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Antioxidative Activity of Philippine Salt-Fermented Shrimp and Variation of Its Constituents during Fermentation

Ernestina M. PERALTA[1], Hideo HATATE[2]*, Daisuke WATANABE[2], Daisuke KAWABE[2], Hisashi MURATA[2], Yoichiro HAMA[3] and Ryusuke TANAKA[4]

[1] *Institute of Fish Processing Technology, College of Fisheries and Ocean Sciences, University of the Philippines, Visayas (Miagao, Iloilo 5023, PHILLIPINES)*

[2] *Faculty of Agriculture, Miyazaki University (1-1, Gakuenkibanadainishi, Miyazaki-shi, Miyazaki 889-2192, JAPAN)*

[3] *Faculty of Agriculture, Saga University (1, Honjo-machi, Saga-shi, Saga 840-8502, JAPAN)*

[4] *Department of Food Science, National Fisheries University (2-7-1, Nagatahon-machi, Shimonoseki-shi, Yamaguchi 759-6595, JAPAN)*

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Abstract: Shrimp (*Acetes sp.*) was mixed with salt in a ratio of 3:1 and allowed to ferment at room temperature (28-30℃). From the shrimp paste samples collected at the initial (1 day) and end (10 days) of fermentation, 85% ethanol extracts were prepared for their antioxidative assays. Both extracts exhibited high free radical scavenging property against 1,1 diphenyl 2-picrylhydrazyl and suppressed peroxidation of methyl linoleate initiated by 2,2′azobis 2-amidinopropane dihydrochloride. These antioxidative activities, however, did not change significantly during fermentation for 10 days, thus suggesting that the observed antioxidative activities could have mainly due to original antioxidants present in shrimp but not to its fermentative products. The salt-fermented shrimp samples contained large amount of polyunsaturated fatty acids including EPA and DHA, although the polar lipids like phospholipids were hydrolyzed during fermentation to produce the corresponding amount of free fatty acids. In addition, free amino acids of the shrimp sample significantly increased during the fermentation and could be responsible for the unique flavor of salt-fermented shrimp paste. Since the salt-fermented shrimp paste was found to obtain potent antioxidative substances and large amount of EPA, DHA, and amino acids, it is expected to act as an effective antioxidant in our body and to be a good source of these nutrients.

Key words: antioxidant, salt-fermented shrimp, EPA, DHA, amino acid

1 Introduction

The Philippines, like other Asian countries such as Thailand and Vietnam, is well known for its fermented fish products that are used as condiment for an array of food preparations. Fish and other types of marine-derived commodity are known to be good dietary source of essential fatty acids like linoleic and linolenic acids, which the body cannot synthesize. Aside from these, fish also contains polyunsaturated fatty acids (PUFA) such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Several researches have shown that health benefits can be derived from the consumption of these PUFA (1, 2). Fish fermentation

*Correspondence to: Hideo HATATE, Faculty of Agriculture, Miyazaki University, 1-1, Gakuenkibanadainishi, Miyazaki-shi, Miyazaki 889-2192, JAPAN

E-mail: a02119u@cc.miyazaki-u.ac.jp

involves the addition of salt to the fish in a ratio of 1:3 and allowed to ferment in earthen jars at ambient temperature (3, 4). In some cases, high temperature incubation is used to enhance hydrolysis of food components. The ability of NaCl (table salt) and elevated temperatures to accelerate lipid oxidation in fish has been well documented by various researches. Under these conditions, fermented fish products will eventually lose their PUFA during fermentation and storage.

Montano *et al*. (5) evaluated the PUFA contents of some traditional fish and shrimp paste condiments found in local markets of Bolinao, Pangasinan, Philippines. They found that shrimp (*Acetes sp.*) paste has the highest content of DHA among the products analyzed and also its content in the shrimp paste did not change by fermentation. This could have been probably due to the presence of antioxidants that prevented its oxidation. Several researchers have found antioxidative activity in a number of fermented products such as yellowfin sole hydrolysates (6), shrimp waste hydrolysates (7), soy sauce (8) and fish sauce (9). However, there is little information about salt-fermented shrimp paste with regard to its antioxidative activity. In this study, we studied antioxidative activity of salt-fermented shrimp paste and variation of its major constituents, lipids and free amino acids, during fermentation.

2 Materials and Methods

2·1 Chemicals

α-Tocopherol, 1, 1-diphenyl 2-picrylhydrazyl (DPPH) and 2, 2′-azobis (2-amidinopropane) dihydrochrolide (AAPH) were purchased from Wako Pure Chemical Ind. (Osaka, Japan). Methyl linoleate was from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). Triton X-100 was from Sigma Chemical (St Louis, MO, USA). All other reagents used in this experiment were of analytical grade.

2·2 Preparation of Salt-Fermented Shrimp Paste

Shrimp (*Acetes sp.*), purchased in a local market in Iloilo, Philippines, was mixed with salt in a weight ratio of 3:1 (shrimp: salt) and allowed to ferment for 10 days at ambient temperature (28-30℃). This process was based on the current practices of most salt-fermented shrimp paste producers in the Philippines. The salt-fermented shrimp samples were collected at the initial (1day) and end (10 days) of fermentation, and stored at -30℃ before use. These shrimp samples are expressed as Day 1 and Day 10 in this paper, respectively.

2·3 Preparation of 85% Ethanol Extract from Salt-Fermented Shrimp Paste

The salt-fermented shrimp sample (5 g) was homogenized and mixed with 5 mL of water and 20 mL of 95% ethanol. After centrifugation at 1500 rpm for 20 min, the upper layer was recovered. The precipitate was again treated with 20 mL of 95% ethanol as described above. The recovered upper layers were combined and adjusted to 50 mL by adding 95% ethanol. When the insoluble materials were observed, the extracted solution was again centrifuged to remove them. The resulting solution, which contained approximately 85% ethanol, was designated as 85% ethanol sample extract.

2·4 Assay of Antioxidative Activity

2·4·1 DPPH Radical Scavenging Activity

Appropriate amount of the 85% ethanol extract was incubated in 2.5 mL of 85% ethanol solution containing 0.1 mM DPPH for 20 min at room temperature. The DPPH radical scavenging activity (%) was calculated from the decrease of absorbance at 517 nm by the addition of 85% ethanol extract toward that of control (without antioxidant).

2·4·2 Inhibition of Methyl Linoleate Peroxidation

Appropriate amount of the 85% ethanol extract was added to a screw cap test tube. The solvent was removed by evaporating under reduced pressure or with nitrogen gas stream. The sample was dispersed with a sonicator and a Vortex mixer in 0.02 M phosphate buffer (pH 7.0) containing 0.02 M methyl linoleate and 1% Triton X-100. The methyl linoleate peroxidation was initiated by 2.5 mM AAPH at 37℃. All the concentrations in this reaction mixture indicated above were the final ones and its initial volume was set to 1.25 mL. The peroxide value (PV) of the sample was periodically measured by the ferric thiocyanate method (10).

2·5 Extraction of Total Lipid from Salt-Fermented Shrimp Paste and Its Lipid Class Analysis

Total lipid (TL) was extracted from the homogenate of *salt-fermented shrimp paste* with a chloroform and methanol mixture according to the method of Bligh and

Dyer (11). The TL was separated on a silica gel rod, Chromorod S III (Dia-Iatron Co. Ltd., Tokyo, Japan) with benzene/chloroform/acetic acid (50/20/0.7) as developing solvent. The composition of lipid classes was determined by an Iatroscan TLC/FID system (Dia-Iatron Co. Ltd., Tokyo, Japan).

2·6 Fatty Acid Analysis

Fatty acid constituents in the TL were converted to fatty acid methyl esters (FAMEs) using 5% hydrochloric methanol (90℃, 1.5 h) (12). These FAMEs were then analyzed by a gas-liquid chromatography using a Shimadzu GC-14A equipped with a capillary column Omegawax 320 (30 m × 0.32 mm, film thickness 0.25 μm) and a flame ionization detector. The carrier gas was helium at a flow rate of 1.6 mL/min and the split ratio was 50 to 1. The injection and detection ports were set to 250 and 260℃ with an oven temperature program of 180℃ to 230℃ at 1℃/min. The FAMEs were identified by comparing the retention times with authentic ones.

2·7 Free Amino Acid Analysis

The free amino acid contents in the salt-fermented shrimp pastes were estimated using the 85% ethanol extracts at Days 1 and 10 since the free amino acids presented in food can be customarily extracted with 85% ethanol-water mixture. The 85% ethanol extract was concentrated and dried in a rotary evaporator. The dried extract was again dissolved with lithium citrate buffer (pH 2.98) (JEOL Ltd., Tokyo, Japan), and filtered through a Millipore filter (0.45 μm). The amino acid content of the filtrate was determined by using an automatic amino acid analyzer system JLC-500/V (JEOL Ltd., Tokyo, Japan).

3 Results

3·1 Antioxidative Activity of Salt-Fermented Shrimp Paste

Table 1 shows DPPH radical scavenging activity of 85% ethanol extracts from salt-fermented shrimp paste samples. The free radical scavenging activity at Day 1 was significant but not as strong as Day 10, with increasing scavenging activity at increased sample concentration.

Figure 1 shows effect of the 85% ethanol extracts on lipid peroxidation. The PV in the control (without antioxidant) increased rapidly, while the increase in PV was strongly suppressed in the addition of the 85% ethanol extracts as well as a known potent antioxidant, α-tocopherol. Both extracts at Days 1 and 10 exhibited almost the same inhibitory effect against lipid peroxidation. With regard to this inhibitory effect, prolongation of the fermentation period up to 10 days did not enhance the initial antioxidative activity at Day 1, although it was confirmed that salt-fermented shrimp

Table 1 DPPH Radical Scavenging Activity of 85% Ethanol Extracts from Salt-Fermented Shrimp Pastes Collected at the Initial (1 day) and End (10 days) of Fermentation.

	* Added amount of sample (mg)		
	22.5	45	90
	**Radical scavenging activity (%)		
Day 1	15.6	33.8	53.8
Day 10	20.3	38.8	59.9

*Added amount of sample was expressed as dry weight of salt-fermented shrimp pastes calculated from their moisture contents. The moisture contents of salt-fermented shrimp samples, determined by the oven method at 105℃, were 55.1% at Day 1 and 56.1% at Day 10.
**α-Tocopherol (100 μg) exhibited 26% of DPPH radical scavenging activity in this assay system.

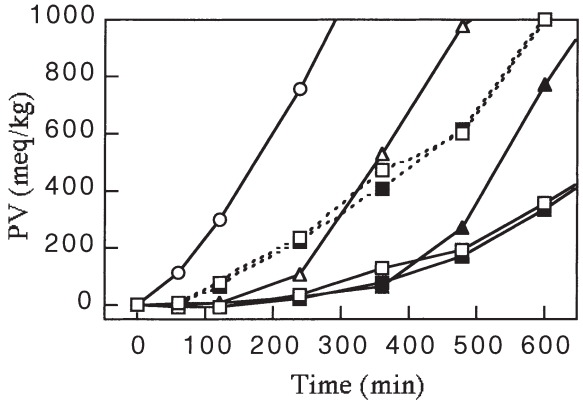

Fig. 1 Inhibition of Methyl Linoleate Peroxidation by 85% Ethanol Extracts from Salt-Fermented Shrimp Pastes Collected at the Initial (1day) and End (10 days) of Fermentation.
—○— Control (without antioxidant), ---□--- 2.5 mg (dry weight) of Day 1, —□— 10 mg of Day 1, ---■--- 2.5 mg of Day 10, —■— 10 mg of Day 10, —△— 6 μg of α-tocopherol, —▲— 12 μg of α-tocopherol.

pastes at Days 1 and 10 showed significant antioxidative activities.

3·2 Lipid Class and Fatty Acid Analyses of Salt-Fermented Shrimp Paste

The TLs extracted from the salt-fermented shrimp pastes at Days 1 and 10 were found to be the same content (1.0 %) and their lipid class analyses were performed. The TLs were separated mainly into four lipid classes, triacylglycerol (TG), free fatty acid (FFA), cholesterol (Cho), and polar lipid (PL) (**Table 2**). Compositional changes were minimal for TG and Cho but large for FFA and PL when Days 1 and 10 were compared. The FFA increased from 29.7% at Day 1 to 47.8% at Day 10 with decreasing the PL from 57.1% to 36.2%.

Table 3 shows the fatty acid composition of the TLs at Days 1 and 10. The major fatty acids in shrimp pastes were 16:0, 18:0, 18:1, 20:5n-3 (EPA) and 22:6n-3 (DHA). Both TLs at Days 1 and 10 were confirmed to contain large amount of PUFA such as DHA and EPA, and their composition did not adversely change during the fermentation. This indicates that the PUFA in the salt-fermented shrimp paste remained intact without significant damage during fermentation up to10 days.

3·3 Free Amino Acid Analysis of Salt-Fermented Shrimp Paste

Table 4 shows the free amino acid content of 85% ethanol extracts at Days 1 and 10. The total free amino acid contents increased from 888.6 mg/100 g net wt. at Day 1 to 1391.7 mg/100 g net wt. at Day 10 due to protein hydrolysis by endogenous and/ or microbial proteases. Taurine content was predominant among the analyzed amino acids, but its content did not significantly change during fermentation for 10 days. On the other hand, noticeable increase in amino acid contents was observed, particularly for asparagine, leucine, alanine and lysine.

Table 2 Lipid Class Composition of Total Lipids Extracted from Salt-Fermented Shrimp Pastes Collected at the Initial (1 day) and End (10 days) of Fermentation.

	*Lipid class (%)			
	TG	FFA	Cho	PL
Day 1	4.2	29.7	9.1	57.1
Day 10	5.7	47.8	10.3	36.2

*TG:Triacylglycerol, FFA:Free fatty acid, Cho:Cholesterol, PL:Polar lipid

4 Discussion

Free radicals and lipid peroxidation products are toxic to biological systems as well as induce food deterioration. Thus, various foods and natural materials have been investigated for their potent antioxidative substances. In this study, we produced the salt-fermented shrimp pastes in our laboratory according to the current procedure in the Philippines and examined antioxidative activity of their 85% ethanol extracts at the initial (1 day) and end (10 days) of fermentation. Both 85% ethanol extracts exhibited significant antioxidative activities against DPPH radical (**Table 1**) and methyl linoleate peroxidation (**Fig. 1**). Although the DPPH radical scavenging activity at Day 10 appeared to be slightly stronger than Day 1, fermentation for 10 days did not effectively increase the initial antioxidative activity. This suggests that the antioxidative activities observed in both extracts could have been due to the

Table 3 Fatty Acid Composition of Total Lipids Extracted from Salt-Fermented Shrimp Pastes Collected at the Initial (1 day) and End (10 days) of Fermentation.

Fatty acid	Day 1	Day 10
	(% of total fatty acids)	
14:0	3.1	3.0
16:0	20.0	19.8
16:1	6.6	6.5
18:0	7.8	7.7
18:1n-9	7.7	7.7
18:1n-7	2.1	2.1
18:2n-6	1.4	1.4
18:3n-3	1.9	1.8
20:0	0.7	0.7
20:1n-9	0.9	0.9
20:4n-6	4.7	4.8
20:5n-3	11.1	11.0
22:4n-3	1.2	1.2
22:5n-3	0.6	1.6
22:6n-3	17.3	17.1
24:1n-9	0.5	0.6
Others	12.4	12.1

original antioxidants present in shrimp but not to its fermentative products. This could also be due to the short fermentation period employed. Production process of the salt-fermented shrimp paste in the Philippines, as practiced, is customarily completed in 10 days unlike fish and soy sauces that are produced by fermenting for several months. Wijewickreme *et al*. (13) and Jing and Kitts (14) measured the antioxidant activity of sugar-lysine Maillard reaction products (MRPs) and found that they exhibited high hydroxyl radical scavenging activity. Thus, the longer the fermentation period the more antioxidative substances like MRPs can be produced.

Lipid class analysis showed that the PL has been hydrolyzed to produce the corresponding amount of FFA during the fermentation (**Table 2**). The PL generally comprises of phospholipids like phosphatidylcholine and phosphatidylethanolamine as major components, suggesting that phospholipases could have participated in liberation of the FFA from the PL. In the Philippines, the salt-fermented shrimp paste is normally produced under the high salt concentration (20-25%) and temperature incubation (30-35℃). Despite these severe fermentation conditions, the fatty acid compositions including EPA and DHA, which are susceptible to active oxygen species, were not adversely affected during the fermentation (**Table 3**). This implies that the salt-fermented shrimp paste could contain some potent antioxidative substances that prevented PUFA from undesirable lipid peroxidation. It has been reported that short chain fatty acids like butyric acid and lipid oxidation products often cause off-flavors during food processing and storage (15). However, since the increased FFA in this study was positively long chain FFA like EPA and DHA and were not oxidatively damaged, they would not cause off-flavor in salt-fermented shrimp paste fermented for 10 days.

Table 4 Free Amino Acid Content of 85% Ethanol Extracts from Salt-Fermented Shrimp Pastes Collected at the Initial (1 day) and End (10 days) of Fermentation.

Amino acid	Day 1	Day 10
	(mg of 100 g wet weight)	
Phosphoserine	6.7	4.6
Taurine	215.2	209.8
Urea	2.2	21.5
L-Threonine	1.6	36.6
Asparagine	10.0	201.1
L-Glutamic acid	25.8	38.2
Glycine	37.9	47.6
L-Alanine	95.9	124.9
L-Citrulline	45.6	83.2
L-Valine	40.1	67.5
L-Methionine	13.1	19.9
L-Isoleucine	28.7	51.4
L-Leucine	62.2	112.7
L-Tyrosine	32.1	50.9
L-Phenylalanine	27.5	47.5
L-Ornithine	4.1	4.7
L-Histidine	14.9	21.1
L-Lysine	76.8	121.7
L-Arginine	80.8	53.6
L-Proline	56.6	61.7
Ammonia	8.0	11.5
Total	885.6	1391.7

Proteins in food are hydrolyzed by bacterial or indigenous enzymes during fermentation to produce simpler compounds such as dipeptides, amino acids and other nitrogenous compounds and they can function as primary antioxidants or synergist in combination with other compounds (16). Several authors have studied synergistic effect of various protein hydrolysates with antioxidants and found that the hydrolysates prepared from sardine myofibril protein (17), bovine (18) and human serum albumins (19) exhibited potent synergism with α-tocopherol against lipid peroxidation. Protein hydrolysates are also known to possess radical scavenging property as observed in dipeptides, carnosine and anserine (20, 21). Kim *et al.* (22) found that the major constituent amino acids of salt-fermented shrimp at 3 months were aspartic acid, glutamic acid, alanine, leucine and lysine. In this study, taurine, asparagine, leucine, alanine and lysine were also recognized as major free amino acids in the salt-fermented shrimp paste (**Tables 4**). Although carnosine and anserine were not detected, some nitrogenous compounds like these free amino acids would be partly responsible for the antioxidative activity. Because there was only a slight difference in the antioxidative activities between Days 1 and 10 (**Table 1** and **Fig. 1**), the increased free amino acids such as asparagine, leucine, alanine and lysine at Day 10 could have not significantly contributed to the initial antioxidative activity at Day 1. Nevertheless, these free amino acids could play an important role in the development of the unique flavor in shrimp

paste.

This study confirmed that the salt-fermented shrimp paste contained potent antioxidative substances, large amount of PUFA and free amino acids. Salt-fermented shrimp paste, when incorporated as dietary food, will serve as effective antioxidant in our body and a good source of EPA, DHA, and a number of essential amino acids. Since this study was limited to the assay of antioxidative activity of shrimp paste, further research should be focused on identification of the substances or compounds that exhibit its antioxidative property.

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