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Food Chemistry 98 (2006) 502–507
Food Chemistry
www.elsevier.com/locate/foodchem

Effects of origins and fermentation time on the antioxidant activities of kombucha

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Received 14 February 2005; received in revised form 17 May 2005; accepted 17 May 2005

Abstract

Effects of kombucha origins and fermentation time on their antioxidant properties were investigated using in vitro free radical scavenging assays. Kombucha from various sources demonstrated different antioxidant activities, and most showed the time-dependent characteristics. The average antioxidant potentials of kombucha after fermenting for 15 days were raised to about 70%, 40%, 49% determined, respectively, by the assays of DPPH, ABTS radical scavenging, and inhibition of linoleic acid peroxidation, while the ferrous ion binding ability was inversely diminished by 81%. The total phenol content increased up to 98% which implied that thearubigin might be subjected to biodegradation during fermentation, resulting in the release of smaller molecules with higher antioxidant activities.

Keywords: Kombucha; Antioxidant activity; DPPH; ABTS; Total phenol content

1. Introduction

Kombucha, also named as tea fungus, has been consumed worldwide as a healthy drink for a very long time especially in China, Russia and Germany (Dipti et al., 2003). Kombucha is a symbiosis of *Acetobacter*, including *Acetobacter xylinum* as a characteristic spieces, and various yeasts, such as the genera of *Brettanomyces*, *Zygosaccharomyces*, *Saccharomyces*, and *Pichia* depending on the source (Mayser, Stephanie Fromme, Leitzmann, & Grunder, 1995). The tea fungus broth is composed of two portions: a floating cellulosic pellicle layer and the sour liquid broth. Acetic acid, ethanol, and gluconic acid are the major components of the liquid broth (Roussin, 1996), other minor constituents such as lactic acid, glucuronic acid, phenolic acid, groups of vitamin B and enzymes are also present (Blanc, 1996; Florensco, 1931). Although there are numerous claims that drinking kombucha is beneficial to health, these individual testimonies are not validated scientifically (Allen, 1998). More rigorous studies on kombucha were not preceded until recent years. Hartmann, Burleson, Holmes, and Geist (2000) reported that in their 3-year longitudinal study, mice fed with kombucha showed significantly increased longevity and behaviours indicative of environmental awareness and responsiveness (Hartmann et al., 2000). Oral administration of kombucha to rats exposed to pro-oxidation species also indicated the potent antioxidant properties of the fermented drink such as decrease of the degree of lipid oxidation and DNA fragmentation (Dipti et al., 2003; Ram et al., 2000). Unbalanced oxidative stress had been known as an inducer of various diseases (Halliwell & Gutteridge, 2001). Many claimed beneficial effects of kombucha such as alleviation of inflammation and arthritis, cancer prevention and immunity enhancement may be associated to its antioxidant activities (Allen, 1998). Dufresne and Farnworth (2000) proposed that some curative

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0308-8146/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2005.05.080

effects of tea fungus present in tea decoction might come from fermentation process but the mechanism remained unclear. It was therefore necessary to elucidate the relationship between fermentation process and antioxidant activities of kombucha. Kombucha was usually prepared statically at ambient temperature for up to 7–10 days but the roles of fermentation time was not seriously considered (Greenwalt, Steinkraus, & Ledford, 2000). Moreover, origins of many home-made kombucha were obtained free from household cultivators in Taiwan, therefore divergence in microbial composition might also affect the antioxidant activity (Chen & Liu, 2000; Liu, Hsu, Lee, & Liao, 1996; Martin, Cristina, Adrien, Ursula, & Michael, 1995; Mayser et al., 1995). Changes of antioxidant ability of kombucha collected from households throughout Taiwan during fermentation were determined in this study, effects of origins of kombucha samples were also investigated.

2. Materials and methods

2.1. Chemicals

α,α-Diphenyl-β-picrylhydrazyl (DPPH), 2,2′-azino-bis-(3-ethylbenzothiazoline- 6-sulfonic acid) diammonium salt (ABTS), ammonium thiocyanate, ferrous chloride, peroxidase, Tween 20 were purchased from the Sigma Chemical Co. (St. Louis, MO). Sodium phosphate monobasic and sodium phosphate dibasic were from Hayashi Pure Chemical Industries Ltd. (Osaka, Japan). Hydrogen peroxide was obtained from the Wako Pure Chemical Co. (Osaka, Japan). Folin–Ciocalteu reagent was from Merck Chemical Co. (Darmstadt, Germany).

2.2. Preparation of the medium

Four grams of black tea leaf (Ten-Ren, Taiwan) were added to 1 l boiling water and kept boiling for 5 min. Sucrose (100 g) was added, the tea was then filtered through a stainless sieve and the decoction was boiled for another 5 min. The sugared tea was immediately distributed into several sterilized 250 ml-jars (each contained 150 ml) under aseptic conditions, and allowed to cool the tea to room temperature.

2.3. Kombucha samples and fermentation

Eight kombucha samples were collected from households located throughout Taiwan, and were alphabetically named from A to H randomly. These cultures were maintained with glycerol (20%, w/w) at −20 °C. After twice activation of the stocked cultures with the medium described above at 30 °C for 5 d, the cultures were used as starter. The fermentation was initiated by adding 10% (v/v) starter to fresh medium and maintained at 30 °C for 15 d. Sampling was performed periodically and the broth was centrifuged at 15,400*g* for 20 min for further analyses.

2.4. Inhibition of linoleic acid peroxidation assay

The inhibition of linoleic acid peroxidation was measured by the method of Mitsuda, Yasumoto, and Iwami (1996). Linoleic acid emulsion was prepared by adding equal amount of linoleic acid and Tween 20, followed by serially dilution to a final concentration of 0.02 M with sodium phosphate buffer (0.2 M, pH 7.0) and homogenization. A 0.02 ml aliquot of each kombucha sample was mixed with 2.5 ml linoleic acid emulsion and 2 ml sodium phosphate buffer and the mixture was incubated at 37 °C for 24 h. Finally, 4 ml ethanol solution (75%), 0.1 ml ammonium thiocyanate and 0.08 ml ferrous chloride (0.02 M in 5.0% HCl) were added to 0.08 ml reaction mixture. After 3 min, the absorbance of the mixture at 500 nm was measured by a spectrophotometer (Hitachi U-2000). The inhibition ratio was calculated as follows: Inhibition ratio (%) = $[1-(A_s-A_b)/(A_c-A_b)]\times 100$, where A_s, A_b and A_c represented absorbances measured for sample, blank and control, respectively.

2.5. DPPH radical scavenging assay

The method used was according to Yen and Chen (1995). 0.025 ml of kombucha was diluted to 4 ml with methanol and then 0.6 ml of 1 mM α,α-diphenyl-β-picrylhydrazyl (DPPH) solution prepared with methanol, was added. The mixture was then incubated at room temperature for 30 min, the absorbance at 517 nm was determined. The scavenging capacity of kombucha was calculated by the following equation: Scavenging effect (%) = $[1-(A_s-A_b)/(A_c-A_b)]\times 100$.

2.6. ABTS radical scavenging assay

The method used was according to Miller, Rice-Evans, Davies, Gopinathan, and Milner (1993). Working solutions of peroxidase, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), and hydrogen peroxide were all prepared with sodium phosphate buffer (5 mM, pH 7.6). ABTS radical was generated by mixing 0.3 ml of peroxidase (4.4 U/ml) and 0.3 ml ABTS (0.1 mM) with 0.4 ml H_2O_2 (50 mM), and were placed in dark for 30 min. A 0.01 ml aliquot of properly diluted kombucha was added to the above solution, and then incubated for 10 min. The absorbance of mixture was detected at 734 nm. The equation used for calculating scavenging capacity of kombucha was the same as that for DPPH radical scavenging assay.

2.7. Ferrous iron chelating assay

Determination of ferrous iron chelation was referred to Dinis, Madeira, and Almeida (1994). Mixture of 0.8 ml kombucha and 0.4 ml ferrous chloride (0.182 mM) was placed in a small tube and reacted for 30 s. Aliquot of 0.8 ml ferrozine (5 mM) was added to the above mixture and the solution was incubated for 10 min. The absorbance at 562 nm was determined. The chelating effect of kombucha was calculated as follows: Chelating efficiency $\% = [1 - (A_s - A_b)/(A_c - A_b)] \times 100$.

2.8. Determination of total phenol content

The polyphenol content of kombucha was measured according to method of Folin and Denis (1915). Kombucha of 0.05 ml was added to 2 ml of 2% sodium carbonate. After 2 min, 0.1 ml Folin–Ciocalteu reagent was mixed with the above solution, the absorbance at 750 nm was then measured after 30 min. The total phenol content was expressed as gallic acid equivalents (GAE, mM) from the calibration curve.

2.9. Statistical analysis

Each experiment was performed in triplicate and repeated twice. The results were expressed as means ± SD using Microsoft Excel software. Statistical comparisons were made by ANOVA procedure followed by a Duncan's multiple range tests, $p < 0.05$ were considered significantly different.

3. Results and discussion

3.1. DPPH radical scavenging assay

Tea decoction, either prepared with black tea or green tea leaf exhibited good antioxidant activity. However, kombucha possessed, although not validated scientifically, some newborn curative effects such as reduction of atherosclerosis, arthritis and inflammation which might be related to its antioxidative activity compared to black tea broth (Allen, 1998). Recent studies demonstrated that kombucha had in vivo antioxidant activities, but the cause of this remained unclear. Therefore, various free radical-generating systems were used to evaluate changes of antioxidative activities of kombucha during fermentation.

The DPPH scavenging abilities of tea decoction from samples A, B, C, and D increased with the fermentation time, and reached values which were about 1.7 times black tea (control) at 15 d. However, for other kombucha samples a slow increase in scavenging ratio with a temporal reduction during the early period of fermentation was observed (Table 1). Different kombucha samples demonstrated various radical scavenging abilities which were probably associated with their normal microbiota. They showed divergences relating to the sources and affected the metabolic fate of culture broth (Liu et al., 1996; Martin et al., 1995; Mayser et al., 1995). The increased potential against DPPH radical might explain the phenomena that kombucha feeding significantly reversed the chromate (VI) or lead-induced oxidative injury in rats (Dipti et al., 2003; Ram et al., 2000).

3.2. ABTS radical scavenging assay

The ABTS radical scavenging capacity of various kombucha samples during fermentation is shown in Fig. 1. All revealed the positive correlation with increased culture time, and reached 1.4 times that of black tea after 15 days, whereas sample H had higher radical scavenging potentials compared to the hierarchy in DPPH scavenging ability. The scavenging effects of tea catechins were generally variable according to radical species. Moreover, properties of antioxidants such as the polarity, ionization state, and steric hindrance also seemed to contribute to their antioxidant abilities (Rice-Evans, Miller, & Paganga, 1996). Since the colour intensity of kombucha decreased gradually during kombucha fermentation (data not shown), this implied that the major brown

Table 1
Effects of kombucha origins on DPPH radical scavenging activities during fermentation

Sample	DPPH radical scavenging activity					
	0[a]	3	6	9	12	15
A	34.3 ± 2.9d	36.8 ± 5.8d	43.4 ± 2.7c	50.5 ± 2.9b	52.6 ± 2.6b	61.2 ± 4.0a
B	34.3 ± 2.9e	43.5 ± 11.3d	50.0 ± 6.3cd	54.3 ± 4.6bc	60.3 ± 5.5ab	64.3 ± 10.1a
C	34.3 ± 2.9f	42.4 ± 3.1e	50.6 ± 4.1d	55.7 ± 3.2c	60.0 ± 4.6b	69.2 ± 3.6a
D	34.3 ± 2.9f	43.4 ± 2.8e	46.7 ± 2.4d	53.3 ± 1.9c	55.9 ± 4.0b	62.2 ± 1.7a
E	36.4 ± 0.8b	30.6 ± 6.1c	26.4 ± 7.8c	41.9 ± 2.9ab	42.6 ± 4.4a	47.8 ± 1.9a
F	36.4 ± 0.8a	31.5 ± 3.7a	29.6 ± 7.2a	33.3 ± 9.2a	35.4 ± 9.2a	39.0 ± 9.1a
G	36.4 ± 0.8ab	33.0 ± 3.1b	32.6 ± 4.5b	35.7 ± 5.1ab	40.0 ± 6.4ab	43.0 ± 9.3a
H	36.4 ± 0.8bc	29.8 ± 6.3c	37.6 ± 4.6bc	43.5 ± 9.4ab	43.9 ± 7.9ab	49.0 ± 3.2a

[a] Number indicates the day when sampling was performed.
[b] Values are means ± SD of triplicate. Lowercase letters in a row represent significant difference ($p < 0.05$).

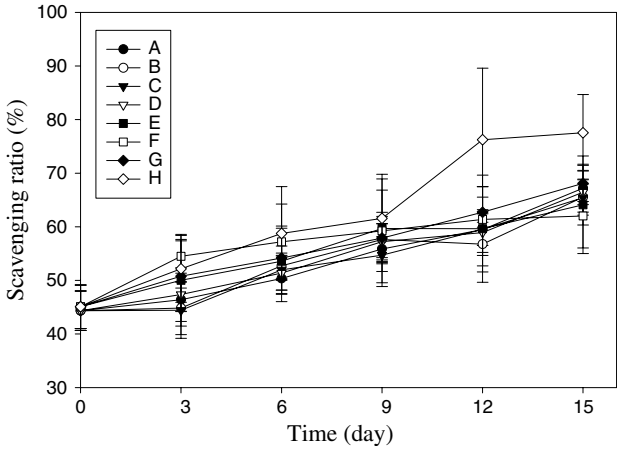

Fig. 1. Effects of kombucha origins on ABTS radical scavenging capacities during fermentation. Data presented were the average of three samples, and error bars represented the standard deviations. Letters A–H represented kombucha origins.

components, mainly thearubigin and less theaflavins, suffered microbial biotransformation or degradation to smaller molecules, and types of metabolites produced might determine their antioxidant ability.

3.3. Inhibition of linoleic acid peroxidation assay

Black tea showed the inhibitory ratio against linoleic acid peroxidation by 65%. Kombucha fermented after three days exhibited a strengthened potential averagely up to 90%, followed by a slow increase throughout the fermentation (Fig. 2). Among them, sample A, B, and C performed better ability, which were similar to those observed in DPPH scavenging (Table 1). The results also corresponded to the studies that oral administration of kombucha to rats challenged with pro-oxidation species such as chromate (VI) and lead, or hepato-toxic drug, paracetamol significantly decreased the malondialdehyde content implicated in lipid peroxidation (Dipti et al., 2003; Pauline et al., 2001; Ram et al., 2000). Kombuchas of sample A, B, and C established better antioxidant properties in inhibition of linoleic acid peroxidation and scavenging of DPPH radical than in elimination of ABTS radical.

3.4. Ferrous iron chelating assay

The chelating efficiency of black tea was about 16%, but it decreased rapidly to about 3% after three days and maintained the level for all samples while fermentation proceeded (Fig. 3). Transition metals such as iron and copper are able to participate in the generation of free radicals. Ferrous iron in the presence of hydrogen peroxide could lead to the formation of highly reactive species, whereas ferric iron together with a reducing compound, such as ascorbic acid, would be reduced and the above reaction could then also occur (Halliwell & Gutteridge, 2001). Tea polyphenols have been recognized as excellent antioxidants mainly due to presence of the 3′–4′ dihydroxy group in the B ring, and galloyl ester in C ring of flavonols which were also important structures in metal ion chelation (Guo, Zhao, Li, Shen, & Xin, 1996; Khokhar & Owusu Apenten, 2003). The above results indicated that kombucha exhibited enhanced antioxidant capacity as compared to black tea, but they were not associated with ferrous iron binding. Moreover, the resonance structures like 4-keto group and 2–3 double bond or modifications such as methylation of 3-OH and 5-OH group in quercetin also determined its iron chelation efficiency (Khokhar & Owusu Apenten, 2003; Guo et al., 1996). Thus, the decreased

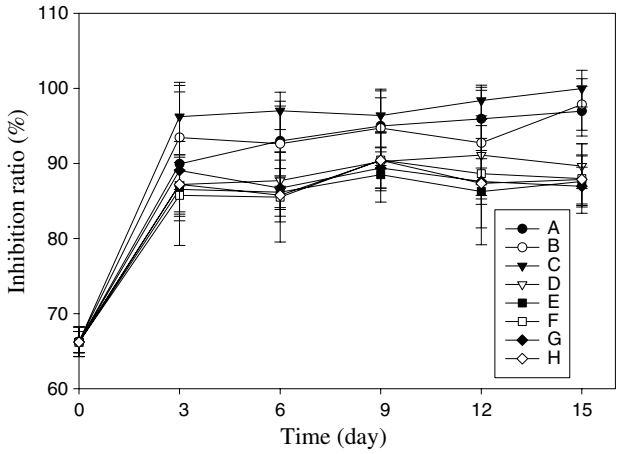

Fig. 2. Effects of kombucha origins on antioxidant activities measured by the thiocyanate method during fermentation. Data presented were the average of three samples, and error bars represented the standard deviations. Letters A–H represented kombucha origins.

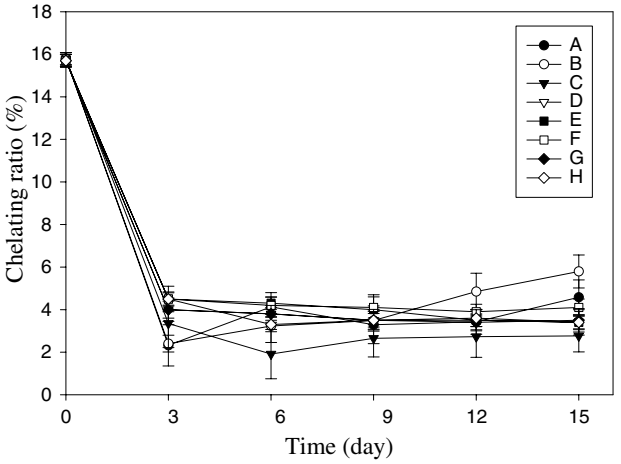

Fig. 3. Effects of kombucha origins on ferrous ion chelating capacities during fermentation. Data presented were the average of three samples, and error bars represented the standard deviations. Letters A–H represented kombucha origins.

ferrous ion chelation power by kombucha might come from the microbial modification of tea polyphenols during the fermentation.

3.5. Determination of total phenol content

As shown in Fig. 4, the total phenol content of kombucha among all samples increased linearly with the fermentation time, and sample C, G, and H had higher content at day 15. The amount was accumulated up to 7.8 mM gallic acid equivalent (GAE) at day 15 of fermentation while it was only about 4 mM GAE for black tea. The brown colour of black tea was mainly from the chromophoric group of thearubigins, consisted of a planar structure formed by oxidative oligomerization of the flavonol precursors as derivatives of catechins. However, the colour became colourless because of the suppression of ionization or destruction of structures (Haslam, 2003). Since the colour of kombucha broth continued to get lighter, this suggested that polyphenols did undergo microbial transformation. The stability of catechins from green tea is pH-dependent; they are very unstable in alkaline solution but stable in acidic solution (Zhu, Zhang, Tsang, Huang, & Chen, 1997). For example, contents of catechin derivatives in green tea or black tea extracts diminished rapidly by 70–80% of the original under pH 7.4 for 60 min, but the antioxidant activities and concentration of polyphenols were only decreased by about 25% (Record & Lane, 2001). These results demonstrated that the disappeared catechins might be polymerized further to molecules with higher molecular weights leading to detection of lower polyphenol contents. During oxidative polyperization of catechin derivatives, the hydroxyl groups on the aromatic ring were responsible for formation of benztropolone or theasinensin, precursors of thearubigins (Haslam, 2003), and were also associated with measurement of polyphenol content. Thus, depolymerization of thearubigins might happen and this explained the phenomena of increased total phenol content that occurred during kombucha fermentation.

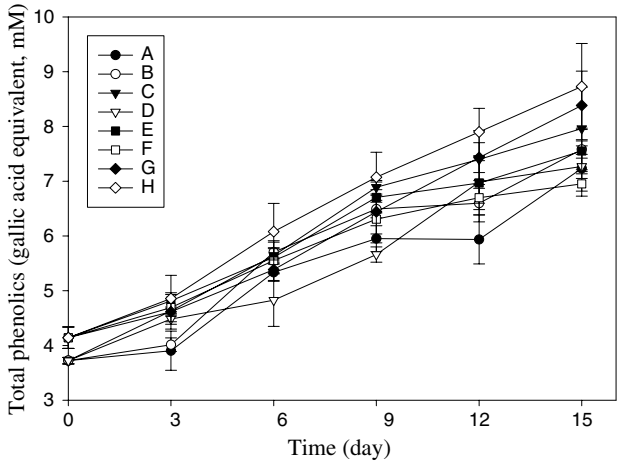

Fig. 4. Effects of kombucha origins on total phenol content during fermentation. Data presented were the average of three samples, and error bars represented the standard deviations. Letters A–H represented kombucha origins.

The ligninolytic enzymes such as lignin peroxidase, manganese peroxidase and laccase produced mainly by white rot fungi, degraded lignin or polycyclic aromatic hydrocarbons in a non-specific, radical based oxidation. The formed cation radical from substrates might undergo spontaneous chemical reactions such as C–C cleavage or hydroxylation resulting in production of more hydrophilic products (Mester & Tien, 2000). The yeast *Candida tropicalis* was employed as a polyphenol degrader in which the induced expression of peroxisomal enzyme such as catalase was the predominant factor (Ettayebi et al., 2003). Moreover, degradation of dietary polyphenol in the colon was regarded as the major pathway of metabolism with gut *Clostridium*, *Bacteroides*, and *Eubacteria*. These gut bacteria were capable of cleaving the C ring of flavonoids, and released phenolic acid as 3-(4-hydroxyphenyl)-propionic acid and 3-hydroxyphenylacetic acid, etc. (Rechner et al., 2004). It was therefore possible that kombucha starters secreted some unknown enzymes that were capable of catalyzing the biodegradation of theaflavins and the hydrolysates were potent antioxidant molecules. However, quantity of total phenol content did not always determine the antioxdative activities of kombucha whereas the types of metabolites produced might have the key effect.

4. Conclusion

Kombucha exhibited increased antioxidant activities during fermentation. The extent of activity depended upon culture period and starter origins, which in turn determined the forms of their metabolites. Although antioxidant properties of most kombucha showed the time-dependent profiles, prolonged fermentation was not recommended because of accumulation of organic acids, which might reach harmful levels for direct consumption (Greenwalt et al., 2000). The identification of extracellular key enzymes and potent metabolites are necessary to elucidate metabolic pathway during kombucha fermentation. Metabolic manipulations may be one of the effective methods to elevate the antioxidant activities and fermentation efficiency of kombucha.

Acknowledgements

The authors are grateful to the National Sceince Council, R.O.C., for support of this work (Project No. NSC 91-2214-E005-008).

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