units were measured using different concentrations (μg) of each enzyme. Reactions were performed at 37 °C for 1 h by incubating enzymes with 10.5 mM of *pNP*- α -Man in 400 mM citrate/phosphate pH 4.0. The *pNP*- α -Man substrate was used as the donor and the acceptor of the mannosyl residue. Ten microliters (10 μl) aliquots of the reaction mixture were quantified by HPLC. Phenol was used as the internal standard

Progress of Yields and Transmannosylation in Optimum Conditions

Following the study of each parameter below (pH, enzyme unit, donor or acceptor concentrations and time), we have noted in optimum conditions, an improvement of transmannosylation yields as the studies went along. The yields (%) increased cumulatively

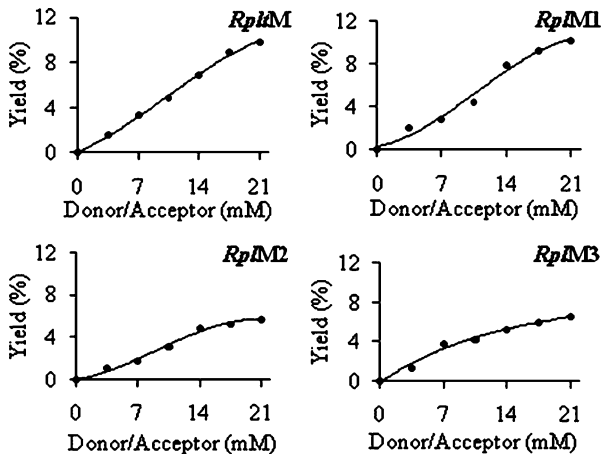


Fig. 4 Effect of donor or acceptor concentrations on transmannosylation activity of α -mannosidases *RplM*, *RplM1*, *RplM2* and *RplM3* from the digestive fluid of *Rhynchophorus palmarum* larvae. The optimum concentrations were measured using various soluble concentrations (mM) of *pNP*- α -Man as the donor and the acceptor of the mannosyl residue. Reactions were performed at 37 °C for 1 h by incubating the optimum enzyme units of each α -mannosidase at different concentrations of *pNP*- α -Man, in 400 mM citrate/phosphate pH 4.0. Ten microliters (10 μl) aliquots of the reaction mixture were quantified by HPLC. Phenol was used as the internal standard

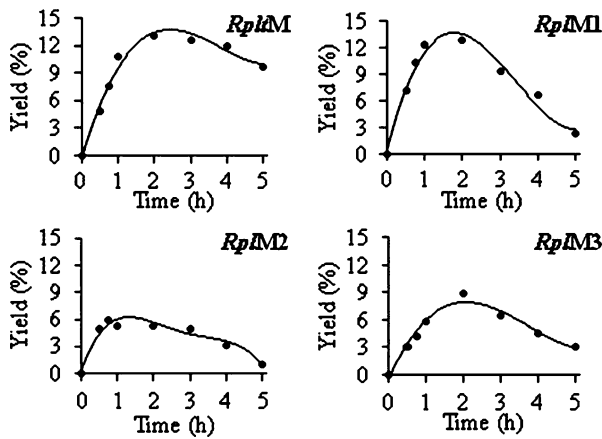


Fig. 5 Time course of transmannosylation reaction catalyzed by α -mannosidases *RplIM*, *RplIM1*, *RplIM2* and *RplIM3* from the digestive fluid of *Rhynchophorus palmarum* larvae. The experiments were performed at 37 °C for different times (0 to 5 h). The optima conditions of enzyme units and donor or acceptor concentrations were fulfilled to incubate the α -mannosidases in 400 mM citrate/phosphate buffer pH 4.0. The *p*NP- α -Man substrate (21 mM) was used as the donor and the acceptor of the mannosyl residue. Ten microliters (10 μ l) aliquots of the reaction mixture were quantified by HPLC. Phenol was used as the internal standard

from optimum pH to optimum time as follow: from 5.87% to 13.08% for *RplIM*, from 6.15% to 12.94% for *RplIM1*, from 4.18% to 5.93% for *RplIM2* and from 4.99% to 8.92% for *RplIM3* (Table 3). When the optimum conditions were fulfilled to perform a unique transmannosylation reaction (assay in triplicate), average yields of around $12.34 \pm 0.75\%$, $12.15 \pm 0.79\%$, $5.59 \pm 0.35\%$ and $8.43 \pm 0.50\%$ were obtained for α -mannosidases *RplIM*, *RplIM1*, *RplIM2* and *RplIM3*, respectively.

Discussion

Enzymes are essential biocatalysts to living organisms. Indeed, different aspects of biological functions have been resolved by studying physiological enzymes [2, 24]. With the aim of diversifying sources of cheap glycosidases for potential biotechnological applications, the hemolymph and the digestive fluid of the larval stage of *R. palmarum*,

Table 3 Yields of transfer product in optimized conditions of the transmannosylation reaction catalyzed by α -mannosidases *RplIM*, *RplIM1*, *RplIM2* and *RplIM3* from the digestive fluid of *Rhynchophorus palmarum* larvae.

Optimum condition	Yield of transmannosylation (%)			
	<i>RplIM</i>	<i>RplIM1</i>	<i>RplIM2</i>	<i>RplIM3</i>
pH	5.87	6.15	4.18	4.99
Enzyme unit	6.06	6.32	4.61	5.21
Donor/acceptor	9.80	10.09	5.64	6.57
Time	13.08	12.94	5.93	8.92
All parameters fulfilled (assay in triplicate)	12.34 ± 0.75	12.15 ± 0.79	5.59 ± 0.35	8.43 ± 0.50

RplIM, *RplIM1*, *RplIM2* and *RplIM3* are α -mannosidases

were screened for glycosidase activities over synthetic and natural substrates. These studies led to the assertion that the biological fluids contain several hydrolytic enzymes and one finds exo and endoglycosidase activities. These hydrolytic activities were better in the digestive fluid suggesting that it would be directly implicated in digestion. However, the lower hydrolytic yields observed by mixing the hemolymph and the digestive fluid suggests that physiologically, the two fluids would not function synergistically. Albeit overall patterns of digestion and digestive enzyme properties correlate well with the phylogenetic position of the insect, each biological fluid displayed an originated physiological function [2]. Those of the digestive fluids were particularly to allow digestion [25, 26].

The glycosidase activity from the digestive fluid of the oil palm weevil (*R. palmarum*) larvae catalyzes predominantly hydrolysis of starch, α -D-mannoside, β -D-glucoside, β -D-galactoside, α -D-glucoside and even α -L-fucoside. The highest α -mannosidase activity may suggest the presence of mannose-rich derivatives in the beetle's diets. As cellulose and hemicelluloses (the major plant cell wall structure, [19]), made up of complex β -linked structures, the cellulase, β -glucosidase and β -galactosidase activities observed were not surprising. However, the lower cellulase activity may be discussed. Indeed, cellulose digestion in insects is rare, because the dietary factor that usually limits growth in plant feeders would be protein quality and not the type of carbohydrate present [2]. Only few insects secrete enzymes able to hydrolyse crystalline cellulose [27, 28] because, insects were generally dependent on symbionts for cellulose digestion [29, 30]. Furthermore, earlier studies have revealed the presence of symbiotic microorganisms in the gut of another pest, namely red palm weevil of the genus *Rhynchophorus* [31].

The efficient degradation of starch may result from the concomitant action of different enzymes. Indeed, three enzymes are known to act specifically on long α -glucan chains such as native starch. α -amylase (EC 3.2.1.1) that catalyses the endohydrolysis of linkages in a random manner, followed by β -amylase (EC 3.2.1.2) that removes successive maltose units, and glucoamylase (EC 3.2.1.3), successive glucose units from the non-reducing ends of the chains. Similar results were reported when studying the α -amylase activity in *Musa domestica* larval midgut [32].

Screening for enzyme activity is a practical approach in enzymology to explore biocatalysts. This approach enabled us to reveal the interesting α -mannosidase activity from the digestive fluid of *R. palmarum* larvae. Our recent investigations have shown that this activity originated from four isoforms of α -mannosidase (EC 3.2.1.24), namely *RpltM*, *RplM1*, *RplM2* and *RplM3* [20, 21]. The present study extends the previous one by determining the potentiality of these enzymes to catalyze transmannosylation reactions. Preliminary tests have shown that these α -mannosidases are able to synthesize oligomannosides by reverse hydrolysis reaction using *p*NP- α -Man either as the donor or the acceptor of mannosyl residue. The ability of other sources of α -D-mannoside mannohydrolases (EC 3.2.1.24) to synthesize mannoconjugates had previously been reported [15, 17, 33]. It seems that the transfer product formed in this study is the *para*-nitrophenyl- α -dimannose. Indeed, similar product was previously obtained by performing assays with Thai and commercial Jack beans α -mannosidases using *p*NP- α -Man as substrate [16].

The experimental conditions were optimized in relation to those factors able to have an influence on the rate of transmannosylation. It must be noted that all the α -mannosidases purified from the digestive fluid of *R. palmarum* larvae operate at an optimum pH for transmannosylation reaction of 4.0. From pH 4.5 the unknown product observed (Fig. 2b) may be a *p*NP derivative due to the nearness of the two retention times. The optimum pHs of transmannosylation (pH 4.0) were different to those of the hydrolysis reactions (pHs 4.5

and 5.0) [20, 21] suggesting that pH 4.0 is favourable to the transmannosylation reactions. These differences could be due to the ionized groups in the active site of the enzymes that enabled hydrolysis and transmannosylation at once [34]. Thus, at pH 4.0, transmannosylation reactions were favoured but not hydrolysis. Dissimilarities between optimum pH of hydrolysis and transglycosylation reactions have also been reported for other glycosidases [13, 34].

Enzyme unit, donor/acceptor concentrations and time course of the reactions were also important parameters in the transmannosylation reactions. Indeed, there are optimum values for each parameter optimizing the yields of synthesis. On both sides of these values, the transmannosylation reactions were affected. The time course of the reactions is particularly important given that the product formed during the transmannosylation reaction decrease gradually as the reaction proceed. It seemed that the transfer product be used as substrate by the same enzyme and progressively hydrolyzed. Due to the possible kind of product formed (*para*-nitrophenyl- α -dimannose), we can suggest that α -mannosidases *RpltM*, *RplM1*, *RplM2* and *RplM3* catalyze the splitting of α -mannosyl residue from non-reducing terminal to liberate α -mannose. This comportment indicates that these enzymes operated by a mechanism involving the retention of the anomeric configuration [35].

In optimal conditions, transmannosylation yields varying from 5.59 ± 0.35 to $12.34 \pm 0.75\%$ were obtained at least within 45 min and 2 h. By the use of *pNP*- α -Man either as the donor and the acceptor of mannosyl residue, these yields were much higher than those reported to date with conventional and commercial sources (Jack bean) of α -mannosidases [16]. The ability of these α -mannosidases to catalyze efficiently synthesis reactions is of great interest because glycosylation is considered to be an important method for the structural modification of compounds with useful biological activities [4].

Several inventions relate to synthetic oligomannosides which replicate the biological properties of natural sugars, their preparation and their use for detecting antibodies and preventing infections [36]. It was from this perspective that mimicking of the high glycan (oligomannose) density on the virus surface has brought a new generation of synthetic Man₉ structure used as a potential immunogen for HIV vaccine development, and as a potential antiviral agent [37]. Elsewhere, it has been reported on the effectiveness recognition of a synthetic trimannoside derivative by a T7 phage peptides, suggesting an alternative issue for the development of inhibitors or drug delivery systems targeting oligosaccharides, as well as further investigations into the function of carbohydrates in vivo [38]. Furthermore, it had been demonstrated the antigenic reactivity of synthetic mannotetraose in relation to antigenic factor from serum of patients with Crohn's disease [39]. Glycosylation also allows conversion of lipophylic compounds into hydrophilic ones, thus improving their pharmacokinetics properties. Sometimes, by attaching a sugar, pharmacodynamic properties are also changed or novel and more effective drug delivery systems obtained [8]. With regard to these considerations above, the ability of the α -mannosidases from the digestive fluid of *R. palmarum* larvae to fasten together mannosyl residues, could serve in the preparation of neomannoconjugates.

To sum up this report, we can note that, screening for enzymatic activity is a practical approach in enzymology to explore new biocatalysts. Furthermore, the interesting transmannosylation properties could suggest the usage of the α -mannosidases *RpltM*, *RplM1*, *RplM2* and *RplM3* from the digestive fluid of the oil palm weevil (*Rynchophorus palmarum*) larvae as an alternative source of synthesizing biocatalysts compared with conventional and commercial α -mannosidases.

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