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CHEMICAL ANALYSIS DURING THE PROCESSING OF DRIED SALTED ANCHOVY

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ABSTRACT

Dried salted anchovy is a rich source of protein which is processed by boiling, salting and drying. During processing the protein undergoes change, e.g. protein myofibril becomes denatured to some extent , and it can be visualised using SDS-PAGE protein pattern. Protein can also react with lipid to form brown colour mainly in high temperature. The result of the analyses shown that there were differences in proximate composition between different samples of anchovy. There were not much difference in sample band pattern for fresh , boiled , boiled and dried anchovy samples. The highest solubility protein was found in the Indonesian dried salted anchovy while the lowest was in fresh sample. The colour of boiled sample was the whitest in comparison to fresh, boiled and dried, Indonesian dried salted anchovy and Japanese boiled. Except for Indonesian dried salted anchovy, brown colour and development of lipid oxidation was not detected.

Key words: Dried salted anchovy, SDS-Page, Browning formation, Lipid oxidation

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INTRODUCTION

Indonesian anchovy known as teri nasi (*Steloporus sp*) is a dried salted commercial exported fish from Indonesia which has high protein value and specific taste and flavour. The demand for this product increases both from South East Asian countries as well as from developed countries such as Japan. In Japan, fish processed into dried and salted fish occupied the third nomination after surimi and frozen product.

In Indonesia anchovy is processed by boiling it in salt solution (3-4 %) and then drying in the open air. Dried fish is left in the open air for just 4-5 hours depending on the water content required by the importing country. Usually anchovy is exported in the form of dried or intermediate moist product.

During processing of dried salted anchovy that involves salting, boiling and drying, the fish can undergo profound changes in the functionality and nutritional quality of protein which are usually undesirable. Thermal denaturation contributes to the alteration of various properties i.e. changes in physical properties which relate to molecular size and shape or conformation (Finley, 1985). Denaturation of myosin (the primary myofibril protein in fish muscle) and sarcoplasmic protein occur to a large extent between 400C and 700C. Fish myofibril decreased in amount and degraded into small protein. The band of protein changes can be visualised using

Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE).

Generally SDS-PAGE is performed to find out the changes in myofibril especially Myosin Heavy Chain (MHC) and Myosin Light Chain (MLC) protein pattern based on migration of protein at a constant rate through a gel of certain pH. In this study, electrophoresis was used to find out the changes in the protein pattern caused by different type of processing e.g. salting, boiling and drying of headless anchovy (*E. japonicus)* or Japanese anchovy and *Stolephorus sp* (Indonesian anchovy).

The density of protein molecular weight (that was visualised by heavy or light band in the SDS-PAGE) can be measured by plotting of log of molecular weight of protein as a function of their migration distance in SDS-PAGE (Copeland, 1994).

Following denaturation, other properties may also change due to exposure to the solvent of specific residues which were previously buried in the inferior native molecules. This will affect the UV absorption spectrum. In this study soluble protein was measured using Biuret method. When protein solution are made strongly alkaline with sodium or potassium hydroxide and then diluted with copper sulphate, a purplish to pinkies violet colour is obtained, the colour depending upon the complexity of the protein. When the protein breaks down to a small compound such as a peptide it will give a very light pink colour (Hames, 1988).

From the denaturated protein, provided sufficient thermal energy is supplied, protein can continue to be degraded or react with other component within the system e.g. the Maillard reaction. This reaction is commonly regarded as the reaction between free amine and carbonyl groups within the food system. The final reaction is melanoidin form which gives brown colour to the food system. In this study brown colour formation was measured using the degree of whiteness samples in the visible wavelength spectrophotometer.

In either the dry or wet salting process, if the concentration of salt is much higher on the outer surface than inside the fish a liquid exudes from the fish meat and consequently some water soluble protein will be lost.

Furthermore the salting and drying process can cause lipid oxidation in fish product due to high concentration of unsaturated fatty acid. The Thiobarbituric Acid (TBA) test is one of the more commonly used method for the detection of lipid oxidation (Gray, 1978). This mechanism was based on investigations which showed that no colour developed for linoleate even at peroxide values of 2000 or greater, but that for fatty acid with tree or more double bonds the molar yield of the TBA colour increased with the degree unsaturation. A red colour appears as a result of the reaction of malonaldehyde with TBA reagent.

In terms of quality, this work was carried out to investigate the effect of processing to the proximate composition, SDS-PAGE of protein, soluble protein, browning colour formation and lipid oxidation.

MATERIAL AND METHODS

Raw Material Preparation

Engraulis japonicus (Japanese anchovy) was supplied by Tokyo University of Fisheries. Anchovies were then prepared according to Indonesian traditional dried salted anchovy method : boiling the fish in 3% salt solution and oven-drying it at 400C for 5 hours. It was assumed that Indonesian open air temperature was 400C.

The sample used were fresh anchovy (1), boiled anchovy (2), the boiled and dried salted anchovy (3), Indonesian dried salted anchovy that was brought from Indonesia as Indonesian

dried salted anchovy (4) and boiled Japanese anchovy bought at Japanese supermaket as Japanese boiled anchovy (5).

Methods of Analysis

Proximate Analysis

All samples were analysed for their protein, moisture and ash content by standard analytical procedures of AOAC (1970). Protein as the total nitrogen content was determined by the Kjeldahl method. Lipid content was determined by oven drying at 1050C to a constant weight. Ash was determined by placing the sample in a muffle furnace of 5250C for about five hours to incinerate until the sample was free from carbon particles.

SDS-PAGE

SDS-PAGE was performed using the modification method of Laemmli (1970). The sample (40 mg) protein was made soluble in 7.5 ml of 2% SDS 8 M urea – 2% merkaptoetanol 20 mM Tris HCl (pH 8.8) by heating in boiling water for 2 minutes. The dissolved sample was dialyzed against 0.1 % SDS – 10 mM Tris HCl (pH 6.8) after stirring overnight at room temperature. The dialyzate was then centrifuged, injecting 20 ul and 40 ul of protein to SDS-PAGE. The gel was conducted in the presence of 0.1% SDS using 4.5 – 12.5% polyacrylamide gel. The protein components were stained with 0.25% Coomassie brilant blue R 250, then destained with 10% isopropanol and 7% acetic acid glacial solution. The molecular weight stained protein bands were determined by plotting of log (molecular weight) of protein as a function of their migration distance in SDS-PAGE (Copeland, 1994). A molecular weight standard (SDS-7 and SDS-6) from Sigma containing myosin heavy chain (205 kDa), β-galaktosidase (116 kDa), phosphorylase-b (97.4 kDa), bovine serum albumin (66 kDa), egg albumin (45 kDa), glyceraldehide (36 kDa), pepsin (34.7 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), trypsin inhibitor (20 kDa) β-lactoglobulin (18.4 kDa), and bovine milk (14.2) was used.

Protein Solubility by Biuret Method

The content of protein in the different samples subjected to SDS-PAGE were measured by Biuret method. Sample preparation was done by dilution of 100 mg sample into 10 ml NaCl 0.5 M. The solution was homogenised and centrifuged at 3500 rpm for 15 minutes, then filtrated. The filtrate was measured using a Hitachi U-1100 Spectrophotometer at 500 nm. Factor can be calculated by plotting the absorbance of 5 mg/ml in the Biuret standard curve.

Protein Solubility in sample:
Absorbance X 6.25 X Dilution X Factor mg/ml sample

Brown Colour Discoloration

The development of brown colour discoloration during the Mailard Reaction was measured using Spectrophotometer at 420 nm. The sample was prepared by dissolving 40 mg headless anchovy in 7.5 ml of 20 mM Tris HCl buffer at pH 8.8 containing 2 % SDS, 8 M urea and 2 % merkaptoetanol, while the degree of whiteness from the samples was measured using Spectrum Colour Sensor (Shimadzu, Japan). The surface colour of different samples were measured and colour parameter were calculated by the L*, a*, and b*, colour system.

Whiteness was calculated using the following equation:
$100 - \{(100 - L^*)2 + a^*2 + b^*2)\}1/2$

Thiobarbituric Acid Test (Sinhuber and Yu, 1977)

0.5 g sample was introduced into a tarred test tube and accurately weighed. Three drops of mixture between BHA and BHT were added following 3 ml of 1 % TBA solution to the test tube followed by adding 17 ml TCA-HCl mixture. The tube was flushed with N2 and then the screw cap was tightly closed. A blank sample was prepared using another tube containing the same quantity of reagent, but without the sample. The tubes were placed in a tube basket and heated in boiling water for 30 minutes. The tubes were then cooled to room temperature using water, and then 15 ml of the colour solution were transferred into a 50 ml conical centrifuge tube. Approximately 5 ml of chloroform was added and then mixed for a few seconds with a vortex mixer. The tube were centrifuged for 10 minutes at 3000 rpm. A part of the aqueous clear colour solution was transferred into a 1 cm cuvet for absorbency measurement at 532 nm in a Hitachi U-1100 Spectro-photometer.

TBA value (mg/kg malonaldehyde) = (Absorbance – 0.0434) / 0.988.

RESULT AND DISCUSSION

Proximate Composition

The proximate composition of different samples are presented in Table 1. It can be seen that the fresh anchovy has a high value of water content, namely about 82 %. After being boiled in salt solution' the fish meat fish still has a high water content, since salt concentration was very light. Water could not be pressed out of the meat. Based on the moisture content, boiled anchovy and Japanese boiled anchovy still have high value of water content and it is highly possible to be spoiled in comparison with Indonesian dried salted anchovy. The protein content of anchovy can also be categorized as a high protein. Lipid content from the fresh to dried sample ranged between 1.30%-4.33% , meaning that anchovy species have a high lipid content. The ash content of the sample however, show high values which resulted from the presence of the bones, head and salt addition.

Table 1. Proximate composition of different anchovy samples. (*)

Sample	Water	Protein	Lipid	Ash
Fresh anchovy	82.10± 0.30	11.60±0.40	1.30±0.03	1.32±0.07
Boiled anchovy	80.06±0.71	12.81±0.24	1.55±0.06	1.05±0.06
Boiled and dried salted anchovy	62.80±1.96	29.60±0.12	2.78±0.24	1.28±0.11
Indonesian dried salted anchovy	31.70±0.66	49.62±1.49	4.33±0.35	11.64±0.14
Japanese boiled anchovy	65.80±0.24	23.01±0.52	2.78±0.26	3.71±0.07

Note:
- Values are means of three replicates ± SD
- Based on wet weight basis.

SDS-PAGE Pattern

Changes in myofibril protein pattern during processing in various sample were examined by polyacrylamide gel electrophoresis with SDS (Figure 1). There were no wide characteristic differences in the myofibril protein pattern especially myosin heavy chain among the fresh, boiled , boiled and dried, Indonesian dried salted anchovy and Japanese boiled samples. It was interesting that Indonesian dried salted anchovy demonstrated similar protein pattern on both myosin heavy chain and myosin light chain to Japanese boiled anchovy (figure 1).

Myofibril protein is the protein which forms myofibril and it covers 66-77% of the total protein in fish. This protein contains myosin and actin which can also exist as actomyosin (Suzuki, 1981). Myosin consists of both myosin heavy chain (MHC) and light chain (MLC). Myofibrillar protein also contains regulating protein such as tropomyosin, troponin, and actin. Myosin heavy chain possesses a molecular weight of 200 kDa, Actin 43 kDa, while Myosin light chain has 25-16 kDa, sub unit tropomyosin 34 kDa.

In the case of 12.5% acrylamide gel, all samples (fresh, boiled, boiled and dried, Indonesian dried sample, Japanese boiled anchovy) performed a heavy band pattern in the upper gel pattern. Based on molecular weight in comparison with SDS 6 marker it was considered as Myosin heavy chain that has a molecular weight of 205 kDa. Some light patterns also appeared in the upper gel. Those SDS patterns indicated that myosin heavy chain has polymerization. These polymerization occurred due to the applied processing methods. During processing endogenous enzymes break down myosin heavy chain into a small compound which has a molecular weight of 120 kDa – 150 kDa (Kataoka *et al*, 1998).

At the lower part of gel on SDS-PAGE (for 5 different samples respectively) there was a heavy band that had been determined to have a molecular weight of 44 kDa (from the calculation by plotting distance of molecular weight). The Indonesian dried salted anchovy and Japanese boiled anchovy showed a rather heavy band . This was assumed as an actin filament which maybe dissociated with tropomyosin. In the samples code of 1, 2 and 3 ; actin and troponin component were less influenced by denaturation of protein because of the processing methods.

The total mobility of sample was at 5.3 cm. It was indicated by BPB distance travelled in SDS 7 marker (figure 1) . While the relative mobility (Rf) for 1^{st}, 2^{nd}, 3^{th}, 4^{th}, 5^{th}, 6^{th}, 7^{th} band appeared in the marker were 1.6 cm, 2.4 cm, 2.95 cm, 3.6 cm, 3.9 cm, 4.7 cm and 5.2 cm (Figure 2) . Hence the molecular weight determination of protein band was calculated as follows :

Molecular Weight Standards using SDS-7 Marker (Figure 2)

1. 1.6 / 5.3 = 0.3018 $\rightarrow$ 66 kDa, log = 4.81 (Bovine serum albumin)

2. 2.4 / 5.3 = 0.4528 $\rightarrow$ 45 kDa, log = 4.65 (Egg albumin)

After plotting in the graph paper, the heavy band in the sample appeared at a distance of 2.2 cm and 44 kDa was estimated as an Actin

3. 2.95 / 5.3 = 0.5566 $\rightarrow$ 36 kDa, log 4.55 (Glyceraldehide-3-P Dehydrogenase)

After plotting in the log graph paper heavy band in the sample appeared at a distance of 2.75 cm and 34 kDa was estimated as a tropomyosin sub unit

4. 3.6 / 5.3 = 0.6792 $\rightarrow$ 29 kDa, log = 4.46 (Bovine Carbonic anhydrase)

5. 3.9 /5.3 = 0.7358 $\rightarrow$ 24 kDa , log = 4.38 (Trypsinogen, bovine pancreas)

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6. 4.7 / 5.3 = 0.8860 → 20 kDa, log = 4.30 (Trypsin inhibitor, soybean)

7. 5.2 / 5.3 = 0.9811 → 14.2 kDa, log = 4.15 (Bovine milk, α lactalbumin)

Mobility of gel (Rf) (X):
Rf (X) = 0.9811 – 0.3018 = 0.6

Molecular Weight Standarts Using SDS-6 Marker (Figure 3)

1. 0.4 / 5.3 = 0.075 → 205 kDa, log = 2.31 (Myosin, rabbit muscle)

2. 0.8 / 5.3 = 0.1500 → 116 kDa , log = 2.06 (β- Galactosidae, *E. Coli*)

3. 1.1 / 5.3 = 0.2000 → 97.4 kDa, log 1.98 (Phosphorylase b, rabbit muscle)

4. 1.7 / 5.3 = 0.3200 → 66 kDa, log = 1.81 (Albumin, Bovine)

5. 2.4 / 5.3 = 0.4500 → 45 kDa, log = 1.65 (Albumin, egg)

6. 2.95 / 5.3 = 0.5600 → 29 kDa, log = 1.46 (Carbonic anhydrase)

Mobility of the gel (Rf) (X):
Rf (X) = 0.67 – 0.075 = 0.085

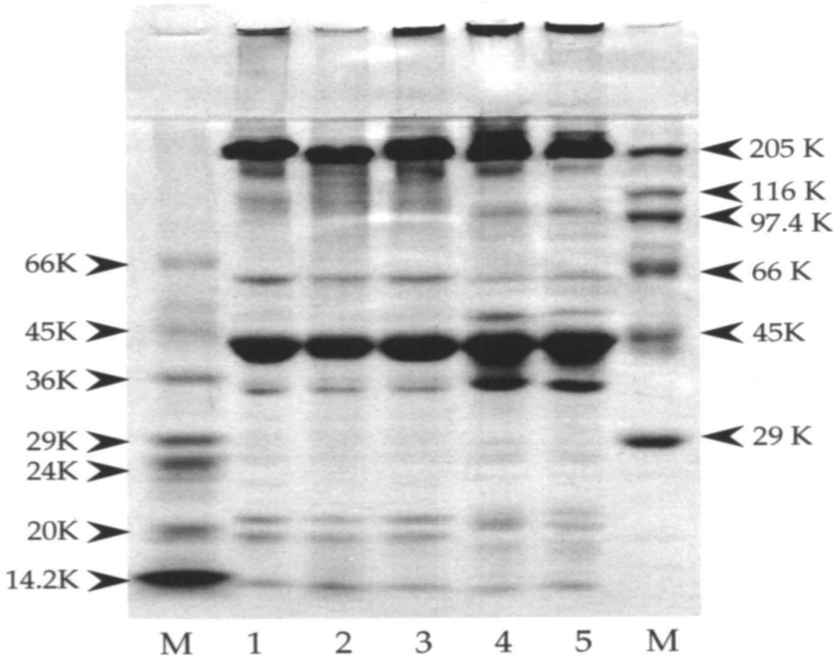

Figure 1: SDS-PAGE Patterns of Anchovy proteins
(M Left lane : molecular weight marker SDS 7 ; 1. Fresh; 2. Boiled ; 3. Boiled and Dried ; 4. Indonesian dried ; 5. Japanese boiled ; M Right lane : molecular weight marker SDS 6)

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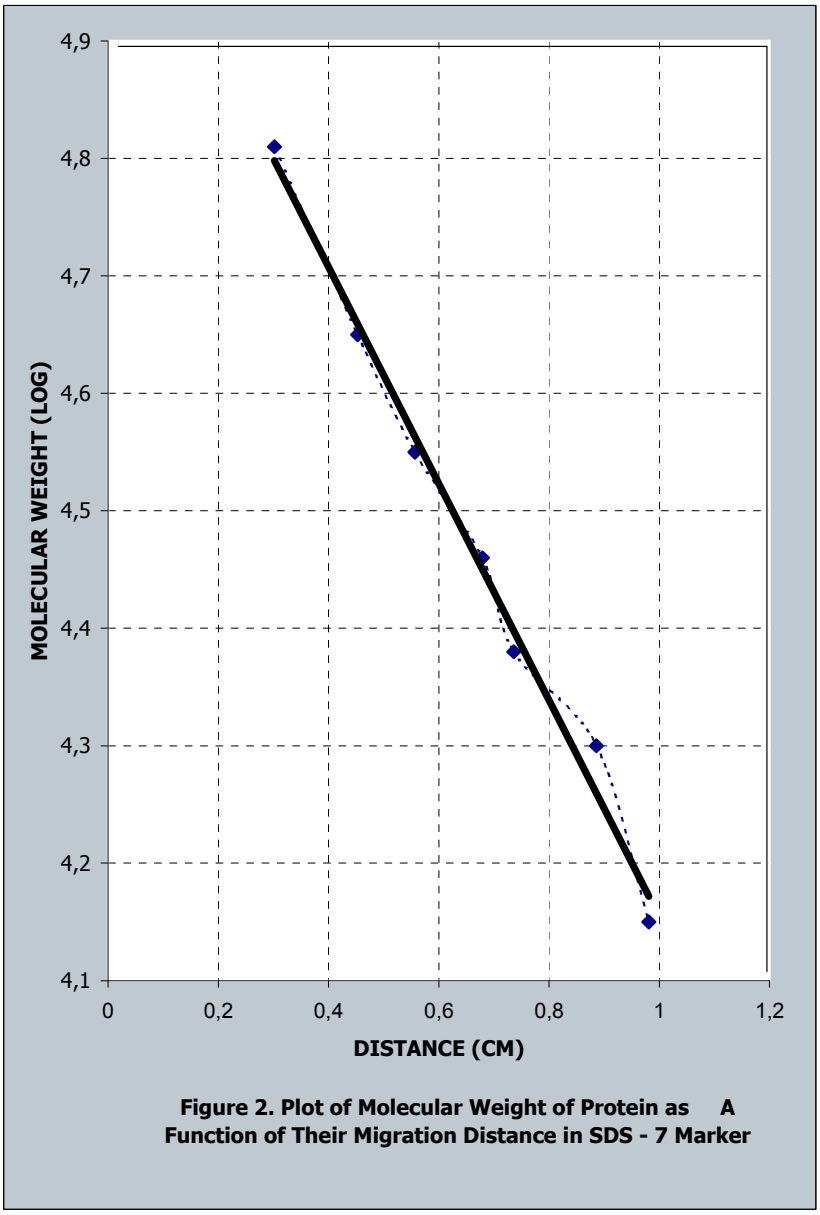

Figure 2. Plot of Molecular Weight of Protein as A Function of Their Migration Distance in SDS - 7 Marker

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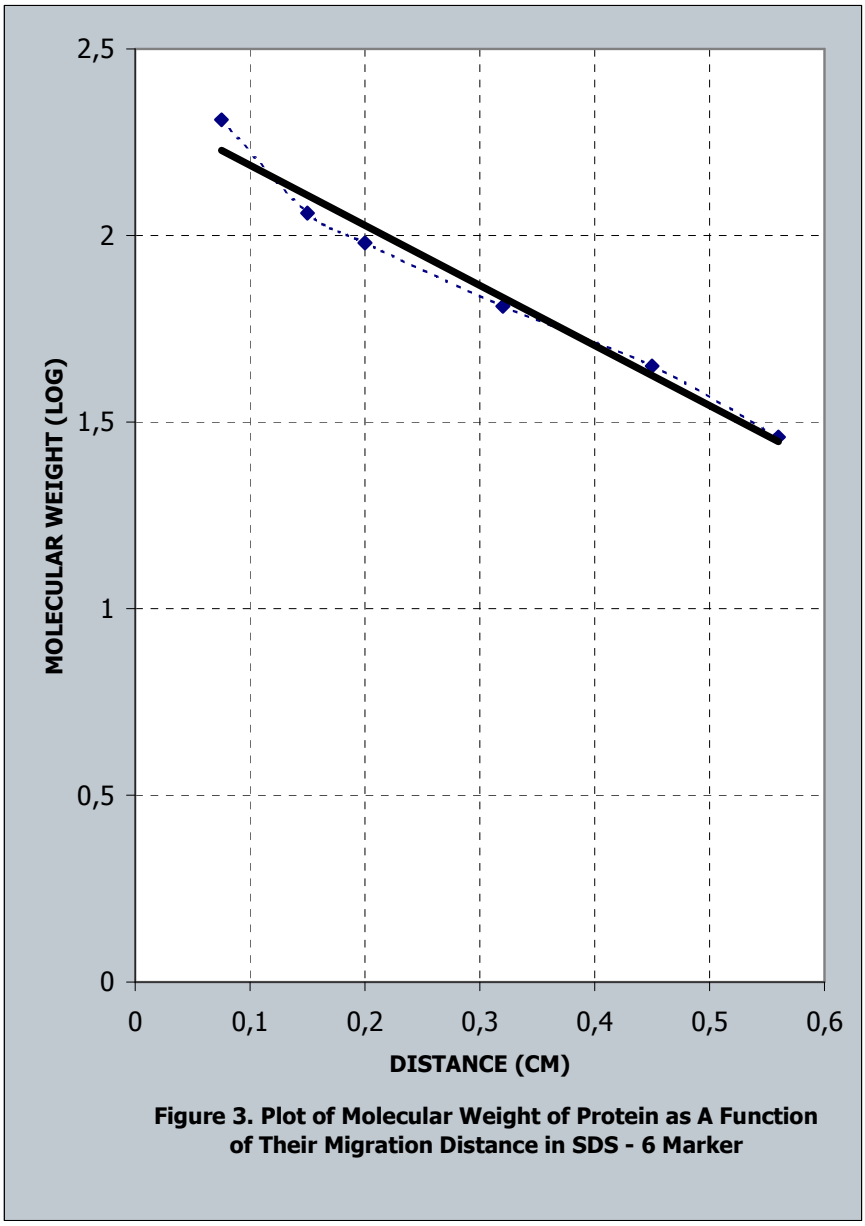

Figure 3. Plot of Molecular Weight of Protein as A Function of Their Migration Distance in SDS - 6 Marker

Protein Solubility

The amount of protein subjected to SDS-PAGE is presented in Table 2.

The soluble protein was 5 mg/ml in the fresh anchovy and the highest value was 13.0 mg/ml in the Indonesian dried salted anchovy. In both boiled samples the value of soluble protein were lower than the dried sample. The highest protein soluble means the lowest quality, since most of protein could not be made soluble. There was a significant difference ($p < 0.01$) between samples. These findings indicated that the drying process would result in the increase of protein solubility in the sample. The increase in the solubility of protein could be attributed mostly to the degradation of myosin in the smaller unit which has lighter molecular weight.

Effect of browning colour formation on the degree of whiteness of anchovy

Measurement of whiteness have been done in two experimental ways.In the first experiment, the whole fish body was used as a sample; in the second experiment anchovy head are removed and flesh body only was used as a sample.

Table 2. Protein Solubility in sample

Sample	Absorbance I	Absorbance II	Protein soluble mg/ml
Fresh anchovy	0.192	0.192	5.0±0.00
Boiled anchovy	0.192	0.197	6.0±0.04
Boiled and dried	0.376	0.371	11.5±0.04
Indonesian dried salted anchovy	0.431	0.433	13.0±0.01
Japanese boiled anchovy	0.284	0.271	8.5±0.01

Note:
- Values are means of duplicates ± SD

Table 3. Anchovy sample whiteness

Sample	Whole anchovy	Anchovy flesh body only
Fresh anchovy	29.00±0.89 (blackish colour appearance)	38.00±1.53 (little bit black appearance)
Boiled anchovy	60.85±1.85 (white appearance)	69.63±0.81 (white appearance)
Boiled and dried anchovy	49.21±1.1 (little black appearance)	66.23±0.78 (white colour appearance)
Indonesian dried salted anchovy	56.84±0.45 (little bit black appearence)	54.41±1.23 (little bit black appearance)
Japanese boiled anchovy	51.47±1.81 (little black appearance)	67.16±0.52 (white appearance)

Note:
Values are means of three replicates ± SD

The highest value of whiteness was achieved in muscle boiled and dried salted anchovy. While the lowest was in whole fresh sample. The degree of whiteness were significantly different between the sample ($p < 0.01$). It was deducted that in the fresh sample especially in the whole anchovy enzymatic activities still worked and developed a black colour in the anchovy flesh. During processing e.g. salting, boiling and drying, enzymatic activities would stop. From the result it could be assumed that the boiling process would increase whiteness in

boiled anchovy and Japanese boiled anchovy, whereas the drying process decreased whiteness of the sample.

The degree of sample whiteness was expected to have negative correlation to the browning development (data presented in Table 4). In this study the lowest sample whiteness i.e fresh material was the lowest browning colour formation. Brown colour was highly developed in the Indonesian dried salted anchovy processed through salting, boiling and drying under the sun. This suggests that the colour developed more rapidly during the drying rather than the boiling process as reported by Fujimaki (1975).

Table 4. Browning Colour Formation (mg/ml)

Sample	Absorbance I	Absorbance II	Absorbance III	Browning mg/ml
Fresh anchovy	-0.016	-0.016	-0.017	Not determined
Boiled anchovy	0.020	0.020	0.020	0.20±0.01
Boiled and dried	0.173	0.176	0.176	0.174±0.01
Indonesian dried salted anchovy	0.484	0.489	0.478	0.483±0.01
Japanese boiled anchovy	0.107	0.108	0.109	0.108±0.01

Note:
Values are means of three replicates ± SD

Lipid Oxidation

The value of lipid oxidation in various samples are presented in Table 5. During processing and storage TBA was not detected in samples except for Indonesian dried salted anchovy. In this sample TBA formed only in small amount. An Indonesian sample was even dried in the open air (sun drying) and susceptible to oxygen attack to develop lipid oxidation. Data presented in Table 5 indicates that oxidation process less occurred less in the sample due to the absence of accelerating factors such as high temperature, so that the formation of peroxide is also slow.

Table 5. Thiobarbituric Acid Test (TBA Value) (mg MA/kg sample)

Sample	Absorbance I	Absorbance II	TBA value Mg MA/kg
Fresh anchovy	0.027	0.047	Not determined
Boiled anchovy	0.012	0.016	Not determined
Boiled and dried anchovy	0.027	0.030	Not determined
Indonesian dried salted anchovy	0.057	0.046	0.082 ± 0.01
Japanese boiled anchovy	0.016	0.025	Not determined

Note:
Values are means of duplicates ± SD

CONCLUSION

In conclusion, anchovy could be categorized as fish with high protein and high lipid content . By using 12.5% acrylamide gel, it was clearly shown that two heavy protein bands appeared at fresh, boiled, boiled and dried, Indonesian dried salted anchovy, and Japanese boiled anchovy, respectively. The first heavy band positioned at 205 kDa was estimated as a myosin heavy chain. The second

heavy chain band had a high molecular weight of 45 kDa and was determined as an actin.

Indonesian dried salted anchovy has the highest soluble protein in comparison with the others. This solubility increased due to salting, boiling and drying processes. These processes also has significant effect on brown colour development and less effect on lipid oxidation.

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