Stability of Muscle Proteins from Some Subtropic Fish

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The stability of actomyosin (AM) from some subtropic fish, tilapia hybrid (*Tilapia nilotica* × *Tilapia aurea*), tilapia (*Tilapia mossambica*), carp (*Cyprimus carpio*), and milkfish (*Chanos chanos*) was investigated. According to the inactivation rate constant (K_D) of AM Ca-ATPase at 0 to 45 and −10 to −40 °C, the stabilities of tilapia hybrid and tilapia AMs were higher than those of milkfish and carp AMs. All AMs of these species were stable at pH 6.5–7.9 and had two maximum Ca-ATPase activities at pH 5.8–6.1 and 9.2, which were lower than other species inhabiting cold areas. No significant difference in K_D values among these species was observed when stored at −40 °C for 6 months. The tilapia hybrid and tilapia were the most stable at storage temperatures of −10, −20, and −30 °C.

Milkfish, tilapia hybrid, tilapia, and carp are the most important aquaculture in Southeast Asia, especially in Taiwan and The Philippines. Consumption of these species is mostly as iced and frozen whole or fillet fish. However, a surplus of these species still exists. Accordingly, fishery technologists have focused their attention on utilization of these species, especially in producing minced fish products. Aggregation of myofibrillar proteins, however, frequently occurred after prolonged frozen storage, which consequently caused a decrease in gelation during preparing minced fish products (Jiang, 1977, 1984; Kurokawa, 1979; Matsumoto, 1979, 1980; Noguchi, 1982; Nosaki et al., 1978; Okada et al., 1974). It was recognized that the stability of muscle proteins varied with species and storage conditions (Suyama and Konosu, 1987). Some constituents, such as lipids (Andou et al., 1979, 1980, 1981a,b; Braddock and Dugan, 1973; Braun and Radin, 1969; Childs, 1973, 1974) and free amino acids and nucleotides (Jiang, 1984; Jiang and Lee, 1985; Jiang et al., 1986, 1987a–c) were also reported to affect the stability of myofibrillar proteins during refrigerated and frozen storage.

The properties of myofibrillar proteins are highly related to the quality of minced fish products (Okada et al., 1974; Seki, 1977). The Ca-ATPase of myofibrillar proteins is reported to be a good index to evaluate the stability of fish muscle proteins during icing, freezing, and subsequent storage (Arai, 1974; Arai and Takashi, 1977). According to the studies on stability of muscle proteins, fish was much more unstable than rabbit and chicken (Arai and Takashi, 1977). The stability of fish muscle proteins is recognized to be highly related to the living environment temperature (Arai, 1974; Arai and Takashi, 1977; Connell, 1961; Seki, 1977; Suzuki, 1981; Suyama and Konosu, 1987). This study aimed to investigate the stability and properties of myofibrillar proteins from some subtropic fish, i.e., milkfish, tilapia hybrid, tilapia, and carp, and consequently provide functionality information for storage and processing of these species.

MATERIALS AND METHODS

The milkfish (*Chanos chanos*), tilapia hybrid (*Tilapia nilotica* × *Tilapia aurea*), tilapia (*Tilapia mossambica*), and carp (*Cyprimus carpio*) were kept alive and transported to the laboratory. After being eviscerated, headed, and washed, samples were used in this study.

Preparation of Actomyosin. Actomyosin (AM) was extracted according to the procedure of Noguchi and Matsumoto (1970). A 10-g portion of meat was blended by using an Waring blender subjoined with a baffle plate for 2 min with 90 mL of chilled buffer solution (0.6 M KCl–0.04 M $NaHCO_3$–0.01 M Na_2CO_3, pH 7.2). The extract was centrifuged at 5000*g*, 0 °C for 20 min. AM was precipitated by diluting with 3 volumes of chilled distilled water and collected by centrifuging at 5000*g*, 0 °C for 20 min. The AM was then dissolved in chilled buffer solution (pH 7.2) and subjected to the following analyses.

Determination of the Stability of Actomyosin. AM (1.0–5.0 mg/mL) was incubated at various temperatures (0, 10, 20, 30, 35, 40, 45 °C). At a definite time interval, all AMs were iced for 5 min and then placed at 25 °C for 5 min. The Ca-ATPase activity was determined according to the procedure of Arai (1974).

Effect of pH on the Stability of Actomyosin. To investigate the effects of pH on the stability of AM, AMs with different pH were prepared with 0.1 M Tris–maleate buffer and then incubated at 25 °C. At definite time intervals, the Ca-ATPase activity was measured to calculate the inactivation rate constant (K_D) of AM ATPase.

Effect of Frozen Storage Temperature on the Stability of Muscle Proteins. For investigating stability under frozen temperature, eviscerated and headed samples were frozen to a body temperature below −18 °C with an air-blast freezer (wind velocity 3.0 m/s, temperature −40 °C). The resulting samples were then stored at −10, −20, −30, and −40 °C for 6 months. At a definite time interval, samples were removed and thawed to 0 °C with running tap water (about 25 °C). The AM was extracted according to the procedure of Noguchi and Matsumoto (1970). Ca-ATPase activity of AM was determined according to the procedure of Arai (1974).

Effect of the pH of Reaction Mixture on Actomyosin Ca-ATPase Activity. Various reaction mixtures with different pHs were prepared with 0.5 M Tris buffer, 0.1 M $CaCl_2$, and deionized water. After AM was added, the pH values of these reaction mixtures were measured again. On the basis of these pH conditions, the Ca-ATPase activity of AM was determined according to the procedure of Arai (1974).

Ca-ATPase Activity. To a 1-mL AM solution (1–5 mg/mL) were added 0.5 mL of 0.5 M Tris–maleate buffer (pH 7.0), 0.5 mL of 0.1 M $CaCl_2$, 7.5 mL of deionized water, and finally 0.5 mL of 20 mM adenosine 5′-triphosphate (ATP) solution (pH 7.0). After ATP was added, the releasing rate of inorganic phosphate at 25 °C between 0- and 3-min reaction was measured to calculate the Ca-ATPase activity. Of 15% trichloroacetic acid 15 mL was added to stop the reaction; the quantity of resulting inorganic phosphate was measured according to Arai (1974). The Ca-ATPase specific activity was defined as micromoles of inorganic phosphate liberated per milligram of protein within 1 min for the reaction at 25 °C. The inactivation rate constant of AM Ca-ATPase activity was calculated according to Arai (1974): $K_D = (\ln C_0 - \ln C_t)/t$, where C_0 = Ca-ATPase activity before incubation

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Table I. Effect of the Incubation Temperatures on the Rate Constant for Inactivation[a] of Actomyosin Ca-ATPase of Milkfish, Tilapia Hybrid, Tilapia, and Carp

temp, °C	$K_D \times 10^5$,[b] s^{-1}			
	A	B	C	D
0	0.520 a	0.356 c	0.448 b	0.396 c
10	0.841 a	0.764 b	0.891 a	0.825 ab
20	1.23 b	1.55 a	1.78 a	1.47 a
30	7.08 a	3.06 c	5.12 b	3.11 c
35	18.0 a	4.12 d	8.15 c	10.1 b
40	41.3 b	28.1 d	37.5 c	58.6 a
45	140 b	96.7 d	124 c	170 a

[a] $K_D = (\ln C_0 - \ln C_t)/t$, where C_0 = Ca-ATPase specific acitivity before incubation, C_t = Ca-ATPase specific activity after t-s incubation, and t = incubation time (s). Concentration of actomyosin: 1.0–5.0 mg/mL; pH 7.0. Means of three determinations from each sample were used to calculate the K_D values and statistical analysis. [b] Key: A, milkfish; B, tilapia hybrid; C, tilapia; D, carp. Values in the same row bearing unlike letters differ significantly ($P < 0.01$).

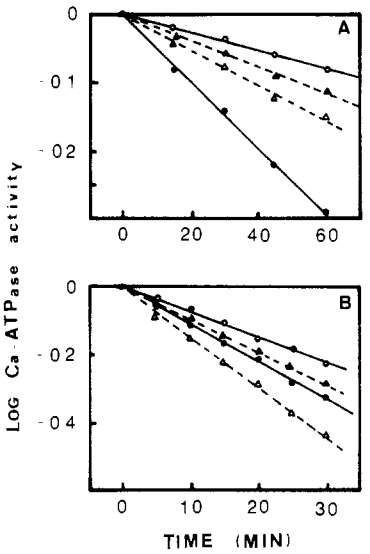

Figure 1. Logarithm of Ca-ATPase activities of milkfish, tilapia hybrid, tilapia, and carp actomyosin as a function of incubation time: (A) 35 °C; (B) 40 °C. Key: ○, tilapia hybrid; ▲, tilapia; ●, milkfish; △, carp.

or day 0 of frozen storage, C_t = Ca-ATPase activity after t seconds of incubation or t days of frozen storage, and t = incubation time (seconds or days).

Statistical Analyses. Duncan's multiple-range test was used for statistical analyses.

RESULTS AND DISCUSSION

Stability of the Extracted Actomyosin. The inactivation rate constants of actomyosin (AM) and myosin Ca-ATPase activities were frequently used for evaluating the thermal stability of fish muscle proteins (Suzuki, 1981; Seki, 1977). The stability of myofibrillar proteins was considered to be highly related to the living environment temperatures of fish (Seki, 1977; Connell, 1961; Suzuki, 1981). In this study, when the stabilities of AM at 40 and 45 °C were compared, carp was the most unstable and then milkfish, tilapia and tilapia hybrid. However, milkfish was the most unstable at 30 and 35 °C (Table I; and Figure 1). The inactivation rate constant of all AMs Ca-ATPase increased remarkedly at 40 and 45 °C (Table I; Figure 1). Comparing the K_D values at 30, 35, 40, and 45 °C, the stability of tilapia hybrid AM was significantly higher than that of carp, milkfish, and tilapia AMs. The K_D values of all AMs increased with the incubation temperature (Table I; Figure 1). When K_D values at 35 °C were compared with those of other species, the AMs of carp, tilapia hybrid, and tilapia had lower values than those of tuna, sea bream, and rainbow trout, which had K_D values of 15.3, 33.8, and 46.1 $\times 10^{-5}$, respectively (Arai, 1974). However, the stabilities of AMs of these species were lower than those of rabbit and whale (Arai, 1974).

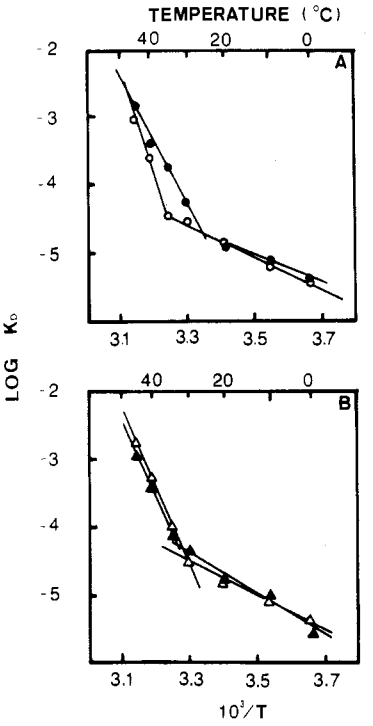

Figure 2. Arrhenius plot of the rate constant for inactivation of actomyosin Ca-ATPase from milkfish, tilapia hybrid, tilapia, and carp at pH 7.0. (Refer to the symbols in Figure 1.)

Table II. Effect of pH on the Rate Constant for Inactivation[a] of Actomyosin Ca-ATPase of Milkfish, Tilapia Hybrid, Tilapia, and Carp

pH	$K_D \times 10^5$, s^{-1}			
	A	B	C	D
5.4	20.3 a	16.2 c	13.1 d	18.5 b
6.5	9.30 a	3.50 c	3.22 c	5.24 b
7.2	5.33 a	2.64 b	2.94 b	2.25 b
7.9	7.27 a	3.70 c	3.28 c	4.14 b
8.8	15.0 a	7.51 c	6.55 c	9.47 b
9.4	19.6 a	15.3 c	16.1 bc	17.8 b

[a] See footnotes, Table I.

From the Arrhenius plot of K_D values as a function of the reciprocal of absolute temperature, the stability of these AMs could be divided into two zones: zone I, milkfish at 0–25 °C, tilapia hybrid and tilapia at 0–35 °C, carp at 0–30 °C; zone II, milkfish at 25–45 °C, tilapia hybrid and tilapia at 35–45 °C, carp at 30–45 °C. The inactivation rate was higher in zone II than at zone I (Figure 2).

Effect of pH on the Stability of Actomyosin. During incubation at 25 °C, all AMs of milkfish, tilapia hybrid, tilapia, and carp were stable at pH 6.5–7.9 (Table II), which were almost the same as those of other species (Suyama and Konosu, 1987).

Effect of Frozen Storage Temperature on Denaturation of Muscle Proteins. Among a variety of freezing conditions, storage temperature and period are the most important factors affecting the protein quality of seafoods (Bito, 1976; Fukuda, 1986; Love, 1962a,b). Accordingly, a quantitative determination of the protein deterioration as functions of storage temperature and period is necessary

Table III. Effect of the Frozen Storage Temperatures on the Rate Constant for Inactivation[a] of Actomyosin Ca-ATPase of Milkfish, Tilapia Hybrid, Tilapia, and Carp

temp, °C	$K_D \times 10^4$,[b] day^{-1}			
	A	B	C	D
−10	63.5 a	29.3 c	30.4 c	34.8 b
−20	26.6 a	11.6 c	10.8 c	15.3 b
−30	7.91 a	5.05 c	4.78 c	6.31 b
−40	3.18 a	2.96 a	2.88 a	3.11 a

[a] $K_D = (\ln C_0 - \ln C_t)/t$, where C_0 = Ca-ATPase specific activity at day 0, C_t = Ca-ATPase specific activity after t days of storage, and t = incubation time (days). Concentration of actomyosin: 1.0–3.0 mg/mL; pH 7.2. Means of three determinations from each sample were used to calculate the K_D values and statistical analysis. [b] Refer to Table I. [c] Values in the same row bearing unlike letters differ significantly ($P < 0.01$).

in studies of protein stability of frozen fish. Recently, Matsumoto et al. (1985) studied the change in myofibrillar proteins Ca-ATPase of mackerel during storage at −15, −20, −25, −30, and −40 °C and found a linear relation between logarithmic Ca-ATPase activity and storage time at various storage temperatures. Moreover, an inactivation rate constant of Ca-ATPase of muscle AM during frozen storage was calculated to compare the stability of muscle protein among different storage temperatures (Fukuda, 1986; Matsumoto et al., 1985).

The inactivation rate constants of Ca-ATPase of muscle AM during frozen storage of eviscerated and headed milkfish, tilapia hybrid, tilapia, and carp were measured and calculated. Although no significant difference in K_D values was observed among these species when stored at −40 °C (Table III), the K_D values of tilapia hybrid and tilapia AMs were significantly lower than those of carp and milkfish AMs when stored at −10, −20, and −30 °C. No significant difference in K_D values was obtained among tilapia hybrid and tilapia stored at these temperatures (Table III). With regard to the stability of muscle proteins at frozen temperatures, tilapia hybrid and tilapia were the most stable, followed by carp and milkfish.

Effect of pH on the Actomyosin Ca-ATPase Activity. All AMs had two identical maximum Ca-ATPase activities at pH 5.8–6.1 and 9.2 (Figure 3). The AMs of tilapia and tilapia hybrid had maximum ATPase activity at pH 5.8, which was lower than that of carp and milkfish (Figure 3) and also other species inhabiting cold areas (Seki, 1977).

The pH of animal muscle is around 7.4, when alive or immediately after slaughter. However, the formation of lactic acid resulting from anaerobic glycolysis decreases the muscle pH to 6.3–6.1, where the myofibrillar proteins have maximum ATPase activity. The hydrolysis of ATP is accelerated and consequently causes an irreversible muscle contraction. The pH of muscle further decreases to 5.4–5.7. Because of the low pH, the glycolytic enzyme activities were greatly inhibited and glycolysis ceased (Nonaka et al., 1976; Fukuda, 1987). In studies on the stability of muscle proteins of chub mackerel, pacific mackerel, and amberfish during frozen storage, much more denaturation of muscle proteins took place in fish frozen during postrigor than those frozen during prerigor was observed (Tsao et al., 1980; Fukuda et al., 1984; Fukuda, 1987). These results indicated that the content and composition of the intermediates resulting from postmortem changes would affect the stability of muscle proteins. According to the studies on the effect of adenosine nucleotides on the stability of frozen fish muscle proteins, high levels of inosine and hypoxanthine resulting from ATP degradation were related to the instability, while high

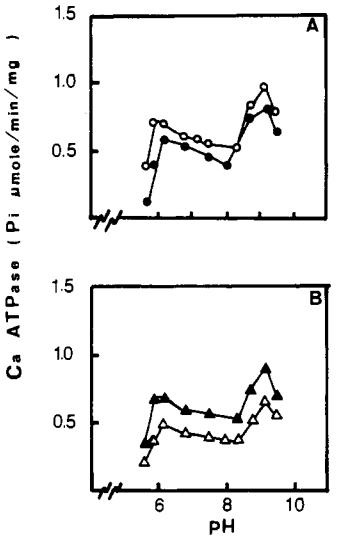

Figure 3. Effect of pH of reaction mixture on the Ca-ATPase of milkfish, tilapia hybrid, tilapia, and carp actomyosin at 25 °C. (Refer to the symbols in Figure 1.)

levels of ATP, ADP, AMP, and IMP were related to the stability of myofibrillar proteins during frozen storage (Jiang et al., 1987b,c).

LITERATURE CITED

Andou, S.; Takama, K.; Zama, K. Interaction between lipid and protein during frozen storage I. Effect of oil dipping on rainbow trout muscle during frozen storage. *Bull. Fac. Fish. Hokkaido Univ.* **1979**, *30*, 282–295.

Andou, S.; Takama, K.; Zama, K. Interaction between lipid and protein during frozen storage II. Effect of non-polar and polar lipid on rainbow trout myofibrils during frozen storage. *Bull. Fac. Fish. Hokkaido Univ.* **1980**, *31*, 201–207.

Andou, S.; Takama, K.; Zama, K. Interaction between lipid and protein during frozen storage III. Interaction between water and lipids surrounding myofibrillar proteins. *Bull. Fac. Fish. Hokkaido Univ.* **1981a**, *32*, 97–104.

Andou, S.; Takama, K.; Zama, K. Interaction between lipid and protein during frozen storage IV. The adaptation of the ultraviolet absorption spectrum method for the determination of protein content in the mixture of lipids and myosin B. *Bull. Fac. Fish. Hokkaido Univ.* **1981b**, *32*, 188–196.

Arai, K. Evaluation of Fish Quality from the Muscle Protein Studies. In *Sakana no Hinshitsu (Quality of Fish)*; Koseisha: Tokyo, 1974; pp 55–80.

Arai, K.; Takashi, R. Studies on muscular proteins of fish—XI. Effect of freezing on denaturation of actomyosin ATPase from carp muscle. *Bull. Jpn. Soc. Sci. Fish.* **1977**, *43*, 533–541.

Bito, M. Studies on the retention of meat color of frozen tuna. *Bull. Tokai Reg. Fish. Res. Lab.* **1976**, *84*, 51–113.

Braddock, R. J.; Dugan, L. R. Reaction of autoxidizing linoleate with Coho salmon myosin. *J. Am. Oil Chem. Soc.* **1973**, *50*, 343–348.

Braun, P. E.; Radin, N. Interaction of lipids with a membrane structural proteins. *Biochemistry* **1969**, *8*, 4310–4215.

Childs, E. A. Interaction of formaldehyde with fish muscle in vitro. *J. Food Sci.* **1973**, *38*, 1009–1012.

Childs, E. A. Interaction of free fatty acids with fish muscle in vitro. *J. Fish. Res. Bd. Can.* **1974**, *31*, 111–116.

Connell, J. J. The relative stabilities of the skeletal muscle myosins of some animals. *Biochem. J.* **1961**, *80*, 503–509.

Fukuda, Y. Effect of freezing conditions on quality of frozen fish meat. *Refrigeration (Japanese)* **1986**, *61*, 18–29.

Fukuda, Y. Degradation of fish quality by freezing and its prevention. *Refrigeration (Japanese)* **1987**, *62*, 1309–1319.

Fukuda, Y.; Tarakita, Z.; Arai, K. Effect of freshness of chub mackerel on the freeze-denaturation of myofibrillar proteins. *Bull. Jpn. Soc. Sci. Fish* **1984**, *50*, 845–852.

Jiang, S. T. Studies on the Denaturation of Mullet Muscle Proteins during Frozen Storage. *Refrigeration (Japanese)* **1977**, *52*, 621–629.

Jiang, S. T. Effect of free amino acids and freezing conditions on protein denaturation of frozen fish. Doctoral Dissertation, University of Rhode Island, 1984.
Jiang, S. T.; Lee, T. C. Changes in free amino acids and protein denaturation of fish muscle during frozen storage. *J. Agric. Food Chem.* **1985**, *33*, 839-844.
Jiang, S. T.; Lan, C. C.; Tsao, C. Y. New Approach to Improve the Quality of Minced Fish Products from Freeze-thawed Cod and Mackerel. *J. Food Sci.* **1986**, *51*, 310-312.
Jiang, S. T.; Tsao, C. Y.; Lee, T. C. Effect of Free Amino Acids on the Denaturation of Mackerel Myofibrillar Proteins in vitro during Frozen Storage. *J. Agric. Food Chem.* **1987a**, *35*, 28-33.
Jiang, S. T.; Hwang, B. S.; Tsao, C. Y. Protein Denaturation and Changes in Nucleotides of Fish Muscle during Frozen Storage. *J. Agric. Food Chem.* **1987b**, *35*, 22-27.
Jiang, S. T.; Hwang, B. S.; Tsao, C. Y. Effect of Adenosine-Nucleotides and their Derivatives on the Denaturation of Myofibrillar Proteins in vitro during Frozen Storage at -20 °C. *J. Food Sci.* **1987c**, *52*(1), 117-123.
Kurokawa, T. Kamaboko-forming Ability of Frozen and Ice Stored Lizard Fish. *Bull. Jpn. Soc. Sci. Fish.* **1979**, *45*, 1551-1556.
Love, R. M. Protein denaturation in frozen fish VI, Cold storage studies on cod using the cell fragility method. *J. Sci. Food Agric.* **1962a**, *13*, 269-273.
Love, R. M. Protein denaturation in frozen fish VII, Effect of the onset and resolution of rigor mortis on denaturation. *J. Sci. Food Agric.* **1962b**, *13*, 534-539.
Matsumoto, J. J. Denaturation of fish muscle proteins during frozen storage. In *Proteins at Low Temperature*; Fennema, O., Ed.; ACS Symposium Series 180; American Chemical Society: Washington, DC, 1979; pp 205-312.
Matsumoto, J. J. Chemical deterioration of muscle proteins during frozen storage. In *Chemical Deterioration of Proteins*; Whitaker, J., Fujimaki, M., Eds.; ACS Symposium Series 123; American Chemical Society: Washingtond, DC, 1980; pp 95-109.
Matsumoto, I.; Ooizumi, T.; Arai, K. Protective effect of sugar on freeze-denaturation of carp myofybrillar proteins. *Bull. Jpn. Soc. Sci. Fish.* **1985**, *51*, 833-840.
Noguchi, S. Characteristics of fish proteins from processing point of view II. Protein denaturation of frozen fish muscle. *New Food Ind. (Japanese)* **1982**, *21*, 34-42.
Noguchi, S.; Matsumoto, J. J. Studies on the Control of the Denaturation of Fish Muscle Proteins during Frozen Storage. *Bull. Jpn. Soc. Sci. Fish.* **1970**, *36*, 1078-1081.
Nonaka, J.; Hashimoto, Y.; Takabashi, T.; Suyama, M. *Marine Food Chemistry (Japanese)*; Koseisha: Tokyo, 1976; pp 52-70.
Nosaki, Y.; Kanazu, R.; Tabata, Y. Freezing Storage of Lizard Fish for Kamaboko Preparation. *Refrigeration (Japanese)* **1978**, *53*, 473-480.
Okada, M.; Yokoseki, M.; Nunomaki, T. Muscle Proteins. In *Minced Fish Meat Products*; Koseisha: Tokyo, 1974; p 38.
Seki, N. Extraction of fish muscle proteins. In *Fish Proteins (Gyoniku Tanpakushitsu)*; Koseisha: Tokyo, 1977; pp 7-23.
Suyama, M.; Konosu, M. Functionality changes of meat during storage and processing.d In *Marine Food* (Suisan *Shokuhin Gaku (Japanese)*; Koseisha: Tokyo, 1987; pp 168-202.
Suzuki, T. *Fish and Krill Proteins, Processing Technology*. applied Science: London, 1981; pp 1-45.
Tsao, C. Y.; Chou, K. C.; Jiang, S. T. Studies on freezing preservation of raw material of minced fish products I. Effect of freshness on quality of frozen mackerel and amberfish. *J. Ch. Agric. Chem. Soc.* **1980**, *18*, 77-85.

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Tobacco-Specific Nitrosamine Accumulation and Distribution in Flue-Cured Tobacco Alkaloid Isolines

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Changes in three tobacco-specific nitrosamines (TSNA) and their alkaloid precursors during growth and the curing process were quantified in seven NC 95 flue-cured isolines with different alkaloid levels. *N'*-Nitrosonornicotine (NNN), *N'*-nitrosoanatabine (NAT), and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) were separated by gas chromatography and measured with a thermionic N-P detector. Mature green leaves contained small amounts (0.6-1.5 $\mu g\ g^{-1}$) of TSNA that increased (0.9-17.8 $\mu g\ g^{-1}$) during curing. Leaves from higher stalk positions and leaves with increased time in the curing process had greater amounts of TSNA. Among the alkaloid isolines, highest significant correlation coefficients between a TSNA and the related alkaloid precursor were 0.95 and 0.76 for the correlations between nornicotine with NNN and anatabine with NAT, respectively. The correlation between nicotine and NNN (r = 0.40) was not significant.

Tobacco-specific nitrosamines (TSNA) in flue-cured tobaccos have not been as extensively studied as they have in other tobaccos and tobacco products. Hecht et al. (1977) quantified (1.31 $\mu g\ g^{-1}$ average) *N'*-nitrosonornicotine (NNN) in Coker 139, a flue-cured genotype, and reported that there was no definitive trend between TSNA and leaf stalk position. Chamberlain and Arrendale (1983) analyzed NNN content in five flue-cured genotypes plus TI-1112 and concluded that there was no significant correlation between NNN levels (70-1000 $\mu g\ g^{-1}$) and the levels of the related alkaloid precursors nornicotine (600-31 400 $\mu g\ g^{-1}$) or nicotine (4000-43 800 $\mu g\ g^{-1}$). Chamberlain et al. (1984) concluded on the basis of effects of nitrogen fertilization on NNN accumulation that there was no significant correlation between nicotine/nornicotine ratios and the levels of NNN in different genotypes of flue-cured tobacco. However, they suggested that some positive correlation existed between the total amount of alkaloids and NNN in cured tobacco. NNN was not detected in green immature tobacco. Chamberlain and Chortyk (1986) and Chamberlain et al. (1987) studied the effect of curing regime on NNN formation in both lamina and midrib tissue of the flue-cured cultivar Speight G-28. It was found that midrib tissue contained a higher concentration of NNN than lamina of air-cured samples of this flue-cured cultivar. Very low levels of NNN were found in midribs of the

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