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ANGIOTENSIN-I CONVERTING ENZYME (ACE) INHIBITORY ACTIVITY AND ANTIOXIDANT ACTIVITY OF FERMENTED FISH PRODUCT *NGARI* AS INFLUENCED BY FERMENTATION PERIOD

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ABSTRACT

Ngari is a traditional salt-free fermented fish product in the north eastern region of India. During the fermentation, breakdown of protein leads to release of the peptides with specific sequences which may have potential anti-hypertensive and antioxidant properties. In the present investigation, influence of fermentation period on anti-hypertensive properties (angiotensin-I converting enzyme (ACE) inhibitory activity) and antioxidant activity of aqueous extract of fermented fish (*ngari*) were evaluated. The ACE inhibitory activity was found to be concentration dependent. Antioxidant properties of *ngari* were assessed which included 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging, ferric reducing antioxidant power and lipid peroxidation inhibition. The antioxidant properties of *ngari* were found to be dependent on protein concentration and period of fermentation. The study indicated that the traditional fermented fish product (*ngari*) could be used in alleviating hypertension and lipid oxidation.

KEY WORDS: **Fermentation, *Ngari*, ACE inhibitory activity and Antioxidant activity**

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INTRODUCTION

Traditional processing of fish such as fermentation, salting, drying and smoking are the principal methods of fish preservation in Southeast Asia[1]. Fermentation is one of the oldest and most economical methods for producing and preserving foods. In addition to preservation, fermented foods can also have the added benefits of enhancing flavor, increasing digestibility and improving nutritional and pharmaceutical value. Among indigenous fermented products, fermented fishery products have been widely consumed in Southeast Asia due to their delicacy and high nutritional properties[2]. Fermentation of fish refers to the breakdown of larger molecules into smaller one with the help of endogenous or exogenous enzymes. Fermented fish products are a good source of proteins, peptides and amino acids. Proteins and peptides derived from fermentation have been found to be physiologically active or bioactive which have regulatory functions in the human body, apart from serving important nutrients [3, 4]. Therefore, interests have been developed to identify biological activities in fermented foods including fish and shellfish[5, 6]. Health related functional properties like ACE inhibitory activity, radical scavenging capacity may present as promising biological benefits of these fermented foods. Angiotensin-I converting enzyme (ACE; EC 3.4.15.1) is a circulating enzyme that participates in the body's renin-angiotensin system and plays an important physiological role in regulating blood pressure. ACE is known as peptidyl dipeptidase A and primarily cleaves a C-terminal dipeptide of substrates. It converts an inactive form of a decapeptide (angiotensin-I) to a potent vasoconstrictor, an octapeptide (angiotensin-II) and also inactivates the catalytic function of bradykinin, which has a vasodilating action[7]. The inhibition of ACE activity is a major target in the prevention of hypertension. High blood pressure is one of the major independent risk factors for cardiovascular diseases, the primary cause of mortality in developed countries and has become the leading cause of death in developing countries[7]. Recently, the search for natural ACE inhibitors as alternatives to synthetic drugs is of great interest to prevent several side effects. ACE-inhibitory activities of fermented food products such as fermented blue mussel sauce[8] and salmon fish sauce[9] have been studied.

Lipid oxidation is the major quality deteriorative process in food products resulting in a variety of breakdown products which produce off-odors and off-flavours. In order to prevent foods from undergoing such deterioration, it is very important to inhibit lipid peroxidation. Antioxidants are used to preserve food products by retarding deterioration as a result of oxidation. Oxidation of biomolecules, including peroxidation, involves a series of free radical-mediated chain reactions. Much attention has been focused on the use of antioxidants, especially natural antioxidants, to inhibit lipid peroxidation and to protect bio-molecules from damage by free radicals. In the past few years, several attempts have been made to identify and characterize the activities of natural antioxidants in different oxidative systems. They could act as inhibitors of lipid peroxidation, direct scavengers of free radicals and agents to chelate transition metal ions that catalyze the generation of radical species[10]. Antioxidant activities of fermented blue mussel[6], Thai traditional fermented shrimp and krill products[11], fermented shrimp bio-waste[12], Philippine salt-fermented shrimp paste[13], fermented fish paste *miso* [27] have been reported. In India, fermented foods are popular among the people of the north-eastern states. *Ngari*, a fermented fish product made from sun-dried non-salted fish. This product is also known as *seedal, sepaa, hidal* and *shidal* in Assam, Tripura, Mizoram, Arunachal Pradesh and Nagaland. The term *ngari* is more often used in Manipur. Fermentation period for *ngari* varies from 6 to 12 months. Fermented fish products apart from serving as a good source of protein might possess bioactivities, especially ACE inhibitory properties and antioxidants activity. Accordingly, these products could be marketed as health foods with high market value. However, no information regarding the bioactivity

of fermented fishery products, especially from traditional fermented fish products from north-east India including *ngari*. Therefore, the objectives of the present study were to evaluate ACE inhibitory and antioxidant activities of *ngari* as a function of fermentation period and protein concentrations.

MATERIALS AND METHODS

(i) Chemicals

Angiotensin converting enzyme (ACE) from rabbit lung ≥2.0 units/mg protein (modified Warburg-Christian), N-[3-(2-Furyl)acryloyl]-Phe-Gly-Gly (FAPGG), 2,2 diphynyl-1-picrylhydrazyl (DPPH), iron (II) chloride, linoleic acid, potassium ferricyanide, bovine serum albumin (BSA), sodium dodecyl sulphate (SDS), N, N'-methylene - bis - acrylamide, 2 - mercaptoethanol, ammonium persulfate (APS), wide range molecular weight sigma marker (6500 – 200000 Da) were procured from Sigma-Aldrich, Inc. (St. Louis, MO, USA). Other chemicals and reagents used were of analytical grade.

(ii) Preparation of ngari

Fresh *Puntius* spp. was used for the preparation of *ngari*. The fishes were sundried for 48 hours and *ngari* was prepared from dried and non-salted fish using traditional method[2, 14]. The fermentation was carried out separately for a period of 6, 8, 10 and 12 months respectively. At the end of 6, 8, 10 and 12 months, bioactive properties of *ngari* were assessed. All samples were packed in airtight plastic jars after fermentation and stored at 4°C until for further analysis. All the analyses were carried within 10 days of desired fermentation period.

(iii) Proximate analysis and pH determination of ngari

The moisture, crude protein, fat and ash content of *ngari* samples were estimated by standard methods[15]. The protein concentration of the aqueous extract of *ngari* was determined using Lowry method [16]. The pH of *ngari* samples were determined by using pH meter (Systronix µ pH system 361, Ahmadabad, India).

(iv) Preparation of aqueous extract from ngari

Ngari (5 g) was mixed with distilled water (100 ml) and the mixture was homogenized at a speed of 10,000 rpm using homogenizer for 3 min. The slurry was centrifuged at 3000Xg for 10 min. at ambient temperature (27±2°C) using a Sorvall Legend XTR centrifuge (Thermo Fisher Scientific Inc., USA) to remove undissolved debris. The supernatant was used for determination of ACE inhibitory and antioxidant activity after determining the protein content.

(v) Angiotensin-I converting enzyme (ACE) inhibitory activity

ACE-I inhibitory activity of *ngari* samples fermented for different time periods and at different protein concentrations was tested using FAPGG as the synthetic substrate[17, 18] with slight modifications. Aqueous extract of *ngari* (200 µl each at protein concentration of 1, 3 and 5 mg/ml protein), ACE enzyme (100 µl of 20 mU enzyme) and substrate (2 ml of 0.5 mM FAPGG substrate) were mixed and the absorbance was monitored at 340 nm for 20 min. using spectrophotometer (UV-VIS spectrophotometer, LaboMed Inc., USA). Hydrolysis of FAPGG by ACE will result in a decrease in absorbance at 340 nm. Slope of the curve was used to calculate the percentage of ACE inhibition. A sample containing FAPGG substrate and the ACE enzyme was used as a control. All experiments were done in triplicates.

$$\% \text{ of ACE inhibition} = 1 - \frac{\text{Slope of the sample curve}}{\text{Slope of the control curve}} \text{ X } 100$$

Where,
Sample is the mixture of enzyme, aqueous extract of *ngari* or inhibitor and substrate
Control is the enzyme-substrate mixture without inhibitor.

(vi) Determination of antioxidant activity

DPPH free radical-scavenging activity

Aqueous extract of *ngari* was diluted with double distilled water to give protein concentrations of 1, 3 and 5 mg/ml protein and were analyzed for DPPH radical scavenging activity[19].

FRAP (ferric reducing antioxidant power)

The ability of the aqueous extract of *ngari* to reduce iron (III) was determined[20]. The FRAP was analyzed as a function of fermentation period and protein concentration. The analyses were carried out in triplicate.

Lipid peroxidation inhibition (%) using linoleic acid model system

Lipid peroxidation inhibition activity of *ngari* was measured using linoleic acid model system[21] at different protein concentrations and fermentation period. The experiments were carried out in triplicate.

(vii) SDS–polyacrylamide gel electrophoresis of fermented ngari

Protein patterns of *ngari* samples fermented for different periods were determined by SDS–PAGE using 4% stacking gel and 10% running gel[22]. Samples (3 g) were solubilized in 27 ml of 0.17 M SDS (85°C). The mixture was homogenized for 1 min. at a speed of 13,000 rpm using an Ultra-turrax homogenizer and incubated at 85°C for 1 h to dissolve total proteins. Protein solution was mixed with sample buffer (100 mM Tris-HCl, 1% SDS, 4% 2-mercaptoethanol, 0.02% coomassie brilliant blue G, 24% glycerol) in 1:1 ratio. The samples were loaded on the gel and the run was carried out at a constant voltage mode. The initial voltage of 30V was maintained until the samples moved out of staking gel. After the entry of samples to resolving gel the voltage maintained was 90V. After the run, the gels were stained using staining solution (0.02% coomassie brilliant blue G-250 prepared in 7.5% acetic acid) followed by destaining with 7.5% acetic acid.

(viii) Statistical analysis

All experiments were conducted in triplicate. The data were subjected to analysis of variance (ANOVA) and the differences between means were evaluated by Duncan's Multiple Range Test. SPSS Version 16.0 (SPSS Inc., Chicago, IL) was used for data analysis.

RESULTS AND DISCUSSION

1. Composition of ngari fermented for different periods

The results of proximate composition of *ngari* as a function of fermentation period are given in Table 1.With increase in fermentation period, the moisture content marginally increased while fat content showed a decreasing trend. Similarly, ash content also decreased. The changes in proximate composition are inter-related and decrease in fat content can be related to hydrolysis of fat and increase in moisture. The composition of *ngari* obtained in the present study compares well with the reported values in the literature[23]. The result indicated that fermented fish samples could be an important source of proteins. The pH of fermented samples showed values in the range of 6.14 to 6.29 over a period of 12 months. The pH of fermented fish product namely *shidal* was 6.1 to 6.2[24] which were closer to the values obtained in the present study.

Table 1
Proximate composition of ngari as influenced by fermentation period

Parameter	Fermentation period (months)			
	6	8	10	12
Moisture (%)	30.36±0.2[a]	31.31±0.18[a]	32.40±1.05[b]	34.08±0.74[c]
Crude protein (%)	38.81±0.07[a]	38.50±0.08[b]	38.97±0.03[c]	39.16±0.07[d]
Fat (%)	16.67±0.18[a]	15.43±0.27[b]	14.99±0.1[c]	14.53±0.22[d]
Total ash (%)	14.14±0.41[a]	14.10±0.38[a]	13.67±0.2[a]	12.44±0.1[b]

Values are means ± SD, n=3; Values with different lower case letters in superscript indicate significant difference between the samples fermented for different periods

2. Angiotensin-I converting enzyme (ACE) inhibitory activity

ACE-inhibitory activity of aqueous extracts of *ngari* as a function of fermentation period and protein concentration is presented in Table 2. With increase in fermentation period the ACE inhibitory activity showed increasing trend at any given protein concentration studied. Significantly higher ACE inhibition was observed at higher protein concentrations by all the *ngari* samples fermented for different periods (p<0.05). There was no significant difference in the ACE inhibitory activity of samples fermented for 10 and 12 months at 5 mg/ml protein concentration (p>0.05). Fermented fish products will have higher content of small peptides and amino acids because of hydrolysis. The ability of peptides to inhibit the activity of ACE is highly dependent on the sequence of amino acid residues and composition[9].

Table 2
Angiotensin I converting enzyme (ACE) inhibitory activity (%) of aqueous extract of fermented ngari at different protein concentrations as influenced by fermentation period

Protein Concentration (mg/ml)	Fermentation period in months			
	6	8	10	12
1	16.10±0.85[aA]	16.67±1.76[aA]	22.60±2.13[aB]	33.62±1.76[aC]
3	31.64±1.76[bA]	44.35±2.13[bB]	46.89 ±2.76[bB]	54.80±2.59[bC]
5	46.89±1.29[cA]	61.58±1.29[cB]	65.82±1.59[cB]	69.77±3.82[cC]

Values are means ± SD, n=3; Values with different lower case letters in superscript indicate significant difference between different protein concentrations; Values with different upper case letters in superscript indicate significant difference within each concentration of samples fermented for different periods

3. DPPH radical scavenging activity

DPPH radical scavenging activity of aqueous extract of *ngar*i increased marginally with the period of fermentation (Table 3). DPPH radical scavenging activity of *ngari* fermented for 12 months was significantly higher from other samples at different protein concentrations (p<0.05). The results of the study indicated that the aqueous extract of *ngari* has hydrogen donating ability (scavenging of DPPH free radicals). Presence of peptides in the *ngari* has the ability to donate the hydrogen atom to free radicals thereby achieving scavenging activity. DPPH is a stable free radical that shows a maximum absorbance at 517 nm in ethanol. When DPPH encounters a proton-donating substance such as an antioxidant, the radical would be scavenged and the absorbance is reduced[25]. Many peptides that are released *in vivo* are bioactive and have a regulatory function as antioxidants[5]. Fermented food products are a good source of peptides which possess antioxidant properties. Size and composition of small peptides affect the antioxidative activity[26]. The results indicated that *ngari* contained substances which were electron donors and could react with free radicals to convert them to more stable products and terminate the radical chain reaction. Similar results have been reported for Japanese fermented fish paste, *miso* [27].

Table 3
DPPH radical scavenging activity (%) of aqueous extract of ngari at different protein concentrations (mg/ml) as influenced by fermentation period

Protein Concentration (mg/ml)	Fermentation period in months			
	6	8	10	12
1	60.94±1.91^{aA}	51.67±9.45^{aA}	59.65±2.24^{aA}	71.32 ±1.19^{aB}
3	66.62±2.23^{bA}	71.25±2.17^{bB}	71.87±0.40^{bB}	79.10±0.55^{bC}
5	75.71±1.82^{cA}	78.87±2.06^{cB}	79.81±0.38^{cB}	83.43±0.37^{cC}

Values are means ± SD, n=3; Values with different lower case superscript letters indicate significant difference between different concentrations; values with different upper case superscript letters indicate significant difference within each concentration of samples fermented for different periods

4. Ferric reducing antioxidant power (FRAP)

FRAP significantly increased with the increase in the protein concentration and period of fermentation (p<0.05) (Table 4). Peptides formed during fermentation as a function of time are likely to have better reducing ability than the peptides formed during the early stage of fermentation. FRAP found in *ngari* samples suggested their capability of providing the electron. The water-soluble fraction from mungoong, a paste made from the cephalothorax of white shrimp exhibited a high antioxidative activity[19]. Higher reducing power of fermented fish paste, *miso* in the early stages of fermentation has been reported[27].

Table 4
Reducing power of aqueous extract of ngari (Abs_{700}) at different protein concentrations (mg/ml) as influenced by fermentation period

Protein Concentration (mg/ml)	Fermentation period in months			
	6	8	10	12
1	0.28±0.01^{aA}	0.42±0.02^{aB}	0.80±0.04^{aC}	0.94±0.01^{aD}
3	0.60±0.01^{bA}	1.10±0.02^{bB}	1.63 ±0.10^{bC}	1.92±0.01^{bD}
5	0.97±0.02^{cA}	1.29±0.05^{cB}	1.90±0.04^{cC}	2.11±0.02^{cD}

Values are means ± SD, n=3; Values with different lower case letters indicate significant difference between different concentrations; Values with different upper case letters indicate significant difference within each concentration of samples fermented for different periods

5. Lipid peroxidation inhibition (%)

Ability of *ngari* samples to protect oxidation of linoleic acid was assessed. All the products fermented for different periods showed inhibition of lipid peroxidation in linoleic acid model system and found to be protein concentration dependent (p<0.05) (Table 5). Lipid peroxidation inhibition was found to be significantly higher in samples fermented for 12 months (p<0.05). Peroxidation of fatty acids can cause deleterious effects in foods by forming complex mixtures of secondary breakdown products of lipid peroxides. Further, intake of these foods can cause a number of adverse effects including toxicity to mammalian cells[28]. The lipid peroxide inhibition of *ngari* samples can be attributed to the ability of peptide to interfere propagation cycle of lipid peroxidation and there by slowing radical mediated linoleic acid oxidation.

Table 5

Lipid peroxidation inhibition (%) in linoleic acid model system by aqueous extract of ngari at different protein concentrations (mg/ml) as influenced by fermentation period

Protein Concentration (mg/ml)	Fermentation period in months			
	6	8	10	12
1	49.18±0.35^{aA}	59.36±1.52^{aB}	58.44±1.18^{aB}	62.37±0.28^{aC}
3	60.21±2.35^{bA}	62.06±1.10^{bA}	65.70±2.21^{bB}	66.62±0.95^{bB}
5	64.841.37^{cA}	66.92±0.84^{cA}	70.43±1.72^{cB}	72.14±0.16^{cB}

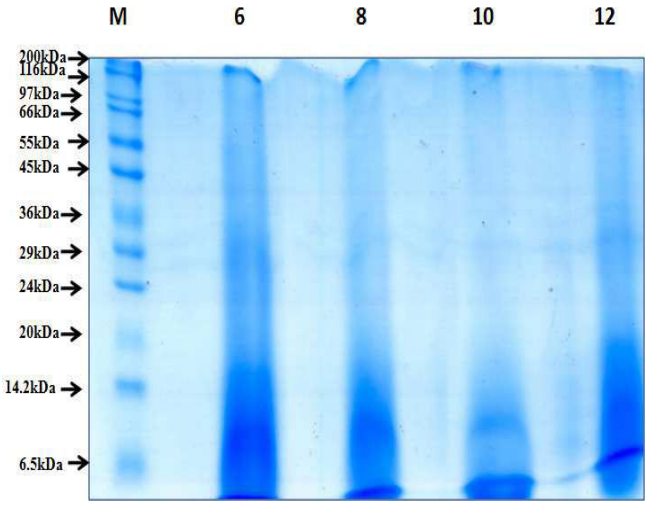
Values are means ± SD, n=3; Values with different lower case superscript letters indicate significant difference between different concentrations; Values with different upper case superscript letters indicate significant difference within each concentration of samples fermented for different periods

6. SDS-PAGE profile

The SDS-PAGE profile for *ngari* fermented for different periods is shown in Figure 1. The presence of low molecular weight bands were evident (in diffused form) from the SDS-PAGE pattern. This is indicative of the release of small peptides during fermentation.

Figure 1

SDS-PAGE profile of ngari samples fermented for different periods



M: standard wide range molecular weight marker; 6, 8, 10 and 12: fermentation period in months

CONCLUSION

In the present study we have reported *in vitro* bioactive properties of traditional fermented fish product, *ngari* from North East India. The fermentation period and protein concentration was found to have positive influence on ACE inhibitory and antioxidant properties of aqueous extract of *ngari.* As the product is salt free, attempt should be made to popularize as health food in other parts of the country.

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