Proc., Symp. on Advances and Priorities in Fisheries Technology, SOFT(I) and CIFT Cochin, 11-13 Feb:322 (1998)

Tissue Proteinase Activity in Structural Proteins of Mackerel

LEEMA JOSE and M.R. RAGHUNATH
Central Institute of Fisheries Technology, Cochin 682 029

Tissue proteinase activity (TPA) in mackerel (*Rastrelliger kanagurta*) was studied. Extraction of sarcoplasmic fraction from muscle did not completely remove TPA from myofibrillar fraction. With inclusion of sodium dodecyl sulphate it was possible to extract more protein into the sarcoplasmic fraction, but TPA was still present in the myofibrillar fraction, though to a lesser extent, in both autolytic and proteolytic modes of assay. Repeated extraction of muscle with low ionic strength buffers could not completely free the myofibrillar fraction from TPA indicating its strong attachment to this fraction.

Key words: Mackerel, tissue proteinase activity, structural proteins

Proteinase activity in the skeletal muscle of fish arises from lysosomes as well as cytosol (Yamashita and Konagaya, 1992). While it meets the necessity of protein turnover in the living animal, tissue proteinase activity (TPA) is responsible for changes like resolution of rigor mortis. Texture degradation of muscle in chum salmon, Pacific whiting and mackerel (Yamashita, 1993; An *et. al.*, 1992; Matsumiya *et. al.*, 1990) due to TPA has been noticed. Deterioration of gel structure in kamaboko is due to TPA (Suzuki, 1981). Presence of numerous proteinases in fish tissues, active at acid, neutral and alkaline pH, is well documented (Jayan *et. al.*, 1997, An *et. al.*, 1992, Matsumiya *et. al.*, 1990, Makinodan *et. al.*, 1984). While isolation and characterization of several of these tissue proteinases have been carried out (Makinodan *et. al.*, 1987, Sakai-Suzuki *et. al.*, 1986) the distribution of TPA is generally taken to reside in the sarcoplasmic fraction of muscle, which is extractable by low ionic strength buffers. Presence of TPA at acid and alkaline pH in the myofibrillar fraction of mackerel (*Rastrelliger kanagurta*) is reported in this paper.

Materials and Methods

Mackerel (*Rastrelliger kanagurta*) was procured in the post-rigor condition. It was headed, eviscerated and washed free of traces of viscera. Skinned muscular tissue was minced at low temperature, frozen and kept at -20°C. Casein (Hammerstein grade) was used for the study. All chemicals used were of analytical grade. Haemoglobin was prepared from bovine blood by washing the red blood cells three times with physiological saline and lysis in distilled water. After removal of cell membranes, crude haemoglobin was dialyzed extensively against distilled water and finally freeze dried.

The mince was blended with water in the ratio 1:4. 2ml of this with 4 ml buffer (Table 1) was incubated at 50°C for one hour. The reaction was stopped by adding 5 ml 11% trichloroacetic acid and the Folin positive material in the supernatant was determined by the method of Herriott (1955) and activity expressed as μM tyrosine released/ml test sample/min. For proteolytic activity assay 2 ml test sample, 4 ml buffer and 1 ml 2% solution of casein or haemoglobin were incubated for 1 h. Termination of reaction and activity expression were done as before. Assays were carried out at pH 3 and 4 with 0.1M citrate-phosphate buffer and at pH 9 and 10 with equimolar Tris-HCl buffer.

For optimization of buffer, minced muscle was homogenised with 4 volumes of one of the chilled buffers in a Polytron homogeniser at 10,000 rpm for 3-4 min keeping the container in an external ice pack. The homogenates were tested for autolytic activity as described above. For fractionation, homogenisation was carried out with chilled 0.2M sucrose + 1mM disodium salt of ethylene diamine tetra acetic acid (EDTA). The homogenate was centrifuged at 15,000 x g for 30 min at 5°C and the supernatant was stored at 5°C. The residue was re-extracted and centrifuged as before and the combined supernatants constituted the sarcoplasmic fraction. Residue from the second extraction was extracted under similar conditions with 8 volumes of sucrose EDTA buffer containing 0.6M NaCl and the supernatant from this extraction constituted the myofibrillar fraction. In extraction with buffers containing sodium dodecyl sulfate (SDS), the first and second extraction buffers contained 0.1% SDS. For repeated extraction the muscle was chopped to small pieces (2-3mm) and suspended in 8 volumes of chilled sucrose EDTA buffer for 30 min with intermittent stirring. The extract was filtered through a fine nylon cloth and the residue was extracted 3 more times under similar conditions. The combined filtrates constituted the sarcoplasmic fraction and the residue from the fourth extraction was homogenized with 8 volumes of sucrose EDTA buffer containing 0.6M NaCl and centrifuged to get the myofibrillar fraction. Protein concentration in different fractions was determined by the biuret reaction (Scopes, 1982)

Results and Discussion

Major TPA in mackerel is at pH 3, 4, 9 and 10 (Jayan *et. al.*, 1997). Four buffers were tested for the optimum for expression of autolytic activity at the above pH (Table 1). Although buffers 2 and 4 were equally good, the presence of mercaptoethanol in the latter interfered with the Folin reaction and hence the former was chosen for further studies.

Table 1. Optimization of buffers for determination of autolytic activity

Buffer	Autolytic activity, μ M tyrosine/ml/min			
	pH 3	pH 4	pH 9	pH 10
30mM Phosphate buffer, pH 7	0.039	0.021	0.046	0.065
0.2M sucrose + 1mM EDTA, pH 7	0.059	0.047	0.042	0.066
0.5% KCl	0.028	0.022	0.038	0.055
40mM phosphate+ 0.5% KCl +10mM 2-mercaptoethanol, pH 7	0.050	0.049	0.037	0.044

Autolytic activity is the activity of the tissue proteinase with the tissue itself as a substrate. As fractionation of muscle into sarcoplasmic and myofibrillar fractions leads to loss of potential substrates in both, the presence of an externally added protein substrate might become necessary for full expression of TPA. Toyohara *et. al.* (1987) and Sakai-Suzuki *et. al.* (1986) used external substrates to measure TPA. Four different protein substrates, bovine serum albumin, haemoglobin, casein and washed carp meat, were tried as external substrates at acid and alkaline pH with the sarcoplasmic fraction as enzyme. All substrates were satisfactory at alkaline pH but at pH 3, only haemoglobin was effective (Table 2). Hence haemoglobin was chosen as the substrate to assay proteolytic activity at acid and casein at alkaline pH range.

Table 2. Proteolytic activity of sarcoplasmic fraction against various substrates at pH 3

Protein substrate	Activity, μM tyrosine/ml/min
Bovine serum albumin	0.00093
Hemoglobin	0.01577
Casein	0
Washed carp meat	0.00319

Autolytic activities in tissue homogenate and in sarcoplasmic and myofibrillar fractions are shown in Table 3. Recovery of autolytic activity at pH 3 and 4 was poor indicating incomplete extraction of TPA. Activity as well as recovery was higher in the sarcoplasmic fraction at pH 9 and10. Removal of myofibrillar fraction appeared to have increased the activity at these pH in the sarcoplasmic fraction. However, the myofibrillar fraction was still not completely free of TPA at any of the above pH showing that TPA was not easily extracted into the sarcoplasmic fraction.

Table 3. Autolytic activities in homogenate, sarcoplasmic and myofibrillar fractions

pH	Homogenate		Sarcoplasmic		Myofibrillar	
	Activity	Recovery %	Activity	Recovery %	Activity	Recovery %
3	0.0087	100	0.002	18.55	0.0009	7.92
4	0.0074	100	0.0028	30.35	0.0028	28.22
9	0.0047	100	0.0037	66.21	0.0007	11.73
10	0.0051	100	0.0077	124.33	0.0023	34.01

Activity is expressed in μ M Tyr/ml/min. Recovery was computed as the ratio of total units in each fraction to total units in the homogenate. Total units = Activity x volume of the fraction

Presence of TPA in both sarcoplasmic and myofibrillar fractions was confirmed by testing proteolytic activity against external protein substrates (Table 4). Addition of external protein substrate was especially helpful in the case of sarcoplasmic fraction, where the activities increased to levels higher than those obtained when native proteins alone were present as substrates. Such increase in activity was not observed with myofibrillar fraction due to the lower concentration of sarcoplasmic fraction compared to the myofibrillar fraction as well as lack of structural proteins in the former, which might have limited the full expression of proteolytic activity. It is also possible

that proteolytic inhibitors are present in the sarcoplasmic fraction, which were diluted on addition of external protein substrates.

Table 4. Proteinase activity of sarcoplasmic and myofibrillar fractions with external protein substrates

pH	Sarcoplasmic		Myofibrillar	
	Activity	% of Autolytic activity	Activity	% of Autolytic activity
3	0.0118	135	0.0085	97.7
4	0.0085	114	0.0065	87.8
9	0.0065	138	0.0035	74.46
10	0.0060	117	0.0062	121.6

Substrate at pH 3 & 4: Hemoglobin, Substrate at pH 9 & 10: Casein. Activities are also expressed as a % fraction of the autolytic activity at each pH shown in Table 3

Table 5. Autolytic activity in sarcoplasmic and myofibrillar fractions of mackerel extracted with buffers containing 0.1% sodium dodecyl sulfate

pH	Sarcoplasmic fraction		Myofibrillar fraction	
	Specific activity	*Total units*	*Specific activity*	*Total units*
3	0.0007	0.2225	0.0009	0.122
4	0.0018	0.561	0.0006	0.075
9	0.0007	0.226	0.0004	0.051
10	0.0016	0.517	nd	nd

Specific activity: μ moles Tyr/min/mg protein. Total units: Activity/ml x volume of fraction. nd : not detected

In view of the apparently strong attachment of tissue proteinases to the myofibrillar fraction, an attempt was made to free them by including a low concentration of detergent SDS in the first extraction buffer. The results are presented in Table 5. It was observed that a higher TPA could be extracted into the sarcoplasmic fraction at all pH tested. Because of the inclusion of SDS in the sarcoplasmic extraction buffer, some amount of myofibrillar proteins was also extracted into this fraction. Both in terms of specific activity and the total units of activity the sarcoplasmic fraction

Table 6. Proteolytic activity in sarcoplasmic and myofibrillar fractions of mackerel extracted with buffers containing 0.1% Sodium dodecyl sulfate

pH	Sarcoplasmic fraction		Myofibrillar fraction	
	Specific activity	Total units	Specific activity	Total units
3	0.031	0.8921	0.039	0.5845
4	0.023	0.6762	0.038	0.5625
9	0.013	0.3703	0.011	0.1666
10	0.016	0.4513	0.018	0.2711

Specific activity : μ moles Tyr/min/mg protein. Total units: Activity/ml x volume of fraction. Hemoglobin used as substrate at pH 3 & 4 and Casein at pH 9 &10

showed higher TPA than the myofibrillar fraction. However, the myofibrillar fraction still had enough detectable TPA at all pH except 10. This shows that not all TPA could be extracted from the tissue, even when a detergent like SDS is included in the buffer. These results were further confirmed by testing the proteolytic activity of the two fractions (Table 6) which showed TPA at pH 10, though not detectable by the autolytic assay.

Table 7. Autolytic activity in fractions of mackerel muscle obtained by repeated washing of muscle.

pH	Sarcoplasmic fraction		Myofibrillar fraction	
	Specific activity	Total units	Specific activity	Total units
3	0.00091	0.2318	0.00055	0.1201
4	0.00110	0.282	0.00034	0.0739
9	0.00089	0.225	0.00002	0.0048
10	0.00062	0.1581	nd	nd

Specific activity : μ moles Tyr/min/mg protein. Total units : Activity/ml x volume of fraction

In view of the difficulty of removing TPA from the structural proteins of mackerel, effect of repeated washing of the muscle on its retention was studied. The results are presented in Tables 7 and 8. It was observed that repeated washing was unable to remove all TPA from the myofibrillar fraction especially at pH 3 and 4. At pH 9 and 10, most of the TPA was removed by washing and was not detected by autolysis at pH 10. However, when tested against external protein substrate, TPA was still present.

Table 8. Proteolytic activity in fractions of mackerel muscle obtained by repeated washing of muscle.

pH	Sarcoplasmic fraction		Myofibrillar fraction	
	Specific activity	Total units	Specific activity	Total units
3	0.0014	0.372	0.0022	0.478
4	0.0027	0.688	0.0019	0.435
9	0.00014	0.035	0.00035	0.077
10	0.0014	0.352	0.00004	0.0094

Specific activity μ moles Tyr/min/mg protein. Total units: Activity/ml x volume of fraction. Hemoglobin used as substrate at pH 3 & 4 and Casein at pH 9 & 10.

These results show that tissue proteinase activity in mackerel at acid and alkaline pH is strongly bound to the myofibrillar fraction, especially at pH 3-4. Although a large portion of the TPA can be removed by repeated extraction with low ionic strength buffers or by washing with buffers containing detergent, complete removal does not occur.

References

An, H., Chung, Y.C. & Mossissey, M.T. (1992) in *Pacific Whiting - Harvesting, Processing, Marketing and Quality Assurance* (Sylvia, G.& Morrissey, T., Eds) p. 36, Oregon State University, Sea Grant College Program, Newport, Oregon

Herriott, R.M. (1955) in *Methods in Enzymology,* Vol. 2 (Colowick, S.P. & Kaplan, N.O., p. 3, Academic Press, N.Y.

Jayan, K., Jose, L., Raghunath, M.R. & Devadasan, K. (1997) in *Technological Advancements in Fisheries* (Hameed, M.S. & Kurup, B.M., Eds) p. 356, School of Industrial Fisheries, Cochin University of Science and Technology, Cochin

Makinodan, Y., Toyohara, H. & Ikeda, S. (1984) *Comp. Biochem. Physiol.,* **79B**, 129

Makinodan, Y., Yokoyama, Y., Kinoshita, M. & Toyohara, H. (1987) *Comp. Biochem. Physiol.,* **87B**, 1041

Matsumiya, M., Mochizuki, A. & Otake, S. (1990) *Bull. Coll. Agric. Vet. Med.,* No. 47, p. 171, Nihon. University, Nichidai. Nojuho

Sakai-Suzuki, J., Tobe, M., Tsuchiya, T. & Matsumoto J.J. (1986) *Comp. Biochem. Physiol.,* **85 B**, 887

Suzuki, T. (1981) *Fish and Krill Protein: Processing Technology,* p.109, Applied Science Publishers Ltd., London

Toyohara, H., Nomata, H., Makinodan, Y. & Shimizu, Y. (1987) *Comp. Biochem. Physiol.,* **86 B**, 99

Yamashita, M. & Konagaya, S. (1992) *Comp. Biochem. Physiol.,* **103 B**, 999

Yamashita, M. (1993) *Bull. Natl Res. Inst. Fish. Sci., Chuosuiken Kenpo,* No. 5, p. 9