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Evaluation of cytotoxicity and immune modulatory activities of soyasaponin Ab: An *in vitro* and *in vivo* study

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ABSTRACT

To improve the immune efficacy of protein subunit vaccines, novel adjuvants are needed to elicit a suitable protective immune response and to promote long term immunologic memory. In this work, soyasaponin Ab, a major constituent among group A soyasaponins in soybeans was purified and prepared from soy hypocotyls. The immunomodulatory effects of soyasaponin Ab both *in vitro* and *in vivo* were investigated, and its pro-immunomodulatory molecular mechanism was also studied. For *in vitro* assays, with mouse macrophage cell line RAW264.7 as the studying model, both cytotoxicity and immune stimulatory activity were investigated to evaluate the potential of soyasaponin Ab as the vaccine adjuvant. The results indicated that soyasaponin Ab could be significantly safer than *Quillaja* saponins (QS). Soyasaponin Ab showed no toxicities over the tested concentration ranges compared to QS. Soyasaponin Ab was proved able to promote releases of inflammatory cytokines like TNFα and IL-1β in a dose-dependent manner. Furthermore, NF-κB signalling was also activated by soyasaponin Ab effectively. In addition, with TLR4 gene expression of RAW264.7 cell inhibited by RNA interference, immune stimulatory effects by soyasaponin Ab dropped down significantly. On the other hand, the *in vivo* experiment results showed that anti-ovalbumin (OVA) IgG, IgG1, IgG2a, IgG2b were significantly enhanced by the soyasaponin Ab and QS groups ($p < 0.05$ or $p < 0.01$). The results suggested that compared to QS, soyasaponin Ab may represent a viable candidate for effective vaccine adjuvant. TLR4 receptor dependent pathway may be involved in immune stimulatory effects of soyasaponin Ab.

Introduction

Soyasaponins belong to one group of complex and structurally diverse oleanane triterpenoids occurring mainly in soybeans (*Glycine max)* (Kitagawa et al. 1976, 1985a,b,) (Fig. 2). Soyasaponins can be categorized into two main groups referred to as group A and group B, according to their respective aglycones, soyasapogenol A and soyasapogenol B. Group A soyasaponins have two sugar chains attached to the C-3 and the C-22 of the aglycone. Group B soyasaponins possess a single sugar chain linked to the C-3. Their absolute chemical structures have been elucidated in detail (Kitagawa et al. 1976, 1985a,b). Soyasaponins exert a wide range of pharmacological activities including anticarcinogenic (Xiao et al. 2007; Zhang and Popovich 2008), capable of lowering plasma cholesterol (Lee et al. 2005; Oakenfull and Sidhu 1990), antiviral (Hayashi et al. 1997; Nakashima et al. 1989), hepatoprotective (Kinjo et al. 1998, 2003), antioxidative (Ishii and Tanizawa 2006), and antimutagenic (Berhow et al. 2000; Jun et al. 2002), among them adjuvant activity is gaining more attention (Oda et al. 2000). A correlation between adjuvant activity and amphipathic structure of soyasaponin was completed on an experimental basis using structurally consecutive analogues. The investigation demonstrated that soyasaponins adjuvant activities increased with the hydrophile–lipophile balance (HLB) value, *i.e.* the length, the number, and the composition of sugar side chains affecting the HLB value would give the overall conformation of each saponin molecule, and the amphipathic structure may define the fundamental adjuvanticity of saponins (Oda et al. 2003).

Soyasaponins can be obtained in large quantities from soybean and many kinds of legume seeds, which could be the great advantage for its practical use as vaccine adjuvants. However, the safety and immune stimulatory activity of soyasaponins have not been

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evaluated in detail. In the present study, a major constituent among group A soyasaponins in soybeans was isolated, and its *in vitro* cytotoxicity and immune modulatory effect as well as its *in vivo* immunoadjuvant activity were investigated.

Materials and methods

Reagents and chemicals

Ovalbumin (OVA, grade V), concanavalin A (conA), lipopolysaccharide (LPS) and saponins from quillaja bark (QS, CFAD-S4521-10G) as partially purified extract with 20–35% of sapogenin content were purchased from Sigma Chemical Co. RPMI-1640 medium was obtained from HyClone, Inc. Foetal calf serum (FCS) was purchased from Hangzhou Sijiqing Biological Engineering Materials Co., Ltd. Goat anti-mouse IgG, IgG1, IgG2a or IgG2b horseradish peroxidase (HRP)-conjugates were obtained from Proteintech Group, Inc.

Animals

ICR mice (6–8 weeks old, mean weight 20 g) were purchased from Shanghai Lab. Animal Research Center (Shanghai, China). They were housed in microlon boxes in a controlled environment (temperature 25 ± 2 °C) and 12 h dark/light cycle with standard laboratory diet and water *ad libitum*. The animals were acclimatized to the laboratory conditions for a week prior to the experimentation and randomly divided into six groups of each six animals. All procedures were performed according to the China legislation on the use and care of laboratory animals and according to the guidelines established by the Shanghai Animal Care and Use Committee and university committee for animal experiments.

Plant material and isolation of soyasaponin Ab

Soy hypocotyls (soybean variety Kenfeng15, Heilongjiang Province, China, harvested in 2010 in Heilongjiang Province, China) were provided by Heilongjiang Jiusan Oil & Fat Co., Ltd. (Harbin, China). Prior to pretreatment, the hypocotyls were milled to a powder and sieved (40 mesh). All other chemicals were of analytical reagent grade, and all solutions were used after redistillation.

Soyasaponin Ab was isolated and identified by our previously reported method (Zhao et al. 2012). Briefly, prior to extraction, the ground hypocotyls were defatted by extraction with petroleum ether (30–60 °C) for 4 h under reflux conditions and air-dried after extraction in the fume hood. The extraction was subsequently carried out with 70% (v/v) aqueous ethanol in an ultrasonic bath. Next, the extracts were concentrated under reduced pressure at 40 °C to remove ethanol, and the residual aqueous phase was applied to a Supelpak^{TM} XAD-2 (Supelco, Bellefonte, PA, USA) column (i.d. 30 × 450 mm). After washing with Millipore water, the column was eluted with 95% (v/v) aqueous ethanol, and the eluate was evaporated to dryness under vacuum at 40 °C and identified as dried crude extract. The extraction of 100 g (dry weight basis) of powdered soy hypocotyls yielded 8.34 g of the crude extract (dry matter) containing 4.88% total soyasaponins after the solid-phase extraction.

Preliminary fractionation of soyasaponin complexes by high-speed counter-current chromatograph (HSCCC) was performed using the same procedure as previously described (Zhao et al. 2012). 100 mg of crude extract was dissolved in 5 ml methanol and loaded on HSCCC. After separation, 22.3 mg soyasaponin complexes with the purity >85% was obtained.

For the final separation of the individual soyasaponins, preparative HPLC (PRE-HPLC) analysis was also carried out as previously described (Zhao et al. 2012). In the step gradient separation, 17 separate fractions were collected, the target compound soyasaponin Ab was obtained in the fraction 6 with the highest yield accounting for 6.32% of the crude extract (18.53% of the loaded soyasaponin complexes).

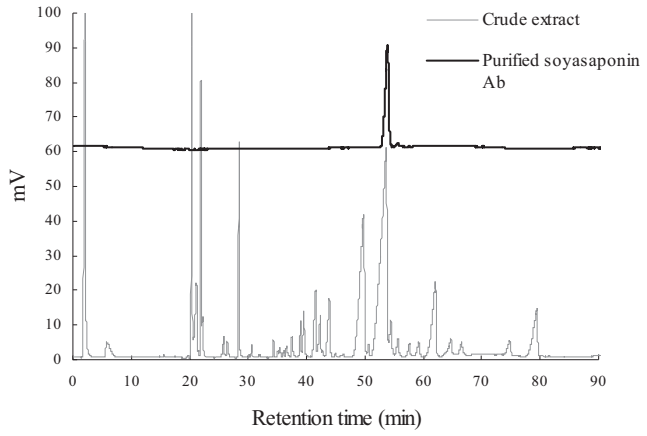

Fig. 1. The HPLC profile of soy germ crude extraction and isolated soyasaponin Ab using RP-HPLC equipped with an evaporative light-scattering (ELS) detection. The eluting components from 10 to 30 min are soy isoflavones, while the compounds after the retention time of 30 min are soyasaponins. Chromatographic conditions were described in the experimental section.

HPLC, LC–MS and NMR analysis of prepared soyasaponin Ab

The purity of the isolated compound was determined by reversed phase-HPLC using UV, evaporative light scattering detection (ELSD) (Fig. 1) described by Decroos et al. (2005). The result indicated the prepared soyasaponin target component purity was superior to 98%.

For LC–MS analysis of soyasaponins samples, UPLC–MS was performed as described previously (Zhao et al. 2012). The structure was elucidated using LC–MS, ^{1}H and ^{13}C NMR spectroscopy (400 MHz D_2O), see support information.

LC–MS: *m/z* 1437.65 for $[M+H]^+$, the molecular formula of the compound is C67H104O33. In this way, by a detailed comparison of the ^{13}C NMR data with literature (Gurfinkel et al. 2005; Shiraiwa et al. 1991), the structure of the purified compound was identified as the fully acetylated group A soyasaponin Ab (Fig. 2).

Haemolytic activity assay

Citrated BALB/c mouse blood was obtained from the animal facility of the Shanghai Laboratory Animal Centre, CAS (SLACCAS). Red blood cells (RBC) were recovered by centrifugation (2000 rpm for 5 min) and washed three times with sterile phosphate-buffered saline (PBS). A micro haemolytic activity assay was carried out using a 0.5% RBC suspension in PBS. A fixed volume of the suspension (100 μl) was mixed in round bottom microplates with a solution of the product to be tested in saline (100 μl). Soyasaponin Ab was assayed at concentrations ranging from 30 to 1250 μg/ml (30, 40, 50, 62.5, 125, 250, 500, 1000 and 1250 μg/ml). Haemolytic activity of *Quillaja* saponin (QS, CFAD-S4521-10G, Sigma) in PBS were also evaluated. Distilled water or PBS buffer was included as positive and negative controls for haemolysis. Microplates were incubated for 30 min at 37 °C and centrifuged at 1000 rpm for 10 min. An aliquot of each supernatant (100 μl) was transferred to a flat-bottom microplates and the optical density (OD) at 405 nm was measured using an ELISA reader (Lab Systems, Finland). Haemolytic activities by saponins were calculated based on the following: percentage of haemolysis = (As − An)/(Ap − An) × 100%. As, An and Ap