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Note

Enzymatic liberation of lycotetraose from the *Solanum* glycoalkaloid α-tomatine

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Abstract—The branched tetrasaccharide, *O*-β-D-glucopyranosyl-(1 → 2)-*O*-[β-D-xylopyranosyl-(1 → 3)]-*O*-β-D-glucopyranosyl-(1 → 4)-D-galactose (lycotetraose) is a key constituent of many biologically interesting natural products. Described herein is a convenient enzymatic preparation of lycotetraose from the readily available *Solanum* glycoalkaloid α-tomatine. The preparation makes use of the recombinant *endo*-glycosidase, tomatinase, from the plant pathogen *Fusarium oxysporum* f. sp. *lycopersici*.

Keywords: Lycotetraose; Tomatinase; *endo*-Glycosidase; Saponin; Enzymatic cleavage

1. Introduction

Saponins are a structurally diverse class of steroidal glycosides found in a wide variety of plants. The general saponin structure consists of either a steroidal or triterpenoid aglycone linked to one or more mono- or oligosaccharide glycone motifs. The branched tetrasaccharide, *O*-β-D-glucopyranosyl-(1 → 2)-*O*-[β-D-xylopyranosyl-(1 → 3)]-*O*-β-D-glucopyranosyl-(1 → 4)-D-galactose, known as lycotetraose, is a recurrent glycone motif found in a variety of saponins structures from a range of plant species. Many of these lycotetraose-based saponins display interesting biological properties including antifungal[1–4] and anticancer activity[3,5–9] as well as inhibition of cAMP phosphodiesterase,[10,11] Na,K-ATPase,[12] tumor promoter-induced lipid metabolism,[13] and human spermatozoan motility.[14] The ability to prepare artificial saponins containing the lycotetraose motif is therefore desirable. Lycotetraose was originally prepared in a 0.9% isolated yield by the partial hydrolysis of α-tomatine under acidic conditions.[17] Chemical syntheses of lycotetraose[15] (11-steps in 10% overall yield) and the lycotetraose-derived saponin desgalactotigonin[16] have previously been reported, but the laborious nature of these preparations makes them impractical for general use. The lycotetraose-based saponin α-tomatine plays an important role in the tomato plant's defence against fungal pathogens, but several pathogenic fungi produce specific glycosidases (tomatinases) that inactivate it by cleavage of one or more sugars from the lycotetraose side-chain.[18] The tomatinase from *F. oxysporum* f. sp. *lycopersici* is a Family 10 *endo*-glycosidase that selectively cleaves the β-linkage between the galactose and tomatidine moiety of α-tomatine, to liberate the fully intact tetrasaccharide from the sterol moiety (tomatidine).[19] The lengthy chemical synthesis of lycotetraose[15] prompted us to investigate its enzymatic preparation from commercially available α-tomatine, using the tomatinase from *F. oxysporum* f. sp. *lycopersici* (Scheme 1). In a similar manner Nohara and co-workers have recently utilized glycyrrhizin hydrolase to access the natural oligosaccharides fabiotriose and mimosatriose during their chemoenzymatic syntheses of novel artificial saponins.[20]

2. Results and discussion

2.1. Over-expression of tomatinase

Recombinant tomatinase (14.5 mg) was prepared from a 500 mL culture of *E. coli* strain BL21(DE3) transformed

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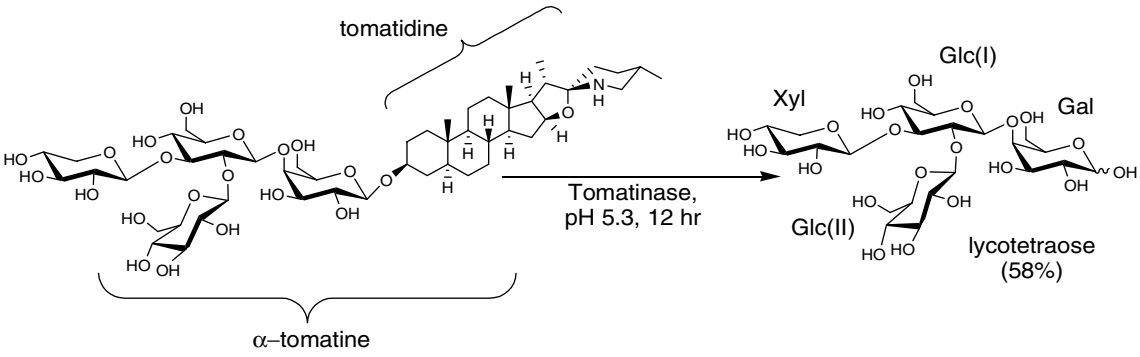

Scheme 1.

with the expression vector (*pFoTom1*) encoding the deca-histidine-tagged protein.[19] As previously described, sufficient tomatinase was expressed as a result of leaky protein expression; induction of protein expression by addition of IPTG has previously been shown to deposit most of the tomatinase as insoluble inclusion bodies.[19] The soluble recombinant protein was easily isolated from the crude cell lysates by nickel affinity chromatography. Imidazole was removed from the eluted protein fractions by extensive dialysis against de-ionized water; dialysis against the standard assay buffer, 20 mM NaOAc pH 5, caused the protein to precipitate. The enzyme was stored as 25% glycerol stocks in un-buffered de-ionized water at −18 °C. In our hands, storage of the enzyme in the absence of buffer salts had no adverse effect on enzyme activity for at least three months.

2.2. Optimized reaction conditions

It was critical to be able to dissolve α-tomatine in the reaction buffer at concentrations that would be appropriate for preparative scale biotransformations. In aqueous solution, α-tomatine is soluble to a maximum concentration of 10 mM up to pH 4.5 and is much less soluble at higher pH, whereas the optimum pH range for tomatinase activity is pH 5.5–7.0.[21] The pH conditions were manipulated to improve substrate dissolution whilst retaining enzyme activity. The best results were achieved when a solution of α-tomatine (10 mM) in 20 mM sodium acetate buffer at pH 4.5 was diluted five-fold into 20 mM NaOAc at pH 5.5 to give a final 2 mM reaction mixture of the enzyme and substrate (corresponding to 2 mg/mL of α-tomatine or 1.3 mg/mL of the tetrasaccharide thereof) at pH 5.3. This initial substrate concentration is almost twice the reported K_m value of 1.1 mM for α-tomatine with wild type tomatinase.[21]

2.3. TLC analysis

During the reaction, enzymatic cleavage of the saponin was monitored by TLC and reaction products were identified by comparison with α-tomatine and the free sterol. When the TLC-separated reaction mixture was visualized by charring with an acidic cerium-(IV)-sulfate/molybdic acid stain, α-tomatine (R_f 0.18) and the liberated sterol (R_f 0.84) were clearly visible, whereas lycotetraose was not readily detected. When the TLC plates were stained and charred using 5% (v/v) of sulfuric acid in ethanol, the tetrasaccharide (R_f 0.24) showed up much more intensely than α-tomatine and the sterol unit did not show up at all. Therefore, biotransformation reactions were routinely monitored by running parallel TLC plates, which were then visualized under these two sets of conditions noted.

2.4. Enzymatic synthesis of lycotetraose

A preparative scale enzymatic synthesis of lycotetraose from 100 mg of α-tomatine was then carried out. By the time the reaction had run to completion (as judged by TLC) most of the sterol had precipitated from the reaction mixture. Isolation of lycotetraose from the crude reaction mixture relied on manipulation of the different physical properties of the reaction components. As the sterol was only partially soluble in the reaction most of it was easily removed by filtration. Unlike lycotetraose, both α-tomatine and tomatidine are soluble in ether. Therefore, an ether extraction was used to remove these components from the aqueous mixture. The aqueous layer was then passed through a mixed-bed ion exchange resin to remove buffer salts and residual protein was finally removed by ultrafiltration to give essentially pure lycotetraose in 58% yield.

When purified in this manner, lycotetraose was isolated as an anomeric mixture (α/β = 37:63). With the aid of COSY and ^{1}H–^{13}C HSQC data, it was possible to assign all of the anomeric centers and C-4 of galactose (Table 1). Unlike the other sugar residues, there was no doubling of the xylose resonances due to the mixed anomeric center at the reducing end hence it was possible to assign all of the xylose ^{1}H and ^{13}C NMR signals. With the obvious exception of the hemi-acetal center, all the other assigned carbon NMR signals for lycotetraose

Table 1. ^{1}H and ^{13}C NMR assignment of the anomeric mixture of lycotetraose

Position		^{1}H (ppm)[a]	J (Hz)[a]	^{13}C (ppm)[a]	α-Tomatine ^{13}C (ppm)[22]
Gal	1	α (5.09, d)	α (3.9)	α 92.7	102.3
		β (4.43, d)	β (7.7)	β 96.9	
	4	α (4.02, d)	α (3.2)	α 81.5	79.8
		β (3.96, d)	β (3.0)	β 80.4	
Glc (I)	1	α (4.519, d)	α (7.9)	α 103.4	104.7
		β (4.523, d)	β (7.7)	β 103.2	
Glc(II)	1	α (4.87, d)	α (7.9)	α 102.4	104.9
		β (4.86, d)	β (8.1)	β 102.4	
Xyl	1	(4.57, d)	(7.7)	103.2	104.8
	2	(3.20, dd)	(9.1, 7.7)	73.8	74.9
	3	(3.32)[b]	Complex	76.3	78.5
	4	(3.49)[b]	Complex	74.0	70.6
	5a	(3.84, dd)	(11.6, 5.4)		
	5b	(3.14, dd)	(11.6, 11.1)	65.7	67.2

[a] α/β Denomination refers to the anomeric configuration of the reducing end sugar.
[b] Multiplicities are unclear due to overlapping with other signals.

are in keeping with those previously assigned to the same tetrasaccharide side-chain incorporated within the parent glycoside, α-tomatine.[22]

3. Conclusions

A straightforward, one-step preparation of lycotetraose from the commercially available α-tomatine has been established using recombinant His_{10}-tagged α-tomatinase and simple purification methods. In future, this should facilitate the preparation and studies of novel lycotetraose-based saponins by the chemical coupling of lycotetraose with other aglycone motifs using standard procedures.[20]

4. Experimental

4.1. General methods

E. coli strain BL21(DE3) containing the recombinant tomatinase plasmid *pFoTom1* was a generous gift from Dr. M. Ruiz-Rubio, Universidad de Cordoba.[19] α-Tomatine and tomatidine were purchased from Sigma. TLC was performed on 250 μm pre-coated glass-backed silica plates (Whatman K6F). Disposable PD-10 desalting columns and FPLC Ni-NTA superflow resin were purchased from Amersham Biosciences and Qiagen, respectively. Ultrafiltrations were carried out using 2 mL Vivaspin concentrators with 10,000 MWCO CTA membranes (VivaScience AG, Germany). ^{1}H NMR spectra were recorded on a Varian Unity Inova spectrometer at 600 MHz and ^{13}C NMR spectra were recorded on a Varian Unity Plus at 100 MHz. ^{1}H and ^{13}C NMR spectra were referenced to MeOH (3.4 and 49.3 ppm) as an internal standard.

4.2. Overexpression and purification of tomatinase

This was carried following a published procedure[19] with some modifications (as described earlier). In this manner 14.5 mg of tomatinase was prepared from a 0.5 L cell culture.

4.3. TLC analysis

Biotransformations were monitored by TLC, eluted in a solvent system consisting of acetic acid, ethyl acetate, MeOH, and water (10:30:20:1).[19,21] The plates were then dipped in either an aqueous solution of cerium-(IV)-sulfate (1%, w/v), molybdic acid (1.5%, w/v), and sulfuric acid (10%, v/v) or sulfuric acid (5%, v/v) in ethanol. Compounds were then visualized by charring.

4.4. *O*-β-D-Glucopyranosyl-(1 → 2)-*O*-[β-D-xylopyranosyl-(1 → 3)]-*O*-β-D-glucopyranosyl-(1 → 4)-D-galactose (lycotetraose)

α-Tomatine (96 mg, 0.093 mmol) was dissolved in 20 mL of 20 mM sodium acetate buffer at pH 4.5 by sonication at room temperature for 10 min and was then diluted by the addition of 80 mL of 20 mM sodium acetate buffer at pH 5.5. A 25% glycerol stock of tomatinase (4.5 mg) was first eluted through a PD-10 desalting column (with buffer at pH 5.5) before addition to the reaction mixture. This was left to stir overnight at 30 °C and a white precipitate had formed. The reaction mixture was filtered through a plug of cotton wool and the filtrate concentrated to 20 mL on a rotary evaporator at 40 °C. This was then washed with ether (4 × 20 mL) and the aqueous layer was then passed through a column of mixed-bed TMD-8 ion exchange resin (20 mL) eluting

with de-ionized water. The protein was then removed from the eluent by centrifugation (4000*g*, 4 °C) in a 2 mL Vivaspin concentrator (note: the Vivaspin cartridge was washed through with three 1 mL aliquots of de-ionized water to remove residual NaN_3 and glycerol in the CTA membrane prior to use). The filtrate was freeze-dried to give an anomeric mixture (α/β = 37:63) of lycotetraose as a white powder (34 mg, 58%); $[\alpha]_D^{28} + 3.0$ (*c* 0.29, water), lit.[17] $[\alpha]_D^{22} + 2$ (*c* 0.5, water), lit.[15] $[\alpha]_D^{26} + 2$ (*c* 1.2, water); δ_C (100 MHz, D_2O) 103.36 (αC-1, Glc(I)), 103.20 (βC-1, Glc(I)), 103.18 (C-1, Xyl), 102.42 (βC-1, Glc(II)), 102.38 (αC-1, Glc(II)), 96.86 (βC-1, Gal), 92.66 (αC-1-Gal), 84.83, 84.76, 81.49 (αC-4, Gal), 80.44 (βC-4, Gal), 79.15, 79.13, 77.11, 76.31 (C-3, Xyl), 76.11, 75.85, 74.74, 74.20, 74.16, 74.00 (C-4, Xyl), 73.80 (C-2, Xyl), 73.09, 72.52, 70.63, 70.30, 70.12, 69.71, 68.41, 65.70 (C-5, Xyl), 62.93, 61.71 (CH_2), 61.53 (CH_2), 61.46 (CH_2), 61.30 (CH_2), 60.99 (CH_2); [found: (ESI), 654.2453 (ammonium adduct), $C_{23}H_{44}NO_{20}$ requires 654.2451]; *m/z* 659 (100%, $M + Na^+$).

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