STUDIES ON THE BEHAVIOR AND THE EFFECT OF SOME PRESERVATIVES IN FISH PRODUCTS—II.

CAUSES OF DECREASE BY HEATING IN RECOVERY RATE OF TYLOSIN ADDED TO FISH MEAT*

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In the previous paper¹⁾, tylosin in cooked fish meat was found poorly recovered when determined by the cylinder-plate assay method using *Sarcina lutea*. This phenomenon took place during a relatively short period of heating at not lower than 50° to 60°C.

The present paper reports that fish meat is responsible for the above phenomenon, although the protein does not destroy the tylosin but inhibits activity by adsorption.

Experimental Methods

Determination of tylosin by the cylinder-plate assay method. This was performed by the same method that described in our previous paper¹⁾.

Determination of tylosin by the dilution method. Into a liquid medium consisting of 0.5% peptone, 0.15% meat extract, and 0.3% yeast extract, the pH being adjusted to 8.0, the lactate of tylosin was added at 0.08, 0.09, 0.1, 0.12, and 0.14 ppm respectively to prepare a standard series. The above medium was poured into 3 test tubes each 10 ml, and was sterilized. The test solution was diluted with the medium above mentioned to prepare a series of dilution for instance 1:20, 1:25, 1:30, 1:35, etc., then poured into test tubes in the same manner as done with the standard series.

One drop each of an overnight broth culture at 32°C of *Sarcina lutea* was inoculated and following an overnight culture at 32°C, the growth of the test organism was examined. And, by referring to the standard series, the concentration of tylosin was calculated. At a certain dilution in which any of the 3 test tubes did not show any growth of *Sarcina*, the concentration in question was regarded to be on the borderline of growth inhibition. In such an instance, the dilutions near them were tested again, repeating the test, if required, until the 3 tubes agreed.

When the test solution was so originally turbid to judge with the unaided eyes the growth of *Sarcina*, one platinum-loopful of the culture was transferred to a new deep agar medium for further culture, and thus the presence or absence of growth

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was confirmed.

Preparation of myosin protein. Horse mackerel meat was added with 5 times volume of 0.6 M KCl. The mixture was stirred well, stood for about 2 hours, centrifuged, and its supernatant was obtained. Diluting the supernatant with water to 5 times its volume, the resultant precipitate was collected by centrifuging. The precipitate was then rinsed well with 0.06 M KCl, dehydrated by adding methanol, dehydrated further with ether.

Preparation of methylester of protein. Two hundred ml of methanol was added to 2 gm of dry protein, into which 2 ml of concentrated hydrochloric acid was then added. This mixture was stood at room temperature for three days and then rinsed several times with methanol to remove hydrochloric acid. It was subsequently rinsed thoroughly with ether, followed by drying. Although methoxyl group of the sample protein was not chemically determined, according to FRAENKEL-CONRAT and OLCOTT², the above procedure is able to render almost all the carboxyl groups of protein into methyl-ester.

Experimental Results and Discussion

1. Substances responsible for the poor recovery in the fish meat. In order to know what substances of the fish meat is responsible for the decreased tylosin recovery by heating, the following fractionation was performed with round head shark meat used as the sample.

Adding 250 ml of water to 50 gm of minced meat of round head shark, the meat was ground well and rendered into an homogeneous suspension, which was employed as the stock solution. Water-soluble fraction was obtained by centrifuging 50 ml of the stock solution at 5,000 rpm for 10 minutes. Adding water to the precipitate, stirring well, and centrifuging, the supernatant was discarded. This procedure was performed over again. The water-insoluble fraction thus obtained was suspended in water and made up to 50 ml.

As the water-soluble fraction contains a water-soluble protein, the following procedure was performed in order to remove the protein. Adding 250 ml of 80% methanol to 50 gm of round head shark meat, this was ground well and centrifuged. The supernatant was obtained and therefrom the methanol was removed under reduced pressure, followed by dilution with water to the original volume. This was called as a methanol-soluble fraction.

The four types of the fractionated solutions thus obtained were adjusted to pH 6.2. Forty ppm of tylosin was added to each solution, followed by heating at 85°C for 30 minutes. And the antibacterial activity was determined by the cylinder-plate assay method. The results are exhibited in Table 1.

Thus, the recovery rate after heating was found to be 85% for the primary

Table 1. The recovery rate of tylosin added to various fractions of fish meat.
(Tylosin added: 40 ppm, pH 6.2, Heating: 85°C 30 min.)

Fraction tested	Recovery rate of tylosin after heating
Original meat suspension*	85
Water soluble fraction	94
Water insoluble fraction	81
80% methanol soluble fraction	100

* 50 g of round shark meat suspended to 250 ml of water.

solution, 94% for the water-soluble fraction, 81% for the water-insoluble fraction, and 100% for the methanol-soluble fraction.

The water-soluble fraction containing water-soluble substances and water-soluble protein showed a recovery rate of 94%. The water-insoluble fraction consisting mostly of myosin protein showed a recovery rate of 81%, nearly the same as the recovery rate observed with the primary solution. Since the methanol-soluble fraction contain other substances than protein, the other substances than protein do not seem to be related to the lowering the recovery rate of tylosin. The aforementioned results may suggest that the fish meat protein is mainly responsible for the lowering the recovery rate of tylosin.

Various additives are usually employed in manufacture of fish sausage. They include such reducing substance as ascorbic acid and such meat pigment fixers as sodium nitrite. In order to see how those substances effect on the recovery of tylosin, the commonly used additives were put into big eye tuna meat, grounded well, and then added with tylosin, followed by heating at 85°C and the determination of tylosin activity (Table 2).

Table 2. Effect of food additives on the recovery rate of tylosin added to fish meat.
(Fish meat used: big eye tuna, Tylosin added: 40 ppm, Heating: 85°C 30 min.)

Additives	Recovery rate of tylosin after heating (%)
No added	43.7
Sodium ascorbate 0.1%	46.7
Monosodium glutamate 0.3%	44.5
Polyphosphates 0.2%	41.7
Sodium nitrite 0.01%	43.7

The results indicate that the substances used as additives to the fish sausage have practically no effect on the rate of recovery after heating. It is likely that such reducing substances as ascorbic acid as used in our present studies have no effect.

2. Recovery of tylosin added to processed proteins. The above mentioned studies have made it clear that the fish meat protein does exert an effect and reduce the recovery rate of tylosin. In order, then, to know what effect other proteins than

fish meat protein have, the following tests were made with egg-white albumin and gelatin. Both those proteins were rendered into a 5% solution and a 10% solution. Forty ppm of tylosin was added to each solution and after being heated at 85°C for 30 minutes the determination was made. The results are presented in Table 3.

Table 3. Decrease of the recovery rate of tylosin added to egg albumin and gelatin.
(Tylosin added: 40 ppm, pH 6.2, Heating: 85°C 30 min)

Protein	Recovery rate of tylosin after heating (%)
5% Egg albumin	57.7
10% "	46.4
5% gelatin	80.8
10% "	66.1

The above results indicate that, even in the instance of the processed proteins, heating under addition of tylosin reduces the recovery rate of tylosin, the rate in the instance of 10% solutions being 46.4% for egg-white albumin and 66.1% for gelatin.

These results may suggest that the other proteins than fish protein also do induce the lowering of the recovery rate of tylosin added and heated.

3. The possibility of tylosin being adsorbed by protein. So long as protein is mainly responsible for the decreased recovery of tylosin with subsequent heating, there seems more likely that tylosin is adsorbed by protein to inactivate than being destroyed in the sample solution.

Thinking thereupon that changing the electric charge of protein might change its adsorbability which might change the recovery of tylosin, experiments were then performed by varying the pH of fish meat sample.

Big eye tuna meat was added with 5 times its volume of a buffer solution, the pH was adjusted to 2.2, 4.0, 6.0, and 8.0, and each of the fish meat suspensions thus prepared were added with 40 ppm of tylosin, followed by heating at 85°C for 30 minutes. Subsequently, the antibacterial activity of tylosin in each sample were measured.

Tylosin is known to be degraded into desmycosin at a pH lower than 5.0, and may be more degraded to substances with far lower antibacterial activity⁸⁾⁴⁾. So, in the case of this group of experiment, the results were checked by a control sample prepared by adding tylosin into the buffer solution alone. The results of the above studies are exhibited in Table 4.

The results indicated in Table 4 are that tylosin heated in buffer solutions of pH 2.2 and 4.0, respectively, declined in the recovery rate to 68.2% and 69.1%, respectively. This finding seems to indicate that tylosin becomes degraded in an acidic solution. Almost no change was observed, on the contrary, at pH 6.0 and pH 8.0.

Table 4. Recovery of tylosin added to fish meat with various adjusted pH.
(Fish meat used: big eye tuna, Tylosin added: 40 ppm, Heating: 85°C 30 min)

pH	A Tylosin found after heating in buffer solution (ppm)	B Tylosin found after heating in meat suspension (ppm)	B/A Recovery rate (%)
2.2	27.2	28.6	105
4.0	27.6	29.7	107
6.0	37.2	23.2	62.3
8.0	36.8	10.4	28.2

Considering the amount of tylosin recovered after heating in the buffer solution to be 100, the tylosin recovery from the fish meat suspension was computed as follows: at pH 2.2, 104%; at pH 4.0, 107%; at pH 6.0, 62.3%; and at pH 8.0, 28.2%. At pH 4.0 or lower, almost the entire amount of tylosin or its degraded product was recovered. That is, as the pH approaches the alkalinity, the recovery rate found lower. This seems to suggest that there is a high possibility for tylosin to become adsorbed by the fish meat protein.

Since tylosin is a basic substance, it may be adsorbed by the carboxyl group of protein, which might be the main cause of its poor recovery. To confirm this point an experiment was carried out with methyl-esterified protein, the carboxyl group of which is sealed by methyl group.

Commercial egg-white albumin and the horse mackerel myosin protein were methyl-esterified as described before, rendered into a 10% solution with buffer at pH 6.2, added with tylosin, and heated at 85°C for 30 minutes, followed by the determination of the recovery rate of tylosin (Table 5).

Table 5. The recovery rate of tylosin added to methylated egg albumin and myosin fraction
(Tylosin added: 40 ppm, pH: 6.2, Heating: 85°C 30 min).

Protein	Recovery rate of tylosin after heating (%)
Egg albumin	45.5
Methylated egg albumin	59.1
Myosin fraction*	78.8
Methylated myosin fraction	83.8

* Prepared from horse mackerel meat.

As presented in Table 5, both methyl-esterified proteins did not show much difference from those original proteins, although the former was slightly higher in recovery than latter. So, the results does not interpret that tylosin is adsorbed by the carboxyl group in particular.

4. Recovery of tylosin adsorbed by fish meat. If tylosin is adsorbed by fish meat protein there might be some possible means to extract the entire amount of tylosin in the sample.

Fish meat added with tylosin was heated at 85°C for 30 minutes, and extracted with buffer at pH 8.0. Effect of repeating the extraction, by adding 10 times of its volume of the buffer and the volume of the buffer solution on the recovery of tylosin was determined.

Table 6. Effect of repeated extraction and volume of extractant on the recovery rate of tylosin from cooked fish meat. (Extractant: M/15 phosphate buffer, pH 8.0)

Condition		Recovery rate of tylosin (%)
Repeated extraction	1	37.9
	2	9.6
	3	<6.0
Volume of extractant	5 times	35.8
	7 "	39.9
	10 "	42.9
	15 "	42.0

As shown in Table 6, by repeating the extracting procedure sum of the recovery was found increasing. As the first extraction was 37.9%, 3.7% of recovery should be expected on the second extraction, if there were no adsorption take place in the sample. The actual recovery, however, was found to be 9.6%, nearly 3 times as high as that of theoretical, which interpretes that some kind of adsorption actually takes place in the fish meat. Under the method mentioned above the total recovery was only around 50%.

Further experiments were carried out by varying the amount of buffer solution for the extraction. The more the amount of the solution, the better was the recovery rate. The recovery was found however, practically constant when the amount of extracting solution exceeded 10 times as much as the volume of the sample. This indicates tylosin in the fish meat cannot be wholly recovered even after repeated extraction procedure, but remains at a certain level.

Extraction with acidic buffer solutions showed slightly higher recovery than the above, though the total recovery did not exceed 45%. Adding common salt, which was expected to replace with the adsorbed tylosin on the fish protein, showed only a slight increase in the recovery.

Extraction with methanol, ethanol, acetone, etc. at 50°C showed slightly higher recovery than aqueous buffer. Benzene, however, showed lower recovery than the buffer.

From the above experiment it was indicated that tylosin adsorbed on fish meat protein is liberated only about 50% by series of extraction. Consequently, more tylosin might be larger liberated if the adsorbed protein be broken down into smaller components. Therefore, a sample protein adsorbing tylosin on it was broken down with a proteinase.

Big eye tuna meat after the addition of tylosin was heated at 85°C for 30 minutes in order that the tylosin be adsorbed on the fish meat. The sample meat was then ground and prepared into a 1:10 suspension with buffer of pH 8.0. Pronase, commercial name of a proteinase produced by the Kaken Kagaku & Co. was added therein at a rate of 0.2% and digestion was thus allowed to take place at a temperature of 50°C. Each aliquot of sample was taken at certain intervals and was assayed for tylosin activity, by both the cylinder-plate assay method and the dilution method. A control was prepared by adding 0.2% Pronase to a buffered solution of tylosin at pH 8.0, which was kept in the same as the sample, thus the effect of the enzyme on tylosin was studied (Table 7).

Table 7. Recovery of tylosin from cooked fish meat by proteolytic digestion.
(Fish meat used: big eye tuna, Tylosin added: 40 ppm, Heating: 85°C 30 min).

Hour of digestion at 50°C	Recovery of tylosin incubated with proteinase		Recovery of tylosin from sample meat by proteinase	
	Cylinder-plate method (ppm)	Dilution method (ppm)	Cylinder-plate method (ppm)	Dilution method (ppm)
0	40.0	39.0—41.4	12.1	25.2—27.0
1	40.3	"	8.8	34.2—36.0
2	39.8	"	10.3	"
3	38.5	"	12.9	39.6—41.4
4	37.0	"	13.1	"
5	36.0	"	15.3	"

As is seen in the Table, in the case of control, the longer the sample was incubated, the less, though very slightly, tylosin was recovered when determined by the cylinder-plate assay method, on the contrary, by the dilution method the theoretical amount of tylosin was wholly recovered. The poor recovery of tylosin when determined by the cylinder-plate assay method may be that a part of tylosin in the solution is adsorbed on the protein component of the enzyme. The complete recovery of tylosin in the same sample when determined by the dilution method may be because the adsorbed tylosin is perfectly liberated due to the dilution with a large amount of medium.

When the tylosin-adsorbing meat was digested with Pronase, the recovery rate, when determined by the cylinder-plate assay method, showed rather a low level at the early stage of digestion, later on, as the digestion progressed, the recovery increased but slightly. Whereas when the same samples were examined by the dilution method, even a sample without digestion the recovery rate was about twice as much high as that demonstrated by the cylinder-plate assay method. With a further progress in digestion, the rate of tylosin recovery rose gradually, and by 3 hours' digestion the theoretical amount of tylosin was recovered.

These phenomena may suggest that tylosin adsorbed on a fish meat is not

liberated even by the proteolytic digestion but is liberated when the sample meat is finely ground with medium to suspension and heated.

The above mentioned observations seem to have practically clarified that the decrease in the rate of recovery of tylosin in fish meat heated at or about 85°C for 30 minutes or so is not because tylosin is destroyed in the meat but because it is so adsorbed by fish meat protein as to become incapable of being extracted. It is likely that even when it can be extracted, tylosin bound to such an extent with protein or peptide as is unable to become dispersed in agar fails to exhibit antibacterial activity.

TOMIYAMA *et al*³⁾, however, reported that tylosin added to fresh muscle tissue could be inactivated by the effect of certain reducing groups upon heating at 100°C. Such observations different from our results may be due to the difference of heating temperature used in the experiment.

Whether tylosin adsorbed by fish meat protein can, in that state, develop antibacterial activity is a very important problem. This will be taken up in our next report.

Summary

Experiments were performed in order to know why the recovery rate of tylosin added to fish meat is declined when determined by the cylinder-plate assay method.

1) Recovery study of tylosin from various fractions of fish meat has indicated that mainly the fish meat protein is responsible and no other soluble fractions of the meat are responsible for lowering the recovery rate of tylosin.

2) Such processed protein as egg-white albumin and gelatin were, like fish meat protein, responsible for lowering the recovery rate of tylosin.

3) Fish meat which pH has been adjusted to 4.0 or lower allows the complete recovery of tylosin added, in contrast, the higher the pH, the lower the rate of recovery.

4) Various procedure of extraction were used for tylosin added to fish meat and heated, but the maximal recovery was around 50%. In contrast, determination by the dilution method after digestion by proteinase permitted the complete recovery of the entire amount of tylosin added.

5) It may be concluded from the above observation that the decline in the recovery rate of tylosin added to fish meat and heated at 85°C for 30 minutes is not because tylosin is destroyed but because it is adsorbed on the fish meat protein.

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