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A facile method for the preparation of α-methylene-γ-butyrolactones from tulip tissues by enzyme-mediated conversion

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ABSTRACT

We developed a facile method of enzyme-mediated conversion of 6-tuliposide to α-methylene-γ-butyrolactone (tulipalin). We used a tuliposide-converting enzyme for the conversion of 6-tuliposides extracted from tulip tissues into the corresponding tulipalins in high yields within 2 h at pH 7.0. The resulting tulipalins were selectively extracted by using several organic solvents.

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All parts of the tulip (*Tulipa gesneriana* L.) plant contain large quantities (0.2–2% w/w fresh weight (F.W.)) of antimicrobial glucose esters, namely, tuliposides (**1**).[1–6] These esters are unstable and are chemically hydrolyzed under basic conditions to give lactones of their aglycones, tulipalin (**2**), which has antimicrobial activities. These compounds are also accumulated in the Lilliflorae plants, such as those belonging to the genera *Erythronium*, *Gabea*, and *Alstromeria*.[7] During the course of our studies on the biosynthesis and the mechanism of action of tuliposides, we discovered a tuliposide-converting enzyme that catalyzes the stoichiometric conversion of tuliposides into tulipalins in tulip tissues.[8] Tulipalins have a common structure—α-methylene-γ-butyrolactone—and are used as synthetic intermediates in the production of several bioactive compounds,[9] antimutagenic agents,[10] insect repellents,[11] electrolyte solution of Li-ion battery,[12] and as monomers of functional bioplastics.[13] Until now, α-methylene-γ-butyrolactones have been prepared according to organic synthesis methods[14] based on petroleum chemistry; however, these methods are not environment friendly. In Toyama Prefecture, Japan, millions of tulip bulbs are produced every year as one of major agricultural products. The petals are cut and used as biomass in order to produce bulbs of excellent qualities. For efficient utilization of this biomass, it is important to develop an environmentally safe approach involving the use of the tuliposide-converting enzyme to convert the tuliposides found in tulip tissues, especially petals, into tulipalins. In this study, we focused on the main tuliposides and tulipalins, that is, 6-tuliposide A (**1a**) and B (**1b**), and tulipalin A (**2a**) and B (**2b**), respectively. We optimized the conditions of the enzymatic transformation of **1a** and **1b** into **2a** and **2b**, respectively, and selectively extracted the products **2a** and **2b** (Scheme 1).

At first, we examined the effect of tuliposide-converting enzyme on the transformation of tuliposides extracted from tulip petals. Freeze-dried petals of the tulip cultivar 'Murasakizuisho' (1 g dry weight (D.W.)) were homogenized in a mortar with 50 mL of cold MeOH at 4 °C. The extract was filtered, concentrated in vacuo, and dissolved in 50 mL of cold water affording a tuliposide-containing extract of the tulip petals. The concentrations of **1a** and **1b** in the extract, as determined by HPLC analysis were 4.0 mM and 4.2 mM, respectively.[15] Subsequently, 5 μL of 1 M potassium phosphate buffer (KPB, pH 7.0) was added to 490 μL of the extract and the mixture was incubated at room temperature with or without the partially purified tuliposide-converting enzyme from the tulip bulb.[16] Even in the absence of the enzyme, 4% and 33% of **1a** and **1b** were chemically converted to **2a** and **2b**, respectively, in 2 h. Increasing the incubation time for more than 3 h resulted in further hydrolysis of **2b**. On the other hand, in the presence of 5U of the enzyme, both **1a** and **1b** were almost quantitatively converted to **2a** and **2b**, respectively, within 2 h. The above-mentioned results strongly suggest that the enzyme has a potential applicability in the production of tulipalins from tuliposides extracted from tulip tissues.

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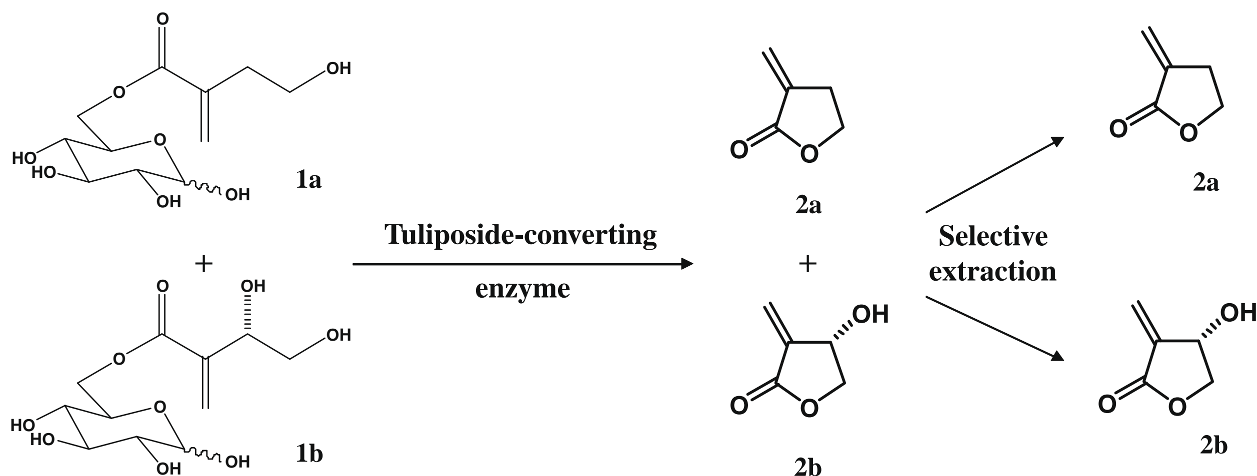

Scheme 1. Enzymatic preparation of α-methylene-γ-butyrolactone (tulipalin).

Since the petals of the tulip cultivar 'Murasakizuisho' contain both **1a** and **1b**, the reaction product obtained was a mixture of **2a** and **2b**. For the separation of **2a** and **2b**, we screened suitable solvents that enabled the isolation of these compounds by simple extraction and concentration, respectively. After the enzymatic conversion of tuliposides in the tulip petal extracts into tulipalins, **2a** was first extracted from the reaction mixture by using a nonpolar organic solvent (solvent-1) under saturating with NaCl.[17] The extraction efficiency of tulipalins is shown in Table 1. The yield of **2a** was higher when aromatic hydrocarbons (entries 4–6) were used than that obtained when an alkane (entry 1) was used as the solvent, and the amount of **2b** co-extracted using these solvents was less than 10%. Extraction with ether- (entry 2) and ester-type (entry 3) solvents also afforded high yields of **2a**; however, significant amount of **2b** was also co-extracted, suggesting that such solvents were not suitable for the selective extraction of **2a**. After extraction of **2a** with toluene, **2b** remaining in the reaction mixture was further extracted with a polar solvent (solvent-2), namely, propyl acetate, 3-methylbutan-1-ol, 2-propanol, 1-butanol (BuOH), and 1:1 mixture of BuOH and acetone and **2b** was obtained in the yields of 62%, 65%, 65%, 87%, and 98%, respectively. Pure **2a** and **2b** can be obtained from the extracts by simple distillation. On the basis of these results, toluene and 1:1 mixture of BuOH and acetone were selected as the most suitable solvents for the selective extraction of **2a** and **2b**, respectively, from the enzyme reaction mixture.

Next, we investigated the bioconversion ability of the tuliposide-converting enzyme extracted from several different tulip tissues. The specific activity of enzyme that was partially purified[18] from the crude extract of roots, stems, leaves, petals, anthers, and pistils of the tulip cultivar 'Murasakizuisho' using DEAE-Toyopearl column chromatography was 18.7, 11.1, 15.4, 68.0, 42.1, and 11.4 U/mg, respectively. These enzymes extracted from these plant parts were used for the enzymatic conversion of tuliposides in the tulip petal extract. The crude enzyme extracted from the roots, bulbs, stems, leafs, petals, anthers, and pistils converted 86%, 91%, 88%, 93%, 94%, 97%, and 93% of **1a** in the petal extract to **2a** and 84%, 82%, 55%, 64%, 83%, 71%, and 74% of **1b** of the same origin to **2b**, respectively. These results show that this enzymatic conversion can be performed using enzymes obtained from any of the abovementioned tulip tissues. We have previously found that the enzyme in each tulip tissue has different substrate specificities.[8] Thus, the differences in the conversion yields were probably due to the substrate specificity of the enzymes toward **1a** and **1b**. The products **2a** and **2b** were selectively extracted using toluene and 1:1 mixture of BuOH and acetone in 70–83% and 88–98% yields, respectively.

A MeOH extract of several tulip tissues was prepared and treated with the tulip bulb enzyme.[19] Table 2 shows the concentrations, conversion yields, and extraction efficiencies of **2a** and **2b** formed from the extracts of various parts of the plant. Although the ratio of **1a**/**1b** was different for each tissue, the enzyme enabled efficient conversion of the reactants into **2a** and **2b** in 2 h. This sug-

Table 1
Extraction of tulipalins (**2a**, **2b**) that were obtained by enzymatic transformation from tuliposides (**1a**, **1b**) from a tulip petal extract using solvent-1

Entry	Solvent-1	Extraction efficiency[a] (%) of	
		2a	**2b**
1	Hexane	8	<0.01
2	Diethylether	70	26
3	Propyl acetate	86	73
4	Xylene	79	5
5	Toluene	84	3
6	Benzene	88	10

[a] Calculated based on the concentrations of **2a** and **2b** in the reaction mixture after enzymatic conversion.

Table 2
The enzymatic preparation of tulipalins (**2a**, **2b**) from tuliposides (**1a**, **1b**) from different tulip tissues

Entry	Tissue	2a			2b		
		Conc.[a] (mM)	Yield[b] (%)	Ext. effi.[c] (%)	Conc.[a] (mM)	Yield[b] (%)	Ext. effi.[c] (%)
1	Root	1.38	98	82	2.43	>99	91
2	Bulb	3.64	93	90	<0.01	N.D.	N.D.
3	Stem	2.73	91	80	0.16	67	89
4	Leaf	3.99	90	85	2.29	>99	71
5	Petal	2.26	96	82	3.47	88	97
6	Anther	0.40	93	73	5.37	80	>99
7	Pistil	10.4	85	84	4.33	>99	78

Tulip petal extract (490 μL) was added to a solution of the enzyme (5 U) extract from the tulip bulb and 1 M KPB (5 μL). After 2 h of incubation at room temperature, NaCl was added to the reaction mixture until saturation, **2a** was extracted with toluene (200 μL × 2, 100 μL × 1), and the **2b** remaining in the mixture was extracted with the same volumes of 1:1 mixture of BuOH and acetone. N.D.: not determined.

[a] Concentration in the reaction mixture.

[b] Calculated on the basis of original concentrations of **1a** and **1b** before enzymatic treatment.

[c] Calculated on the basis of the concentrations of **2a** and **2b** in the reaction mixture after enzymatic treatment.

gests that this system can be applicable in the case of all the tulip tissues. Further, the products **2a** and **2b** were selectively extracted with toluene and 1:1 mixture of BuOH and acetone, respectively, and were recovered in sufficiently high amounts. Studies are under way to develop methods for the production of tulipalins by the enzymatic conversion of tuliposide-related compounds extracted from other plant tissues.

In conclusion, we developed a novel approach for the production of α-methylene-γ-butyrolactones (tulipalins) from tuliposides, which are compounds found in tulip tissues, by enzyme-mediated conversion for successive extraction. The reaction proceeds at room temperature in an aqueous solution. Since tuliposide-related compounds are found in several easily available plants, the application of enzymatic conversion is an attractive option for the production of tulipalins. In order to use this system for manufacturing α-methylene-γ-butyrolactones from plant biomass, further studies are now under way. Further, plant tissues are also being screened for the identification of tissues that specifically accumulate **1a** or **1b** that enable us to obtain pure **2a** and **2b** without any selective extraction.

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15. The reaction product was analyzed by high-performance liquid chromatography (HPLC; Shimadzu, Japan) at 208 nm, with an ODS-100 V column (4.6 × 150 mm) at a flow rate of 0.65 mL/min using an elution solvent consisting of 10 mM of H_3PO_4 in 10% MeOH.
16. Two grams (D.W.) of freeze-dried bulbs of the tulip cultivar 'Murasakizuisho' were pounded in a mortar into which 20 mL of 10 mM potassium phosphate buffer (KPB, pH 7.0) was added and the solution was centrifuged (10,000*g*, 15 min, 4 °C) to obtain a cell-free extract. The extract was loaded onto a DEAE-Toyopearl chromatography column (Tosoh, Japan), which had been equilibrated with a 10 mM KPB solution, fractionated by elution with 100 mM KPB, and the active fractions were collected. The obtained crude enzyme solutions having specific activity of 100 U/mg were used for enzymatic conversions. One unit (U) of the enzyme activity was defined as described previously.[8]
17. After enzymatic conversion, NaCl was added to the mixture until saturation, **2a** was extracted with solvent-1 (200 μL × 2, 100 μL × 1), and the **2b** remaining in the mixture was extracted with solvent-2 (200 μL × 2, 100 μL × 1). Both the extracts were combined and analyzed by gas chromatography (DB-5 column; Agilent technologies).
18. Samples of freeze-dried roots (1.3 g D.W.), stems (2.2), leaves (2.2), petals (1.2), anthers (2.0), and pistils (1.2) of the tulip cultivar 'Murasakizuisho' were disrupted in a mortar; subsequently, 150 mL of 10 mM KPB was added and the mixture was stirred vigorously at 4 °C for 1 h. After filtration and centrifugation (21,500*g*, 15 min, 4 °C), the obtained supernatant was mixed with 4 g of DEAE-Toyopearl equilibrated with 10 mM KPB and stirred for 1.5 h at the same temperature. The gels were placed in a small column and washed with 20 mL of 10 mM KPB. The fractions exhibiting **1a**-converting activity were eluted using 100 mM KPB or 100 mM KPB + 100 mM NaCl, combined, concentrated by Centriprep 100 (Millipore, USA), and used for enzymatic conversion. Five microlitre of 1 M KPB and 490 μL of the tulip petal extract were mixed with the crude enzyme extracted from the roots (0.86 U), bulbs (5), stems (0.62), leaves (3.2), petals (3.0), anthers (3.6), and pistils (0.84) and incubated for 2 h. After adding NaCl to the reaction mixture until saturation, **2a** and **2b** were extracted with toluene and 1:1 mixture of BuOH and acetone, as described.[16]
19. The tissues (50–200 mg F.W.) were disrupted using a multi-beads shocker (Yasui-Kikai) in 1 mL of MeOH and the solution was centrifuged (10,000*g*, 4 °C). The supernatant was evaporated and the residue was dissolved in 1 mL of cold water. Five microlitre of 1 M KPB and 490 μL of the tissue extract were mixed with the enzyme extracted from the bulbs (5 U) and incubated for 2 h. The reaction product was extracted with toluene and 1:1 mixture of BuOH and acetone and was analyzed as described.[16]