Research article

Identification and confirmation of 14-3-3 \( \zeta \) as a novel target of ginsenosides in brain tissues

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1. Introduction

Ginseng is one of the most widely consumed herbal nutritional products in the world [1]. Pharmacological studies showed that ginseng contains various chemical components, including ginsenosides, ginseng polysaccharides, peptides, fatty acids, and amino acids [2]. Ginsenosides are the primary active components of ginseng and various ginsenosides have been identified and isolated. According to the chemical structure of their aglycones, ginsenosides can be mainly divided into two categories, namely, the protopanaxadiol group (e.g., Rb2, Rb1, Rd, Rc, Rh2, and Rg3) and protopanaxatriol groups (e.g., Re, Rg1, Rf, and Rh1) [3]. The original form of ginsenosides can be metabolized by intestinal bacteria and liver metabolism, mainly into deglucose-based sub-saponins or aglycones [4,5]. In the protopanaxatriol group, Re and Rg1 are bio-transformed into 20(S)-protopanaxatriol (PPT) via F1 and Rh1. In the protopanaxadiol group, Rd and Rb1 are converted into 20(S)-protopanaxadiol (PPD) by ginsenoside compound K (CK) and Rh2 [6,7]. After oral administration, ginsenosides and their metabolites are absorbed through the intestinal tract into the systemic circulation. Previous studies have shown that ginsenosides could...
penetrate the blood–brain barrier (BBB), directly target the neur-
on, and may cause pharmacological effects in the central nervous
system (CNS). For instance, through rapid HPLC-MS/MS analysis,
Liu et al [8] found the distribution of ginsenosides Rg1, Re, Rb1, Rc,
and Rd in brain tissues after intravenous injection of ginseng ex-
tracts. As for the aglycones of ginsenosides, the low water solubility of
PPD resulted in poor oral bioavailability and limited its effective
delivery to the brain. However, compared with unformulated PPD,
the prepared nanocrystals can significantly improve PPD’s oral
bioavailability and drug concentration in the brain after oral
administration in rats [9,10].
Ginseng affects the nervous system, and ginsenosides have a
neuroprotective effect and enhance cognitive performance and
memory [11]. Nowadays, ginsenosides have become a research
hotspot in the treatment of nervous system diseases because of
their effectiveness, low toxicity and minor side effects. For example,
ginsenoside Rg1 has good antiaging effects [12]. Ginsenosides Rh2
and Rg3 are promising low-toxic candidates for cancer prevention
and treatment [13]. Ginsenoside Ro considerably suppresses tumor
growth with outstanding significant side effects on immune organs and
body weight [14]. At present, a large number of studies have proved
that ginsenosides have positive pharmacological effects on the CNS.
Zhang et al [15] reported that ginsenoside Rd3 and its four degly-
cosylated derivatives- Rg3, Rh2, CK, and PPD - have antidepressant
activities in mice models. Chen et al [16] showed that ginsenoside
Rb1 vigorously prevents loss in the integrity of BBB after cerebral
ischemia by negatively regulating NOX4-derived reactive oxygen
species (ROS) production and local inflammation. Zhu et al [17]
proved that Rg1 may be a useful compound to prevent age-related
impairment related to learning and memory. Hou et al [18]
demonstrated that long-term Rh2 administration might contribute
to spatial learning by enhancing the role of newly generated hip-
cocampal cells in the dentate gyrus (DG) of mice or a parallel
mechanism of the enriched environment. However, the immediate
protein target of ginsenosides in the brain remains unknown.
To identify the protein targets for ginsenosides in brain tissues,
we fixed ginsenosides to 4% cross-linked agarose beads with
covalent-linked diamino dipropylamine, and used affinity chro-
matography which is a classic target recognition method [19].
From the initial screening, we identified five proteins as potential targets
for ginsenosides in brain tissues, including the 14-3-3ζ protein, the
14-3-3 ε protein, actin, creatine kinase B-type, and ATP synthase
subunit beta, among which the 14-3-3ζ protein was of further
study. We applied BLI to detect the direct interaction of ginsenosides
and the 14-3-3ζ protein and found that their metabolite PPD dis-
played the strongest direct interaction with 14-3-3ζ protein. The
interaction of PPD with 14-3-3ζ protein was proved through BLI
and ITG cocrystallization and site-directed mutagenesis analysis
identified the binding sites between PPD and the 14-3-3ζ protein.

2. Methods
2.1. Extraction of high-purity ginseng total saponins
High-purity ginseng total saponins were extracted as previously
described [3,20]. In brief, the ginsenosides Re, Rh1, Rg2, Rg1, Rb2,
Rc, Rg3, F1, Rb1, and Rd were measured in high-purity ginseng total
saponins (Supplementary Figure 1). According to the colorimetric
method and the charged electrosol detection response, the esti-
mated total saponin contents were approximately 107% and 90%,
respectively [21]. Re, Rg1, Rb1, F1, Rh1, Rb2, CK, PPD , Rd, and PPT
standards (purity ≥98%) were obtained from the National Institute
of Food and Drug Control (Beijing, China).

2.2. Experimental animals
The mice needed for brain tissue were obtained from SLAC
Laboratory Animal Co., Ltd. (Shanghai, China). All animal proced-
ures were approved by the Institutional Animal Care and Use
Committee of Nanjing University of Chinese Medicine and carried
out in accordance with the Guidelines of Accommodation and Care
for Animals formulated by the Chinese Convention for the Protec-
tion of Vertebrate Animals Used for Experimental and Other Sci-
entific Purposes. The minimum number of animals required to
obtain consistent data were used.

2.3. Affinity chromatography
The ginsenoside-bead conjugate was synthesized as described
previously [22], with minor modifications. The detailed steps are
described in the Supplementary Methods.

2.4. Cloning and protein expression
The construct containing the 14-3-3ζ gene was generated by
PCR from the human genome, and a hexahistidine tag was added to
the N-terminus of the gene, followed by a TEV cleavage site. The
gene was subcloned into the pET28a vector and transformed into
Escherichia coli BL21(DE3). The transformed BL21(DE3) cells were
grown in Luria-Bertani medium containing 50 µg/ml Kanamycin at
37 °C until the OD600 reached approximately 0.6. Then, the tem-
perature was changed to 25 °C and E.coli cells were induced with
isopropyl-d-1-thiogalactopyranoside (IPTG) to a final concentra-
tion of 0.5 mM for 18 h [23].

2.5. Protein purification
The detailed procedures of protein purification are described in
the Supplementary Methods. After purification of nickel column,
the 14-3-3ζ protein was digested overnight at 4 °C with TEV pro-
tease at a ratio of 1:30 (protease : protein) [24] to remove the label.
Under denaturing conditions, the molecular weight was estimated
using a protein marker (Bio-Rad, California, USA) as a reference
protein, and protein purity was evaluated by sodium lauryl sulfate-
polyacrylamide gel electrophoresis (SDS-PAGE) on a 15% poly-
acrylamide gel (Supplementary Figure 2). The concentration of 14-
3-3ζ protein was then detected at the absorbance of 280 nm by
using a molar absorptivity constant calculated using the ExPASY/ProtParam tool (https://web.expasy.org/protparam/) [25].

2.6. Site-directed mutagenesis
The open reading frame (ORF) of 14-3-3ζ was amplified using the
cDNA of HCM cells (BeNa Culture Collection, Beijing, China) as a
template and the primers (Supplementary Table 1). The amplicon
of 14-3-3ζ was digested with BamHII and SalI, and then cloned into
SalI /BamHI -digested expression vector pET28a to generate pET-14-
3-3ζ. Using site-directed mutagenesis with pET-14-3-3ζ as tem-
plate, we constructed pET56, pET127 and pET128, which have
mutations of arginine at position 56 to alanine (14-3-3ζ R56A,
Mutant 1, Supplementary Figure 3), arginine at position 127 to
alanine (14-3-3ζ R127A, Mutant 2, Supplementary Figure 4) and
tyrosine at position 128 to alanine (14-3-3ζ Y128A, Mutant 3,
Supplementary Figure 5), respectively [3]. The detailed steps are
described in the Supplementary Methods.
2.7. Biolayer interferometry

The binding affinities between PPD and WT or the mutants of human 14-3-3ζ protein were measured using the BLI technique for testing. Measurements were carried out at 30 °C as previously described [26]. The detailed process is described in the Supplementary Methods. The final Kon and Koff rate constants were used to calculate the affinity of protein and small molecule (KD = Koff/Kon). All experiments were repeated in thrice.

2.8. Isothermal Titration Calorimetry

ITC experiments were executed using a VP-ITC instrument (MicroCal, Northampton, MA) [27]. The detailed process is described in the Supplementary Methods. The KD values listed represent the average of at least three independent measurements.

2.9. Protein crystallization and structural elucidation

Crystallization was performed using the sitting drop vapor diffusion method. Exactly 10 mg/ml 14-3-3ζ was incubated with PPD in a molar ratio of 1:10 at room temperature for 1 h. Moreover, 0.3 μL of proteolysis solution containing 10 mg/ml 14-3-3ζ and 5 mg/ml PPD in 50 mM NaCl, and 20 mM Tris at pH 8.0 was mixed with 0.3 μL of well solution containing 0.1 M sodium citrate tribasic dehydrate at pH 5.6, 0.2 M ammonium acetate, and 30% w/v PEG 4000. The crystallization drop was incubated at 290 K against 40 μL of well solution. The crystals of 14-3-3ζ-PPD complex appeared in one day and reached the maximum size of 150 μm × 100 μm × 80 μm after seven days. The crystals were directly flash-frozen in liquid nitrogen. Diffraction data were collected from the beamline BL17U1 of the Shanghai Synchrotron Radiation Facility (SSRF) at 100 K [28]. Images were processed with imosflm [29]. The structure of the 14-3-3ζ-PPD complex was resolved through molecular replacement implemented in Phaser [30]. The coordinates of the structure (PDB code: 6ejl) were used as a search model. Then, the model was briefly refined using Phenix [31] and then manually adjusted using Coot [32]. The final model was refined to Rfree=0.2772. All crystallographic figures were created using PYMOL (http://www.pymol.org/) [25].

2.10. Statistical analysis

The data in this study were expressed as the mean of the number of experiments shown ± the standard error of the mean (S.E.M.) and analyzed using the statistical software package of Social Science Statistics Software Package version 10.1.

3. Results

3.1. Primary screening for the protein targets of ginsenosides in brain tissues by affinity chromatography

High-purity ginseng total saponins were used as ligands, and their glycosyl group was used to attach to the resin by oxidation with sodium periodate (Fig. 1A). The beads attached to the saponins were then incubated with the brain tissue extracts and washed extensively to remove non-specifically bound proteins. Tightly bound proteins were eluted under high denaturing conditions and analyzed by SDS-PAGE (Fig. 1B). The results showed that two protein strips (Bands 1 and 3) in the brain tissues had a certain affinity with the control resin, and 10 protein strips (Bands 1 - 10) had a certain affinity with the attached resin in the ginseng total saponin (Fig. 2A). Bands 2 and 4 - 10 were analyzed by mass spectrometry. Bands 4 - 8 were successfully identified using the Mascot search engine, and the results are shown in Supplementary Figures 6-10. The potential target proteins of the top Mascot score for each band were 14-3-3 protein zeta (Supplementary Figure 11), 14-3-3 protein epsilon (Supplementary Figure 12), actin (Supplementary Figure 13), creatine kinase B-type (Supplementary Figure 14), and ATP synthase subunit beta (Supplementary Figure 15) (Fig. 2B). The direct interaction between ginsenosides and 14-3-3ζ was considered as a priority in subsequent research.

Fig. 1. General experimental procedure for affinity chromatography of small molecule. (A) Sodium periodate oxidation method is used to immobilize ginsenosides on the resin. (B) To enrich target proteins, we incubated protein lysates with the matrix-ligand chemical complex. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) analysis were used to detect the target proteins in the elution mixture.
Fig. 2. Affinity chromatography was used to identify the protein target of ginsenosides in brain tissues. (A) Ginseng total saponins (GTS) were used as the ligand, while the negative control pull-down adopted sucrose as the ligand. SDS-PAGE coupled with Coomassie blue staining was used to visualize the eluted mixtures. Bands of varying abundance between GTS and control samples were excised and then identified by LC-MS/MS. (B) Mass spectrometry data were converted into a format of Mascot universal and then resolved via the Mascot search engine to recognize proteins from the database of peptide sequence. Based on the sequence coverage and Mascot score, the most potential proteins for each band were listed.

Fig. 3. Interaction of 14-3-3 ζ protein with ginsenosides. (A) The representative association and dissociation curves of the 14-3-3 ζ protein to ginsenosides and aglycones (PPD and PPT) were obtained by BLI analysis. (B) The direct binding reaction of the 14-3-3 ζ protein with ginsenosides and aglycones (PPD and PPT) were measured using the BLI technology (mean ± SEM, n = 3). (C) PPD binding to human 14-3-3 ζ protein was observed by BLI kinetic analysis. The PPD concentrations were set to 16.9, 33.9, 67.8, 135.5 and 271 μM. The results were obtained from at least three experiments. (D) ITC analysis for the 14-3-3 ζ protein binding to PPD. The ITC data displayed represents three replicates, and fitting errors are reported. PPD, 20(S)-protopanaxadiol; PPT, protopanaxatriol; SEM, standard error of mean.
3.2. PPD bound directly to the 14-3-3ζ protein

First, direct binding was measured by BLI, where human 14-3-3ζ was fixed on a biosensor, and the wavelength shift was detected in real time after adding or diluting small molecules. At a concentration of 271 μM, the direct binding response of 14-3-3ζ to Re, Rg1, Rb1, Rh1, Rd, F1, GK, Rh2, PPD, and PPT is shown in Fig. 3A and B. Ginsenosides including Rb1, Rh1, Rd, Re, and F1 barely interacted with the 14-3-3ζ protein. Rg1, CK, Rh2, and the aglycones (PPD and PPT) exhibited significant affinity to the 14-3-3ζ protein. Among these compounds, PPD had the strongest binding activity to the 14-3-3ζ protein and was used for subsequent affinity and binding conformational analysis.

The ratios of intermolecular $K_{on}$, $K_{off}$, and binding constants ($K_0 = K_{off}/K_{on}$) were determined using BLI technique [33]. The BLI kinetics analysis suggested that the affinity ($K_0$) of PPD for 14-3-3ζ protein was $4.68 \pm 0.12E-5$ M. The $K_{on}$ and $K_{off}$ ratios of the 14-3-3ζ protein to PPD were $7.47 \pm 0.21E2$ 1/Ms and $3.49 \pm 0.10E-2$ 1/s, respectively (Fig. 3C). To further verify the direct interaction of 14-3-3ζ to PPD and assess the physicochemical parameters of this interaction, we performed ITC analysis to detect the affinity of the binding partners in their natural states [34]. Exactly 271μM of PPD was titrated into 50μM of 14-3-3ζ protein. The affinity $K_0$ of the PPD to 14-3-3ζ was $3.10 \pm 0.20E-6$ M, as measured by three independent experiments and calculated with a very suitable 1:1 binding model. The thermodynamic parameters for the interaction suggest that it is driven by helium and has a low entropy component ($\Delta H = -1927 \pm 178.8$ cal/mol, $\Delta S = 18.7$ cal/mol/deg) (Fig. 3D).

3.3. Crystallographic study of the PPD—14-3-3ζ complex

Co-crystallization of the 14-3-3ζ protein with PPD (Fig. 4A) yielded the best crystal that diffracted to a resolution of 2.6 Å (Fig. 4B) and belonged to the space group P212121, with unit-cell parameters of $a = 70.045$, $b = 72.259$, and $c = 129.312$ (Table 1).
By using the previously reported structure of the 14-3-3-ASK1 fusion protein (PDB entry 6ejl) as a search model, the structure was determined by molecular replacement. A final \( R_{\text{work}} \) of 0.2364 and \( R_{\text{free}} \) of 0.2772 were refined to the model. Table 1 summarizes the details of data collection and optimization statistics.

3.4. Crystal structure of the PPD–14-3-3 \( \zeta \) complex

The crystal structure suggests the 14-3-3 \( \zeta \) protein formed a homodimer (Fig. 4C). The initial Fo-Fc map shows extra density near the residues Arg56, Arg127 and Tyr128, suggesting the position of the bound PPD. However, considering the relatively weak interaction, the electron density was not clear enough to locate the exact position of the individual atoms of the PPD molecule (Fig. 4D).

3.5. R56, R127, and Y128 residues were critical sites for the interaction of 14-3-3 \( \zeta \) protein with PPD

According to the crystal structure of 14-3-3 \( \zeta \) protein with PPD, we generated a series of mutants of the 14-3-3 \( \zeta \) proteins R56A, R127A, and Y128A (Fig. 5A). The affinities of the wild-type (WT) and the 14-3-3 \( \zeta \) protein with PPD were measured with BLI (Fig. 5B) and the mutant forms of 14-3-3 \( \zeta \) (Fig. 5C–E). As shown in Fig. 5F, the \( K_D \) of WT and three mutants with PPD were 4.52 ± 0.12E-5 M, 2.35 ± 0.11E-3 M, 2.28 ± 0.10E-4 M, 1.36 ± 0.11E-4 M, respectively. The affinities of all the three mutants with PPD were reduced compared with the that of WT, suggesting that the R56, R127, and Y128 residues are critical sites for the binding of PPD to 14-3-3 \( \zeta \).

4. Discussion

Identifying protein targets for natural products is very challenging but very significant for the understanding of their related functions. Some experimental approaches can be used to identify the protein targets of small molecules, and affinity chromatography remains one of the most selective and versatile forms [35,36]. In comparison with other newly developed target recognition methods [37], affinity chromatography only depends on the ability of the compounds binding to the target protein, instead of specific biological parameters (i.e., thermodynamic [38] or proteolytic [39] measurements) that are only useful for a subset of the compounds. However, affinity chromatography requires an active group on a small molecule that binds to the solid support; hence, spatial hindrance may occur around the active group and prevent the protein to interact with the ligands [40].

To address this issue, we used high-purity ginseng total saponins as the ligands, where the sugar attachment sites of...
ginsenosides at C-3, C-6, or C-20 positions are different from each other [3]. From the initial screening, we identified five proteins as potential targets for ginsenosides in brain tissues, including the 14-3-3 ζ protein, the 14-3-3 ε protein, actin, creatine kinase B-type, and ATP synthase subunit beta. Among these proteins, creatine kinase has been identified and reported in our previous study [3]. ATP synthase subunit beta is involved in the last step of oxidative phosphorylation of the respiratory chain, which is mainly related to energy. The 14-3-3 proteins are a group of acidic soluble proteins (27-32 kDa) with the highest abundance in the brain [41]. In recent years, 14-3-3 proteins have drawn increasing attention as effective drug targets of various diseases. More binding partners, mostly polyproteins, for 14-3-3 are found to be effective for 14-3-3 related increases. 14-3-3ζ plays key roles, such as synaptic plasticity, learning and memory, and neuronal differentiation, in the CNS [42]. Natural products act as valuable sources of drugs and lead structures for new drug discovery, but many of their direct protein targets remain unknown. We applied the BIJ technique to detect the direct interaction of 14-3-3ζ to parent ginsenosides (i.e., Rd, Rb1, Re, and Rg1) and their metabolites (i.e., Rh2, Rh1, F1, PPT, CK, and PPD) (Fig. 3A and B). In subsequent research, we chose PPD, the most potent ligand among all the compounds tested, to study the interaction with the 14-3-3ζ protein. The BLI kinetics analysis (Fig. 3C) and ITC assays (Fig. 3D) both showed that PPD had the modest binding affinity to the 14-3-3ζ protein. Crystallographic study showed that PPD directly bind to the active site of the 14-3-3ζ protein and the main interactions occurred between the residues R56, R127, and V128 of the 14-3-3ζ protein and a portion of PPD (Fig. 4). Moreover, mutating any of the above residues resulted in a significant decrease of affinity between PPD and the 14-3-3ζ protein (Fig. 5). These results indicate that 14-3-3ζ is a cellular target of PPD, and R56, R127, and Y128 residues are located in the binding site.

The 14-3-3 proteins are involved in many types of neural processes, such as learning and memory and synaptic function [43]. The 14-3-3ζ protein is related to various neurological diseases and signaling pathways. For example, the isotype 14-3-3ζ causes the oligomerization of its target proteins (such as phosphorylated tau), and high levels of 14-3-3ζ increase the levels of phosphorylated tau, which is a marker associated with Alzheimer's disease [44]. The 14-3-3ζ protein is localized in amyloid plaques in human brains with spongiform encephalopathies [41]. In the spinal cerebellar ataxia type 1 (SCA1) of polyglutamine repetitive disease, after Akt phosphorylation, 14-3-3ζ could bind to and stabilize ataxin-1, thereby slowing its normal degradation [45]. Li et al [46] demonstrated that 14-3-3ζ mediated tau phosphorylation in HEK-293 cells by Ser9-phosphorylated GSK3β. In summary, it is possible that ginsenosides and their metabolites may exert their neural activities based on 14-3-3ζ protein. In this study, we report the first crystal structure of a natural compound PPD in complex with its target protein 14-3-3ζ. Further investigation of binding properties by co-crystallization and amino acid site-directed mutation confirmed the binding site on 14-3-3ζ [47]. However, how the PPD exerts neural activity through 14-3-3ζ needs further investigation.

5. Conclusion

In this study, we demonstrated that the 14-3-3ζ protein is a target protein of ginsenosides in brain tissues. The metabolites of ginsenosides could be directly bound to the 14-3-3ζ protein, among which PPD displayed the highest affinity. Our findings help to better understand the mechanisms of ginseng neuronal activities and generate relevant implications in developing small-molecule compounds that can bind to 14-3-3ζ based on the structure of dammarane-type triterpenoid with a four-trans-ring rigid steroid skeleton.

Conflicts of interest

The authors have declared that there is no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jgr.2020.12.007.

Contributors

F. C., J.Z. and Y.Z. conceived and designed the experiments. F.C., L.C. and W.L. performed the experiments. F.C., L.C., W.L., Z.Z., J.L., W.Z., Z.Z., J.Z., and Y.Z. analyzed the data and drafted the manuscript. All authors contributed to the revision of this manuscript and approved the final manuscript.

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