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Macromolecular interactions of triterpenoids and targeted toxins: Role of saponins charge

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ABSTRACT

Macromolecular interaction of protein toxins with certain plant triterpenoids holds potential for application in tumor therapy. The ability of only certain saponins to enhance the endosomal escape of toxins specifically in tumor cells was evaluated and set into correlation with the electrophoretic mobility. Saponins from *Saponaria officinalis* Linn, were selected as a lead to understand this evolutionarily conserved principle in detail. Agarose gel electrophoresis was utilized to procure pure saponin fractions with different electrophoretic mobility, which were tested for their ability to enhance the toxicity by live cell monitoring. Five fractions (SOG1–SOG5) were isolated with a relative electrophoretic mobility of (−0.05, 0.41, 0.59, 0.75 and 1.00) and evaluated using thin layer chromatography, HPLC, and mass spectroscopic analysis. Cytotoxicity experiments revealed highest effectiveness with SOG3. Live cell imaging experiments with SOG3 revealed that this saponin with a specific REM of 0.59 could assist in the lyso/endosomal release of the toxic payload without affecting the integrity of plasma membrane and could lead to the induction of apoptosis. This charge dependent enhancement was also found to be highly specific to type I ribosome inactivating proteins compared to bacterial toxins.

Charge interaction of plant toxins and saponins with tumor cells, plays a major role in toxin specific modulation of response. The finding opens up newer ways of finding protein saponin interaction conserved evolutionarily and to test their role in endosomal escape of therapeutic molecules.

1. Introduction

Tumor cell-targeted toxins are recombinant proteins that consist of a targeting domain and an enzymatically active domain. They are capable of identifying the tumor cells and thereafter enabling the entry of protein into the target cells along with a bacterial or plant toxin thereby killing the tumor cells. An array of toxins have been a matter of research in the recent years and some are now under clinical approval. The apoptotic pathways induced by different toxins are varied in most cases. Many toxins are restricted in their efficacy because of lysosomal degradation and in this context plant triterpenoids (saponins) have shown great promise [1,2].

Amongst various secondary metabolites obtained from plants, saponins possess potent pharmacological and biomedical applications [3,4]. Medicinal properties attributed to saponins include their anti-inflammatory, hypo-lipidemic, expectorant, antiulcer, androgenic and anabolic properties; other attributes of saponin include their applications as vaccine adjuvants and nutritional supplements [5,6]. Presence of saponins in variable quantities is reported in more than 90 plant families [7,8]. While there are some generalized effects elicited by saponins due to their detergent-like properties, the majority of functions ascribed to saponins are clearly structure-dependent as has been shown for instance in case of their ability to enhance the effect of targeted toxins in tumor therapy [9].

Saponins have also shown effectiveness in reducing oxidative and nitrosative stress and therefore the ability to suppress the development of malignancies and related diseases [10]. In our working group we have been evaluating the role of saponins

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in enhancement of the endosomal escape of therapeutic toxins. Saponins can lead to a more than 10,000 fold enhancement of target specific toxicity of toxins *in vitro* and a more than 20 fold improvement *in vivo.*

In our previous work, we have successfully utilized agarose gel electrophoresis in isolation of saponin fractions of saponinum album. Thereafter a single saponin was isolated just by a single HPLC run [11]. This indicated a strong electrochemical interaction between the saponins and the targeted toxins, moreover no previous studies attempted to evaluate this interaction. Therefore in the present study we tried to isolate a number of purified saponins using their electrochemical migration and tested their efficacy to augment the target specific enhancement of toxicity of various toxins.

There is evidence generated that the charge/mass attributes of saponin may play a critical role in the enhancement of toxicity of targeted toxins *via* enhancement of endosomal escape. Therefore, in the present study we have done a charge dependent separation of saponins from the roots of *Saponaria officinalis* Linn. (Fam. Caryophyllaceae). We attempted to evaluate differently charged saponin fractions in real time to see their effects on toxicity enhancement of Saporin-EGF. *S. officinalis* is the source of toxic protein Saporin as well as a rich source of saponins. It is a common ethnic belief that the lethal combination of toxin and saponin is an evolutionary tool used by the plant for its self-defense. Therefore, based on this evolutionarily conserved aspect it was selected for saponin isolation and evaluation in the present work. Live cell imaging experiments were carried out to evaluate the cellular events occurring in the presence of appropriately charged saponin fraction.

2. Materials and methods

2.1. Plant material

Dried roots of *S. officinalis* were purchased from Galke, (Gittelde, Germany). The roots were powdered using a mechanical grinder and subjected to extraction with 90% methanol (*v*/*v*) by hot percolation (45–50 °C) with continuous shaking over a magnetic stirrer (Heidolph, Germany). After 24 h the extract supernatant was filtered and filtrate was vacuum concentrated (Buchi, Switzerland) to reduce it to one third of the original volume. The remaining extract was filtered and to this acetone was added (1:1, *v*/*v*), following this the mixture was gently shaken and then stored at 4 °C for 3 h. Thereafter the precipitate was collected past centrifugation at 800 *g* for 10 min. followed by further washing using acetone. This process was repeated thrice and finally the precipitate was collected (SpnA) and lyophilized (Heto, Germany). SpnA was used for purification and isolation by agarose gel electrophoresis. The isolated fractions were labeled as SOG1–SOG5. SO signifying *S. officinalis* while G standing for gel fractionation. The isolated fractions were thereafter tested for their self-toxicity and the ability to enhance the toxicity of the targeted toxin SE consisting of a plant ribosome inactivating protein Sap-3 and epidermal growth factor (EGF) in HER-14 cells [12].

2.2. Electrophoretic separation of SOG1–SOG5

SpnA (20 mg) was dissolved in 80% glycerol solution (1.0 mL). The gel was loaded with 100 μL of this solution which was used as loading solution.

2.2.1. Effect of different buffers and pH on separation of saponins

Slab gel electrophoresis of saponins was initially carried out at room temperature for different buffers. In the first instance different buffers at reported pH values were tested. These buffers included collidine-acetic acid buffer (CA), 0.07 M pH 6.9, pyridine-acetic acid buffer 0.2 M (pH 4.9), sodium tetraborate 0.2 M (pH 9.2), sodium boric acid buffer 1 mM (pH 8.0), Tris acetate EDTA buffer pH 7.2 (1 mM EDTA, 5 mM sodium acetate and 40 mM Tris in 2.0 L distilled water), Tris borate- EDTA buffer (pH 7.4). All these buffers were tested using a 1% agarose gel.

2.2.2. Effect of temperature and the percentage composition of agarose gel

In the optimized buffer solution, different percentages of gel were tested. A 0.5%, 1%, 1.5% and 2% agarose gel was prepared and the separation pattern was evaluated. Furthermore, different percentages of gels were tested at 4 ± 1 °C and at 25 ± 2 °C.

2.3. Running conditions

Electrophoresis was carried out in a horizontal slab gel apparatus (BioRad, Germany). The dimensions of the gel were 10 cm × 6 cm × 1 cm (LBH). Sample wells were made by using a modified Lucite comb. The power source for the apparatus was a Consort EV261 (Consort, Germany). Electrophoresis was carried out at 100 mA, 120 V for 1 h without any gradient. All the running parameters were optimized after numerous test runs (data not reported) [13]. Saponins were detected under UV light (Intas, Germany). The five bands of saponins were carefully excised, and placed in separate tubes. The relative electrophoretic mobility was calculated for all the bands with reference to electrophoretic migration of bromophenol blue [11].

2.4. Recovery of saponins from gel

Saponins from the different bands were collected by modifying the methodology reported by Thakur et al. [13]. In brief, the bands were cut and crushed using a cell douncer (Sigma, Mexico). Thereafter, 300 μL of 80% methanol was added to the centrifuge tube (Millipore, Germany) and subjected to sonication and vigorous shaking. The solution was filtered and to the filtrate 400 μL of acetone was added to precipitate the saponins. The tube was subjected to centrifugation at 16 × 1000 *g* for 30 min. The supernatant was discarded and the pellet was collected and dried in a rotary vacuum evaporator.

As a control for the recovery process, fresh agarose gel was prepared and a small piece of gel without any sample was excised and subjected to the recovery process as described above. No precipitate was obtained but the solution was used for thin layer chromatography and HPLC as agarose gel control (AgC).

2.5. Experimental and analytical procedures

Thin layer chromatography (TLC) of SOG1–SOG5 and AgC was carried out to assess the preliminary purity and identification of the saponins. Chromatography was performed on methanol pre-washed and pre-activated (at 50 °C for 45 min) silica gel F_{254} aluminum foil backed HPTLC plates, mobile phase was n-butanol: acetic acid: water (4:1:5, *v*/*v*) in a twin-trough chamber up to 80 mm under laboratory conditions. The chromatogram was then dried under stream of hot air and visualized past derivatization with anisaldehyde sulfuric acid reagent and heating at 105 °C for 3 min. Furthermore, a 2D TLC was also performed for SO-G3, where the plate was initially developed as described above, dried completely under stream of hot air and placed in perpendicular direction for a 2D development in chloroform: methanol: water (7:3:1, *v*/*v*), followed by detection with anisaldehyde sulfuric acid reagent as described above.

After dissolving in 20% methanol, saponins (0.5 mL, 40 mg) were subjected to an UltraSep ES PHARM RP18E (7 μm, 250 mm × 4 mm)