Free and Soluble Bound Phenolic Acids in Some Cruciferous Vegetables

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Hydroxybenzoic, and hydroxycinnamic acids of free, glucosidic, and ester forms were obtained separately from fresh leaves of six cruciferous vegetables and analyzed by GC and GC-MS. Three hydroxybenzoic acids (*p*-hydroxybenzoic, vanillic, and protocatechuic acids) and eight hydroxycinnamic acids (*cis*-, and *trans*-isomers of *p*-coumaric, ferulic, caffeic, and sinapic acids) were identified in all the vegetables used, of which *cis*-, and *trans*-sinapic acids were the most prominent and *trans*-ferulic acid was next abundant in each phenolic acid fraction of the vegetables. The hydroxybenzoic acids were relatively higher in amounts in the glucosidic fractions. On the other hand, most of the hydroxycinnamic acids were found in the alkaline hydrolyzates of each vegetable extract, indicating that the hydroxycinnamic acids are mainly contained as esters.

In our previous experiments^{1,2)}, volatile phenols such as phenol, guaiacol, and their ethyl and vinyl derivatives were identified in the salted products of some cruciferous vegetables (Nozawana, Hiroshimana and Takana). In the case of salted Takana leaves (Takana-zuke), relative amounts of the phenols increased with their storage time, and the phenols are considered being responsible for the characteristic flavor of the aged Takana-zuke (furuzuke). It has been known that ethyl or vinyl derivatives of phenol and guaiacol are produced from their corresponding phenolic acids such as *p*-coumaric and ferulic acid through microbial³⁾ or thermal⁴⁾ degradation. It is presumed that the precursors of the volatile phenols occurring in the salted cruciferous vegetables are probably their corresponding phenolic acids. However, little information is available on the composition of phenolic acids in the fresh leaves of these cruciferous vegetables. The present experiments were conducted to clarify the phenolic acid composition in some cruciferous vegetables for pickling use. It has been found that naturally occurring phenolic acids are

usually contained as free acids, esters, glycosides, and insoluble form⁵⁻⁷⁾ bound tightly to cell wall polysaccharide, of which insoluble bound phenolic acids were not analyzed in the present study. Although HPLC method⁸⁾ for direct analysis of esters and/or glycosides of phenolic acids has been developed, it is not so easy to obtain the authentic specimens. In the present study, phenolic acids of glucosidic, and ester forms were hydrolyzed beforehand and the liberated phenolic acids were analyzed by GC and GC-MS as well as free acids after their trimethylsilylation.

Materials and Methods

Plant materials

The vegetables used in the present experiments were Takana (*Brassica juncea* L. var. *integrifolia*), Nozawana (*B. campestris* L. var. *rapa*), Hiroshimana (*B. campestris* L. var. *pekinensis*), Mana (*B. campestris* L. var. *pekinensis*), Kyomizuna (*B. campestris* L. var. *japonica*) and Mibuna (*B. campestris* L. var. *japonica*), which were obtained from Tohoku Seeds Co. Ltd., Utsunomiya, in Nov. 1986.

These were stored at -25°C for 5 days until use. The average weights (g/plant) of each vegetable were : Takana, 340 ; Nozawana, 330 ; Hiroshimana, 1040 ; Mana, 390 ; Kyomizuna, 770 ; Mibuna, 960.

Chemicals

p-Hydroxybenzoic, vanillic, protocatechuic, *p*-coumaric, ferulic, and caffeic acids, N, O-Bis (trimethylsilyl) trifluoroacetamide (BSTFA), Trimethylsilylchlorosilane (TMCS) and dimethylformamide (DMF) were purchased from Tokyo Kasei Kogyo Co., Ltd. Tokyo. Sinapic acid was obtained from Aldrich Chemical Co. Milwaukee, USA. All the hydroxycinnamic acids used were *trans*-isomers. β -Glucosidase (sweet almonds, 1 000 units/mg) was obtained from Biozyme Laboratories Ltd., South Wales, England.

Extraction and fractionation of phenolic acids

The materials (125 g) boiled in 500 ml of 70 % ethanol for 30 min were pulped in a homogenizer and left for 60 min at room temperature. The pulped materials were filtered and washed five times using appropriate volume of 70% ethanol. After the removal of ethanol from the extract, the aqueous concentrate was washed with *n*-hexane to remove free fatty acids, and then extracted with ethyl acetate. The ethyl acetate extract was evaporated to dryness and the residue was dissolved in 5 ml of methanol. This solution was used for the analysis of free phenolic acids. The residual aqueous solution was divided into two equal volumes, of which one was incubated with β -glucosidase (30 mg) in 0.1 M acetate buffer (pH 5.2) at 37°C for 60 min, and the other was

Fig. 1 Gas chromatograms of the TMS-derivatives of phenolic acids of free, glucosidic, and ester forms obtained from the Takana leaves

(A), Free phenolic acid fraction; (B), glucoside fraction; (C), ester fraction.

1, malic acid ; 2, *p*-hydroxybenzoic acid ; 3, vanillic acid ; 4, *cis*-*p*-coumaric acid ; 5, protocatechuic acid ; 6, unknown ; 7, unknown ; 8, *cis*-ferulic acid ; 9, *trans*-*p*-coumaric acid ; 10, *cis*-caffeic acid ; 11, unknown, 12, *cis*-sinapic acid ; 13, *trans*-ferulic acid ; 14, *trans*-caffeic acid ; 15, unknown ; 16, *trans*-sinapic acid ; 17, unknown.

GC conditions are described in the text.

mixed with 2 M sodium hydroxide solution under stream of nitrogen at room temperature for 2.5 h. Both reaction mixtures were extracted with ethyl acetate after their pH adjustment to about 2.2. The respective extract was evaporated to dryness and the residue was dissolved in 5 ml of methanol. The former solution was used for the analysis of phenolic acids of glucosidic form and the latter for those of ester one.

Preparation of trimethylsilyl derivatives of phenolic acids

The methanolic solutions (0.25 ml) were evaporated to dryness, and 0.16 ml of DMF, 0.02 ml of methyl myristate (10 mg/ml DMF) as the internal standard, 0.02 ml of TMCS and 0.1 ml of BSTFA were added to the residue. The mixture was heated at 65°C for 15 min, and analyzed immediately by GC and GC-MS.

GC and GC-MS conditions

A Shimadzu 6AM gas chromatograph with a flame ionization detector was used. A glass column packed with 5% OV-1 coated on chromosorb WAW (60 ~ 80 mesh) was employed. The column temperature was programmed from 100 to 260°C at a rate of 5°C/min, and both detector and injector were maintained at 270°C. Nitrogen was used as the carrier gas with a flow rate of 40 ml/min. Mass spectra were obtained at an electron energy of 70 eV by use of a Hitachi M-80 mass spectrometer. GC conditions were the same as in the above except for the carrier gas (helium).

Results and Discussion

Identification of phenolic acids in the fresh leaves of the cruciferous vegetables

Phenolic acids contained as free, glucosidic, and ester forms in the fresh leaves of Nozawana, Hiroshimana, Takana, Kyomizuna, Mibuna and Mana were analyzed by GC and GC-MS after their trimethylsilylation. Fig. 1 shows the gas chromatographic profiles of the trimethylsilyl derivatives obtained from the fractions of free acids, glucosides, and esters in the Takana leaves as a representative.

Three hydroxybenzoic acids, namely, *p*-

hydroxybenzoic, vanillic, and protocatechuic acids and four hydroxycinnamic acids, that is, *trans*-isomers of *p*-coumaric, ferulic, caffeic, and sinapic acids were identified in all the vegetables examined by comparing their mass spectra and retention times with those of the authentic specimens. The compounds of peak nos. 4, 8, 10 and 12 gave the same major mass fragments as those of the *trans*-isomers of *p*-coumaric, ferulic, caffeic, and sinapic acids, respectively, though their relative retention times were not identical with those of the *trans*-isomers corresponded. Under the employed GC conditions, retention times of the *cis*-isomers were shorter than those of the *trans*-isomers⁹⁾. Mass spectra of the *cis*-, and *trans*-isomers of the hydroxycinnamic acids have the same major fragment ions¹⁰⁾. Therefore, it appeared to be no doubt that the above unknown compounds are *cis*-isomers of the four hydroxycinnamic acids. These phenolic acids are known as common constituents in plants. The component of peak 1, which was the most prominent in the hydrolyzates of ester fractions prepared from the six vegetables used, was confirmed as trimethylsilylated malic acid from the mass spectrum recorded (*m/z* 147, *m/z* 73, *m/z* 233, *m/z* 335, *m/z* 245, *m/z* 190, *m/z* 133). It is well known that in crucifers, malic¹¹⁾, or tartaric acid¹²⁾ often forms ester with some hydroxycinnamic acids, such as ferulic and sinapic acids. Since large amount of malic acid was found in the alkaline hydrolyzates, it may be contained as an ester

Table 1 Losses of phenolic acids during the alkaline hydrolysis

phenolic acid	losses (%) ^a
<i>p</i> -hydroxybenzoic acid	9.4
Vanillic acid	6.6
Protocatechuic acid	62.2
<i>p</i> -Coumaric acid	11.8
Ferulic acid	10.4
Caffeic acid	76.6
Sinapic acid	24.8

^a) Means of triplicate samples

of the hydroxycinnamic acids in the cruciferous vegetables, though it was not confirmed in the present study. Tartaric acid was not detected in all the samples. This may be partly owing to its relatively lower solubility to ethyl acetate used as an extracting solvent. The components of peak nos. 6, 7, 9, 15, and 17, which were detected from all the vegetables and appeared to be trimethylsilylated components

from their mass fragmentation patterns, were not confirmed in the present study.

Quantification of phenolic acids of free, glucosidic, and ester forms in fresh leaves of the cruciferous vegetables

Since certain hydroxycinnamic acids are known to be unstable in an alkaline solution¹³⁾¹⁴⁾, losses of the identified phenolic acids under the conditions of the alkaline hydrolysis

Table 2 Contents^a of free phenolic acids in the cruciferous vegetables (I~VI)^b

phenolic acids	I	II	III	IV	V	VI
<i>p</i> -Hydroxybenzoic	0.3	0.1	0.1	0.2	0.2	0.1
Vanillic	0.1	0.2	0.3	0.1	0.1	0.2
<i>cis-p</i> -Coumaric	2.9	0.6	3.1	0.6	4.0	2.6
Protocatechuic	0.4	0.5	0.3	0.4	0.7	0.6
<i>cis</i> -Ferulic	0.1	0.1	0.3	0.2	0.1	0.3
<i>trans-p</i> -Coumaric	— ^c	—	—	—	—	—
<i>cis</i> -Caffeic	—	—	—	—	—	—
<i>cis</i> -Sinapic	52.5	24.8	27.6	14.6	13.8	23.5
<i>trans</i> -Ferulic	3.8	2.2	0.9	1.1	1.7	1.8
<i>trans</i> -Caffeic	—	—	—	—	—	—
<i>trans</i> -Sinapic	22.8	21.3	8.9	3.4	0.4	0.2

^a) The values were the means of duplicate samples and expressed as mg/kg fresh weight.

^b) The vegetables were : I, Takana; II, Nozawana; III, Hiroshimana IV, Kyomizuna; V, Mibuna and VI, Mana.

^c) Not detected.

Table 3 Contents^a of glucosidic phenolic acids in the cruciferous vegetables (I~VI)^b

phenolic acid	I	II	III	IV	V	VI
<i>p</i> - Hydroxybenzoate	1.6	1.9	0.7	0.1	0.1	0.8
Vanillate	2.2	2.3	2.8	1.4	0.6	2.4
<i>cis-p</i> -Coumarate	1.2	1.4	0.5	1.4	0.2	0.9
Protocatechuate	1.6	2.7	1.5	2.1	2.6	2.1
<i>cis</i> -Ferulate	0.8	0.3	0.5	0.3	0.2	0.6
<i>trans-p</i> -Coumarate	9.6	7.2	4.6	10.2	12.2	8.8
<i>cis</i> -Caffeate	— ^c	—	—	—	—	—
<i>cis</i> -Sinapate	15.7	9.9	10.8	19.5	22.4	6.4
<i>trans</i> -Ferulate	8.3	3.6	1.8	3.2	2.2	1.1
<i>trans</i> -Caffeate	0.9	0.8	0.4	0.6	0.7	0.2
<i>trans</i> -Sinapate	24.6	14.8	6.4	23.6	12.7	5.8

^a) The values were the means of duplicate samples and expressed as mg/kg fresh weight.

^b) See footnote in Table 2.

^c) Not detected.

were examined. The results are shown in Table 1, indicating that large amounts of *o*-dihydroxy derivatives of benzoic and cinnamic acids are degraded during the alkaline treatment. The losses of protocatechuic and caffeic acids were about 62% and 77%, respectively. About 25% of sinapic acid was also lost. The losses of the other phenolic acids were nearly 10%. DABROWSKI and SOSULSKI¹⁴⁾ reported that losses during the treatment with 2 N sodium hydroxide for 4 h under nitrogen at room temperature were 83% for caffeic, 23% for sinapic, 18% for ferulic, 13% for *p*-coumaric, 9% for vanillic, and 9% for *p*-hydroxybenzoic acids. The values in Table 1 were similar to those reported by them¹⁴⁾ except for caffeic and ferulic acids.

On the other hand, relationships between the ratios of peak areas of the identified phenolic acids to that of the internal standard and their concentrations were linear in the range 5 ~ 50 μ g for *p*-hydroxybenzoic, vanillic, and protocatechuic acids and in the range 20 ~ 300 μ g for *p*-coumaric, ferulic, caffeic, and sinapic acids (*trans*-isomers), indicating the accuracy of the gas chromatographic determination.

Tables 2, 3 and 4 show the contents of the identified phenolic acids of free, glucosidic, and ester forms, in which the values were expressed

as mg/kg fresh weight without correction of the losses during the alkaline treatment. As shown in Table 2, 8 phenolic acids were detected in the free acid fractions from the six vegetables, where *cis*-sinapic acid was the most predominant ranging from about 14 to 53 mg/kg, and was followed with its *trans*-isomer (3 ~ 23 mg/kg) except for Mibuna and Mana. In the case of the latter two vegetables, the contents of *trans*-sinapic acid were lower than those of *cis*-*p*-coumaric and *trans*-ferulic acids. *trans*-*p*-Coumaric, and both *cis*-, and *trans*-caffeic acids were not found in the fractions.

On the other hand, 10 phenolic acids were detected from the glucoside fractions of all the vegetables, of which *cis*-, and *trans*-sinapic acids, *trans*-*p*-coumaric acid and *trans*-ferulic acid were the major components (Table 3). *cis*-Caffeic acid was not detected in the fractions. Glucosides of sinapic and *p*-coumaric acids have been found in *Brassica* seed meals²⁰⁾. However, little report on the contents of glucosidic phenolic acids in fresh leaves of cruciferous vegetables⁵⁾ has been available. Another noticeable point was that the amounts of *p*-hydroxybenzoic, vanillic, and protocatechuic acids in the glucosidic form were relatively higher than those in the other forms.

Table 4 Contents^a of esterified phenolic acids in the cruciferous vegetables (I~VI)^b

phenolic acid	I	II	III	IV	V	VI
<i>p</i> -Hydroxybenzoate	0.2	0.1	tr ^c	tr	tr	0.2
Vanillate	0.7	0.5	0.9	0.7	0.4	1.1
<i>cis</i> - <i>p</i> -Coumarate	8.3	5.1	3.3	7.1	6.2	15.2
Protocatechuate	0.7	0.6	0.7	0.2	0.7	1.3
<i>cis</i> -Ferulate	13.2	12.8	18.6	6.4	23.2	19.5
<i>trans</i> - <i>p</i> -Coumarate	17.1	27.2	14.8	7.7	43.2	16.5
<i>cis</i> -Caffeate	0.6	0.2	0.4	0.3	0.6	0.3
<i>cis</i> -Sinapate	18.3	77.2	32.2	9.6	31.1	38.3
<i>trans</i> -Ferulate	84.8	18.8	54.6	39.3	59.6	63.2
<i>trans</i> -Caffeate	32.2	13.2	29.1	19.5	44.4	35.2
<i>trans</i> -Sinapate	148.3	129.8	133.6	98.7	108.8	168.1

^{a)} The values were the means of duplicate samples and expressed as mg/kg fresh weight.

^{b)} See footnote in Table 2.

^{c)} The value was lower than 0.05.

As to this, LEUNG *et al.*¹⁵⁾ studied the phenolic components of sunflower flour and described that *p*-hydroxybenzoic, vanillic, and syringic acids might exist in glycosidic form to a larger extent than in ester form.

Table 4 shows the contents of the phenolic acids released by alkaline hydrolysis. All vegetables examined had considerably higher amounts of both *cis*- and *trans*-isomers of *p*-coumaric (3 ~ 43 mg/kg), ferulic (6 ~ 85 mg/kg), caffeic (0.2 ~ 44 mg/kg), and sinapic acids (10 ~ 168 mg/kg), of which *trans*-sinapic acid was distinguished from the other in its large content ranging from 99 to 168 mg/kg fresh weight. In cruciferous plants, sinapic acid is known to be contained as the ester of choline (sinapine)¹⁶⁾, malate or glucose¹⁷⁾, which are hydrolyzable easily with dilute alkali at room temperature^{7,20)}. Since the phenolic acids contained as ester form in the above vegetables also prepared by a moderate alkali hydrolysis of the aqueous extracts, they are probably bound with such compounds as malic acid, glucose or choline. This should be confirmed by further study.

It is surprising that a considerable amount of *trans*-caffeic and small amounts of *cis*-caffeic and protocatechuic acids were detected in the alkaline hydrolyzates from all the vegetables, because these *o*-dihydroxy phenolic acids were lost much during the alkaline hydrolysis. Thus, it is presumed that some mechanism involved in such stabilization of *o*-dihydroxy phenolic acids may be included in the vegetable extracts.

On the other hand, considerable amounts of *cis*-isomers of the hydroxycinnamic acids were also detected in the free and soluble bound phenolic acid fractions of the vegetables. It has been reported that *cis*-isomers of hydroxycinnamic acids can be partially converted from their *trans* form by the action of UV light¹⁸⁾ or an alkaline treatment¹⁹⁾. However, it appeared to be unreasonable that the presence of a large amount of *cis*-sinapic acid in the free phenolic acid fractions of the vegetables is attributed to such *cis-trans* isomerization, since the fractions were treated by neither UV light nor

alkali. The reason why the free phenolic acid fractions had abundant *cis*-isomer of sinapic acid could not be confirmed in the present study.

From the above results, it became clear that the hydroxycinnamic acids in the cruciferous vegetables examined are mainly contained as ester form like some *Brassica* seeds^{20,21)} and their compositions are similar among the vegetables.

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アブラナ科野菜中の遊離及び可溶性
結合型フェノール酸

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高菜・野沢菜・広島菜などの塩漬製品中に生成する揮

発性フェノール類の前駆体は、これら野菜中に含まれるフェノール酸と見られることから、今回、6種のアブラナ科野菜について、遊離型・配糖体型・エステル型各フェノール酸の同定と定量を試みた。全部で3種のヒドロキシ安息香酸 (*p*-ヒドロキシ安息香酸, バニリン酸, プロトカテキュ酸) 及び8種のヒドロキシ桂皮酸 (シス-及びトランス-*p*-クマル酸, 同フェルラ酸, 同コーヒー酸, 同シナピン酸) が同定された。この中、シス及びトランス-シナピン酸はどの野菜でも各形態のフェノール酸中、最も多く含まれていた。各形態別の主要なフェノール酸は、遊離型では、シス-シナピン酸 (14~53 mg/kg), 配糖体型では、シス- (6~22 mg/kg), 及びトランス (6~25 mg/kg) シナピン酸及びトランス-*p*-クマル酸 (5~12 mg/kg), またエステル型では、トランス-シナピン酸 (99~168 mg/kg), トランス-フェルラ酸 (19~85 mg/kg), トランス-コーヒー酸 (13~44 mg/kg) 及びトランス-*p*-クマル酸 (8~43 mg/kg) であった。3種のヒドロキシ安息香酸はいずれの野菜でも低含量であったが、これらは配糖体型として存在する量が高かった。以上の結果から、これらの野菜中のフェノール酸の主体は4種のヒドロキシ桂皮酸であり、主にエステルの形態で存在することが明らかにされた。