SUPPLEMENTARY MATERIAL

Preparation of highly purified timosaponin AIII from rhizoma anemarrhenae through an enzymatic method combined with preparative liquid chromatography

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Abstract:

Timosaponin AIII (TAIII) exhibits extensive pharmacological activities and has been reported as a potent antitumour agent for various human cancers. In the present study, a potential industrial process for producing TAIII that involves biotransformation directly in the crude extract liquid of rhizoma anemarrhenae (RA) was developed. \textbeta-D-glycosidase was used to transform Timosaponin BII (TBII) into TAIII, and monofactor experiments were conducted to optimise the enzymolysis conditions. In addition, AB-8 macroporous resin column chromatography, preparative liquid chromatography, and crystallisation technique were applied for yielding TAIII crystals with a purity >97%. Approximately 7 g of TAIII with a high purity of >97% was obtained from 1 kg of RA through this five-step preparation method, which can be used to produce TAIII on a large scale.

Keywords: \textbeta-D-glycosidase, enzymolysis, preparative liquid chromatography, Timosaponin AIII
Experimental

Reagents and materials

Rhizoma anemarrhenae slices were purchased from Shanghai Kangqiao Medicinal Materials Electuary Co., Ltd. (Shanghai, China, 20120827), and identified by Experiment Center for Teaching and Learning, Shanghai University of Traditional Chinese Medicine, and the voucher specimen number was KQ0227. TAIII and TBII were purchased from Yuanye Biology technology Co., Ltd. (Shanghai, China). β-D-glycosidase enzyme was purchased from Baofeng Bio-Chemical Co., Ltd. (Shanghai, China). AB-8 macroporous resin was purchased from Qingyang Biology Co., Ltd. (Shanghai, China). Methanol used for HPLC was from Sinopharm Chemical Reagent Co., Ltd. (Merk, Shanghai, China), and ultra-pure water was prepared using a Milli-Q Ultrapure water purification system (Millipore, Bedford, MA, USA). All other chemicals were of analytical grade.

HPLC-ELSD conditions

TAIII was analysed through HPLC with an AlltimaC18 column (250 mm×4.6 mm, 5 μm) by using an Alltech 3300 ELSD. The mobile phase was an isocratic mode of elution with methanol-water (85:15, v/v) at a constant flow rate of 1.0 mL/min and the injection volume was 20 μL. The ELSD system was set to a probe temperature of 60 °C and a gain of 1.0, and the nebulizer N₂ gas was adjusted to 1.5 L/min.

Preparation of RA crude extract

RA slices (1 kg) were subjected to a chemical extraction process through a reflux method using 70% ethanol at 70 °C twice. After filtration, the extracting liquors were combined and evaporated to obtain the crude extract solution with the ratio of raw material to extract volume being 1.6 g/mL, while the content of TBII was 0.05 g/mL. TAIII was not detected in the crude extract of RA (Fig. S2B). The sample solution was stored at 4 °C prior to use.

Enzymolysis of TBII present in the crude extract solution

Approximately 20 g of the crude extract (containing 1 g of TBII and no TAIII) was immersed in 400 mL of an acetic acid–sodium acetate buffer solution and then hydrolysis was conducted using β-D-glycosidase at pH 4.0 for 3 h while the vapour-bathing constant temperature vibrator
was maintained at 50 °C. The enzymatic hydrolysate was extracted three times by using 400 mL of water-saturated butanol and subsequently purified twice using an ammonia solution. The extracts were evaporated in vacuo until dry at 60 °C.

**Purification through AB-8 macroporous resin column chromatography**

Purification was performed in a glass column (50 × 2.5 cm id) packed with 100 g (dry mass) of AB-8 resins. Because TAIII is insoluble, samples of the enzymolysis products (containing 0.9 g of TAIII) were first dissolved in 95% ethanol (100 mL), mixed with 50 g (dry mass) of AB-8 resins, and condensed. The adsorbents for TAIII were then loaded on the top of the well-packed column and the bed volume (BV) of the total wet resin was approximately 200 mL. The adsorbents were eluted with water and 20%, 50%, and 80% ethanol (each at 10 BV) in the given order at a flow rate of 2 mL/min. The effluents of 80% ethanol were collected and dried to yield TAIII.

**Separation through preparative liquid chromatography**

Preparative liquid chromatography separation was performed on the Reveieris® X2 Flash Chromatography system (Grace, USA) using a C18 column (310 × 160 mm, 40 µm, 45 g) through a simple dry-pack method. The C18 column was washed with 100% methanol extensively before use and then pre-equilibrated with 75% methanol for separation. The purified fraction (0.5 g) obtained through the purification process with the AB-8 macroporous resin was dissolved in methanol, mixed with 1.5 g of silica gel (300–400 mesh; Qingdao Haiyang Chemical Corporation Co., Ltd., Qingdao, China) and then evaporated until dry. The dried sample was placed in a solid sample loader and gradient flushed using a water–methanol (A-B, v/v) solution in series as follows: 0–15 min, 70%–77% B; 15–20 min, 77%–80% B; 20–35 min, 80% B; 35–75 min, 85% B. The flow rate was set at 20 mL/min. All chromatographic experiments were conducted at an ambient temperature. The fractions corresponding to TAIII were collected and subjected to HPLC for determining its purity.

**Crystallisation technique**

Approximately 100 mg of the purified fraction from preparative liquid chromatography was dissolved in a minimised quantity (approximately 12 mL) of 95% ethanol by using a water bath at 80 °C. Subsequently, ether was added constantly until the solution was turbid. The target compound crystallised after the solution was maintained at an ambient temperature for 2 days.
White amorphous powder was obtained after filtering the solution followed by drying. Sample fractions were subjected to analytical HPLC-ELSD for determining the TAIII purity, and the chemical structure of TAIII was identified by analysing IR, ESI-MS (Agilent 6460A mass detector), $^1$H-NMR, and $^{13}$C-NMR (400 NMR spectrometer, Bruker Avance, Switzerland) spectra.

**Figures and table captions:**

Fig. S1. The results of monofactor experiments. (A) Effect of pH on enzymolysis; (B) Effect of temperature on enzymolysis; (C) Effect of reaction time on enzymolysis; (D) Effect of β-D-glycosidase amount on enzymolysis.

Fig. S2. HPLC-ELSD chromatograms of TAIII products in different process. (A) TAIII standard solution; (B) The crude extract; (C) TAIII in enzymolysis products; (D) TAIII products after purification through macroporous resin; (E) TAIII products after purification through preparative liquid chromatography; (F) TAIII crystals.

Fig. S3. $^1$H-NMR of TAIII

Fig. S4. $^{13}$C-NMR of TAIII

Table S1. $^{13}$C-NMR data of TAIII’s key carbons

Fig. S1

![Graph](image1)

Fig. S2

![Graph](image2)
Table S1. $^{13}$C-NMR data of TAIII’s key carbons

| carbon | $\delta_C$, type | carbon | $\delta_C$, type |
|--------|------------------|--------|------------------|
| 1      | 30.59,CH$_2$     | 21     | 14.65,CH$_3$     |
| 2      | 26.63,CH$_2$     | 22     | 110.37,C         |
| 3      | 74.81,CH         | 23     | 25.77,CH$_2$     |
| 4      | 30.06,CH$_2$     | 24     | 26.09,CH$_2$     |
| 5      | 36.10,CH         | 25     | 27.15,CH         |
| 6      | 26.44,CH$_2$     | 26     | 65.34,CH$_2$     |
| 7      | 26.51,CH$_2$     | 27     | 16.06,CH$_3$     |
| 8      | 35.28,CH         | 1'     | 100.89,CH        |
| 9      | 40.04,CH         | 2'     | 81.52,CH         |
|   |     |   |     |
|---|-----|---|-----|
| 10| 35.00, C | 3' | 76.27, CH |
| 11| 20.95, CH₂ | 4' | 69.65, CH |
| 12| 40.13, CH₂ | 5' | 75.67, CH |
| 13| 40.73, C   | 6' | 61.59, CH₂ |
| 14| 56.27, CH  | 1'' | 103.91, CH |
| 15| 31.71, CH₂ | 2'' | 75.42, CH |
| 16| 78.41, CH  | 3'' | 77.92, CH |
| 17| 62.54, CH  | 4'' | 71.46, CH |
| 18| 16.46, CH₃ | 5'' | 77.3, CH  |
| 19| 23.86, CH₃ | 6'' | 62.32, CH₂ |
| 20| 42.37, CH  |   |       |