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Changes of phenolic acids and antioxidant activities during potherb mustard (*Brassica juncea*, Coss.) pickling

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Abstract

Phenolic acids in potherb mustard (*Brassica juncea*, Coss.) were determined and the effects of pickling methods on the contents of total free phenolic acids, total phenolic acids, total phenolics, and antioxidant activities were investigated. Gallic acid, protocatechuic acid, *p*-hydroxybenzoic acid, vanillic acid, caffeic acid, *p*-coumaric acid, ferulic acid, and sinapic acid were identified in the present study. The contents of total free phenolic acids, total phenolic acids and total phenolics in fresh potherb mustard were 84.8 ± 0.58 μg/g dry weight (DW), 539 ± 1.36 μg/g DW, and 7.95 ± 0.28 mg/g DW, respectively. The total free phenolic acids increased during the pickling processes, but the total phenolic acids, total phenolics, and antioxidant activities decreased. However, after 5 weeks of fermentation, all the pickling methods retained over 70% of total phenolic contents and above 65% of antioxidant capacities. The results indicated that pickling processes were relatively good methods for the preservation of phenolic acids and antioxidants for potherb mustard.

Keywords: Potherb mustard; Pickling; Phenolic acid; Antioxidant activity

1. Introduction

Potherb mustard (*Brassica juncea*, Coss.), belonging to the family of *Cruciferae*, is a leafy vegetable cultivated in China (Liu, 1994). A single plant of potherb mustard may have as many as 150–200 leaves clustered together in a compact bunch of 20–30 cm diameter. Potherb mustard is consumed not only in cooked and raw fresh vegetables, but also in salt-preserved or pickled forms. Pickled potherb mustard is a traditional fermented vegetable product and is widely consumed by all social groups in China (Li, 1988). The quality characteristics of potherb mustard pickles are due to its typical flavour and taste, and the major flavour components are mainly derived from glucosinolate hydrolysis. The predominant glucosinolates in potherb mustard are sinigrin, gluconapin, glucocochlearin and gluconasturtin (Zhao, Tang, & Ding, 2007). During pickling, these glucosinolates can be hydrolysed to the products of isothiocyanates or indole-3-carbinol (Kameoka & Hashimoto, 1980; Masuda, Harada, Tanaka, Nakajima, & Tabeta, 1996), resulting in alleviation of the pungent flavour and endowing the pickled vegetable with a special savoury flavour. Interestingly, the glucosinolate hydrolysis products such as isothiocyanates and indole-3-carbinol are capable of modulating enzyme activity and preventing certain cancers (Fahey, Zhang, & Talalay, 1997; Greenwald, 2004).

Phenolic compounds in vegetables are also beneficial to the health of human beings (Tomás-Barberán & Robins, 1997; Verhoeven, Goldbohm, van Poppel, Verhagen, & van den Brandt, 1996). Their high antioxidant capacities are thought to have links with the inhibition of oxidative damage diseases, such as coronary heart disease, stroke, and cancers (Block, Patterson, & Subar, 1992; Huang & Ferraro, 1992; Powles & Ness, 1996). The main flavonols in potherb mustard are quercetin and kaempferol, and flavonol contents range from 6.51 to 14.9 mg/g dry weight,

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which are positively correlated with antioxidant activities. The contents of antioxidant compounds and antioxidant activities are lower in pickled leaf mustard compared with fresh samples (Wang & Zhu, 2006a). Antioxidant activities in pickled products are also significantly correlated with their contents of total phenolics (Wang & Zhu, 2006b).

As a high dietary intake of cruciferous vegetables is thought to be desirable for preventing certain cancers (Suzuki, Ohnishi-Kameyama, Sasaki, Murata, & Yoshida, 2006), development of methods for retaining these functional compounds is expected to aid in the production of cruciferous vegetable products with greater health benefits.

Potherb mustard is one of the most popular and important cruciferous vegetables in China. However, information on the chemical components, especially the phenolic acids, of fresh and pickled potherb mustard is limited. In this study, we analysed the phenolic acids in the potherb mustard, and their changes during the pickling processes were monitored. The antioxidant activities of these foods evaluated with different methods were also investigated.

2. Materials and methods

2.1. Chemicals and reagents

Standards of gallic acid, protocatechuic acid, *p*-hydroxybenzoic acid, vanillic acid, caffeic acid, *p*-coumaric acid, ferulic acid, sinapic acid, 2,4,6-tris(2-pyridyl)-*s*-triazine (TPTZ), 6-hydroxyl-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonate) ($ABTS^{+}$), and Folin–Ciocalteu phenol reagent were purchased from Sigma (St. Louis, MO). All other chemicals used were of analytical grade.

2.2. Materials

Fresh potherb mustard was harvested from a farm field in the rural area of Hangzhou City, Zhejiang Province, in March 2007, and transported to our laboratory within 2 h.

2.3. Pickle preparation

On arrival, the mustard leaves were washed and drained as whole plants, then wilted in the sun to a water content of about 85%. Three treatments were conducted:

(1) 5 g/100 g NaCl: 500 g of sodium chloride was added to 10 kg of mustard leaves, mixed well, packed into earthenware pot in layers, and then pressed tightly.
(2) 5 g/100 g NaCl + starter culture: sample was treated as in treatment 1, except that 3.6×106 cfu/g of *Lactobacillus plantarum* (obtained from the Institute of Plant Protection and Microbiology, Agricultural Academy of Zhejiang Province, China) was inoculated as starter culture;
(3) 10 g/100 g NaCl: sample was treated as in treatment 1, except that 1 kg of sodium chloride was added to 10 kg of mustard leaves. The pots were sealed and the leaves allowed to ferment for 5 weeks. The experiments were conducted in duplicate.

2.4. Sampling

The first sample was taken on the day of pickling before the potherb mustards were salted, and represents the fresh sample in the present study. Other samples were taken on a fixed day every week during the fermentation process. Two plants were randomly selected from the centre and bottom of the pots, and three leaves were selected from the outer, middle and centre of each plant. The leaves were pooled, frozen with liquid nitrogen and then freeze dried. The samples were ground and stored in plastic bags at −20 °C until analysis.

2.5. Extraction of phenolic acids

Phenolic acids was extracted and hydrolysed using a modified method previously described by Mattila and Kumpulainen (2002). Two grams of freeze dried potherb mustard powder were extracted with 80 ml of methanol and 10% acetic acid (85/15, v/v) and ultrasonicated at room temperature for 30 min. The mixture was made up to 100 ml with distilled water, and mixed, and 1 ml was filtered through a 0.45 μm cellulose acetate filter (Millipore Corp., Bedford, MA) for the HPLC analysis of free phenolic acids.

After the sample was analysed for free phenolic acids, 120 ml of distilled water and 50 ml of 10 M NaOH were added to the sample. The sample was then flushed with nitrogen, sealed, and stirred for 4 h at room temperature using a magnetic stirrer. The solution was then adjusted to pH 2 using 6 M HCl, and liberated phenolic acids were extracted five times with 150 ml of a mixture of cold diethyl ether (DE) and ethyl acetate (EA, 1/1, v/v) by manually shaking and centrifuging. DE/EA layers were combined, evaporated to dryness, and dissolved in 15 ml of methanol. HPLC was performed after the samples were filtered through a 0.45 μm cellulose acetate filter.

After the above alkaline hydrolysis, 25 ml of concentrated HCl was added into the sample and incubated in a water bath (85 °C) for 30 min. After acid hydrolysis, the sample was allowed to cool, and adjusted to a pH of 2. The DE/EA extraction performed was similar to that for alkaline hydrolysis. The evaporated extract was then dissolved in 15 ml of methanol, filtered through a membrane filter (see above), and analysed by HPLC. After HPLC quantification, the results from alkali and acid hydrolysates were calculated to represent total phenolic acids.

2.6. HPLC analysis

HPLC analyses of phenolic acids were carried out on a Waters 2695 HPLC system equipped with a 2996

photodiode array (Waters Co., Milford, MA). The phenolic acid solution of 10 μl was injected onto a Diamonsil™ C18 reversed phase column (250 × 4.6 mm, 5 μm particle size, Dikma Technologies, Beijing, China). The column thermostat was set at 40 °C. The elution profile was that used by Subba Rao, Muralikrishna (2002), with minor revision. Solvent A was 4% acetic acid, and solvent B was methanol (A/B = 80/20, v/v), with a flow rate of 1 ml/min. After each run, the column was washed with 100% methanol and equilibrated to initial conditions for 15 min. UV–visible spectral measurements were made over the range of 200–400 nm. Chromatograms were recorded at 260 nm for gallic acid, protocatechuic acid, *p*-hydroxybenzoic acid and vanillic acid, and at 320 nm for caffeic acid, *p*-coumaric acid, ferulic acid and sinapic acid. Phenolic acids were identified by the retention time and the UV–visible spectra of standards. Quantification of phenolic acids was carried out by an external standard method using calibration curves. The amount of each phenolic acid was expressed as microgram per gram dry weight (μg/g DW).

2.7. Total phenolic contents (TPC)

TPC was estimated calorimetrically using the modified Folin-Ciocalteau method (Sellappan, Akoh, & Krewer, 2002). An aliquot of 0.2 ml of free phenolic acid extract was added to 0.8 ml of water, 5 ml of 0.2 N Folin-Ciocalteau reagent, and 4 ml of saturated sodium carbonate solution (75 g/l) and mixed in a screw-top test tube. The absorbance was measured at 765 nm with a Shimadzu UV–visible 2550 spectrophotometer (Shimadzu Co., Kyoto, Japan), after incubation for 2 h at room temperature. Quantification was based on the standard curve, established with 100, 200, 300, 400 and 500 mg/l of gallic acid, and the results were expressed as gallic acid equivalent in milligrams per kilogram dry weight (mg GAE/kg DW). The results were the averages of triplicate analyses.

2.8. ABTS free radical scavenging assay

The ABTS free radical scavenging assay followed the method of Re et al. (1999). To oxidise the colorless ABTS to the blue-green $ABTS^{\cdot+}$ radical cation, ABTS (7 mM) was mixed with potassium persulfate and kept for 12–16 h at room temperature in the dark. On the day of analysis, the $ABTS^{\cdot+}$ solution was diluted with ethanol to an absorbance of 0.70(±0.02) at 734 nm. Then 0.05 ml of sample extract was added to 0.95 ml of $ABTS^{\cdot+}$ solution, stirred for 30 s and allowed to stand for 15 min at room temperature. The absorbance was then determined at 734 nm. A calibration curve was made by absorbance reduction with different concentrations of Trolox. A control consisted of 0.05 ml of 80% methanol and 0.95 ml of $ABTS^{\cdot+}$ solution. The stable ABTS radical scavenging activity of the extracts was expressed as Trolox equivalent antioxidant capacity (TEAC) milligrams per gram DW. The radical stock solution was prepared fresh daily.

2.9. Ferric reducing ability assay (FRAP)

The ferric reducing ability of sample extracts was measured according to a modified protocol developed by Benzie and Strain (1996). To prepare the FRAP reagent, 0.1 M acetate buffer (pH 3.6), 10 mM TPTZ, and 20 mM ferric chloride (10:1:1, v/v/v) were well mixed. Sample extracts of 0.1 ml were added to 1.9 ml of reagent. The reaction was monitored for up to 10 min and the readings at the absorption maximum (593 nm) were taken every 15 s, using the Shimadzu UV–visible 2550 spectrophotometer. Trolox solution was used to perform the calibration curves. Result was also expressed as TEAC mg/g DW.

2.10. Statistical analysis

The significant differences among the treatments were determined at the 95% level using the Tukey test of means. SPSS 10.0 was used as the statistic software (SPSS Inc., Chicago, Il).

3. Results and discussion

3.1. Phenolic acids and total phenolics contents in potherb mustard

Efficient separations of potherb mustard phenolic acids were achieved; Fig. 1 shows the HPLC chromatogram of the free phenolic acids. By comparison with standards, gallic acid, protocatechuic acid, *p*-hydroxybenzoic acid, vanillic acid, caffeic acid, *p*-coumaric acid, ferulic acid, and sinapic acid were identified in the fresh potherb mustard. Although chlorogenic acid is the main phenolic acid in many other foods (Mattila & Kumpulainen, 2002), it was not detected in potherb mustard. Flavonols of quercetin and kaempferol, which were found in another publication (Wang & Zhu, 2006a), were not identified in this investigation. It should be mentioned that, though most of the compounds were identified, there were still some peaks in Fig. 1, which were not elucidated by this analysis method.

Among the detected phenolic acids, sinapic acid was the most abundant component, while gallic acid was the least (Table 1). The content of total free phenolic acids was 84.8 ± 0.58 μg/g DW. After alkaline hydrolysis, the content of total phenolic acids increased to 440 ± 1.22 μg/g DW, which implied that most of the phenolic acids existed as ester forms in potherb mustard (Mattila & Kumpulainen, 2002; Xu, Ye, Chen, & Liu, 2007). The contents of total phenolic acids in the acid hydrolysis fractions were relatively low (99.2 ± 0.75 μg/g DW), but were still higher than those of free forms (Table 1). The results indicated that alkaline hydrolysis can liberate most of the bound phenolic acids, which was in accordance with the report of Mattila and Kumpulainen (2002).

The content of total phenolic acids, calculated from the results of alkali and acid hydrolysis, was 539 ± 1.36 μg/g DW (Table 1). Because we have not found any other

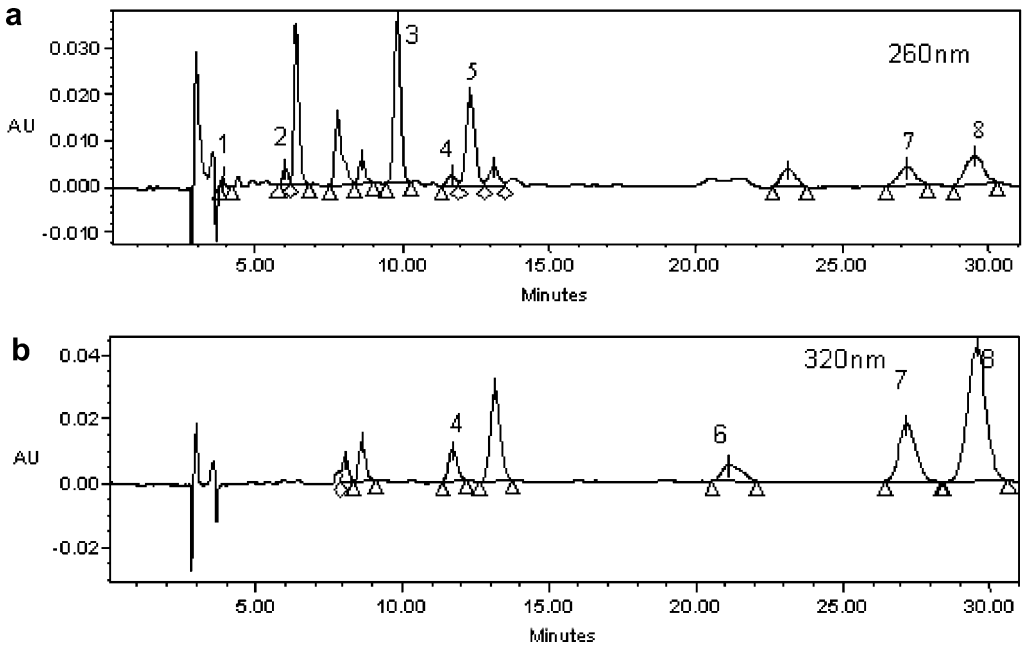

Fig. 1. HPLC chromatogram of the free phenolic acids in fresh potherb mustard, detected at 260 nm (a) and 320 nm (b). Peak numbers of 1–8 were identified as gallic acid, protocatechuic acid, *p*-hydroxybenzoic acid, vanillic acid, caffeic acid, *p*-coumaric acid, ferulic acid, and sinapic acid, respectively.

Table 1
Contents of phenolic acids and total phenolics in fresh potherb mustard (μg/g DW)

Phenolic forms	Gallic acid	Protocatechuic acid	*p*-Hydroxybenzoic acid	Vanillic acid	Caffeic acid	*p*-Coumaric acid	Ferulic acid	Sinapic acid	Total
Free	0.5 ± 0.01	0.7 ± 0.01	2.6 ± 0.05	1.6 ± 0.02	2.7 ± 0.12	13.4 ± 1.01	20.7 ± 0.25	42.6 ± 0.38	84.8 ± 0.58
Alkaline hydrolysis	1.1 ± 0.01	1.8 ± 0.02	10.2 ± 0.29	6.5 ± 0.12	11.8 ± 0.13	72.1 ± 1.17	85.6 ± 0.46	190 ± 1.18	440 ± 1.22
Acid hydrolysis	0.9 ± 0.01	1.1 ± 0.02	5.6 ± 0.21	4.3 ± 0.06	10.6 ± 0.15	25.6 ± 0.58	30.4 ± 0.54	20.7 ± 0.49	99.2 ± 0.75
Total phenolic acids[a]	2.0 ± 0.01	2.9 ± 0.03	15.8 ± 0.25	10.8 ± 0.13	22.4 ± 0.15	97.7 ± 1.23	116 ± 1.07	211 ± 1.09	539 ± 1.36
Total phenolic content (mg/g DW)[b]			7.95 ± 0.28						

[a] Calculated from the alkaline and acid hydrolysis of phenolic acids.
[b] Estimated by the Folin-Ciocalteau method.

reports referring to the phenolic acids in potherb mustard, these results were not comparable. However, levels of other kinds of phenolics in this vegetable, quercetin and kaempferol, were 0.25–0.89 and 0.53–1.76 mg/g DM, respectively (Wang & Zhu, 2006a), suggesting that the contents of total flavonols were higher than those of total phenolic acids in potherb mustard. In plant foods, it has been proposed that flavonoids account for approximately two-thirds of the dietary phenols, and phenolic acids account for most of the remaining third (Robbins, 2003; Scalbert & Williamson, 2000). However, when estimated by the Folin-Ciocalteau method, the content of total phenolics was 7.95 ± 0.28 mg/g DM, which was in the range of previously reported results (6.51–14.9 mg/g DM) (Wang & Zhu, 2006a).

3.2. Changes of phenolic acids and total phenolic contents during pickling

The contents of total free phenolic acids, total phenolic acids and total phenolics were monitored during the 5-week potherb mustard pickling. Fig. 2a shows that the contents of free phenolic acids increased in all three pickling treatments, with those in the low-salt treatment samples (5% NaCl) increasing more than those in the high-salt treatment samples (10% NaCl). Moreover, when the starter culture of *L. plantarum* was inoculated in the low-salt treatment samples, the content of free phenolic acids increased even higher. Based on the results of the experiments, when pickled for 2 weeks, the titratable acidities were 1.74%, 1.55%, and 1.48% in the samples of 5% NaCl + starter culture, 5% NaCl, and 10% NaCl, respectively. It might be deduced that the higher acidity liberated more free phenolic acids from the bound forms.

Starter cultures of lactobacillus have been proposed for sauerkraut, to reduce the amount of salt required for fermentation (Breidt, Crowley, & Fleming, 1995; Harris, Fleming, & Klaenhammer, 1992). In another study of potherb mustard pickling, when the sample was treated with relatively low salinity (5 g/100 g NaCl compared to 8 g/100 g and 10 g/100 g NaCl), there was an extremely rapid development of brine acidity. Furthermore,

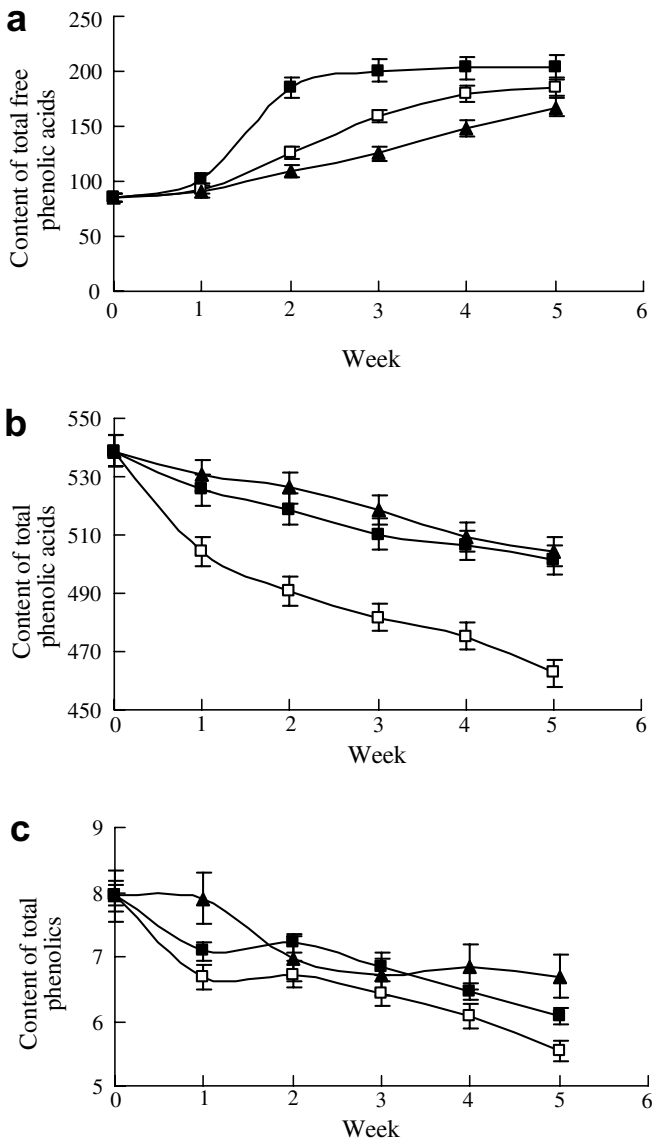

Fig. 2. Changes of total free phenolic acids ((a) μg/g DW), total phenolic acids ((b) μg/g DW) and total phenolics ((c) mg/g DW) during potherb mustard pickling. (□) 5 g/100 g of NaCl; (■) 5 g/100 g of NaCl + starter culture; and (▲) 10 g/100 g of NaCl.

differences in the titratable acidities could be observed between the trials of 5 g/100 g NaCl with or without *Bacillus coagulans* B179 inoculation (Zhao & Ding, in press). In the present investigation, the starter culture of *L. plantarum* also effectively promoted lactic acid fermentation and thus increased the titratable acidity, which in turn lowered the sample pH and increased the liberation of free phenolic acids.

However, the contents of total phenolic acids (Fig. 2b) and total phenolics (Fig. 2c) decreased in all treatments during the pickling processes, indicating the tendency of phenolics degradation, which was in accordance with the report of Wang and Zhu (2006b). Generally, the order of the decrease rates for the three treatments was 5 g/100 g NaCl > 5 g/100 g NaCl + starter culture > 10 g/100 g NaCl, which implied that the higher salt content in the sample was a benefit to the phenolic stability, and higher acidity might be another positive factor. The knowledge that phenolic degradation is mainly caused by polyphenol oxidase is well known. The results indicated that the higher salt content and acidity were obviously inhibitors of the enzyme in the present experiment. It was noteworthy that, though the contents of total phenolic acids and total phenolics decreased, the rates were relatively limited. For example, after 5 weeks of pickling, the content of total phenolics for the trial of 10 g/100 g of NaCl decreased from 7.95 to 6.69 mg/g DW, a loss of about 15.8% of phenolics. For the most severely degraded sample, the trial of 5 g/100 g of NaCl, 30.3% of phenolics were lost. Wang and Zhu (2006b) observed 10.3–34.7% of total phenolic losses during potherb mustard pickling, the variation depending on the vegetable variety (Wang & Zhu, 2006b). Compared to other processing methods, which lose the majority of the phenolics (Amakura, Umino, & Tonogai, 2000; Fang, Zhang, Sun, & Sun, 2006; Rossi et al., 2003), pickling was a relatively good method for phenolics preservation for potherb mustard. The preservation factors might be the high-salt content, high acidity and almost anaerobic conditions in the sealed pots for fermentation.

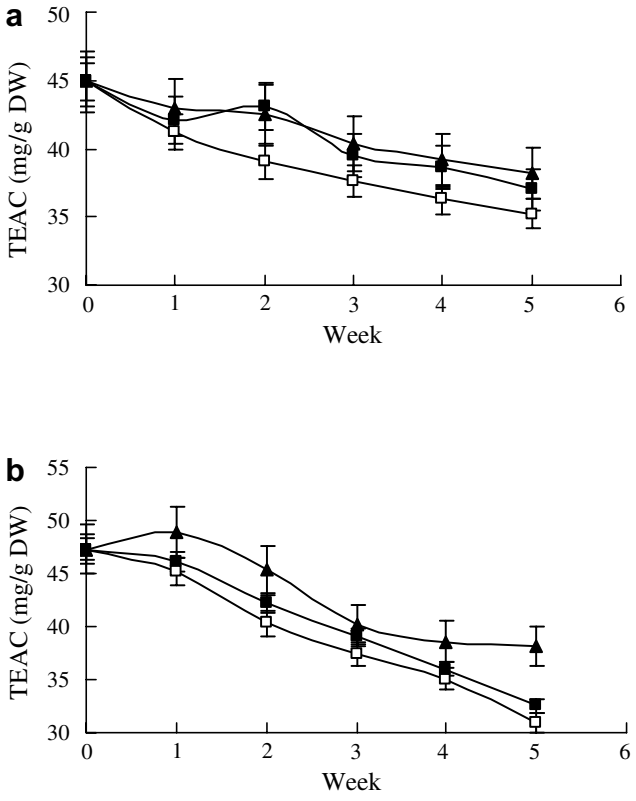

Fig. 3. Changes of ABTS (a) and FRAP (b) values during potherb mustard pickling. (□) 5 g/100 g of NaCl; (■) 5 g/100 g of NaCl + starter culture; and (▲) 10 g/100 g of NaCl.

Table 2
Correlation coefficients (r^2 values) of total phenolic acids (TPA) and total phenolic content (TPC) to ABTS assay and FRAP assay

Sample	5 g/100 g NaCl		5 g/100 g NaCl + starter culture		10 g/100 g NaCl	
	TPA	TPC	TPA	TPC	TPA	TPC
ABTS	0.989	0.924	0.908	0.949	0.988	0.743
FRAP	0.890	0.834	0.922	0.849	0.863	0.796

3.3. Changes of antioxidant activities during potherb mustard pickling

The antioxidant capacities of potherb mustard extracts were evaluated according to the ABTS decoloration method and the FRAP assay. Fig. 3 shows that the treatment with 10 g/100 g NaCl had the highest antioxidant capacities and the treatment with 5 g/100 g NaCl had the lowest. The results also showed that the antioxidant capacity of all the sample extracts decreased during potherb mustard pickling. For example, after 5-week fermentation, the antioxidant capacity of the trial of 10 g/100 g NaCl decreased from 44.9 to 38.2 TEAC mg/g DW by the ABTS method, and from 47.3 to 38.1 TEAC mg/g DW by the FRAP assay, i.e. 15.0% and 19.4% of antioxidant capacity loss for the ABTS and FRAP methods, respectively. Wang and Zhu (2006b) also observed a decrease (8.49–19.1%) of antioxidant capacity during potherb mustard pickling.

The decrease of antioxidant capacities might be caused by the decrease of phenolics. The correlation coefficients among total phenolic content (TPC), total phenolic acid, ABTS assay and FRAP assay are shown in Table 2. It can be seen that the lowest correlation coefficient (r^2 value) is 0.744 (correlation coefficient between TPC and ABTS assay for the treatment of 10 g/100 g NaCl) and the highest is 0.989 (correlation coefficient between TPC and ABTS assay for the treatment of 5 g/100 g NaCl). The correlation coefficients in each case are significant ($p < 0.05$), which means that the antioxidant capacities of the potherb mustard extracts are mainly due to the total phenolic content and total phenolic acids, both in the fresh or pickled samples. We did not determine the flavonols in the present study, but there was also a positive correlation between the antioxidant activities and flavonols in this kind of vegetable (Wang & Zhu, 2006a).

In the study of low-salt potherb mustard pickle fermentation, the addition of a starter culture of *B. coagulans* B179 to 5 g/100 g salinity fermentation can effectively improve the desired native lactic acid bacteria growth and inhibit the growth of undesirable fungi, resulting in a shortened fermentation period and enhanced pickle quality (Zhao & Ding, in press). From the viewpoint of total phenolic acid and antioxidant activity, the addition of a starter culture of *L. plantarum* had no significant influence on the pickled potherb mustard. The three pickling treatments in the present investigation all retained acceptable levels of phenolics (70–84% of TPC), and antioxidant capacities (78–85% for ABTS and 65–80% for FRAP assay). The treatment of higher salt concentration (10% NaCl) retained the highest content of phenolics and antioxidant capacities. However, consumers nowadays prefer to lower their sodium intake, and more lactic acid bacteria in the inoculated sample were another healthy factor attractive to consumers. Based on these standpoints, the low-salt (5% NaCl) and starter culture (*L. plantarum*) inoculated treatment seemed a better method for potherb mustard fermentation.

4. Conclusion

Phenolic acids have received considerable attention as potentially protective factors against cancer and heart diseases, in part because of their potent antioxidative properties and their ubiquity in a wide range of commonly consumed foods of plant origin (Breinholt, 1999; Shahidi & Naczk, 1995). The results of the present study showed that pickling processes were relatively good methods for the preservation of phenolic acids in potherb mustard, and most of the antioxidant capacities remained after 5 weeks of fermentation, which suggested that pickled potherb mustard was not only a delicious vegetable product, but also a good source of antioxidants.

Acknowledgements

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