Korean J.Plant.Res. 5(1) 78~85(2002)

Constituents Analysis of Amino Acid and Antioxidative Activity from Cultivated Callus and Rhizome in *Rhodiola sachalinensis*

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ABSTRACT

The material of *Rhodiola sachalinensis* collected from an alpine region of the west-northern China. For analysing the effect, I used *Rhodiola sachalinensis's* rhizome and cultivated callus. In EtOAc, BuOH, H_2O separation the plant showed strong antioxidative activity, but not in Hexane. The radical scavenging effect of EtOAc(RC_{50}, 35(g), BuOH(RC_{50}, 43(g), H_2O(RC_{50}, 50(g) fraction and MeOH extract(RC_{50}, 50(g) of the *Rhodiola sachalinensis* was comparable to that of synthetic antioxidant BHA(RC_{50}, 14(g) and α-Tocopherol(RC_{50}, 12(g). Total amino acid concentration of plant of In nature condition were 18,009ppm, and major components were arginine, glutamic acid, aspartic acid and valine. The ratio of essential/total amino acid on plant of *In nature* condition was 46.93%. Total amino acid concentration of callus of *In vitro* condition were 32,435ppm, and major components were valine, histidine, lysine and leucine. The ratio of essential/total amino acid on callus of *In vitro* condition was 56.07%.

Key Words :*Rhodiola sachalinensis*, antioxidative activity, amino acid, callus, In vitr

INTRODUCTION

Rhodiola sachalinensis, which is distributed in West and North Asia, belongs to an angiosperm division, a sedum family, and a *Rhodiola* genus, and there are about 90 kinds of them in the world. Among them 73 kinds are growing in China (Kim et al.,1994).

The plants grows in Dongbei, Huabei, Xizang, and Xinjiang, especially in Xizang 34 kinds and 2 variations grow. It is a perennial herb and has fleshy rootstocks. It' s good for lack of vigor, longevity, oxygen deficiency, chilliness, fatigue, copy of microwave, improving attention and effects of working, geriatric deseases, physical strength, mental capacity, high or low blood pressure, memory, all kinds of nervousness, the coronary arteries, powerlessness, glycosuria, hemoptysis, pneumonia, a bruise, a burn (Kim and Park, 1997; Xiao and Kim, 1995). An aerobe including human beings live by processing energy metabolism with oxygen(O_2), but if the oxygen within body gets stressed by physical, chemical, biological action, it turns into harmful active oxygen species such as, superoxide anion radical (· O_2^-), peroxide of hydrogen

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(H_2O_2), and hydroxy radical (· OH), causes fatal biological difficulty, even deaseases and death. A living body is considered to be evolved developing antioxidative mechanism as a self-protection device for eliminating active oxygen species, but development of the species beyond defensive function of tissues damages factors of immune system like protein, DNA, ferment, and T-cells, and causes various deseases. Also it attacks the unsaturated fat acid which is an element of cell organism membrane, so this leads to peroxidation and accumulation of peroxide lipid, which causes aging and various deseases (Sies, 1985; Niki and Shimazaki, 1987; Steinberg et al., 1989; Okuda & Yoshikawa, 1990; Fukuzawa and Takaishi, 1990; Halliwell, 1991; Neuzil et al. 1993).

As the hypothesis that Active oxygen species is the cause of aging and disease of adult people is recently acknowledged, development of antioxidant which is known to control the active oxygen species is vigorously under way. Studies of antioxidative ferment such as, superoxide dismutase, peroxidase, catalase, glutathione peroxidase, and of a low molecule antioxidant such as, tocopherol, ascorbate, carotenoid, glutathione are going on (Pratt and Watts, 1964; Chang et al., 1977; Hammerschmidt and Pratt, 1977; Ryu et al.,1988). Synthesized antioxidant including BHT, BHA, and Troxol C are developed in abundant, and used in the field of medical supplies and food (Kitagara et al., 1992; Hatano, 1995; Masaki et al., 1995).

But there are some reports about the synthesized antioxidant, which reports that consumers don' t like to use them and they cause cancer in experimented animals (Branen, 1975), so its use is gradually being limited. Instead natural antioxidant is being made(Du and Xie, 1995; Kim et al., 1998; Sandberg, 1998; Yoshikawa et al., 1997; Yu et al., 1993), but the developed materials can' t outdo BHT and BHA in effect and expenses. Therefore, development of safer and more effective natural antioxidant is necessary.

Because plants do photosynthesis, oxygen consistency of their organism is very high. So they are likely to produce active oxygen species, and adapt themselves to the change in their surroundings (They cannot move, even if the surroundings are not good). Due to the reason, plants are expected to defense themselves by producing various active oxygen species. In this experiment antioxidative activity of *Rhodiola sachalinensis*, a herb of China, is examined by the DPPH free radical elimination method (Choi et al., 1993), and refining and separating is done in order to identify the structure of antioxidant from the ethyl acetate separation and butanol separation, which their activities are strong.

MATERIALS AND METHODS

Materials

The material is *Rhodiola sachalinensis* collected from an alpine region of the west-northern China in August, 1997. I germinated its seeds in March, 1998, and used young leaf explants grown about 5cm and its young leaves as cultivation materials. For analysing the effect, We used *Rhodiola sachalinensis*' s root and cultivated callus.

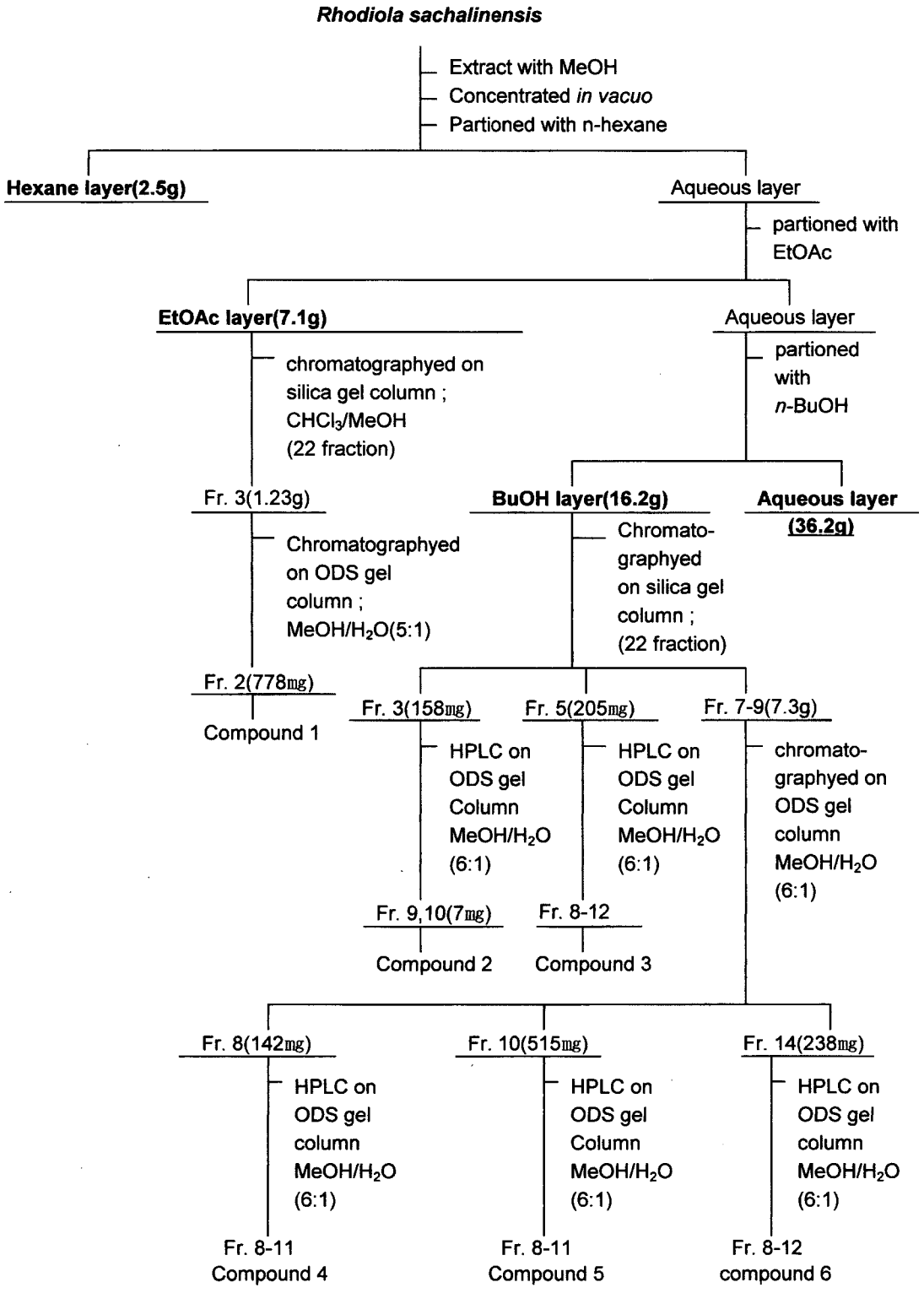
Methods of Constiuent Analysis

1. The Analyzing Method of antioxidative activity

1) Extraction and separation

Underground parts of *Rhodiola sachalinensis* dried in the shade in room temperature were cut in 2cm, and extracted with MeOH (10L) for 24 hours two times. They were concentrated and dried in a water bath with a decompression device, so MeOH (62g) were extracted.

For solvent separation of the extracts, they were suspended in distilled water. After that they were separated with a solvent using separation flask (3L) and



Scheme 1. Isolation Scheme of compounds 1~6.

Table 1. Operating conditions of amino acid autoanalyzer for analysis of amino acids.

Items	Conditions
Instrument	SYKAM Gmbh Amino Analyzer S7130 (Made in Germany)
Column	Cation Separation column(150(4mm), SYKAM
Buffer solution	pH 2.2~10.8, Sodium citrate
Flow rate	Buffer 0.5mL/min, Ninhydrin 0.25mL/min
Analysis time	64min
Reactor temp.	120℃
Column temp.	51~110℃
Detector	UV/Vis detector(570nm), (440nm:proline)
Injection volume	100μL

n-Hexane, Ethyl acetate (EtOAc), and n-Butanol (BuOH) in order. As a result, Hexane separation 2.5g, EtOAc separation 7.1g, BuOH separation 16.2g, and finally H_2O separation 36.2g were gained.

And then we measured antioxidative activity by dissolving some separation parts in MeOH and by using DPPH free radical elimination (Scheme 1).

2) Reagents and Experiment devices

Reagents

We used DPPH (1,1-dipheny1-2-2-picrylhydrazyl) and α-tocopherol (vitamine E) of Sigma, and BHA (3-tert-butyl-4-hydroxyanisole) of Kanto. Except the finest methanol (MeOH) of Daejung, which was used for first extraction from the plant, the other solvents were for ACS grade or HPLC of TEDIA and CARLO ERBA.

In order to measure absorbance, MILTON ROY company spectronic 20D made in U.S.A spectrophotometer was used.

Experiment devices

Rotary Evaporate(RE 47 YAMATO)
Circulator(SACO-41 SAN CHEON BIO TECH)
Sonicator(Branson 5510)
Spectrophotometer(MILTON ROY company spectronic 20D made in U.S.A)
Water Bath(KSB-55)
Turbing pump(MINIPULS 3 GILSON)
C18 column(YMC co.)
HPLC(Waters co.)

3) Measuring antioxidative activity by DPPH free radical elimination

We measured the antioxidative activity by DPPH free radical elimination for refining separation, which Choi et al. (1993) used. Samples of various consistency were dissolved in MeOH of 4mL, and DPPH solution (1.5 × 10M DPPH in MeOH) of 1mL was added. And then they were in room temperature for 30 min. and measured absorbance in 517nm.

We represented the amount of samples needed to reduce the absorbance of the counter group with no sample to half as RC_{50}. And I compared them with the existed antioxidant, α-tocopherol and BHA.

2. Method of an amino acid constituent analysis

For analyzing an amino acid element, Rhodiola sachalinensis roots dried in natural condition and cultured callus were grinded. The grinded Rhodiola sachalinensis roots of 300mg were put into the sample tube, and purged with N2 for 5 min. after adding HCl (6N) of 5mL for oxidation prevention.

After screwing tight the tube cap, they were left in heating block for 24 hours and hydrolyzed. Then they were left in cold temperature and HCl of the sample of 100 μl was eliminated from the heating block.

Following is the result of the amino acid analysis(Table 1) by passing the samples through SYKAM Gmbh Amino Analyzer S7130 (made in Germany).

RESULTS AND DISCUSSION

1. Antioxidative activity

In order to divide the separation parts extracted from *Rhodiola sachalinensis* with MeOH, I did the experiment as following:

Hexane separation products wasn' t activated, and the dividing process of EtOAc and MeOH is as following. First EtOAc separation parts were dissolved in little amount of MeOH with open column chromatography (Φ5cm × L50cm), which had silica gel and used a solvent.

The solvent was made of $CHCl_3$ and MeOH, and the proportion was lowered by subtracting 1L from each proportion as 100:0, 90:10, ..., 0:100, and then we adjusted the volume as 500mL, and got 22 separation parts.

Each fraction was tested, and only activated fractions were concentrated and examined with TLC plate. The result was that fraction 3 was activated. For more precise refining the fractions went through ODS gel open column chromatography with column (Φ2cm × L50cm) and a solvent, MeOH/H_2O (5:1), so Fraction 2 (compound 1) came out.

Because BuOH fraction was more in amount than EtOAc, I used bigger column (Φ6cm × L60cm) and separated. After charging them in Si-gel column and dissolving in small amount of MeOH, by subtracting 1L from each EtOAc and MeOH proportion. And the volume was adjusted as 500mL, so 22 fractions came out.

We did the activity test with each fraction, and concentrated only activized fractions by vacuum rotation. And we identified them with TLC plate, and the result was that fraction 3, 5, and 7-9 were activized. Fraction 3 and 5 were concentrated by vacuum rotation. We poured 1mL of them per minute in a test tube with HPLC ODS gel column was attached and the solvent, MeOH/H_2O (6:1) was put. As a result fraction 3 was activized in 9 and 10 tubes, and fraction 5 was activized in 8-9 tubes. So we could get compound 2 and 3.

There was a large amount of fraction 7-9, so they were gone through ODS gel open column chromatography with Φ2cm×L50cm open column and MeOH/H_2O (6:1). In 8, 10 and 14 fraction there were activities, we poured 1mL of them per minute in a test tube with HPLC ODS gel column was attached and the solvent, MeOH/H2O (6:1) was put.

Fraction 8 and 10 were activized in 8-11 tubes, and fraction 14 was activized in 8-12 tubes. These are called compound 4, 5, and 6. Compound 2-5 were detected with HPLC, and foreign matters came out, so we kept refining for complete pure compounds. H2O fraction was tested with DPPH reagent, but the activity wasn' t strong.

Herb medicines including plants for medicine have been used for prevention and healing of disease, but the constituent have not been defined yet. Recently some constituents are bad for human body, and there are lots of antioxidative activity elements, which is not good, some reports said(Yeo et al., 1995).

These elements are considered to be related to the effect of those herb medicine. The activity might depends on the culture environment and other factors. This experiment used Chinese *Rhodiola sachalinensis* to use them. And I examined the DPPH free radical activity by using MeOH extractions. Table 2 shows the results.

Table 2. DPPH free radical scavenging activities of methanol extracts from the aerial parts of *Rhodiola sachalinensis* and their solvent fractions.

Fractions	RC_{50}[z](μg)
MeOH extract	45
Hexane fraction	-
EtOAc fraction	35
BuOH fraction	43
H_2O fraction	50
BHA	14
α-Tocopherol	12

[z] Amount required for 50% reduction of DPPH after 30 min.

The extracted materials of underground parts of the plant showed strong activity, and were separated with a solvent, Hexane, $CHCl_3$, EtOAc, BuOH, and H_2O to examine the activity of each fraction. Hexane fraction didn' t show the activity, but EtOAc, BuOH, and H_2O showed strong activity.

So it could be concluded that the antioxidative activity material can be combined with materials of strong polarity. Although these are not completly refined crude fractions, showed relatively high activity and there exist various materials.

In Korea there are active studies about antioxidative activity with extractions of herb medicine including vegetables(Lee et al., 1992; Kim et al., 1995; Yeo et al., 1995; Choi, 1996; Kwak et al., Jhee and Yang, 1996), but the experiment methods are different from each study. Ultimately all the experiments showed similar results, so this experiment is meaningful in comparing with other results indirectly. For more direct comparison a further study needed.

2. Amino acid constituent

There are 17 kinds of amino acid(Table 3), and natural plants contain 18,009ppm, and cultured callus contain 32,435ppm amino acid.

Table 3.

Cultured callus contain more amino acid than natural plants, and this countributes to the growth environments and culturing condition.

Natural plants contain amino acid as following: arginine 2,384ppm (13.2%), glutamic acid 10.6%, aspartic acid 10.4%, valine 8.0%, leucine 7.6%, alanine 7.3%, lysine 6.0%, glysine 5.9%, histidine 5.1%, iso-leucine 5.0%, threonine 4.5%, serine 4.2%, cysteine 3.9%, phenylalanine 3.5%, tyrosine 2.0%, methionine 1.4%, NH_3 1.4%.

Cultured callus contain: valine 3,666ppm (11.4%), histidine 10.5%, lysine 9.7%, leucine 8.9%, NH_3 7.4%, arginine 7.2%, iso-leucine 6.7%, alanine 6.4%, aspartic acid 5.6%, glutamic acid 5.1%, tyrosine 4.9%, phenylalanine 3.8%, glysine 3.2%, methionine 3.2%, threonine 2.2%, cysteine 2.2%, serine 1.6%.

Essential amino acids of natural plants are valine, leucine, lysine, histidine, iso-leucine, threonine, phenylalanine, methoinine, and cultured callus have valine, histidine, lysine, leucine, iso-leucine, phenylalanine, methionine, threonine.

Generally cultured callus has more essential amino acids (18,187ppm, 2.5 times) than natural plants (7,371ppm). The proportion of essential amino acid to total amino acid of natural plants was 40.93%, and the

Table 3. Amino acid concentrations of *Rhodiola sachalinensis* root in *In nature* condition and callus derived from *Rhodiola sachalinensis* shoot tip in In vitro condition.

Amino acid	Plant of *In nature* condition		Callus of *in vitro* condition	
	Conc.(ppm)	(%)	Conc.(ppm)	(%)
ASP	1,865	10.4	1,794	5.6
THR	801	4.5	715	2.2
SER	758	4.2	520	1.6
GLU	1,913	10.6	1,651	5.1
GLY	1,069	5.9	1,040	3.2
ALA	1,322	7.3	2,054	6.4
CYS	705	3.9	715	2.2
VAL	1,442	8.0	3,666	11.4
MET	253	1.4	1,027	3.2
ILE	892	5.0	2,171	6.7
LEU	1,367	7.6	2,860	8.9
TYR	366	2.0	1,599	4.9
PHE	621	3.5	1,235	3.8
HIS	921	5.1	3,380	10.5
LYS	1,074	6.0	3,133	9.7
NH_3	256	1.4	2,392	7.4
ARG	2,384	13.2	2,340	7.2
[z]TAA	18,009	100	32,435	100
[y]EAA	7,371		18,187	
EAA/TAA(%)	40.93		56.07	

[z]TAA : Total amino acid

[y]EAA : Total essential amino acid(THR+VAL+ILE+LEU+PHE+HIS+LYS)

one of cultured callus was 56.07%. There was a report that *Rhodiola rosea* contains arginine and glutamic acid the most, and Ca content was the largest(Bao and Xu,1992), and those results were the same as this experiment.

According to Brekhman of Russia (1992), *Rhodiola rosea* was developed to be used for health in special situations such as improving endurance of spacemen and athletes, and as a adaptogen.

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(Received Apr. 15, 2002)
(Accepted Apr. 25,2002)