STUDIES ON THE EFFICIENT EXTRACTION AND CHEMICAL TRANSFORMATION DURING PROCESSING OF PLANT POLYPHENOLS

（植物ポリフェノールの効率的抽出と加工過程
における化学的変換に関する研究）

Sumei Huang

2008

The United Graduate School of Agricultural Sciences,
Kagoshima University

CONTENTS

Chapter		Page
1	**GENERAL INTRODUCTION**	1
1.1	Classification of plant polyphenols	1
1.2	Importance of plant polyphenols	2
1.3	Extraction techniques of plant polyphenols	3
1.4	Processing of polyphenols materials	4
1.5	Objectives of the research	6
2	**EXTRACTION OF POLYPHENOLS USING BEAN PROTEIN**	9
2.1	Extraction of antocyanins using soybean protein	10
2.1.1	Materials and Methods	11
2.1.2	Results and Discussion	16
2.2	Extraction of various polyphenols using defatted soybean protein	30
2.2.1	Materials and Methods	31
2.2.2	Results and Discussion	33
2.3	Extraction of tea polyphenols using various bean proteins	39
2.3.1	Materials and Methods	40
2.3.2	Results and Discussion	43
2.4	Conclusion	47

3 HEATING PROCESSING OF PLANT MATERIALS CONTAINING POLYPHENOLS 56

3.1 Heating processing of tea polyphenols 57

3.1.1 Materials and Methods 58

3.1.2 Results and Discussion 60

3.2 Heating processing of materials containing hydrolaysable tannin 66

3.2.1 Materials and Methods 67

3.2.2 Results and Discussion 68

3.3 Conclusion 71

4 FERMENTATION OF PLANT MATERIALS CONTAINING POLYPHENOLS 76

4.1 Fermentation of materials containing hydrolysable tannin With individual strain of various microorganisms 77

4.1.1 Materials and Methods 77

4.1.2 Results and discussion 81

4.2 Fermentation of tea extract with the strain of various fungi 92

4.2.1 Materials and Methods 92

4.2.2 Results and discussion 93

4.3 Conclusions 96

5 GENERAL DISCUSSION 102

5.1 Extraction of polyphenols using bean protein 102

5.2 Heating processing of plant materials containing polyphenols 104

5.3 Fermentation of plant materials containing polyphenols with microorganism 106

SUMMARY 109

ACKNOWLEDGEMENTS 112

REFERENCES 114

Abbreviations

DMSO	Dimethyl sulfoxide
DPPH	1, 1 –diphenyl 1–2–picrylhyrazyl
HPLC	high performance liquid chromatography
IC_{50}	50 % inhibitory concentration
TBA	tetrabutrylammonium chloride
rpm	round per minute
DW	dry weight

CHAPTER 1

GENERAL INTRODUCTION

1.1 Classification of plant polyphenols

Polyphenols are general substances which have two or more hydroxyl groups (-OH) combined with the aromatic ring. Polyphenols are a group of the popular phytochemicals widely distributed in various plant parts such as leaves, flowers and fruits. Up to now, about 8000 phenolic structures of plant polyphenols are known (Bravo 1998). Generally, polyphenols can be classified into monomer group and polymer group (Fig. 1). The monomer group is mainly constituted of flavonoids (such as catechins, anthocyanins etc., all of them have a C6-C3-C6 carbon structure) and phenolic acids (such as chlorogenic acid, gallic acid etc.). The polymer group (tannins) consists of two major groups i.e. the condensed tannins (proanthocyanidin) and the hydrolysable tannins (gallotannin and ellagtannin). The condensed tannins are usually composed of flavan 3-ol units, mainly (+) catechin, (-)-epcatechin or related compounds, which are condensed by C-C bonds. The hydrolysable tannins are generally esters of phenolic acids and polyol such as glucose. Gallotannins are also called galloylglucoses, which are galloyl esters of glucose; ellagitannins are derivatives of hexahydroxydiphenic acid, which become lactonized to ellagic acid during hydrolysis (Haslam 1998).

1.2 Importance of plant polyphenols

In recent years, polyphenols have received great attention because it was found that the consumption of foods rich in polyphenols may decrease the risk of cancers, stroke and coronary disease (Kris-Etherton et al. 2002, Stoner and Mukhtar 1995). Among the polyphenols, tea polyphenols have been shown to have effects on the inhibition to carcinogen-induced several tumors (skin, lung, forestomach, esophagus, duodenum, colon tumors etc.) in rodents (Huang et al. 1992, Fujiki et al. 1996, Liao et al. 1995, Hirose et al. 1997); and anthocyanins have advantages of the prevention of oxidative stress and take an important role in protecting against cardiovascular disease (Ghiselli et al. 1998) and liver injury (Obi et al. 1998), antimutagenicity (Yoshimoto at al. 1999) and promoting effect on rhodopsin synthesis (Matsumoto et al. 2003) and so on. The prevention of many simple flavonoids to cancer and atherosclerosis are also reported (McAnlis et al. 1999, Hollman et al. 1996). Isoflavones such as daizein and genistein, which are mainly derived from soybean, are suggested to play a role in the prevention of breast cancer and osteoporosis (Adlercreutz and Mazur 1997, Tapiero et al. 2002). Anti-inflammation, hemostatic and astringent effects of tannins are well known from ancient times (Nishioka 1983). Recently, tannins were also reported to have the effects of inhibiting the generation of superoxide radicals, anti-allergy, anticarcinogenic and antimutagenic effects as well as flavonoids (Chung et al. 1998).

1.3 Extraction techniques of plant polyphenols

Following the increase of polyphenol consumption, more and more polyphenol products or functional food materials of polyphenols are required to be supplied. Obtainment of these polyphenols products are considerably depended on extraction techniques of polyphenols. Many techniques such as CC (column chromatography), HPLC (high performance liquid chromatography), HSCC (high speed counter-current chromatography) and CE (capillary electrophoresis) are reported to be used for the polyphenols separation (Sao and Deng. 2004, Yoshida et al. 1998). These techniques have some advantages, but they also have some drawbacks such as the use of a lot of organic solvent which are harmful to human health, unfriendly to environment, time-consuming, high cost etc.. Recently, some alternative methods that eliminate or reduce significantly the use of organic solvents such as SPE (solid-phase extraction), MAE (microwave-assisted extraction), SFE (supercritical fluid extraction) and PLE (pressurized liquid extraction) (Lucena et al. 2005, Papagiannopoulos et al. 2002, Huang and Zhang 2004, Mendiola et al. 2007) are gradually used for extraction of active ingredients from plant materials in food and medicinal industries, but these procedures still have many drawbacks such as time-consuming and labouring-intensive operations. Few studies have attempted to extract polyphenols by some methods without use of organic solvent (Ishimaru and Nonaka 2001, Li et al. 2005), however, the studies on this aspect are only done a little. Therefore, it is

necessary to carry out further studies on the development of extraction techniques of polyphenols and establish better and novel techniques which are simple, rapid, low cost, and safe compared with current techniques.

1.4 Processing of polyphenol materials

Usually, polyphenols are directly obtained from the wild or conventional cultured plants (Sugimoto et al. 2001, Wang et al. 1996). Recently, many polyphenols have been reported to be isolated from some tissue cultured plant materials (hairy roots, callus) such as roots of *Sanguisorba officinalis* (Ishimaru et al. 1990), hairy roots of *Geranium thunbergii* (Ishimaru et al. 1991), callus of *Sapium sebiferum* (Neera and Ishimaru 1992). Although most of polyphenols have been mainly obtained from the raw plant materials, some polyphenols have also been isolated from the processed food materials (Esaki et al. 1998, 1999, Miyake et al. 2003, Zhou et al. 2005,).

In fact, many studies proved that the constituents (including polyphenols) in the processed food can be changed compared with those in the raw materials after the processing procedures such as heat processing (boiling, broiling frying, steaming, grilling etc.), oxidizing enzymatic fermentation and microbial fermentation (Kumazawa and Masuda 2001, Takenaka et al. 2006). Yet, amongst the studies on the processed products, only few of them such as processed tea (Peterson et at. 2004, Jin et al. 2001, Angayarkanni et al. 2002), processed soybean products

(natto, miso, soy sauce, tempeh etc.) (Sumi 2007, Kawano 2007, Kataoka 2005) and grape wine (Louw et al. 2006) are received much attention, the studies on the processing of many plant materials containing polyphenols are not done so much.

To prevent food spoilage and contamination, lots of foods are consumed after thermal (heating) processing such as cooking, boiling and steaming. The influence of thermal processing on the components (such as vitamin, carotenoids etc.) of food was widely investigated (Awuah et al. 2007, Rattanathanalerk et al. 2005), but the data on the influence of thermal processing on polyphenols are not so much (Takenaka et al. 2006, Brenes et al. 2002). In order to get better acquainted with the functionalities of the thermal processed food, it is necessary to investigate the chemical transformations of polyphenols during heating processing. In the present research, the heating processings of several plant materials containing polyphenols (*S. sebiferum, Phyllanthus urinaria* and *Camellia sinesis*) were attempted and the changes of polyphenol composition and chemical structures were investigated during the processing.

From ancient times, people used microorganisms (bacteria and fungi) to convert agricultural commodities into various fermented foods (such as natto, soy sauce, pu-er tea etc.). It was reported that the processing of fermentation with microorganism has great influence on the chemical constituents and bioactivities of fermented products (Esaki et al. 1998, 1999, Miyake et al. 2003, Lin et al. 2006, Zhu et al. 2007). Although there are many reports on the constituents of many fermented products,

these reports are limited in a narrow range of microorganism and materials. For many fermented products, the composition of polyphenols is still unclear; further studies on the clarification of chemical transformations of polyphenols during the fermentation of many plant materials with various microorganisms are necessary. In this research, the fermentations of some plant materials containing polyphenols (*S. sebirerum, P. urinaria* and *C. sinesis*) with bacteria or fungi were carried out and the metabolic patterns of polyphenols during the fermentation were clarified.

1.5 Objectives of the research

The general aim of this research is to establish an efficient and safe methodology of polyphenol extraction and clarify the chemical transformations of polyphenols during various processing procedures.

The following are the objectives of this study:

1) To establish extraction methodology of polyphenols through investigation of the effects of bean protein on the extraction of various polyphenols.
2) To investigate the changes in the chemical structures and composition of polyphenols during heat processing of various plant materials containing polyphenols

3) To investigate the changes in the chemical structures and composition of polyphones during the fermentation of various plants materials containing polyphenols with bacilli or fungi

This dissertation is the compilation of the results of the study conducted at the Faculty of Agriculture of Saga University, Japan with the above-mentioned objectives (Huang et al. 2004a, 2004b and 2004c, Huang et al. 2006, in press).

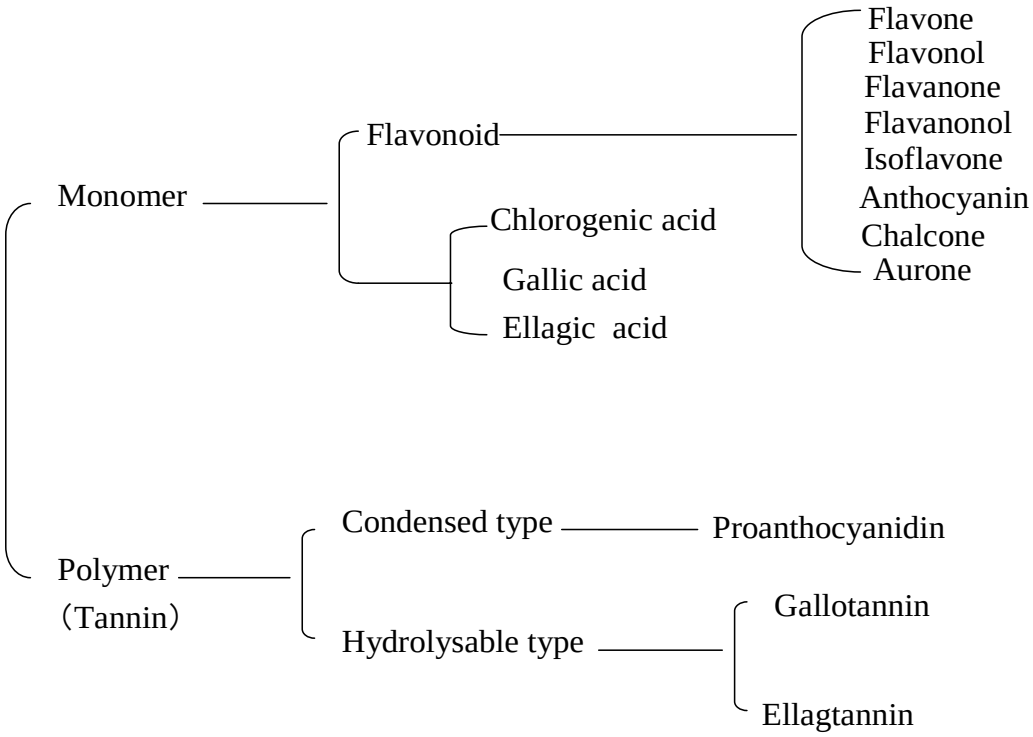


Fig. 1 Classification of polyphenols

CHAPTER 2

EXTRACTION OF POLYPHENOLS USING BEAN PROTEIN

For polyphenols extraction, lots of organic solvent and various column chromatographies are necessary using traditional techniques, which are complicated and always cost lots of time (Sao et al. 2004). More efficient techniques are expected to be developed.

Since ancient times, people used polyphenols (especially tannins) as tanning agent to make leather in terms of their properties, binding with protein and formatting protein–polyphenol complexes. Recently, to clarify the influence of protein on the bioactivities of polyphenols, this property of polyphenols has been used in the medical science (Rawel et al. 2001, de Freitas and Mateus 2001). In addition, few studies have attempted to use this property in the separation of polyphenols (Ishimaru and Nonaka 2001, Ushijima et al. 2001).

Soybean protein is a very important vegetable protein and very easily prepared (precipitated at pH4.5) from defatted soybean flours or soybean by water extraction. Furthermore, soy protein isolate as functional ingredient gains increasingly acceptance in recent years because of its excellent functional properties and good nutritional values (Li et al. 2007). According to the properties of combination between polyphenols and protein, Ishimaru et al. succeeded in extracting tea polyphenols by using soybean protein through the formation of tea catechins–soybean protein complex (Ishimaru and Nonaka 2001, Ushijima et al. 2001). Although this

method is safe and efficient to extraction of catechins, its feasibility to the extraction of other polyphenols still needs to be proved.

Since all of the polyphenols have the common structure of phenolic structure and hydroxyl groups as catechins, they probably have the same property of conjugating to the proteins easily. In this chapter, using soybean protein, extraction of several representative polyphenols such as anthocyanins, phenolic acid, condensed tannins and hydrolysable tannins (including gallotannins and ellagitannins) were conducted and the effect on extraction of tea catechins using protein from several bean species was also investigated. In addition, to investigate the antioxidative activities of the prepared polyphenols–protein complexes, DPPH (1, 1 -diphenyl 1-2-picrylhyrazyl) radical scavenging activities were also determined.

2.1 Extraction of anthocyanins using soybean protein

Anthocyanins (in Greek *anthos* means flower, and *kyanos* means blue) are naturally occurring plant pigments that impart colour to fruits, vegetables, and ornamental plants. They belong to flavonoids, which are one subgroup of polyphenols. By the year of 2000, more than 500 anthocyanins have been found (Harborne and Williams 2001). The basic chemical structure of popular anthocyanins is shown in Fig. 2. Generally, aglycones of anthocyanins are divided into six classes according to the number

of the hydroxyl groups in B ring i.e. pelargonidin, cyanidin, peonidin, delphinidin, petunidin and malvidin (Fig. 2).

According to recent reports, as a class of important antioxidant, anthocyanins possess many physiological functions such as free radical scavenging properties, antimutagenicity (Hollman et al. 1996), anitumor, lowering serum cholesterol effects, improvement of sight effects, protection on liver injury etc. (Igarashi 2000). Therefore, there is a broad prospect to exploit the anthocyanins as functional elements in functional food, and the demands for products containing anthocyanins will increase with the increase of consumption of functional food. Hence, how to obtain anthocyanins efficiently will become important subject, which depends on the methods of extraction in great degree. In this study, the extraction of anthocyanins from 15 plant species using soybean protein was attempted and DPPH radical scavenging activities of the anthocyanins–soybean protein complexes were also examined.

2.1.1 Materials and methods

2.1.1.1 Materials and preparation of samples

Preparation of anthocyanins extracts

Red leaves of *S. sebiferum* (Chinese Tallow), the fruits of Yeddo hawthorn (*Rhaphiolepis umbellata*) and bayberry (*Myrica rubra*) were respectively collected in October, November and July, 2003 in the Saga University (Japan); fruits of several berries (i.e. strawberries, blueberry, cranberry and raspberry), seeds of black

bean, tubers of purple sweet potato, peel of red potatoes, the peels from several plant species (i.e. peaches, red onions and eggplants), leaves of red cabbage were purchased at the market. All of these plant materials were extracted with MeOH (containing 0.1 % HCl) at room temperature for 16 hours. The extracts were filtered through filter paper (ADVANTEC No. 3) . The filtrates were concentrated under reduced pressure at 40 ℃ and then lyophilized. The lyophilized filtrates were used as anthocyanin extracts. In addition, the purple corn pigment used in the experiment was purchased from SANEIGEN FFI Inc. in Japan.

Preparation of defatted soybean protein extract

The commercial defatted soybean (The Riken Agricultural Production, Fukuoka) (50 g, DW) was mixed with 500 ml distilled water and the mixture was autoclaved (121 ℃, 15 min). After cooling to room temperature, the autoclaved mixture was filtered through microcloth (Calbiochem.) and the filtrate was used as defatted soybean protein extract.

Preparation of anthocyanins-soybean protein complexes

Three aliquots (each 100 mg) of each anthocyanins extract (respectively from the materials of 14 plant species) as well as three aliquots (each 10 mg) of purple corn pigment were mixed with defatted soybean protein extract, respectively. The values of pH of the three mixtures were adjusted to 5.3, 4.5, and 2.5 with 2 N HCl, respectively. The precipitate occurred in the different acid

conditions. Then each of the mixtures was centrifuged for 10 min at 25 ℃ (TOMY MX-300, 6500 rpm). The supernatant was filtered through the Milipore filter (Millex ® LH, 0.45 μm) and then subjected to HPLC (Shimadzu LC 6A) analysis (conditions of HPLC were described in 2.1.1.2-a). The precipitate was washed with 50 ml distilled water for two times. Finally, the precipitate (anthocyanins-soybean protein complex) was lyophilized.

As the control sample, defatted soybean protein extract without mixing with anthocyanins extract was also precipitated under the same pH conditions as described above. And the supernatant was also subjected to HPLC analysis; the precipitate was lyophilized and used as the precipitate of soybean protein.

2.1.1.2 Analysis of chemical constituents

a. *HPLC analysis of anthocyanins*

Anthocyanin extract (8 mg) was dissolved in 5 ml acetate buffer (pH 2.5). After filtrating through milipore filter (Millex ® LH, 0.45 μm), the extract solution was subjected to HPLC ananlysis. According to the results of HPLC analysis, the amount of anthocyanins in 100 mg anthocyanin extract or 10 mg purple corn pigment was calculated using cyanidin chloride (EXTRASYNTHESE, France) as an equivalent compound. HPLC conditions of anthocyanin analysis were as follows: column: Capcellpak C18 ACR (4.6 mm *i.d.* × 250 mm), mobile phase: 1.5 % H_3PO_4 – 1 % H_3PO_4/20 % AcOH/25 % CH_3CN (70: 30 → 25: 85, in 30 min), flow rate: 0.8 ml/min, column temperature: 40 ℃, detection: 520 nm.

The amount of anthocyanins in the supernatant of the mixture of anthocyanin extract and defatted soybean protein extract were analyzed by HPLC. The adsorption rate of anthocyanins to anthocyanins–soybean protein complexes from various anthocyanin extracts was calculated according to the following formula:

$$\text{Adsorption rate (\%)} = 100 \times (1 - A/B)$$

In this formula, A is the amount of anthocyanins in the supernatant of the mixture of anthocyanins extract and defatted soybean protein extract; B is the amount of anthocyanins in 100 mg anthocyanin extract or in 10 mg purple corn pigment.

Anthocyanins–soybean protein complex (20 mg) was extracted with 1 ml acetate buffer (pH 2.5) for 16 hours at room temperature in the dark. The mixture was centrifuged and the supernatant after filtration was subjected to HPLC analysis. The content of eluted anthocyanins from anthocyanins–soybean protein complex was calculated according to the following formula:

$$\text{Content (\%)} = 100 \times C/D$$

Here, C is the amount of eluted anthocyanins from the anthocyanins–soybean protein complex; D is the dry weight of the complex.

b. *HPLC analysis of isoflavones and tannins*

The anthocyanins–soybean protein complexes (each 20 mg) from *S. sebiferum* as well as soybean protein precipitates (each 20 mg) were extracted with 1 ml acetate buffer (pH 2.5) at room

temperature in the dark, and the extracts after filtration were subjected to HPLC analysis, respectively. HPLC conditions were as follows: column: TOSOH ODS 80Ts (4.6 mm *i.d.* ×250 mm), mobile phase: 1 mM tetrabutrylammonium chloride (TBA) (adjusted to pH 2.9 with AcOH)−CH_3CN (90:10→20:80, in 30 min), flow rate: 0.8 ml/min, column temperature: 40 ℃, detection: 280 nm. Isoflavones (i.e. daidzin, glycitin, genistin, daidzein and genistein) (Fig. 3) and tannins i.e. gallic acid (G), geraniin (18) and chebulagic acid (19) (Fig. 10−1 and Fig. 10−2.) were used as standards.

2.1.1.3 Measurement of DPPH radical scavenging activity

The anthocyanins−soybean protein complexes (each 20 mg) as well as soybean protein precipitate (each 20 mg) were extracted with 5 ml acetate buffer (pH 2.5) at room temperature for 16 hours and then the mixtures of complex and acetate buffer were centrifuged at 6500 rpm for 10 min. Each supernatant (0.5 ml) was mixed with equivalent 0.02 % (W/V) ethanol solution of DPPH. After violently shaking, the mixture (with DPPH) was allowed to stand for 20 min at room temperature in the dark. Then, the absorbance of the resultant mixture was measured at 517 nm using V−530 UV/VIS spectrophotometer. In addition, the mixture of acetate buffer (0.5 ml) and equivalent 0.02 % (W/V) ethanol solution of DPPH was used as control; the mixture of EtOH and acetate buffer was used as blank control. The DPPH radical scavenging activity was calculated according to the following formula:

Radical scavenging rate (%) = 100 − 100 × absorbance of the sample/ (absorbance of control − absorbance of blank control)

2.1.1.4 Isolation and identification of anthocyanins from *S. sebiferum*

Red leaves of *S. sebiferum* were collected in Saga University in October, 2003. These leaves were extracted with 70 % EtOH containing 0.1% HCl. The extract was concentrated under reduced pressure using a rotary evaporator and then lyophilized. The lyophilized extract (ca. 1.0 g) was dissolved in 5 ml MeOH. The dissolved extract was subjected to Sephadex LH-20 column chromatography (eluted with MeOH containing 0.1% HCl) and DIAION HP-20 (eluted with EtOH containing 1% AcOH) to afford the anthocyanin (isolated) of *S. sebiferum*.

2.1.2 Results and Discussion

The amount of anthocyanins-soybean complexes

As shown in Fig. 4, all the prepared complexes showed reddish or purple colorations, while the precipitates of soybean protein did not. These colorations of the complexes were considered to be originated from the anthocyanins which were bound to soybean protein.

Although the amount of anthocyanins-soybean protein complexes ranged from 556 mg to 976 mg, the average amount of anthocyanins-soybean protein complexes was about 800 mg, which

was similar to the average amount of the precipitates of soybean protein (Fig. 5). At pH 4.5, the highest amount of the complex from each plant was obtained, it was suggested that this pH condition was the most effective on the formation of the precipitates during the preparation of the complexes. There was no distinct difference between the amount of each complex and that of the precipitate of soybean protein at pH 4.5. These results indicated that there was no influence on the formation of the precipitate of soybean protein in the procedure of mixing plant extract and soybean protein extract.

Adsorption rates of anthocyanins adsorbed in anthocyanins-soybean protein complexes

Fig. 6 showed that the adsorption rates of anthocyanins adsorbed in anthocyanins-soybean complexes were affected by the pH conditions. The maximum adsorption rates of anthocyanins from most of plant extracts were observed at pH 2.5.

Under the same pH condition, the adsorption rates of anthocyanins to different complexes were different (Fig. 6). As shown in Fig. 5, the complexes from most of plant extracts showed high adsorption rates of anthocyanins (over 30 %), while those from raspberry extracts showed very low adsorption rates of anthocyanins (less than 10 %). The difference of contents and compositions of anthocyanins in various plants was presumably a main reason for the difference of anthocyanin adsorption rates to the complexes. It also suggested that most of anthocyanin materials from the plants were potent for preparation of

anthocyanins–soybean protein complexes.

Among all the adsorption rates of anthocyanins, that from *S. sebiferum* extract was the highest (about 50 % at pH 2.5), followed by those from black bean extract and blue berry extract (nearly 50% at pH 2.5). Therefore, as anthocyanin materials for the preparation of the anthocyanins–soybean protein complexes, *S. sebiferum*, black bean and blue berry were better than others plants.

Contents of eluted anthocyanins from anthocyanins–soybean protein complexes

As shown in Fig. 7, the highest content (0.23 %, DW) of eluted anthocyanins was observed in the complex from black bean prepared at pH 2.5. Under the same pH condition, the complex from black bean showed the highest content of eluted anthocyanins among all of the complexes. Although the highest adsorption rate of anthocyanins was observed in the complexes from *S. sebiferum* (Fig. 6), the content of eluted anthocyanins from these complexes was not high (e.g. about 0.05 % at pH 2.5) (Fig. 7). It showed that the anthocyanins adsorbed in the complexes from black bean were easy to be eluted, while those adsorbed in the complexes from *S. sebiferum* were difficult to be eluted. It was presumed that the elution of anthocyanins from the complexes was affected by the compositions or chemical structures of anthocyanins from various plants materials.

DPPH radical scavenging activities of anthocyanins-soybean protein complexes

As shown in Fig. 8, high DPPH radical scavenging rates were observed in the complexes from *S. sebiferum*, black bean, and red onion, respectively. Especially, the DPPH radical scavenging rate (about 90 %) of each complex from *S. sebiferum* was much higher than those of the complexes from other plants.

The complexes with low contents of eluted anthocyanins (e.g. the complexes from raspberry or the complexes from red potato) tended to show the low DPPH radical scavenging activities (Fig. 8). It indicated that the eluted anthocyanins from the complexes had a positive relation to the DPPH radical scavenging activities of the complexes. On the other hand, the complexes from *S. sebiferum* showed the lower contents of eluted anthocyanins than the complexes from black bean, while they showed significantly stronger DPPH radical scavenging activities than those complexes from black bean. Similar tendency was observed in the complex from red onion. Thus, besides the anthocyanins, other compounds such as tannins and flavones with strong DPPH radical scavenging activity were also probably adsorbed in the *S. sebiferum* complexes or red onion complexes.

The DPPH radical scavenging activities of the complexes from the same plant were almost identical under three different pH conditions. It indicated that pH condition of preparation of complexes had little influence on the DPPH radical scavenging activities of the complex. The DPPH radical scavenging activities

of all the anthocyanins-soybean protein complexes were stronger than those of the precipitates of soybean protein. Therefore, the preparation of anthocyanins-soybean protein complex was effective on the improvement of functionalities of soybean protein; and also the anthocyanins-soybean protein complex is expected to be a new type of functional food which possesses the functionalities originated from both soybean protein and anthocyanins. In addition, red leaves from *S. sebiferum*, red onion, blueberry and black bean seemed to be potential anthocyanin materials for preparation of anthocyanins-soybean protein complexes.

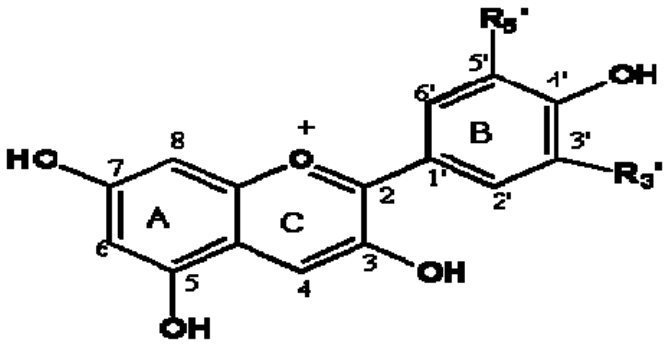
Contents of isoflavones and tannins in the complexes from S. sebiferum

Since the strong DPPH radical scavenging activities from *S. sebiferum* complexes were observed, it was presumed that other polyphenols were also adsorbed in the complexes and attributed to the DPPH radical scavenging activities. Therefore, in these complexes, tannins and isoflavones which are respectively originated from *S. sebiferum* and soybean protein were analyzed. As shown in Table 1, isoflavones (daizin and genistin) were detected at the similar level in both complexes from *S. sebiferum* and precipitates of defatted soybean protein. And the other phenolic compounds such as gallic acid, geraniin and chebulagic acid were detected in the complexes with high contents (geraniin: 2.809-3.454 %, chebulagic acid: 0.810-1.220 %), but undetectable in

the soybean protein precipitates. Since the precipitates of defatted soybean protein showed very low DPPH radical scavenging activities, it was suggested that isoflavones had little effect on DPPH radical scavenging activity. It revealed that the high DPPH radical scavenging activities of the complexes from *S. sebiferum* were mainly originated from the phenolic compounds (gallic acid, geraniin and chebulagic acid) adsorbed in the complexes. Therefore, the anthocyanins-soybean protein complexes from *S. sebiferum* are expected to be superior functional foods which possess the functionalities originated from not only soybean protein but also anthocyanins and other polyphenols.

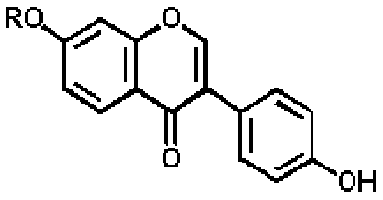
Identification of anthocyanin from S. sebiferum

Since the anthocyanins-soybean protein complexes from *S. sebiferum* showed very strong DPPH radical scavenging activities, it is important to elucidate the chemical structure of the anthocyanin from *S. sebiferum*. For this purpose, the isolation and identification of anthocyanin from the red leaves of *S. sebiferum* were carried out. The isolated anthocyanin from *S. sebiferum* was identified as cyanidin 3-glucoside by the comparison of the spectral data (UV and Mass) of the authentic standard compound (cyanidin 3-glucoside) (Fig. 9).

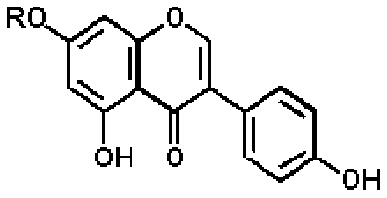


	R3'	R5'
pelargonidin	H	H
cyanidin	H	OH
peonidin	H	OCH_3
delphinidin	OH	OH
petunidin	OH	OCH_3
malvidin	OCH_3	OCH_3

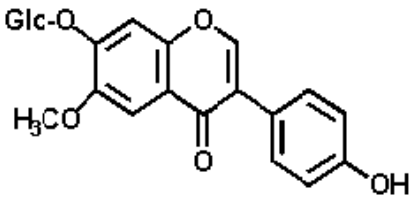
Fig. 2 Structures of the six common anthocyanidins



daidzein: R=H
daidzin: R=glc



genistein:R=H
genistin: R=glc



glycitin

Fig. 3 Chemical structures of isoflavones from soybean

Fig. 4 Anthocyanins–soybean protein complexes from various plant extracts

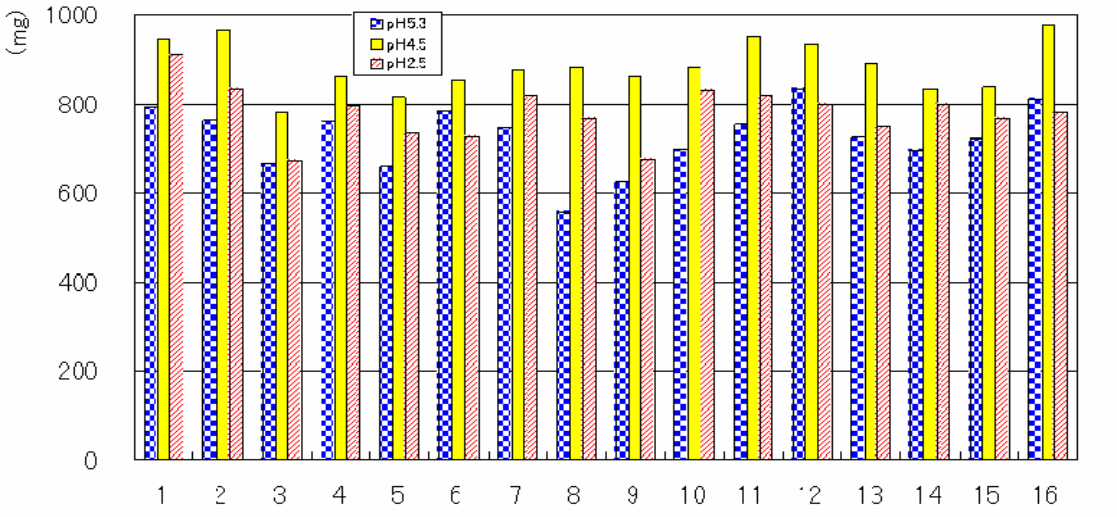


Fig. 5 Amounts of the anthocyanins-soybean protein complexes and the precipitates of soybean protein

1. defatted soyprotein 2. *S. sebiferum* (Chinese Tallow)
3. *Rhaphiolepis umbellate* (yeddo hawthorn)
4. *Myrica rubra Sieb.et Zucc* (bayberry)
5. *Prunus persica (L.) Batsch* (peach)
6. *Fragaria x ananassa Duchesne* (strawberry)
7. blueberry 8. cranberry 9. raspberry
10. black bean 11. purple sweet potato
12 red potato 13. red onion 14. red cabbage
15. eggplant 16. purple corn

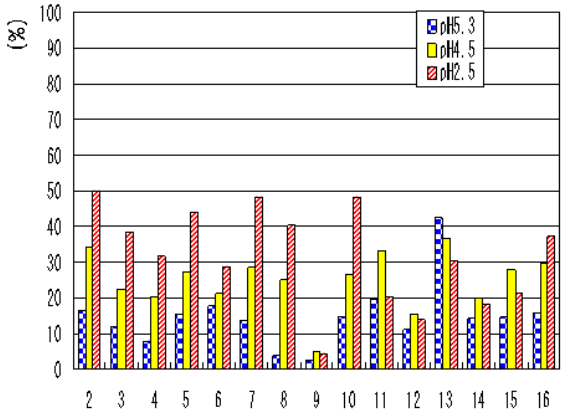


Fig. 6 Adsorption rates of anthocyanins adsorbed in anthocyanins-soybean protein complexes (DW)

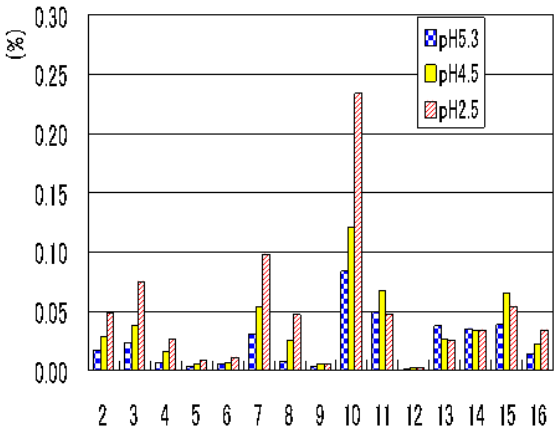


Fig. 7 Contents of eluted anthocyanins from anthocyanins-soybean protein complexes (DW)

2. *S. sebiferum* (Chinese Tallow)

3. *Rhaphiolepis umbellate* (yeddo hawthorn)

4. *Myrica rubra Sieb.et Zucc* (bayberry)

5. *Prunus persica (L.) Batsch* (peach)

6. *Fragaria x ananassa Duchesne* (strawberry)

7. blueberry 8. cranberry 9. Raspberry

10. black bean 11. purple sweet potato 12. red potato

13. red onion 14. red cabbage 15. eggplant 16. purple corn

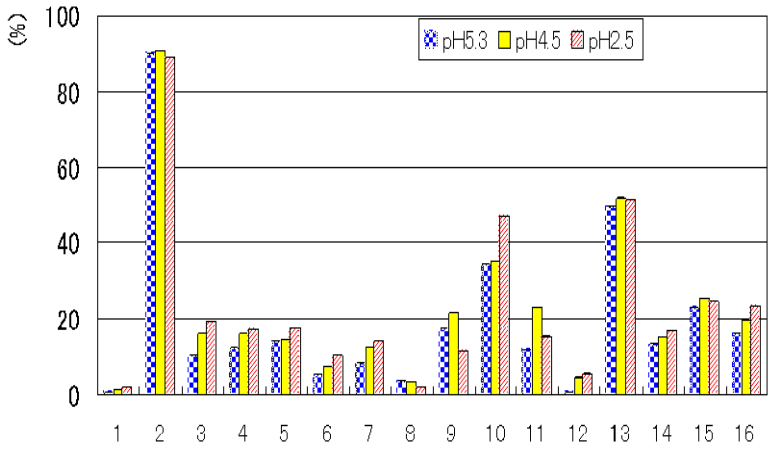


Fig. 8 DPPH radical scavenging rates of anhocyanins-soybean protein complexes and the precipitates of soybean protein

1.defatted soyprotein 2. *S. sebiferum* (Chinese Tallow)
3. *Rhaphiolepis umbellate* (yeddo hawthorn)
4. *Myrica rubra Sieb.et Zucc* (bayberry)
5. *Prunus persica (L.) Batsch* (peach)
6. *Fragaria x ananassa Duchesne* (strawberry)
7. blueberry 8. cranberry 9. raspberry
10. black bean 11. purple sweet potato 12. red potato
13. red onion 14. red cabbage 15. eggplant 16. purple corn

Table 1 Contents of phenol s in the complexes from *S. sebiferum* and in the precipitates of defatted soybean protein (% as dry weight)

	Complexes (from *S. sebiferum*)			Protein precipitates (from soybean)		
	pH 5.3	pH 4.5	pH 2.5	pH 5.3	pH 4.5	pH 2.5
Daidzin	0.177	0.104	0.104	0.092	0.093	0.116
Daidzein	n.d	n.d	n.d	0.008	0.009	0.010
Genistin	0.140	0.187	0.218	0.146	0.200	0.184
Genistein	n.d	n.d	n.d	0.003	0.003	0.004
Glycitin	n.d	n.d	n.d	0.016	0.015	0.018
Gallic acid	0.169	0.142	0.157	n.d	n.d	n.d
Geraniin	2.809	3.411	3.454	n.d	n.d	n.d
Chebulagic acid	0.810	1.203	1.220	n.d	n.d	n.d

n.d. : not detected

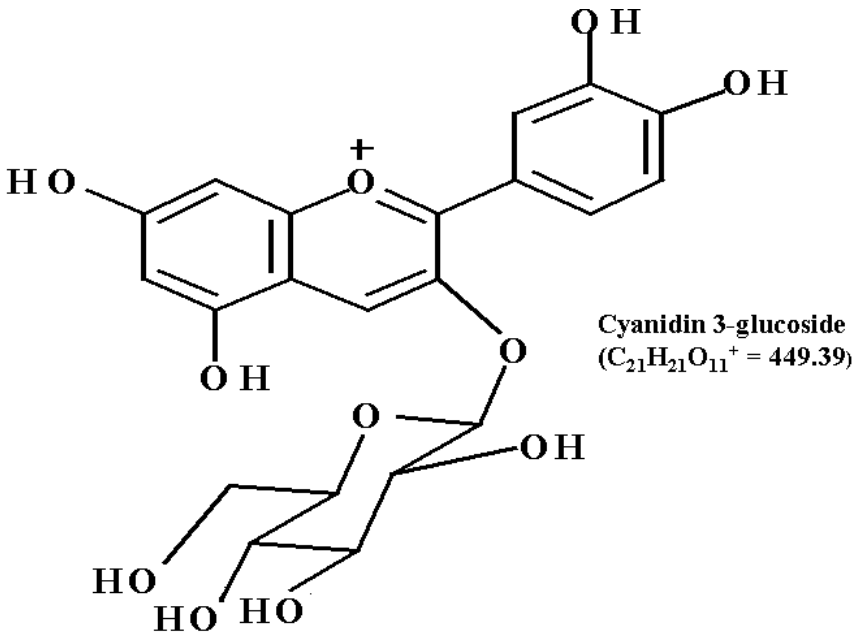


Fig. 9 Chemical structure of anthocyanin isolated from red leaves of *S. sebiferum*

2.2 Extraction of various polyphenols using of defatted soybean protein

Both of tea catechins (Ishimaru and Nonaka 2001) and anthocyanins (Huang et al. 2004a) were extracted successfully by using defatted soybean protein, which indicates that using defatted soybean protein for extraction of polyphenols is one of feasible methods. In order to widely utilize this method for extraction of various polyphenols, it is necessary to reveal what correlation exists between the chemical structure of polyphenol and the conjugational ability of polyphenol to soybean protein.

Some studies (Hagerman and Butler, 1978, Bacon and Rhodes, 2000, de Freitas and Mateus, 2001) describing influence of polyphenol structure on the binding of polyphenol to protein have been reported. Some reports have shown that the conjugations of polyphenols to protein were main hydrophobic combinations (Oh et al. 1980, Sarker et al. 1995) and the conjugational positions were mainly located on the aromatic rings or the hydroxyl groups combined with the aromatic rings of polyphenols (Siebert et al. 1996). However, these studies have limited to interactions of a particular protein or narrow range of polyphenols, interactions of polyphenols and soybean protein have not been reported.

In order to elucidate the relationships of chemical structures of polyphenols and the conjugation of polyphenols to proteins, the effects of 22 polyphenols on the conjugations of the polyphenols to defatted soybean protein were investigated. These polyphenols respectively belong to phenolic acids, condensed type tannins,

hydrolysable type tannins including gallotannins and ellagitannins.

2.2.1 Materials and methods

2.2.1.1 Materials and preparation of samples

Twenty-two polyphenols (Fig. 10-1 and Fig. 10-2) i.e. (+)-catechin (**C**) (**1**), (-)-epicatechin (**EC**) (**2**), (-)-epicatechin 3-*O*-gallate (**ECG**) (**3**), (-)-epigallocatechin 3-*O*-gallate (**EGCG**) (**4**), procyanidin B-1 (**5**), procyanidin B-3 (**6**), procyanidin C-1 (**7**), procyanidin B-1 3-*O*-gallate (**8**), procyanidin B-2 3'-*O*-gallate (9), gallic acid (**10**), methylgallate (**11**), bergenin (**12**), 6-*O*-galloylbergenin (**13**), 1,2,3,6-tetra-*O*-galloyl-*β*-D-glucose (**14**), 1,2,3,4,6-penta-*O*-galloyl-*β*-D-glucose (**15**), 1-desgalloyleugeniin (**16**), eugeniin (**17**), geraniin (**18**), chebulagic acid (**19**), castalagin (**20**), vescalagin (**21**), rosmarinic acid (**22**) were dissolved in 10 % (V/V) dimethyl sulfoxide (DMSO) (0.3 ml) solution, respectively. And then various solutions containing 0.5 mM of the polyphenol were obtained, which were used as polyphenols solutions. In the twenty-two polyphenols, **1-4** are catechins; **5- 9** belong to condensed polyphenols; **10**, **11**, **22** are phenolic acids; others are included in hrdrolysable tannins : **12-15** are members of gallotannins and 16-21 belong to ellagitannins.

The defatted soybean protein extract, which was prepared as mentioned in 2.1.1.1, was adjusted to pH 4.5 and centrifuged at 8000 rpm for 5 min. The precipitate was used as defatted soybean protein precipitate after lyophilisation.

Aliquots of the defatted soybean protein precipitate (each 10 mg, DW) were mixed with the polyphenols solutions prepared as above (each 0.3 ml), respectively. The mixtures were allowed to stand for one hour at room temperature and then centrifuged at 10,000 rpm for 5 min. The supernatants after filtration were subjected to HPLC analysis (as described in 2.1.1.2-b); the precipitates were washed with water for two times and lyophilized. These lyophilized precipitates were used as polyphenol-defatted soybean protein complexes.

2.2.1.2 HPLC analysis of polyphenols in the complexes

The polyphenol-defatted soybean protein complexes from 22 polyphenols (each 10 mg, DW) were extracted with 50 % aqueous acetone (0.3 ml) for 16 hours at room temperature in the dark. After filtration, each extract was subjected to HPLC analysis (as described in 2.1.1.2-b). As control, the precipitate of defatted soybean protein was also extracted and analysed in the same ways.

The adsorption rate of the polyphenol was calculated according to the following formula:

$$\text{Adsorption rate (\%)} = 100\,(1 - A1/A2)$$

Here, A1 is the amount of polyphenol in the supernatant; A2 is the amount of polyphenol in 0.3 ml polyphenol solution from 22 polyphenols.

The elution rates of 22 polyphenols were calculated according to the following formula:

$$\text{Elution rate (\%)} = 100 \times B1/B2$$

Here, B1 is the amount of polyphenol extrated from the complex; B2 is the amount of polyphenol in 0.3 ml polyphenol solution from 22 polyphenols.

2.2.2 Results and discussion

The adsorption rates and elution rates of 22 polyphenols are shown in Table 2. catechins and condensed tannins showed higher adsorption rates than hydrolysable tannins when they have the same number of galloyl moiety. For example, the adsorption rates of **3**, **4**, **8** and **9** (catechins or condensed type tannins) were 77.6%, 80.4%, 67.5% and 69.5%, respectively, while that of **12** (hydrolysable type tannin) was only 10.5%, although all of these polyphenols (**3**, **4**, **8**, **9** and **12**) had one galloyl moiety.

The adsorption rates of **15**, **14**, **13** and **12** (gallotannins) were 84.2 %, 74.1 %, 49 % and 10.5 %, respectively; and the number of galloyl moieties in these polyphenols (**15**, **14**, **13** and **12**) was five, four, two and one, respectively. It indicated that the adsorption rate of the gallotannins increased with the number of galloyl moieties in them.

The hydrolysable tannins (**14**, **15**, **17**, **18** and **19**) with a galloyl moiety which are esterified at C-1 of the glucose, showed very high adsorption rate (74.1-84.2%), while **12** and **13** which have a galloyl moiety esterified at C-2 of the glucose, showed relatively low adsorption rate (10.5% and 49.0%, respectively). It suggested that the existence of galloyl moiety esterified at C-1 of the glucose could increase the conjugation of polyphenol to soybean protein.

These results indicated that galloyl moieties were very

important for the conjugation of polyphenols to soybean protein.

Although **14** and **16** have the same number of galloyl units and similar molecular weight (788 and 786, respectively), the adsorption rate of **14** (74.1%) was higher than that of **17** (59.1%). Similar phenomenon was observed between **15** and **17** (Table 2). It was presumed that the formation of HHDP (hexahydroxydiphenoyl) unit which is formed from two galloyl units linkage each other through their aromatic carbon atoms, could decrease the conjugation of polyphenol to soybean protein. For this reason, gallotannins (i. g. **14** and **15**) were easier to conjugate to soybean protein than ellagitannins (i. g. **16** and **17**) when they have the same number of galloyl units and similar chemical structures.

The adsorption rates of **20** and **21** (ellagitannins) were only 58.9% and 49.5% respectively, although they have many hydroxyl groups and a large molecular weight as well as other ellagitannins (i. g. **18** and **19**) which showed high adsorption rate (about 83%). It is presumed that the open-chain glucose structure of **20** and **21** was the main reason that resulted in the decrease of the adsorption rates.

The adsorption rates of phenolic acids (**10**, **11** and **22**) were lower than those of most of other type polyphenols (condensed type tannins and hydrolysable type tannins). The influences of molecular weight and the number of hydroxyl groups in polyphenols on conjugation were still unclear.

The elution rates of most of polyphenols (except for **20** and **21**) were near to their adsorption rate to defatted soybean protein. It

showed that most of polyphenols adsorbed to defatted soybean protein were easily extracted from the complexes.

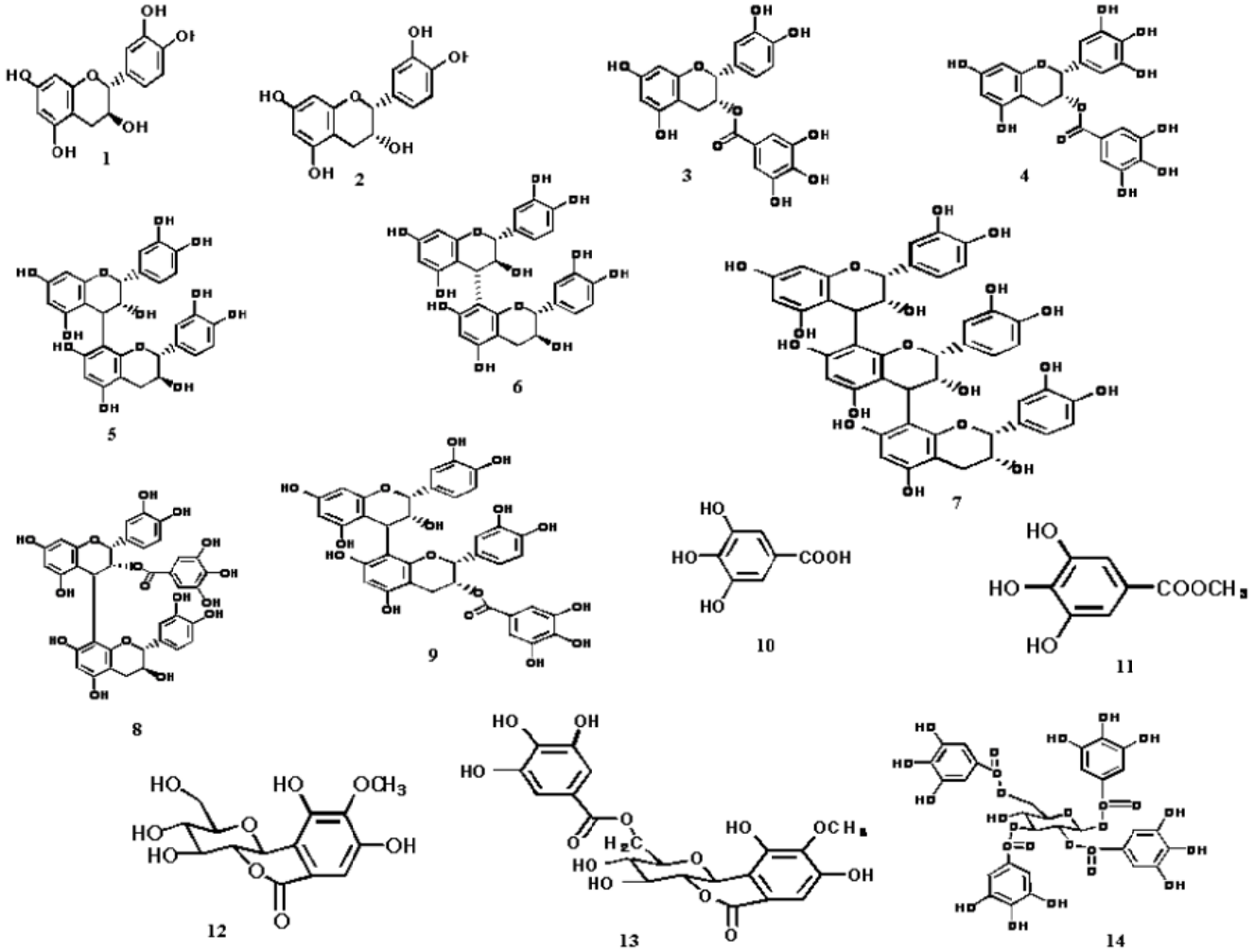


Fig. 10-1 Chemical structures of 22 polyphenols (1-14)

1. (+)-catechin (C), **2.** (-)-epicatechin (EC), **3.** (-)-epicatechin 3-*O*-gallate (ECG), **4.** (-)-epigallocatechin 3-*O*-gallate (EGCG), **5.** procyanidin B-1, **6.** procyanidin B-3, **7.** procyanidin C-1, **8.** procyanidin B-1 3-*O*-gallate, **9.** procyanidin B-2 3'-*O*-gallate, **10.** gallic acid (G), **11.** methylgallate, **12.** bergenin, **13.** 6-*O*-galloylbergenin, **14.** 1, 2, 3, 6-tetra-*O*-galloyl - β-D-glucose

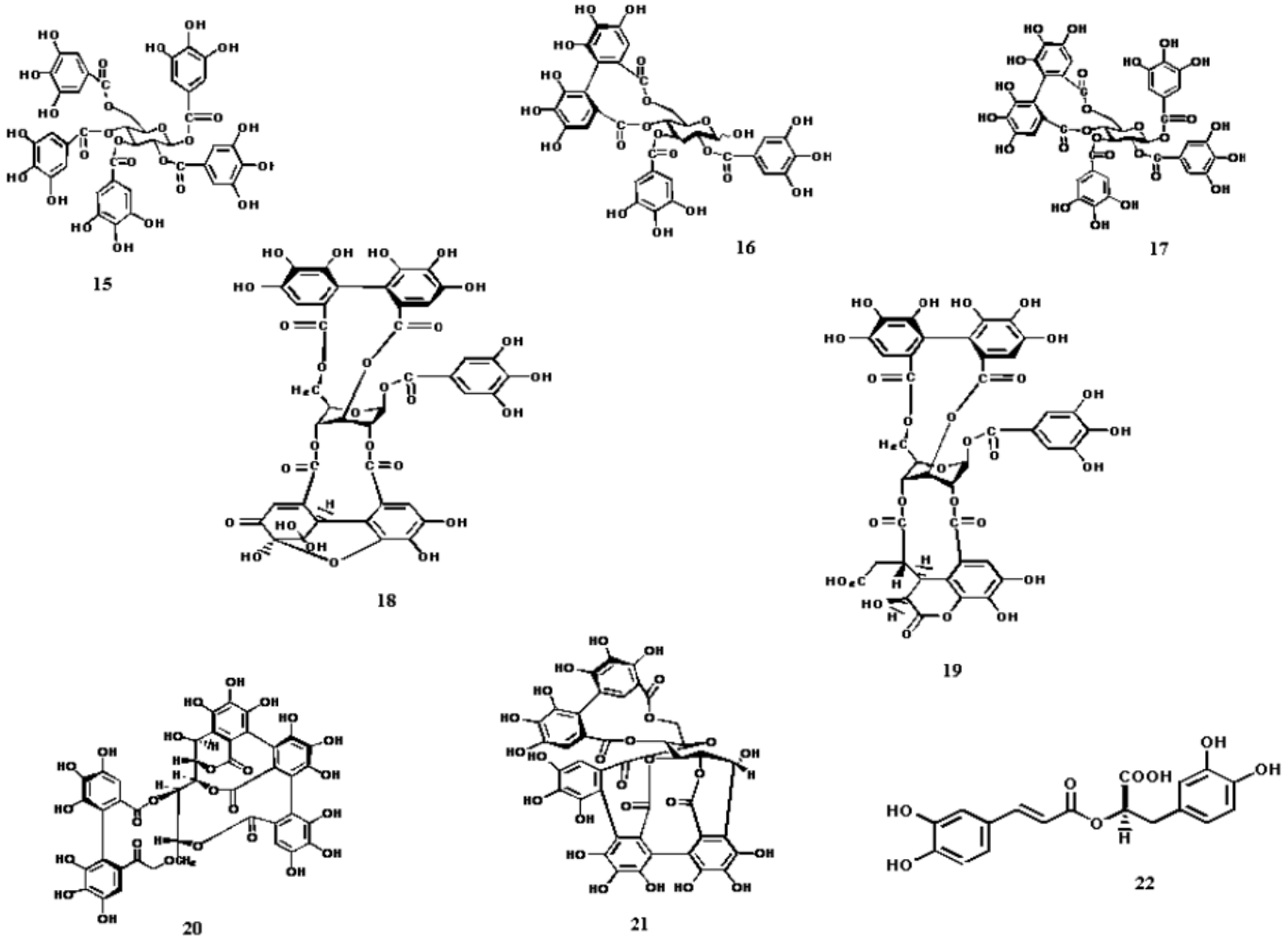


Fig. 10-2 Chemical structures of 22 polyphenols (15-22)

15. 1, 2, 3, 4, 6-penta-*O*-galloyl -*β*-D-glucose, **16.** 1-desgalloyleugeniin, **17.** eugeniin, **18.** geraniin, **19.** chebulagic acid, **20.** castalagin, **21.** vescalagin, **22.** rosmarinic acid

Table 2 Parameters related to conjugational abilities of various polyphenols to defatted soybean protein

polyphenols	MW	adsorption(%)	Elution (%)
1	290	30.3	30.3
2	290	27.7	27.6
3	442	77.6	71.9
4	458	80.4	69.3
5	578	32.2	26.6
6	578	33.5	28.6
7	866	39.0	29.0
8	730	67.5	57.5
9	730	69.6	52.7
10	170	17.8	16.6
11	184	24.7	19.6
12	328	10.5	10.8
13	480	49.0	40.3
14	788	74.1	66.9
15	940	84.2	73.7
16	786	59.1	53.4
17	938	83.0	50.8
18	952	82.7	73.3
19	954	83.4	71.4
20	934	58.9	20.1
21	948	49.5	12.2
22	360	30.2	22.7

1. 10 mg of autoclaved soybean protein was mixed with 0.3 ml of polyphenol solution (final concentration, 0.5 mM). The mixture was centrifuged at 10,000 rpm for 5 min. Polyphenols in the precipitates were extracted with 50 % aq. Me_2CO for 16 hours, and then were analyzed by HPLC.

2. MW: molecular weight

2.3 Extraction of tea polyphenols using various bean protein

Today, tea is consumed throughout the world and widely available in foods, beverage and cosmetic products because of its' various physiological functions (Wang et al. 2000). How to improve the functions of tea and how to explore some tea products with some new functions are the hot topics in recent years. On the other hand, legumes have gotten increasing attention for their many physiological functions such as cholesterol reduction and anti-fatness (Anderson et al. 1995). Tea catechins-soybean protein complex, a new food material, was successfully prepared by using soy bean protein and showed antibacterial activities and other bioactivities in the rats' experiments (Ishimaru and Nonaka 2001, Alim et al. 2003, 2004).

Although soybean protein was proved to be good material for the extraction of polyphenols (such as anthocyanins and tea catechins) (Ishimaru and Nonaka 2001, Huang et al. 2004a), the effects of the protein from other beans on the extraction of polyphenols are still unclear. In order to obtain better protein materials from other beans (besides soybean protein) for the extraction of polyphenols, in this study, the extraction of tea polyphenols by using bean protein from six bean species was attempted.

2.3.1 Materials and methods

Chemicals

Caffeine and tea polyphenols i.e. **G**, **C**, **EC**, **ECG**, **EGCG**, (−)−gallocatechin (**GC**), (−)−epigallocatechin (**EGC**) were purchased from Sigma Co. Ltd. in Japan. All of them were used as authentic standards. The chemical structures of them were shown in Fig. 10−1 and Fig. 11, respectively.

Preparation of tea extract

Dried green tea (*C. sinensis*) leaves (25 g, from Kagoshima Prefecture, in Japan) were extracted with 500 ml hot water (85 ℃) for 2 hours. Then the extract was filtered through 4−fold gauze and used as tea extract.

Preparation of the extracts of bean protein

six bean species i.e. *Azuki* bean, black bean, *Uzura* bean, *Taishoukintoki* bean, *Dainagon* bean, soybean and defatted soybean were used as materials of bean protein (Fig. 12).

Various bean species (each 50 g, DW) after crushing were respectively mixed with 500 ml water and autoclaved (121 ℃, 15 min). The autoclaved mixtures were filtered through 4−fold gauze, and the filtrates were used as extracts of degenerated bean protein.

The mixtures of various bean species (each 50 g, DW) and 500 ml water were mixed and homogenized after steeping for 16 hours at room temperature. The homogenized mixtures were filtered through the filter paper set in the BÜchner funnel, and the filtrates were

used as extracts of non-degenerated bean protein.

Preparation of bean protein-catechins complexes

The extract of degenerated bean protein as well as the extract of non-degenerated bean protein was mixed with tea extract, respectively. The mixtures were adjusted to pH 4.5 and precipitates occurred in this condition. Then the mixtures were centrifuged at 10000 rpm for 5 min. The supernatants after filtration were subjected to HPLC analysis (as described in 2.1.1.2-b). The precipitates (complexes) were washed with water for two times and then lyophilized. The lyophilized precipitates were used as bean protein-catechins complexes (Fig. 13.).

As controls, the extracts of degenerated and non-degenerated bean protein were also adjusted to pH 4.5 and the precipitates occurred. The supernatants were subjected to HPLC analysis. The precipitates were lyophilized and used as the precipitates of degenerated and non-degenerated bean protein, respectively.

HPLC analysis of catechins from bean protein-catechins complexes

The bean protein-catechins complex (20 mg) was extracted with 50 % aqueous acetone (5 ml) for 16 hours at room temperature in the dark. The extraction was subjected to HPLC analysis (as described in 2.1.1.2-b) after filtration.

The adsorption rate of catechin was calculated according to the following formula:

$$\text{Adsorption rate (\%)} = 100 - 100 \times C1/C2$$

Here, C1 is the amount of catechin in the supernatant of the mixture of bean protein and tea extract; C2 is the amount of catechin in the tea extract.

The content of eluted catechins from bean protein-catechins complex was calculated according to the following formula:

$$\text{Content (μg/mg)} = D1/D2$$

Here, D1 is the amount of catechin extracted from bean protein-catechins complex; D2 is the dry weight of bean protein-catechins complex.

Measurement of DPPH radical scavenging activity

The bean protein-catechins complexes (each 20 mg) as well as the precipitates of degenerated and non-degenerated bean protein (20 mg) were respectively extracted with 5 ml acetate buffer (pH 2.5) for 16 hours in the dark at room temperature, and the extractions were centrifuged at 10000 rpm for 5 min. Aliquots of the supernatant were diluted with acetate buffer (pH 2.5), and the final concentrations of the diluted solutions were equalled to 1/2, 1/4, 1/8, 1/16, 1/32 of the original concentration of the supernatant, respectively. The supernatant (0.5 ml) as well as the diluted solution (0.5 ml) was mixed with equivalent 0.02 % (W/V) ethanol solution of DPPH. The mixture of acetate buffer (pH 2.5) (0.5 ml) and 0.02 % (W/V) ethanol solution of DPPH (0.5 ml) was used as control; the mixture of EtOH and acetate buffer was used as blank control.

After violently shaking, the mixtures (with DPPH) were allowed to stand for 20 min in the dark at room temperature. Then absorbencies of mixtures were measured at 517 nm, and the DPPH radical scavenging rates (%) of the samples were calculated by using the formula mentioned in 2.1.1.3. The DPPH radical scavenging activity was expressed as IC_{50} value (mg/ml, concentration required to scavenge 50% DPPH free radicals) calculated from a log-dose inhibition curve (Yokozawa et al. 1998).

2.3.2 Results and discussion

Amounts of various bean protein-catechins complexes

Prepared bean protein-catechins complexes and bean protein precipitates are shown in Fig. 13. The amounts of the complexes from non-degenerated bean protein (except for non-degenerated defatted soybean protein) were much larger than those of the complexes from degenerated beans protein (Fig. 14). Among the complexes from degenerated bean protein, those from defatted soy bean and black bean showed larger yield; and among the complexes from non-degenerated beans protein, those from black bean and soybean showed larger yields (Fig.14). Therefore, to obtain large amount of complexes, soybean and black bean seemed to be better than other beans.

Adsorption rates of catechins adsorbed in various bean protein-catechins complexes

Adsorption rates of catechins to all of the complexes (including those complexes from degenerated bean protein and non-degenerated bean protein) were almost at the same level (Fig. 15). The adsorption rates of catechins ranged from 29 % to 89 %. Among all the catechins, EGCG showed the highest adsorption rate (56.8 %-89.2 %) in each complex, ECG showed higher adsorption rate compared to other catechins and caffeine. Since both of EGCG and ECG possess galloyl moiety, these results suggested that the catechins with galloyl moiety were easily conjugated with bean protein.

Contents of catechins in various bean protein-catechins complexes

Among all the catechins, EGCG showed the highest content in each complex, ECG also showed higher content compared to other catechins and caffeine (Fig. 16). Since similar tendency was also observed in the adsorption rates of EGCG and ECG, these results showed that the catechins with galloyl moiety (EGCG and ECG) were easily conjugated to the proteins from various beans and also easily extracted from the complex. The complexes from the degenerated bean protein showed significantly higher contents of catechins than those from the non-degenerated bean protein. It indicated that the conjugations of catechins to degenerated bean protein were more efficient than those of catechins to non-degenerated bean protein. Among the complexes from

degenerated bean protein, the complexes from black bean, *Azuki* bean, soybean and defatted soybean showed higher content of catechins. Therefore, the degenerated proteins from black bean, *Azuki* bean, soybean and defatted soybean were considered as efficient materials for extraction of catechins.

DPPH radical scavenging activities of various bean protein-catechins complexes

DPPH radical scavenging activities of the precipitates from various bean proteins were very weak compared with those of the complexes (Fig. 17 and Fig. 18). The complexes from degenerated bean protein showed remarkably stronger DPPH radical scavenging activities than those from non-degenerated bean protein (Fig. 17). Among the complexes from degenerated bean protein, the complexes from *Azuki* bean, *Taishoukintoki* beans, black bean and defatted soybean showed very strong DPPH radical scavenging activities, with IC_{50} values of 0.069 mg/ml, 0.082 mg/ml, 0.247 mg/ml and 0.229 mg/ml, respectively. Obviously, the complexes with higher contents of catechins showed stronger DPPH radical scavenging activities. It indicated that the DPPH radical scavenging activities of the complexes were mainly originated from the catechins adsorbed in the complexes.

The protein precipitates from *Azuki* bean, black bean, *taishoukintoki* bean, *Dainagon* bean and *Uzura* bean showed very weak DPPH radical scavenging activities, while the protein precipitates from soybean and defatted soybean hardly showed

DPPH radical scavenging activity (Fig. 18). It was presumed that anthocyanins originally contained in theses beans (*Azuki* bean, black bean, *Taishoukintoki* bean, *Dainagon* bean and *Uzura* bean) were conjugated with the protein precipitates showing weak DPPH radical scavenging activities. In addition, the DPPH radical scavenging activities of the degenerated protein precipitates were stronger than those of the non-degenerated protein precipitates (Fig.18). It suggests that the degenerated bean proteins might easily conjugate with the anthocyanins while non-degenerated bean proteins did not.

These results indicated that anthocyanins from beans also might be adsorbed in the complexes, and enhanced the DPPH radical scavenging activities of the complexes. Hence, the stronger DPPH radical scavenging activities of the complexes from degenerated *Azuki* bean and *Taishoukintoki* bean were originated from not only catechins but also anthocyanins of the beans; similar results were also obtained in the complexes from black bean, *Dainagon* bean and *Uzura* bean.

All of the complexes from degenerated bean proteins showed very strong DPPH radical scavenging activities, especially the complexes from *Azuki* bean, *Taishoukintoki* bean and black bean. Therefore, the degenerated proteins from all of the beans were suitable for extraction of catechins, especially degenerated proteins from *Azuki* bean, *Taishoukintoki* bean and black bean were better than those from other bean. Moreover, these complexes are expected to be novel functional food materials which possess

functionalities originated from bean anthocyanins, bean proteins and tea catechins.

2.4 Conclusion

In this chapter, various polyphenols such as anthocyanins, catechins, hydrolysable tannins, condensed tannins and some monomer polyphenols were successfully extracted using soybean protein. It was found that galloyl moieties of polyphenols are very important for the conjugation of polyphenols to bean protein. For the extraction of catechins, the degenerated bean protein was more efficient than non-degenerated bean protein. *Azuki* bean, *Taishoukintoki* bean and black bean which contain anthocyanins are considered to be better protein materials for the extraction of polyphenols.

In conclusion, an efficient methodology was established using bean protein via preparation of polyphenols-bean protein complexes. Furthermore, these complexes are anticipated to be used as a new type of food materials which possess functionalities originated from bean protein and polyphenols.

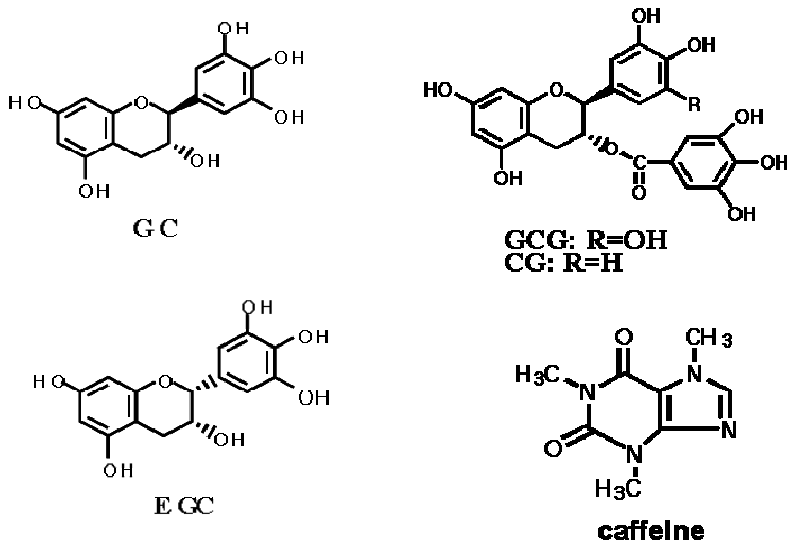


Fig. 11 Chemical structures of tea catechins and caffeine

(−)−gallocatechin (**GC**) (−)−gallocatechin 3−*O*−gallate (**GCG**)
(−)−catechin 3−*O*−gallate (**CG**) (−)−epigallocatechin (**EGC**)

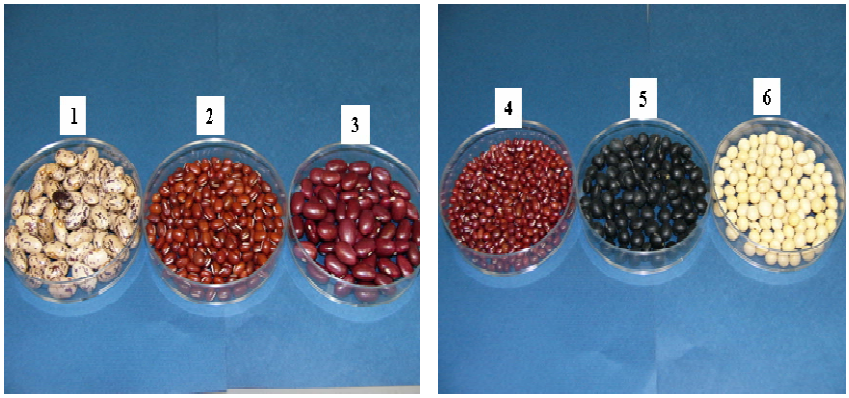


Fig. 12 Various beans used for extraction of catechins

1. *Uzura* bean 2. *Dainagon* bean 3. *Taishoukintoki* bean
4. *Azuki* bean 5. black bean 6. soybean

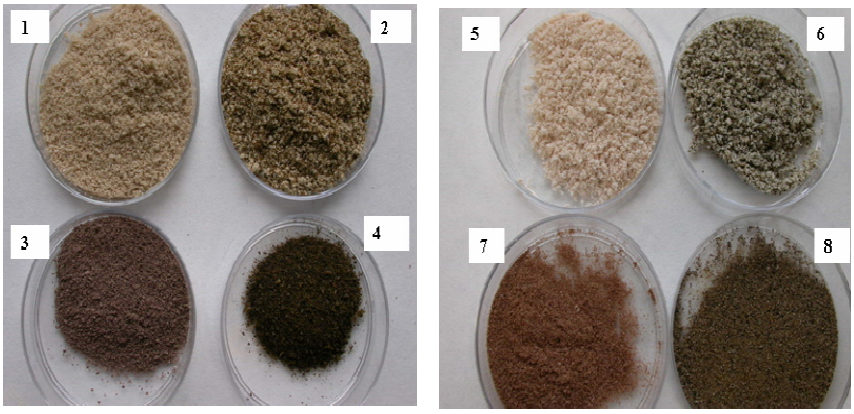


Fig. 13 Protein precipitates and protein-catechins complexes from various beans

1. non-degenerated *Uzura* bean protein precipitate
2. non-degenerated *Uzura* bean protein-catechins complex
3. degenerated *Uzura* bean protein precipitate
4. degenerated *Uzura* bean protein-catechins complex
5. non-degenerated *Taishoukintoki* bean protein precipitate
6. non-degenerated *Taishoukintoki* bean protein-catechins complex
7. degenerated *Taishoukintoki* bean protein precipitate
8. degenerated *Taishoukintoki* bean protein-catechins complex.

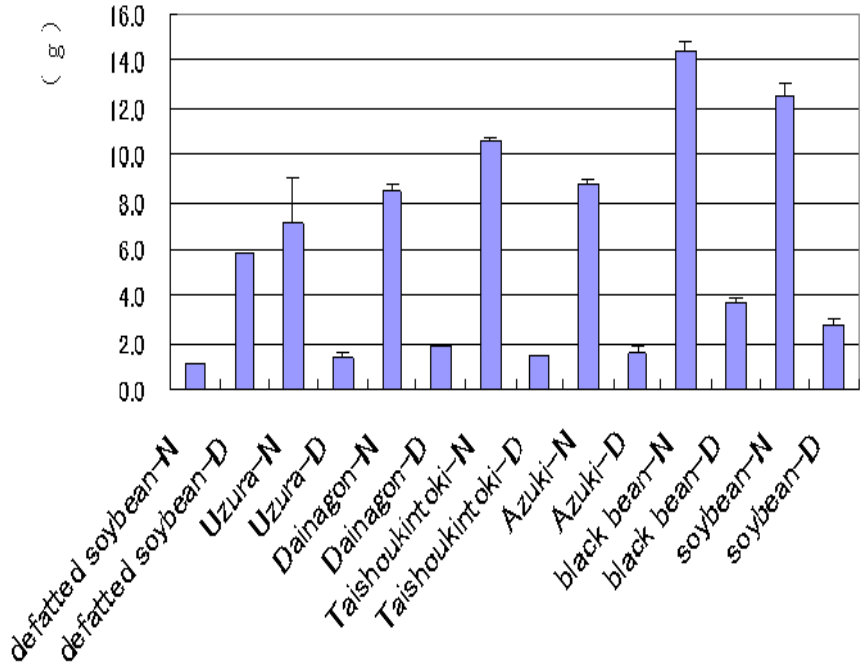


Fig. 14 Amounts of protein-catechins complexes from various bean (DW)

1. (-N :non-degenerated bean protein, -D : degenerated bean protein)
2. **C**: (+)-catechin, **EC**: (-)epicatechin,
   EGC: (-)-epigallocatechin,
   ECG: (-)-epicatechin 3-*O*-gallate,
   EGCG: (-)-epigallocatechin3-*O*-gallate

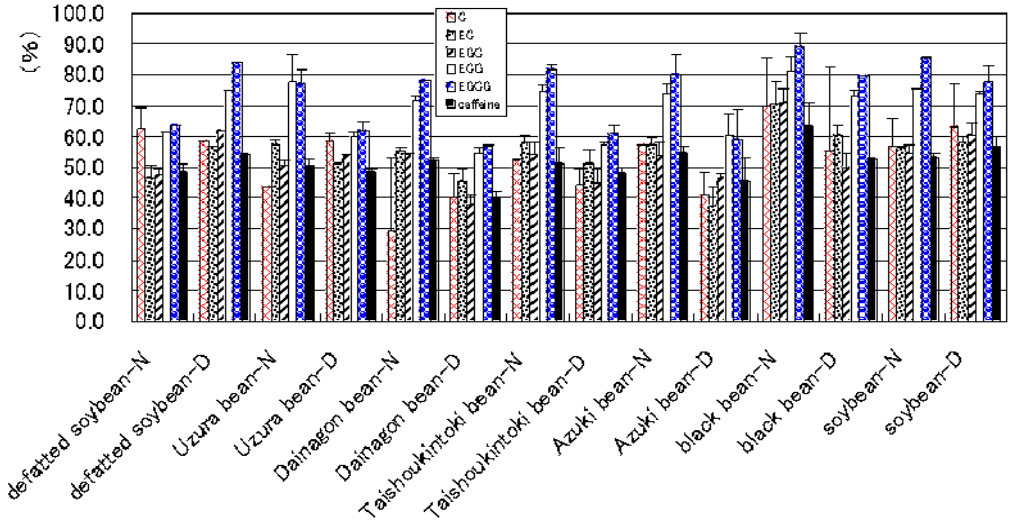


Fig. 15 Absorption rates of catechins adsorbed in protein-catechins complexes from various beans

1. (-N :non-degenerated bean protein, -D : degenerated bean protein)
2. **C**: (+)-catechin, **EC**: (-)epicatechin,
   EGC: (-)-epigallocatechin,
   ECG: (-)-epicatechin 3-*O*-gallate,
   EGCG: (-)-epigallocatechin 3-*O*-gallate

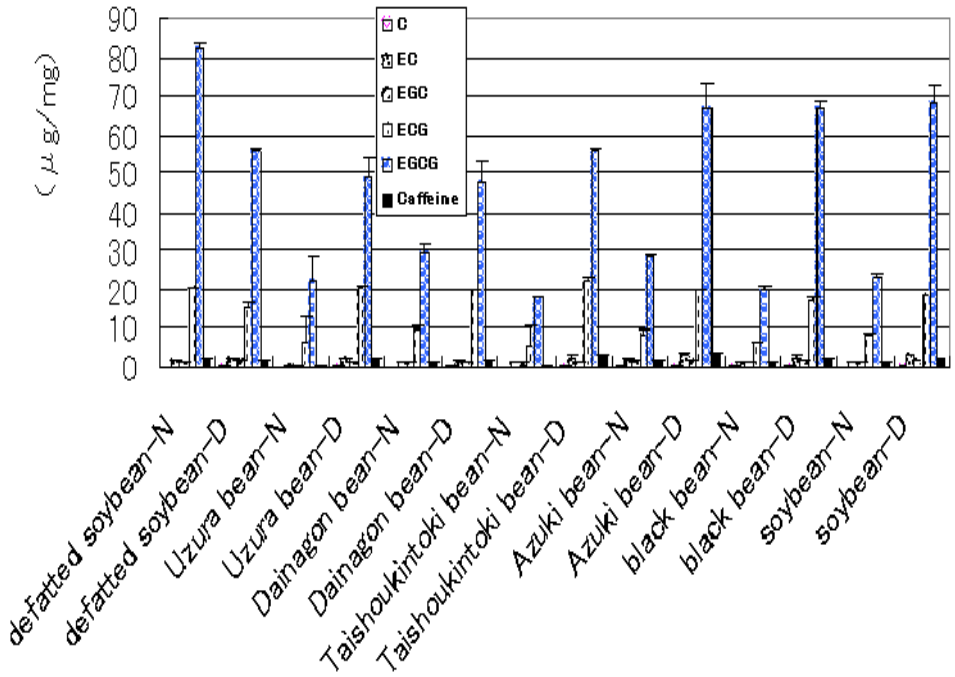


Fig. 16 Contents of catechins from various bean protein-catechins complexes (DW)

1. (-N :non-degenerated bean protein, -D : degenerated bean protein)
2. **C**: (+)-catechin, **EC**: (-) epicatechin,
   EGC: (-)-epigallocatechin,
   ECG: (-)-epicatechin 3-*O*-gallate,
   EGCG: (-)-epigallocatechin 3-*O*-gallate

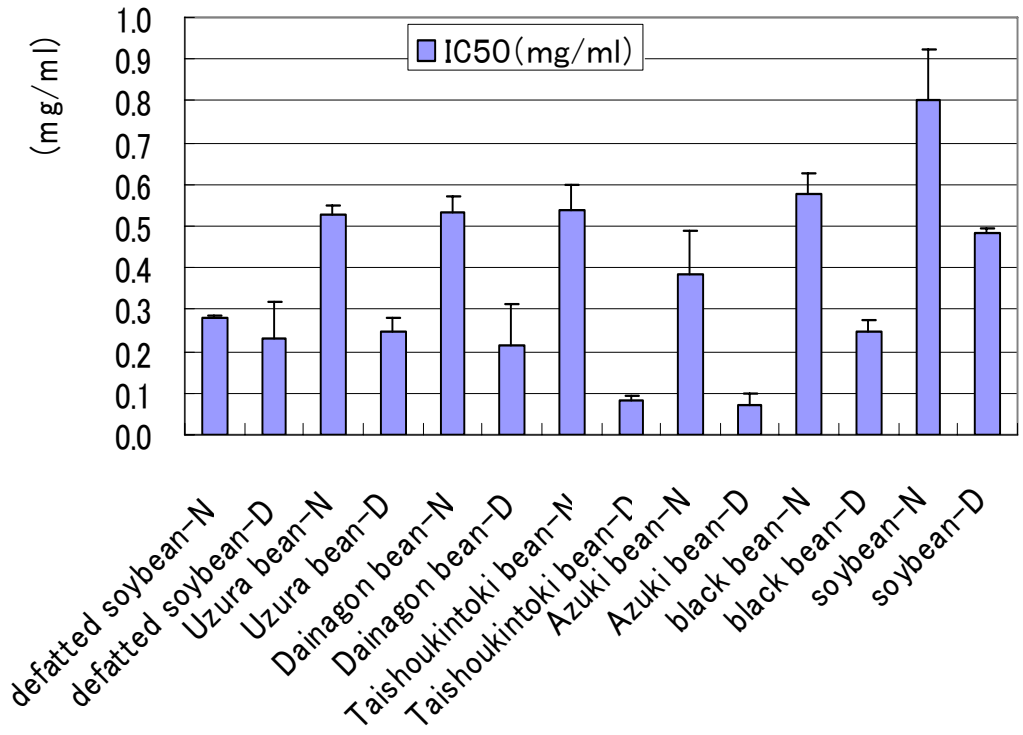


Fig. 17 DPPH scavenging activities of various bean protein-catechins complexes

1. (-N :non-degenerated bean protein, -D : degenerated bean protein)
2. **C**: (+)-catechin, **EC**: (-) epicatechin,
   EGC: (-)-epigallocatechin,
   ECG: (-)-epicatechin 3-*O*-gallate,
   EGCG: (-)-epigallocatechin 3-*O*-gallate

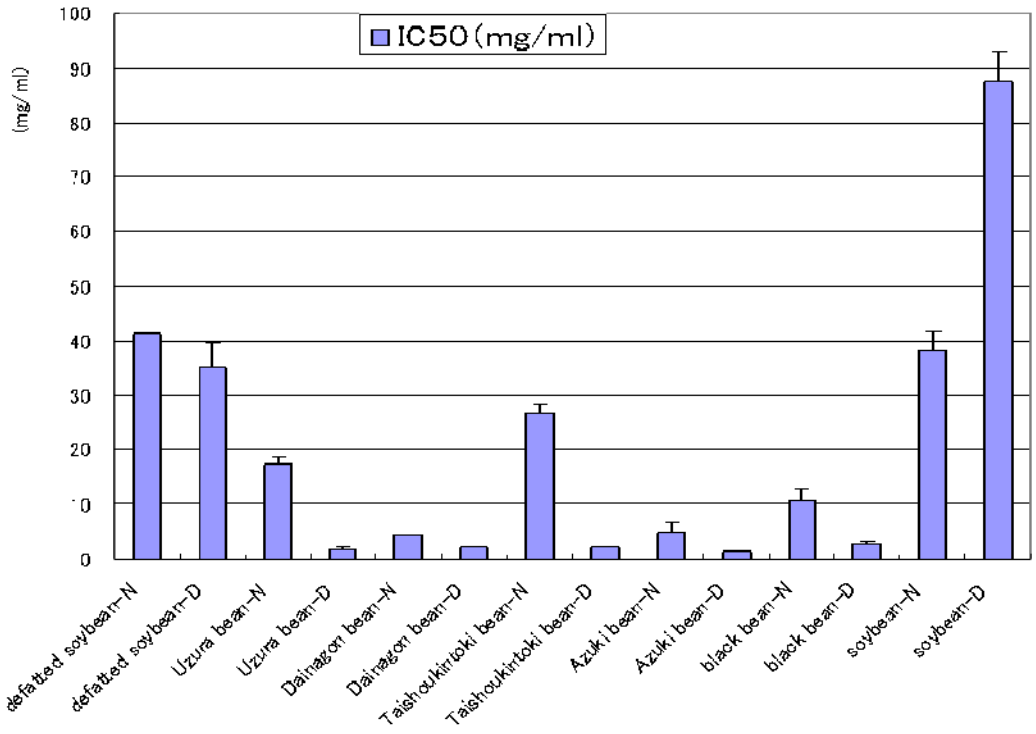


Fig. 18 DPPH scavenging activities of protein precipitates from various beans (IC_{50})

(-N: non-degenerated bean protein, -D: degenerated bean protein)

CHAPTER 3

HEATING PROCESSING OF PLANT MATERIALS CONTAINING POLYPHENOLS

The influence of heating processing on the components of food was widely investigated (Bergamo et al. 2003, Jensen et al. 1995, Rohn et al. 2007). The nutritional components such as vitamin, carotenoids were reported to be degraded during the heat processing in many studies (Awuah et al. 2007, Rattanathanalerk et al. 2005). In recent years, there are also some reports on the influence of various domestic thermal processing (i.e. frying, microwave heating, cooking, boiling and roasting processes) on the changes in contents of some monomer polyphenols (Ioku et al. 2001, Brenes et al. 2002, Stintzing et al. 2006, Mathias et al. 2006). However, the influences of autoclave processing on the changes in chemical structures of polyphenols are rarely reported (Rohn et al. 2007). In this experiment, in order to clarify the chemical transformations of catechins and hydrolysable tannins, the materials from several plants (*C. sinesis*, *S. sebiferum* and *P. urinaria*) containing catechins or hydrolysable tannins were autoclaved. The changes in the contents and chemical structures of polyphenols were investigated and the reaction products were also indentified during autoclave processing.

3.1 Heating processing of tea polyphenols

Generally, in various tea (such as green tea and black tea) leaves, the contents of epicatechin derivatives i.e. EGCG (4%–9% of dry weight of tea leaves) and ECG (0.8%–1.8% of dry weight of tea leaves) are very high, while the contents of GCG [(–)–gallocatechin 3–*O*–gallate] (less than 0.1% of dry weight of tea leaves) and CG [(–)–catechin 3–*O*–gallate] (about 0.02% of dry weight of tea leaves) are very low (Nishitani and Sagesaka 2004).

Recently, it was reported that GCG and CG were more effective than EGCG and ECG against cholesterol adsorption (Ikeda et al. 2003). However, little is known about the functionality of GCG and CG. These gallocatechin type tea polyphenols (GCG and CG) are considered to be originated from the epimerization of EGCG and ECG during procedure of thermal manufacture of commercial tea drinks (Xu et al. 2003). However, these conversions of EGCG and ECG have not been reported in the tea leaves and tea aqueous extracts.

In order to further elucidate the changes in structures and composition of catechins in various tea materials during heating processing, various tea materials (i.e. tea leaves, tea aqueous extract and commercial tea drinks) were autoclaved. And the changes in contents and composition of catechins were investigated in this experiment.

3.1.1 Materials and methods

Chemicals

Caffeine and tea polyphenols i.e. **G**, **GC**, **C**, **EGC**, **EC**, **EGCG**, **ECG**, (−)−gallocatechin 3−*O*−gallate (**GCG**), (−)−catechin 3−*O*−gallate (**CG**) were purchased from Sigma Co. Ltd. in Japan. All of them were used as authentic standards. The chemical structures of them were shown in Fig. 10−1 and Fig. 11

Conditions of HPLC analysis

The HPLC conditions were identical to those mentioned in 2.1.1.2−b.

Preparation of non−autoclaved and autoclaved tea leaves and HPLC analysis

The fresh tea leaves were collected in Ureshino city of Saga prefecture (Japan) in June, 2003. Some of the fresh leaves were autoclaved and then dried at 60 ℃ overnight (as autoclaved leaves); others were dried immediately at 60 ℃ overnight (as non−autoclaved leaves). Both of non−autoclaved and autoclaved leaves from fresh tea leaves (each 8 g, DW) were extracted with 100 ℃ water (each 20 ml) for 6 min. The extracts were subjected to HPLC analysis after filtration through Millex LH filter® (0.45 μl), respectively.

Two of aliquots of commercial tea leaves (each 1 g, DW) were respectively mixed with 1 ml water. One of aliquots was

autoclaved and dried at 60 ℃ overnight. These dried commercial green tea leaves were used as autoclaved commercial tea leaves. Another of aliquots was directly dried at 60 ℃ and used as non-autoclaved commercial tea leaves. Both of the non-autoclaved and autoclaved commercial tea leaves (each 40 mg) were extracted with 50 % MeOH (2 ml) for 16 hours. The extracts were subjected to HPLC analysis after filtration through Millex LH filter ® (0.45 µl), respectively.

Preparation of non-autoclaved and autoclaved tea drinks and tea extract and HPLC analysis

Five tea drinks i.e. CATECHIN-SHIKI RYOKUCHA, PAN FIRED JAPAN Tea, NAMACHA, MAROCHA CHABANOKOU and FAN OOLONGCHA were purchased from market in Saga, Japan. Each drink was divided into two aliquots. One of the aliquots was subjected to HPLC analysis after filtration as non-autoclaved tea drink. Another of aliquots was autoclaved and then subjected to HPLC analysis after filtration as autoclaved tea drink.

The commercial green tea leaves (2 g, DW) were extracted with 100 ℃ water (100 ml) for 5 min and then filtered through the filter paper (ADVANTEC No. 3). The filtrate was divided into two aliquots. One of the aliquots was subjected to HPLC analysis as non-autoclaved tea extract. Another of aliquots was autoclaved and then subjected to HPLC analysis as autoclaved tea extract.

3.1.2 Results and Discussion

Transformations of catechins in tea leaves after autoclaving

In non-autoclaved tea leaves, EGCG (1.22% from the fresh tea leaves, 5.74% from the commercial tea leaves, DW) and ECG (0.80% from the fresh tea leaves, 1.93% from the commercial tea leaves, DW) were the main gallate type epicatechins, and GCG and CG were not detected (Fig. 19A and Fig. 20A). On the contrary, in the autoclaved leaves, high levels of GCG (2.58% from the fresh tea leaves, 2.92% from the commercial tea leaves, DW) and CG (0.24% from the fresh tea leaves, 0.57% from the commercial tea leaves, DW) were detected. In addition, the decreases of EGCG and ECG were also observed in the tea leaves after autoclaving (Fig. 19 and Fig. 20). In the case of the commercial tea leaves, after autoclaving, the content of EGCG decreased from 5.74% to 3.37%, and that of ECG decreased from 1.93% to 1.36% (Fig. 20).

Xu et al. pointed out that epimerisation of catechins (such as EGCG and ECG) might occur and converse to their isomers (such as GCG and CG) during manufacturer processing of commercial tea drinks (such as canned and bottled tea drinks) (Xu et al. 2003). Thus, it was considered that EGCG and ECG (in the non-autoclaved tea leaves) were epimerised and conversed to GCG and CG (in the autoclaved tea leaves) during autoclaving processing in this experiment. Significant increase of GC and C were also observed in the tea leaves after autoclaving (Fig. 19 and Fig. 20). It suggested that the conversions of non-gallate type epicatechin derivatives (EGC and EC) to their epimers (GC

and C) also occurred during autoclave processing. It showed that the epimerisation occurred in epicatechin derivatives (EGCG, ECG, EGC and EC) during autoclave processing of tea leaves.

Although the non-autoclaved tea leaves were treated at 60℃, GCG and CG were not detected in them after treatment (Fig. 19 and Fig. 20). It is suggested that high temperature is necessary for the epimerization of catechins.

It has been reported that the contents of GCG (less than 0.1% of dry weight of tea leaves) and CG (about 0.02% of dry weight of tea leaves) are generally very low in green tea, Oolong tea or black tea (Nishitani and Sagesaka 2004). In this experiment, the contents of GCG and CG in the autoclaved tea leaves were about 29 times as those of GCG and CG in tea leaves reported in the literature (Nishitani and Sagesaka 2004). Accordingly, it is considered that autoclave processing is an efficient method for production of tea leaves with high contents of GCG and CG. In addition, these autoclaved tea leaves are expected to be used as new materials in food and pharmaceutical fields since they contain higher contents of GCG, CG, GC and C compared to the original tea leaves.

Transformations of catechins during autoclave processing of tea aqueous extract

As shown in Table 3, GCG (0.59 mg/ml) and CG (0.08 mg/ml) were detected in the autoclaved tea extract, while they were not detected in the non-autoclaved tea extract. Increases of GC (0.14 mg/ml) and C (0.08 mg/ml) were also observed after

autoclave processing. In addition, the decrease of EGCG concentration (0.53 mg/ml) was near to the increase of GCG concentration (0.59 mg/ml). Similar trends were also observed for other pairs, i.e. ECG and CG, EC and C, EGC and GC. These results showed that the epicatechin derivatives (EGCG, ECG, EGC and EC) also epimerised and conversed to their isomers (GCG, CG, GC and C) during autoclave processing of tea aqueous extract. Xu et al. also obtained the similar results in the thermal processing of canned and bottled tea drinks, in which epicatechin derivatives (EGCG, ECG, EGC and EC) were added.

Changes in the concentration of catechins in commercial tea drinks during autoclave processing

As shown in Table 3, GCG and CG, which were undetectable in non-autoclaved tea leaves and extract, were detected in all of the non-autoclaved tea drinks at the same levels as EGCG and ECG, respectively. It indicated that epimerisation of epicatechin derivatives have occurred during manufacture processing of commercial tea drinks. Except for GC, all of the catechins in the commercial tea drinks degraded in different degree after autoclaving. These results suggested that epimerisation of most epicatechin derivatives had reached maximum level during manufacture processing of commercial tea drinks and further thermal processing only caused the degradation of most catechins.

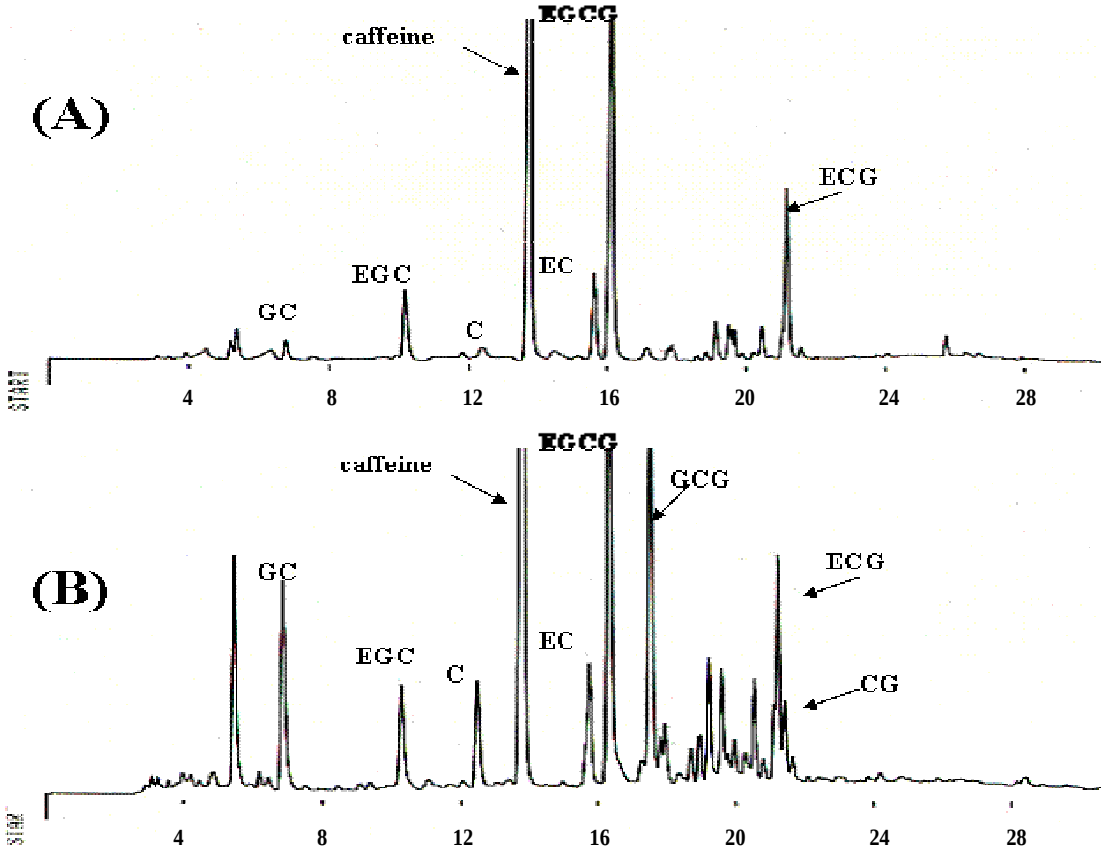


Fig. 19 HPLC profiles of hot water extract from fresh tea leaves

(A) non-autoclaved (B) autoclaved

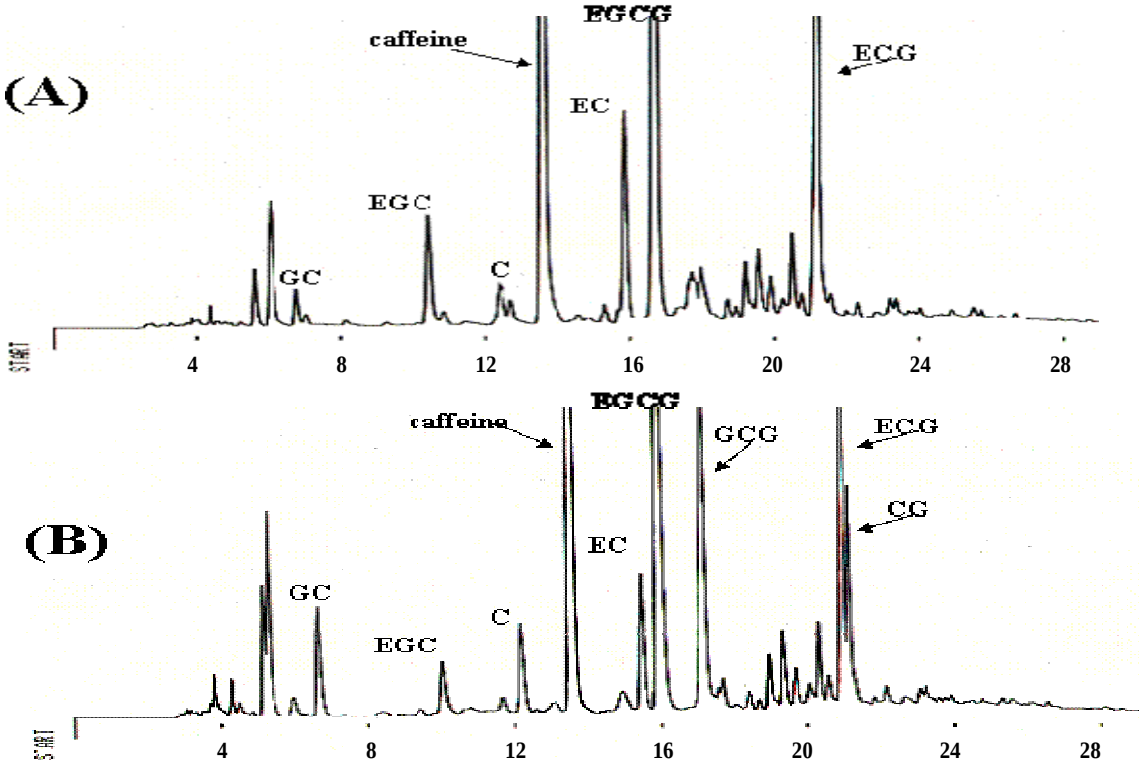


Fig. 20　HPLC profiles of MeOH extract from commercial tea leaves

(A) non-autoclaved　(B) autoclaved

Table 3 Catechins and caffeine concentrations (mg/ml) in tea extracts and various tea drinks

Tea drinks and extracts	GC	EGC	C	EC	EGCG	GCG	ECG	CG	Caffeine
CATECHIN-SHIKI RYOKUCHA	0.06	0.03	0.04	0.03	0.24	0.34	0.07	0.11	0.11
CATECHIN-SHIKI RYOKUCHA-A*	0.07	0.02	0.04	0.03	0.17	0.19	0.06	0.04	0.10
PAN FIRED JAPAN Tea	0.03	0.04	0.03	0.04	0.10	0.10	0.07	0.01	0.09
PAN FIRED JAPAN Tea-A*	0.06	0.02	0.03	0.02	0.08	0.08	0.02	0.02	0.10
NAMACHA	0.04	0.02	0.03	0.01	0.09	0.13	0.03	0.01	0.08
NAMACHA-A*	0.04	0.02	0.02	0.01	0.06	0.06	0.01	0.01	0.09
MAROCHA CHABANOKOU	0.06	0.04	0.03	0.03	0.13	0.16	0.09	0.05	0.10
MAROCHA CHABANOKOU-A*	0.09	0.03	0.03	0.03	0.10	0.11	0.04	0.02	0.10
FAN OOLONGCHA	0.02	0.01	0.01	0.01	0.07	0.07	0.04	0.02	0.09
FAN OOLONGCHA-A*	0.03	0.02	0.01	0.01	0.06	0.08	0.03	0.02	0.10
Extract of commericial tea leaves **	0.04	0.21	0.03	0.2	0.92	-	0.22	-	0.31
Extract of commericial tea leaves ** A*	0.18	0.08	0.11	0.13	0.49	0.59	0.17	0.08	0.38

A＊ autoclaved

＊＊ 2 g (dry weight) of tea leaves were extracted with 100 ml hot water (100 ℃) for 5 min.

3. 2 Heating processing of materials containing hydrolysable tannin

It was known that transformations of catechins occurred during heating processing (Huang et al. 2004c). However, the transformation of hydrolysable tannins during heating processing was rarely reported.

Geraniin is known as the main hydrolysable tannin of *G. thunbergii* (a traditional herb) and usually used for the remedy of gastric ulcer and medicine for intestinal disorders, possesses many bioactivities such as antihypertensive activity (Cheng et al. 1994), effects of cancer prevention (Fujiki et al. 2003), HIV (human immunodeficiency virus) inhibitory activity (Notka et al. 2003). Although *S. sebiferum* and *P. urinaria* also contain a large amount of geraniin as well as *G. thunbergii,* they have still not widely utilized in pharmacy and food fields. In China and India, *P. urinaria* is usually used for the remedy of B virus hepatitis, since it possesses various bioactivities i.g. antibacterial activity and anti-inflammation activity (Cheng et al. 1995). From ancient times, *S. sebiferum* has been used as diuretic, antiphlogistic and insectifuge in China. Recently, some components of antihypertensive activity (Hsu et al. 1994), anti-herpes activity (Kane et al. 1988) and antitumor (Liu et al. 1988) were isolated from this plant.

In this experiment, in order to clarify the influence of heating processing on the composition and chemical structures of hydrolysable tannins, the leaves of *S. sebiferum* and *P. urinaria* were autoclaved and the DPPH scavenging activities of

processed materials were also determined.

3.2.1 Materials and methods

Chemicals

Gallic acid (**G**), geraniin (**18**), corilagin and brevifolin carboxylic acid were isolated from Euphorbiaceous plants (Saijo et al. 1989); Ellagic acid was purchased from Sigma Co. Ltd., Japan. All of them were used as authentic standards. The chemical structures of them were shown in Fig. 10-1, Fig. 10-2 and Fig. 21.

Conditions of HPLC analysis

The HPLC conditions were identical to those mentioned in 2.1.1.2-b.

Preparation of non-autoclaved and autoclaved leaves and HPLC analysis

The fresh leaves of *S. sebiferum* and *P. urinaria* were respectively collected in Saga University in June and November, 2005. The fresh leaves (each 5 g) were autoclaved and then dried at 60℃ overnight. The dried leaves (each 0.02 g) after grounding were extracted with 2 ml MeOH for 16 hours at room temperature in the dark. The extracts (each 3 μl) were subjected to HPLC analysis after filtration through Millex LH filter ®(0.45 μl). As controls, the fresh leaves of the two plants (each 5 g) without autoclave treatment were directly dried at 60℃ overnight. The

non-autoclaved leaves (each 0.02 g) were also extracted with 2 ml MeOH and the extracts were also subjected to HPLC analysis after filtration.

In addition, geraniin (1.2 mg) were dissolved with 2 ml H_2O using sonicator and then the solution was filtered through Millex LH filter® (0.45 µl). The filtrate was divided into two aliquots. 6µl of an aliquot was subjected to HPLC analysis; another aliquot was autoclaved and cooled to room temperature prior to HPLC analysis.

Measurement of DPPH radical scavenging activity

Autoclaved leaves and non-autoclaved leaves of *S. sebiferum* and *P. urinaria* (each 0.03 g, DW) were respectively extracted with 3 ml MeOH for 16 hours at room temperature in the dark. The extract was filtered through filter paper and DPPH radical scavenging activity of the filtrate was measured as described in 2.1.1.3.

3.2.2 Results and Discussion

The contents of polyphenols in *S. sebiferum* and *P. urinaria* leaves (autoclaved and non-autoclaved leaves) were shown in Table 4. In non-autoclaved leaves, the contents of geraniin were 55.63 mg/g (dry weight) in leaves of *P. urinaria* and 55.42 mg/g in those of *S. sebiferum,* respectively, while the contents of other polyphenols were very low. It showed that geraniin is the main polyphenol in the non-autoclaved leaves of the two plants. In

the autoclaved leaves, remarkable decrease of geraniin and increase of other four polyphenols (gallic acid, corilagin, ellagic acid and brevifolin carboxylic acid) were observed (Table 4).

In addition, the concentration of geraniin in geraniin aqueous solution decreased from 0.5 mg/ml (before autoclaving) to 0.1 mg/ml after autoclaving, simultaneously, the productions of gallic acid (0.03 mg/ml), corilagin (0.05 mg/ml), ellagic acid (0.01 mg/ml) and brevifolin carboxylic acid (0.01 mg/ml) were also confirmed in the autoclaved aqueous solution of geraniin (Fig. 22 A and B).

These results suggested that the four polyphenols were originated from geraniin during autoclaved processing. It is deduced that autoclave processing caused the hydrolysis reaction of the five ester linkages of geraniin and the reaction products of the hydrolyzation were converted to various compounds (including the four polyphenols) through reaction of dehydrogenation or decarboxylation. Okada et al. reported the decrease of geraniin and the productions of corilagin and ellagic acid during the decoction of *G. thunbergii*, while the production of brevifolin carboxylic acid was not observed (Okuda et al. 1979). Hence, it is concluded that production of brevifolin carboxylic acid only occurred during the processing of geraniin at high temperature such as autoclave processing.

As shown in Fig. 23, both the autoclaved leaves and non-autoclaved leaves showed the strong DPPH radical scavenging activities. It suggests that the four polyphenols in

the autoclaved leaves also possess strong activities as well as geraiin in the non-autoclaved leaves.

It is well known that ellagic acid is widely used as food antioxidant or the constituent of cosmetics since it possesses strong antioxidative activity; recently, many bioactivities of elllagic acid such as antitumor effect (Bhosle et al. 2005), antivirus effect (Shin et al. 2005) and inhibitory effect to proliferation of cancer cells (Losso et al. 2004), were also reported. Corilagin was thought to possess cancer prevention effect (Fujiki et al. 2003) and hypertensive effect (Cheng et al. 1995). It has been suggested that brevifolin carboxylic acid may play a role in protection against dioxin toxicity (Amakura et al. 2003). In this experiment, brevifolin carboxylic acid which has not been detected from fresh materials of *S. sebiferu*, was detected in the autoclaved leaves of the two plants. Since autoclaved leaves of two plants contain these four polyphenols, while non-autoclaved leaves mainly contain geraniin, different bioactivities are expected in the autoclaved leaves (compared to those of non-autoclaved leaves). Taking into account the stability of these four polyphenols in heating processing, the autoclaved leaves containing amounts of the four polyphenols are potential to become novel materials in food or pharmaceutical industries which need heating processing.

3.3 Conclusion

During the autoclave processing of tea leaves and aqueous tea extracts, epicatechin derivatives (EGCG, ECG, EGC and EC) epimerised and converted to their isomers (GCG, CG, GC and C). By autoclave treatment, tea leaves containing high content of GCG and CG were obtained, which are expected as new materials in food and pharmaceutical fields. In addition, it is considered that autoclave treatment of green tea leaves is an efficient way for the production of GCG and CG.

It was also clarified that geraniin were remarkably degraded and transformed into four polyphenols (gallic acid, corilagin, ellagic acid and brevifolin carboxylic acid) during autoclave processing of *S. sebiferum* and *P. urinaria* leaves. During autoclave processing, the five ester linkages of geraniin were cleaved and the four polyphenols formed from the reaction products of this cleavage through reaction of dehydrogenation or decarboxylation. The DPPH radical scavenging activities of these four polyphenols seemed to be almost identical with that of geraniin. Taking into account the stability of these polyphenols in heating process, autoclaved leaves of these two plants seemed to be good materials for new food ingredients.

Therefore, autoclave processing is a practical and efficient method for transformation of various polyphenols. In this experiment, valuable findings were provided for the development of new functional materials containing polyphenols.

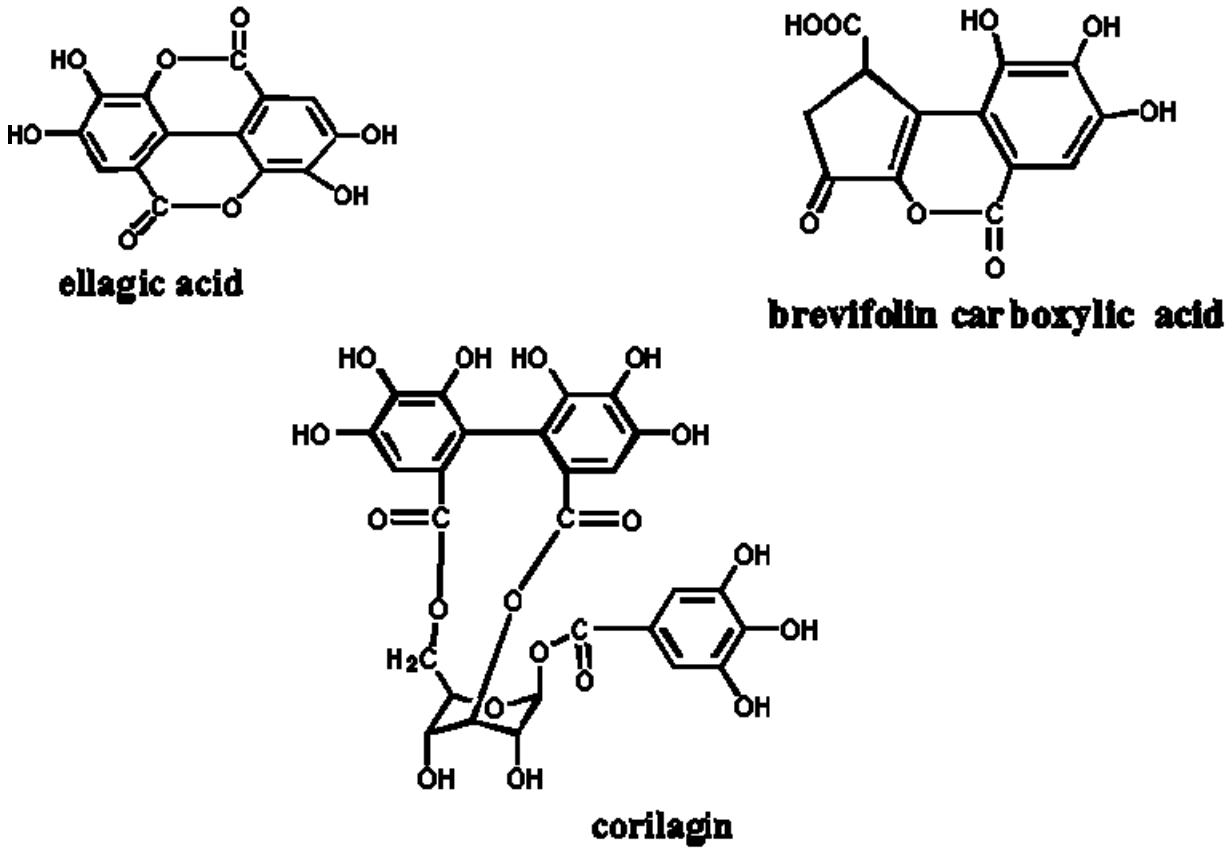


Fig. 21 Chemical structures of polyphenols from *S. sebiferum* and *P. urinaria*

Table 4 Contents (mg/g, as dry weight) of Polyphenols in *S. sebiferum* and *P. urinaria* leaves

plant leaves	gallic aid	geraniin	corilagin	ellagic acid	brevifolin carboxylic acid
S. sebifenum	0.63	55.42	_	4.96	_
S. sebifenum *	11.32	0.95	23.63	13.03	14.39
P. urinia	1.08	55.63	_	_	_
P. urinia *	19.15	1.70	29.11	18.90	25.93

* autoclaved

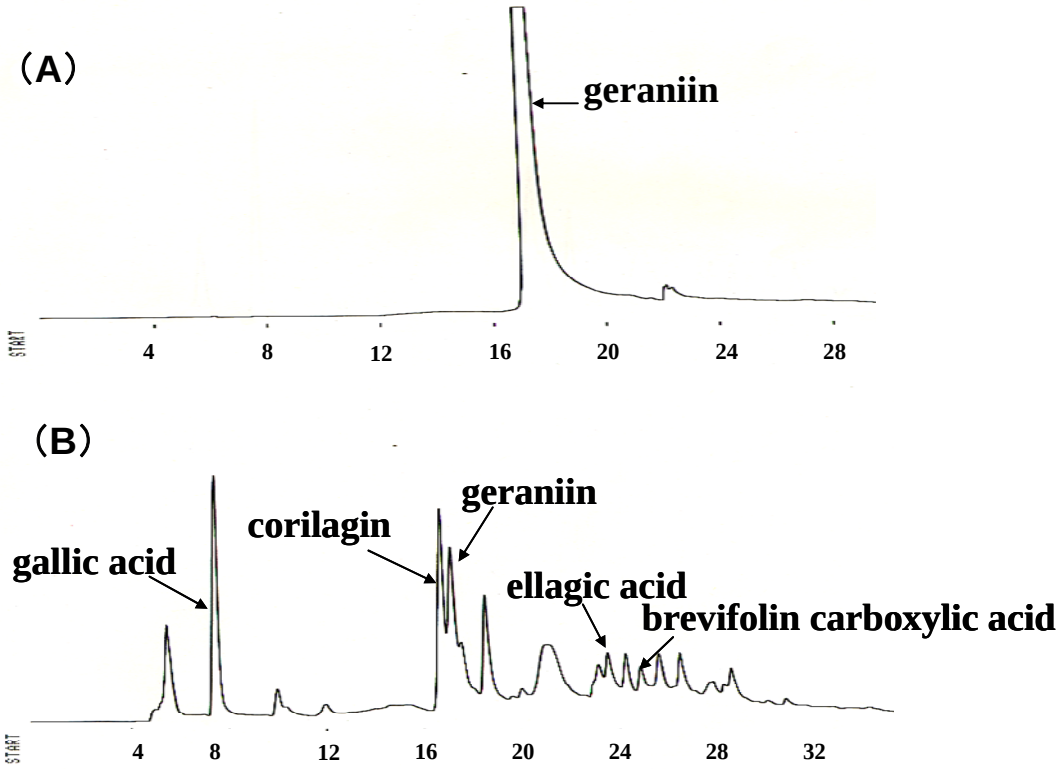


Fig. 22 HPLC profiles of geraniin aqueous solution

(A) non-autoclaved (B) autoclaved

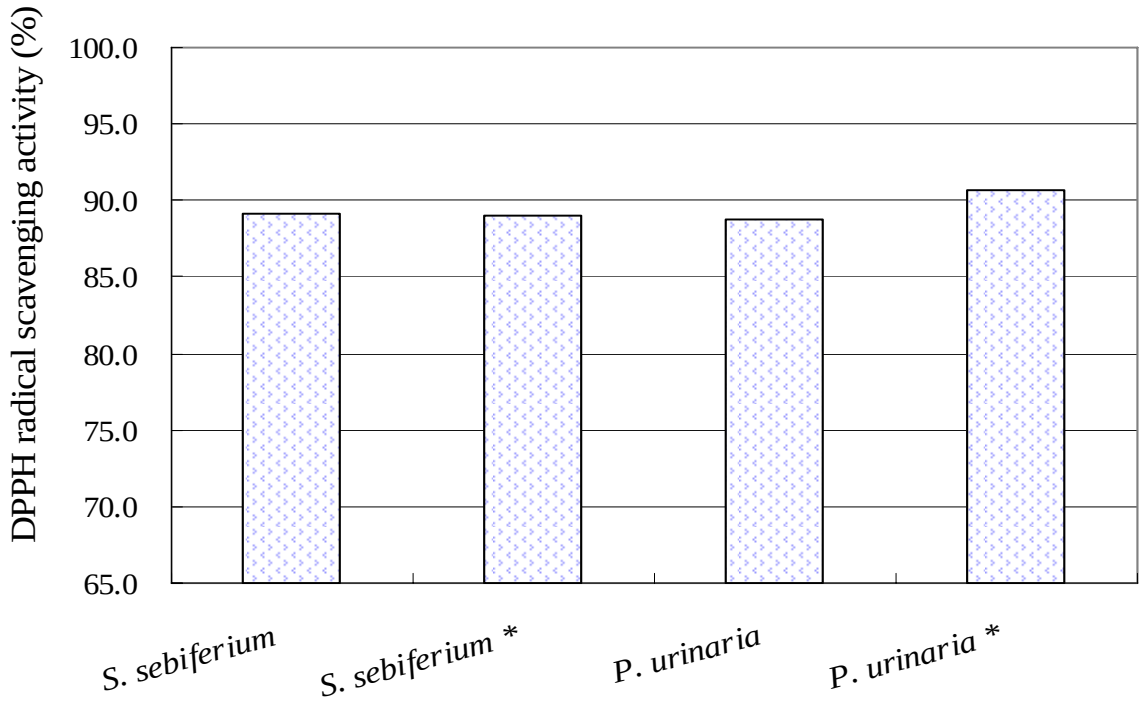


Fig. 23 DPPH radical scavenging activities of the extracts from *S. sebiferum* and *P. urinaria* leaves

* autoclaved

CHAPTER 4

FERMENTATION OF PLANT MATERIALS CONTAINING POLYPHENOLS

Fermented foods are popular all over the world because the nutritional value, sensory properties and functional qualities of food materials may be improved during the fermentation with microoganisms (Blandino et al. 2003). It was reported that the processing of fermentation with microorganisms has great influence on the chemical constituents of various fermented products (Yin et al. 2004, Wang and Murphy 1994). It is believed that the microorganisms play important roles in the changes of bioactivities and the formation of new chemical constituents during fermentation (Zhou et al. 2005, Zhu et al. 2007 and Chang et al. 2007).

Usually, the fermentations of most of traditional fermented products are natural and various microoganisms such as some bacteria, fungi and yeasts are frequently found in these products (Blandino et al. 2003). The roles of individual strain of bacteria or fungi on the metabolism of chemical constituents are usually unclear since various microorganism strains are mixed during the fermentation. In order to more effectively utilize microorganisms in food fermentation and obtain the desirable fermented products, it is important to clarify the effect of individual strain of various microorganisms on metabolism of the chemical constituents during fermentation.

The individual strains of few fungi (e. g. *Aspergillus*) and

bacteria (e. g. *Bacillus subtilis*) are widely used in the fermentation of some legume products (Terlabie et al. 2006, Chang et al. 2007). It has been rather known about compositions in these fermented products and the transformations of chemical constituents caused by the individual strain of microorganisms (such as *A. saitoi, B. subtilis natto etc.*). However, there are few reports on the transformations of polyphenols during the fermentation with individual microorganism.

To clarify the metabolic pattern of polyphenols caused by the individual microbial strains, the fermentations of some materials containing polyphenols (i.e. green tea extracts, the leaves and aqueous extracts of *S. sebiferum* and *P. urinaria*) with the individual strain of bacteria or fungi were attempted. During fermentation, the changes in the concentration of chemical constituents and newly formed metabolites were investigated. In addition, the DPPH scavenge activities of fermented materials were also determined.

4.1 Fermentation of materials containing hydrolysable tannin with individual strain of various microorganisms

4.1.1 Materials and methods

Chemicals

The chemicals were identical to those described in 3.2.1.

Plant materials

The fresh leaves of*S. sebiferum* and *P. urinaria* were identical to those mentioned in 3.2.1.

Microorganisms

B. subtilis natto Naruse (*B. subtilis natto* N.) was purchased from Chemistry Institute of Naruse Co., Tokyo, Japan. *B. subtilis natto* Marumiya (*B. subtilis natto* M.) was prepared from Marumiya Natto. *B. subtilis* 168 and *B. subtilis* W23 were maintained at the Laboratory of Applied Microbiology, Faculty of Agriculture, Saga University. These four strains of bacteria were respectively maintained on the YEB (Vervliet et al. 1975) solid medium in the darkness at 25 ℃.

Three strains of fungi (*Penicillium sp.*, *Fusarium solani* and *Rosellinia necatrix*) were provided by the Laboratory of Food Chemistry, Faculty of Agriculture, Saga University. The fungi were maintained on PDA (potato dextrose agar) medium (EIKEN CHEMICAL Co., LTD.) slants and preserved at 2-8℃.

Conditions of HPLC analysis

The HPLC conditions were identical to those mentioned in 2.1.1.2-b.

Preparation of fermented leaves with the strain of various bacteria and HPLC analysis

The four strains of bacteria (*B. subtilis natto* N., *B. subtilis natto* M., *B. subtilis* 168 and *B. subtilis* W23) were respectively inoculated to sterilized Petri dishes with solid YEB medium and cultured for three days in the darkness at 25 ℃.

The fresh leaves of *S. sebiferum* and *P. urinaria* were autoclaved and cooled to room temperature. The autoclaved leaves (ca. 1.0 g, fresh weight) of the two plants after cooling were respectively placed over the bacteria which have been cultured for three days on solid YEB medium, and then incubated (fermented) in the dark at 25 ℃. The leaves of *S. sebiferum* were fermented for 7, 14 and 21 days; and the leaves of *P. urinaria* were fermented for 4, 7 and 14 days. The fermented leaves were moved from the pretri dish and then dried at 60℃. The dried leaves (0.12 g) after grounding were extracted with 2 ml MeOH for 16 hours at room temperature in the dark and the extracts (each 3 μl) were subjected to HPLC analysis after filtration through Millex LH filter ® (0.45 μl). As controls, the autoclaved leaves without incubation with bacteria, which were placed into the Petri dishes with or without solid YEB medium, were also incubated simultaneously in the same conditions and subjected to HPLC analysis as described above.

Preparation of fermented extracts with the strain of various fungi and HPLC analysis

1) *Preparation of various fungi cultures*

Penicillium sp., *F. solani* and *R. necatrix* were respectively inoculated into glass flask with 50 ml of 1/2 MS liquid medium (Murashige and Skoog 1962) containing 30 g/L sucrose and then incubated at 25℃ on a Multi Shaker at 80 rpm for five days. Mycelial masses of the fungi in the liquid media

were used for the fermentation of the aqueous extracts of two plants.

2) *Preparation of plant aqueous extracts*

The fresh leaves of *S. sebiferum* (600 g) and *P. urinaria* (300 g) were homogenized with 400 ml and 300 ml of distilled water, respectively. The mixtures were filtrated through 4-fold gauze. The filtrate (100 ml/flask) was transferred to glass flask, autoclaved and then cooled to room temperature.

3) *Fermentation of the aqueous extracts with the strain of various fungi and HPLC analysis*

Two mycelial masses (about 5 mm in diameter) of *Penicillium sp., F. solani* or *R. necatrix* were respectively inoculated into the cooled autoclaved extract (100 ml) and incubated at 25℃ at 80 rpm for 28 days. During incubation processing, 2 ml of the extract was taken from glass flask and filtered through Millex LH filter® (0.45 μm) every 7 days, and then the filtrate (1 μl) was subjected to HPLC analysis. As the control, the extract without inoculation with fungus was also incubated simultaneously in the same conditions and subjected to HPLC analysis.

Measurement of DPPH radical scavenging activity

The treated leaves (each 0.03 g, DW) with or without the strain of bacteria were extracted with 3 ml MeOH for 16 hours at

room temperature in the dark. The extract was filtered through the filtrate paper. The DPPH radical scavenging activity of the filtrate was determined (as described as 2.1.1.3). In addition, the DPPH radical scavenging activities of the extracts treated with or without the strain of fungi were also determined every 7 days.

4.1.2 Results and discussion

Changes in the contents of polyphenols in the leaves treated with bacteria

Changes in the contents of chemical constituents in the bacterial fermentation leaves are shown in Fig. 24 and Fig. 25. In chapter 3, it was described that the four polyphenols (gallic acid, corilagin, ellagic acid and brevifolin carboxylic acid) were the main constituents in the autoclaved leaves of *S. sebiferum* and *P. urinaria*. These polyphenols remarkably decreased in the bacterial fermentation leaves of both plants during the first 7 days compared to those in the control samples (the leaves without inoculation with bacteria). Gallic acid and corilagin were hardly detected after 7 days fermentation in all the bacterial fermentation samples. Compared to gallic acid and corilagin, ellagic acid and brevifolin carboxylic acid were slightly stable during the fermentation with bacteria. The results showed that the four polyphenols were easily degraded by the four strains of *bacillus* Genus. For the degradations of polyphenols, no significant difference was observed in all the bacterial fermentation samples.

Changes in the concentrations of polyphenols in fermented extracts with fungi

The concentrations of the four polyphenols in the fungal fermentation extracts of two plants were shown in Fig. 26, Fig.27, Fig. 28 and Fig. 29. Gallic acid slightly increased during the initial stage (0-14 days) of fermentation in the fermented extracts of both plants, and then it decreased; while in the control samples no significant change was observed for gallic acid throughout the fermentation. Corilagin remarkably decreased and was hardly detected after 7 days fermentation in the fungal fermentation extracts of both plants; and the concentration of ellagic acid decreased 50%-82.1% after 7 days fermentation. Compared to the three polyphenols, brevifolin carboxylic acid changed not so much in the concentration, especially in those fungi fermented extracts of *S. sebiferum,* the maximum decrease of brevifolin carboxylic acid was only 11% after 28 days fermentation with fungi.

Tannase was found to be produced by some bacteria and several genera of fungi such as *Aspergillus*, *Fusarium* and *Penicillium* (Belmares et al. 2004). It was supposed that *Penicillium sp., F. solani* and *R. necatrix* also excreted tannase or some tannase-like enzymes during the fermentation of extracts in this experiment. These enzymes caused the degradations of some polyphenols (e.g. corilagin) and released gallic acid and other related compounds. For this reason, the increase of gallic acid was observed in all of the fungal fermentation extracts, while it was not observed in the control

samples.

DPPH radical scavenging activities

Fig. 30 shows the DPPH radical scavenging activities of the MeOH extracts of the fermented samples with the strain of *bacillus* genus. During the whole fermentation period, the DPPH radical scavenging activities of the bacterial fermentation samples decreased (from 89.1–90.4% to 50.2–79.5%) with the fermentation time, while that of the control samples changed little and stayed at a high level (about 90%). It indicated that the four polypheols were the main constituents that contributed to the DPPH radical scavenging activities of the autoclaved leaves.

The DPPH radical scavenging activities of the extracts fermented with the strain of fungi were shown in Fig. 31. During fermentation, the DPPH radical scavenging activities of the extracts treated with *Penicillium sp.*, or *R. necatrix* slightly decreased with fermentation time. In contrast, the increase of DPPH radical scavenging activity was observed in the extracts treated with *F. solani* from 7 days to 14 days of fermentation. And those extracts showed the strongest DPPH radical scavenging activity (about 95%) on the 14th day. The detail reasons of the increase of DPPH radical scavenging activity are not clear; some metabolic compounds are assumed to be produced during the fermentation with *F. solani* and caused the increase of DPPH radical scavenging activity of the fermented extracts.

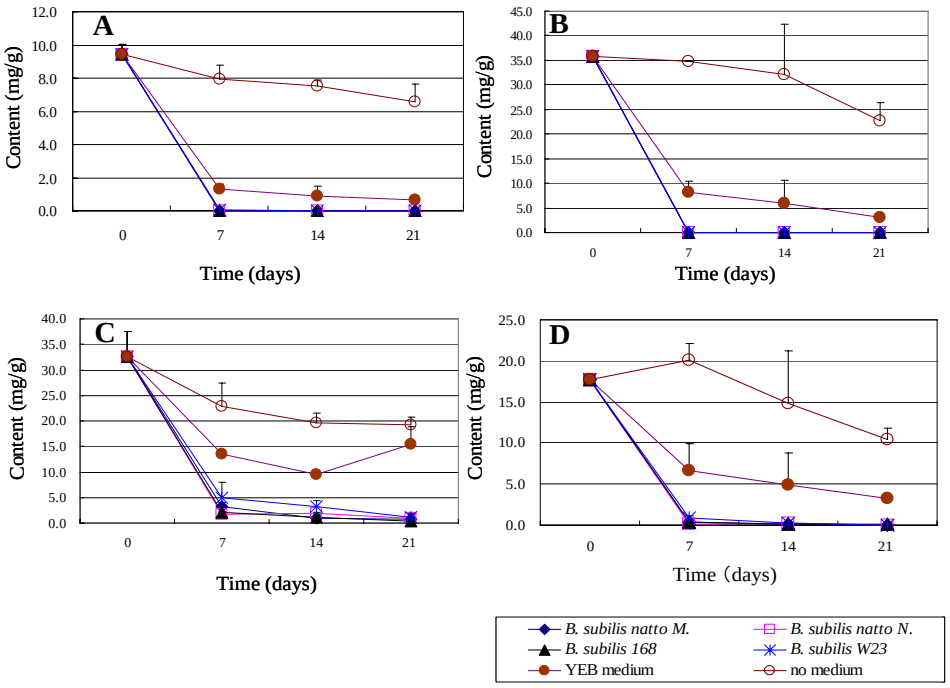


Fig. 24 Contents of polyphenols in *S. sebiferum* leaves fermented with the strain of *B. subtilis* (n=3)

A: gallic acid B: corilagin C: ellagic acid

D: brevifolin carboxylic acid

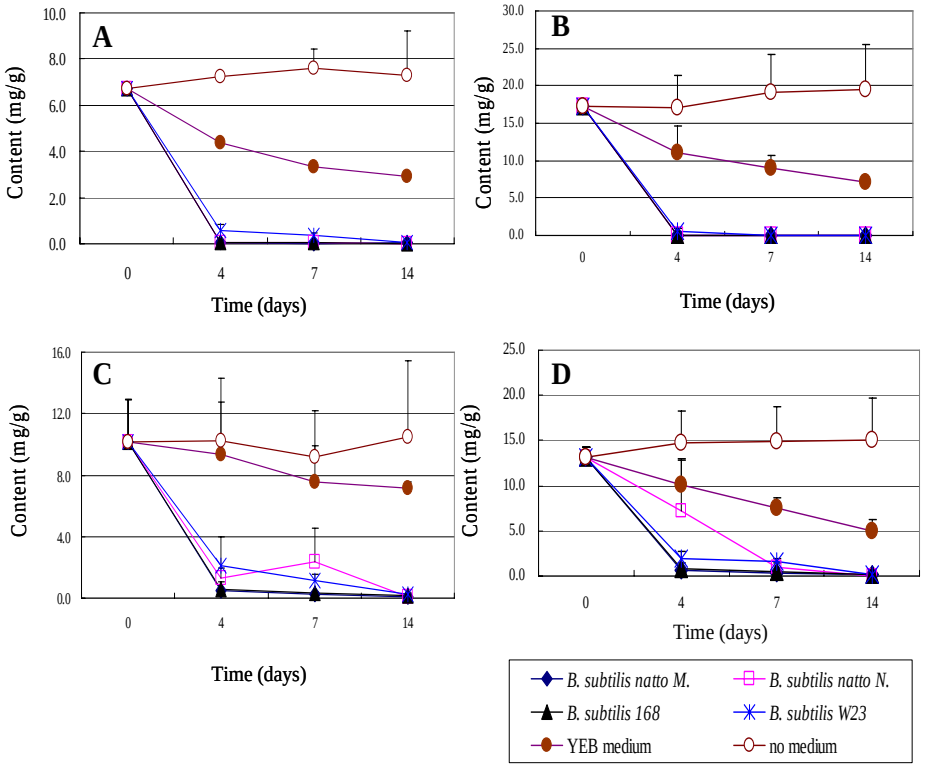


Fig. 25 Contents of polyphenols in *P. urinaria* leaves treated with the strain of *B. subtilis* (n=3)

A: gallic acid B: corilagin C: ellagic acid

D: brevifolin carboxylic acid

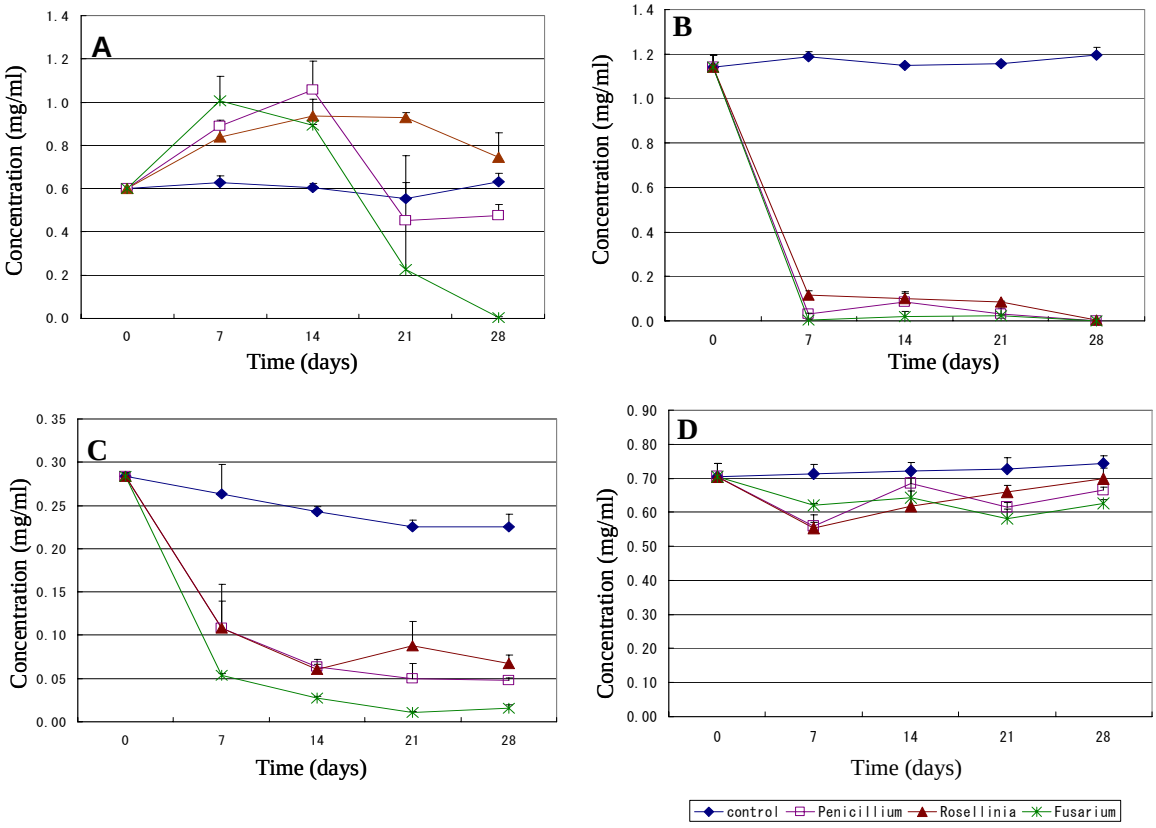


Fig. 26 Concentrations of polyphenols in the extracts of *S. sebiferum* fermented with *Penicillium sp.*, *F. solani* or *R. necatrix* (n=3)

A: gallic acid B: corilagin C: ellagic acid

D: brevifolin carboxylic acid

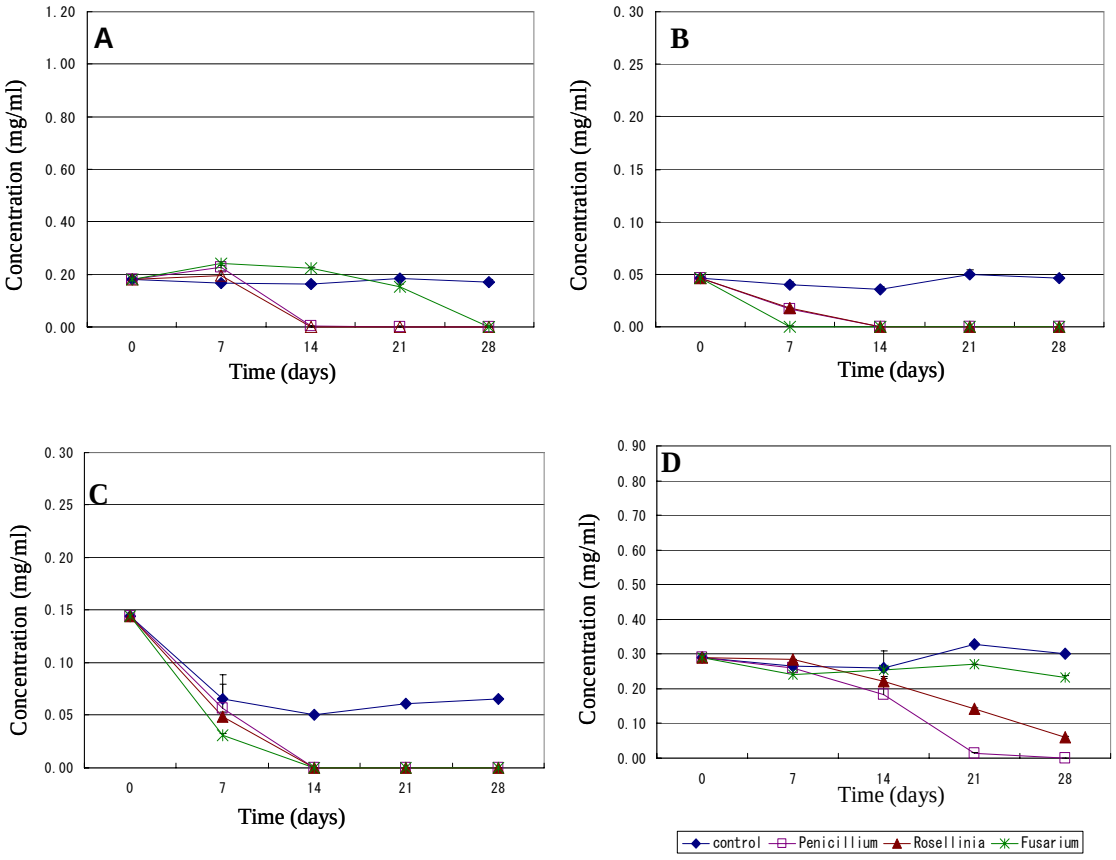


Fig. 27 Concentrations of polyphenols in the extracts of *P. urinaria* fermented with *Penicillium sp.*, *F. solani* or *R. necatrix* (n=3)

A: gallic acid B: corilagin C: ellagic acid

D: brevifolin carboxylic acid

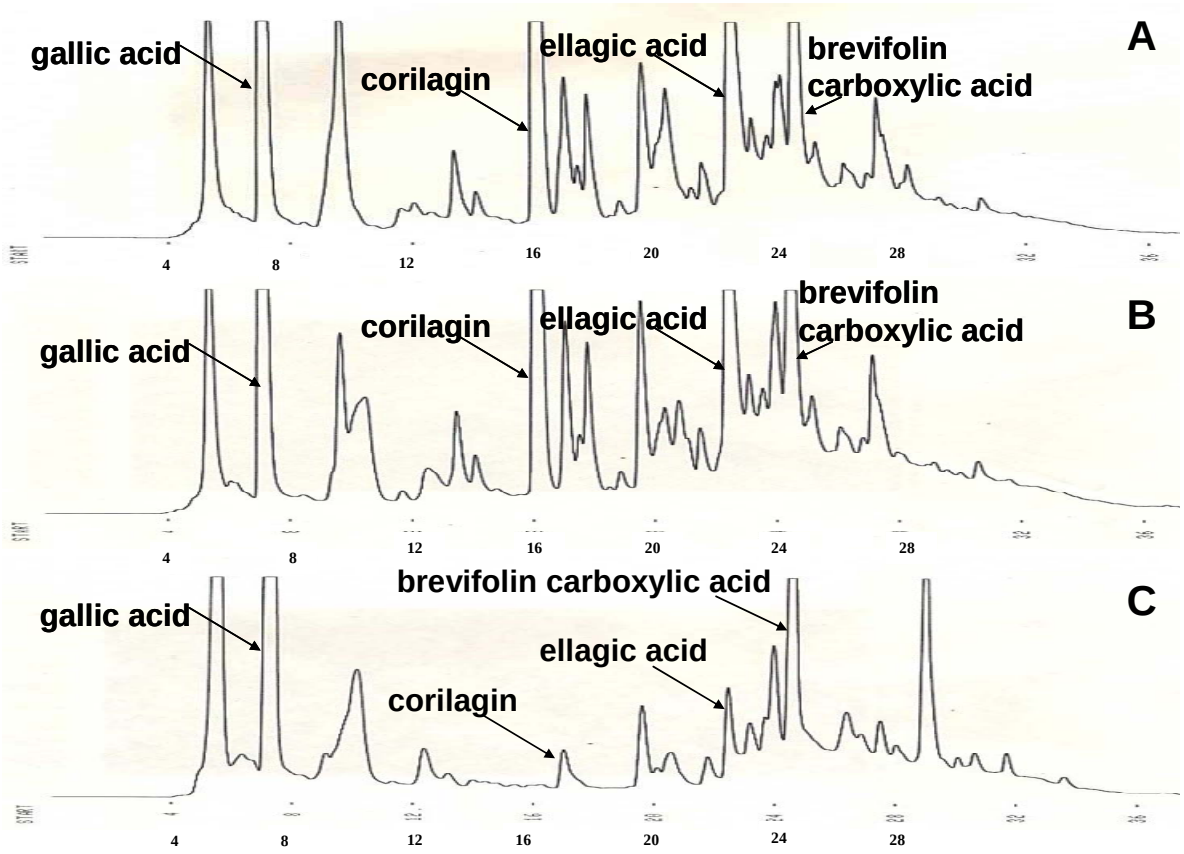


Fig. 28 HPLC profiles of the autoclaved extracts from *S. sebiferum leaves*

A: before fermentation

B: incubated for 14 days without inoculation fungus

C: fermented with *F. solani* for 14 days

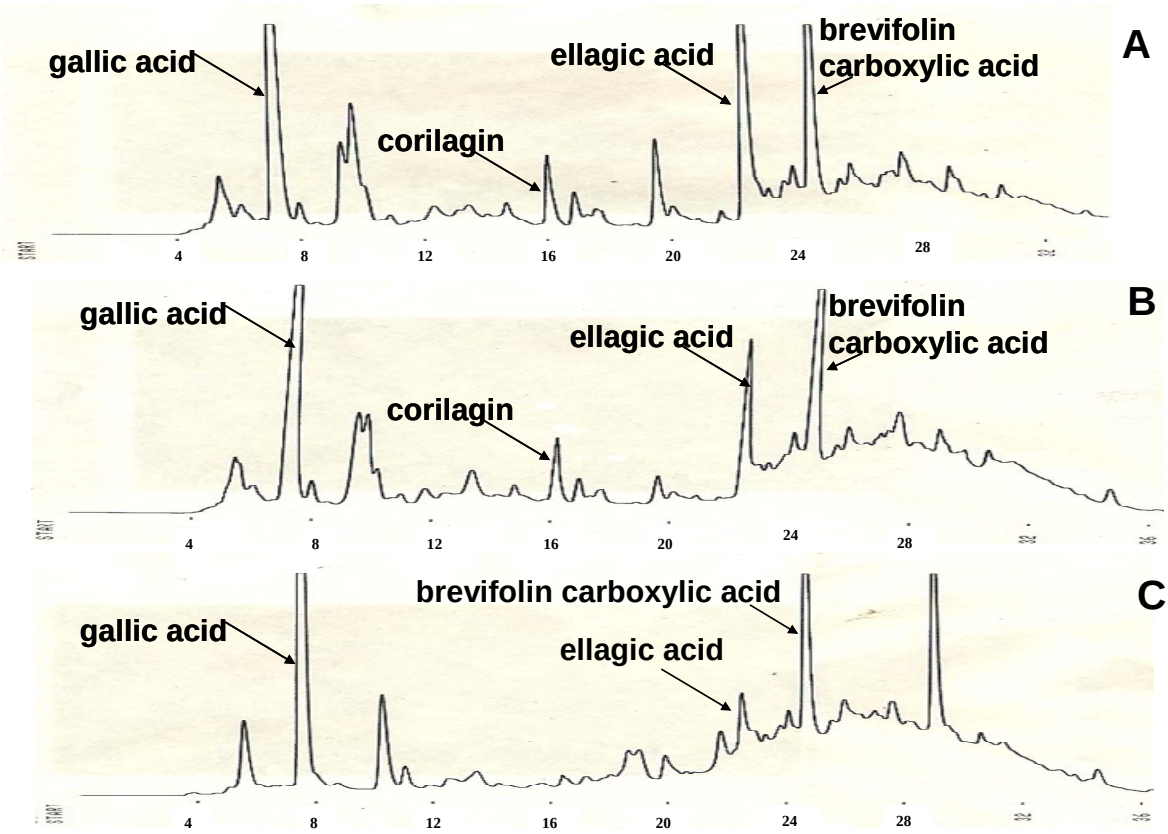


Fig. 29 HPLC profiles of the autoclaved extracts from *P. urinaria* leaves

A: before fermentation

B: incubated for 14 days without inoculation of fungus

C: fermented with *F. solani* for 14 days

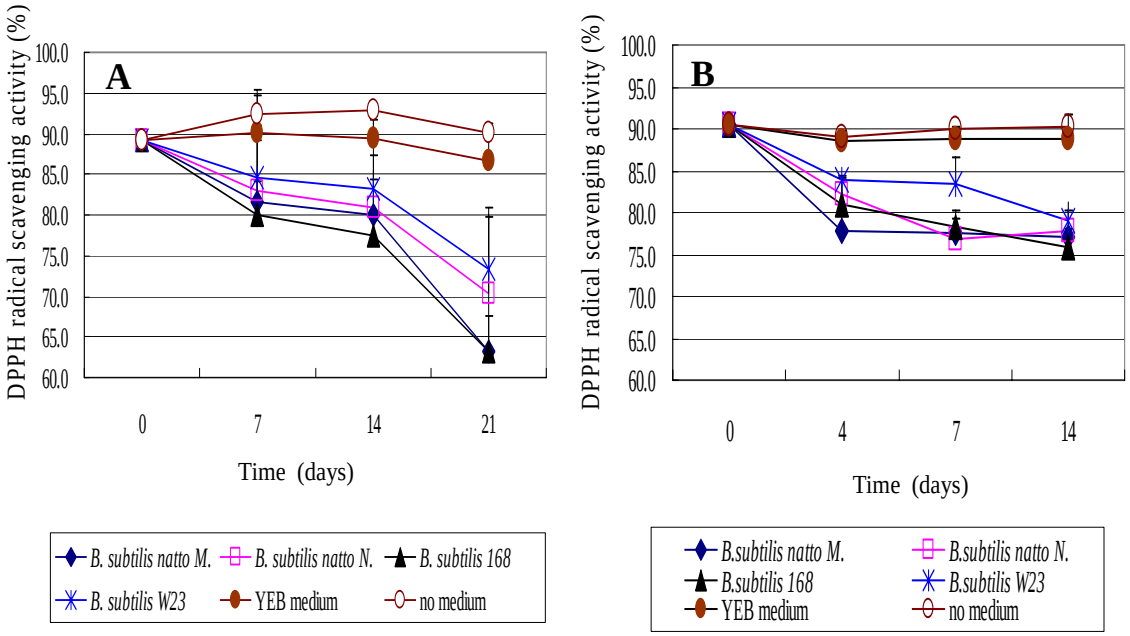


Fig. 30 DPPH radical scavenging activities of leaves treated with the strain of *B. subtilis* (n=3)

A : *S. sebiferum* B : *P. urinaria*

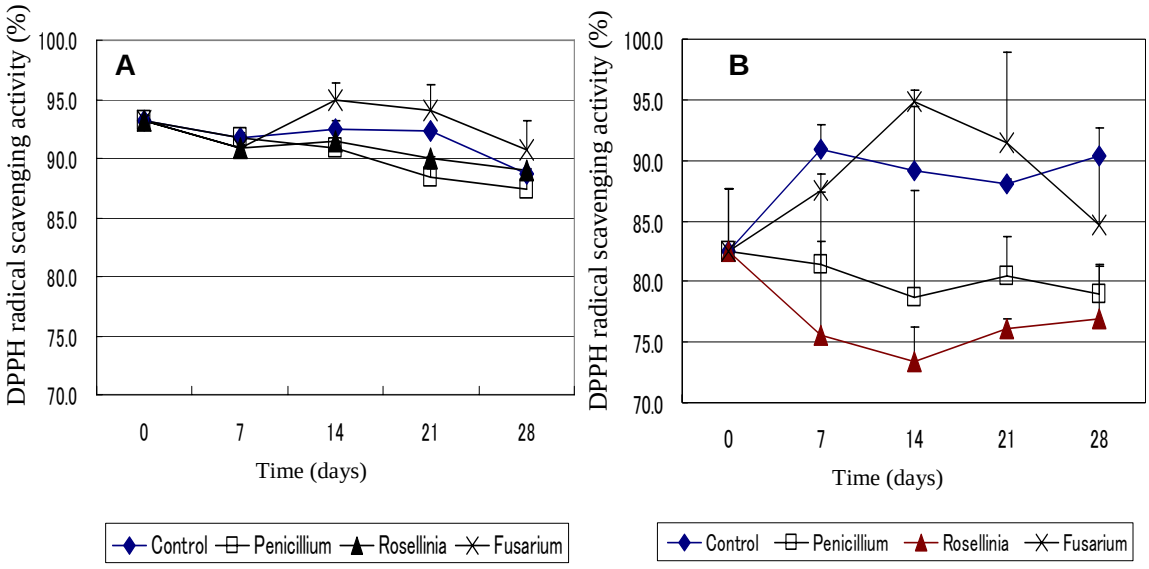


Fig. 31 DPPH radical scavenging activities of extracts fermented with *Penicillium sp.*, *F. solani* or *R. necatrix* (n=3)

A : *S. sebiferum* B : *P. urinaria*

4.2 Fermentation of tea extract with the strain of various fungi

4.2.1 Materials and methods

Materials and chemicals

Green tea leaves (AOYAGI, coarse tea) were purchased in a market of Saga, in Japan. *Penicillium sp., F. solani* and *R. necatrix* were identical to those described in 4.1.1. The authentic standards of caffeine, G, GC, EGC, C, EC, EGCG, GCG, ECG, CG and rutin were purchased from sigma Co. Ltd, Japan. The structures of these authentic standards were illustrated in Fig. 10-1, Fig. 11 and Fig. 32.

Fermentation of tea extract with the strain of various fungi and HPLC analysis

1) *Preparation of various fungi cultures*

The fungi culture was prepared as described in 4.1.1.

2) *Preparation of tea aqueous extract*

Green tea leaves (80 g, DW) were added to 1000 ml boiling water and steeped for two hours at 60℃ without loss of steam. The extract was filtered through 4-fold gauze. The filtrate (100 ml/flask) was transferred to glass flask, autoclaved and cooled to room temperature.

3) *Fermentation of tea aqueous extracts with the strain of various fungi*

The fermentation of tea extract with the strain of various fungi was performed as described in 4.1.1.

4) *HPLC analysis*

HPLC conditions were identical to those mentioned in 2.1.1.2-b.

Isolation of metabolites from tea aqueous extract fermented with F. solani

1500 ml of tea extract prepared using 150 g (DW) green tea leaves was fermented with *F. solani* in the same conditions as described above. After fermentation for 21 days, the tea extract was filtered through 4-fold gauze and then concentrated to 100 ml *in vacuo*. The filtrate was successively subjected to DIAION HP20ss, Sephadex LH-20, Preparative C18 125 Å and ODS-G3 column chromatographies to afford rutin (14 mg) (Ömür Demirezer et al. 2006) and blumenol B (7 mg) (Miyase et al. 1988). The chemical structures of these compounds were illustrated in Fig. 32 and ^{1}H and ^{13}C-NMR spectra of these compounds were shown in Fig. 33 and Fig. 34.

4.2.2 Results and Discussion

The concentrations of chemical constituents in tea extracts during fermentation with *Penicillium sp., F. solani* or *R.*

necatrix were shown in Fig.35. Gallate type catechins (EGCG, ECG, GCG and CG) rapidly decreased in the tea extracts fermented with the strain of three fungi and were hardly detected after 7 days fermentation, while they gradually decreased in the control samples. With the degradation of gallate type catechins, non-gallate type catechins (GC, EC, C and EGC) slightly increased during the first 7 days of the fermentation and the concentration of G also significantly increased to the maximum levels (0.8 – 0.9 mg/ml, on the 7th day). In the control samples, the increase of non-gallate type catechins and G was not observed, on the contrary, these catechins slowly decreased throughout the fermentation, and the concentration of G stayed at a very low level (less than 0.2 mg/ml) throughout the fermentation. Several genera of fungi such as *Aspergillus*, *Fusarium* and *Penicillium* were found to be able to produce tannase (Belmares et al. 2004). Thus, it is supposed that the increase of non-gallate type catechins and G in the fermented extracts with fungi was presumably resulted from the esterase, which originated from the three strains of fungi and led to the degradation of gallate ester linkages of gallate type catechins.

The concentration of caffeine stayed at a high level (0.55–0.75 mg/ml) in all of the extracts throughout the fermentation. This observation indicated that caffeine was very stable during the fermentation processing with the existence of these three fungi.

The concentration of rutin in the tea extract treated with *F. solani* increased from 0.14 mg/ml at the beginning up to 0.32 mg/ml on the 28th day of the fermentation, while in those treated with *Penicillium sp.* or *R. necatrix,* it decreased gradually throughout the fermentation (Fig. 35 and Fig. 36). From the tea extract fermented with *F. solani,* blumenol B was also isolated together with rutin. As blumenol B was not detected in the crude green tea extract (data is not shown), this compound seemed to be newly produced during the fermentation with *F. solani*. Although blumenol B has been isolated from *Epimedium grandifolorum* MORR. var. *thunbergianum* (MIQ.) NAKAI. (Miyase et al. 1988), there is no report on the isolation of blumenol B from tea products. In this experiment, it was isolated from the tea extract for the first time.

In this experiment, by the fermentation with the strain of three fungi, the gallate type catechins in the tea extract drastically decreased because of the degradation of gallate ester linkages of them. Only in the tea extract treated with *F. solani*, accumulation of rutin and blumenol B was observed. Although the enzymes of *F. solani* which caused the accumulation of rutin and blumenol B were not identified yet, this fungus seemed to be an interesting strain for biotransformation of various phytochemicals.

4.3 Conclusions

It was found that four polyphenols (gallic acid, corilagin, ellagic acid and brevifolin carboxylic acid) were easily degraded during the fermentation of *S. sebiferum* and *P. urinaria* leaves with *B. subtilis*. During the fermentation of *S. sebiferum* and *P. urinaria* extracts with fungi, corilagin was the most easily degraded by fungi, while brevifolin carboxylic acid was more stable than other three polyphenols.

In the fermentated tea extracts with fungi, gallate type catechins (EGCG, ECG, GCG and CG) degraded, and transformed into gallic acid and corresponding non-gallate type catechins. The accumulations of rutin and blumenol B were confirmed only in the extract treated with *F. solani*. From the tea extract treated with *F. solani*, blumenol B was also isolated together with rutin. This is the first time for isolation of blumenol B from tea products.

The clarification on the degradation pattern of polyphenols during fermentation with microorganisms, will give basic data for investigation of chemical constituents in the fermented products containing polyphenols.

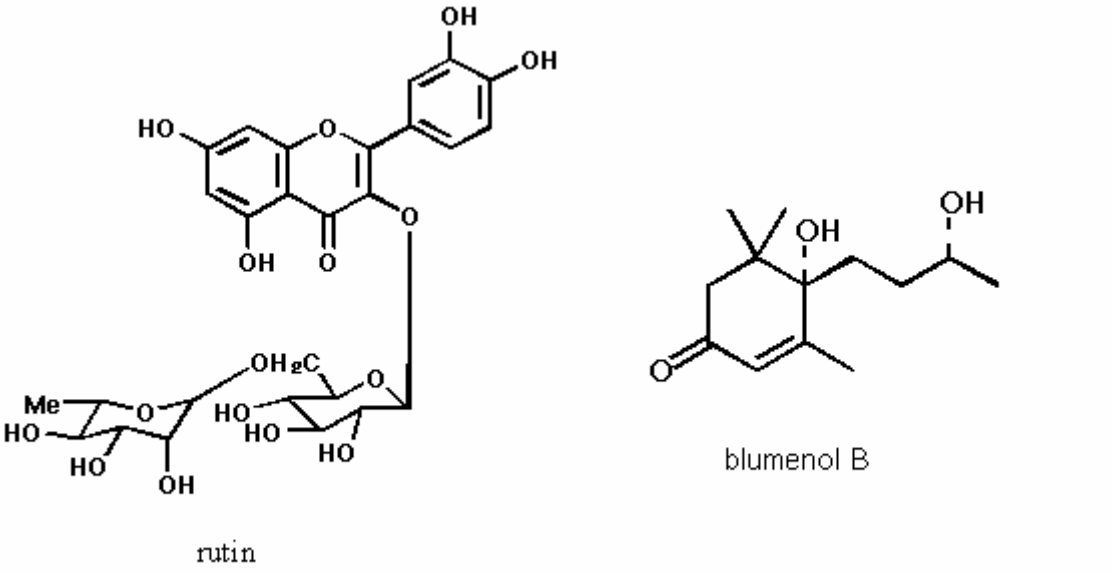


Fig. 32 Structures of rutin and blumenol B

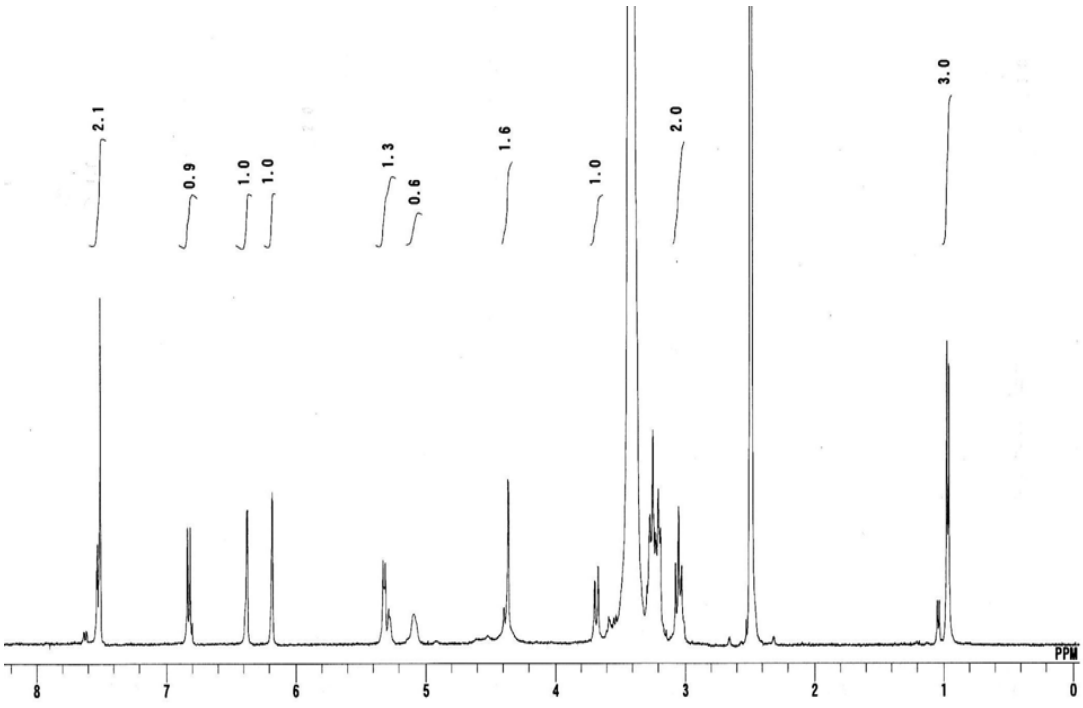


Fig.33 ^{1}H-NMR spectrum of rutin

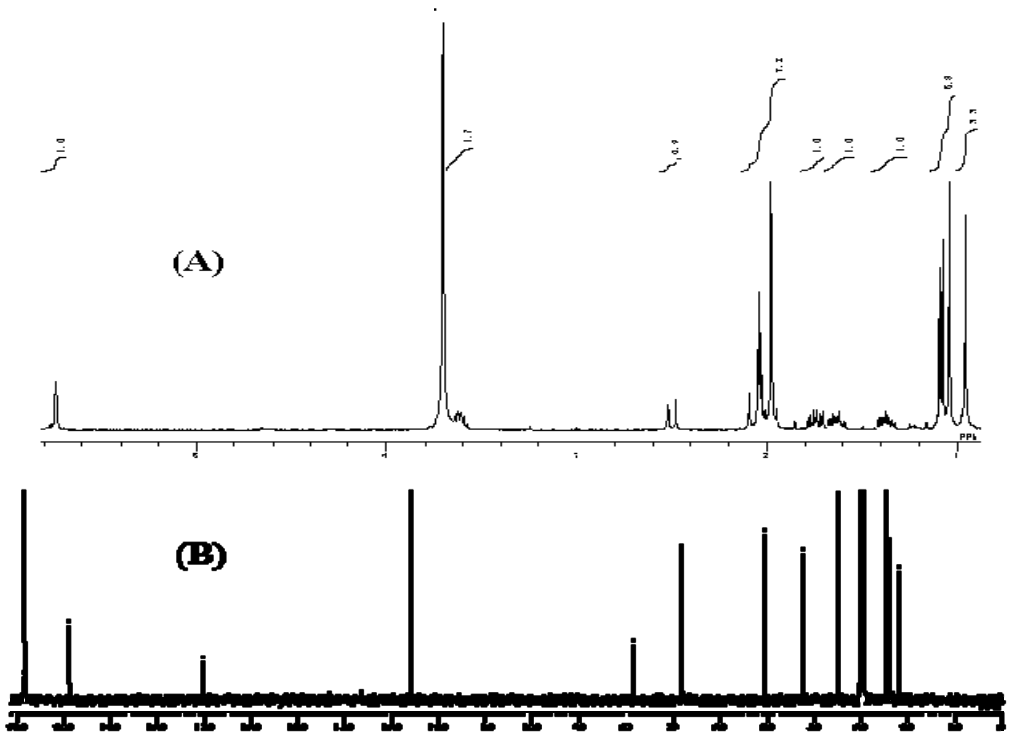


Fig.34 ^{1}H-NMR and ^{13}C-NMR spectra of blumenol B

A : ^{1}H-NMR B : ^{13}C-NMR

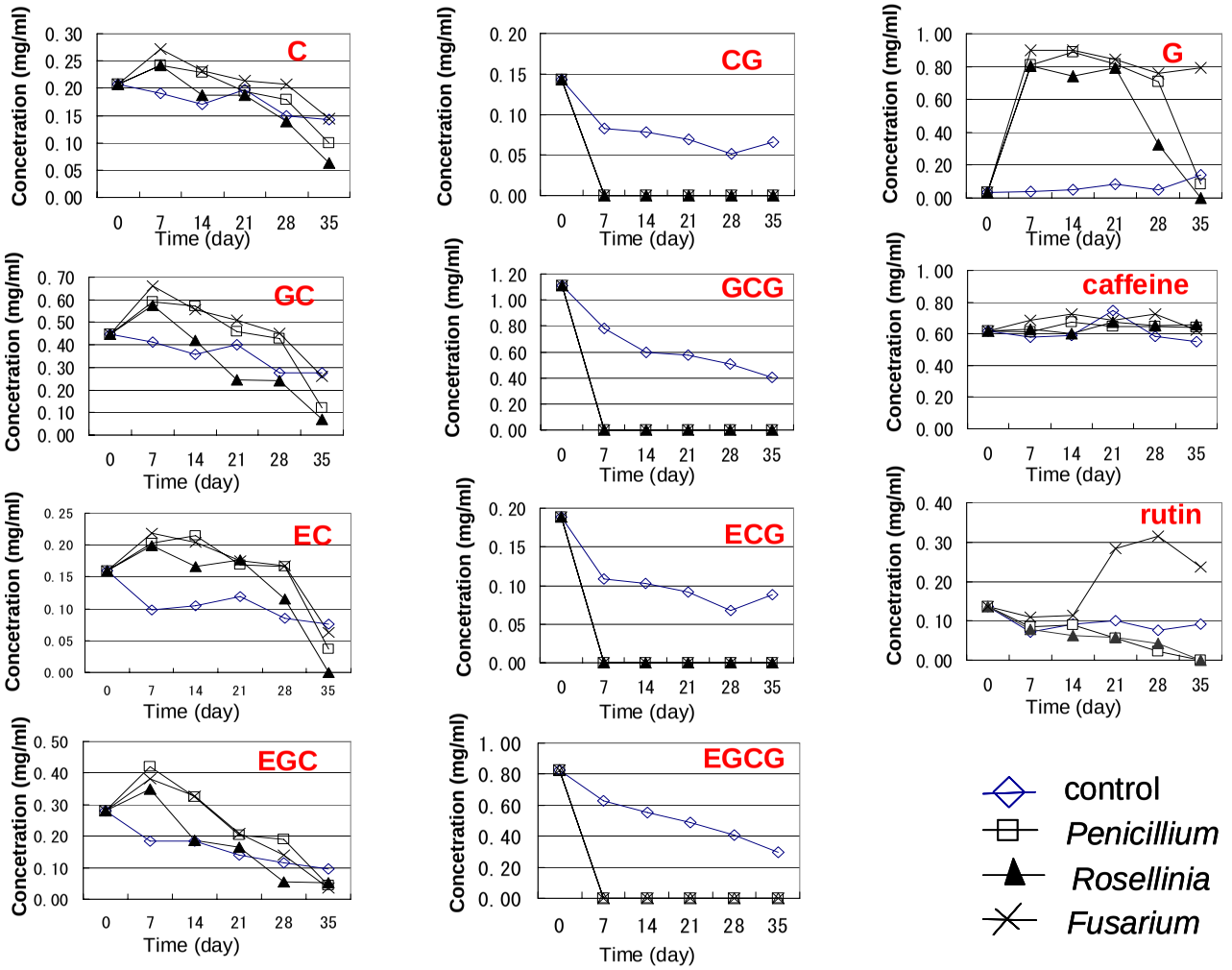


Fig. 35 Concentrations of constituents in tea extracts fermented with *Penicillium sp.*, *R. necatrix* or *F. solani*

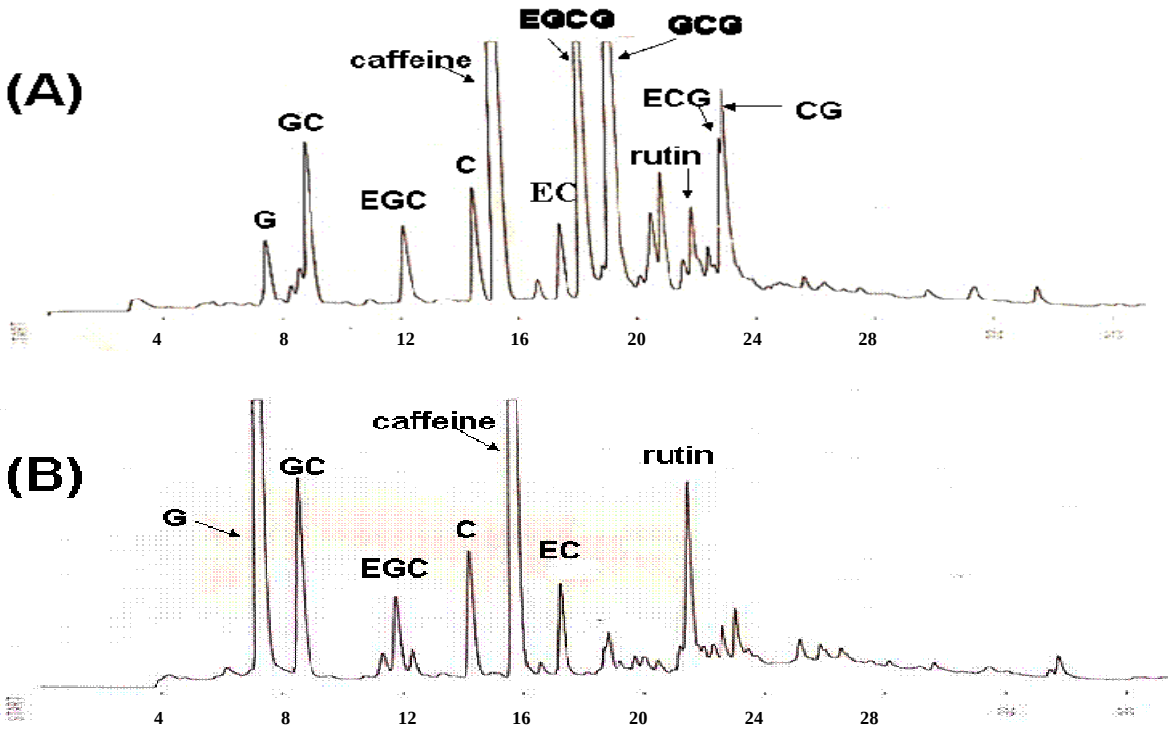


Fig. 36 HPLC profiles of tea extracts

(A) before fermentation (B) fermented with *F. solani* for 21 days

CHAPTER 5

GENERAL DISCUSSION

5.1 *Extraction of polyphenols using bean protein*

It is well known that polyphenols may interact with protein mainly through hydrogen bonding and hydrophobic bonding, and lead to precipitation (Hagerman and Butler 1978, Sarni-Manchado et al. 1999). According to this character of polyphenol, Ishimaru et al. succeeded in the extraction of tea catechins using soybean protein (Ishimaru and Nonaka 2001). But there is no report on the extraction of the other phenolic compounds using this method.

In this research, various polyphenols (such as anthocyanins, hydrolysable tannins, condensed tannins, phenolic acid etc.) were successfully extracted using soybean protein by the formation of polyphenol-soybean protein complexes. It is proved that soybean protein is feasible for the extractions various polyphenols.

Some studies describing influence of polyphenol structure on the binding of polyphenols to protein have been reported (Hagerman and Butler, 1978, Bacon and Rhodes, 2000, de Freitas and Mateus, 2001). However, there is no report about the influence of polyphenols structures on conjugation of polyphenols to soybean protein. In this research, it was found that the conjugational abilities of the gallotannins to soybean protein increased with the number of galloyl moieties of gallotannins (Chapter 2). Similar result was also observed in binding of galloyltannins to other protein (histone, bovine serum albumin, casein and gelatin) (He et al. 2006). Besides

numbers of galloyl moiety, esterified position of them were also very important for the conjugation of polyphenols to soybean protein. In conclusion, galloyl moiety is very important for the conjugations of polyphenols to soy bean protein. Thus, for the extraction of polyphenols, soy bean protein seems very good material, particularly for the extraction of those polyphenols with galloyl moiety.

Besides polyphenols structure, the conjugation of polyphenols to protein is also affected by the property of protein (Sims et al. 1995). The property of protein from different bean species may be different, and also the effect of different protein on the extraction of polyphenols may be different. Six bean species were used as different protein materials for the extraction of catechins. The degenerated bean protein was more efficient than non-generated bean protein for the extraction of catechins. *Azuki* bean, *Taishoukintoki* bean and black bean seem to be very good protein materials for the extraction of catechins, since the complexes from these beans showed very strong DPPH radical scavenging activities which were presumably contributed to not only tea catechins but also anthocyanins from these beans. These beans are also presumed to be the potential protein materials for other polyphenols extraction.

It was suggested that tea catechins-soybean protein complexes possess the bioactivities from both soybean protein and tea catechins (Alim et al. 2003, 2004). The polyphenols-bean protein complexes obtained in this research also presumably have the bioactivities from both bean protein and polyphenols. Therefore,

these complexes are expected to be used as new materials for food additives and pharmaceutical resources.

In this study, an efficient method of polyphenol extraction was established, which give new opinions for the extraction of polyphenols.

5. 2 *Heating processing of plant materials containing polyphenols*

The change in contents of polyphenols during heating processing has been widely investigated in many studies (Chuda et al. 1998, Brens et al. 2002, Ioku et al. 2001, Rohn et al. 2007). The relationship of chemical structure and thermal stabilities of some phenolic compounds have been also elucidated in few studies (Stintzing et al. 2006, Mathias et al. 2006). Although the newly formed products (reaction products) during thermal processing are reported in some studies (Xu et al. 2003, Buchner et al. 2006, Takenaka et al. 2006), there are very few studies on the transformation of polyphenols during heating processing.

In this study, it was found that the epicatechin derivatives (EGCG, ECG, EGC, EC) epimerised and converted to their isomers (GCG, CG, GC, C) during autoclave processing of tea leaves and their aqueous extract (in Chapter 3). The high temperature is thought to be necessary for the epimerisation of epicatechin derivatives. Xu et al. obtained the similar results during the thermal processing of commercial tea drinks, in which epicatechin derivatives were added (Xu et al. 2003), while there is no report on the the epimerisation of epicatechin derivatives in tea leaves and aqueous.In this study, the contents of GCG and CG in the autoclaved tea leaves were about 29

times as those of GCG and CG in the tea leaves reported in the literature (Nishitani and Sagesaka 2004). Since GCG and CG were reported to be more effective than EGCG and ECG against cholesterol adsorption (Ikeda et al. 2003), the autoclaved tea leaves are expected as a novel functional material in food and pharmaceutical fields. Furthermore, for the production of GCG and CG, autoclave treatment of tea leaves seems to be a very efficient method.

Geraniin, a hydrolysable tannin which is contained in *S. sebiferum* and *P. urinaria* leaves were degraded and converted to four polyphenols (gallic acid, corilagin, ellagic acid and brevifolin carboxylic acid) by autoclave treatment. It is deduced that the ester linkages of hydrolysable tannin are easily cleaved through hydrolyzation reaction and new compounds were produced through reaction of dehydrogenation or decarboxylation during autoclave processing. The productions of corilagin and ellagic acid during the decoction of *G. thunbergii* have been reported, while the production of brevifolin carboxylic acid was not observed (Okuda et al. 1979). It suggested that production of brevifolin carboxylic acid only occurred during the processing of geraniin at high temperature.

The clarification of chemical constituents of the autoclaved plant materials containing polyphenols in this study, will offer useful information for the studies on the functionalities of heating processed products.

5. 3 *Fermentation of plant materials containing polyphenols with microorganism*

Although many microorganisms such as bacteria, fungi and yeasts were identified from many traditional fermented products, the roles of the strain of individual microorganism on the transformation of many chemical constituents are still not clear (Zhou et al. 2004, Blandino et al. 2003). In this research, the individual strain of microorganisms was used for the fermentation of plant materials containing polyphenols.

The strains of *B. subtislis* are used for production of many fermented soy foods such as Japanese natto, Thai thua-nao, Indian kinema and West African dawadawa (Steinkraus 1995, Ibe et al 2001, Kuo et al. 2006). Some new constituents were also isolated and identified from the soybeans fermented with *B. subtislis (natto)* (Toda et al. 1999), while there are few reports on the effluence of *B. subtislis* on metabolism of other polyphenols during the fermentation of plant materials.

In this research, during the fermentation of autoclaved leaves of *S. sebiferum* and *P. urinaria* with the strain of four *B. subtislis*, respectively, four polyphenols (gallic acid, corilagin, ellagic acid and brevifolin carboxylic acid) were easily degraded by *B. subtislis* (in Chapter 4). Bairagi et al. reported that tannins were degraded in the fermented duckweed (*Lemna polyrhiza*) leaves with *B. subtislis* (Bairagi et al. 2002). It was reported that tannase can be produced by some bacteria (Das Mohapatra et al. 2006). It is presumed that the four *B. subtislis* strains also produced some tannase which caused the degradations of the four polypnenols in this research.

Three strains of fungi (*Penicillium sp., F. solani* and *R. necatrix*) were selected for the fermentation of aqueous extracts of green tea

and *S. sebiferum* and *P. urinaria.* Amongst these fungi, genera *Penicillium* and *Fusarium* were detected in some traditional fermented products, while genus of *Rosellinia* was rarely used for fermentation (Zhou et al. 2004, Blandino et al. 2003). The influences of them on the metabolic pattern of polyphenols were investigated.

As mentioned in Chapter 4, corilagin were easily degraded by three fungi, but brevifolin carboxylic acid was stable throughout the fermentation. In the fermentation of tea extracts, gallate type catechins were easily degraded by the fungi, and converted into gallic acid and corresponding non-gallate type catechins. Compared with the gallate type catechins, non-gallate type catechins were more stable during the fermentation. It suggested that polyphenol with ester linkage was easily to be degraded. It is well known that tannase can also be produced by many fungi (Belmares et al. 2004). Thus, *Penicillium sp., F. solani* and *R. necatrix* also presumably produced tannase which play the main role in the cleavage of the ester linkages of polyphenols with ester linkages.

It is interesting to note that, accumulation of blumenol B and rutin was confirmed in the fermented tea extract with *F. solani*; while the phenomenon was not observed in the fermented extracts with *Penicillium sp.* or *R. necatrix* (in Chapter 4). The mechanism of the accumulation of these compounds is still unclear and is necessary to be further investigated and clarified. In addition, *F. solani* seems to be a potential strain for biotransformation of various phytochemicals by utilizing in the fermentation of plant materials containing polyphenols.

In this study, the degradation patterns of several polyphenols

during the fermentation with the individual strain of several microorganisms were clarified. The results obtained will be very important as basic data for the studies on chemical constituents of some fermented products especially those containing polyphenols such as condensed tannins or hydrolysable tannins.

SUMMARY

Anthocyanins from 15 plant species were successfully extracted using soybean protein via preparation of anthocyanin-soybean protein complex. The complex from red leaves of *S. sebiferum,* fruits of blueberry or black bean showed high adsorption rate (about 50%) of anthocyanin. The strongest DPPH radical scavenging activity (about 90%) was observed in the complexes from red leaves of *S. sebiferum*. It was considered that the strongest DPPH radical scavenging activity was contributed to anthocyanins and tannin constituents (geraniin and chebulagic acid) from *S. sebiferum*.

In order to clarify the influence of chemical structures of polyphenols on the conjugation of polyphenols to soybean protein, the polyphenols-soybean protein complexes from 22 polyphenols were also prepared. It was also found that the adsorption rate of the gallotannins increased with the number of galloyl moieties of them. The existence of galloyl moiety esterified at C-1 of the glucose could increase the conjugation of polyphenol and soybean protein. Therefore, it was considered that galloyl moieties were very important for the conjugation of polyphenols to soybean protein. The open-chain glucose structure of hydrolysable tannin seems to be the main reason that resulted in the decrease of the adsorption rate.

The degenerated bean protein and non-degenerated bean protein from six bean species were used for the extraction of tea catechins. The complexes from degenerated bean proteins showed not only higher content of catechins but also stronger DPPH radical scavenging activities than those from non-degenerated bean

proteins. Therefore, degenerated bean protein was more efficient for the extraction of catechins compared with non-degenerated bean protein. The complexes from *Azuki* bean, *Taishoukintoki* bean, black bean and defatted soybean showed higher adsorption rates and higher contents of catechins compared to the complexes from other beans, in addition, stronger DPPH radical scavenging activities were also observed in these four complexes. Thus, these four bean species were considered to be the potential protein resources for the extraction of tea catechins.

During autoclave processing, epicatechin derivatives (EGCG, ECG, EGC and EC) were epimerised and converted to their isomers (GCG, CG, GC and C). High temperature seems necessary to this epimerization. The tea leaves containing high level of GCG (2.58-2.92%, DW) and CG (0.24-0.57%, DW) were obtained by autoclave treatment and they are expected to be new material in food and pharmaceutical fields.

Geraniin, a hydrolysable tannin contained in the leaves of *S. sebiferum* and *P. urinaria*, was degraded and transformed into four polyphenols (gallic acid, corilagin, ellagic acid and brevifolin carboxylic acid) during the autoclave processing. DPPH radical scavenging activities of these four polyphenols seemed to be almost identical with that of geraniin. Taking into account the stability of these polyphenols in heating process, autoclaved leaves of these two plants seemed to be good materials for new food ingredients.

The four polyphenols were rapidly decreased during the fermentation of the autoclaved leaves of *S. sebiferum* and *P. urinaria* with the strain of *B. subtilis.* The DPPH radical scavenging activities

of those fermented leaves also decreased with the decrease of the four polyphenols. In the fungal (*Penicillium sp.*, *F. solani* or *R. necafrix*) fermentation extracts of *S. sebiferum* and *P. urinaria*, corilagin were the most easily degraded, and brevifolin carboxylic acid was the most stable among the four polyphenols.

In the fermented tea extracts with the strain of fungi (described as above), decreases of gallate type catechins (EGCG, ECG, GCG and CG) as well as increases of non-gallate type catechins (GC, EC, C and EGC) and gallic acid were observed after 7 days fermentation. Esterase activities originated from these fungi seemed to be strong during fermentation and they caused the rapid degradation of gallate ester linkage of gallate type catechins. Only in the tea extract treated with *F. solani*, rutin concentration increased (from 0.14 mg/ml to 0.32 mg/ml), while it decreased throughout fermentation in other fungal fermentation samples. From the tea extract treated with *F. solani*, blumenol B which has not been detected in various tea products was also isolated together with rutin.

In this research, an efficient extraction method of polyphenols was established by using bean protein, and valuable findings were provided for extraction of polyphenols in the food and pharmaceutical fields. Clarification of the transformations of polyphenols during autoclave processing and fermentation processing with microorganisms, will provide the basic data for the studies on the functionalities and chemical constituent analysis of processed products containing polyphenols.

ACKNOWLEDGEMENTS

I would like to express my profound gratitude and indebted to my reverend supervisor, Dr. Kanji Ishimaru, Associate Professor of Faculty of Agriculture, Saga University, for giving me access to his laboratory and guiding me throughout the course. I am also grateful to him for his valuable advice, constant encouragement, and daily careful supervision not only in my research but also in the life of my family in Saga.

I am very grateful to Dr. Shiro Isshiki, Associate Professor of Faculty of Agriculture, Saga University and Dr. Yusuke Sakata, Professor of Faculty of Agriculture, Kagoshima University, for their guidance, constructive comments and hearty encouragement during this study.

I also wish to acknowledge helpful comments and suggestions from Dr. Fumio Hashimoto, Associate Professor of Faculty of Agriculture, Kagoshima University and Dr. Shuji Fujita, Professor of Faculty of Agriculture, Saga University.

Deep gratitude is also expressed to Professor Yosuke Tashiro, Professor Fumio Kato, Professor Kohzo Kanda, Associate Professor Toyoaki Anai and Dr. Kiyohiko Seki, Faculty of Agriculture, Saga University for their helpful suggestions, encouragement and kind support.

I am very much grateful to Dr. Norihiko Terahara, Professor of Faculty of Health and Nutrition, Minami-Kyushu University, Dr. Toshihiro Fujioka, Professor of Pharmaceutical Sciences, Fukuoka University and Dr. Takashi Tanaka, Associate

Professor of Graduate School of Biomedical Sciences, Nagasaki University, for their constant technical assistance and support throughout the research work.

I am also deeply grateful to Dr. Norie Nishikawa for valuable advice in my research and sincerely help in my life. In addition, thanks are also extended to the members of Dr. Ishimaru's laboratory for their collaboration in the study and help in various ways in my life in Saga.

I am very grateful to Government of Japan for their generous financial support by providing me with the Monbusho Scholarship without which my study could not be completed.

It is a pleasure to thank my husband, Wu shengjin, my parents and families for their help and encourage during this study.

In fact, I am grateful to every people for helping me to reach this present stage. My acknowledgements extend beyond those listed here.

REFERENCES

Adlercreutz, H. and Mazur, W. 1997 Phyto-oestrogens and Western diseases. *Ann. M.* **29** : 95-120

Alim, P., Nishikawa, K., Tanaka, N., Ishimaru, K., Kurayama, T., Fukuda, N. and Maeda, H. 2003 Antibacterial activities of extracts and constituent in the complex of tea catechins and soybean protein. *Jpn. J. of Food Chem.* **10**: 161-164

Alim, P., Nishikawa, K., Tanaka, N., Ishimaru, K., Kurayama, T., Fukuda, N. and Maeda, H. 2004 Effect of complex of tea catechins and soybean protein on lipid metabolism in rats. *Jpn. J. of Food Chem.* **11** : 60-66

Amakura, Y., Tsutsumi, T., Sasaki, K., Yoshida, T. and Maitani, T. 2003 Screening of the inhibitory effect of vegetable constituents on the aryl hydrocarbon receptor-mediated activity induced by 2, 3, 7, 8-tetrachlorodibenzo-*p*-dioxin. *Biol. Pharm. Bull.* **26** : 1754-1760

Anderson, J. W., Johnstone, B. M. and Cook-Newivell, M. E. 1995 Meta-analysis of the effects of soy protein intake on serum lipids, *New England J. Med.* **333** : 276-282

Angayarkanni, J., Palaniswamy, M., Murugesan, S. and Swaminathan, K. 2002 Improvement of tea leaves fermentation with *Aspergillus* spp. Pectinase. *J. Biosci. Bioeng.* **94** : 299-303

Awuah, G.B., Ramaswamy, H. S. and Economides, A. 2007 Thermal

processing and quality: Principles and overview *Chem. Eng. Prog.* 46 : 584-602

Bairagi, A., Sarkar Ghosh, K, Sen. S. K., Ray, A. K. 2002 Duckweed (*Lemna polyrhiza*) leaf meal as a source of feedstuff in formulated diets for rohu (*Labeo rohita Ham.*) fingerlings after fermentation with a fish intestinal bacterium. *Bioresour. Technol.* **85** : 17-24.

Bacon, J. R. and Rhodes, M. J. C. 2000 Binding affinity of hydrolysable tannins to parotid saliva and to proline-rich proteins derived from it. *J. Agric. Food Chem.* **48** : 838-843.

Belmares, R., Contreras-Esquivel, J.C., Rodriguez-Herrera, R., Coronel, A.R. and Aguilar, C.N. 2004 Microbial production of tannase: an enzyme with potential use in food Industry. *Lebensmittel-Wissenschaft und -Technologie* **37** : 857-864

Bergamo, P., Fedele, E., Iannibelli, L. and Marzillo, G. 2003 Fat-soluble vitamin contents and fatty acid composition in organic and conventional Italian dairy products *Food Chem.* 82 : 625-631

Bhosle, S. M., Huilgol, N. G. and Mishra, K. P. 2005 Enhancement of radiation-induced oxidative stress and cytotoxicity in tumor cells by ellagic acid. *Clin. Chim. Acta* **359** : 89-100

Bravo, L. 1998 polyphenols: chemistry, dietary sources, metabolism, and nutritional significance. *Nutr. Rev.* **56** : 317-333

Brenes, M, Garcia, A, Dobarganes, M. C., Velasco, J., Romero, C. 2002 Influence of thermal treatments simulating cooking processes on the polyphenol content in virgin olive oil. *J. Agric. Food Chem*. **50** : 5962-7

Buchner, N., Krumbein, A., Rohn, S. and Kroh, L. W. 2006 Effect of thermal processing on the flavonols rutin and quercetin. *Rapid Commun. Mass Spectrom.* **20** : 3229-3235

Chang, T. S., Ding, H. Y., Tai, S. S. and Wu, C. Y. 2007 Metabolism of the soy isoflavones daidzein and genistein by fungi used in the preparation of various fermented soybean foods. *Biosci. Biotechnol. Biochem*. **71** : 1330-1333

Chen, Y. X., Guo, S. H. and Zhang, D. F. 1995 Experimental study on anti-duck hepatitis B viral effect of *Phyllanthus urinaria* of different areas and combined therapy with other drugs. *Zhong guo Zhong Xi Yi Jie He Za Zhi* **15**: 225-227

Cheng, J. T., Chang, S. S. and Hsu, F. L. 1994 Antihypertensive action of geraniin in rats. *J. Pharm. Pharmacol.* **46** : 46-49.

Cheng, J. T., Lin, T. C. and Hsu, F. L. 1995 Antihypertensive effect of corilagin in the rat. *Can. J. Physiol. Pharmacol.*, **73** : 1425-1429

Chuda, Y., Suzuki, m., Nakata, T. and tsushida, T. 1998 Contents and cooking loss of three quinic acid derivatives from garland (*Chrysanthemum coronarium* L.) *J. Agric. Food Chem*. **46** : 1437-1439

Chung, K., Wong, T. Y., Wei, C., Huang, Y. and Lin, Y. 1998 Tannins and Human Health: A Review. *Crit. Rev. Food Sci. Nutr.* **38** : 421-464

Das Mohapatra, P. K., Mondal, K. C., Pati, B. R. 2006 Production of tannase through submerged fermentation of tannin-containing plant extracts by Bacillus licheniformis KBR6. *Pol. J. Microbiol.* **55** : 297-301

De Freitas, V. and Mateus, N. 2001. Structural features of procyanidin interactions with salivary proteins. *J. Agric. Food Chem.* **49** : 940-945

Esaki, H., Onozaki, H., Morimitsu, Y., Kawakishi, S. and Osawa, T. 1998 Potent antioxidative isoflavones isolated from soybeans fermented with *Aspergillus saitoi*. *Biosci. Biotechnol. Biochem.* **62** : 740-746

Esaki, H., Kawakishi, S., Morimitsu, Y. and Osawa, T. 1999 New potent antioxidative o-dihydroxyisoflavones in fermented Japanese soybean products. *Biosci. Biotechnol. Biochem.* **63** : 1637-1639

Fujiki, H., Suganuma, M., Okabe, S., Komori, A., Sueoka, E., Sueoka, N., Kozu, T. and Sakai, Y. 1996 Japanese green tea as a cancer preventive agent in humans. *Nutr. Rev.* **54** : S67-S70

Fujiki, H., Suganuma, M., Kurusu, M., Okabe, S., Imayoshi, Y., Taniguchi, S. and Yoshida, T. 2003 New TNF-α releasing inhibitors as cancer preventive agents from traditional herbal medicine and combination cancer prevention study with EGCG and sulindac or tamoxifen. *Mutat. Res.* **523-524** :

119-125

Ghiselli, A., Nardini, M., Baldi, A. and Scaccini, C. 1998 Antioxidant activity of different phenolic fractions separated from an Italian red wine. *J. Agric. Food Chem.* **46** : 361-367

Hagerman, A. E. and Butler, L. G. 1978 Protein precipitation method for the quantitative determination of tannins. *J. Agric. Food Chem.* 26 : 809-812

Harborne, J. B. and Williams, C. A. 2001 Anthocyanins and other flavonoids. *Nat. Prod. Rep.* **18** : 310-333

Haslam, E. 1989 plant polyphenols; Cambridge University Press: Cambridge, U.K.

He, Q., Shi, B. and Yao, K. 2006 Interactions of gallotannins with proteins, amino acids, phospholipids and sugars *Food Chem.* **95** : 250-254

Hirose, M., Mizoguchi, Y., Yaono, M., Tanaka, H., Yamaguchi, T. and Shirai, T. 1997 Effects of green tea catechins on the progression or late promotion stage of mammary gland carcinogenesis in female Sprague-Dawley rats pretreated with 7,12 dimethylbenz(a)anthracene. *Cancer Lett.* **112** : 141-147

Hollman, P. C. H., Hertog, M. G. L. and Katan, M. B. 1996 Role of dietary flavonoids in protection against cancer and coronary heart disease. *Biochem. Soc. Trans.* **24** : 785-789

Hsu, F. L., Lee, Y. Y. and Cheng, J. T. 1994 Antihypertensive activity of 6-O-galloyl-D-glucose, a phenolic glycoside from *Sapium sebiferum*. *J. Nat. Prod.* **57** : 308-312

Huang, J. and Zhang, Z. 2004 Microwave-assisted extraction of quercetin and acid degradation of its glycosides in Psidium guajava leaves. *Anal. Sci.* **20** : 395-397

Huang, M. T., Ho, C. T., Wang, Z. Y., Ferraro, T., Finnegan-Olive, T., Lou, Y.R., Mitchell, J. M., Laskin ,J. D., Newmark, H. and Yang, C.S. 1992 Inhibitory effect of topical application of a green tea polyphenol fraction on tumor initiation and promotion in mouse skin. *Carcinogenesis* **13** : 947-954

Huang, S., Tanaka, N. and Ishimaru, K. 2004a Preparation and functional evaluation of anthocyanin-soybean protein complex. *Journal of the Japanese Society for Food Science and Technology* **51** : 18-22

Huang, S., Shigetomi, N., Tanaka, N., Terahara, N., Fujioka, T., Yoshida, M. and Ishimaru, K. 2004b Preparation and functional evaluation of plant polyphenol-bean protein complex. *Journal of the Japanese Society for Food Science and Technology* **51** : 626-632

Huang, S., Inoue, K., Li, Y., Tanaka, T. and Ishimaru, K. 2004c Ananlysis of catechins in autoclaved tea leaves and drinks. *Jpn. J. Food Chem.* **11** : 99-102

Huang, S., Seki, K., Kanda, K., Kato, F., T anaka, T., Kouno, I. and Ishimaru, K. 2006 Processing of plants containing geraniin

and analysis of the products. *Jpn. J. Food Chem.* **13** : 118-124

Ibe, S., Kumada, K., Yoshiba M. and Onga., T. 2001 Production of Natto Which Contains a High Level of Isoflavone Aglycons. *Journal of the Japanese Society for Food Science and Technology* **48** : 27-34

Igarashi, K. 2000 Physiological functions of food component anthocyanins. *Foods Food Ingredients J. Jpn.* **187** : 17-29

Ikeda, I., Kobayashi, M., Hamada, T., Tsuda, K., Goto, H., Imaizumi, K., Nozawa, A., Sugimoto, A. and Kakuda, T. **2003** Heat-epimerized tea catechins rich in gallocatechin gallate and catechin gallate are more effective to inhibit cholesterol absorption than tea catechins rich in epigallocatechin gallate and epicatechin gallate., *J. Agric. Food Chem.* 51 : 7303-7307

Ioku K., Aoyama Y., Tokuno A., Terao J., Nakatani N.andTakei Y. 2001 Various cooking methods and the flavonoid content in onion. *J. Nutr. Sci. Vitaminol.* **47** : 78-83

Ishimaru, K., Hirose, M., Takahashi, K., Koyama, K. and Shimomura, K. 1990 Tannin production in root culture of *Sanguisorba officinalis*. *phytochemistry* **29** : 3827-3830

Ishimaru, K. and Shimomura, K. 1991 Tannin production in hairy root culture of *Geranium thunbergii. phytochemistry* **30** : 825-828

Ishimaru, K., Arakawa, H. and Neera, S.. 1994 *Sapium sebiferum*

(Chinese Tallow):In Vitro culture and production of tannins and other phenolic compounds. *Biotechnology in Agriculture and Forestry.* **28** : 426-444

Ishimaru, K. and Nonaka, G. 2001 Rapid purification of catechins using soybean protein. *Journal of the Japanese Society for Food Science and Technology* **48** : 664-670

Ishimaru, K., Tanaka, N. and Nonaka, G. 2002 Isolation of tea catechins using soybean protein or tempe protein. *Res. Adv. in Phytochem.* **3**: 1-8

Jensen, S. K., Liu, Y. G. and Eggum, B. O. 1995 The effect of heat treatment on glucosinolates and nutritional value of rapeseed meal in rats *Anim. Feed Sci. Technol.* **53** : 17-28

Jin, C., Yan, Z. and Jianwei, L. 2001 Processing procedures of brick tea and their influence on fluorine content. *Food Chem. Toxicol.* **39** : 959-962

Kane, C. J., Menna, J. H. and Yeh, Y. C. 1988 Methyl gallate, methyl-3,4,5-trihydroxybenzoate, is a potent and highly specific inhibitor of herpes simplex virus in vitro. I. purification and characterization of methyl gallate from *Sapium sebiferum. Biosci. Rep.* **8** : 85-94

Kataoka, S. 2005 Functional effects of Japanese style fermented soy sauce (shoyu) and its components *J. Biosci. Bioeng.* **100** : 227-234

Kawano, K. 2007 The course of Miso development and its expected effectiveness. *The Japanese Journal of Taste and Smell Research* **14**：137-144

Kris-Etherton, P. M., Hecker K. D., Bonanome A., Coval S. M., Binkoski A. E., Hilpert K. F., Griel A. E. and Etherton T. D. 2002 Bioactive compounds in foods: their role in the prevention of cardiovascular disease and cancer . *Am. J. Med.* **113**：71-88

Kumazawa, K. and Masuda, H. 2001 Change in the flavor of black tea drink during heat processing. *J. Agric Food Chem.* **49**：3304-3309

Kuo, L. C., Cheng, W. Y., Wu, R. Y., Huang, C. J. and Lee, K. T. 2006 Hydrolysis of black soybean isoflavone glycosides by Bacillus subtilis natto. *Appl. Microbiol. Biotechnol.* **73**：314-320

Li, P., Wang, Y., Ma, R. and Zhang, X. 2005 Separation of tea polyphenol from Green Tea Leaves by a combined CATUFM-adsorption resin process. *J. Food. Process. Eng.* **67**: 253-260

Li, Y., Chen, Z. and Mo, H. 2007 Effects of pulsed electric fields on physicochemical properties of soybean protein isolates. *LWT - Food Science and Technology* **40**：1167-1175

Liao, S., Umekita, Y., Guo, J., Kokontis, J. M. and Hiipakka, R. A. 1995 Growth inhibition and regression of human prostate and breast tumors in athymic mice by tea epigallocatechin gallate. *Cancer Lett.* **25**： 239-243

Lin, C. H., Wei, Y. T. and Chou, C. C. 2006 Enhanced antioxidative activity of soybean koji prepared with various filamentous fungi. *Food Microbiol.* **23** : 628-633

Liu, S. Q., Pezzuto, J. M., Kinghorn, A. D. and Scheld, H. W. 1988 Additional biologically active constituents of the Chinese tallow tree *(Sapium sebiferum). J. Nat. Prod.* **51** : 619-620

Losso, J. N., Bansode, R. R., Trappey, A II. , Bawadi, H. A. and Truax, R. 2004 In vitro anti-proliferative activities of ellagic acid. *J. Nutr. Biochem.* **15** : 672-678

Louw, C., La Grange, D., Pretorius, I.S. and van Rensburg, P. 2006 The effect of polysaccharide-degrading wine yeast transformants on the efficiency of wine processing and wine flavour *J. Biotechnol.* **125** : 447-461

Lucena, R., Cardenas, S., Gallego. M., Valcarcel, M. 2005 Autoanalyzer for continuous fractionation and quantitation of the polyphenols content in wines. *J. Chromatogr. A.* **1081** : 127-131.

Mathias, K., Ismail, B., Corvalan, C. M. and Hayes, K. D. 2006 Heat and pH effects on the conjugated forms of genistin and daidzin isoflavones. *J. Agric. Food Chem*. **54** : 7495-7502

Matsumoto, H., Nakamura, Y., Tachibanaki, S., Kawamura, S., Hirayama, M. 2003 Stimulatory effect of cyanidin 3-glycosides on the regeneration of rhodopsin. *J. Agric. Food Chem*. **51** : 3560-3.

McAnlis, G. T., McEneny, J., Pearce, J. and Young, I. S. 1999 Adsorption and antioxidant effects of quercetin from onions, in man. *Eur. J. Clin. Nutr.* 53 : 92-96

Mendiola, J. A., Herrero, M., Cifuentes, A. and Ibanez, E. 2007 Use of compressed fluids for sample preparation: food applications. *J. Chromatogr. A.* **1152** : 234-246

Miyake, Y., Minato, K., Fukumoto, S., Yamamoyo, K., Oya-Ito, T., Kawakishi, S. and Osawa, T. 2003 New Potent Antioxidative Hydroxyflavanones Produced with *Aspergillus saitoi* from Flavanone Glycoside in Citrus Fruit. *Biosci. Biotechnol. Biochem.* **67** : 1443-1450

Miyase, T., Ueno, A., Takizawa, N., Kobayashi, H. and Oguchi, H. 1988 Studies on the glycosides of *Epimedium grandifolorum* MORR. var. *thunbergianum* (MIQ.) NAKAI. III. *Chem. Pharm. Bull.* **36** : 2475-2484

Neera, S. and Ishimaru, K. 1992 Tannin production in cell culture of *Sapium sebiferum*. *Phytochemistry* **31** : 833-836

Nishioka, I. 1983 Chemistry and biological activities of Tannins. *Yakugaku Zasshi* **103** : 125-142

Nishitani, E. and Sagesaka, Y. M. 2004 Simultaneous determination of catechins, caffeine and other phenolic compounds in tea using new HPLC method. *J. Food Comp. Anal.* **17** : 675-685

Notka, F., Meier, G. R. and Wagner, R. 2003 Inhibition of

wild-type human immunodeficiency virus and reverse transcriptase inhibitor-resistant variants by *Phyllanthus amarus*. *Antiviral Res.* ***58*** : 175-186

Obi, F., Usenu, I. and Osayande, J. 1998 Prevention of carbon tetrachloride-induced hepatotoxicity in the rat by *H. rosasinensis* anthocyanin extract administered in ethanol. *Toxicology* **131** : 93-98

Oh, H. I., Hoff, J. E., Armstrong, G. S. and Haff, L. A. 1980 Hydrophobic interaction in tannin-protein complexes. *J. Agric. Food Chemi.* **28** : 394-398

Okuda, T., Mori, K., Ishino, M. 1979 Constituents of *Geranium thunbergii* SiEB. et Zucc. VIII. Transformations of geraniin upon decoction. *Yakugaku Zasshi* **99** : 505-509.

Ömür Demirezer L., Gürbüz F., Güvenalp Z., Ströch K. and Zeeck A. 2006 Iridoids, flavonoids and monoterpene glycosides from *Galium verum subsp. Verum. Turk. J. Chem.* **30** : 525-534

Papagiannopoulos, M., Zimmermann, B., Mellenthin, A., Krappe, M., Maio, G. and Galensa, R. 2002 Online coupling of pressurized liquid extraction, solid-phase extraction and high-performance liquid chromatography for automated analysis of proanthocyanidins in malt. *J. Chromatogr. A.* **958** : 9-16.

Peterson, J., Dwyer, J., Jacques, P., Rand, W., Prior, R. and Chui, K. 2004 Tea variety and brewing techniques influence flavonoid content of black tea. *J. Food Compost. Anal.* **17** : 397-405

Rattanathanalerk, M., Chiewchan, N. and Srichumpoung, W. 2005 Effect of thermal processing on the quality loss of pineapple juice. *J. Food Process. Eng.* **66** : 259-265

Rawel, H.M., Kroll, J. and Rohn, S. 2001. Reactions of phenolic substances with lysozyme — physicochemical characterisation and proteolytic digestion of the derivatives. *Food Chemistry* **72** : 59-71

Rohn, S., Buchner, N., Driemel, G., Rauser, M. and Kroh, L. W. 2007 Thermal degradation of onion quercetin glucosides under roasting conditions. *J. Agric. Food Chem.* **55** : 1568-1573

Saijo, R., Nonaka, G., and Nishioka, I. 1989 Tannins and related compounds. LXXXVII. Isolation and characterization of four new hydrolysable tannins from the leaves of *Mallotus repandus*. *Chem. Pharm. Bull.* **37** : 2624-2630

Sao, R. T. and Deng, Z. 2004 Separation procedures for naturally occurring antioxidant phtochemicals. *J. Chromatogr.* **812** : 85—89

Sarker, D. K., Wilde, P. J. and Clark, D. C. 1995 Control of surfactant-induced destabilization of foams through polyphenol-mediated protein-protein interactions. *J. Agric. Food Chem.* **43** : 295-300

Sarni-Manchado, P., Cheynier, V. and Moutounet, M. 1999 Interactions of grape seed tannins with salivary proteins. *J. Agric. Food Chem.* **47** : 42-47

Shin, M. S., Kang, E. H. and Lee, Y. I. 2005 A flavonoid from medicinal plants blocks hepatitis B virus-e antigen secretion in HBV-infected hepatocytes. *Antiviral Res.* **67** : 163-168

Siebert, K.J., Troukhanova, N.V. and Lynn, P.Y. 1996 The nature of polyphenol-protein interactions. *J. Agric. Food Chem.* **44** : 80-85

Sims, C. A., Eastridge, J. S. and Bates, R. P. 1995 Changes in phenols, color, and sensory characteristics of muscadine wines by pre- and post-fermentation additions of PVPP, casein, and gelatin. *Am. J. Enol. Vitic.* **46** :155-158

Steinkraus, K. H. 1995 Indigenous Fermented Foods Involving an Alkaline Fermentation (2nd edn. ed.), Marcel Dekker, NY 349-362

Stintzing F. C., Hoffmann M. and Carle R. 2006 Thermal degradation kinetics of isoflavone aglycones from soy and red clover. *Mol. Nutr. Food Res*. **50** : 373-7

Stoner, G. D. and Mukhtar, H. 1995 Polyphenols as cancer chemopreventive agents. *J. Cell. Biochem.* **22** Suppl. : 169-180

Sugimoto, N., Kikuchi, H., Yamazaki, T. and Maitani, T. 2001. Polyphenolic constituents from the leaves of *Rubus suavissimus*. *Nature Medicines* **55** : 219

Sumi, H. 2007 The history and medical food of Bacillus subtilis natto. *The Japanese journal of taste and smell research* 14: 129-136

Takenaka, M., Nanayama, K., Isobe, S. and Murata, M. 2006 Changes in caffeic acid derivatives in sweet potato (*Ipomoea batatas L.*) during cooking and processing. *Biosci. Biotechnol. Biochem.* **70**: 172-177

Tapiero, H., Ba, G. N. and Tew, K. D. 2002 Estrogens and environmental estrogens. *Biomed. Pharmacother.* **56**: 1-9

Terlabie, N. N., Sakyi-Dawso, E. and Amoa-Awu. W. K. 2006 The comparative ability of four isolates of Bacillus subtilis to ferment soybeans into dawadawa. *Int. J. Food Microbiol.* 106 :145-152.

Toda, T., Uesugi, T., Hirai, K., Nukaya, H., Tsuji, K. and Ishida H. 1999 New 6-*O*-acyl isoflavone glycosides from soybeans fermented with Bacillus subtilis (natto). I. 6-*O*-succinylated isoflavone glycosides and their preventive effects on bone loss in ovariectomized rats fed a calcium-deficient diet. *Biol. Pharm. Bull.* **22**: 1193-1201

Ushijima, K., Nozawa T., Tanaka, N., G. Nonaka, and Ishimaru, K. 2001 Purification of catechins using Tempe protein. *Journal of the Japanese Society for Food Science and Technology* **48**: 913-917

Vervliet, G., Holsters, M., Teuchy, H., Van Montagu, M. and Schell, J. 1975 Characterization of different plaque-forming and

defective temperate phages in *Agrobacterium* strains. *J. Gen. Virol.* **26** : 33-48

Wang, H. and Murphy, P. A. 1994 Isoflavone composition of American and Japanese soybeans in Iowa: effects of variety; crop year, and location, *J. Agric. Food Chem.* **42** : 1674-1677

Wang, H., Provan, G. J. and Helliwell, K. 2000 Tea flavonoids: their functions, utilisation and analysis. *Trends in Food Science & Technology.* **11** : 152-160

Wang, Y., Ohtani, K., Kasai, R. and Yamasaki, K. 1996 Flavonol Glycosides and phenolics from leaves of *Cordia dichotoma*. *Natural Medicines* **50** : 367

Xu, Z. J., Leung, L. K., Huang, Y. and Chen, Z. Y. 2003 Epimerisation of tea polyphenols in tea drinks *J. Agric. Food Chem*. **83** : 1627-1621

Yin, L. J., Li, L. T., Li, Z. G., Tatsumi, E. and Saito, M. 2004 Changes in isoflavone contents and composition of sufu (fermented tofu) during manufacturing. *Food Chemistry* **87** : 587-592

Yoshimoto, M., Okuno, S., Yoshinaga, M., Yamakawa, O., Yamaguchi, M. and Yamada, J. 1999 Antimutagenicity of sweet potato (*Ipomoea batatas*) roots. *Biosci. Biotechnol. Biochem* **63** : 537-541

Yoshida, T., Hatano, T. and Okuda, T. 1998 Chromatography of tannins : IV. Separation of labile oligomeric hydrolysable tannins and related polyphenols by centrifugal partition

chromatography. *J. Chromatogr. A.* **467**: 139-147

Yokozawa, T., Chen, C. P., Dong, E., Tanaka, G. and Nishioka, I. 1998 Study on the inhibitory effect of tannins and flavonoids against the 1,1-diphenyl-2-picrylhydrazyl radical. *Biochem. Pharmcol.* **56** : 213-222

Zhou, Z., Zhang, Y., Xu, M. and Yang, C. 2005 Puerins A and B, two new 8-C substituted flavan-3-ols frm pu-er tea. *J. Agric. Food Chem*. **53** : 8814-8617

Zhou, Z., Li, J. H. and Zhao, L. 2004 Study on main microbes on quality formation of Yunnan pu-er tea during pile fermentation process. *Journal of Tea Science* **24** : 211-218

Zhu, Y. P., Fan, J. F., Cheng, Y. Q. and Li, L. T. 2007 Improvement of the antioxidant activity of Chinese traditional fermented okara (Meitauza) using *Bacillus subtilis* B2. *Food Control*, In Press, Corrected Proof, Available online 24 July