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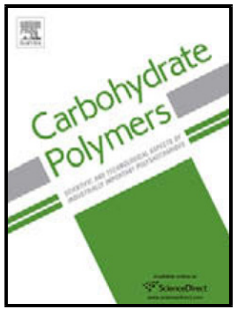

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One-pot bi-enzymatic cascade synthesis of puerarin polyfructosides

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Highligts

- Regioselectivity of fructosyl transfer to puerarin varies between levansucrases
- *Bs*_SacB synthesizes puerarin-4'-O-β-D-fructofuranoside
- *Gd*_LsdA synthesizes β-D-fructofuranosyl-(2→6)-puerarin and linear oligofructosides
- *Bs*_SacB transforms β-D-fructofuranosyl-(2→6)-puerarin into polyfructosides
- Cascade *Gd*_LsdA/*Bs*_SacB reactions yield polyfructosides directly from puerarin

Abstract

Enzymatic glycosylation is an efficient way to increase the water solubility and the bioavailability of flavonoids. Levansucrases from *Bacillus subtilis* (*Bs*_SacB),

Gluconacetobacter diazotrophicus (*Gd*_LsdA), *Leuconostoc mesenteroides* (*Lm*_LevS) and *Zymomonas mobilis* (*Zm*_LevU) were screened for puerarin (daidzein-8-C-glucoside) fructosylation. *Gd*_LsdA transferred the fructosyl unit of sucrose onto the glucosyl unit of the acceptor forming β-D-fructofuranosyl-(2→6)-puerarin (P1a), while *Bs*_SacB, *Lm*_LevS and *Zm*_LevU synthesized puerarin-4'-*O*-β-D-fructofuranoside (P1b) and traces of P1a. The *Gd*_LsdA product P1a was purified and assayed as precursor for the synthesis of puerarin polyfructosides (PPFs). *Bs*_SacB elongated P1a more competently forming a linear series of water-soluble PPFs reaching at least 21 fructosyl units, as characterized by HPLC-UV-MS, HPSEC and MALDI-TOF-MS. Simultaneous or sequential *Gd*_LsdA/*Bs*_SacB reactions yielded PPFs directly from puerarin with the acceptor conversion ranging 82-92%. The bi-enzymatic cascade synthesis of PPFs in the same reactor avoided the isolation of the intermediate product P1a and it is appropriate for use at industrial scale.

Keywords: fructo-conjugates, fructosyltransferase, flavonoid, levansucrase, puerarin.

1. Introduction

Puerarin (daidzein-8-C-glucoside) is an isoflavone that exhibits vasodilator, anti-oxidant, anti-cancer, and anti-inflammatory properties. The poor water solubility of puerarin limits its absorption *in vivo*, impeding optimal pharmacological applications (Zhou, Zhang, & Peng, 2014). Enzymatic glycosylation has emerged as an efficient way to increase the water solubility of flavonoids and consequently other physicochemical and biological properties like stability, bioavailability and bioactivity. The alternative chemical method requires glycosyl activation and multiple steps of protection/deprotection to control the regioselectivity, while the enzymatic reaction is regio- and stereoselective, and more friendly to the environment (Desmet et al., 2015).

In nature, glycosylated forms of flavonoids are produced by Leloir-type glycosyltransferases. The requirement of nucleotide-activated sugars as donor substrates is a disadvantage for biotechnological applications. More attractively, glycosyltransferases from Glycoside-

Hydrolase (GH) families 13, 70, 32 and 68 transfer the glucosyl or fructosyl moiety of the cheap and abundant substrate sucrose. GH13 and GH70 enzymes have been widely used for the *in-vitro* glucosylation of natural flavonoids (Hofer, 2016; Malbert et al., 2018, 2014; Slámová, Kapešová, & Valentová, 2018). However, examples of flavonoid fructosylation by GH32 or GH68 (clan GH-J) enzymes remain scarce (Herrera-González et al., 2017). To our knowledge, there is no report of enzymatic synthesis of puerarin polyfructosides (PPFs).

Sucrose-transforming fructosyltransferases from plants and fungi (GH32 members) are not appropriate for the production of PPFs as they synthesize short-chain fructans and their acceptor range is generally restricted to sugars (Trollope, Görgens, & Volschenk, 2015; Van Den Ende, Lammens, Van Laere, Schroeven, & Le Roy, 2009). Family GH68 includes fructosyltransferases from over 500 bacterial and archaeal species. Inulosucrase (EC 2.4.1.9) and levansucrase (EC 2.4.1.10) produce linear/branched fructan chains elongated via β-(2→1) and β-(2→6) linkages, respectively. Bacterial inulin and levan contain from two to thousands fructosyl units. Fructans are water-soluble fibbers with prebiotic effects in humans and animals. High-molecular-mass levan finds additional applications in several non-food industries (Öner, Hernández, & Combie, 2016).

Levansucrases catalyse the transfer of the fructosyl unit of sucrose onto a variety of natural acceptors including water, glucose, fructose, sucrose, and fructans. The double-displacement (ping-pong) reaction occurs with an overall retention of the anomeric β-configuration of the fructosyl residue. Variations in size distribution are often observed in the fructans produced by levansucrases of different origins. For instance, levansucrase from *Bacillus subtilis* (*Bs*_SacB) elongates the products 1-kestotriose and 6-kestotriose using non-processive (distributive) and processive mechanisms that yield short levans (average mass 7.2 kDa) and long levans (average mass 2,300 kDa), respectively (Raga-Carbajal et al., 2016; Raga-Carbajal, López-Munguía, Alvarez, & Olvera, 2018). Levansucrase from *Gluconacetobacter diazotrophicus* (*Gd*_LsdA) accumulates 1-kestotriose, whereas the processive elongation of 6-kestotriose results in levan polymerization (Hernández et al., 1995).

Several GH68 enzymes have shown the ability to fructosylate non-conventional acceptors such as other sugars, aromatic and aliphatic alcohols, and phenolic compounds (Hernández, Quintero, & Musacchio, 2019). Enzymatic fructosylation of puerarin was initially attempted

in the presence of co-solvents (ethanol or DMSO) that dissolve the isoflavone. By this way, permeabilized cells of *Lysinibacillus fusiformis* (Wang et al., 2014) and *Microbacterium oxylans* (Yu et al., 2010) synthesized puerarin-7-*O*-fructoside, while β-fructosidase from *Arthrobacter nicotianae* formed mono- and di-β-D-fructofuranosyl-(2→6)-puerarin (Wu, Chu, Wu, Zhang, & He, 2013). Recently, Núñez-López et al. (2019) reported that *Gd*_LsdA fructosylates puerarin and other phenolic compounds in an aqueous medium.

In this study, it was first revealed that the regioselectivity and efficiency of the fructosyl transfer from sucrose onto puerarin vary among levansucrases of different origins. A second hypothesis was that PPFs can be produced by the cascade reaction of two levansucrases of different regioselectivity but complementary transferase activities. *Gd*_LsdA synthesized the precursor β-D-fructofuranosyl-(2→6)-puerarin, which was elongated in a distributive manner by the more efficient polymerase *Bs*_SacB yielding a linear series of PPFs with up to 21 fructosyl units. Then, PPFs were produced directly from natural puerarin in a one-reactor system using either simultaneous or sequential *Gd*_LsdA/*Bs*_SacB reactions. The incorporation of two or more β(2→6) linked fructosyl units highly enhanced the water solubility of puerarin.

2. Materials and Methods

2.1 Chemical materials

Puerarin and levan from *Erwinia herbicola* were supplied by Carbosynth Limited (Compton, UK), sucrose, dextran standards, dimethylsulfoxyde (DMSO), acetonitrile (ACN) and formic acid were supplied by Sigma Aldrich Inc. (MO, USA).

2.2 Enzymes and activity determination

Levansucrase from *Gluconacetobacter diazotrophicus* SRT4 (*Gd*_LsdA) was purified according to Hernández et al. 1995. Levansucrase from *Bacillus subtilis* (*Bs*_SacB) was supplied by Ozyme™ (France). Recombinant levansucrases from *Zymomonas mobilis subsp. mobilis* ATCC 10988 (*Zm*_LevU) and *Leuconostoc mesenteroides* NRRL B-512 F (*Lm*_LevS) were produced in *Escherichia coli*.

The enzymatic activity was determined by measuring the reducing sugars released from sucrose (100 g/L) in 50 mM citrate-phosphate buffer (pH 5.8) using the 3,5-dinitrosalicylic acid (DNS) method (Sumner & Howell, 1935). The reaction temperature was 42 °C for *Gd*_LsdA, *Bs*_SacB and *Zm*_LevU), and 30 °C for *Lm*_LevS. One unit is defined as the amount of enzyme releasing 1 μmol of glucose per min at initial rates under the described reaction conditions.

2.3 Synthesis of puerarin fructo-conjugates

P1a and P2a were synthesized by *Gd*_LsdA (0.1 U/ml) in reaction with 146 mM sucrose and 25 mM puerarin. P1b was produced by *Bs*_SacB (0.5 U/ml) using 500 mM sucrose and 25 mM puerarin. PPFs were synthesized using either purified P1a as precursor or directly from puerarin. In the first case, *Gd*_LsdA (0.5 U/ml) or *Bs*_SacB (0.5 U/ml) was incubated with 500 mM sucrose and 25 mM P1a. In the second case, two different cascade reactions were performed: i) Simultaneous enzyme addition: *Gd*_LsdA and *Bs*_SacB were mixed prior to the reaction at rates 0.75-0.75 U/mL (1:1), 0.5-1 U/mL (1:2) and 1-0.5 U/mL (2:1) and incubated with 500 mM sucrose and 10 mM puerarin, ii) Sequential enzyme addition: *Gd*_LsdA (0.1 U/mL) was incubated with sucrose (146 mM) and puerarin (25 mM) for 6 h and inactivated 5 min at 95 °C, and then *Bs*_SacB (0.5 U/mL) was added with/without extra sucrose (292 or 500 mM).

All reactions were in duplicates. Donor and acceptor substrates were mixed in 50 mM citrate-phosphate buffer (pH 5.8) at 42 °C. After 24 h, the reactions were stopped by heating at 95 °C for 10 min. The acceptor products were quantified by HPLC and the conversion percentage was determined according to Núñez-López et al. (2019).

2.4 Chromatography analysis

The HPLC-UV-MS analysis of puerarin and its fructo-conjugates was performed on an Ultimate 3000 series chromatograph equipped with a Dionex 340 UV/VIS detector and coupled with a simple quadruple mass spectrometer (MSQ Plus, Thermo Scientific). Puerarin and its fructo-conjugates were detected using UV at 254 nm and separated by a Phenomenex PFP C18 column (Luna 5μm, 100 A, 250 x 4.6 mm, USA) maintained at 40ºC. Samples were

eluted using a mixture of solvent water/formic acid 0.05% (eluent A) and acetonitrile/formic acid 0.05% (eluent B) by the following gradient (95/5 at 0 min, 75/25 at 16 min, 5/95 during 5 min and finally 95/5 during 10 min) at a flow rate of 1 mL per min. The mass spectrometer was used in negative and positive mode with a voltage cone at 50, 80 and 110 V, the temperature of the electrospray ionization (ESI) ion source was 450ºC and the gas carrier was nitrogen. The mass spectrometer scanned from m/z 100 to 1,500. The data acquisition and processing were performed using Chromeleon™ 7.2 data systems.

The molar mass (MM) distribution of puerarin fructo-conjugates was determined by high-pressure size exclusion chromatography (HPSEC) using two columns (Shodex OH-Pak 805 and 802.5) connected in series at 70 °C and equipped with RI and UV detector. Elution was performed using a solution of 450 mM sodium nitrate and 1% (v/v) ethylene glycol in 50 mM sodium acetate as eluent at a flow rate of 0.25 mL per min for 85 min. MM distribution was estimated by comparing to the migration of the standards levan from *Erwinia herbicola* (1000 kg/mol) and commercial dextrans (3.5, 6, 68 and 400-600 kg/mol).

2.5 Purification of puerarin fructo-conjugates

Puerarin fructo-conjugates were purified by flash chromatography on a Reveleris® Flash Chromatography System (Grace, USA) with a Reveleris ® C18 column (40 µm, 120 g, Lot #16052035), using ultra-pure water (eluent A) and acetonitrile (eluent B) under the following gradient (v/v): 100/0 at 0 min, 75/25 at 11.2 min, 30/70 during 0.9 min and finally keep 100/0 during 1.8 min, the flow rate was 85 mL per min. Sugars eluted with water and puerarin fructosides of different sizes were recovered with increasing gradient of acetonitrile. The detection wavelength was set at 254 nm and the evaporative light scattering detector (ELSD) was set at 20 mV to follow the separation.

2.6 NMR structural characterization

NMR was acquired on an Advance 500 MHz spectrometer (Bruker) operating at 500 MHz for ^{1}H and 125 MHz for ^{13}C. The data were processed using Topspin 3 Software. All measurements were performed at 298 K and chemical shifts (supplementary material Table

S1) were given in ppm relative to the residual signal of DMSO-d_6 (δH ppm : 2.50 ppm, δC ppm : 39.5 ppm).

2.7 MALDI-TOF-MS analysis of puerarin fructo-conjugates

The MM of puerarin fructo-conjugates was determined by MALDI-TOF-MS in a Waters® MALDI-Micro MX-TOF Mass spectrometer. The measurements were performed with the mass spectrometer in positive reflection mode using an accelerating voltage of 12 kV. Mass spectra were acquired from 400 (m/z) to 3000 (m/z). Samples were dissolved in water (1 mg/mL) and incubated 10 min at 50°C. A 0.5 mL of sample solution was mixed with 0.5 ml of the matrix solution (2,5-dihydroxybenzoic acid 10 mg/mL in H_2O:EtOH 0.5:0.5; v/v) and a total of 1 µL was applied to a stainless steel sample slide and dried at room temperature.

2.5 Water solubility of isolated puerarin fructo-conjugates

The water solubility of puerarin and puerarin monofructoside (P1a) was determined at 25 °C as described by Núñez-López et al. (2019). Puerarin difructoside (P2a), puerarin oligofructosides (POFs, 3-12 fructosyl units, predominantly 3-5) and puerarin polyfructosides (PPFs, 3-21 fructosyl units, predominantly 12-15) were synthesized from the precursor P1a and isolated by Flash Chromatography. P2a (1.84 g), POFs (2.01 g) and PPFs (1.06 g) were mixed with ultrapure water (1 g). Each solution was incubated at 25 °C and 250 rpm for 2 h and then centrifuged 5 min at 12000g. The very high viscosity of the medium impeded to evaluate the solubilization of P2a, POFs and PPFs at higher concentrations.

3. Result and Discussion

3.1 Synthesis of puerarin fructosides by levansucrase from four bacterial species

Levansucrases from *Bacillus subtilis* (*Bs*_SacB), *Gluconacetobacter diazotrophicus* (*Gd*_LsdA), *Leuconostoc mesenteroides* (*Lm*_LevS), and *Zymomonas mobilis* (*Zm*_LevU) were assayed for their capacity to transfer the fructosyl moiety of sucrose onto puerarin. The highest percentage of acceptor conversion was achieved by *Gd*_LsdA (88.7 %) and *Bs*_SacB

(24.8 %) (Table 1). In the case of *Lm*_LevS and *Zm*_ LevU, less than 10 % of puerarin was transformed into products.

Table 1. Fructosylation of puerarin by levansucrases of different origins

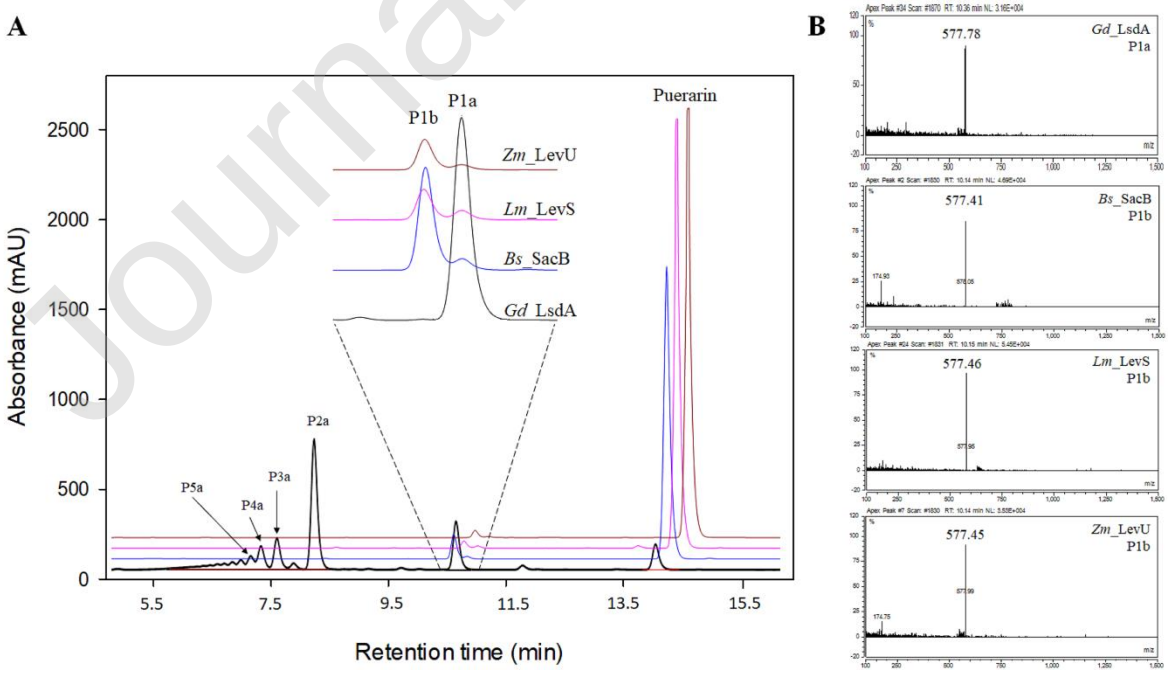
Bacteria	Enzyme name	Puerarin	
		Conversion (%)[a]	Type of fructosides[b]
Bacillus subtilis	*Bs*_SacB	24.8 ± 0.1	Mono
Gluconacetobacter diazotrophicus	*Gd*_LsdA	88.7 ± 0.4	Mono to ≥ penta
Leuconostoc mesenteroides	*Lm*_LevS	7.3 ± 2.2	Mono
Zymomonas mobilis	*Zm*_LevU	9.0 ± 2.5	Mono

Each enzyme (0.5 U/mL) was reacted with sucrose (500 mM) and puerarin (10 mM) in citrate-phosphate buffer (50 mM, pH 5.8) for 24 h. The reaction temperature was 42 °C except for *Lm*_LevS (30 °C). All the experiments were performed in duplicates.

[a] Puerarin conversion was calculated from the amount of acceptor remaining after the reaction. Conversion $=100*([\text{Puerarin}]_{\text{initial}} - [\text{Puerarin}]_{\text{final}})/ [\text{Puerarin}]_{\text{initial}}$.

[b] The type of fructoside is defined according to mass values determined by mass spectrometry.

The acceptor reactions of *Bs*_SacB, *Lm*_LevS and *Zm*_LevU exhibited similar HPLC-UV chromatograms (Fig. 1A). Puerarin fructosylation resulted in two products, designated as P1a and P1b, with retention times of 10.36 and 10.19 min, respectively. The molecular ions at m/z 577.18 ($M_{\text{Puerarin-Fru}}-H$) for P1a and at m/z 577.41 for the most abundant P1b (Fig. 1B) revealed the synthesis of two monofructoside isomers. By contrast, the HPLC-UV chromatogram of the *Gd*_LsdA reaction showed the occurrence of P1a, a major product P2a,



and a short series of further elongated fructosides in decreasing amounts (P3a, P4a, P5a and others) (Fig. 1A). The synthesis of P1a as unique monofructoside highlights the strict regioselectivity of *Gd*_LsdA compared to the other tested levansucrases.

Fig. 1. Puerarin fructosylation by the four levansucrases in study. A) Superimposition of HPLC-UV chromatograms. B) MS chromatograms of puerarin mono-fructosides. Peaks were identified as P1a and P1b (monofructosides), P2a (difructoside), P3a (trifructoside), P4a (tetrafructoside), and P5a (pentafructoside). The reaction conditions are described in Table 1.

3.1.1 Structural characterization of products P1a, P2a and P1b

*Gd*_LsdA and *Bs*_SacB were used for the preparative synthesis, purification and structural characterization of the products P1a, P2a and P1b. The chemical structure of P1a, P2a (supplementary material Fig. S1-S4), and P1b (supplementary materials Fig. S5-S8) was determined by ^{1}H NMR, ^{13}C NMR and 2D NMR spectroscopy.

P1a was identified as β-D-monofructofuranosyl-(2→6)-puerarin, confirming that *Gd*_LsdA transfers the fructosyl moiety to the O6 position of the glucosyl residue of the isoflavone (Núñez-López et al. 2019). Levansucrases fructosylate glucose via a β(2→1) linkage regenerating sucrose or a β(2→6) linkage producing the isomer blastose, which is not used as donor (Homann et al., 2007; Raga-Carbajal et al., 2018). The synthesis of P1a is thus consistent with the linkage specificity of levansucrases but, with exception of *Gd*_LsdA, the presence of the daidzein group prevented the efficient fructosylation of the acceptor at its glucosyl unit.

The molecular ion at m/z 739 ($M_{Puearin\text{-}Fru\text{-}Fru}$−H) for the *Gd*_LsdA product P2a confirms the transfer of two fructosyl units to puerarin. The ^{13}C spectrum revealed 33 signals (three overlapped), of which 13 carbon signals were in the range 110–175 ppm and assigned to puerarin, and 17 signals were in the range 60-110 ppm and assigned to the three monosaccharides (two fructose units and one glucose unit). Six additional signals for the fructosyl unit were identified. Two of these signals with chemical shift at 104.20 and 104.34 ppm characteristic of the carbon atom C2’’’’ and C2’’’ of the fructosyl moiety confirm the presence of two fructose unit. The HMBC spectrum (supplementary material Fig. S4) of P2a

was then used to deduce the position of fructosylation. A long-range correlation between the carbon atom C2'''' of fructose (δ_C 104.20 ppm) and the proton H6''' (δ_H 3.55) of the fructose unit, demonstrate that P2a corresponds to β-D-difructofuranosyl-(2→6)-puerarin. Notably, P2a is neither a donor nor an efficient acceptor of *Gd*_LsdA and thus accumulates in the reaction mixture after prolonged incubation (see Fig.1).

The ^{13}C spectrum of monofructoside P1b synthesized by *Bs*_SacB revealed 27 signals (three overlapped). Twelve signals were in the range 60-110 ppm being characteristic of the carbons of the fructosyl and glucosyl moieties. The other 13 signals (110-176 ppm) can be assigned to the isoflavone (supplementary materials Fig. S6). A large downfield shift (+ 6.1) was observed for carbon C3' and C5' and (+ 3.66) for carbon C1' and an upfield shift (- 2.75) for carbon C4'. The ^{1}H-NMR spectrum (supplementary material Fig. S5) revealed the disappearance of the peak at 9.53 ppm, which corresponds to 4' -OH and a displacement of the signals at 7.25 ppm (+ 0.45) characteristic of H3' and H5', as deduced by comparing to the spectra of puerarin and P1a (Fig. 2). The position of the attached fructosyl unit was confirmed using HSQC and HMBC analyses. From these results, P1b was identified as puerarin-4'-*O*-fructofuranoside. The transfer of the fructosyl unit onto the 4' O-position of the phenolic cycle of puerarin by the enzymes *Bs*_SacB, *Lm*_LevS and *Zm*_LevU (Fig. 3) occurred with efficiencies ranging 7-25 %. In previous studies, *Lm*_LevS (Kang et al., 2009) and *Bs*_SacB (Mena-Arizmendi et al., 2011) catalyzed the fructosyl transfer onto the aromatic compound hydroquinone (benzene-1,4 diol) yielding 4-hydroxyphenyl-β-D-fructofuranoside, with acceptor conversion below 12 %.

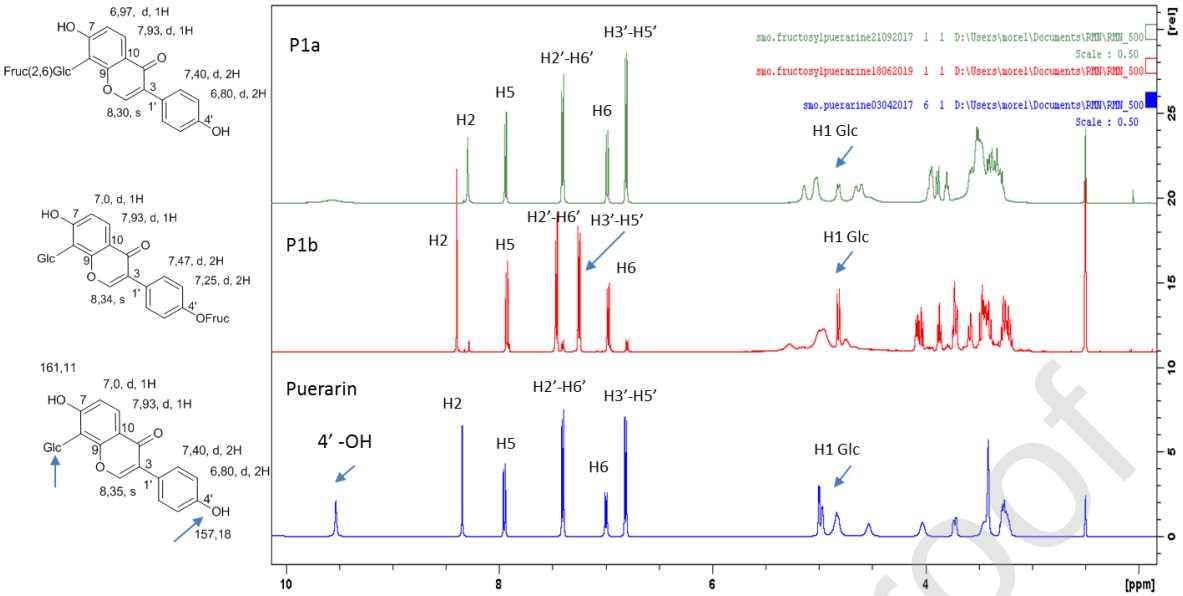
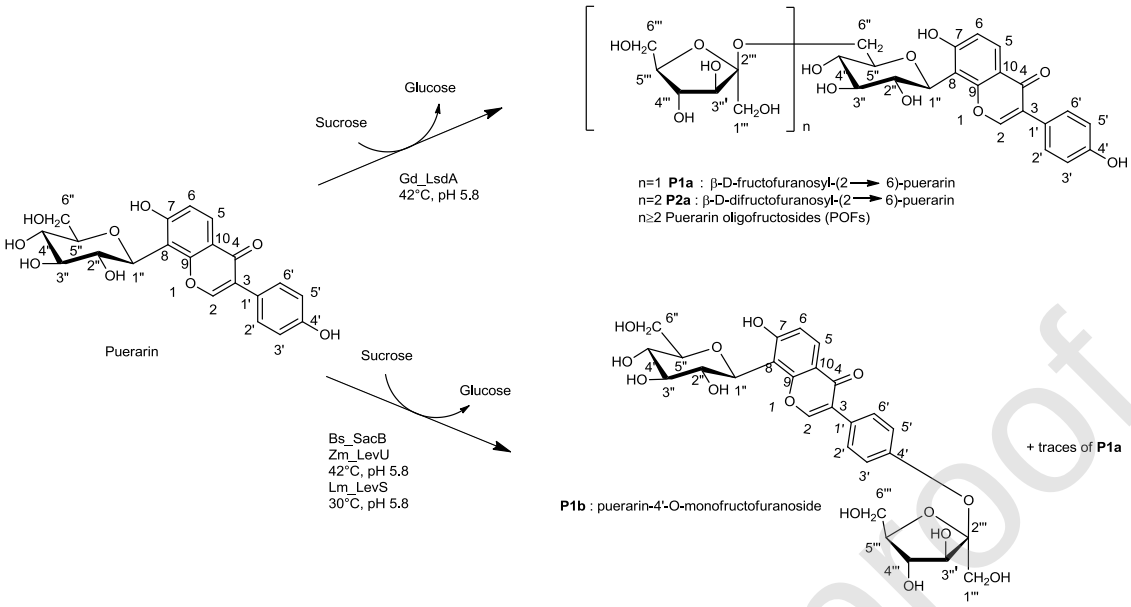


Fig. 2. Superimposition of the ^{1}H NMR spectra of puerarin, P1a and P1b

Three positional isomers of puerarin monofructosides have been reported so far. The synthesis of β-D-fructofuranosyl-(2→6)-puerarin (Núñez-López et al., 2019; Wu et al., 2013), puerarin-7-*O*-β-fructofuranoside (Wang et al., 2014; Yu et al., 2010), and puerarin-4'-*O*-β-fructofuranoside (this work) reveals variations in the regioselectivity of puerarin fructosylation by GH68 enzymes. The occurrence of only β-fructosyl products is in agreement with the strict stereoselectivity of retaining fructosidases. The 3D-structure resolution of *Gd*_LsdA and *Bs*_SacB in complex with puerarin, as well as molecular docking simulations and mutagenesis studies would be of interest to study the acceptor interactions with these two representative levansucrases, and identify the structural determinants at the origin of their regiospecificity.

Fig. 3. Schematic synthesis of puerarin fructosides by *Gd*_LsdA, *Bs*_SacB, *Lm*_LevS and



*Zm*_LevU.

3.2 Synthesis of puerarin polyfructosides (PPFs) using P1a as acceptor

P1a [β-D-monofructofuranosyl-(2→6)-puerarin] that is produced efficiently by *Gd*_LsdA was purified by flash chromatography and assayed as a precursor for the synthesis of PPFs using the same enzyme and the more competent polymerase *Bs*_SacB. Purified P1a (10 mM) was incubated with either *Gd*_LsdA or *Bs*_SacB at different sucrose concentrations (0.5, 1 or 1.5 M). *Bs*_SacB transformed 93-95 % of initial P1a regardless of the numeric variation of donor molecules in the reaction (Table 2). By contrast, the acceptor conversion by *Gd*_LsdA (55.2 %) decreased as the sucrose concentration was increased to 1.5 M, a condition that favors fructosylation over donor hydrolysis. This result reflects a competition between sucrose and P1a for binding the acceptor subsite. Sucrose is known to be not only the natural donor but also the preferred acceptor of *Gd*_LsdA (Hernández et al., 1995).

HPLC-UV chromatograms show remarkable differences in the size distribution of the puerarin fructo-conjugates synthesized from the acceptor P1a (Fig. 4). *Gd*_LsdA synthesized a linear series of puerarin oligofructosides (POFs) (P2a, P3a, P4a, P5a, ...) remaining P2a as the major product, while *Bs*_SacB produced PPFs of longer fructan chains (overlapped peaks

with retention times 4.2-7.8 min) and at a higher yield. *Bs*_SacB also synthesized the monofructoside P1b from remaining traces of puerarin.

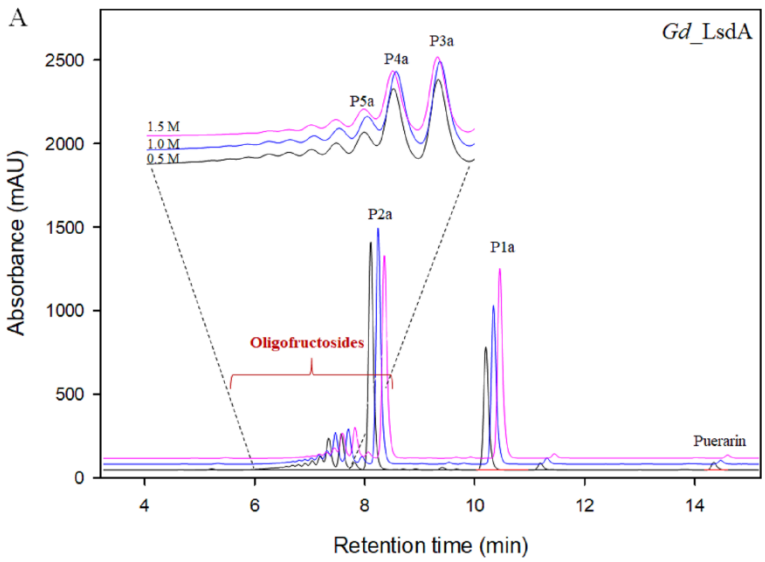
Table 2. P1a fructosylation by *Gd*_LsdA and *Bs*_SacB at different sucrose concentrations

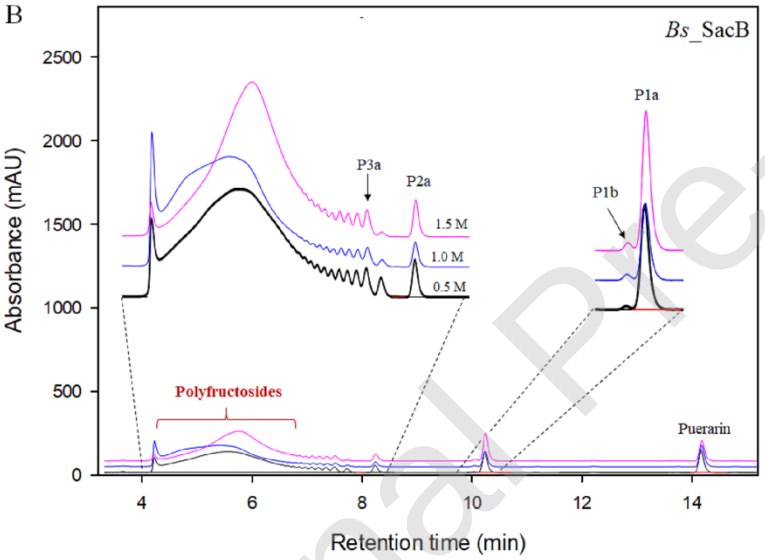
Levansucrase	P1a conversion (%)[a]		
	0.5[b]	1.0[b]	1.5[b]
*Gd*_LsdA	72.1 ± 1.6	65.9 ± 1.6	55.2 ± 1.9
*Bs*_SacB	93.2 ± 1.7	95.9 ± 0.1	93.4 ± 0.8

Each enzyme (0.5 U/mL) was incubated with purified P1a (10 mM) and sucrose (0.5-1.5M) in 50 mM phosphate buffer (pH 5.8) at 42 °C during 24 h under agitation.
[a]Acceptor conversion $=100*([P1a]_{initial} - [P1a]_{final})/[P1a]_{initial}$
[b]Sucrose concentration (M)

In parallel, the course of sucrose transformation into fructans in the presence or absence of P1a was monitored by HPAEC-PAD (supplementary material Fig. S9). *Gd*_LsdA accumulated high levels of 1-kestotriose and 1,1-kestotetraose. The enzyme also synthesized 6-kestotriose and 6,6-kestotetraose, which acted as preferred acceptors for levan polymerization. By contrast, *Bs*_SacB produced an array of growing oligosaccharides and a higher yield of levan polysaccharide. The presence of P1a did not cause a visible variation in the yield or the spectra of the fructan products synthesized by either enzyme, due to the fact that compared to the initial concentration of sucrose, the amount of fructosyl units transferred to P1A is negligible.

The HPLC-UV chromatograms of the *Bs*_SacB products obtained using puerarin (Fig. 1) or P1a (Fig. 3) as acceptors show notable variations. Only 25% of the initial puerarin content was converted into products and the main monofructoside P1b was not further elongated. In contrast, the enzyme used P1a as a convenient precursor for the synthesis of PPFs with the conversion value reaching 95 %. The incorporation of a fructosyl unit to the glucosyl unit of puerarin through the formation of a β(2→6) linkage appears to fulfill the necessary requirements for the proper accommodation and effective binding of P1a in the acceptor subsites of *Bs*_SacB, *Gd*_LsdA and probably other levansucrases.





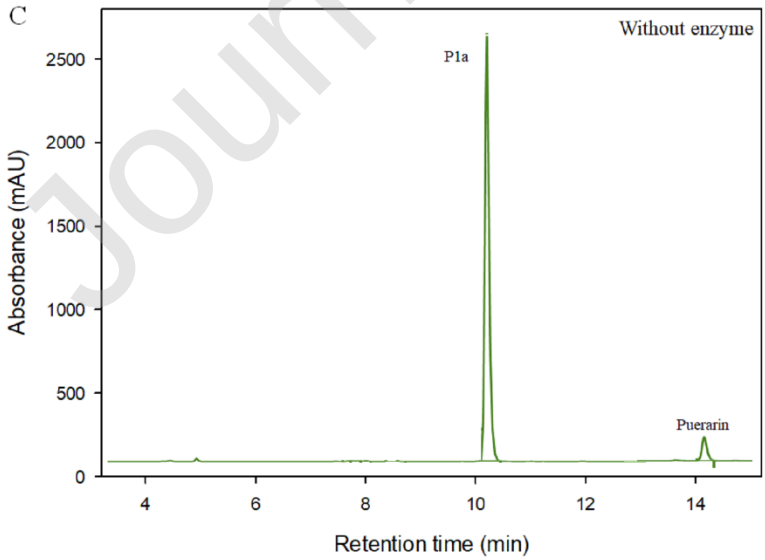


Fig. 4. HPLC-UV-MS chromatograms of P1a elongation by (A) *Gd*_LsdA and (B) *Bs*_SacB. Each enzyme (0.5 U/mL) was incubated with P1a (10 mM) and sucrose (0.5-1.5M) in 50 mM phosphate buffer (pH 5.8) at 42 °C during 24 h under agitation. The chromatogram of purified P1a used as acceptor is shown (C). Puerarin oligofructosides (POFs) produced by *Gd*_LsdA comprises P2a (2 fructosyl units) as the major component, followed by decreasing concentrations of P3a, P4a, P5a and other very small peaks. The linear chains of puerarin polyfructosides (PPFs) produced by *Bs*_SacB reach at least 21 fructosyl units being the predominant range 12-15.

3.2.1 Size estimation of puerarin fructo-conjugates synthesized from precursor P1a

Puerarin fructo-conjugates were synthesized at a preparative scale from sucrose (500 mM) and P1a (25 mM) by *Gd*_LsdA and *Bs*_SacB with conversion efficiencies of 70 % and 73 %, respectively. The products of the two independent reactions were purified by flash chromatography and their mass distribution was characterized by HPSEC-RI using dextran for calibration and MALDI-TOF MS.

The HPSEC chromatogram of the *Gd*_LsdA products (Fig. 5A) showed a sharp peak matching to P2a and a shorter but wider peak (retention times 55-65 min) corresponding to a population of POFs with average molar mass of 1400 $g.mol^{-1}$ at peak apex. More interestingly, the *Bs*_SacB, products (Fig. 5B) were distributed as a small P2a peak and a larger peak (retention times 52-60 min) corresponding to PPFs with average molar mass of 3500 $g.mol^{-1}$ at peak apex.

The MALDI-TOF MS analysis of the *Gd*_LsdA acceptor products (Fig. 5C) revealed a spectrum of POFs with molecular masses ranging from m/z 601.2 to 2545.8, consistent with the distributive incorporation of a maximum of 13 fructosyl units. The number average molecular weight (Mn) and weight average molecular weight (Mw) of POFs synthesized by *Gd*_LsdA were 953.1 and 1042.2, respectively. *Bs*_SacB yielded PPFs of a wider mass range covering from m/z 601.2 to 3842.2 (Fig 5D). The latter value reveals that the fructan chain incorporated onto puerarin reached at least 21 fructosyls units. The average values for Mn and Mw were 1508.4 and 1818.1, respectively.

The size distribution of PPFs observed in the HPLC-UV-MS (Fig. 4), HPSEC-RI (Fig. 5 A, B) and MADI-TOF chromatograms (Fig. 5 C, D) revealed that *Bs*_SacB polymerizes the precursor P1a more efficiently than *Gd*_LsdA, which otherwise tends to accumulate the immediate product P2a. In both cases, the elongation of the fructan chain occurred via a distributive mechanism, in which the product dissociates from the enzyme after every fructosyl transfer.

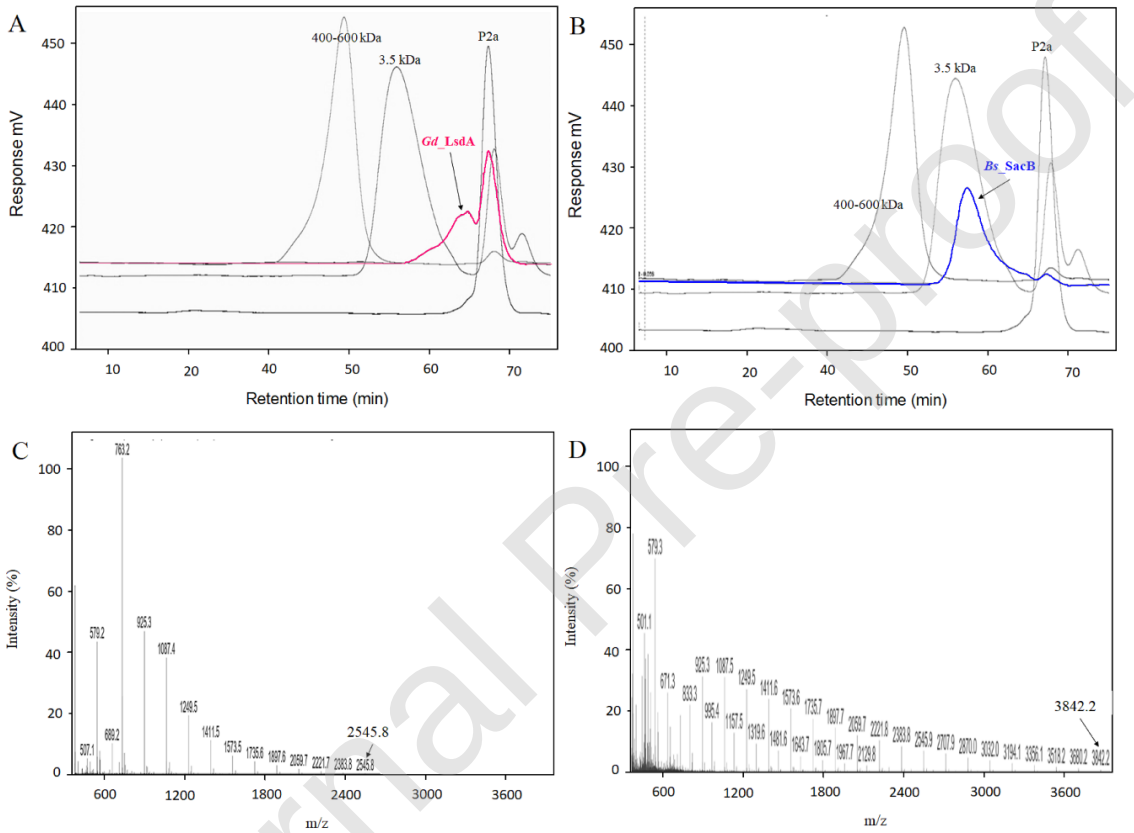


Fig. 5. Mass distribution of puerarin fructo-conjugates from P1a elongation. A) HPSEC-RI analysis of puerarin fructo-conjugates synthesized by *Gd*_LsdA and B) *Bs*_SacB using sucrose (500 mM) as donor and purified P1a (25 mM) as acceptor. P2a and dextrans were used as reference for molar mass determination. The chromatograms corresponding to *Gd*_LsdA and *Bs*_SacB are highlighted in pink and blue, respectively. MALDI-TOF MS analysis of PPFs produced by C) *Gd*_LsdA and D) *Bs*_SacB

*Gd*_LsdA and *Bs*_SacB display a single domain 5-bladed β-propeller fold enclosing the substrate-binding central cavity. In the two enzymes, the donor sucrose accommodates similarly at the bottom of the deep funnel-shaped pocket (Martínez-Fleites et al., 2005; Meng

& Fütterer, 2003), but there are important differences in the predicted area (*Bs*_SacB 3783 $Å^2$, *Gd*_LsdA 5773 $Å^2$) and volume (*Bs*_SacB 6263 $Å^3$, *Gd*_LsdA 10100 $Å^3$) of their entire active site cavity (Fig. 6). In this sense, *Gd*_LsdA offers a wider gate that may facilitate a more rapid release of the intermediate product P2a to the medium delaying its binding to the acceptor subsite for the next fructosylation cycles. On the other hand, the 3D structures of *Gd*_LsdA and *Bs*_SacB do not superimpose well at the rim and surface of the active site pocket (Martínez-Fleites et al., 2005), which include the regions responsible for the acceptor binding, orientation and elongation (Ortiz-Soto, Porras-Domínguez, Seibel, & López-Munguía, 2019). Rational mutagenesis of amino acids lining the surface of the active site cavity of *Gd*_LsdA may contribute to increase the enzyme affinity for the acceptor P2a. There is no report on enhanced formation of levan polysaccharide by a mutated variant of *Gd*_LsdA or another FOS-yielding levansucrase.

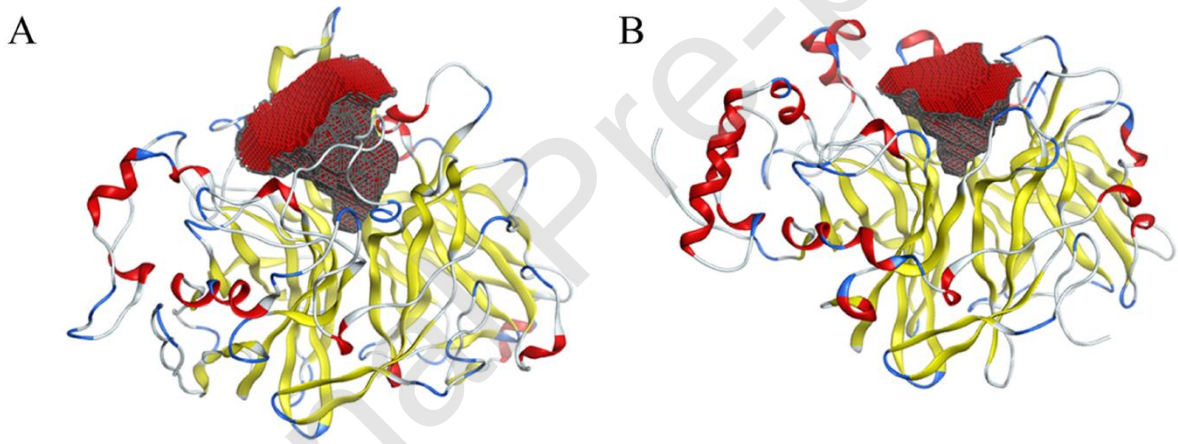


Fig. 6. Representation of the active site cavity in the 3D structure of levansucrases from (A) *Gluconacetobacter diazotrophicus* (*Gd*_LsdA; PDB code 1W18) and (B) *Bacillus subtilis* (*Bs*_SacB; PDB code 1OYG). The surface area (brown) and volume (red) for the entire active site cavity of *Gd*_LsdA (5773 Å2, 10100 Å3) and *Bs*_SacB (3783 Å2, 6263 Å3) were determined using CavityPlus (Xu et al., 2018), CASTp (Tian, Chen, Lei, Zhao, & Liang, 2018), and UCSF Chimera (Pettersen et al., 2004). The 3D structures are shown as ribbon diagrams.

3.3 One-pot, bi-enzymatic cascade synthesis of PPFs

P1a was the sole monofructoside synthesized from puerarin by *Gd*_LsdA. The conversion efficiency of the acceptor reaction was 93% using 146 mM sucrose and 25 mM puerarin in the absence of co-solvents (Núñez-López et al., 2019). On the other hand, *Bs*_SacB fructosylted the precursor P1a to longer chains, compared to *Gd*_LsdA. In an attempt to avoid the isolation of the intermediate product P1a, the possibility to produce PPFs directly from puerarin was evaluated by combining the complementary activities of *Gd*_LsdA and *Bs*_SacB in the same reactor.

The one-pot bi-enzymatic cascade reaction (Fig. 7) was performed through two operating methods: (i) either by using the two enzymes sequentially or (ii) by using them simultaneously.

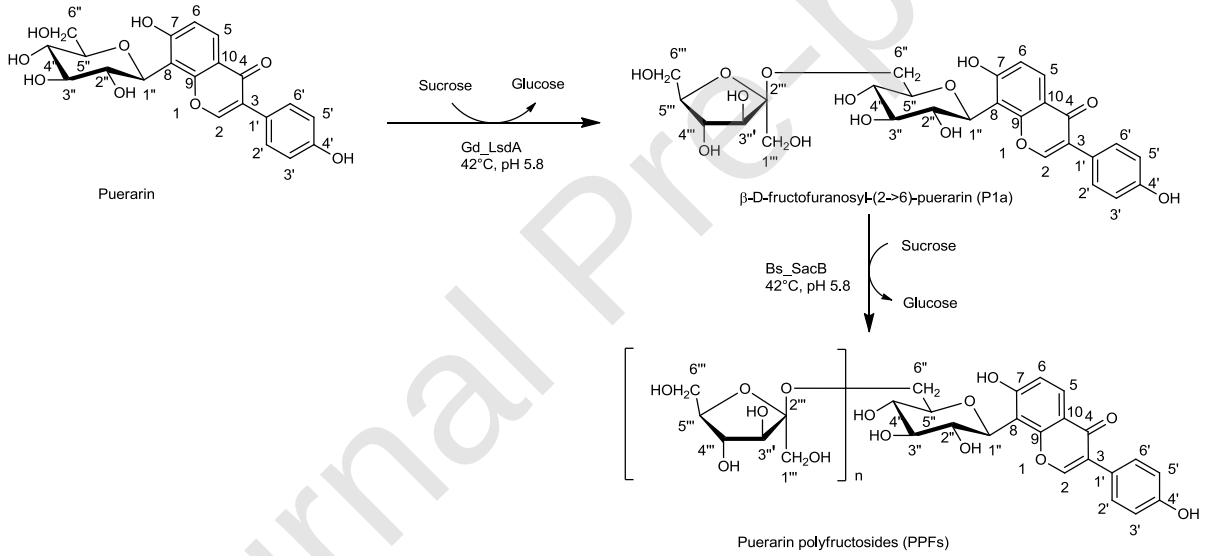


Fig. 7. Schematic synthesis of puerarin polyfructosides by the cascade reaction of *Gd*_LsdA and *Bs*_SacB. The number of fructosyl units (n) in PPFs ranges from 2 to at least 21, predominantly 12-15.

In the first method, *Gd*_LsdA initiated the reaction for 6 h (step one) and then *Bs*_SacB was incorporated with or without extra sucrose (step two). In the second method, *Gd*_LsdA and *Bs*_SacB were both incorporated in the reactor at the start of the reaction, with different activity ratios (1:1, 2:1 or 1:2) (Table 3).

High percentages of puerarin conversion (82-92%) were achieved, except for the sequential reaction in which extra fructosyl donor was not supplied at step two. When the reaction was performed under the same conditions for step one [*Gd*_LsdA (0.1 U.mL^{-1}), sucrose (146 mM) and puerarin (25 mM)] but adding *Bs*_SacB (0.5 U.mL^{-1}) together with sucrose (292 mM) the acceptor conversion increased from 38 to 89 % (Table 3).

Table 3. One-pot, bi-enzymatic synthesis of PPFs using puerarin as acceptor

Operating method	Enzyme step1 (U/mL)	Sucrose step 1 (mM)	Acceptor puerarin (mM)	Enzyme step 2 (U/mL)	Sucrose step 2 (mM)	Acceptor conversion * (%)
Sequential use of enzymes	*Gd*_LsdA 0.1	146	25	*Bs*_SacB 0.5	0	38.1 ± 0.4
	*Gd*_LsdA 0.1	146	25	*Bs*_SacB 0.5	292	88.9 ± 0.9
	*Gd*_Lsd*A* 0.1	146	25	*Bs*_SacB 0.5	500	84.8 ± 0.1
Simultaneous use of enzymes	*Gd*_LsdA 0.75 *Bs*_SacB 0.75	500	10	-	-	85.9 ± 0.3
	*Gd*_LsdA 1.0 *Bs*_SacB 0.5	500	10	-	-	92.4 ± 0.7
	*Gd*_LsdA 0.5 *Bs*_SacB 1.0	500	10	-	-	82.2 ± 0.6

Each mixture of donor (sucrose) and acceptor (puerarin) was prepared in 50 mM citrate-phosphate buffer (pH 5.8) and incubated with the enzymes at 42 °C under constant agitation. For the sequential use of enzymes, *Gd*_LsdA was reacted with the substrates for 6 h and inactivated 5 min at 100°C (step 1), then *Bs*_SacB was incorporated with or without supplementary sucrose (step 2). For the simultaneous use of enzymes, *Gd*_LsdA and *Bs*_SacB were added to the substrate mixture from the beginning of the reaction.

[a]The conversion was calculated from the amount of puerarin remaining after 24 h of reaction. Acceptor conversion = $100*([\text{puerarin}]_{\text{initial}} - [\text{puerarin}]_{\text{final}})/ [\text{puerarin}]_{\text{initial}}$.

When the two enzymes were incorporated simultaneously in the reactor at the reaction start, maximum conversion (92.4 %) was obtained with 1 U/mL^{-1} *Gd*_LsdA, 0.5 U/mL^{-1} *Bs*_SacB, 500 mM sucrose and 10 mM puerarin (Table 3). This value is slightly superior to that achieved with the best sequential treatment (89 %), which otherwise used ten times less *Gd*-LsdA and a closer molar proportion donor/acceptor (439/25). Finally, the one-pot bi-enzymatic systems (either sequential or simultaneous) were comparable in terms of conversion with the acceptor reaction performed with *Bs*_SacB and pure P1a (93.2 %) but avoided the isolation of the intermediate product P1a, an advantage of prime interest for biotechnological applications.

3.4 Increased water solubility of di-β-D-fructofuranosyl-(2→6)-puerarin (P2a), POFs and PPFs

In previous experiments, the isomers β-D-fructofuranosyl-(2→6)-puerarin (P1a) (Núñez-López et al., 2019) and puerarin-7-*O*-β-fructofuranoside (Wang et al., 2014) were found to be 23 and 5.6 times more soluble in water than puerarin, respectively. Those results indicated that the solubility of puerarin monofructosides is influenced by the position of the fructosyl moiety. Here, the water solubility of puerarin fructo-conjugates containing linear β(2→6)-linked fructan chains of different sizes was investigated. The solubility at 25ºC of P2a (>1840 g/L) and POFs (> 2018 g/L) synthesized by *Gd*-LsdA and PFFs (>1062 g/L) synthesized by *Bs*-SacB was higher than that of P1a (16.2 g/L) and puerarin (0.7 g/L). The high viscosity of the limpid aqueous solutions of purified P2a, POFs and PPFs impeded to further increase their concentration until reaching the saturation point. P2a (2 fructosyl units), POFs (3-12 fructosyl units, predominantly 3-5) and PPFs (3-21 fructosyl units, predominantly 12-15) have an increased number of hydroxyl groups which can form hydrogen bonds with water, compared to the precursor P1a. Noteworthy, difructosylation of puerarin enabled to improve by at least 2620-fold the water solubility of the flavone. Our finding reveals that the solubility, and by consequence the bioavailability, of puerarin can be tremendously enhanced by attaching two or more fructosyl units via β(2→6) linkages.

We describe for the first time the enzymatic synthesis of PPFs as novel products using a bi-enzymatic cascade and demonstrate the interest of conducting one-pot synthesis. In comparison to puerarin, the increased water solubility of PPFs must enhance their absorption *in vivo*, broadening the spectrum of potential pharmacological applications. Additionally, it will be interesting to investigate if the fructan chain provides PPFs with prebiotic properties.

4. Conclusion

In this study, four levansucrases of different origins were found to fructosylate the non-conventional acceptor puerarin in an aqueous medium, although with variations in their regioselectivity and conversion efficiency. The enzyme from *Gluconacetobacter diazotrophicus* (*Gd*_LsdA) efficiently synthesized β-D-fructofuranosyl-(2→6)-puerarin (P1a), while the three other levansucrases formed puerarin-4'-*O*-β-D-fructofuranoside (P1b)

and only traces of P1a with low conversion rates. Purified P1a was converted into water-soluble polyfructosides (PPFs) reaching at least 21 fructosyl units by the competent polymerase activity of *Bacillus subtilis* levansucrase (*Bs*_SacB). More attractively, simultaneous or sequential *Gd*_LsdA/*Bs*_SacB reactions yielded PPFs directly from puerarin in one reactor, using sucrose as a cheap fructosyl donor, and avoiding the isolation of intermediate precursor P1a. The one-pot bi-enzymatic procedure described in this paper is appropriate to scale up the cascade production of PPFs, new products with potentially healthy properties.

Author contributions

Gema Núñez-López: Investigation, Writing original draft

Sandrine Morel: Conceptualization, Methodology, Investigation, Visualization, Writing-review & editing

Lázaro Hernández: Methodology, Visualization, Writing-review & editing

Alexis Musacchio: Data curation, Formal analysis

Lorena Amaya-Delgado: Resources, Funding acquisition

Anne Gschaedler: Resources, Funding acquisition

Magali Remaud-Simeon: Project administration, Supervision, Funding acquisition in France.

Javier Arrizon: Project administration, Supervision, Visualization, Funding acquisition in Mexico.

Conflict of interest

The authors declare that they have no conflict of interest.

Compliance with ethical standards

This article does not contain any studies with human participants or animals performed by any of the authors.

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