In Vitro Biosynthesis of Strictosidine Using *Lonicera japonica* Leaf Extracts and Recombinant Yeast

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Strictosidine is a key intermediate in the biosynthesis of the terpenoid indole alkaloid (TIA) pathway. It results from a condensation reaction, catalyzed by strictosidine synthase (STR), between tryptamine and secologanin. We have now developed a useful method, based on enzyme-assisted synthesis, to produce strictosidine. Our procedure utilizes leaf extracts from Japanese honeysuckle *Lonicera japonica* Thunb. as a secologanin source. In these experiments, an enzyme extract was prepared from transgenic yeast *Saccharomyces cerevisiae* that expresses the *Catharanthus roseus STR* (CrSTR) coding region. Strictosidine was then isolated with a 38% yield based on the initial amount of tryptamine in the enzymatic reaction.

Keywords: Catharanthus roseus, honeysuckle *Lonicera japonica*, secologanin, strictosidine, strictosidine synthase

Terpenoid indole alkaloids (TIAs) are a large class of natural products that include the anti-cancer agents vinblastine, vincristine and camptothecin; the anti-hypertensive drug ajmalicine; and the sedative serpentine. Strictosidine, the precursor for the majority of TIAs, is formed by the condensation of tryptamine with monoterpene secologanin, as catalyzed by strictosidine synthase (STR) (Treimer and Zenk, 1979; Kutchan, 1993) (Fig. 1). Several STR clones have been isolated from members of the Apocyanaceae family -- *Catharanthus roseus* (L.) G. Don (McKnight et al., 1990; Pasquali et al., 1992) and *Rauvolfia serpentina* (L.) (Kutchan et al., 1988); and from the Rubiaceae family, e.g., *Ophiorrhiza pumila* (L.) (Yamazaki et al., 2003). C. *roseus STR*, encoded by a single-copy gene containing a signal peptide of 31 amino acids, localizes in the vacuole as multiple isoforms (Pasquali et al., 1992; de Waal et al., 1995). *STR* genes have been expressed in *E. coli* (Kutchan, 1989; Roessner et al., 1992), insect cells (Kutchan et al., 1994), and yeast (Geerlings et al., 2001). This demonstrates the practicality of producing strictosidine in heterologous systems. However, it is necessary to provide tryptamine and secologanin precursors to those systems for the production of strictosidine.

Secologanin, a secoiridoid glucoside, is derived mostly from the triose phosphate/pyruvate pathway (Contin et al., 1998), and is converted from loganin by secologanin synthase (SLS) (Irmler et al., 2000). This compound is abundant in several plant families -- Apocyanaceae, Caprifoliaceae, and Verbenaceae (Hallard et al., 1998; Rastrelli et al., 1998). *Lonicera japonica* Thunb., a woody ornamental plant belonging to the Caprifoliaceae, is native to East Asian countries, such as Korea and Japan, and contains a large number of iridoid glucosides, including loganin and secologanin (Tomassini et al., 1995; Kakuda et al., 2000; Machida et al., 2002). Its flower buds or aerial parts are utilized as herbal medicine because of their detoxicant, anti-inflammatory, and antipyretic properties (Lee et al., 2001; Kwak et al., 2003; Tae et al., 2003; Kang et al., 2004; Kim et al., 2005).

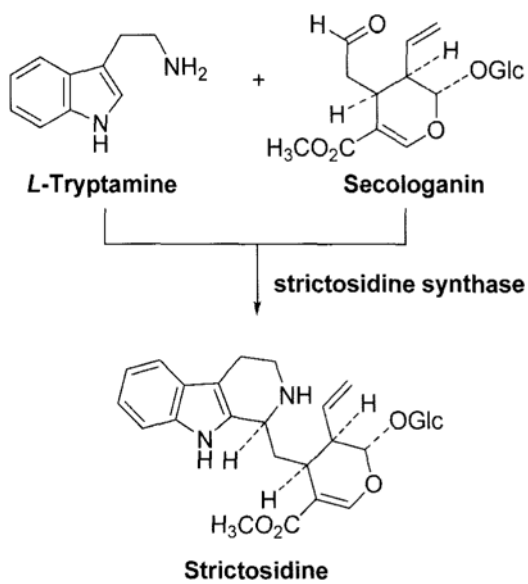

Figure 1. Biosynthesis of strictosidine from secologanin and tryptamine.

Because of significant pharmacological interest in TIAs, strictosidine is of great value as a starting material for further studies of TIA biosynthesis. However, it is not feasible to obtain this compound from plant tissues, and no commercial sources are currently available. Thus, we here present a rapid and inexpensive cell-free system for producing strictosidine with a crude enzyme extract prepared from yeast cells expressing the C. *roseus* STR gene. In addition, we demonstrate that *L. japonica* Thunb. can serve as an alternative source of secologanin in this cell-free system because commercial supplies of that glucoside are expensive and of limited availability in the market.

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MATERIALS AND METHODS

Plant Material and Preparation of Leaf Extract

Mature leaves were collected from natural populations of *L. japonica* Thunb. in Daejeon, Korea, from June to September of 2006. They were ground with liquid nitrogen into a fine powder using a mortar and pestle, and secologanin was extracted as described by Hallard et al. (1998), with some modifications. The dried tissue (1 g) was suspended in 20 mL water, and incubated for 2 min in boiling water. These samples were then homogenized at 10-s intervals for 5 min. Their homogenate was centrifuged at 5000*g* for 20 min at room temperature, and the pellets were extracted again as described above. The combined supernatant was then concentrated in a rotary evaporator and stored at -20°C.

Vector Construction

C. roseus STR cDNA was amplified from leaf RNAs via reverse-transcriptase polymerase chain reactions (RT-PCRs). Total RNA from plants of soil-grown *C. roseus* cv. Pacifica Deep Orchid was extracted with TRIZOL reagent (Life Technologies, UK), as described by the manufacturer. Here, 2 mg of RNA was reverse-transcribed at 42°C using MMLV reverse transcriptase (FINNZYMES, Finland) and an oligo(dT) primer. The resulting cDNA (1 mL) served as template for PCR-amplifications with the following gene-specific primers: 5'-GGTACCGGATCCATGGCAAACTTTTCTGGATCT-3' (*Bam*HI site underlined) and 5'-GCATGCCTCGAGCTAGCTAGAAA-CATAAGAATTTCC-3' (*Xho*I site underlined). Amplification was performed using *Pfu* DNA polymerase (Stratagene, USA) under the following conditions: 5 min denaturation at 95°C; then 25 cycles of denaturation at 95°C for 30 s, annealing at 53°C for 30 s, and extension at 72°C for 30 s. The resulting PCR product was cloned into pCR®Blunt (Invitrogen, USA) to create pCRCrSTR before being sequenced. The *CrSTR* gene was excised by *Bam*HI/*Xho*I digestion and sub-cloned into the *Bam*HI/*Xho*I site of a shuttle vector pYES2 (Invitrogen), thereby generating the construct pYES2CrSTR.

Expression of Recombinant *CrSTR* and Preparation of Crude Enzyme Extracts

Saccharomyces cerevisiae strain Y2805 was transformed with pYES2CrSTR according to a lithium chloride method, and the transformed cells were selected on a synthetic complete medium without uracil (SC-Ura). The transformed yeast cells were inoculated in 15 mL SC-Ura containing 2% glucose, and incubated overnight at 30°C. These overnight cells were then inoculated in 250 mL of SC-Ura supplemented with 2% galactose, and again incubated at 30°C for 30 h. The cells were harvested by centrifugation at 1500*g* for 5 min, and stored at -80°C. Frozen cells were re-suspended in a buffer containing 50 mM sodium phosphate (pH 7.4), 1 mM EDTA, 5% glycerol, and 1 mM PMSF, and were lysed using acid-washed glass beads. The lysate was centrifuged, and the resulting supernatants were used as enzyme sources for strictosidine synthesis. Total protein was measured according to the assay of Bradford (1976).

Synthesis, Purification, and Identification of Strictosidine

Enzymatic reactions were carried out in a total volume of 500 μL of 100 mM potassium phosphate buffer (pH 6.5) with a mixture of 1 mM tryptamine (Sigma, USA), 2 mM secologanin (Fluka, Switzerland), and 100 μL cell lysate (0.2 mg of total protein). When using the *Lonicera* leaf extract, our enzymatic reaction was started by adding 600 μL cell lysate (1.2 mg of total protein) to a mixture of 10 mg extract and 1 mM tryptamine in 100 mM potassium phosphate buffer (pH 6.5) to achieve a total volume of 3 mL. The reaction mixture was incubated at 30°C for 3 h and then lyophilized. This lyophilized sample was extracted with 3 mL methanol three times, and the combined methanol was evaporated under reduced pressure. The residue was analyzed with a Silica $60F_{254}$ TLC plate (Merck, Germany) using acetone/methanol/diethylamine (7:2:1) as the eluting solvent. HPLC was performed on an HP1050 model (Hewlett-Packard, Germany) that was equipped with a UV detector, and fragments were separated on either a reverse-phase analytical column (Gromsil 120 ODS-3 CP, 10 μm particle size, 250 x 4.6 mm) or a preparation column (Gromsil 120 ODS-4HE, 10 μm, 250 x 10 mm), with a step gradient of solvent A (water:TFA:acetonitrile = 95:0.01:5, v/v/v) to solvent B (water:TFA:acetonitrile = 5:0.01:95, v/v/v). Gradient conditions included Time 0 to 5 min, 90% A; Time 15 to 20 min, 70% A; Time 25 to 35 min, 5% A; and Time 37 min, 90% A, with a flow rate of 1.0 mL min^{-1}. Injection volumes were 20 μL for the analytical column and 400 μL for the preparation column. Peaks were monitored by absorption at 210 nm. LC-MS was performed on an HP1050 model that was coupled to a Finnigan LCQ mass spectrometer (Finnigan, USA). This was equipped with an electrospray ionization (ESI) source operated in the positive ion mode to generate the $[M + H]^+$ ion of strictosidine.

RESULTS AND DISCUSSION

Heterologous Expression of CrSTR in Yeast Cells

In both prokaryotes and eukaryotes, signal sequences guide proteins for the secretary pathway and select different translocation systems for actual transport across the membrane (Martoglio and Dobberstein, 1998; Martoglio, 2003). CrSTR contains an amino-terminal signal peptide of 31 amino acids, and it correctly targets to the vacuoles when expressed in transgenic tobacco plants (McKnight et al., 1991). To prepare strictosidine synthase, we expressed the *CrSTR* gene in a yeast, *S. cerevisiae*, under the control of the *GAL1* promoter. In transformed yeast strain Y2805, STR activity was found exclusively inside the cells, probably within the vacuole. This finding is distinct from that of Geerlings et al. (2001), who reported that CrSTR activity was observed predominantly in the culture medium. Such a contrast was probably due to incorrect functioning of a plant vacuolar-targeting sequence within the heterologous recipient yeast. The discrepancy may have resulted from differences in components between expression systems, including the type of promoter, culture conditions, and yeast strains employed.

Strictosidine Synthesis Using Enzyme-Coupled Reactions

Strictosidine was synthesized through a single-step enzymatic reaction, using commercially available substrates and the crude yeast cell lysate that harbored the STR protein. The formation of strictosidine in our reaction mixture was initially verified by TLC analysis. R_f values for those two substrates, tryptamine and secologanin, were 0.76 and 0.40,

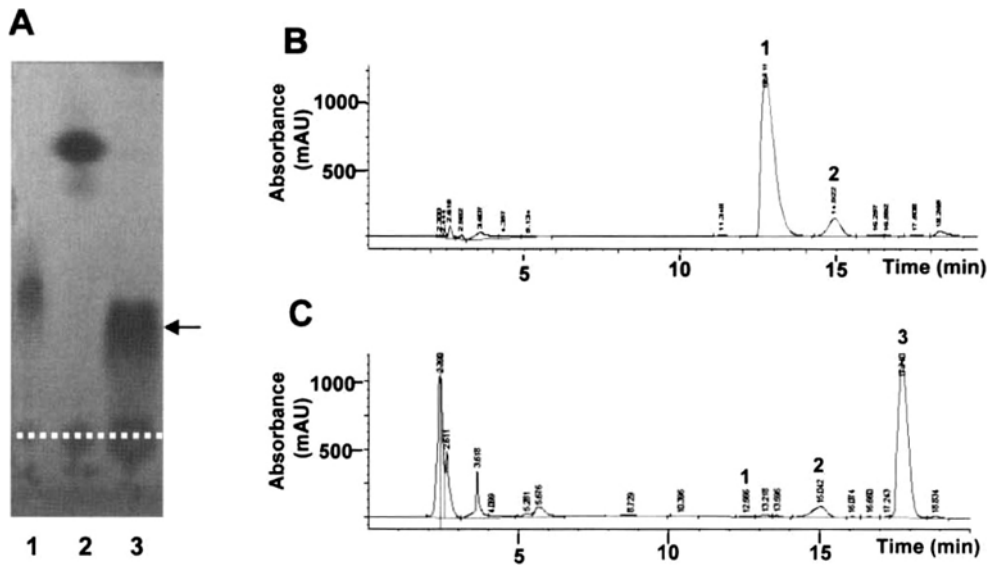

Figure 2. Detection of strictosidine from enzymatic reaction using commercially authentic secologanin and tryptamine. (**A**) Strictosidine formation was determined by TLC. Lane 1, secologanin; Lane 2, tryptamine; Lane 3, crude product of enzymatic reaction. (**B**) HPLC chromatogram of crude product from negative control reaction [using cell lysate of Y2805(pYES2)]. (**C**) HPLC chromatogram of crude product from enzymatic reaction [using cell lysate of Y2805 (pYES2CrSTR)]. Peak 1, tryptamine; Peak 2, secologanin; Peak 3, strictosidine.

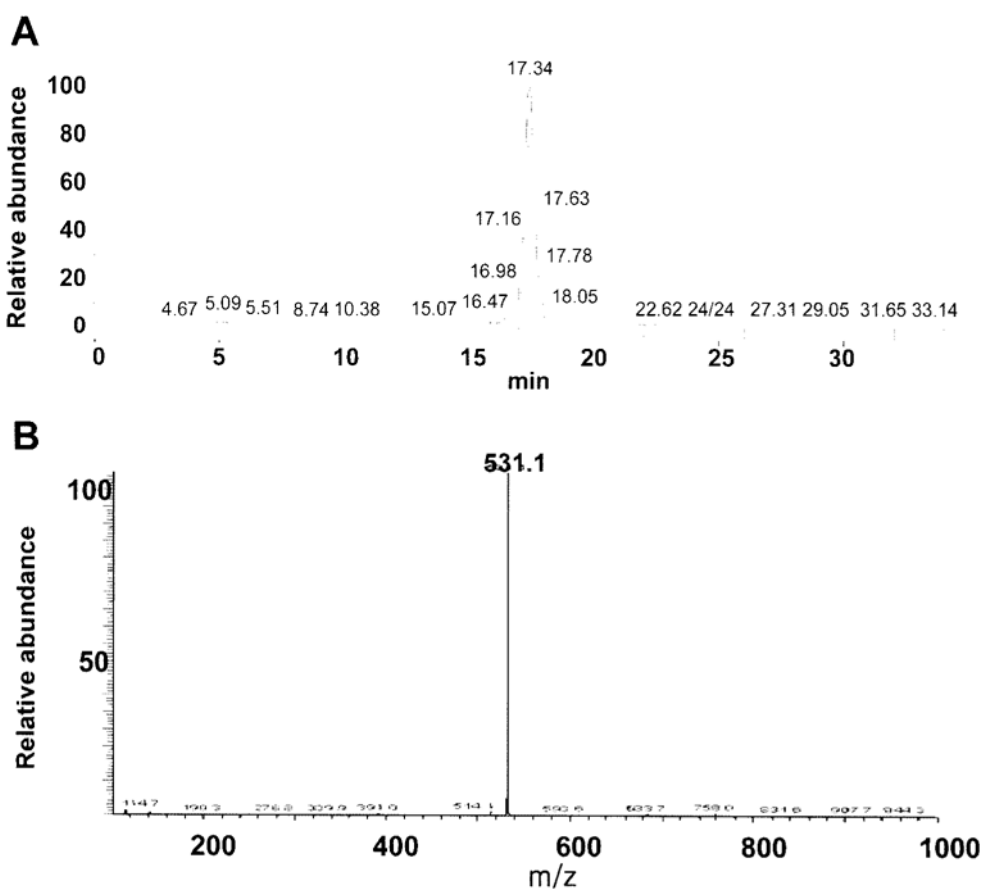

Figure 3. Identification of purified strictosidine by LC-MS analysis. (**A**) Positive-ion electrospray mass spectra obtained in full-scan mode from HPLC-purified strictosidine. (**B**) MS spectrum of peak at 18.00 min.

respectively (Fig. 2A). One major compound newly appeared at R_f = 0.33 after 3 h of the enzymatic reaction; its existence was confirmed by HPLC. A control reaction carried out with cell lysate bearing the pYES2 vector showed only two major peaks, which corresponded to the starting substrates -- tryptamine (Peak 1) and secologanin (Peak 2) (Fig. 2B). Incubation of the cell lysate containing the recombinant pYES2CrSTR vector with substrates yielded a new compound (Peak 3) with a retention time of 17.34 min, together with reduced amounts of tryptamine and secologanin (Fig. 2C). The portion having retention times of 17.2~18.5 min was then collected in order to isolate this new compound from our preparative HPLC. HPLC-electrospray ionization mass spectrometry (HPLC-MS/ESI), in a positive ion mode, was conducted to identify that third compound, which had a molecular weight $[M+H]^+$ of m/z = 531.1 that matched the molecular weight of protonated strictosidine ($C_{27}H_{34}N_2O_9$, MW = 530.57) (Fig. 3). We called this new compound strictosidine.

Japanese Honeysuckle Leaf Extract as a Secologanin Source

Due to limited market sources, we instead searched for a substitute for commercially available secologanin, investigating Japanese honeysuckle (*L. japonica* Thunb.) as a possibility. Mature leaves were subjected to hot-water extraction, and the presence of secologanin in that extract was first verified by HPLC-MS/ESI (Fig. 4). We identified a positive ion $[M+H]^+$ of secologanin ($C_{17}H_{24}O_{10}$, MW = 388.37) at m/z 388.9. No peak corresponded to strictosidine, indicating that the extract could be used as an alternative source of secologanin for strictosidine production without interference from any intrinsic strictosidine. A major peak appeared at

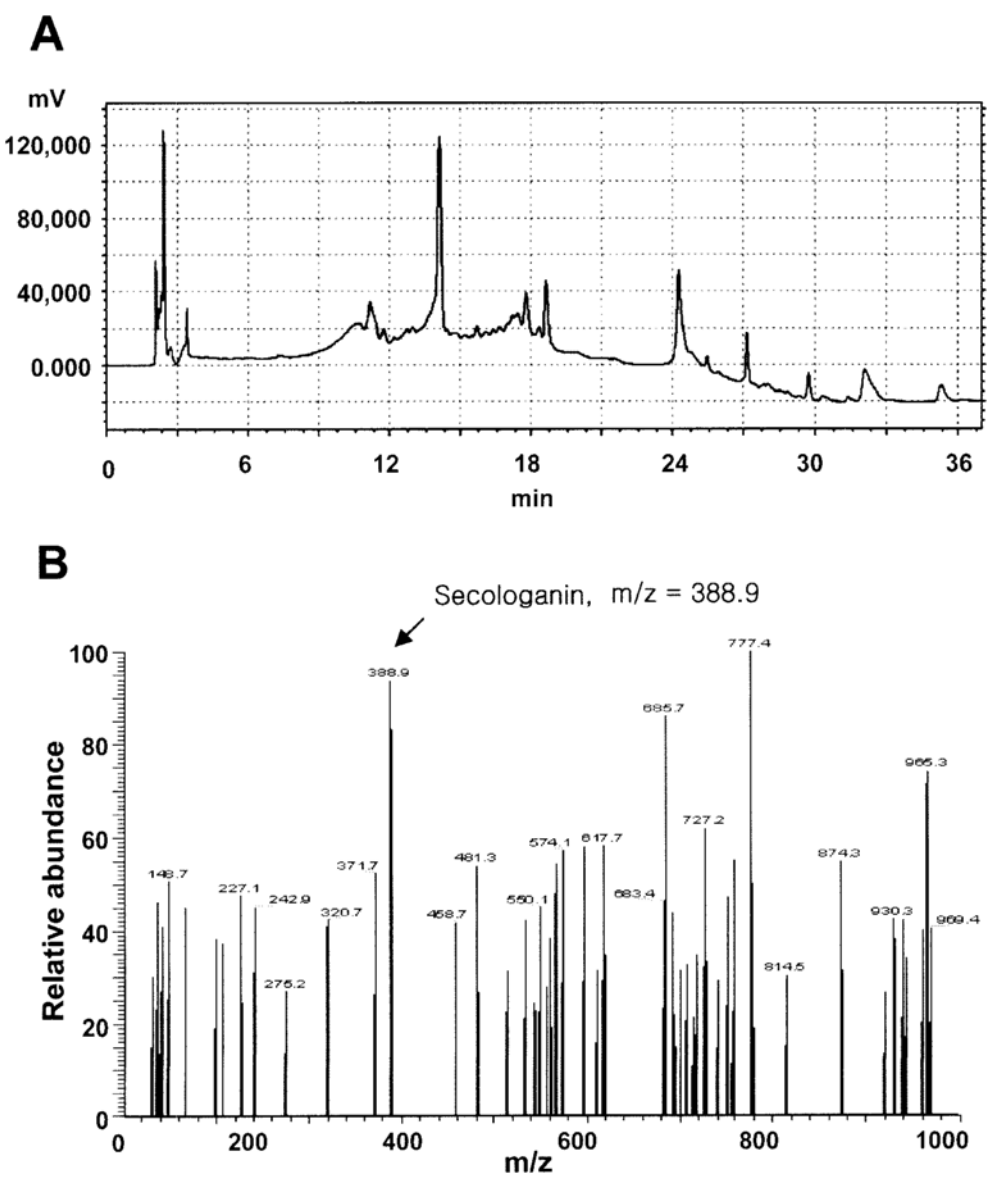

Figure 4. Metabolite profiles of *L. japonica* leaf extract. (**A**) HPLC chromatogram of methanol extract from mature leaves, detected at 210 nm. (**B**) MS spectrum of methanol extract from mature leaves. Protonated secologanin molecule (m/z = 388.9) was detected at 15.00 min. No peaks corresponding to tryptamine and strictosidine were identified.

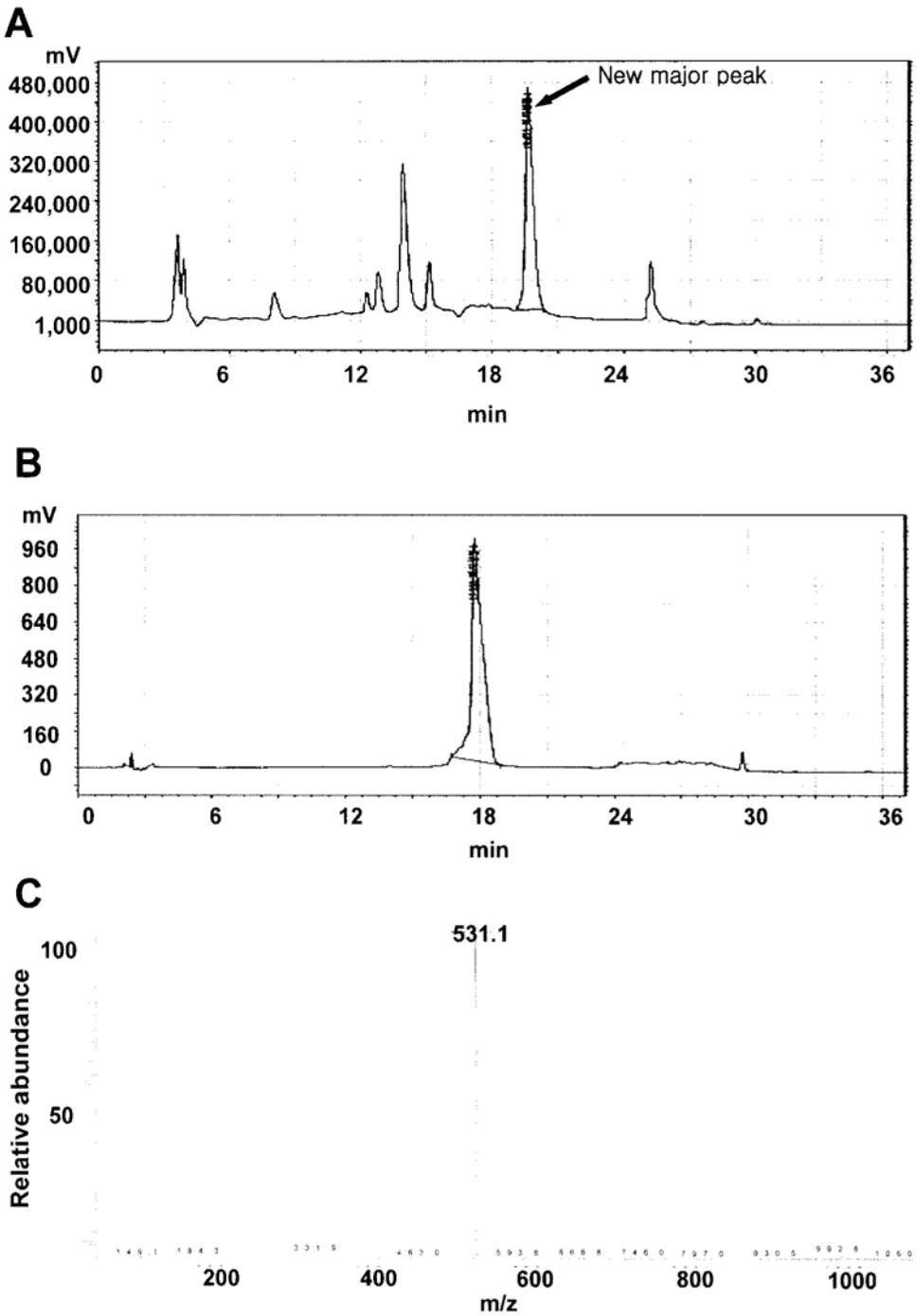

Figure 5. Enzymatic strictosidine synthesis using *L. japonica* leaf extract as secologanin source. (**A**) HPLC chromatogram of crude product condensed after enzymatic reaction. (**B**) HPLC chromatogram of purified strictosidine. (**C**) MS spectrum of peak at 18.00 min.

the retention time of 18.00 min from the enzymatic reaction that used a mixture of 10 mg leaf extract and 1 mM tryptamine (Fig. 5A). This peak was purified (Fig. 5B) and confirmed to be strictosidine by HPLC-MS/ESI (Fig. 5C). The HPLC purification provided 0.6 mg of strictosidine, meaning that this isolation yield was 38%, based on the amount of tryptamine supplied. Even though strictosidine is one of the most important intermediates for TIA biosynthesis, there had not been an easy way to synthesize it or any commercial sources for its supply prior to our research. Therefore, our results conclusively demonstrate that single-step biotransformation in a cell-free system is an efficient means for producing large quantities of this compound. Likewise, we have shown that crude leaf extracts from Japanese honeysuckle, *L. japonica* Thunb., are an economically suitable substitute for more expensive secologanin. These results open up the possibility for further studies on TIA biosynthesis.

ACKNOWLEDGEMENTS

This work was supported by a grant to JRL from the Korea

Science and Engineering Foundation through the Plant Metabolism Research Center of Kyung Hee University funded by the Korean Ministry of Science and Technology, and by a grant to JHP from the Korea Science and Engineering Foundation through the Advanced Materials Research Center for Better Environment at Hanbat National University, Korea.

Received February 1, 2007; accepted April 4, 2007.

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