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Enzymatic synthesis and anti-coagulant effect of salicin analogs by using the *Leuconostoc mesenteroides* glucansucrase acceptor reaction

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Abstract

Glucansucrases from *Leuconostoc mesenteroides* catalyze the transfer of glucosyl units from sucrose to other carbohydrates by acceptor reaction. We modified salicyl alcohol, phenol and salicin by using various glucansucrases and with sucrose as a donor of glucosyl residues. Salicin, phenyl glucose, isosalicin, isomaltosyl salicyl alcohol, and a homologous series of oligosaccharides, connected to the acceptors and differing from one another by one or more glucose residues, were produced as major reaction products. By using salicin and salicyl alcohol as acceptors, B-1355C2 and B-1299CB-BF563 dextransucrases synthesized most widely diverse products, producing more than 12 and 9 different kinds of saccharides, respectively. With phenol, two acceptor products and oligosaccharides were synthesized by using the B-1299CB-BF563 dextransucrase. Salicyl derivatives, as acceptor products, showed higher anti-coagulation activity compared with that of salicin or salicyl alcohol that were used as acceptors.

Keywords: Glucansucrase; Acceptor reaction; *Leuconostoc mesenteroides*; Anti-coagulation; Salicin; Salicin analogs

1. Introduction

A number of biologically active compounds are glycosides. Sometimes, the glycosidic residue is crucial for their activities (Ruggiero et al., 2003);

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in other cases glycosylation improves pharmacokinetic parameters (Schottelius et al., 2002). Recent developments in molecular glycobiology brought better understanding of aglycon versus glycoside activities and made it possible to develop new, more active or more effective glycodrugs. Glycosylation process also increases drug solubility, lowers drug toxicity by decreasing the amount of a drug required for a given therapeutic effect and improves tissue-specific drug targeting (Schweizer and Hindsgaul, 1999).

It is well known that glucansucrases catalyze the transfer of glucosyl units from sucrose to other carbohydrates. This reaction is the so-called *acceptor reaction*, and the added carbohydrates are called *acceptors* (Koepsell et al., 1953). More than 30 different carbohydrates are known to act as acceptors, so it is possible to utilize glucansucrases to synthesize new kinds of carbohydrates by the acceptor reaction. Furthermore, different kinds of acceptor products are obtained from the same acceptor by changing glucansucrases elaborated by different species of microorganisms.

Salicin ($C_{13}H_{18}O_7$) is a natural product extracted from several species of *Salix* (willow) and *Populus* (poplar), and was also found in *Gaultheria procumbens* (wintergreen) and in *Betula lenta* (sweet birch), the volatile oils of which consist almost entirely of methyl salicylate (Jourdier, 1999). The pharmacokinetic and pharmacological properties of salicin have made it an ideal antipyretic prodrug (Akao et al., 2002). Glucosylations using cultured plant cells have been the subject of increasing attention (Shimoda et al., 2002). There are several reports on the glucosylation of salicyl alcohol by cultured plant cells showing that only benzyl hydroxyl group was glucosylated to give isosalicin (Syahrani et al., 1998). However, the reaction products were mostly synthesized by a single glucose addition to substrate. Also, little attention has been paid to the glycosylation of hydroxyl groups of phenolic compounds by dextransucrase or glucosyltransferase using the inexpensive, widely available substrate, sucrose.

In this report, we synthesized various structures of salicin analogs by using glucansucrases with sucrose as a glucosyl donor and salicyl alcohol, salicin or phenol as an acceptor. We also found the inhibition effect of the salicin analogs for blood coagulation.

2. Materials and methods

2.1. Materials

Salicin, salicyl alcohol (2-hydroxybenzyl alcohol), heparin and phenol were purchased from Sigma-Aldrich (Milwaukee, WI).

2.2. Preparation of purified glucansucrases

Each *Leuconostoc mesenteroides* was grown in 3 l LWG medium with the pH and temperature kept at 5.5 and 28 °C, respectively (Kim and Kim, 1999). LWG medium is composed of 2% (w/v) glucose, 2% (w/v) K_2HPO_4, 0.5% (w/v) peptone, 0.5% (w/v) yeast extract, and 1% (v/v) mineral solution [2% (w/v) $MgSO_4{\cdot}7H_2O$, 0.1% NaCl, 0.1% $FeSO_4{\cdot}7H_2O$, 0.1% $MnSO_4{\cdot}H_2O$, 0.13% $CaCl_2{\cdot}2H_2O$] (Kim and Kim, 1999). After fermentation, the culture was harvested, centrifuged and concentrated using hollow fiber (30 K cut-off, Millipore, Japan) (Kim and Kim, 1999). Dextransucrases and alternansucrase were purified as described by Kim and Kim (1999).

2.3. Preparation of salicin analogs

Equal volume of 300 mM sucrose and an acceptor (50 mM salicin, 50 mM salicyl alcohol or 250 mM phenol in 20 mM sodium phosphate buffer, pH 6.8) was added to each glucansucrase (final activity: 1 U ml^{-1}) and reacted at 28 °C until all sucrose was consumed. Wet immobilized yeast (10 g) was added into the reaction digest (50 ml) to remove monosaccharides and the mixture was kept at 37 °C for 12 h. The immobilized yeast was removed by filtration, and the supernatants were concentrated to about 10 times by vacuum rotary evaporation. Reaction products were then analyzed by using TLC as described previously (Kim et al., 1997). Whatman K5F plate (Whatman Inc., Clifton, NJ) with two to three ascents of 85:15 (v/v) acetonitrile/water was used. The carbohydrates on TLC plate were visualized by dipping the plates into 5% (v/v) H_2SO_4 in methanol containing 0.3% (w/v) *N*-(1-naphthyl) ethylenediamine, followed by drying and heating for 10 min at 121 °C (Kim et al., 1997). The quantitative amounts of the carbohydrates were determined by TLC densitometry, using a Bio-Rad scanning densitometer (GS-670, Bio-Rad Laboratories, Hercules, CA).

2.4. Fractionation of the enzyme reaction products by Bio Gel P2 and Superdex G-10 column chromatographies

About 1.2 ml of the reaction digest (10 mg ml^{-1}) was loaded onto Bio-Gel P2 (fine) column (1.5 cm × 120 cm), and eluted with deionized water at a flow rate of 0.05 ml/min, collecting 1.0 ml fractions (Yoon and Robyt, 2002). The carbohydrate composition of the fractions was analyzed by TLC as described above with Whatman K5 plates (Kim et al., 1997). The fractions of the same composition were pooled and concentrated to 1 ml, loaded onto Superdex G-10 column (1.5 cm × 100 cm), and eluted with deionized water at a flow rate of 0.03 ml min^{-1}, collecting 0.5 ml fractions. The carbohydrate composition of the fractions was also determined by TLC as described above with Whatman K5 plates (Kim et al., 1997).

2.5. Purification of the reaction products by descending paper chromatography

The enzyme reaction products fractionated by Bio-Gel P2 and Superdex G-10 column chromatographies were further purified by preparative descending paper chromatography. An appropriate amount (200–250 μl) of the pooled concentrates was loaded onto Whatman 3 MM paper (23 cm × 56 cm). The paper was then irrigated with 10:4:3 (v/v/v) of ethyl acetate–pyridine–water for 8 h at room temperature (Yoon and Robyt, 2002). Each separated compound was visualized on the paper by using a silver nitrate method, followed by cutting each purified compound from the paper (Yoon and Robyt, 2002). The compounds were then eluted with deionized water, and then concentrated to about 0.5 ml by vacuum rotary evaporation for further analyses.

2.6. Mass analysis of reaction products by matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF MS)

An amount of 1 μl of each compound (2 mg ml^{-1}) and/or the reaction products were transferred to the probe and mixed with 1.0 μl of 0.1 M 2,5-dihydroxybenzoic acid (DHB) in 70% (v/v) acetonitrile/water solution. The solvent was evaporated under airflow. The molecular masses of the samples were analyzed by MALDI-TOF MS, using a Dynamo instrument (Thermo Bio-Analysis, Ltd., Paradise, UK) with a nitrogen laser (337 nm). Ions were detected in a positive mode at an acceleration voltage of 20 kV.

2.7. The structure analysis of salicin analogs

We used several methods to determine the structure of the salicin analogs. To identify each component of salicin analogs, each purified product was mixed with an equal volume of 1 M hydrochloric acid. After hydrolysis at 100 °C for 30 min, the hydrolyzate was vacuum dried and re-dissolved in water. An amount of 1 μl of the reaction solution was spotted on the silica gel plate and developed twice in acetonitrile/water (85/15, v/v). To identify the acceptor reaction product contained in the oligosaccharide mixture in the reaction digest, each fraction was mixed with glucansucrases (3 U/ml) and 100 mM sucrose in 20 mM sodium acetate buffer (pH 5.2) in the proportion of 1:1:1 (v/v/v). Then it was reacted at 28 °C for 12 h. Each acceptor reaction product obtained from the above reaction was also identified by using TLC and compared to the original oligosaccharide mixture. Also, the salicin analogs were digested with debranching enzymes (dextranase, isoamylase, α- or β-glucosidase) to determine the structure. Dextranase hydrolyzes α-(1 → 6)-D-glucosidic linkages in dextran and isomaltooligosaccharides. Isoamylase hydrolyzes α-(1 → 6)-D-glucosidic branch linkages in glycogen, amylopectin and their β-limits dextrins. α- or β-glucosidase hydrolyzes terminal, non-reducing D-glucose residues with release of D-glucose. The hydrolyzate was also identified by using TLC and compared to the original oligosaccharide mixture.

2.8. Anticoagulant activity assay by measurement of clotting times

Blood was drawn from healthy rabbit. Each salicin, salicyl alcohol, aspirin and salicin analogs was dissolved in PBS buffer (pH 7.4) to make 10% solution. Then 1.4 ml blood was mixed with 0.1 ml (10% w/v) each solution and incubated for 10 min. The time needed for clotting was measured after the addition of activator. If there was still no clotting observed after

30 min, the sample was defined as no blood clot formation (NC). Heparin was used as a positive control.

3. Results and discussion

3.1. Modification and purification of salicin analogs

In addition to catalyzing the synthesis of dextran from sucrose, glucansucrase also catalyzes the transfer of glucose from sucrose to other carbohydrates that are present or are added to the digest. The added carbohydrates are called acceptors and the reaction is called an acceptor reaction. Salicin, salicyl alcohol and phenol were modified by using acceptor reactions of glucansucrases from *L. mesenteroides* (Fig. 1). Different glucansucrases synthesize different acceptor products. Glucansucrases using salicin, salicyl alcohol and phenol produced more than 15, 9 and 3 kinds of acceptor products, respectively. After purification of each compound using GPCs and paper chromatography method, we analyzed the structure of each product: first, mass spectrometry was used to determine the mass of product, and then estimated the sugar composition of each product. For example, the mass of P1 was Mr 448. Thus, based on the calculation of [salicin (286) + glucose (180) − H_2O (18) = 448], P1 was assigned as salicylalcohol-isomaltoside or -maltosides. After enzymatic hydrolysis of P1 with isoamylase into glucose and salicin were produced. Thus, we assigned P1 as salicylalcohol isomaltosides. In addition, we performed acid hydrolysis of each product to identify sugar composition. Also, an acceptor reaction using each purified salicin analog (or acceptor reaction product) was performed and the resulted products were compared with those of salicin acceptor reaction on TLC. Based on these experiments, we could assign each structure of acceptor reaction product of salicin and salicin analog. The final structures of salicin analogs are summarized in Table 1.

When the acceptor is a monosaccharide or disaccharide, there is usually a series of oligosaccharides produced as acceptor products. There are actually two classes of acceptors: those that give a homologous series of oligosaccharides, each differing one from the other by one glucose residue; and those acceptors that only form a single acceptor product containing one glucose residue more than the acceptor (Koepsell et al., 1953). Fu and Robyt found that B-512FM glucansucrase transfers D-glucose to C-6 hydroxyl of both the nonreducing-end and the reducing-end residues of G3–G8. G3, thus, gave two tetrasaccharides, 6^3-α-D-glucopyranosyl maltotriose and 6^1-α-D-glucopyranosyl maltotriose. The former acceptor product was also an acceptor giving a homologous series of isomaltodextrins attached to C-6 hydroxyl of the glucose residue at the non-reducing end. The acceptor product with glucose attached to the reducing-end residue, however, was not an acceptor (Fu and Robyt, 1990). All products from salicin acceptor reaction were formed by the addition of glucosyl unit to free hydroxyl group of non-reducing end of the acceptor.

Cote and Robyt found that alternansucrase formed both α-1,6 and α-1,3 glycosidic bonds with acceptors, although not all the acceptors reacted with equal efficiency (Cote and Robyt, 1982). With salicin (salicyl alcohol glucoside), the highest acceptor reaction efficiency was obtained by B-512FMCM dextransucrase based on the amount sum of P1 to P14, excluding glucooligosaccharides. The most numbers of products were obtained by using 1355C2 alternansucrase acceptor reaction. B-1355C2 alternansucrase synthesized salicyl alcohol nigerosyl glucoside and this is possibly the ability of alternansucrase forming α-1,3 and α-1,6 linkages in alternan. Other dextransucrases can synthesize nigerose, yet there was no product containing α-1,3 linkages among the structure determined oligosaccharides. The largest acceptor product of salicin was salicyl alcohol isomaltoheptoside (P14) synthesized by 512FMCM dextransucrase. Interestingly, the glucosyl residues from some of salicin were transferred by dextransucrase and formed isosalicin (salicyl glucoside) and then glucosyl groups of sucrose were transferred to the 6-hydroxyl groups to form salicyl isomaltooligosaccharides (P7, P11). In the case of salicyl alcohol, the highest acceptor reaction was obtained by using B-1299CB-BF563 dextransucrase (Table 1), while the rest of the enzyme reactions were less efficient and showed similar reaction products of B-1299CB-BF563 (data not shown). The major reaction product was isosalicin (salicyl glucoside). Some of the glucosylated products resulted from the transfer of glucosyl residue of isosalicin. The resulting salicin was then used as an acceptor and formed salicyl alcohol oligosaccharides (PA3, PA5, PA7, and PA8). In general,

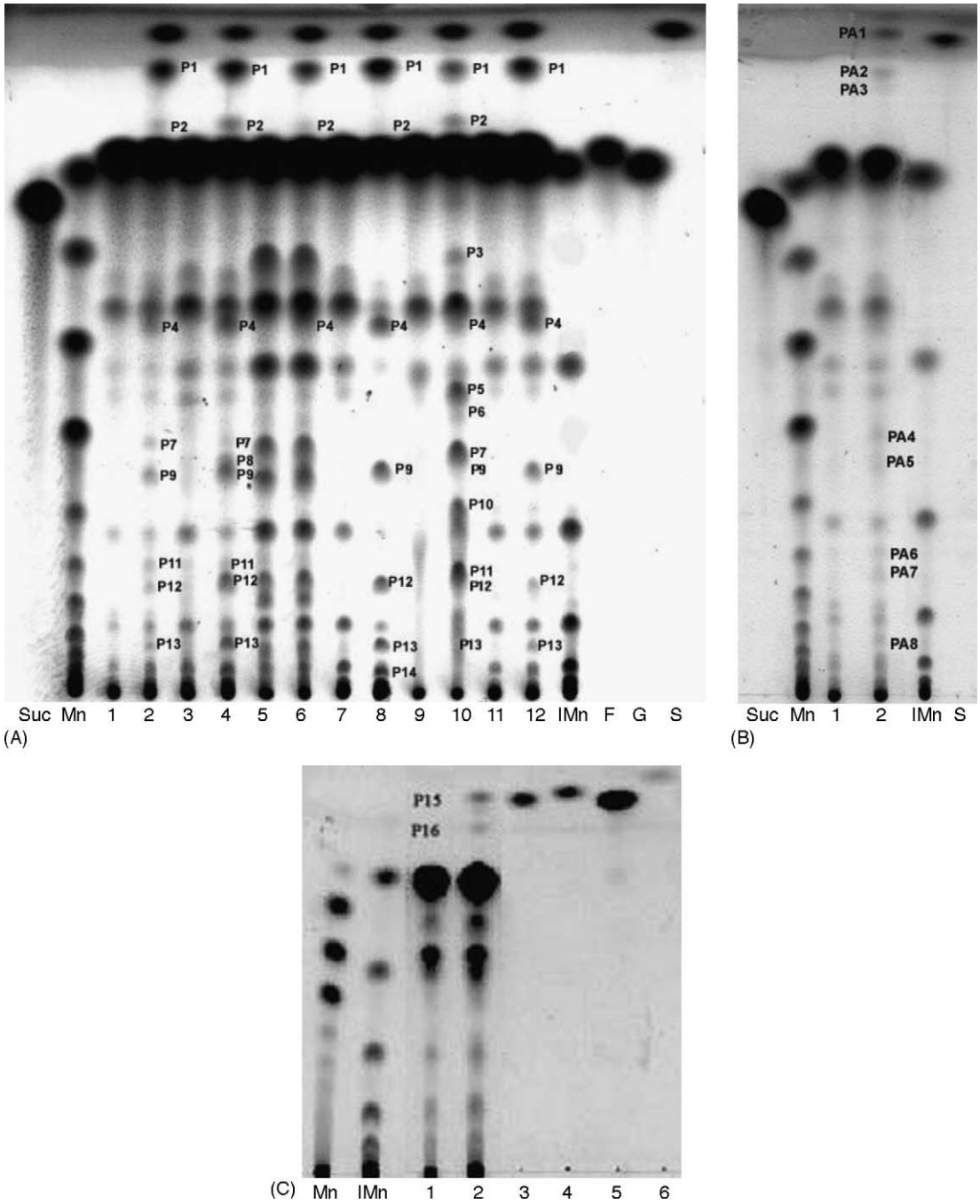

Fig. 1. Acceptor reaction products of salicin, salicyl alcohol and phenol with sucrose by using various glucosyltransferases from *L. mesenteroides*. Odd numbered lanes: reaction with sucrose and glucansucrase; even numbered lanes: reaction with sucrose, acceptor and glucansucrase. (A) Acceptor reaction digests with salicin lanes 1/2, with 1299CB dextransucrase; lanes 3/4, with 1299CB-BF563 dextransucrase; lanes 5/6, with 512F dextransucrase; lanes 7/8, with 512FMCM dextransucrase; lanes 9/10, with 1355C2 alternansucrase; lanes 11/12, with 742CB4 dextransucrase. Mn: maltodextrin series, IMn: isomaltodextrin series, S: salicin, A: salicyl alcohol. (B) Acceptor reaction digests with salicyl alcohol lanes 1/2, with 1299CB-BF563 dextransucrase. (C) Acceptor reaction digests with phenol lanes 1/2, with 1299CB-BF563 dextransucrase lane 3, phenyl α-D-glucopyranoside; lane 4, phenyl β-D-glucopyranoside; lane 5, salicin; lane 6, salicyl alcohol.

Table 1
The compositions of salicin, salicyl alcohol and phenol acceptor reaction products by using *L. mesenteroides* glucansucrases with sucrose

	Salicin reaction products	Glucansucrases							Salicyl alcohol	
		1299 CB4	1299 CB-BF563	512F	512 FMCM	1355 C2	742 CB		Reaction products	1299 CB-BF563
P1	Salicyl alcohol isomaltoside	25.8	17.3	7.81	35.9	9.13	25.7	PA1	Salicyl glucoside (isosalicin)	22.1
P2	Unknown product	3.44	5.16	1.63	3.27	4.35		PA2	Salicyl isomaltoside	5.12
P3	Salicyl alcohol nigeroside					5.36		PA3	Salicyl alcohol isomaltoside	3.56
P4	Salicyl alcohol isomaltotrioside	7.12	9.88	12.5	17.3	2.72	14.5	PA4	Salicyl isomaltotetroside	3.99
P5	Salicyl alcohol nigerosyl glucoside					8.75		PA5	Salicyl alcohol isomaltotetroside	2.92
P6	Unknown product					1.84		PA6	Salicyl isomaltopentoside	1.25
P7	Salicyl isomaltotetroside	1.88	1.6			8.3		PA7	Salicyl alcohol isomaltopentoside	1.21
P8	Unknown product		5.49		9.21		5.01	PA8	Salicyl alcohol isomaltohexoside	4.78
P9	Salicyl alcohol isomaltotetroside	3.26	2.4			1.25			Glucooligosaccharides[a]	50.9
P10	Salicyl alcohol nigerosyl isomaltoside					9.52			Polymer	4.18
P11	Salicyl isomaltopentoside	1.63	4.87			8.57			Phenol reaction products	1299 CB-BF563
P12	Salicyl alcohol isomaltopentoside	1.53	2.31		6.32	5.7	2.02			
P13	Salicyl alcohol isomaltohexoside	2.1	5.93		5.73	3.06	2.08	P15	Phenyl α-glucopyranose	8.06
P14	Salicyl alcohol isomaltoheptoside				7.76			P16	Phenyl isomaltoside	5.1
	Glucooligosaccharides[a]	51.1	42.7	89.3	12.3	29.05	48.6		Glucooligosaccharides[a]	80.6
	Polymer	2.12	2.37	1.25	2.26	2.38	2.12		Polymer	6.27

[a] Glucooligosaccharides: the DP of these oligosaccharides is higher than the DP of P14, PA8 and P16.

three types of salicin derivatives were formed (Fig. 2). By using phenol with B-1299CB-BF563 dextransucrase, two acceptor reaction products were determined, phenyl α-glucopyranose and phenol isomaltoside.

3.2. Measurement of clotting times

An anticoagulant slows the rate of blood clot formation (Table 2). Blood clots can cause severe and life-threatening problems. Heparin, dextran sulfate (Sakamoto et al., 2002) and chitosan ester (Drozd et al., 1992) are used to prevent formation of blood clots (after surgery and in other settings) and in circumstances to help dissolve blood clots already formed (deep vein thrombosis, pulmonary embolism, and other situations involving excessive blood clotting). Aspirin (acetylsalicylic acid) is one of the most popular drugs in the world. Since its clinical introduction in 1899, we have become familiar with this drug and its many surprising effects, including reduced risks of cardiovascular disease and possibly colorectal cancer, as well as its analgesic, anti-inflammatory and anti-platelet actions (Paterson and Lawrence, 2001). Aspirin belongs to a family of compounds called the salicylates, the simplest of which is salicylic acid. Salicylic acid is the principal metabolite of aspirin (Paterson and Lawrence, 2001).

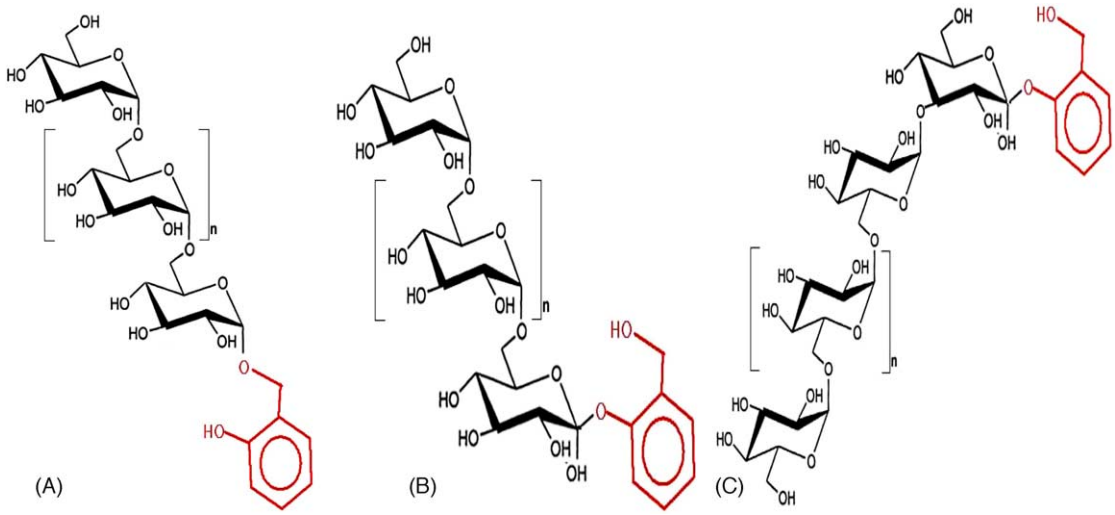

Fig. 2. Types of acceptor reaction products of salicin and salicyl alcohol by using glucansucrases and sucrose. (A) Type A; salicyl β-isomaltooligosaccharides ($0 \leq n \leq$ at least 3: PA2, $n=0$; PA4, $n=2$; PA6, $n=3$; P7, $n=2$; P11, $n=3$), (B) type 2; salicyl alcohol β-isomaltooligosaccharides ($0 \leq n \leq 5$; P1, PA3, $n=0$; P4, $n=1$; P9, PA5, $n=2$; P12, PA7, $n=3$; P13, PA8, $n=4$; P14, $n=5$), (C) type C; salicyl alcohol nigerosyl isomaltooligosaccharides ($0 \leq n \leq 2$; P3, $n=0$; P5, $n=1$; P10, $n=2$).

Table 2
Effect of salicyl derivatives for blood clot formation

Time (min)	Control	Aspirin	Salicin	Salicyl alcohol	Isosalicin (salicyl glucoside)	Salicyl isomaltoside
10	C[a]	C	C	NC[b]	NC	NC
30	C	C	C	C	NC	NC

[a] C: clot formation.
[b] NC: no clot formation.

Many of the salicylates share the same properties as aspirin, although its anti-platelet action is specific. Yet, salicin, salicyl alcohol or aspirin has low solubility and an ill effect on the stomach. Thus, the additions of glucose to salicin or salicyl alcohol could possibly increase the solubility and change or improve these characteristics.

To find the anticoagulation effect of modified salicin or salicyl alcohol, blood was mixed with aspirin, salicin, salicyl alcohol, salicyl glucoside and salicyl alcohol isomaltoside, and the time needed for clotting was measured. Aspirin and salicin showed blood clots within 10 min under our experimental condition and within 20 min salicyl alcohol formed blood clot. Salicyl glucoside and salicyl isomaltoside did not form blood clot, even after 30 min had passed. We also checked the anticoagulation effect of heparin as a positive control. Heparin (0.5 U/ml), a positive control, showed slow blood clotting within 10 min under our experimental condition and 0.05 U/ml of heparin showed complete blood clotting within 10 min under same condition. This result suggests that 1% of salicyl alcohol isomaltoside is more effective for anticoagulation than 0.5 U/ml of heparin for anticoagulant activity.

One-step enzymatic glycosylation is useful for the preparation of glycosides rather than chemical glycosylation that requires tedious steps including the protection and the deprotection of hydroxyl groups of sugar moieties. Thus, biocatalytic approaches would be superior to chemical synthesis, since a position-specificity is expected from an enzymatic reaction. Moreover, various novel structured carbohydrates and their derivatives can be easily synthesized. The physical property and function studies of each novel structured carbohydrate as an anti-cariogenic and an anti-coagulant are in progress.

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