Studies on Jellyed Meat of Tuna—III Proteolytic Activity of the Jellyed Meat of Yellowfin Tuna*

Shiro KONAGAYA** and Keishi AMANO**

(Received July 10, 1973)

The proteolytic activity of the jellyed meat of yellowfin tuna was examined. Extracts of the jellyed meat exhibited maximal hemoglobin splitting activity at pH 3. The pH-activity curves of the jellyed and normal meat extracts were qualitatively similar. The activity of the jellyed meat in the advanced stage of degradation of the muscle architecture was found to be three-fold greater than that of the normal meat. The activity of the muscle in an earlier stage was about one and a half-fold higher than the normal level. The extract of the muscle adjacent to the jellyed portion where no abnormality could be observed from the external appearance showed about the same level of activity as that in the jellyed portion of the same fillet.

Since the degraded fish muscle such as jellyed, milky and wormy meat is always associated with the presence of a specific protozoan parasite which varies with the species of fish, it has been believed that the parasite has some causative relation to the degradation of muscle.^{1,2)} WILLS³⁾ and other workers^{1,4,5)} hypothesized that the parasite secretes a powerful proteolytic enzyme, which causes breakdown of the muscle tissue. PATASHNIK *et al.*¹⁾, TSUCHIYA *et al.*⁴⁾ and ASAKAWA⁵⁾ demonstrated a comparatively high proteolytic activity at acidic pH in the extract of milky halibut, jellyed swordfish and jellyed tuna, respectively. However, so far there is no sufficient evidence to warrant their hypotheses that the activity be derived from protozoan parasite. It must be of interest to characterize the proteolytic enzyme in the jellyed or milky fish, in particular, if the property of the enzyme coincides with that of cathepsins existing in the normal muscle tissue, since it may be a possible way of examining the relationship between the proteolytic enzyme and the parasite.

In this paper, the quantitative assay procedure of the proteolytic activity in the jellyed meat (Azuki***) of yellowfin tuna was developed and a comparison of the activity was made in muscle samples varying in the degree of abnormality.

Experimental

Materials Jellyed (Azuki) fillets of yellowfin tuna were separated by sight into three groups of varying degree of the abnormality: slightly infected fillet, fillet in intermediate grade of the infection, and heavily infected fillet. The tunas were caught with

* 東海区水産研究所業績 B 581 号

** Tokai Reg. Fish. Res. Lab., Kachidoki 5, Chuo-ku, Tokyo, Japan (小長谷史郎, 天野慶之, 東海区水産研究所)

*** A common name for one of the abnormally degraded condition of fish muscle⁶⁾.

a purse seine net in June, 1971, off the south coast of Mie Prefecture, gutted on board and iced. Within 48 hr after capture they were brought to the Tokyo Central Wholesale Fish Market, and filleted. Body weight of the fish ranged from 44 to 55 kg. Three pairs of fillets from six different fish were used for comparison of the proteolytic activity.

The slightly infected meat was similar to the normal meat in external appearance as well as elasticity when sensed by fingers, except that a number of very small pockets¹⁾ which are generally considered the forerunners of jellification were seen on the cut surface. In the intermediately and heavily infected fillets, the degree of degradation varied with different portions of the fillet. The severity of the condition was greatest in the vicinity of the vertebra (Fig. 1, C, Deep dorsal muscle), where the muscle tissue had lost the

Fig. 1. Sampled portions of the muscle of yellowfin tuna.

A: Superficial-dorsal muscle, B: Interior-dorsal muscle, C: Deep-dorsal muscle, T: Caudal muscle.

eligible fish dealers. All the specimens were stored at -40°C until use.

Hemoglobin substrate Five grams of hemoglobin (Hb) powder (bovine Hb, Calbiochem Co., Ltd.) were stirred in 100 ml of 0.05 N NaOH in the presence of 30 g of urea at 30°C for 1 hr. During this treatment, the Hb powder was completely solubilized. To remove urea from the mixture*, it was dialyzed against four changes of 2 l of water for 40 hr. The dialyzed solution was concentrated by partial freezing to final Hb concentration of 4% and used as substrate. The final concentration of urea in the dialyzed Hb solution was not over 0.01 M.

Preparation of extract One part of sample meat was homogenized with 1.5 or 3 parts of cold 0.5% KCl solution in a Waring blender for 2 min. The homogenate, after standing for 2 hr below 3°C , was centrifuged at $7,200 \times g$ for 30 min. The supernatant was filtered through a fluted filter paper (Toyo Roshi No. 2), and used for the assay. The filtrate was dialyzed against 20 times its volume of 0.5% KCl for 20 hr, if necessary.

* High concentration of urea may decrease the enzyme activity⁷⁾.

elasticity and was partially destroyed, and some of the heavily infected fillets showed liquefaction. However, none of the abnormality was found in the caudal muscle (Fig. 1, T) of infected fillets so far examined. Specimens were, therefore, taken from four different portions of a fillet as shown in Fig. 1, and subjected to the activity assay. In addition, the reference specimens were similarly collected from two different yellowfin tuna of which muscle was judged normal by some

Protein concentration in the extract was determined by FOLIN-LOWRY method⁸⁾.

Detection of proteolytic activity with Hb The procedure used for the assay of proteolytic activity was essentially that of ANSON.⁹⁾ To 3 ml of buffer solution as described later were added 1 ml of the muscle extract and 1 ml of 4% dialyzed urea-denatured Hb solution. Control incubation system was prepared in the same manner except that water was substituted for Hb. Another control system was composed of buffer and Hb, and water in place of the muscle extract. The mixture was incubated for 5 hr at 37°C with continuous stirring. Acetate-hydrochloric acid buffer (WALPOLE buffer¹⁰⁾) was used in pH range between 1.5 and 2.5, phosphate-citric acid buffer (MACILVAIN buffer¹⁰⁾) between 2.5 and 9.0, and borate buffer (ATKINS-PANTIN buffer¹⁰⁾) between 8.0 and 10.0. The reaction was stopped by the addition of 5 ml of 5% trichloroacetic acid (TCA) solution to the reaction mixture. After standing overnight at room temperature, the mixture was filtered through a filter paper (Toyo Roshi No. 5A) and the filtrate was served for estimation of the products of enzymic reaction. The estimation was made on 1 ml aliquot of the filtrate according to the method of FOLIN-CIICALTEU¹¹⁾ as modified by HAGIWARA¹²⁾ using 5 ml of 0.55 M Na₂CO₃ and 1 ml of the diluted (1:2) phenol reagent. The optical density (OD) of the FOLIN-CIICALTEU color was measured at 660 m μ (10 mm cuvette). The proteolytic activity was expressed as reading at 660 m μ . The OD value of 0.1 corresponds to 0.563 μ mole tyrosine per 1 ml of the TAC filtrate.

Extractability of enzyme Each of jellied and normal meat (35 g) was homogenized in a Waring blender with 75 ml of 0.5% KCl for 2 min. The homogenate was centrifuged at 7,200 $\times$ g for 30 min. The sediment was repeatedly extracted in the same manner. The residue after the 4th extraction was frozen at -40°C and then thawed with water at 5°C. This freezing-thawing treatment was repeated more than three times. Finally the thawed residue was extracted with 0.5% KCl as mentioned above. Each of the extract obtained was made up to 100 ml with 0.5% KCl. The Hb-splitting activity in 1 ml aliquot of each extract was assayed after pH was adjusted to 3.0. In addition, total nitrogen in 1 ml of each extract was determined by KJELDAHL method.

Influence of incubation time and enzyme concentration on Hb-splitting activity The extract (sample to solvent ratio of 2: 3) was diluted to 3: 1, 1: 1, and 1: 3 with 0.5% KCl. Reaction was run using 1 ml of the original extract or each dilution, and 1 ml of 4% Hb as substrate, at pH 3.0 (MACILVAIN buffer) and at 37°C for the period of incubation ranging from 30 min to 10 hr.

Influence of dialysis on activity A thirty milliliters portion of the extract (1: 3) was placed in a cellophan tubing (Viking Co., Ltd.) and dialyzed against three changes of 600 ml of 0.5% KCl for 24 hr. The Hb-splitting activity in the dialyzed and undialyzed extracts was compared by 2 hr incubation at pH 3.0 and at 37°C.

Standardized assay procedure The standardized assay procedure adapted for the

comparison of Hb-splitting activity in the muscles varying in the degree of abnormality is as follows: To 3 ml of buffer (pH 3.0, MACILVAIN buffer) was added 1 ml of undialyzed extract (sample to solvent ratio of 1:3) and 1 ml of 4% Hb as substrate. The mixture was incubated with continuous stirring for 2 hr at 37°C. The reaction was stopped by the addition of 5 ml of 5% TCA. Estimation of the reaction products in the TCA-filtrate was made as described above for detection of proteolytic activity. Relative activity was expressed as the OD value of the FOLIN-CIOCALTEU color at 660 m μ for 1 ml of the TCA-filtrate.

Results

Detection of proteolytic activity of the jellied and normal meat extract In Fig. 2, the OD of the FOLIN-CIOCALTEU color of an aliquot of the TCA-filtrate was plotted against pH of the actual reaction mixture. Maximal proteolysis was observed at pH 3 for both assay mixtures of the jellied and normal meat extracts. In contrast to a far less degree of proteolysis in the control system for the normal meat extract, a considerable progress of proteolysis was observed in the control system for the jellied meat. This proteolytic activity may be referred as autolytic activity,^{13,14} since the meat extract provided both enzyme and substrate protein. Because of the high autolytic activity of the jellied meat extract, it may not be reasonable to compare the level of proteolytic activity in the extract of muscle differing in degree of the jellification by whole proteolytic activity. Therefore,

Fig. 2. Over-all proteolytic activity in the extracts of the jellied and normal meats. —●—: Complete assay mixture for the jellied, —●—: Control system for the extract of the jellied, —○—: Complete assay mixture for the normal, —○—: Control system for the extract of the normal, —×—: Control system for Hb substrate.

Fig. 3. Hemoglobin-splitting activity in the extracts of the jellied meat and normal meat.

the activity on added Hb substrate, which is ordinarily termed Hb-splitting activity, was taken as measure of the proteolytic activity in muscles varying in degree of jellification. Fig. 3 shows the pH-activity curves for Hb-splitting activity of the jellied and normal meat extracts. The optimum pH for maximum Hb-splitting activity was at pH 3 for both meat extracts. This is within the range of optimum pH value for cathepsin D in the muscle tissue of various animals.¹⁸⁾ There is a marked difference between the level of the Hb-splitting activity of the jellied meat extract and that of the normal meat extract. No measurable proteolysis was recognized in the alkaline range at an incubation temperature of 37°C.

Extractability of enzyme Since the muscle tissue of the jellied meat has been loosened or disintegrated, and was very much susceptible to homogenization,¹⁸⁾ there was a suspicion that the higher level of proteolytic activity in the jellied meat extract is associated with the increased extractability of enzyme. The Hb-splitting activity as well as total nitrogen content in each of the extracts obtained by repeated extractions was examined (Table 1).

Table 1. Extractability of proteolytic enzyme.

Extraction number	Jellied		Normal	
	Activity (OD)	Total-N (mg/ml)	Activity (OD)	Total-N (mg/ml)
1	0.144	5.95	0.044	5.96
2	0.074	1.09	0.020	1.20
3	0.030	0.23	0.009	0.26
4	0.022	0.08	0.007	0.08
5*	0.032	0.05	0.008	0.04
Total	0.302	7.40	0.088	7.54

* The residue was extracted after freezing-thawing treatment.

The activity and total nitrogen content in respective extracts were expressed as percentage of total activity and total nitrogen extracted through the repeated extraction, respectively. The results are given in Fig. 4. The percentage activity in the jellied meat extract as well as in the normal meat extract decreased as extraction proceeded. The percentage extractability of nitrogenous compounds was paralleled with the change in the activity. The fact appears to show that the

Fig. 4. Extractability of proteolytic enzyme in the jellied and normal meats.

extractability of enzyme is not different between the jellied meat and the normal meat under the experimental conditions.

On the other hand, there has been reported some evidence for "particle-bound" state of cathepsin D in muscle tissue,¹⁷⁻¹⁹⁾ however, the cathepsin is solubilized by freezing-thawing treatment of muscle prior to the extraction.¹⁹⁾ Since the frozen samples were used in this experiment, it is probable that the extractability did not differ between the samples varying in the degree of abnormality, and a comparison of the activity level in the muscle could be made by single extraction procedure, although, only 50% of the total activity was extracted during the first extraction.

Influence of dialysis on Hb-splitting activity According to SLIWINSKY²⁰⁾, two factors interfere with the assay of proteolytic activity in beef muscle. The first is chromogenic materials absorbing in the ultraviolet region, which were extracted along with the enzyme. The second is some inhibitory compounds present in the undialyzed extract. When FOLIN-CIocalTEU method^{11,12)} is used for the assay of reaction products, the first problem is probably eliminated¹³⁾. Therefore, influence of dialysis on the activity was examined. The results are given in Table 2. During the course of the dialysis, the final volume of the inner solution (extract) was increased due to dilution, however, the total activity was unchanged. Evidently, both extracts of the jellied and normal meat did not contain sufficient dialyzable substances which inhibit or activate the reaction.

Table 2. Influence of dialysis on proteolytic activity in extract.

Condition of fillet	Treatment	Control (OD)	Assay mix (OD)	Difference
Heavily jellied	Undialyzed	0.403	0.565	0.162
	Dialyzed	0.183	0.339	0.156 (0.168)*
Normal	Undialyzed	0.093	0.155	0.062
	Dialyzed	0.067	0.121	0.054 (0.057)*

* Value corrected by a factor for the increase in volume of the extract during dialysis.

Influence of incubation time and enzyme concentration on Hb-splitting activity To determine the optimal condition of the assay of Hb-splitting activity with respect to reaction time and enzyme concentration, a series of activity assay was made using different dilutions of extracts of both normal and jellied meat. As shown in Fig. 5, the activity of the original extract (sample to solvent ratio of 2:3) of the jellied meat was not linear with time, however, a linear relation was found with the dilutions of 3:1, 1:1 and 1:3 up to 2 hr. The activity in undiluted (sample to solvent ratio of 2:3) and diluted (1:1) extract of the normal meat were linear with time during the first 4 hr. The activity measured after 2 hr incubation was also approximately linear to the dilution, except the undiluted jellied meat extract.

Fig. 5. Influence of incubation time and enzyme concentration on Hb-splitting activity.

Fig. 6. Comparison of Hb-splitting activities in the heavily (▨), intermediately (▤) and slightly (▥) activities infected meat, and normal meat (□).

Comparison of Hb-splitting activities in the jellied and normal meats A comparison of Hb-splitting activities at pH 3.0 in the muscles of yellowfin tuna varying in the degree of abnormality, and in the four different muscle portions of the same fillet (Fig. 1) is graphically given in Fig. 6. In all three groups of samples, the activity did not vary greatly from portion to portion in the same fillet. There are, however, distinct differences in the activity between the muscle of the normal fish and that of the infected fish. The activities in the intermediately and heavily infected muscles were about three times higher than that in the normal muscle. The activity in the slightly infected muscle was about one and a half times the normal level. These results would suggest that proteolytic enzyme

plays a significant role in the muscle degradation. On the other hand, the fact that the degradation occurs most severely in the vicinity of the vertebra, where the body temperature is maximum,^{21,22)} probably indicates the temperature dependence of the degradation and participation of proteolytic enzyme in the muscle degradation process. It seems reasonable to assume that in the case of the slightly infected muscle the degradation rate is low due to the low proteolytic activity, and the body temperature is lowered before the muscle degradation proceeds retaining the possibility of further degradation depending upon the conditions given (unpublished results).

Discussion

Although TSUCHIYA *et al.*⁵⁾ and other workers^{2,6)} demonstrated the proteolytic activity in extract of degenerated muscle such as jellied and milky fish, a question remains still unsolved as to whether the proteolytic enzyme is released by the parasite as WILLS³⁾ inferred. This is likely due to difficulty in isolation or culture of the parasite to determine directly if the parasite produces proteolytic enzyme. If proteolytic enzyme in the jellied meat can be shown to be different from cathepsins which exist in the normal muscle, it should be possible to demonstrate the extent of relationship between the high proteolytic activity in the jellied meat and the presence of parasite. Furthermore, the abnormal degradation of fish muscle tissue, jellification, occurring post-mortem will be elucidated. In this respect this study was begun to obtain quantitative data on the proteolytic activity in the jellied meat (Azuki) of yellowfin tuna.

Many meat researchers postulated a relationship between autolysis, especially proteolysis, and increased tenderness of muscle during post-mortem aging, and they considered autolytic degradation of muscle tissue, mainly fibrous components involving the connective tissue, to be attributed to the catheptic action. On the other hand, BODWELL *et al.*²³⁾ and other workers^{24,25)} reported that the major substrates for cathepsins are the sarcoplasmic proteins. ZENDER²⁶⁾ and RODOUCO-THOMAS *et al.*²⁷⁾ showed that autolysis occurring in meat over long period of storage is accompanied by the degradation of the fine structure of the muscle fiber. The recent chemical and microscopic studies²⁸⁻³²⁾ showed that three distinct changes occurring in the myofibrillar structure probably relate to the increased tenderness of aged meat. The first is a loss of adhesion of myofibrils. The second is a loss of association of actin and tropomyosin within the I-band region. The last is a loss of material from the Z-discs leading in some cases to complete dissolution of the structure. ASHLEY *et al.*³³⁾ and STROMER *et al.*³⁴⁾ independently demonstrated the similar phenomenon, the disappearance or partial destruction of Z-discs of myofibrils, after the myofibrils were treated with trypsin. However, despite of numerous works, the conclusive evidence has not yet been obtained on the participation of proteolytic enzyme

in these structural changes.

The authors¹⁶⁾ previously described that the structural changes of myofibrils occurring during jellification of muscle of yellowfin tuna are similar to those occurring during meat aging. The present results suggest the participation of proteolytic enzyme in the jellification of the fish muscle, however, they do not give any answer to how the proteolytic enzyme is related to the presence of parasite. A new question arose as to the pH-dependence of the activity. Although the proteolysis would be expected to occur in the range of pH of the post-mortem muscle (pH 5 to 7), the maximal proteolysis was observed at pH 3 for both of the jellied and normal muscles, and as the pH is increased from 3, the reaction rate is drastically decreased.

Acknowledgements

The authors wish to express their gratitude to Dr. Y. MAKINODAN, Kyoto Univ., for his helpful suggestion.

References

- 1) M. PATASHNIK and H. S. GRONINGER: *J. Fish. Res. Bd. Canada*, **21**, 335-346 (1964).
- 2) K. MATSUMOTO: *Referential Data Book, No. 7, Japan Frozen Foods Inspection Corporation*, Tokyo (1954).
- 3) A. G. WILLS: *Australian J. Sci. Res., (Ser. B)*, **2**, 379-398 (1949).
- 4) Y. TSUCHIYA and Y. TATSUKAWA: *Tohoku J. Agric. Res.*, **7**, 347-349 (1957).
- 5) S. ASAKAWA: *Report of Nankai Reg. Fish. Res. Lab.*, No. 5, 176-186 (1957).
- 6) S. KONAGAYA, M. BITO and K. AMANO: *This Bull.*, **36**, 597-605 (1970).
- 7) B. M. WOJTCWICZ and P. ODENSE: *Canadian J. Biochem.*, **48**, 1050-1053 (1970).
- 8) O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL: *J. Biol. Chem.*, **193**, 265-275 (1951).
- 9) M. L. ANSON: *J. Gen. Physiol.*, **22**, 79-89 (1938).
- 10) K. CHITANI and Y. KONDO: in "Jikkenkagaku-koza" (ed. by S. AKABORI and S. FUNAHASHI) Vol. 24, Maruzen Co. Ltd., Tokyo 1958, pp. 201-229.
- 11) O. FOLIN and V. CIOCALTEU: *J. Biol. Chem.*, **73**, 627-650 (1927).
- 12) B. HAGIWARA: in "Kosokenkyuho" (ed. by S. AKABORI). Vol. 2, Asakura shoten, Tokyo 1956 pp. 242.
- 13) T. R. KASZALKA and L. L. MILLER: *J. Biol. Chem.*, **235**, 665-668 (1960).
- 14) K. A. CALDWELL: *J. Agr. Food Chem.*, **18**, 276-279 (1970).
- 15) "Tissue Proteinases" (ed. by A. J. Barrett and J. T. Dingle) North-Holland Publishing Co., Inc., Amsterdam (1971). ix-x,
- 16) S. KONAGAYA and K. AMANO: *This Bull.*, **37**, 904-911 (1971).
- 17) K. A. CALDWELL and O. K. GROSJEAN: *J. Agr. Food Chem.*, **19**, 108-111 (1971).
- 18) P. REDDI, L. M. CONSTINIDES and H. A. DYMSZA: *J. Food Sci.*, **37**, 643-648 (1972).
- 19) F. C. PARRISH and M. E. BAILEY: *J. Agr. Food Chem.*, **15**, 88-94 (1967).
- 20) R. A. SLIWINSKY, D. M. DOTY and W. A. LANDMANN: *Agr. Food Chem.*, **7**, 788-791 (1959).
- 21) F. G. CAREY and J. M. TEAL: *Proc. Nat. Acad. Sci.*, **56**, 1464-1469 (1969).
- 22) S. KONOGAYA, K. YAMABE and K. AMANO: *This Bull.*, **35**, 410-416 (1969).
- 23) C. E. BODWELL and A. M. PEARSON: *J. Food Sci.*, **29**, 602-607 (1964).
- 24) J. G. SHARP: *J. Sci. Food Agric.*, **14**, 468-479 (1963).
- 25) A. J. LUTLO-BOSA and H. F. MACRAE: *J. Food Sci.*, **34**, 401-404 (1969).

- 26) R. ZENDER, C. LATASTE-DOROLLE, R. A. CALLET, P. ROWINSKY and R. F. MOUTON: *Food Res.*, **23**, 305-326 (1958).
- 27) C. RADOUCO-THOMAS, C. LATASTE-DOROLLE, R. ZENDER, R. BUSSET, H. M. MEYER, and R. F. MAUTON: *ibid.*, **24**, 453-482 (1959).
- 28) C. L. DAVEY and K. V. GILBERT: *J. Food Sci.*, **33**, 2-7, 343-348 (1968), **34**, 69-74 (1969).
- 29) C. L. DAVEY and R. M. DICKSON: *ibid.*, **35**, 56-60 (1968).
- 30) M. FUJIMAKI, N. ARAKAWA, A. OKITANI and O. TAKAGI: *ibid.*, **30**, 937-943 (1965).
- 31) T. FUKAZAWA and T. YASUI: *Biochim. Biophys. Acta*, **146**, 534-537 (1967).
- 32) T. FUKAZAWA, E. J. BRISKY and F. TAKAHASHI: *J. Food Sci.*, **34**, 606-610 (1969).
- 33) C. A. ASHLEY, K. R. PORTER, D. E. PHILPOTT and G. M. HASS: *J. Exptl. Med.*, **94**, 9-20 (1952).
- 34) M. H. STROMER, E. D. GOLL and L. E. ROTH: *J. Cell Biol.*, **34**, 431-445 (1967).