Fitoterapia 114 (2016) 51–55

Contents lists available at ScienceDirect

Fitoterapia

journal homepage: www.elsevier.com/locate/fitote

Immunochromatographic strip assay for detection of bioactive *Ganoderma* triterpenoid, ganoderic acid A in *Ganoderma lingzhi*

Seiichi Sakamoto [a], Nao Kikkawa [a], Toshitaka Kohno [a], Kuniyoshi Shimizu [a,b], Hiroyuki Tanaka [a,*], Satoshi Morimoto [a]

[a] Department of Pharmacognosy, Graduate School of Pharmaceutical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan
[b] Department of Agro-Environmental Sciences, Graduate School of Agriculture, Kyushu University, 6-10-1 Hakozaki, Higashi-ku, Fukuoka 812-8581, Japan

ARTICLE INFO

Article history:
Received 17 July 2016
Received in revised form 23 August 2016
Accepted 25 August 2016
Available online 26 August 2016

Keywords:
Colloidal gold
Ganoderic acid A
Ganoderma lingzhi
Immunochromatographic assay
Monoclonal antibody

ABSTRACT

Ganoderic acid A (GAA) is one of the major *Ganoderma* triterpenes produced by medicinal mushroom belonging to the genus *Ganoderma* (Ganodermataceae). Due to its interesting pharmacological activities, *Ganoderma* species have been traditionally used in China for the treatment of various diseases. Herein, we developed a colloidal gold-based immunochromatographic strip assay (ICA) for the rapid detection of GAA using highly specific monoclonal antibody against GAA (MAb 12A) conjugated with gold nanoparticles. Using the developed ICA, the detection of GAA can be completed within 15 min after dipping the test strip into an analyte solution with the limit of detection (LOD) for GAA of ~500 ng/mL. In addition, this system makes it possible to perform a semi-quantitative analysis of GAA in *Ganoderma lingzhi*, where high reliability was evaluated by enzyme-linked immunosorbent assay (ELISA). The newly developed ICA can potentially be applied to the standardization of *Ganoderma* using GAA as an index because GAA is major triterpenoid present much in the mushroom.

1. Introduction

Ganoderma lingzhi (Ganodermataceae), a medicinal mushroom that is known as Reishi in Japan and Lingzhi in China, has been traditionally used in China for thousands of years. *Ganoderma lingzhi* (*G. lingzhi*) is a wood-degrading mushroom, in which the hard fruiting body comprises a pileus, spore, and stipe [1]. To date, a wide range of diseases have been targeted using *Ganoderma* extracts, including hepatitis B [2,3], cancer-related fatigue and immune disorders [4,5], and neurasthenia [6]. However, it is primarily used to target cancer [7] as *Ganoderma* extracts have been known to exhibit anticancer activity against a variety of cancer cells, e.g., MCF-7 and MDA-MB-231 breast cancer cells [8,9], 95-D lung cancer cells [10], PC-3 prostate cancer cells [9], and HUC-PC and MTC-11 bladder cancer cells [11]. The major active compounds that exert these activities are the *Ganoderma* triterpenoids; to date, >100 ganoderic acids have been isolated from the *Ganoderma* species [1]. Among them, ganoderic acid A (GAA, Fig. 1) is the most abundant triterpenoid present in the *Ganoderma* species [12]. Moreover, GAA has been found to suppress the growth and invasive behavior of the human breast cancer cell line MDA-MB-231. The *anti*-proliferate effects of GAA are thought to be mediated via the down-regulation of expression of cyclin-dependent kinase 4, which is related to the regulation of the G_1/G_0 transition in the cell cycle, whereas the *anti*-invasive effects of GAA are thought to be mediated via the inhibition of AP-1/NF-κB-dependent secretion of urokinase plasminogen activator (uPA), which controls cell adhesion and migration [13]. Recently, a potential therapeutic strategy using GAA in combination with chemotherapeutic agents for cancer treatment has been proposed, because GAA was found to enhance the chemosensitivity of HepG2 human liver cancer cells to cisplatin by the inhibition of signal transducers and activators of transcription 3 (STAT3) phosphorylation through the suppression of JAK1 and JAK2 [14]. As such, GAA has attracted much attention as the subject of pharmacokinetic and oral bioavailability studies [15,16]. Furthermore, the quality control of *Ganoderma* extracts is of great interest because the quality of the plant directly reflects the effects of *Ganoderma* itself; therefore, accurate, sensitive, and rapid detection methods for GAA quantification are required. Recently, we have produced a highly specific monoclonal antibody (MAb) to GAA (MAb 12A) and applied it to the development of an indirect competitive enzyme-linked immunosorbent assay (icELISA) for the detection of GAA in *G. lingzhi*, in which limit of detection (LOD) for GAA in icELISA was ~6.1 ng/mL [17]. The icELISA enabled the accurate and sensitive detection of GAA in *Ganoderma* extracts; however, the method required five steps and 5 h to complete one assay. The need to undertake field work

* Corresponding author at: Department of Pharmacognosy, Graduate School of Pharmaceutical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan.
E-mail address: htanaka@phar.kyushu-u.ac.jp (H. Tanaka).

http://dx.doi.org/10.1016/j.fitote.2016.08.016
0367-326X/

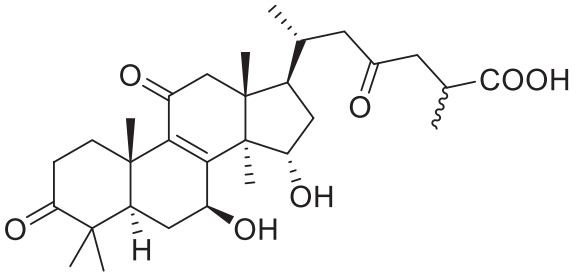

Fig. 1. Structure of ganoderic acid A (GAA).

outside of the laboratory means that these long and complicated steps require modification.

Immunochromatographic strip assay (ICA) is a simple and rapid analytical method that can be completed within 15 min after being immersed in an analytes. In this study, therefore, we have developed an ICA using gold nanoparticles conjugated with MAb 12A, which exhibited a LOD of ~500 ng/mL. Considering the practical uses of the developed system, ICA has many advantages over icELISA despite the fact that LOD for GAA in ICA is greater than that in icELISA. The application of MAb 12A in a simple and rapid ICA is described in this study.

2. Materials and methods

2.1. Chemicals and reagents

GAA (≥99%) was obtained from ChromaDex (Irvine, CA, USA). A gold colloidal solution with a mean diameter of 15 nm was purchased from the Tanaka Kikinzoku Kogyo (Kanagawa, Japan). Mouse serum albumin (MSA; ≥96%) and bovine serum albumin (BSA; ≥ 98%) were obtained from Sigma-Aldrich (St. Louis, MO, USA) and Nacalai Tesque (Kyoto, Japan), respectively. An *anti*-mouse IgG rabbit antibody was obtained from MP Biomedicals (Solon, OH, USA). All other chemicals were standard commercial products of analytical reagent grade.

2.2. Sample preparation of G. lingzhi

Sample preparations of *G. lingzhi* were performed by following our previous report [17]. Briefly, three parts of *G. lingzhi* (the pileus, stipe, and spore) were individually ground, passed through a 0.56 mm mesh, and measured 50 mg. *Ganoderma* extracts containing ganoderic acids were prepared by sonication of the solids in 1.0 mL of various concentrations of aqueous ethanol (100%, 75%, 50%, 25%, and 0%) for 30 min. The supernatants obtained after centrifugation at 12,000 rpm for 10 min were collected in small test tubes. The extraction process was repeated five times and the resultant extracts were combined and dried at 60 °C. Each residue was then dissolved in 1.0 mL of methanol and appropriately diluted as a sample for the ICA test.

To prepare a GAA standard solution, GAA was dissolved in methanol to yield a final concentration of 1 mg/mL and diluted 20-fold with distilled water to prepare a mother GAA solution (50 μg/mL) in 5% (v/v) methanol.

2.3. Preparation of colloidal gold-conjugated MAb 12A

The colloidal gold-conjugated MAb 12A was prepared by following our previous report [18] with slight modifications. Potassium carbonate (2% (w/v), 20 μL) was added to a gold colloidal solution with a mean diameter of 15 nm (1 mL; Tanaka Kikinzoku Kogyo, Kanagawa, Japan) to obtain a solution with pH 9.0. The MAb 12A was dissolved in phosphate-buffered saline containing 0.05% (v/v) Tween 20 (PBS-T) to yield a final concentration of 2 mg/mL; 10 μL of this solution, which is equivalent to 20 μg of MAb 12A, was added to the colloidal gold solution and left for 10 min. Subsequently, a 10% (w/v) BSA solution (111 μL) prepared in a 100 mM Tris buffer (pH 8.0) was added to the resultant colloidal gold solution to stabilize the conjugates, and the mixture was incubated for 1 h. The pellets obtained after centrifugation at 7000 rpm for 30 min at 4 °C were then resuspended in a 1% (w/v) BSA solution in a 100 mM Tris buffer (pH 8.0, 1 mL) and subjected to a second centrifugation at 7000 rpm for 30 min at 4 °C. The resultant pellets were concentrated in a 1% BSA (w/v) solution (30 μL). Following this, 10% (w/v) sucrose in water (6 μL), 1% (v/v) Tween 20 (3 μL), and distilled water (3 μL) were added to prepare the detection reagents. The detection reagent (10 μL) was applied to a glass fiber conjugate pad (Millipore Temecula, CA, USA) and dried for 2 h before the test strip was assembled.

2.4. Preparation of test capture reagents

GAA-MSA conjugates were prepared by cross-linking the carboxylic acid of GAA with the primary amines of MSA lysine residues using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) [19]. MSA (4.1 mg) and EDC (6.0 mg) was dissolved in an MES buffer (pH 4.7, 1.5 mL) consisting 100 mM [2-(N-morpholino)ethanesulfonic acid] and 0.9% (w/v) sodium chloride.

Subsequently, GAA (0.5 mg) dissolved in 40% (v/v) pyridine (0.5 mL) was added dropwise to the MSA and EDC solution, and the mixture was stirred at room temperature for 3 h. The resultant mixture was then dialyzed five times against distilled water at 4 °C and lyophilized to obtain GAA-MSA conjugates (2.4 mg), which were used as test capture reagents. A matrix-assisted laser desorption/ionization time-of-flight mass spectrometry analysis revealed that they were five GAA molecules bound to MSA molecule. An *anti*-mouse IgG rabbit antibody was used as a control capture reagent.

2.5. Preparation of chromatographic strip

The test (GAA-MSA conjugates) and control (anti-mouse IgG rabbit antibody) capture reagents were dissolved in a 50 mM carbonate buffer (pH 9.6; 2 μg/mL) and 1% (w/v) sodium dodecyl sulfate in 10 mM phosphate buffer (pH 7.0; 1 μg/mL), respectively. Subsequently, each capture reagent (1 μL) was applied to a strip of nitrocellulose membrane (Millipore Temecula, CA, USA) and dried by incubation at room temperature for 1 h. The membrane was then immersed in PBS containing 1% (w/v) BSA for 2 h to avoid non-specific adsorption, washed twice with PBS-T for 10 min, dried, and cut into single test strips that were 6.0 mm in width and 60 mm in length. Each nitrocellulose membrane test strip comprised an adsorbent pad, a conjugate pad containing the detection reagent, and a sample pad (Fig. 2). The sample solution (400 μL) was transferred to a small test tube into which the lower edge of a test strip was immersed. After the test strips were immersed into the analytes, the sample migrated upward; the results were judged ~15 min when a spot was observed in the control zone. To evaluate concentration-dependent manner of GAA in ICA developed, solutions containing various concentrations of GAA (3.90 ng/mL–5.00 μg/mL) were prepared in 5% (v/v) methanol and the LOD for GAA was evaluated by visual observation.

3. Results and discussion

The quality control of *Ganoderma* extracts has recently been attracted much attention because such extracts have recently been used worldwide. GAA is one of the major *Ganoderma* triterpenes that possesses various pharmacological activities; therefore, it can be used as an index for the standardization of *Ganoderma* extracts. In the present study, we have developed an ICA for the detection of GAA that permits a rapid quantitative/qualitative analysis, thereby overcoming the principle disadvantage of the icELISA method.

In the preparation of the chromatographic strip, the 1% (w/v) BSA solution helps to immobilize the captured reagents on the nitrocellulose