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Research Article

Overproduction of anthocyanin in ginseng hairy roots enhances their antioxidant, antimicrobial, and anti-elastase activities

Sora Jin · Seounggun Bang · Min-A Ahn · Kyubin Lee · Kyunghwan Kim · Tae Kyung Hyun

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Abstract Genetic engineering is a potential approach to improve secondary metabolism in plants. In order to elucidate the effect of production of anthocyanin pigment 1 (*PAP1*) overexpression on the bioactivity of ginseng, we analyzed its antioxidant, antimicrobial, and anti-elastase activities in this study. Our results showed that *PAP1* overexpression increased the production of polyphenolic compounds including anthocyanins. The antioxidant, antimicrobial, and anti-elastase activities were stronger in anthocyanin-overproducing ginseng hairy roots (AOX) than in wild ginseng hairy roots. Using a different solvent system (0, 30, 70, and 100% (v/v) EtOH), we revealed that variations in the contents of the polyphenolic compounds were highly correlated with changes in the antioxidant and antimicrobial activities of AOX. The antioxidant, antimicrobial, and anti-elastase effects of AOX highlight genetic engineering as a powerful approach to enhance the therapeutic properties of plants. Our results show that AOX could potentially have various functional applications in the cosmetic and pharmaceutical industries.

Keywords Antioxidant activity, Anthocyanin, Antimicrobial activity, Ginseng hairy root

Introduction

Anthocyanins are glycosylated polyphenolic compounds synthesized in the cytoplasm and stored in vacuoles (Chanoca et al. 2015). Although anthocyanins are well known as water-soluble flavonoid pigments with colors ranging from orange and red to purple and blue in flowers, seeds, fruits, and vegetation, they are important in attracting pollinators, seed dispersal by promoting fruit consumption, as well as plant protection against biotic and abiotic stresses owing to their antioxidant properties (Liu et al. 2018). There are numerous papers regarding the health benefits of anthocyanins because of their antioxidant, anti-inflammatory, and anti-cancer effects (Alappat and Alappat 2020), indicating that they are integrally involved interactions between humans and nature. In higher plants, anthocyanin biosynthesis is modulated by the transcriptional complex MYB-bHLH-WD40 (MBW complex) comprising DNA-binding R2R3 MYB transcription factors, basic helix-loop-helix (bHLH) proteins, and WD40 repeat proteins (Xu et al. 2015). Of the three proteins that form the MBW complex, R2R3 MYB transcription factors act as a master regulator of the entire set of anthocyanin biosynthesis genes including chalcone synthase, chalcone isomerase, flavanone 3-hydroxylase, and flavonol synthase (Mehrtens et al. 2005), indicating that the R2R3-MYB transcription factors could be significant in increasing anthocyanin in crops for value-added traits. Ginseng (*Panax ginseng* C.A. Meyer) is the most widely consumed herbal plant because of its various pharmacological benefits such as neuroprotective, internal secretion adjustment, protective cardiovascular, anti-aging, anti-tumor, and immunomodulatory effects (Choi et al. 2013; Kim 2018). Although ginsenosides, triterpene saponins, are well known as major bioactive ingredients of ginseng, anthocyanins in ginseng berry have been reported to directly inhibit the activity of tyrosinase in α-melanocyte-stimulating hormone-stimulated B16F10 cells (Jin et al. 2019). Moreover, the whitening activity of ginseng has been shown to be improved by Production of Anthocyanin Pigment 1 (PAP1, Arabidopsis R2R3-MYB type transcription factor)-dependent anthocyanin accumulation in ginseng hairy roots (Jin and Hyun 2020),

S. Jin · S. Bang · M.-A. Ahn · T. K. Hyun (✉)
Department of Industrial Plant Science and Technology, College of Agricultural, Life and Environmental Sciences, Chungbuk National University, Cheongju 28644, Republic of Korea
e-mail: taekyung7708@chungbuk.ac.kr

K. Lee · K. Kim
School of Biological Sciences, College of Natural Sciences, Chungbuk National University, Cheongju 28644, Republic of Korea

indicating that the accumulation of anthocyanins in ginseng roots can improve its pharmaceutical properties.

In this study, we examined the antioxidant, antimicrobial, and anti-elastase activities of *PAP1*-overexpressing ginseng hairy roots to understand the effect of anthocyanins on the pharmaceutical properties of ginseng. This study is expected to garner further interest in the use of anthocyanin-overproducing ginseng roots as a novel functional resource in the cosmetic and pharmaceutical industries.

Materials and methods

Plant materials and extraction

In a previous study, we generated *PAP1*-overexpressing ginseng hairy roots using Agrobacterium rhizogenes R1601 (Jin and Hyun 2020). The anthocyanin-overproducing ginseng hairy roots (AOX) and transgenic controls (TC) were cultured on agar-solidified B5 medium at 22 °C in the dark. Four-week-old AOX and TC were harvested, ground, and freeze-dried. The materials were then soaked in different extraction solvents including water, 30, 70, and 100% (v/v) EtOH for 24 h and sonicated in an ultrasonic bath. Each solvent extract was evaporated to produce a dried powder extract.

Determination of total phenolic and flavonoid content

In order to determine total phenolic content (TPC), each extract was mixed with 2 N Folin–Ciocalteu reagent and incubated with 20% Na_2CO_3 for 15 min. The absorbance of the resultant blue colored solution was recorded at a wavelength of 725 nm. The TPC for each extract was calculated using the equation obtained from the standard gallic acid graph and expressed in milligram gallic acid equivalents (μg GAE/mg extract).

Total anthocyanin content (TAC) for each extract was quantified as described by Jin and Hyun (2020). The absorbance was determined at 530 and 657 nm and the TAC was calculated as (A530 – 0.25*A657) / mg of dry weight.

Chemical-based assays to quantify antioxidant activity

The antioxidant activities of various extracts were determined by monitoring the disappearance of 1,1-dephenyl-2-picryl-hydrazyl (DPPH) at 520 nm and the reducing power assay at 750 nm, as described by Jin et al. (2019). The concentration of sample required to reduce DPPH absorbance by 50% (RC_{50}) was calculated for each sample.

In order to determine the ferric reducing antioxidant power (the ability to reduce Fe^{3+} to Fe^{2+}), different concentrations of samples were mixed with sodium phosphate buffer (0.2 M, pH 6.6) and potassium ferricyanide (1%, w/v). After incubation at 50 °C for 20 min, the reaction was stopped by adding 10% trichloroacetic acid. Then, 0.5 ml of reaction mixture was mixed with same volume of distilled water and 0.1 ml of 0.1% (w/v) ferric chloride. The absorbance of the sample was measured at 750 nm.

The final assay solution contained 150 μl of 0.08 μM fluorescein, 25 μl of phosphate buffer (blank), Trolox standard (6.25 ~ 50 μM), and each extract. After incubation at 37 °C for 10 min in the dark, 25 μl of 0.12 g/ml fresh 2,2'-azobis(isobutyramidine) dihydrochloride was added. A SpectraMax Gemini EM microplate reader was used with fluorescence filters (excitation at 485 nm and emission at 525 nm). The fluorescence of the mixture solution was recorded every minute for 90 min. Area under the curve was calculated for each sample by integrating the relative fluorescence curve. ORAC values were expressed as μM of Trolox equivalents (μM TE).

Determination of antimicrobial activity

The antimicrobial activity of each extract was tested against eight bacterial species: gram-positive *Kocuria rhizophila* (KACC 14744), *Micrococcus luteus* (KACC 14819), *Listeria monocytogenes* (KACC 19115), and *Staphylococcus aureus* (KACC 1916) as well as gram-negative *Enterobacter cloacae* (KACC 11958), *Salmonella enteritidis* (KACC 12021), *Salmonella enterica* subsp. enterica (KACC 10769), and *Pseudomonas aeruginosa* (KACC 2004). The minimum inhibitory concentration (MIC) was determined by an anti-bacterial assay performed with the two-fold serial dilution method using 96 U-bottom microtiter plates. The lowest concentration showing growth inhibition in comparison with the control was defined as MIC.

Neutrophil elastase inhibition assay

In vitro inhibitory effects of each sample against neutrophil elastase were determined using Neutrophil Elastase Inhibitor Screening Kit (BioVision, Milpitas, CA, USA) according to the manufacturer's instructions.

Statistical analysis

The results were presented as mean ± standard error (SE)

of the indicated number of experiments (n ≥ 3). One-way analysis of variance (ANOVA) followed by Duncan's multiple-range test was used to determine statistically significant differences between the groups. $P < 0.05$ was considered statistically significant.

Results and discussion

Influence of ethanol concentration on the extraction of phenolic and anthocyanin compounds

Selecting the solvent is one of the most important steps in extraction of bioactive compounds from plant materials. Among pigments, chlorophylls and carotenoids are hydrophobic or nonpolar, whereas anthocyanins are polar molecules (Mattioli et al. 2020; Pérez-Gálvez et al. 2020). Therefore, polar solvents including ethanol and methanol are frequently used for extraction of polyphenolic compounds such as anthocyanins. Considering safety and final potential use in the industry (Wendakoon et al. 2012), we used different concentration of EtOH to compare the extraction efficiency of polyphenolic compounds from AOX and TC. As shown in Figure 1A, the 30% EtOH extract of AOX contained the highest TPC of 100.71 ± 4.17 μg GAE/mg of extract, whereas the lowest TPC of 27.10 ± 0.89 μg GAE/mg of extract was observed in the 100% EtOH extract of TC. Furthermore, TAC was only detected in AOX samples in the following order: 70% EtOH > 30% EtOH > 0% EtOH (water) > 100% EtOH (Fig. 1B). This indicates that a binary-solvent system (EtOH/water) is more effective than a mono-solvent system (water or EtOH) in the extraction of polyphenolic compounds from ginseng hairy roots. It is known that the solvent polarity significantly affects the extraction yield of phytochemicals from plant tissues (Kim 2020). The TPC and TAC changed based on solvent polarity (Fig. 1), indicating that ginseng hairy roots contain diverse polyphenolic compounds with varying polarity.

Antioxidant properties of AOX

Although reactive oxygen species (ROS) play an important role as intracellular signalling molecules, an imbalance between ROS-generating and ROS-scavenging systems causes oxidative stress, which induces lipid peroxidation, and disrupts DNA, RNA, as well as protein functions (Darbandi et al. 2018). Therefore, antioxidant therapy using free radical scavengers, such as polyphenolic compounds, has been receiving increasing attention as a useful strategy to restore the impaired balance between ROS and antioxidant systems. We hypothesised that that an increase in polyphenolic compounds caused by *PAP1* overexpression

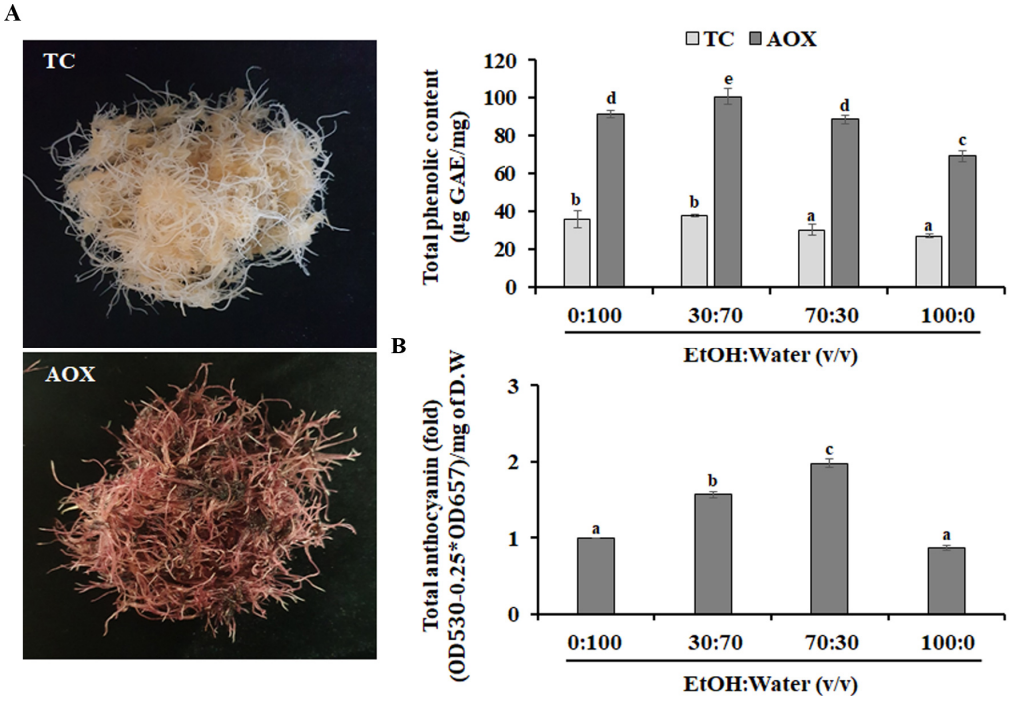

Fig. 1 Effects of *PAP1* on total phenol (A) and total anthocyanin contents (B) in ginseng hairy roots. Anthocyanin-overproducing ginseng hairy roots: AOX; Transgenic control ginseng hairy roots: TC. Different letters denote significant differences ($p < 0.05$, Duncan's test)

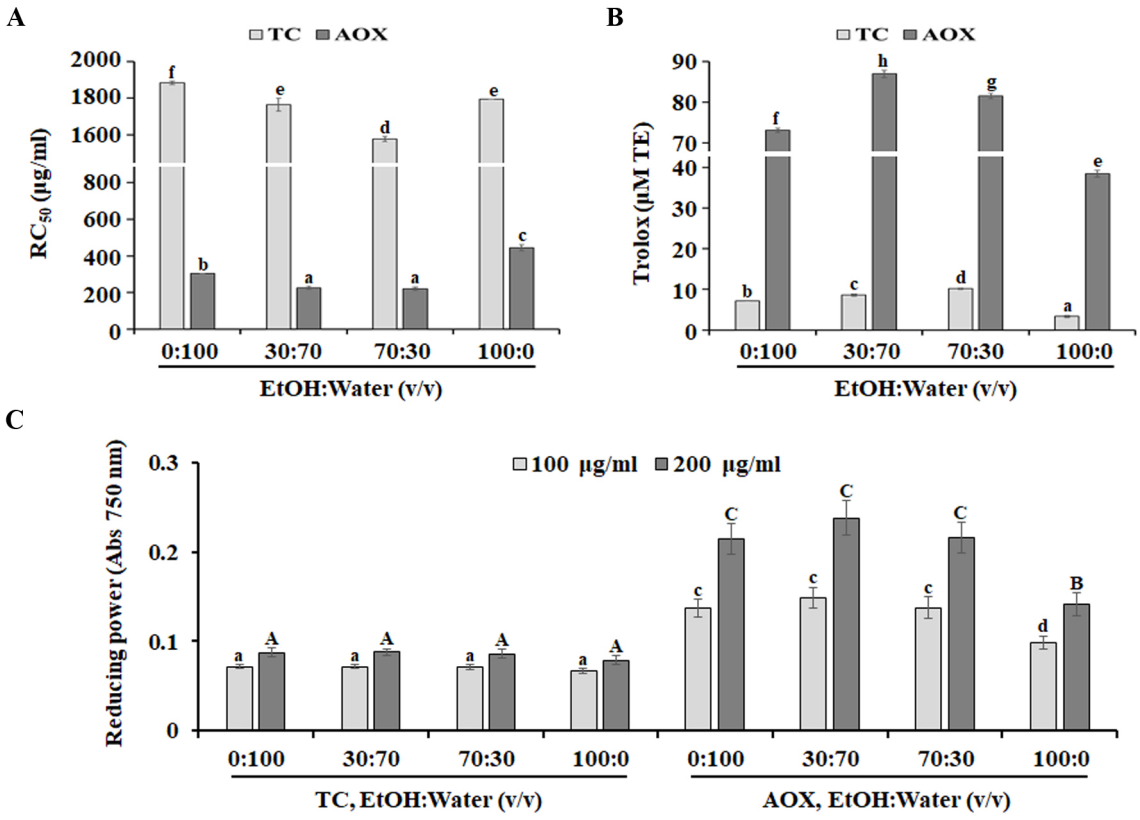

Fig. 2 Antioxidant activity of anthocyanin-overproducing ginseng hairy roots. The antioxidant activity was measured based on DPPH-free radical scavenging activity (A), ORAC assay (B), and reducing power (C). DPPH-radical scavenging activities were expressed as RC_{50} values (μg/ml). ORAC values for each extract are calculated in μM of Trolox equivalents. Values are averaged from triplicate experiments and represented as mean ± SE. Different letters denote significant differences ($p < 0.05$, Duncan's test). Anthocyanin-overproducing ginseng hairy roots: AOX; Transgenic control ginseng hairy roots: TC

influences the antioxidant properties of ginseng hairy roots. As shown in Figure 2A, AOX extracts exhibited a higher level of DPPH-free radical scavenging activity (RC_{50} between 220.9 and 444.5 μg/ml) compared with that exhibited by TC (RC_{50} between 1577.8 and 1883.9 μg/ml). Furthermore, AOX and TC extracts at 40 μg/ml exhibited ORAC values of 38.5 ~ 87.0 μM TE and 3.3 ~ 10.2 μM TE, respectively (Fig. 2B). Moreover, 200 μg/ml of AOX extracts had OD700 values ranging from 0.14 to 0.24, which were higher than those of TC (0.08 to 0.09) indicating stronger activity of AOX extracts. AOX extracts that were extracted with 30 and 70% EtOH exhibited strong DPPH-free radical scavenging activity and high ORAC values, whereas those extracted with 100% EtOH exhibited the lowest antioxidant activity (Fig. 2). These results indicate that the polyphenolic constituents are responsible for the antioxidant activities of plants, thereby validating our hypothesis that *PAP1* overexpression can improve the antioxidant properties of ginseng hairy roots.

Antimicrobial activity of anthocyanin-overproducing ginseng hairy roots

Despite advancements in modern medicine, infectious diseases remain a major public health problem (Cos et al. 2006). The emergence and dissemination of multidrug-resistant human pathogens have also become a significant public health hazard. The World Health Organization has recognized medicinal plants as a potential source to obtain various antimicrobial agents (Cheesman et al. 2017). Therefore, there has been revived interest in phytochemicals with antimicrobial activities to treat infectious diseases. In order to investigate the antimicrobial activity of ginseng hairy roots, we determined the MIC of each extract using the serial two-fold dilution method (Table 1). Overall, the 30 and 70% EtOH AOX extracts were more effective than the others. The 70% EtOH AOX extract was most active against *Staphylococcus aureus* (MIC = 250 μg/ml) compared to all the bacteria tested. *S. aureus* is the causative agent of multiple infectious human diseases such as bacteremia, skin, prosthetic device, and pulmonary infections (Tong et

Table 1 Antimicrobial activity of anthocyanin-overproducing ginseng hairy roots

		MIC (μg/ml)[1)]							
Sample	EtOH:Water (v/v)	S.a[2)]	M.l	K.r	L.m	S.e	E.c	S.s	P.a
TC[3)]	0:100	-	-	1000	1000	1000	1000	-	-
	30:70	-	-	1000	1000	-	1000	-	-
	70:30	1000	-	1000	1000	-	1000	-	-
	100:0	-	-	1000	-	-	1000	-	-
AOX	0:100	-	-	-	1000	500	1000	-	-
	30:70	1000	-	500	500	500	500	1000	-
	70:30	250	-	500	500	500	500	1000	-
	100:0	-	-	1000	-	-	1000	-	-
AMP		7.8	7.8	15.6	31.2	31.2	7.8	62.5	7.8

[1)]MIC values against bacteria were determined using the two-fold serial dilution method.
[2)]S.a.: *Staphylococcus aureus* 1916; M.l.: *Micrococcus luteus* 14819; K.r.: *Kocuria rhizophila* 14744; L.m.: *Listeria monocytogenes* 19115; E.c.: *Enterobacter cloacae* 11958; S.e.: *Salmonella enteritidis* 12021; S.s.: *Salmonellaentrica* sub sp.enterica 10769; P.a.: *Pseudomonas aeruginosa* 2004
[3)]TC: transgenic control; AOX; anthocyanin-overproduction line; AMP: ampicillin

al. 2015), indicating that AOX is a potential resource to obtain non-antibiotic agents against *S. aureus*. Anthocyanins are antimicrobial agents known to destroy the structure of pathogenic bacteria by inducing cytoplasmic leakage (Ma et al. 2019), indicating that the high level of antimicrobial activity in the 30 and 70% EtOH AOX extracts could be because of the presence of high levels of anthocyanins in extracts.

Anti-elastase activity of anthocyanin-overproducing ginseng hairy roots

Wrinkles and loss of skin elasticity are typical phenomena of skin aging, which is caused by the loss of structure of extracellular matrix (ECM) (Trojahn et al. 2015). Degradation of ECM is mainly caused by increasing activity of aging-related enzymes including elastase (a serine proteinase), which is primarily responsible for the breakdown of elastin in ECM (Pientaweeratch et al. 2016). Therefore, the inhibitors of elastase can be potential cosmetic ingredients to prevent skin aging. As shown in Figure 3, 70% EtOH AOX extract exhibited the largest inhibitory effect on the elastase activity compared with that of the other extracts. AOX extracted using 70% EtOH (100 μg/ml) significantly inhibited elastase activity (20.5 ± 0.3%), whereas AOX extracted using water (0% EtOH) showed low inhibitory effects (8.6 ± 1.2%). Similarly, 70% EtOH TC extract exhibited higher inhibitory activity than other TC extracts. Interestingly, AOX extracts contained higher levels of TPC and TAC than those in TC extracts; however, there was no dramatic difference in the elastase inhibitory activities

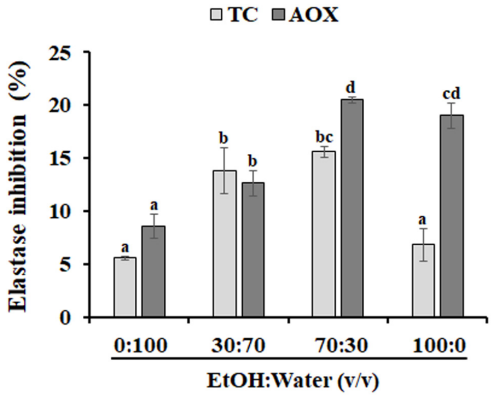

Fig. 3 Anti-elastase activity of anthocyanin-overproducing ginseng hairy roots. Values represent the mean ± SE from triplicate experiments. Different letters denote significant differences ($p < 0.05$, Duncan's test). Anthocyanin-overproducing ginseng hairy roots: AOX; Transgenic control ginseng hairy roots: TC

between the AOX and TC extracts, except the 100% EtOH extracts. These results indicate that polyphenolic compounds in ginseng hairy roots are not the major anti-elastase compounds. Ginsenosides have been reported as the main anti-aging ingredients in ginseng (Lai et al. 2018). Although some of polyphenolic compounds also act as inhibitors of aging-related enzymes, this finding suggests that the variation in anti-elastase activities between solvent systems could be because of the presence of other active compounds such as ginsenosides.

Conclusion

In order to determine the biological activities of AOX, we

analyzed the antioxidant, antimicrobial, and anti-elastase activities of extracts prepared using different solvent systems. We found that AOX extracted using 30 and 70% EtOH have strong antioxidant and antimicrobial activities. Although the effects of AOX extracts have been established only *in vitro*, these results indicate that overproducing anthocyanins through genetic engineering is a promising strategy to improve the pharmaceutical properties of ginseng hairy roots.

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