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Enzymatic synthesis of L-DOPA α-glycosides by reaction with sucrose catalyzed by four different glucansucrases from four strains of *Leuconostoc mesenteroides*

Seung-Heon Yoon [a,b], D. Bruce Fulton [b], John F. Robyt [a,b,*]

[a] Laboratory of Carbohydrate Chemistry and Enzymology, Iowa State University, Ames, IA 50011, USA
[b] Department of Biochemistry, Biophysics, and Molecular Biology, Iowa State University, Ames, IA 50011, USA

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ABSTRACT

L-DOPA α-glycosides were synthesized by reaction of L-DOPA with sucrose, catalyzed by four different glucansucrases from *Leuconostoc mesenteroides* B-512FMC, B-742CB, B-1299A, and B-1355C. The glucansucrases catalyzed the transfer of D-glucose from sucrose to the phenolic hydroxyl position-3 and -4 of L-DOPA. The glycosides were fractionated and purified by Bio-Gel P-2 column chromatography, and the structures were determined by ^{1}H NMR spectroscopy. The major glycoside was 4-*O*-α-D-glucopyranosyl L-DOPA, and the minor glycoside was 3-*O*-α-D-glucopyranosyl L-DOPA. The two glycosides were formed by all four of the glucansucrases. The ratio of the 4-*O*-α-glycoside to the 3-*O*-α-glycoside produced by the B-512FMC dextransucrase was higher than that for the other three glucansucrases. The glycosylation of L-DOPA significantly reduced the oxidation of the phenolic hydroxyl groups, which prevents their methylation, potentially increasing the use of L-DOPA in the treatment of Parkinson's disease. The use of one enzyme, glucansucrase, and sucrose as the D-glucosyl donor makes the synthesis considerably simpler and cheaper than the formerly published procedure using cyclomaltodextrin and cyclomaltodextrin glucanyltransferase, followed by glucoamylase, and β-amylase hydrolysis.

1. Introduction

Parkinson's disease (PD) is a neurodegenerative disease that develops from the cell death of dopaminergic neurons in the mid-brain.[1] The loss of the dopaminergic neurons occur because of the oxidation by a reactive oxygen species and/or the action of nitric oxide,[1–6] which affects neuronal toxins,[2–4] and also by the expression of inflammatory cytokines[2,7] and aggregated cytotoxic proteins.[2,8] Many dopamine agonists and supplements are used to relieve PD symptoms. Dopamine agonists, such as pramipexole (Mirapex),[9,10] ropinirole (Requip),[11] and rotigotine (Neupro),[12] activate dopamine receptors and compensate for the decreased dopamine signal caused by neuronal cell death. The dopamine agonists eventually become ineffective, however, unless dopamine or a dopamine precursor such as 3,4-dihydroxyphenyl-L-alanine (L-DOPA) is transported into the mid-brain. Since dopamine cannot pass through the blood–brain barrier, its natural precursor, L-DOPA, which can pass though the barrier, is prescribed as a pharmaceutical treatment of PD.[1,4] L-DOPA is converted to dopamine in the brain by L-tyrosine decarboxylase.[1,4]

Although L-DOPA is the most effective drug for the treatment of PD,[13,14] it must be used carefully, because it has some deleterious side effects.[4] When L-DOPA is administered, the 3-phenolic group of L-DOPA is methylated in the peripheral tissues by catecholamine-*O*-methyltransferase to produce 3-*O*-methyl-L-DOPA, which reaches ~13.8 times the concentration of L-DOPA in plasma.[15] The conversion to 3-*O*-methyl-L-DOPA not only reduces the availability of L-DOPA but also inhibits the transport of L-DOPA from the blood though the blood–brain barrier, producing cytotoxic effects and damage to the neuronal cells.[16,17] In clinical trials, L-DOPA significantly reduces the apparent PD symptoms in the early stages of PD. It has been observed, however, that the use of L-DOPA for about five years resulted in more than 50% of the PD patients experiencing severe motor fluctuations.[15] The worsening of PD, which occurs with the use of L-DOPA, has been associated with the oxidation of dopamine in the brain.[1–7]

Based on the physicochemical properties of L-DOPA in the treatment of PD, it has been suggested that the blocking or derivatization of one of the two phenolic hydroxyl groups on the aromatic ring of L-DOPA would eliminate the enzymatic- and/or auto-oxidation, as well as the enzymatic methylation of L-DOPA.[18] A very mild, biological derivatization of L-DOPA is glycosylation, which would eliminate both oxidation and methylation, as well as making L-DOPA much more water soluble. This then would very considerably increase the long-term use of L-DOPA in treating PD.

Recently dopamine, L-DOPA, and D,L-DOPA-glycosides were prepared by a transglycosylation reaction or a reverse hydrolysis

* Corresponding author. Tel.: +1 515 294 1964; fax: +1 515 294 0453.
E-mail address: jrobyt@iastate.edu (J.F. Robyt).

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reaction of glucoamylase and β-glucosidase in a micro-aqueous reaction system.[19,20] The glycosylation was carried out in 100 mL of diisopropyl ether, containing 0.3–2.2 mL of 0.01 M buffer (pH 4–8), and 1 mmol of D-glucose (0.182 g) or other carbohydrates as glycosyl donors. Enzymes (0.18–0.137 g) were added, and the mixture was incubated for 72 h at 68 °C under reflux.

In the present work, we have synthesized L-DOPA α-glycosides by enzymatic transglycosylation reactions of L-DOPA with one of the relatively simplest and cheapest glucosyl donors, sucrose. The transglycosylation reactions were catalyzed by four different glucansucrases that catalyze the so-called acceptor reactions,[21] giving L-DOPA α-glycosides.

2. Experimental

2.1. Materials

Four different kinds of glucansucrases [EC 2.4.1.5] were obtained by growing constitutive mutants, *Leuconostoc mesenteroides* B-512FMC,[22,23] B-742CB,[23,24] B-1299CA,[24,25] and B-1355C,[22,24] in a medium containing D-glucose instead of sucrose as a carbon source.[26] The four glucansucrases were each concentrated, fractionated, and dialyzed against 20 mM pyridinium acetate buffer (pH 5.2) by a size-exclusion, hollow-fiber, ultra-filtration system, with a molecular-weight cut off of 30,000 Da (Amicon, Beverly, MA), using the culture supernatants as previously described.[26] L-DOPA was purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). Bio-Gel P-2 (fine) resin was purchased from Bio-Rad Laboratories (Hercules, CA). Whatman K5 TLC plates were obtained from Fischer Scientific Co. (USA). All other chemicals were of reagent grade. Immobilized yeast was prepared, as previously described.[27]

2.2. Synthesis of L-DOPA α-glycosides by reaction with glucansucrases and sucrose

Four different glucansucrases (80 U, where 1 U = 1 μmole of D-glucose released from sucrose/min) were added to each 80 mL of substrate solution composed of 20 mM L-DOPA and 40 mM sucrose in 20 mM pyridinium acetate buffer (pH 5.2), which was degassed with a vacuum pump for 20 min and then incubated at 23 °C for 24 h under vacuum, with slow stirring. The pH of the digest was then decreased to 3.0 by addition of 1.0 M HCl to stop the reaction.

The reaction digest was concentrated to 10 mL with a vacuum rotary evaporator at 23 °C. Ten milliliters (one volume ratio) of EtOH was added slowly to 10 mL of the concentrated reaction digest to precipitate the dextran that was produced as a by-product, and then it was kept at 4 °C for 24 h. The resulting precipitate was removed by centrifugation at 10,000*g* for 30 min, and the supernatant was concentrated again to 10 mL by vacuum rotary evaporation to remove the EtOH. Immobilized yeast (8 g, wet weight) was added to the enzyme digests and incubated at 23 °C for 24 h under vacuum to remove fermentable carbohydrates. The insoluble materials and immobilized yeast were removed by centrifugation at 10,000*g* for 30 min, followed by glass filtration. The filtrate was concentrated to ~4 mL by vacuum rotary evaporation, the pH was adjusted to 3, and then the mixture was placed under nitrogen. The reaction digests that were obtained at each reaction step were analyzed by TLC.[28] An appropriate amount (2–5 μL) of sample was added to a Whatman K5 silica gel TLC plate and irrigated 2–3 times over ~18-cm path length with 3:1 volume proportions of CH_3CN and H_2O. The carbohydrates on the TLC plate were visualized by dipping the plate into a solution containing 0.3% (w/v) *N*-(1-naphthyl)ethylenediamine and 5% (v/v) sulfuric acid in MeOH, followed by drying and subsequent heating in an oven at 130 °C for 10 min. The quantitative amounts of the compounds on the TLC plate were determined by scanning densitometry.[28]

2.3. Fractionation and purification of L-DOPA α-glycosides by Bio-Gel P-2 column chromatography

Approximately 1 mL of the concentrated reaction products was added to a Bio-Gel P-2 column (2.5 × 110 cm) and eluted with acid–water (pH 3.0).[18] The flow rate was 0.4 mL/min and the fractions collected were 3.0 mL each. The amount of carbohydrate contained in each of the fractions was analyzed by TLC[28] by the micro phenol–sulfuric acid method.[29] The L-DOPA-glycoside fractions were pooled and concentrated by vacuum rotary evaporation.

2.4. Molecular structure analysis of the reaction products by ^{1}H NMR spectroscopy

The reaction products were dried by vacuum rotary evaporation at ~21 °C and were exchanged three times with 3 mL of D_2O. The final dried products were dissolved in 0.5 mL of pure D_2O and put into 5-mm NMR tubes. All spectra were acquired at 25 °C using a 500-MHz or a 700-MHz Bruker NMR spectrometer. The ^{1}H chemical shifts of L-DOPA and the L-DOPA-glycosides were compared with chemical shifts previously reported.[18]

3. Results and discussion

3.1. Synthesis of L-DOPA α-glycosides by the acceptor reactions of dextransucrases

Previously, we have synthesized L-DOPA α-glycosides (3-*O*-α-D-glucopyranosyl L-DOPA and 4-*O*-α-D-glucopyranosyl L-DOPA) by enzymatic transglycosylation reactions of L-DOPA with cyclomaltohexaose (α-cyclodextrin), catalyzed by *Bacillus macerans* cyclomaltodextrin glucanyltransferase (CGTase), [EC 2.4.1.19].[18] In the present study, we have synthesized the same two L-DOPA α-glycosides, but with a much simpler and cheaper substrate, sucrose, catalyzed by four different *L. mesenteroides* B-512FMC, B-742CB, B-1299A, and B-1355C glucansucrases [EC 2.4.1.5], hereafter referred to as B-512FMC dextransucrase, B-742CB dextransucrase, B-1299A dextransucrase, and B-1355C alternansucrase.

Three of the dextransucrases synthesize three structurally different dextrans and show different specificities: B-512FMC dextransucrase synthesizes dextran with α-(1→6)-linked D-glucose residues in the main chains with 5-6% α-(1→3)-linked single D-glucose residues and long α-(1→6)-linked D-glucose chains branched onto the main α-(1→6) chains; B-742CB dextransucrase synthesizes α-(1→6)-linked D-glucose residues in the main chains, with single D-glucose residues α-(1→3)-linked branches on every D-glucose residues in the main chains and a few main chains with α-(1→3)-linked branches onto main chains; B-1299A dextransucrase synthesizes α-(1→6)-linked D-glucose residues in the main chains with 33% α-(1→2)-linked single branched D-glucose residues and 1% main chains linked to main chains by α-(1→2) branch linkages; B-1355C alternansucrase synthesizes alternating α-(1→6) and α-(1→3)-linked D-glucose residues in the main chains with 7% D-glucose and main chains branched by α-(1→3) linkages onto the main chains.[21] All four of the glucansucrases also carry out acceptor reactions that transfer D-glucose residues to other carbohydrates and to some noncarbohydrate compounds containing hydroxyl groups.[21]

In the presence of L-DOPA, all four of the glucansucrases produced D-fructose, D-glucose, isomaltulose, leucrose (α-D-glucopyranosyl-(1→5)-D-fructopyranose), isomaltodextrins (isomaltose, isomaltotriose, and isomaltotetraose), and new products

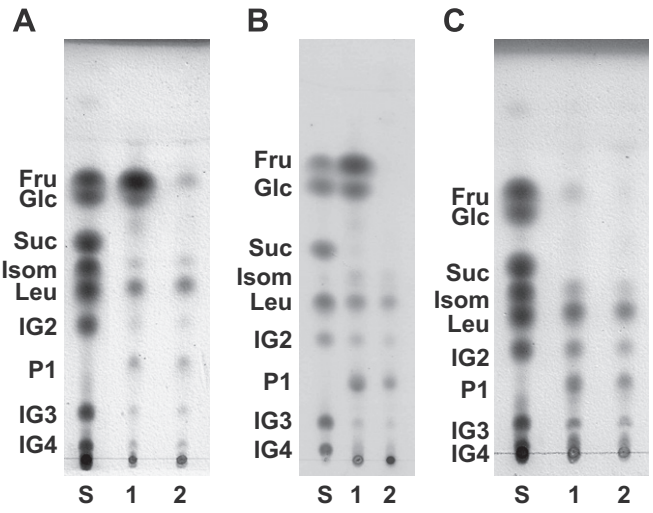

Figure 1. TLC of the glucansucrase reaction products obtained from 20 mM L-DOPA and 40 mM sucrose. (A) B-512FMC dextransucrase reaction products; (B) B-742CB dextransucrase reaction products; (C) B-1299A dextransucrase and B-1355C alternansucrase reaction products. Lane S, carbohydrate standards; lane 1, enzyme reaction products; lane 2, immobilized yeast-fermented products from lane 1; lane 1 in C, immobilized yeast-fermented products from dextransucrase B-1299A reaction products; and lane 2 in C, immobilized yeast-fermented products from alternansucrase B-1355C reaction products. P1 indicates L-DOPA-glycosides. The standards contain Fru, Glc, Suc, Isom (isomaltulose), Leu (leucrose), IG2 (isomaltose), IG3 (isomaltotriose), and IG4 (isomaltotetraose).

(P1-512FMC, P1-742CB, P1-1299A, and P1-1355C) by reaction of 20 mM L-DOPA and 40 mM sucrose (Fig. 1A—lane 1 and B—lane 1).

Addition of immobilized yeast removed the fermentable carbohydrates, D-fructose and D-glucose, leaving the other known carbohydrates (leucrose, isomaltulose, and isomaltodextrins) and the new products (see, Fig. 1A—lane 2, B—lane 2, and C—lanes 1, and 2).

3.2. Fractionation of the L-DOPA α-glycosides by Bio-Gel P-2 column chromatography

The dextransucrase reaction product mixtures (0.5 mL), containing L-DOPA α-glycosides (P1-512FMC, P1-742CB, P1-1299A, and P1-1355C) were each loaded onto a Bio-Gel P-2 column (2.5 × 110 cm) and eluted with acidified water (pH 3.0). The reaction mixture was fractionated into more than five compounds (Fig. 2), and each fraction was analyzed by TLC and compared with the known carbohydrates (Fig. 3). Fraction I was well separated from other known carbohydrates and identified as a new reaction product, L-DOPA glycosides (Fig. 3). Fraction II was D-fructose that was not completely removed by yeast fermentation. Fraction III was leucrose and Fraction IV was a mixture of isomaltulose, leucrose, and isomaltose (Fig. 3). The main component of Fraction V

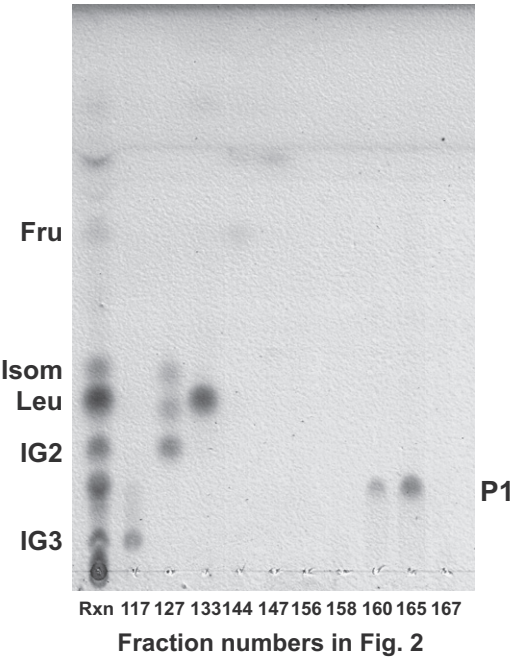

Figure 3. TLC analysis of the fractions obtained from the Bio-Gel P-2 column chromatography shown in Figure 2. Designation of carbohydrates and product is the same as in Fig. 1. The results are representative of all four glucansucrase reactions with L-DOPA and sucrose.

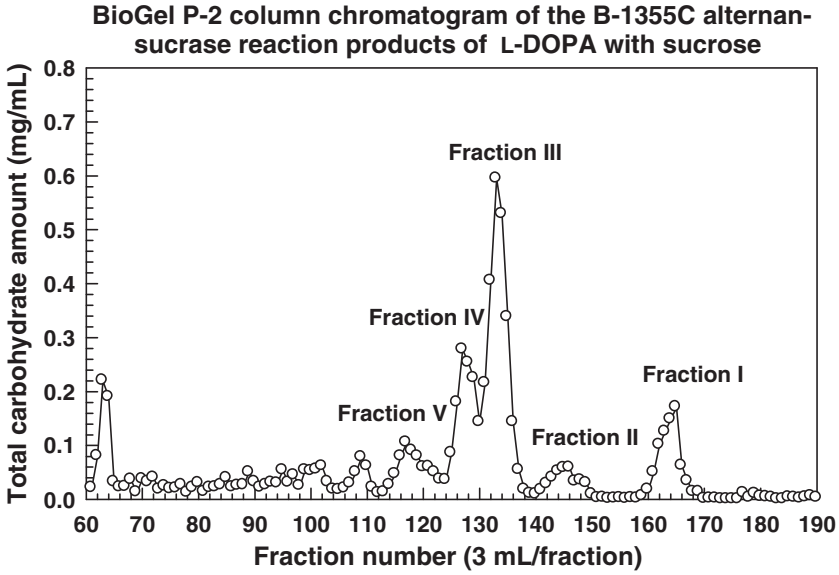

Figure 2. Bio-Gel P-2 (fine) column (2.5 × 110 cm) chromatogram of the alternansucrase B-1355C reaction products L-DOPA with sucrose, followed by the subsequent immobilized yeast fermentation. Fraction I = L-DOPA-glycosides, Fraction II = D–D-glucose + D-fructose, Fraction III = leucrose, Fraction IV = mixture of leucrose, isomaltulose, and isomaltose, Fraction V = isomaltotriose. The results are representative of all four glucansucrase reactions with L-DOPA and sucrose.

was isomaltotriose (Fig. 3). The elution pattern of the Bio-Gel P-2 column was compared with CGTase reaction products,[18] and Fraction I was identified as L-DOPA-glycoside(s). The four-dextransucrase reaction products were fractionated by the same Bio-Gel P-2 column, and essentially gave identical fractionation patterns that were found to be the same compounds by TLC analysis.

3.3. ^{1}H NMR spectroscopic analysis of L-DOPA-glycosides

L-DOPA glycosides produced by four different glucansucrases were analyzed by 1D ^{1}H NMR spectroscopy (Fig. 1). All of the L-DOPA-glycosides gave the same ^{1}H NMR peak patterns and chemical shifts (Fig. 4B–E). When the ^{1}H NMR chemical shifts of the L-DOPA-glycosides were compared with those of the L-DOPA-glycoside (4-*O*-α-D-glucopyranosyl L-DOPA) that was obtained by CGTase transglycosylation, the results were identical (Table 1) except for the occurrence of weak peaks due to the minor reaction products (italic numbers in Fig. 4). From the results of Figure 4 and Table 1, it was evident that the four different glucansucrases all produced 4-*O*-α-D-glucopyranosyl L-DOPA as a major acceptor reaction product and 3-*O*-α-D-glucopyranosyl L-DOPA as a minor reaction product (Fig. 4, peaks identified with italic numbers) as in the reaction products of the CGTase transglycosylation reaction with L-DOPA.[18] But, the relative amount of 3-*O*-α-D-glucopyranosyl L-DOPA was different for each of the enzyme reaction products when the relative amounts of the two glycosides were calculated by integration of the ^{1}H NMR peaks of the L-DOPA products (Fig. 4 and Table 2). Dextransucrase B-512FMC, which makes the simplest dextran, produced ~1% (w/w) 3-*O*-α-D-glucopyranosyl L-DOPA and 99% (w/w) 4-*O*-α-D-glucopyranosyl L-DOPA, whereas the other dextransucrases and alternansucrase, which make more complicated glucans than B-512FMC dextransucrase, produced ~2–3% (w/w) more of 3-*O*-α-D-glucopyranosyl L-DOPA (Table 2). These results indicate that glucansucrases that catalyze more complicated glucan structures have a slightly wider specificity range in their transglycosylation acceptor reactions.

3.4. Properties of the two L-DOPA α-glycosides as pharmaceutical agents in decreasing the symptoms of Parkinson's disease

It has previously been known that glycosylation of some pharmaceuticals changes the physicochemical properties of the drugs, such as toxicity, membrane transport properties, especially, the crossing of the blood–brain barrier membrane, biological activity, ligand selectivity, and so forth. High doses of retinoic acid caused resorption of implantation sites in pregnant mice, whereas the glycoside (β-glucuronide) of retinoic acid did not have that toxicity at the same dose.[30] The clinical application of opioid peptides has been limited, even though those compounds had good analgesic activity because they did not penetrate the blood–brain barrier. When the deltophins and the dermorphins (ligands for δ- and μ-opioid receptors, respectively) were glycosylated at the hydroxyl group of the C-terminal L-threonine residue, the glycosylated

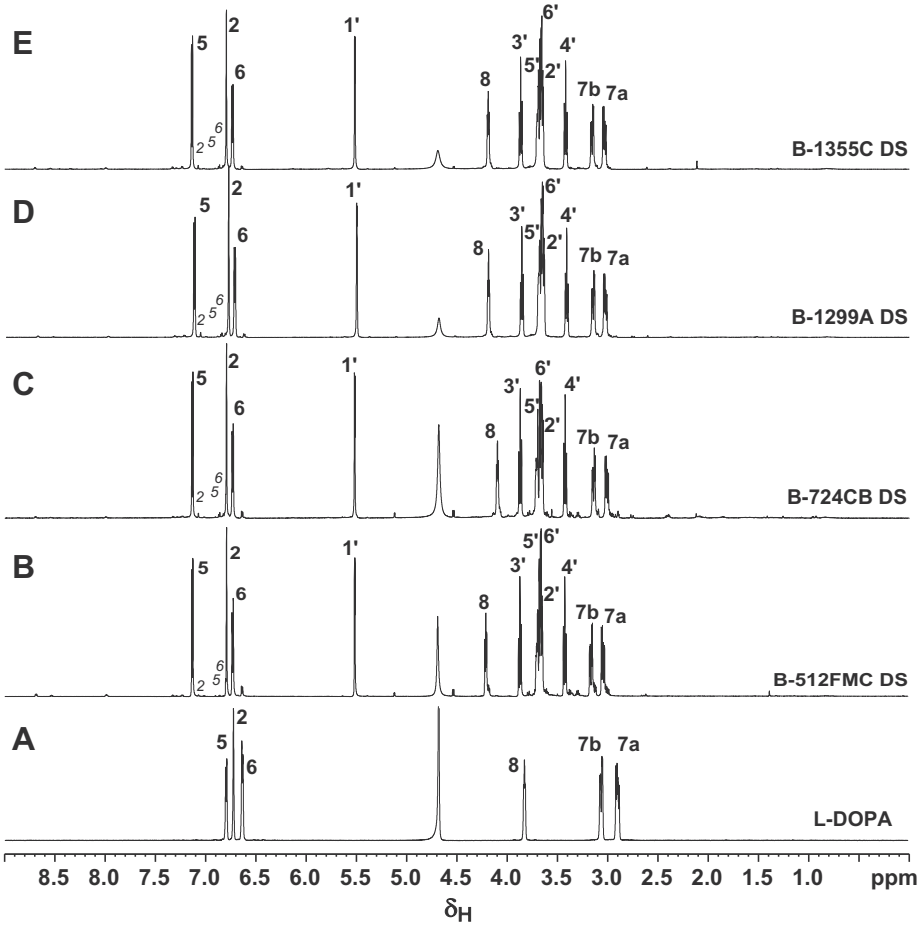

Figure 4. ^{1}H NMR spectra of L-DOPA (A) and L-DOPA-glycosides obtained by (B) B-512FMC, (C) B-742CB, and (D) B-1299A dextransucrases, and (E) B-1355C alternansucrase. Unprimed numbers refer to the ^{1}H attached to the carbons of L-DOPA and the primed numbers refer to the ^{1}H attached to the α-D-glucose carbons. Numbers are from the major reaction product (4-*O*-α-D-glucopyranosyl L-DOPA), and the small italic numbers are from the minor reaction product (3-*O*-α-D-glucopyranosyl L-DOPA).

Table 1
^{1}H NMR chemical shifts and coupling constants for L-DOPA and L-DOPA-glycosides produced by four different kinds of glucansucrases

	Position[a]	L-DOPA	B-512FMC Dextransucrase	B-742CB Dextransucrase	B-1299A Dextransucrase	B-1355C Alternansucrase	CGTase[b]
L-DOPA	H-2	6.73	6.72	6.80	6.78	6.79	6.78
	H-5	6.80	7.13	7.14	7.13	7.13	7.12
	(*J*, Hz)[c]	(d, 8.0)	(d, 8.3)	(d, 8.4)	(d, 8.3)	(d, 8.4)	(d, 8.3)
	H-6	6.64	6.73	6.74	6.72	6.73	6.72
	(*J*, Hz)[c]	(d, 7.9)	(d, 8.3)	(d, 8.5)	(d, 8.3)	(d, 8.5)	(d, 8.3)
	H-7a	2.90	3.04	3.01	3.03	3.03	2.98
	(*J*, Hz)	(dd, 19.0)	(dd, 20.7)	(dd, 20.0)	(dd, 20.0)	(dd, 20.1)	(dd, 20.0)
	H-7b	3.06	3.16	3.14	3.15	3.15	3.12
	(*J*, Hz)[c]	(m, 22.4)	(m, 22.7)	(m, 22.5)	(m, 22.5)	(m, 22.5)	(m, 22.5)
	H-8	3.82	4.21	4.10	4.19	4.19	4.03
	(*J*, Hz)[c]	(m, 12.3)	(m, 13.1)	(m, 12.9)	(m, 12.8)	(m, 13.0)	(m, 12.9)
D-Glucose	H-1		5.52	5.52	5.51	5.51	5.51
	($J_{1,2}$, Hz)		(3.61)	(3.67)	(3.43)	(3.60)	(3.65)
	H-2		3.65	3.66	3.65	3.65	3.64
	H-3		3.87	3.87	3.86	3.86	3.86
	H-4		3.42	3.43	3.42	3.42	3.41
	H-5		3.70	3.71	3.69	3.70	3.70
	H-6		3.66	3.67	3.65	3.66	3.65
	H-6′		3.69	3.69	3.68	3.68	3.67

[a] The proton position numbers refer to Figure 4 in Ref. 18.
[b] 4-*O*-α-D-Glucopyranosyl L-DOPA obtained from L-DOPA by reaction with cyclomaltohexaose catalyzed by cyclomaltodextrin glucanyltransferase (CGTase) transglycosylation (from Ref. 18).
[c] Spin–spin coupling constants of peaks: d, doublet; dd, doublet–doublet; m, multiplet.

Table 2
Relative amounts of the two L-DOPA-glycosides produced by four different glucansucrases compared with the synthesis by *Bacillus macerans* CGTase cyclomaltohexaose

Glycosides	B-512FMC Dextransucrase	B-742CB Dextransucrase	B-1299CA Dextransucrase	B-1355C Alternansucrase	CGTase[a]
3-*O*-α-D-Glucopyranosyl L-DOPA (%)	1.1	2.6	2.9	2.2	3.5
4-*O*-α-D-Glucopyranosyl L-DOPA (%)	98.9	97.4	97.1	97.8	96.5

[a] From ^{1}H NMR data in Ref. 18.

analogs showed increased blood–brain barrier penetration, and they retained high receptor selectivity and remarkable in vivo activity.[31,32] One of the glycosides, morphine-6-β-D-glucuronide, is a more attractive analgesic than morphine itself, because it has a 100-fold higher affinity for μ-opioid receptors and lower side effects than those of morphine.[33]

L-DOPA is given to PD patients as a pharmaceutical to counteract the symptoms of PD. It must pass though the blood–brain barrier into the brain where it is converted into dopamine by enzymatic decarboxylation. After continuous administration, over a period of time, L-DOPA is decreased in the peripheral tissues by enzymatic and non-enzymatic oxidation of the phenolic hydroxyl groups, and also by enzymatic methylation of these groups.

Previously, we have shown that L-DOPA α-glycosides are more resistant to non-enzymatic oxidation than L-DOPA and have a significantly longer half-life than L-DOPA.[18] It was found that L-DOPA α-glycoside was more than two times more stable than L-DOPA using a simulated oxidative tolerance experiment that contained hydrogen peroxide and ferrous ion, which are components that are found in biological systems. It, therefore, is highly likely that the L-DOPA α-glycosides would also be resistant to enzymatic oxidation by glycosylation, as the phenolic hydroxyl group is blocked by D-glucose, and further the L-DOPA α-glycosides would not bind to the active sites of the oxidases and the methylating enzymes due to steric hindrance produced by the covalently linked D-glucosyl residues at position-3 or -4 of the phenyl ring.

3.5. Conclusions

L. mesenteroides B-512FMC dextransucrase synthesizes L-DOPA α-glycosides using a simpler enzyme and the 200 times cheaper D-glucosyl donor, sucrose, compared to the more complicated synthesis of L-DOPA α-glycosides, using *Bacillus macerans* cyclomaltodextrin glucanyltransferase (CGTase) and cyclomaltohexaose, followed by hydrolysis with glucoamylase and β-amylase reactions.[18] Glycosylation by B-512FMC dextransucrase occurs predominantly (98.9%) at the C-4 phenolic hydroxyl group of L-DOPA. Glycosylation at the C3- and C4-phenolic hydroxyl groups of L-DOPA would prevent both oxidation and methylation and, therefore, would not have to be separated for pharmaceutical use.

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