Postmortem Degradation of Fish Muscle Proteins

1. Nature of proteolysis and bacterial contribution

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어육단백질의 사후분해

1. 단백질분해의 본질과 세균기여

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백색 육어류의 사후 기간중에 일어나는 단백질 분해의 본질을 규명하려는 방도로써 가자미류(English sole, *Paraphyrus vetulus*)와 볼락류(rockfish, *sebastodes caurinus*)의 어육을 시료로 하여 맨 먼저 가자미 전 어체를 빙장하는 동안 취하여 어육 slurry를 만든 후 20℃에서 유지시키는 동안의 단백질 분해율을 측정하였다. 근육자가소화효소 cathepsin에 의한 단백질 분해도를 규명키 위하여 어육 slurry 일부는 0.5Mrad 감마선조사로써 무균화시키면서 세균 번식활동에서 오는 단백질 분해가능성을 제거시켰다.
어육을 기계적으로 분쇄하여 얻은 slurry를 무균화한 후 20℃ 수조에서 17시간 동안 유지시켰는데도 불구하고 단백질 분해가 전혀 검측되지 안했음은 cathepsin 작용이 없었음을 뜻한다. 이와 반면에 비조사구 slurry의 경우, 20℃ 수조에서의 유지기간중 총균수의 증가에 따라 약간의 단백질 분해가 이뤄졌으나 slurry로 만들어지기 전의 어체의 부패도가 후기에 접어 들어 총균수가 최고선에 도달하였을 때 비로소 현저한 단백질 분해를 가져 왔다. 이는 부패초기에는 부패세균에 의한 단백질 분해효소의 합성이 거의 없음을 시사한다.

이어서 볼락어육을 무균적으로 취하여 그 일부는 단백질 분해력이 강한 Pseudomonad균을 접종하고, 나머지는 각각 0, 0.5, 그리고 2.0Mrad의 감마선에 조사한 후 0°~2℃에서의 저장기간중에 일어나는 단백질 분해과정을 규명하였다. 세균번식이 없는 무균어육의 염용성(0.5M KCl) 총질소질과 70% 에타놀 용해성 아미노태 질소량은 저장기간중 약간 감소된 반면 Pseudomonad균을 접종한 어육의 질소질의 증가는 총균수 증가에 평행하였다.

이로써 어류의 사후 기간중에 일어나는 단백질 분해는 정상적인 어류부패과정의 일부분이기는 하나 부착 세균의 번식이 진행되어 총균수가 최고선에 도달할 때까지 단백질 분해는 지연되며 최소한 백색 어육에 관한한 사후기간중 cathepsin에 의한 단백질 분해에의 기여도는 거의 무시될 수 있음이 확인되었다.

INTRODUCTION

Our present knowledge on the the separate contribution of autolysis and bacterial action to post-mortem degradation of fish muscle proteins is mostly from comparative storage studies of normally spoiling fish and sterile fish samples. Partman (1954) reviewed the results of earlier workers(Reed *et al.*, 1929; Kamarov, 1933; Nickerson and Proctor, 1935; Beatty and Collins, 1939; Nickerson *et al.*, 1950; and Proctor *et al.*, 1950)and concluded that the actions of microflora developing in normally spoiling fish are largely responsible for the protein breakdown and that, in white flesh fish at least, autolytic proteolysis independent of bacterial action is very slight.

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This was confirmed in recent studies based on free amino acid changes occurring in fish muscle postmortem(Hodgkiss and Jones, 1955; Jones, 1955; Shewan and Jones, 1957; de Silva and Hugbes, 1960; and Bramstedt, 1962). Although there is some evidence that in fatty fish such as mackerel proteolysis independent of bacterial action may be significant(Nickerson *et al.*, 1950), such protein degradation even in the fatty fish can preceed only so far and more striking changes in appearance, flavor, and texture of fish are caused by bacteria(Shewan, 1962).

In normally spoiling fish, proteins are not attacked by bacteria until fish enter into an advanced stage of spoilage(Beatty and Collins, 1939; and Tarr, 1954). On the other hand, Shewan and Jones(1957) noted with cod that during the early period bacteria multiply at the expense of non-protein nitrogen (NPN) components of fish muscle and that a near complete disappearance of the NPN components coincides with the advanced fish spoilage. During the time sequence of fish spoilage, *Pseudomonas* spp. increase at the expense of other groups of microflora and emerge as predominant species by the time fish enter into the advanced spoilage (Shewan and Liston, 1956; de Silva, 19 60; and Shewan, 1962). The predominance of *Pseudomonas* spp. appears to take place regardless of the type and size of the initial fish microflora (Liston *et al.*, 1963). In view of foregoing points, it would be important to know the possible relationship between the gradual disappearance of NPN components and the onset of proteolysisin spoiling fish muscle and the role of *Pseudomonas* spp. in the the preteolysis occurring in fish muscle postmortem. This study is the first of a series of investigation on the postmortem degradation of fish muscle proteins in terms of sequential changes in biochemical conditions occurring in spoiling fish muscle and the response of spoilage bacteria to the different environmental conditions, designed specifically to test the nature of proteolysis of fish muscle and the possible involvement of bacteria in it.

METHODS AND MATERIAL

Proteolysis in fish muscle during storage in ice

Preparation of fish homogenate samples: English sole (*Paraphyrus vetulus*) were caught by Otter trawl. The fish of 30 to 35 cm in length were killed immediately by stabing with scaple in the nape region, eviscerated, and packed in ice aboard. The fish were transported to the processing labolatory, received, and stored in a walk-in cold room (0-2°C). They were reiced every fourth day during 31 days of storage.

At intervals of 0, 1, 2, 4, 8, 17 and 31 days of storage, four fish were removed and filleted immediately at room temperature(22°C)by method similar to commercial practice with the exception that the fillets were not rinsed with water. Two samples each weighing 25 gm were cut from each fillet and homogenized with ten timts their volume of pre-chilled (0°C), sterile distilled water in a waring blender for two minutes at 0°C. The remaining portion of the fillets were allowed to stand at room temperature for two hours and then samples were prepared as before.

Estimation of preteolysis: 100ml. each homogenate was transferred into sterile, dry French square bottles in duplicate. One bottle from each pair was exposed to 0.5 Mrad of gamma radiation at 1°C, while the other was kept at 0°C as a control. After the irradiation, the control and irradiated fish homogenate samples were paced in a 20°C(±0.1°) water bath. At intervals of 0, 2, 4, 8 and 17 hr of incubation, a 5 ml aliquot was drawn from each sample and mixed with 5 ml of 10% trichloroacetic acid (TCA) in a test tube. The mixture was allowed to stand at room temperature (22°C) for 2 hr before being heated for 30 min in a 45°C water bath, then filtered through Whatman No. 50 paper to obtain a clear filtrate. The amino nitrogen (amino-N) in the TCA filtrate was determined by the ninhydrine

method, as modified by Moore and Stein(1954), and the results were expressed as μ Mole leucin-N per gm fish flesh.

Total bacterial count: An aliquot sampled at each interval was used for estimating total bacterial population by the drop-plate method of Gaudy *et al.*, (1963), using sea water MacLeod agar(MacLeod *et al.*, 1954) and pepton water as medium and diluent, respectively. Colonies on plates were counted after 2 days of incubation at 22°C and the results expressed as logarithmic number of bacteria per gm fish flesh.

Proteolysis in sterile fish muscle and muscle incubated with a proteolytic Pseudomonad

Preparation of sterile fish muscle: Live rockfish (*Sebastodes caurinus*) caught by Otter trawl also were transported live and kept in a seawater aquarium until being used. This species was chosen primarily because the fish are round, non-fatty fish and could provide sterile muscle required for this study when carefully excised aseptically.

A small wooden hood was constructed having a slant sea-through glass top, two sliding windows on both sides of lower front for hands, and a side window to pass fish in and out of the hood. The field of hood was shined with an UV germicide disinfector unit for 30 min prior to operation. The live fish, one at a time, were stunned by a blow on the head and placed inside the hood. The muscle of 10 to 15 gm was excised from the dorsal region aseptically and transferred into dry, sterile four oz wide-mouth bottles provided with plastic screw caps. The exact weight of muscle was determined by weighing the bottles before and after the transfer of samples.

Proteolytic Pseudomonad: Culture No. 1021 used in this study was chosen among the stock culture of fish isolates and maintained at the College of Fisheries, University of Washington. The strain was isolated from skin of fish on 6th hour of ice storage (Liston and Kasemsarn, 1962). It is actively motile, Gram-negative rod, and typically aerobic, and produces green pigment. According to the Shewan's scheme(Shewan, 1959), it belongs to Pseudomonad I. The strain peptonizes litumus milk with alkaine reaction, liquefies getaline rapidly and its cell-free growth medium hydrolyzes fish proteins, milk proteins and gelatine equally well (Chung, 1963).

The proteolytic *Pseudomonad* was grown in nutrient broth at 22°C overnight and transferred into the fresh medium twice before being diluted with peptone water to the cell density of approximately 10^7 cells per ml.

Inoculation and storage of muscle samples: The aseptically excised muscle samples were divided into inoculated and sterile groups. A portion of the sterile samples was irradiated at the dose of 0.5 and 2.0 Mrad to insure the sterility of muscle during storage. Thus the sterlie samples were divided into three groups; 0, 0.5, 2.0 Mrad irradiated. For the inoculated group 2 ml of the cell suspension was spread on the surface of unirradiated sterile muscle samples to give approximately 10^6 cells per gm fish flesh. All samples were stored at 0-2°C.

Estimation of proteolysis: At intervals of 0, 1, 5, 10, 20, 40, and 60 days, two bottle were withdrawn from each group and the muscle homogenized with two times the volume of sterile, prechilled (at 0-2°C) 0.5*M* KCl solution in a waring blender for 2 min and the homogenate centrifuged (11,000 x g) at 0°C for 10 min. An aliquto of the supernatant was used for total nitrogen (Total-N) determination by Kjeldahl method(A. O. A. C., 1970) and the results expressed as μ Mole N per gm fish flesh. At the same time an aliquto of the same supernatant was made to 70% in ethanol using absolute ethanol and the ethanol filtrate obtained similarly as previously mentioned in TCA filtrate preparation. The amino-N soluble in 70% ethanol was determined by the same ninhydrin method as before. The ratio between amino nitrogen and total nitrogen for each

storage interval was computed as an index of proteolysis.

Total bacterial count: The total viable count was estimated also from an aliquot sampled at each interval by the standard pour plate technique, using phosphate buffer (pH 7.2) water as diluent and sea water MacLeod agar (MacLeod *et al.*, 1954) as medium. Colonies on plates again were counted after 2 days of incubation at 22°C and the results expressed as logarithmic number of bacteria per gm fish flesh.

RESULTS AND DISCUSSIONS

Protein breakdown in fish muscle during ice storage

The changes in viable count in the fish muscle during ice storage and in its homogenate, both irradiated and unirradiated, during incubation at 20°C are shown in Table 1. The viable cells in the fish muscle on 0 day of ice storage (approximately 4 hours of ice storage after capture) were a little over 37,000 per gm fish flesh (4.57 in logarithm of number). The viable population, after the first two days of lag period, started to increase, reaching above 10^7 cells per gm fish flesh by the 31st day.

In the homogenates prepared from the fish of each ice storage interval, the viable population continued to increase very rapidly during the subsequent 17 hours of incubation at 20°C after a short initial lag. It is noted that the increase was higher with the fish homogenates of low bacterial population prior to incubation; thus in the homogenate prepared from 0-day fish, the viable population increased nearly one thousand fold, while the increase in the homogenate from 31-day fish was only ten fold. No viable count was obtained in the homogenates irradiated at 0.5 Mrad, even after 17 hours of incubation at 20°C. A dose of 0.5 Mrad is apparently sufficient to inactivate the fish microflora of levels as

Table. 1 Bacteria in the muscle tissue of English sole stored in ice

(Log No./*gm* muscle)

Sample group	Irradiated at (Mrad)	*	Storage (day) 0	1	2	4	8	17	31
Fillets homogenized with no holding time	0.0	0 hr	4.76	4.60	4.62	4.72	5.26	5.80	7.42
		2	4.50	4.40	4.55	4.54	5.18	5.65	7.40
		4	4.65	4.50	4.40	4.70	5.50	6.67	7.51
		8	5.34	5.20	5.26	5.40	6.00	7.15	7.77
		17	7.32	7.20	7.25	7.40	7·60	7.97	8.50
Fillets homogenized with no holding time	0.5	0	0**	0	0	0	0	0	0
		2	0	0	0	0	0	0	0
		4	0	0	0	0	0	0	0
		8	0	0	0	0	0	0	0
		17	0	0	0	0	0	0	0
Fillets homogenized after 2hours standing at 20°C	0.0	0	4.65	4.58	4.60	4.66	5.28	5.90	7.42
		2	4.48	4.30	4.34	4.52	5.12	5.80	7.38
		4	4.55	4.51	4.46	4.72	5.45	6.60	7.50
		8	5.25	5.15	5.22	5.30	6.11	7.15	7.74
		17	7.30	7.22	7.26	7.32	7.45	7.80	8.40
Fillets homogenized after 2 hours standing at 20°C	0.5	0	0	0	0	0	0	0	0
		2	0	0	0	0	0	0	0
		4	0	0	0	0	0	0	0
		8	0	0	0	0	0	0	0
		17	0	0	0	0	0	0	0
Fish muscle			4.75	4.40	4.48	4.60	5.20	5.80	7.40

*Incubation of homogenate at 20°C.

**Zero plate count indicates less than 20 viable cells/*gm* muscle.

hight as 10^7 cells per gm fish flesh. The changes of vaible count in the homogenates prepared from fillets after holding at room temperature for two hours were not significantly different from those in samples prepared immediately after filleting.

The changes of TCA soluble amino-N in the fish muscle during ice storage and in its homogenates during subsequent incubation at 20°C are shown in Table 2. The amino-N content in O-day fish muscle ranged 63 to 64 u Moles per 100 gm fish flesh and it decreased slightly during subsequent ice storage until the 31-day, when the values increased over the initial levels. Also no significant increase of amino-N was noted in the homogenates during 17 hours of incubation at 20°C unless the fish from which homogenate samples were prepared had been stored in ice longer than 17 days. As representative of four sample groups, the changes of amino-N in unirradiated samples prepared from fillets with no holding time are shown in Fig. 1.

From the fact that amino-N values did not increase above initial levels until the level of developing microflora reached above 10^7, it appears that protein are not attacked by bacteria until the late stages of fish spoilage. Shewan and Jones (1957) reported that there occurs initially a gradual disappearance of non-protein nitrogenous (NPN) components before the onset of proteolysis in spoiling cod muscle. The same observations were made by Jay and Kontou (1967) with spoiling beef at 7°C.

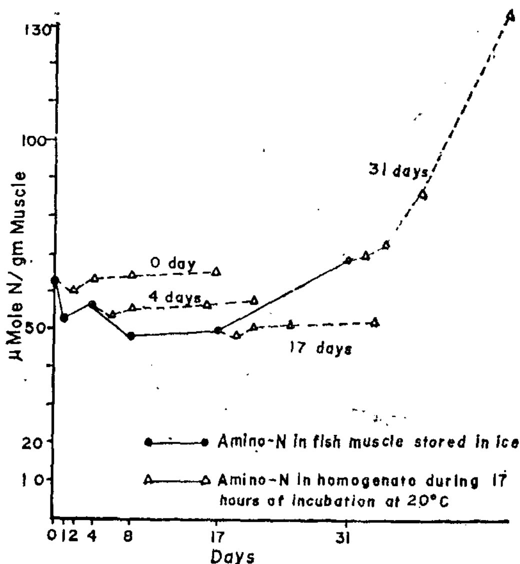

Fig. 1. Amino-N in the muscle tissue of English sole stored in ice and during subsequent incubation of its homogenate at 20°C. (Each point represents four separate determinations).

The delay phenomenon of proteolysis by bacteria in spoilng fish is more evident from the finding that the amino-N value of homogenates prepared from fish having viable bacterial population less than 10^7 did not increase appreciably during 17 hours of incubation at 20°C due to an apparent absence of proteinases elaboraled by bacteria. Mechanical disruption of fish muscle by homoginization process and subsequent incubation of the homogenate at high temperature would have greatly enhanced the endogenous muscle proteinase activity, if there were any such muscle enzyme acting upon fish muscle proteins under the condition provided. This apparent absence of so-called cathepsin activity is contrary to the suggestions of Silbert and Schmitt (1965) that fish muscle cathepsin activity could contribute significantly to the postmortem degradation of fish muscle proteins.

The increase of amino-N in the unirradiated 31-day fish homogenate is much greater that in the irradiated 31-day fish homogenate (Table 2) and this is apparently due to the fact that in the irradiated homogenate, proteolysis was limited to the amount of proteinases produced by bacteria before the radiation treatment and survived 0.5 Mrad of gamma radiation, whereas in the unirradiated sample bacteria continued to produce more proteinases during the 17 hours of incubation at 20°C.

The results of this study indicate, firstly, that the proteolysis, as measured by increase of amino-N soluble in TCA, was shown to be due entirely to bacterial action and secondly, that the proteolysis independent of bacteria action is negligible in white flesh fish such as English sole

Table 2. Amino N in the muscle of English sole stored in ice (μ *Mole* N/*gm* muscle)

Sample Group	Irradiated at (Mrad)	*	Storage (day) 0	1	2	4	8	17	31
Filleets homogenized with noeholding time	0.0	0 hr	63.21	53.01	54.37	56.34	48.16	49.69	68.25
		2	60.68	51.56	53.71	53.76	47.73	48.25	69.94
		4	63.73	51.46	53.86	55.86	46.73	50.73	72.76
		8	64.15	52.06	55.00	56.11	48.59	51.72	86.41
		17	65.26	53.28	55.13	57.75	49.25	52.04	133.98
Filletshomogenized with no holding time	0.5	0	64.52	53.34	54.07	55.44	48.50	48.72	70.35
		2	63.42	52.92	53.97	52.60	47.56	47.55	74.50
		4	60.48	53.49	53.12	54.28	46.10	48.51	75.12
		8	63.00	53.89	53.75	55.13	48.90	47.56	87.78
		17	64.26	53.81	54.01	55.34	50.62	53.44	109.41
Fillets homogenized after 2hours holding at 20°C	0,0	0	64.40	54.07	56.86	56.91	48.59	59.53	67.93
		2	62.58	52.00	55.97	55.84	48.38	47.34	71.92
		4	63.31	52.97	55.72	57.54	47.93	49.50	75.07
		8	64.35	53.95	56.04	58.64	48.94	50.25	83.37
		17	65.10	54.70	57.00	50.16	49.55	55.05	126.23
Fillets homogenized afte 2hours holding at 20°C	0.5	0	64.25	53.23	56.33	58.43	48.73	47.25	67.73
		2	62.26	52.71	52.39	54.74	46.89	46.01	70.34
		4	64.47	53.34	55.02	56.89	45.75	47.77	74.36
		8	67.20	53.38	55.92	57.85	47.34	48.67	88.20
		17	67.48	54.54	56.76	58.52	49.15	52.29	105.52

*Incubation of homogenate at 20°C.

during the practical storage condition.

Proteolysis in sterile fish muscle inoculated with a fish spoilage Pseudomonad

The changes in total viable counts in each group are shown in Table 3. In the inoculated samples the counts increased from 10^6 on 0 day to 10^{10} cells per gm fish flesh by the 10th day and the viable population entered into the stationary phase thereafter. By the 60th day, the count was declining. The unirradiated sterile samples were free of viable bacteria only for 10 days due to apparent contamination and the count increased from 10^2 on the 20th day to 10^4 cells per gm fish flesh on the 40th day. The sterile samples irradiated at 0.5 Mrad, however, remained sterile until the 60th day and no bacteria were found in the 2 Mrad irradiated samples throughout entire 60 days of storage.

Although objective test was not a part of this study, it was noted that the inoculated samples deteriorated (in appearance and odor)rapidly. By the 5th day, the samples were distinctively putrefactive and bacterial growth on the surface became evident by the 10th day. This was followed by liquefaction of muscle samples and by the 60th day more than half of the muscle was liquefied. The unirradiated sterile samples became slightly putrid by the 40th day, but no liquefaction of muscle was noted. The irradiated sterile samples developed no obvious changes attributable to bacterial spoilage, however, the 2.0 Mrad irradiated samples had a strong off-odor which persisted during the storage with progressive darkening on the surface of sample, begining 10th day.

Total-N soluble in 0.5 *M* KCl and amino-N soluble in 70% ethanol(less proteins precipitated with 70% ethanol) of 0 day samples ranged from 362.0 to 373.1 and from 61.0 to 63.3 u Moles per gm fish flesh, respectively. In the inoculated samples both values increased very rapidly with storage, reaching 1,812.7 and 686.8 μ Moles per gm fish flesh by the 60th day of storage (Table 4) and these increases were in parallel with increases of bacterial number in the samples (Table 3). On the other hand, the total-N and

Table 3. Bacteria in the muscle tissue of rockfish at 0-2°C (Log No. /*gm* muscle)

Sample group	Irradiation(Mrad)	Storage at 0-2°C (days) 0	1	5	10	20	40	60
Inoculated muscle	0	6.5	6.7	9.7	10.0	10.6	10.3	9.8
Sterile muscle	0	0	0	0	0	2.1	4.6	4.3
Sterile muscle	0.5	0	0	0	0	0	0	2.5
Sterile muscle	2.0	0	0	0	0	0	0	0

Table 4. Amino-N and total-N in the muscle tissue of rockfish at 0-2°C (μ *Moles* N/*gm* muscle)

Sample group	Irradiation (Mrad)	Analysis	Storage at 0-2°C (days) 0	1	5	10	20	40	60
Inoculated muscle	0	amino-N	63.3	63.1	108.0	221.8	363.5	619.2	686.8
		total-N	373.1	365.1	470.4	906.8	1,421.7	1,786.7	1,812.7
Sterile muscle	0	amino-N	61.0	56.3	55.3	60.0	56.4	56.2	58.7
		total-N	372.3	350.1	348.9	363.5	339.0	328.8	344.0
Sterile muscle	0.5	amino-N	61.2	56.7	52.2	49.8	46.6	50.3	51.4
		total-N	362.0	350.2	338.3	308.3	305.6	298.3	304.1
Sterile muscle	2.0	amino-N	61.0	55.2	51.2	49.2	47.6	48.9	48.3
		total-N	369.7	345.4	326.0	305.8	298.1	291.9	288.2

amino-N of the sterile samples steadily decreased during the storage with the exception of the unirradiated sterile samples in which the values increased slightly on the 60th day due probably to bacterial action (Table 3 and 4). The proteolytic action of Pseudomonad inoculated into the sterile fish muscle is evident from the increase in total-N extactable by 0.5*M* KCl and from corresponding increase increase in amino-N soluble in 70% ethanol. The overall increase of amino-N was more than tenfold whereas total-N increased only by sixfold.

When amino-N content was expressed as per cent of the total-N at each sampling interval, the increase of the ratio of amino-N to total-N occured only in the inoculated samples, corresponding to the bacterial action, while the ratio in sterile sample groups remained unchanged

Table 5. Ratio between amino-N and total-N in the muscle tissue of rockfish at 0-2°C(amino-N expressed as per cent of total-N)

Sample group	Irradiation(Mrad)	Storage at 5 0—2°C (days) 0	1	5	10	20	40	60
Inoculated muscle	0	16.9	17.3	22.9	24.5	31.8	34.6	37.9
Sterile muscle	0	16.4	16.1	15.8	16.5	16.6	17.1	17.1
Sterile muscle	0.5	16.9	16.2	15.4	16.2	15.3	16.9	16.9
Sterile muscle	2.0	16.5	16.0	15.7	16.1	16.0	16.8	16.8

throughout storage (Table 5). This ratio may serve as a useful index of proteolysis in a system involving a simultaneous protein breakdown and assimilation of NPN components by microflora developing in spoiling fish muscle.

The results of this camparative storage study of sterile fish samples and the samples inoculated with a proteolytic Pseudomonad support the results obtained with English sole and confirm the nature of proteolysis occurring in spoiling fish muscle and apparant involement of bacteria in it as revealed in preceding study with English sole.

SUMMARY

Two experiments were conducted to study the nature of protein degradation in fish muscle postmortem, first one with English sole (*Paraphyrus vetulus*) followed by another with rockfish (*Sebastodes* spp.). In the first one, proteolysis was measured by the increase of amino-N in gutted fish during storage in ice and in the homogenates prepared from fish of different ice storage during 20°C-incubation. In order to test the possible involvement of fish muscle a cathepsin, a portion of each homogenate sample was exposed to 0.5 Mrad of gamma radiation to destroy viable microorganisms prior to the incubation. Proteolysis was not detected until viable count reached a level above 10^7 cells per gm fish flesh, corresponding to 31 days of ice storage. Even if fish flesh were mechanically disrupted by means of homogenization and subsequently incubated at 20°C, proteloysis attributable to muscle cathepsin was not detected.

In the second with rockfish muscle aseptically prepared from freshly killed fish, the samples were inoculated with a proteolytic strain of fish spoilage Pseudomonad or irradiated at 0, 0.5 and 2.0 Mrad. The four samle groups were stored at 0-2°C to compare the spoilage pattern of sterile and non-sterile muscle. In sterile muscle both total-N (extracted in 0.5*M* KCl) and amino-N (soluble in 70% ethanol) declined slightly while the inoculated muscle showing increase in parallel with the increase of number of inoculated bacterium.

The results indicate that proteolysis is a part of normal fish spoilage and the onset of proteolysis is delayed until viable count reaches its maximum level. Contribution of fish muscle cathepsin to protein degradation in white flesh fish muscle post-mortem is nil.

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