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Studies on the Electrophoretic Pattern of Proteins in Frozen Stored Fish

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To find out the effect of frozen storage on fish proteins, two species *Megalaspis cordyla* (Linnaeus) and *Labeo rohita* (Hamilton - Buchanan) were stored for 90 days at -18°C after freezing at –40°C. Samples were collected at 15-day intervals and Salt Soluble Protein (SSP), Water Soluble Protein (WSP) and Non Protein Nitrogen (NPN) content of the samples were determined. Electrophoretic pattern of both SSP and WSP of frozen stored samples were compared with that of the fresh sample. SSP, WSP and NPN content showed significant decrease during 90 days frozen storage period. However, the electrophoretic pattern of WSP remained almost the same throughout the frozen storage period with some minor bands becoming less intense with storage period. Electrophoretic pattern of SSP showed a major change in the myosin heavy chain, which became narrower and less intense with the concomitant appearance of high molecular weight protein aggregate at the top of the gel.

Key words: ***Labeo rohita, Megalaspis cordyla,*** **salt soluble proteins, water soluble proteins, electrophoresis, frozen storage**

Fish is considered to be among the most perishable of foodstuff as a result of a complex series of chemical, bacteriological and histological changes that occur in the muscle tissue. These inter-related processes are usually accompanied by the gradual loss or development of different compounds that affect fish quality. Good quality fish that has been properly frozen and packaged can be normally held at –20°C to –30°C for more than one year without appreciable loss in consumer acceptability (Dyer, 1968; Makie *et al.*, 1986). Ideally there should be no distinguishable difference between fresh and frozen fish after thawing. However, quality changes are evident from denaturation and solubility loss (Sikorski *et al.*, 1976; Shenouda, 1980) as well as deteriorative changes in colour (Bunda and Hultin, 1983) and texture (Dingle *et al.*, 1977, Gill *et al.*, 1979). Also the utility of fish as a raw material for fabricated products may be limited by loss of protein functionality (Colomenero and Borderias, 1983).

Sarcoplasmic proteins play a major role in species identification in fish and fish products, whenever the identification of fish species by sensory evaluation becomes difficult. Myofibrillar proteins play a major role in the rheological properties of surimi and surimi based products. In the present study, fish proteins were examined in an attempt to define any changes in their properties as a result of freezing and to use this information to monitor any changes occurring during frozen storage.

Materials and Methods

Fresh *Megalaspis cordyla* (horse mackerel) and *Labeo rohita* (rohu), size ranging from 250-500 g, were obtained from the harbour and market, respectively and brought to the laboratory, dressed, washed with potable water and frozen at – 40°C in a blast

freezer for 6 h. It was then dip glazed, wrapped in polythene bags and stored at −18°C in cartons. After initial sampling (fresh), samples were taken periodically viz. on the 15th, 30, 45, 60, 75 and 90th day and thawed at 5°C and analysed.

For extraction of water-soluble proteins about 15 g meat was taken and minced thoroughly keeping the temperature at 4°C. From this 5 g meat was homogenized with 10 ml cold distilled water for 2 min at 4°C. The suspension was centrifuged at 10000 rpm for 10 min at 4°C and the supernatant was transferred to a 50 ml standard flask. The volume was made up with cold distilled water.

The residue of the above extract was mixed with about 40 ml Dyer's buffer (5% NaCl in 0.02 M $NaHCO_3$, pH 7.0) and stirred at low speed using magnetic stirrer for 1 h. at 4°C. Salt Soluble Protein was extracted following the method of Dyer *et.al* (1950). The suspension was then centrifuged at 10000 rpm for 20 min and the supernatant made up to 50 ml using buffer.

Total nitrogen (TN), Non-protein nitrogen (NPN), Water Soluble Nitrogen (WSN) and Salt Soluble Nitrogen (SSN) were determined using microkjeldal method (AOAC, 1984).

Electrophoresis of Proteins: The protein samples were mixed with an equal volume of sample buffer (Tris buffer pH 6.7) containing 2% SDS, 5% β- mercaptoethanol and 10% glycerol. The samples were heated in a boiling water bath for 5 min. To each sample 5µl of 0.025% bromophenol blue solution was added and SDS-PAGE was conducted following the method of Laemmli (1970) on a 7.5% poly acrylamide disc gel. The running buffer was Tris Glycine containing 0.1% (w/v) SDS. Electrophoresis was carried out at 3mA per gel tube till the marker dye reached the bottom of the gel. The gel was stained for proteins with 0.2% (w/v) Coomassie Brilliant Blue R-250 in methanol: water: acetic Acid (46:46:8(v/v/v)) and destained till the bands appeared as dark blue discs and preserved in 7% acetic acid solution. Molecular weights of the protein bands were determined according to the methods of Weber and Osborn (1969) and Davis and Stark (1970) using a high molecular weight (MW) protein kit, SDS- 6H (Sigma Chemical Co., St. Louis, MO)

Results and discussion

The values of SSN on different days of frozen storage are shown in Table 1. The SSN of fresh water fish *L. rohita* was 48.85% of TN, which decreased to 36.76% on 95 d of frozen storage. In the case of *M.cordyla* an

Table 1. Salt soluble nitrogen content in frozen-stored fish

Species	SSN as % of TN days of frozen storage						
	Fresh	15	30	45	60	75	95
Labeo rohita	48.85 ^{a} ± 2.5	44.86 ^{b} ± 1.28	44.15 ^{b} ± 0.75	42.82 ^{bc} ± 1.08	40 ^{c} ± 2.01	38.04 ^{cd} ± 0.74	36.76 ^{d} ± 0.74
Megalaspis cordyla	48.73 ^{a} ± 1.41	46.80 ^{b} ± 0.79	42.72 ^{c} ± 0.67	41.56 ^{cd} ± 0.79	41.1 ^{de} ± 0	39.06 ^{f} ± 0.79	37.11 ^{g} ± 0.50

Superscripts a,b,c,...,f. indicate the results of pairwise comparison, different superscripts showing significantly different means ($P<0.05$).

Table 2. Water Soluble Nitrogen Content in frozen-stored fish

Species	WSN as % of TN Days of frozen storage						
	Fresh	15	30	45	60	75	95
Labeo rohita	24.84 ^{ab} ± 0.39	24.94 ^{b} ± 0.68	24.22 ^{ac} ± 0.31	23.75 ^{cde} ± 0.4	24.09 ^{cd} ± 0.15	23.55 ^{de} ± 0.15	23.43 ^{e} ± 0.13
Megalaspis cordyla	21.1 ^{a} ± 0.22	20.72 ^{a} ± 0.82	19.42 ^{b} ± 0.44	18.91 ^{bc} ± 0.55	19.09 ^{bc} ± 0.67	18.44 ^{cd} ± 0.16	18.53 ^{cd} ± 0.16

Superscripts a,b,c,...,f. indicate the results of pair wise comparison, different superscripts showing significantly different means ($P<0.05$).

initial 48.73% of SSN decreased to 37.11% on 95 th d of frozen storage. Thus SSN decreased significantly ($P<0.05$) by 24.75% and 23.8% in *M. cordyla*, *L. rohita*, respectively in 3 months frozen storage at -18°C. A decrease in the SSP during frozen storage was reported by several workers (Joseph and Perigreen, 1980; Sarma *et al.*, 1998). This is mainly attributed to the denaturation of proteins especially the myofibrillar proteins.

The values of WSN on different days of frozen storage are shown in Table 2. WSN showed a gradual decreasing trend in *L. rohita*. This is against the findings of Devadasan *et al.*, (1978), where no significant difference in WSN was observed during frozen storage. The loss in WSN may be due to drip loss. A decreasing trend for WSN was also observed in *M. cordyla*.

The values of NPN on different days of frozen storage are shown in Table 3. NPN showed an overall significant decline ($P<0.05$) by 24% and 27% in *M.cordyla*, *L.rohita*, respectively possibly due to drip loss.

Figures 1 and 2 show the electrophoretic pattern of WSP extracted from *L rohita* and M *cordyla*. A total number of 15 bands were obtained for the WSP from *L rohita* and the molecular weights for each band is shown in table 4. 10 bands were obtained for *M cordyla* and the molecular weight of each band is shown in table 5. The WSP obtained from fresh water fish *L rohita* contained a number of higher molecular mass proteins while these were absent in WSP of the marine fish *M cordyla*. This may be due to the difference in the species.

Table 3. Non – Protein Nitrogen Content in frozen-stored fish

Species	NPN as % TN Days of frozen storage						
	Fresh	15	30	45	60	75	95
Labeo Rohto	16.09 ^{a} ± 0.28	15.45 ^{b} ± 0.27	15.5 ^{bc} ± 0.21	14.17 ^{d} ± 0.07	13.73 ^{de} ± 0.54	12.24 ^{f} ± 0.15	11.71 ^{g} ± 0.07
Megalaspis cordyla	10.57 ^{a} ± 0.15	10.05 ^{b} ± 0.34	10.09 ^{b} ± 0.08	9.27 ^{c} ± 0.29	8.59 ^{d} ± 0.21	8.31 ^{d} ± 0.29	8.01 ^{d} ± 0.09

Superscripts a,b,c,...,f. indicate the results of pair wise comparison, different superscripts showing significantly different means ($P<0.05$).

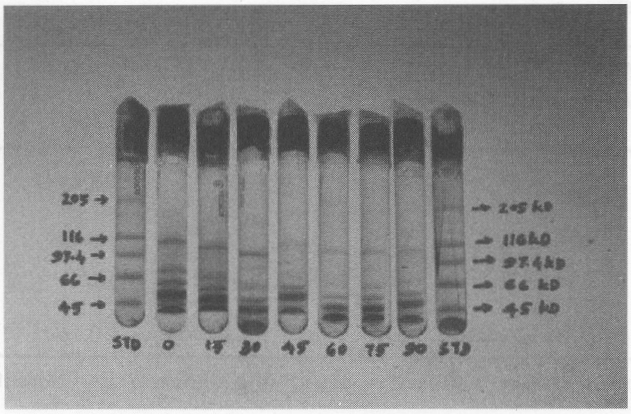

Fig. 1. Electrophoretic Pattern of Water Soluble Protein (*L. rohita*)

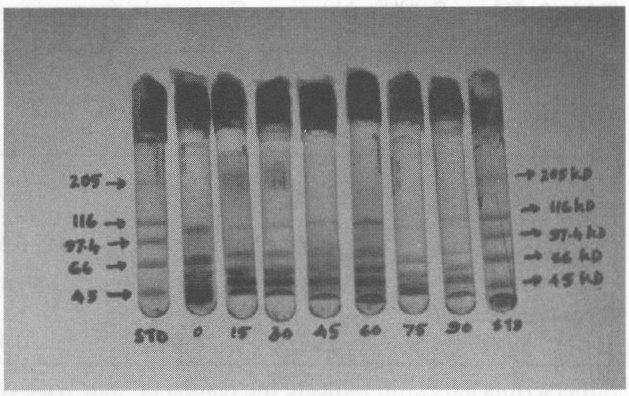

Fig. 2. Electrophoretic Pattern of Water Soluble Protein (*M. cordyla*)

Since 7.5% polyacrylamide gel was used, only those proteins with molecular weight higher than 40 kD could be separated. In both the species major bands in the electrophoretic pattern of water-soluble proteins remained almost the same during frozen storage. However in L.rohita protein bands with molecular weight 178 and 169 kD from 60th day sample onwards and 266 kD in the 90th day sample could not be seen. In *M.cordyla*, protein band with molecular weight 63 kD could not be seen from the 45th day sample onwards. These bands which could not be seen during frozen storage gave only a narrow and faint band in the fresh sample also. There was a significant difference in the water-soluble nitrogen content during frozen storage. Owusu-Anash and Hultin (1992) reported that, relative to the control samples there were decreases in all water-soluble protein bands in the electrophoretic pattern of frozen samples. Dyer and Dingle (1961) found detectable change in the electrophoretic patten of the water-soluble protein fraction from frozen cod stored for 7 weeks at –12^{0}C. Sarcoplasmic protein insolubilization has been reported upon frozen storage of a white fish (Award *et al.*, 1969) and cod fillets (Yowell and Flurkey, 1986). Tiecco (1981) reported a gradual disappearance of the slowest migrating band in frozen beef held

Table 4. Molecular weights of the WSP bands extracted with distilled water (*L rohita*)

Band No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Mol. Wt. In kD.	226	178	168	133	126	112	89	84	75	71	67	56	47	42	40

Table 5. Molecular weights of the WSP bands extracted with distilled water (*M cordyla*)

Band No.	1	2	3	4	5	6	7	8	9	10
Mol. Wt. In kD.	112	89	75	71	63	56	53	47	42	40

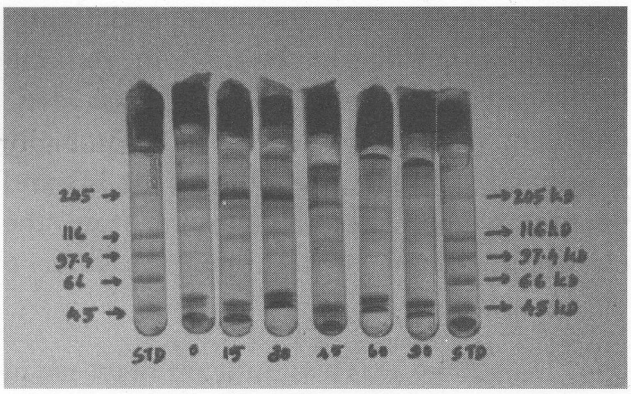

Fig. 3. Electrophoretic Pattern of Salt Soluble Protein (*L. rohita*)

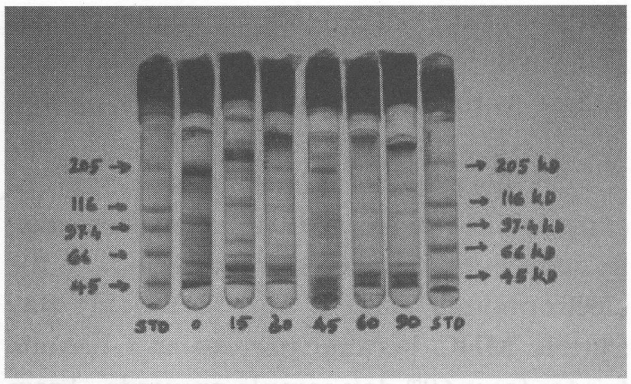

Fig. 4. Electrophoretic Pattern of Salt Soluble Protein (*M. cordyla*)

at -12^{0}C which started fading in samples frozen stored for 15 days and had disappeared completely in samples stored for 70 days. LeBlanc and LeBlanc (1989) observed difference in the SPP electrophorograms of cod fillets frozen at -12^{0}C for 10 months. LeBlanc *et al.* (1994) studied storage changes in SPP from cod and haddock fillets using capillary electrophoresis. They found that the electrophoretic pattern was different for the two species and the protein bands of both high and low molecular mass changed during frozen storage. However, in the present study no additional bands could be seen during frozen storage, which is in confirmation with the findings of Devadasan *et al.*, (1978).

Fig. 3 and Fig. 4 show the electrophoretic pattern of SSP on different days of frozen storage of fresh water fish *L rohita* and the marine fish *M cordyla*. The molecular weight of protein bands obtained for the fresh samples are shown in tables 6 and 7. A total number of 18 bands were obtained for *L rohita* and a total of only 15 bands were obtained in the case of *M cordyla* showing the species difference. A general decrease in the intensity of bands was observed with increase in frozen storage period. This is in agreement with the results obtained by Ohnishi and Rodger (1980). With the increase in the storage period extractability also decreased. Major changes were found in the

Table 6. Molecular weights of salt soluble protein bands(in Kilo Daltons) extracted with Dyer's buffer (*L rohita*)

Band No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Mol. Wt. In kD.	335	316	200	178	168	158	150	133	100	94	79	70	63	60	56	50	45	40

Table 7. Molecular weights of salt soluble protein bands (in Kilo Daltons) extracted with Dyer's buffer (*M cordyla*)

Band No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Mol. Wt. In kD.	200	158	141	133	112	100	94	84	79	63	60	50	47	42	35

myosin heavy chain (MHC, Mol. Wt. 200 kD), which became narrower and less intense with the increase in storage period.

In the case of *L.rohita* reduction in extractability decreased at a consistent rate through out the frozen storage period. In the electrophoretic pattern also in the 45th day sample MHC became narrow and became fainter from 60th day sample onwards. From 45th day sample onwards a new protein band at the top of the gel could be seen, which became more intense in the following day sample. This may be due to the formation of protein aggregates, which could not enter the gel. Mathews *et al.* (1980) in studying frozen stored minced cod observed an initial decrease in the amount of MHC and the concomitant appearance of a band of material, which did not enter 8.75% polyacrylamide gel. Similar high molecular weight proteins were also observed by Lim and Haard, (1984) in frozen minced Greenland halibut.

In *M cordyla*, MHC became narrower from the 45th day sample onwards and its intensity also decreased from the 60th day sample onwards. Here the rate of decrease in the extractability of salt soluble protein was more during the initial period. From the 15th day sample onwards a new band with apparent molecular weight of 290 kD could be seen, which disappeared in the 60th day sample. Concurrently a new protein band at the top of the gel had also been noticed from the 30th day onwards, the intensity of which increased in the following day samples. The intensity of protein band with molecular weight 94 kD decreased heavily from the 60th day sample onwards and those with 84 kD from the 30th day sample onwards. These may be due to the reduction in the extractability with frozen storage period. Owusu-Ansah and Hultin (1992) reported that the bands with molecular weights corresponding to those of MHC (200 kD), M- proteins (180-195 kD), probably C-proteins (140 kD) and others (151 kD and 92 kD) were completely absent from the electropherogram of test samples after 6 weeks storage.

Thus there was a significant decrease in the SSP of both the species during frozen storage. WSP and NPN also registered a gradual decrease during frozen storage. Electrophoretic pattern of water-soluble protein remained unchanged in both the species except for a few less intense bands, which could not be seen properly in the subsequent frozen storage period. In the electrophoretic pattern of the salt soluble protein, MHC showed a major change in both the species with the concomitant appearance of a protein band at the top of the gel, which may be the aggregate formed.

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