



# Bioactive Properties of Fermented Anchovy (*Stolephorus indicus*) Fish Sauce and its Stability

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## ABSTRACT

Hydrolytic breakdown of fish proteins by fermentation process was applied for utilizing low value fish in preparation of bioactive materials. In the present investigation, fermented anchovy (*Stolephorus indicus*) fish sauce was prepared and its bioactivity including antihypertensive and antioxidant activities was assessed as a function of protein concentration. The moisture, crude protein, crude fat and ash content of anchovy sauce were  $68.32 \pm 0.95$ ,  $16.44 \pm 0.60$ ,  $1.23 \pm 0.04$  and  $14.52 \pm 0.94$  per cent, respectively. The pH and NaCl content were  $5.58 \pm 0.03$  and  $24.75 \pm 0.33$  per cent, respectively. ACE inhibitory activity of anchovy sauce increased significantly with protein concentration ( $p < 0.05$ ) and was stable during storage at ambient and refrigerated temperature. DPPH radical scavenging activity of sauce samples decreased with increase in the protein concentration whereas ferric reducing antioxidant power and lipid peroxidation inhibition in linoleic acid model system in fish sauce significantly increased with increasing protein concentration ( $p < 0.05$ ). *In vitro* significance of fish sauce which is a natural source of bioactive peptides with desirable properties against oxidation and hypertension has been demonstrated which may be beneficial for consumers and processors from economical and health point of view.

**Key Words:** Anchovy sauce, Bioactive properties, Fish fermentation, Storage stability.

## INTRODUCTION

Increased health awareness and concern among seafood consumers lead to considerable change in demand for healthy food in addition to food for hunger and nutrition (Kearney, 2010). Functional and health promoting nutraceuticals trending at top in the food industries (Valls *et al*, 2013). Health problems such as hypertension related to cardiovascular dysfunctions are faced by people worldwide. Angiotensin I converting enzyme regulates blood pressure and cardiovascular function by converting angiotensin I to angiotensin II making ACE inhibition mechanism as important in hypertension related disorders where synthetic ACE inhibitors are mostly used (Miller and Arnold, 2019). Oxidation of lipids in food which on development lead to off-

flavours, odours and dark colour is of great concern when food industries are considered as it may lead to cancer, heart dysfunction. Antioxidants can prevent deterioration of food and are often used in synthetic form due to higher efficiency but there is a concern about their safety aspects.

There is increasing interest in deriving bioactive peptides from low value fish for their utilization as food ingredients and the search for natural ACE inhibitory compounds and antioxidants. Bioactive peptides are small subunits of native protein derived from food which in addition to their nutritional value exerts some physiological effect on body but inactive and can be activated by processes such as fermentation and hydrolysis (Phadke *et al*, 2014;

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Phadke *et al*, 2016). Fermentation is one of the oldest and most economical methods for producing and preserving fish for shelf life extension along with enhancing a unique taste, flavour and nutrition and bioactivities due to protein breakdown by microbial proteases (Kwon *et al*, 2010). Fish sauce is the hydrolyzed product of fish tissues which consist of protein and lipid. Fermented fish products like anchovy fish sauce (liquid form) produced from anchovy which have little commercial value and comprises of fair quantity of landings apart from serving as a good source of protein might possess bioactivities, especially natural antioxidants, which provide health benefits and could be marketed as highly valued. Although fermented fish products are of major importance in Asia, studies of these products and their bioactivities remain few. Hence, an attempt has been made to study the properties of bioactive peptides from fermented fish products with evaluation and characterization of bioactive properties and stability of these bioactivities during storage.

## MATERIALS AND METHODS

### Raw material used for preparation of fish sauce

Fresh Indian anchovies (*Stolephorus indicus*) with length varied from 7 to 10 cm and weight from 3 to 6 g, caught off Mangalore coast were procured from the fish landing centre in iced condition using fish: ice ratio of 1:1. Commercial fish sauce samples (two from anchovy paste in different ratio and one from oyster extract) prepared in Thailand were procured from local supermarkets in Mangalore and stored at ambient temperature (27±2°C) until further use.

### Chemicals and reagents

Chemicals and reagents used in the present study were of AR or GR grade. Angiotensin converting enzyme (ACE) from rabbit lung ≥2.0 units / mg protein (modified Warburg-Christian), N-[3-(2-Furyl) acryloyl]-Phe-Gly-Gly (FAPGG), captopril, 2,2 diphenyl-1-picrylhydrazyl (DPPH) were procured from Sigma Chemical Co., USA.

### Preparation of anchovy fish sauce

Anchovy fish were washed with water, descaled, cut into 1 to 1.5 cm small size pieces and ground into fine paste which was mixed with salt and fermented in glass jar for 12 m. After liquefaction, formed self-brine was filtered through 4 layers of muslin cloth and heat inactivated at 85±2°C for 15 min. The filtrate was referred as Anchovy fish sauce.

### Proximate composition, pH and NaCl contents of anchovy sauce

Proximate composition (moisture, crude protein, crude fat and ash) of anchovy fish sauce were analysed by the standard method as described in AOAC (2005). Anchovy sauce samples protein in protein concentration (mg/ml) evaluation was carried out using method described by Lowry *et al* (1951) using bovine serum albumin as a standard. The pH was measured using pH meter (Systronix µ pH system 361, Ahmedabad, India) without diluting sauce samples. Sodium chloride (NaCl) content of anchovy sauce was estimated by Volhard's titrimetric method (AOAC, 2005).

### ACE I inhibitory activity

ACE I inhibitory activity of fish sauce samples was determined by the method as described by Elavarasan and Shamasundar (2013) using FAPGG substrate.

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

Where, Sample is the mixture of enzyme, fish sauce or inhibitor and substrate; Control is the enzyme-substrate mixture without inhibitor

### DPPH (2, 2-Diphenyl-1-picrylhydrazyl) radical scavenging activity

DPPH radical scavenging activity of fish sauce was determined in triplicate using appropriate control according to the method described by Yen and Wu (1999) at an absorbance of 517 nm. It was calculated as;

$$\text{DPPH free radical scavenging activity (\%)} = 1 - \frac{\text{Abs}_{\text{Sample}}}{\text{Abs}_{\text{Control}}} \times 100$$

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Appropriate control was maintained using DDW.

### Ferric reducing antioxidant power (FRAP) assay

The ability of samples to reduce iron (III) was determined by the method as described by Oyaiza (1986) taking absorbance at 700 nm in triplicate.

### Lipid peroxidation inhibition (LPI) assay in linoleic acid model system

Lipid peroxidation inhibition of samples with known protein concentration was measured using linoleic acid model system according to the method as described by Osawa and Namiki (1985) and it was expressed as follows;

$$\text{Lipid peroxidation inhibition (\%)} = 1 - \frac{\text{Abs}_{\text{Sample}}}{\text{Abs}_{\text{Control}}} \times 100$$

### Storage stability of anchovy fish sauce in relation to ACE inhibitory and antioxidant activity

Stability of fish sauce samples in relation to their DPPH radical scavenging activity and ferric reducing antioxidant power was assessed at 0, 2 and 4 wk during their storage at  $4 \pm 2^\circ\text{C}$  and  $27 \pm 2^\circ\text{C}$  and ACE inhibitory activity of fish sauce was assessed at 5 mg/ml protein concentration at 0 and 4 wk storage period by the methods described earlier.

### Statistical analysis

All data were stated as mean  $\pm$  standard deviation for triplicate determination. One-way analysis of variance (ANOVA) was used to analyze the data (Keppel, 1973). Significant difference between the means were determined by Duncan multiple range test at  $p < 0.05$  level using the statistical software. Statistical Package for the Social Sciences (SPSS) Version 16.0 (SPSS Inc., Chicago, IL) and data analysis tool in Microsoft Excel-2007 were used.

## RESULTS AND DISCUSSION

### Composition of anchovy sauce

The fish sauce sample had moisture, crude

protein, total fat and ash contents of 68.32, 16.44, 1.23 and 14 per cent, respectively similar to commercial anchovy sauce samples (Lee *et al.*, 2013) with little lesser crude protein which may be attributed to differences in origin of fish, condition of fermentation such as temperature, pH and salt concentration. The NaCl content and pH of anchovy sauce were found to be 24.75 per cent and 5.3 to 6.7, respectively.

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

Anchovy sauce prepared in the laboratory exhibited higher ACE inhibitory activity (71.14-84.96%) at different protein concentration when compared with commercial fish sauce samples ( $p < 0.05$ ) and was found to be concentration dependent as shown in Table 1. Smaller size of peptides due to fermentation resulted to ACE I inhibitory action. Amino acid sequence, composition, nature of substrate, additives during fermentation influenced ACE inhibitory action along with implications due to presence of dipeptides with tryptophan at C-terminal position in fish sauce samples (Okamoto *et al.*, 1995).

### DPPH radical scavenging activity

All the fish sauce samples exhibited DPPH radical scavenging activity and the activity decreased with increase in protein concentration (Table 2) indicating their ability to donate hydrogen due to presence of peptides formed during fermentation with varied DPPH radical scavenging may have arisen due to varying size and composition of peptides formed with similar results in Japanese fermented fish paste (Giriet *al.*, 2012). DPPH radical scavenging activity of fish sauce samples was lower than that of synthetic antioxidants such as BHA, BHT and  $\alpha$ -tocopherol which were  $96.16 \pm 0.03$ ,  $92.14 \pm 0.02$  and  $87.91 \pm 1.25$  per cent, respectively. They can be used at higher concentration than synthetic antioxidants because of their less restrictive toxicological parameters (Giri *et al.*, 2012).

**Table 1: ACE inhibitory activity of fish sauce samples at different protein concentrations.**

Protein Concentration (mg / ml)	ACE inhibitory activity (Per cent)			
	S1	S2	S3	AS
1	65.44±0.70 <sup>a</sup>	52.85±1.40 <sup>a</sup>	17.88±3.52 <sup>a</sup>	71.14±1.41 <sup>a</sup>
3	68.70±0.70 <sup>b</sup>	67.48±0.70 <sup>b</sup>	19.10±0.70 <sup>a</sup>	77.24±3.73 <sup>b</sup>
5	77.65±0.71 <sup>c</sup>	73.58±2.82 <sup>c</sup>	32.12±3.52 <sup>b</sup>	84.96±0.71 <sup>c</sup>

Values are expressed as mean±standard deviations, n=3; Different lower case letters in superscript indicate significant difference between the treatments ( $p<0.05$ ); S1 and S2: Commercial anchovy fish sauce sample made with anchovy (Thai fish sauce and Blue dragon fish sauce); S3: Commercial oyster sauce made from oyster extract (Pantai oyster sauce); AS: Anchovy sauce prepared in the laboratory

### Ferric reducing antioxidant power (FRAP)

Fish sauce samples exhibited FRAP and found to be dependent on protein concentration (Table 3) which are well comparable with synthetic and natural antioxidants (BHA, BHT and  $\alpha$ -tocopherol) at lower (200 ppm) concentration which may be due to peptides formed during early stages of fermentation and such concentration dependent values were also found in Thai fermented shrimp and krill products (Faithong *et al*, 2010). FRAP values for BHA, BHT and  $\alpha$ -tocopherol were 1.234±0.03, 1.090±0.05 and 0.626±0.01, respectively.

### Lipid peroxidation inhibition (LPI) in linoleic acid model system for fish sauce samples

Fish sauce showed inhibition of lipid peroxidation in linoleic acid model system which was dependent on protein concentration ( $p<0.05$ ) (Table 4). Fish sauce samples showed LPI at higher

concentration as compared to that of synthetic antioxidants. BHA, BHT and  $\alpha$ -tocopherol exhibited LPI values of 65.24±1.56, 78.51±0.45 and 70.96±0.94 per cent, respectively. It could be attributed to the ability of peptides in the fermented products to interfere propagation cycle of lipid peroxidation and there by slowing radical mediated linoleic acid oxidation (Giriet *al*, 2012).

### Storage stability of fish sauce

ACE inhibitory activity of anchovy sauce at ambient and refrigerated temperature exhibited values ranged between 80.49±0.91 to 85.37±0.10 with no significant difference in the ACE inhibitory activity for fish sauce samples stored at ambient (27±2°C) and refrigerated (4±2°C) temperatures ( $p>0.05$ ) indicating that all the samples were stable during storage in relation to ACE inhibition. The DPPH radical scavenging activity of fish

**Table 2: DPPH radical scavenging activity of fish sauce samples at different protein concentrations.**

Protein Concentration (mg / ml)	DPPH radical scavenging activity (Per cent)			
	S1	S2	S3	AS
1	89.47±0.32 <sup>a</sup>	88.36±0.21 <sup>a</sup>	53.08±0.52 <sup>a</sup>	74.64±1.65 <sup>a</sup>
3	80.46±1.30 <sup>b</sup>	81.77±1.07 <sup>b</sup>	49.27±2.88 <sup>b</sup>	71.03±1.15 <sup>b</sup>
5	76.37±1.05 <sup>c</sup>	77.89±0.78 <sup>c</sup>	50.94±0.91 <sup>c</sup>	67.98±0.42 <sup>c</sup>

Values are expressed as mean±standard deviations, n=3; Different lower case letters in superscript indicate significant difference between the treatments ( $p<0.05$ ); S1 and S2: Commercial anchovy fish sauce sample made with anchovy (Thai fish sauce and Blue dragon fish sauce); S3: Commercial oyster sauce made from oyster extract (Pantai oyster sauce); AS: Anchovy sauce prepared in the laboratory

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**Table 3: Ferric reducing antioxidant power (FRAP) of fish sauce samples at different protein concentrations.**

Protein Concentration (mg / ml)	Absorbance 700nm			
	S1	S2	S3	AS
1	0.768 (0.049) <sup>a</sup>	0.607 (0.017) <sup>a</sup>	0.833 (0.001) <sup>a</sup>	0.446 (0.001) <sup>a</sup>
3	1.833 (0.004) <sup>b</sup>	0.995 (0.004) <sup>b</sup>	1.876 (0.002) <sup>b</sup>	0.968 (0.002) <sup>b</sup>
5	2.939 (0.002) <sup>c</sup>	2.312 (0.001) <sup>c</sup>	2.926 (0.004) <sup>c</sup>	1.373 (0.001) <sup>c</sup>

Values are expressed as mean±standard deviations, n=3; Different lower case letters in superscript indicate significant difference between the treatments ( $p < 0.05$ ); S1 and S2: Commercial anchovy fish sauce sample made with anchovy (Thai fish sauce and Blue dragon fish sauce); S3: Commercial oyster sauce made from oyster extract (Pantai oyster sauce); AS: Anchovy sauce prepared in the laboratory

**Table 4: Lipid peroxidation inhibition in linoleic acid model system by fish sauce samples at different protein concentrations.**

Protein Concentration (mg / ml)	Lipid Peroxidation Inhibition (%)			
	S1	S2	S3	AS
1	38.55 (3.25) <sup>a</sup>	35.16 (3.61) <sup>a</sup>	47.20 (2.69) <sup>a</sup>	62.73 (0.75) <sup>a</sup>
3	51.24 (2.12) <sup>b</sup>	46.25 (3.10) <sup>b</sup>	55.39 (0.59) <sup>b</sup>	69.76 (0.73) <sup>b</sup>
5	61.22 (1.30) <sup>c</sup>	51.47 (0.57) <sup>c</sup>	63.49 (0.47) <sup>c</sup>	74.33 (0.84) <sup>c</sup>

Values are expressed as mean±standard deviations, n=3; Different lower case letters in superscript indicate significant difference between the treatments ( $p < 0.05$ ); S1 and S2: Commercial anchovy fish sauce sample made with anchovy (Thai fish sauce and Blue dragon fish sauce); S3: Commercial oyster sauce made from oyster extract (Pantai oyster sauce); AS: Anchovy sauce prepared in the laboratory

sauce samples did not show significant variations during storage at different temperatures (Table 5). Similarly no significant changes in ferric reducing antioxidant power of fish sauce samples were noted during storage at different temperatures (Table 6).

### CONCLUSION

Fish sauce samples exhibited the ACE inhibitory activity and could be used as a source of natural ACE inhibitors. Fish sauce contained the mixture of peptides having the ability to transfer H-atom / electron and could be effectively used to terminate the free radical-induced chain reaction and in reduction of ferric ions to ferrous. *In-vitro* significance of fish sauce for prevention of lipid oxidation in food and oxidative stress related diseases have been demonstrated which are the natural sources of antioxidants and could be used

as an alternative source for synthetic antioxidants. The bioactivities of fish sauce were not affected as a function of storage period. This has relevance in promoting the products as beneficial health foods.

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**Table 5: DPPH radical scavenging activity of anchovy sauce at ambient (27±2°C) and refrigerated (4±2°C) temperatures as a function of storage period.**

Protein Concentration (mg / ml)	DPPH radical scavenging activity (Per cent)					
	Storage period (weeks)					
	0		2		4	
	A	R	A	R	A	R
1	74.64±1.65 <sup>c</sup>	74.64±1.61 <sup>c</sup>	69.99±6.64 <sup>a</sup>	69.58±4.01 <sup>a</sup>	69.30±2.11 <sup>a</sup>	71.31±1.65 <sup>b</sup>
3	71.03±1.14 <sup>b</sup>	71.03±1.41 <sup>b</sup>	74.91±6.64 <sup>c</sup>	74.29±1.53 <sup>c</sup>	71.59±0.12 <sup>b</sup>	74.49±0.60 <sup>c</sup>
5	67.98±0.41 <sup>a</sup>	67.98±0.41 <sup>a</sup>	69.37±2.05 <sup>a</sup>	71.52±3.14 <sup>b</sup>	69.68±2.38 <sup>a</sup>	72.69±3.66 <sup>b</sup>

A: Ambient temperature (27±2°C), R: Refrigerated temperature (4±2°C); Values were expressed as mean±standard deviations, n=3; Different small letters in superscript indicates significant difference between the protein concentrations (p<0.05)

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