



Neuroactive and other free amino acids in seed and young plants of *Panax ginseng*

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Abstract

The seeds and one to three years old plants of Asian ginseng (*Panax ginseng* C.A. Meyer) were analyzed for their free amino acid contents. The neuro-excitatory β -ODAP (β -*N*-oxalyl-L- α , β -diaminopropionic acid), suggested to be the cause of the crippling neurologicalism, was the major component in the seed extract (70% of the total free amino acids detected) and showed the highest concentration (0.43% by wt) compared to that in the different parts of young plants. β -ODAP concentration was higher in the shoots as compared to roots and declined in older plants. The amount of β -ODAP in the roots may be considered as an indirect measure of age and quality. Another neuro-active non-protein amino acid, GABA (γ -aminobutyric acid), increased dramatically after germination and reached highest concentration in different parts of 3 year-old plants. Glutamine and arginine were the two major free proteinogenic amino acids in the ginseng plants and together they constituted over 50% of all the free amino acids detected in the root.

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1. Introduction

Ginseng roots have been used in Asian countries since ancient times as herbal medicine for enhancing physical strength and longevity. Preparations of ginseng root are becoming increasingly popular in Europe and North America. Most preparations contain root or root extracts of *Panax ginseng* C.A. Meyer, a plant originating from Korea and North China. The North American species *Panax quinquefolius* L. is considered less potent. Tea of ginseng leaves is also commercialized.

The multifunctional effects of ginseng have been studied mostly in mice, rats or with cultured cell lines. Ginseng root extracts were reported to have antioxidant activity (Hu and Kitts, 2001), to show a dose dependent stimulation of inducible nitric oxide synthase (NOS) and NO production (Park et al., 2001; Friedl et al.,

2001), to be anti-carcinogenic and to reduce cancer incidence (Yun et al., 2001; Shibata, 2001), to affect neuronal function in preventing dementia and also in enhancement of cognitive functions (Mantle et al., 2000; Rudakewich et al., 2001). However, the efficacy of the above mentioned activities from ginseng roots has not been conclusively established and some adverse effects and interactions with drugs were reviewed recently (Coon and Ernst, 2002).

The major pharmacologically active components of *Panax* are considered to be ginsenosides (a diverse group of saponins) and more than 30 different ginsenosides have been identified (Harkey et al., 2001). While most research focused on ginsenosides in the roots, other constituents and other parts of the ginseng plants have been studied in less detail. However, recent studies showed that the extract of *P. ginseng* berries has anti-hyperglycemic, anti-obese effects and also significantly reduced plasma cholesterol levels in mice (Attele et al., 2002). Ginsenoside Re of this extract plays a significant role in antidiabetic action but is not associated with the changes of body weight and plasma cholesterol. This

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suggests that other constituents in the extract contributed to those effects.

Recently, dietary non-protein amino acids were implicated as potential factors in human disease of unknown etiology. Some of the over 900 naturally occurring non-protein amino acids, mostly from plant origin, can cause clinical disorders or poisoning, or can have beneficial effects as anti-carcinogens or by enhancing insulin production (Rubenstein, 2000). The naturally occurring non-protein amino acid β -ODAP (β -*N*-oxalyl-L- α , β -diaminopropionic acid; synonym BOAA: β -oxalyl-amino-L-alanine) was suggested to be the cause of neuro-lathyrism, a primary upper motor neuron disease, after over-consumption of grass pea seeds (*Lathyrus sativus* L.) during drought triggered famine episodes in Ethiopia, India and Bangladesh (Getahun et al., 1999). β -ODAP has been detected in older ginseng roots of different species and of different origins including *P. ginseng*, *P. notoginseng* and *P. quinquefolius* (Long et al., 1996). Nunn and coworkers (1997) also reported the presence of β -ODAP in 15 out of 18 commercial ginseng preparations (capsules, liquid and tablets) sold retail in the United Kingdom. The precursor of β -ODAP in grass pea was confirmed to be a heterocyclic non-protein amino acid BIA, β -(isoxazolin-5-on-2-yl)-L-alanine, by in vivo ^{14}C - and ^{15}N - tracer techniques (Lambein et al., 1990; Kuo et al., 1998) and also by in vitro enzymatic breakdown (Ikegami et al., 1999). The precursor BIA itself was found to be an agonist at non-NMDA type receptors and a weak neurotoxin in mouse cortical explants (Riepe et al., 1995).

In this paper we report the analysis of β -ODAP and other free amino acids in the rarely studied seeds and young plants of *P. ginseng*.

2. Results and discussion

2.1. β -ODAP and other non-protein amino acids

The results of amino acid analyses from 70% ethanol extracts of seeds and young plants of *P. ginseng* analysed by HPLC are presented in Table 1. The same samples were also analyzed by automatic amino acid analyser to confirm the identity of some peaks.

A high concentration of the neuro-excitatory amino acid β -ODAP (4.3 mg/g or 0.43%) was found in ginseng seed, comparable to the level of β -ODAP in high toxin landraces of *L. sativus* L. seeds from India (Raipur 0.69%), from Ethiopia (Elena 0.53%) and from Bangladesh (Jamalpur 0.46%). β -ODAP was the predominant component of the free amino acids in seed extracts: 70% of the free amino acids detected. Besides being present in the seeds of 21 *Lathyrus* species, mainly *L. sativus*, *L. cicera* and *L. clymenum*, β -ODAP was also found in some other genera of leguminous plants including 17

Acacia species and 13 *Crotalaria* species (Quereshi et al., 1977). Ginseng is the only non-legume plant in which β -ODAP is known to be present.

β -ODAP has been reported to show strong agonistic activity on recombinant AMPA (α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptors and has facilitating and/or prolonging effects on glutamatergic transmission by inhibition of glutamate transport (Kusama et al., 2000). This finding on recombinant receptors is supported by the results of another group (LaBella and Piccoli, 2000) working with rats, suggesting that β -ODAP increases the extracellular level of glutamate by a mechanism not related to its effect on the non-NMDA receptors, which might involve inhibition of glutamate transport in the brain of freely moving rats. Besides its neurotoxic properties, β -ODAP was reported to be gliotoxic causing the lysis of glial cells in neonatal rat astrocytes (Bridges et al., 1991) and to specifically inhibit tyrosine aminotransferase in mice (Shasi Vardhan et al., 1997). To date the molecular mechanism of β -ODAP leading to the upper motor neuron disease neuro-lathyrism is not yet fully understood. As its function in plants producing it, β -ODAP was proposed to be the carrier molecule for zinc ions in grass pea (Lambein et al., 1994) and recently it was also suggested to act as a hydroxyl radical scavenger (Zhou et al., 2001).

β -ODAP was also found in various parts of young ginseng plants: 3.38 mg/g in 1-year-old whole plants, while in 2-year-old plants 1.09 mg/g was found in the root and 1.54 mg/g in the shoot. In 3 year-old plants, β -ODAP was concentrated in the leaves and buds (2.09 mg/g), but less in stem (0.58 mg/g) and root (0.59 mg/g). The concentration of β -ODAP in the underground parts of older *P. ginseng* was reported to vary from 0.29 to 0.40% depending on the origin of the material, and it was mostly concentrated in the rhizome, rather than in the main roots (Long et al., 1996). β -ODAP concentrations were much lower in commercial preparations ranging between 0.004% (liquid) and 0.10% (capsules) (Nunn et al., 1997), this level being comparable to that of the low toxin lines of *L. sativus* seeds (Kuo et al., 2000). High intake of *L. sativus* seeds (*khesari* in India and *guaya* in Ethiopia) as the sole staple food in a diet for several months can cause human neuro-lathyrism in up to 6% of the population, especially for malnourished poor peasants. Ginseng is a rather precious commodity and the roots or its extracts and other commercial preparations used as herbal tonic have never been consumed in high quantity as staple food and thus the possibility to develop neuro-lathyrism from over-consumption is negligible to non-existent. Traditionally the value of the ginseng roots is judged by their age. From our present investigation it follows that low β -ODAP in older roots may be an indirect measure of age and quality.

Table 1

Neuroactive amino acids β -ODAP, GABA and other free amino acids (mg/g \pm S.D.)^a in 70% ethanol extracts of seeds and 1-year-, 2-year-, 3-year-old plants of *Panax ginseng* C.A. Meyer analyzed by HPLC

	Seed	1 year	2 years	3 years			
		Whole plant	Root	Stem + leaves	Root	Stem	Leaves + buds
Asp	0.124 \pm 0.007	0.527 \pm 0.018	0.335 \pm 0.013	0.547 \pm 0.062	0.607 \pm 0.006	0.193 \pm 0.018	0.861 \pm 0.074
β -ODAP	4.294 \pm 0.030	3.381 \pm 0.335	1.096 \pm 0.052	1.537 \pm 0.020	0.596 \pm 0.015	0.579 \pm 0.027	2.094 \pm 0.115
α -AAA	ND ^b	0.117 \pm 0.004	0.157 \pm 0.011	0.194 \pm 0.031	0.086 \pm 0.012	0.215 \pm 0.023	ND
Glu	0.327 \pm 0.021	0.374 \pm 0.016	0.270 \pm 0.005	0.213 \pm 0.048	0.230 \pm 0.008	0.293 \pm 0.018	0.380 \pm 0.020
Ser	0.012 \pm 0.001	1.371 \pm 0.034	0.245 \pm 0.014	0.799 \pm 0.028	0.465 \pm 0.011	0.385 \pm 0.065	0.546 \pm 0.083
Asn	0.036 \pm 0.007	3.517 \pm 0.237	0.751 \pm 0.008	1.318 \pm 0.081	2.136 \pm 0.043	1.013 \pm 0.060	0.937 \pm 0.060
Gly	0.019 \pm 0.002	0.085 \pm 0.005	0.058 \pm 0.005	0.112 \pm 0.008	0.142 \pm 0.005	0.107 \pm 0.016	0.163 \pm 0.015
Gln	0.050 \pm 0.006	10.053 \pm 0.524	2.599 \pm 0.016	3.119 \pm 0.263	14.363 \pm 0.393	5.961 \pm 0.112	9.314 \pm 0.730
β -Alanine	ND	0.146 \pm 0.010	0.019 \pm 0.009	0.090 \pm 0.040	0.053 \pm 0.017	ND	ND
His	ND	0.176 \pm 0.017	ND	0.113 \pm 0.006	0.968 \pm 0.062	0.224 \pm 0.037	ND
Taurine	ND	0.092 \pm 0.003	0.241 \pm 0.044	0.128 \pm 0.030	0.221 \pm 0.045	0.047 \pm 0.011	0.169 \pm 0.019
Citrulline	ND	ND	ND	ND	ND	0.370 \pm 0.036	ND
Arg	0.573 \pm 0.001	3.257 \pm 0.104	9.104 \pm 0.297	1.437 \pm 0.059	9.572 \pm 0.284	1.196 \pm 0.032	7.966 \pm 0.276
Thr	0.014 \pm 0.003	0.709 \pm 0.053	0.197 \pm 0.001	0.208 \pm 0.017	0.679 \pm 0.016	0.141 \pm 0.002	0.240 \pm 0.023
GABA	0.051 \pm 0.010	1.175 \pm 0.085	0.972 \pm 0.006	2.284 \pm 0.182	1.778 \pm 0.041	2.335 \pm 0.043	2.774 \pm 0.260
Ala	0.205 \pm 0.017	1.258 \pm 0.074	1.448 \pm 0.023	0.973 \pm 0.049	2.720 \pm 0.694	1.741 \pm 0.067	2.197 \pm 0.367
Pro	0.056 \pm 0.008	0.272 \pm 0.029	0.260 \pm 0.005	0.624 \pm 0.026	1.106 \pm 0.021	0.761 \pm 0.050	0.675 \pm 0.117
Ethanolamine	0.082 \pm 0.008	0.800 \pm 0.096	1.030 \pm 0.031	0.985 \pm 0.049	0.557 \pm 0.052	0.664 \pm 0.096	0.826 \pm 0.095
Tyr	0.083 \pm 0.014	0.274 \pm 0.025	0.310 \pm 0.002	0.748 \pm 0.064	1.339 \pm 0.011	0.851 \pm 0.073	2.118 \pm 0.290
Val	0.011 \pm 0.013	1.422 \pm 0.042	0.416 \pm 0.009	0.650 \pm 0.027	0.623 \pm 0.040	0.512 \pm 0.042	0.631 \pm 0.019
Ile	0.014 \pm 0.004	0.939 \pm 0.038	0.233 \pm 0.012	0.426 \pm 0.012	0.654 \pm 0.027	0.309 \pm 0.023	0.574 \pm 0.038
Leu	0.033 \pm 0.012	0.954 \pm 0.067	0.341 \pm 0.019	0.618 \pm 0.030	1.251 \pm 0.003	0.504 \pm 0.008	1.052 \pm 0.054
Phe	ND	0.645 \pm 0.082	0.286 \pm 0.003	0.455 \pm 0.021	0.985 \pm 0.008	0.214 \pm 0.027	0.532 \pm 0.035
Trp	0.067 \pm 0.004	0.547 \pm 0.121	0.961 \pm 0.021	0.213 \pm 0.066	1.406 \pm 0.023	0.163 \pm 0.045	0.464 \pm 0.011
Lys	0.041 \pm 0.004	1.011 \pm 0.024	0.589 \pm 0.015	0.254 \pm 0.033	0.682 \pm 0.019	0.061 \pm 0.009	0.322 \pm 0.044

^a N = 4 (average of four analyses; two extractions and each extraction was injected twice).

^b ND: not detected; β -ODAP: β -N-oxalyl-L- α , β -diaminopropionic acid; α -AAA: α -amino adipic acid; GABA: γ -aminobutyric acid.

Another non-protein amino acid γ -aminobutyric acid (GABA), was found to be present in the seed in low concentrations (0.05 mg/g), while it gradually increased after germination and reached the highest concentration in 3-year-old plants. The presence of GABA and β -ODAP in older ginseng root was previously reported (Ye and Xing, 2001). GABA is known as inhibitory neurotransmitter in the central nervous system and three types of GABA receptors (A, B and C) have been identified on the basis of their pharmacological and electrophysiological properties (Enz, 2001). Whether GABA also plays a role in the complex ginseng effects needs to be studied.

Some other non-protein amino acids such as α -amino adipic acid (α -AAA) which is a recognized gliotoxin (Bridges et al., 1991), β -alanine, taurine, citrulline and ethanolamine were also found in low concentrations in some samples.

BIA, β -(isoxazolin-5-on-2-yl)-L-alanine, the biosynthetic precursor of ODAP in grass pea, was detected neither in seeds nor in young plants of *P. ginseng*. Likewise, BIA was not found in *Crotalaria* and *Acacia* species where ODAP was present (Lambein et al., 1990). Perhaps another alternative pathway for the biosynthesis of ODAP might exist in these three plant genera or BIA is

a short-lived intermediate and is under our detection limit.

One of the major peaks in the stem extracts of 3-year-old plants that was eluted at 10.9 min with an absorption max at 240 nm after PITC derivatization was not identified. This unknown non-protein amino acid was not detected in the seed but was found in other parts of the ginseng plants at much lower concentrations. After mild hydrolysis in 1 N HCl at 100 °C for 1 h this unknown peak completely disappeared while the peak of glutamic acid increased by 15-fold and other amino acids including serine, glycine, arginine and alanine increased to a lesser extent. This indicated that the unknown peak might be a γ -glutamyl peptide mentioned by other authors (Ye and Xing, 2001). Several minor peaks in the seed and plant extracts remain to be identified.

2.2. Free protein amino acids

In general, levels of free protein amino acids are low in the seeds. Arginine, glutamic acid and alanine are the major peaks next to β -ODAP (Table 1).

In 1-year-old plants, glutamine (10.05 mg/g) is by far the predominant amino acid followed by asparagine (3.52 mg/g) and arginine (3.26 mg/g). In 2-year-old

plants, arginine (9.10 mg/g) is dominant in the root while glutamine (3.12 mg/g) is quantitatively predominant in the shoot. In 3-year-old plants, glutamine is the most abundant amino acid, and together with arginine they made up about 55% of the total free amino acids in the root, 38% in the stem and 50% in the leaves and buds. Arginine was reported to be a major nitrogenous compound, representing approximately 60.6% of the free amino acid nitrogen and 13.6% of the total nitrogen, in three-year-old ginseng roots (Park et al., 1990) and an arginase of very high molecular weight was purified from the 3-year-old ginseng roots (Hwang et al., 2001).

L-Arginine is not only the substrate of arginase but also the substrate of nitric oxide synthase (NOS) and its high concentrations in the *Panax* root which is the major part used for herbal medicine might have some impact on the production of NO after consumption besides the reported effect of polysaccharides isolated from aqueous extract of *P. ginseng* root (Park et al., 2001; Friedl et al., 2001). The arginine present in ginseng root may support the increase in NO-production stimulated by other ginseng root components.

3. Conclusion

Although older ginseng roots (above 4 years) are normally used commercially for herbal medicines, other parts of the plant might also contain some constituents which have important pharmacological or physiological impact such as the berry extracts investigated recently and should be considered for further evaluation.

4. Experimental

4.1. Amino acid analysis by HPLC

4.1.1. Sample preparation and PITC derivatization

Seeds and different parts of ginseng plants were ground separately by hand with a mortar and pestle, except the three years old root parts that were homogenized by a small electric laboratory mill. 200 mg of each sample were weighed and extracted by 70% EtOH overnight together with 50 μ l of internal standard (100 μ mol/ml DL-allylglycine, Sigma). The extracts were centrifuged at 34 800 g for 20 min. The pellets were washed and centrifuged twice. The supernatants were pooled and concentrated under vacuum to dryness. To this, 0.5 ml deionized H₂O were added to make the aqueous extracts.

50 μ l aliquots of ginseng extract were derivatized with phenylisothiocyanate (PITC) as before (Khan et al., 1994) and dissolved in 500 μ l buffer A. After Millipore filtration (0.22 μ m), 20 μ l were injected. A standard

amino acid mixture (Food hydrolysate A 9656, Sigma) and purified β -ODAP was prepared as above as standards. The absorbance at 254 nm was used for calculations. The results were analyzed by Millennium software (Waters, version 1.10).

4.1.2. HPLC

A Waters 625 LC system with Waters 991 photodiode array detector was used. For reversed-phase HPLC, an Alltima C18 column (250 \times 4.6 mm I.D., 5 μ m particle size) from Alltech (USA) was used. A guard column cartridge (Alltima C18, 5 μ m) was directly connected to the column. The column temp was 43 $^{\circ}$ C and the buffer gradient system included buffer A: 0.1 M NH₄OAc, pH 6.5, and buffer B: 0.1 M NH₄OAc-ACN-MeOH (44:46:10; v/v/v), pH 6.5. The flow rate of 1 ml/min and the gradient program with an analysis time of 50 min were as previously described (Khan et al., 1994).

4.2. Amino acid analysis by automatic amino acid analyzer

4.2.1. Sample preparation

All plant materials (each 0.3–0.5 g) were dried at 40 $^{\circ}$ C and finely chopped into small pieces. An extract of each plant was prepared by macerating the plant material with 75% EtOH. The extracted solutions were filtered and evaporated to dryness in vacuum below 40 $^{\circ}$ C and then dissolved in 20 ml of 0.02 N HCl (pH 1.7–1.8). After filtration through a cellulose acetate filter (0.45 μ m), 50–100 μ l of each sample were injected. A standard amino acid mixture (AN-B type, Wako Pure Chemical Industries), glutamate and tryptophan (Sigma) were prepared as above as standards.

4.2.2. Amino acid analyzer

A Hitachi 835-10 automatic amino acid analyser in addition to post-column ninhydrin reaction under standard operating conditions (2.6 \times 250 mm column, #2619 resin, 33–68 $^{\circ}$ C, Li-citrate buffer system, pH 3.0–7.0, flow rate 0.275 ml/min) was used as previously described (Murakoshi et al., 1984). The results were calculated by Chromato-Integrator (Hitachi D-2500).

4.3. Source of plant material

Mature seeds of *P. ginseng* C.A. Meyer (Araliaceae) were collected from the medicinal plant garden of Nihon University College of Pharmacy, Funabashi, Japan. One to three year-old fresh, young plants of *P. ginseng* were collected from a ginseng cultivating farm at Mochizuki-machi, Nagano Prefecture, Japan.

Young plants were washed, separated into different parts, dried at 40 $^{\circ}$ C, weighed and then stored at –20 $^{\circ}$ C before analysis. Seeds were kept in a cool and dry place before analysis.

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