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Enzymatic synthesis of primeverosides using transfer reaction by *Trichoderma longibrachiatum* xylanase

N. Kadi, J. Crouzet *

UMR Ingenierie Des Réactions Biologiques-Bioproduction CC 23, Université de Montpellier II, Place E. Bataillon, 34095 Montpellier Cedex 05, France

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Abstract

Enzymatic synthesis of two phenyl xylopyranosyl glucopyranosides, through transfer reaction by *Trichoderma longibrachiatum* endoxylanase, was achieved in the presence of *n*-hexane used as solvent, phenyl glucoside (10 mM) as acceptor and xylan (2 g/l) as donor. Kinetic study showed that only one compound, identified by ^{1}H and ^{13}C NMR and heteronuclear 2D (^{1}H–^{13}C) chemical shift correlation as phenyl primeveroside (phenyl 6-*O*-β-xylopyranosyl-1-β-D-glucopyranoside), was synthesized when the reaction time was beyond 1 h. Benzyl and hexyl primeverosides were obtained under the same conditions. When several phenyl glucoside concentrations, from 5 to 50 mM, were used with 2 g/l of xylan, a phenyl primeveroside isomer, identified as phenyl 4-*O*-β-xylopyranosyl-β-D-glucopyranoside, accumulated in the medium whereas the production of phenyl primeveroside decreased. Only phenyl primeveroside was produced when several xylan concentrations from 2 to 10 g/l were used with 10 mM of phenyl glucoside and its concentration in the reaction mixture increased with the increase of xylan concentration.

Keywords: Disaccharide glycosides; Primeverosides; Enzymatic synthesis; Xylanase; Transfer reaction; Kinetics; FAB^{+}; ^{1}H and ^{13}C NMR

1. Introduction

The widespread occurrence of glycosylated aroma compounds and glycoside hydrolast in plants is well established, as recently summarized by Sarry and Günata (2004). More particularly, 6-*O*-β-xylopyranosyl-β-D-glucopyranosides or primeverosides were identified in several plants in leaves, fruits, roots or flowers. Some of them, (*Z*)-hex-3-enyl, benzyl, 2-phenylethyl, methyl salicylate, linalyl, geranyl, *trans*- and *cis*-linalool 3,6-oxide primeverosides, known as the main precursors of the floral aroma of tea (*Camellia sinensis*), developed during processing (Guo et al., 1993, 1994; Moon et al., 1994; Nishikitami, Kubota, Kobayashi, & Sugawara, 1996; Nishikitami, Wang, Kubota, Kobayashi, & Sugawara, 1999; Wang, Yoshimura, Kubota, & Kobayashi, 2000; Wang, Kurasawa, Yamaguchi, Kubota, & Kobayashi, 2001). Primeverosides were more generally present in leaves of plants belonging to the *Camellia* family; eugenyl primeveroside was isolated in *Camellia sasanqua* (Yamada, Aoki, Tamura, & Sakamato, 1967).

Moreover, vomifoliol primeveroside was identified in apples (Schwab & Schreier, 1990), whereas benzyl and phenylethyl primeverosides were components of *Alangium platanifolium* leaves (Otsuka, Takeda, & Yamasaki, 1990), and a derivative of salicin was present as primeveroside in *Alangium premnifolium* leaves (Kijima et al., 1997). 2-Phenylethyl and *n*-hexyl primeverosides were found in roots of *Rehmannia glutinosa* var. *purpurea* (Nishimura, Sasaki, Morata, Chin, & Mitsuhashi, 1990). The presence of small amounts of primeverosides in Cupuacu fruit was indicated by the identification of xylose in the terminal position in the glycosidic extract

* Corresponding author. Fax: +467 14 42 92.
E-mail address: jean.crouzet@univ-montp2.fr (J. Crouzet).

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obtained from this fruit (Boulanger & Crouzet, 2000). Bornyl primeveroside identified, besides linalyl primeveroside, in *Gardenia jasmoïdes* (Watanabe et al., 1994) would be hydrolyzed into volatile borneol during flower opening by the action of endogenous enzymes (Watanabe et al., 1993).

β-Primeverosidase was isolated and purified from fresh tea leaves (Guo et al., 1996; Ijima et al., 1998; Ogawa et al., 1997). This enzyme acts as a diglycosidase (Sarry & Günata, 2004) and hydrolyzes primeverosides and several naturally-occurring diglycosides to give the corresponding disaccharides and aglycones during tea aroma formation (Ma et al., 2001). More recently, β-primeverosidase from tea was cloned and the mature form of the enzyme was able to hydrolyse β-primeverosides (Mizutani et al., 2002).

To the best of our knowledge, only some primeverosides have yet been chemically synthesized: eugenyl primeveroside (Yamada, Aoki, Tanaka, & Munakata, 1967) *p*-nitrophenyl β-D-primeveroside (Sone & Misaki, 1986), benzyl, 2-phenylethyl, linalyl, geranyl, and *trans*- and *cis*-linalyl 3,6-oxide primeverosides (Matsumura, Takahashi, Nishikitami, Kubota, & Kobayashi, 1997). Enzymatic synthesis of *p*-nitrophenyl β-D-primeveroside, by enzymatic transfer from xylobiose to *p*-NP-β-D-glucopyranoside using *Aspergillus niger* β-D-xylosidase, was reported by Murata, Shimida, Watanabe, Sakata, and Usui (1999). Two isomers, PNP-4-*O*-β-D-xylopyranosyl-β-D-glucopyranoside and PNP-3-*O*-β-D-xylopyranosyl-β-D-glucopyranoside, were also produced in minor amounts.

In a previous work (Kadi, Belloy, Chalier, & Crouzet, 2002), we reported the enzymatic synthesis of β-D-xylosides, β-D-xylobiosides and β-D-xylotriosides by transfer reaction between xylan, xylotriose or xylobiose and several aroma compounds, using *Trichoderma longibrachiatum* endoxylanase. The results obtained indicated that, when the syntheses were in the presence of *n*-hexane (90%, v/v) as solvent, only one xylose unit could be transferred, indicating that the synthesis of disaccharide glycosides by transfer of a xylose unit to a glycoside was possible.

The aim of the present work was the study of conditions for the production of primeverosides during the transfer reaction catalyzed by *Trichoderma longibrachiatum* endoxylanase, using xylan as donor and glucosides as acceptors in the presence of *n*-hexane as solvent.

2. Material and methods

2.1. Enzyme

Xylanase XL-200 of *Trichoderma longibrachiatum* was obtained as a gift from Saf-Isis (Soustons, France).

2.2. Products

n-Hexane (95% purity) was from Prolabo (Paris, France). TFA reagent [*N*-methylbis-(trifluoroacetamide)] was obtained from Sigma. Water, conductivity 18.2 Ω cm, was produced by a Purelab Plus system (US Filter). *p*-NP xyloside, *p*-NP glucoside, hexyl, heptyl, benzyl and phenyl glucosides used in the present work (purity 97–100%) were from Sigma. Benzyl primeveroside was a gift of Prof. Kobayashi (University of Ochinamuzi, Japan). Xylan, from birch wood (>90% xylose residues), was obtained from Sigma.

2.3. Xylanase activity measurement

Hydrolysis was carried out by incubation of 250 µl of xylan solution (10 g/l) in sodium acetate buffer (0.1 M, pH 5.0) and 250 µl of diluted (1/1000) xylanase XL-200 preparation in the same buffer for a total volume of 0.5 ml at 40 °C during 10 min. The liberated reducing sugars were determined by the Somogyi–Nelson method (Somoyi, 1952) with xylose as standard. The activity was expressed in International Units (IUs). One International Unit is defined as the enzyme amount catalyzing the release of 1 µmol of xylose per minute. The activity of xylanase XL-200 preparation was 48. 10^3 IU/ml.

2.4. Enzymatic synthesis

In a standard experiment, a heterogenous system constituted of 9 ml of *n*-hexane and 1 ml of xylanase XL-200 preparation, containing phenyl glucoside (10 mM) and xylan (2 mg), was agitated by magnetic stirring (400 rpm). The reaction was carried out at 50 °C. The reaction was stopped by heating in boiling water for 10 min, and *n*-hexane (solvent) was evaporated. The reaction mixture was dissolved in 10 ml of distilled water and enzyme was elimined by centrifugation (15 min, 25,000*g*). The mixture was flash-purified on an RP 18 cartridge equilibrated with water, 3 × 2 ml of water were used for washing and elution was performed using 3 × 2 ml of methanol. The methanol extract was derivatizated as indicated below.

The kinetic reaction study was achieved with 10 mM glucoside, xylan 2 g/l and for 0–5 h reaction time.

For the acceptor concentration study, 0–50 mM of phenyl glucoside for a constant xylan concentration (2 g/l) was used. For the donor concentration study, 0–10 g/l of xylan for a constant phenyl glucoside concentration (10 mM) was used. In both cases the reaction time was 3 h.

The enzymatic synthesis of hexyl and benzyl primeverosides was carried out under the following standard conditions: time 3 h, glucoside 10 mM, xylan 2 g/l.

2.5. Trifluoroacetylation

An aliquot of the methanolic solution, obtained after elution of the RP 18 column, was concentrated to dryness in a screw-capped vial at 60 °C under a stream of nitrogen. Anhydrous pyridine (20 µl) and 20 µl of trifluoroacetylating (TFA) reagent: [*N*-methylbis (Trifluoroacetamide)] were added. The tightly-closed vial was heated at 60 °C for 20 min with stirring, and then allowed to cool to room temperature (Chassagne, Crouzet, Baumes, Lepoutre, & Bayonove, 1995).

2.6. Gas chromatography analysis

A DB-5 MS fused silica capillary column, 30 m × 0.25 mm i.d., 0.25 µm bonded phase (J&W Scientific, Folson, CA), was used. The column temperature was isothermal at 125 °C during 5 min then raised from 125 to 220 °C at 3 °C/min, and increased to 280 °C at 6 °C/min; injector and detector temperatures were 280 and 300 °C, respectively. The flow rate for the carrier gas, hydrogen, was 1.8 ml/min. Split mode injection with a 1/10 ratio, and a makeup of 30 ml/min of nitrogen were used. TFA hexyl and heptyl glucosides were the internal standards used for quantitative determination.

2.7. Gas chromatography–mass spectrometry analysis (GC–MS)

EI–MS spectra were recorded by coupling a Varian 3400 (Walnut Creek, CA) gas chromatograph equipped with a DB-5 MS fused silica capillary column (30 m × 0.25 mm i.d., 0.25 µm bonded phase), to an Automass 020 (Unicam, Argenteuil, France) mass spectrometer. Injections were about 1 µl. The transfer line was maintained at 290 °C and the injector temperature was 280 °C. The column temperature programming was the same as that used in GC experiments, Helium at 1.2 ml/min was the carrier gas. Source temperature was 200 °C, and mass spectra were scanned at 70 eV in the *m/z* range 60–600 mass units.

2.8. Positive fast atom bombardement (FAB)

Positive FAB spectra were obtained using a mass spectrometer, Jeol DX 300 (Laboratoire de mesures physiques, Université de Montpellier 2, France). Xenon was used as inert gas and nitrobenzyl alcohol as matrix.

2.9. Nuclear magnetic resonance (NMR)

Spectra were recorded with a multinuclear Bruker Advance DRX 400 spectrometer (Laboratoire de mesures physiques, Université de Montpellier 2, France), operating at 400 MHz for ^{1}H, from 0 to 10 ppm, and 100 MHz for ^{13}C, from 0 to 200 ppm. The solvent was deuterated water and chemical shifts were given relatively to tetramethylsilane (TMS), used in small amount, as internal standard in both measurements. For ^{13}C NMR, 6144 scans were cumulated after total irradiation of ^{1}H nuclei. For the heteronuclear, two dimensional (^{1}H–^{13}C) chemical shift correlation experiment, 0–200 ppm for ^{13}C and 0–7.5 ppm for ^{1}H were scanned.

3. Results and discussion

3.1. Phenyl xylopyranosyl glucopyranoside synthesis

As indicated by gas chromatography of TFA derivatives obtained after 1 h of reaction (Fig. 1A), three compounds, **1**–**3**, were produced during the reaction. The retention times of the TFA derivatives, 26.6, 27.6 and 29.3 min, respectively, were more important than those of hexyl and heptyl glycosides (compounds a and b) used as internal standards, and that of the unreacted phenyl glucoside (compound c). The same order of magnitude was obtained for an authentic sample of benzyl primeveroside, retention time (30.6 min). However, after 3 h of reaction, only the compound **2** was present in the medium at 27.6 min (Fig. 1B).

Identical GC–EI–MS mass spectra of TFA derivatives of compounds **1** and **2** were obtained; fragment ions at *m/z* 69 and 94 were characteristic of the phenyl aglycone moiety and fragments ions at *m/z* 177, 193, 265, 319 and at *m/z* 193, 278, 307, 421 indicated the presence of a glucose unit and *n*-xylose unit, respectively. Moreover, two peaks at *m/z* 389 $(M + 1)^{+}$ and *m/z* 411 $(M + Na)^{+}$, in the positive FAB spectra of the reaction mixture, were detected. These spectrometric data indicated that the compounds **1** and **2** were isomers with a molecular mass of 388, and had glucose and xylose units linked to a phenyl moiety.

Regarding compound **3**, produced in smaller amount, the mass spectrum different from the other two but cannot be analyzed. This compound, may be a triglycosylated derivative or a polyxyloside, but was not further considered.

3.2. Phenyl xylopyranosyl glucopyranoside production kinetic study

According to these preliminary results, a kinetic study was achieved (Fig. 2). The compound **1** concentration, determined by GC after TFA derivatisaton using TFA hexyl and heptyl glucosides as internal standards, increased rapidly to 72 mg/l for 30 min of reaction then decreased quickly near to zero after more than 1 h of reaction. The concentration of compound **2** decreased very slowly, from 45 mg/l for 30 min reaction time. After 5 h, its concentration represented about 50% of

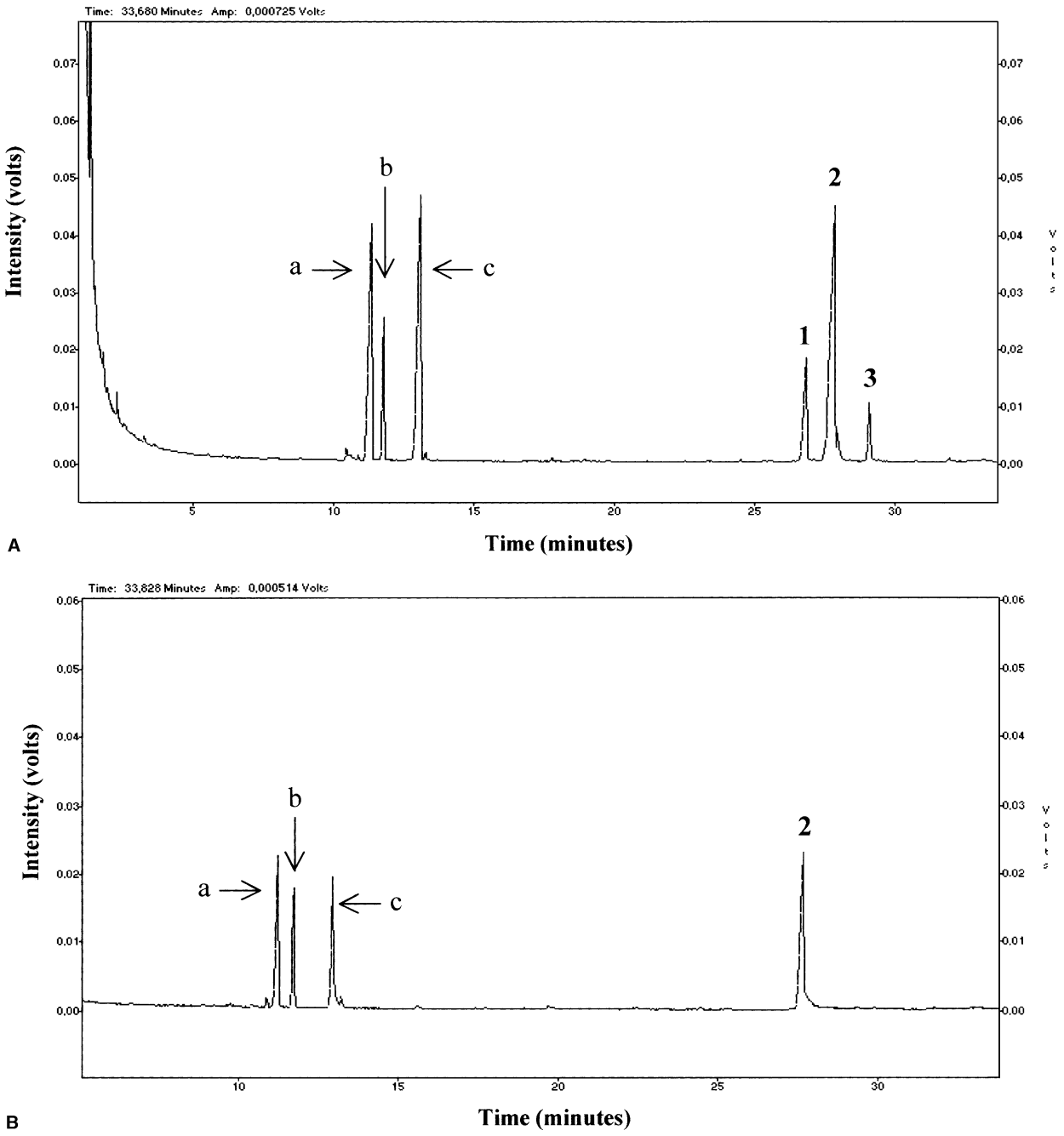

Fig. 1. (A) Gas chromatogram of TFA derivative of the compounds separated from the reaction medium: 9 ml of *n*-hexane and 1 ml of *Trichoderma longibrachiatum* xylanase XL-200 enzymatic preparation containing phenyl glucoside (10 mM) and xylan (2 mg/l) after 1 h: (a) hexyl glucoside (internal standard); (b) heptyl glucoside (internal standard) and (c) unreacted phenyl glucoside and (**1**–**3**) compounds produced by enzymatic synthesis. (B) Gas chromatogram of TFA derivative of the compounds separated from the reaction medium: 9 ml of *n*-hexane and 1 ml of *Trichoderma longibrachiatum* xylanase XL-200 enzymatic preparation containing, phenyl glucoside (10 mM) and xylan (2 mg/l), after 3 h: (a) hexyl glucoside (internal standard); (b) heptyl glucoside (internal standard); (c) unreacted phenyl glucoside and (**2**) compound produced by enzymatic synthesis.

that maximum concentration. After 1 h of reaction, compound **2** was the major product of the reaction, and compound **1** was only present in trace amount.

It can be assumed that the secondary hydrolysis of compound **2** by parasite activities present in xylanase XL 200 preparation was carried out at a slower rate than the hydrolysis of its isomer. Gais, Zeissler, and Maidonis (1988) reported that longer reaction times normally led to a decrease in the yield of galactosides produced by diastereoselective transfer from phenyl-β-galactopyranoside or

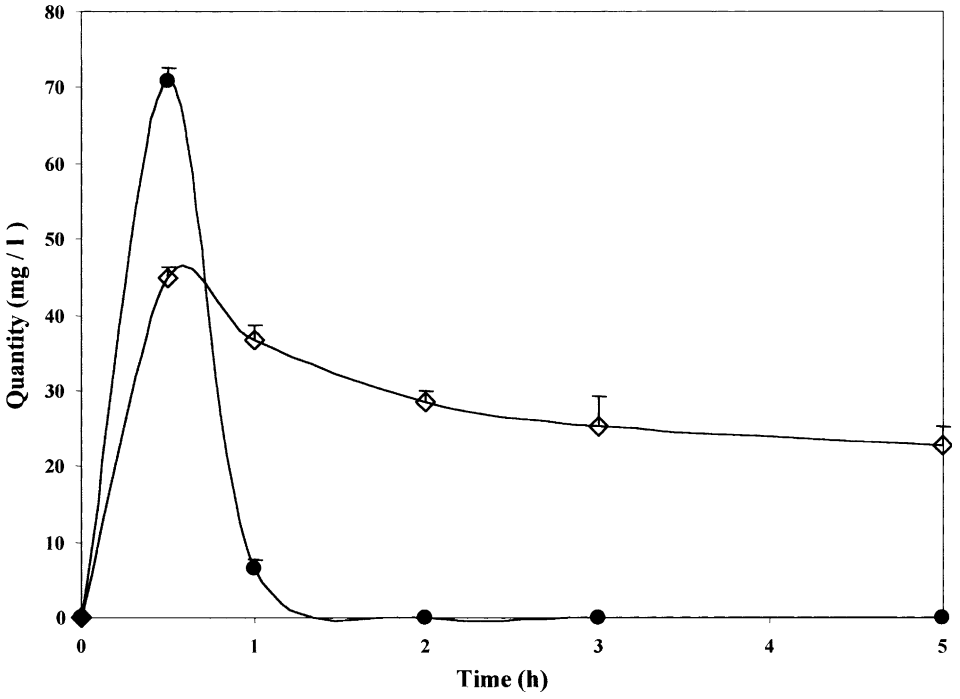

Fig. 2. Kinetics of transfer reaction from xylan to phenyl glucoside: 9 ml of *n*-hexane and 1 ml of xylanase XL-200 enzymatic preparation containing, phenyl glucoside (10 mM) and xylan (2 mg/l): (●) compound **1**, (◇) compound **2** (phenyl primeveroside).

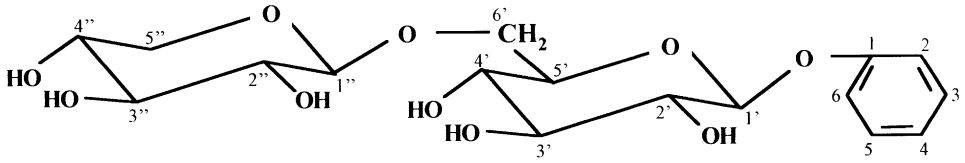

Fig. 3. Structure of compound **2**: phenyl 6-*O*-β-xylopyranosyl-β-D-glucopyranoside (phenyl primeveroside).

β-D-galactose to several meso diols. This decrease was the result of a competitive β-galactosidase-catalyzed hydrolysis. A kinetically-controlled enantioselective transfer of a β-galactosyl moiety to 1-phenylethanol was indicated by Matsumura, Yamazaki, and Toshima (1997). The authors showed that the *R*-enantiomer was more quickly galactosylated than the *S*-enantiomer but that *R*-1-phenylethyl-β-D-galactoside was hydrolyzed much faster than the corresponding *S*-compound.

Compound **2** can be considered as the kinetic product of phenyl disaccharide glycoside enzymatic synthesis. The kinetic observation that the compound which was the most easily synthesized, was the most easily hydrolyzed, characterized a transfer reaction.

3.3. Compound **2** identification

The identification of compound **2**, isolated in pure form after 3 h of reaction (Fig. 1B) was made possible by the 2D NMR. The resonance peaks of the anomeric C-1′ and of the anomeric proton, H-1′, corresponding to the glucose unit (Fig. 3, Table 1), were located at 101.5 ppm and at 5.05 ppm, respectively. H-1′ gave a doublet corresponding to one proton with a coupling constant $J = 7.4$ Hz. These results indicated, as expected, a β-linkage between the glucose and aglycone moieties. A good agreement was found for the values obtained for C-1″ at 103.1 ppm and H-1″, doublet corresponding to one proton at 4.32 ppm ($J = 7.8$ Hz) and those previously reported for the xylose unit in geranyl primeveroside in D_2O (Guo et al., 1993). So it could be concluded that a β-linkage was present between xylose and glucose units, and that the compound obtained by enzymatic synthesis for a reaction time longer than 1 h was the phenyl 6-*O*-β-xylopyranosyl-β-D-glucopyranoside or phenyl primeveroside.

3.4. Compound **1** identification

Concerning the compound **1**, 2D RMN analysis was done on a sample obtained by enzymatic synthesis in the presence of phenyl glycoside in excess (50 mM), xylan (2 g/l) in 1 ml of enzymatic preparation and 9 ml of *n*-hexane, for 30 min of reaction time (see *infra*: acceptor

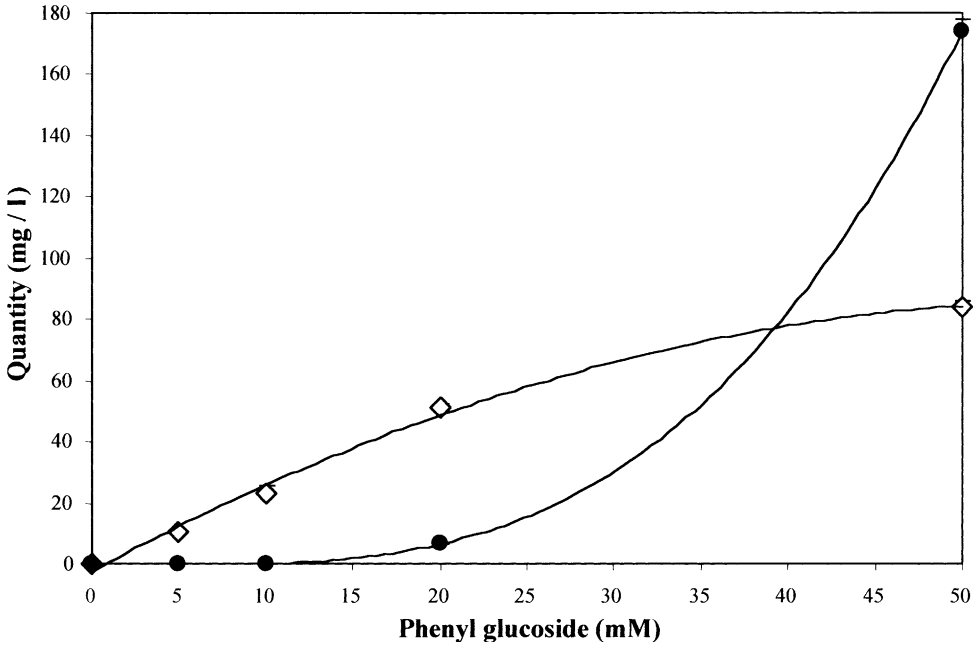

Fig. 4. Effect of phenyl glucoside acceptor molarity (0–50 mM) on phenyl xylosyl glucosides enzymatic synthesis, using *Trichoderma longibrachiatum* xylanase XL-200 enzymatic preparation. Incubation was done at 50 °C for 3 h under agitation in the presence of xylan donor at 2 g/l: (●) compound **1** and (◇) compound **2** (phenyl primeveroside).

Table 1
Phenyl primeveroside ^{1}H NMR and ^{13}C NMR data in D_2O at 400 and 100 MHz, respectively

Position ^{1}H (Fig. 4)	Chemical shift (ppm)	Proton number	Multiplicity	Position ^{13}C (Fig. 4)	Chemical shift (ppm)
1′	5.05	1	d($J = 7.40$ Hz)	1′	101.5
2′,3′,5″a	3.10–3.30	3	br	2′	73.9
				3′	76.4
4′,5′,2″	3.40–3.60	5	br	4′	70
3″, 4″	″				
5′	″	1	m	5′	75.6
6′a, 5″b	3.72–3.85	2	m	6′	68.5
6′b	4.07	1	d($J = 10.8$ Hz)		
1″	4.32	1	d($J = 7.8$ Hz)	1″	103.1
				2″	73.2
				3″	75.9
				5″	63.1

d: doublet, m: multiplet, br: broad peak.

concentration effect). The sample contains 90% of this compound. The spectrum showed resonance peaks of the anomeric C-1′, and of the anomeric proton H-1′ corresponding to the glucose unit, located at 102.6, and at 4.96 ppm ($J = 7.4$ Hz), respectively. The values obtained for C-1′ and H-1″ were at 103.1, and 4.31 ppm ($J = 7.75$ Hz), respectively. These data were indicative of the presence of β-linkages between glucose and aglycone units and xylose and glucose units.

A chemical shift of 78.4 ppm was found for the C-4′ of glucose unit of this compound, indicating a difference of 8.4 ppm relatively to the chemical shift for the C-4′ of primeveroside glucose unit (70.0 ppm). These results were in agreement with those reported by Murata et al. (1999); the authors indicated a chemical shift of 80.7 for C-4′ of *p*-NP 4-*O*-β-glucopyranosyl-β-D-glucopyranoside and 72.1 for C-4′ of *p* NP 6-*O*-β-glucopyranosyl-β-D-glucopyranoside. Similar differences, 8.9–10 ppm, were also reported between the chemical shifts of C-4′ obtained for 2-phenylethyl 4-*O*-β-glucopyranosyl-β-D-glucopyranoside (80.5 ppm) or 2-phenylethyl 4-*O*-α-glucopyranosyl-β-D-glucopyranoside (81.1 ppm) and chemical shifts of C-4′ for several diglycosides possessing β-(1 → 6) bonds between the sugar units (Ma et al., 2001). The values given by these authors for chemical shift for C-4′ of 2-phenylethyl primeveroside, gentiobioside and vicianoside were, 71.0, 71.2, and 71.6 ppm, respectively.

The chemical shifts of C-2′ (71.9 ppm), and C-3′ (75.8 ppm) for compound **1** were close to those observed for the similar carbon atoms of phenyl primeveroside

(Table 1). Moreover, the chemical shifts of C-6′ were different for compound **1** (71.3 ppm) and phenyl primeveroside (68.5 ppm).

These data indicated that the hydroxyl group bound to C-4′ was involved in a linkage, whereas the hydroxyl groups bound to C-2′, C-3′ and C-6′ were free; thus compound **1** was identified as phenyl 4-*O*-β-xylopyranosyl-β-D-glucopyranoside.

3.5. Acceptor concentration effect

The effect of acceptor (phenyl glucoside) concentration, 0–50 mM, for a xylan concentration of 2 g/l and a reaction time of 3 h, is given Fig. 4. When the phenyl glucoside concentration was less than 15 mM, only phenyl primeveroside was present in detectable amount; the isomer appeared when the concentration of the acceptor was between 15 and 20 mM and increased with the increase of acceptor concentration. When this concentration was 50 mM, the quantity of the isomer (175 mg/l) produced was about twice that of primeveroside (70 mg/l). It could be postulated that, in the presence of an excess of acceptor, phenyl primeveroside isomer accumulates in the reaction medium, indicating a modification of the enzyme selectivity or a modification in the relative secondary hydrolysis rate.

According to the literature, yield and selectivity of the transfer reaction are dependent of the nature of the enzyme and of acceptor concentration (Drouet, Zhang, & Legoy, 1984; Nilsson, 1987; Nilsson, 1988; Sauerbrei & Thiem, 1992). Murata et al. (1999) showed that, when *p*-NP-β-D-glucopyranoside was used, as acceptor and xylobioside as donor, *Aspergillus pulverentus* β-xylosidase elevated the synthesis of *p*-NP-6-*O*-β-xylopyranosyl-β-D-glucopyranoside to its isomers, *p*-NP-*O*-β-xylopyranosyl-β-D-glucopyranoside and *p*-NP-3-*O*-β-xylopyranosyl-β-D-glucopyranoside. For some acceptors, the enzymatic synthesis, of β-(1 → 3) digalactosyldipeptide derivative was favoured relatively to that of β-(1 → 6) regioisomer when the synthesis was operated using *Escherischia coli* β-D-galactosidase, in the presence of an excess of acceptor. Moreover, the structure of the aglycone and the configuration of the glycosidic linkage can influence the regioselectivity for digalactoside formation (Nilsson, 1987).

3.6. Xylan concentration effect

When 10 mM phenyl glucoside for several xylan concentrations, from 2 to 10 g/l and a reaction time of 1 h were used, only phenyl primeveroside was produced (Fig. 5). The concentration of phenyl primeveroside in the reaction medium increased with the increase of xylan concentration. The primeveroside produced was 3-fold higher when xylan, 10 g/l was used than when the synthesis was operated for a xylan concentration of 2 g/l. Under these conditions, phenyl primeveroside accumulated to 65 mg/l.

It can be assumed that, in the presence of an excess of xylan, phenyl primeveroside synthesis was increased, or that hydrolysis rate of its isomer was more important, or that the two phenomena occurred simultaneously. It was previously shown (Baker, Turner, & Webberley, 1994) that, using a fixed concentration of acceptor (*p*-NP β-D

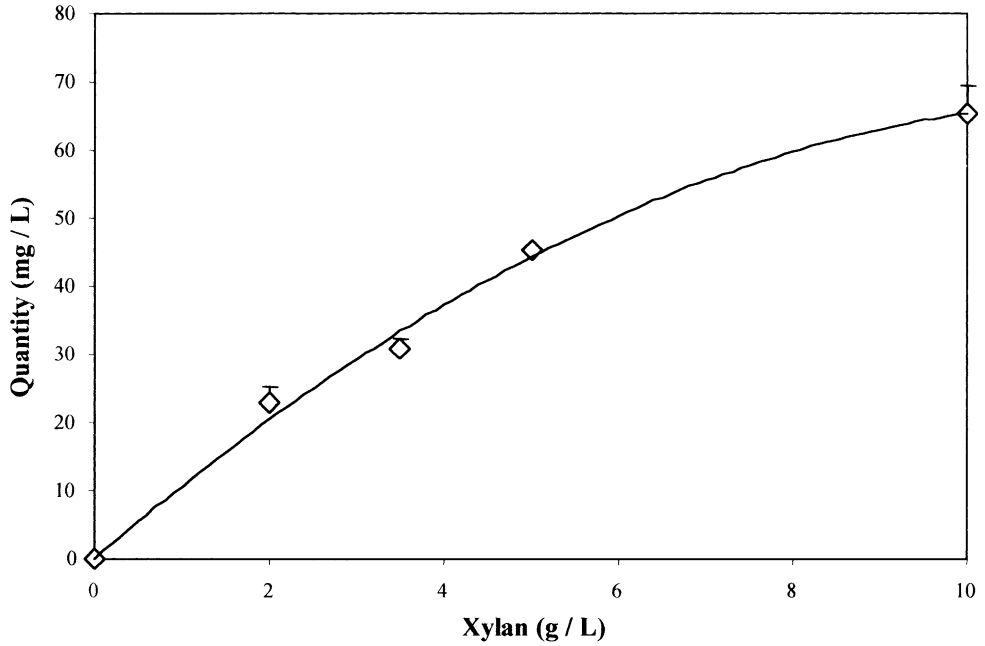

Fig. 5. Effect of xylan donor concentration (2–10 g/l on phenyl xylosyl glucoside enzymatic synthesis, using *Trichoderma longibrachiatum* xylanase XL-200 enzymatic preparation (1 ml) and 9 ml of *n*-hexane. Incubation was done at 50 °C for 3 h under agitation in the presence of phenyl glucoside 10 mM: (◇) compound **2** (phenyl primeveroside).

Table 2
Benzyl and hexyl primeveroside gas chromatography and mass spectrometry (EI and FAB^+) data

Compound	GC	GC–IE–MS		FAB^+	
	Rt (min)	Aglycone-moiety	Saccharidic moiety	M + 1	M + 23
Hexyl primeveroside	27.1	50(93), 51(63), 57(65), 69(97), 85(100)	165(4), 177(7), 193(73), 265(6), 278(7), 307(7), 319(15), 421(4)	397	419
Benzyl Primeveroside	30.7	50(41), 91(100), 92(16), 107(10), 108(4)	177(3), 193(24), 265(2), 278(1), 307(2), 319(5), 421(1)	403	425

Rt: retention time.

galactose) and adding an excess of donor (*N*-acetyl L-serine glucoside) with β-glycosidase as catalyst, only formation of β-(1 → 6) galactopyranosyl-glucopyranoside regioisomer occurred with a 8% yield.

However, the increase of xylan concentration used was limited by its solubility in the reaction medium and by the increase of viscosity. It was previously reported (Park, Lee, & Lee, 1999; Shin, Kong, Lee, & Lee, 2000) that the transglycosylation reaction was made more difficult when high viscosity was reached.

One possibility for obtaining an excess of xylan relative to the glucoside acceptor, was the continuous addition of the donor during the reaction, as previously indicated for digalactoside enzymatic synthesis (Bay, Namane, & Cantacuzene, 1993).

3.7. Benzyl and hexyl primeverosides synthesis

Several primeverosides were obtained by enzymatic synthesis under standard conditions: xylan (2 g/l), benzyl or hexyl glucosides (10 mM) in 90% (v/v) *n*-hexane in the presence of xylanase XL-200 enzymatic preparation (10%), at 50 °C and over 3 h. The mass spectra obtained by GC–EI–MS of TFA derivatives of the benzyl and hexyl primeverosides indicated the presence of a glucose unit and a xylose unit, similar to those of phenyl primeveroside and the fragment ions characteristic of the benzyl and hexyl aglycone moiety. The identification of benzyl and hexyl primeverosides was confirmed by FAB^+ data (Table 2).

GC quantification of phenyl, benzyl and hexyl primeverosides, synthesized under the same conditions, shows the syntheses of 26.6 ± 2.2, 81.5 ± 1.3, 100 ± 5.3 mg/l of these compounds, respectively. The yield differences observed were probably due to differences of glucoside reactivities, or differences in primeveroside secondary hydrolysis by parasite activities present in the xylanase XL-200 preparation.

4. Conclusion

The results obtained in the present work confirm the previously reported data concerning the transfer of one xylose unit by *Trichoderma longibrachiatum* endoxylanase. They open the door to specific biosynthesis of several aroma compound disaccharide glycosides naturally present in food products. The enzymatic synthesis of primeverosides and other disaccharidic derivatives of aroma compounds, such as rutinosides, vicianosides or gentiobiosides, using enzymes possessing the transfer activities needed, is of particular interest because of the difficulties encountered in their chemical synthesis. Aroma compound glycoconjugates obtained can be recognized as natural if natural products are used for their enzymatic synthesis. Moreover, the synthesized glycosides can be used as reference compounds for identification of glycoconjugates present in natural products. One other application, in the food industry, is the protection of aroma compounds, during processing, storage or final preparation of food, by covalent binding.

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