Quantitative proteomics analysis for effect of *Acanthopanax senticosus* extract on Neuroinflammation

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**Abstract:** *Acanthopanax senticosus*, a traditional herb commonly found in Northeastern Asia, has been used for treating neurodegenerative diseases. However, the molecular and cellular mechanisms of its effect on neuroinflammation have not been investigated. In the current study, quantitative proteomics approach was applied to investigate the effect of *Acanthopanax senticosus* extract (ASE) on nitrosative stress and inflammatory response in BV-2 microglial cells stimulated with lipopolysaccharide (LPS). The results showed ASE inhibited LPS-induced nitric oxide (NO) production, while no significant toxicity appeared in the cells. Proteomic quantitative analyses using 2D-DIGE (Difference in Gel Electrophoresis) combined with liquid chromatography–electrospray ionization tandem mass spectrometry (LC–ESI-MS/MS) revealed that 17 proteins showed significant changes responding to ASE. Furthermore, signal transduction pathways and network modulated by the ASE were predicted using Ingenuity Pathway Analysis (IPA). These findings indicated the ability of ASE to suppress LPS-induced nitrosative stress in BV-2 cells, and may provide important insights into the molecular mechanism underlying potentially beneficial effect of *Acanthopanax senticosus*.

**Keywords:** *Acanthopanax senticosus* extract; Microglia; BV-2 cells; 2D-DIGE.

**INTRODUCTION**

Microglia are erythromyeloid-lineage cells in the central nervous system that are primarily involved in maintenance of the brain and the spinal cord parenchyma tissue homeostasis (Griffiths et al., 2009; Aguzzi et al., 2013; Sugama et al., 2009; Bal-Price et al., 2001). They can be activated by bacterial endotoxins such as lipopolysaccharides (LPS) upon phagocytosis of invading bacteria, and produce pro-inflammatory mediators including nitric oxide (NO), which is known to play diverse roles in the regulation of biological processes ranging from development to disease (Moss et al., 2001; Streit et al., 2005; Saijo et al., 2011; Brown et al., 2003). There is evidence that excess NO is thought to induce nitrosative stress and contribute to neuronal injuries leading to the progression of neurodegenerative diseases including cerebral ischemia, Parkinson’s disease, Alzheimer’s disease, Huntington’s disease and multiple sclerosis (Sun et al., 2010; Glass et al., 2010; Liu et al., 2013; Tansey et al., 2010; Amy et al., 2010). Therefore, agents that attenuate chronic microglial activation and suppress production of neurotoxic proinflammatory molecules are of interest for development of novel therapeutic approaches to various neurodegenerative diseases (Aldskogius et al., 2001).

With the long history of human civilization, many botanicals possessing antioxidant and antimicrobial properties have been offered beneficial effects on antagonizing inflammatory responses and preventing neurological diseases (Yamagishi et al., 2005; Kim et al., 2001; Lee et al., 2003; Lee et al., 2000; Bate et al., 2004). As one of the most prominently used traditional medicine used in Northeastern Asia, *Acanthopanax senticosus* has shown its therapeutic effects on diabetes (Zhou et al., 2012), ischemia, and ROS production (Chen et al., 2002). *In vivo*, ASE was shown to reduce nitric oxide (NO) synthesis by suppressing iNOS expression to attenuate endotoxic shock (Lin et al., 2008). These findings suggest that ASE may offer beneficial effects on prevention of neuroinflammation and neurodegenerative diseases. The goal of this study is to investigate the effects of ASE on neuroinflammatory responses in lipopolysaccharide (LPS)-activated murine BV-2 microglial cells using proteomic analysis.

Two dimensional difference in-gel electrophoresis (2D-DIGE) complemented with liquid chromatography tandem mass spectrometry (LC-MS/MS) is a classical proteomic approach, which can visualize and detect differential fold-changes of large numbers of proteins on one gel. It can provide quantitative information, since it minimizes gel-to-gel variation, improves spot matching, reduces number of gels needed, and permits quantitative analysis of small sample amounts (Marouga et al., 2005; Calamia et al., 2012; Komatsu et al., 2006; Sharma et al., 2013; Hirano et al., 2007). Therefore, such an approach has been mainly applied in discovering novel biomarkers and understanding the molecular mechanisms of action of specific molecules.
In the present study, we examined the effect of ASE on LPS-stimulated microglial BV2 cells. Using 2D-DIGE in couple with liquid chromatography tandem mass spectrometry (LC-MS/MS), we identified multiple molecular targets of ASE in LPS-stimulated BV-2 cells. Furthermore, we predicted signal transduction pathways and protein network that are modulated by ASE using Ingenuity Pathway Analysis (IPA). These findings indicated the ability of ASE to attenuate LPS-induced nitrosative stress in BV-2 cells and may provide important insights into the molecular events underlying the treatment of ASE and allow the identification of novel therapeutic targets.

EXPERIMENTAL

Materials

ASE were purchased from Xi’an Acetar Biology and Technology Co., Ltd. (Xi’an, China). Dulbecco’s modified Eagle’s cell culture medium (DMEM) was obtained from Gibco (Invitrogen Co., San Diego, CA, USA). Sequencing-grade trypsin was obtained from Promega (Madison, WI, USA). Fetal bovine serum (FBS) was obtained from Hangzhou Sijiqing Company (Hangzhou, China). Lipopolysaccharide (LPS) (rough strains) from Escherichia coli F583 (Rd mutant), N-omega-Nitro-L-Arginine Methyl Ester Hydrochloride (L-NAME) and methylthiazolyldiphenyl-tetrazolium bromide (MTT) were from Sigma-Aldrich (St. Louis, MO). CyDye DIGE Fluor minimal dyes and DryStrip gels (24 cm, pH 3-10) were purchased from GE Healthcare (Buckinghamshire, UK).

Assessing cell viability

A MTT assay was performed to access cell viability. Cells were treated with specified concentrations of ASE. Then, the conditioned medium was removed and 100μL of MTT reagent (0.5mg/mL) dissolved in DMEM was added to each well. The plates were incubated for 3 h at 37°C. The formazan particles were dissolved with 100μL DMSO, and absorbance was measured at 540 nm using a microplate reader (Biotek Synergy 2, Winooski, VT).

Nitric Oxide (NO) determination

After cell treatment for 16 hours, aliquots of conditioned medium (50μL) was collected to incubate with 50μL of Griess reagent (1% (w/v) sulfanilamide and 0.1% (w/v) N-(1-naphthyl) ethylenediamine in 5% (v/v) phosphoric acid) for 10 min at room temperature covered in dark. Absorbance was measured at 543 nm using the BioTek Synergy 2 micro-plate reader and NO concentration was determined from a standard curve of sodium nitrite dilution series.

Protein extraction

The cell samples from the control and experimental groups were frozen in liquid nitrogen and then crushed by using a mortar and pestle. All samples were lysed with a lysis buffer containing 7M urea, 2M thiourea, 4% w/v CHAPS and 30mM Tris, pH 8.5. All samples were centrifuged at 15,000×g for 15 min at 4°C and the supernatant was collected. Protein concentration was determined with the BCA protein assay kit following the
manufacturer’s instructions. The samples were then aliquoted and stored at -80 °C for further analysis.

![Image](image1.png)

**Fig. 3:** Progenesis SameSpot analysis of the 2-D DIGE results. A total of 17 spots were found occurring significant protein level changes between LPS-treated and LPS+ASE-treated samples (p<0.05, fold change >1.5) (A) A representative 2-D DIGE gel showing the differentially expressed spots. (B) An example spot of down-regulated by ASE (Spot #13, p = 1.263e-004, fold change = 2.0).

**Labeling proteins with CyDye**

Labeling of proteins for 2D-DIGE was performed according to manufacturer’s protocol (ratio of 50µg protein to 400 pmol CyDye™ dyes). To ensure that there were no dye-specific labeling artifacts, sample replicates in different gels were labeled with either Cy3 or Cy5 dye, whereas the pooled internal reference sample, a mixture of same amounts of all analyzed samples, was labeled with Cy2 dye.

**Two-dimensional electrophoresis**

The labeled protein samples were pooled and brought up to a final volume of 420µL with rehydration buffer (8 M urea, 4% CHAPS, 2% IPG buffer, pH range 3-10, 20 mM dithiothreitol (DTT). The first-dimension isoelectric focusing (IEF) was performed in an IPGphor IEF unit on 24-cm IPG strips pH 3-10. After IEF, the proteins were reduced and alkylated by successive 15 minutes treatments with equilibration buffer containing 1.0% (w/v) DTT, followed by 2.5% (w/v) iodoacetamide. Proteins on the strips were further resolved by 12% SDS-PAGE. All gels were run at 1 Watt/gel in the dark overnight.

**Gel image acquisition and DIGE data analysis**

The gel images were acquired on an Ettan DIGE imager scanner. Excitation and emission wavelengths were set specifically for each dye according to the manufacturer’s recommendations. After imaging for CyDyes, The gels were stained by the Coomassie staining method. DIGE data was processed by SameSpots software (Version 4.5; Totallab Nonlinear USA Inc. Durham, North Carolina) (Karp et al., 2008).

**Protein preparation for LC-MS/MS**

Protein spots of interest were manually excised from stained gels and digested by the enzyme solution containing sequencing-grade modified porcine trypsin for 16 h at 37°C. The peptides were desalted by using ZipTip C18 pipette tips (Millipore, Bedford, MA, USA). Full-scan MS and MS/MS experiments were carried out with Thermo Scientific LTQ™ Orbitrap-XL in positive ion reflector mode. Approximately 4µL of sample was loaded and the peptides were separated and eluted from the analytical column with a gradient of acetonitrile from a 10% to 60% for 100 min at 250nL/minute. The Sorcerer 2IDA (Sage-N research Inc., San Jose, California) was used to query the data against the IPI-mouse database. Raw data were copied to the sorcerer and searched against the database.

![Image](image2.png)

**Fig. 4:** Effect of ASE in LPS-induced microglial BV-2 cells on canonical pathways and signaling network. (A) Top 14 canonical pathways participated by the differentially expressed proteins responding to ASE treatment in BV-2 cells. (B) The top protein network responding to ASE treatment in BV-2 cells. The symbols labeled in red and green represent up-and down-regulation, respectively and the intensity of the colors indicate the degree of regulation. Solid lines in the network imply direct interactions between proteins, and dashed lines indicate indirect interactions.

**STATISTICAL ANALYSIS**

Data shown represent the mean and standard error. Statistical analysis was performed with one-way analysis of variance (ANOVA). Data were considered statistically significant at p < 0.05.

**Bioinformatic analysis**

The identified proteins were uploaded into the Ingenuity Pathway Analysis (IPA) software version 7.6. Canonical pathways and protein-protein network were predicted using IPA (Kramer et al., 2014).

**RESULTS**

**The effect of ASE on cells viability and NO production in LPS-stimulated BV2 cells**

To determine the effect of ASE in LPS-simulated BV-2 cells, we first conducted dose titrations for ASE. BV-2 cells were treated with various concentration of ASE (0, 10, 20, 30, 40µg/mL) in the presence of LPS. A Griess assay showed that NO level significantly increased by LPS induction compared with the untreated control (fig. 1A). Whereas administration of ASE decreased NO production and more inhibitions were seen as the
concentrations of ASE increased. MTT assay result indicated no significant cell death was caused by addition of ASE (fig. 1B). ASE treatment alone had neither cytotoxicity to the cells nor showed any significant effects on NO production (fig. 2). As a positive control, treatment with a known NO inhibitor L-NAME ensured the inhibitory response to the LPS-stimulated BV-2 cells. Following these findings, we chose to use 20 $\mu$g/mL concentrations for the next set of 2D-DIGE experiments designated to identify molecular targets of ASE in LPS-stimulated BV2 cells.

**Modulation of ASE on protein expression in LPS-stimulated microglial BV-2 Cells**

To determine the global proteomic changes after ASE treatment in LPS-stimulated BV-2 cells, we next performed 2D-DIGE followed by LC-MS/MS analysis. Three samples (untreated, LPS-treated, treated with LPS + ASE) in biological triplicates were lysed and labeled by CyDyes. After 2D-DIGE separation, images of the gels were subjected to quantitative analysis using the SameSpots software. A total of 935 protein spots were detected, of which 17 spots were significantly altered (fold change > 1.5, p<0.05) by the treatments. These spots were depicted on a representative DIGE gel image shown in fig. 3A. Representative quantification of spot #13 (fig. 3B) were presented as an examples of down-regulation in the LPS + ASE treatment group. Next, the 17 differential spots were excised on the corresponding Coomassie staining gel for protein identification by LC-MS/MS. The identified proteins are listed in table 1.

**DISCUSSION**

In order to get more insight on the mechanisms for the action of ASE under LPS-induced microglial activation, we predicted canonical pathways and protein-protein interaction network affected by ASE in LPS-stimulated BV-2 cells using IPA data on differentially expressed proteins. The top canonical pathways revealed the proteins altered by ASE are mainly involved in superoxide radicals degradation, pentose phosphate pathway (oxidative branch), nrf2-mediated oxidative stress response, 14-3-3-mediated signaling, and gap junction signaling (fig. 5). Protein-protein interaction network, which is associated with ASE treatment, were further examined by IPA analysis. The results showed that 14 out of the 17 proteins are involved in this network. Among these proteins, 3 proteins are down-regulated (shaded green) and 11 proteins are up-regulated (shaded red). They mainly play roles in cell death and survival, protein synthesis and free radical scavenging. These results indicated that ASE treatment can significantly decrease neuroinflammation by regulating expression of many proteins that are involved in multiple oxidative stress-related pathway, consistent with their known antioxidant effects.

**CONCLUSIONS**

In the present study, we applied quantitative proteomics to investigate the effect of ASE in LPS-induced BV-2 cells. Our findings provide detailed mechanistic insights.
into the multiple protein targets of ASE. Moreover, based on the expression levels of the differentially expressed proteins, we predicted the signal transduction pathways and network associated with these proteins. These studies provide molecular evidence for the underlying mechanism of their multi-modal action of ASE and may aid in better understanding of the molecular mechanisms of its preventive effect against neurological diseases.

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REFERENCES


