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Studies on Fish Sauce

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(Figs. 1–2, Tables 1–4)

Fish sauce is a liquefied protein food made from fish by digestion with enzymes contained originally in fish or added from outside. Although only the Shottsuru of Akita Prefecture and a few other products are typical in Japan, a large variety of fish sauces are known and widely used in Southeast Asia. These are the Nuoc-mam of Vietnam, the Nampla of Thailand, the Patis of the Philippines and so on. Fish sauce is one of the important sources of animal protein and minerals for the peoples in those regions. Among them, Nuoc-mam of Vietnam may be the most well known product. Its production was estimated to be around 67,000 tons in 1935. It takes up an important position in that country[1].

And yet, researches on the quality or the manufacturing methods of Nuoc-mam are still very few. One of those is the improvement of manufacturing method suggested by THANH [2]. The preservation methods and some chemical analyses of Nuoc-mam were also reported by CHOM[3–5]. Recently the amino acid composition of commercial Nuoc-mam was reported by ABE[6]. Therefore, we intended to examine further the temperature conditions of the autolysis process of the fish sauce production and the effects of the addition of protein hydrolyzing enzyme or rice malt "Koji" on the quality of the product in order to contribute a more effective Nuoc-mam production in Vietnam.

EXPERIMENTAL AND RESULTS

Materials and preparation of fish sauce. Sand lance (Ikanago, *Ammodytes personatus*), Japanese anchovy (Katakuchi iwashi, *Engraulis japonicus*), Sardine (Maiwashi, *Sardinopus melanosticta*), Mackerel (Masaba, *Pneumatophorus japonicus*) and Saury (Samma, *Colorabis saira*) were purchased from the fish market and used as raw materials. Each fish was cleaned twice with water before using.

In the case of sand lance and anchovy, the whole body was used. Each 1 kg of fish was mixed thoroughly with 20 weight % of sodium chloride, put into jars and stored at temperatures of 2°, 10°, 30° and 50°C, respectively. In addition to these, 6 other specimens were also prepared, as follows. Anchovy was added with 35% of "Koji" prepared in our laboratory or 0.5 % of pronase purchased from Riken Kagaku Co. and sand lance added with 35 % of "Koji", and ripened at 30°C and 50°C, respectively, in order to examine the effects of these additives on products. The storage periods were set at 150 days for anchovy and 100 days for sand lance.

In the case of mackerel, sardine and saury, the fish were beheaded without evisceration and minced with a chopper. Large bones and fins were removed at this stage. Each 1 kg of minced meat was mixed with 30 % of salt and stored at 50°C for 63 days. The manufacturing conditions and compositions of materials are shown in Table 1.

Table 1. Composition of materials and manufacturing conditions.

Sample No.	Raw material	Composition (%)				Storing Temperature(°C)	Storing Period (day)
		Fish	NaCl	Koji	Pronase		
A–50–1	Anchovy	80	20	–	–	50	150
A–50–2	"	45	20	35	–	50	150
A–50–3	"	80	20	–	0.5	50	150
A–30–1	"	80	20	–	–	30	150
A–30–2	"	45	20	35	–	30	150
A–30–3	"	80	20	–	0.5	30	150
A–10	"	80	20	–	–	10	150
A–2	"	80	20	–	–	2	150
B–50–1	Sand lance	80	20	–	–	50	100
B–50–2	"	45	20	35	–	50	100
B–30–1	"	80	20	–	–	30	100
B–30–2	"	45	20	35	–	30	100
B–10	"	70	20	–	–	10	100
B–2	"	70	20	–	–	2	100
C–50	Mackerel	70	30	–	–	50	63
D–50	Saury	70	30	–	–	50	63
E–50	Sardine	70	30	–	–	50	63

During the aging period, the salted mashes were occasionally stirred and the oil that floated out at the top of the mixture was removed. After aging, the whole liquefied amount of the digested mixture was obtained by filtration through sheets of cloth and Toyo filter paper No. 2, successively. The resulting filtrate was further centrifuged to remove the tiny particles and oil, if neccessary The yields of fish sauces are shown in Table 2.

Some properties of prepared fish sauces. The total nitrogen and α-amino nitrogen of the products were determined by the methods of KJELDAHL and Van SLYKE, respectively. Volatile base nitrogen evaluated by the CONWAY 's microdiffusion method[7]. The content of creatine and creatinine was estimated colorimetrically using JAFFE's reaction[8]. The sodium chloride content was measured by titration with 0.1 *N* silver nitrate. These results are all summarized in Table 2. The ratio of liquefied protein nitrogen to total nitrogen of raw materials are also shown in the same table.

In the case of anchovy fish sauce, 25.3 % of protein was liquefied after 150 days aging at a temperature of 50°C. The liquefied portion decreased to 17.6 %, 6.3 % and 2.9 % as the storage temperature lowered into 30°C, 10°C and 2°C in order, respectively. Similar tendency was observed in the case of sand lance fish sauce. The yields of fish sauces decreased according to the decline of the liquefied protein ratio.

Table 2. Yield and some properties of fish sauces prepared.

Sample No.	Material (Total N %)	Yield of fish sauce ml	pH	Specific gravity	Solid %	NaCl %	Total N mg/ml	α-Amino N mg/ml	Liquefied protein ratio %	Volatile base N %	Creatine & creatinine %
A-50-1	Anchovy	318	5.35	1.15	37.8	19.8	23.5	10.4	25.3	–	–
A-50-2	(2.96)	800	4.06	1.16	36.1	18.7	9.0	5.0	24.3	–	–
A-50-3		316	5.32	1.12	37.5	19.3	24.8	12.4	26.5	–	–
A-30-1		226	5.68	1.12	36.8	18.4	23.0	11.2	17.6	–	–
A-30-2		666	5.03	1.10	35.2	18.7	10.8	6.8	24.3	–	–
A-30-3		245	5.46	1.18	36.8	17.9	24.2	12.5	20.0	–	–
A-10		97	5.67	1.15	36.4	19.8	19.1	8.4	6.3	–	–
A-2		46	5.72	1.15	37.2	19.1	18.5	5.8	2.9	–	–
B-50-1	Sand lance	297	5.22	1.11	36.1	18.3	26.2	11.1	25.6	–	–
B-50-2	(2.93)	714	4.65	1.16	36.7	18.2	11.2	5.0	27.3	–	–
B-30-1		254	5.46	1.12	35.4	19.3	25.6	12.2	22.2	–	–
B-30-2		650	5.00	1.12	36.7	17.8	12.0	7.2	26.6	–	–
B-10		80	5.78	1.13	38.1	18.6	21.1	10.0	5.8	–	–
B-2		35	5.82	1.11	38.2	18.6	20.0	8.3	2.4	–	–
C-50	Mackerel (2.88)	285	5.72	1.22	46.0	27.5	26.9	12.6	26.6	5.3	5.0
D-50	Saury (3.20)	170	5.77	1.18	45.9	28.1	24.2	10.7	12.9	5.1	3.7
E-50	Sardine (2.91)	317	5.52	1.22	46.1	27.8	23.3	10.4	25.4	5.6	4.5

The amounts of liquefied protein in the "Koji" added specimens kept at 50°C were almost the same as those of the non-added ones, yet they considerably higher at 30°C in anchovy and in sand lance products. Fish sauces aged at 50°C with "Koji" showed an obvious browning.

The addition of pronase in anchovy fish sauce slightly increased the solubilized protein ratio at 30°C but not at 50°C. Any distinct acceleration effect on the protein hydrolysis was not observed in this pronase addition.

With regard to the flavors of the products, sand lance and anchovy fish sauces prepared either at 50°C and 30°C gave a typical fish sauce flavor similar to that of Nuoc-mam or Shottsuru. Both specimens produced at the temperature of 10°C and 2°C possessed a fishy taste and odor resembling to that of the raw materials. The flavor of pronase added anchovy fish sauce was indistinguishable from that of the non-added one. The "Koji" added specimen had a flavor considerably different from that of usual fish sauce, and it rather resembled to that of soy sauce. Although considerably high yields were obtained, a remarkable odor of rancid oil was detected in the case of mackerel, sardine and saury fish sauce only after 63 days ripening. This may be attributable to the high fat content of these fish. Therefore this kind of fish seemed unsuitable for fish sauce materials.

Amino acid composition of 80 % ethanolic soluble fraction of fish sauce. For examination of the free amino acid composition of fish sauces, 80 % ethanolic soluble fraction was prepared as follows. The samples of the fish sauce were added with 4 volumes of absolute ethanol. The arisen precipitate was separated by centrifugation and washed further with 4 volumes of 80 % ethanol for 2 times. The combined ethanolic solutions were evaporated *in vacuo* in order to remove ethanol, and then made to the original volume with water. The free amino acid composition of this solution was deter-

mind by the Hitachi 034 type amino acid analyzer. The whole amino acid composition of the same solution was also determined by the same method after hydrolysis with 6 *N* HCl in the usual manner. The results are shown in Table 3. The percentage ratio of each free to whole amino acid are also set forth in the same table.

Table 3. Amino acid composition of 80 % ethanolic extract of fish sauces prepared.

Material	Anchovy																		Sand lance	
Sample No. Additive	A–50–1 –			A–50–2 Koji			A–50–3 Pronase			A–30–1 –			A–30–2 Koji			A–30–3 Pronase			B–30–1 –	B–30–2 Koji
	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	b	b
Taurine	433	458	100	456	–	–	445	442	99.3	292	283	96.9	546	586	100	312	332	100	345	–
Aspartic acid	1129	499	44.2	1440	600	41.7	1467	526	36.0	1055	515	48.8	1106	520	47.0	940	622	63.8	282	741
Threonine	1138	954	83.8	2260	1264	100	1522	1155	75.7	1026	814	79.3	1339	1612	100	1153	1067	94.2	983	1618
Serine	913	712	78.0	936	920	98.3	1092	744	68.2	782	590	75.4	1039	1112	100	777	675	87.1	714	1371
Glutamic acid	3123	878	28.1	4736	488	10.3	3872	909	23.5	2628	777	29.6	3084	1059	34.4	2582	1098	42.5	772	1579
Proline	1523	820	53.9	1784	1120	62.9	1961	1251	63.8	1006	500	49.7	1585	1026	64.7	1451	1356	93.4	574	1007
Glycine	1542	461	29.9	1072	296	27.6	1791	382	21.3	1155	346	29.9	1472	546	37.1	1173	456	38.8	389	747
Alanine	2191	1924	87.8	2616	1904	72.8	2603	1966	75.5	1465	1155	78.9	2538	2211	87.1	1913	1777	92.8	1501	2190
Valine	1794	1749	97.5	2152	1584	73.6	2323	2246	96.7	1394	1220	87.5	2584	2011	77.8	1648	1676	100	1638	1924
Methionine	172	642	–	432	424	–	801	955	–	133	472	–	120	753	–	420	550	–	668	910
Isoleucine	1371	1320	96.3	1623	1168	71.9	1709	1686	98.5	924	782	84.6	2171	1598	73.6	1092	1100	100	1328	1755
Leucine	2223	2175	97.8	2448	1624	66.3	2150	2203	100	1352	1146	84.8	3397	2657	78.2	1233	1254	100	1707	2593
Tyrosine	200	219	100	824	496	60.0	281	232	82.0	140	104	74.2	733	486	66.6	126	132	100	160	604
Phenylalanine	770	690	89.7	848	392	46.2	1231	1114	90.5	624	481	77.2	1319	759	57.6	906	884	97.6	742	929
Lysine	2964	2512	84.8	1504	696	46.3	2948	2658	90.1	2823	1862	66.0	2078	1838	88.5	2251	2105	93.5	1400	1969
Histidine	1743	1708	98.0	576	328	56.9	1025	842	82.1	1428	1132	79.3	1585	766	48.3	902	835	92.7	434	403
Arginine	1784	1530	85.7	160	96	60.0	1545	1419	91.8	1446	1076	74.4	1419	1146	80.8	993	1041	100	1234	2060
Ammonia	712	413	58.0	808	392	48.5	639	469	73.8	613	305	49.8	819	406	49.6	583	370	63.4	267	416
Total	25725	19664	76.4	25756	13792	54.0	29405	21199	72.1	20286	13560	66.8	28934	21092	72.9	20444	17331	84.8	15138	22860

a, whole amino acid mg in fish sauce extract; b, free amino acid mg in fish sauce extract; c, ratio of free to whole amino acid in % (b/a).

In all the fish sauce samples examined, the contents of free glutamic acid, aspartic acid, proline and glycine were remarkably low, especially glutamic acid. However, these amino acids appeared in considerable quantity after hydrolysis. This suggests that peptides containing these amino acid moieties remain in unhydrolyzed state in the fish sauce.

With regard to the temperature effect, the total free amino acid content of the product aged at 50°C was about 45 % higher than the one of those aged at 30°C. No remarkable difference was observed in the amino acid composition between these specimens.

Free amino acid content of "Koji" added fish sauce prepared at 30°C was remarkably higher than that of non-added specimen, and reverse situation was observed in the specimens prepared at 50°C. In the case of "Koji" added specimens, the contents of basic amino acids such as lysine, histidine and arginine in 50°C aged product were remarkably lower than those in 30°C aged one. As fish sauce prepared at 50°C showed a distinct browning, the decreace of basic amino acids may be reasonably explained by Maillard's reaction with carbohydrates present in "Koji".

Little difference was found in the amino acid content and composition between control and the pronase added specimens.

Free amino acid composition of "Koji" added sand lance fish sauce resembled to that of Shottsuru reported by ABE[6], except for the concentration of some amino acids such as glutamic acid.

Fractionation of amino acids and peptides. As a considerable part of amino acids remained in bound form in the prepared samples, the constituents were examined as follows. The ethanolic extract of anchovy fish sauce was separated into 4 fractions using ion-exchange resins as shown in Fig. 1. Fraction 1, 2 and 3 were constituted by strongly acidic, neutral and basic, and acidic amino acids and peptides, respectively. The amino acid compositions before and after hydrolysis of these fractions are shown in Table 4.

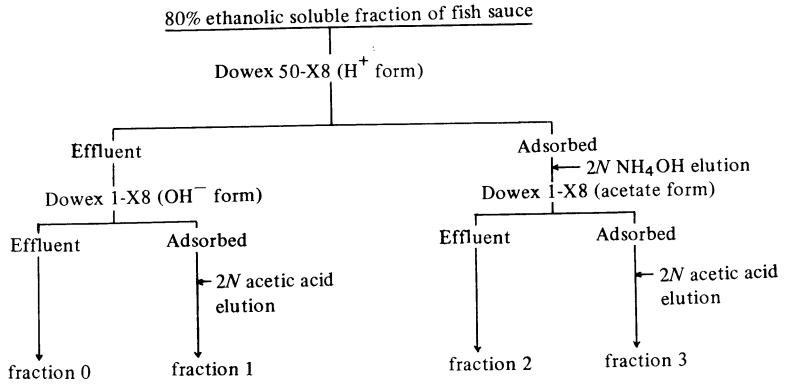

Fig. 1. Fractionation of fish sauce extract.

Table 4. Amino acid compositions of fraction 1, 2 and 3 (μmole/ml of fish sauce)

Fraction No.	Amino acids	Before hydrolysis	After hydrolysis
1	Taurine	9.5	11.2
	Glutamic acid	–	28.6
	Others	–	traces
2	Aspartic acid	–	2.1
	Threonine	23.1	26.5
	Serine	21.2	25.8
	Glutamic acid	–	2.8
	Proline	14.6	20.6
	Glycine	16.3	39.1
	Alanine	53.5	57.0
	Valine	44.6	46.3
	Methionine	14.0	–
	Isoleucine	26.3	28.4
	Leucine	37.6	41.6
	Tyrosine	1.3	1.3
	Phenylalanine	11.8	12.5
	Lysine	36.8	54.5
	Histidine	23.6	29.2
	Arginine	27.7	34.6
3	Aspartic acid	10.9	13.3
	Glutamic acid	18.0	29.4
	Others	–	traces

In fraction 1, taurine alone was detected before hydrolysis. But after hydrolysis, a remarkable amount of glutamic acid and traces of some other amino acids appeared. This suggested that in this fraction glutamic acid may be present in cyclyzed form pyroglutamic acid or in peptide.

In fraction 2, glycine, lysine, arginine and proline increased in fairly large amount after hydrolysis. This may suggest that fraction 2 contained peptides having these amino acid residues.

The principal constituents of fraction 3 were free glutamic acid and aspartic acid.

Isolation of pyroglutamic acid. Constituents of fraction 1 were examined by paper chromatography using Toyo filter paper No. 50 and a solvent mixture of 1-butanol, acetic acid, formic acid and water (4:1:1:2, v/v) in descending. Spots were visualized with ninhydrin and o-tolidine-NaClO reagent. With ninhydrin, only taurine was detected at R*f* 0.1. Three spots, of which R*f* values were 0.1, 0.6 and 0.7, respectively, showed up with o-tolidine-NaClO reagent. These components were separated by preparative paper chromatography using the same solvent system.

The substance of R*f* 0.1 was identified as taurine by thin layer chromatography. The substance of R*f* 0.7 was very small in quantity and gave aspartic acid, serine, glutamic acid, alanine and glycine after hydrolysis. This substance was assumed to be peptide.

When the compound of R*f* 0.6 was subjected to hydrolysis with 2 *N* HCl at 100°C for 1 hr, it afforded glutamic acid. R*f* values of this compound in thin layer chromatography using several solvent systems agreed well with those of authentic pyroglutamic acid. From these results, it may reasonably be concluded that this compound is pyroglutamic acid. Its concentration in the fish sauce was 27.4 μmoles in 1 m*l* of the sample.

Separation of peptides in fraction 2. According to GILBERTI and NIEDERWIESER[9], peptides can be isolated from free amino acids if their copper complexes are treated with DEAE-Sephadex A-25 (in carbonate form) column. Using this method in a slightly modified form, free amino acids and peptides of this fraction were separated into 5 fractions. Each of the obtained peptide fraction was subjected to DNP-derivatization by the method of SANGER and THOMPSON[10]. From these fractions, DNP-glycylalanine, DNP-glycylproline and DNP-alanylglycine were isolated by preparative thin layer chromatography and identified after hydrolysis[11]. Concentrations of these dipeptides in anchovy fish sauce were estimated to be 4.9, 4.9 and 1.2 μmoles/m*l*, respectively.

Formation of pyroglutamic acid from glutamine in fish sauce. As mentioned above, fish sauces prepared in this study contained a considerable quantity of pyroglutamic acid. Therefore the effect of temperature on the formation of pyroglutamic acid from glutamine or glutamic acid in fish sauce was examined as follows. One hundred and fifty μmoles of glutamine and glutamic acid were added to each 5 m*l* portion of anchovy fish sauce and these were kept at 30°C and 50°C for 20 days, respectively. Aqueous solutions of these amino acids were used as control. During the standing period, the amounts of glutamine and glutamic acid in sample solutions were estimated by an amino acid analyzer at intervals. The results are shown in Fig. 2.

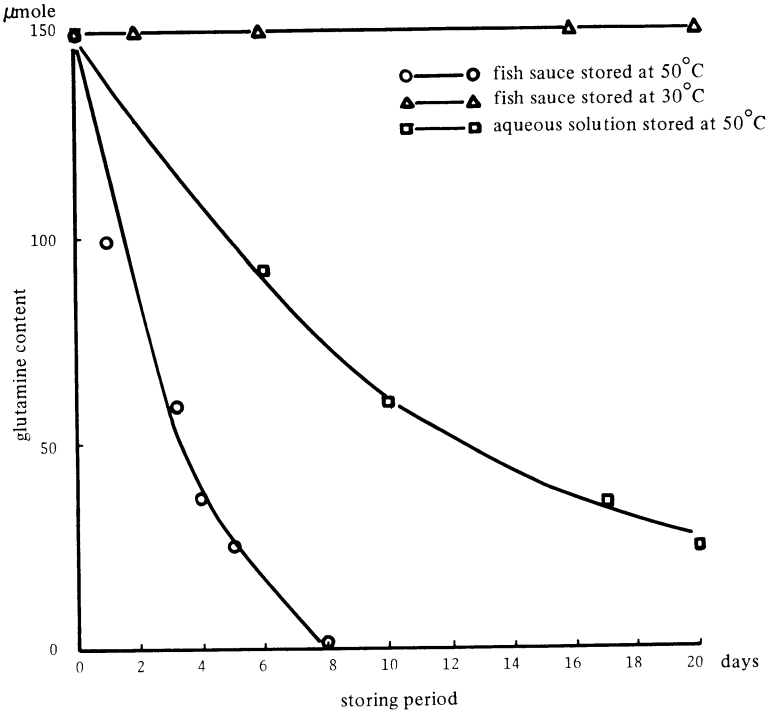

Fig. 2. Decrease of added glutamine in fish sauce.

The quantity of glutamic acid in fish sauce did not change after storage for 20 days both at 30°C and 50°C. On the other hand, glutamine in fish sauce kept at 50°C disappeared completely after 8 days. In a control aqueous solution stored at 50°C, 60 % and 80 % of glutamine disappeared after 10 and 20 days, respectively. When these aqueous solutions were subjected to hydrolysis with 2 *N* HCl at 100°C for 1 hr, free glutamic acid equivalent to the added glutamine was recovered. These results suggest that glutamine in fish sauce is transformed into pyroglutamic acid during the aging period.

Storage at 30°C for 20 days did not affect on the contents of glutamine both in fish sauce and in aqueous solution.

DISCUSSION

Fish sauces were prepared from several kinds of common fish under some conditions and their yields and properties were examined in order to investigate the effects of temperature and additives on their quality and production. The yields of liquefied protein were 25.2 and 26.6 % of the total protein of fish materials in the case of anchovy riped at 50°C for 150 days and sand lance aged for 100 days, respectively. As the aging temperature decreased, the yield of solubilized protein decreased. Below 10°C only 2 to 3 % of total protein could be obtained as fish sauce.

In free amino acid composition, the fish sauces prepared in this study were extremely deficient in glutamic acid. But on the other hand they were considerably rich in lysine and arginine compared with the commercial Shottsuru and Nuoc-mam reported by Abe[6].

The distribution of nitrogen in the fish sauce prepared in this study resembled fairly well to that of Shottsuru reported by ABE and TSUYUKI[12].

In the amino acid composition of 80 % ethanolic extract of fish sauce, aspartic acid, glutamic acid, proline and glycine remarkably increased after hydrolysis. This suggested that a considerable part of these amino acids remained in bound form after autolysis of the fish body under these conditions.

The occurrence of a fairly large amount of pyroglutamic acid in the prepared fish sauce was established. This compound was assumed to be derived from glutamine or glutamic acid during the digesting process as in the hydrolysis products of soy beans[13]. The transformation of glutamine into pyroglutamic acid in fish sauce was also established, especially at higher temperature as 50°C. In fish sauce kept at 30°C for 20 days, formation of this compound was not observed, although its presence in a large quantity in fish sauce made at the same temperature was confirmed. This may be due to the shortness of aging period. Because of its bitter taste, occurrence of pyroglutamic acid in fish sauce is undesirable. Moreover, its formation causes a decrease of free glutamic acid which is considered to be the principal origin of the meaty or palate-satisfying taste of fish sauce. Therefore it is neccessary to find a method of preventing the formation of pyroglutamic acid during digestion.

The presence of peptides was confirmed by the isolation of glycylglycine, glycylproline and alanylglycine. Occurrence of some acidic peptides containing glutamic acid as major component was presumed but could not be confirmed.

The addition of "Koji" showed a considerable effect on the liquefication of protein and the formation of free amino acids at 30°C. However, in the specimen prepared at 50°C, the amount of free basic amino acids decreased remarkably. As this product revealed an extreme brown color, basic amino acids may be consumed in the Maillard's reaction with carbohydrate contained in "Koji".

The amino acid composition of "Koji" added sand lance fish sauce resembled to that of the commercial Shottsuru reported by ABE[6] except for the concentration of glutamic acid, lysine and arginine. But the taste of "Koji" added product was distinctly different from those of the usual fish sauces such as Shottsuru, and rather similar to that of soy sauce.

The effect of pronase on protein hydrolysis in fish sauce was examined, because this enzyme was reported to accelerate the preparation of Shottsuru remarkably[14]. Yield and quality were not remarkably different for pronase added and control specimens.

Fish sauce prepared from mackerel, saury and sardine smelled of rancid oil only after 64 days autolysis. This must be caused by the high oil content of these fish. Therefore these kinds of fish are unsuitable for fish sauce.

Further investigation on liquefication of fish protein and on the quality of product should be neccessary in order to contribute to the production of fish sauce in Southeast Asia.

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魚醤油の製造に関する研究

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市販のイカナゴ，カタクチイワシ，マイワシ，サバおよびサンマの5種を原料として，魚醤油を製造し，温度条件，蛋白質分解酵素および麹添加の品質に与える影響を調べた。その結果，50℃，30℃，10℃および2℃の各温度条件下における魚肉蛋白質の液化率は温度が高い程よく，50℃で150日熟成後のカタクチイワシ，100日後のイカナゴを原料としたものでは，それぞれ25.3％および26.6％であった。10℃以下では液化率は数％にすぎなかった。またアミノ酸の遊離比ではグルタミン酸，グリシン，アスパラギン酸，プロリンが著しく劣り，これらを構成成分とするペプチッドが分解不完全のまま残存することが知られた。

グルタミン酸は魚醤油製造工程中に相当量環状化してピログルタミン酸に変化することが確認された。この外，未分解のまま残存するペプチッドとして，グリシルプロリン，アラニルグリシンおよびグリシルグリシンを分離，同定した。

麹の添加は30℃の熟成条件下では蛋白質液化率と遊離アミノ酸の生成にかなりの効果があったが，50℃条件下では逆に減少した。これは製品が著しく褐変することから，アミノカルボニル反応によるものと推定された。

プロナーゼの添加は無添加のものと比べ，遊離アミノ酸組成と蛋白質液化率に顕著な効果は認められなかった。

マイワシ，サバおよびサンマでは原料魚の脂質含量が高く，油焼け臭を発生し，魚醤油原料としては不適当と思われた。