Binding of Carbonyls to Fish Actomyosin

Srinivasan Damodaran* and John E. Kinsella

Binding of carbonyls to fish actomyosin was studied. The binding affinity and the binding capacity of actomyosin decreased as salt concentrations were increased. The addition of tripolyphosphate at 0.1% and elevated temperatures enhanced the binding affinity. Increasing the chain length of the carbonyl increased the binding affinity, suggesting that hydropholic forces were involved in actomysin–carbonyl interactions.

Coventionally fish protein concentrate is prepared by organic solvent extraction (Finch, 1970). But, the organic solvent treatment results in loss of functional properties of the isolated fish proteins and thus impairs effective utilization in many food products (Bass and Caul, 1972; Moorjani and Vasantha, 1973; Spinelli et al., 1972). The recent aqueous extraction techniques (Chu and Pigott, 1973) are preferred for fish protein concentrate production due to its low cost and retention of many desirable functional properties of fish protein. But, the incomplete removal of lipids during aqueous extraction (Shenouda and Pigott, 1974) results in off-flavor development and loss of nutritional quality of fish protein during storage. The off-flavor development is largely due to formation of trimethylamine and carbonyl compounds derived from oxidation of polyunsaturated lipids in fish. The type and mechanism of formation of various off-flavor compounds from the oxidation of unsaturated lipids have been reviewed (Kinsella and Damodaran, 1980).

Information concerning interaction of off-flavor carbonyls with fish proteins is very limited. In order to facilitate successful removal of off-flavors from fish protein concentrate, basic information on the factors affecting the interaction of carbonyls with fish protein is needed. Using methods previously reported we studied the interaction of alkanones with fish actomyosin.

MATERIALS AND METHODS

Isolation of Actomyosin. Actomyosin from trout was isolated by a modified salt extraction method (Shenouda and Pigott, 1974, 1975). All buffer systems used in this study contained 0.1 mM dithiothreitol to prevent polymerization of actomyosin via sulfydryl oxidation. The temperature was maintained at 0–4 °C throughout the extraction procedure. For the preparation of myofibrils, portions of muscles from trout (obtained from Tunison Laboratory of Fish Nutrition, Cortland, NY) were cut and washed with 5 mM phosphate buffer, pH 7.4, containing 4 mM NaCl, 1 mM $MgCl_2$, 0.1 mM EGTA [ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid] and 1mM dithiothreitol. The muscles were then homogenized in a blender with the above buffer solution and the homogenate centrifuged at 16000g. The supernatant was discarded. The muscle pellet was then rehomogenized and centrifuged twice with the above buffer solution to remove all soluble sarcoplasmic proteins. The myofibrils thus obtained were suspended in 5 mM phosphate buffer, pH 7.4, containing 40 mM NaCl. An equal volume of glycerol was added to the above suspension and stirred at 2 °C for a few hours. Under these conditions the myofibrils could be stored at −20 °C.

Institute of Food Science, Cornell University, Ithaca, New York 14853.

The above myofibril preparation was used as the starting material for the preparation of actomyosin as needed for the binding studies. The myofibrils were separated from the glycerol solution by adding 5 mM phosphate buffer to dilute out the glycerol, followed by centrifugation at 16000g.

For isolation of actomyosin, the washed myofibrils were suspended in 5 mM phosphate buffer, pH 7.4, containing 40 mM NaCl. The ionic strength of the above solution was incresed to 0.6 M by adding calculated amounts of NaCl. The solution was stirred gently at 4 °C for 1 h and then centrifuged at 45000g for 90 min. The supernatant containing actomyosin was used as such for the binding studies with ketones. The protein concentration was determined by the Lowry method (Lowry et al., 1951) using bovine serum albumin as the standard.

Measurement of Binding. Interaction of ketones with fish actomyosin at 0.6 M ionic strength was studied by the equilibrium dialysis method (Damodaran and Kinsella, 1981a). Acrylic cells of equal volume separated by a membrane (Spectropor-2, 12 000 M_r exclusion) were used. In a typical experiment 3 mL of actomyosin solution and 3 mL of buffer containing a known concentration of the ligand (ketone) were placed in compartments separated by the membrane. The cells were shaken at the required temperature for at least 24 h to reach equilibrium. After equilibrium 1 mL of the solution from each side of the membrane was withdrawn and placed in a vial containing 1 mL of isooctane. The ligand from the aqueous phase was extracted into the isooctane phase by shaking. The concentration of the ligand in the isooctane phase was determined by gas chromatography as described previously (Damodaran and Kinsella, 1980). The difference in the concentration of the ligand on either side of the membrane in the dialysis cell represented the amount of the ligand bound to the protein. The concentration of the ligand on the buffer side (no protein) represented the free ligand concentration which is in equilibrium with the bound ligand.

For binding studies with insoluble actomyosin suspensions, the experiments were done as follows: To 1-mL aliquots of actomyosin in buffer containing 0.6 M NaCl 3 mL of 5 mM phosphate buffer containing various concentrations of the ligand was added. Since actomyosin is insoluble at low ionic strength, the decrease in the ionic strength from 0.6 to 0.15 M NaCl resulted in precipitation of actomyosin, forming a uniform suspension in the solution. The vials were capped tightly and incubated for 4 h at the required temperature with shaking to reach equilibrium between the free ligand in the solution and the ligand bound to the insoluble actomyosin suspension. After equilibration, the solutions were centrifuged at 10000g for 10 min. One milliliter of the supernatant was transferred to a vial containing 1 mL of isooctane. The

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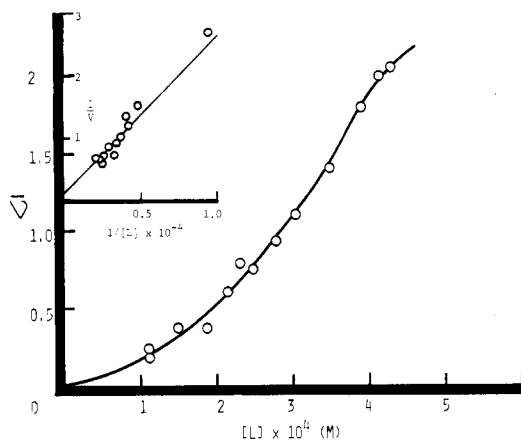*J. Agric. Food Chem.*, Vol. 31, No. 4, 1983 **857**

Figure 1. Binding of 2-nonanone to fish actomyosin at 0 °C in 5 mM sodium phosphate buffer, pH 7.4, containing 0.6 M NaCl and 0.1 mL dithiothreitol. $\bar{\nu}$ is the number of moles of 2-nonanone bound to 10^5 g of actomyosin and [L] is the free 2-nonanone concentration in molarity. The insert shows double-reciprocal plot of the same data.
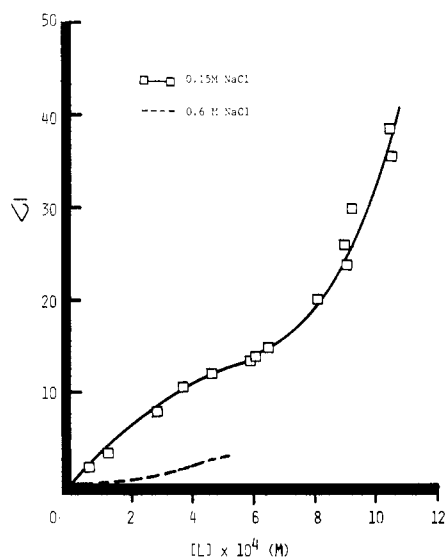

Figure 2. Effect of ionic strength on the binding of 2-nonanone to fish actomyosin at 0 °C.
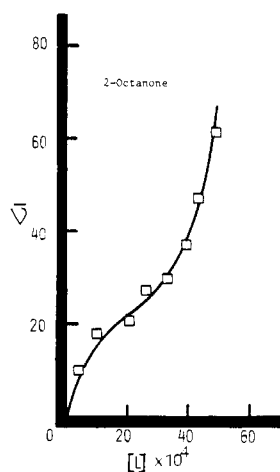

Figure 3. Binding of 2-octanone to fish actomyosin at 0 °C in 5 mM phosphate buffer, pH 7.4, containing 0.15 M NaCl and 0.1 mM dithiothreitol.

ligand was extracted from the aqueous phase into the issoctane phase by shaking. The concentration of the ligand in the isooctane phase representing the free ligand concentration was determined by gas chromatography.
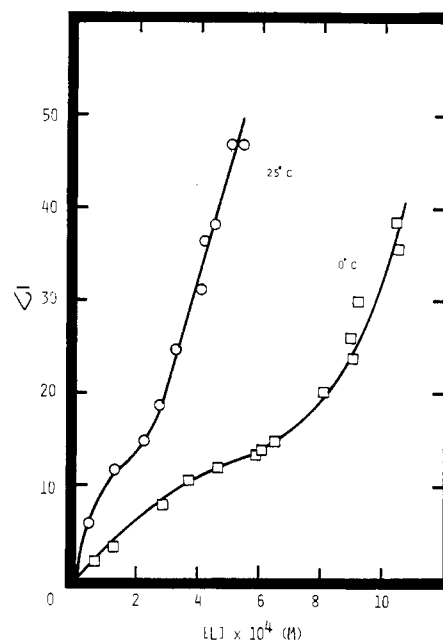

Figure 4. Effect of temperature on the binding of 2-nonanone to fish actomyosin in 5 mM phosphate buffer, pH 7.4, containing 0.15 M NaCl and 0.1 mM dithiothreitol.
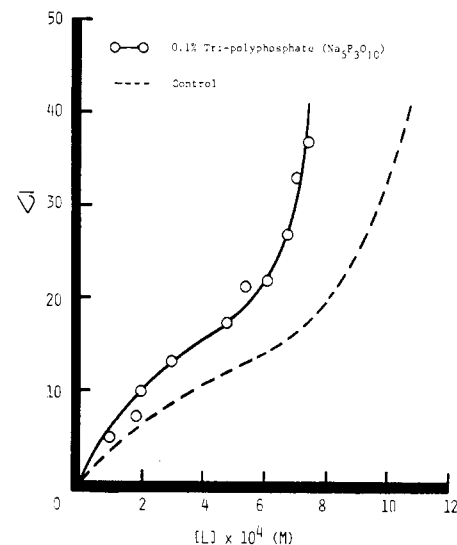

Figure 5. Effect of tripolyphosphate ($Na_5P_3O_{10}$) on the binding of 2-nonanone to fish actomyosin in phosphate buffer containing 0.15 M NaCl and 0.1 mM dithiothreitol. The tripolyphosphate concentration was 2.72 mM (0.1%).

Knowing the concentration of the ligand initially present and the free ligand concentration after equilibrium, we calculated the amount of ligand bound to insoluble actomyosin. From the protein concentration in the system the number of moles of ligand adsorbed per mole of actomyosin (assuming a M_r of 100 000) was calculated.

The binding data were analyzed according to the double-reciprocal equation (Steinhardt and Reynolds, 1969)

$$1/\bar{\nu} = 1/n + 1/(nK[L])$$

where $\bar{\nu}$ is the number of moles of ligand bound per mole of protein, [L] is the free ligand concentration which is in equilibrium with the bound ligand, n is the total number of binding sites, and K is the intrinsic binding constant.

RESULTS AND DISCUSSION

Effect of Ionic Strength. The binding isotherm of 2-nonanone to actomyosin in 5 mM phosphate buffer, pH

7.4, containing 0.6 M NaCl is shown in Figure 1. Since the solubility of 2-nonanone is very low at 0.6 M NaCl, binding ratios above 2 could not be obtained. Apparently, the sigmoidal behavior of binding suggests that the binding may be a cooperative process. A double-reciprocal plot of the same data is presented in the insert of Figure 1. The intercept suggests that in the soluble form at 0.6 M ionic strength there are about 13 binding sites in actomyosin for 2-nonanone. The intrinsic binding constant, calculated from the slope of the curve in Figure 1B, is about 386 M⁻¹ for 2-nonanone. This value is low compared to 930 M⁻¹ and 1800 M⁻¹ for soy protein and bovine serum albumin, respectively (Damodaran and Kinsella, 1981a, 1980).

Actomyosin is completely in the soluble form at 0.6 M ionic strength. Hence, the above binding affinity may not represent the real conditions in the fish muscle where it is in the form of protein fibers. When the ionic strength is lowered to 0.15 M, actomyosin becomes insoluble and forms a uniform suspension. Interaction of this actomyosin suspension with off-flavors may approximate the conditions in fish muscle or fish protein concentrate.

The interaction of 2-nonanone with actomyosin at 0.15 M ionic strength is shown in Figure 2. At 0.15 M ionic strength the binding affinity was significantly greater than at 0.6 M. Double-reciprocal plots of these data indicated that at 0.15 M ionic strength the initial number of binding sites in actomyosin was about 50 (on the basis of 100 000 M_r) and the intrinsic binding constant was about 600 M⁻¹. Since the interaction of a ligand with an insoluble suspension is a surface adsorption phenomenon and not a true binding at the molecular level, the increase in the number of binding sites at 0.15 M ionic strength may be attributable to nonspecific adsorption of 2-nonanone to the protein aggregate. However, from the binding curves shown in Figure 2 it may be deduced that if an actomyosin sample contained higher amounts of bound off-flavor carbonyls (for example, in fish muscle or FPC), solubilization of the protein preparation at 0.6 M ionic strength would greatly decrease the amount of carbonyls bound to the protein. In other words, the off-flavor carbonyls orginally bound to the protein would come out as free ligand which might be removed by dialysis or ultrafiltration.

Effect of Chain Length. The binding of 2-octanone to actomyosin at 0.15 M ionic strength is shown in Figure 3. Double-reciprocal plots of the same data indicated that the binding constant for 2-octanone was about 110 M⁻¹ compared to 600 M⁻¹ for 2-nonanone. This suggests that the binding constant decreased about 5-fold for a decrease in the chain length by one methylene group. This is different from the results obtained for soy protein and bovine serum albumin (Damodaran and Kinsella, 1981a, 1980) in which the binding constant decreasewd 3-fold for each methylene group decreased in the chain length of the ligand. This discrepancy may be attributable to the fact that the experiments with actomyosin were done with insoluble protein suspensions whereas with the other two proteins the experiments were done with protein solutions. The dependency of the binding affinity of ketones on the chain length suggests that the interaction may be hydrophobic in nature. Binding studies with 2-heptanone did not show any appreciable affinity of the ligand for actomyosin. This indicates that the major off-flavor compounds bound to fish tissues may be higher molecular weight carbonyls and other compounds.

Effect of Temperature. The effect of temperature on the binding of 2-nonanone to actomyosin at 0.15 M ionic strength is shown in Figure 4. At 25 °C the binding affinity increased dramatically compared to that at 0 °C.

The increase in the binding affinity at higher temperatures may be due to unfolding of actomyosin which may result in exposure of more hydrophobic regions which were originally buried in the interior of the molecule. Shenouda and Pigott (1975) reported that the formation of an actin–lipid complex was enhanced and stabilized at higher temperatures. This was attributed to conformational changes in actin which may expose more hydrophobic regions for formation of the complex. Furthermore, since hydrophobic interactions exhibit positive enthalpy and entropy changes (Scheraga, 1979), such interactions would be more stabilized at higher temperatures.

Effect of Polyphosphates. Polypohosphates are usually used during fish storage to enhance the water binding capacity of fish muscle and thus reduce drip loss. In order to determine whether the use of polyphosphates has any effect on the binding of carbonyls to actomyosin, the interaction between 2-nonanone and actomysin in the presence of tripolyphosphate (2.7 mM) ($Na_5P_3O_{10}$) was studied. At 0.1% concentration (2.7 mM) tripolyphosphate enhanced the binding affinity of 2-nonanone for actomyosin (Figure 5). The observed effect of tripolyphosphate may be explained in two ways: The decrease in the water activity upon addition of polyphosphate may in turn result in decreased solubility of 2-nonanone in the system and hence "salt out" the ligand onto the insoluble protein matrix. On the other hand, phosphates are known to enhance the structure of liquid water (Dandliker and de Saussure, 1971). Since hydrophobic interactions are a direct consequence of the structural state of liquid water, the enhancement in the hydrogen-bonded water structure in the presence of tripolyphosphate may enhance the hydrophobic interaction between 2-nonanone and actomyosin.

These data indicate that the binding of lipid oxidation products to fish muscle may be accentuated by treatment with polyphosphates and higher storage temperatures. On the other hand, the binding affinity as well as the binding capacity decreased at higher NaCl concentrations. The dependence of the binding affinity on the chain length of the carbonyl suggests that hydrophobic forces are mainly responsible for actomyosin–carbonyl interactions. This suggests that treatment of fish proteins under conditions which destabilize hydrophobic interactions, e.g., addition of chaotropic salts, may facilitate removal of off-flavor carbonyls (Damodaran and Kinsella, 1981b).

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Chemical Characterization and Sensory Evaluation of a Dietary Sodium–Potassium Fish Sauce

Supshorn Chayovan, Ramu M. Rao,* Joseph A. Liuzzo, and Mahmood A. Khan[1]

Uneviscerated ground fish (flounder and trout with 1.6 and 9.2% fat, respectively) were fermented in a mixture of sodium and potassium salts in different fish to salt ratios. Desired ratios of these salts in the fermented fish sauce were obtained by mixing appropriate volumes of individual salt-based sauce. Chemical analyses included total and ammonia nitrogen, salt, pH, carbonyls, amines, and amino acids. In all sauce samples micromoles per liter concentrations of carbonyls such as butanal, octanol, 2,4-decadienal, 2-undecenal, tetradecanal, etc. and amines (mono-, di-, and trimethylamine) were obtained. Amino acids such as lysine, histidine, arginine, aspartic acid, threonine, etc. ranged in concentrations between 10^{-4} and 10^{-3} mmol/L of sauce. The pH of sauce made from flounder and trout ranged from 5.0 to 6.1 and 4.9 to 5.6, respectively. Sensory analyses indicated that a mixture of NaCl and KCl (NaCl:KCl = 50:50) could provide as replacement for NaCl generally used in fish sauce fermentation.

Fish sauce is a clear brown liquid, rich in NaCl and soluble nitrogen compounds. The concentration of NaCl in these sauces ranges between 25 and 32% in the finished products.

The importance of NaCl in human diets is generally recognized. However, there has been considerable concern about the amount of salt in the diet. Much of this concern centers on questions about dietary sodium and hypertension, or high blood pressure (Dahl, 1972; Meneely, et al., 1957; Meneely and Ball, 1958; Institute of Food Technologists, 1980; Kempner, 1948; Vanderstoep, 1978; Tobian, 1960).

The requirement of NaCl as a taste and flavor enhancer in the human diet is so great that many people find it a hardship to be subjected to a salt-restricted meal. However, numerous publications have reported that naturally occurring KCl can replace sodium salt without affecting the sensory qualities of foods (Frank and Mickelson, 1969; Tucker et al., 1957; Michelsen et al., 1977).

A recent work by Chayovan et al. (1983) has shown that an organoleptically acceptable dietary sodium–potassium fish sauce, which can be used by people who are on a sodium-restricted diet, can be prepared. Therefore, this investigation was undertaken as a continuation of this study to formulate a fish sauce fermented in an optimum mixture of NaCl and KCl salts, to evaluate the sensory acceptability, and to identify and quantify certain chemical and flavor constituents of such a sauce.

A review of literature indicated that commercially produced fish sauces using 100% NaCl are fermented for at least 9 months to develop the desirable flavors. Therefore,

Department of Food Science, Louisiana Agricultural Experiment Station, Louisiana State University Agricultural Center, Louisiana State University, Baton Rouge, Louisiana 70803.
[1]Present address: Department of Food and Nutrition, University of Illinois, Urbana, IL 61801.

Table I. Formulation of Fish Sauce[a]

sample	KCl, g	KCL:fish	NaCl, g	NaCl:fish	H₂O, L
		Flounder			
1	600	1:3.3			0.5
2	800	1:2.5			0.5
3			600	1:3.3	0.5
4			800	1:2.5	0.5
		Trout			
1	600	1:3.3			0.5
2	800	1:2.5			0.5
3			600	1:3.3	0.5
4			800	1:2.5	0.5

[a] Sample weight: 2 kg. Storage temperature: 37 °C.

the present investigation is limited to sensory evaluation and chemical characterization of test samples that were fermented for 6 and 9 months.

MATERIALS AND METHODS

Sample Collection and Preparation of Fish Sauce. Two varieties of fish—flounder, a lean fish with low fat content (1.6%), and trout, a fatty fish with 9.2% fat—were selected for the preparation of fish sauce. The methods of fish procurement and the preparation of fish sauce were according to the procedures followed by Chayovan et al. (1983). The desired ratios of sodium and potassium salts in the fish sauce were obtained by mixing appropriate volumetric amounts of the sodium- and potassium-based sauces (Table I).

Chemical Analyses. Chemical analyses included total nitrogen, ammonia nitrogen, salt content, and pH of 1-, 3-, 6-, and 9-month samples and carbonyls, amines and amino acids of 6- and 9-month samples.

Total Nitrogen. Total nitrogen in the fish samples was determined by the Kjeldahl–Gunning modification method as outlined in AOAC (1975).

Ammonia Nitrogen. The principle used for the determination of ammonia nitrogen was as follows: Ammonia