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# Application of amylomaltase for the synthesis of salicin-α-glucosides as efficient anticoagulant and anti-inflammatory agents

Prakarn Rudeekulthamrong [a], Jarunee Kaulpiboon [b, *]

[a] Department of Biochemistry, Phramongkutklao College of Medicine, Phramongkutklao Hospital, Bangkok 10400, Thailand
[b] Department of Pre-Clinical Science (Biochemistry), Faculty of Medicine, Thammasat University, Pathumthani 12120, Thailand

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ABSTRACT

The focus of this study was the synthesis of α-glucosyl derivatives of salicin by a transglucosylation reaction. The reaction was catalyzed by recombinant amylomaltase using tapioca starch as a glucosyl donor. Several reaction parameters, such as the enzyme-substrate concentrations, pH, temperature and incubation time, were optimized. Using the optimum conditions, at least three products with retention times ($R_t$) of 6.2, 9.2 and 14.1 were observed. The maximum yield of glucosylated salicin derivatives was 63% (w/w) of the total products. The structures of the glucosylated salicin derivatives were confirmed to be salicin-α-D-glucopyranoside, salicin-α-D-maltopyranoside and salicin-α-D-maltotriopyranoside through a combination of enzyme treatments, mass spectrometry and NMR analyses. The glycosidic bond between glucose units consisted of an α-1,4-configuration. The water solubility of salicin-α-D-glucopyranoside, salicin-α-D-maltopyranoside and salicin-α-D-maltotriopyranoside was 3-, 5- and 8-fold higher, respectively, than that of salicin, whereas their relative sweetness values were lower than that of sucrose. Interestingly, the long-chain salicin-α-D-glucosides showed greater anticoagulant and anti-inflammatory activities than salicin. In addition, the synthesized salicin-α-D-glucosides were able to tolerate acidic and high temperature conditions, but not α-glucosidase or human digestive enzymes. Therefore, these salicin-α-D-glucosides should be applied by the injection route to achieve greater bioavailability than is possible by the oral route.



## 1. Introduction

Amylomaltase (EC 2.4.1.25), an intracellular 4-α-glucanotransferase (4αGTase), was first identified in *Escherichia coli* as an enzyme involved in maltose metabolism [1,2]. The enzyme was subsequently reported to be expressed in most thermophilic and archaea bacteria, such as *Thermus aquaticus* [3], *Aquifex aeolicus* [4] and *Pyrobaculum aerophilum* IM2,[5] and corresponded to the D-enzyme in plants, such as potato [6]. Amylomaltase catalyzes an intermolecular transglucosylation reaction, transferring glucosyl residues from donor to acceptor as free OH groups of C4 to produce longer linear oligosaccharides. In addition, this enzyme generates a unique intramolecular transglucosylation reaction to yield cycloamyloses or large-ring cyclodextrins (LR-CDs) with a degree of polymerization (DP) of 16 or more. The enzyme has four different activities: disproportionation, cyclization, coupling and hydrolysis [7,8]. The first two activities are the main activities. Amylomaltase has many potential applications. Firstly, it is used in the production of LR-CDs via a cyclization reaction. An LR-CD can form a inclusion complex with guest molecules by trapping them into a hydrophobic cavity, thus resulting in a change in the solubility, stability and biological properties of the guest molecules [7]. Secondly, amylomaltase is used to modify starch and produce a thermo-reversible starch gel with a gelatin-like property that can be used as a low fat or vegetarian heavy cream substitute in dairy products [5,9,10]. Finally, the enzyme can be used in the production of linear oligosaccharides and glucoside products through an intermolecular transglucosylation reaction. For example, short-chain isomaltooligosaccharides (IMOs), such as DP2-DP6, with prebiotic activity were reported to be synthesized by a combination of amylomaltase and transglucosidase [11].

Salicin ($C_{13}H_{18}O_7$: 2-hydroxymethyl-phenyl β-D-glucopyranoside) is an alcoholic β-glucoside extracted from several species of *Salix* (willow) and *Populus* (poplar). In addition, salicin was also found in *Gaultheria procumbens* (wintergreen) and in *Betula lenta* (sweet birch), the volatile oils of which consist almost entirely of

* Corresponding author.
*E-mail address:* jkaulpiboon@yahoo.com (J. Kaulpiboon).

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methyl salicylate [12]. Salicin is used as an analgesic, anti-inflammatory and antipyretic agent [13]. Glucosylation of salicin has been the subject of increasing attention to improve its pharmacokinetic parameters [14]. There are several reports on the glucosylation of salicyl alcohol by cultured plant cells showing that the benzyl hydroxyl group was glucosylated to yield isosalicin [15]. However, the reaction products were mostly synthesized by the addition of a single glucose to the substrate. Until now, there have been, to our knowledge, only two reports on the glucosylation of salicyl alcohol or salicin using bacterial enzymes. In 2005, Seo et al. [12] successfully modified salicyl alcohol, phenol and salicin using *Leuconostoc mesenteroides* glucansucrase (EC 2.4.1.5) with sucrose as a glucosyl donor. Salicin, phenyl glucose, isosalicin, salicyl β-isomaltooligosaccharides, and salicyl alcohol β-isomaltooligosaccharides were the major products. Later, *Deinococcus geothermalis* (DGAS) and *Neisseria polysaccharea* (NPAS) amylosucrases (EC 2.4.1.4) were used to synthesize glucosyl and maltosyl salicin from sucrose and salicin substrates [16]. Two salicin α-glucosides from NPAS amylosucrase were detected and identified as glucosyl and maltosyl salicin. In contrast, DGAS amylosucrase synthesized only one glucosyl salicin product.

Hence, in this study, more attention has been paid to the synthesis of a long carbohydrate salicin glucoside chain by amylomaltase using inexpensive, widely available substrates, such as tapioca starch, which is a major economic crop in Thailand. In our previous study, the amylomaltase gene was directly isolated from soil DNA, cloned into a pET19b vector, and expressed in *E. coli* BL21(DE3). The ORF of this gene consisted of 1572 bp, encoding an enzyme of 523 amino acids [17]. Although it showed 99% sequence identity to amylomaltase from *Thermus thermophilus* ATCC 33923, this enzyme is unique regarding its alkaline optimum pH. The enzyme produced cycloamyloses or LR-CD products in the range of DP23- > DP50 through its intramolecular transglucosylation activity [18]. The prebiotic α-1,6-isomaltooligosaccharides were successfully synthesized from tapioca starch by the co-action of this amylomaltase and transglucosidase through their intermolecular transglucosylation reactions [19]. In this study, we focus on the synthesis of new salicin derivatives using *Thermus* sp. amylomaltase with salicin and tapioca starch substrates in an effort to increase the value of the agricultural product. In addition, a comparison of the inhibitory effects of the salicin analogs on blood coagulation and inflammation is also performed.

## 2. Materials and methods

### 2.1. Materials

β-Salicin was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). D(+)-Glucose, maltooligosaccharides (MOSs) with a degree of polymerization (DP) of 2–7, potato starch, rat intestinal acetone powder, α-amylase, heparin and bovine serum albumin (BSA) were obtained from Sigma-Aldrich (USA). The Bio-Gel®P-2 Gel was obtained from Bio-Rad Laboratories (USA). *A. niger* α-glucosidase (EC 3.2.1.20) was obtained from Amano Enzyme Inc. (Nagoya, Japan). Tapioca starch was a gift from Siam Modified Starch Co., Ltd. (Thailand). Rabbit blood without anticoagulant was purchased from the National Laboratory Animal Center, Mahidol University (Thailand). All other chemicals used were of analytical grade.

### 2.2. Amylomaltase preparation

*Escherichia coli* cells harboring the p19bAMY plasmid were grown in LB medium containing 100 μg/ml ampicillin at 37 °C for 24 h. Enzyme expression was induced by adding 0.8 mM IPTG and the intracellular crude amylomaltase fraction was obtained after cell sonication. The enzyme was purified using a HisTrap FF™ column, as previously described [17]. The active fractions were pooled and assayed for enzyme activity using a starch transglucosylation assay [17], and the protein concentration was determined by the Bradford method [20] using BSA as the standard.

### 2.3. Donor specificity

The appropriate donor was selected by incubating 1% (w/v) salicin and 40 U/ml amylomaltase in 0.2 M phosphate buffer at pH 6.0 with 1% (w/v) of the different glucosyl donors (starches and maltoheptaose) in a 160 μl reaction at 70 °C for 24 h. The transfer products were analyzed using thin-layer chromatography (TLC) with the mobile phase described in Section 2.5. The densities of the salicin-α-glucoside spots on the TLC plate were measured using a densitometer using the GS-800 program (Bio-Rad Laboratories, CA, USA).

### 2.4. Optimization of the production of salicin-α-glucosides (β-Sa-α-$G_n$)

The optimum conditions for the production of salicin-α-glucosides were considered and defined in terms of obtaining the highest percentage yield of salicin-α-glucoside products, as determined from the HPLC results. The effects of varying the salicin concentration (0–2% (w/v)), tapioca starch concentration (0–2% (w/v)), amylomaltase concentration (40–120 U/ml, starch transglucosylation activity), pH (5.0–8.0), temperature (50–90 °C), and incubation time (0–29 h) were all investigated. After completion of the incubation period, all tested reactions were inactivated by boiling at 100 °C for 10 min prior to HPLC analysis. The conversion yield of glucosylated salicin was calculated from the ratio of the peak area of the salicin-α-glucosides to the peak area of the total products in the HPLC profile using equation (1):

$$\text{Conversion yield } (\%) = \frac{\text{peak area of } \beta - \text{Sa} - \alpha - \text{G}_\text{n}}{\text{peak area of total product}} \times 100 \quad (1)$$

### 2.5. Product analysis

The transglucosylation products were detected and identified using HPLC and TLC analyses. For the HPLC analysis, the reaction mixture of transglucosylation products was filtered using a 0.45-μm filter and analyzed with a 5 μm SphereClone™ NH2 column (4.6 × 250 mm) (Phenomenex, USA) connected to a Shimadzu LC-9A system with a SPD-6AV UV–VIS detector at 265 nm [16]. The column was eluted with 75% acetonitrile as the mobile phase using a flow rate of 1.0 ml/min at room temperature. TLC was performed with silica gel 60 $F_{254}$ plates (Merck, Germany) after activation at 95 °C for 5 min. A 10-μl aliquot of each reaction mixture was loaded onto a plate and developed with a solvent system of 5:5:3 (v/v/v) n-butanol-ethanol-water in a TLC tank. Salicin-α-glucosides were detected by spraying the plates with a mixture of concentrated sulfuric acid and methanol (1:2, v/v), and then heating them at 110 °C for 20 min [21].

### 2.6. Larger scale production of salicin-α-glucosides

Larger-scale production was undertaken in a reaction volume of 48 ml using the optimized reaction conditions to obtain a greater quantity of the salicin-α-glucoside products for the subsequent

studies. Tapioca starch (2% (w/v)) was incubated with 120 U/ml (starch transglucosylation unit) of amylomaltase in 0.2 M phosphate buffer at pH 6.0 and 70 °C for 3 h. After completion, the reaction mixture was stopped by boiling it for 10 min. The reaction products were dried in a lyophilizer (Biotechnologies Inc., Germany) before being applied to a Bio-Gel®P-2 Gel column.

### 2.7. Purification of the salicin-α-glucosides

The mixture of dried salicin-α-glucoside products was dissolved with distilled water and clarified by centrifugation before being applied to a Bio-Gel®P-2 Gel column (1.2 × 97 cm). The column was equilibrated and eluted with distilled water at a flow rate of 8 ml/h, and 1-ml fractions were collected during this process. The carbohydrates in the fractions were measured using the DNS assay [22]. The positive fractions were then analyzed by HPLC, and each pure fraction was collected and lyophilized to analyze the products.

### 2.8. Structural analysis of salicin-α-glucosides

#### 2.8.1. Mass spectrometry (MS) analysis

The transfer products of interest were dissolved in a 50% (v/v) methanol solution, and their masses were determined on a microTOF spectrometer (Bruker, Germany). The products were introduced into a mass spectrometer for processing in the ESI-TOF-MS system, which ionizes the sample by electrospray ionization (ESI) in the sodium positive ion mode using a capillary voltage of 5000 V. A 4.0 L/min flow of nitrogen gas at a temperature of 150 °C was used to nebulize the analytic solution to droplets at a nebulizer pressure of 1.0 bar. The ions were detected using linear time-of-flight mass spectrometry (TOF-MS), and the mass to charge (m/z) spectra of the products were detected as pseudo-molecular ion peaks $[M+Na]^+$, from which the molecular weights were calculated using a Bruker Daltonics Data Analysis 3.4 software program.

#### 2.8.2. Nuclear magnetic resonance (NMR) analysis

The structures of the salicin-α-glucosides synthesized using amylomaltase were identified with $^1$H- and $^{13}$C-NMR using a Bruker AVANCE™ III HD 600 NMR Spectrometer and standard Bruker NMR software. The spectrometer was operated at 600 MHz at ambient temperature. Chemical shifts were measured with sodium-4,4-dimethyl-4-sila-pentane sulfonite (DSS) as the internal standard. The purified products (2 mg) were freeze-dried and dissolved in 1 ml of $CD_3OD$ containing 0.1% DSS prior to $^1$H- and $^{13}$C-NMR analysis.

### 2.9. Physical and biological properties of the salicin-α-glucoside products

#### 2.9.1. Water solubility

Excess salicin and salicin-α-glucosides were mixed with 200 μl of water in micro-centrifuge tubes at room temperature. After 20 min of mixing with a vortex mixer at 25 °C, each of the samples was diluted and filtered through a 0.45-μm membrane for HPLC analysis to determine the concentrations.

#### 2.9.2. Sweetness

The relative sweetness of 1% (w/v) of the tested salicin-α-glucosides was determined by measuring the Brix values using a refractometer (RX-1000, Atago Co., Ltd., Tokyo, Japan) [23]. Sucrose was used as the reference compound to plot the standard curve of the concentrations (%, w/v) versus the degrees Brix (°Bx). One degree Brix is defined as 1 g of sucrose in 100 ml of solution.

#### 2.9.3. Acidic resistance

The acidic resistance of the salicin derivatives was investigated in 100 mM acetate buffer, pH 3.0, containing 2% (w/v) salicin-α-glucosides synthesized from amylomaltase or original salicin. The reaction mixture was incubated at 37 °C for 24 h. The remaining salicin and salicin derivatives were determined by HPLC [23].

#### 2.9.4. Digestive enzyme treatment

The resistance of the products to digestive enzymes was tested to evaluate the route of drug administration. One milligram of the salicin-α-D-maltotriopyranoside product was mixed and dissolved in 0.5 ml of 0.2 M acetate buffer, pH 5.5, followed by an incubation with 0.5 ml of α-glucosidase (10 U/ml), α-amylase (10 U/ml) and rat digestive enzymes (10 mg/ml, I1630-Sigma-Aldrich) at 37 °C for 0, 1 and 24 h. After incubation for each of these time intervals, the reaction was halted by boiling and a 10-μl aliquot of each reaction was then analyzed by TLC (System II).

#### 2.9.5. Anticoagulant activity

The whole blood clotting time was determined using the method of Seo et al., [12] with slight modifications. This experiment was performed in clean glass tubes immediately after blood was drawn from a healthy rabbit of the National Laboratory Animal Center, Mahidol University (Thailand). Then, 0.1 ml of a 10% (w/v) solution of salicin and the salicin derivatives in phosphate buffer (pH = 7.4) was incubated with 1.4 ml of rabbit blood for 5, 10, 15 and 20 min. The time required for clotting was calculated immediately after salicin and the salicin derivatives were added. If clotting was not observed after 20 min, the sample was defined as not forming a blood clot (NC). Heparin (0.5 and 50 U/ml) was used as a positive control.

#### 2.9.6. β-Glucuronidase inhibition assay

For this assay, 100 μl of 2.5 mM *p*-nitrophenyl-β-D-glucopyranosiduronic acid in 0.1 M acetate buffer (pH = 7.4) was incubated with 1 mg of salicin and the salicin derivatives for 5 min, followed by the addition of 0.1 ml of β-Glucuronidase (Merck, France). The reaction mixture was then incubated for 30 min, followed by the addition of 2 ml of 0.5 N NaOH to halt the reaction. The amount of reaction product formed was measured at 410 nm and calculated using equation (2):

$$\text{Anti} - \text{inflammatory activity } (\%) = \frac{(1 - A_{410} \text{ of test sample})}{A_{410} \text{ of control sample}} \times 100 \quad (2)$$

A 1 mM salicylic acid solution was used as a reference drug for comparison [24].

### 2.10. Statistical analyses

All data are expressed as the means ± SD from at least three separate experiments, and the differences were calculated using Student's *t*-test (GraphPad Prism software version 4). *P*-values < 0.05 were considered statistically significant.

## 3. Results and discussion

### 3.1. Donor specificity

A 1% (w/v) salicin solution was used as the acceptor and 1% (w/v) potato starch, tapioca starch and maltoheptaose ($G_7$) were used as glucosyl donors in a transglucosylation reaction with recombinant amylomaltase (40 U/ml) in 0.2 M phosphate buffer at pH 6.0 at 70 °C for 24 h to determine the potency of different glucosyl donors

for salicin. The products were analyzed by TLC. Upon analysis of the product by TLC, at least three products with higher $R_f$ values than the sugar standard were observed (Fig. S1). These transglucosylation products were believed to be the salicin-α-glucosides, which were synthesized by an amylomaltase-catalyzed transglucosylation reaction from tapioca starch to the salicin acceptor. In addition, the transglucosylation yields were also determined from the relative ratio of the salicin-α-glucoside-spot density to total product-spot density. The salicin-α-glucoside yields of potato starch, tapioca starch and $G_7$ were 36, 36 and 20% (w/w) of the total products, respectively. Based on this experiment, tapioca starch, an inexpensive and widely available substrate, was chosen as a suitable glucosyl donor for transglucosylation to a salicin acceptor.

### 3.2. Optimization of salicin-α-glucoside synthesis

The parameters of the reaction were sequentially and independently optimized by changing some of the conditions of the transglucosylation reaction to produce the highest yield of the salicin-α-glucosides. These parameters included the substrate concentrations, enzyme concentrations, pH, temperature and incubation time. The products obtained from each reaction were analyzed by HPLC and TLC, compared to the salicin standard (Fig. 1), and quantified using equation (1). As a result, the optimum condition for the synthesis of salicin-α-glucosides was an incubation of 1% (w/v) salicin and 2% (w/v) tapioca starch with 120 U/ml amylomaltase in 0.2 M phosphate buffer at pH 6.0 and 70 °C for 3 h (Fig. S2). Under these conditions, the yield of the salicin-α-glucoside products was up to 63% (w/w) (18.9 mg/ml) of the total products, which were increased more than 1.8-fold compared to the yield before optimization. This increase in product yield represented an increase in all salicin-α-glucoside, β-Sa-α-$G_{1\text{-}3}$, products. The concentrations of β-Sa-α-$G_1$, β-Sa-α-$G_2$ and β-Sa-α-$G_3$ were 9.9, 5.4 and 3.6 mg/ml, respectively, compared with the standard curve of each salicin-α-glucoside. In general, the synthesis of salicin glucosides by the action of other bacterial enzymes has been reported. For example, the incubation of *Leuconostoc mesenteroides* 1299 CB-BF563 glucansucrases with sucrose and salicin could catalyze the synthesis of salicyl alcohol β-isomaltooligosaccharides, with yields of 47% (w/w) [12]. The optimum conditions for generation of the salicin transfer product by the *Neisseria polysaccharea* (rNPAS) and *Deinococcus geothermalis* (rDGAS) amylosucrases with salicin and sucrose substrates were 35 °C (pH = 6) and 45 °C (pH = 8), respectively [16]. Their HPLC analyses showed that two glucosylated salicin products (designated as glucosyl and maltosyl salicin) were generated in the reaction with the rNPAS enzyme, but only one product (glucosyl salicin) was generated in the reaction with the rDGAS enzyme. The yield of the salicin transfer products was over 80% in the rNPAS reaction, but the yield of the rDGAS reaction (70%) was lower than the rNPAS reaction. Accordingly, the salicin-α-glucoside yield obtained in this study was quite sizeable as compared to the group involved in the bacterial enzyme-catalyzed synthesis. Specifically, we learned that our amylomaltase is a unique enzyme that acts differently than

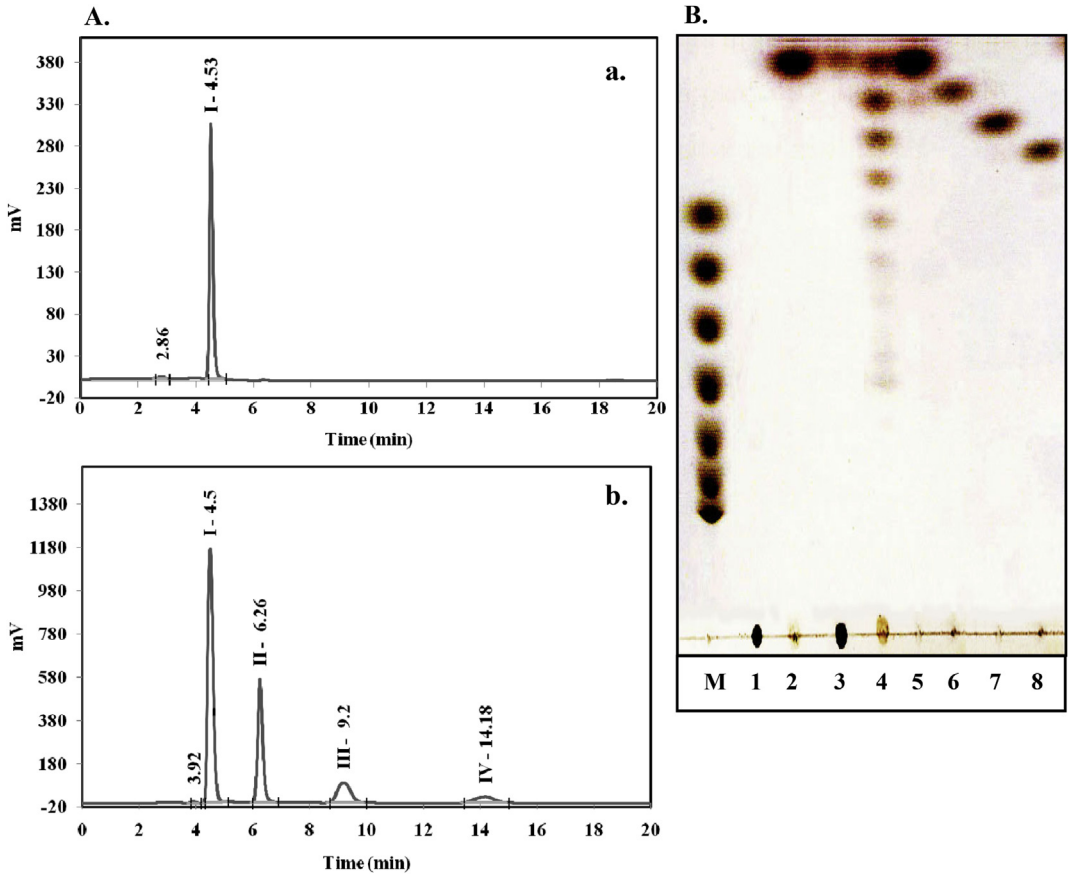


**Fig. 1.** HPLC and TLC analyses of the products from the reaction of recombinant amylomaltase with the salicin acceptor and tapioca starch donor. (A.) HPLC analysis: (a.) reaction at 0 h and (b.) reaction at 3 h under the optimal conditions. (B.) TLC analysis: Lane M - $G_1$-$G_7$ standards, Lane 1–2% (w/v) tapioca starch, Lane 2 - 1% (w/v) salicin, Lanes 3 and 4 - products from the enzymatic reaction at 0 and 24 h, Lane 5 - salicin (HPLC peak I), Lane 6 - purified product 1 (HPLC peak II), Lane 7 - purified product 2 (HPLC peak III), and Lane 8 - purified product 3 (HPLC peak IV).

other enzymes in the transglycosylation of oligosaccharides to salicin for the synthesis of novel salicin-α-D-maltotriopyranosides.

### 3.3. Large scale preparation, purification and structural analysis of the synthesized salicin-α-glucosides

The reaction mixture was incubated under the optimal conditions and scaled up to 48 ml to prepare a larger amount of the synthesized salicin-α-glucosides for the structural analysis. The reaction mixture was then concentrated with a lyophilizer. The concentrated reaction products were loaded onto a Bio-Gel-P2 column and separately analyzed by HPLC before mass analysis. The molecular weights of the purified products were confirmed with ESI-TOF MS in the positive mode. There were intense signals at *m*/*z* 471.28 (Product 1), 633.25 (Product 2) and 795.38 (Product 3), respectively (Fig. S3). These three reaction products corresponded to the molecular weight of salicin-α-D-glucopyranoside (β-Sa-α-$G_1$) [16], salicin-α-D-maltopyranoside (β-Sa-α-$G_2$) [16] and salicin-α-D-maltotriopyranoside (β-Sa-α-$G_3$), respectively.

The structures of the salicin-α-glucosides were also analyzed by $^{13}$C- and $^{1}$H-NMR to identify the structure of salicin glucoside products, as shown in Tables 1 and 2 (Figs. S4 and S5). For β-Sa-α-$G_1$ (Product 1; $R_t = 6.2$ min), the $^{13}$C-NMR analysis displayed nineteen carbon signals. Seven were assigned as salicyl alcohol. Another twelve signals were assigned to the glucosyl group. The C4′ signal of the glucose I unit (Product 1) changed greatly from 71.34 ppm (Salicin) to 80.90 ppm (+9.56 ppm) because of the glucosylation between glucose I and II. The type of glucosidic linkage was also investigated using $^{1}$H-NMR and was based on the coupling constants of the anomeric protons, as shown in Table 2 β-Sa-α-$G_1$ gave a double signal at 4.88 and 5.23 ppm, with coupling constants (*J*) of 8.0 and 4 Hz, respectively, which suggested that the type of glucosidic linkage was one β-configuration and one α-configuration. When β-Sa-α-$G_1$ was analyzed in terms of its structure and in combination with its molecular weight, we concluded that the β-Sa-α-$G_1$ product was salicin-α-D-glucopyranoside (Fig. 2A).

Likewise, the structural analysis of β-Sa-α-$G_2$ (Product 2) and β-Sa-α-$G_3$ (Product 3) and the analysis of the $^{13}$C-NMR spectra of β-Sa-α-$G_2$ and β-Sa-α-$G_3$ were also performed and revealed twenty-five and thirty-one carbon signals, respectively (Table 1). Thirteen of the signals were assigned to the salicin group, and the remaining twelve and eighteen signals were construed as two and three units of glucose. Compared to the C4″-glucose II signals of Product 1, the C4″-glucose II signals of Product 2, and C4″-glucose II and C4‴-glucose III signals of Product 3 showed downfield chemical shifts of 9.82, 9.79 and 9.89 ppm, respectively. These downfield shifts indicated that β-Sa-α-$G_2$ (Product 2) and β-Sa-α-$G_3$ (Product 3) had two and three glucosidic linkages with salicin, respectively. One of the glucosidic linkage was between the salicin group and glucose, and the other was between glucose and glucose. In addition, the $^{1}$H-NMR spectrum of β-Sa-α-$G_2$ revealed that β-Sa-α-$G_2$ had one β-configuration and two α-anomeric configurations, based on the signals at 4.87, 5.18 and 5.24 ppm ($J = 8.0$, 4.0 and 4.0 Hz). Similarly, the structure of β-Sa-α-$G_3$ was one β-configuration and three α-configurations that were confirmed by the signals of the anomeric proton at 4.87, 5.18, 5.25 and 5.28 ppm ($J = 8.0$, 4.0, 4.0 and 4.0 Hz, respectively) by $^{1}$H-NMR analysis (Table 2). During the course of the integrated NMR and MS analysis, Products 2 and 3 were found to be salicin-α-D-maltopyranoside and salicin-α-D-maltotriopyranoside (Fig. 2B and C).

**Table 1**
$^{13}$C-NMR analysis of salicin and the salicin transglucosylation products.

| Carbon atom | $^{13}$C-NMR (δ, ppm) | | | |
|---|---|---|---|---|
| | Salicin[a] | Product 1 | Product 2 | Product 3 |
| C-salicyl alcohol | | | | |
| 1 | 156.99 | 157.06 | 157.06 | 157.09 |
| 2 | 132.03 | 132.21 | 132.22 | 132.29 |
| 3 | 129.75 | 129.92 | 129.91 | 129.93 |
| 4 | 123.62 | 123.80 | 123.81 | 123.83 |
| 5 | 129.85 | 129.97 | 129.97 | 129.95 |
| 6 | 116.96 | 117.04 | 117.06 | 117.14 |
| 7 | 60.98 | 60.98 | 60.98 | 60.99 |
| C-glucose I | | | | |
| 1′ | 103.29 | 103.19 | 103.13 | 103.27 |
| 2′ | 75.05 | 74.70 | 74.70 | 74.73 |
| 3′ | 77.96 | 77.75 | 77.73 | 77.77 |
| 4′ | 71.34 | 80.90 | 80.92 | 80.86 |
| 5′ | 78.21 | 76.86 | 76.85 | 76.89 |
| 6′ | 62.53 | 62.04 | 62.08 | 62.13 |
| C-glucose II | | | | |
| 1″ | | 102.94 | 102.94 | 102.89 |
| 2″ | | 74.19 | 73.81 | 73.81 |
| 3″ | | 75.11 | 74.97 | 74.96 |
| 4″ | | 71.56 | 81.38 | 81.35 |
| 5″ | | 74.84 | 73.41 | 73.41 |
| 6″ | | 62.73 | 62.19 | 62.20 |
| C-glucose III | | | | |
| 1‴ | | | 102.71 | 102.74 |
| 2‴ | | | 74.28 | 74.30 |
| 3‴ | | | 75.13 | 75.15 |
| 4‴ | | | 71.53 | 81.45 |
| 5‴ | | | 74.81 | 74.80 |
| 6‴ | | | 62.77 | 62.80 |
| C-glucose IV | | | | |
| 1⁗ | | | | 102.65 |
| 2⁗ | | | | 73.43 |
| 3⁗ | | | | 75.00 |
| 4⁗ | | | | 71.59 |
| 5⁗ | | | | 73.92 |
| 6⁗ | | | | 62.25 |

[a] Jung et al. [16].

**Table 2**
$^{1}$H-NMR chemical shift values and coupling constants for the glucosidic protons of salicin and the salicin transglucosylation products.

| Compound | Glucosidic protons | | | |
|---|---|---|---|---|
| β-Salicin (β-Sa)[a] | 4.86[b] (d,8.0)[c] | | | |
| Product 1 (β-Sa-α-$G_1$) | 4.88 (d[d],8.0) | 5.23 (d,4.0) | | |
| Product 2 (β-Sa-α-$G_2$) | 4.87 (d,8.0) | 5.18 (d,4.0) | 5.24 (d,4.0) | |
| Product 3 (β-Sa-α-$G_3$) | 4.87 (d,8.0) | 5.18 (d,4.0) | 5.25 (d,4.0) | 5.28 (d,4.0) |

[a] Jung et al. [16].
[b] Chemical shifts (δ) were given in ppm.
[c] The coupling constants (Hz) are presented in parentheses.
[d] Doublet.

### 3.4. Physical and biological properties of the salicin-α-glucoside products

#### 3.4.1. Water solubility

The solubility of each of salicin-α-glucoside was compared to that of the original salicin. Excess salicin and salicin glucosides were mixed with water and incubated at 25 °C for 20 min. The soluble part of the sample was analyzed by HPLC to determine the concentrations. Based on the result, the solubility of salicin was 25.42 mg/ml, whereas the solubilities of β-Sa-α-$G_1$, β-Sa-α-$G_2$ and β-Sa-α-$G_3$ were 74.32, 132.36 and 209.57 mg/ml, respectively, corresponding to levels that were 3, 5 and 8 times higher than that of salicin. This result implied that the glucosylation process could be used to increase the solubility of salicin.

#### 3.4.2. Sweetness

The sweetness values of salicin and the synthesized β-Sa-α-$G_1$,

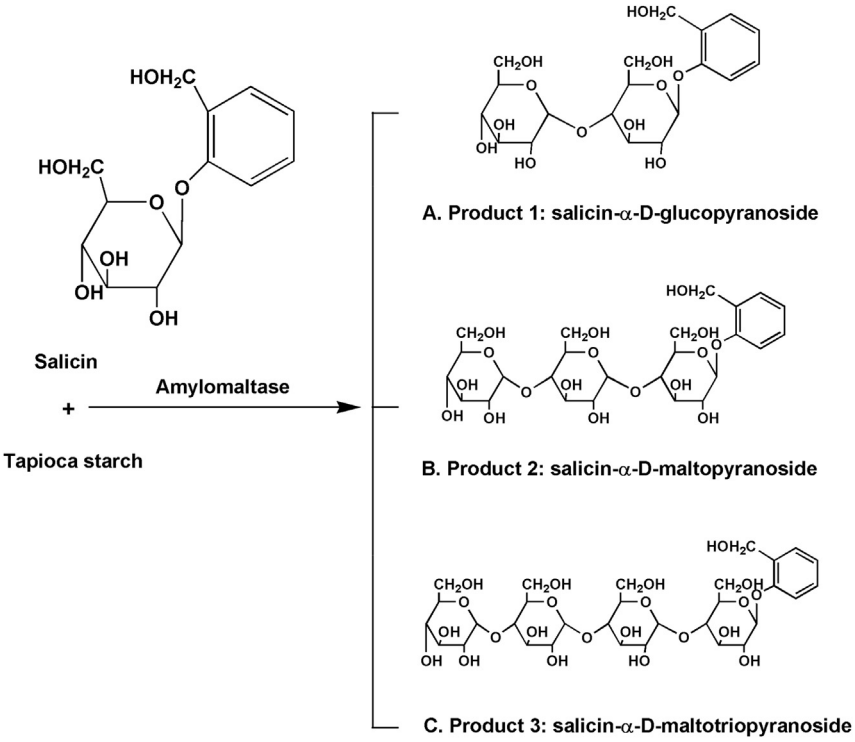


**Fig. 2.** Structures of salicin and the salicin-α-D-glucosides. A. Salicin-α-D-glucopyranoside. B. Salicin-α-D-maltopyranoside. C. Salicin-α-D-maltotriopyranoside.

β-Sa-α-$G_2$ and β-Sa-α-$G_3$ products were 0.25, 0.20, 0.1 and 0.05, respectively, compared to the sweetness value of sucrose, which is set to 1.0. Based on these results, the sweetness decreased as the saccharide chain lengths of the salicin glucosides increased. Practically, the sweetness depends on the chemical structure, the degree of polymerization of the oligosaccharides present, and the levels of mono- and di-saccharides in the mixture [25]. According to Roberfroid and Slavin [26], the sweetness of an oligosaccharide decreases with longer oligosaccharide chain lengths. A low sweetness level is quite useful when the glucosides are used as a sucrose substitute in a functional material where the use of sucrose is restricted by its high sweetness property [14,27].

### 3.4.3. Acidic resistance

Salicin and all salicin derivatives showed low degradability in an acidic buffer at pH 3.0 after an incubation at 37 °C for 24 h (Fig. S6). Their high stabilities under acidic conditions suggests that the synthesized salicin-α-glucosides could resist the gastric acidity of humans and could be used in acidic materials, such as fruit juices, alcoholic beverages, soft drinks, sparkling water and acidic drugs.

### 3.4.4. Digestive enzyme treatment

The route of drug administration was preliminarily investigated by a digestive enzyme treatment, as described in Section 2.9.3. After a 1-h treatment, β-Sa-α-$G_3$ was degraded by α-glucosidase and rat digestive enzymes, such as α-glucosidase, maltase, isomaltase, α-amylase, sucrase, among others, yielding β-Sa-α-$G_1$ and β-Sa-α-$G_2$. After a longer treatment period of 24 h, glucose and salicin products were observed (Fig. S7). This result suggested that salicin derivatives should be introduced into the body by intravenous or subcutaneous routes rather than by an oral route to avoid the digestive tract. In addition, this result also confirmed that each glucose was linked with salicin through an α-configuration, making it easier to digest with α-glucosidase and rat digestive enzymes. In contrast, original salicin, which is linked by a β-configuration between salicyl alcohol and glucose, could not be digested with either enzyme. This result is related to the fact that the amylomaltase is specific for the α-1,4-glucosidic bond [28].

### 3.4.5. Anticoagulant activity

The time required for clotting was measured to determine the anticoagulant effects of salicin, the salicin derivatives, and aspirin (Table S1). Aspirin and salicin produced blood clots within 5 min, whereas β-Sa-α-$G_1$ and β-Sa-α-$G_2$ caused blood clots to form within 15 min β-Sa-α-$G_3$ formed blood clots within 20 min under the described conditions. In addition, we also checked the anticoagulant effect of heparin as a positive control. Heparin (0.5 U/ml) produced slow blood clotting within 5 min and complete blood clotting within 10 min, whereas heparin (50 U/ml) did not form blood clots, even after 20 min had passed. Typically, the typical dose of heparin that is used to treat and prevent blood clots in the veins and arteries in the lungs is a final concentration of $\geq$0.5 U/ml blood [29,30]. This result suggests that a dose of 0.67% (w/v) of the salicin derivatives has a greater potential to inhibit the formation of blood clots than 0.5 U/ml of heparin. The anticoagulant action of the salicin-α-glucosides in this study might be the result of the inhibition of cyclooxygenase (COX) enzymes [31], which are involved in thromboxane synthesis in the blood coagulation system.

### 3.4.6. β-Glucuronidase inhibition assay

Anti-inflammatory activity was determined using the β-glucuronidase inhibition assay. Salicin, β-Sa-α-$G_1$, β-Sa-α-$G_2$, and β-Sa-α-$G_3$ showed moderate anti-inflammatory activity measuring 15, 28, 32 and 40% of salicylic acid (control = 100%), respectively. The results also showed that the long carbohydrate chains of salicin were more effective at inhibiting β-glucuronidase than shorter carbohydrate chains. It is known that β-glucuronidase is mainly located in lysosomes of neutrophils and plays an important role as mediator of the initiation and progression of inflammation [32]. This

result might explain how the modified salicin inhibited β-glucuronidase and how it is related to the inhibition of other inflammatory mediators, such as prostaglandins, lipoxins and leukotrienes [32].

## 4. Conclusions

The enzyme from the p19bAMY recombinant cells containing the amylomaltase gene from *Thermus* sp. could be used to synthesize salicin-α-D-glucosides by transferring one or more glucose residues from the glucosyl donor, tapioca starch, to the salicin acceptor through the transglucosylation reaction. Under the optimal conditions, the yield of the obtained salicin glucosides was 63% (w/w) of the total products. The acceptor products of salicin were confirmed to be salicin-α-D-glucopyranoside, salicin-α-D-maltopyranoside and salicin-α-D-maltotriopyranoside by ESI-TOF MS and NMR analyses. Moreover, the products obtained from this study also exhibited properties of a potential anticoagulant and anti-inflammatory agent.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.carres.2016.06.011.

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