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Effect of Spore-forming *Eumycetes* on the Chemical Change of Korean Ginseng Components

Ⅱ. Spectroscopic Studies on Free Fatty Acid and Total Saponin in the Root of *Panax ginseng* C. A. Meyer†

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胞子形成眞菌類가 韓國産 人蔘成分의 化學的 變化에 미치는 影響

第 2 報: *Panax ginseng* C. A. Meyer 의 뿌리에 存在하는 遊離脂肪酸과 total saponin 의 分光學的 硏究†

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Abstract

Free fatty acid and total saponin, which were extracted from the spent media and colony of the strain *Phizopus delemar* Rh-1, were qualitatively examined by GLC and spectrosco pic methods and compared with those of intact ginseng media. The 18, 18, and 14 free fatty acids were respectively identified from the control, spent media and colony. Among them, $iC_{12:0}$, $C_{16:2}$, $C_{17:0}$, $C_{19:3}$ and $C_{20:0}$ acids were not detectable from the colony. In add ition, $C_{16:1}$ and $C_{20:0}$ peaks were not found in the spent media and control, respectively. The isible absorption spectra of free fatty acids and total saponins in the control, spent media and colony were invariable. In contrast, however, the infrared and ultraviolet absorption spectra showed significant spectral variations, particularly in the ir finger-print region, demonstrating that the ginseng components were considerably utilized by the strain *Rhizopus delemar* Rh-1.

Introduction

Korean ginseng(*Panax ginseng* C.A. Meyer), wh ich is a perennial plant growing naturally in Korea under the family *Araliaceae*, has been used by the Orientals as a mysterious panacea for nearly 5000 years[1]. Since Garriques[2] first isolated panaquilon, a glycoside from American ginseng (*Panax quinquefolium* L.) in 1854, scientific rese-

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arch on ginseng has been intensely carried out by a large number of investigators. Now Korean ginseng is generally recognized as an efficacious medicinal herb[1,3,4] and becoming increasingly popular in all over the world.

The vulnerability of ginseng to insects and diseases such as damping off and bacteria root rot, is one of the most serious problems in the production of ginseng as well as the preservation. Ginseng root is easily rotted by microbial propagation even after harvest, particularly under a humid condition. The noxious microorganisms as well as various methods and techniques of controlling the diseases of ginseng have been reported[5~7]. Despite some progresses made for the diseases control, however, the investigation of exterminable or preventive methods against ginseng diseases remains yet to be done.

Apart from the intense microbiological studies on ginseng, research on the chemical change of ginseng root affected by spore-forming *Eumycetes*, which are highly detrimental to ginseng products, has not been reported. It is within the bounds of possibility that the active principles of ginseng may be destroyed or consumed by microorganisms and the quality of ginseng be deteriorated. Although some physiological phenomena of microorgani sms dffer from those of higher animals, it seems to be still imperative that microorganism should be used as a diagnostic tool for the investigation of efficacious ginseng components. Thus the microbial studies on the chemical change of ginseng components may not only be valuable for providing an indirect information on the true nature of ginseng efficacies but also on a rating criterion of ginseng quality. To this end, we have isolated and identified the strain *Rhizopus delemar* Rh-1, which revealed the strongest propagating power on the root of *Panax ginseng* C. A. Meyer among the 9 species, as described in our previous report[8]. The objective of this paper was to report spectroscopic evidences for the chemical change of Korean ginseng root affected by the spore-forming *Eumycetes*.

Materials and Methods

Materials

Fresh 4 to 6-year-old ginseng root was directly obtained from the ginseng fields of Kanghwa, Kimpo, Keumsan and Punggi, immediately after harvest. The root was thoroughly washed with water and air-dried at about 15°C using an electric fan. The material was then sealed up with polyethylene film and kept in refrigerator at −20°C. The rotten root and soil were also collected from the same spot of ginseng fields. in order to isolate spore-froming *Eumycetes*.

Isolation and identification of microorganisms

The strain *Rhizopus delemar* Rh-1 was isolated and identified by same method as our previous paper[8]. The rotten and uninjured ginseng roots together with soil were kept in an incubator of 30°C to make microorganisms grow and maintained humidity by covering their surface with a wet cloth. The cultured ginseng root was transferred into sterilized water and shaken vigorously with gyratory agitation. Microorganisms in the supernatant were inoculated on malt-extract agar medium containing 5% crushed ginseng root and cultured for 48 hrs at 30°C. The newly formed spores were diluted with sterilized water, followed by an ordinary procedure for pure isolation. The isolated strains were inoculated on culture media as shown in Table 1. The morphological state of sporangiophore, sporangia, chlamydospore, zygospore, rhizoid, stolen, etc. was examined with optical microscope during culture. In addition, the physiological characteristics were not only studies on media -and temperature-dependent growth rates of the strains but also on sugar fermentability. To examine sugar fermentability, same mold species were inoculated on culture media supplemented with various sugars.

Growth of microorganisms

Malt-extract agarmedium was used for the growth of the strain *Rhizopus delemar* Rh-1. For experiments, the strain was inoculated on intact and neat ginseng root (850 *g*) and cultured for 3 days at 30°C.

Table 1. Culture media for the isolation of molds

Ingredient \ Media*	Ⅰ	Ⅱ	Ⅲ	Ⅳ
Glucose	30 *g*	50 *g*	Malt extract was added to Czapeck's solution agar.	Oryzanin was added to the Pfeffer's solution.
$NaNO_3$	3 *g*	—		
KH_2PO_4	1 *g*	5 *g*		
KCl	0.5 *g*	—		
$MgSO_4 \cdot 7H_2O$	0.5 *g*	2.5 *g*		
$FeSO_4 \cdot 7H_2O$	0.01 g	—		
$FeCl_3$	—	trace		
Agar	1.5 *g*	20 *g*		
Distilled water	1000 *ml*	1000 *ml*		

* Ⅰ : Czapeck's solution agar
Ⅱ : Pfeffer's media
Ⅲ : Malt infusion Czapeck's solution agar
Ⅳ : Pfeffer's-oryzanin agar

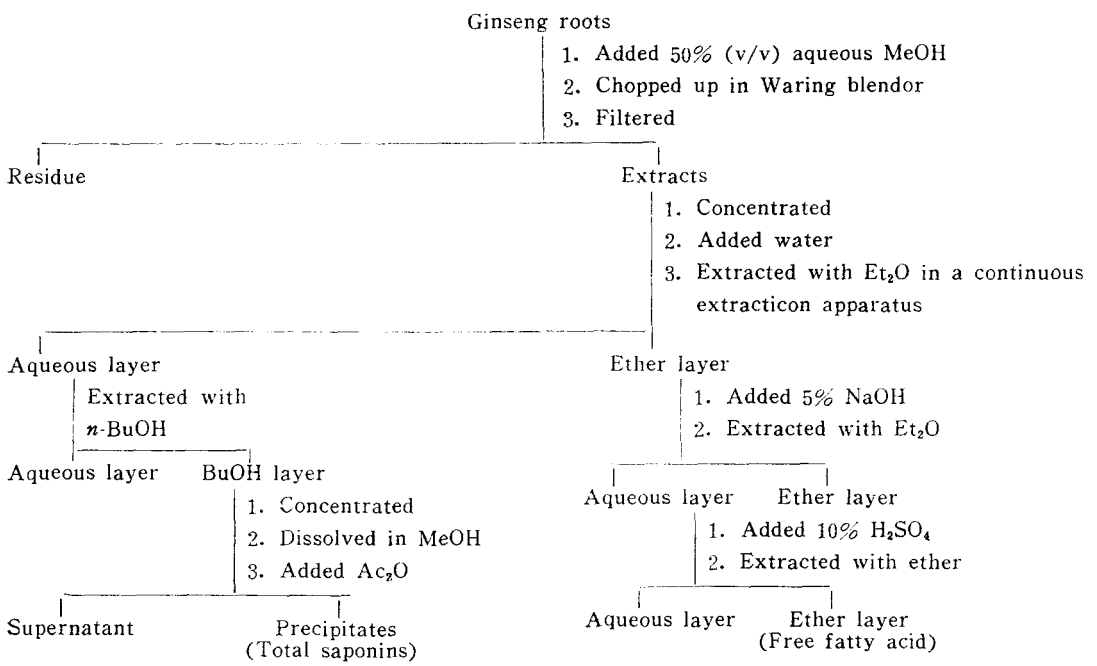

Fig. 1. Extraction of ginseng components

Extraction of ginseng components

Ginseng components were extracted by a modification of the procedures of Shibata[9] and Lee[10]. The 800 *g* each of uninjured ginseng root for control and spent medium separated out from mycelia were used for the extraction of ginseng components as shown in Fig. 1, except the mycelia (10 *g*) separated from the rotten root of ginseng. The mycelia were powdered in a mortar, followed by the same procedure as Fig. 1.

The sample was placed in Waring blendor containing 300 *ml* of 50% (v/v) aqueous methanol and chopped up into fine granules. The material was allowed to stand for 24 hrs at room temperature and filtered. The extracts were concentrated on a rotary evaporator under a reduced pressure. 50*ml* of water was added to the extracts, and then the mixture extracted with ether in a conti-

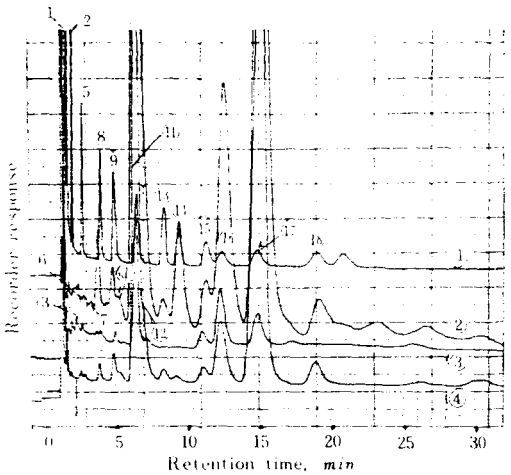

Fig. 1. Gas chromatogram of free fatty acids
①, authentic sample; ②, spent media
③, colony; ④, control

nuous extraction apparatus for 24 hrs. The ether layer was separated and aqueous layer extracted with *n*-butanol. The butanol layer was evaporated to dryness *in vacuo*. To the ether layer, was added 5% (v/v) sodium hydroxide solution and the mixtur extracted 3 times with ether. The ether extracts were washed with water, dried with anhydrous sodium sulfate and evaporated on a rotary evaportor to dryness. The aqueous layer was acidified with 10% (v/v) sulfuric acid solution and extracted 3 times with ether. The ether extracts were then washed with water, followed by usual workup.

Preparation of the methyl ester of free fatty acids

The methyl ester of free fatty acids was prepared by the boron trifluoride method of A.O.A.C.[11]. Fatty acid was introduced into a 50 *ml* reaction flask. After the addition of BF_3-MeOH reagent (5 *ml*) to the acid, condenser was attached and boiled for 2 min. To the reaction mixture, was added heptane (5 *ml*) thru condenser and boiled one minute longer. After the removal of heat, condenser was removed and enough saturated aqueous sodium chloride solution added into neck of flask in order to float the methyl ester of fatty acid in heptane. The heptane soulution (1 *ml*) was transferred into test tube and dried with small amount of anhydrous sodium sulfate to give rise a final volume of 2~3 *ml*. For gas-liquid phase chromatography, each sample was concentrated at 50°C under a reduced pressure and dissolved in chloroform (100 *μl*).

Spectrometry

The visible and ultraviolet absorption spectra were recorded on a Beckman Model 25 and the infrared absorption spectra on a Perkin-Elmer Model 735B. The gas-liquid phase chromatography was performed on a Hitachi Model 163 using a column of 15% DEGS (80~100 mesh Uniport B) under the following operation conditions:

Detector	: flame ionization detector
Column	: Stainless steel(3 *mm* I.D.×2.5 *m*)
Column temperature	: 180°C
Injection temperature	: 260°C
Flow rate	: N_2; 45 *ml*/min H_2; 35 *ml*/min Air; 600 *ml*/min
Sensitivity	: $10^2 \times 34$
Chart speed	: 10 *mm*/min
Sample injection volume	: 2 *μl*

Results and Discussion

The strain *Rhizopus delemar* Rh-1 was inoculated on ginseng media and cultured. Three groups-spent media, colony and control - were separately subjected to the isolation of free fatty acid and total saponin as shown in Fig. 1. The isolates were qualitatively examined by GLC and spectroscopic methods, and the spectra were compared. Water is reported as a superior solvent to alcohol for the extraction of ginseng components, except saponins which are more soluble in ethanol than water[12]. Methanol is a comparable solvent to ethanol, and we used 50%(v/v) aqueous methanol in this experiment in order to increase the amount of both water-and alcohol-soluble ginseng components.

The 18, 18 and 14 fatty acids were identified from the spent media, control and colony, respectively, by gas-liquid phase chromatographic method using a column of 15% diethylene glycol succinate (80~100 mesh Uniport B) at 180°C (Table 2 and Fig. 2). It seems likely that the fatty acids $C_{8:0}$, $C_{10:0}$, $C_{11:0}$, $C_{12:0}$, $C_{13:0}$, $iC_{14:0}$ and $C_{18:0}$ were not affected by the strain *Rhizopus delemar* Rh-1, The acid $C_{20:0}$ was found only from the spent media. This may

be due to biological degradation and/or to an ingestional selectivity of the acids by microorganism. The acid $iC_{12:0}$, $C_{16:2}$, $C_{17:0}$, $C_{18:3}$ and $C_{20:0}$ were not detectable from the colony but from the spent media and control. In contrast with the spent media and control, the colony showed greater peak area of $C_{11:0}$ and $C_{18:1}$ acids than those of $C_{12:0}$ and $C_{18:2}$ acids, respectively. The spent media and control showed greater peak area of $C_{18:2}$ acid than that of $C_{16:0}$ acid, while it was in the reverse order in case of the colony. And the greater peak area of $C_{15:0}$ acid than that of $C_{14:0}$ acid was not observed from the spent media and colony but the control. Compared with the control, the spent media showed distinctive increase in the peak of $C_{17:0}$ acid, and the colony did not show the acid at all. Cook and Ann[13] reported the absence of $nC_{18:3}$ acid in *Panax ginseng* C. A. Meyer. In accord with Lee's report[14], however, the presence of $nC_{18:3}$ acid was confirmed from this experiment.

Table 2. Methyl esters of free fatty acids in ginseng root, spent media and colony

Peak No.	Fatty acid		
	Ginseng root (control)*	Spent media**	Colony***
1	$C_{8:0}$	$C_{8:0}$	$C_{8:0}$
2	$C_{10:0}$	$C_{10:0}$	$C_{10:0}$
3	$C_{11:0}$	$C_{11:0}$	$C_{11:0}$
4	$iC_{12:0}$	$iC_{12:0}$	—
5	$C_{12:0}$	$C_{12:0}$	$C_{12:0}$
6	$C_{13:0}$	$C_{13:0}$	$C_{13:0}$
7	$iC_{14:0}$	$iC_{14:0}$	$iC_{14:0}$
8	$C_{14:0}$	$C_{14:0}$	$C_{14:0}$
9	$C_{15:0}$	$C_{15:0}$	$C_{15:0}$
10	$C_{15:1}$	$C_{15:1}$	$C_{15:1}$
11	$C_{16:0}$	$C_{16:0}$	$C_{16:0}$
12	$C_{16:1}$	—	$C_{16:1}$
13	$C_{16:2}$	$C_{16:2}$	—
14	$C_{17:0}$	$C_{17:0}$	—
15	$C_{18:0}$	$C_{18:0}$	$C_{18:0}$
16	$C_{18:1}$	$C_{18:1}$	$C_{18:1}$
17	$C_{18:2}$	$C_{18:2}$	$C_{18:2}$
18	$C_{18:3}$	$C_{18:3}$	—
19	—	$C_{20:0}$	—

* Identified fatty acids from ginseng-root media (conrol)

** Identified fatty acids from ginseng culture media which were separated out from colony

*** Identified fatty acids from colony

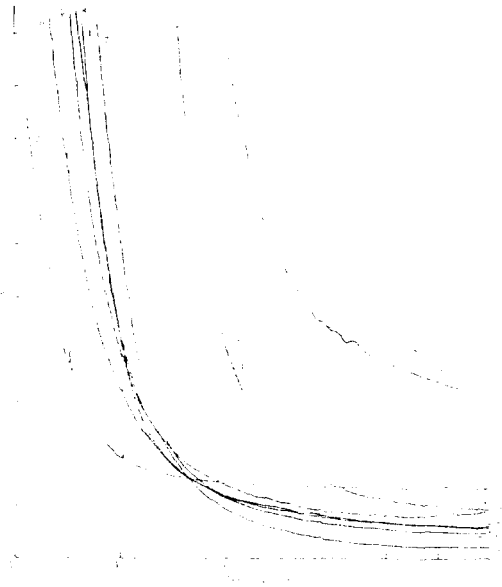

Fig. 2. Visible absorption spectra of free fatty acid and total saponin

①, free fatty acid in colony; ②, saponin in colony; ④, free fatty acid in control; ⑤, saponin in control; ⑦, free fatty acid in spent media; ⑧, saponin in spent media

The visible absorption spectra of free fatty acid and total of the spent media, colony and control were just alike and indistinguishable as shown in Fig. 3. In contrast to the visible spectra, however, the ultraviolet spectra showed singificant spectral variations, suggesting that ultraviolet absorption spectroscopy would be an efficient tool for the verification of chemical change of the ginseng components affected by microorganism. Compared with the control, the spent media and colony gave significant shift and intensity variation of ultraviolet absorption bands (Fig. 4, 5 and 6). In particular, the chemical change of free fatty acid in the control was more remarkable than that of total saponin.

The free fatty acid of the spent media, colony and control also showed variant infrared absorptions as shown in Fig. 7. In the control the hydroxyl stretching band at 3400 cm^{-1} was disappeared with lapse of time whereas the spent media and colony showed the strong absorption band.

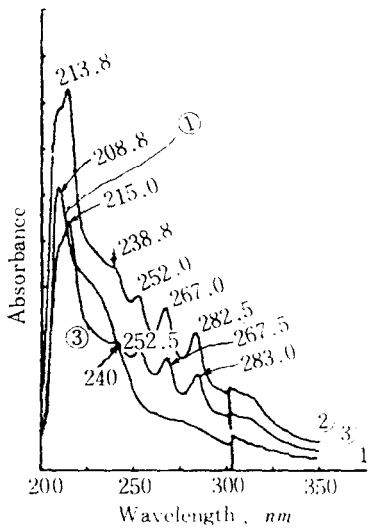

Fig. 3. Ultraviolet absorption spectra of free fatty acid and lipids

①, free fatty acid of colony; ②, lipids of control; ③, free fatty acid of control

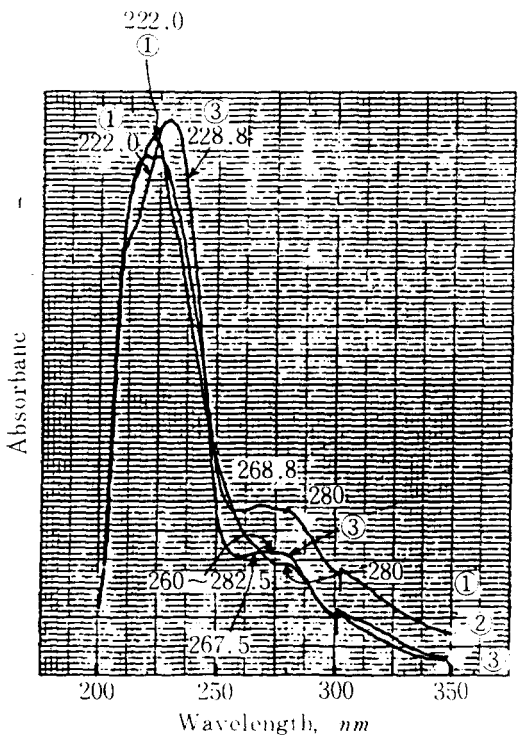

Fig. 4. Ultraviolet absorption spectra of free fatty acid, lipids and total saponin

①, saponin of control; ②, lipids of spent media; ③, free fatty acid of spent media

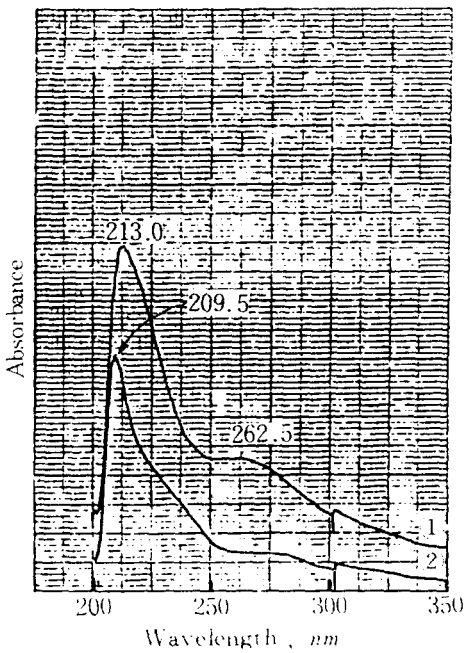

Fig. 5. Ultraviolet absorption spectra of total saponin

①, colony; ②, spent media

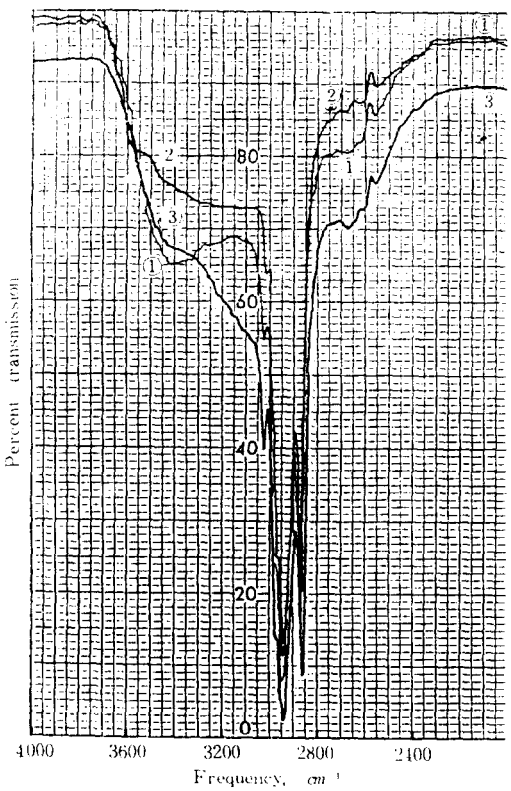

Fig. 6. Infrared absorption spectra of free fatty acid

①, control; ②, colony; ③, spent media

The carbonyl stretching band at 1710 cm^{-1} was greatly shifted to 1740cm^{-1} in case of the colony, and particularly the absorption frequencies were quite different from others in the finger print region (Fig. 8a and 7)· In the colony the 1415 cm^{-1} band (the dimeric OH in plane bending of carboxylate coupled with C-O stretching) was disappeared, whereas the 1160 cm^{-1} and 1090 cm^{-1} bands which were not observed from the spent media and control were strongly appeared (Fig. 9) [10, 15-24]. The hydroxyl bending vibration (out-of-plane) of COOH dimer at 940 cm^{-1} was completely disappeared from the spent media and colony. And the 800 cm^{-1} band, which was not observed from the spent media, was reappeared in the colony.

In the total saponin of colony the 1630 cm^{-1} band ($\nu_{C=C}$ of ginsenoside- Ro, -Rb_1, Rb_2, -Rc and -Rd) [18,20,24] was greatly shifted to 1720 cm^{-1} ($\nu_{C=O}$ of 6-membered ring), and this is presumably due to the biological oxidation of unsaturated double bond (Fig. 10 and 11) [21,25,26]. The 1410 cm^{-1} and 1310 cm^{-1} bands (coupling between in-plane OH bending and C-O stretching of COOH dimer) were respectively appeared in the colony and spent media. However, the 1075 cm^{-1} band (the ring frequency

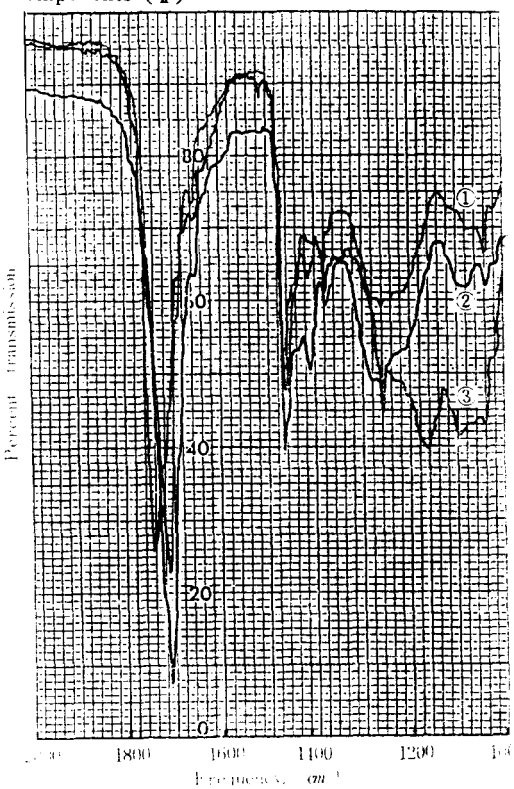

Fig. 7a. Infrared absorption spectra of free fatty acid
①, control; ②, colony; ③, spent media

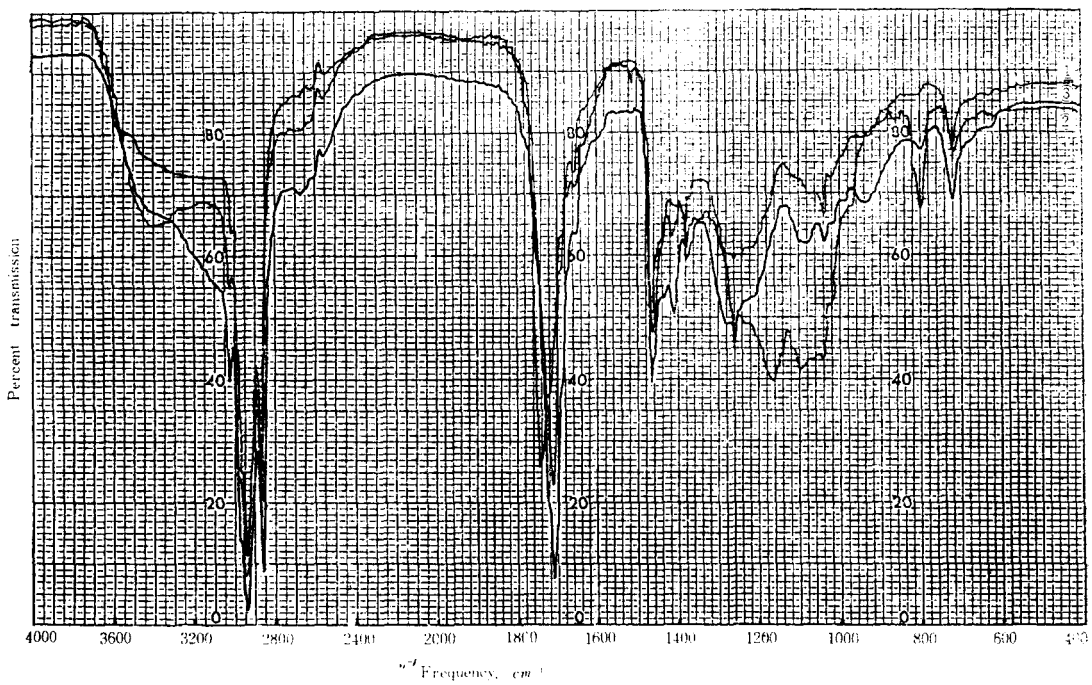

Fig. 7b. Infrared absorption spectra of free fatty acid
①, control; ②, colony; ③, spent media

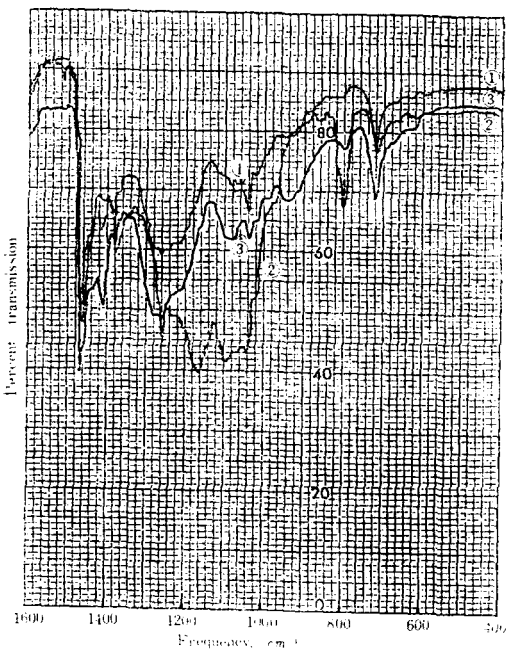

Fig. 8. Infrared absorption spectra of free fatty acid
①, control; ②, colony; ③, spent media

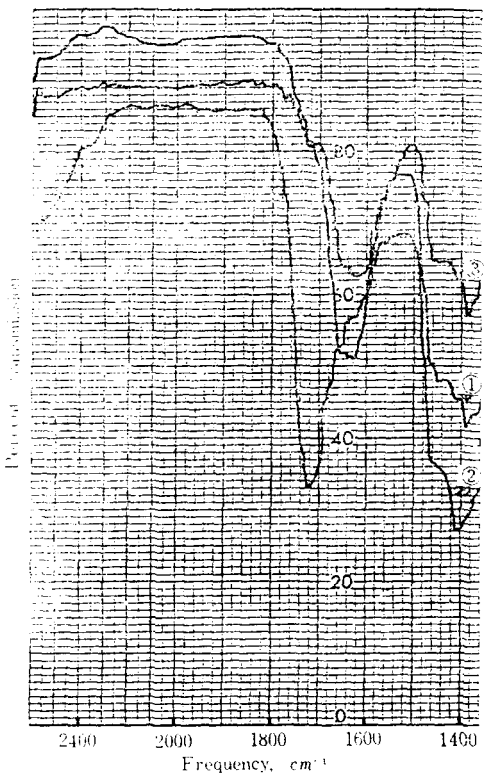

Fig. 10. Infrared absorption spectra of total saponin
①, control; ②, colony; ③, spent media

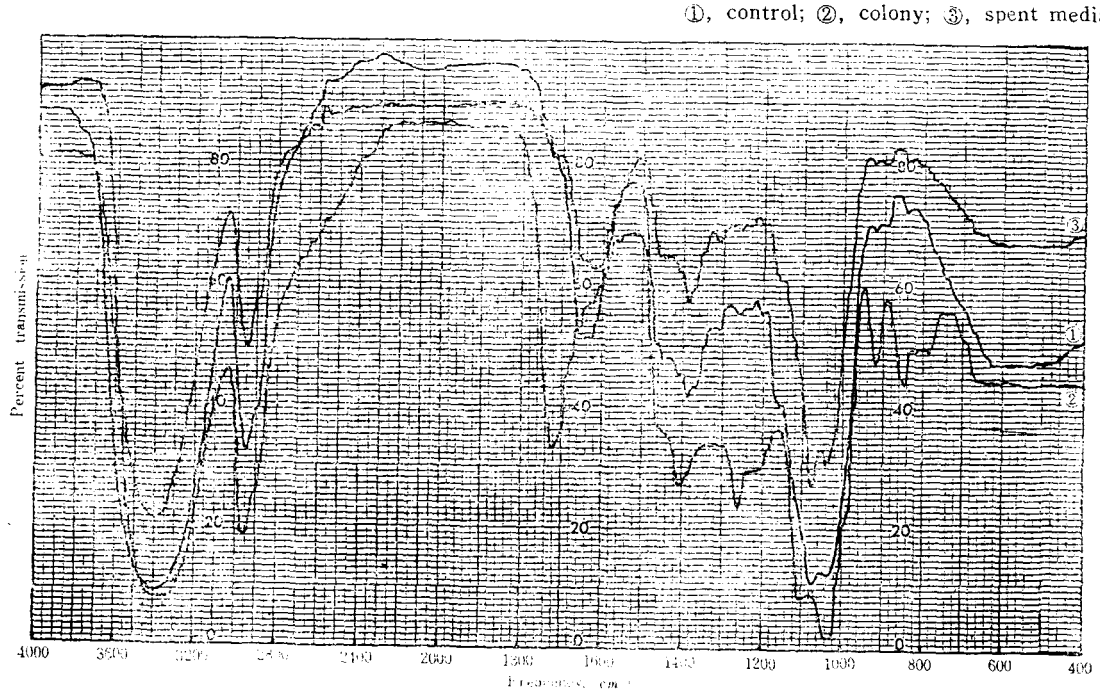

Fig. 9. Infrared absorption spectra of total saponin
①, control; ②, colony; ③, spent media

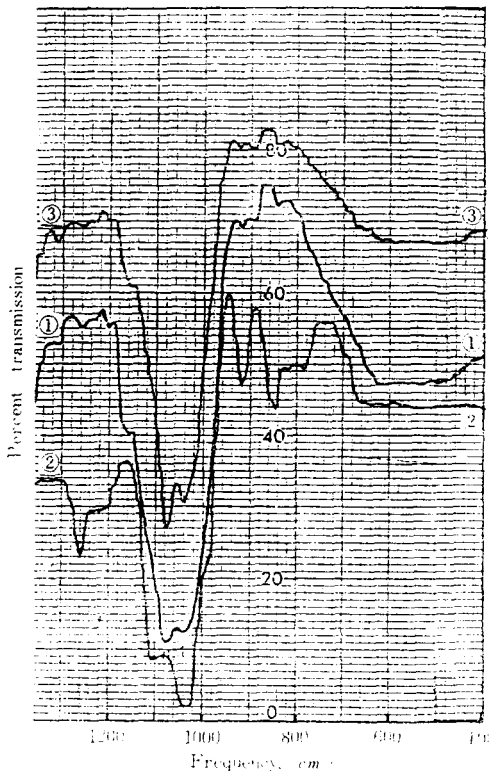

Fig. 11. Infrared absorption spectra of total saponin
①, control; ②, colony; ③, spent media

of sugar)[15] was disappeared in the colony. In addition, the 920 cm^{-1} (δ_{OH} out-of-plane of COOH dimer and the ring vibration of pyranose) and 845 cm^{-1} (CH_2 rocking of sugar and δC_1-H of sugar) bands were appeared only in the colony (Fig. 12).

Despite some spectral data indicating the contamination of free fatty acid with impurities, the ultraviolet and infrared spectra as well as the gas chromatographic data clearly demonstrated that the free fatty acid and total saponin of ginseng were metabolized by microorganism and also suggest that the propagation of microorganism on the root of ginseng would degrade the quality of ginseng. A close examination of each one of purified free fatty acids and saponin components, which are thought to be highly effective to the microbial growth, is under way in this laboratory.

要 約

人蔘에 繁殖하는 眞菌類가 人蔘의 遊離脂肪酸과 saponin의 化學的 變化에 미치는 影響을 調査하기 위하여 前報에서 分離한 *Rhizopus delemar* Rh-1을 人蔘에 接種, 培養한 다음 그 spent media와 colony 別로 遊離脂肪酸과 saponin을 抽出하여 이 抽出物의 可視線, 紫外線 및 赤外線의 分光分析과 가스크로마토그래피로 얻은 分析結果는 다음과 같다.

즉, 人蔘의 遊離脂肪酸 19種을 가스크로마토그래피法으로 同定하였으며 그 중 colony에서는 $iC_{12:0}$, $C_{16:2}$, $C_{17:0}$, $C_{18:3}$ 및 $C_{20:0}$酸을, spent media에서는 $C_{16:1}$酸을 對照群에서는 $C_{20:0}$酸을 檢出할 수 없었다.

可視分光分析法으로는 微生物에 依한 遊離脂肪酸과 saponin의 化學的 變化를 確認할수 없으나 紫外線과 赤外線의 스펙트럼에서는 吸收帶의 移動 乃至는 消長관계를 알수 있었다.

또한 saponin의 化學的 變化를 紫外線과 赤外線의 分光分析法과 가스크로마토그래피法으로 確認하였다.

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