Protein Hydrolysis and Proteinase Activity during the Ripening of Salted Anchovy (*Engraulis encrasicholus* L.). A Microassay Method for Determining the Protein Hydrolysis

M. Manuela Hernández-Herrero,* Artur X. Roig-Sagués, Emilio I. López-Sabater,†
José J. Rodríguez-Jerez, and M. Teresa Mora-Ventura

Laboratori d'Higiene, Inspecció i Control dels Aliments, Facultat de Veterinària, Universitat Autònoma de Barcelona (U.A.B.), 08193 Bellaterra, Barcelona, Spain

The protein hydrolysis and proteinase activity during the ripening of salted anchovy were studied. A rapid, simple, and inexpensive microassay method for determining the protein hydrolysis by trinitrobenzenesulfonic acid (TNBS) has been developed. A linear relationship was observed between proteolysis determination by the TNBS method and ripening time in the fish muscle and in the brine ($r = 0.99$). A linear relationship was also observed between the ratio nonprotein nitrogen and total nitrogen (NPN/TN) and ripening time ($r = 0.98$). Proteolysis by the TNBS method and NPN/TN determination could be considered as objective methods to follow and assess the ripening process of an anchovy. A value of proteolysis by the TNBS method of 240 mM leucine in the fish muscle and/or 200 mM leucine in the brine would indicate the ripening point. The crude enzyme prepared of fish muscle and brine showed that alkaline proteinases dominate.

Keywords: *Anchovy; proteolysis; TNBS; proteinase activity; ripening*

INTRODUCTION

Ripened semipreserved anchovies are prepared from fish of the *Engraulis encrasicholus* (L) species, belonging to the Clupeidae family, by a process of salting and ripening. This process goes back to ancient times and it is a common tradition in some Mediterranean countries and in Argentina, where the species used is *Engraulis anchoita* (Triqui and Reineccius, 1995). Salting of anchovies can be divided into two stages: the first corresponds to the diffusion of salt into the fish and elimination of water from it. The second stage, longer and slower than the first, is ripening which renders a product with tender consistency and the characteristic pleasant aroma and taste (Filsinger, 1987). The ripening process takes at least 2 months for *E. encrasicholus* of the Mediterranean Sea (Campello, 1985) and 8−12 months for *E. anchoita* (Filsinger et al., 1982) at a temperature between 16 and 20 °C.

Ripening involves a series of complex biochemical processes, which can be grouped broadly into proteolysis, lipolysis, and lipid oxidation. The physical and chemical changes that occur during ripening determine the overall organoleptic qualities of salted anchovy. Such organoleptic qualities are traditionally assessed by sensory evaluation of the flavor, odor and texture, and the overall quality of salted anchovy by experienced judges or trained consumer panels. However, personal preferences of judges and their inability to assess a wide range of samples without taste fatigue make sensory evaluation subjective. Sensory analysis is essential in assessing salted anchovy quality before marketing, but it is inadequate when used alone to compare research data from different laboratories. The fish industry is interested in developing indices of salted anchovy ripening and quality that might supplement sensory evaluation, although is recognized that such indices cannot be used to assess quality.

Some attempts have been made to develop a suitable method such as using the total ester index (Filsinger et al., 1982), the evolution of basic volatile total nitrogen (Filsinger et al., 1984; Hernandez-Herrero, 1997), the ratio of free amino acids to total amino acids (Baldrati et al., 1977), the ratio of nonprotein to total nitrogen (Campello, 1985; Durand, 1982; Perez-Villarreal and Pozo, 1992), or the estimation of free fatty acids (Roldan et al., 1985). However, these parameters have not been very successful in predicting the quality of the final product or the stage at which anchovy develops optimum flavor and should go to market. As a result, visual inspection and tasting are still the usual methods of following the process.

It is recognized that the ripening of salted anchovy takes place via enzymatic pathways. The importance attributed to tissular enzymes versus microbial enzymes is controversial. Research has indicated that intestines of the anchovy are particularly important. Partial evisceration, as practiced in the factories, eliminate part of the digestive enzymes; otherwise a bitter taste develops in the fish (Durand, 1982). However, a perfect evisceration with rigorous rinsing leads to slower ripening, and the fish does not acquire the characteristic flavor (Alm, 1965; Voskresensky, 1965; Durand, 1982). The concentration of enzymes vary with the season. Fish anchovy is caught in the period from May to August (during the heavy-feeding stage) when it contains sufficient enzyme to promote satisfactory ripening. The traditional ripening procedure is time-consuming and

* Author to whom correspondence should be addresssed [telephone (34) 93 5811460; fax (34) 93 5812006; e-mail mhernandez@quiro.uab.es].

† Departament de Ciencia i Tecnologia dels Aliments, Campus de Miramarges, Sagrada Familia 7, Universitat de Vic, 08500 Vic, Barcelona, Spain.

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demands a large storage capacity. However, enzymes could be added to accelerate the ripening process. Several methods involving enzyme addition have been patented. The main advantage with the traditional long-time storage method is that enzyme activities gradually decline during storage (ripening and shelf life), giving a quite stable final product with very little residual enzyme activity. To obtain stable products with rapid methods, it is necessary to reduce the residual activities as much as possible (Gildberg, 1993).

This investigation was undertaken mainly to study the protein hydrolysis and proteolytic activity during the ripening of salted anchovy and to study the possibility of using some of these parameters as an objective method to follow and assess the anchovy ripening process.

MATERIALS AND METHODS

Preparation of Anchovy Samples. Fifteen batches of ripened semipreserved salted anchovies (*Engraulis encrasicholus* subsp. *mediterraneus*) were analyzed. Fresh anchovy were purchased from the local landing center (Barcelona, Spain). These fish samples were caught in the Northeastern Spanish Mediterranean coast. After being caught, the fish were held with ice for at least 24 h before they arrived at the laboratory where they were prepared following the traditional method described below. The fish samples were manually beheaded, partially gutted in the same operation, and packed in 500-cm^3 cans. A layer of salt was first put in the container then a layer of fish and so on until the container was filled with alternate layers of salt and fish finishing with a layer of salt. Cans were stored for ripening at 20 °C for 9 weeks. Samples were removed every week from the same processing batch until the ripening phase was finished.

Sensory Analyses. Sensory analyses of the ripening process were determined according to Filsinger et al. (1982). Five parameters were analyzed: flesh adherence to backbone, odor, color, flavor, and texture. The maturation scale has its minimum at zero, representing sensory characteristics of raw fish, just before the beginning of the ripening process. The scale reaches the scale maximum at point 8, which means an overripening stage. Point 6 expresses the optimum level of ripening.

Chemical Analyses. *Determination of Degree of Hydrolysis of Protein by Trinitrobenzenosulfonic Acid (TNBS).* Basically, the TNBS method is a spectrophotometric assay of the chromophore formed by the reaction of TNBS with primary amines. The reaction takes place under slightly alkaline conditions (Adler-Nissen, 1979). TNBS also reacts slowly with hydroxyl ions whereby the blank reading increases; this increase is stimulated by light (Fields, 1971).

1. Preparation of Samples. The fish muscle samples were solubilized as follows: 10 g of fish muscle was homogenized with 90 mL of buffer (Borax buffer: 2% (w/v) of sodium dodecyl sulfate (SDS), 0.477 (w/v) $Na_2B_4O_7 \cdot 10H_2O$, pH 8.9 adjusted with HCl) and was shaken thoroughly in screwed-capped 100-mL flasks in a thermostatic bath at 75 °C for 15 min (to prevent proteolytic degradation) and then at 60 °C for 2 h. Thereafter, this solution was diluted 10 and 100 times with standard buffer. The sample should be dispersed in hot sodium dodecyl sulfate rather than buffer alone. The brine samples were obtained by filtration through a No. 1 Whatman filter paper. Then, this solution was diluted 100 and 200 times with distilled water.

2. Microtiter Plate Assay. The following reagents were used: 0.2125 M phosphate buffer (0.2125 M NaH_2PO_4 is added to 0.2125 M Na_2HPO_4 until pH is 8.2 ± 0.02) and 0.1% TNBS solution (covered with aluminum foil; the solution must be prepared immediately before use); leucine standard in Borax buffer or distilled water was prepared regularly.

The reaction was started by the addition of 10 μL of a sample containing between 0.25 and 4 mM of standard solutions, 80 μL of phosphate buffer, and 80 μL of 0.1% TNBS solution. After incubation at 42 °C, the absorbance at 405 nm was directly read using a microtiter plate reader 340 ATTC (STL Labinstruments, Salzburg). Readings were realized every 5 min for 1 h. All the experiences were repeated 8 times.

Nitrogen Compounds. Total nitrogen (TN) was determined in 0.5 g of fish muscle samples and 3 mL of brine using the macro-Kjeldahl method (Ministerio de Sanidad y Consumo, 1985). Nonprotein nitrogen (NPN) was extracted using trichloroacetic acid (TCA). To determine NPN, 25-g portions of fish muscle and 10 mL of brine were homogenized with 25 and 10 mL of 7.5% TCA, respectively, and centrifuged at 3000 rpm for 10 min. The supernatant was filtered through a No. 1 Whatman filter paper. The precipitate of fish muscle and brine was suspended with 25 and 10 mL of 7.5% TCA, respectively, and centrifuged again. The supernatant was filtered and combined with the first supernatant. Ten milliliters of this solution was used for the measurement using the macro-Kjeldahl method mentioned above.

Proteinase Activity. Ten grams of fish muscle was homogenized with 100 mL of cooled distilled water. The homogenates were centrifuged (17000*g*, 15 min at 4 °C) and the soluble proteinase activity was determined in the supernatants. The enzyme extracts were stored at −18 °C. The brine extracts were obtained by filtration through a No. 1 Whatman filter paper.

Total proteinase activity was measured using casein or haemoglobin as the substrate. A typical assay was performed as follows: 1 mL of enzymatic extract and 3 mL of casein or haemoglobin (20 mg/mL of appropriate buffer) were mixed and incubated at 50 °C under the specific conditions of the experiment (pH and NaCl concentration). The reaction was stopped at time 0 and 60 min by adding 1 mL of mixed solution to 4 mL of 2% SDS for the assay with fish muscle extracts and 0.5 mL of mixed solution to 9.5 mL of 2% SDS for the assay with brine extracts. Thereafter, solutions were heated at 75 °C for 15 min (for inhibiting the proteolytic enzymatic activity). The effect of pH on the protein hydrolysis was tested with 0.1 M sodium acetate−HCl (pH 3 and 5.8) and 0.1 M Tris−HCl (pH 9). The effect of NaCl concentration (18%) on the total proteinase activity was carried out by using the buffers with adequate concentrations of NaCl. Protein hydrolysis was determined by the aforementioned microassay method. One unit (*U*) was defined as the amount of enzyme that liberates 1 μM of leucine/min.

Statistical Analyses. Analyses of variance or Kruskal−Wallis analyses were performed, previously verifying continuous distribution and variance homogeneity (Domenech and Riba, 1990) to determine the differences between the experimental periods of storage. Regression analysis was performed to obtain the correlation between variables. All statistical analyses were performed using the Statistical Package for Social Sciences (SPSS for Windows, SPSS Inc., Chicago, IL).

RESULTS AND DISCUSSION

Proteolysis by the TNBS Method. Proteolysis results in the formation of new α-amino groups, which can be measured by the reaction of anchovy extracts with TNBS reagents. This method is specially suitable for checking of blocking or unblocking of amino groups in proteins and peptides (Fields, 1971; Hatakeyama et al., 1992). An assay for proteolysis determination using TNBS has also previously been reported by Adler-Nissen (1979). This author performed the reaction of TNBS for 60 min at 50 °C and the absorbance was measured at 340 and 420 nm. In our study, it was concluded that a period of at least 60 min at 42 °C should elapse to ensure reproducible results (Figure 1). Moreover, measurements were done at 405 nm since this is one of the wavelengths available for the microtiter plate reader that we used, and absorption of the products is comparably high enough to be determined

Table 1. Sensory Analyses and Protein Hydrolysis during the Ripening of Salted Anchovy[a]

	weeks of ripening									
	0	1	2	3	4	5	6	7	8	9
sensory analyses	0.00	2.87	3.74	3.96	4.75	5.11	5.34	5.79	5.91	6.03
TNBS muscle (mM leucine)	135.06	132.96	145.26	158.91	168.26	186.96	201.23	214.45	221.53	238.59
TNBS brine (mM leucine)		85.90	113.18	122.27	133.71	148.19	160.81	174.64	190.02	198.90
protein nitrogen muscle (%)	2.71	3.27	3.24	3.12	3.06	3.02	2.92	2.89	2.91	2.85
protein nitrogen brine (%)		0.14	0.16	0.13	0.15	0.15	0.11	0.12	0.09	0.09
NPN muscle (%)	0.68	0.53	0.57	0.57	0.61	0.67	0.66	0.69	0.73	0.75
NPN brine (%)		0.47	0.50	0.57	0.60	0.65	0.64	0.63	0.65	0.66
NPN/TN muscle(%)	20.06	13.32	15.14	15.53	16.60	18.26	18.04	19.17	20.15	20.85
NPN/TN brine (%)		75.93	78.54	80.25	79.70	80.88	84.14	85.08	87.97	88.48

[a] On all cases, 15 samples were tested.

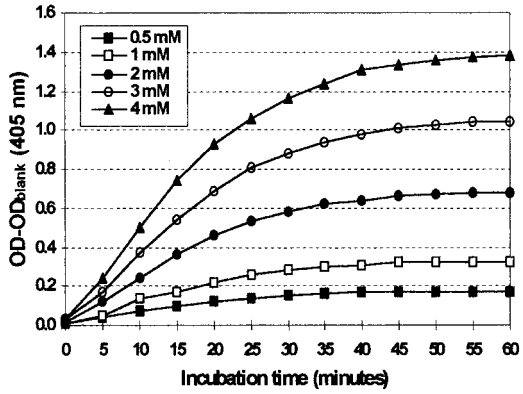

Figure 1. Absorbance evolution with different leucine concentrations incubated at 42 °C for 60 min.

at that wavelength. On these conditions, the linearity of the test was observed between 0.25 and 4 mM of leucine and the correlation was very significant after a regression analysis of the variance ($r = 0.9997$ and $p < 0.01$). Incorporation of SDS in the TNBS method has been reported by Adler-Nissen (1979), who used this agent for denaturing protein in the samples. The use of mercaptoethanol to prevent protein aggregation is ruled out because this agent reacts with TNBS. Reaction of a cheese extract with TNBS is a common method for determining the concentration of free amino groups in cheese. However, the TNBS method has been criticized as it underestimates the level of proteolysis in cheese containing high levels of ammonia, with which TNBS reacts slowly (Farkye and Fox, 1990).

The protein hydrolysis of fish muscle decreased slightly from 135 to 133 mM of leucine during the first week of the ripening process because nitrogenous substances chiefly of low molecular weight diffuses from the fish into the brine. After the first week, protein hydrolysis of fish muscle and brine increased gradually ($p < 0.05$) throughout the ripening time (Table 1). A higher correlation was obtained between fish muscle proteolysis and time ($r = 0.991$; $p < 0.01$). However, this correlation is higher ($r = 0.998$; $p < 0.01$) when it was only considering the ripening process between the first and ninth week (Figure 2). Moreover, a linear relationship was also observed between the proteolysis of the brine and ripening time ($r = 0.995$; $p < 0.01$) (Figure 2). Consequently, proteolysis determination by the TNBS method in the fish muscle and in the brine would allow one to follow and assess the ripening process of the anchovy. A value of 240 mM leucine in the fish or 200 mM leucine in the brine would indicate the ripening point. Proteolysis determination in the brine would allow one to obtain the samples by nondestructive methods. Moreover, the use of microtiter wells allows

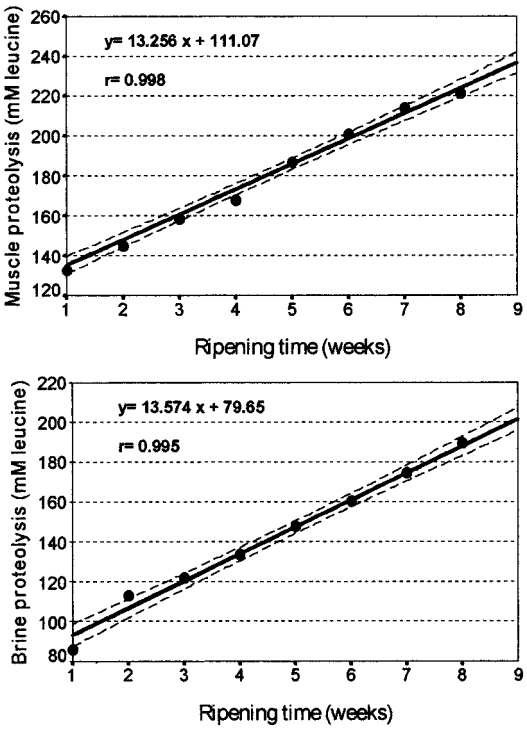

Figure 2. Protein hydrolysis determined by the TNBS method in fish muscle and brine versus time during the ripening of salted anchovy. The area between the dashed lines is the 95% confidence limits of the regression estimate.

large numbers of samples to be assayed; the amounts of the samples and the reagents are small, and the following measurement of absorbance can be rapidly and simultaneously done by using a microtiter plate reader. The following expression relates the proteolysis quantification (x) of fish muscle (1) and brine (2) to the time (y):

$$y = 13.256x + 111.07 \quad r = 0.998 \qquad (1)$$

$$y = 13.574x + 79.65 \quad r = 0.995 \qquad (2)$$

Nitrogen Compounds. The protein nitrogen (PN) content in the fish muscle decreased markedly ($p < 0.05$) after 6 weeks and then gradually to 2.85% after 9 weeks. However, the PN content in the brine remained constant after 6 weeks and then decreased appreciably until the ninth week (Table 1). After 9 weeks, 88% of the total nitrogen of the brine was NPN compounds. Protein gradually loses their colloidal properties and diffuses from the fish into the brine, where they undergo further changes. Despite a considerable protein degradation, the ripened anchovy maintains its structure and is easily cut into fillets.

Table 2. Proteinase Activity (*U*/mg of protein) in Fish Muscle and Brine Extracts at Three Different pHs and 18% NaCl Concentration during the Ripening of Salted Anchovy[a]

		weeks of ripening									
proteinase activity	pH	0	1	2	3	4	5	6	7	8	9
fish muscle	3	1.23	1.39	1.67	2.33	1.88	1.84	3.22	1.83	2.17	3.21
	5.8	0.61	0.99	1.93	0.82	1.72	1.25	2.03	1.67	1.05	2.72
	9	1.98	1.91	3.01	3.05	2.91	3.78	3.30	4.15	4.30	4.77
brine	3		1.04	0.29	0.73	1.18	0.73	1.10	0.85	0.77	0.72
	5.8		1.62	1.33	2.82	1.59	1.06	3.54	1.61	3.04	2.40
	9		4.16	2.45	2.60	1.58	1.93	2.18	1.42	3.53	2.61

[a] On all cases, 15 samples were tested.

The nonprotein nitrogen compounds (NPN) of fish muscle decreased appreciably ($p < 0.05$) from 0.68% to 0.53%, during the first week of the ripening process. Thereafter, the NPN increased gradually ($p < 0.05$) throughout the ripening time (Table 1). However, the NPN content of the brine increased markedly ($p < 0.05$) until the sixth week and remained constant (Table 1). During salting, the exchange of matter in the system is accomplished mainly by the movement of salt molecules; but during the ripening period nitrogenous substances, mainly of low molecular weight (NPN compounds), diffuses from the fish into the brine (Voskresensky, 1965). In this study, during the first week of the ripening, the 75.93% of total nitrogen of the fish muscle that diffuses into the brine was NPN compounds. Differences of the increasing rates between TNBS and NPN in the muscle has been observed. According to observations of Baldrati et al. (1977), it could be so that an increase of TNBS in the muscle is caused by hydrolysis of peptides to amino acids because NH_2 groups increased faster than NPN.

The correlation between NPN/TN in the fish muscle and time observed was $r = 0.621$ ($p < 0.01$). However, a higher correlation was obtained between anchovy NPN/TN and time ($r = 0.987$; $p < 0.01$) when it was only considering the ripening process between the first and ninth week (Figure 3). Moreover, a linear relationship was also observed between the NPN/TN of the brine and ripening time ($r = 0.979$; $p < 0.01$) (Figure 3). NPN/TN determination in the fish muscle and in the brine would also allow one to follow and assess the ripening process of the anchovy. The NPN/TN determination in the brine has the advantage that samples could be obtained by nondestructive methods, but their correlation coefficient is lower than the correlation coefficient of fish muscle. The following expression relates the NPN/TN content (x) of fish muscle (1) and brine (2) to the time (y):

$$y = 0.898x + 12.96 \quad r = 0.987 \qquad (1)$$

$$y = 1.544x + 74.61 \quad r = 0.979 \qquad (2)$$

The ratio of nonprotein and total nitrogen has already been proposed as an objective method to follow and assess the ripening process of the anchovy (Campello, 1985; Durand, 1982; Perez-Villarreal and Pozo, 1992). These authors observed a high correlation between NPN/TN of fish muscle and ripening time, and they proposed a value of NPN/TN between 33% and 40% as indicative of the ripening point. However, in our work, a value of NPN/TN of 21% in the fish muscle and/or 89% in the brine would indicate the ripening point.

Proteinase Activity. The activity of alkaline proteinases in the fish muscle increased gradually throughout the ripening process. However, the activity of acidic proteinases (pH 3 and 5.8) showed a high variability (Table 2). The proteinase activity at three different pHs in the brine was also very variable. Within a species, the amount of endogenous enzyme present in a tissue can be dramatically influenced by intraspecific factors such as biological age, diet, exercise, water salinity, hydrostatic pressure, season and feeding activity at the time of capture (Haard, 1992; Makinodan et al., 1984; Wheaton and Lawson, 1985; Martinez and Gildberg, 1988).

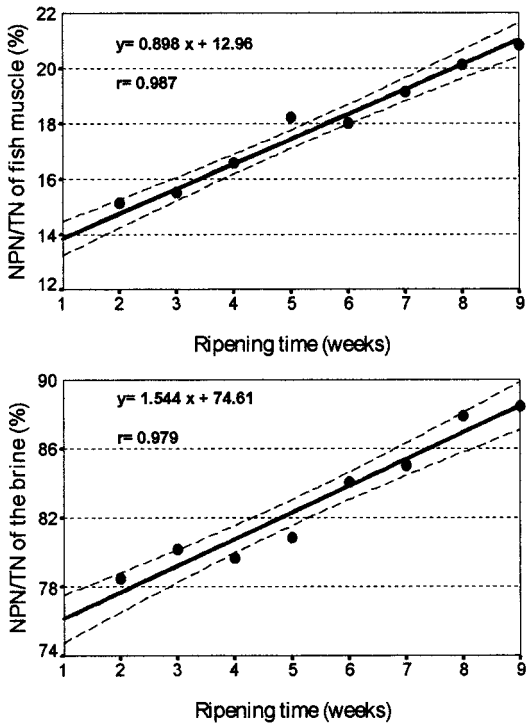

Figure 3. Ratio nonprotein nitrogen and total nitrogen (NPN/TN) in fish muscle and brine versus time during the ripening of salted anchovy. The area between the dashed lines is the 95% confidence limits of the regression estimate.

The proteinase activity at pH 3 and 9 in the fish muscle was 2 or 3 times higher than those of brine, but a similar proteinase activity was observed in the fish muscle and brine at pH 5.8 (Table 2). The crude enzyme prepared of fish muscle showed that alkaline proteinases dominate. The activity at pH 9 was about 2 times higher than the activity at pH 3 and 5.8. A similar finding was cited by Yatsunami and Takenaka (1996) in a study of changes in proteinase activity in ripened salted sardine. However, the crude enzyme prepared of brine showed a high proteinase activity at pH 5.8 and 9 (Table 2). Brine contains enzymes from muscle and the internal organs. It is assumed that enzyme in brine also decomposes the protein during the ripening of salted anchovy.

In digestive extracts of anchovy, maximum enzymatic activity points were detected in the acidic range at pH 1.8−2 and at pH 3−4 and in the alkaline range at pH 8.25−9.46 (Establier and Gutierrez, 1978). It was shown that the fish stomach contains substantial amounts of proteinases active at less acid conditions than mammalian pepsins. It has been proven that many fishes secrete at least two pepsins with different pH optima (Noda and Murakami, 1981). Pepsin I has pH optimum in the pH range of 3−4, whereas pepsin II is most active in the pH range of 2−3. The pH of the salted ripened anchovy during its production was 5.75−5.80 and fairly from the optimal values of alkaline and acid proteinase. Probably, the activity of both proteinases appearing in the ripened anchovy is very suppressed by the pH shift from their optimal value and by the presence of a high concentration of NaCl. The proteolytic activity of the acid and alkaline proteinases is inhibited strongly in the presence of 15−20% NaCl. Only one alkaline proteinase (a trypsin-like proteinase) and one acid proteinase (a pepsin-like proteinase) were stable for 3 months in the presence of 15−20% NaCl, although all other proteinases in salted sardine disappeared almost completely within a week (Noda et al., 1982). The suppressed proteinase activity causes a long period of ripening for salted anchovy. Endogenous enzymes play an essential role in ripened anchovy preparation and protein hydrolysis is probably caused mainly by trypsin-like enzymes (Heu et al., 1991; Noda et al., 1982). Trypsin is known to be more stable in acid than alkaline conditions and the slightly acidic pH of salted ripened anchovy may be one important factor in the persistence of the enzyme (Orejana and Liston, 1981). In our study, the enzyme activity and protein hydrolysis was not suppressed when the ripening period is completed. It is therefore necessary to store these products at refrigerating temperature.

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