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Efficient enzymatic synthesis and antibacterial activity of andrographolide glycoside

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ABSTRACT

19-*O*-β-Galactosyl andrographolide, a potential novel antibacterial agent, was synthesized through enzymatic transgalactosylation of andrographolide in co-solvent systems. Organic solvents and their contents have important influences on the regioselective galactosylation of andrographolide catalyzed by β-galactosidase from bovine liver in co-solvent systems. β-Galactosidase showed high activity and stability in 5–15% (v/v) DMSO with 22–52% total molar yields of andrographolide glycosides. The addition of hydrophilic DMSO not only greatly promoted the solubility of the substrate, but also improved the reaction efficiency of the process. β-Galactosidase displayed absolute regioselectivity toward the 19-position of andrographolide. The solubility of andrographolide glycoside in water was 42.1 mg ml^{-1}, which is about 702 times that of andrographolide. The glycosylated andrographolide showed antibacterial activity against five representative species of food-borne pathogenic bacteria [with minimal inhibitory concentrations (MICs) as low as 8 μg ml^{-1}], whereas andrographolide exhibited no such activity. These results indicate an enzymatic modification was not only facile and green, but an effective method for the preparation of an andrographolide monoglycoside.

1. Introduction

Andrographolide ($C_{20}H_{30}O_5$, Scheme 1), a diterpene lactone [1], is the main and most medicinally active component isolated from *Andrographis paniculata* (Burm.f.) Nees, an important herbal medicine traditionally used to treat different range of diseases in Asian countries [2–4]. Andrographolide is found in the whole plant but is most concentrated in the leaves (about 0.5–1.0%, dry weight) and has multiple pharmacological activities such as anti-bacterial, anti-inflammatory, antiviral, and anti-allergic activities [3–5]. However, the solubility of andrographolide in water was maximal at a concentration of 60 μg ml^{-1}. Moreover, andrographolide has a poor solubility in most organic solvents. The low solubility of andrographolide is disadvantageous not only by limiting its pharmacological use in potential therapeutics but also for its modification. Andrographolide glycosides are also active components from *A. paniculata*, and have good solubility in water, but their contents are very low (about 0.01–0.03%, dry weight) [5]. In fact, andrographolide glycosides derivatives can be achieved by glycoside modification of andrographolide.

Glycosylation has been used to modify the hydrophilicity, bioactivity and chemical properties of lipophilic natural products [6]. Glycosylation strategy has been established well for the improvement of therapeutic efficacy by enhancing oral absorption, selectivity and water-solubility of the parent agents, etc. [7]. For instance, the water-solubility of the antitumor drug—geldanamycin was markedly improved upon glycosylation modification [8]. Despite the many excellent chemical methods for glycosylation, methods for the direct regioselective glycosylation of andrographolide are limited owing to the presence of multi active hydroxyl groups in the reactant (Scheme 1). In general, arduous and tedious protection–deprotection steps and environmentally unfriendly catalysts were involved in traditional chemical methods. Use of enzymes as the alternative to chemical catalysts offered many new opportunities for the regioselective glycosylation, because of excellent selectivity, simplicity, mild reaction conditions and being environmentally benign [9]. Process simplification resulted in an overall reduction in energy use and waste production because of avoiding protection–deprotection steps (Fig. 1).

However, our pre-experiments showed that enzymatic glycosylation reaction in buffer was not efficient due to the low solubility of the andrographolide. Enzymatic glycosylation in hydrophilic organic solvents provides numerous industrially attractive advan-

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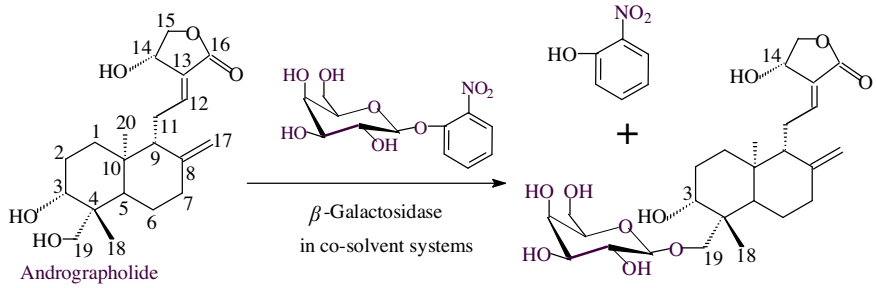

Scheme 1. Enzymatic regioselective galactosylation of andrographolide catalyzed by β-galactosidase from bovine liver.

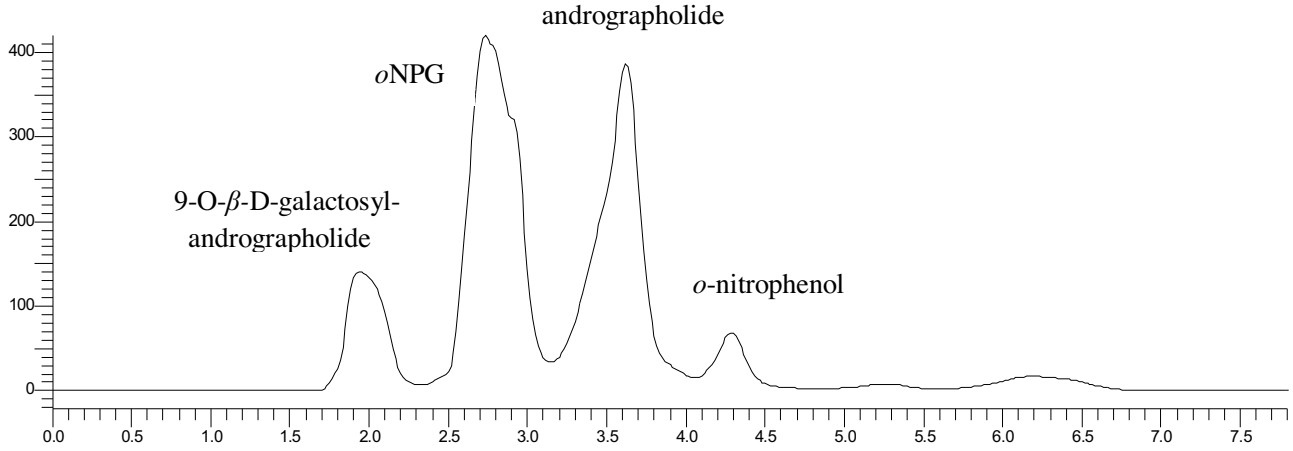

Fig. 1. HPLC of 19-*O*-β-D-galactosyl-andrographolide (1.9 min), *o*NPG (2.7 min), andrographolide (3.6 min) and *o*-nitrophenol (4.3 min).

tages, such as the increased solubility of substrate, reversal of the thermodynamic equilibrium of the hydrolysis reactions, and elimination of microbial contamination [10,11]. Obviously, the denaturation of enzymes in hydrophilic media is a major disadvantage. The synthetic utility of glycosidase would be considerably improved if its catalytic activity could be maintained in the presence of hydrophilic media.

Thus, an efficient solvent-resistant glycosidase in the presence of a certain percentage of hydrophilic organic solvent may play a pivotal role in a drastic reaction process. To the best of our knowledge, there have been no reports on the application of β-glycosidase to andrographolide glycoside syntheses. Herein we demonstrate that the β-galactosidase from bovine liver can be efficiently used for the glycosylation of andrographolide using *o*-nitrophenyl-β-D-galactoside (*o*NPG) as donor in hydrophilic organic solvents containing systems (Scheme 1). The activities of andrographolide glycoside derivatives as new antibacterial agents are also explored.

2. Materials and methods

2.1. Chemical and biological materials

The β-galactosidase from bovine liver (≥0.15 U mg^{-1} protein) was obtained from Sigm–Aldrich. Andrographolide (purity >99%) was purchased from Nanjing TCM Institute of Materia Medica, Nanjing, China. *o*-Nitrophenyl-β-D-galactoside (*o*NPG) was from Guangzhou Genebase Bioscience Co., Ltd., China. Dimethyl sulfoxide (DMSO), tetrahydrofuran (THF), pyridine and acetone (all HPLC grade) were purchased from Sinopharm (Shanghai, China). All other chemicals were obtained from commercial sources and of the highest purity available.

Antimicrobial activities were assessed using five bacteria [*Vibrio parahemolyticus* ATCC 17802, *Listeria monocytogenes* ATCC 19115, *Salmonella enteritidis* CMCC 50041, *Staphyloccocus aureus* CICC 10307, *Escherichia coli* CICC 10372]. These test microbes were obtained from the American Type Culture Collection (ATCC), China Medical Culture Collection Center (CMCC, Beijing) and China General Microbiology Culture Collection Center (CGMCC, Beijing).

2.2. Enzyme activity assay

The enzyme powder was dissolved in phosphate buffer (100 mM, pH 7.0) at 4 °C to form the enzyme solution (10 mg ml^{-1}). Enzyme solution (20 μl) was added to 0.6 ml phosphate buffer (100 mM, pH 7.0) containing *o*NPG (50 mM). The reaction was conducted for 30 min at 45 °C, and then stopped by adding 5.38 ml 1 M Na_2CO_3. The released *o*-nitrophenol was assayed at 420 nm. One unit of glycosidase activity was defined as the amount of enzyme required to catalyze the release of 1 μmol *o*-nitrophenol per minute under the conditions given. The specific activity of the enzyme was 5 U ml^{-1}.

2.3. Solubility determination

Andrographolide or andrographolide glycoside was mixed with 0.5 ml of solvent in an Eppendorf tube at 45 °C. An ultrasonic cleaner (Type NP-B-400-15; Newpower Co., Ltd., Kunshan, China) was used to maximize the solubility of each compound. After 1 h sonication and centrifugation at 10,000 × *g* for 20 min to remove insoluble material, the sample was diluted and filtered through a 0.45 μm membrane, and used for HPLC analysis to determine the sample solution concentration.

Table 1
Effects of solvents on the galactosylation of andrographolide catalyzed by β-galactosidase.[a]

Reaction media	log *P* [13]	Dielectric constant [14]	Solubility (mg ml^{-1})[b]	Reaction time (h)	Glycosylation (%)[c]
PBS (pH 6.5)	–[d]	80.2 (20 °C)	$6.1 \pm 0.2 \times 10^{-2}$	60	<1
DMSO	−1.30	47.2 (20 °C)	25.5 ± 1.5	60	10.5 ± 0.4
Acetone	−0.23	21.0 (20 °C)	5.9 ± 0.4	60	7.4 ± 0.6
Ethanol	−0.24	24.5 (25 °C)	20.1 ± 0.8	60	<5
Acetonitrile	−0.34	37.5 (21 °C)	15.2 ± 0.6	60	<5
THF	0.49	7.5 (22 °C)	18.5 ± 0.9	60	5.0 ± 0.7
Pyridine	0.71	13.3 (20 °C)	10.8 ± 0.8	60	<5
Butanol	0.88	7.8 (19 °C)	2.1 ± 0.3	60	<5
10% (v/v) DMSO–PBS	–	–	–	48	40.0 ± 2.3
10% (v/v) acetone–PBS	–	–	–	48	18.4 ± 1.1
10% (v/v) ethanol–PBS	–	–	–	48	13.5 ± 0.9
10% (v/v) acetonitrile–PBS	–	–	–	48	14.6 ± 1.6
10% (v/v) THF–PBS	–	–	–	48	13.2 ± 0.8
10% (v/v) pyridine–PBS	–	–	–	48	10.3 ± 0.5
10% (v/v) butanol–PBS	–	–	–	48	5.2 ± 0.8

[a] Reaction conditions: andrographolide 0.1 mmol, *o*-NPG 0.6 mmol, NaH_2PO_4–Na_2HPO_4 buffer (100 mmol l^{-1}, pH 6.5)–organic solvent (ϕ = 10%), 0.75 U β-galactosidase, 45 °C, 200 rpm, total volume 5 ml.
[b] The solubility of andrographolide in each solvent was determined by HPLC analysis of saturated solutions at 45 °C.
[c] Glycosylation efficient was calculated on the bases of remaining acceptor.
[d] No data or not determined.

2.4. General procedure for enzymatic glycosylation of andrographolide

In a typical experiment, β-galactosidase (final concentration 0.15 U ml^{-1}) was added to the reaction mixture (5 ml) containing 0.1 mmol andrographolide, 0.6 mmol *o*NPG and phosphate buffer (100 mM, pH 6.5) in 15% (v/v) DMSO. The reaction was conducted at 45 °C with shaking at 200 rpm. Aliquots were withdrawn at intervals, held at 100 °C for 5 min to denature the enzyme, and then diluted by 10 times with water/methanol (30/70, v/v) prior to HPLC analysis. A control reaction was performed in which no enzyme was added to the above procedure; no chemical glycosylation of andrographolide was detectable in the absence of enzyme. All experiments were performed in triplicate, and the results are reported as the mean ± standard deviation.

2.5. HPLC analysis

The reaction mixture was analyzed by RP-HPLC on a SinoChrom ODS-BP column (4.6 mm × 150 mm, 5 μm, Dalian Elite Analytical Instruments Co., Ltd., China) using a Hitachi L-7110 pump and a Hitachi L-7420 UV detector (Hitachi Co., Ltd., Tokyo, Japan) at 225 nm. The mobile phase was a mixture of water and methanol (30/70, v/v) at a flow rate of 1.0 ml min^{-1}. The retention times for 19-*O*-β-D-galactosyl-andrographolide, *o*NPG, andrographolide, and *o*-nitrophenol were 1.9, 2.7, 3.6 and 4.3 min (Fig. 1), respectively. All data are averages of experiments performed at least in duplicate, and no more than 2% deviation was observed.

Table 2
Effect of DMSO contents on enzymatic galactosylation of andrographolide.[a]

Medium	pH	Maximal yield (%)	19-Regioselectivity (%)[b]
PBS	6.50	<1	>99
1%(v/v)DMSO–PBS	6.52	5.0 ± 0.2	>99
5%(v/v)DMSO–PBS	6.60	22.3 ± 1.4	>99
10%(v/v)DMSO–PBS	6.75	40.5 ± 1.8	>99
15%(v/v)DMSO–PBS	6.85	51.5 ± 2.4	>99
20%(v/v)DMSO–PBS	6.92	42.8 ± 2.1	>99

[a] Reaction conditions: andrographolide 0.1 mmol, *o*-NPG 0. 6 m mol, NaH_2PO_4–Na_2HPO_4 buffer (100 mmol l^{-1}, pH 6.5)–organic solvent, 0.75 U β-galactosidase from bovine liver, 45 °C, 200 rpm, reaction time 48 h, total volume 5 ml.
[b] Regioselectivity was defined as the ratio of the HPLC peak area corresponding to the indicated product to that of all the products formed.

2.6. The stability of β-galactosidase in DMSO-containing systems

The β-galactosidase (final concentration 0.5 U ml^{-1}) was added to various systems in universal bottles and incubated at 45 °C with shaking at 200 rpm. Every 3 h, incubated β-galactosidase was sampled, and the remaining activity was measured as described above. The stabilities of β-galactosidase are expressed as the remaining activity compared with the initial activity in a buffer system (taken as 100%).

2.7. Structure determination of andrographolide glycoside

The product was separated and purified through flash column chromatography using ethyl acetate/methanol (6/3, v/v) as the eluent. The product structure was determined by ^{13}C NMR and ^{1}H NMR (Bruker AVANCE AV-500 NMR spectrometer, Germany). CD_3OD was used as a solvent.

19-*O*-β-D-Galactosyl-andrographolide ^{1}H NMR (δ): H1 (1.85, m, 1H; 1.29, m,1H), H2 (1.78, m, 2H), H3 (3.39, *t*, *J* = 8.1, 1H), H5 (1.31, m, 1H), H6 (1.87, m, 1H; 1.38, m, 1H), H7 (2.43, m, 1H; 2.03, m, 1H), H9 (1.93, m, 1H), H11 (2.60, m, 2H), H12 (6.85, td, *J* = 6.8, 1.7 Hz, 1H), H14 (5.01, d, *J* = 6.1 Hz, 1H), H15 (4.16, dd, *J* = 10.2, 2.2 Hz, 1H;

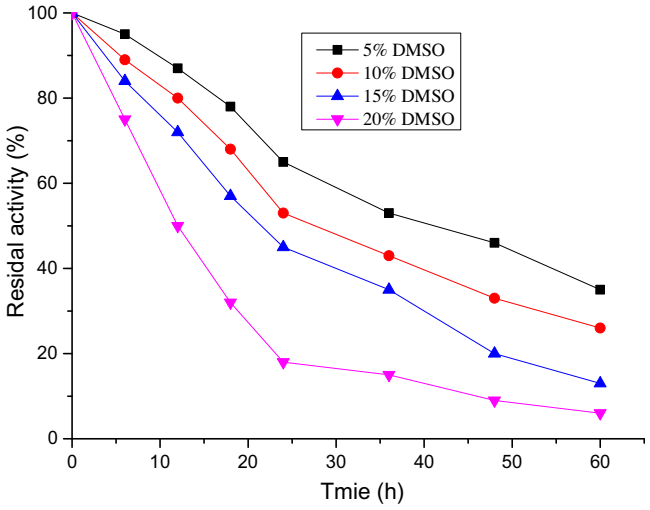

Fig. 2. The deactivation profile of β-galactosidase across a range of time in different media.

Table 3
Antibacterial activity of andrographolide and its derivatives.

Compound	Solubility[a] (mg ml^{-1})	MIC in broth (μg ml^{-1})[b]				
		S1	S2	S3	S4	S5
Andrographolide	6.0×10^{-2}	>128[c]	>128	>128	>128	>128
19-*O*-β-Galactosylandrographolide	42.1	8	32	16	8	16
Gentamicin sulfate[d]	nd[e]	1	0.5	2	nd	1
Benzylpenicillin sodium[d]	nd	4	2	nd	2	nd

[a] The solubility of andrographolide and 19-*O*-β-galactosyl andrographolide was determined by HPLC analysis of saturated solutions at 45 °C.
[b] The calculated average MIC values are presented. S1: *Escherichia coli* CICC 10372; S2: *Staphyloccous aureu*CICC 10307; S3: *Vibrio parahemolyticus*ATCC 17802; S4: *Listeria monocytogenes* ATCC 19115; S5: *Salmonella enteritidis* CMCC 50041.
[c] MIC > 128 μg ml^{-1} was considered to be inactive.
[d] Gentamicin sulfate and benzylpenicillin sodium were used as positive controls.
[e] nd: not determined.

4.46, dd, J = 10.2, 6.1 Hz, 1H), H17 (4.67, s, 1H; 4.88, s, 1H), H18 (1.22, s, 3H), H19 (4.15, d, J = 11.0 Hz, 1H; 4.91, d, J = 11.0 Hz, 1H), H20 (0.75, s, 3H); H2′ + H3′ + H4′ + H5′ + H6′ (3.62–3.93, m, 6H), H1′ (4.51, d, J = 7.5 Hz, 1H). ^{13}C NMR (δ): C1 (38.2), C2 (29.0), C3 (80.9), C4 (40.6), C5 (56.4), C6 (25.2), C7 (38.9), C8 (148.8), C9 (57.4), C10 (39.9), C11 (25.7), C12 (149.4), C13 (129.8), C14 (66.7), C (76.1), C16 (172.6), C17 (109.2), C18 (23.4), C19 (67.6), C20 (15.5); C6′ (60.7), C4′ (68.3), C2′ (70.3), C3′ (72.5), C5′ (74.9), C1′ (102.7).

2.8. Antibacterial activity test

The minimum inhibitory concentrations (MICs) of andrographolide and its derivatives were determined using a liquid dilution method performed in 96 well micro-trays [12].

3. Results and discussion

3.1. Effect of organic solvents on enzymatic galactosylation of andrographolide

The solubility of andrographolide in water was very low. Relatively high concentrations of andrographolide were achieved in hydrophilic organic solvents (Table 1). The andrographolide concentration in DMSO was over 418 times greater than that in PBS (pH 6.5). To demonstrate an application of glycosidase, we examined the performance of β-galactosidase in hydrophilic solvents such as DMSO, acetone, THF and pyridine, which have been employed to overcome the solubility of andrographolide. Initially, galactose and lactose were tested as galactosyl donors. Unfortunately, no galactosylated derivative was formed. So the active glycosyl donor *o*NPG was used in this work.

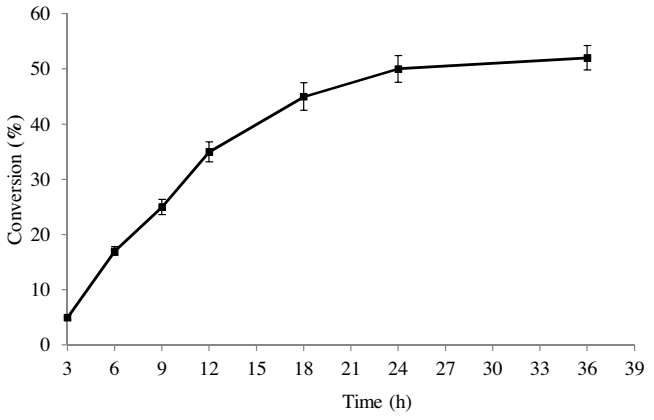

Fig. 3. Time course of β-galactosidase-catalyzed glycosylation of andrographolide. The reaction conditions were as follows: andrographolide 0.1 mmol, *o*-NPG 0.6 mmol, NaH_2PO_4–Na_2HPO_4 buffer (100 mmol l^{-1}, pH 6.5)–organic solvent (ϕ = 15%), 0.75 U β-galactosidase from bovine liver, 45 °C, 200 rpm, total volume 5 ml.

Enzyme-catalyzed reactions in non-aqueous media are affected by the nature of the solvent [10,11]. In general, polar solvents may be employed to overcome the solubility problem. However, these solvents usually strip the essential water off of the enzymes, thereby inactivating the biocatalyst [15]. In our system, the reaction media had an obvious effect on enzymatic glycosylation of andrographolide (Table 1). As shown in Table 1, β-galactosidase was unstable and inactivated substantially in organic solvents. Glycosylation efficiency was disappointingly low because of the β-galactosidase deactivation. The detrimental impacts of organic solvents on the enzyme may be relieved by decreasing their contents. When organic solvent except DMSO is used as cosolvent, enzymatic glycosylation efficiency in 10% (v/v) organic solvent–PBS (pH 6.5) systems was not significantly improved. But, very interestingly, 40% glycosylation rate occurred when DMSO was used as the cosolvent. The catalytic performance of β-galactosidase correlated well with log P and the dielectric constants of organic solvents in co-solvent systems, among which the enzyme displayed the highest activity in DMSO-containing system.

To better understand β-galactosidase-mediated glycosylation reactions conducted in the DMSO–PBS and to further optimize the reaction, the reactions were carried out in the DMSO co-solvent system as a function of the DMSO content and reaction time.

3.2. Effect of DMSO contents on enzymatic galactosylation of andrographolide

The solubility of the substrate was greatly improved in DMSO compared to buffer. DMSO was found to be freely miscible with buffer in all proportions tested. Besides, pH values of reaction media are not significantly affected by DMSO addition (1–25%, v/v) (Table 2). The effect of the concentration of DMSO on the galactosylation reaction was then examined to determine the optimal concentration in the co-solvent mixture.

A higher galactosylation of andrographolide was achieved when DMSO (15%, v/v) was added to phosphate buffer than in phosphate buffer alone (Table 2). This result occurred because the addition of DMSO led to an improvement in the solubility of the andrographolide, enhancing the mass transfer of the substrates and products to and from the active site of the enzyme, thus resulting in enhanced galactosylation activity. However, with too much DMSO present in the reaction system (>15%, v/v), the galactosylation activity of β-galactosidase dropped sharply, which may be attributed to more serious inactivation of the enzyme by DMSO (Fig. 2).

3.3. Stability of β-galactosidase in DMSO-containing systems

From both a practical and a theoretical viewpoint, it was important to understand the influence of the DMSO-containing system on the thermal stability of the enzyme. A comparative study was

thus performed on β-galactosidase stability by incubating it in the 5–20% (v/v) DMSO–PBS at 45 °C and a range of incubation time from 1 to 60 h, followed by measurement of its residual activity (Fig. 2).

After incubation in 5%, 10% and 15% (v/v) DMSO–PBS for 24 h at 45 °C, β-galactosidase retained approximately 65%, 53% and 45% of its original activity, respectively; however, when incubated in 20% (v/v) DMSO-PBS under the same condition, β-galactosidase retained only 15% of its original activity. The better stability of β-galactosidase in ≤15% (v/v) DMSO solvent system suggests that β-galactosidase can potentially be used as an industrial catacyst for enzymatic glycosylation in water-miscible organic solvents. The reusability of biocatalysts is one of the essential factors for cost reduction. For practical application, β-galactosidase should be immobilized (e.g. on a macroporous acrylic resin) and recycled. Upon completion of the reaction, the reaction mixture can be filtered or centrifugated to acquire the immobilized enzyme. Then the immobilized β-galactosidase is used in the next batch reaction composed of new substrates, highlighting the cost-effectiveness of the enzyme.

3.4. Time course of andrographolide glycosylation

To gain a deeper insight into the enzymatic process, the time courses of the synthesis of *O*-galactosylated andrographolide derivative was investigated. The time course of β-galactosidase-catalyzed glycosylation of andrographolide under the optimum conditions is depicted in Fig. 3. The reaction proceeded at a high rate during the first 12 h. A substantial deceleration of the reaction was observed when the reaction time extended beyond 18 h. Several issues might account for the decrease in enzyme activity over time, including increasing inactivation by the solvent and the *o*-nitrophenyl byproduct of *o*NPG hydrolysis or product. After 36 h, about 50% yield of the desired product was achieved.

Analysis of the product structures indicated that, within the range examined, reaction media and reaction time had little effect on regioselectivity; only the hydroxyl groups attached to the C-19 of andrographolide participated in glycoside bond formation (detailed NMR information shown in Section 2.7).

β-Galactosidase, also known as a lactase, is considered to be one of the most widely used industrial enzymes [16,17]. Recently, in view of the potential of β-galactosidase transgalactosylation, a new field of development for galactose containing compounds is being extensively studied due to their promising role in the food industry, cosmetics and medicine [16–19]. Despite the fact that β-galactosidase is abundant in nature, the most common enzyme sources are microorganisms, and the synthesis of galactosides almost entirely microbial enzymes have been used [16–18]. In present study, the β-galactosidase of animal origin, produced by bovine liver, was used to synthesize andrographolide glycoside. As mentioned above, β-galactosidase displayed a high efficiency, stability and regioselectivity. These results suggest that β-galactosidase can potentially be used as an industrial catalyst for enzymatic glycosylation.

3.5. Solubility and antibacterial activities of andrographolide glycoside

The solubility of andrographolide glycoside in water was dramatically higher than that of andrographolide (Table 3). The apparent solubility of andrographolide glycoside in water was 42.1 mg ml^{-1}, which is about 702 times that of andrographolide. This suggests that the attachment of a galactosyl residue to andrographolide greatly enhanced the aqueous solubility.

Food-borne illnesses usually result from food contaminated with pathogenic bacteria. Spread of food-borne pathogenic bacteria is now a critical problem in public health and there is an urgent need for new antibacterial agents [20]. Natural products are known to be a rich source of antimicrobial compounds with novel mechanisms and chemical structures that may have the potential to provide new and effective therapies against bacteria resistant to drugs currently in use [21]. Andrographolide and its glycoside derivative (19-*O*-β-galactosyl andrographolide) were evaluated for their antibacterial action against five representative species of food-borne pathogenic bacteria that are the most common in food or most dangerous to human health [20]. In vitro antibacterial activity was assessed by measuring minimum inhibitory concentration (MIC) values via the liquid dilution method performed in 96 well plates [12]. Andrographolide did not demonstrate measurable antibacterial activity, but the glycosylated andrographolide derivatives exhibited relatively broad antibacterial spectra against most of the strains of food-borne pathogenic bacteria that were tested (Table 3). The most powerful activities were against the *L. monocytogenes* (G^+) and *E. coli* (G^-) (MIC = 8 μg ml^{-1} in both cases), which are comparable to the activities of the established antibiotics gentamicin and benzylpenicillin against these strains (Table 3). These results highlight the critical role of the glycoside moiety on the lactone-ring in determining the antibacterial activity of andrographolide derivatives.

Further work is warranted to determine whether the 19-glycosylated derivatives of andrographolide reported here have a novel mechanism of antibacterial action and hence would be attractive lead compounds for development of a valuable class of new antibacterial agents. The results also indicate that enzymatic modification is a good method for the preparation of andrographolide derivatives with new properties for new applications.

4. Conclusions

The synthesis of andrographolide glucoside derivatives can be successfully conducted with excellent regioselectivity and moderate yield by means of β-galactosidase-catalyzed glycosylation in DMSO-containing systems. The solubilities and antibacterial activities of andrographolide glycoside were higher than those of andrographolide. Enzymatic modification of andrographolide offers simplicity, excellent selectivity and environmentally benign processes. If further scale-up is possible, the reaction may be attractive for industrial application.

Acknowledgements

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