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Characterization of Vasculoprotective Bioactive Compounds in Zhejiang Rosy Rice Vinegar, A Traditional Chinese Fermented Vinegar

Zhu L[1*], Zhou YQ[1], Liu Y[2], Feng W[2], Matsumoto N[3], Suzuki E[3], Wang X[1] and Hasumi K[3*]

[1]State Microbial Technology of Zhejiang Province, Institute of Plant Protection and Microbiology, P. R. China

[2]Zhejiang Wuweihe Food Co., Ltd., Huzhou, P. R.China

[3]Department of Applied Biological Science, Tokyo NoKo University, Tokyo, 183-8509, Japan

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Corresponding author:
Liying Zhu and Keiji Hasumi,
State Microbial Technology of Zhejiang Province, Institute of Plant Protection and Microbiology, Zhejiang Academy of Agricultural Sciences, P. R. China and Department of Applied Biological Science, Tokyo NoKo University, Tokyo, 183-8509, Japan,
Email: zhuliying@hotmail.co.jp; hasumi@cc.tuat.ac.jp

ABSTRACT

Zhejiang rosy rice vinegar (ZRRV), traditional fermented vinegar manufactured from rice as a sole raw material, is believed to have vasculoprotective effects. To confirm its folklore health benefits, we investigated the presence of inhibitors of soluble epoxide hydrolase (sEH) and 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) in ZRRV. Among eighteen commercially available brands of Chinese traditional fermented vinegar including 4 ZRRV brands, one brand of ZRRV showed a significant inhibitory activitytoward N-terminal phosphatase (Nterm-phos) of sEH. We isolated 48 strains of filamentous fungi from the mash of ZRRV and wereassayed for their ability to produce inhibitors of sEH Nterm-phos and HMG-CoA reductase. Two isolates, *Penicillium citrinum* and *Talaromycesspectabilis*, manifested significant inhibitory activity toward sEH Nterm-phos. Another strain, *Aspergillus terreus*, was a producer ofmonacolin K (lovastatin), a potent inhibitor of HMG-CoA reductase. Our results provide a biochemical basis for the folklore health benefits of ZRRV and suggest that traditionally fermented foods are a treasure box of microbial resources of bioactive substances.

INTRODUCTION

Vinegar is a common food ingredient that has been consumed daily for a long time. Acetic acid is the ingredient to identify the product, of which the minimum of 4% is required in USA and China. Other constituents of vinegar include vitamins, mineral salts, amino acids, polyphenolic compounds and nonvolatile organic acids, which are depended on the starting materials and the fermentation methods. It has also been recognized fortheir health-promoting and disease-preventing properties such as anti-infective, antioxidant, vasculoprotective, antitumor and blood glucose controlling activities [1].

The factors in vinegar responsible for the beneficial impacts have been widely studied. Acetic acid is one of the factors to account for the anti-infective and anti-hyperglycemic effects. Human intervention trails have indicated that the white vinegar consumptions, excluding the effect of other ingredients such as polyphenol, are effective in glycemic control by increasing glucagon like peptide 1 (GLP-1) secretion via free fatty acid receptor 2 (FFAR2) and activating AMP-activated protein kinase to increase fatty acid oxidation and decrease hepatic gluconeogenesis [2], of which the equivalent to acetic acid doses is between 0.5 and 2.0 g per meal. On the other

hand, Melanoidins, the final products of Mallard reaction with health benefits of both antioxidants andprebiotic effect to promote the growth of bifidobacteria, are recently detected in several traditional vinegars, including traditional balsamic vinegar [3,4], Zhenjiang aromatic vinegar [5] and Shanxi aged vinegar [6]. These findings suggest the melanoidins in these traditional vinegars contribute to their health beneficial effects despite they are structure-unknown [7] and subsequently unquantified. Moreover, ligustrazine, a bioactive alkaloidfound in Zhenjiang aromatic vinegar, shows vasculoprotective activities, of which the content in 6-year-old vinegar reached 696.63 mg/L while 34.7 mg/L in the raw vinegar [8]. For the antitumor effects, Seki et al. found that the oral administration of fermented vinegar (more than 0.5% of a chemically defined diet for 72 days), which is produced from traditional Japanese liquor post-distillation slurry, can significantly reduce colon tumor size [9]. These data suggest there are variable physiological active compounds in traditional fermented vinegars.

The history of vinegar in China has been more than 5000 years. The health effect of vinegar is recorded in the oldest medical prescription which is speculated to be written in the 4th or 3rd century BC. Moreover, rice vinegar has been clearly documented as one of the drugs in the traditional Chinese medicine books from the dynasties of Wei and Jin (3rd to 6th century) [10]. Zhejiang rosy rice vinegar (ZRRV) is traditional rice-fermented vinegar produced in Zhejiang province, located in the southeast of China, of which rice is the sole raw material. Unlike other traditional starter-aided, solid-state vinegar fermentations such as Zhenjiang aromatic vinegar and Shanxi aged vinegar, the brewage of ZRRV is a natural liquid fermentation process except for the two-week solid-state fermentation in the beginning without a starter. The fermentation process takes 5 to 8 months and completely relies on the environmental microorganisms and ambient temperature. ZRRV is believed to have many health-promoting effects, such as stimulation of bone and muscle growths as well asanti constipation, antiperspirant, antihypertensive and antihyperlipidemic effects [11]. However, the functional factors responsible for the health benefits remain unknown.

Recently, the screening methods for vasculoprotective compounds have been developed. Statins are the competitive inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase and have been used for hypercholesterolemia treatment [12]. Monacolin K (lovastatin) is a natural statin of fungal origin that is produced by several fungi such as *Aspergillus terreus* and *Monascus* spp. [13]. *Monascus*-fermented products with richer ingredients of monacolin K and other statins, for example the red yeast rice, has become a cholesterol-lowering dietary supplement sold worldwide [14]. It has been reported that a strain of *Monascus* which can secretemonacolin K was isolated from ZRRV mash [11].

Soluble epoxide hydrolase (sEH) is a homodimer enzyme with a C-terminal hydrolase (Cterm-EH) and N-terminal phosphatase (Nterm-phos) and is highly promising pharmacological target for anti-inflammation drugs [15]. The Cterm-EH hydrolase epoxides to corresponding diols with less physiological activation, whereas the Nterm-phos hydrolyzes phosphorylated lipids such as isoprenoid phosphates and lysophosphatidic acid that stimulate cell growth. Inhibition of Cterm-EH increases levels of the epoxy-fatty acids (EpFAs), the eicosanoids metabolites formed by cytochrome P450, which have anti-inflammatory effects and can prevent the development of cardiovascular diseases, neuroinflammatory diseases [16], fatty liver, and diabetes [17]. On the other hand, Nterm-phos is a potential therapeutic target in hypercholesterolemia-related disorders [18] and plays a significant role in cellular lysophosphatidic acid hydrolysis [19]. Since it has the potential therapeutic applications, high-throughput screening methods of sEH inhibitors are established including the assay with fluorescent substrates, 3-phenyl-cyano（6-methoxy-2-naphthalenyl）methyl ester-2-oxiraneacetic acid (PHOME) for Cterm-EH activity [20] and Attophos for Nterm-phos activity [18]. In fact, successful application of PHOME for Cterm-EH measurement led to the discovery of novel compounds including non-urea compounds [21] and fulvestrant [22], which are different to the well-known sEH inhibitors, such as AUDA which contains urea as the primary pharmacophore.

In this study, we investigated bioactive compounds in ZRRV as well as other traditional fermented vinegars available in the market. Among 18 traditional vinegars, one brand of ZRRV showed a significantly potent ability to inhibit Nterm-phos activity of sEH. Moreover, 48 filamentous fungi were isolated from ZRRV mash and subjected to the screening assays.

Characterization of Vasculoprotective Bioactive Compounds in Zhejiang Rosy Rice Vinegar, A Traditional Chinese Fermented Vinegar. Nutrition and Food Science Journal. 2019; 2(1):118.

MATERIALS AND METHODS

Vinegar collection and isolation of fungi from the mash of ZRRV fermentation

Eighteen brands of commercially available vinegar were purchased from local supermarkets around Hangzhou for screening for sEH inhibition. The brands, manufactures and their characteristics of fermentation are shown in Table 1.

The batches of fresh ZRRV mash were collected from a local ZRRV workshop every day on the stage of solid fermentation. Samples were immediately kept on ice and transferred to the laboratory for isolation of microbial strains. Filamentous fungi were isolated by preparing serial dilutions in 0.85% (w/v) sterile physiological saline and plated on Potato-dextrose-agar (PDA) (Hangzhou Microbial Reagent Co., Ltd) with 50 mg/L rose bengal. After 5 days incubation at 25°C, colonies were isolated randomly based on the morphological differences, streaked on PDA plates and incubated at 25°C for 3-5 days. Single colonies on the streaking plates were subsequently sub-cultured in a new PDA plate. The sub-cultures were repeated until a pure culture was obtained for strain storage.

The isolates were inoculated in the a liquid medium containing 10 g/L glucose, 11 g/L glycerol, 50 g/L soy bean powder, 8 g/L peptone, 1 g/L $NaNO_3$, 0.5 g/l$Zn(NO_3)_2$ and 5 g/L olive oil [23]. The culture for screening sEH inhibitors was performed in 100-ml Erlenmeyer flasks with 10 ml medium on the thermostatic shaker (180 rpm, 25°C) for 11 days. Broth was obtained by centrifuging cultures at 15,000 rpm for 10 minutes at 4°C.

For fungal identification, isolates were classified by phylogenetic analysis of the ITS region of the nuclear ribosomal RNA genes. In brief, the mycelia were collected after 30 hours incubation in potato dextrose broth (Hangzhou Microbial Reagent Co., Ltd) at 28°C with shaking at 180 rpm. DNA was extracted using HP Fungal DNA Kit (Omega Bio-Tek), and the ITS region was amplified using the primer pair ITS1 (5' TCCGTAGGTGAACCTGCGG 3') and ITS4 (5' TCCTCCGCTTATTGATATGC 3'). The PCR products were sequenced by a commercial sequencing facility (Shanghai Sangni Biosciences Corporation, Shanghai), and the sequence obtained were compared with known sequences using BLAST service (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Table 1: Commercial traditional fermented vinegars used in this study [5,26].

No.	Brandname[a]	Manufacturer[a]	Fermentation			
			Type[a]	Materials[a]	Period[b]	Starter Use[b]
V1	Rosy Vinegar	Hangzhou Xihushenggu brewing food Co., Ltd.	Liquid	Rice	5-8 months	No
V2	Red rosy Zhejiang Vinegar	Ningbo Zuocanwang Seasoning Food Co., Ltd.	Liquid	Rice	5-8 months	No
V3	Jiangnan Rice Vinegar	Shaoxing Renchang Sauce workshop Co., Ltd.	Liquid	Rice	5-8 months	No
V4	Aromatic Vinegar	Foshan Haitian Seasoning Food Co., Ltd.	Solid	Glutinous rice, wheat bran	Unknown	Unknown
V5	Zhenjiang Aromatic Vinegar	Hengfeng Sauce and Vinegar Co., Ltd.	Solid	Glutinous rice & wheat bran	30 days	Yes
V6	Zhenjiang Aromatic Vinegar	Zhenjiang Hengkang Seasoning Co., Ltd.	Solid	Glutinous rice & wheat bran	30 days	Yes
V7	Royal Feast Vinegar	Hengfeng Sauce and Vinegar Co., Ltd.	Solid	Glutinous rice & wheat bran	30 days	Yes
V8	Aged aromatic vinegar	NanjinXiaoerhe Food Co., Ltd.	Solid	Glutinous rice, wheat bran , wheat & pea	Unknown	Unknown
V9	Shuita Aged Aromatic Vinegar (10-years aged)	Shanxi Shuita Aged Vinegar Co., Ltd.	Solid	Sorghum, wheat, pea & wheat bran	20 days	Yes
V10	Aromatic Vinegar	Fujian JinjiangJinguanFood Co., Ltd.	Liquid	Glutinous rice, red yeast rice & wheat bran	Unknown	Unknown
V11	Ginger and Garlic Aromatic Vinegar	Guiyang Weichunyuan Food Co., Ltd.	Solid	Wheat bran, rice, ginger juice, & garlic juice	Unknown	Unknown
V12	Rosy Zhejiang Vinegar	Hangzhou Food and Brewing Co., Ltd.	Liquid	Rice	5-8 months	No

No.	Brandname[a]	Manufacturer[a]	Fermentation			
			Type[a]	Materials[a]	Period[b]	Starter Use[b]
V13	Shanxi Aged Vinegar (3-years aged)	Shanxi Aged Vinegar Group Co., Ltd.	Solid	Sorghum, pea, wheat bran & rice hull	20 days	Yes
V14	Zhenjiang Aged Vinegar	Zhenjiang Danhe Vinegar Co., Ltd.	Solid	Wheat bran & glutinous rice	30 days	Yes
V15	Feast Aromatic Vinegar	Zhenjiang Danhe Vinegar Co., Ltd.	Solid	Glutinous rice & wheat bran	30 days	Yes
V16	Healthy Vinegar	Shanxi Aged Vinegar Group Co., Ltd.	Solid	Sorghum, pea, wheat bran, honey, red dates, peanuts, licorice & hawthorn	20 days	Yes
V17	Dumpling Vinegar	Beijing ErshangWeizikang Food Co., Ltd.	Solid	Rice, wheat bran, garlic & ginger	Unknown	Unknown
V18	Aged Vinegar	Shanxi Yishou Seasoning Food Co., Ltd.	Solid	Sorghum, wheat bran & wheat	20 days	Yes

[a] According to the label instructions; [b] According to the general process of Zhejiang rosy rice vinegarlasted for 5-8 months (described in this study), Zhenjiang aromatic Vinegar fermented for 30 days [8] and Shanxi aged vinegar [31] of which the fermentation period is 20 days.

Sample preparation for screening assay

One milliliter of vinegar or fungal culture supernatant was freeze-dried and resuspended in 1 ml of 90% methanol at 4°C overnight. The supernatant of the methanol extract was stored at 4°C for HPLC analysis. Regarding the sEH assay, supernatant of the methanol extract was evaporated *in vacuo* and resuspended in the assay buffer containing 25 mMBisTis-HCl, pH7.0, 1 mM $MgCl_2$ and 0.1 mg/ml bovine serum albumin.

Analysis for sEH activity

sEH activity was determined by calculating the initial velocity of substrates turnover based on the kinetic measurement [24]. The maximum slope of products curve indicated the corresponding sEH activity. The inhibitory rate of sample was calculated as the percentage of the difference between the activities of the control (100%) and the sample. The assay for sEH activity was described as previously [25]. Briefly, sEH solution was pre-incubated with sample solution or buffer (as control, of which activity is 100%) for 10 minutes at 30°C in a black polystyrene 96-well plate. Kinetic measurement was started just after the substrate addition. In 100 µl assay system, the final concentrations of 12.5 µM PHOME and 80 ng/ml human recombinant sEH was used for Cterm-EH assay. For Nterm-phos assay, the final reaction mixture contained 5 mMp-nitrophenyl phosphate and 300 ng/ml mouse sEH. The fluorescence in the Cterm-EH assay was measured at 330 nm (excitation) and 465 nm (emission) with 60-sec intervals for 20 minutes (1420 Multilabel Counter, Perkin Elmer, US),and the absorbance at405 nm was used for Nterm-phos assay with 60-sec intervals for 120 minutes (MTP-500, CORONA ELECTRIC, Japan).

HPLC analysis of monacolin K

The analysis of monacolin K was conducted according to the method described previously [26-29]. The standard curve of monacolin K was made with the following procedures. The hydroxyl acid form of monacolin K was prepared by 2-hour incubation with 0.2 MNaOHat 60°C.The solution was neutralized by 0.05 M HCl. The concentration of monacolin K standard solution was adjusted to 0.1 mg/ml in acetonitrile [30].

All samples were separated by reverse-phase HPLC (L-2000 series LaChrom ELITE, Hitachi) on an Inertsil PREP-ODS column (6×250 mm, GL Science, Tokyo, Japan) with a linear gradient of methanol (10 to100% in 20 minutes) in 0.1% H_3PO_4 at a flow rate of 1 ml/min. The column temperature was 30°C and the sample injection volume was 20 µl. A photodiode array detector L-2455 (Hitachi, Ibaraki, Japan) was set at 205-350 nm. The final concentration of monacolin K in the samples was calculated by the internal standards method [31].

RESULTS

ZRRV showed sEH inhibition activity

Eighteen brands of Chinese traditional fermented vinegar were collected from local supermarkets (Table 1) and subjected to the screening assays for sEH inhibition. In the first run of the screening for sEH inhibition activities, 1 µl of methanol extract

of vinegar was mixed with the reaction buffer to generate 1% of vinegar in 100 µl of a reaction system, of which buffer was used as the control. The samples that gave more than 40% inhibition for Cterm-EH and/or more than 20% for Nterm-phos were selected for further examination. As shown in Figure 1a and Supplementary Table S1, only one brand of ZRRV, V2, produced by the Ningbo Zuocanwang Seasoning Food Company, reached the selection criteria (21% inhibition of Nterm-phos), while none of any tested samples showed inhibition of Cterm-EH over 40%. In the experiment using varying concentrations of the extract (Figure 1b), Nterm-phos inhibition by V2 increased along with the increasing concentration and reached 50% when vinegar extracts was added at 4% (v/v).

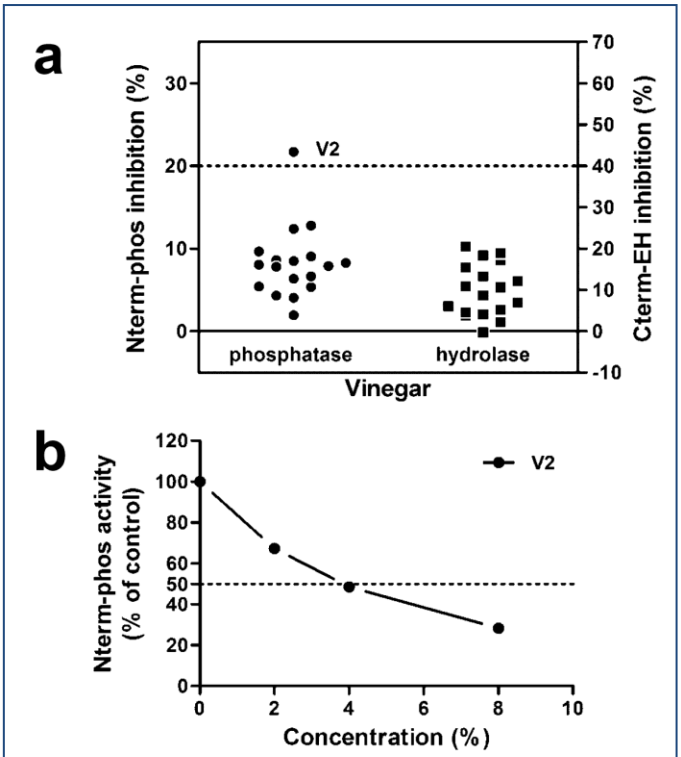

Figure 1: The screening results of the traditional fermented vinegars on sEH activities.

1% (V/V) assay samples of the 18 vinegars were added to 100 µl reaction system of sEH for the first screening (a). Samples gave more than 20% inhibition (dashed line) of Nterm-phos or 40% inhibition of Cterm-EH were selected for the next screening (b) to test whether the inhibition is concentration-dependent. The No. 2 vinegar (V2) listed in Table 1 passed the first screening and subjected to the second screening assay.

Chinese traditional fermented vinegar does not containmonacolin K

HPLC method has been developed as a reliable method to detect monacolin K in a complex mixture, such as *Monascus*-fermented products based on its characteristic tri-peaked UV absorption spectrum detected by photodiode array detector [26-28]. Here we employed three monacolin K standards, hydroxy acid, lactone and sodium salt, to determine the presence of monacolin K in 18 brands of Chinese vinegar. However, the HPLC analysis showed that none of the tested vinegar contained any forms of monacolin K (data not shown), suggesting the vasculoprotective effect of ZRRV might be different to that of the fermented red yeast rice (Hongqu) that contains monacolin K.

Isolation and identification of filamentous fungi in ZRRV mash

Since ZRRV demonstrated higher inhibitory activity toward Nterm-phos, we focused on filamentous fungi involved in ZRRV fermentation for their ability to produce natural sEH inhibitors since filamentous fungi are well-known to produce a large number of secondary metabolites. PDA plates were used to isolate filamentous fungi from ZRRV mash during the stage of solid fermentation. After purification by repeating steak cultures, forty-eight isolates were obtained and identified by the ribosomal RNA sequence analysis for theirinternal transcribed spacer (ITS) region (Table 2). *Penicillium* and *Aspergillus* were abundant species among the isolates: 19 out of 48 were classified as *Penicillium* and 18 *Aspergillus*. Apart from the two main genera, *Monascus pilosus* was also identified.

Fungal isolates produce bioactive compounds inhibiting sEH activity

Forty-eight isolates of filamentous fungi were cultured in PDA for 11 days and the culture supernatant of each isolate wascollected and assayed forinhibition of sEH. As expected, extracts from the isolates 2010F2 and 2011F2 showed concentration-dependent inhibition of Nterm-phos (Figure 2a and 2b). Based on the phylogenetic analysis of the ITS sequences, 2010F2 and 2011F2 were classified as *Penicillium citrinum* and *Talaromyces spectabilis*, respectively.

A fungal isolate can produce monacolin K

Subsequently, the capability of monacolin K production was screened using the forty-eight isolates. Comparing to the HPLC

spectra of the standardthree forms of monacolin K (Figure 3a and 3b), the broth of the isolate 2011F5 displayed the same specific UV-spectrum of monacolin K (Figure 4b) with the retention time 20.02 (Figure 4c), which was comparable to that of the hydroxy acid form of monacolin K (Figure 3a), suggesting that the isolate 2011F5 could produce monacolin K. Based on the analysis of the ITS sequence, 2011F5 was identified as *A. terreus*.

Table 2: Identification of the fungal strains isolated from ZRRV mash.

No.	Isolate	species	Identity %	Accession No.
1	2010F1	*Penicillium brevicompactum*strain S3	99	KJ145421.1
2	2010F2	*Penicillium citrinum* strain a1s2_d37	100	KC344960.1
3	2010F3	*Penicillium ochrosalmoneu*m NRRL 35499	100	NR_121328.1
4	2010F4	*Eupenicilliumochrosalmoneum* isolate NRRL 35496	99	EF626958.1
5	2010F5	Uncultured fungus clone L042885-122-065-B01	100	GQ999347.1
6	2010F6	*Penicillium brevicompactum* isolate XF44	100	KJ780804.1
7	2010F7	*Aspergillus ruber* strain SRRC 2172	100	AY373891.1
8	2010F8	*Tilletiopsis* sp. GX3-1C	100	FJ037727.1
9	2010F9	*Penicillium* sp. 9 BRO-2013	99	KF367506.1
10	2010F10	*Aspergillus tubingensis* strain USMF07	100	KF434096.1
11	2010F11	*Monascus ruber* strain JCM 22614	99	JN942658.1
12	2010F12	*Monascus albidulus*	99	DQ978994.1
13	2010F13	*Penicillium expansum* strain Pe1614M	100	KP670440.1
14	2010F14	*Penicillium* sp. 9 BRO-2013	99	KF367506.1
15	2010F15	*Penicillium commune* strain ATCC 10428	99	AY373905.1
16	2010F16	*Monascus ruber* strain JCM 22614	99	JN942658.1
17	2010F17	*Penicillium expansum* strain Pe1614M	100	KP670440.1
18	2010F18	*Penicillium expansum* strain MHTS9	100	KP204877.1
19	2010F19	*Aspergillus fumigatus* strain SGE57	99	JQ776545.1
20	2010F20	*Penicillium* sp. GA6	99	KJ415574.1
21	2010F26	*Monascus ruber* strain JCM 22614	99	JN942658.1
22	2010F27	*Penicillium* sp. F731	99	KM249071.1
23	2010F31	*Penicillium ochrosalmoneum* NRRL 35499	100	NR_121328.1
24	2010F32	*Penicillium commune* strain ATCC 10428	99	AY373905.1
25	2010F34	*Aspergillus oryzae* strain AS-A9	99	HQ285528.1
26	2011F1	*Penicillium expansum* ATCC 7861	99	NR_077154.1
27	2011F2	*Talaromycesspectabilis* strain LTBF 008 1	99	KC344960.1
28	2011F3	*Monascus ruber* strain JCM 22614	99	JN942658.1
29	2011F4	*Aspergillus fumigatus* strain PT-B5	99	HQ285569.1
30	2011F5	*Aspergillus terreus* strain AKF2	99	KJ685810.1
31	2011F6	*Aspergillus fumigatus* strain 095623	100	EU664467.1
32	2011F7	*Aspergillus oryzae* strain QRF378	99	KP278186.1
33	2011F8	*Aspergillus oryzae* strain AS-A9	99	HQ285528.1
34	2011F9	*Penicillium* sp. 9 BRO-2013	99	KF367506.1
35	2011F10	*Aspergillus* sp. DX-SEL2	99	KC871017.1
36	2011F11	*Monascus ruber* strain JCM 22614	100	JN942658.1
37	2011F12	*Penicillium expansum* strain NRRL 6069	99	DQ339562.1
38	2011F13	*Penicillium* sp. F731	99	KM249071.1
39	2011F14	*Aspergillus flavus* strain AD-B3	99	HQ285520.1
40	2011F15	*Penicillium expansum* strain NRRL 6069	99	DQ339562.1
41	2011F16	*Aspergillus fumigatus* strain Ppf10	99	EF495242.1
42	2011F17	*Aspergillus fumigatus* strain KARVS04	99	KC119200.1
43	2011F18	*Aspergillus oryzae* strain QRF378	100	KP278186.1
44	2011F19	*Aspergillus* sp. BM6	100	KJ567461.1
45	2011F20	*Aspergillus* sp. DX-SEL2	99	KC871017.1

No.	Isolate	species	Identity %	Accession No.
46	2011F21	*Penicillium* sp. 9M3	99	KF914642.1
47	2011F22	*Aspergillus fumigatus* strain PT-B5	99	HQ285569.1
48	2011F24	*Aspergillus oryzae* strain SJ-A4	99	HQ285576.1

The fungal isolates were identified by phylogenetic analysis of the ITS region of the nuclear ribosomal RNA genes. The mycelia were collected after 30 hours incubation in potato dextrose broth and followed by DNA extraction. The ITS region was amplified using the primer pair ITS1and ITS4. The PCR products were sequenced by a commercial sequencing facilityand the sequence obtained were compared with known sequences using BLAST service.

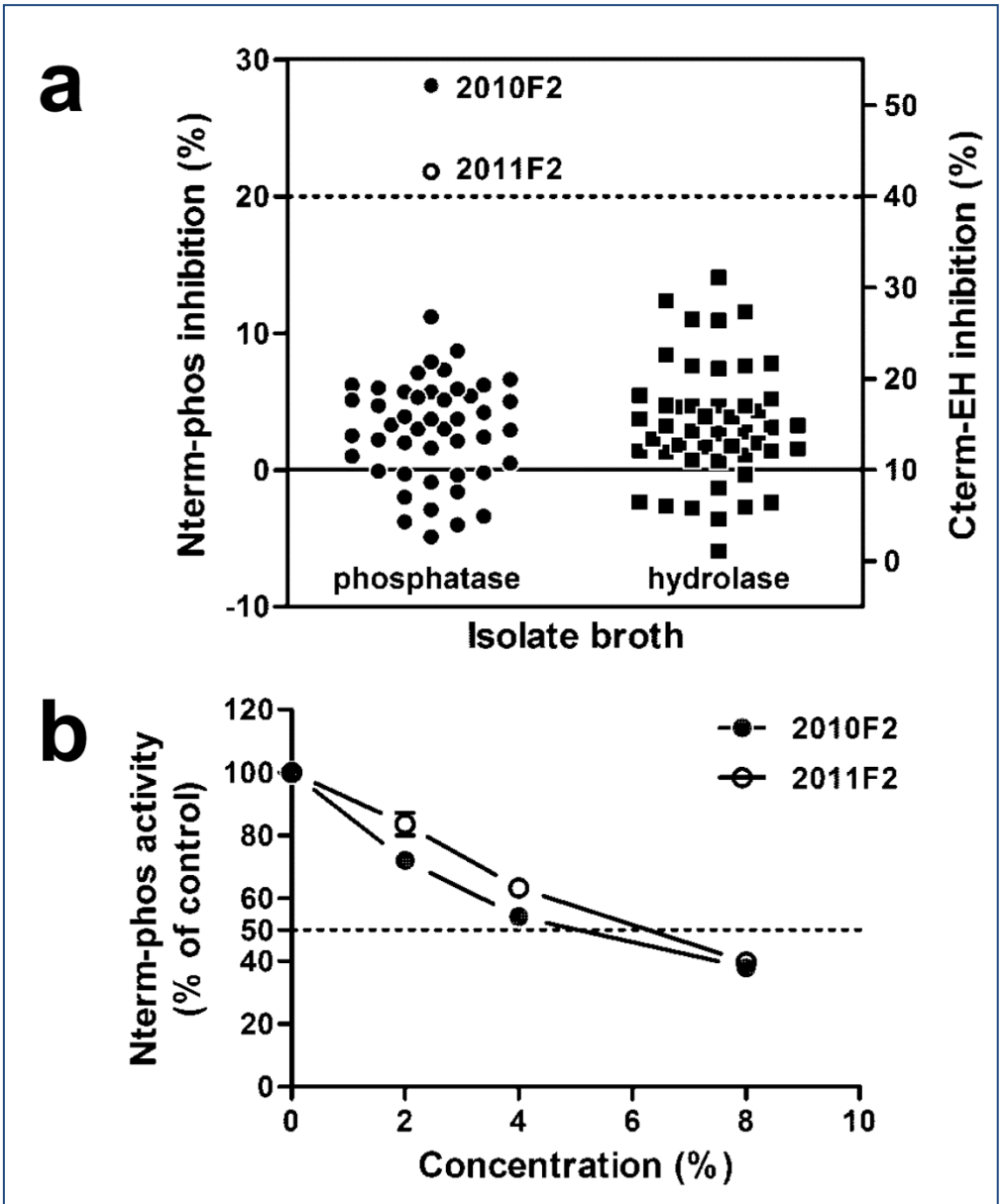

Figure 2: The screening results of the isolate broths on sEH activities.

1% (V/V) assay samples of the 48 fungal broths were added to 100 µl reaction system of sEH for the first screening (a). Samples gave more than 20% inhibition (dashed line) of Nterm-phos or 40% inhibition of Cterm-EH were selected for the next screening (b), which displayed that the inhibition of the selected sample was concentration-dependent.

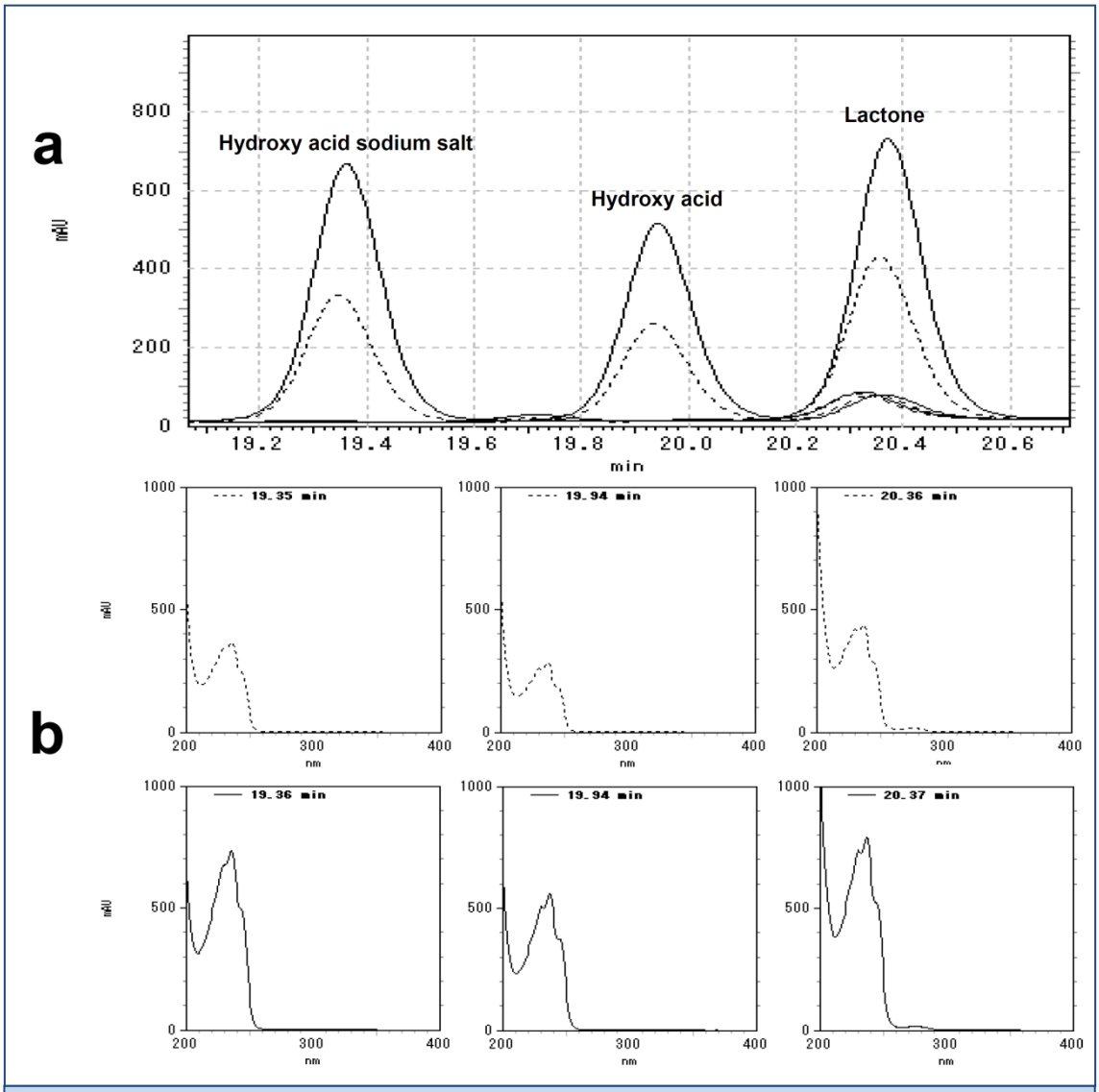

Figure 3: HPLC chromatograms of the standards of monacolin K used in this study.

a: Retention times of 3 monacolin K forms; b: UV-spectra of 3 monacolin K forms at 236 nm of DAD analysis (the operation parameter of 236nm is not displayed in the monitor). Methanol and 0.1% H_3PO_4 were used as the mobile phase with a linear gradient of Methanol from 10 to 100% in 20 minutes. 0.1 mg/ml of each standard solution in acetonitrile was injected in 10 µl (dashed line) and 20 µl (solid line) for DAD analysis at 236 nm.

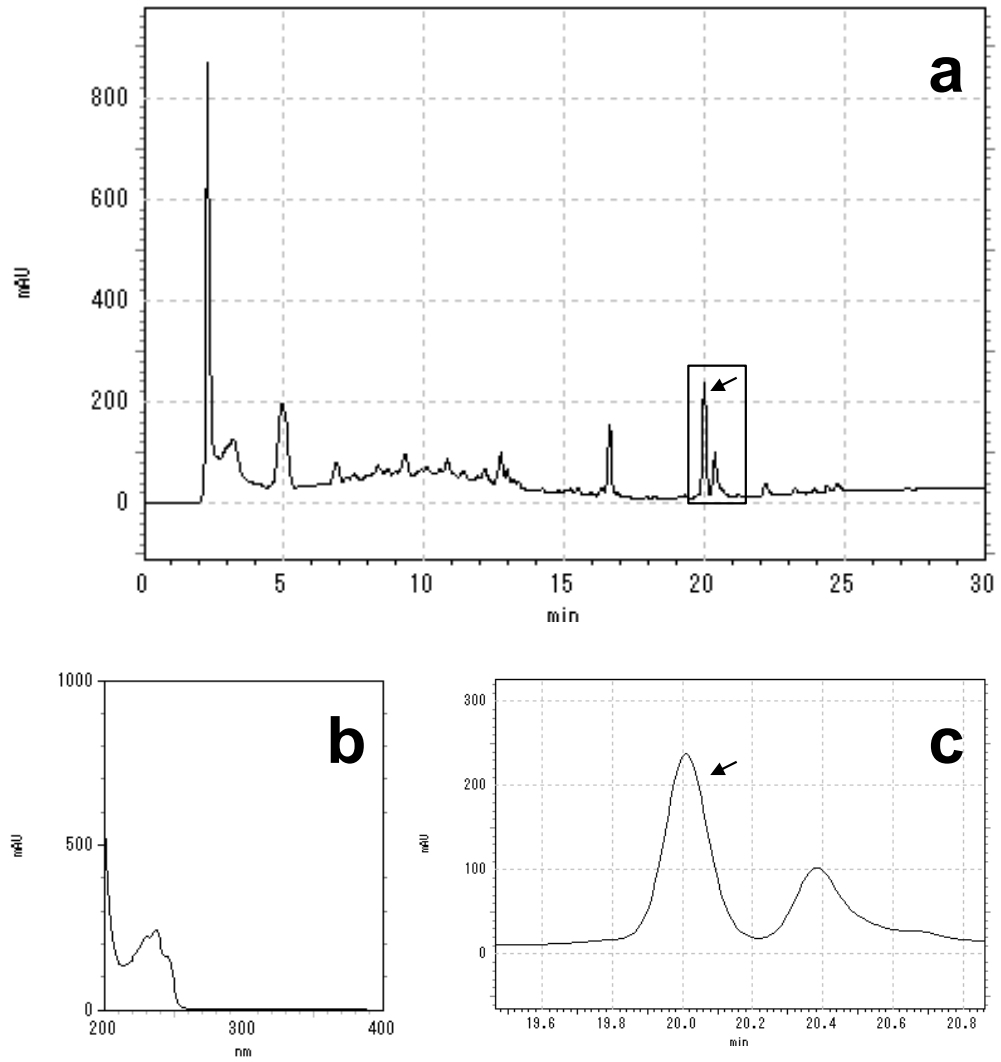

Figure 4: HPLC chromatogramsof monacolin K in the broth of fungus isolate 2011F5.

The peak arrowed in box (a) showed the same retention time as that of monacolin K hydroxyl acid (c). The UV-spectrum of this peak at 236 nm of DAD analysis (the operation parameter of 236nm is not displayed in the monitor)(b) displayed the characteristic spectrum of monacolins. One ml of the broth cultured for 11 days at 25 °C, 180 rpm was freeze-dried. 20 µl of the methanol extract of the lyophilized broth was injected for HPLC analysis after centrifugation.

Table 3: Commercial traditional fermented vinegars and their inhibition rate of sEH

No.	Vinegar Name	Manufacturer	Fermentation Type	Inhibition of sEH (%)[a]	
				Nterm-EH	Cterm-phos
1	Rosy Vinegar	Hangzhou Xihushenggu brewing food Co., Ltd.	Liquid fermentation	4.3±2.7	2.1±7.1
2	Red rosy Zhejiang Vinegar	Ningbo Zuocanwang Seasoning Food Co., Ltd.	Liquid fermentation	21.7±1.7	0.3±2.4
3	JIangnan Rice Vinegar	Shaoxing Renchang Sauce workshop Co., Ltd.	Liquid fermentation	8.6±1.4	3.8±2.6
4	Aromatic Vinegar	Foshan Haitian Seasoning Food Co., Ltd.	Liquid fermentation	5.4±0.6	4.6±1.3
5	Zhenjiang Aromatic Vinegar	Hengfeng Sauce and Vinegar Co., Ltd.	Solid fermentation	9.1±3.1	15.5±1.2
6	Zhenjiang Aromatic Vinegar	Zhenjiang Hengkang Seasoning Co., Ltd.	Solid fermentation	5.3±3.3	6.0±4.5
7	Royal Feast Vinegar	Hengfeng Sauce and Vinegar Co., Ltd.	Solid fermentation	8.5±0.5	12.1±0.4

No.	Vinegar Name	Manufacturer	Fermentation Type	Inhibition of sEH (%)[a]	
				Nterm-EH	Cterm-phos
8	Aged aromatic vinegar	NanjinXiaoerhe Food Co., Ltd.	Solid fermentation	7.8±1.8	10.8±7.6
9	Shuita Aged Aromatic Vinegar (10-years aged)	Shanxi Shuita Aged Vinegar Co., Ltd.	Solid fermentation	9.6±0.6	20.5±0.2
10	Aromatic Vinegar	Fujian Jinjiang Jinguan Food Co., Ltd.	Liquid fermentation	4.1±1.0	4.1±2.6
11	Ginger and Garlic Aromatic Vinegar	GuiyanWeichunyuan Food Co., Ltd.	Solid fermentation	12.4±0.6	5.1±5.0
12	Rosy Zhejiang Vinegar	Hangzhou Food and Brewing Co., Ltd.	Solid fermentation	12.8±2.2	6.9±4.5
13	Shanxi Aged Vinegar (3-years aged)	Shanxi Aged Vinegar Group Co., Ltd.	Solid fermentation	8.1±4.0	18.3±7.3
14	Zhenjiang Aged Vinegar	Zhenjiang Danhe Vinegar Co., Ltd.	Solid fermentation	6.6±2.4	17.2±4.7
15	Feast Aromatic Vinegar	Zhenjiang Danhe Vinegar Co., Ltd.	Solid fermentation	1.9±4.1	18.9±2.8
16	Healthy Vinegar	Shanxi Aged Vinegar Group Co., Ltd.	Solid fermentation	8.2±2.8	10.5±2.8
17	Dumpling Vinegar	Beijing ErshangWeizikang Food Co., Ltd.	Solid fermentation	6.3±2.7	8.6±3.0
18	Aged Vinegar	Shanxi Yishou Seasoning Food Co., Ltd.	Solid fermentation	7.9±1.1	13.3±6.4

[a]Data are mean ± S. D..

The sEH activity was determined by calculating the initial velocity of substrates turnover based on the kinetic measurement.The sEH solution was pre-incubated with sample solution or buffer (as control,of which activity is 100%) for 10 minutes at 30°C in a black polystyrene 96-well plate.Kinetic measurement was started just after the substrate addition. The fluorescence in the Cterm-EH assay was measured at 330 nm (excitation) and 465 nm (emission) with 60-sec intervals for 20 minutes (1420 MultilabelCounter, PerkinElmer, US),and the absorbance at405 nm was used for Nterm-phos assay with 60-sec intervals for 120 minutes

DISCUSSION

ZRRV is one of the well-known Chinese vinegar with a long history comparable to that of Chinese rice wine in view of their same birthplace. Moreover, ZRRV is one of the few to maintain its own traditional process for large-scale production and to be used in traditional Chinese medicine until now. In this study, we found that one brand of ZRRV, out of 18 brands of commercially available traditional fermented vinegar, contains inhibitory activity toward Nterm-phosof sEH. Cterm-EH of sEH can be a pharmacological target to treat hypertension, inflammation, and pain [32], while the biological role of Nterm-phosof sEH is not well known. Although the research is limited by the lack of a potent inhibitor of Nterm-phos, recent findings suggested the complementary biological roles of this activity are important in the development of therapy for cardiovascular diseases [18]. To our knowledge, this is the first report that traditional fermented vinegar has an ability to inhibit sEH activity. Furthermore, we also found two filamentous fungiout of 48 isolates from ZRRV mash produced inhibitory activity toward Nterm-phos, suggesting the presence of rich bioactive substances in ZRRV and a potential new means for the discovery of sEH inhibitors.

Filamentous fungi are indispensable for the production of traditional fermented foods, in which fermentation starts from starch-rich raw materials, because of their potent capability not only for saccharification but also for the production of secondary metabolites responsible for the flavors and health effects of the end products. For the ZRRV brewing, filamentous fungi are particularly important and are required to make them grow on the steamed rice as much as possible during the solid-state fermentation since no starter is used in the brewage. It is believed that the more ambient molds grow, the better the saccharification and brewing proceeds. Since the liquid-state fermentation just follows the solid-state fermentation in the same pot, the metabolites produced in the solid-state were possibly to be brought into liquid mash and preserved. The continuity of fermentation might also result in sustained enzyme action from solid-state to liquid-state, leading to the production and accumulation of certain metabolites, nevertheless the filamentous fungi are living or dying after water adding. However, the processes for the production of other traditional vinegars, such as Zhenjiang aromatic vinegar and Shanxi aged vinegar, lack spatial continuity of fermentation between

saccharification and acetic acid fermentation. Furthermore, the addition of rice husks into the vinegar mash is generally required to provide a good ventilation environment for the growth of acetic acid bacteria in the solid-state fermentation. This makes an environment different from that for the saccarification, leading to a challenge for maintaining enzyme actions and for accumulating metabolites. Therefore, the feature of the ZRRV brewing process that includes the growth of ambient molds and the maintenance of the continuity of fermentation seems to be responsible for the production of diverse and unique bioactive substances, which contributes to folklore health promoting effects of ZRRV.

In this study, we isolated *P. citrinum* (2010F2) and *T. Spectabilis* (2011F2) from the ZRRV mash and found that they produced sEH inhibitors. The genus *Penicillium* is well known due to its ability to produce second metabolites, such as penicillin and statin (compactin) [23]. Our result that the broth of *P. citrinum* inhibits the activity of sEH Nterm-phos implies its potential application for drug discovery. *Talaromyces* has been confirmed to be the genus *Byssochlamys* by the phylogenetic analysis [33], and now *T. spectabilis*is regarded as the synonym of *B. spectabilis*, which is the anamoph of *Paecilomyces variotii*. *P. variotii* has been used as an industrial strain for tannase production and biofiltration of toluene [34]. Recently, one isolate of *P. Variotii* was found to degrade formaldehyde [35]. Our result indicated another prospect of the application of *B. spectabilis*.

Additionally, strain 2011F5, isolated from the ZRVV mash and identified as *A. terreus*, was revealed to produce the hydroxylacid form of monacolin K (lovastatin) by HPLC analysis. Monacolin K is one of the natural statins that has been used as a successful medicine for lowering cholesterol levels in blood and decreasing the risk of heath attack [36]. *A. terreus* ACTT 20542 is the industrial strain which is isolated from soil in Spain [37] and strain TUB F-514 has been successfully used in solid-state fermentation of lovastatin in India [38]. Recently, *A. terreus* was identified in the stage of saccharification during the production of Maotailiquor [39]. Since the process of the Maotai liquor production is also a simultaneous process of saccharification with natural inoculation, it is most likely that *A. terreus*is involved in the natural saccharification for traditional food fermentation.

In summary, ZRRV, the rice-fermented traditional vinegar, was revealed to have a potential vasculoprotective effect. Not only ZRRV itself, but also the cultured broths from two filamentous fungi strains isolated from its mash were found to inhibitsEH Nterm-phos. On the other hand, monacolin K was found in the broth of one strain belonging to *A. terreus* isolated from ZRRV mash. Currently, the work for chemical structural identification of the bioactive compounds is ongoing in our laboratory. Our results suggest that traditional fermented foods with the folklore health benefits, such as ZRRV, are worth to be studied. Both the traditional fermented foods and the microorganisms involved in their fermentation are the resources of bioactive substances discovery.

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