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Transglycosylation reactions performed by glycosyl hydrolases from the marine anaspidean mollusc *Aplysia fasciata*

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Abstract

The search for glycosyl hydrolytic activities from natural sources and the investigation of their transglycosylation potential can provide enzymes with new interesting catalytic activities for the synthesis of oligosaccharides. We focused our attention on the sea hare *Aplysia fasciata* Poiret, 1789, a common Mediterranean opistobranch mollusc, describing the results on the presence of different glycosyl hydrolases found in this organism. The activities of interest are present in the visceral mass and in the hepatopancreas extracts which were highly active with respect to those from other marine molluscs, namely an exo-α-D-glucosidase activity and a β-galactosidase, were investigated. The former is able to produce panose in high percentage by transglycosylation from maltose, or α-D-glucosides of α-methyl mannopyranoside and 2-deoxyglucose using *p*-nitrophenyl α-D-glucopyranoside as donor. The β-galactosidase from hepatopancreas produced in transgalactosylation reactions the β-Gal-(1-4)-GlcNAc as the most abundant product and Gal-Xyl disaccharides of biological importance using *p*-nitrophenyl β-D-galactopyranoside as donor and GlcNAc or benzyl α-xyloside as acceptors, respectively.

Keywords: Glycosidases; Isomaltose; Panose; Marine organisms; *Aplysia fasciata*

1. Introduction

Glycosidases which usually hydrolyze glycosidic linkages are also able, under certain conditions, to catalyze the stereospecific formation of these bonds by transglycosylation reactions [1]. Their synthetic potential appeared to be of great interest in these years as an alternative to both glycosyl-transferases and to non-enzymatic methodologies in oligosaccharide synthesis [2]. Genetic engineering manipulation of glycosidases, with the creation of their glycosynthase version, is a recent progress in the application of the synthetic potential of these biocatalysts [3].

Pyranosidic structures possessing several hydroxyl groups are often the most interesting acceptors for transglycosylation reactions catalyzed by glycosidases, hence the regioselectivity of the carbohydrate transfer is a topic of great concern. In this context the effort for the identification of different enzymes, each forming any desired glycosidic bond, is of current significance [4]. Recently an extensive screening for the presence of these enzymes (and the analysis of regioselectivity of their reactions) was conducted on several dozen enzymatic preparations mostly from commercial sources. The need for such an investigation was the construction of a library of different glycosidases [5].

Glycosyl hydrolases from marine sources have been mainly investigated for the hydrolysis of polysaccharides. Hepatopancreas glycosidases of *Aplysia kurodai* have been in fact previously studied but only α-L-fucosidase activity was reported [6] as a useful hydrolytic tool in the structural identification of fucosaccharides. Laminarin or fucoidan are typical examples in which enzymatic transformation can produce low molecular weight compounds that exhibit anticancer and anticoagulative activities. Following this approach the production of biologically active oligosaccharides is another interesting application of enzymatic hydrolytic activities. Examples were reported in the literature [7,8] for the exploitation of β-1,3-D-glucanase from the marine mollusc *Spisula sachalinensis*.

Hence the biodiversity in marine organisms make them an optimal and almost unknown source of new enzymes with

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different specificities for the formation of glycosidic bonds. The investigation of natural sources, coupled nowadays with genetic engineering techniques for easy production and modification of enzymes, will allow the construction of potent biocatalytic tools.

After a preliminary investigation on the presence of glycosyl hydrolases in different marine organisms (data not shown), we focused our attention on the sea hare *Aplysia fasciata*, a large mollusc belonging to the order *Anaspidea* which is easily collectable and very common in Mediterranean marine habitats [9]. In this paper we describe the identification of different glycosyl hydrolytic activities in *A. fasciata* and a preliminary analysis of their transfer potential in order to assess the interest in the synthetic field.

2. Materials and methods

2.1. Materials

Nitrophenyl glycosides and polysaccharides were obtained from Sigma (St. Louis, MO). Bio-Gel P2 was from Bio-Rad (Richmond, CA). Reverse-phase silica gel and TLC silica gel plates were from E. Merck (Darmstadt, Germany). All other chemicals were of analytical grade. Protein concentration was determined using the Bradford assay system (Biorad) with bovine serum albumin as standard. Acetylation of compounds was performed with pyridine/Ac_2O at room temperature. The solvents were removed by an N_2 stream, and the reaction mixture was purified by silica gel chromatography or preparative TLC. HPLC was performed on a Milton Roy apparatus equipped with UV detector (Waters Associates, USA). NMR spectra were recorded on Bruker instruments at 400 or 300 MHz. Samples for NMR analysis were dissolved in suitable solvents using the downfield shift of the signal of the solvent as internal standard. ESI-MS spectra were obtained on a Q-Tof mass spectrometer, Micro (Micromass).

2.2. Biological sample and homogenate preparation

Twenty animals were collected and each of them was carefully dissected in order to separate the external part (300 g total, including oral tentacles, rhinophores, eyes, foot, mantle and parapodia) from the visceral mass (98 g total, including digestive, excretory, blood-vascular, and reproductive systems) and hepatopancreas (52 g total). Each part was homogenized in ca. 2.5–3 v/w of 50 mM acetate buffer (pH 5), and clear protein solutions were obtained by centrifugation: external part, 310 ml, 0.95 mg/ml; visceral mass, 260 ml, 1.43 mg/ml; hepatopancreas, 170 ml, 1.19 mg/ml. Hepatopancreas and the visceral mass extracts were dialyzed and concentrated by ultrafiltration to a final concentration of 6 and 10 mg/ml, respectively.

2.3. Enzymatic assay

Glycosyl hydrolase activities were measured by using the *p*-nitrophenyl (PNP) substrates and the amount of *p*-nitrophenol formed was determined spectrophotometrically at 405 nm at alkaline pH. The reaction mixture (0.5 ml) contained 0.9 mM PNP substrate in the appropriate buffer at the chosen temperature. The reaction was initiated by addition of an appropriate amount of the protein solution. Aliquots (0.05 ml) were withdrawn at intervals, the reaction was stopped with the addition of 1 M sodium carbonate (0.45 ml) and the increase in absorbance at 405 nm was measured. One unit corresponds to the amount of enzyme hydrolyzing one nanomole of substrate in 1 min/mg protein.

Preliminary screening of glycosidase activities on crude extracts was performed by using PNP substrate 0.9 mM in *K*-acetate buffer 50 mM (pH 5.5) at 35 °C; subsequently, α-glucosidase activity was assayed under standard condition at 37 °C in *K*-phosphate buffer 50 mM (pH 6.3) by using 0.9 mM *p*-nitrophenyl α-D-glucopyranoside.

2.4. Heat, acidity and organic solvent effects on α-D-glucohydrolytic activity

The activity was measured as a function of pH at 35 °C in the presence of 0.9 mM substrate as described above. In particular, 50 mM *K*-acetate (pH 4.0, 4.7, and 5.2) and 50 mM *K*-phosphate (pH 5.6, 6.5, 7.1, and 7.4) were used. The activity was determined as a function of the temperature in the range 30–55 °C, in 50 mM *K*-phosphate (pH 6.3), in the presence of 0.9 mM substrate. Thermal stability against irreversible processes was measured in 50 mM *K*-phosphate buffer (pH 6.3). Protein samples (1 mg/ml) were incubated at 34 and 39 °C; aliquots were withdrawn at intervals, cooled in ice, and the residual enzymatic activity was measured under standard conditions. Stability in the presence of organic solvent (10%, v/v) was measured in 50 mM *K*-phosphate buffer (pH 6.3) at 34 °C. The protein concentration was 1 mg/ml. Aliquots were withdrawn at intervals and the residual enzymatic activity was measured under standard conditions.

3. Enzymatic reactions

3.1. Hydrolysis reactions

Curdlan (13.5 mg), xylan from birchwood (22 mg) or fucoidan from *Fucus vesiculosus* (18 mg) were suspended in 1 ml of 50 mM sodium acetate buffer (pH 5.6) and 400 μl of hepatopancreas extract (6 mg/ml) was added. The product formation was monitored by TLC using EtOAc: CH_3COOH:2-propanol:$HCOOH$:H_2O (25:10:5:1:15) as solvent system at different time intervals.

Maltose and isomaltose, maltotri- to maltohexaose, sucrose, trehalose, soluble starch and pullulan (1–5 mg) were dissolved in 1 ml of 50 mM phosphate buffer (pH 6.4) and

the reaction was started by adding 100 μl of visceral mass extract, 1.43 mg/ml. The formation of products was monitored at different time intervals by TLC.

3.2. β-Galactosidase activity in the hepatopancreas extract

p-Nitrophenyl β-D-galactopyranoside (**1**) (Fig. 1) (0.033 mmol) dissolved in acetate buffer 50 mM, pH 5.6 (500 μl), was added to 2-acetamido-2-deoxy-D-glucopyranose (**2**) (Fig. 1) (0.16 mmol) and to 500 μl of hepatopancreas extract (1.19 mg/ml of total protein); the reaction was placed in a sealed vial at 32 °C under stirring. At total consumption of the donor **1** (1 h) as judged by TLC in EtOAc/MeOH/H_2O (70:20:10), the product purification (preparative TLC) furnished 20% yield of a mixture of disaccharides containing mainly (>80%) the β-1-4 regioisomer (**3**) (Fig. 1).

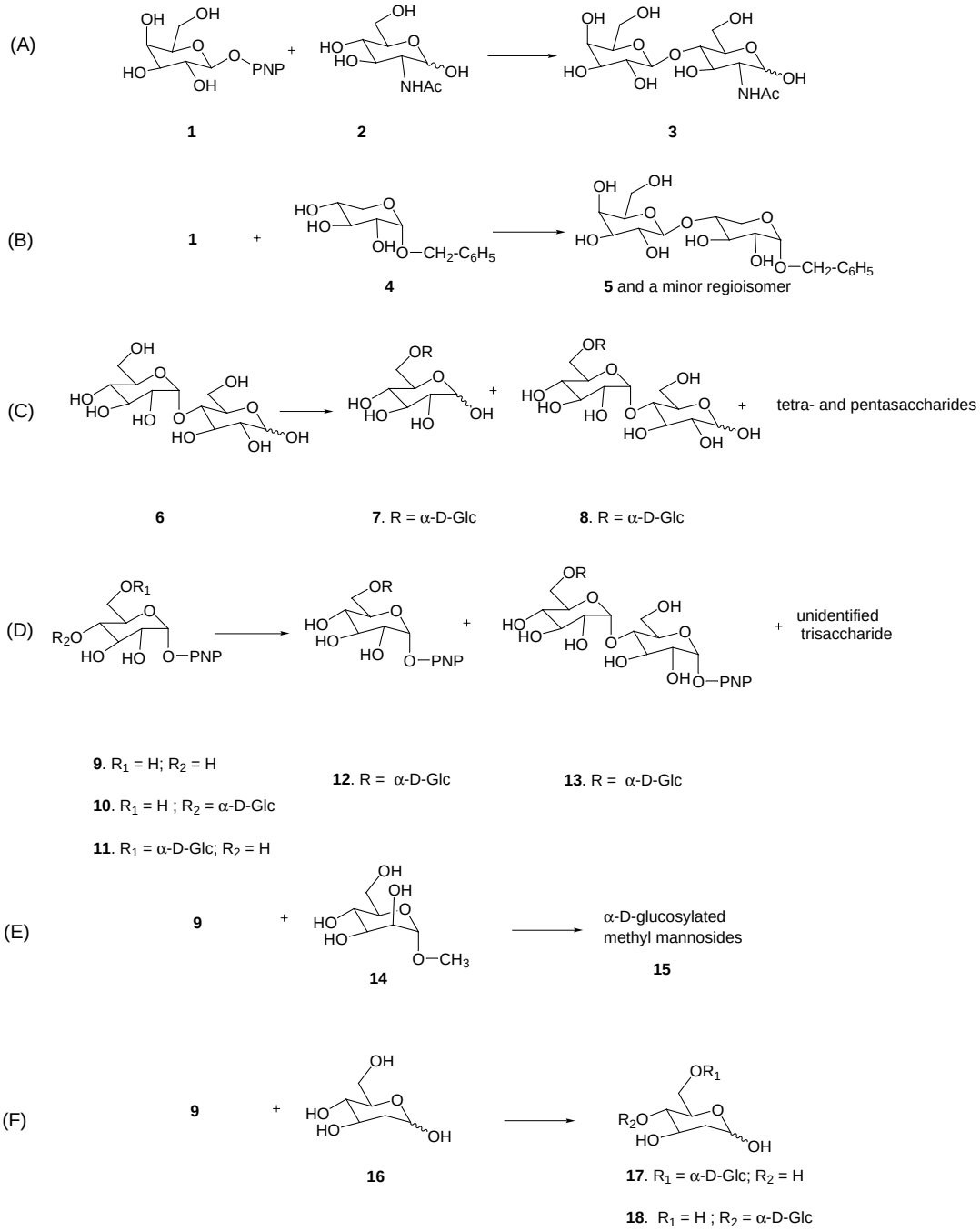

Fig. 1. Transglycosylation reactions (A–F) performed by β-galactosidase (A and B) and α-D-glucosyl hydrolase (C–F) found in hepatopancreas and visceral mass extracts of *A. fasciata*. D is a schematic representation of multiple reactions using **9**–**11** as substrates and giving different products (see text section for details). PNP: *p*-nitrophenyl.

Benzyl α-xyloside (**4**) (Fig. 1) (200 mg, 0.83 mmol) and *p*-nitrophenyl β-D-galactopyranoside (**1**) (Fig. 1) (50 mg, 0.16 mmol) were dissolved in 2 ml of 50 mM acetate buffer (pH 5.6), and 200 μl of crude hepatopancreas extract (6 mg/ml total protein) were added. The reaction was placed in a sealed vial at 32 °C under stirring. At the total consumption of the donor (2 h) reverse phase (prepacked Lobar RP-18) column chromatography eluted with a gradient of aqueous methanol (0–50%), furnished 31 mg (48% yield) of a mixture of two galactosylated xylosides which were unseparable in TLC (both as native and even as acetylated derivatives). Mono- and bidimensional NMR spectroscopy of this material however allowed the structural identification of the most abundant regioisomer as described in Section 4.

3.3. Maltose and p-nitrophenyl glycosides oligomerization

Maltose (**6**) (Fig. 1) (360 mg, 1.0 mmol) was dissolved directly in the crude homogenate (1 ml) from visceral mass containing 1870 U of α-D-glucohydrolytic activity per mmol of maltose. The reaction was started at 35 °C under agitation in a sealed vial. After 48 h an aliquot (500 μl) of the reaction was purified by a series of Biogel-P2 columns and/or silica gel column chromatography of acetylated derivatives, obtaining different oligosaccharides as described in Section 4. The reaction was also conducted at 0.01, 0.1, 1 and 1.7 M maltose concentration; in these cases 0.34 mmol of maltose was dissolved in the appropriate amount of 50 mM phosphate buffer (pH 6.4) containing 640 U of α-D-glucohydrolytic activity (see Fig. 2).

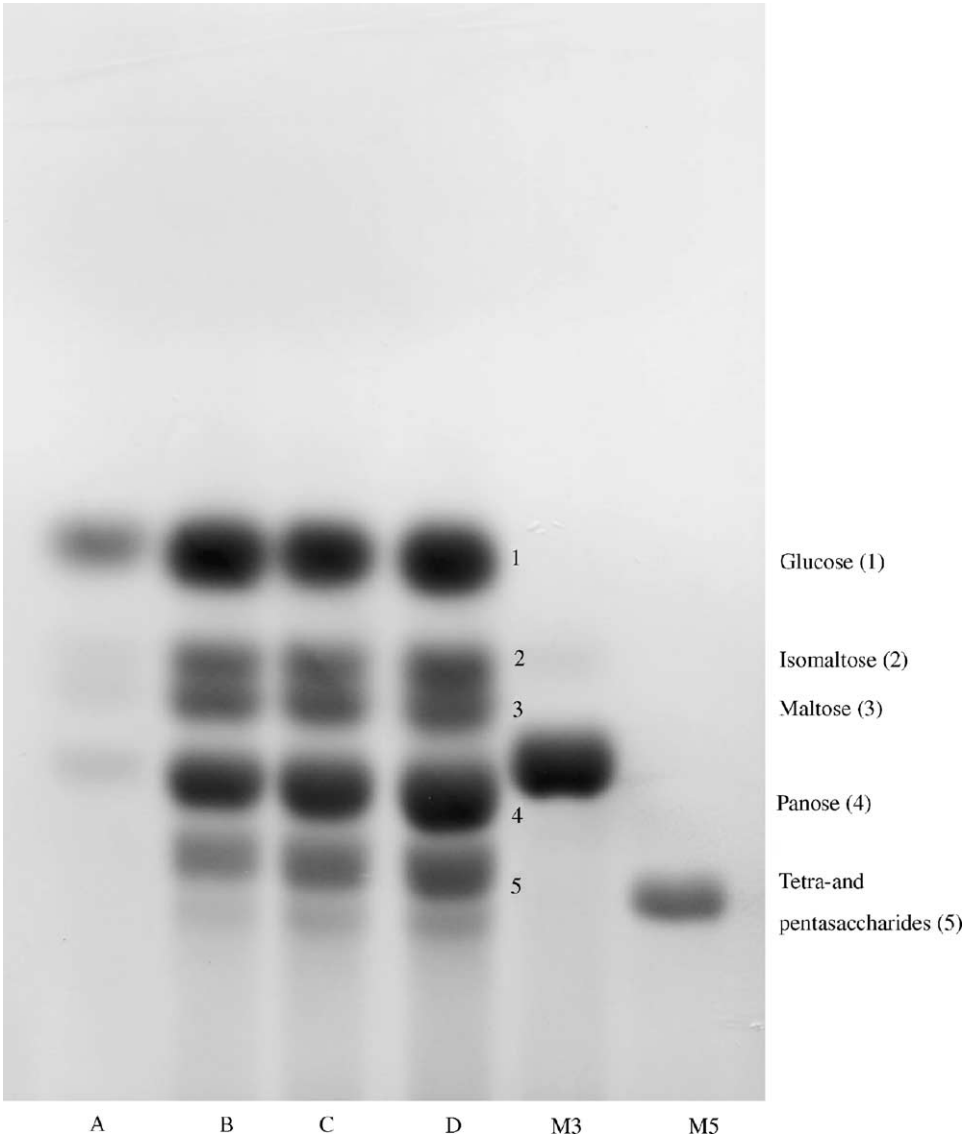

Fig. 2. Silica gel TLC of different reactions for oligomerization of maltose after 24 h. A–D refer to 0.01, 0.1, 1 and 1.7 M maltose solution. M3 and M5 refer to maltotriose and maltopentaose respectively. TLC solvent:EtOAc:CH_3COOH:2-propanol:HCOOH:H_2O (25:10:5:1:15). The numbers define the spot identification (see text).

p-Nitrophenyl α-D-glucopyranoside (**9**) (Fig. 1) (0.132 mmol, 40 mg) was added at different time intervals (35 min) in four aliquots of 10 mg to 1 ml of 50 mM phosphate buffer (pH 6.4) containing 640 U of α-D-glucohydrolytic activity. After purification of the reaction mixture, conversion of the aryl glucoside was 75%. The 20% of glucose theoretical value was found in the products purified by reverse phase silica gel chromatography and analyzed by NMR spectroscopy. Glucose theoretical value is the amount of free glucose that can be obtained after hydrolysis of substrate at the substrate conversion obtained. The HPLC analysis of reaction mixture was conducted on a Carbohydrate Analysis (Waters Associates) column using acetonitrile/water (85:15) as eluant (1 ml/min), with UV detection. *p*-Nitrophenyl α-maltoside (**10**) (Fig. 1) or *p*-nitrophenyl α-isomaltoside (**11**) (Fig. 1) (0.01 mmol, 5 mg) were separately dissolved in 1 ml of 50 mM phosphate buffer (pH 6.4) containing 200 U of α-D-glucohydrolytic activity. Aliquots (10 μl) of the reaction mixture were spotted on TLC at intervals. The products of these reactions were identified by comigration in HPLC with authentic standards.

3.4. Transglucosylation reaction using methyl α-mannoside and 2-deoxyglucose

p-Nitrophenyl α-D-glucopyranoside (**9**) (Fig. 1) (0.066 mmol) was added to 0.33 mmol of methyl α-D-mannopyranoside (**14**) (Fig. 1) in 2 ml of 50 mM phosphate buffer (pH 6.4) containing 640 U of α-D-glucohydrolytic activity from the visceral mass. The reaction was complete (donor consumption) in 2 h. The products **15** (Fig. 1) were purified by Biogel P2 and preparative TLC obtaining a total yield of ca. 4%. ^{1}H NMR spectra of the two compounds in D_2O indicated a methyl α-D-mannopyranoside substructure with an α-linked glucose unit.

p-Nitrophenyl α-D-glucopyranoside (**9**) (Fig. 1) (0.2 mmol) were added to 1 mmol of 2-deoxyglucopyranose (**16**) (Fig. 1) in 6 ml of 50 mM phosphate buffer (pH 6.4) containing 1920 U of α-D-glucohydrolytic activity. The reaction was complete (donor consumption) in 1.5 h. The products (total yield of ca. 30%) were purified by reverse phase and silica gel chromatography after acetylation obtaining two compounds 4-*O*-α-D-glucopyranosyl 2-deoxyglucopyranose (**17**) and 6-*O*-α-D-glucopyranosyl 2-deoxyglucopyranose (**18**) in 7:3 ratio as calculated by weight.

4. Results and discussion

A preliminary investigation on the presence of different glycosyl hydrolases in the external part, visceral mass and hepatopancreas crude extracts from *A. fasciata* was performed using chromophoric substrates and the results are summarized in Table 1. The extracts from hepatopancreas and from visceral mass were rich in glycosyl hydrolases; their activities were in the range found in other marine molluscs [10]. By contrast, the external part extract did not hydrolyze any of the substrates tested. Interestingly the β-D-galactosidase (289 U/mg protein) and the α-D-glucosidase (1313 U/mg protein) enzymes from hepatopancreas and visceral mass, respectively were highly active. To test the potential of these biocatalysts in synthesis, a preliminary analysis of the hydrolytic potential and of the feasibility of transfer reactions from different substrates to interesting acceptors was performed.

5. Hepatopancreas extract of *A. fasciata*

The crude extract from the hepatopancreas of *A. fasciata* contained mainly β-glycosyl hydrolase activities in addition to α-L-fucosidase activity (Table 1). The hydrolytic potential of this extract was analyzed using as substrates curdlan (a β-1-3-glucan homopolymer produced by *Agrobacterium faecalis*), xylan from birchwood and fucoidan from *Fucus vesiculosus*. Product formation was followed by TLC. While curdlan was actively hydrolyzed to glucose with no formation of oligosaccharides, the other two polymers remained intact also after long incubation times indicating the absence of any xylanolytic activity and no action of the α-fucosidase activity present in the extract on fucoidan.

The β-galactosidase activity found in the hepatopancreas extract (Table 1) of *A. fasciata* was by an order of magnitude higher with respect to other mollusc extracts [10], and was used to test the transfer of a galactose moiety to 2-acetamido-2-deoxy-D-glucopyranose (GlcNAc) (**2**) (reaction A in Fig. 1). The possible products are of interest since the Gal-GlcNAc disaccharidic moiety is the typical terminal sequence of N-linked oligosaccharides [11,12]. The reaction was performed by using a five molar excess of **2** reacted with *p*-nitrophenyl β-D-galactopyranoside (66 mM)

Table 1
Glycoside hydrolases activities contained in protein extracts of hepatopancreas and visceral mass of *A. fasciata*

Substrates[a]	U/mg of protein[b]	
	Visceral mass	Hepatopancreas
PNP α-L-Fuc	3	49
PNP β-D-Fuc	0	92
PNP α-D-Gal	5	25
PNP α-D-Glc	1314	32
PNP β-D-Gal	72	289
PNP β-D-Glc	209	102
PNP β-D-Man	216	109
PNP β-D-NAG	12	150
PNP α-D-Man	0	0
PNP β-D-Xyl	9	0

[a] Fuc, fucose; Gal, galactose; Glc, glucose; Man, mannose; NAG, *N*-acetylglucosamine; Xyl, xylose.

[b] One unit corresponds to the nanomole of substrate consumed in 1 min/mg protein.

as donor. After total consumption of the donor (1 h), the product was purified and characterized by NMR. The presence of a diagnostic signal at ca. 78 ppm, as found in the ^{13}C NMR spectrum of β-Gal-(1-4)-GlcNAc regioisomer **3** (Fig. 1) [11], confirmed that the most abundant transfer was to position 4 of GlcNAc; the remaining signals agreed with the structure proposed. However the ^{1}H NMR spectrum indicated the presence, in trace amounts, of other regioisomers. The regioselectivity of this reaction is interesting since most β-galactosidases afford β-Gal-(1-6)-GlcNAc as the most abundant transfer product [12].

A similar reaction was conducted by using a xylose-based acceptor in order to obtain Gal-Xyl disaccharides. Gal-Xyl is a substructure present in the xyloglucan oligosaccharides possessing several biological activities [13] and in saccharides present in the region between glycosaminoglycan chains and protein in serine-linked connective tissue proteoglycan [14]. Benzyl α-xyloside (**4**) (Fig. 1) was used as acceptor to give in 48% yield two β-Gal derivatives in a ca. 2:1 ratio. Unfortunately the two regioisomers were inseparable by TLC both as native material and as acetylated derivatives but the structure of the most abundant compound was defined by NMR spectroscopy (COSY and ^{1}H–^{13}C spectra) of the acetylated mixture. The most abundant regioisomer was characterized by the β-Gal-(1-4)-Xyl disaccharidic sequence as seen by the clearly distinguishable ^{1}H–^{13}C correlation at 3.78/76.6 due to the H4 of xylose assigned by COSY spectra. The remaining signals are in agreement with structure proposed (**5**) (Fig. 1) also in comparison with previously characterized 4-penten-1-ol derivatives [15]. The complexity of the spectra of the two compounds in the mixture did not allow a clear definition of the minor regioisomer.

The heterogeneous nature of the hepatopancreas crude extract containing other β-glycosyl hydrolases hampers any discussion about the regioselectivity observed in these two transgalactosylation reactions. However, it is of interest that galactose transfer is possible on at least two different pyranosidic structure analyzed (xylose and GlcNAc) and a high percentage of β-Gal-(1-4)-GlcNAc is obtained using the latter acceptor. No further effort was made for the purification of the β-galactosidase or improvement of the reaction yield at this stage.

6. Visceral mass extract of *A. fasciata*

The crude extract from the visceral mass of *A. fasciata* showed the highest hydrolytic activity on *p*-nitrophenyl α-D-glucopyranoside. Preliminary investigation showed that this activity had an optimal pH at 6.3 in 50 mM phosphate buffer and, at this pH, an optimal temperature of 40 °C. The activity was completely retained (95%) after 24 h at 35 °C while at 39 °C the half-life was about 11 h. The analysis of activity in presence of 10% of water-soluble organic solvents indicated good resistance of the enzyme to DMSO (80% residual activity after 24 h), high sensitivity to acetonitrile and DMF (total loss of activity after few minutes) and an intermediate resistance to acetone (50% residual activity after 24 h). These data were interesting because of the low solubility of aryl substrates usually used as donors in transglycosylation reactions.

The hydrolytic potential of this extract was also qualitatively studied by following the product formation by TLC. Di-, tri-, tetra- penta- and hexasaccharides of maltose type (α-1-4-linked) were very quickly hydrolyzed producing glucose; sucrose was also rapidly converted into glucose and fructose, while trehalose, isomaltose, soluble starch and pullulan were resistant to hydrolysis. However, these compounds slowly produced glucose to a much lesser extent after long incubation time (days). In the reaction using maltose as substrate the formation of oligosaccharides was noticed.

Thus the crude hydrolytic activity was analyzed for the transfer potential in the oligomerization of maltose and other aryl glucosides used as substrates and acceptors in the reaction mixture (reactions C–F in Fig. 1). The possible products from maltose are extremely relevant for their nutritional value as well as those from aryl glucosides in applications as chromophoric substrates [16].

As far as the maltose is concerned, TLC analysis after 24 h reaction time (Fig. 2) showed the formation of oligomers containing two to five glucose units (C, Fig. 1). A similar pattern of products was obtained by using different maltose concentrations ranging from 0.01 to 1.7 M. In the reaction conducted at 1 M maltose solution (1870 U of enzyme/mmol of maltose), all the maltose was consumed in ca. 48 h and the amount of free glucose in the reaction mixture afforded 40% of the theoretical value, the remaining glucose being converted in higher molecular weight compounds (di-, tri-, tetra-, and pentasaccharides in 28:49:20:3 ratio) which were separated by a combination of Biogel-P2, as native material, and silica gel chromatography, after formation of acetylated derivatives.

The disaccharide was identified as isomaltose (6-*O*-α-D-glucopyranosyl-D-glucose) (**7**) (Fig. 1) by comparison of ^{1}H and ^{13}C NMR spectra of the acetylated derivative with an authentic standard sample of isomaltose; diagnostic signals due to the –CH_2OH unit of glucose at the reducing end (67.2 and 67.1 α/β anomers) were found as in the spectra of isomaltose.

The most abundant trisaccharidic compound **8** (Fig. 1) showed four anomeric signals in the ^{1}H NMR spectrum of native material. Two of them at 5.40 and 4.95 ppm showed peak areas in a 1:1 ratio, while the peaks for α- and β-anomeric protons at the reducing end were found at 5.25 and 4.65 ppm, respectively. These anomeric protons and the shape of the remaining overlapping signals from 4.0 to 3.2 ppm were identical to the ^{1}H NMR spectrum of panose run at 400 MHz and differ from that of isopanose [17]. The pattern of carbon signals in the ^{13}C NMR spectrum of the

trisaccharide showed very close similarity to those reported for panose substructure at the trisaccharidic reducing end of a pentasaccharide enzymatically obtained from a *Leuconostoc mesenteroides* dextrans-sucrase [18]. Finally the analysis of the COSY, TOCSY and ^{1}H–^{13}C NMR correlation spectra of the acetylated derivative of this trisaccharide showed unambiguously the panose structure; in fact following the coupling pattern of the first glucose molecule starting with signals of free anomers (H1α, 6.25/88.7; H1β, 5.75/91.1), the chemical shifts of the C6 hydrogen atoms were found at 4.44–4.24 (62.6 ppm for ^{13}C), a typical value for acetylated hydroxyl groups. Owing to the glycosylation shift, the H4 signal of this glucose was found at 3.99 ppm. Starting from C6/H6 signals of the central glucose molecule at 65.2/3.50–3.71 in the TOCSY and ^{1}H–^{13}C spectra, it has been possible to assign the anomeric signals at 5.34/95.6 for this unit while the hydroxymethylene hydrogens of the terminal glucose unit (C1/H1, 5.16/95.9 ppm) were found at 4.26–4.09/61.8 ppm. Taken together these data clearly indicated that this trisaccharidic component was panose (*O*-α-D-glucopyranosyl-(1-6)-*O*-α-D-glucopyranosyl-(1-4)-D-glucose).

Tetra- and pentasaccharidic nature of the remaining products was established both by their Rfs in TLC (Fig. 2) which were similar to maltotetraose and maltopentaose, respectively, and by mass spectroscopy (ESI-MS positive ions) of acetylated derivatives.

The oligomerization reaction was also conducted on an analytical scale using *p*-nitrophenyl α-D-glucopyranoside (reaction D, from **9** to **12** and **13**, Fig. 1) as substrate and acceptor. At 75% conversion of the total aryl substrate, 20% of converted glucose was present in the mixture of products: *p*-nitrophenyl α-isomaltoside (**12**) (80%), *p*-nitrophenyl α-panoside (**13)** (10%) and other minor disaccharidic derivatives (10%) as established by HPLC analysis. The structure of **12** was unambiguously confirmed by ^{13}C NMR spectrum (D_2O) that showed the two diagnostic methylene signals at 61.4 and 66.3 ppm and the two anomeric signals due to α-glucosyl linkages at 98.6 and 97.5. As far as the *p*-nitrophenyl trisaccharide (**13**) is concerned the spectrum of its acetylated derivative showed three anomeric signals at 94.1/5.75, 95.2/5.38, 95.8/5.16 with $J_{1,2}$ consistent with the α-linkage. The analysis of the COSY and ^{1}H–^{13}C spectra unambiguously confirmed the panose structure above described for the free carbohydrate moiety.

When the disaccharide *p*-nitrophenyl α-maltoside was used as substrate and acceptor (reaction D in Fig. 1; from **10** to unidentified trisaccharide), the absence of maltose at the early stage of reaction clearly indicated the exo-glucosidase activity of the enzyme. Interestingly the disaccharidic portion of the reaction mixture after 15 min showed the presence of only trace amount of *p*-nitrophenyl α-isomaltoside (**12**) and the formation of additional regioisomer(s) (α-1-3 or α-1-2) which comigrated with *p*-nitrophenyl α-maltoside in TLC. The HPLC retention time of the trisaccharide(s) formed in this reaction in the initial stage was not the same as found for *p*-nitrophenyl α-panoside previously formed using *p*-nitrophenyl α-D-glucopyranoside. However, trace amount of *p*-nitrophenyl α-panoside (**13**) could be detected in the later stages of this reaction, before complete hydrolysis to glucose.

The *p*-nitrophenyl α-isomaltoside (reaction D in Fig. 1; **11** as substrate) acted as the worst substrate. After 5 h incubation, under the same conditions used for *p*-nitrophenyl α-maltoside, it was almost completely unreacted and only traces of glucose were formed.

The transfer capability of this glucosyl hydrolase was also tested on heteropyranosidic acceptors such as methyl α-mannopyranoside (reaction E in Fig. 1) and 2-deoxyglucose (reaction F in Fig. 1), and both proved to be acceptors of the α-D-glycosyl unit in different positions. The α-D-glucosylated methyl mannosides (**15**) were formed in low amounts (4%) and no further efforts were made towards their fine structural characterization.

When 2-deoxyglucose was used as acceptor, the yield of the reaction was higher (ca. 30%) and two compounds were formed. Their structures were established by ^{1}H, COSY and ^{1}H–^{13}C NMR spectra of acetylated derivatives. Both compounds were characterized by the presence of the 2-deoxyglucose unit with H1/C1 (6.1–6.2/90.4 ppm α and 5.7–5.8/90.6–90.9 ppm β) and H2/C2 (2.3–1.7/33–34 ppm). In the most abundant product (70%) two clearly distinguishable set of signals were present for the terminal hydroxymethylene units at 4.22–4.05/61.6 (acetylated position) and at 3.70–3.59/66.7 (glucosylated position) leading to the proposed structure (6-*O*-α-D-glucopyranosyl-2-deoxyglucose, the 2-deoxy analog of isomaltose **17**). The minor compound besides the H1 and H2 pattern indicated above showed the 2-deoxyglucose H4 signal at 3.91/72.6 confirming its 4-*O*-α-D-glucopyranosyl-2-deoxyglucose structure, the 2-deoxy analogue of maltose **18** (Fig. 1).

α-D-Glucosidases catalyze both the liberation of glucose (from nonreducing ends of α-glucosides, α-linked oligosaccharides and α-glucans) and/or its transfer, thus exhibiting clear transglycosylation activity, a characteristic exploited in industry to produce oligosaccharides. However transglycosylation activities of exo-type glycosidases sometimes play physiological important roles in gene regulation involved in the carbohydrate utilization. In fact isomaltose, among other α-linked oligosaccharides, is the strongest inducer in *A. oryzae* and *A. nidulans*. The latter organism possesses an α-glucosidase with a strong transglycosylation activity converting maltose to isomaltose, panose and higher transglycosylation products [19] in dilute solutions. In our case the formation of oligosaccharidic products were observed from dilute (0.01 M) to concentrated (1.7 M) maltose solutions (Fig. 2). Oligosaccharides were also highly formed at ca. 3 M maltose (data not shown); in this reaction only 30% of the converted glucose was present as free monosaccharide at the end of reaction (total maltose consumption).

A discussion about the regioselectivity observed using the crude enzymatic preparation from *A. fasciata* cannot be easily made at this stage although the extract contained only α-L-fucosidase and no other α-D-glycohydrolytic enzymes; however the transfer observed in our reaction suggests that the enzymatic activity(ies) behaves similar to those reported in *A. oryzae* and *A. nidulans* and differs from the α-glucosidase from *B. stearothermophilus* (showing a preferential transfer to C4 of maltose) and from the yeast α-glucosidase (catalyzing the formation of α-1-3, α-1-4 and α-1-6 linkages) [20]. The high panose content in the final stage of the reaction using concentrated maltose solution (Fig. 2) could indicate the preferential transfer to C6 and/or the less efficient hydrolysis for isomalto and panose structures (end-products). In the presence of high maltose concentrations the C6 transfer to a maltose molecule has been recognized as responsible for the formation of panose as previously demonstrated for *A. oryzae* α-glucosidase [21] using radiolabeled compounds. In dilute solution using aryl substrates, *p*-nitrophenyl α-glucopyranoside (0.03 M) also formed their isomaltoside (major compound) and panoside derivatives as end-products but in the initial stages of this reaction the formation and rapid hydrolysis of different disaccharides was noticed (data not shown). Using *p*-nitrophenyl α-maltoside (0.01 M), the enzyme in the initial stage of the reaction lead to the formation of PNP α-glucopyranoside, only trace amount of *p*-nitrophenyl isomaltoside and the formation of different disaccharide(s); as expected a *p*-nitrophenyl trisaccharide was also formed but its structure was different from the expected panoside. Thus in this case, at least in the early stage of the reaction, a C6 glucose transfer on the substrate *p*-nitrophenyl α-maltoside forming *p*-nitrophenyl trisaccharide with panose structure can be ruled out. The panoside however could be detected in this reaction at a later stage indicating it to be the trisaccharidic end-product. The role of end-product for the disaccharide *p*-nitrophenyl isomaltoside is also demonstrated by the high resistance of this compound to hydrolysis under similar conditions. Owing to these results a final conclusion about the real mechanism of formation of trisaccharidic products cannot be made; in particular the initial formation of a trisaccharide with a structure different from panose lead to the conclusion that a more complex arrangement of anomeric linkages must be responsible for panoside formation instead of a simple C6 transfer on to the maltosyl substructure; a great role in the accumulation of these products could be played by their different specificity for the hydrolysis reaction or by the ability of the enzyme to follow intramolecular hydrolysis/transglycosylation pathways as described following careful experimentation during lactose–allolactose isomerization [22]. Finally the enzymatic activity from *A. fasciata* was able to transfer glucose on C6 and C4 of 2-deoxyglucose forming the corresponding disaccharides which are interesting compounds for substrate-enzyme interaction studies [23]. The glucose was also transferred to different positions on α-methyl mannopyranoside in low yield.

7. Conclusion

The crude extracts of different parts of *A. fasciata* contained enzymes interesting for synthesis; in this paper we obtained encouraging results demonstrating the utility of the high activities found in the hepatopancreas and in the visceral mass. The use of the α-glucohydrolytic activity in the latter for the preparative chemo-enzymatic synthesis of chromophoric derivatives of isomaltooligosaccharides is under investigation. This activity and other enzymes present (β-galactosidase, β-mannosidase, β-*N*-acetyl glucosaminidase) are also interesting from the synthetic point of view, as demonstrated for the formation of β-galactosides, and will be the subject of further reports when purified material will permit a more accurate analysis of the regioselectivity of the reactions.

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