EFFECT OF SELECTED PRE TREATMENTS DURING FROZEN STORAGE ON THE PROPERTIES OF PROTEINS FROM INDIAN OIL SARDINE

(*Sardinella longiceps*)

A THESIS SUBMITTED TO THE UNIVERSITY OF MYSORE,
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DOCTOR OF PHILOSOPHY

IN

BIOTECHNOLOGY

BY

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July 2005

Dedicated to My Beloved Parents

Kochaha and Chechi

Declaration

I hereby declare that the thesis entitled "**Effect of selected pre treatments during frozen storage on the properties of proteins from Indian oil sardine (*Sardinella longiceps*)"** which is submitted herewith for the degree of **Doctor of Philosophy** in **Biotechnology** of the **University of Mysore, Mysore** is the result of the work done by me in the Department of Protein Chemistry and Technology, Central Food Technological Research Institute, Mysore, India under the guidance of Dr. V. Prakash during the period 2001- 2005.

I further declare that the results of this work have not been previously submitted for any degree or fellowship.

Date: 11th July 2005 **Mr. Sijo Mathew**

Place: Mysore

Certificate

I hereby certify that the thesis entitled "**Effect of selected pre treatments during frozen storage on the properties of proteins from Indian oil sardine (S*ardinella longiceps*)"** submitted by **Mr. Sijo Mathew** for the degree of **Doctor of Philosophy** in **Biotechnology** of the **University of Mysore, Mysore** is the result of research work carried out by him in the Department of Protein Chemistry and Technology, Central Food Technological Research Institute, Mysore, India under my guidance and supervision during the period 2001- 2005.

I further declare that the results of the work have not been submitted either partially or fully to any other degree or fellowship.

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Date: 11th July 2005
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LIST OF ABBREVIATIONS

A	absorbance
A°	angstrom
ADP	adenosine di phosphate
AMP	adenosine mono phosphate
Arg^{714}	This refers to amino acid arginine and its position in the sequence analysis as shown in the superscript. This same system is followed wherever other amino acids and their position in the sequence is indicated in the text such as tryptophan, glycine, phenylalanine, methionine and cysteine
ATP	adenosine tri phosphate
ATPase	adenosine tri phosphatase
c	concentration of protein (mg/ml)
$CaCl_2$	calcium chloride
$CaSO_4$	calcium sulfate
CD	circular dichroism
cm	centimeter
dm	decimeter
DSC	differential scanning calorimetry
DTT	dithiothreitol
DVB	dynamic viscoelastic behaviour
EB	extraction buffer
EC	emulsion capacity
EDTA	ethylene diamine tetra acetic acid
EEZ	exclusive economic zone
EGTA	ethyleneglycol tetra acetic acid
FDA	food and drug administration
Fig.	figure
FPLC	fast protein liquid chromatography

g	gram
G´	storage modulus
G´´	loss modulus
HMM	heavy mero myosin
IMP	inosine mono phosphate
hrs	hours
Hz	hertz
kD	kilodalton
kCal	kilocalories
Kg	kilogram
kJ	kilo joules
l	litre
LMM	light mero myosin
M	molar concentration
m	meter
mA	milli ampere
mg	milli gram
$MgCl_2$	magnesium chloride
MHC	myosin heavy chain
min	minute
ml	milli liter
mm	milli meter
mmol	milli moles
mol	mole
MPa	mega pascal
MRW	mean residue weight
MW	molecular weight

N	normality
NAD	Nicotinamide adenine di nucleotide
NADP	Nicotinamide adenine di nucleotide phosphate
NAM	natural actomyosin
NaCl	sodium chloride
ng	nanogram
nm	nanometer
P	pressure
PAGE	polyacrylamide gel electrophoresis
PER	protein efficiency ratio
PMSF	phenyl methyl sulfonyl fluoride
rpm	revolutions per minute
SDS	sodium dodecyl sulfate
sec	second
SH	thiol groups
SR	sarcoplasmic reticulum
SS	disulfide
S1	myosin subfragment 1
T	absolute temperature
Tanδ	rheological transition
TCA	trichloro acetic acid
TEMED	N,N,N',N', -tetramethyl ethylene diamnine
T_m	thermal denaturation temperature
Tris	tris (hydroxymethyl) amino methane
TGase	trans glutaminase
UV	ultraviolet
v/v	volume by volume

v_e	elution volume
v_t	total volume
w/v	weight by volume
w/w	weight by weight
WAC	water absorption capacity
WHC	water holding capacity
$ZnCl_2$	zinc chloride
$ZnSO_4$	zinc sulfate
ΔA	difference in absorbance
°C	degree Celsius
µ	chemical potential
µ g	microgram
µ l	microlitre
µ mole	micromole
%	percentage
λ_{max}	emission maximum (wavelength)
x g	times acceleration due to gravity
$E^{1\%}_{1cm\ \lambda max}$	absorption coefficient of 1% solution in 1cm path length cell at its absorption maximum
ω	angular velocity
ε	molar extinction coefficient ($M^{-1}cm^{-1}$)
θ_{MRW}	mean residue molar ellipticity

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*Reciprocal plot indicates the double reciprocal plot normally known as Lineweaver- Burk plot for enzyme substrate interactions.

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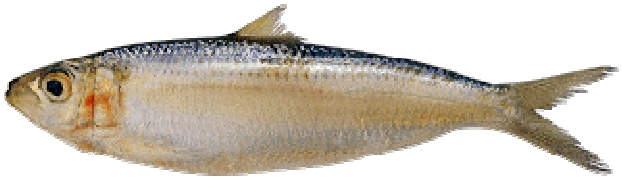
INTRODUCTION

1 INTRODUCTION

Fish is a staple food for large population in the developing countries. Asia is by far the most important region for the direct human consumption of fish. Total fish production of world amounts to 132 Million ton in the year 2002 (FAO, 2004). The marine fish production of India contributes to 2.5% of world fisheries production (CMFRI, 2002). The fish production statistics is shown in Table 1. In India about 60 to 75% of the marine catch comes from the west coast and 25 to 40% from east coast. The pelagic fishes contribute about 40 to 60%, the demersal fishes 35 to 45%, the crustaceans 15 to 20% and the molluscs 5 to 15% of the total marine landings which is being exploited from ~8085 km coast line and 2.02sq km EEZ. There are about 200 commercially important species in Indian waters; the major landings are oil sardine, mackerel, penaeid prawn, Bombay ducks, scianids, lesser sardines, pomfrets, elasmobranches and cephalopods. The Indian production of oil sardines is estimated at 0.34 million tons (CMFRI, 2002). Percentage contribution by oil sardine to total Indian fish production is 13%. Sardines and anchovies are distributed in temperate zones worldwide and have representative species or populations in most of the temperate boundary current systems (Gran *et al*., 1998). Sardinella are found in subtropical and tropical zones and have representative species or populations in most of the subtropical and tropical boundary systems (Jerome *et al*., 2003). The total world production of oil sardine (*Sardinella longiceps)* amounts to 4.09 million tons during the year 2002. The catch of oil sardine fluctuates widely every year. This fishery is of utmost importance in two maritime states namely; in Kerala and Karnataka coast of south India with the bulk of landings coming from the former.

The commercial fishery mainly comprises of less than 1 year old fishes. This fish matures when it is 150 to160 mm long (Fig. 1). Even though the fish is known to spawn from May to October, only a few spawning grounds have been located. This species spawn once in the season and twice in the lifetime. Fecundity of the species is ~50,000 eggs per individual. Juveniles appear abundantly in the near shore water during July to September. The fish feed mainly on planktonic microorganisms. The different items of plankton in its diet are dominated by the phytoplankton varied



Trade Name	Indian oil sardine
Scientific name	**Sardinella longiceps**
Gear used	Boat seine, Purse seine and Gill net
Peak season	August to December
Distribution	South-West coast of India
Areas of abundance	Kerala and Karnataka coasts

Fig. 1: Photograph of Indian oil sardine with the distribution of species

Table 1: World fish production as reported by FAO during 2004

Source	World production (Tons)	Indian production (Tons)
Capture	93,190,654	3,770,912
Culture	39,798,571	2,191,704
Total	132,989,225	5,962,616
Indian Oil sardine	409,894	313,843

Source: FAO (2004)

according to their abundance during the different periods of the year in the environment. Copepods, nauplii, crustacean remains, larval bivalves, tintinnids, dinoflagellates *Fragilaria oceanica* and certain other diatoms are the major items ascribed to its intake.

1.1 Crafts and gears for the exploitation of fishery

From very early times this fishery has been exploited by the indigenous crafts and gear, which naturally set a limitation on the region fished and the duration of fishing. Along the southern Kerala the bulk of catches are made from the one boat, Boat seine 'Thangu Vala' and 'Ayila Vala' (gillnet used for catching big sized sardines and mackerels) and some purse seines. Along northern Kerala the 'Patten kolli' (Boat seine) is the major gear followed by the gillnet 'Mathi chala vala' and the cast net. About 100 purseiners are in operation along the entire Kerala coast. Along Karnataka coast, the most important gear now is the purse seine. More than 450 purseiners are in operation. The other gear operated are shore seines known as 'Rampani', 'Yendi' and 'Cast net'. Generally the small sized oil sardine belonging to the zero year class measuring 70 to 100 mm in length dominate the commercial catches from August to Nov. The higher sized fishes from 100 to 200 mm dominate the catches in the later months during peak season, which generally extends up to January. The fishery dwindles in the later months and comes to close by May.

1.2 Utilization of sardine and the products from sardine

The seasonal abundant catch of sardine is being used for the production of various products and the extraction of oil. The composition and nutrient content of sardine meat is shown in Table 2 & 3. From this table it is clear that sardine meat is a good source of protein and vitamins. The white meat from sardine is comparatively less in fat content in consideration with red meat. It's a good source of calcium and phosphorous. Various product styles are accepted for preservation and utilization of this important fishery resource is described below.

Table 2: Composition of red meat and white meat of oil sardine

Components	Red meat	White meat
Composition (gram%)		
Moisture	64.6	72.8
Carbohydrate	0.9	0.5
Fat	14.1	4.8
Ash	1.4	1.7
Protein	19.1	20.7
Calorific value (kcal/100g)	206.9	127.2
Minerals (mg/100g)		
Sodium	149	131
Potassium	361	404
Calcium	103	112
Inorganic Phosphorous	306	370
Iron	11	4
Essential amino acids (g/100g)		
Lysine	8.7	7.4
Leucine	6.4	5.7
Isoleucine	5.3	4.7
Phenylalanine	3.8	4.9
Methionine	4.9	5.4
Tyrosine	4.8	3.8
Threonine	5.7	5.1
Valine	6.8	5.8

Source: Mukundan *et al*. (1985)

Table 3: Nutrient composition of oil sardine

Components	Quantity/100g
Vitamin A	229 I.U
Thiamin	0.8 mg
Riboflavin	0.23 mg
Niacin	8.2 mg
Vitamin B_{12}	11.0 mcg
Pantothenic acid	1.1 mg
Calcium	73.0 mg
Phosphorous	240.0 mg
Iron	2.3 mg
Zinc	1.0 mg
Sodium	100.0 mg
Potassium	55.0 mg

Source: Nettleton (1985)

1.2.1 Canning of sardine

Canned sardine are mainly supplied to the defense personnel. Small quantity being sold internal markets and exported. Sardines are dressed properly and washed thoroughly to remove all slime, dirts and blood with special reference to stomach cavity. Then they are immersed in 15% salt solution for 15 min. Brined fishes are autoclaved at 0.35 kg/sq.cm steam pressure for 35 to 40 min. and allow the cook drip to drain off. This helps to bring the required shrinkage, carting of excess water content, inactivation of the enzymes and partial sterilization. Cooked and cooled fishes are then packed in 301 x 206 cans with the head and tail ends alternatively facing the bottom so as to ensure compact packing. Usually 200 g is packed in one can. Commercially also they are packed in quarter dingley cans with fill weight being about 106 g.

Hot refined groundnut oil at 90-95°C is then added to the can leaving a head space of 5 to 10 mm. Exhausted for 8 to 10 minutes with steam, double seamed and autoclaved at 0.84 kg/sq.cm steam pressure for 70 to 75 minutes. Then they are cooled in chlorinated ice water. Oil sardine generally kept in ice for 2 days also, which can be used for canning. Oil sardine are also canned in natural style (brine) or in tomato sauce or curry also. Sardines which are smoked after brining also can be used for canning.

1.2.2 Sun drying

Fishes were dipped for a few minutes in 15 to 20% salt solution and spread over coir or bamboo mats and dry in open beach. These fishes are easily prone to bacterial and fungal attacks and the high content of salt is what acts as preservative and subsequent low moisture.

1.2.3 Dry curing

The ventral side of the fishes are split open; gills and intestine removed and washed clean. Weighed quantity of salts is scrubbed into body/split/scored flesh surfaces and stacked in curing tanks (1:3 to 1:10). Wooden planks and some heavy weight is placed over the fishes to keep them down. The salts draws out water from fish muscles and form self brine. Restack after 24 hours and allowed to remain for another 24 hours. After this a light rinse with fresh potable water to remove the adhering salts crystals and dried on mats for 2-3 days.

1.2.4 Wet curing

This method is most suited for oil sardine. The fish is allowed to remain under self brine until they are removed to the market. The advantage in this case is that as long as the fish are under the self-brine atmospheric oxygen is excluded and consequent oxidation of the fat is prevented.

1.2.5 Colombo curing

Medium sized fishes are used for this. The fishes are gutted, degilled and cleaned. They are salted as in the case of dry curing. The salted fishes are taken out and pieces of dried pods of Gorukkapuli (*Garcinia cambogea,* Malabar tamarind) placed in the body cavities and stacked in wooden barrels. When the barrels are full they are filled with saturated brine, closed water tight and exported. The fishes retain their qualities as long as they are under the brine.

1.2.6 Smoke curing

The big sized sardines are dressed (removal of head, gut and fins) and the body is split open so as to have maximum surface area. The dressed sardines are brined and air dried which will be ready for smoking process. Smoking process is normally carried out in smoking klins and smoke is generated using coconut husk or

any other suitable material. The brined fishes are hung in a smoking klin and generated smoke is allowed to deposit on the fish. Temperature of klin, smoking duration and type of wood used for generation of smoke will have bearing on the final quality of smoked product.

1.2.7 Smoked sardine flakes

This consists of beheading, gutting and cleaning the fish in running water. They are then dipped in saturated brine containing 3% sodium propionate and 0.1% butylated hydroxy anisole for 15 minutes. The brined fishes are drained for 10 minutes spread on wire mesh trays and smoked for six hours at 70- 80°C using coconut husk and shell and saw dust for producing smoke. The smoked fishes are then cooled, the skin of which would have become loose at this stage is peeled off manually and then flaked off from both sides of the backbone. About 25 g are rolled up in clean coloured cellophane sheets and marketed. The moisture content of the product is normally around 30%.

1.2.8 Oil from sardine

Oil sardine used for the extraction of body oil from fish. Usually this is carried out on a cottage industry scale at many isolated places close to landing centers. This is also obtained as a by product of the fishmeal industry. The ISI specification for the oil produced from sardine is shown in Table 4. Traditionally the fishes are taken in large mild steel vats and heated over flame with or without addition of water. When the fish get cooked, the oil floats on the top. This is scooped off and the cooked mass after cooling is transferred into gunny bags and pressed in screw press. All the free water and oil get entangled in the cooked flesh get pressed out and are collected in large cement tanks. The press liquor is allowed to stand for two to three days when the oil floats on the top and is ladled off. The oil fractions are pooled and heated on fire to drive away any free moisture. The stick water is concentrated and used as fine manure. The oil thus obtained is dark in colour with a somewhat strong pungent

Table 4: Chemical characteristics of sardine oil according to Indian Standards Institution specifications

Characteristics	Requirement Grade 1	Requirement Grade 2
Free fatty acids as percentage oleic acid (w/w Max)	1.0	3.0
Moisture (% by weight Max)	0.5	0.5
Iodine value (Wijs)	145-180	145-180
Saponification value	185-205	185-205
Unsaponifiable matter (% w/w, Max)	2.0	2.0
Refractive Index (40°C)	1.4739-1.4771	1.4739-1.4771

Source: IS 5734-1970

smell. This is used mostly for painting wooden country crafts as a protective coating. The composition of fatty acids in the oil from sardine is shown in Table 5.

1.3 Composition of fish meat

Muscle is composed of numerous proteins. Based on solubility, muscle proteins can be classified as sarcoplasmic proteins (water soluble) which are soluble in low ionic strength buffer (I = 0.05 - 0.15), myofibrillar proteins which are soluble in high ionic strength buffers (I = 0.5 -1.0) and are thus called salt soluble proteins, stroma proteins which are insoluble irrespective of salt concentration. The myofibril fraction contribute 60 -70% of total proteins in fish, and the most abundant protein in the myofibril is myosin. Thus the properties of myosin largely affects the function of muscle as well as the characteristics of muscle as food. Since fish muscle normally contains a lower portion of stroma proteins than the myosystems of land animals, it normally contains more myofibrillar proteins than other meats (Ochiai and Chow, 2000).

The major part of the water in the meat is held physically within sub cellular (myofibrillar) structures, and is reasonable to postulates that difference in muscle structure will affect the water distribution and thereby the water holding capacity of the meat (Bertram *et al*., 2002). The myofibrillar proteins from fish are more stable at neutral pH and it affects not only the denaturation rate at high temperatures but also the denaturation rate during frozen storage (Matsumoto and Naguchi, 1992).

1.3.1 Actomyosin

Even though the structure of fish myosin is similar to other mammalian myosin, it is unstable than the latter and studies on the Mg^{2+}ATPase of actomyosin and Ca^{2+}ATPase of myofibrils suggest that the stability of fish myosin is species specific and associated with the temperature of its habitat (Hashimoto *et al*., 1982). Myosin is responsible for the heat induced gel formation of fish meat (Samejima *et al*., 1981). Factors affecting the protein stability or interactions may also affect gel

Table 5: Fatty acid profile of lipids from oil sardine muscle and skin (in weight %)

Fatty acid	Muscle	Skin
10:0 and lower	0.6 ± 0.4	0.51 ± 0.29
12:0	0.2 ± 0.1	0.3 ± 0.1
13:0	0.16 ± 0.07	ND
14:0	8.8 ± 1.5	9.9 ± 1.5
15:0	0.9 ± 0.2	0.9 ± 0.2
16:0	18.3 ± 3.4	18.4 ± 2.8
17:0	2.8 ± 0.9	2.9 ± 1.0
18:0	6.9 ± 1.2	6.9 ± 0.9
14:1	0.5 ± 0.3	0.6 ± 0.4
16:1	10.6 ± 2.5	11.4 ± 2.3
18:1	9.7 ± 2.1	11.0 ± 2.2
20:1	1.3 ± 0.9	1.4 ± 1.0
16:2	3.7 ± 1.0	3.6 ± 0.8
18:2	2.9 ± 0.9	2.9 ± 0.8
18:3	1.1 ± 0.6	0.9 ± 0.7
18:4	3.0 ± 1.0	3.3 ± 1.0
20:2	ND	0.8 ± 0.6
20:4	2.8 ± 1.0	2.7 ± 1.0
20:5	12.4 ± 2.1	12.7 ± 2.9
22:3	0.7 ± 0.4	ND
22:4	1.3 ± 0.6	1.1 ± 0.5
22:6	11.6 ± 3.7	8.1 ± 3.1
Total saturated acids	37.7 ± 2.5	38.8 ± 2.6
Total monoens	22.2 ± 4.44	24.3 ± 4.6
Total poly unsaturated	40.1 ± 5.36	36.8 ± 4.4

ND – not detectable

Source: Nair and Nair (1985)

properties of and have considerable implications for fish processing and storage (Togashi *et al*., 2002).

Myosin is highly asymmetric hexameric protein. The properties of myosin depend on the source of origin. Each myosin molecule consists of two globular head regions and a rod like tail portion that is coiled coil of α-helices. The crystallographic structure of myosin head is shown in Fig. 2. The rod portion is responsible for the assembly of myosin to form the functional thick filament structure. Hydrophobic interactions are partially involved in the loss of protein extractability with time of storage (Owusu-Anash and Hultin, 1986).

Depending on the ionic strength of the medium, myosin could have two forms, monomeric and filamentous. Myosin is a monomer in high salt medium such as 0.5 M NaCl, while it easily transforms into filamentous form by self-association at reduced ionic strength at physiological levels (Konno *et al*., 1997). The tail portion of myosin is termed rod, which is responsible for the salt soluble nature of myosin or for the filament formation of the myosin molecule. Periodic arrangement of negatively and positively charged amino acids in the rod region generates electrostatic forces necessary for forming filaments (Maita *et al.*, 1991). Solubilization of myosin filaments by salt occurs under the conditions where electrostatic bridge formation between myosin molecules was inhibited. At a high concentration, salt ions readily form electrostatic bonds with corresponding charged amino acid residues of myosin. The bond formed with salt decreases the attraction of myosin molecules and results in inhibition of myosin filament formation (Konno *et al.*, 1997). Thermal denaturation of myofibrils monitored by measuring ATPase enzyme activity loss was well determined by the number of hydroxyl groups (Ooizumi *et al*., 1981). Two isoforms of myosin heavy chain (MHC) are known to exist in mammalian cardiac tissue, and it is within this myosin subunit that ATPase activity resides. Although the MHC-α and MHC –β isoforms differ by less than 0.2% in total molecular mass and amino acid sequence, this difference results in significantly altered ATP hydrolysis rate and velocity of myosin actin cross bridge cycling interactions and can therefore influence the rate and

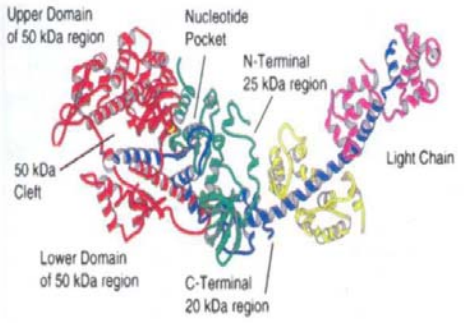


Fig. 2: Ribbon diagram of S 1 fraction of chicken skeletal muscle myosin. (Source: Rayment,1996)

efficacy of energy utilization for the generation of contractile force (Cuda *et al*., 1997; Piao *et al*., 2003).

The role of light chains on the structure and activity of smooth muscle myosin revealed that interaction among the light chains and heavy chains may be required for full regulation of the conformation and motor activities of smooth muscle myosin (Katoh and Morita, 1996). The importance of secondary interactions and covalent bonds in the aggregation of myofibrillar proteins have been described for several different species and conditions (Careche *et al*., 1998; Laird *et al.,* 1980; Lim and Haard, 1984; Tejada *et al*., 1996). Myosin and actin are the proteins that are most likely to be inextricable, not only in salt but also in solutions that rupture secondary interactions and disulfide bonds (Careche *et al*., 1998; Le Blanc and Le Blanc, 1989; Lim and Haard, 1984; Tejada *et al*., 1996).

Natural actomyosin of bigeyesnapper contained myosin and actin as the major components (>95%), with trace amounts of troponin, tropomyosin and myosin light chain. The high molecular weight protein bands at the top of the gel indicated the aggregation of myosin possibly formed during fish handling (Vissessanguan *et al*., 2003). The protein oxidation may results in the loss of ε-NH_2 groups of lysine residues which are the cross linking site in proteins, through a deamination process (Levine *et al*., 1990).

1.3.2 ATPase activity an index of myosin denaturation

Fish myosin are generally unstable, and rapidly form aggregates with concomitant decrease in ATPase activity (Ochiai and Chow, 2000). Heat induced formation of myosin oligomers precedes inactivation of myosin ATPase (Kimura *et al*., 1980). By taking myofibrillar ATPase as a parameter many researchers estimated the extent of denaturation or deterioration of muscle foods. For example total Ca^{2+}ATPase activity (sp.activity multiplied by protein concentration) in fish meat paste (surimi) is in good relationship with its gel forming ability (Katoh *et al*., 1979). Sensitivity of myofibrillar ATPase to the thermal denaturation is in parallel with the

loss of ATPase activity during frozen storage. The inactivation of myofibrillar Ca^{2+}ATPase follows basically a first order rate reaction as a function of frozen storage time (Matsumoto *et al*., 1985). However the structural changes of myosin caused by freezing is considered to be different from that caused by heating (Azumma and Konno, 1998). In particular, the rod portion is believed to remain unchanged during frozen storage.

Several workers have suggested that determination of ATPase activity might be a more accurate index of change in quality of chill stored fish than measurement of metabolites (Watabe *et al*., 1989). ATPase activity varies from fish to fish (Gopakumar, 2000). High Ca^{2+} ATPase activity was noted for surimi of high quality and this agrees well with another excellent quality criterion of surimi, the gel strength. A large decrease in gel strength of surimi gels from various species accompanied a decrease in the Ca^{2+}ATPase activities of the surimi gels during low temperature setting between 10ºC and 30ºC (Numakura *et al*., 1989).

Ca^{2+}ATPase reduced to 0.02 μM/min/mg protein from 0.12 μM/min/mg protein within 6 days in case of ice storage and from 0.135 to 0.02 within 4 days during frozen storage. Ca^{2+}ATPase activity decreased by 3 times during first week of frozen storage at -20ºC (Chan *et al*., 1995). Loss in Ca^{2+}ATPase activity was 80% in 40 days of storage in the case of both sodium acetate treated and untreated shrimps (Shamasunder and Prakash, 1994b).

Milkfish (*Chanos chanos*) actomyosin pressurized to 200 MPa at 4°C lost about 80% of its original Ca^{2+}ATPase activity and almost completely inactivated upon pressurization at 300MPa for 5 minutes (Ko, 1996). Flying fish (*Gypselurus opistiopus*) and sardine (*Sardinops melanostictus*) actomyosin ATPases were also inactivated, the activity in sardine being more sensitive than that of flying fish (Ko *et al*., 1991). Pressure sensitive component responsible for the loss of actomyosin ATPase activity is the myosin subfragment-1 which most readily unfolds exposing hydrophobic groups with a concomitant loss of solubility and decreased helical content (Ishizaki *et al*., 1995). The habitat temperature of the fish species has got a good correlation to the denaturation of actomyosin Ca^{2+}ATPase as revealed from

Alaska pollack (*Theragra chalcograma*) and tilapia (*Oreochromis aureus*) myofibrillar Ca^{2+}ATPse (Ashie and Lanier, 2000). Polyols imparted varying levels of baroprotection to fish (*Theragra chalcograma*) muscle Ca^{2+}ATPase activity and found no correlation between number of equatorial hydroxyl groups in the sugar or polyols and baroprotection (Ashie and Lanier, 2000).

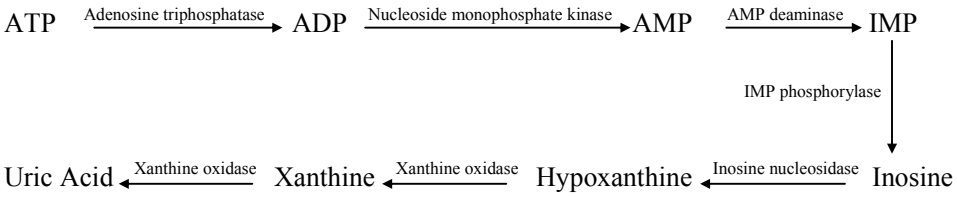
Caldesmon is an actin binding protein that inhibits actin activated ATPase activity of myosin in solution (Soube *et al*., 1981) and force production in muscle fiber system (Brenner *et al*., 1991; Pfitzer *et al*., 1993). Inhibition of actin activated ATP hydrolysis by caldesmon parallels the decrease in binding of S1 ATP to actin (Chalovich *et al*., 1987; Hemric and Chalovich, 1988; Velaz *et al*., 1989; Velaz *et al*., 1990). An important component of the inhibition of ATPase activity by caldesmon appears to be the inhibition of binding of myosin S1 to actin tropomyosin (Chalovich *et al*., 1987; Sen and Chalovich, 1998).

Caldesmon inhibits the binding of myosin S1 to actin and actin tropomyosin with and effectiveness that are related to the strength of the S1 actin interaction (Yan *et al*., 2003). The inhibitory action of caldesmon on actin activated ATPase activities of myosin in solution or the force generated in contracting the muscle fiber may be caused by a reduction of active sites for myosin binding (Yan *et al*., 2003).

When cod (*Gadus morhua*) stored in ice, ATPase enzyme activity was lost in two weeks and when stored at 15ºC the activity was lost within one week itself (Yamanka and Mackie, 1971). The authors concluded that loss of ATPase activity is a potential index for evaluating the quality of ice stored but not frozen stored cod. It is also possible that by products of lipid oxidation, like malonaldehyde, could cause denaturation, unfolding or cross linking of muscle proteins. Such structural changes, in turn may affect some quality aspects of muscle foods. As suggested by Wang *et al*., (1997) antioxidant treatment during washing was effective in preventing loss of myosin ATPase activity and maintained a higher available sulfhydryl content in fresh beef heart surimi, but not in frozen storage.

1.3.3 Nucleotide degrading enzyme activity of ATPase

Following is the schematic representation of ATP degradation (Gill, 2000):



Since the degradation of ATP and its metabolites occurred in a more or less predictable manner, formula developed for freshness index K value

$$K\% = \frac{[\text{Inosine}] + [\text{Hypoxanthine}]}{[\text{ATP}] + [\text{ADP}] + [\text{AMP}] + [\text{IMP}] + [\text{Inosine}] + [\text{Hypoxanthine}]} \text{ X100}$$

- - - (1)

The higher the K value the poorer the quality of fish.

1.3.4 Rigor mortis and ATP

Rigor mortis of the muscle is known to occur when the ATP decreases below a certain critical concentration (Iwamoto *et al.*, 1987). ATP is enzymatically degraded through a series of intermediates in seafood, resulting in rigor mortis (Ashie *et al*., 1996). The ATP, ADP and AMP were significantly negatively correlated with peneterometer reading of anaesthetized fish. Higher concentration of ATP, ADP and AMP were positively correlated with low peneterometer reading (Firm texture) (Maria *et al*., (1992).

1.3.5 Advantages of ATP in post mortem muscle

The faster the rate at which muscles were frozen the slower would be the rate of post mortem glycolysis and of ATP break down and that this will be reflected in less exudation (or drip) on thawing. Magnesium sulfate was injected pre slaughter in

doses sufficient to cause relaxation of the animal, the rate of ATP break down was slowed even more and the retention of *in vivo* water holding capacity was enhanced to an even greater extent and is reflected in extent of drip. When ATP breakdown was arrested then the degree of drip upon thawing would be less still. The presence of ATP in the muscle tissue of a freshly slaughtered animal allows the muscle to retain its elasticity by inhibiting the molecular interaction between the myofibrillar proteins, and as a consequence it can retain a higher water holding capacity (Guylinder and Lowrie, 1999). Following the death of fish, the conversion of ATP to IMP is usually complete within 1 or 2 days and this is presumed to be totally autolytic process. The formation of hypoxanthine from ATP is fast and depend on endogenous enzymes. Then the formation of hypoxanthine to xanthine and ultimately to uric acid is slow and mainly by microbial enzyme activity. The post mortem depletion of ATP and ADP permits actin and myosin to interact, forming permanent actomyosin cross links (Ashie *et al*., 1996). In most post harvest fish the initial catabolism of ATP normally results in accumulation of inosinemonophosphate, which contribute to the overall flavor of meat.

It is always advisable to assess the quality of fish and fishery products complementary with other quality index such as sensory test, gel strength or others along with ATPase activity measurement to arrive at an objective evaluation of the products edibility (Gopakumar, 2000). Helical content of myosin molecule decreased by only a few percent even after the ATPase activity completely disappeared. Hence, the loss of ATPase activity does not necessarily imply that the myosin head has been unfolded. It only indicated that the myosin head undergoes some conformational change (Chan *et al*., 1995).

The loss of water holding capacity arising from the formation of actomyosin when *in vivo* ATP level falls and rigor mortis ensues and the efficacy of pyrophoshphate in improving it in minces, suggests that there would be some benefit in keeping the ATP high postmortem (Lowrie, 1998). It was suggested that destruction of sulfhydryl groups located in subfragment-1 of myosin could cause loss of Ca^{2+}ATPase activity (Wells and Young, 1979). There was a close relationship

between Ca^{2+}ATPase activity and sulfhydryl content in fresh beef heart surimi but not for frozen stored surimi (Wang *et al*., 1997).

The nucleotides ATP, ADP and IMP have been shown to exert a protective effect on fish actomyosin stored at –20ºC. While nucleotide catabolites inosine and hypoxanthine destabilized these proteins (Jiang *et al*., 1987). This finding may help to explain why fresh fish with consequent higher concentrations of ATP, ADP and IMP are more stable during frozen storage than less fresh fish (Fukuda *et al*., 1984).

ATP showed similar extent of protection to myosin as actin. This directly binds to myosin head and cause conformational change, resulting in stabilization of this protein. Actually, S-1structure is considerably stabilized in the presence of Mg^{2+}ATP through conformational change (Sivaramakrishnan and Bruke, 1982).

In presence of just 0.6 mM of $ZnSo_4$ the concentration of sugars needed to protect certain enzymes against drying or freezing could be lowered by magnitude of two orders (Mac Donald and Lanier, 1991). At 0.6 mM concentration of $ZnSO_4$ inhibited the Ca^{2+}ATPase activity of tilapia actomyosin to zero (Mac Donald *et al*., 1996).

1.4 Functional properties of proteins in general

Proteins play several functional roles in the expression of sensory attributes of various foods. Traditionally proteins of animal origin have been used in conventional and fabricated food. The functional role of protein that contribute to the sensory quality of the product does not arise from a single physicochemical property rather it is a manifestation of a complex interaction of multiple properties. The major functional properties of proteins and its functional role in food systems are shown in Table 6. The functional properties are fundamentally related to their physico-chemical and structural properties such as size, shape, amino acid composition/sequence, net charge, charge distribution, hydrophobicity/ hydrophilicity ratio, secondary, tertiary and quaternary structural arrangements, number of microdomain structures and

Table 6: Functional role of proteins in food systems

Function	Mechanism	Food system	Protein source
Solubility	Hydrophilicity	Beverages	Whey proteins
Viscosity	Water binding, Hydrodynamic size, shape	Soups, Gravies, Salad dressing	
Water binding	H-bonding, ion hydration	Meat sausages, cakes, breads	Muscle proteins, egg proteins
Gelation	Water entrapment and immobilization, net work formation	Meats, gels, cakes, bakeries, cheese	Muscle proteins, egg and milk proteins
Cohesion/adhesion	Hydrophobic, ionic and H-bonding	Meats, sausages, pasta baked goods	Muscle proteins, egg proteins, whey proteins
Elasticity	Hydrophobic bonding, disulfide cross links	Meats, Bakery	Muscle proteins
Emulsification	Adsorption at interfaces, film formation	Sausages, bologna, soup, cakes	Muscle proteins, egg proteins, milk proteins
Foaming	Interfacial adsorption film formation	Whipped toppings, ice creams, cakes, desserts	Egg proteins, milk proteins
Fat and flavour binding	Hydrophobic bonding, entrapment	Simulated meats, bakery, doughnuts	Milk proteins, egg proteins

Source: Kinsella *et al.* (1985)

adaptability of domain structures or the structure of whole molecule to changes in environmental conditions (Damodaran, 1994).

In addition to the intrinsic molecular factors, several extrinsic factors such as the method of isolation, pH, ionic strength, the redox potential of food system, and interactions with other food components also affect the functional properties of proteins (Kinsella, 1982). The various functional properties of food proteins are manifestations of two molecular aspects of proteins namely protein surface related properties and hydrodynamic properties (Damodaran, 1989). The surface related properties are governed by the hydrophobic, hydrophilic and steric properties of the protein surface and the properties related to hydrodynamic properties of proteins are governed by size, shape and flexibility of proteins (Damodaran, 1994). The lack of standardized method to evaluate both the molecular properties of proteins and the functional properties also has confounded proper understanding of structure-function relationship of proteins (Kinsella, 1982).

Emulsion capacity is generally defined as the maximum amount of lipid emulsified by a protein dispersion. A molecule that contains a moiety that is soluble in water and another that is soluble in nonpolar solvents is termed amphiphilic. Immiscible liquids may be stabilized against coalescence by the addition of these types of molecules, generally referred to as emulsifiers. Proteins are capable of coating lipid droplets and can provide an energy barrier to both particle association and phase separation. One of the ways that proteins minimize their energy is by folding into structures of low free energy. These structures generally result when the interactions of polar groups with water are maximized and the interaction of nonpolar groups with water is minimized (Mangino, 1994). Solubility, in addition to surface hydrophobicity, plays a role in emulsifying properties of proteins (Kinsella *et al*., 1985). Highly insoluble proteins display very poor emulsifying properties. Since the stability of protein films at the oil water interface requires favourable interaction of the protein chain with the both the oil and aqueous phases, an optimum balance of hydrophilic and hydrophobic groups that maintains the protein in solution is needed for better emulsifying properties. Partial denaturation of proteins does not always

cause lowering of stability but resulting in improvement of emulsifying properties of proteins also (Kato and Nakai, 1980).

Foams are complex two-phase colloidal systems containing at least initially a continuous liquid phase and a gas phase dispersed as bubbles or air cells. Proteins that are effective in foaming must rapidly diffuse to the interface, adsorb and then reorient to form a viscous film to maintain discrete bubbles until stabilizing interactions develop (Dickinson *et al*., 1988; Prins, 1988). This ability is dependant on the intrinsic factors including the structure and conformation of the protein, which in turn depends on environmental factors such as pH, ionic strength and protein interactions (Damodaran, 1990). The foaming properties as well as other surface active properties of proteins correlate well with surface hydrophobicity (German and Philips, 1994). Structural features of proteins that are conducive to forming rapid and large volumes of foam are not necessarily ideal for promoting the protein-protein interactions that give rise to viscoelastic films and hence stable foams (Kinsella and Whitehead, 1988). The intrinsic factors that determine the structure and flexibility of a protein, namely, electrostatic interactions, hydrogen bonds, hydration and hydrophobic effects and disulfide bonds also determine interfacial behaviour and the foaming properties of a single protein and the interactions between protein mixtures at the bubble surface (German and Philips, 1994).

The solubility of a protein under a given set of conditions is the thermodynamic manifestation of the equilibrium between protein-protein and protein-solvent interactions. Solubility is directly related to the physico-chemical nature of protein surface (Damodaran, 1994). Solubility of proteins is an important criterion for many functional properties like gelation, emulsification capacity and viscosity. The factors responsible for solubility of proteins from fish are ionic strength, pH and temperature (Yang and Frowning, 1990). The myofibrillar proteins generally require higher ionic strength solutions usually more than 0.3 M and solubility in such solvent is taken as an index of protein conformation.

Gelation is an important functional property of food proteins. Gels act as a medium for holding water, flavors, and other nutrients and impart unique rheological

and textural qualities of food systems (Kinsella, 1976). A gel is an intermediate state between a protein solution and a protein precipitate, formed above a certain level of concentration with just the right balance of protein-protein and protein-solvent interactions. High ionic strength tends to reduce the electrostatic repulsion between proteins due to the shielding of ionizable groups by mobile ions. When the protein-solvent interaction is favourable, the protein can reduce its total energy by surrounding itself with solvent molecules; when interactions are repulsive, the solvent is generally excluded (Tanaka, 1981).

Viscoelasticity can be characterized either by transient experiments, such as creep compliance or stress relaxation, or by dynamic measurement wherein, the material is subjected to an oscillatory (usually sinusoidal) deformation, and the magnitude and phase lag of the resultant stress wave are detected. For a perfectly elastic response the stress in phase with the applied strain; for a purely viscous system the stress is precisely 90° out of phase. When the deformation is a shear strain the modulus determined is then a shear modulus and is designated such using the capital letter G. In dynamic mechanical spectroscopy two fundamental parameters are measured; G′ the storage modulus, a measure of energy stored elastically, and G″ the loss modulus a measure of the energy dissipated as heat (viscous component). G′ is determined from the component of the stress which is in phase with the strain, and G″ from the component which is 90° out of phase. The loss angle (δ) is the phase angle between the applied strain and resultant stress (Ziegler and Foegeding, 1990)

The physical integrity of a gel is maintained by counter balancing forces of attraction and repulsion between the polymer molecules and between the polymer network and the surrounding solvent (Ziegler and Foegeding, 1990). Three of these forces are the rubber elasticity, the polymer-polymer affinity, and the hydrogen ion pressure. The total pressure acting on the gel is the sum of these three components as termed as osmotic pressure of the gel since it determines whether the gel tends to take up or expel fluid (Tanaka ,1981).

1.5 Surimi

Surimi is a highly functional, pure fish protein water cryoprotectant combination prepared from washed fish flesh (Pigott and Tucker, 1990). Surimi is obtained from fish skeletal muscle that is washed with water and blended with cryoprotectants (Park *et al*., 2004). The art of preparing a washed, minced fish flesh that can be used as a base for preparing formulated food products was developed in Japan several centuries ago. The washed flesh, when combined with certain condiments, mixed or kneaded and steamed formed a fish gel that was called kamaboko. Today there are many kamaboko products sold in Japan under the category neriseihin (kneaded seafood) include; original kamaboko - the washed fish flesh is mixed with flavouring and gelling ingredients, shaped, steamed and cooled. This make it easy to slice with a knife; broiled kamaboko chikuwa - kamaboko skewered, broiled, cooled and sold in the form of a cylindricall roll; fried kamaboko satsumage - kamaboko shaped, fried, cooled and sold in many shapes and forms; ham and sausage kamaboko - the first analog kamaboko products that stimulated other products using fish as a substitute for conventionally used pork.

India has got nine surimi plants with an installed capacity of 324 million ton. The varieties used for production of surimi are threadfin breams, croaker, goatfish and ribbonfish. The export of frozen surimi from India amounts to a quantity of 26302 tons that earned a foreign exchange equivalent to US$ 3.7 million (MPEDA, 2001).

Efficient washing of the flesh is the most important factor in preparing a surimi that will have the maximum gelling properties for the given raw material. Minced flesh contains approximately two thirds myofibrillar protein, which possesses the desired functional properties. The remaining one third consists of oil, blood, enzymes and sarcoplasmic proteins, which impede gel formation of final surimi product. The purpose of washing is to increase the quality of surimi and extend the storage life by removing this fraction, thus increasing the concentration of the functional component, actomyosin. The gel properties of surimi are dependant on the actomyosin that is present in its native state. There is a considerable variation in the

functional properties of surimi made from the minced flesh of different species (Pigott and Tucker, 1990). Water washing of mince is a necessary operation to prevent myofibrillar protein denaturation during frozen storage and the gel strength continues to improve as the number of water washing cycles increases (Matsumoto and Naguchi, 1992). Cryoprotectants are compounds that protect or stabilize a product during freezing and thawing. The addition of cryoprotectants is important to minimize rate of denaturation and there by exhibiting desired functionality. Sugars, sorbitol, phosphates and salt are the commonly used materials. Although the amount of cryoprotectants added varies considerably between producers, the average quantities added are approximately 4-5% sugars 0-5% sorbitol, 0-3% salts and 0-0.3% phosphates. It is possible to control the gelling properties and allow the underutilized fish species for surimi production.

The levels of nutrients in surimi are not identical to those of natural seafood. There are nutritional advantages as well as disadvantage involved. It increased protein and PER and decreased fat, cholesterol and moisture. The lower protein content is due to washing and addition of cryoprotectants. The actual protein level may improve due to the dewatering step. Cholesterol content is usually significantly reduced, often as much as 50% or more, as the lipid components are washed out. Unless fat is added to the final product, the fat content is extremely low. Gel forming ability decreased markedly after the addition of the extract of all water soluble substances (Matsumoto and Naguchi, 1992).

The presence of cryoprotectants is important to ensure maximum functionality of frozen surimi because freezing induces protein denaturation and aggregation (Mac Donald *et al*., 2000). There are two kinds of temperature tolerance with respect to the myofibrillar proteins of fish, one the thermal stability at temperatures above the freezing point of surimi, which is affected by the initial fish quality and the control of temperatures and other factors during the process of surimi manufacture and the other one is the stability during frozen storage, which determines the shelf life of frozen surimi and both these are known to be species specific (Matsumoto and Naguchi, 1992). An important physical property of muscle proteins is gel-forming ability

(Lanier, 2000). Gel strength calculated on the basis of the force multiplied by deformation using a unit of g.cm is used by the surimi industry as an index of quality (Mac Donald *et al*., 2000).

1.5.1 Changes during processing

During frozen storage, both fiberosity and hardness tend to increase as well as changes in texture, there is loss of muscle protein functionality, which is of particular concern in product such as surimi where it is important to preserve functionality to obtain high quality gels (Tejada *et al*., 2003). In Hake most pronounced changes occurred in properties relating to aggregation during frozen storage, given that side to side mechanisms may be preferentially involved in myosin aggregation during freezing in model systems of isolated myosin (Ramirez *et al*., 2000). During frozen storage hydrophobic and hydrogen bonds buried inside the protein molecules becomes exposed and broken from their native arrangement and thus bring about the conformational changes leading to loss in functionality (Benjakul *et al*., 2003). Nevertheless, a different type of aggregation among myosin molecules may be occurring, as oligomers formed by carp myosin retaining ATPase activity after heating, in which rod aggregation is restricted to the region near the S1/rod junction has been documented (Tazawa *et al*., 2002).

Frozen fish can be used as a potential year round raw material for surimi manufacture. During frozen storage at –20°C free SH content of sardines (*Clupea pilchardus*) decreased by 20% from an initial value of 80.6 mM/kg protein related to loss of cyst(e)ine (Garcia-Arias *et al*., 2003). Endogenous metalloprotease is predominantly responsible for myosin degradation in the muscle paste (Park *et al*., 2003). Conformational changes in globular head of myosin probably must have resulted in the exposure of reactive sulfhydryl groups, which were prone to oxidation or disulfide interchange and masking of sulfhydryl groups by protein aggregates was also contributed to the decrease in free sulfhydryl group availability during storage (Benjakul *et al*., 2003). Ca^{2+}ATPase from the sarcoplasmic reticulum (SR) and cytochrome oxidase from inner mitochondria membranes are transmembrane

enzymes, and the activity of these enzymes is known to depend on the membrane lipid environment. Freezing of muscle from cod resulted in an increase in SR Ca^{2+}ATPase activity indicating membrane disruptions and leaking of proteins from the intracellular organelles. During frozen storage SR Ca^{2+}ATPase activity decreased (Godiksen *et al*., 2003). Products of oxidation and hydrolysis are known to denature soluble proteins (Mackie, 1993). Subzero temperature fluctuations may lead to formation of large ice crystals, allowing ice to melt and refreeze on already existing ice crystals and thus could be expected to have severe effects on membrane integrity (Godiksen *et al*., 2003; Mackie, 1993). The lower α-helical content of LMM from Walley pollack suggests that the denaturation of proteins might have occurred during the preparation (Togashi *et al*., 2002). Formation of disulfide bond also contributes to the decrease in salt soluble protein during frozen storage (Owusu-Anash and Hultin, 1986). There was a dip in the extractability pattern of proteins in salt solution after 8 weeks of storage (Owusu-Anash and Hultin, 1986). The increase in salt concentration of the unfrozen phase also contributes to the denaturation of proteins in association with physicochemical changes observed during frozen storage. The covalent bonds formed during frozen storage are mainly disulfide bonds and there was a loss of myosin heavy chain or ATPase or both in the myofibrils extracted from the frozen stored samples (Le Blanc and Le Blanc, 1989).

The activity measurements of sarcoplasmic reticulum Ca^{2+}ATPase and cytochrome oxidase indicated that cold stored at relatively close storage temperatures – 20°C and – 30°C, had a high sensitivity of the enzymes against frozen storage temperatures and is an important and very valuable property of enzymes to qualify them as frozen storage indicators. The fact that enzyme stability at fluctuating subzero temperatures was increased in ice stored fish may be used to develop a test system that can determine whether a frozen storage fish has been in ice stored before freezing (Godiksen *et al*., 2003).

Denaturation and aggregation of muscle proteins are associated with the formation of disulfide as well as the formaldehyde formation (Badii and Howell, 2001; Jiang *et al*., 1988). The decrease in ATPase activity of fish muscle observed

during frozen storage. The decrease in ATPase activity was possibly due to the conformational changes of myosin globular head as well as the aggregation of this portion (Okada *et al*., 1986). Denaturation of myofibrils was caused by the increased salt concentration in unfrozen phase (Tkahashi *et al*., 1993). Rearrangement of protein through protein-protein interactions was also presumed to contribute to the loss in ATPase activity (Benjakul and Bauer, 2000). The surface hydrophobicity of actomysoin from threadfin bream and bigeye snapper increased during frozen storage, during extended frozen storage, the protein underwent conformational changes in which the hydrophobic portions exposed and the hydrophobic interactions leading to the aggregation and loss in solubility. Hydrophobic amino acids residues of myosin molecules in white muscles fishes were more easily exposed by freezing compared to those in red muscle fish (Niwa *et al*., 1986).

The maximum shear stress values for striped mullet (*Mugil cephalus*) surimi observed with a calcium concentration of 0.4% (Ramirez *et al*., 2003). Addition of 0.2% calcium levels reported for the preparation of surimi from Atlantic croaker, Mexican flounder and Northern King fish (Morales *et al*., 2001). Continuous denaturation of proteins during frozen storage lead to the lower water holding capacity of proteins and the ice crystal formed resulted in the tissue damage and leakage of various organelles. As a result, water could be released from muscle more easily, particularly when the frozen storage time increased (Benjakul *et al*., 2003).

Rheological changes from sol to gel are referred to as setting when salted surimi, is maintained at low temperature and is important for the enhancement of gel strength. Transglutaminase enzyme plays a major role in the setting phenomenon of salted fish paste. Transglutaminase is a calcium-activated enzyme and the setting ability of meat depends on the species. Settings involve complex reactions by forming intra and inter molecular interaction of myosins. Unfolding of α –helix in myosin molecules initiate setting (Ogawa *et al*., 1995). Yongsawatdigul and Park (1996) also reported that slow ohmic heating 1ºC/min induced MHC cross linking of Alaska pollock surimi. Addition of 0.2% calcium chloride improved gel strength through enhancing the covalent cross linking reaction of MHC (Yongsawatdigul *et al*., 2002).

Setting is induced by hydrophobic and a sulfhydryl-disulfide exchange reactions of actomyosin (Niwa *et al*., 1983). During thermal gelation proteins undergoes structural changes like exposure of functional groups followed by protein-protein interactions. Setting of surimi resulted in the formation of a high amount of cross linked myosin heavy chain (Wan *et al*., 1994).

1.5.2 Additives to the surimi

1.5.2.1 Polyhydric alcohols

The term sugar alcohol refers to chemical compounds containing three or more hydroxyl groups and is synonymous with another customary term polyhydric alcohol. The calorie value ascribed to sugar alcohols should in theory be the same [approximately 4 kcal/g (17.6 kJ/g)] as determined for dietary carbohydrates in general. However, the incomplete absorption of sugar alcohols, especially from larger single doses, suggests that perhaps a lower calorie value should be given to those substances. Values of 2.0-2.4 kcal/g, for example have later been proposed for sugar alcohols (Makinen, 1994).

Virtually all sugar alcohols share the same type of carbon skeleton with other natural, dietary carbohydrates, and the sugar alcohols can even be assayed as sugars in chemical total sugar analyses. All sugar alcohols can be converted chemically or enzymatically to the corresponding aldoses and ketoses, which in turn are reducible to sugar alcohol form. Some of the unique properties of sugar alcohols that make them biologically unique are as follows:

The absence of reducing carbonyl groups: This fact makes sugar alcohols chemically somewhat less reactive than the corresponding aldoses and ketoses. The sugar alcohols thus avoid certain chemical reactions that take place at a higher rate with several aldoses and ketoses. The relative chemical inertness is also reflected in the fact that in human oral cavity the sugar alcohols are less reactive and do not normally participate in extensive acid formation and in dental plaque. The reducing

power, in spite of the relative inertness and the absence of reducing carbonyl groups, the sugar alcohols do actively participate in metabolic reactions where their extra hydrogen bonds can be deposited in the metabolites such as coenzymes (NADP and NAD, for example) and other acceptors to form chemically reduced products of metabolism.

Complex formation: By virtue of their polyoxy nature, many sugar alcohols form interesting although chemically weak complexes with several polyvalent cations. For various physiologic and nutritional purposes the complexes with Ca^{2+}, Fe^{3+}, Cu^{2+} and possibly several trace elements in general, are important.

Hydrophilicity: The presence of maximum possible number of hydroxyl groups in carbohydrate structure makes virtually all sugar alcohols very hydrophilic (although solubility of galactitol and D-mannitol in water is lower). At least some lower homologues can compete with water molecules for the hydration layer of proteins, other biomolecules, and also metal cations (with out true complex formation). The consequences of this can be seen in the fact that in aqueous solutions- and this may be valid biological fluids as well – the sugar alcohols indirectly strengthen hydrophobic interactions between proteins, stabilizing them against thermal and other denaturation or damaging processes. In chemical parlance also glucose and sucrose are polyhydric alcohols. Such compounds can act as catalysts in the hydrolysis of esters and other compounds. This reaction may be attributed to nucleophilic reactivity of those carbohydrates, as a result of the anion formed by ionization of a hydroxyl group. Therefore, the sugar alcohols also form such highly basic alkoxide ions (with pKa values in the range of 12 to 13; such anions have an exceptionally strong solvation energy).

There are several physiologic corollaries of the above qualities of sugar alcohols, one of them being their osmotically active nature. This quality is not only affects the function of sugar alcohols as food, but also in numerous intracellular events and medical applications as in the intravenous use of D-mannitol in the lowering of intracranial pressure, and in renal function studies. The use of D-mannitol as a diuretic should be specifically mentioned. Because of their polyol nature, some

sugar alcohols (D-mannitol, for example) with the right configuration can act as free radical scavengers in biological and experimental systems. Under certain chemical conditions sugar alcohols can function as chaotropic agents. Chaotropic agents break up organized water structures in aqueous systems. Such agents have the capacity to affect reactions that obtain their energy from the release of structured water to the bulk solvent. At least in theory these substances could be used to disperse suspensions of dental plaque and salivary deposits.

1.5.2.2 Carbohydrates as cryoprotectants

Surimi, the refined myofibrillar component of fish muscle, requires the inclusion of a cryoprotective component prior to freezing to ensure long term stability of the proteins in frozen storage. This in turn assures good functionality of the materials in food manufacture expressed primarily as gel forming potential with its manifestations of texture formation and water binding properties. Large changes in functionality (as measured by gel forming ability, water holding capacity and protein solubility) take place during storage at temperatures as low as –28^{o}C (Park *et al*., 1987a,b). Fennema (1982) noted that all proteins would be expected to have an optimum stability at a temperature just above the freezing point of water. At higher temperatures, thermal denaturation would ensue as a result of destabilization of hydrogen bonds and increased molecular motions. At lower temperatures, despite enhanced intramolecular hydrogen bonding, hydrophobic interactions would weaken to the point that a net destabilization may be expected to occur. This optimum temperature of stability might actually be below the freezing point, but the onset of ice crystal formation could induce changes in the protein environment that effect denaturation. Muscle protein expresses their functionality only when the salt soluble proteins are fully extracted (solubilized), and cryoprotection is only possible when stronger association of the cryoprotectant and the protein molecules occur. Thus both the expression and cryoprotection of muscle protein functionality will optimally occur in a minced or comminuted muscle system.

Watanabe *et al*., (1988) demonstrated the cryoprotective ability of certain surfactants, particularly certain polyoxyethylene sorbitan esters and sucrose esters, in preventing loss of gel forming ability in surimi. A cryoprotective effect has been attributed to triglycerides, in that free fatty acids which may be released through hydrolysis of phospholipids and react to denature proteins are thought instead to preferentially react with triglyceride, thus indirectly protecting the proteins (Wessels *et al*., 1981). Sucrose and sorbitol are used in the ratio of 1:1 about 8% of the leached muscle serve as the common cryoprotectant. These carbohydrates were chosen because of their relative low cost, good availability. However these additives may impart minor sensorial changes, which may not be significant in changing the profile of the product. Many other low molecular weight sugars and polyols have been tried as cryoprotectants for proteins during storage. Lactitol and lactulose reportedly have low sweetness.

1.5.2.3 Metal ions

The current FDA list includes the following minerals; calcium, iron, magnesium, potassium, zinc, copper, manganese, and few others as safe within certain limits. In addition, the FDA has stated that calcium supplementation up to 2,500 mg per day is considered safe. Numerous forms of calcium are available for supplementation. Most studies have found that calcium bioavailability from various salts to be virtually identical. A recent perspective epidemiological investigation of over 45,000 men over 40 years of age found that high calcium intakes, including calcium from supplements, lead to a decline in symptomatic kidney stones (Curhan *et al*., 1993).

Calcium chloride has been of much interest to meat scientists as well as meat processors due to its ability to tenderize meat (Nurmahmudi and Sams, 1997). Calcium chloride tended to be prooxidative at low concentrations (0.05% for chicken and 0.15% or less for beef) and antioxidative at higher concentrations (Cho and Rhee, 1995). St. Angelo *et al*., (1991) observed a prooxidative effect of calcium chloride (0.3%) in lamb, hypothesized that calcium chloride may increase the activity of

lipases and lipid oxidizing enzymes. On the other hand, Graf and Panter (1991), who showed an antioxidative effect of calcium chloride (0.02% - 0.1%) in chicken, suggested that calcium and some other polyvalent ions may displace iron from its binding site on phospholipids thus preventing lipid oxidation and warmed over flavor development.

Stabilization effect of certain salts of the Hofmeister or lyotropic series on proteins is due to the strengthening of the protein intermolecular hydrophobic interactions and the property of a salt that affects hydrophobic interactions are quantified by its molal surface tension increment (Melander and Horvath, 1977). In both cryoprotection and protection from drying it has been found that a remarkable synergism exists between many protective compounds and certain divalent cations, notably Zn^{2+} (Carpenter *et al*., 1987a,b). In the presence of just 0.6 mM $ZnSO_4$ the concentration of sugar needed to protect certain enzymes against drying or freezing could be lowered by two orders of magnitude (Hazen *et al*., 1988).

The addition of $CaCl_2$ and pre incubation conditions significantly affects the textural properties of threadfin bream surimi gels. At any added Ca^{2+} concentration the breaking force of samples set at high temperature (40ºC) were higher than those set at low temperature (4ºC and 25ºC) and also without setting (Yongsawatdigul *et al*., 2002). Ca^{2+} concentration is the major factor related to the gelling properties of Walley pollack surimi paste (Wan *et al*., 1994). Lee and Park (1998) found that the addition of 0.2% calcium compounds improved the shear stress of pacific whiting where as the lower concentration (0.05 to 0.1%) effectively increased the gel texture of Alaska pollock. Species dependence of optimum calcium chloride was attributed to the inherent varied concentrations of Ca^{2+} in muscle among species (Lee and Park, 1998). Breaking force of threadfin bream surimi gel was highest when 0.2% calcium chloride was added and set at 40ºC for 2hrs. Effect of Ca^{2+} was more pronounced at 40ºC than at 4ºC and 25ºC because activity of transglutaminase was greater at 40ºC (Yongsawatdigul *et al*., 2002). Addition of $CaCl_2$ more than 2 mM to diluted surimi pastes increased the breaking strengths to approximately 200 and 150% for set and

cooked gels respectively and the addition of EGTA completely inhibited the gelation (Wan *et al*., 1994).

The concentrations of calcium chloride and pre incubation conditions significantly affected the protein patterns on SDS-PAGE of surimi. MHC decreased with an increase in the concentration of calcium. At 0 and 0.05% MHC intensity at various settings conditions was comparable. When 0.1% Ca^{2+} was added, MHC intensity of gels pre incubated at 25ºC for 4 hrs and 40ºC for 2 hrs were less than those of the control and those pre incubated at 4ºC. MHC completely disappeared when 0.2% Ca^{2+} was added and pre incubated at 25ºC for 4hrs and 40ºC for 2hrs. Ca^{2+} had a more pronounced effect on the disappearance of MHC when pre incubated at 25ºC for 4hrs and 40ºC for 2 hrs (Yongsawatdigul *et al*., 2002). Reduction of MHC reached 90% of its original content when 0.06% of $CaCl_2$ was added in to hoki surimi incubated with crude hoki TGase (Kimura *et al*., 1991). Cross linked MHC of Alaska pollock surimi was maximally formed in the presence of 0.02% Ca^{2+}, and further increase in Ca^{2+} concentration did not increase MHC cross links (Wan *et al*., 1994). Changes of MHC at various conditions corresponded well with breaking force of surimi gels. An increased breaking force at higher Ca^{2+} concentrations was due to an increase in the catalytic activity of endogenous transglutaminase. MHC of control also decreased with added Ca^{2+} concentration. Endogenous transglutaminase catalyzed cross linking of MHC even without incubation. Slow temperature increase in a 3 cm dia casing allows the catalytic activity of endogenous TGase to progress before thermal inactivation occurs (Yongsawatdigul *et al*., 2002).

Zinc exists as a divalent cation Zn^{2+} that has a completely filled d shell with 10 d electrons. This electronic configuration has four important consequences. First because of the filled d shell Zn^{2+} has no ligand filed stabilization energy when co-ordinated by ligand in any geometry. For ions with partially filled d shells, this electronic energy term can favour certain arrangement of ligands over others. Second in terms of hard-soft acid base theory, Zn^{2+} is considered as a borderline acid. Because of this zinc can interact strongly with a variety of ligand types including sulfur from cysteine, nitrogen from histidine, oxygen from glutamate, aspartic acid

and water. Third divalent zinc ion is not redox active, neither the potential oxidized form Zn^{3+} nor the potential reduced form Zn^{+}, is accessible under physiological conditions. Finally zinc is relatively labile in kinetic terms; it undergoes ligand exchange reactions relatively rapidly.

The generation of a tetrahedral site from a combination of cysteine and histidine residues will automatically have a preferential affinity for zinc over most other common metal ions (Berg and Merkle, 1989). Most metal ions with partially filled d shells lose ligand field stabilization energy upon going from an octahedral site in aqueous solution to a tetrahedral site in a protein. This effect, combined with the borderline activity of zinc causes tetrahedral site with a combination of cysteine and histidine ligands to bind zinc more tightly than other metal ions such as Fe^{2+}, Ni^{2+} and Co^{2+} by factors of two orders of magnitude or more (Berg and Shi, 1996).

Metallothionein is a unique metalloprotein in which cysteine constitute one third of its amino acids and histidine and aromatic amino acids are all completely absent. All 20 cysteine residues bind seven zinc atoms such that each metal atom has a complement of four cysteine ligands. Classically zinc has not been thought to be associated with biological redox reactions, because the metal is not readily oxidizable or reducible in solution. However, it is distinct possibility that a change in the redox status of a donor atom of a zinc compound might alter the over all oxidoreductive properties of the complexes and hence its biological reactivity. In metallothionein, four thiolates interact with each of the seven zinc ions to form two clusters (Maret and Vallee, 1998).

Phosphates are salts of phosphoric acid. This may be condensed with either sodium or potassium but the sodium salts are the most common. Although there are several different kinds of phosphate salts, the ones most effective in seafood treatments are sodium phosphates (sodium tripolyphosphates) and sodium pyrophosphate. The pyrophosphate contains two phosphorous atoms and the polyphosphates contains three. A third phosphate sodium hexameta phosphate is used in canned seafood to prevent the formation of struvite crystals.

Polyphosphates do several things; they help to prevent loss of water or drip from cut fish and shellfish, which improve yield and physical appearance; and they prevent oxidation and minimize texture changes (Shimp, 1985). They are effective during both refrigerated storage and freezing. Too much polyphosphates however will extract proteins from the flesh to bond and this property improves the appearance and texture of thawed fish. It also means that frozen fillets need to be separated by plastic film if they are to be easily separated later.

Polyphosphates act primarily on the surface of fish or shellfish and do not penetrate the flesh. For this reason flat fish, whose surface area is large relative to their weight, take up more polyphosphates than round fish. It is difficult to assess how much polyphosphate is taken up by the fish because of the large variation in the natural phosphate content. Polyphosphate treatment also increases the uptake of natural phosphate content. Polyphosphate treatments also increase the uptake of water in the flesh. Unscrupulous operators often use excessive phosphates to increase the weight of products.

The antioxidant effect of polyphosphates were more when the fishes were cooked. The presence of polyphosphates seemed to be the main cause of phosphate hydrolysis. Polyphosphates are good antioxidants in the cooked fish because they remain as polyphosphates longer and are therefore, still be able to chelate (Regenstein and Weilmeier, 2004).

1.6 Mechanism of cryoprotection of Surimi

Physicochemical factors important for the cryoprotection of surimi would include effects of leaching, temperature tolerance of the myofibrillar proteins, pH effects and the freezing/frozen storage/thawing procedures employed which include the addition of cryoprotective substances (Matsumoto and Naguchi, 1992). Funatsu *et al* (1993) found that sorbitol enhanced the softness of thermally induced gels by controlling the cross linking reactions of myosin heavy chain that take place during

setting (Kimura *et al*., 1991). Sorbitol was able to disassemble the myosin filaments and the hydroxyl groups were responsible for decrease in turbidity of myofibril suspension (Konno *et al*., 1997).

The effect of sugars was reported to be sugar concentration dependent and sugar specific (Kumazawa *et al*., 1990; Matsumoto and Arai, 1986). Quantitative protective effect of sugars against thermal denaturation of myofibrils also indicated same pattern (Ooizumi *et al*., 1981). In functional and textural deterioration during storage there appears to be an increase in quantity of bonds or interactions and/or in the incidence of stronger bonds or interactions. The number of disulfide and nondisulfide covalent bonds increases with time (Torrejon *et al*., 1999). Sorbitol promoted solubilization of myofibrillar proteins at physiological concentrations of NaCl (Konno *et al*., 1997).

The cryoprotective effect of dicarboxylic acids appears to be closely related to their solubility in water and melting point: the higher the solubility, or the lower the melting point, the higher will be cryoprotective properties. The molecular structure as it influences the hydration capacity of the molecule, is important to its cryoprotective effect, similar to the case of sugar alcohols. The di or poly functional structure of the additive molecule is essential for a good cryoprotective effect (Matsumoto and Naguchi, 1992).

There exists some common principle with respect to the relationship between structure and cryoprotective ability of amino acids where glutamic acids and aspartic acids are the most effective and the -COOH group of the side chain can be replaced by $-NH_2$, -SH, -OH, $-SO_3H$ groups without losing the cryoprotective effect but introduction of hydrophobic group into the side chain inhibits the cryoprotective effect (Matsumoto and Naguchi, 1992).

Phosphates mainly to enhance the cryoprotective effect of sugars, perhaps by the buffering effect of polyphosphates on muscle pH and /or the chelation of metal ions rather than imparting any direct cryoprotective effect on their own. The molecule

has to posses one essential groups, either –COOH, or –OH and more than one of the following supplementary groups: -COOH, -OH, -SH, -NH_2, -SO_3H, -OPO_3H_2 and the functional groups (both essential and supplementary ones) should suitably placed and oriented relative to each other and it must be comparatively small. The size request have been violated by the positive cryoprotective effect of some materials like polydextrose, but the cryoprotective effect of maltodextrin decreased with increase of the chain length; samples beyond 50 glucose units are hardly cryoprotective. Chemically reactive substances are not suitable as cryoprotectant, though the reactivity of additives is not so pronounced at low temperature (Matsumoto and Naguchi, 1992).

Arakawa and Timasheff (1982a) concluded that the denatured structure of proteins is thermodynamically less favorable in sugar solution than water in its stabilization property. The cryoprotectant molecules interact and bond with the protein molecules via functional groups on the surfaces. Water molecules are hydrated on to the other remaining functional groups of the cryoprotectant; thus hydrated cryoprotectant molecules cover each protein molecule. The higher effectiveness of anionic compounds as cryoprotectant additives may be due to the fact that at neutral pH, the muscle proteins possess a negative net surface charge due to the prevalence of exposed anionic residues. Thus it would require less anionic additive to block the remaining cationic surface groups than cationic additives to block the more prevalent anionic surface groups of the protein. Additionally, the anionic groups tend to become more hydrated than cationic groups. Thus, an ionic coating is more extensively achieved when anionic additives are used. Matsumoto and Naguchi, (1992) indicated that actomyosin filament suspensions to which sodium glutamate is added show stretched filaments separated from one another, while those to which glucose is added are curled or lightly entangled. Stretching and separation of the filaments indicates a mutual repulsion due to ionic coating of the same kind of ionic charge, namely, an anionic one, along the protein molecules.

Based on the above literature an in depth study was undertaken to characterize the proteins from sardine mince. The characterization of proteins from oil sardine is necessary to understand the composition, structure and stability of these proteins and their behaviour from the association-dissociation and denaturation point of view. It also throws light on the stabilization-destabilization of the structural integrity of the proteins such that one can use the information for applications using biotechnological approaches. The major protein responsible for various functionality of mince was isolated. This actomyosin was characterized in terms of physical, chemical, enzymatic and biophysical properties. The interaction of various compounds like metal ions and polyhydric alcohols on the structural and enzymatic activities were taken up with a view for stabilization improvements which can be implemented in the fish mince and also to understand the mechanism of interaction of these compounds to the proteins of sardine mince. The influence of selected additives to the fish mince was also undertaken to give an indepth picture during storage under low temperature conditions. The influence of these additives on the physical, chemical and functional properties of the mince is investigated in detail.

SCOPE AND OBJECTIVES

SCOPE AND OBJECTIVES

Proteins from fish muscle are considered as very nutritious owing to its easy digestibility and its association with polyunsaturated fatty acids in fish muscle. Fish muscle proteins are mainly contributed by actin and myosin. In comparison to other muscle proteins, fish proteins are less stable for processing and storage. Because of this fact a good amount of this high value proteins ends up in fishmeal plants or go as manure. Preparation of shelf stable products from under utilized species and its quality improvements are very important. Preparation of surimi and its storage life enhancement will helps an efficient utilization of this unexplored resources. Surimi is the stabilized fish mince, frozen stored and used as a raw material for the production of various convenient products.

Stabilization of proteins under various storage conditions attracted the attention of protein chemists. Since fish proteins are of less stable in character, stabilization of the same required a very deep understanding of the structure and function. Various additives like low molecular weight carbohydrates, sugars and sugar alcohols were used to prevent the denaturation of protein during frozen storage. The extent of protection induced by these compounds to fish muscle proteins is the limitation in keeping the mince for a considerable time.

The commonly used cryoprotectants to surimi are sucrose and sorbitol which impart a sweet taste to the products. Mannitol is a polyhydric alcohol whose potentials to protect the proteins from various denaturing conditions are not studied in detail. Being a low molecular weight carbohydrate and with structural similarity to sorbitol, this can act as a good cryoprotectant. Mannitol is being produced from microbial sources and can be used at low cost. Various metal ions influence the structure - activity of enzymes and proteins. Sometimes metals act as cofactors for these proteins or it can be inhibitory too. Various divalent cations can be helpful in inducing better storage stability to fish proteins. Calcium can be a useful additive to synergies the protective effect. Addition of calcium compounds also helps to restore the nutritional quality of mince prepared from fish muscle. Specific effect of each these compounds

and its optimum concentration are very important for stabilization in the system. Divalent metal ions such as zinc also are of importance in the structural integration of proteins. Zinc is considered to be an important micronutrient. The specific alteration in the structure and properties of fish actomyosin in presence of zinc ions is an important phenomenon to be looked in.

Actin and myosin are the major functional component of fish muscle. The quality and functionality of fish muscle based products mainly based on these two structural proteins from muscle tissue. The specific interaction of polyhydric alcohols to these proteins will be useful to understand the mechanism of action of these compounds with actomyosin complex. The different concentrations of the above compounds and actomyosin help to derive a clear idea of this interaction. The enzymatic, structural, biochemical and biophysical approaches are proved to be good tools for the extensive understanding of this phenomenon.

The various additives and treatment to extend the storage stability and improve the quality of proteins requires a thorough understanding of structural details of proteins and its effect on functionality. The specific nature of interaction between these compounds and proteins helps to formulate a convenient formulation for the enhancement of shelf life of product with a clear mandate on quality attributes. This also helps us to find out the mechanism of interaction of these small molecules with selected protein that can lead to a better understanding of interaction of multimeric proteins and ligand molecules. The specific objectives can be briefly summarized as follows:

Detailed analysis of the proteins from Indian oil sardine (*Sardinella longiceps*) in terms of its physicochemical and functional attributes will be studied to characterize the system. Isolation and characterization of major protein fraction the actin myosin complex to understand the exact mechanism of action of various additives to system will be undertaken.

Understanding the mechanism by *in vitro* effects of selected polyhydric alcohols and specific metal ions on the properties of isolated actomyosin for

application from a biotechnological angle on protein- protein stabilization and protein-protein interactions are also the objectives of this study.

Pretreatment using selected polyhydric alcohol/cryoprotectant such as mannitol on frozen storage of *Sardinella longiceps* from the point of view of stabilization of macromolecules and measurement of altered physical, chemical and enzymatic properties of the proteins are investigated. Effect of selected salts at several concentrations on the biochemical, biophysical and enzymatic properties of the proteins for utilization of the results in biotechnological processes such as stabilization and destabilization of the proteins/enzymes during prolonged low temperature storage.

The mechanism of extension of proteins quality during processing and storage by the addition of various compounds perhaps would provide a clear understanding of the nature of interaction and range of utilization of these techniques for various biotechnological applications helps to improve products. The use of alternative additives helps to reduce the cost of production and make it more cost effective process for storage and utilization. Mannitol can be a good alternative cryoprotectant, which can protect the proteins during low temperature storage and also the use of divalent metal ions like calcium help to prevent deterioration of proteins thus enhancing the quality. This synergistic approach could be of immense application in quality.

The specific interaction of various polyols with actomyosin may clearly lead us to find out the mechanism of interaction of these with the major protein fraction of muscle. Polyols like sorbitol and mannitol can have a very specific interaction pattern on the actomyosin molecules and helps for its further utilization. Various metal ions like zinc can helps to impart the changes in structure and activity of actomyosin from fish muscle and also the role of zinc as a micronutrient needs to be revealed in such studies. The interaction of this divalent cation with actomyosin will help to conclude the use of low levels of metal ions as additive to various muscle systems for enhancing the quality of the meat.

MATERIALS AND METHODS

3 MATERIALS AND METHODS

3.1 Materials

3.1.1 Fish

Indian oil sardine (*Sardinella longiceps*) is a pelagic shoaling fish. Fresh sardine was procured from purseiners caught along the coast of Mangalore, India. The fishes were iced in the ratio 1:1 and transferred to the laboratory immediately and washed thoroughly with chilled water to remove dirt and slime and stored with ice (1:1) till further processing.

3.1.2 Chemicals

Sulfuric acid, hydrochloric acid, copper sulfate, potassium sulfate, sodium hydroxide, zinc chloride, zinc sulfate, calcium sulfate, calcium chloride dihydrate, ammonium molybdate, ferrous sulfate, sodium dihydrogen phosphate, disodium hydrogen phosphate, potassium dihydrogen phosphate, dipotassium hydrogen phosphate, ethylene diamine tetra acetic acid, potassium chloride, bromocresol green, methyl red, standard buffers and trichloroacetic acid were procured from E.Merck (India) Ltd, Mumbai, India. Ammonium persulfate (A 3678), glycine (G 7126), acrylamide (A 3553), bisacrylamide (N,N,methylenebisacrylamide, M 7279), N,N,N,N,tetramethyl ethylenediamine (E 5134), sodium dodecyl sulfate (L 3771), 2-mercpatoethanol (N 7154), urea (U 1250), adenosine triphosphate disodium salt (A 7699), N-bromosuccinimide (B 9252), bovine serum albumin (A 2153), myosin (M 7266), myosin hevay chain, rabbit (M 7659) actomyosin (A 6394), sepharose 6B (6B-100), ANS (A 1028), Tris hydroxymethyl amino methane (T 4661), sorbitol (S 1876), mannitol (M 9546), glycerol (G 5516), sucrose (S 7903), adenosine triphosphatase from porcine cerebral cortex (A 7510), adenosine triphosphatase from canine kidney (A 0412), chicken ovalbumin (A 5503), trypsin inhibitor (T 9003) and brilliant blue G-250 (B 5133) were procured from Sigma chemical company, St Louis, MO, USA. Spectrapor dialysis membranes were from Spectrum Medicine

Industries, Inc, California, USA. Blue dextran was from Pharmacia fine chemicals, Uppasala, Sweden. Coomassie brilliant blue was from Bio-Rad Laboratories, Richmond, USA. Sodium potassium tartarate and Folin-phenol reagent were from Sisco Research laboratories Pvt. Ltd., Bombay India. All chemicals used in this study were of the highest purity available. Quartz triple distilled water was used in all the experiments.

3.2 Methods

3.2.1 Preparation of mince

Head, scales, fins and the viscera of Indian oil sardine was removed and washed thoroughly to remove the blood and other undesirable things. All the operations during the preparation of mince were carried out at 10-12°C. Eviscerated fishes were washed with chilled water and used for meat picking. Meat picking was done mechanically with the help of a reciprocating meat picker having a pore diameter of 5 mm. Picked meat was washed with chilled water (1:3 ; meat : water) for three times. Picked meat was mixed with chilled water and stirred mechanically for 5 minutes. The mixture was allowed to stand for 5 minutes and the supernatant was removed. Excess water was removed by a basket centrifuge (Remi, India). Prepared meat was minced using a mincer. The mincer was helpful to remove the scales and bones that found entry to picked meat. The minced meat was mixed with the selected cryoprotectant at various concentrations (w/w) using a silent cutter. The mixing was continued till the complete mixing of additives and mince was done. The product was packed in polythene bags and loaded to air blast freezer which was pre cooled to - 35ºC and frozen for 90 min. at - 35ºC. Frozen samples were kept at - 20ºC for monitoring the storage stability. The various steps for the preparation of mince from sardine are shown in Figure 3 as a flow chart. Depending upon the additive either metal ion or cosolvent it is added at desirable concentration and mince prepared according to the above procedure and frozen stored and samples were recovered at predetermined intervals.

Fresh sardine

↓

Washed thoroughly in chilled potable water to remove slime and dirt

↓

Beheading, gutting and semi dressing of fish followed by washing with chilled potable water to remove blood and viscera

↓

Meat picking with the a reciprocating meat picker with 5mm pore size

↓

Washing of picked meat with chilled potable water for three times in the ratio 1:3 (meat: water) to remove the fat, myoglobin and sarcoplasmic fractions. Removed the excess water by centrifuging in a basket type centrifuge

↓

Washed meat was minced with meat mincer

↓

The minced meat was mixed with various additives and frozen at –35°C for 90 minutes

↓

Stored at –20°C

Fig. 3: Flow chart for the preparation of mince from Indian oil sardine (*Sardinella longiceps*).

3.2.2 Preparation of actomyosin

Actomyosin from a pelagic, planktivorous, shoaling fish *Sardinella longiceps* was prepared according to a modified procedure of Margassian and Lowey, (1982). Fresh sardines were brought to the lab in iced condition. From the eviscerated fish meat picking was done manually. Picked meat was used for isolation of actomyosin as depicted in Figure 4. The isolated actomyosin was kept at a temperature of 4ºC under refrigerated conditions. The prepared actomyosin used within three days of its preparations.

3.2.3 Freeze drying

Sample for the estimation of water absorption capacity and for the scanning electron microscopy were prepared by freeze drying of the mince. The frozen samples were freeze-dried in Virtis freeze dryer continuously still the moisture content of the samples was less than 2%. Mince was frozen and brought down the temperature to -35ºC and the flask containing frozen samples were connected to the freeze drier which maintain the temperature of -50ºC and vacuum. Freeze drying continued till there is no further removal of moisture from the samples are observed. Samples were sealed in airtight containers and desiccated. These freeze dried powder was used for further study.

3.3 General methods of estimations

3.3.1 Moisture

Moisture content of meat, mince and actomyosin were determined according to the method described in AOAC (1995). A known quantity of fish mince was thawed and weighed exactly. These samples were dried in hot air oven at 105 ± 2ºC for 16 hrs. The samples were cooled in desiccators and its weight recorded. This was repeated till a constant weight was obtained. In case of actomyosin accurately weighed samples were placed in the hot air oven and kept at 105 ± 2ºC and the above procedure was continued till the entire moisture content was removed.

3.3.2 Total Nitrogen

25 g fish meat + potassium phosphate buffer (pH 6.5, 0.15 M) in the ratio 1: 6 stirred for 10 minutes at 4°C. Strain off the supernatant. Repeat this two times. For the third time centrifuge at 6,500 x g for 10 minutes at 4°C and collect the pellets

↓

Pellets + potassium phosphate buffer (pH 6.5,0.15 M) containing 0.3 M KCl, 0.001 M ATP, 0.025 M EDTA and 0.005 M $MgCl_2$ added in the ratio 1:10 (pellets: buffer) and stirred for 15 minutes at 4°C. Centrifuge the slurry at 10,000 x g for 20 minutes at 4°C

↓

Make up the volume to three times of supernatant with deionised water and allowed to stand for 3 hours at 4°C and centrifuge at 7,000 x g for 10minutes at 4°C

↓

Dissolve the precipitate in potassium phosphate buffer (pH 6.5, 0.15 M) containing 1.0 M KCl so as to get a final concentration of 0.6 M KCl and allowed to stand for 30 minutes at 4°C

↓

This is diluted 10 times by deionised water and allowed to stand for 30 minutes at 4°C. Centrifuge the slurry at 7,500 x g for 15 minutes at 4°C

↓

Pellets were collected and dialyse overnight against deionised water at 4°C. Centrifuge the solution at 10000 x g for 15 minutes and store the pellets at 4°C.

Fig. 4: Flow chart for the isolation of actomyosin from sardine meat.

The total nitrogen of the fish meat was determined by micro-kjeldahl method described in AOAC (1995). To 1.0-1.5 g of thawed fish mince/fish actomyosin, 15 ml of concentrated sulfuric acid and 100 mg of digestion mixture (K_2SO_4: $CuSO_4$: SeO_2: 10:1:0.25) was added. The digestion was carried out at 400ºC using Gerhardt digestion system. The samples were digested till it turns colourless. After cooling to room temperature the clear solution was quantitatively transferred to a 50 ml volumetric flask and the volume made up with deionised quartz triple distilled water. The distillation of samples was carried out using Gerhardt vapodest distillation unit. An aliquot (5ml) was taken in the distillation tubes. To this 20 ml of 40% NaOH was added and allowed the steam to pass through. The steam distillate was trapped in 10 ml of 2% boric acid containing 0.01 ml of mixed indicator (0.033% (w/v) methyl red and 0.167% (w/v) bromocresol green) and titrated against N/70 hydrochloric acid. Oven dried analytical grade ammonium sulfate was used as standard for determining the acid factor. Total protein was calculated by multiplying the total nitrogen content by a factor of 6.25 and expressed as percentage protein in the sample.

3.3.3 Total lipid

The crude lipid content of the meat was determined by Soxhlet extraction method (AOAC, 1995). About 1 g of moisture free meat sample was taken in a Whatman thimble. The thimble was plugged with cotton and placed in a soxhlet extraction unit. Petroleum ether AR grade (B.P. 40°–60°C) was used as the extraction solvent. Extraction was carried out by heating the soxhlet unit at 60°C on a thermostatically controlled water bath. Extraction was continued for 16 hours. After the extraction, the preweighed receiver flask containing the extracted fat was dried initially on a water bath at 98 –100°C and then using an oven at 100 ± 2°C till constant weight was obtained. After complete drying, the receiver flask was cooled in desiccators and weighed exactly. The difference in the initial and final weight of receiver flask was determined and fat content of meat calculated on dry weight basis using following formula.

$$\text{Crude fat (\%)} = \frac{\text{Final weight} - \text{Initial weight}}{\text{Sample weight}} \times 100 \qquad \text{- - - (2)}$$

3.3.4 Extraction of proteins from mince

Total extractable proteins from fresh and frozen mince were extracted using phosphate buffer (pH 7.5, 0.05 M) containing 1.0 M sodium chloride. This buffer here afterwards referred as extraction buffer (EB). Chilled buffer was added to the mince (15:1) and homogenized with virtis homogeniser at 3000 rpm for one minute. Temperature of the mixture was maintained at 4ºC during homogenization. The homogenate was centrifuged at 10,000 x g for 10 min using refrigerated centrifuge (Biofuge, 15R, Haereus, Germany) at 4ºC. The supernatant was collected and was used for further studies.

The protein concentration of extract was determined routinely by the ultraviolet absorption spectra at absorption maximum, 280 nm in double beam UV spectrophotometer (Shimadzu UV 1601) against respective blanks. These absorbance values were converted to concentration units (mg/ml) using an extinction coefficient, $E^{1\%}_{1cm,280nm}$ values. The extinction coefficient were obtained from the plot of absorbance values versus protein concentration in mg/ml. The protein concentration in this calibration graph was determined by micro-kjeldahl method (AOAC, 1995).

3.3.5 Measurement of pH

The pH of the solution and buffers were measured using a Control Dynamics pH meter (CD Instruments, Bangalore, India). The pH was measured at 28°C. The pH meter was calibrated with standard pH buffers from Merck, India, before every measurement.

3.3.6 Ultra violet absorption spectra

Ultra violet absorption of solubilised fish actomyosin was recorded with Shimadzu UV 1601 (Shimadzu, Japan) double beam spectrophotometer in the range 200-350 nm. Clear protein solutions of absorbance 0.25 to 0.3 at 280 nm were used in quartz cuvettes and the spectra was recorded.

3.3.7 Fluorescence spectra

The fluorescence emission spectra of the solubilized proteins from mince/ fish actomyosin were recorded using Shimadzu RF-5000 (Shimadzu, Japan) spectrofluorimeter at 25ºC. Clear protein solution having an absorbance value of 0.08-0.1 at 280 nm and is equivalent to 0.15-0.17 mg/ml protein concentration was used for the measurement. Excitation and emission slit width were kept at 5 nm. The emission spectra were recorded in the range of 300-400 nm after exciting with a wavelength of 280 nm. All the fluorescence measurements were recorded 10 sec after excitation.

3.3.7.1 Fluorescence spectra of actomyosin

Intrinsic fluorescence emission spectra of solubilized actomyosin in presence of various concentrations of metal ions and also in presence of cosolvents was recorded for an emission range of 300-400 nm after exciting the protein at 280nm using a specrtofluorimeter (Shimadzu RF-500, Japan). Emission spectra were recorded after 10 sec of excitation of the proteins. Excitation and emission slits were kept at 5 nm. The protein concentration was adjusted to an absorbance of 0.1 at 280 nm equivalent to 0.2 mg/ml protein concentrations before the experiment. The fluorescence spectra were recorded at a constant temperature of 25°C with the help of circulating water bath that is connected to instrument.

3.3.8 Circular Dichroic spectra measurement

The secondary structure of sardine proteins and isolated actomyosin determined by circular dichroic spectra by using a spectropolarimeter (Jasco 810C, Jasco, Tokyo, Japan). The protein extracted from sardine mince dissolved in Tris-HCl buffer (0.1 M; pH 7.0) containing 0.5 M KCl used for secondary structural determination. Solubilized actomyosin in presence of different concentrations of metal ions and cosolvents was used for determination of secondary structure. The far UV-CD spectra of proteins were recorded for the protein in presence of zinc salts, calcium salts and cosolvents using quartz cuvettes of 0.1 cm path length. Dry nitrogen

gas has purged in to the instrument before and during use. Mean residue ellipticity values were calculated using a value of 115 for mean residue weight.The molar ellipticity values were obtained at 1nm interval by using the equation (Adler *et al.*, 1973).

$$(\theta)_{MRW} = \frac{(\theta)_{obs} \times MRW}{10 \times d \times C} \qquad - - - \quad (3)$$

where, $(\theta)_{obs}$ is observed ellipticity, d is path length in cm, C is protein concentration in g/ml and MRW is mean residue weight of the protein. The secondary structural analysis was done with the help of programme in the instrument (Yang *et al*., 1986).

3.3.9 Thermal denaturation measurements

The proteins from the mince were extracted with phosphate buffer (0.05 M, pH 7.5) containing 1.0 M NaCl. A specific volume (0.3ml) of protein solution with a known protein concentration (0.5-0.6 mg/ml) was equilibrated to 25ºC in special thermal melting cuvettes and melting curve was followed at 287 nm in the temperature range of 25ºC-90ºC at 1ºC interval using Gilford response II spectrophotometer (Cibacorning, Cleveland, Ohio, USA). The curves were smoothened using the software provided with the instrument and melting temperature was obtained. The first derivative plot of the thermal denaturation profiles were used to analyze the apparent T_m of proteins.

3.3.10 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Sodium dodecyl sulfate polyacrylamide gel electrophoretic pattern of proteins extracted with the buffer (0.125 M Tris-HCl, 4% SDS, 2% 2-mercaptoethanol, 20% glycerol, 0.02% bromophenol blue) were carried out in 10% polyacrylamide gel according to Laemmlli (1970). Electrophoresis was carried out using polyacrylamide gel slabs of 10 x 8 cm (length x width) in a vertical slab gel electrophoresis apparatus. A discontinuous gel of acrylamide concentration T%=10% and C% = 2.5% was used (T% is the concentration of total monomer present in the solution and C% is the

contribution of cross linker, bis acrylamide, to the total monomer concentration). For polymerization of gel, TEMED was used as initiator and ammonium persulfate as catalyst. The gels were cast with a thickness of 0.75 mm. Minced meat/fish actomyosin mixed well with treatment buffer (0.125 M Tris-HCl, 4% SDS, 2% 2-mercaptoethanol, 20% glycerol, 0.02% bromophenol blue) in the ratio 1:1 and boiled on a boiling water bath for 2 minutes, cooled and centrifuged at 8000 x g for 5 minutes. Clear supernatant collected was loaded to the wells and run under constant voltage of 100 mA. Run was terminated when the marker dye touched the bottom of gel. After the run the gels were stained either by coomassie stain or by silver staining. Molecular weight of the proteins were determined by measuring the relative mobility of standard proteins used as markers by the method of Weber and Osborne (1969):

$$R_f = \frac{D_p \times L_2}{L_1 \times D_d} \qquad - - - - \quad (4)$$

where, D_p = distance moved by protein band from top (cm); D_d = distance moved by indicator dye (cm); L_1 = length of gel after destaining and L_2 = length of gel before staining (cm), R_f = relative mobility .The molecular weight of different proteins bands were determined by a plot of log molecular weight of standard proteins versus R_f by interpolation of graph.

Coomassie blue staining is carried out as follows; Destaining solution: combine 1.6 litrs of methanol, 400 ml glacial acetic acid, 2 litrs distilled water. Mix well and store below 25°C; Coomassie blue staining solution: Dissolve 2 g coomassie brilliant blue R-250 in 500 ml methanol, 100 ml glacial acetic acid (17.4M) and 400 ml distilled water. Mix well filter through whatman no.1 filter paper and store below 25°C. Place the gel in 200 ml coomassie blue staining solution in covered tray. Stain it for one hour at 25°C on a mixing platform. Decant the used staining solution. Destain the gels with destaining solution until desired back ground is achieved.

Silver staining of the protein gels was carried out as per the following procedure. This is as much as 100 times more sensitive than dye staining. Bands containing 10-100 ng of protein can be easily seen. The procedure followed was to fix the proteins in the gel

in about 400 ml of 40% methanol, 10% acetic acid (v/v) for 30 min to overnight followed by fixing twice in 400 ml 10% ethanol, 5% acetic acid (v/v) for 15-30 min. After fixing the proteins the gels was soak for 3-10 min in 200 ml of fresh oxidizer solution (0.0034M potassium dichromate, 0.0032 N nitric acid) and wash the gels three to four times for 5-10 min in 400 ml water, until the yellow color has been washed out. Soak the washed gel in 200 ml fresh silver reagent (0.012 M silver nitrate) for 15-30 min. and wash with 400 ml water for 1-2 min. The gel was soak in developer (0.28 M sodium carbonate, 1.85% paraformaldehyde) solution until satisfactory staining has been obtained and stops development with 5% acetic acid (v/v).

3.3.11 Gel filtration

Gel filtration profile of the proteins extracted in phosphate buffer (0.05 M, pH 7.5) containing 1.0 M NaCl and the actomyosin dissolved in the same buffer was carried out using a Sepharose 6B gel packed into a glass column (1.2x100 cm). Gel column was equilibrated against the buffer before introduction of samples. Extracted proteins were dialysed extensively against the same buffer at 4ºC. A known amount of proteins were loaded to the column. The elution was carried out at a rate of 30 ml per hour flow rate. Fractions were collected using fraction collector (Redifrac, Amersham Pharmacia). Proteins content of the collected fractions were monitored by the absorbance at 280 nm using spectrophotometer (Shimadzu, UV-Mini, 1240). Absorbance values were plotted against the elution volume to get the profile of extracted proteins. Void volume of the column was determined with blue dextran.

3.3.12 Fast Protein Liquid Chromatography

The profile of isolated actomyosin from sardine was recorded by fast protein liquid chromatography system (Amersham Pharmacia) using superose 6 HR column. Isolated protein was loaded to a pre-equilibrated column with phosphate buffer (0.05 M; pH 7.5) containing 0.5 M NaCl with flow rate of 0.5 ml per minute. Actomyosin

was dissolved in phosphate buffer (0.05 M; pH 7.5) containing 0.5 M NaCl and eluted with the same buffer. The absorbance of fractions were recorded at 280 nm.

3.3.13 Water absorption capacity

The water absorption capacity of freeze dried mince was determined according to the method of Sosulski (1962). To the finely powdered freeze dried material (0.1-0.3g) 5 ml of distilled water was added in a preweighed glass tubes. The contents of the tubes were mixed well with the help of cyclomixer. The tubes were allowed to stand for 30 min with occasional stirring. After the stipulated time the tubes were centrifuged at 6000 x g for 15 min., supernatant was discarded and the tubes were dried in a hot air oven at 50ºC for 30 min. in an inclined position of 45º angle. The difference in weight was noted down and the water absorption capacity was expressed as grams of water absorbed per gram of freeze dried material.

3.3.14 Emulsion capacity

Emulsion capacity of proteins was determined by the method of Pearce and Kinsella (1978). Proteins were extracted with phosphate buffer (0.05 M, pH 7.5) containing 1.0 M NaCl by a homogenizer for one minute. Temperature of the mixture was kept 4ºC during the process of extraction. The slurry was centrifuged at 8000 x g at 4ºC for 20 min. using a refrigerated centrifuge (Biofuge 15R, Haeraeus, Germany). The supernatant was used for emulsion capacity determination. To 10 ml of protein extract 5 ml of super refined groundnut oil was added and mixed thoroughly at 6000 rpm for 10 sec using Ultra turrax homogenizer. Homogenization was continued at a speed of 11,000 rpm with addition of oil at a flow rate of 0.5-0.6 ml/sec until phase inversion was recorded. Protein content of the extract was determined by the method of Lowry *et al*. (1951). Emulsion capacity was expressed as ml oil/ mg proteins.

3.3.15 Scanning electron microscopy

Scanning electron microscopic studies of the freeze dried fish mince were carried out using LEO 435 VP, Cambridge model surface scanning electron microscope. Before loading the samples into the system, the samples were coated with

gold using poloron SEM coating system E-5000. Average coating time was 2-3 min. Thickness of the coating was 200- 300nm, which was calculated using the formula

$$T = 7.5\ It \qquad - \ - \ - \ (5)$$

where, I = current in mA, t = time in min. ,T = Thickness in Aº.The coated samples were loaded on the system and the image viewed under 20 kV potential using 35 mm Picoh camera.

3.3.16 ATPase activity

ATPase activity of fish proteins determined was calcium activated ATPase. Ca^{2+}ATPase activity of the proteins from mince was determined by the method of Perry (1955). ATPase activity of mince was carried out after extracting the proteins with glycine-NaOH buffer (pH 9.0, 0.2 M) containing 0.5 M KCl. The reaction mixture consists of 0.03ml of ATP (50 mM), 0.2ml of calcium chloride (0.1 M), 1.0 ml of buffer and 0.2 ml of muscle extract. Reaction was started by the addition of ATP solutin to the mixture. The mixture was incubated at 25 ± 2ºC for15 min. in a shaking water bath (Grant OLS 200,Grant Instruments (Cambridge) Ltd, England). The reaction was stopped with the addition of 1 ml of 15% TCA. The solution was centrifuged at 6000 x g for 20 min. The inorganic phosphorous content of the supernatant was estimated by the method of Taussky and Shorr (1953). To 3 ml of sample containing 4-8 µg of phosphorous add 2 ml of ferrous sulfate molybdate reagent (5 g of $FeSO_4.7H_2O$ dissolved in 1% ammonium molybdate in 1 N sulfuric acid) was added and the intensity of colour read at 660 nm. The colour develops within 1 minute and stable up to 2 hr. Standard graph is plotted against Pi content using potassium dihydrogen phosphate and straight line is obtained. ATPase activity was expressed as M Pi/mg protein/min.

3.3.16.1 ATPase activity of actomyosin

Actomyosin was solubilized in buffer containing 0.5 M KCl in the ratio of 1:10. Centrifuged at 7000 x g for 15 minutes at 4ºC in an inverter high speed

refrigerated centrifuge (Kuboto 6930, Kuboto corporation Tokyo). Protein content of the supernatant was estimated by the method of Lowry *et al.,* (1951). ATPase activity of the actomyosin determined by the method of Perry (1955). The enzyme activity was determined at different temperatures and pH (glycine-NaOH buffer of pH 9.0, 0.2 M and Tris- HCl buffer of pH 7.0, 0.1 M). To illustrate the effect of divalent cations on the enzyme activity, different concentration of metal ions ($1x10^{-5}$ M to $1x10^{-2}$ M) was taken in the reaction mixture (0.2 ml of actomyosin solution, 0.2 ml of 0.1 M $CaCl_2$ the solution was made up to 2 ml with buffers). In the case of polyhydric alcohols the concentration of polyhydric alcohols were maintained at 1-10% in the reaction mixture. Reaction started with the addition of 0.05 ml of 50 mM ATP and incubated for 15 min. in a shaking water bath (Grant OLS 200,Grant Instruments (Cambridge) Ltd, England) and stopped by the addition of 1 ml of 15% TCA. The precipitate was removed by centrifuging at 6000xg for 15 min. Inorganic phosphorous released during the reaction was estimated in the supernatant by the method of Taussky and Shorr (1953) and the ATPase activity of actomyosin expressed as M Pi/mg protein/min. Quartz triple distilled water was used through out the study.

3.3.17 Dynamic viscoelastic measurements

Dynamic viscoelastic behavior of mince was measured using a controlled stress rheometer (Rheometric, UK) in oscillatory mode. A 4 cm parallel plate measuring geometry was used for the measurement. The gap was adjusted to 2000 μ. Measurement was made by applying small amplitude oscillations with frequency of 1 Hz. apparent stress was compared with resultant strain. When the material is an ideal elastic solid the stress is in phase with strain, when it is an ideal liquid there is a phase difference of 90° and when the material is a viscoelastic, the phase angle is some where between these two. The results of such measurements are expressed as the storage modulus (G′) and loss modulus (G′′). The storage modulus characterizes the rigidity of the sample (the energy expended in deforming an elastic solid is stored and is recoverable when the stress is released) the loss modulus characterizes the resistance of the sample to flow (energy expended in inducing flow is dissipated as heat and therefore lost). The ratio of G′′/ G′ gives tanδ values, which is continuously

recorded and gives the sol-gel transition temperature. Tanδ is a measure of the ratio of energy lost to energy stored in a cyclic deformation; a small tanδ means the material is highly elastic. About 4.0 g of mince ground with different additives and kept on the plate and raised to contact with measuring geometry. After equilibration time of 10 sec the measurements were started at a heating rate of 1ºC/min for a temperature of 30º-90ºC.

3.3.18 Methods of protein estimation

The protein content of solution was measured by Folin Ciocaltue's phenol reagent method and Bradford method depending on the samples as follows:

3.3.18.1 Protein estimation by Folin - Phenol reagent

The protein content of extracts was measured according to the procedure of Lowry *et al*., (1951). Reagent A: 2% Na_2CO_3 in 0.1 N NaOH; Reagent B: 0.5% $CuSO_4.5H_2O$ in 1% sodium potassium tartarate; Reagent C: Alakaline copper solution. Mix 50 ml of reagent A with 1ml of reagent B. Discard after 1 day. Reagent D: diluted Folins reagent.

To a sample of 5 to 100 μg of protein in 0.2 ml or less in a 3 to 10 ml test tube, 5 ml of reagent C is added. Mix well and allow to stand for 10 min. or longer at room temperature. 0.5 ml of reagent D is added very rapidly and mixed within a second or two. After 30 min. or longer the sample absorbance is recorded at 660 nm. Standard graph was plotted against known protein concentrations of BSA.

3.3.18.2 Protein estimation by Bradford method

The protein in the solution was estimated by the method of Bradford (1976). A stock solution of 100 ml 95% ethanol, 200 ml 88% phosphoric acid, 350 mg serva blue G were prepared and this is stable indefinitely at room temperature. The working solution was prepared as follows 425 ml distilled water, 15 ml 95% ethanol, 30 ml

88% phosphoric acid and 30 ml Bradford stock solution were mixed and filtered through Whatman No.1 filter paper and stored at room temperature in brown glass bottles.

The protein content was estimated with 100 µl protein solution (or add buffer to make to 100µl) and 1.0 ml of Bradford working solution was added and vortex it. Read at A_{595} after 2 min but before 1 hour. Standard curve was plotted between 2.5 µg to 15 µg BSA.

3.3.19 Surface hydrophobicity

Hydrophobicity of fish actomyosin in presence of various concentrations of metal ions and cosolvents were determined by the help of fluorescent dye ANS according to the method of Kato and Nakai (1980). Stock solution of ANS was prepared in the respective buffers at concentration of 8 mM at 25°C. To 4 ml of protein solutions of concentration in the range of 0.001 to 0.025%, 20 µl of ANS solution was added and the emission intensity recorded at wave length of 470 nm after exciting at 390 nm. Fluorescence intensity recorded 30 sec after excitation. Temperature of the solutions was kept constant with the help of circulation water bath attached to the instrument. A plot of fluorescence intensity versus the protein concentration was recorded and the initial slope of the plot, $S_{0,}$ was taken as hydophobicity index of protein.

3.3.20 Free thiol group estimation

Free SH content of fish actomyosin determined by the method Ellman (1959) as modified by Beveridge *et al*. (1974) using Ellman's reagent**.** Samples were incubated for 15 min at 25°C with 1 mM of Ellman's reagent in respective buffers. Absorbance was recorded at 412 nm with appropriate blanks. The total number of thiol groups was calculated using the molar extinction coefficient of 13500 $M^{-1}cm^{-1}$ at 412 nm for the thiobenzoate that was stochiometrically released by the reaction of Ellman's reagent with the sulfhydryl groups.

RESULTS AND DISCUSSION

CHAPTER - 1

PROPERTIES OF PROTEINS FROM OIL SARDINE MINCE AND CHARACTERIZATION OF ISOLATED PROTEIN FRACTION FROM OIL SARDINE

PROPERTIES OF PROTEINS FROM OIL SARDINE MINCE AND CHARACTERIZATION OF ISOLATED PROTEIN FRACTION FROM OIL SARDINE

The Indian oil sardine (*Sardinella longiceps*) is a pelagic shoaling fish with high fat content and dark meat component (Gopakumar, 1997). This marine fish is available from Indian Ocean and a large amount is being caught along Indian coast. Water washing of separated meat is an essential step to minimize denaturation of myofibrillar protein during frozen storage. The improved gel strength is related to the number of water washing cycles (Matsumoto and Naguchi, 1992). The number of washing cycles and volume of water employed in surimi production will vary with fish species, initial condition of fish and type of processing unit that is continuous or batch operation (Lee, 1984). The different unit operations in the process of surimi may alter the properties of major protein fractions. Hence the functional and physiochemical characterization proteins from mince is necessary to understand for its better utilization and also to improve the protein stabilization during frozen storage.

A) Functional and physicochemical properties of proteins from sardine mince

The composition of mince is very important factor in deciding the quality and use of mince for various purposes. The composition of mince varies with season and the processing steps followed to produce the mince from fresh fish. Composition of fish mince prepared from fresh oil sardine is given in Table 7. The average total crude protein content of the prepared mince was 16.12 ± 1.8% on wet weight basis. The moisture content of the mince was found to be higher than whole fish (sardine) that will be in the range of 65-70%. The higher moisture content in the mince is mainly due to uptake of water by the major protein fractions during water washing process. Removal of water after washing operations using centrifuge could not reduce the moisture content below 78% and indicates strong interactions between protein and water. The moisture content of the prepared mince was 78 ± 3%. The amount of protein and moisture present in the mince influences the physicochemical and functional properties of mince (Hultin and Kelleher, 2000). The various

Table 7: Proximate composition, physicochemical, functional and enzymatic properties of mince from Indian oil sardine (*Sardinella longiceps*)

Properties	Values
Protein	16.12 ± 1.8% wet weight
Moisture	78 ± 3%
Lipid	2.20 ± 0.52%
ATPase activity	0.23 ± 0.03 μM Pi/mg protein/min
Emulsion capacity	0.13 ± 0.01ml oil/mg protein
Water absorption capacity	3.65 ± 0.25g water/g dried mince
Apparent T_m	57 ± 1.5°C
Extractable proteins of the total proteins in phosphate buffer (0.05 M, pH 7.5) containing 1.0 M NaCl	64 ± 3%

physicochemical properties of mince prepared from sardine are studied and tabulated in Table 7.

ATPase activity is the major enzymatic property of mince. This is considered as an index of quality of mince. The myosin molecules present in the mince mainly contribute for this enzymatic activity. Myosin head is responsible for ATPase activity of mince. ATPase activity of sardine mince was found to be 0.23μM Pi/mg protein/min (Table 7). The enzymatic activity was comparable to the enzyme activity of other fishes from the literature. Suzuki (1981) reported the enzyme activity of other species like mackerel (0.12-0.19), yellow fin tuna (0.22-0.38), white marlin (0.17-0.26), yellowtail (0.16-0.22) and big eye tuna (0.13-0.16).

Functional properties of proteins are those properties that enable proteins to contribute to the desirable characteristics of food. Functional properties affect the sensory quality and physical behaviour of foods during preparation, processing and storage. Several factors influence the functional properties of proteins like the inherent properties of proteins (native or denatured), degree of purification, pH, temperature and ionic concentration (Kinsella, 1982). Functional properties of proteins from fish mince assume greater significance as they are meant for preparation of various products, which are directly affected by the functionality. There is considerable variation in the functional properties of surimi made from the minced flesh of different species (Pigott and Tucker, 1990). The functional properties of sardine mince were determined to characterize the system, which is helpful to understand the possible use of the mince for various purposes.

The emulsion capacity of proteins extracted from prepared mince was 0.13 ± 0.01 ml oil/mg protein (Table 7). This was comparable to the emulsion capacity values of other teleost fishes and was more compared to shellfish proteins (Shamasundar and Prakash, 1994b). Since the proteins present in the mince are the major contributor of emulsion capacity, it is expressed in terms of the amount of oil needed per mg of proteins.

Solubility profile of proteins from mince in the given solvent is considered to be a good indicator for various functional properties. It is generally accepted the minimum ionic strength required to solubilise myofibrillar proteins should be higher than 0.3M. In the present study the term solubility and extractability is interchangeably used. Table 7 shows the amount of extractable proteins getting into solution using phosphate buffer (0.05 M, pH 7.5, containing 1.0 M NaCl) was found to be 64 ± 3%. Extractability of proteins in the high ionic strength buffer is a good indicator of the functionality of mince. The major contributor to the mince is actin and myosin. The extractability was determined as percentage of proteins extracted in the buffer with respect to the total proteins present in the mince.

Water absorption capacity is an important functional property of fish mince. This property is contributed mainly by the proteins present in the mince. Proteins have inherent property to hold water. The capacity of proteins to retain water depends on the structure and status of proteins. Water absorption capacity of sardine mince was found to be 3.65 g water/g of freeze dried mince (Table 7). The hydration of protein molecules is influenced by number of charged residues it posses and conformational status. The functional properties of fish meat change with the decrease of freshness (Pacheco-aguilar *et al.*, 2003).

Foaming capacity of proteins is a surface active property and depends on the size as well as structure of protein molecules. Proteins from sardine mince did not show any capacity to foam. Removal of smaller molecular weight proteins in the process of washing might have resulted in the loss of foaming capacity of mince.

In Fig.5 is shown the thermal denaturation profile of proteins extracted from sardine mince in the extraction buffer (0.05M phosphate buffer, pH 7.5 containing 1.0 M NaCl) was followed in the range of 25-95°C (Fig.5). The unfolding properties of proteins were monitored at 287 nm. The apparent T_m of proteins was 57 ± 1.5°C (Table 7). The increase in temperature of the protein resulted in unfolding and further leads to aggregation under the experimental conditions.

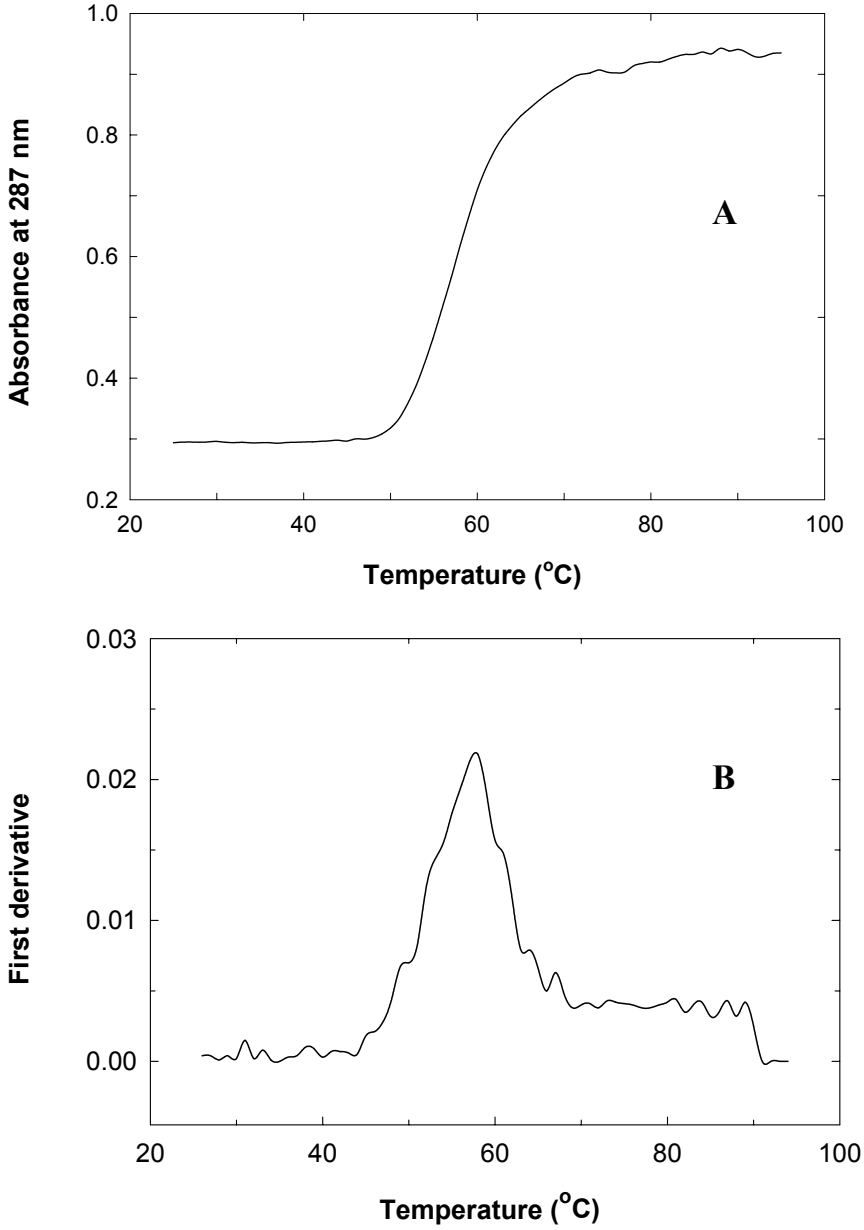


Fig. 5: Thermal denaturation profile of fish proteins during heating from 25 - 95°C at pH 7.5. A) The absorbance at 287 nm was monitored during heating in thermal cuvettes with a heating rate of 1°C per minute B) first derivative plot of absorbance *vs* temperature.

The gel filtration profile of total proteins from sardine mince is given in Fig.6. The major peak possibly an actomyosin complex eluted at an elution volume of 65 ml. The peak eluting at an elution volume of 120 ml is considered as low molecular weight component and the concentration is much lower than the high molecular weight component namely actomyosin. The lower concentration of low molecular weight components is due to the fact that the sarcoplasmic proteins were leached during the process of water washing and subsequent relative increase in the concentration of actin and myosin in the mince. Myosin and actin are the major contributors to the functionality of muscle. Higher content of myosin and actin is necessary to give better textural properties for prepared products from mince.

The gel filtration technique can be used to monitor association - dissociation phenomenon as a function of storage or processing conditions. In order to obtain subunit composition of different protein fractions SDS electrophoresis technique under reduced condition is normally followed. The SDS-PAGE pattern of total proteins from fresh sardine mince is given in Fig.7. The pattern reveals multiple bands in the molecular weight range 205 kD to 20 kD. The band appearing at 205 kD is myosin heavy chain and at 45 kD is actin components. The number of bands with molecular weight range less than 45 kD are few indicating most of the low molecular weight components present in the mince have been leached during washing process.

Intrinsic fluorescence emission spectra of proteins extracted from the mince recorded in the range of 300-400 nm showed typical tryptophan fluorescence with an emission maximum at 335 nm (Fig.8). The excitation wavelength was fixed at 280 nm. Fluorescence emission at 335 nm is mainly contributed by the presence of tryptophan residues present in the proteins. The fluorescence emission profile of proteins was influenced by the structure of proteins. The secondary structure of proteins can be determined by far UV-CD spectra of proteins.

Far UV-CD spectra of proteins from sardine mince predominantly showed helical structure (Fig.9). The secondary structure of total proteins showed 60% α-helix, 29% aperiodic structure and the rest was β-strucutre. The helical structure is

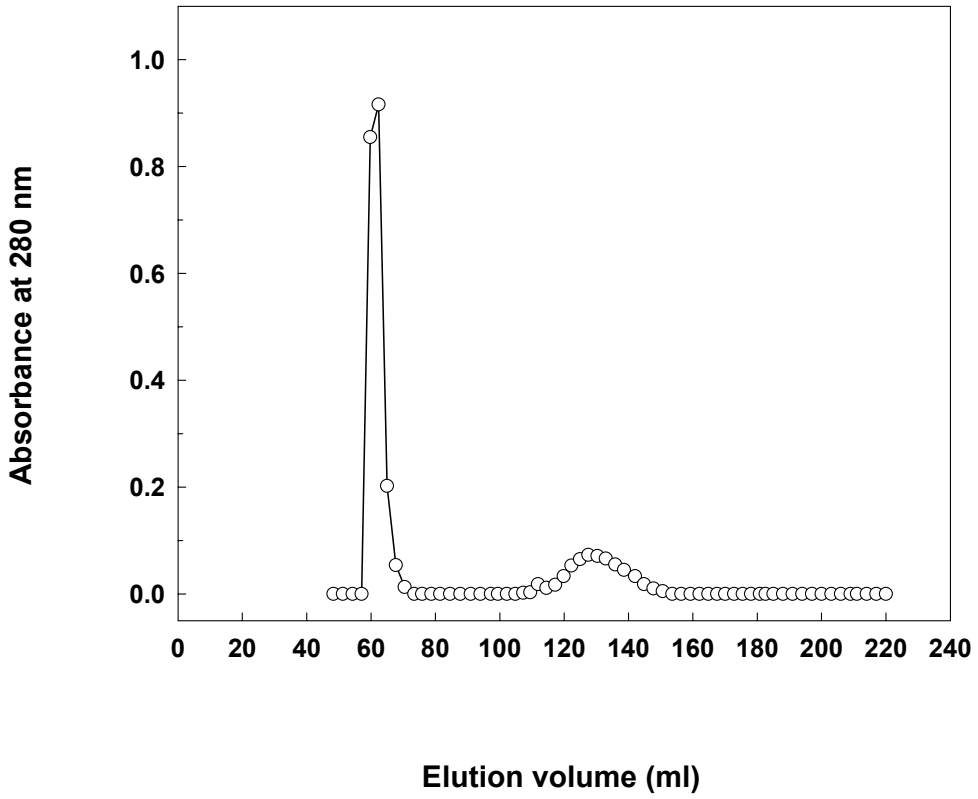


Fig. 6: Gel filtration profile of proteins extracted from sardine mince. Proteins were extracted by phosphate buffer of pH 7.5; 0.05 M containing 1.0 M sodium chloride and eluted through Sepahrose 6B column using the same buffer. Absorbance at 280nm was measured of each fraction and plotted against elution volume.

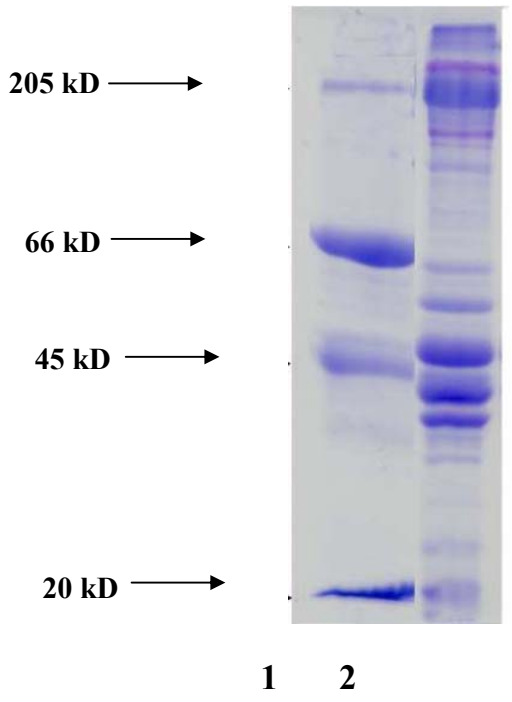


Fig. 7: SDS-PAGE pattern of proteins extracted from sardine mince. Lane 1) Standard proteins - Myosin (205 kD), BSA (66 kD), Ovalbumin (45 kD) and Trypsin inhibitor (20 kD); Lane 2) protein extracted from sardine mince.

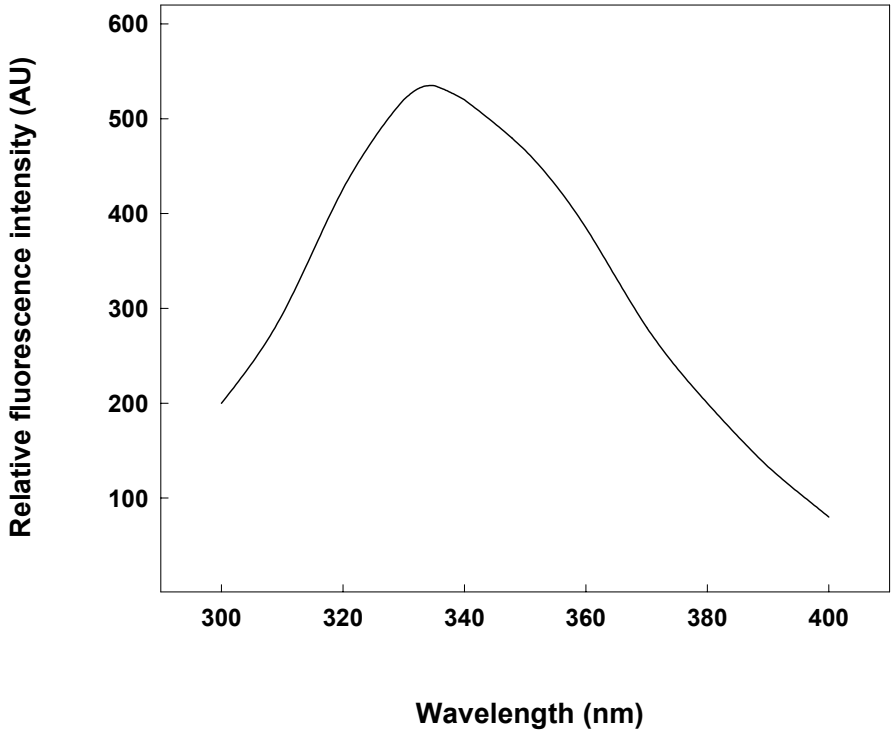


Fig. 8: Intrinsic fluorescence emission spectra of proteins extracted from sardine mince. The proteins were extracted in phosphate buffer of pH 7.5 (0.05 M) containing 1.0 M sodium chloride from sardine mince. Emission spectra recorded over a range of 300-400 nm, excitation wavelength was fixed at 280 nm. The protein concentration of the solution was 0.1 OD/ml at 280 nm.

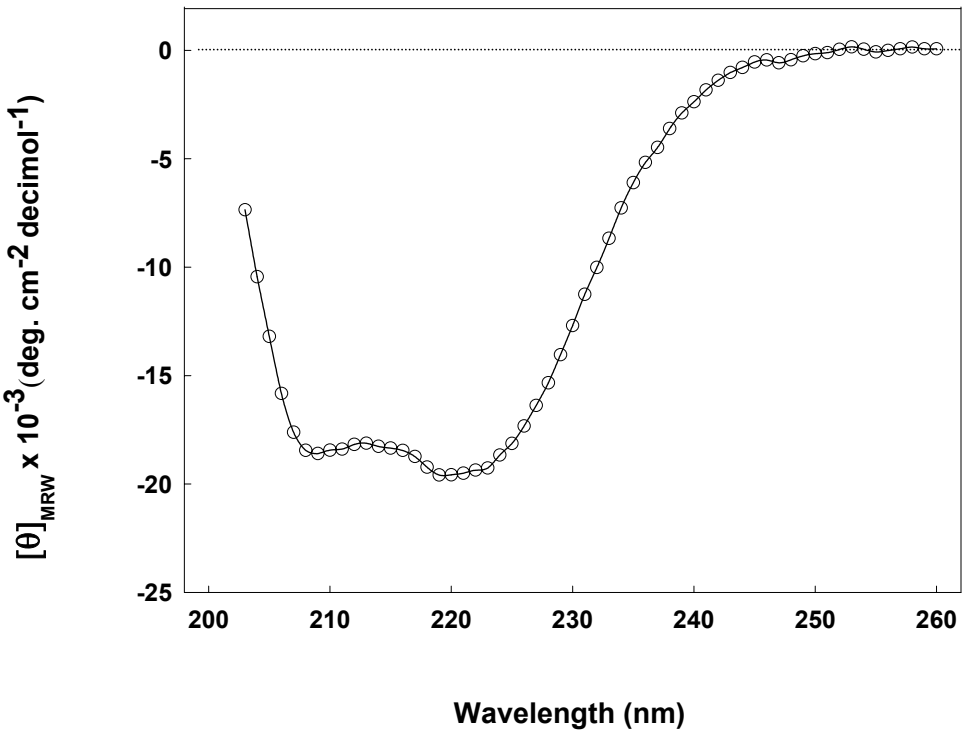


Fig. 9: Far UV-CD spectra of proteins extracted from sardine mince. The buffer used for extraction of proteins was Tris-HCl buffer (pH 7.0; 0.1 M) containing 0.5 M KCl. The spectra was recorded at 25°C.

mainly contributed by the myosin molecule. It has been reported that myosin head contain 50% helix and the tail portion containing 100% helical structure (Harrington and Rodgers, 1984). Since myosin is the major contributor of whole proteins, it showed higher helix content. Myosin head consists of 50% helix and the tail portion is a coiled coil of helixes with 100% helical structure. Secondary structural conformation is important for optimum functionality of proteins. Integrity of myosin molecule is important for enzymatic, functional and physicochemical quality parameters of the proteins from fish mince. A change in conformation results in the altered functionality and may affect quality of end product. This analysis on overall indicated and is interpreted with caution, as it is a mixture of proteins.

Dynamic viscoelastic behaviour of sardine mince

Gelation is an important functional property of food proteins. Gels act as a medium for holding water, flavors, and other nutrients and impart unique rheological and textural qualities of food systems (Kinsella, 1976). When the protein-solvent interaction is attractive, the protein can reduce its total energy by surrounding itself with solvent molecules; when interactions are repulsive, the solvent is excluded (Tanaka, 1981). A gel is an intermediate state between a protein solution and a protein precipitate, formed above a certain level of concentration with just the right balance of protein-protein and protein solvent interactions.

Since gels are viscoelastic materials, they posses both solid like elastic and fluid like viscous behavior, they can be characterized by dynamic rheological tests. Under sinusoidal oscillatory tests the material under study is in an enclosure of sample geometry (e.g. parallel plates or concentric cylinder) and the samples is subjected to a small amplitude sinusoidal shear. Stress and strains can be calculated from the force (torque) and displacement, respectively. The most common parameters used are G′, G″ and tanδ. G′, the storage modulus, is a measure of the energy recovered per cycle of the sinusoidal shear deformation, where as G″, the loss modulus, is an estimate of the energy dissipated as heat per cycle. The ratio of the loss and storage modulus is termed loss tangent or tanδ, which is useful parameter in

describing the phase change during gel formation. The tanδ value will be diminished as a material becomes more elastic in nature. Generally these parameters are measured at a constant oscillatory frequency (ω) as functions of temperature, or at a constant temperature as functions of ω, which is also termed mechanical spectrometry.

The dynamic viscoelastic behavior of sardine mince in the temperature range of 30°-90°C is given in Fig.10. The dynamic viscocelastic behaviour of sardine mince in the temperature range of 30°- 90°C was carried out with 0.4 M NaCl and without NaCl. The storage modulus (G′) of the profile is depicted in Fig.10A and the loss modulus (G′′) depicted in Fig.10B. It is evident that in the absence of NaCl the value of G′ and G′′ decreased with increase in temperature. This clearly indicates the absence of structure formation (net work) and resulting promotion of aggregation by thermal energy. The DVB profile of sardine mince in presence of 0.4 M NaCl showed a substantial increase in G′ and rapid increase was observed between 50°-70°C. The increase in G′ value beyond 50°C is mainly due to the opening up of S1 fraction of myosin promoting hydrophobic interaction and disulfide bond formation (Lanier, 2000). The sharp increase in loss modulus occurred in the range of 50°-70°C. The initial solubilization/hydration of myofibrillar proteins is an essential prerequisite for gelation process. The minimum NaCl concentration required for hydration (by modifying native structure) is 0.35-0.4 M.

The composition of fish meat is predominantly moisture and proteins and in some fishes it is also accompanied by lipid. The range of these components in fish meat is 15-24% protein, 0.1-22% lipid, 1-3% carbohydrates, 0.8-2% inorganic salts and 66-84% water (Suzuki, 1981). It is not uncommon to find wide seasonal variation in the composition of fish meat. In the present investigation the composition of sardine mince showed a lower lipid content of 2.20 ± 0.52%, because of repeated washing process than the unwashed mince. Washing does remove some of the solubles along with it some amount of fat globules also is released from the matrix which is going off with the water. The prime objective of water washing is to reduce the lipid content and to remove sarcoplasmic proteins and coloring pigments. The

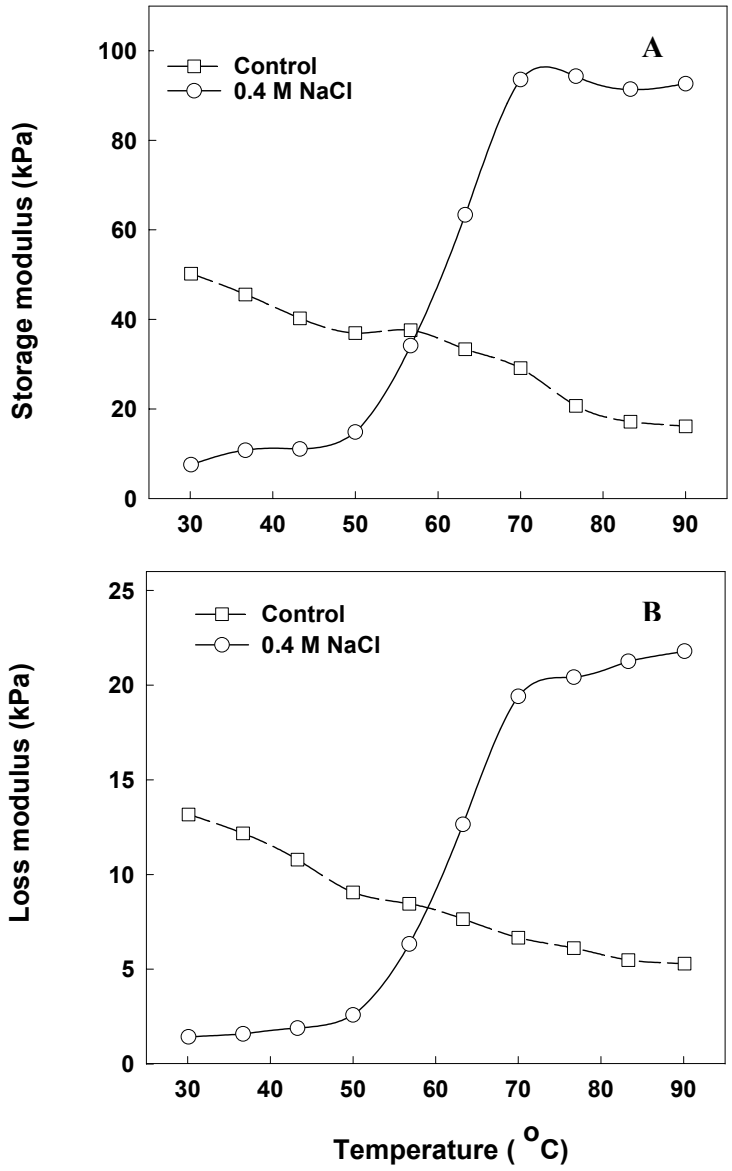


Fig. 10: Dynamic viscoelastic behaviour of sardine mince in the temperature range at 30-90°C. Elastic and viscous components are determined by measuring the resultant strain after applying stress to the mince. A) Storage modulus, the elastic component of sardine mince control and in presence of 0.4 M NaCl; B) Loss modulus, the viscous component of sardine mince control and in presence of 0.4 M NaCl.

increase in moisture content of meat after three washings is mainly due to hydrophilic residues of myofibrillar proteins. Thermal denaturation profile of proteins extracted from sardine mince in the extraction buffer (EB) recorded an apparent T_m of 57 ± 1.5°C (Table 7). Increase in temperature resulted in conformational changes in the protein structure, protein–protein interaction and aggregation of molecules. During thermal treatment proteins undergoes structural changes like exposure of functional groups followed by protein-protein interactions (Wan *et al.,* 1994). Denaturation and aggregation of muscle proteins are associated with the formation of disulfide (Jiang *et al.,* 1988). Sano *et al*. (1990b) suggested that the transformation of myosin in the temperature range of 51ºC – 80ºC is due to the interaction amongst the head portion of myosin molecules.

Fluorescence emission spectra of proteins from the sardine mince showed typical tryptophan fluorescence spectra with an emission maximum at 335 nm when excited with wavelength of 280 nm (Fig.8) as described earlier. It has been reported that phenylalanine, tyrosine and tryptophan residues of proteins exhibit fluorescence emission in the range 320-330 nm where the dominance of fluorescence by tryptophan is always observed when tryptophan is present (Cantor and Schimmel, 1980). It is well known that proteins show fluorescence in the 300-400nm range after excitation in the range of 280 nm to 290 nm and an emission maximum at 336 nm. Changes in the profile of fluorescence spectra are generally attributed to the changes of protein structure if there is structural change. Fluorescence emission spectra of proteins extracted from sardine mince was not altered in presence of calcium salts as shown in Fig.11and12. In the presence of divalent cation zinc the intensity of fluorescence decreased showing the possibility of some structural alteration to the proteins from sardine mince in presence of zinc salts (Fig.13-14). Adenosine triphsophatase activity of sardine mince was found to be 0.23 μM Pi/mg protein/min when the assay was carried out at pH 9.0 (Table 7). Ca^{2+} activated ATPase activity of myosin showed the maximum activity at pH 9.0. ATPase activity is taken as an index of native structural integrity of myosin molecules. The myofibrillar ATPase enzyme activity is more accurate index of change structure of fish proteins (Watabe *et al*., 1989). The ATPase enzyme activity of sardine mince was comparable with the

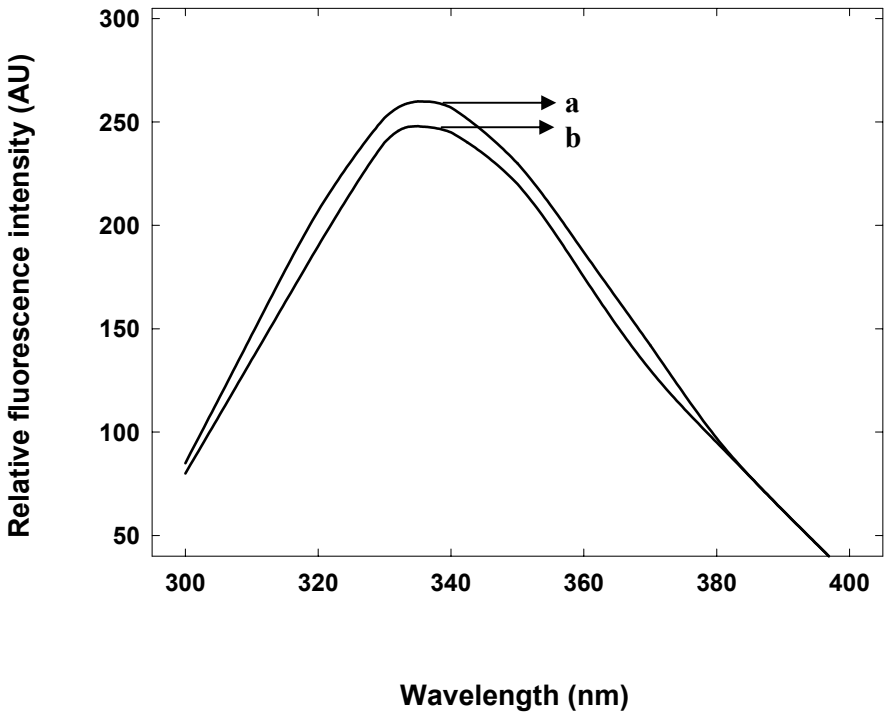


Fig. 11 A: Intrinsic fluorescence emission spectra of proteins extracted in Tris-HCl buffer of pH 7.0, 0.1 M containing 0.5 M potassium chloride from sardine mince as a function of calcium chloride concentration. Emission spectra recorded over a range of 300-400 nm, excitation wavelength fixed at 280 nm. Absorbance of the protein solution was adjusted to 0.1 at 280 nm before fluorescence measurement. a) Control and $1x10^{-5}$ M $CaCl_2$ (no difference in the fluorescence spectra) **and b) $1x10^{-4}$ M, $1x10^{-3}$ M and $1x10^{-2}$ M $CaCl_2$** (no difference in the fluorescence spectra)**.**

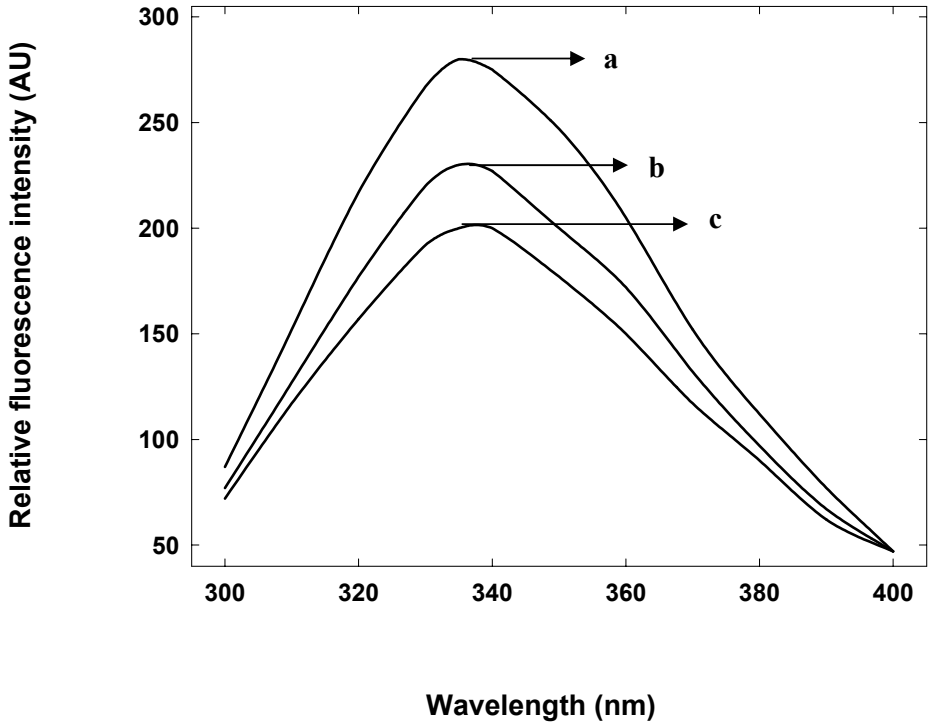


Fig. 11 B: Intrinsic fluorescence emission spectra of proteins extracted in glycine-NaOH buffer of pH 9.0, 0.2 M containing 0.5 M potassium chloride from sardine mince as a function of calcium chloride concentration. Emission spectra recorded over a range of 300-400 nm, excitation wavelength fixed at 280 nm. Absorbance of the protein solution was adjusted to 0.1 at 280 nm before fluorescence measurement a) Control and $1x10^{-5}$ M $CaCl_2$(no difference in the fluorescence spectra)**; b) $1x10^{-4}$ M and c) $1x10^{-3}$ M and $1x10^{-2}$ M $CaCl_2$** (no difference in the fluorescence spectra)**.**

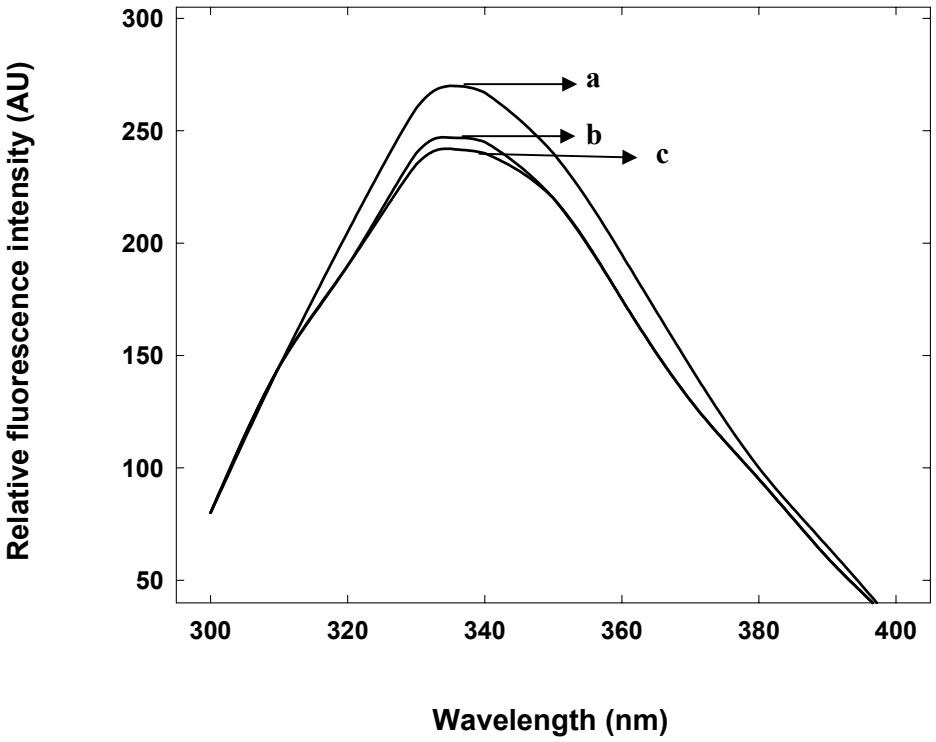


Fig. 12 A: Intrinsic fluorescence emission spectra of proteins extracted in Tris-HCl buffer of pH 7.0, 0.1 M containing 0.5 M potassium chloride from sardine mince as a function of calcium sulfate concentration. Emission spectra recorded over a range of 300-400 nm, excitation wavelength fixed at 280 nm. Absorbance of the protein solution was adjusted to 0.1 at 280 nm before fluorescence measurement a) Control and $1x10^{-5}$ M $CaSO_4$(no difference in the fluorescence spectra)**; b) $1x10^{-4}$ M and c) $1x10^{-3}$ M and $1x10^{-2}$ M $CaSO_4$**(no difference in the fluorescence spectra)**.**

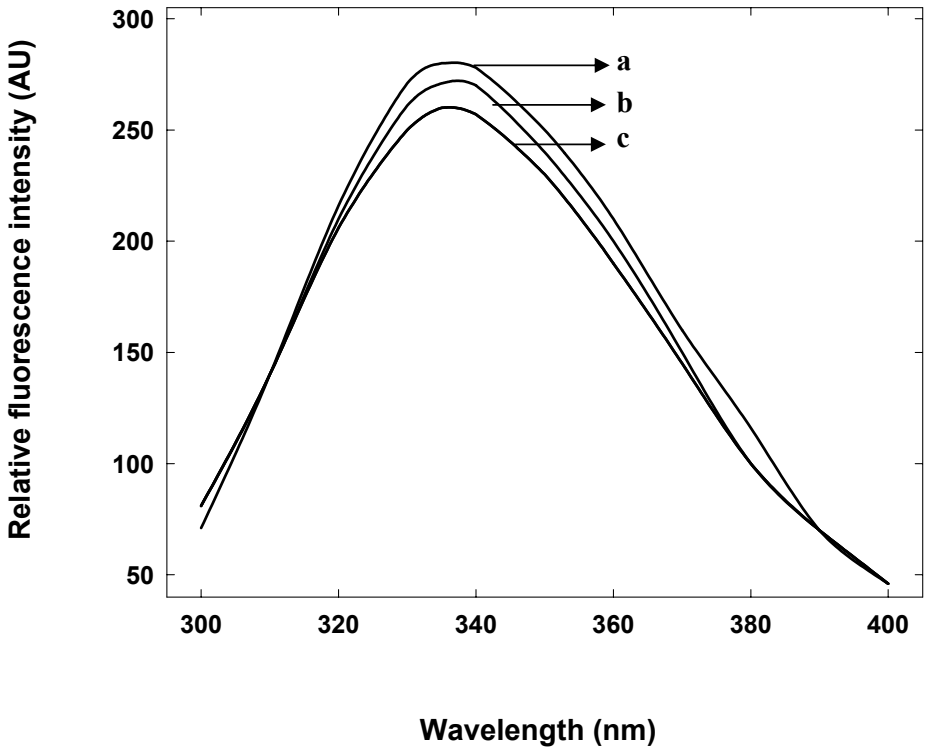


Fig. 12 B: Intrinsic fluorescence emission spectra of proteins extracted in glycine-NaOH buffer of pH 9.0, 0.2 M containing 0.5 M potassium chloride from sardine mince as a function of calcium sulfate concentration. Emission spectra recorded over a range of 300-400 nm, excitation wavelength fixed at 280 nm. Absorbance of the protein solution was adjusted to 0.1 at 280 nm before fluorescence measurement. a) Control and $1x10^{-5}$ M $CaSO_4$ (no difference in the fluorescence spectra)**; b) $1x10^{-4}$ M and c) $1x10^{-3}$ M and $1x10^{-2}$ M $CaSO_4$** (no difference in the fluorescence spectra)**.**

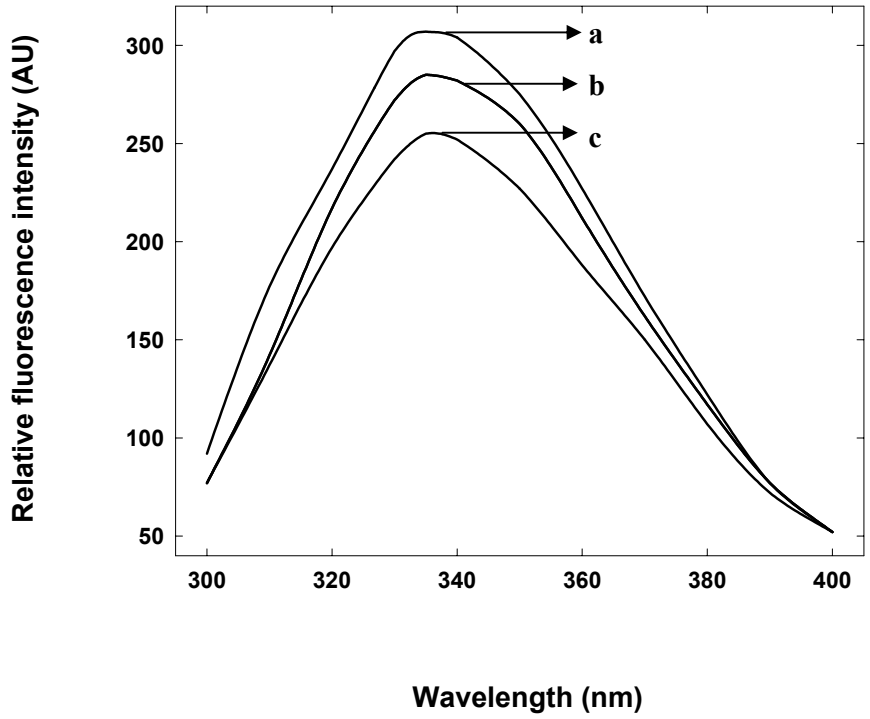


Fig. 13 A: Intrinsic fluorescence emission spectra of proteins extracted in glycine-NaOH buffer of pH 9.0, 0.2 M containing 0.5 M potassium chloride from sardine mince as a function of zinc chloride concentration. Emission spectra recorded over a range of 300-400 nm, excitation wavelength fixed at 280 nm. Absorbance of the protein solution was adjusted to 0.1 at 280 nm before fluorescence measurement. a) Control and $1x10^{-5}$ M $ZnCl_2$ (no difference in the fluorescence spectra)**; b) $1x10^{-4}$ M $ZnCl_2$ and $1x10^{-3}$M $ZnCl_2$** (no difference in the fluorescence spectra) **and c) $1x10^{-2}$ M $ZnCl_2$.**

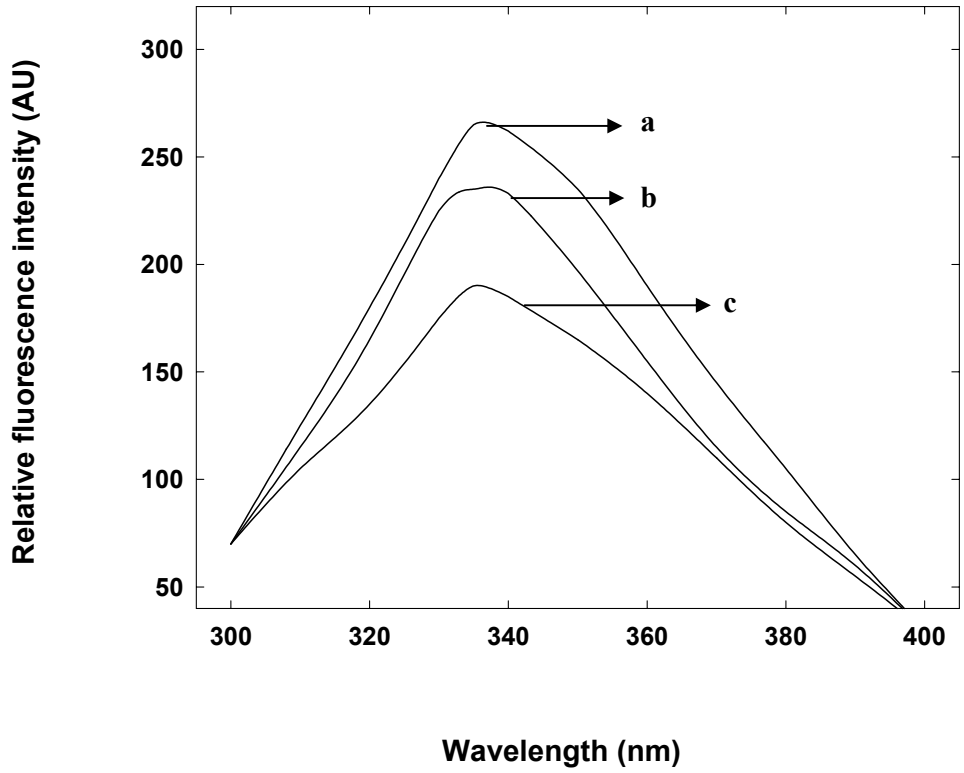


Fig. 13 B: Intrinsic fluorescence emission spectra of proteins extracted in Tris-HCl buffer of pH 7.0, 0.1 M containing 0.5 M potassium chloride from sardine mince as a function of zinc chloride concentration. Emission spectra recorded over a range of 300-400 nm, excitation wavelength fixed at 280 nm. Absorbance of the protein solution was adjusted to 0.1 at 280 nm before fluorescence measurement. a) Control and $1x10^{-5}$ M $ZnCl_2$ (no difference in the fluorescence spectra)**; b) $1x10^{-4}$ M $ZnCl_2$ and $1x10^{-3}$ M $ZnCl_2$** (no difference in the fluorescence spectra) **and c) $1x10^{-2}$M $ZnCl_2$.**

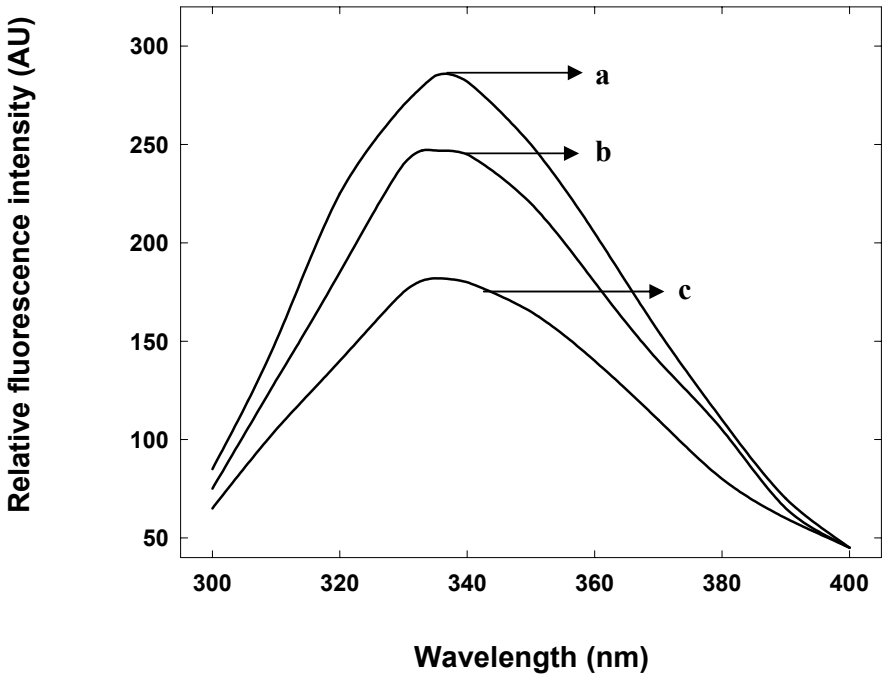


Fig. 14 A: Intrinsic fluorescence emission spectra of proteins extracted in glycine-NaOH buffer of pH 9.0, 0.2 M containing 0.5 M potassium chloride from sardine mince as a function of zinc sulfate concentration. Emission spectra recorded over a range of 300-400 nm, excitation wavelength fixed at 280 nm. Absorbance of the protein solution was adjusted to 0.1 at 280 nm before fluorescence measurement. a) Control and $1x10^{-5}$ M $ZnSO_4$(no difference in the fluorescence spectra)**; b) $1x10^{-4}$ M $ZnSO_4$ and $1x10^{-3}$ M $ZnSO_4$** (no difference in the fluorescence spectra) **and c) $1x10^{-2}$ M $ZnSO_4$.**

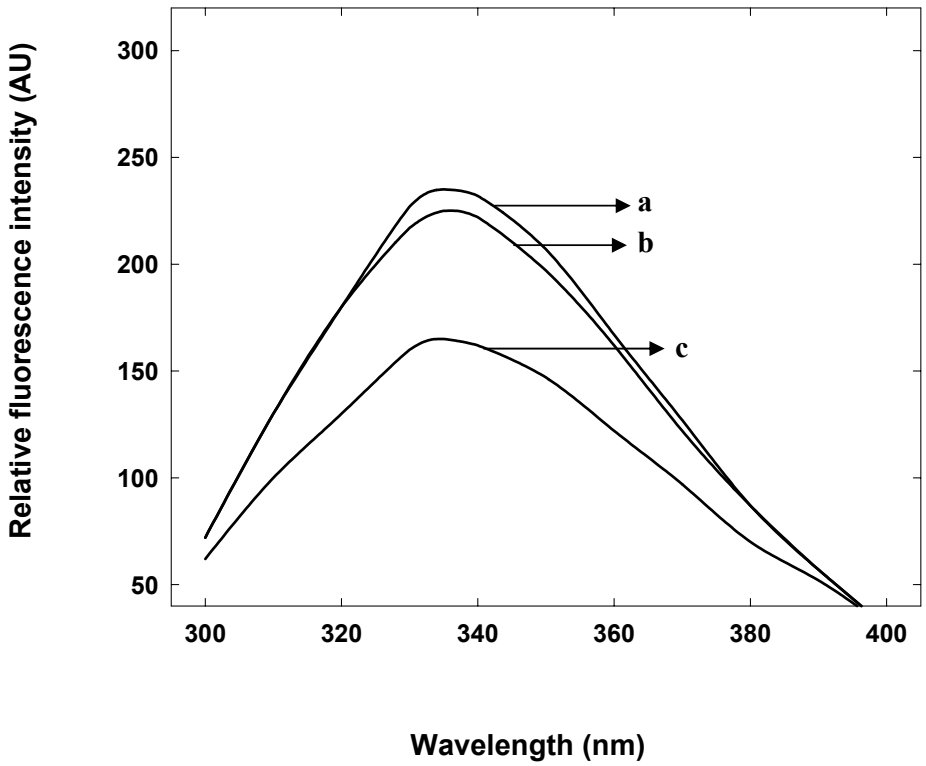


Fig. 14 B: Intrinsic fluorescence emission spectra of proteins extracted in Tris-HCl buffer of pH 7.0, 0.1 M containing 0.5 M potassium chloride from sardine mince as a function of zinc sulfate concentration. Emission spectra recorded over a range of 300-400 nm, excitation wavelength fixed at 280 nm. Absorbance of the protein solution was adjusted to 0.1 at 280 nm before fluorescence measurement. a) Control; b)$1x10^{-5}$ M $ZnSO_4$ and $1x10^{-4}$ M $ZnSO_4$ (no difference in the fluorescence spectra) **and c) $1x10^{-3}$ M $ZnSO_4$ and $1x10^{-2}$ M $ZnSO_4$** (no difference in the fluorescence spectra)**.**

ATPase activity of fish meat from other species. Presence of ATPase enzyme is generally taken as an index of native structural integrity of myosin molecule.

Functionality of fish mince mainly arises from myofibrillar proteins. The emulsion capacity of sardine mince in the present study was 0.13 ± 0.01 ml oil/ mg protein. This was comparable with the values reported for other fish and shellfish. An emulsion is a three-phase system, wherein, proteins act as an emulsifier to reducing the interfacial energy of water oil system (Dickinson and Sainsby, 1982). Proteins have unique surface properties due to their large molecular weight and tertiary structure, and their amphiphilic properties make them suitable as potent surfactant (Kato *et al.,* 1993). The capacity of proteins to form emulsion depends on the nature and quality of proteins present in the mince. Any change in structure of proteins will affect the emulsification capacity. To stabilize an emulsion, the hydrophobic domain of the proteins should ideally be oriented towards the oil phase. It was reported that the increase in surface hydrophobicity by the exposure of hydrophobic residues lead to an increase in the emulsion capacity of fish muscle proteins (Kristinsson and Hultin, 2003a; Li-Chan *et al.,* 1984). The functional properties like emulsion capacity depends on the amount of proteins solubilized in the given solvent/buffer. Hence, extractability/solubility of proteins in the buffer is very important for exhibiting good functionality.

The total extractable proteins from sardine mince in high ionic strength buffer was 64 ± 3% (Table 7). The major components of proteins are actin and myosin, which are extractable in the buffer containing salts. The alteration of protein extractability is a useful factor, which may be used to determine the textural quality of frozen fish muscle, as protein aggregation is accompanied by a significant decrease in their solubility (Badii and Howell, 2001; Careche *et al.,* 1998; Howell *et al.,* 1996). Factors affecting the protein stability or interactions may also affect gel properties and have considerable implications for fish processing and storage (Togashi *et al.,* 2002). Depending on the ionic strength of the medium, myosin could have two forms, monomeric and filamentous. Myosin is a monomer in high salt medium such as 0.5 M NaCl, while it can easily transform into filamentous form by self-association at

reduced ionic strength at physiological levels (Konno *et al.,* 1997). The tail portion of myosin is termed rod, is responsible for the salt soluble nature of myosin or for the filament formation of the myosin molecule. Periodic arrangement of negatively and positively charged amino acids in the rod region generates electrostatic forces necessary for forming filaments (Maita *et al.,* 1991). Solubilization of myosin filaments by salt occurs under the conditions where electrostatic bridge formation between myosin molecules is inhibited. At a high concentration, (0.5-1.0 M) salt ions readily form electrostatic bonds with corresponding charged amino acid residues of myosin. The bond formed with salt decreases the attraction of myosin molecules and results in inhibition of myosin filament formation (Konno *et al.,* 1997).

The capacity of fish mince to hold water is another important functional property that has commercial relevance. Water holding capacity is the ability of the meat to retain water during application of external forces, such as cutting, heating, grinding, or pressing. As the fish quality decreases the water holding capacity also decreases (Hsing *et al.,* 1990). The water absorption capacity of sardine mince was 3.65 ± 0.25 g water/g dried material (Table 7). Reduction water holding capacity is due to a loss of the myofibrillar protein integrity. The major part of the water in the meat must therefore be held physically within sub cellular (myofibrillar) structures, and is reasonable to postulate that difference in muscle structure will affect the water distribution and thereby the WHC of the meat (Bertram *et al.,* 2002). Gel forming ability of sardine mince as measured by small strain test shown that the mince prepared from sardine was not able to form gels in the absence of salts. The rapid decline in tanδ demonstrated the development of elastic structure while the increase in G′ indicated the formation of rigid gel matrix. Hermansson *et al.,* (1986) observed two distinct morphologies in bovine myosin gels, depending on pH and ionic strength. At pH 5.5-6.0 and low ionic strength (0.25 M KCl) fine stranded network were formed, while at high ionic strength (0.6 M KCl) coarsely aggregated structures were produced. High ionic strength tends to reduce the electrostatic repulsion between proteins due to the shielding of ionizable groups by mobile ions (Tanaka, 1981). The study reveals that use of 0.4 M NaCl could enhance gel rigidity appreciably.

B) Isolation and characterization of actomyosin from oil sardine

Myofibrillar proteins constitute nearly 70% of total proteins in fish muscle. Actin and myosin are the major proteins present in the myofibrils of fish. Actin and myosin forms a complex resulting in actomyosin during postmortem. The actin myosin complex was isolated from the fresh sardine to characterize the system. Since these are the major proteins present in the muscle, any alteration in the quality is directly correlated with the structure and function of this complex. Studies on the interaction of actomyosin with various additives will help to understand the system and the mechanism of interaction of these compounds for better biotechnological applications which is described in detail in Chapter 2 and 3.

The actomyosin isolated from fresh sardine meat contained 94.2 ± 1% moisture and rest 5.81 ± 0.7% protein (Table 8). For all characterization fresh actomyosin is isolated and stored at 4°C maximum for 48 hours. The gel filtration was carried out with Sepharose 6B column. The proteins were eluted with phosphate buffer 0.05 M, pH 7.5 containing 1.0 M sodium chloride. The gel permeation chromatography showed a prominent peak eluted at an elution volume of 63 ml was actomyosin complex (Fig.15). The elution profile of actomyosin was carried out with fast protein liquid chromatography (FPLC) system to confirm the purity of preparation. FPLC pattern of protein showed a single peak eluting at 12.5 ml (Fig.16). The SDS-PAGE of isolated actomyosin (Fig.17) showed multiple bands corresponding to myosin heavy chain, actin and two light chains of myosin. Myosin molecule is a hexamer with two heavy chains corresponds to 205 kD and four light chains with 16-20 kD. The composition of light chains and heavy chains vary with species. For comparison purposes the SDS-PAGE of actomyosin from chicken and myosin from chicken is also given in Fig.17.

The results of SDS-PAGE, gel filtration and FPLC showed that the prepared actomyosin contains myosin and actin and the absence of low molecular weight proteins in the preparation was confirmed. The ultraviolet absorption spectra of actomyosin have shown two peaks at 280nm and 215nm. The prominent peak arising

Table 8: Proximate composition, physicochemical and enzymatic properties of actomyosin isolated from Indian oil sardine

Parameters	Values
Moisture	94.2 ± 1 %
Protein (N x 6.25)	5.81 ± 0.7 %
ATPase activity	0.26 ± 0.03 μ M Pi/mg protein/min
Free SH content	122 ± 12 μ M SH / g of AM
Secondary structure	
α - helix	65 ± 3%
β - structure	3 ± 2%
Aperiodic	32 ± 4%

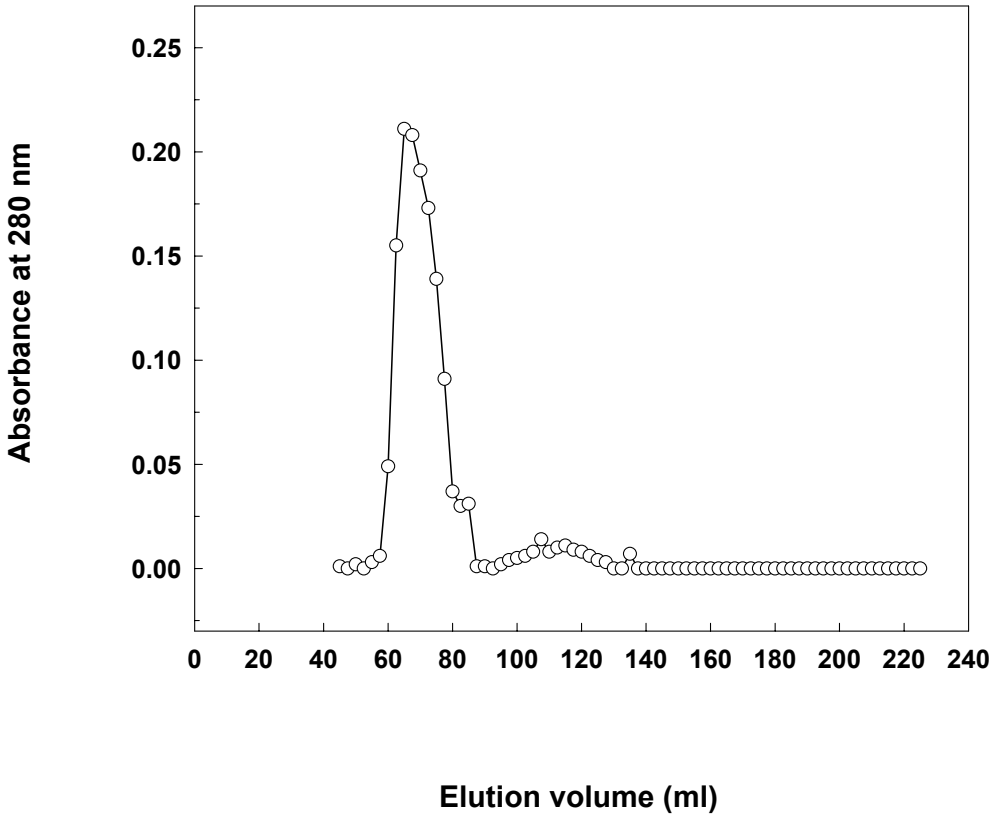


Fig. 15: Gel filtration profile of isolated actomyosin from *Sardinella longiceps* on Sepharose 6B column. Actomyosin was dissolved in phosphate buffer (0.05 M, pH 7.5) containing 1.0 M sodium chloride and eluted with the same buffer and absorbance was recorded at 280 nm.

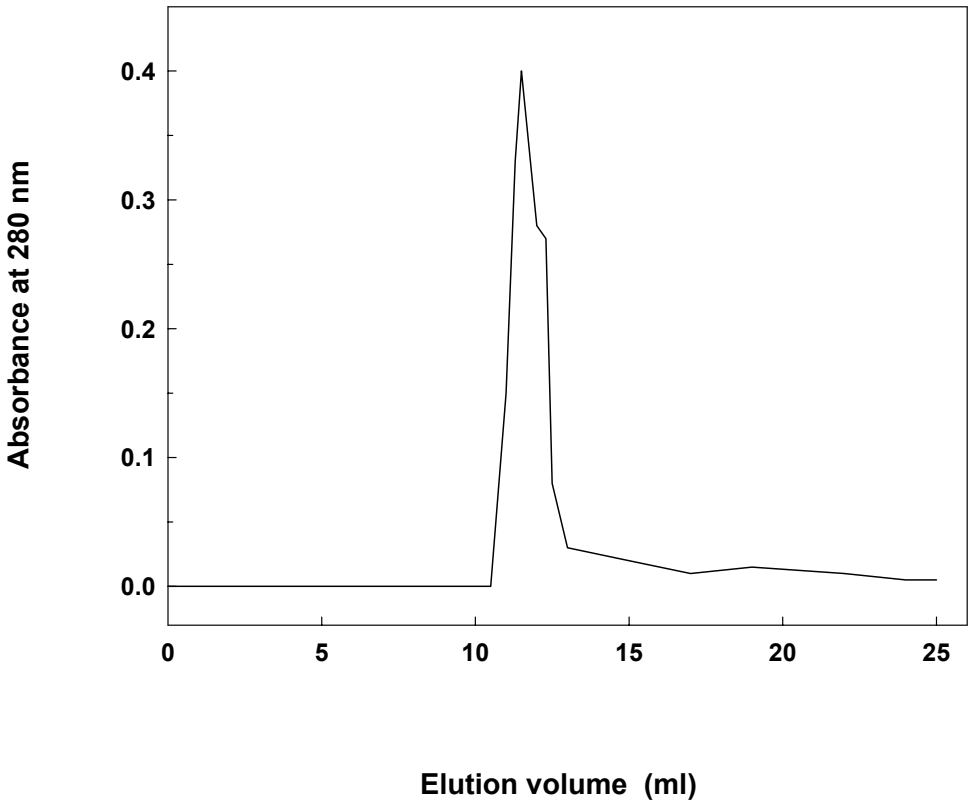
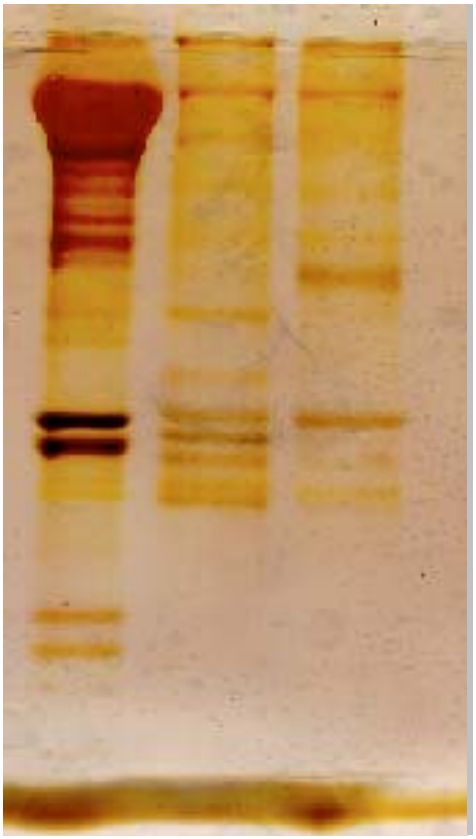


Fig. 16: FPLC pattern of fish actomyosin carried out on Superose 6HR column. Fish actomyosin was dissolved in phosphate buffer (0.05 M; pH 7.5) containing 0.5 M NaCl and eluted with the same buffer. The absorbance of fractions were recorded at 280 nm.



1 2 3

Fig. 17: SDS-PAGE pattern of actomyosin isolated from oil sardine. 1) actomyosin from fish: 2) actomyosin from chicken; 3) myosin from chicken.

at 280nm is mainly by aromatic aminoacids. The free SH content of actomyosin was estimated to 122 μM SH/g of actomyosin. The free SH content was in accordance with the SH content of fish protein from other species (Suzuki, 1981). Myosin molecules are having two free SH groups on the upper 50 kD domain. This particular domain is responsible for the ATP hydrolyzing action of myosin molecules. The isolated actomyosin was further characterized by various biophysical techniques.

Intrinsic fluorescence emission spectra of protein isolated was recorded over a range of 300-400 nm after exciting the proteins at 280 nm. Fluorescence emission spectra of actomyosin showed the emission peak at 335 nm, which corresponds to the fluorescence spectra dominated by tryptophan residues (Fig.18). Myosin has tryptophan residues both in its rod and head region (Kato and Konno, 1993). The secondary structures of actomyosin were followed by the far UV-CD spectra. Far UV-CD spectra of fish actomyosin showed two troughs one at 208 nm and one at 222 nm. Secondary structural estimation showed that the protein is a prominently α-helical in nature (Fig.19). The secondary structural analysis of actomyosin indicates that it has 65 ± 3% α-helical content, 3 ± 2% β- structure and 32 ± 4% aperiodic structure. Myosin molecules have two heads and a tail. Head of myosin have 50% helix in the structure. The long tail portion of myosin molecule is a coiled coil that contributes to the majority of helical structure.

Actomyosin complex has the prominent ATP degrading activity. The active site for this enzyme located at the head portion of myosin. ATPase activity of myosin in presence of actin is activated by calcium ions (Perry, 1955). This enzyme activity is considered as an index of integrity of actomyosin complex. Ca^{2+} activated ATPase activity of actomyosin is studied in detail to characterize the system.

The actomyosin ATPase enzyme activity as a function of pH is shown in Fig.20. The effect of pH on the ATPase activity of actomyosin was assessed in the range of pH 5.0-10.5. The results indicated it is having optimum activity both at pH 7.0 and 9.0. The activity at pH 9.0 is nearly twice the activity at pH 7.0. The results obtained in the present study are comparable to available literature in terms of overall

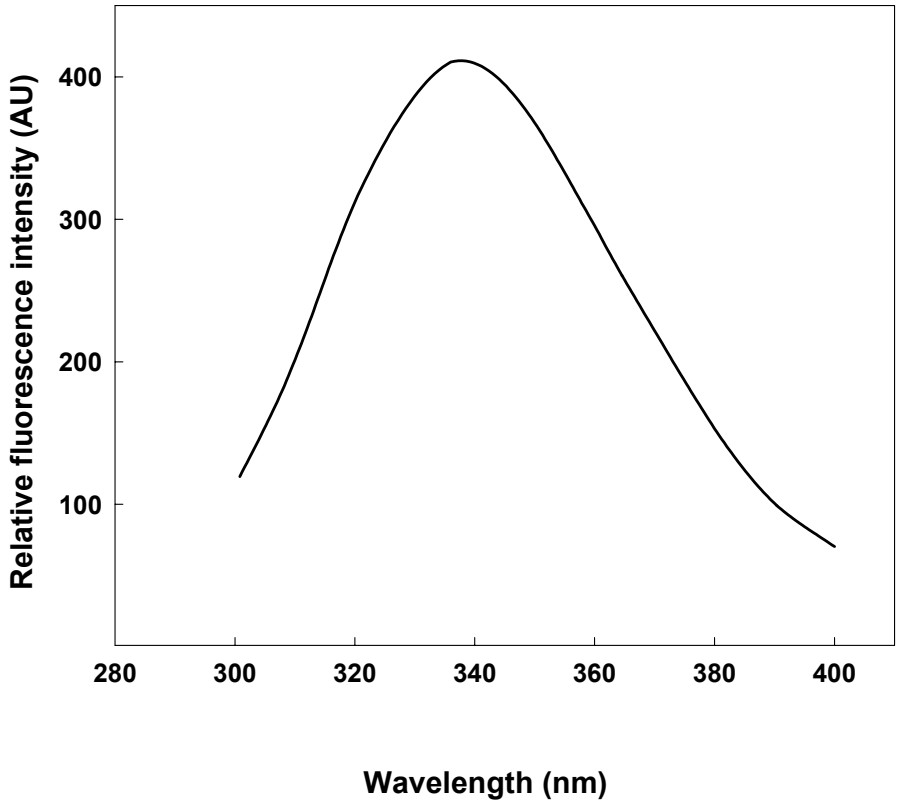


Fig. 18: Intrinsic fluorescence emission spectra of isolated actomyosin from *Sardinella longiceps*. The fluorescence spectra was determined with Tris HCl buffer (pH 7.0, 0.1 M) containing 0.5M KCl. Fluorescence emission spectra was recorded over a range of 300-400 nm with excitation wavelength at 280 nm.

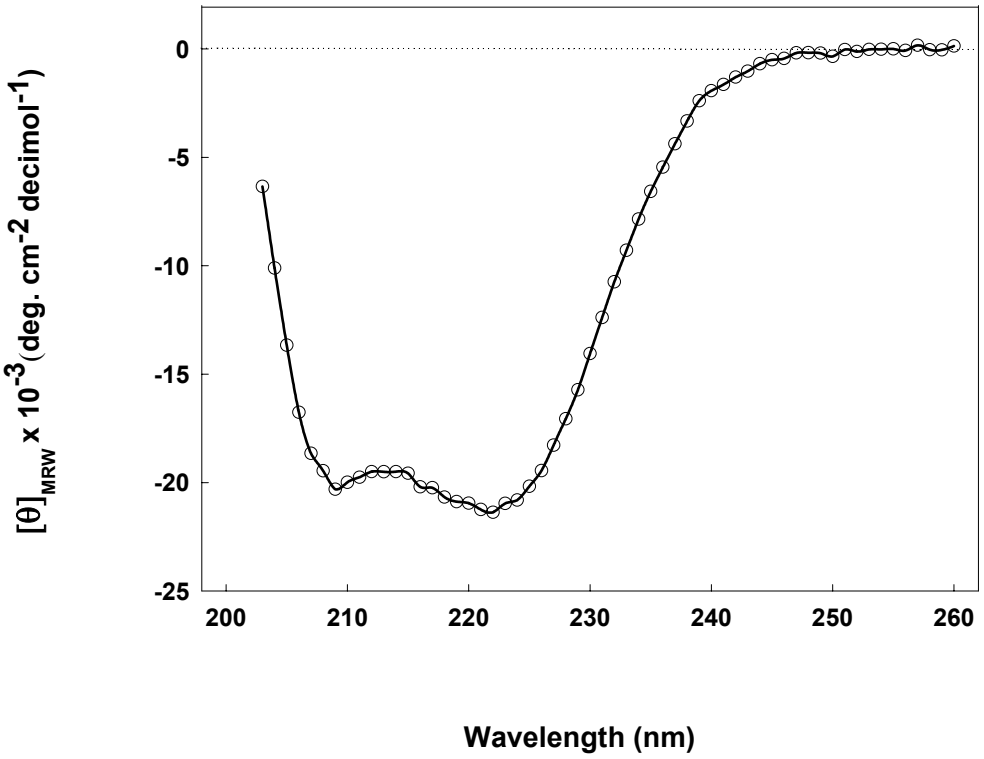


Fig. 19: Far UV-CD spectra of fish actomyosin isolated from *Sardinella longiceps* in 0.1 M Tris-HCl buffer of pH 7.0 containing 0.5 M KCl at 25°C.

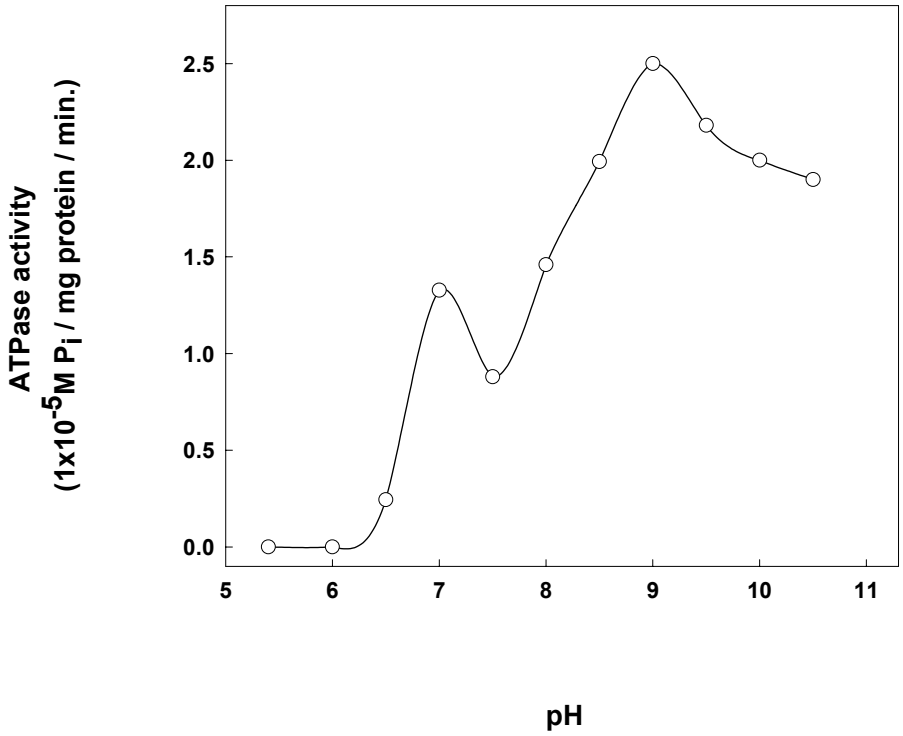


Fig. 20: ATPase enzyme activity of actomyosin as a function of pH. ATPase activity is expressed as moles of inorganic phosphate released per mg of actomyosin per minute at 25°C.

bimodal peak of the profile. Perry (1955) reported pH optimum for myosin ATPase enzyme at 9.0 and at neutral pH a moderate enzymatic activity was also observed for myosin. Since the activity was observed at two pH 7.0 and 9.0 further it was decided to study the effect of time of incubation, temperature and substrate concentration. The rate of ATP hydrolysis varied at the two selected pH values as shown in Fig.21. In the alkaline pH the rate of ATP hydrolysis was pronounced than at neutral pH.

The ATPase activity of actomyosin was determined as a function of temperature at pH 9.0 and pH 7.0 (Fig.22). At lower temperature the activity was remained almost same. The maximum activity was at 25ºC. The temperature dependence of activity followed the same pattern in both pH conditions. Activity showed slight reduction when the temperature increased to 30ºC. Further increase in temperature resulted in drastic reduction of the activity and it decreased with increase in temperature up to 45ºC. It has reached near zero values at temperature of 45ºC. Conformational changes in the myosin head during heating is the reason for drastic reduction in the activity of enzyme. Further ATPase activity of fish actomyosin was investigated as function of substrate concentration at 25°C (Fig.23). The results indicated at pH 9.0 maximum activity observed with a substrate concentration of $8x10^{-4}$ M, where as, at pH 7.0 maximum activity was observed at a substrate concentration of $1x10^{-3}$ M. From the results it is evident the optimum pH, temperature, substrate concentration and time for actomyosin from sardine was 9.0, 25°C, $8x10^{-4}$ M and 15 min respectively. The ATPase activity of actomyosin isolated was found to be 0.26 ± 0.03 μM Pi/mg protein/min.

The composition of actomyosin from fresh sardine meat had 94.2 ± 1% moisture and rest 5.81 ± 0.7% protein (Table 8). Actomyosin prepared from fish meat contain actin, myosin, tropomyosin and troponin and is called natural actomyosin (Suzuki, 1981). UV spectra of fish actomyosin showed a distinct peak 220 nm and 280 nm. The absorbance at 280 nm is contributed by aromatic amino acids and at 220 nm is mainly contributed by peptide bonds. In the ultra violet spectra of protein solutions absorbance at 250 nm indicate the presence of disulfide bonds and the strong absorbance below 230 nm is due to peptide bonds (Plummer, 1971; Schimid,

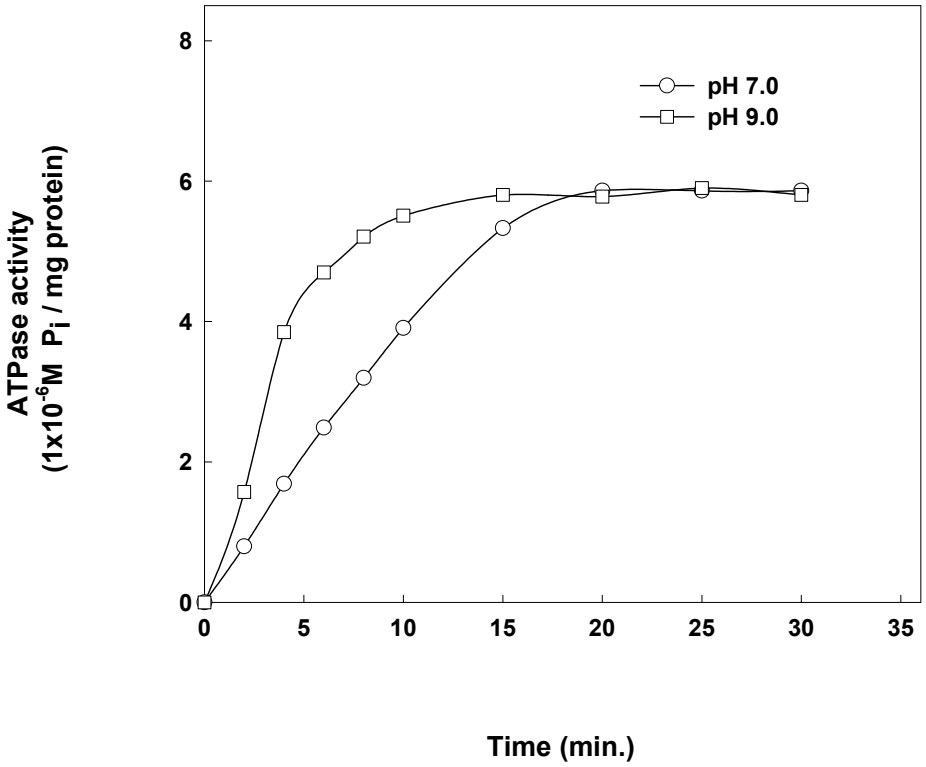


Fig. 21: ATPase activity of actomyosin from sardine as a function of time at two selected pH 9.0 and 7.0. Activity was determined with glycine-NaOH (pH 9.0; 0.2 M) and in Tris-HCl (pH 7.0; 0.1 M) containing 0.5 M KCl. Activity of enzyme is expressed as moles of inorganic phosphate released per mg of actomyosin.

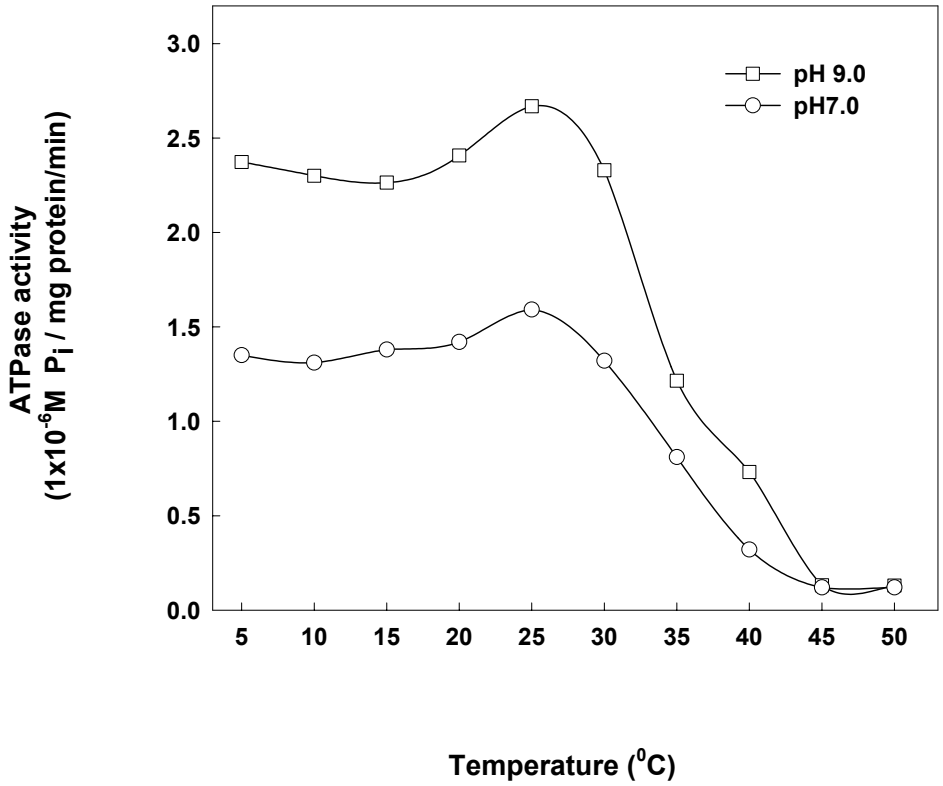


Fig. 22: ATPase activity of actomyosin as a function of temperature at two selected pH of 9.0 and 7.0. Activity was determined with glycine-NaOH (pH 9.0; 0.2 M) and Tris-HCl (pH 7.0; 0.1 M) containing 0.5 M KCl. Activity of enzyme is expressed as moles of inorganic phosphate released per mg protein per minute.

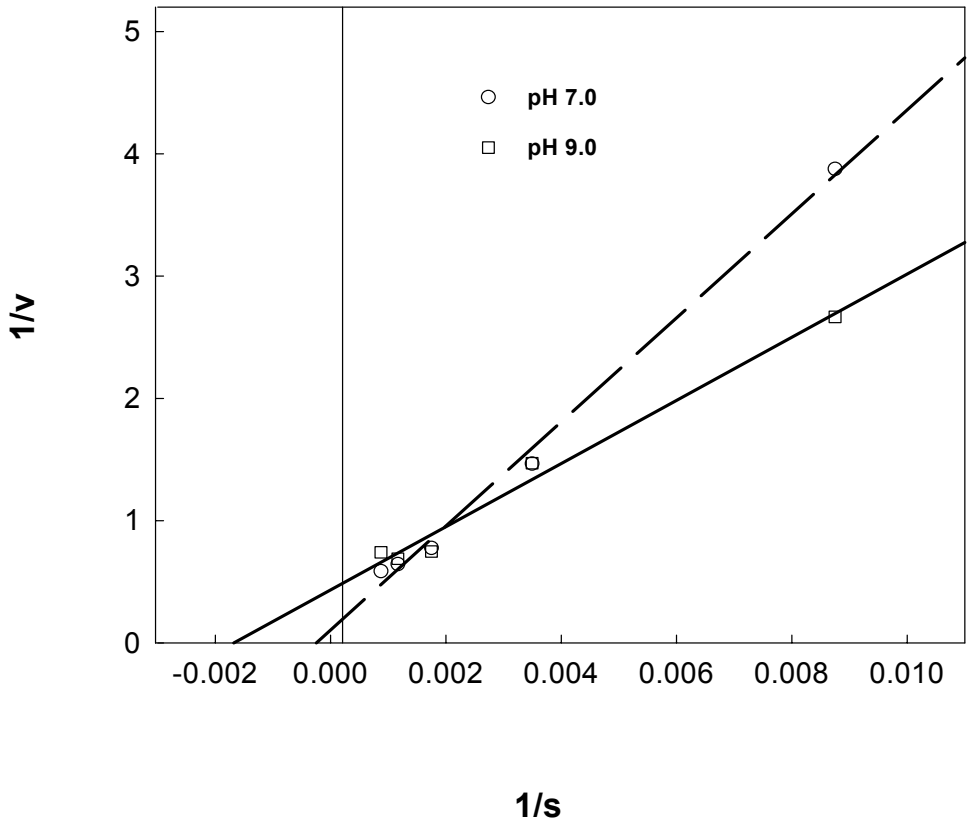


Fig. 23: Lineweaver-Burk plot of ATPase enzyme of sardine actomyosin at pH 7.0 and 9.0. Activity was expressed as moles of inorganic phosphate released per mg protein per minute.

1997). The absorbance peak in the range 275-280 nm indicates presence of aromatic amino acids residues. Free SH content of actomyosin was found to be 122 ± 12 μM SH/g of AM. The free SH content was in accordance with the SH content of fish protein from other species (Suzuki, 1981). Freshly prepared myosin molecules have about 42 free SH groups (Buttukus, 1970). There are two active SH groups present in the two α-helices lying under the nucleotide binding pocket and are more reactive than other 11 SH groups (Rayment *et al.,* 1993a). The nucleotide-binding site is located in a pocket at the interface of the 25 kD N-terminal and the central 50 kD tryptic fragment (Rayment, 1996). The polypetides form the α-helices lying under the nucleotide-binding site. This highly conserved segment in the sequence contains the two sulfhydryl groups Cys^{707} and cys^{697}. The α- carbons of cys^{697} and cys^{707} are approximately 18Aº apart suggests that a rearrangement or conformational change in this area must occur upon nucleotide binding (Rayment *et al.,* 1993a).

Fluorescence spectra of actomyosin showed typical tryptophan fluorescence (Fig.18). Myosin has tryptophan residues both in its rod and head region (Kato and Konno, 1993). Secondary structural estimation showed that the protein is a prominently α-helical and aperiodic in nature (Fig.19). The myosin rod portion is almost 100% α-helix and the globular head has less than 50% α-helix, thus rod has been contributing to the majority of secondary structure of actomyosin (King and Lehrer, 1989; Harrington and Rodgers, 1984).

Fish myosins are typically characterized by a more unstable structure than mammalian myosin as indicated by their lower thermal resistance to aggregation, higher tendency to lose light chains, increased tryptic digestibility and lower thermal inactivation of ATPase (Hamai *et al.,* 1989; Kato *et al.,* 1993; Ochiai *et al.*, 1989; Yamamoto *et al.,* 1989). The loss of ATPase enzyme activity of actomyosin isolated from sardine at higher temperature could have been due to the aggregation of myosin molecules. Changes in conformation of myosin at a temperature around 40ºC results in the loss of Ca^{2+} activated ATPase activity of myosin and aggregations of tail portion of the myosin molecules are prominent at this temperature (Benjakul *et al.,* 2001; Chen, 1995; Sano *et al.,* 1990b).

ATPase activity of actomyosin from fresh sardine increased with increase in substrate concentration. ATPase activity reached a maximum value in the presence of $1x10^{-3}$ M of ATP. The ATPase activity of actomyosin isolated was 0.26 ± 0.03 μM Pi/mg protein/min. The enzyme activity was similar to the values reported in the literature for sardine, carp and yellowtail (Arai *et al*., 1973).

From the above results and discussion it is evident that sardine mince prepared from fresh sardine has functional properties which can be utilized for product development using these properties. The advantage of higher solubility and an elevated emulsion capacity are some of the indicators in this direction. The understanding of the physicochemical, functional and the various enzymatic parameters of both the total proteins and isolated actomyosin would further help in deriving the rationale and for better understanding of the mechanism of interaction of selected salts and metal ions with the proteins especially actomyosin during frozen storage of fish mince.

CHAPTER - 2

INFLUENCE OF DIVALENT CATIONS ON THE STRUCTURE AND ATPase ENZYME ACTIVITY OF ACTOMYOSIN FROM OIL SARDINE

INFLUENCE OF DIVALENT CATIONS ON THE STRUCTURE AND ATPase ENZYME ACTIVITY OF ACTOMYOSIN FROM OIL SARDINE

Frozen storage of mince needs special attention in order to minimize protein denaturation. Commercially cryoprotectants are added to extend the shelf life of frozen foods. They may be added during either processing or formulation of the mince. A wide variety of compounds have the ability to cryoprotect the proteins during frozen storage. These include sugars, aminoacids, synthetic polymers, inorganic salts and metal ions. In order to optimize the concentration of cryoprotectant to be used there is a need to understand the mechanism of interaction between major protein fractions namely actomyosin and chosen cryoprotectant. In the present chapter results of interaction of actomyosin with divalent cations on the structure of actomyosin have been studied with a view to elucidate the mechanism of action.

Fish muscle is composed of numerous proteins. The myofibril fraction constitutes 40-70% of whole muscle proteins, and the most abundant protein in the myofibril is myosin (Ochiai and Chow, 2000). Myosin is one of the principal protein components of numerous contractile systems and comprises almost 50% of total protein in skeletal muscles (Harrington and Rodgers, 1984). Myosin is the most abundant molecular motor, which plays both structural and enzymatic role in muscle contraction and intracellular motility (Rayment *et al.,* 1993b). Myosin molecule is highly asymmetric hexamer. Each myosin molecule consists of two globular head regions and a rod like tail portion that is coiled coil of α-helices. The properties of myosin depends on the source of origin. Eventhough the structure of fish myosin is similar to other mammalian myosin, it is unstable than the latter and studies on the Mg^{2+} ATPase of actomyosin and Ca^{2+} ATPase of myofibrils suggest that the stability of fish myosin is species specific and associated with the temperature of its habitat (Hashimoto *et al*., 1982).

A) Effect of zinc salts on the structure and enzyme activity of sardine actomyosin

Various treatments and additives have been used to improve the functionality of fish myosin (Kristinsson and Hultin, 2003a,b; Ramirez *et al.*, 2003; Sultanbawa and Li-Chan, 1998). Use of certain divalent metal ions to reduce the amount of sugars required for cryoprotection has been reported by some authors (Carpenter *et al*., 1987a; Hazen *et al*., 1988). Zinc is one of the major micronutrient required for eukaryotes. Zinc has got two important properties that make it well suited for its role as structural element in nucleic acid binding or other gene regulatory proteins (Berg and Shi, 1996). The generation of tetrahedral site from a combination of cysteine and histidine residues will automatically have a preferential affinity for zinc over most other common metal ions (Berg and Merkle, 1989). Classically zinc has not been thought to be associated with biological redox reactions, because the metal is not readily oxidizable or reducible in solutions. It is a distinct possibility that a change in the redox status of a donor atom of a zinc compound might alter the overall oxidoreductive properties of the complexes and hence its biological activities (Maret and Vallee, 1998). Zinc is an essential micronutrient and present in most marine fishes (Nettleton, 1985). This study has been taken up to elucidate the role of zinc compound on the structure and function of fish actomyosin isolated from a pelagic fish. In the present study the effect of zinc chloride and zinc sulfate of different concentrations on the physicochemical and enzymatic properties of actomyosin have been studied.

The effect of zinc sulfate at different concentrations on the ATPase enzyme activity of sardine actomyosin as a function of temperature at pH 7.0 is shown in Fig.24A. Three different temperatures *viz*. 25°C, 37°C and 45°C were chosen for the study. The time of incubation was held at 15 min. based on the optimum time interval for the hydrolysis. At 25°C there was a progressive reduction in activity with increase in zinc sulfate concentration and at $1x10^{-2}$ M there was 100% loss of enzyme activity. At 37°C and 45°C of incubation the activity reduced significantly even at $1x10^{-5}$ M concentration. At a concentration of $1x10^{-4}$ M both at 37°C and 45°C activity reduced

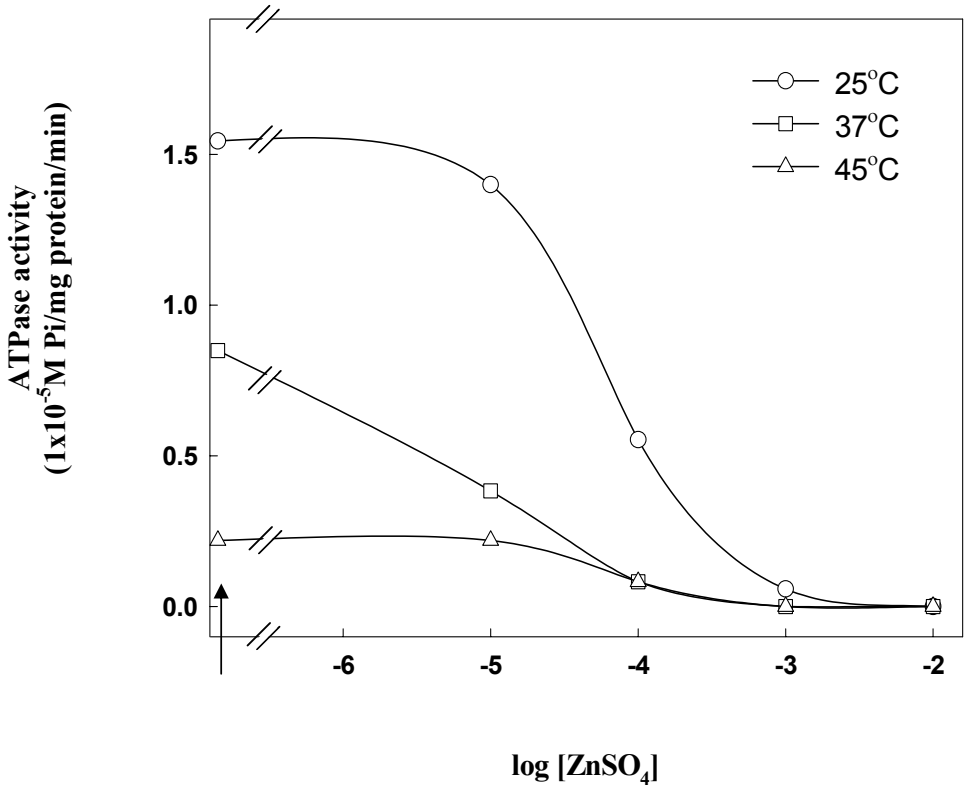


Fig. 24 A: ATPase activity of actomyosin from *Sardinella longiceps* in presence of different concentrations of $ZnSO_4$ at pH 7.0 as a function of temperature. The enzyme activity was determined with Tris-HCl buffer of pH 7.0 (0.1 M) containing 0.5 M KCl at three different temperatures of 25°C, 37°C and 45°C. Enzyme activity is expressed as moles of inorganic phosphate released per mg of protein per minute. Arrow (↑) indicates the control values without $ZnSO_4$.

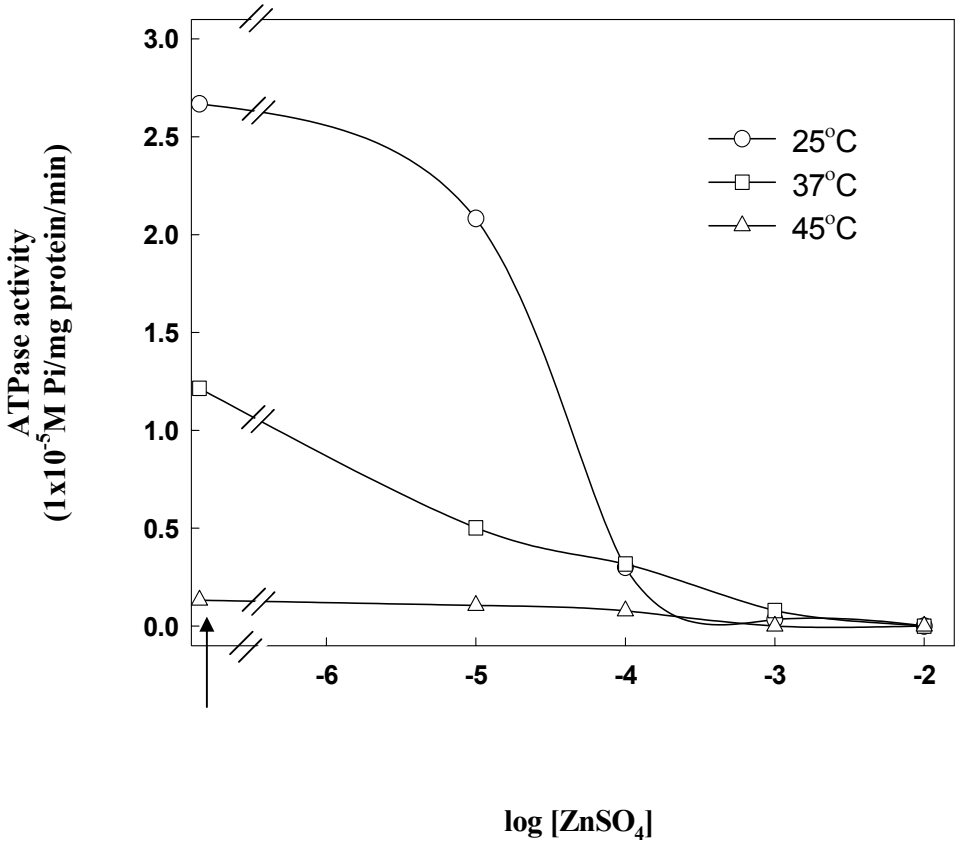


Fig. 24 B: ATPase activity of actomyosin from *Sardinella longiceps* in presence of various concentrations of $ZnSO_4$ at pH 9.0 as a function of temperature. Enzyme activity was carried out with glycine-NaOH buffer pH 9.0 (0.2M) containing 0.5 M KCl at temperatures of 25°C, 37°C and 45°C. Activity expressed as moles of inorganic phosphate released per mg of proteins per minute. Arrow (↑) indicates the control values without $ZnSO_4$.

by 100%. This clearly indicates that zinc sulfate has more inhibitory effect at 37°C and 45°C than at 25°C.

The effect of zinc sulfate at different concentration at pH 9.0 on the ATPase enzyme activity is shown in Fig. 24B. From the figure it is evident that at pH 9.0 the inhibitory effect of zinc sulfate at 37°C and 45°C has followed the same pattern as that of observed at pH 7.0. However, the absolute values of enzyme activity are higher at pH 9.0 suggesting pH optimum for actomyosin ATPase enzyme is pH 9.0.

The effect of pH and temperature in presence of different concentrations of zinc chloride on the ATPase enzyme activity profile have been studied. Fig.25A depicts the effect of different concentration of zinc chloride on the ATPase enzyme activity as a function of temperature at pH 7.0. As in case of zinc sulfate the inhibitory effect of zinc chloride on ATPase enzyme activity could be observed with increase in concentration. The inhibitory effect was more profound when the incubation temperature was 45°C. In all the three temperatures, it was found that zinc chloride at higher concentrations ($1x10^{-3}$ M and $1x10^{-2}$ M) could completely inhibit the enzyme activity.

Influence of zinc chloride at pH 9.0 was investigated as a function of temperature and concentration of salt and shown in Fig.25B. Presence of zinc chloride at a concentration of $1x10^{-5}$ M did not bring any significant change in the activity at 25°C compared to control samples but a concentration of $1x10^{-4}$ M of zinc chloride decreased the activity by 42% and at $1x10^{-3}$ M concentrations the activity was reduced to 12% of original activity. Higher concentrations of salt completely inactivated the activity. When the activity studies were monitored in presence of $1x10^{-5}$ M zinc chloride it is reduced by 75% at 37°C and 97% at 45°C. The reduction in the activity was 88% at 37°C and 98% at 45°C in presence of $1x10^{-4}$ M zinc chloride. From these results it is observed that at higher concentrations (above $1x10^{-3}$ M zinc chloride) completely inactivated the ATPase activity of actomyosin.

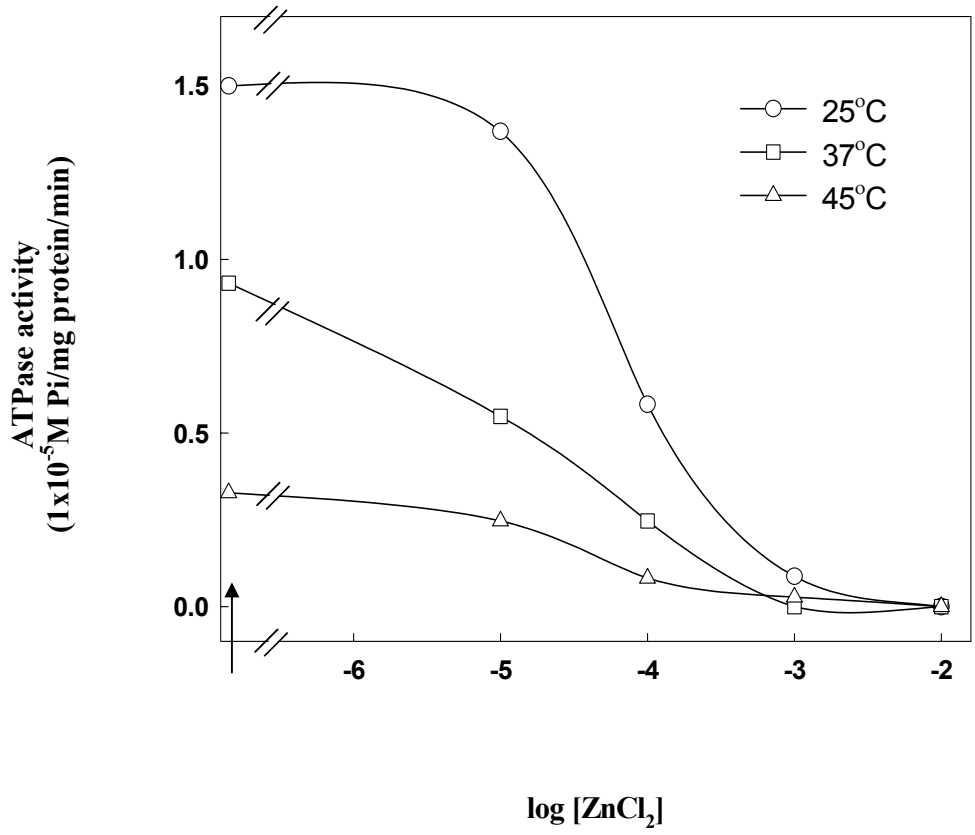


Fig. 25 A: ATPase activity of actomyosin from *Sardinella longiceps* in presence of different concentrations of $ZnCl_2$ at pH 7.0 as a function of temperature. Activity was determined with Tris-HCl pH 7.0 (0.1 M) containing 0.5M KCl. Activity was carried out at three different temperatures of 25°C, 37°C and 45°C. Enzyme activity was expressed as moles of inorganic phosphate released per mg of protein per minute. Arrow (↑) indicates the control values without $ZnCl_2$.

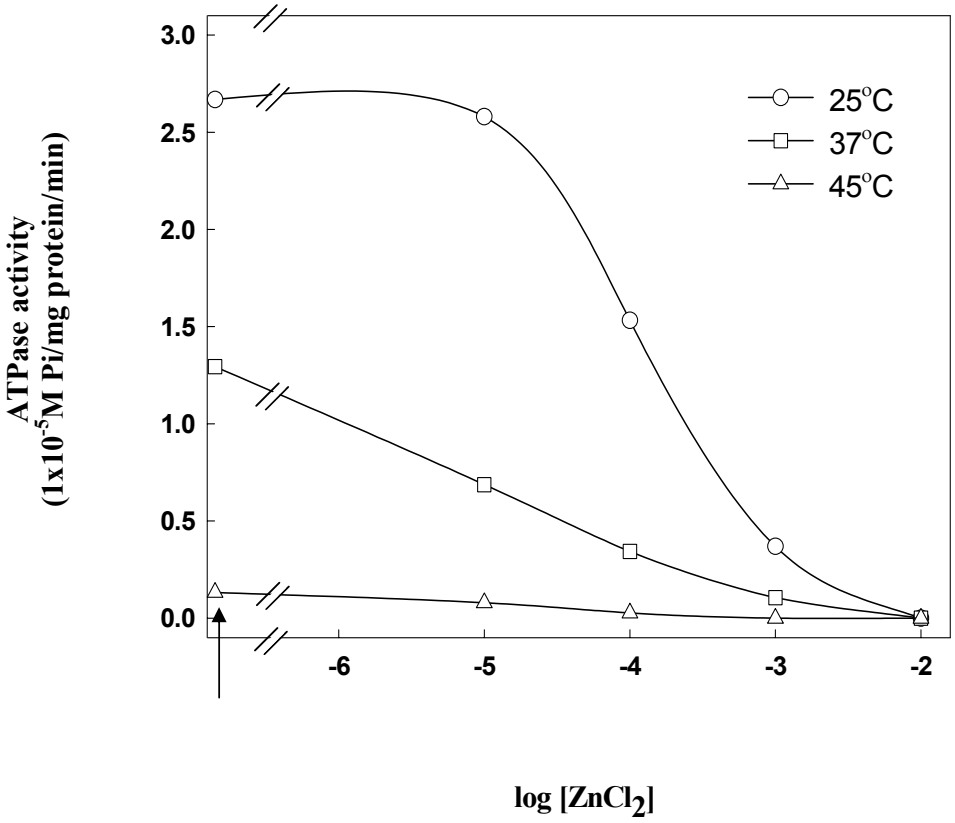


Fig. 25 B: ATPase activity of actomyosin from *Sardinella longiceps* in presence of various concentrations of $ZnCl_2$ at pH 9.0 containing 0.5 M KCl as a function of temperature. Enzyme activity was carried out with glycine-NaOH buffer pH 9.0 (0.2 M) at temperatures of 25°C, 37°C and 45°C. Enzyme activity was expressed as moles of inorganic phosphate released per mg protein per minute. Arrow (↑) indicates the control values without $ZnCl_2$.

The free SH content of actomyosin from sardine at different concentration of zinc chloride and zinc sulfate is given in Table 9. A progressive reduction in free SH groups with increase in concentration of zinc chloride and zinc sulfate is evident. The free SH content decreased from a value of 122 ± 12 μM/g actomyosin to 80 μM/g actomyosin in presence of $1x10^{-3}$ M zinc sulfate and 75 μM/g actomyosin in presence of $1x10^{-3}$ M zinc chloride. Reduction in the SH content of actomyosin is in concurrence with the inhibitory activity of zinc compounds can be either due to the blocking of SH groups in the active site of 50 kD ATP binding subunit of myosin molecules or by altering the structural arrangement of the molecule by the presence of these metal ions. The structural changes induced to the actomyosin by the presence of zinc are being followed by various biophysical techniques.

Intrinsic fluorescence emission spectra of actomyosin in presence of zinc salts were assessed at the pH 7.0 and pH 9.0 at 25°C. Emission spectra recorded in the wavelength of 300-400 nm after exciting at 280 nm. The intensity reduced significantly with increase in concentration of zinc sulfate at pH 7.0 (Fig.26A). The effect of zinc sulfate at different concentration on fluorescence emission spectra of actomyosin at pH 9.0 is given Fig.26B. With increase in zinc sulfate concentration the relative fluorescence intensity reduced significantly. The effect of zinc chloride on the fluorescence emission spectra of actomyosin at pH 7.0 is shown in Fig.27A and at pH 9.0 is shown in Fig.27B. However at pH 9.0 the effect of zinc chloride at different concentration on relative fluorescence intensity was minimum. There was small shift (3 nm) in the emission maximum in presence of higher concentrations of zinc salts in solutins. The effect of zinc chloride and zinc sulfate on the fluorescence emission spectra appears to be pH sensitive. In acidic conditions myosin rod may fully dissociate to due to electrostatic repulsion within the coiled coil while it may not dissociate in alkaline pH. Also it is reported that conformational changes in globular head and to form a molten globule in both acid and alkaline pH (Kristinsson and Hultin, 2003b). One distinct observation in the emission spectra was that zinc salts at a concentration of $1x10^{-5}$ M, pH 7.0 did not show difference in terms of relative fluorescence intensity compared to that of control. The reduction in the fluorescence intensity may due to the formation of aggregates by the actomyosin molecules, which

Table 9: Free SH content of fish actomyosin in presence of zinc salts in Tris-HCl buffer (0.1 M; pH 7.0 containing 0.5 M KCl). Free SH content of actomyosin was determined with Ellman's reagent and expressed as micromoles of free SH per gram of actomyosin.

Concentration of salts (M)	Zinc chloride	Zinc sulfate
Control (in buffer only)	122 ± 12	122 ± 12
$1x10^{-5}$	116 ± 11	119 ± 9
$1x10^{-4}$	112 ± 8	107 ± 6
$1x10^{-3}$	75 ± 6	80 ± 7

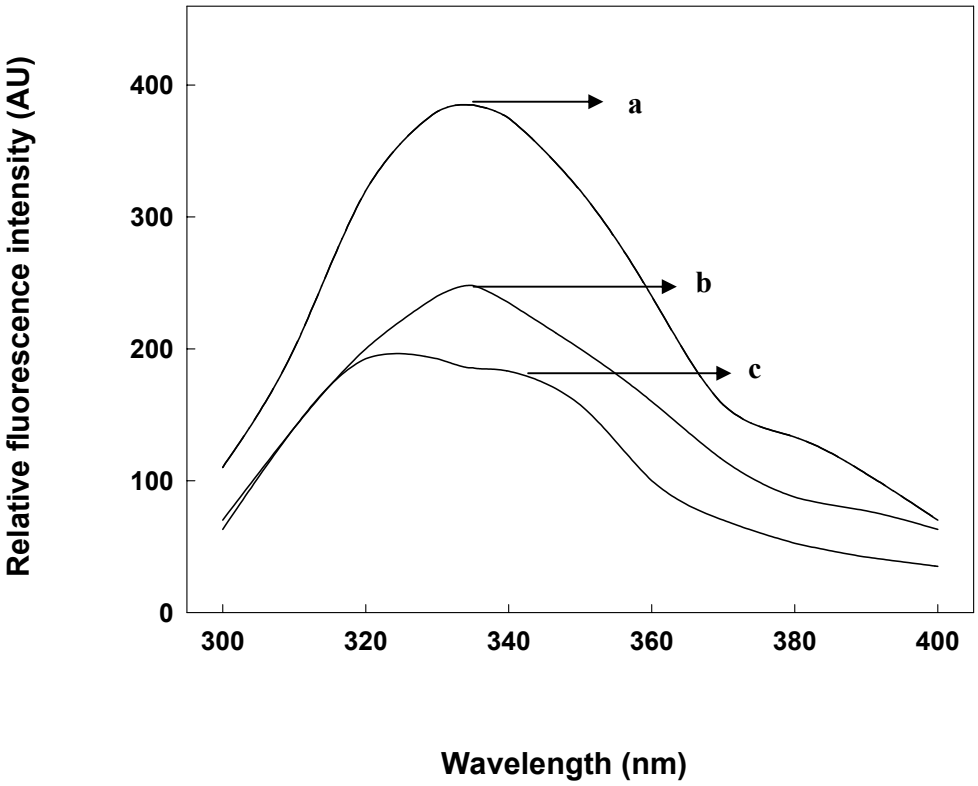


Fig. 26 A: Intrinsic fluorescence emission spectra of actomyosin in the presence of different concentrations of $ZnSO_4$ at pH 7.0 (Tris-HCl; 0.1 M) containing 0.5M KCl. Emission spectra recorded over a range of 300-400 nm. The excitation maximum was 280 nm. a) Control and $1x10^{-5}$ M $ZnSO_4$ (no difference in the fluorescence spectra)**; b) $1x10^{-4}$ M $ZnSO_4$ and c) $1x10^{-3}$ M and $1x10^{-2}$ M $ZnSO_4$** (no difference in the fluorescence spectra)**.**

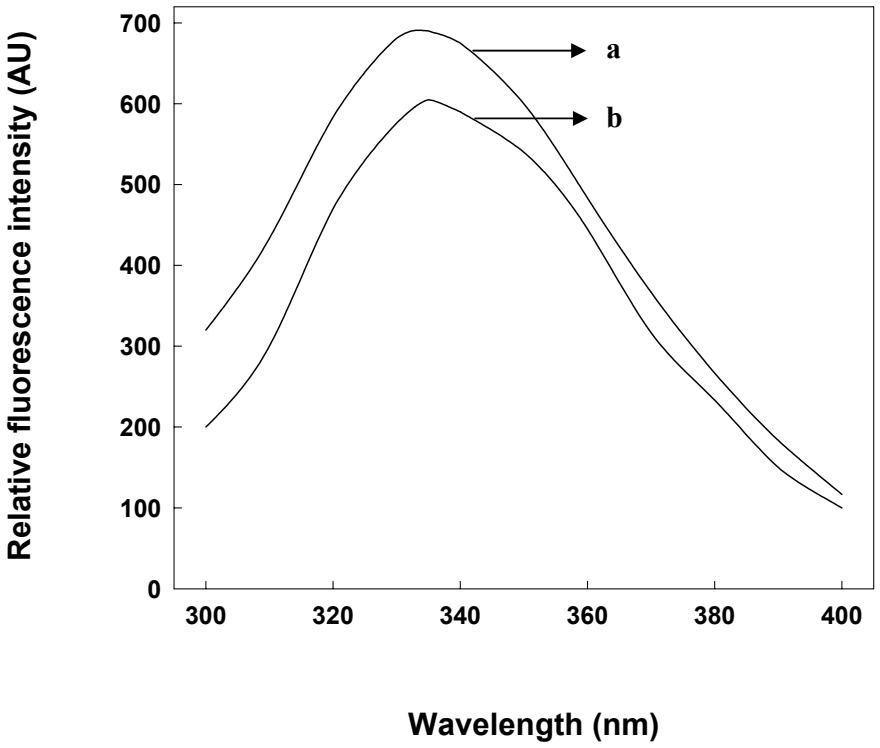


Fig. 26 B: Intrinsic fluorescence emission spectra of fish actomyosin in presence of different concentrations of $ZnSO_4$ at pH 9.0 (glycine-NaOH buffer; 0.2 M) containing 0.5 M KCl. Emission spectra recorded over a range of 300–400 nm. Excitation maximum was fixed at 280 nm. a) Control and b) $ZnSO_4$ at concentrations of $1x10^{-5}$ M, $1x10^{-4}$ M, $1x10^{-3}$ M and $1x10^{-2}$ M (no difference in the fluorescence spectra)**.**

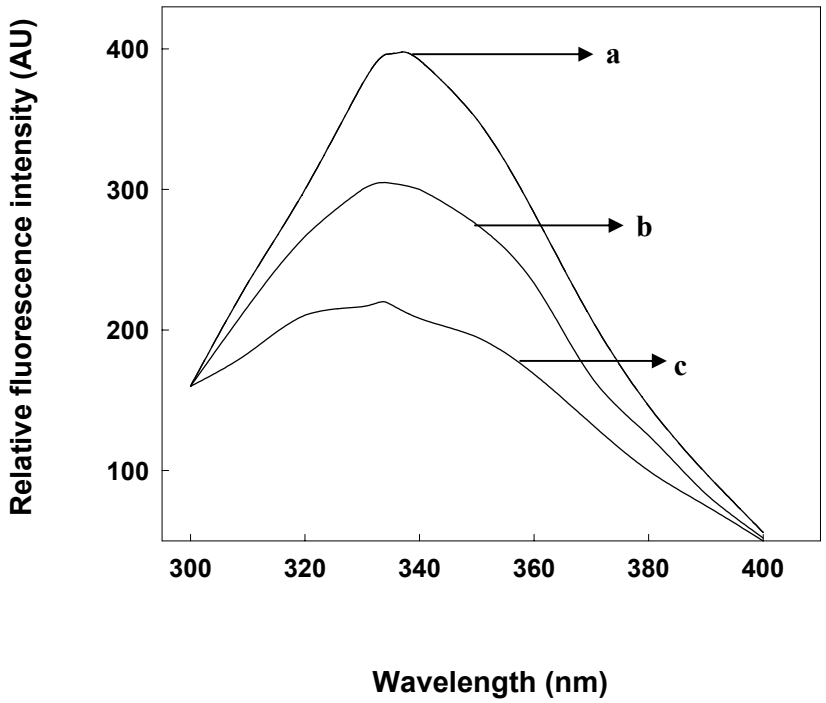


Fig. 27 A: Intrinsic fluorescence emission spectra of fish actomyosin in presence of different concentrations of $ZnCl_2$ at pH 7.0 (Tris-HCl; 0.1 M) containing 0.5 M KCl. Emission spectra were recorded over a range of 300–400 nm. Excitation maximum was fixed at 280 nm. a) Control and $1x10^{-5}$ M $ZnCl_2$ (no difference in the fluorescence spectra)**; b) $1x10^{-4}$ M $ZnCl_2$ and c) $1x10^{-3}$ M and $1x10^{-2}$ M $ZnCl_2$** (no difference in the fluorescence spectra)**.**

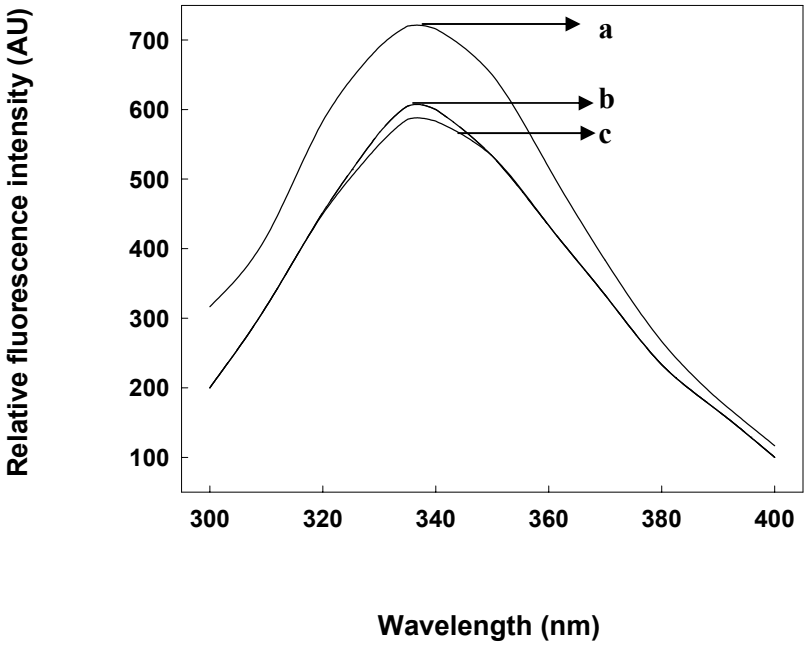


Fig. 27 B: Intrinsic fluorescence emission spectra of fish actomyosin in the presence of different concentrations of $ZnCl_2$ at pH 9.0 (glycine-NaOH; 0.2 M) containing 0.5 M KCl. Emission spectra recorded at a range of 300–400 nm. Excitation maximum was fixed at 280 nm. a) Control; b) $1x10^{-5}$ M $ZnCl_2$ and $1x10^{-4}$ M $ZnCl_2$ (no difference in the fluorescence spectra) **and c) $1x10^{-3}$ M and $1x10^{-2}$ M $ZnCl_2$**(no difference in the fluorescence spectra)**.**

may reduce the exposed tryptophan residues of the proteins trapped perhaps between the aggregated molecules. Alteration in the exposure of different amino acid residues will bring about changes in the hydrophobic and hydrophilic exposure of proteins. The exposure of hydrophobic groups can be determined by measuring the binding of dye ANS to the amino acid residues of the selected protein molecules.

The hydrophobicity index varied with the nature of compounds used. Hydrophobicity value of actomyosin decreased with increase in zinc concentration (Table 10). The reduction in the hydrophobicity of actomyosin followed the inhibitory pattern of enzyme activity due to the presence of zinc ions. zinc sulfate brought out more reduction in the hydrophobicity than zinc chloride in solutions. Since at higher concentrations of zinc compounds i.e. above $1x10^{-4}$ M the solutions were turned turbid the hydrophobicity values were not measured. The changes in conformations resulted in the exposure of hydrophobic residues of protein. Zinc ions might have induced the aggregation of proteins, which in turn effected the change in surface hydrophobicity values. Altered exposure of aminoacid residues and the subsequent changes in the hydrophobic nature of actomyosin molecules might have been due to the change in the secondary structure of protein molecules in the presence of these divalent cations. The turbidity development in presence of ANS and the zinc ions indicates an aggregation of molecules. The tail portion of myosin molecules is more prone to aggregation that can be followed by secondary structural determination of protein molecules. Since the major structure of tail is α-helix, the changes in the secondary structure of proteins is clearly indicated that the aggregation of process is accompanied by structural changes also.

The secondary structural analysis of fish actomyosin in presence of zinc sulfate at pH 7.0 is shown in Fig.28 and in presence of zinc chloride is shown in Fig.29. Far UV-CD spectra of fish actomyosin showed two troughs one at 208 nm and one at 222 nm. The analysis of data indicated that actomyosin has high helical content of 65%. The tail portion of myosin molecule contributes to the majority of helicity, which is a coiled coil structure. The α-helical content of proteins decreased in presence of zinc sulfate. In presence of $1x10^{-5}$ M zinc sulfate α-helicity reduced to

Table 10: Hydrophobicity values of fish actomyosin in presence of zinc salts* in Tris -HCl buffer (0.1 M; pH 7.0) containing 0.5 M KCl.

Concentration of salts (M)	Zinc chloride	Zinc sulfate
Control(in buffer only)	422 ± 20	422 ± 20
$1x10^{-5}$	389 ± 19	365 ± 15
$1x10^{-4}$	378 ± 23	289 ± 17

* Measurements above $1x10^{-4}$ M concentrations could not be performed due to the presence of turbidity in the solution.

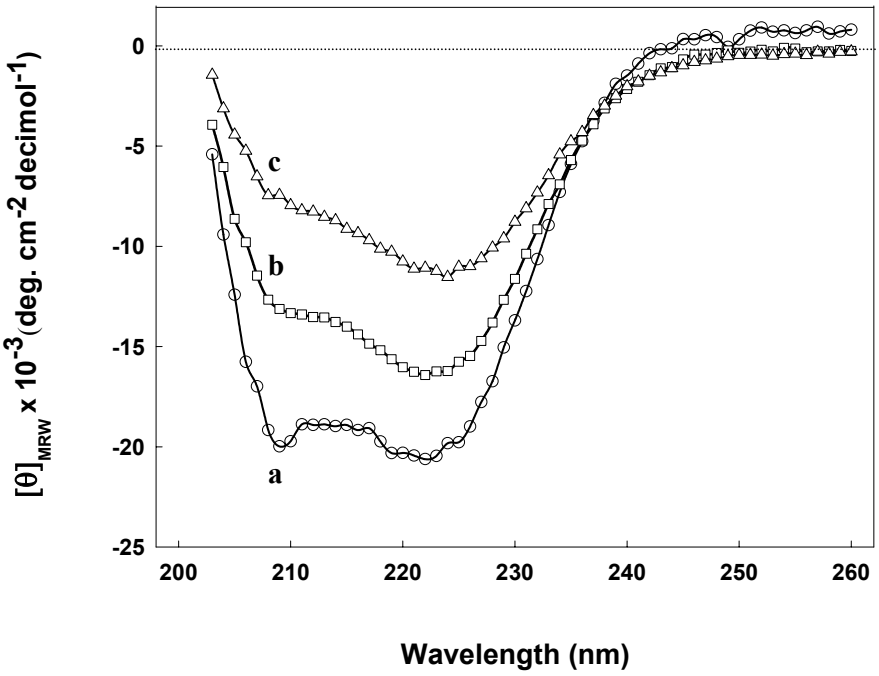


Fig. 28: Far UV-CD spectra of fish actomyosin in Tris-HCl buffer (pH 7.0) containing 0.5 M KCl as a function of $ZnSO_4$ concentration at 25°C. The concentrations of $ZnSO_4$ are a) Zero (Control); b) $1x10^{-5}$ M $ZnSO_4$ and c) $1x10^{-4}$ M and $1x10^{-3}$ M $ZnSO_4$ (no difference in the CD spectra)**.**

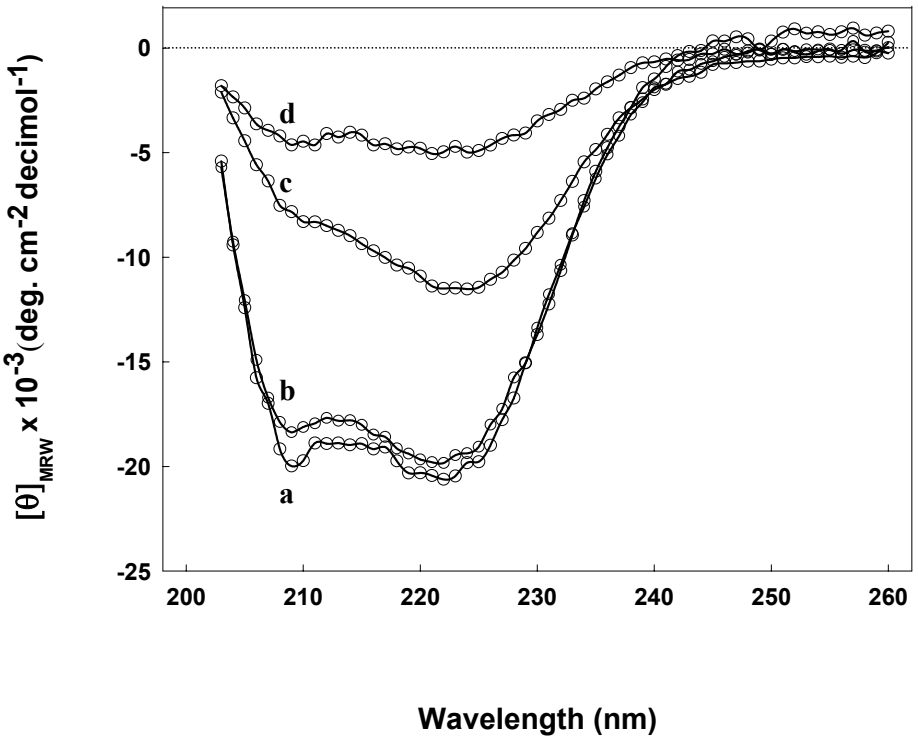


Fig. 29: Far UV-CD spectra of fish actomyosin in Tris-HCl buffer (pH 7.0) containing 0.5 M KCl as a function of $ZnCl_2$ concentration at 25°C. The concentrations of $ZnCl_2$ are a) Zero (Control); b) $1x10^{-5}$ M $ZnCl_2$ and c) $1x10^{-4}$ M and d) $1x10^{-3}$ M $ZnCl_2$.

Table 11: Secondary structure of fish actomyosin in presence of different concentrations of $ZnSO_4$ in 0.1 M Tris-HCl buffer containing 0.5 M KCl at pH 7.0

Zinc sulfate (M)	Secondary structure (%)		
	α-helix	β- structure	Aperiodic
Control (in buffer only)	65 ± 3	3 ± 1	32 ± 2
$1x10^{-5}$	51 ± 2	15 ± 1	34 ± 2
$1x10^{-4}$	39 ± 2	29 ± 1	32 ± 2
$1x10^{-3}$	39 ± 2	28 ± 1	33 ± 2

Table 12: Secondary structure of fish actomyosin in presence of different concentrations of $ZnCl_2$ in 0.1 M Tris-HCl buffer containing 0.5 M KCl at pH 7.0.

Zinc chloride (M)	Secondary structure (%)		
	α-helix	β- structure	Aperiodic
Control (in buffer only)	65 ± 3	3 ± 1	32 ± 2
$1x10^{-5}$	62 ± 2	8 ± 1	30 ± 2
$1x10^{-4}$	40 ± 2	27 ± 1	33 ± 2
$1x10^{-3}$	21 ± 2	43 ± 1	36 ± 2

51% and a further increase in concentration of zinc sulfate to $1x10^{-3}$ M reduced it to 39% (Table11). There was concomitant increase in β-structure with reduction in α-helicity and the aperiodic structure remained same. Zinc chloride was more potent in bringing down the secondary structural changes in fish actomyosin. The change in the molar ellipticity is considerably larger in presence of zinc chloride than in presence of zinc sulfate. The α- helicity of actomyosin molecules reduced from 65% to 21% in the presence of $1x10^{-3}$ M zinc chloride (Table 12). The β-structure increased with increase in concentration of zinc chloride and the aperiodic structure also shown an increase with increase in zinc chloride. The binding of zinc allows relatively short stretches of polypeptide chains to fold into well-defined units that are well suited to participating in macromolecular interactions (Berg and Shi, 1996). Reduction in α-helicity conformation of actomyosin correlated well with decrease in ATPase activity. Reduced exposure of hydrophobic groups and fluorescence intensity, which indicates the role of zinc in inducing the conformational changes of actomyosin. The conformational change could lead to aggregation and altered solubility profile. In order to delineate the effect of zinc salts on the solubility of actomyosin a detailed investigation on the precipitation profile have been studied.

The effect of zinc sulfate on the extent of precipitation of actomyosin at pH 7.0 and 9.0 is shown in Fig.30. Low concentrations of zinc sulfate did not bring considerable changes in the precipitation of actomyosin. At pH 7.0 it brought only 5% precipitation and at pH 9.0 it did not make any difference in the precipitation of actomyosin. A steep increase the precipitation of proteins observed when the concentration of zinc sulfate increased to $1x10^{-4}$ M and further increase in zinc sulfate resulted a marginal reduction in precipitation at pH 7.0. At pH 9.0 the extent of precipitation remained almost same when the zinc sulfate concentration increased up to $1x10^{-4}$ M and the increase in precipitation was gradual up to a concentration of $1x10^{-3}$ M zinc sulfate and after that it showed a considerable increase in precipitation with increase in concentration of zinc sulfate.

The precipitation profile of actomyosin in presence of zinc chloride is shown in Fig.31. Precipitation of actomyosin in presence of zinc chloride also followed same

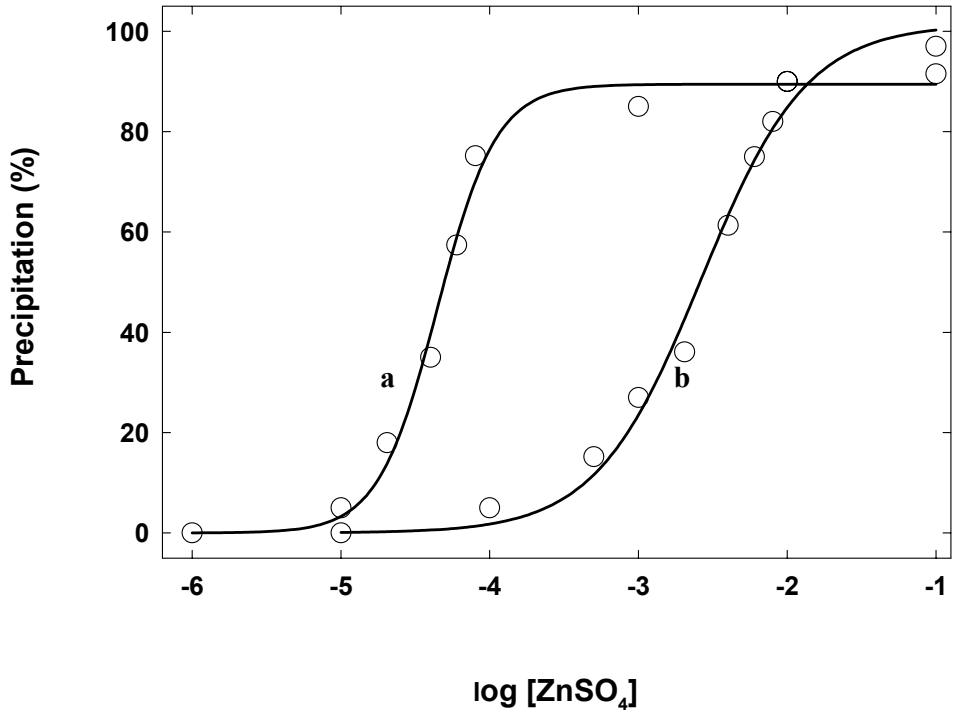


Fig. 30: Precipitation profile of actomyosin in presence of $ZnSO_4$ at 25°C. a) Tris- HCl buffer (0.1 M) pH 7.0 containing 0.5M KCl and b) glycine-NaOH buffer (0.2M) pH 9.0 containing 0.5 M KCl.

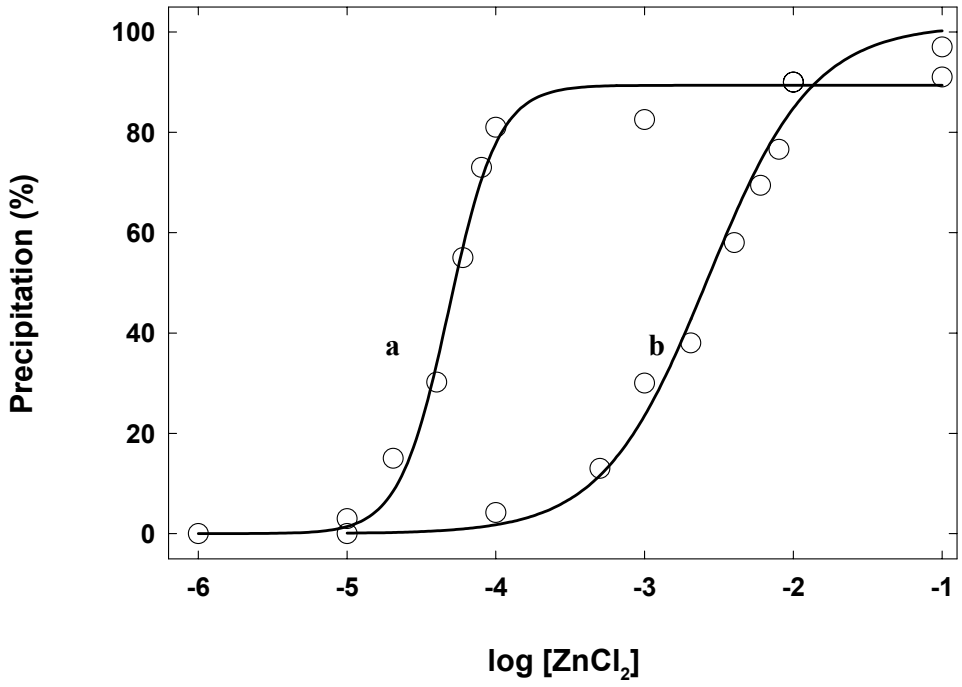


Fig. 31: Precipitation profile of actomyosin in presence of $ZnCl_2$ at 25°C. a) Tris-HCl buffer (0.1M) pH 7.0 containing 0.5 M KCl and b) glycine- NaOH buffer (0.2 M) pH 9.0 containing 0.5 M KCl.

pattern as that of zinc sulfate. At pH 7.0 the presence of $1x10^{-5}$ M zinc chloride did not make any significant change in precipitation of fish actomyosin. It resulted in increase in precipitation when the concentration is increased to $1x10^{-4}$ M zinc chloride (Fig.31). At pH 9.0 the increase in precipitation of actomyosin was not significant up to a concentration of $1x10^{-4}$ M zinc chloride. When the concentration of metal ion was increased to $1x10^{-3}$ M zinc chloride it resulted in 30% precipitation of protein. Ten times increase in concentration of zinc salt i.e. to $1x10^{-2}$ M zinc chloride resulted in 90% precipitation of proteins. The precipitation profile of actomyosin in presence of zinc salts followed a sigmoid pattern and at pH 7.0 it reached a plateau above $1x10^{-4}$ M concentration of both salts. At pH 9.0 the curve showed plateau region above $1x10^{-2}$ M concentrations of zinc salts.

The above results infer that ATPase enzyme activity of actomyosin from sardine decreased in presence of zinc salts. ATPase enzyme activity of actomyosin was not affected at very low concentrations of zinc. But higher concentrations of zinc salts in the reaction mixture showed a decrease in the activity of actomyosin and completely inhibited at a concentration of $1x10^{-3}$ M. The decrease in ATPase activity can induces a protective effect of actomyosin during further storage and processing. The reduction in ATPase activity resulted to have higher concentration of the ATP molecules in the meat, which has protective effect on muscle protein as described in literature. The nucleotides ATP, ADP and IMP have been shown to exert a protective effect on fish actomyosin stored at -20ºC, while nucleotide catabolites ionosine and hypoxanthine destabilized these proteins (Jiang *et al*., 1987). The loss of water holding capacity arising from the formation of actomyosin when *in vivo* ATP level falls and rigor mortis ensues and the efficacy of pyrophoshphate in improving it in minces, suggests that there would be some benefit in keeping the ATP high postmortem (Lowrie, 1998). The reduction in the ATP splitting activity of actomyosin in turn help to keep the concentration of ATP in the muscle at high levels. The presence of ATP in the muscle tissue of a freshly slaughtered animal allows the muscle to retain its elasticity by inhibiting the molecular interaction between the myofibrillar proteins, and as a consequence it can retain a higher water holding capacity (Guylinder and Lowrie, 1999). The reduction in the splitting of ATP thus

could induce positive effect on the quality of muscle proteins during handling and storage.

The two different zinc salts, which have been selected to study the effect on the enzyme activity, showed inhibitory effect. This inhibitory effect was evident in both pH conditions of 7.0 and 9.0. To determine whether the activity of enzyme was affected by temperature the reaction was carried out at 25°C, 37°C and 45°C. With increase in temperature from 25°C to 45°C the activity of ATPase decreased by 80%. Fish myosin is generally unstable and easily and rapidly form aggregates with concomitant decrease of ATPase activity (Ochiai and Chow, 2000). Heat induced formation of myosin oligomers precedes inactivation of myosin ATPase (Kimura *et al*, 1980). The decrease in activity by the increase in temperature of reaction is mainly contributed by the structural or conformational changes. The structural changes might have been contributed by the residues present at the active site of myosin that is responsible for the ATPase activity of actomyosin complex. The nucleotide binding pocket of myosin has a highly conserved segment contains the two sulfhydryl groups which are more reactive than the other sulfhydryl and termed SH1 and SH2 respectively in order of their chemical reactivity. The two reactive sulfhydryl groups were separated by a α-helix. The segment that follows the reactive sulfhydryl group consists of a small free stranded antiparallel β sheets that include residues Arg^{714} to Tyr^{717}, Tyr^{758} to Gly ^{761} and Lys ^{764} to Phe^{767} and is associated with two short helices.

Free SH content of the actomyosin decreased with increase in concentration of zinc salts. The importance of free SH content in the myosin ATPase enzyme has been demonstrated as early as 1946 by Engelhardt. Wang *et al*. (1997) suggested a close relationship between Ca^{2+}ATPase activity and sulfhydryl content in fresh beef heart surimi but not for frozen stored surimi. Zinc can interact strongly with a variety of ligand types including sulfur from cysteine, nitrogen from histidine, oxygen from glutamate, aspartic acid and water (Berg and Shi, 1996). The generation of a tetrahedral site from a combination of cysteine and histidine residues will automatically have a preferential affinity for zinc over most other common metal ions (Berg and Merkle, 1989). Binding of zinc to the free SH groups present at the active

site of the nucleotide binding cleft of myosin S1 induced the decrease in ATPase activity. The reduction in SH content correlated well with decrease in activity of actomyosin by zinc salts. This clearly indicates the relationship between these two parameters. Reduction in the SH content of actomyosin in concurrence with the inhibitory activity of zinc compounds can be either due to the blocking of SH groups in the active site of 50 kD ATP binding subunit of myosin molecules or by altering the structural arrangement of the molecule by the presence of these metal ions.

Intrinsic fluorescence intensity is a good indicator of structural changes in actomyosin molecules. Change in the microenvironment of tryptophan residues or a change in exposure of these residues lead to a drastic change in the fluorescence profile from the proteins. In the present study, the effect of zinc salts at pH 7.0 and 9.0 on the relative fluorescence intensity of actomyosin have been assessed. This reduction in the intensity might have been due to the masking of tryptophan residues present in the active nucleotide binding site of myosin molecule. Myosin has tryptophan residues both in its rod and head regions (Kato and Konno, 1993). It was cited in the literature that the change in tail conformation also brought changes in fluorescence profile. The reduction in the fluorescence intensity may due to the formation of aggregates by the actomyosin molecules, which reduced the exposed tryptophan residues of the proteins. Alteration in the exposure of different amino acid residues will bring about changes in the hydrophobicity of proteins. There was reduction in the surface hydrophobicity of actomyosin in presence of zinc salts. There was variation in the hydrophobic index of actomyosin between the salts used to inhibit the enzyme activity of actomyosin. The turbidity development at higher concentrations of zinc salts in presence of ANS indicates aggregation of actomyosin molecules.

The changes in structure of protein molecules due to the presence of these salts studied by secondary structural determination of protein molecules. α-helix is the major secondary structure of actomyosin. The secondary structure of actomyosin was altered in presence of zinc salts. The decrease in α-helicity of protein molecules with decrease in ATPase activity clearly showed that the structural changes are the

major contributor for the inhibitory effect of these salts. Since the major part of tail is of α-helix, the changes in the α-helicity of proteins clearly indicated a cation-induced aggregation of protein molecules.

The effective reduction in ATPase activity of actomyosin is mainly due to zinc induced conformational changes of the protein in solutions. The relationship between ATPase activity and structure of actomyosin was investigated by various authors (Chan *et al*., 1995; Ogawa *et al*., 1996; Togashi *et al*., 2002). Loss of helicity indicated the metal ion have induced the aggregation of fish actomyosin, which is mainly responsible for the inhibition of the activity and the changes in fluorescence and hydrophobic pattern of molecules in the given conditions. The aggregations of molecules are mainly contributed by the structural changes induced to tail portion of molecules. These changes will bring alterations in the solubility profile of actomyosin in buffers. The precipitation of protein molecules was determined in presence of zinc compounds. Actomyosin was precipitated by the presence of zinc salts. Precipitation effect of zinc salts was more at pH 7.0. At alkaline pH (pH 9.0) the precipitation was less and the extent of precipitation was 27% proteins at a concentration of $1x10^{-3}$ M of zinc sulfate. Zinc chloride also followed the same pattern of precipitation. At pH 7.0 conditions were more favourable for precipitation of actomyosin by zinc salts.

Low concentrations of some other metal ions like copper and silver also reported to have inhibitory effect on the ATPase activity of myosin and magnesium ions have been reported to inhibit the activity while the calcium ions are essential for activity of actin activated ATPase (Perry, 1955). When Ca^{2+} ions bind to the SR Ca^{2+}ATPase it underwent a conformational change with the appearance of a new thermal transition at 60°C with a 50°C transition in DSC profile (Pang and Chen, 2004). It has been hypothesized that proteins fold so as to pack the apolar domains in a nearly crystalline state and the denaturation often results in protein unfolding their interior hydrophobic surfaces (Chan *et al*., 1995). Chan *et al*. (1992) reported that fish myosin lost its helical content with simultaneous exposure of interior hydrophobic surface when the proteins were thermally denatured. The binding of zinc to the actomyosin induced conformational changes to the actomyosin molecules in

solutions. This metal ion forms complexes with the free SH groups in the active site of myosin head leads to the loss of enzyme activity to the actomyosin molecules. The inhibitory activity of zinc compounds is pH and temperature dependent. From the results of interaction studies of zinc ions with actomyosin clearly indicated that these ions induce the structural alteration with the inhibition of ATPase enzyme activity. Also it brings down the solubility profile of proteins by the process of aggregation of actomyosin. Thus the presence of zinc ion in the fish muscle system may result in the loss of functionality of proteins and may reduce the quality of the product at higher concentrations. The role of other divalent cations like calcium on the structure and activity of actomyosin need to be investigated for the potential use of these compounds as stabilizer molecules.

B) Effect of divalent cation calcium on the structure and enzyme activity of actomyosin from sardine

Calcium is the fifth most abundant element on the earth and is ubiquitous in biological organisms. The calcium binding proteins can be classified in two groups. The first group includes enzymes stabilized by calcium ions, some of these using the calcium ions in catalysis. The second group comprises proteins that bind calcium ions reversibly and, as a result, modulate the action of other proteins or enzymes (Glusker, 1991). In some cases bound calcium ions found to stabilize the proteins against thermal or chaotropic denaturation and also protects against the proteolytic degradation (McPhalen *et al*., 1991). Incorporation of divalent metal ions like calcium improved the shear stress of surimi (Lee and Park, 1998; Morales *et al*, 2001). Myosin molecules showed ATP degrading action in presence of calcium ions. It is important to know whether the presence of calcium brings about any change in the structure of actomyosin for its use in biotechnological applications. Calcium chloride is a soluble and most commonly used salt of calcium. The effect of different concentrations of calcium salts like calcium chloride and calcium sulfate on the structure and ATPase enzyme activity of fish actomyosin was studied.

The effect of calcium chloride on the ATPase activity of actomyosin is shown in Fig.32. Calcium dependence of ATPase activity was carried out at two selected pH

conditions of 7.0 and 9.0. In both the pH conditions the activity was enhanced significantly at a concentration of $1x10^{-2}$ M calcium chloride. Lower concentrations of calcium chloride were not able to enhance the enzymatic activity. At concentration of $1x10^{-4}$ M calcium chloride the activation was only 38% of maximum activity at pH 7.0 and only 18% of maximum activity at pH 9.0. Higher concentrations of calcium inhibited the enzyme activity. At any given concentration of calcium chloride above $1x10^{-2}$ M at pH 9.0 the activity was much higher than that was at 7.0.

The effect of calcium sulfate at different concentrations and pH on the ATPase enzyme activity of actomyosin is shown in Fig.33. The maximum activity was recorded at a concentration of $1x10^{-3}$ M, pH 9.0 and at pH 7.0 there was a steep increase in activity with increase in concentration from $1x10^{-4}$ M to $1x10^{-2}$ M.

Intrinsic fluorescence emission spectra of fish actomyosin in presence of both calcium salts (calcium chloride and calcium sulfate) at pH 7.0 are shown in Fig.34A & 35A. The fluorescence spectra of actomyosin was not altered in presence of either of the calcium salts. There was a slight reduction in the intensity of fluorescence but it was not significant. The fluorescence emission spectra at pH 9.0 in presence of calcium chloride is shown in Fig.34B and in presence of calcium sulfate is shown in Fig.35B. Fluorescence spectra remained unchanged in presence of calcium salts. There was no change in emission maxima by the presence of different concentrations of calcium salts.

The far UV-CD spectra of actomyosin in presence of calcium chloride are shown in the Fig.36. The secondary structural content of actomyosin was not affected significantly in presence of calcium chloride. The far UV-CD spectra of actomyosin in presence of calcium sulfate are shown in Fig.37. The structure of actomyosin remained almost same with increase in concentration of calcium salts. There were no significant changes in the secondary structure of actomyosin with addition of calcium ions.

It is evident from the fluorescence and CD spectra that the addition of calcium ions have not altered the structure of fish actomyosin. Activity of ATPase enzyme is

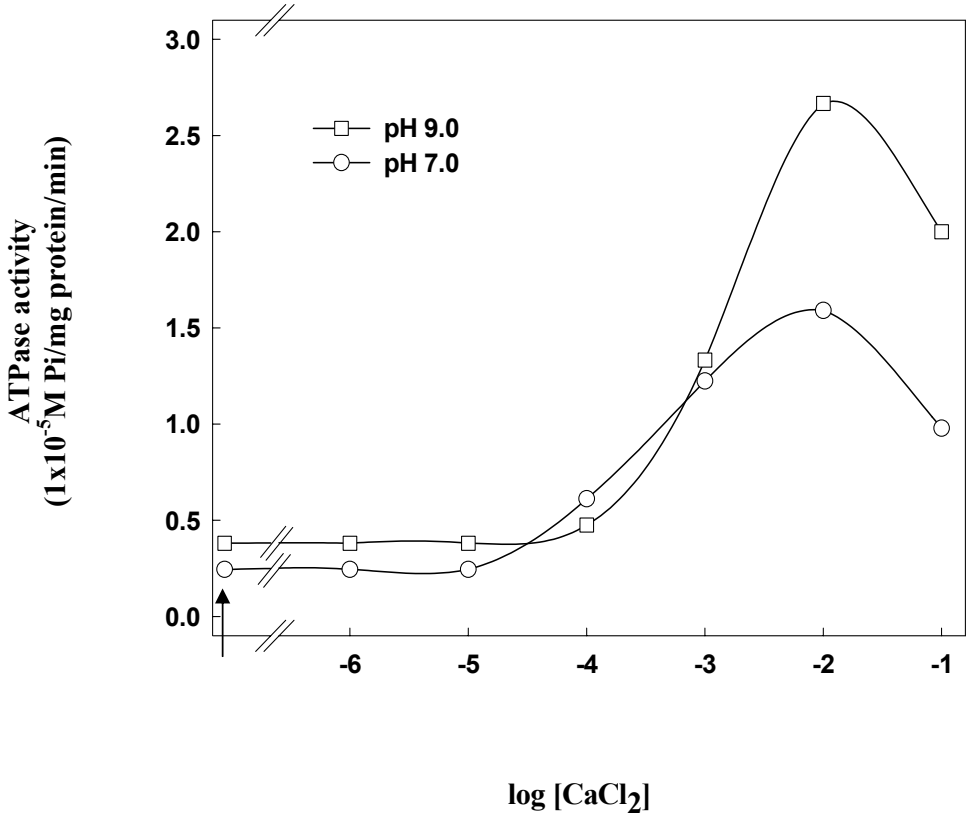


Fig. 32: ATPase activity of actomyosin from *Sardinella longiceps* in presence of different concentration of $CaCl_2$ at pH 7.0 (Tris-HCl buffer pH 7.0 (0.1 M) containing 0.5 M KCl) and pH 9.0 (glycine-NaOH buffer pH 9.0 (0.2 M) containing 0.5 M KCl). Enzyme activity was expressed as moles of inorganic phosphate released per mg protein per minute. Arrow (↑) indicates the control values without $CaCl_2$.

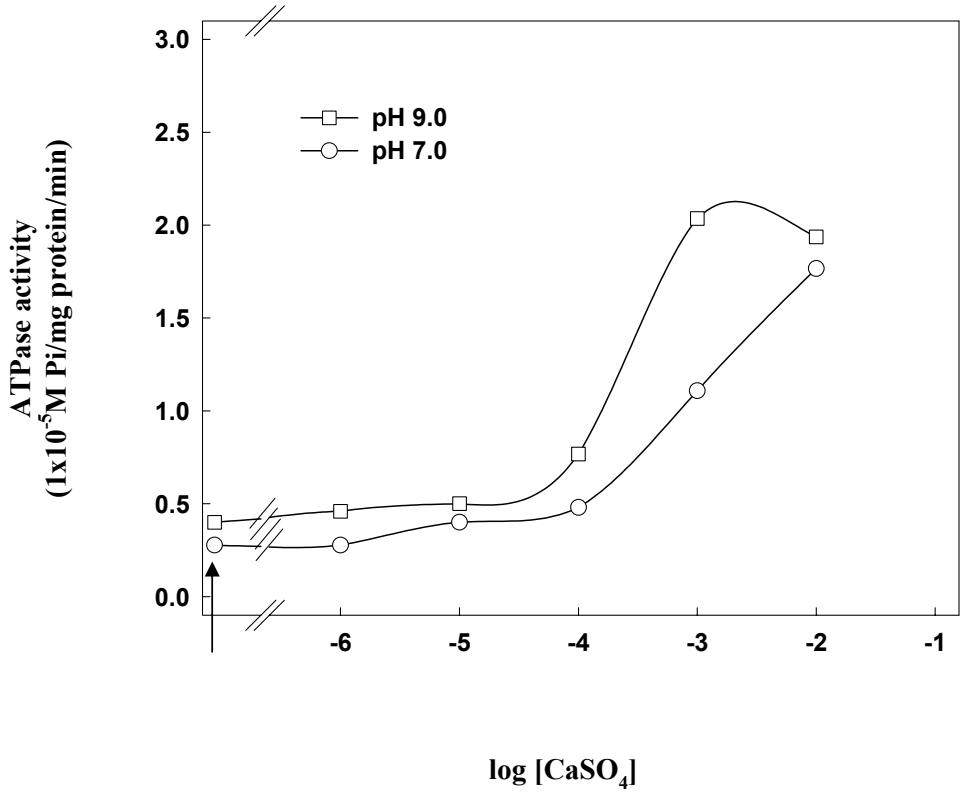


Fig. 33: ATPase activity of actomyosin from *Sardinella longiceps* in presence of various concentration of $CaSO_4$ at pH 7.0 (Tris-HCl buffer pH 7.0 (0.1 M) containing 0.5 M KCl and pH 9.0 (glycine-NaOH buffer pH 9.0 (0.2 M) containing 0.5 M KCl. Enzyme activity was expressed as the moles of inorganic phosphate released per mg protein per minute. Arrow (↑) indicates the control values without $CaSO_4$.

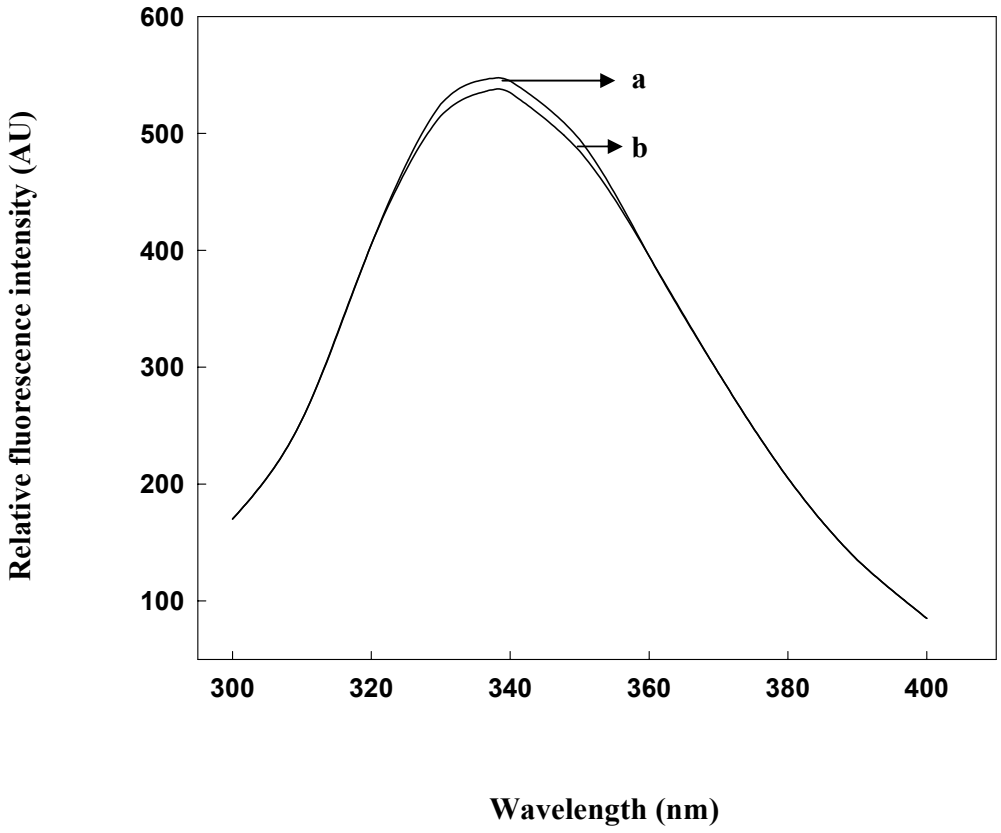


Fig. 34 A: Intrinsic fluorescence emission spectra of fish actomyosin in presence of different concentrations of $CaCl_2$ at pH 7.0 (Tris-HCl; 0.1 M) containing 0.5 M KCl. Emission spectra were recorded over a range of 300-400 nm. Excitation maximum at 280 nm. a) Control and b) $CaCl_2$ $1x10^{-5}$ M, $1x10^{-4}$ M, $1x10^{-3}$ M, $1x10^{-2}$ M and $1x10^{-1}$ M (no difference in the fluorescence spectra)**.**

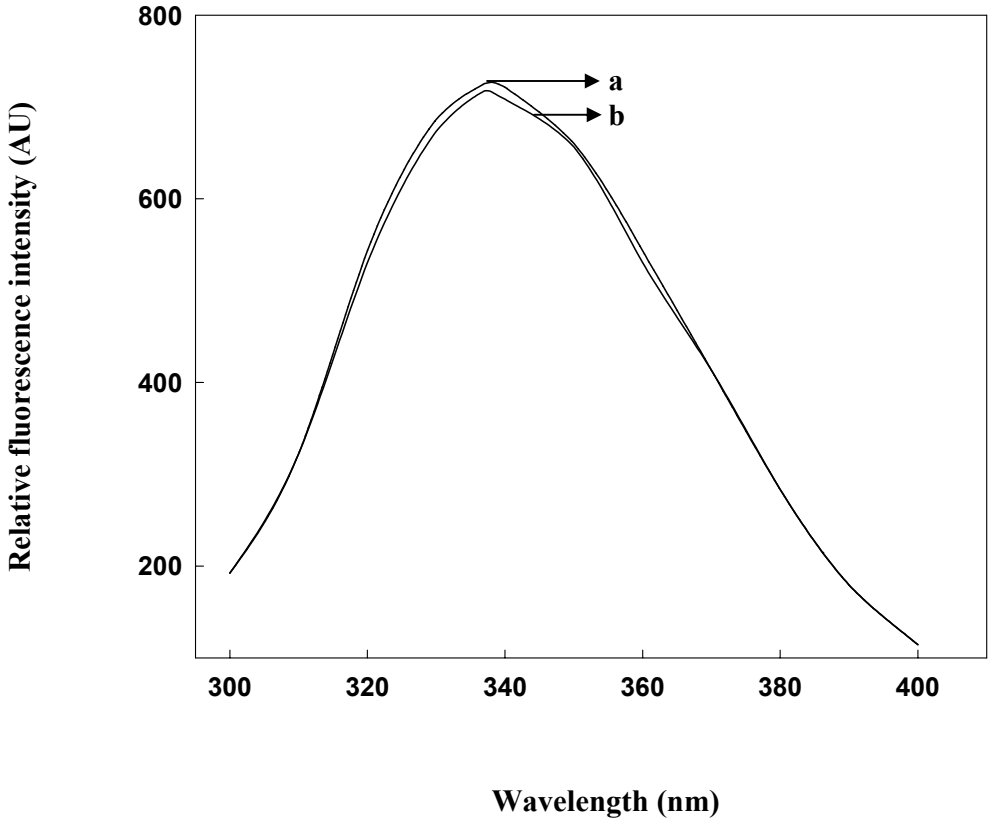


Fig. 34 B: Intrinsic fluorescence emission spectra of fish actomyosin in presence of different concentrations of $CaCl_2$ at pH 9.0 (glycine-NaOH; 0.2 M) containing 0.5 M KCl. Emission spectra was recorded over a range of 300-400 nm. Excitation maximum at 280 nm. a) Control and b) $CaCl_2$ $1x10^{-5}$ M, $1x10^{-4}$ M, $1x10^{-3}$ M, $1x10^{-2}$ M and $1x10^{-1}$ M (no difference in the fluorescence spectra)**.**

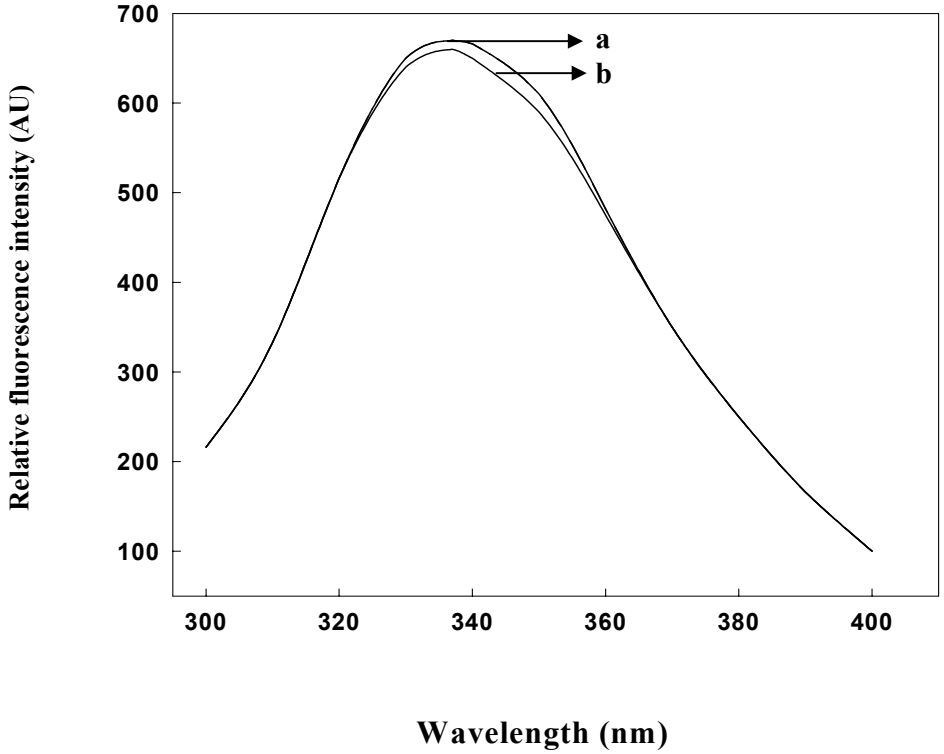


Fig. 35 A: Intrinsic fluorescence emission spectra of fish actomyosin in presence of different concentrations of $CaSO_4$ at pH 7.0 (Tris-HCl; 0.1 M) containing 0.5 M KCl. Emission spectra was recorded over a range of 300-400nm. Excitation maximum was 280 nm. a) Control and b) $CaSO_4$ $1x10^{-5}$ M, $1x10^{-4}$ M, $1x10^{-3}$ M, $1x10^{-2}$ M and $1x10^{-1}$ M (no difference in the fluorescence spectra)**.**

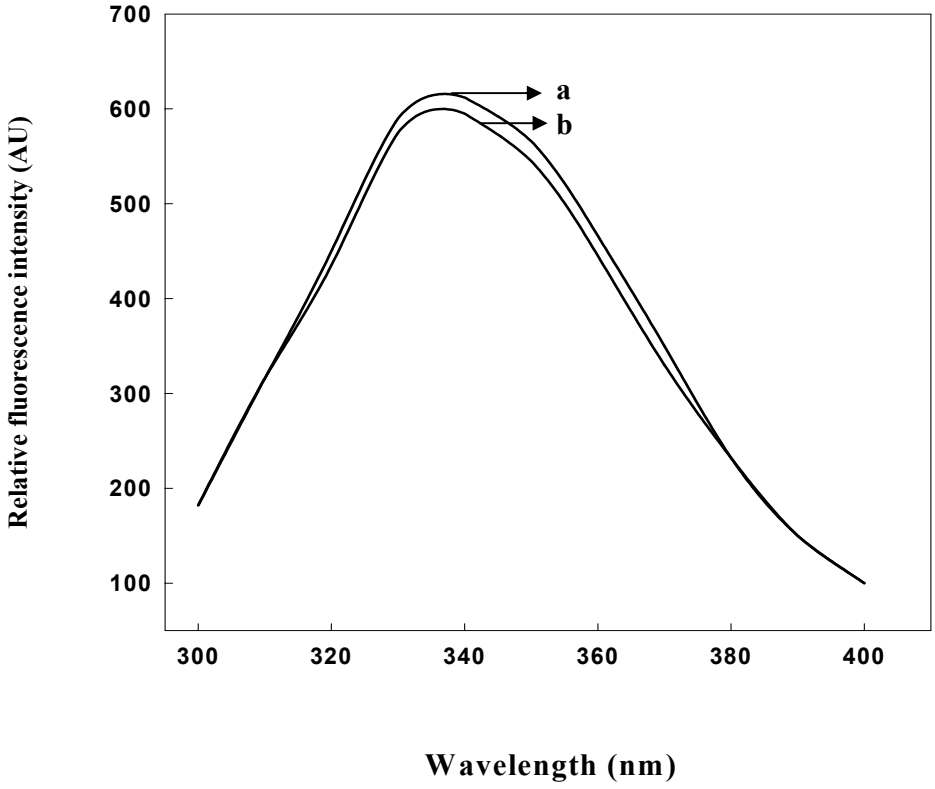


Fig. 35 B: Intrinsic fluorescence emission spectra of fish actomyosin in presence of different concentrations of $CaSO_4$ at pH 9.0 (glycine-NaOH; 0.2 M) containing 0.5 M KCl. Emission spectra was recorded over a range of 300-400 nm. Excitation maximum was 280 nm. a) Control and b) $CaSO_4$ $1x10^{-5}$M, $1x10^{-4}$ M, $1x10^{-3}$ M, $1x10^{-2}$ M and $1x10^{-1}$ M (no difference in the fluorescence spectra)**.**

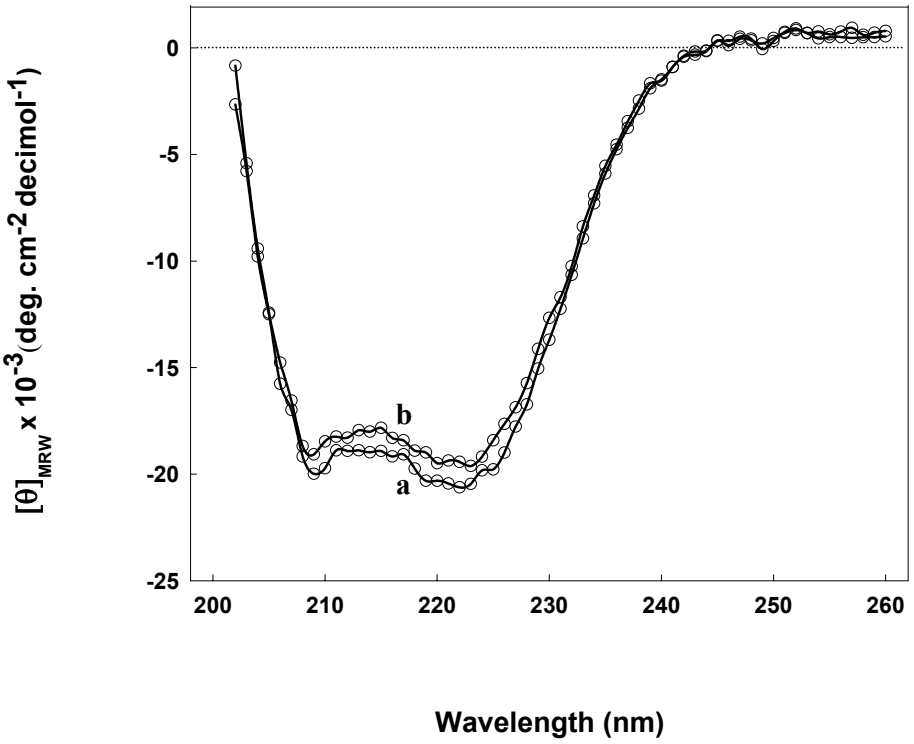


Fig. 36: Far UV-CD spectra of fish actomyosin in presence of different concentrations of $CaCl_2$ in 0.1 M Tris - HCl buffer pH 7.0 containing 0.5 M KCl at 25^{o}C. a) Control and b) $CaCl_2$ 1x10^{-5} M, 1x10^{-4} M, 1x10^{-3} M and 1x10^{-2} M (no difference in the CD spectra)**.**

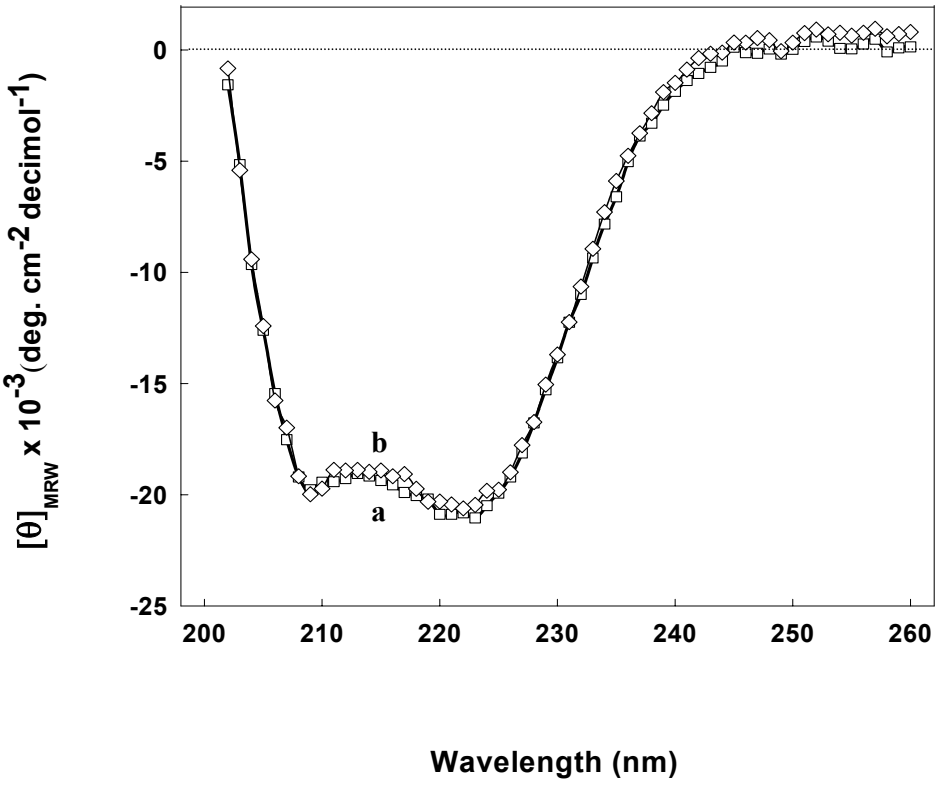


Fig. 37: Far UV-CD spectra of fish actomyosin in presence of different concentrations of $CaSO_4$ in 0.1 M Tris-HCl buffer pH 7.0 containing 0.5 M KCl at 25°C. a) Control and b) $CaSO_4$ $1x10^{-5}$ M, $1x10^{-4}$ M, $1x10^{-3}$ M and $1x10^{-2}$ M (no difference in the CD spectra)**.**

increased in the presence of higher concentrations of calcium chloride. Lower concentrations of calcium ions is found to be not effective in increasing the enzyme activity of actomyosin. Hence lower concentrations of calcium salts can be used as a stabilizing agent during frozen storage. Addition of zinc salts were found to decrease the enzyme activity. It also alters structure of actomyosin. The utility of these results would be reflected in the keeping quality of fish mince during storage at low temperature where one desires the structural integrity to be retained for ultimately having a product which is also of functionally stable. These results would thus have wide application in incorporating such additives in the enhancement of shelf life of fish mince and its quality.

CHAPTER - 3

INFLUENCE OF POLYHYDRIC ALCOHOLS ON THE STRUCTURE AND ATPase ENZYME ACTIVITY OF ACTOMYOSIN FROM OIL SARDINE

INFLUENCE OF POLYHYDRIC ALCOHOLS ON THE STRUCTURE AND ATPase ENZYME ACTIVITY OF ACTOMYOSIN FROM OIL SARDINE

In this chapter the effect of polyhydric alcohols mainly sorbitol and mannitol on the structure and enzyme activity of actomyosin have been studied. In order to stabilize proteins in fish mince during frozen storage sucrose and sorbitol at various concentrations are commercially used. Sugars and sugar alcohols are used as cryoprotective agents for fish proteins during storage. Sugars added to myosin filament suspensions or myofibrils form hydrogen bonds with some amino acids residues including charged amino acids of myosin rods via hydroxyl groups of sugars and sorbitol (Konno *et al*., 1997). There are several physiological corollaries of the qualities of sugar alcohols, one of them being their osmotically active nature. This quality not only affects the function of sugar alcohols as food, but also in numerous intracellular events and medical applications. Because of their polyol nature, some sugar alcohols (D-mannitol, for example) with the right configuration can act as free radical scavengers in biological and experimental systems. Sorbitol promoted the soulbilization of myofibrillar proteins at physiological concentrations of NaCl (Konno *et al*., 1997). The influence of two selected polyhydric alcohols sorbitol and mannitol on the structure and enzyme activity of actomyosin from sardine was studied in detail using biophysical and enzymatic tools to understand the mechanisms of action.

ATPase activity of actomyosin was monitored as a function of polyhydric alcohol concentration, pH and temperature. The polyhydric alcohols selected was sorbitol and mannitol at a concentrations range up to 10% in the reaction mixture. The pHs selected were pH 7.0 and pH 9.0 where the ATPase activity showed higher activity peaks.

Effect of sorbitol at different concentrations and temperature at pH 9.0 on the activity profile of ATPase enzyme activity is shown in Fig.38A. The activity of enzyme decreased linearly with increase in sorbitol concentration with increase in incubation temperature. The activity reduced and more significantly at 45°C. At a concentration of 8-10% at 25°C, pH 9.0 the activity was almost nil. So it is evident at pH 9.0 both temperature and sorbitol concentration had significant effect on the

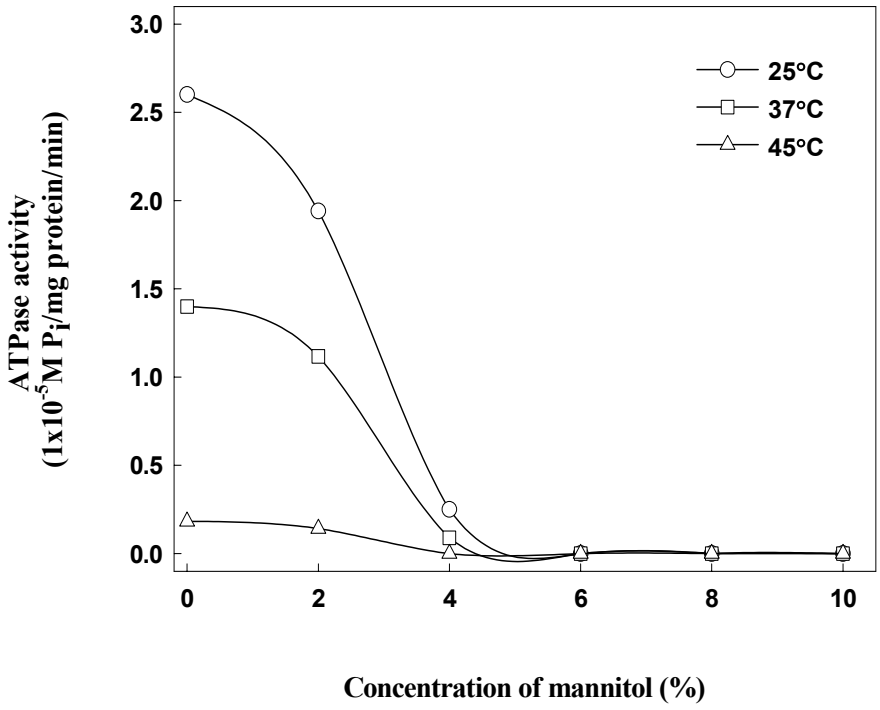


Fig. 39 A: ATPase activity of fish actomyosin determined in the presence of different concentration of mannitol as a function of temperature at pH 9.0. The activity was carried out in glycine-NaOH buffer (0.2 M) containing 0.5 M KCl at temperatures of 25°C, 37°C and 45°C. Activity expressed as moles of inorganic phosphate released by per mg of protein per min.

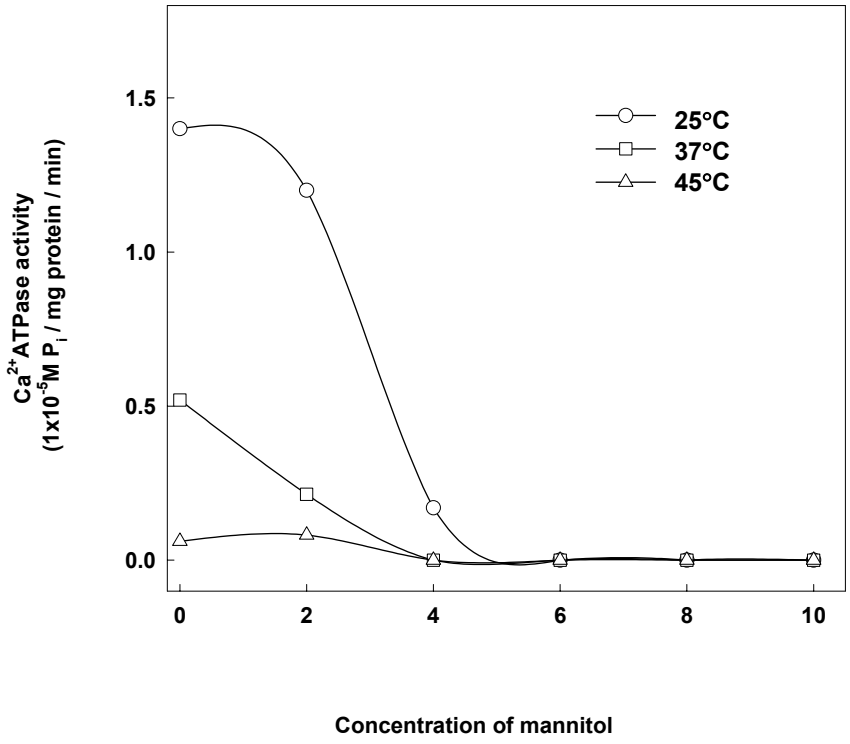


Fig. 39 B: ATPase activity of fish actomyosin determined in presence of different concentrations of mannitol as a function of temperature at pH 7.0. Activity was carried out in Tris-HCl buffer (0.1 M) containing 0.5 M KCl at temperatures of 25°C, 37°C and 45°C. Activity expressed as moles of inorganic phosphate released by per mg of protein per min.

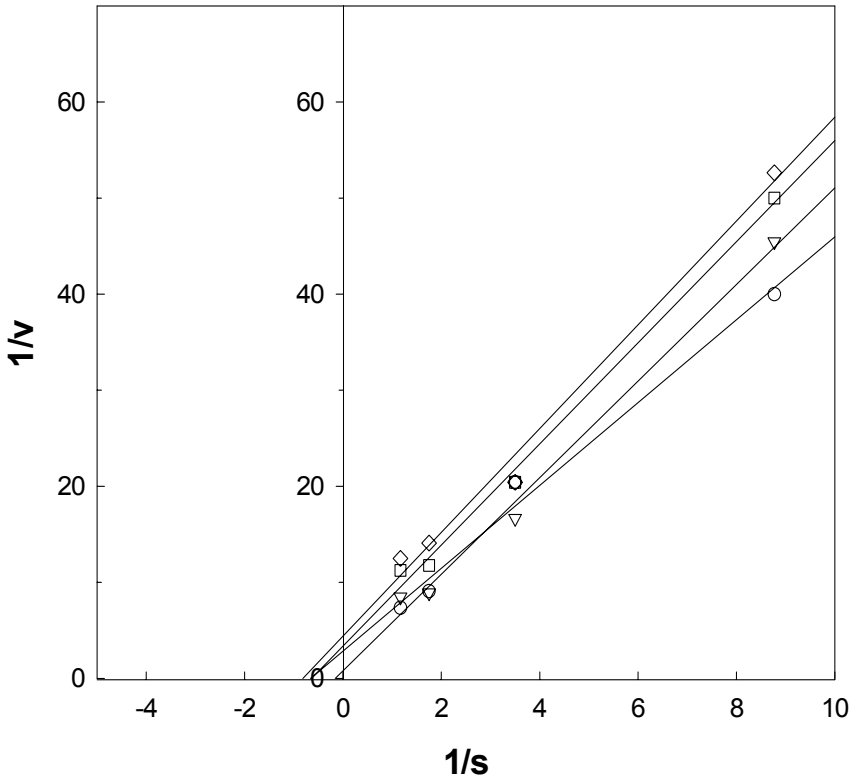


Fig. 38 C: Reciprocal plot of ATPase activity of fish actomyosin in presence of different concentration of sorbitol at pH 9.0. The activity was carried out in glycine-NaOH buffer (0.2 M) containing 0.5 M KCl at temperature of 25°C. O 0%; ∇ 2% ; □ 3%; ◇4%

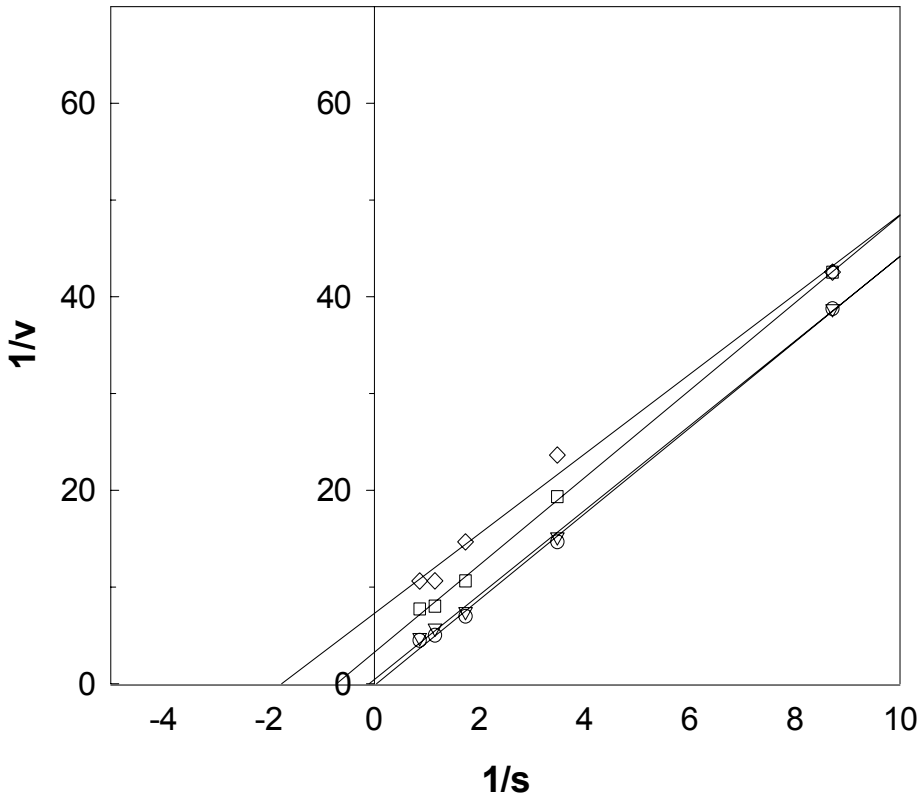


Fig. 38 D: Reciprocal plot of ATPase activity of fish actomyosin in presence of different concentration of sorbitol at pH 7.0. The activity was carried out in Tris-HCl buffer (0.1 M) containing 0.5 M KCl at temperature of 25°C. **O 0%; ∇ 2%; □ 3%; ◇ 4%**

activity profile of ATPase enzyme activity. Effect of different concentrations of sorbitol and incubation temperature at pH 7.0 (Fig.38B) showed a similar pattern to that of pH 9.0. However the absolute values of enzyme activity was lower at pH 7.0 at different concentrations of sorbitol and temperature as compared to that at pH 9.0. The double reciprocal plot of ATPase activity in presence of various concentrations of sorbitol is shown in Fig.38C &D.

The effect of mannitol on the ATPase activity of actomyosin were investigated as a function of temperature and pH at 25°C. The concentration of mannitol used, temperature and pH conditions were same as with sorbitol. The inhibitory action of mannitol was more prevalent than sorbitol in the experimental conditions.

The ATPase enzyme activity reduced considerably with increase in mannitol concentration and temperature at pH 9.0 (Fig.39A). The inhibitory effect was maximum at 4% level at pH 9.0 and different temperature of incubation. The activity profile of ATPase enzyme at pH 7.0 as a function of mannitol concentration and different temperatures is shown in Fig.39B. Relatively the given substrate concentration and temperature of incubation at pH 9.0 could yield higher activity than at pH 7.0. At both the pH the inhibitory effect, as a function of concentration of mannitol were similar. Fig.39C&D shows the double reciprocal plot of ATPase activity in presence of mannitol.

The intrinsic fluorescence emission spectra of actomyosin from sardine in presence of sorbitol and mannitol as cosolvents have been studied. The spectrum has been obtained at pH 7.0 and pH 9.0 as a function of different concentrations of sorbitol and mannitol. At pH 7.0 different concentrations of sorbitol did not have effect on the emission maximum and relative fluorescence intensity of actomyosin (Fig.40A). Similarly at pH 9.0 different concentrations of sorbitol did not have influence on the emission spectra in terms of emission maximum and relative fluorescence intensity (Fig.40B). The effect of mannitol at different concentrations on the fluorescence emission spectra did not reveal any change in relative fluorescence intensity and emission maximum at pH 7.0 and 9.0 (Fig. 41A & B).

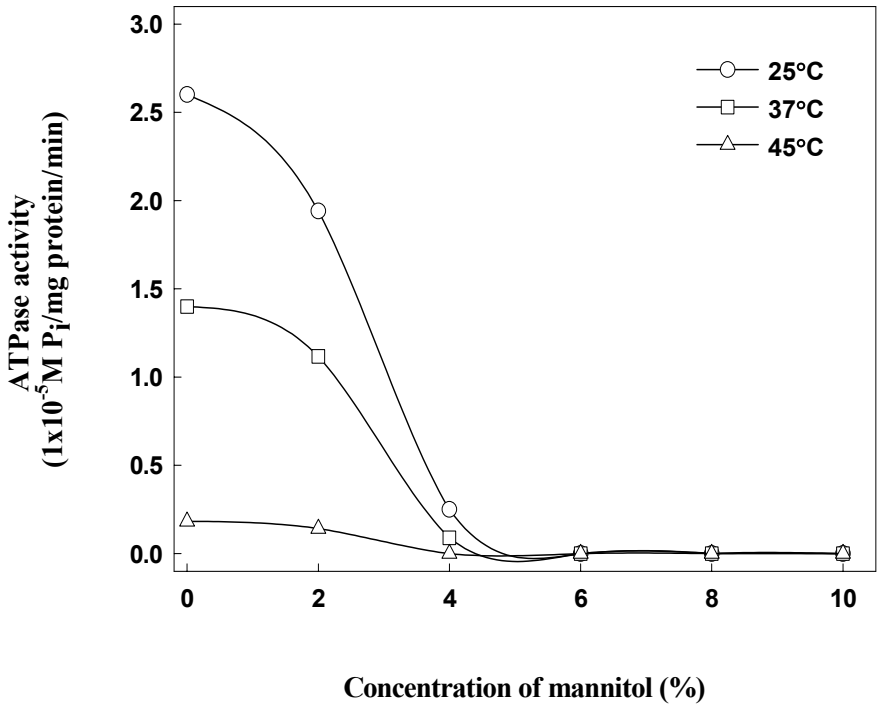


Fig. 39 A: ATPase activity of fish actomyosin determined in the presence of different concentration of mannitol as a function of temperature at pH 9.0. The activity was carried out in glycine-NaOH buffer (0.2 M) containing 0.5 M KCl at temperatures of 25°C, 37°C and 45°C. Activity expressed as moles of inorganic phosphate released by per mg of protein per min.

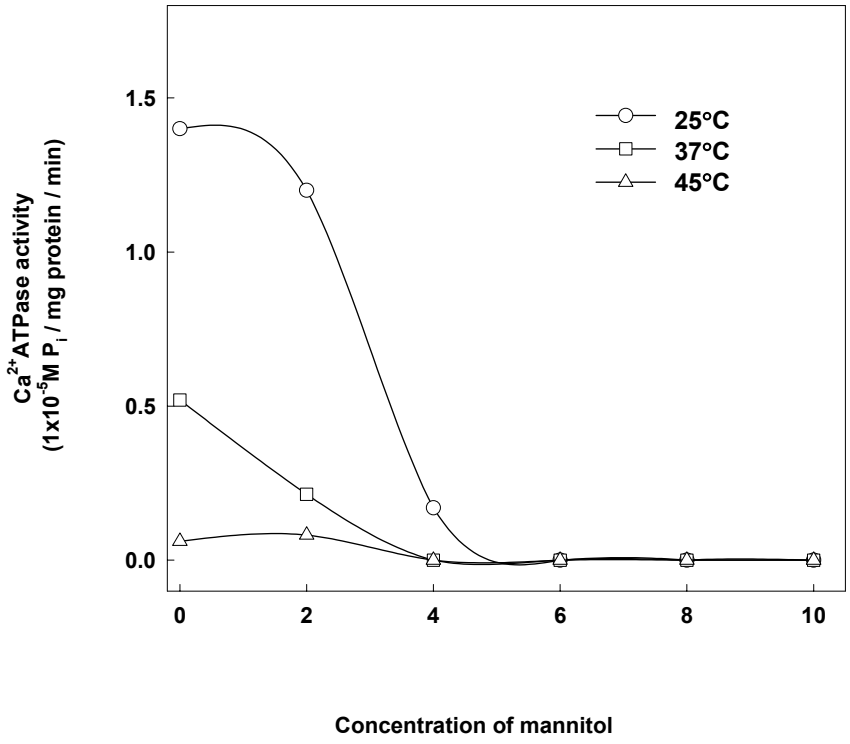


Fig. 39 B: ATPase activity of fish actomyosin determined in presence of different concentrations of mannitol as a function of temperature at pH 7.0. Activity was carried out in Tris-HCl buffer (0.1 M) containing 0.5 M KCl at temperatures of 25°C, 37°C and 45°C. Activity expressed as moles of inorganic phosphate released by per mg of protein per min.

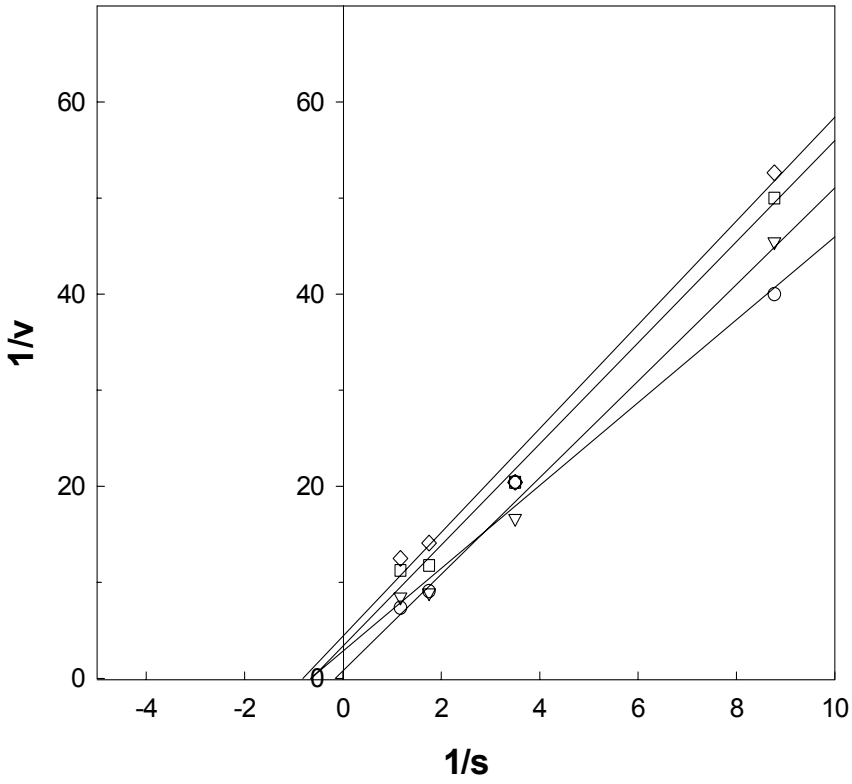


Fig. 38 C: Reciprocal plot of ATPase activity of fish actomyosin in presence of different concentration of sorbitol at pH 9.0. The activity was carried out in glycine-NaOH buffer (0.2 M) containing 0.5 M KCl at temperature of 25°C. O 0%; ∇ 2% ; □ 3%; ◇4%

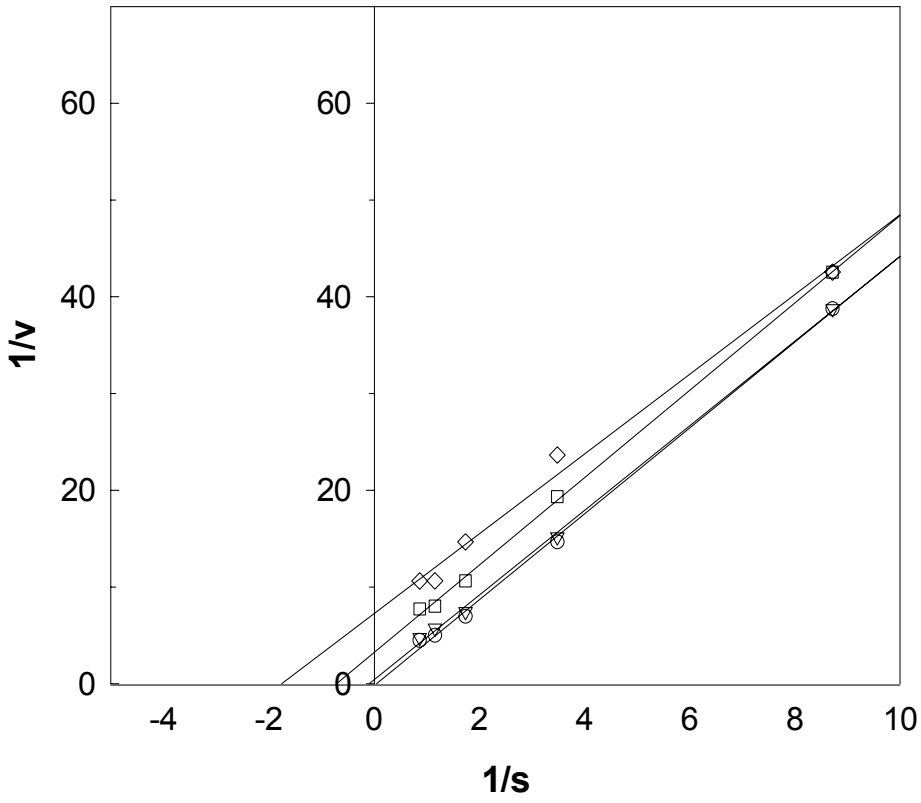


Fig. 38 D: Reciprocal plot of ATPase activity of fish actomyosin in presence of different concentration of sorbitol at pH 7.0. The activity was carried out in Tris-HCl buffer (0.1 M) containing 0.5 M KCl at temperature of 25°C. **O 0%; ∇ 2%; □ 3%; ◇ 4%**

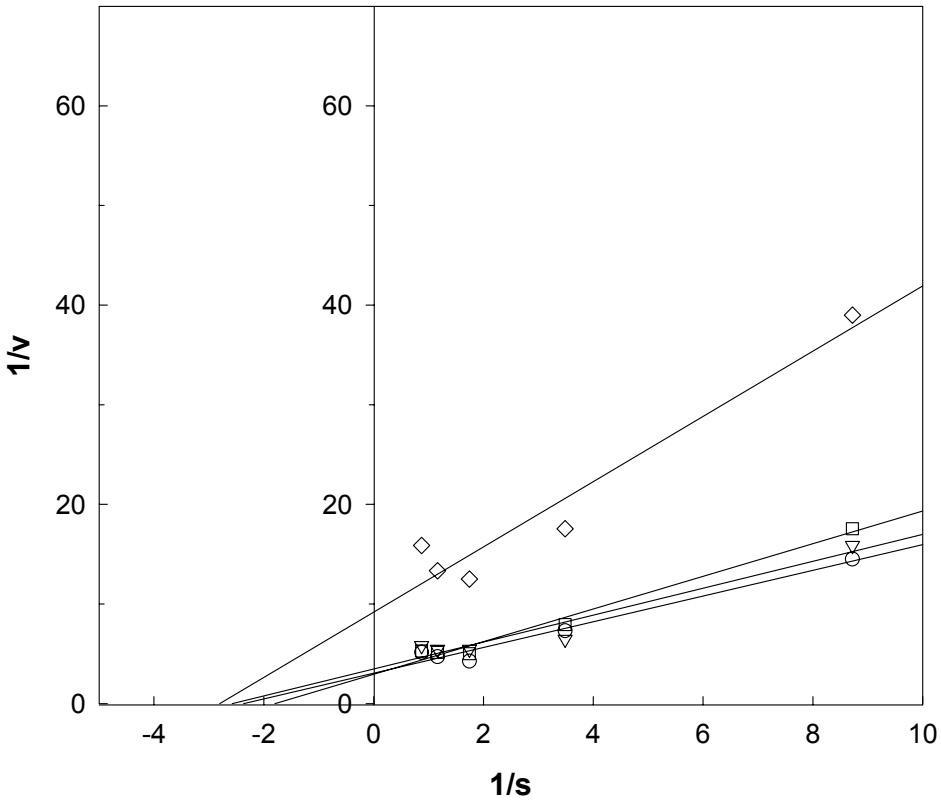


Fig. 39 C: Reciprocal plot of ATPase activity of fish actomyosin in presence of different concentration of mannitol at pH 9.0. The activity was carried out in glycine-NaOH buffer (0.2 M) containing 0.5 M KCl at temperature of 25°C. O 0%; ∇ 2% ; □ 3%; 4%

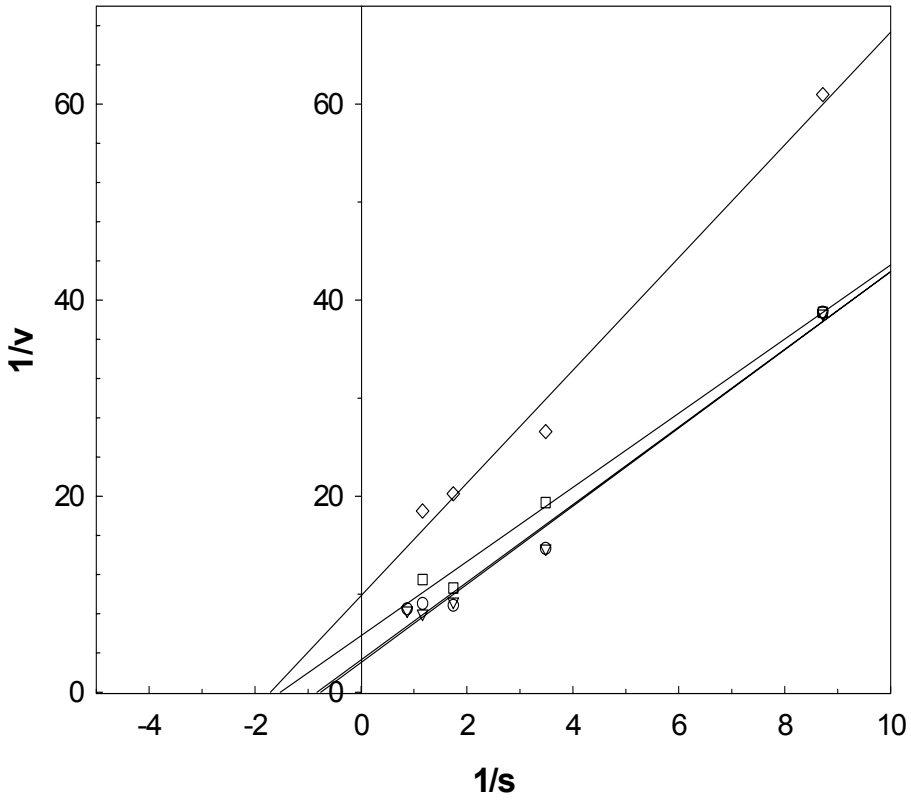


Fig. 39 D: Reciprocal plot of ATPase activity of fish actomyosin in presence of different concentration of mannitol at pH 7.0. The activity was carried out in Tris-HCl buffer (0.1 M) containing 0.5 M KCl at temperature of 25°C. O 0% ; ∇ 2%; □ 3% ; ◇ 4%

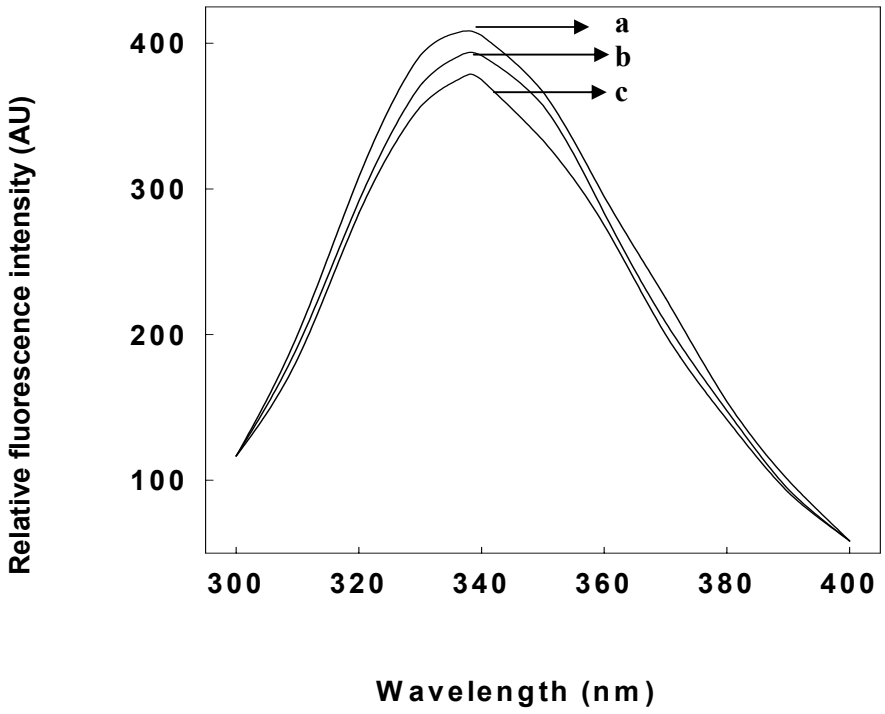


Fig. 40 A: Intrinsic fluorescence emission spectra of fish actomyosin in presence of different concentrations of sorbitol in Tris- HCl buffer (0.1 M) pH 7.0 containing 0.5 M KCl at 25°C. Emission spectra recorded over a range of 300-400 nm. The excitation maximum was at 280 nm. a) Control; b) 2%, 4% and 6% sorbitol (no difference in the fluorescence spectra) **and c) 8% and 10% sorbitol** (no difference in the fluorescence spectra)**.**

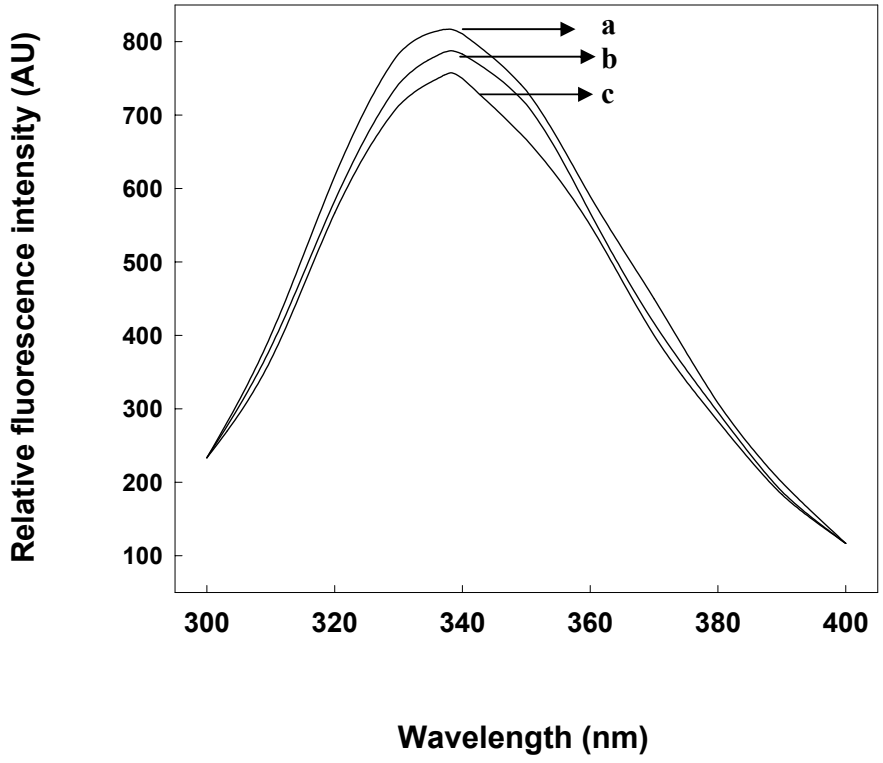


Fig. 40 B: Intrinsic fluorescence emission spectra of fish actomyosin in presence of different concentrations of sorbitol in glycine-NaOH buffer (0.2 M) pH 9.0 containing 0.5 M KCl at 25°C. Emission spectra recorded over a range of 300-400 nm. The excitation maximum was at 280 nm. a) Control; b) 2%, 4% and 6% sorbitol (no difference in the fluorescence spectra) **and c) 8% and 10% sorbitol** (no difference in the fluorescence spectra)**.**

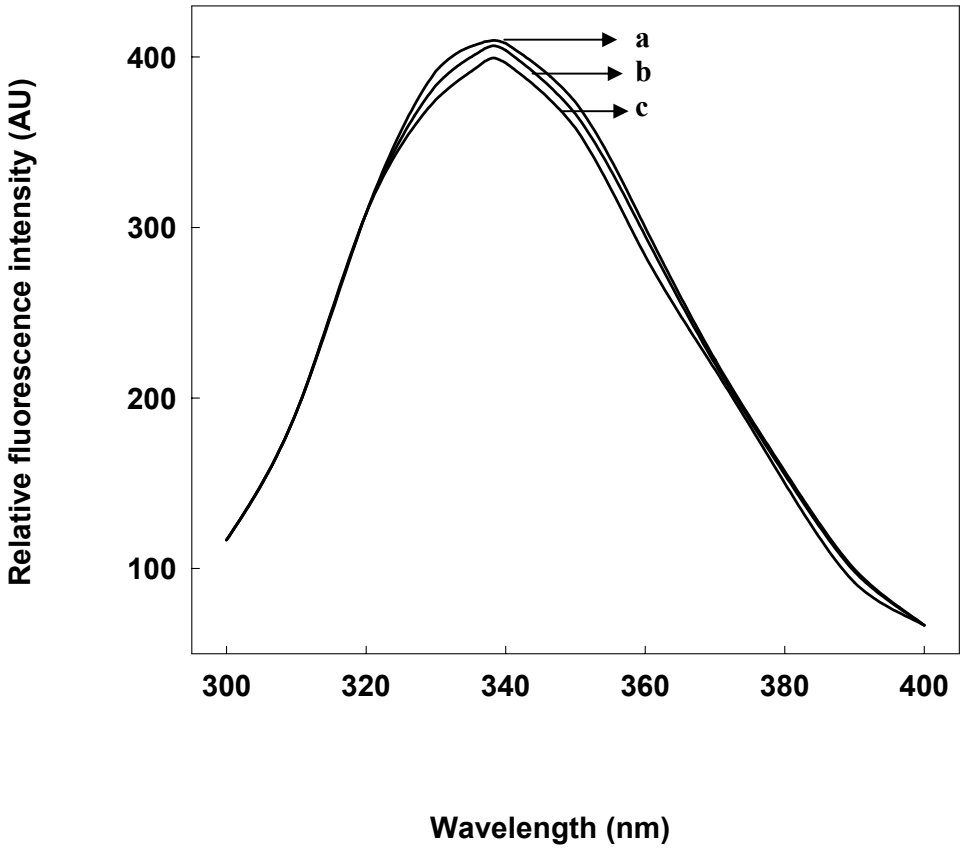


Fig. 41 A: Intrinsic fluorescence emission spectra of sardine actomyosin in the presence of different concentrations of mannitol in Tris-HCl (0.1 M) buffer pH 7.0 containing 0.5 M KCl at 25°C. Emission spectra recorded over a range of 300-400 nm. Excitation maximum was fixed at 280 nm. a) Control; b) 2% and 4% mannitol (no difference in the fluorescence spectra) **and c) 6%, 8% and 10% mannitol** (no difference in the fluorescence spectra)**.**

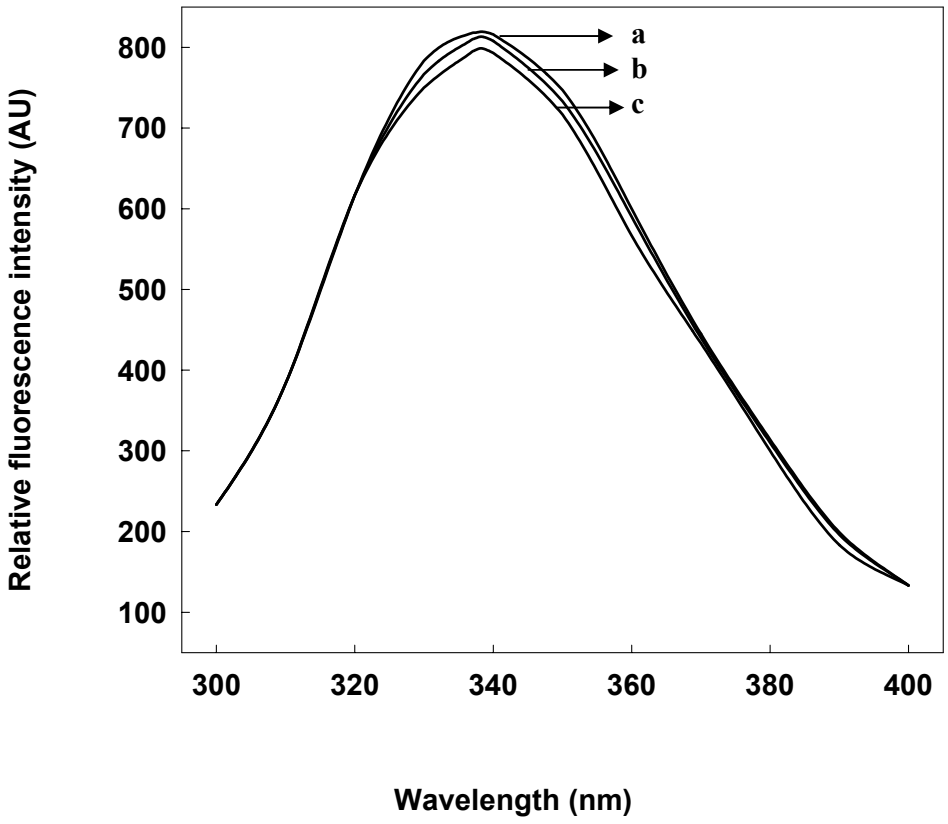


Fig. 41 B: Intrinsic fluorescence emission spectra of sardine actomyosin in the presence of different concentrations of mannitol in glycine-NaOH (0.2 M) buffer pH 9.0 containing 0.5 M KCl at 25°C. Emission spectra recorded over a range of 300-400 nm. Excitation maximum was fixed at 280 nm. a) Control; b) 2% and 4% mannitol (no difference in the fluorescence spectra) **and c) 6%, 8% and 10% mannitol** (no difference in the fluorescence spectra)**.**

The effect of cosolvents (sorbitol and mannitol) at different concentrations on the hydrophobicity index of actomyosin is given in Table 13. A reduction in hydrophobicity index was observed with increase in concentration of sorbitol and mannitol. However the reduction appears to be marginal and not significant. The free SH content of fish actomyosin decreased with increased concentration of polyhydric alcohols (Table 14). The free SH content of fish actomyosin was recorded as 122 μM SH/g actomyosin. The free SH content decreased with increase in concentrations of polyhydric alcohols as expected. The presence of 2% sorbitol did not affect the free SH content of fish actomyosin. A further increase in sorbitol concentration decreased the values and brought it to 97 μM SH/g actomyosin by the presence of 6% sorbtiol. The reduction in free SH was more affected by the presence of 4% and 6% sorbitol. The reduction was steeper in presence of mannitol. Presence of 2% mannitol effected in a reduction of free SH content by 5%. A further increase to 4% mannitol effected the reduction by 21%. The free SH content valued reduced to 88 μM SH/g actomyosin i.e. 88% of the original value in presence of 6% mannitol in solution. The reduction in free SH contents followed the pattern of inhibition in activity of ATPase by the presence of polyhydric alcohols.

The effect of sorbitol and mannitol on two different concentrations on the secondary structure of proteins has been studied using far UV-CD spectra. The far UV-CD spectrum of actomyosin in presence of sorbitol at 5 and 10% concentration is shown in Fig.42. There is a very small difference in the secondary structure in the presence of sorbitol (Table 15). The effect of mannitol on the secondary structure of actomyosin also investigated and shown in Fig.43. Here also, the presence of cosolvents did not make significant difference in the structure of proteins (Table 16). The structure showed that the actomyosin is prominently a helical protein and the reduction in helical content is very less even when the ATPase activity was inactivated completely. The tail portion of myosin molecules, which is a coiled coil structure mainly, contributes to helicity. The site of ATP hydrolysis is situated at the head portion of myosin molecules.

Table 13: Hydrophobicity index of fish actomyosin in presence of different concentrations of cosolvents at pH 7.0 (Tris-HCl, 0.1 M).

Concentration of cosolvent (%)	Cosolvent	
	Sorbitol	**Mannitol**
Control (in buffer only)	422 ± 20	422 ± 20
2	404 ± 17	423 ± 5
4	400 ± 10	416 ± 12
6	396 ± 11	396 ± 8
8	388 ± 8	387 ± 4
10	373 ± 4	377 ± 6

Table 14: Free SH content of fish actomyosin in presence of different concentrations of cosolvents at pH 7.0(Tris-HCl buffer) (Free SH content expressed as μM SH/ g of protein)

Concentration of cosolvents (%)	sorbitol	mannitol
Control (in buffer only)	122 ± 12	122 ± 12
2	128 ± 3	117 ± 4
4	105 ± 10	97 ± 3
6	97 ± 4	88 ± 6

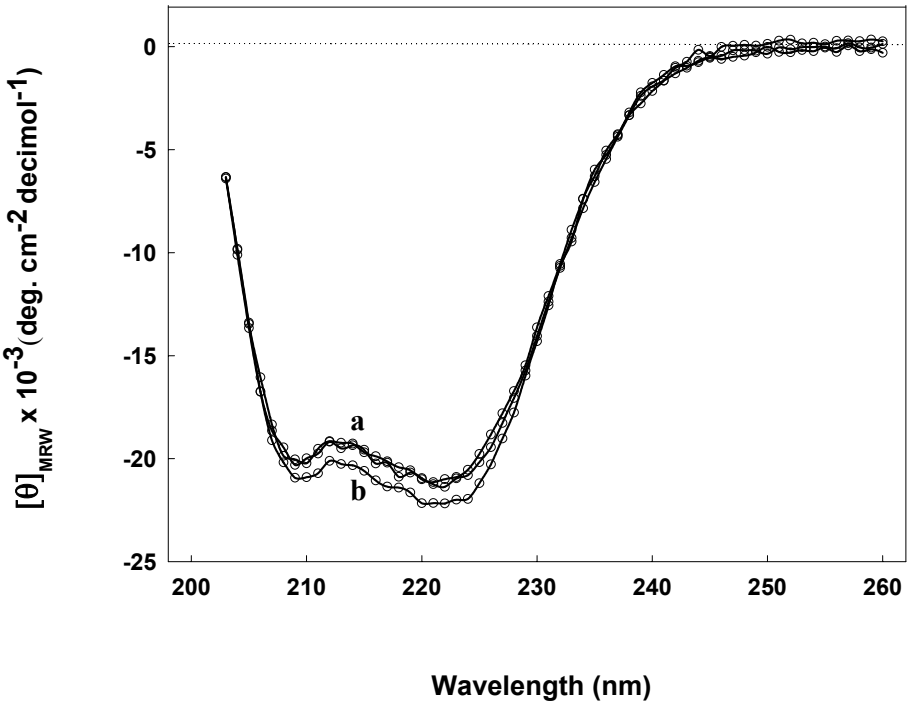


Fig. 42: Far UV-CD spectra of fish actomyosin in presence of different concentrations of sorbitol 5% and 10% in 0.1M Tris-HCl buffer pH 7.0 containing 0.5 M KCl at 25°C. a) Control and 5% sorbitol (no difference in the CD spectra) **and b) 10% sorbitol.**

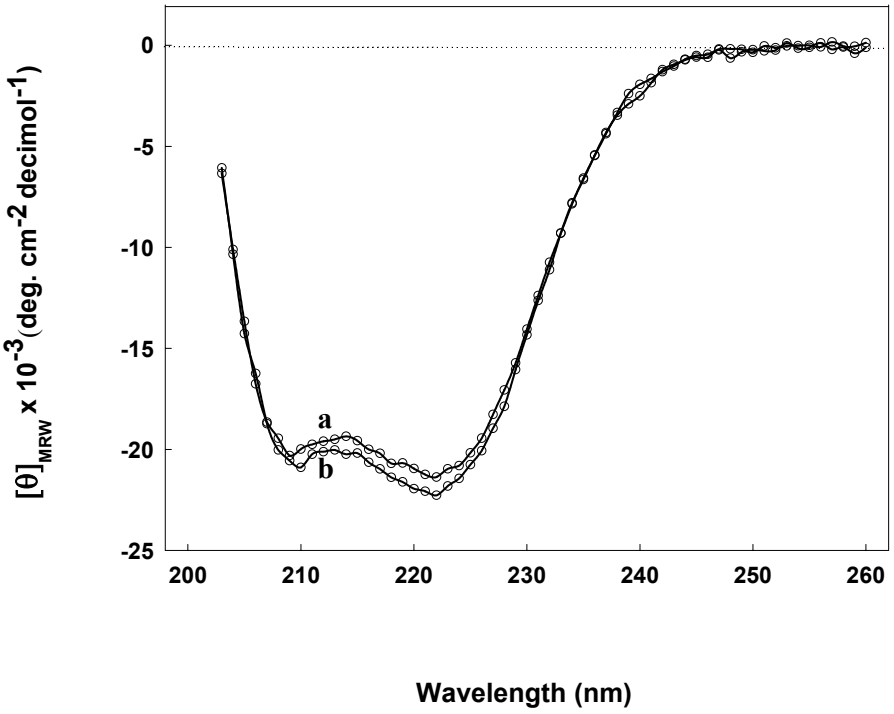


Fig. 43: Far UV-CD spectra of fish actomyosin in the presence of different concentrations of mannitol 5% and 10% in 0.1 M Tris-HCl buffer pH 7.0 containing 0.5 M KCl at 25°C. a) Control and b) 5% and 10% mannitol (no difference in the CD spectra)**.**

Table 15: Secondary structure of fish actomyosin in presence of different concentrations of sorbitol in 0.1 M Tris-HCl buffer at pH 7.0 containing 0.5 M KCl

Concentration of Sorbitol (%)	Secondary structure (%)		
	α-helix	β- structure	Aperiodic
Control (in buffer only)	65 ± 3	3 ± 1	32 ± 2
5.0	65 ± 2	4 ± 1	31 ± 2
10.0	68 ± 2	4 ± 1	28 ± 2

Table 16: Secondary structure of fish actomyosin in presence of different concentrations of mannitol in 0.1 M Tris-HCl buffer at pH 7.0 containing 0.5 M KCl

Concentration of Mannitol (%)	Secondary structure (%)		
	α-helix	**β- structure**	**Aperiodic**
Control (in buffer only)	65 ± 3	3 ± 1	32 ± 2
5.0	69 ± 2	4 ± 1	27 ± 2
10.0	68 ± 2	4 ± 1	28 ± 2

Polyhydric alcohols used in this study (sorbitol and mannitol) resulted in the decrease of ATPase activity of actomyosin. The effect of mannitol on the activity was more pronounced than sorbitol. Mannitol showed higher inhibitory action than sorbitol at pH 7.0 and 9.0. The nature of inhibition was evident for both sorbitol and mannitol at the pH conditions where the activity was measured. The nucleotide binding site of myosin is located in a pocket at the interface of the 24 kD N-terminal and the central 50 kD tryptic fragments, the nucleotide lies parallel to the plane of a seven stranded β-sheet, where the α-and β-phosphates interact with the P loop from the 50 kDa segment (Rayment, 1996). Highsmith *et al.* (1998) reports that the aggregation induced by polyethylene glycol is by osmotic pressure, which reduces the pool of water available for hydration.

The changes in intrinsic fluorescence emission spectra of actomyosin were recorded in presence of these cosolvents. Intensity of intrinsic fluorescence emission spectra of fish actomyosin decreased marginally in presence of polyhydric alcohols. Since the pattern of fluorescence spectra revealed that there is no influence on the aromatic amino acid residues of proteins. Hydrophobicity of actomyosin decreased marginally with increase in concentrations of polyhydric alcohols. The exposure of hydrophobic residues might have been hindered by interaction of OH groups of the cosolvent molecules. The hydrophobic proteins orient more readily than less hydrophobic ones at the surface with their polar groups directed towards the aqueous phase and their hydrophobic groups towards the non aqueous phase to lower the surface free energy (Kato and Nakai, 1980). The structural changes will bring out difference in surface hydrophobicity of myosin molecules. Kristinsson and Hultin (2003b) reported that unfolding of myosin molecule by extremes of pH and subsequent refolding increased the hydrophobicity. Changes in the exposure of different amino acid residues will bring about changes in the hydrophobicity of proteins. Exposure hydrophobic residues due to structural changes will increase the surface hydrophobicity. Since the difference in the fluorescence emission spectra was not significant, the structural changes due to the presence of these polyhydric alcohols is minimized.

The presence of cosolvents did not make significant difference in the structure of proteins. There was no direct relation between the secondary structure and ATPase enzyme activity of actomyosin. The site of ATP hydrolysis is mainly situated at the head portion of myosin molecules. Helical content of myosin molecule increased by only a few percent even after the ATPase activity is completely absent. Hence, the loss of ATPase activity does not necessarily imply that the myosin head has been unfolded and indicated that the myosin head undergo some conformational change (Chan *et al.*, 1995).

The α- helicity of actomyosin remained almost same in presence of cosolvent molecules deduced from far UV-CD spectra shown in Fig.42 & 43. Rayment *et al*. (1993a) proposed that myosin subfragment S1 comprises of helical structure that extends between Asp^{327} and Ile^{340} will form the top of nucleotide binding pocket. In the present investigation a slight increase in helicity as induced by cosolvents, which may extend the nucleotide binding pocket at Asp^{327} and Ile^{340}. The structural changes of actomyosin during various other treatments were reported in literature. The helicity of actomyosin decreased during setting (low temperature gelation), which induces gelation of myosin molecule by the unfolding of long helical tail portion (Ogawa *et al.,* 1995). Cold water fish myosin lost the active sites of ATPase enzyme activity with the unfolding of secondary structure during preparation (Ogawa *et al.,* 1996). Polyethylene glycol potentiates the binding of a myosin product complex to actin, and this accounts for the increase in stiffness in fibers incubated with the phosphate analogues as well as the k_m for the acto S1 ATPase activity (Chinn *et al*., 2000).

Actomyosin from fish varied in its characteristics from other vertebrate actomyosin. ATPase activity of fish actomyosin was less stable to temperature as compared to other vertebrate actomyosin. Polyhydric alcohol induced inhibition of ATPase is of importance in dealing with post mortem muscle systems. Retaining the structural integrity of actomyosin even after the inhibition of ATPase activity is an interesting phenomenon of muscle protection, which helps to enhance the storage quality of proteins without altering the required properties for further processing.

The mechanism of stabilization of proteins by sugars and sugar alcohols are described and various models were put forward in literature. The nonpolar solute stabilizer interaction is better described by the term preferential solvation of the solute by the stabilizer (Carrillo-Nava *et al*., 2004). The preferential exclusion of sucrose and the increased protein chemical potential indicate that any state of protein perhaps which has an increased surface area should be thermodynamically less favorable than more compact states. For example the preferential exclusion of sucrose from the α-chymotrypsin, chymotrypsinogen, ribonuclease and tubulin has been shown to be due to the increase in the surface tension of water and not due to any specific property of individual proteins or sugars (Arakawa and Timasheff, 1982a; Kita *et al*., 1994; Lee and Timasheff, 1981; Lin and Timasheff, 1996; Timasheff, 1993). Further more it is perhaps the unfavorable interactions of peptide backbones, and not the side chains with sucrose that give rise to the increased protein chemical potential in the cosolvent solutions (Liu and Bolen, 1995).

The results of these interaction studies clearly reveal that cosolvents (sorbitol and mannitol) could be used to improve the functionality and keeping quality of fish mince during frozen storage. Both the polyhydric alcohols can be used as additive at low concentrations to improve the quality of fish mince. Mannitol perhaps is a better option than sorbitol as mannitol inhibits ATPase enzyme activity at lower concentrations than sorbitol and also it will not affect the structure of actomyosin at this low concentration. Such biotechnological approaches of addition of small quantities of cosolvents would improve the enhancement of quality and shelf life of fish mince.

CHAPTER - 4

EFFECT OF MANNITOL ON THE STRUCTURE AND FUNCTION OF PROTEINS FROM SARDINE MINCE DURING FROZEN STORAGE

EFFECT OF MANNITOL ON THE STRUCTURE AND FUNCTION OF PROTEINS FROM SARDINE MINCE DURING FROZEN STORAGE

Indian oil sardine (*Sadinella longiceps*) is a pelagic fish with high fat content is normally used for fishmeal and oil production especially during glut season. In order to utilize oil sardine resources for human consumption it is needed to evolve strategies for improving the keeping quality of sardine either by processing or by better preservation. Production of surimi (water washed fish flesh added with cryoprotectants) is good option for efficient utilization of oil sardine resources for human consumption. Because of the intrinsic nature of muscle like dark muscle, high fat content and high quantity of endogenous enzymes necessitates special attention for stabilization of proteins during frozen storage. Texture is one of the most important quality factors of fish as food. Structural links between the muscle cells and connective tissue of fish provide the necessary integrity for the flesh to withstand the effect of post harvest handling, processing and storage (Bremner, 1992).

Various additives are used to reduce the freeze denaturation of muscle proteins. During frozen storage emphasis is given to additives, which doesn't induce any alteration of the product profile. The effect of certain salts from Hoefmeister series on proteins is known to be protective agents from denaturing proteins (Jencks, 1969). Salts such as acetates, sulfates, phosphates and citrates are known to protect proteins and minimize denaturation (Badii and Howell, 2002; Shamasundar and Prakash, 1994b,c). Other additives like polyhydric alcohols also used for the commercial production of surimi promoting stabilization of proteins during frozen storage. Stabilization of proteins from fish during frozen storage improves the functional and sensory characteristics, which is desirable in the product formulation. From the results as described in the Chapter 3 polyhydric alcohols like mannitol, could induce desirable structural changes and may have bearing in stabilization of proteins during frozen storage. In this chapter the effect of mannitol on the frozen storage stability of sardine mince as protein stabilizer is dealt with.

Certain additives like polyhydric alcohols bring in significant change in the proteins during storage and it also used commercially within the ambient of the regulatory system applicable to each country. The three dimensional structure of proteins can be stabilized by both covalent and noncovalent interactions. Normally cosolvents are used as additives to bring in stabilization and macroscopic property changes to products not only sensorial part of product and its deterioration during storage but also bring in desired effect of stabilization.

Cryoprotectants are compounds that protect or stabilize a product during freezing and frozen storage. Cryoprotectants are compounds that extend the shelf life and quality of frozen foods (Park *et al*., 2004). The presence of cryoprotectants is important to ensure maximum functionality of frozen surimi because freezing induces protein denaturation and aggregation (Mac Donald *et al*., 2000). Sugars, sorbitol, phosphates and salts are the commonly used as cryoprotectants in surimi industry. Although the amount of each added additive varies considerably between producers, the average quantities added are approximately 4-5% sugars 0-5% sorbitol, 0-3% salts and 0-0.3% phosphates. Polyhydric alcohols are known to protect the proteins and enzymes during low temperature exposure. In this chapter the effect of selected polyhydric alcohol mannitol on the stabilization of sardine proteins is described. The effect of polyhydric alcohol, mannitol, on the structure and activity of sardine actomyosin is already described in Chapter 3. To analyse the effect of different concentrations of mannitol on the properties of mince from sardine and also to assess the structural, functional and physicochemical properties of proteins during frozen storage as a function of time was investigated.

Enzymatic, physicochemical and structural properties of fish mince proteins during storage

ATPase enzyme activity of proteins from frozen mince was monitored during storage. The profile of ATPase enzyme activity during frozen storage of mince is shown in Fig.44. Immediately after freezing, ATPase activity was less in the case of untreated samples than the samples treated with mannitol. ATPase activity is a good indicator of the integrity of myosin molecules (Roura and Crupkin, 1995). ATPase

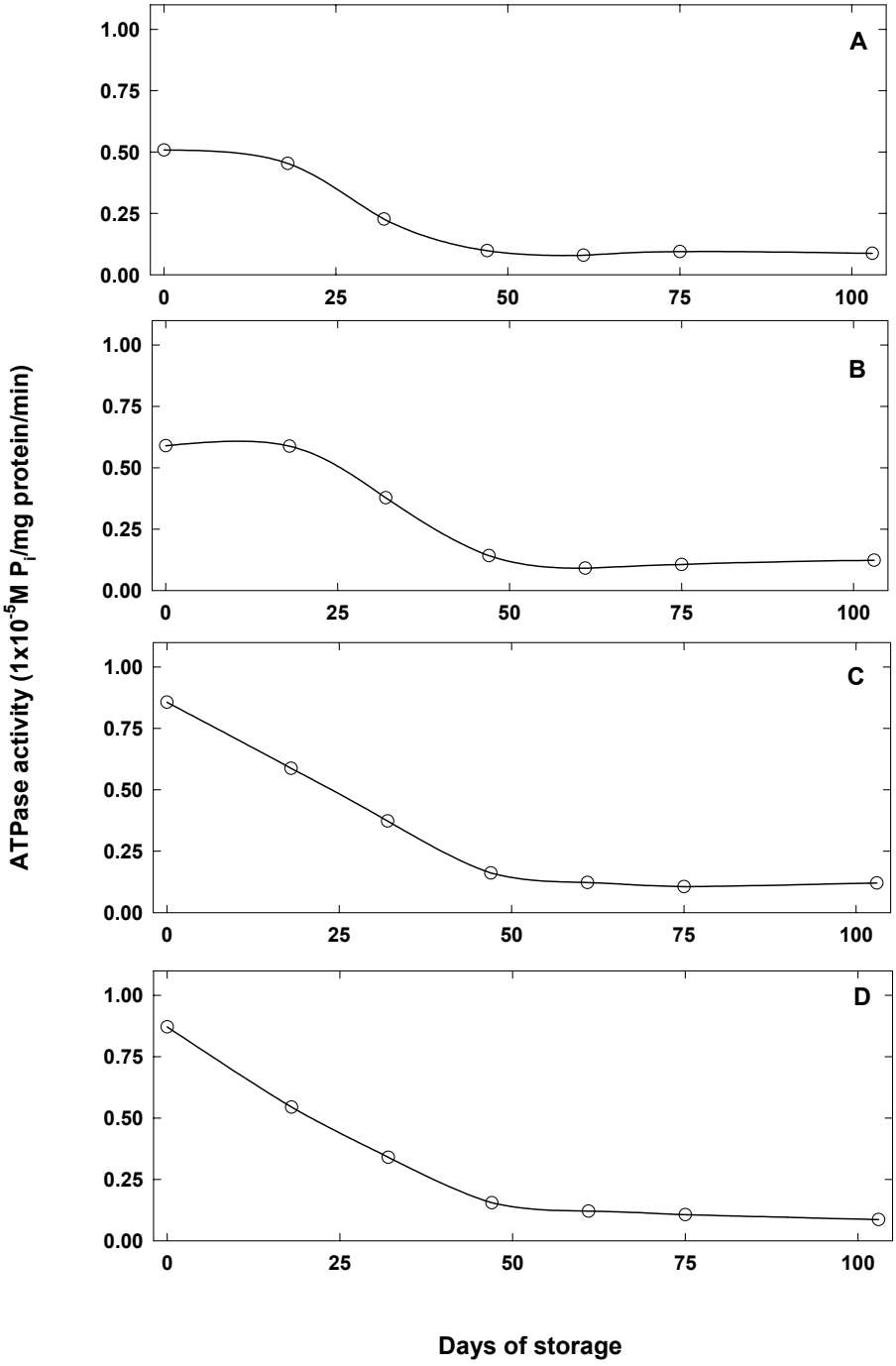


Fig. 44: ATPase activity of proteins extracted in glycine-NaOH pH 9.0 (0.2 M) containing 0.5 M KCl from sardine mince treated with different concentrations of mannitol during frozen storage as a function of time. A) Control; B) 2% mannitol; C) 4% mannitol and D) 6% mannitol.

activity from frozen mince reduced during frozen storage resulted sharp decrease in ATPase activity occurred up to 50 days of storage. Mannitol treated samples have shown higher activity up to 60 days of storage. After 75 days of storage ATPase activity of mince attained an asymptotic value (Fig.44). The reduction in activity was 80% after 50 days of storage. The higher ATPase activity of extract from mannitol treated samples than control samples during freezing clearly showed that this polyhydric alcohol protected the myosin and actin from freeze denaturation.

If one look at the interaction of actomyosin with mannitol it is evident that mannitol at 4% inhibits the ATPase enzyme activity. On the contrary same concentration in mince has shown considerable higher activity even after 50 days of storage. This is due to the fact that during extraction of ATPase enzyme from the mince mixed with mannitol, dilution is occurs by 10 times wherein concentration of mannitol reduced to 0.4%. This reduction in mannitol concentration in all probability has shows higher ATPase enzyme activity. From previous experiments it was observed that mannitol at a concentration of 1% did not affect ATPase enzyme activity of extract.

Depending upon the nature and concentration of the cosolvents as well as the storage conditions one can see that the effect of both stabilization and the properties of macromolecules not altered as a result of the treatment. The protein profile of the samples with and without mannitol were analysed by SDS-PAGE after extracting the proteins with sample buffer is shown in Fig.45. The major bands were contributed by myosin and actin components of mince. There was no significant change in the protein pattern due to the treatment with mannitol at different concentrations. With increase in concentration up to 6% mannitol in the mince, the protein profile remained unaltered. During storage under low temperature the intensity of bands corresponding to actin and myosin increased and resulted in the lower resolution of bands. During frozen storage there was aggregation of proteins, especially the high molecular weight myosin heavy chain (Fig.45A-F). Actin molecules were the one that predominantly showed aggregation during storage. In order to quantify the various proteins in the system with a clear mandate of separating, molecular weight standards are being run

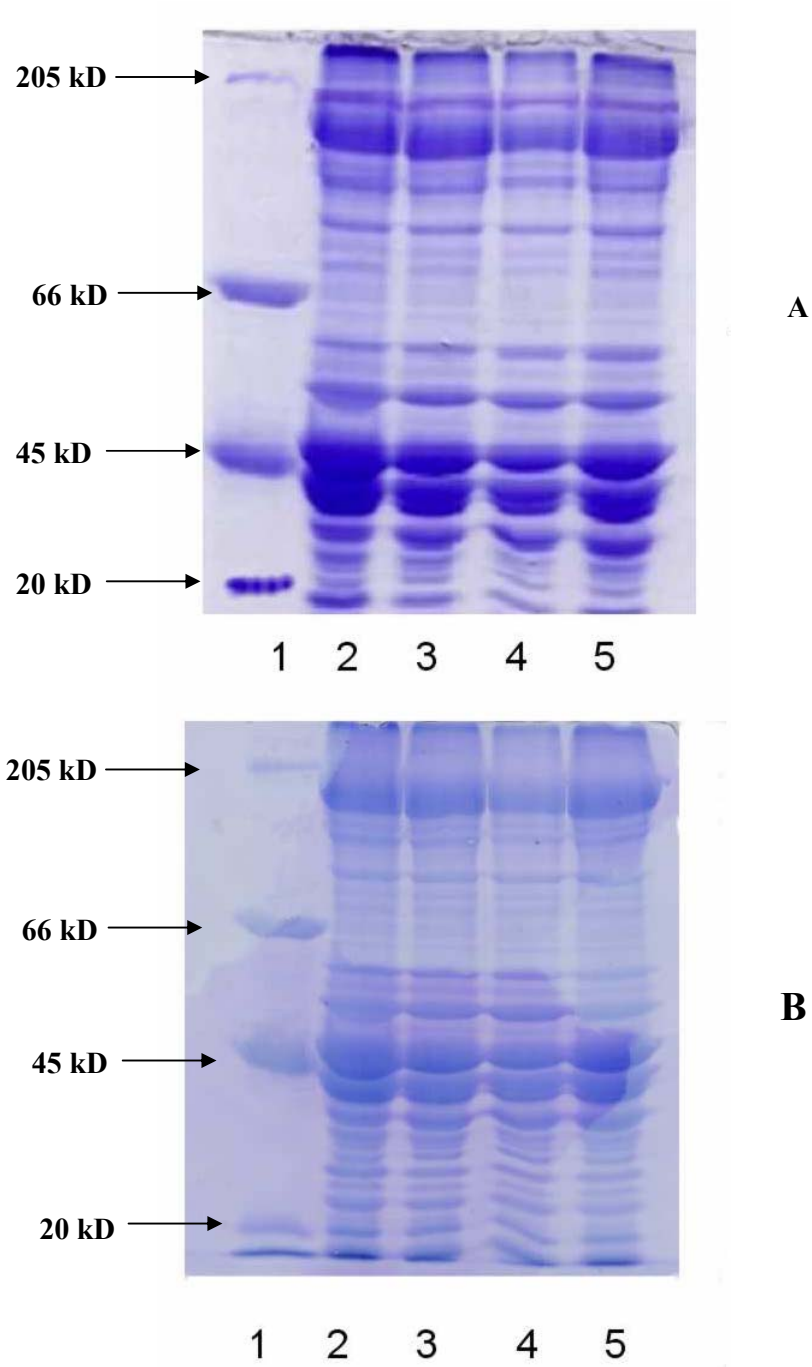


Fig. 45 A, B: SDS-PAGE pattern of proteins extracted from sardine mince treated with mannitol during frozen storage. A) immediately after freezing; B) 30 days of storage. 1) Standard proteins - Myosin (205 kD), BSA (66 kD), Ovalbumin (45 kD) and Trypsin inhibitor (20 kD); 2) Control; 3) 2% mannitol; 4) 4% mannitol; 5) 6% mannitol.

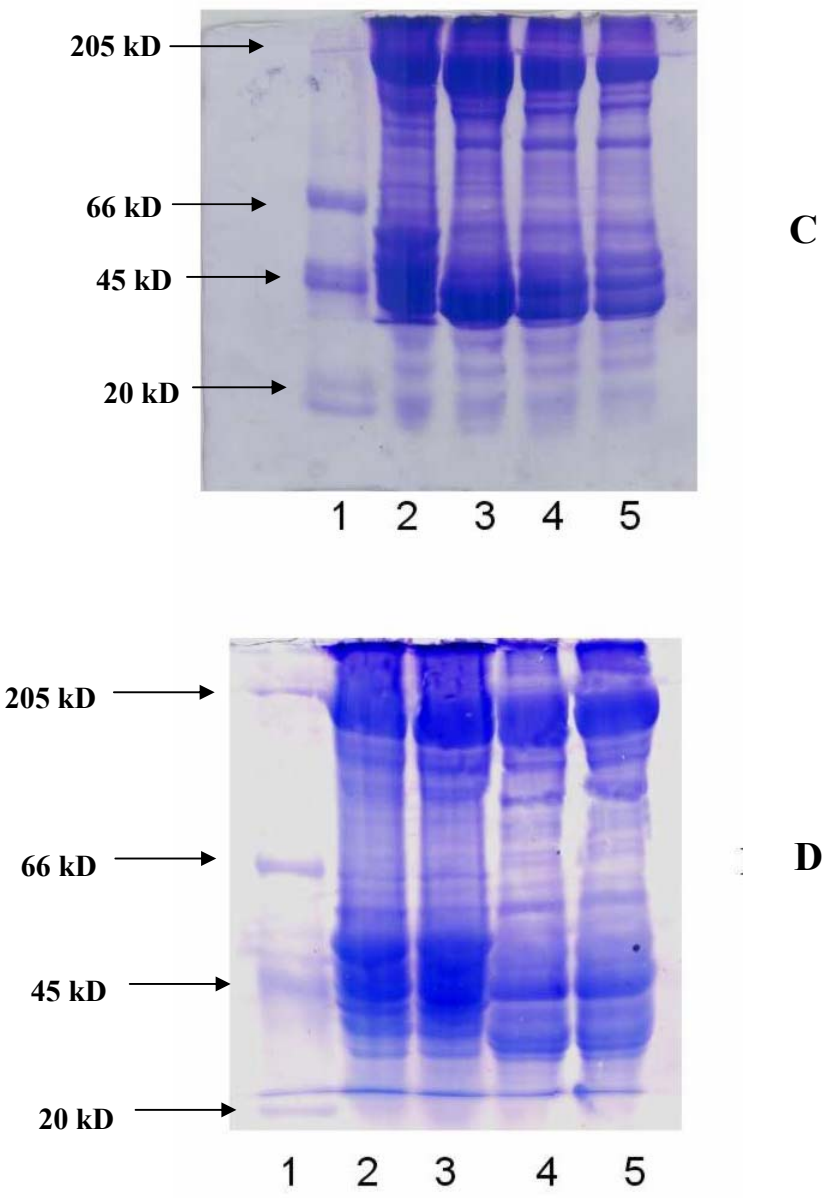


Fig. 45 C, D: SDS-PAGE pattern of proteins extracted from sardine mince treated with mannitol during frozen storage. C) 50 days; D) 72 days of storage. 1) Standard proteins - Myosin (205 kD), BSA (66 kD), Ovalbumin (45 kD) and Trypsin inhibitor (20 kD); 2) Control; 3) 2% mannitol; 4) 4% mannitol; 5) 6% mannitol.

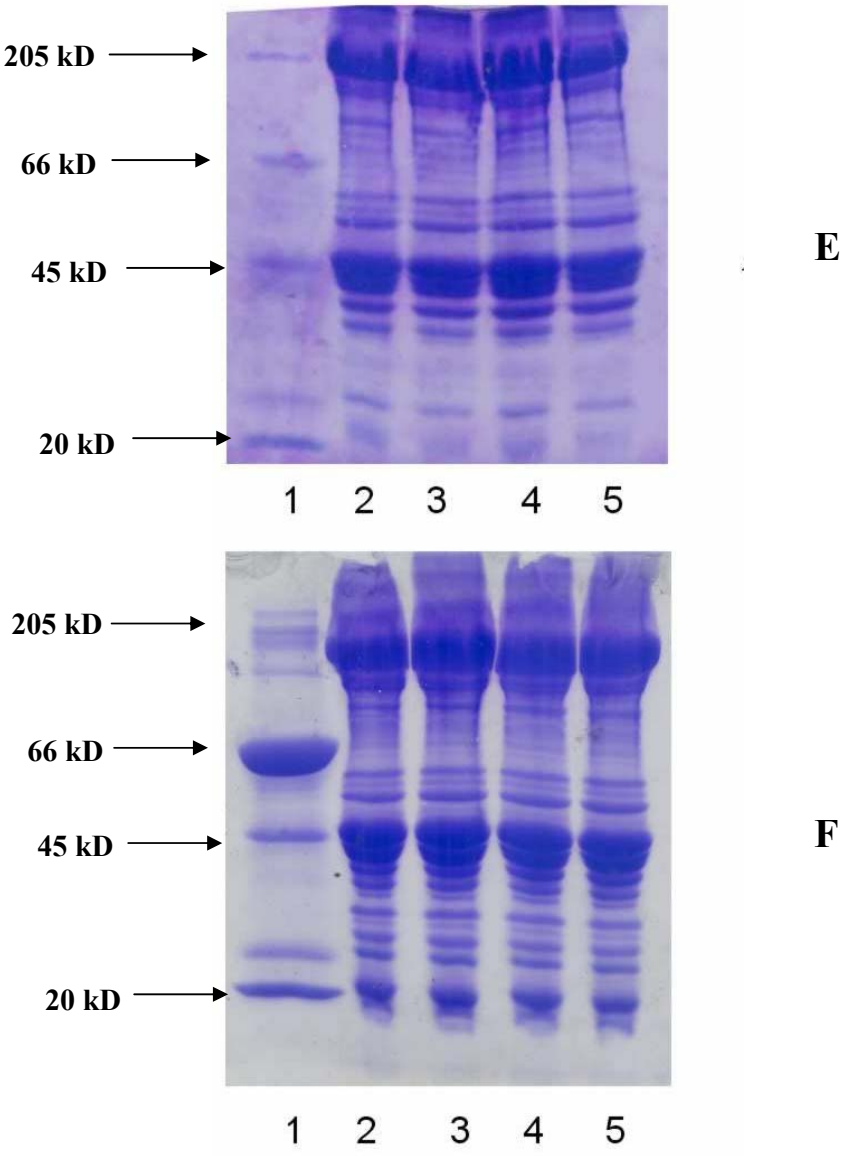


Fig. 45 E, F: SDS-PAGE pattern of proteins extracted from sardine mince treated with mannitol during frozen storage. E) 100 days; F) 120 days of storage. 1) Standard proteins - Myosin (205 kD), BSA (66 kD), Ovalbumin (45 kD) and Trypsin inhibitor (20 kD); 2) Control; 3) 2% mannitol; 4) 4% mannitol; 5) 6% mannitol.

to benchmark the molecular weight parameter (myosin, BSA, ovalbumin and trypsin inhibitor) in comparison with standard the molecular weight indicates and falls within the range of 40-45 kD. The rate of aggregation in presence of mannitol 4% and 6% were less than control samples. Since the low molecular weight protein bands did not show any change in the pattern it could be concluded that the proteolytic activity was considerably low in the minced meat. Washing of meat with water has leached all sarcoplasmic protein fractions, which include proteolytic enzymes and myoglobin, thus resulted in low proteolytic activity of mince during frozen storage. The composition of proteins in mince influences the behaviour of mince proteins during heating. During heating the proteins undergo denaturation and this can be monitored by the change in apparent T_m of proteins. This also helps to understand the behavior of proteins in presence of various additives and also during treatments with them.

The gel filtration profiles of proteins from sardine mince during different periods of frozen storage have been investigated. The gel filtration profile of proteins from mince (without manntiol) is shown in Fig.46A. At the end of 120 days of storage concentration of low molecular weight components have increased eluting at an elution volume of 130 ml. There was no change in the elution volume of mannitol treated samples (Fig.46B-H), which indicates the ability of mannitol to protect the proteins from aggregation. The process of aggregation and conformational changes are able to bring changes in the elution pattern of proteins. The gel filtration profile of proteins from fishes like cod indicates dissociation process of high molecular weight protein components during frozen storage (Ohinishi and Rodeger, 1980). The area under the low molecular weight protein under equiconcnentrations of loading of both control and treated indicates not only an increase in the area of low molecular weight with additives but small change in elution volume (125 ml). Eventhough this is quite non significant but it was worth noting. The structure and functionality of mince is being contributed by the subunit composition and the presence of other proteins present in the system. The individual protein components and subunit composition can be better understood by analyzing the electrophoretic pattern of proteins under reduced conditions.

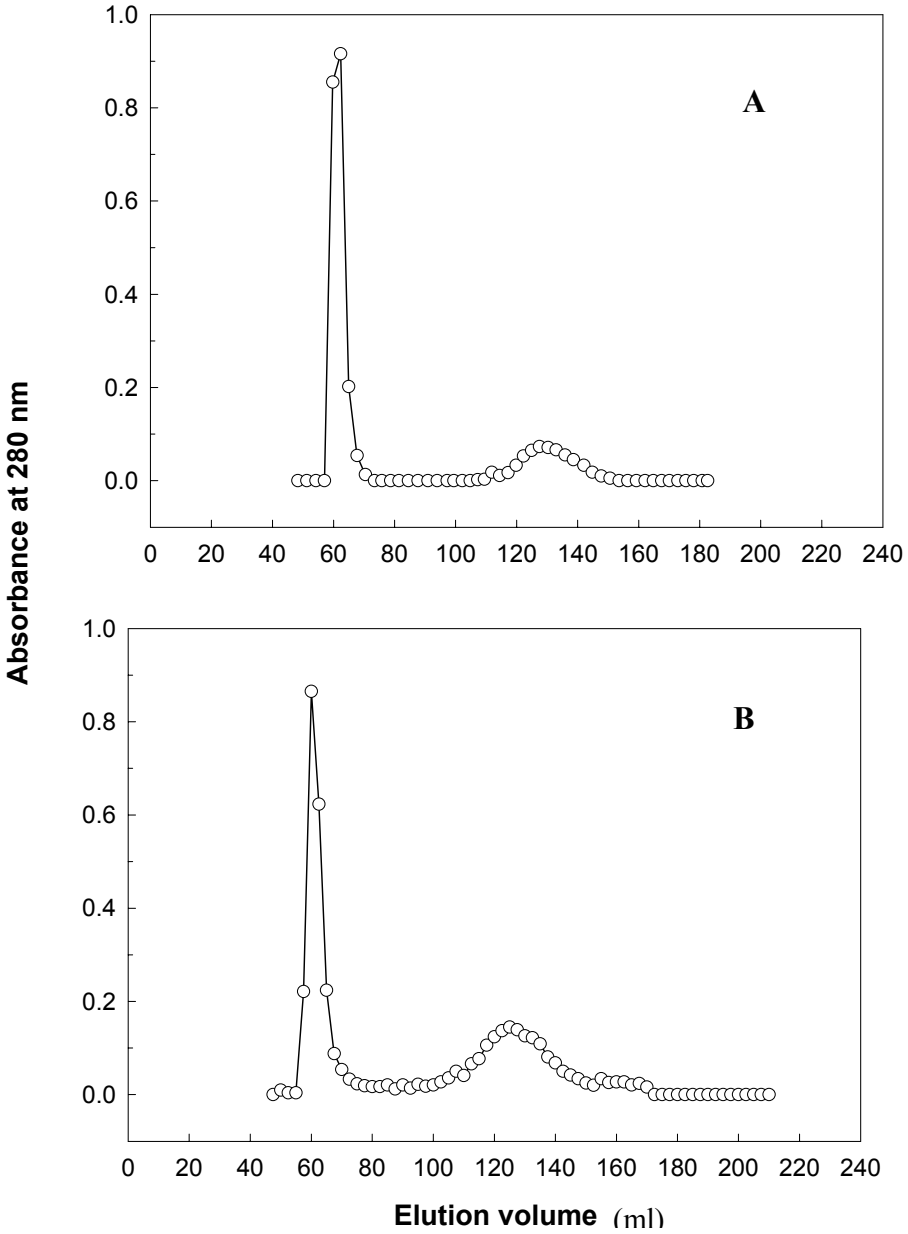


Fig. 46 A, B: Gel filtration profile of proteins extracted with phosphate buffer of pH 7.5 containing 1.0 M NaCl from sardine mince as a function of storage. Extracted protein were loaded on to Sepharose 6B column and eluted with the above buffer. A) immediately after freezing and B) 120 days of storage.

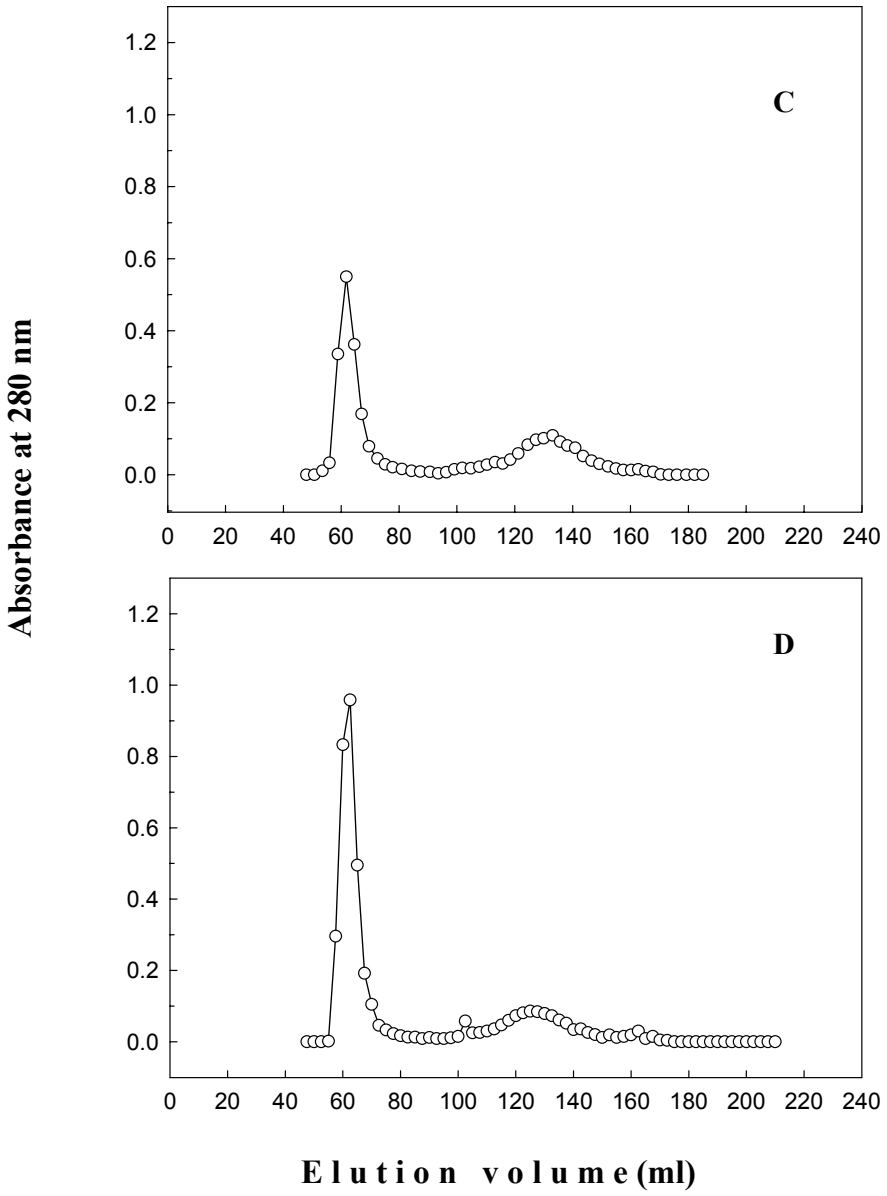


Fig. 46 C, D: Gel filtration profile of proteins extracted with phosphate buffer of pH 7.5, containing 1.0 M NaCl from sardine mince treated with 2% mannitol and frozen stored. Extracted proteins were loaded on to Sepharose 6B column and eluted with the above buffer. C) immediately after freezing and D) 120 days of storage.

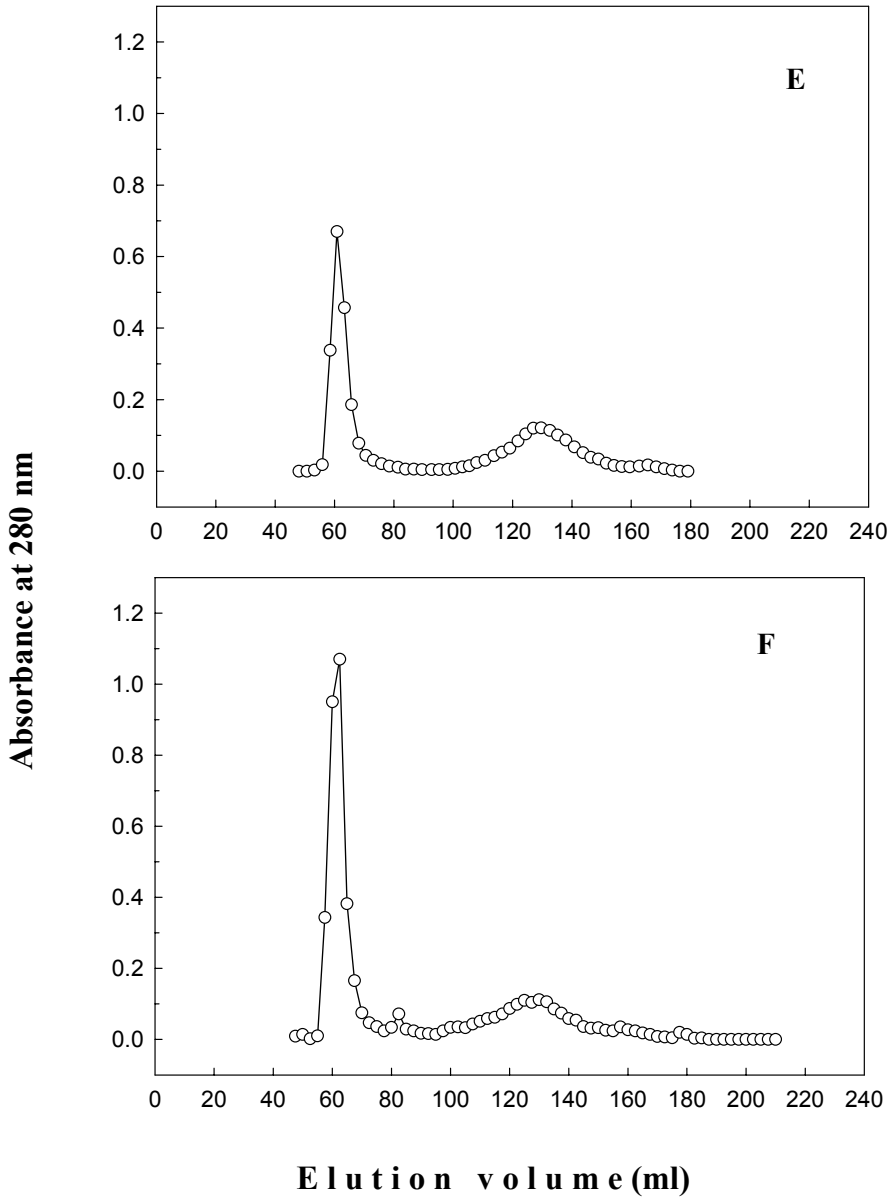


Fig. 46 E, F: Gel filtration profile of proteins extracted with phosphate buffer of pH 7.5, containing 1.0 M NaCl from sardine mince treated with 4% mannitol and frozen stored. Extracted proteins were loaded on to Sepharose 6B column and eluted with the above buffer. E) Immediately after freezing and F) 120 days of storage.

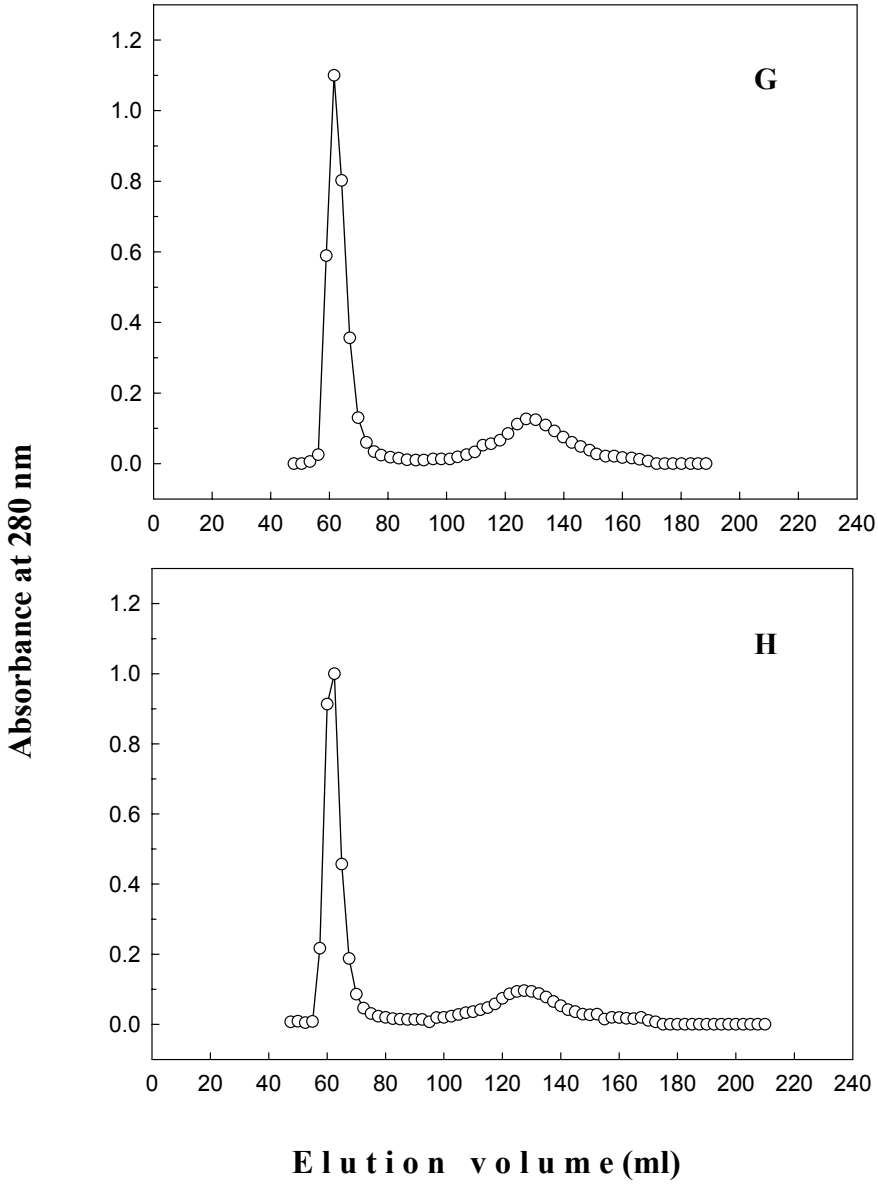


Fig. 46 G, H: Gel filtration profile of proteins extracted with phosphate buffer of pH 7.5, containing 1.0 M NaCl from sardine mince treated with 6% mannitol and frozen stored. Extracted proteins were loaded on to Sepharose 6B column and eluted with the above buffer. G) immediately after freezing and H) 120 days of storage.

The qualitative nature of SDS-PAGE and because of large number of bands it is very difficult to interpret the association-dissociation and aggregation of proteins through the techniques of gel filtration and or by SDS-PAGE. It can be concluded that the aggregation of the proteins is unequivocally certain both from the results of gel filtration and SDS-PAGE. The proteins extracted from sardine mince showed an apparent T_m of 57 ± 1.5ºC (Table17). Apparent T_m of proteins increased by 1.9ºC in presence of mannitol. All the samples treated with mannitol shown higher values in apparent T_m than control samples. Maximum increase was shown by the presence of 2% mannitol. The effect of mannitol in stabilization is seen by the T_m recorded that especially at lower concentrations where a clear bimodal pattern at 2% where as in the control where in and above 2% such bimodal transitions occur at (57°C and 71°C) during storage is not observed and it may be due to change in the surface properties of the proteins at these concentrations of mannitol below this concentrations the net amount of significantly in the bimodal spectra. This is also supported by partial specific volume measurements of other proteins in this concentration region of cosolvents (Sathish, 2000; Gopalakrishna, 2000). At higher concentration of cosolvents the bimodality in terms of T_m values as a function of storage was observed. Apparent T_m as recorded during frozen storage decreased with storage time. Control samples shown a gradual decrease in the values of apparent T_m with increase in storage time. In presence of 2% mannitol the decrease was rapid and it reached to values less than control samples. Higher concentration of mannitol in the samples shown to keep the apparent T_m values more than control samples during storage. Samples with 4% mannitol was able to keep the higher values up to 60 days of storage, there after it decreased rapidly and reached values almost near to control samples. The apparent T_m values did not change even after 75 days of storage and noticed no significant change with control samples. A concentration of 4% mannitol was able to protect the structure of actomyosin, the major protein fraction during frozen storage to some extent as revealed by T_m values. T_m values of proteins depend on several parameters including heating rate, sample size, shape and on denaturation temperature (Poulter *et al*., 1985). Structure and stability of proteins contribute to the functional properties of proteins. Functionality of mince is an essential requirement to

Table 17: Apparent T_m (°C) of sardine proteins in presence of different concentration of mannitol during frozen storage

Days of frozen storage	Cosolvent concentration			
	Control (without mannitol)	**2% mannitol**	**4% mannitol**	**6% mannitol**
0	57.0 ± 1.5	58.9 ± 1.3	58.5 ± 1.2	58.0 ± 0.9
10	57.5 ± 1.2	58.9 ± 1.1	58.3 ± 1.4	58.0 ± 1.0
20	56.7 ± 1.4	58.8 ± 1.4	57.8 ± 1.1	57.6 ± 1.2
35	56.9 ± 1.1	56.8 ± 1.0	58.0 ± 1.3	57.2 ± 1.0
49	56.8 ± 1.3	55.0 ± 1.3	58.5 ± 1.1	57.2 ± 0.9
67	57.0 ± 1.0	55.4 ± 1.2	58.6 ± 0.8	56.8 ± 1.1
77	55.8 ± 1.2	54.7 ± 1.4	56.3 ± 1.2	55.2 ± 1.3
100	55.0 ± 1.1	54.1 ± 0.7	55.8 ± 1.2	55.0 ± 1.1

produce quality products. The storage quality of mince is being monitored by the changes in functional properties during processing and storage.

The functional, physicochemical and hydrodynamic properties of proteins will be influenced by changes in structure of mince. The physical structure of mince is important to maintain the texture and quality of mince. The changes in the structure of mince and its proteins can be monitored by scanning electron microscopy. Microstructure of mince in presence of various concentrations of mannitol and the effect of frozen storage was assessed with the help of scanning electron microscopy is shown in Fig.47-50. There was change in the microstructure of mince in presence of various concentration of mannitol. Mince treated with 6% mannitol showed more porous structure during frozen storage (Fig.50). There was no evidence of fragmentation of myofibrils was observed during storage was observed by phase micrograph (Geensik *et al*., 2000). The structural integrity of mince remained almost similar during the period of storage. SEM is not able to give a clear picture of structural changes in the mince during frozen storage. The porosity of surface of muscle increased with increase in concentration of mannitol in the mince. During frozen storage there was a reduction in the surface porosity of meat, which clearly showed the aggregation phenomenon of proteins during frozen storage. This loss of structure was prevalent in control samples (Fig.47A-D). It is evident from the micrographs that mannitol concentration of 4% was able to protect the structure up to 70 days of storage and it reduced marginally during further storage (Fig.49). Higher concentration of mannitol was able to retain this structure even after 120 days of storage. The structural changes in the actomyosin complex may directly affect different functional properties of the minced meat.

Functional properties of fish mince proteins during storage in presence of mannitol

Emulsion capacity of extracted proteins from mince was studied during storage under low temperature. Emulsion capacity of samples treated with 6% mannitol was more than control samples (Table 18). Lower concentrations of mannitol induced decrease in emulsion capacity. The decrease in emulsion capacity of

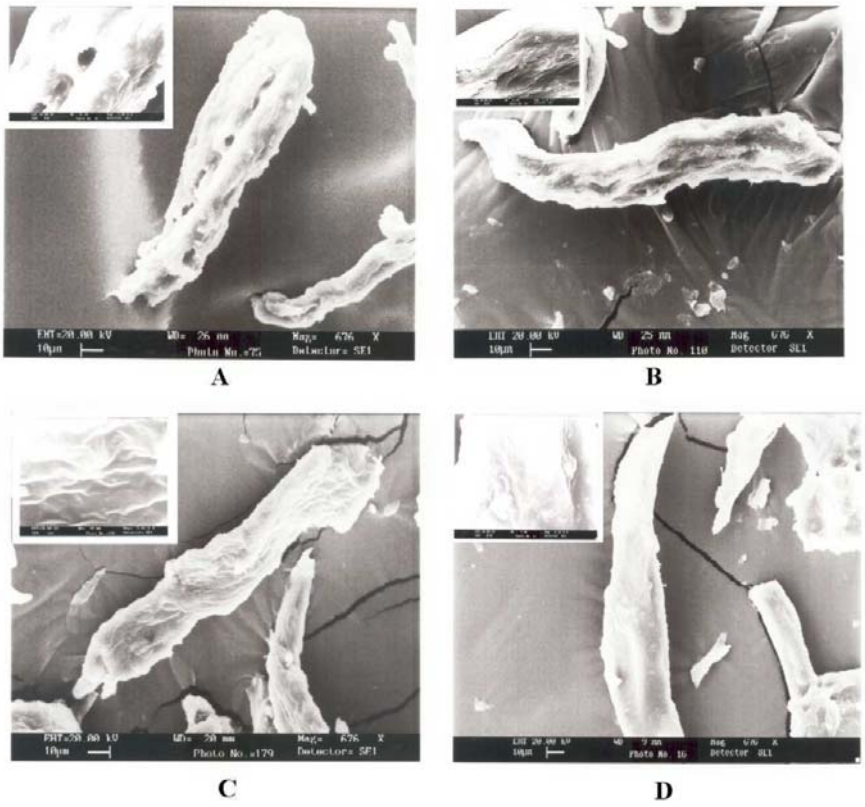


Fig. 47: Scanning electron micrographs of sardine mince during frozen storage. Micrographs were recorded at a magnification of 500. Inset Micrographs at a magnification of 4000. Samples were drawn from frozen storage and thawed to room temperature gradually. A) Immediately after freezing (control); B) 30 days; C) 70 days and D) 120 days of storage.

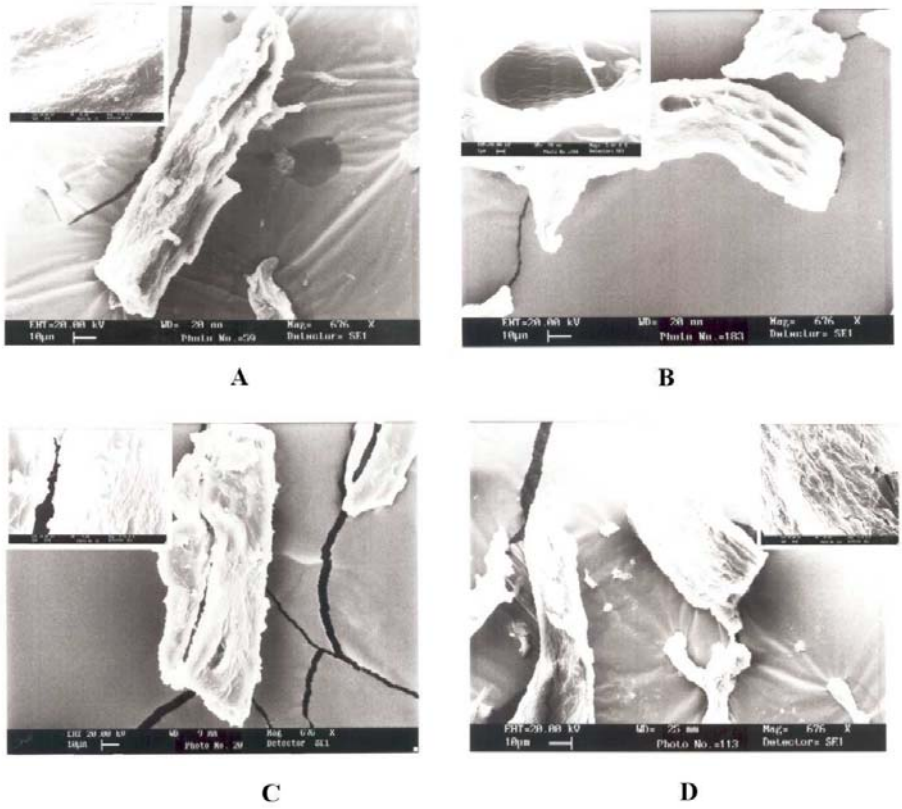


Fig. 48: Scanning electron micrographs of sardine mince treated with 2% mannitol during frozen storage. Micrographs were recorded at a magnification of 500. Inset: Micrographs at a magnification of 4000. Samples were drawn from frozen storage and thawed to room temperature gradually. A) Immediately after freezing (control); B) 30 days; C) 70 days and D) 120 days of storage.

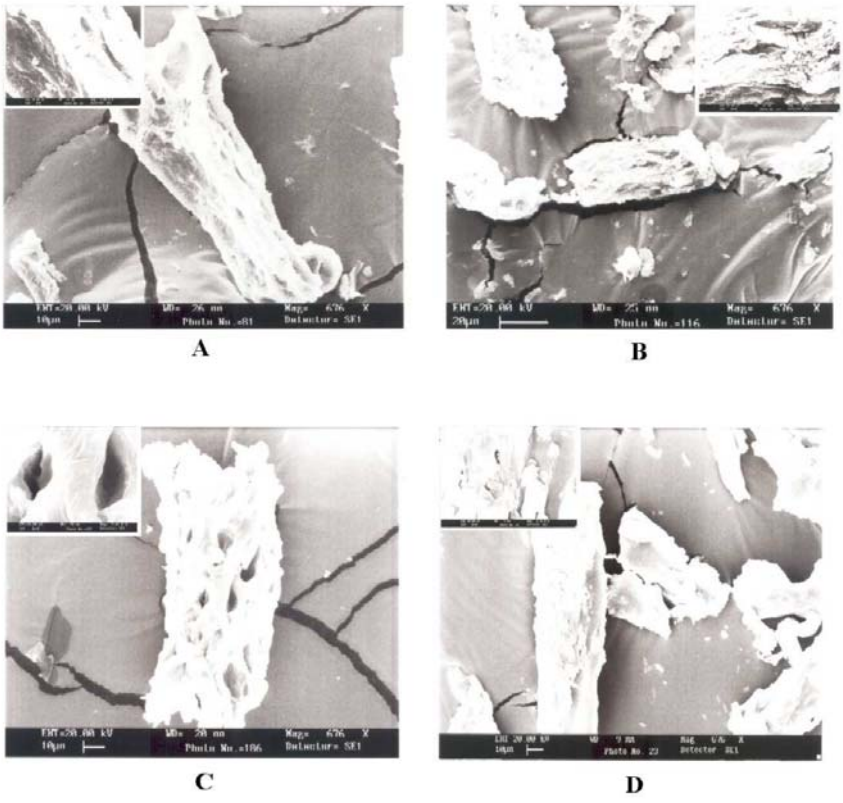


Fig. 49: Scanning electron micrographs of sardine mince treated with 4% mannitol during frozen storage. Micrographs were recorded at a magnification of 500. Inset: Micrographs at a magnification of 4000. Samples were drawn from frozen storage and thawed to room temperature gradually. A) Immediately after freezing (control); B) 30 days; C) 70 days and D) 120 days of storage.

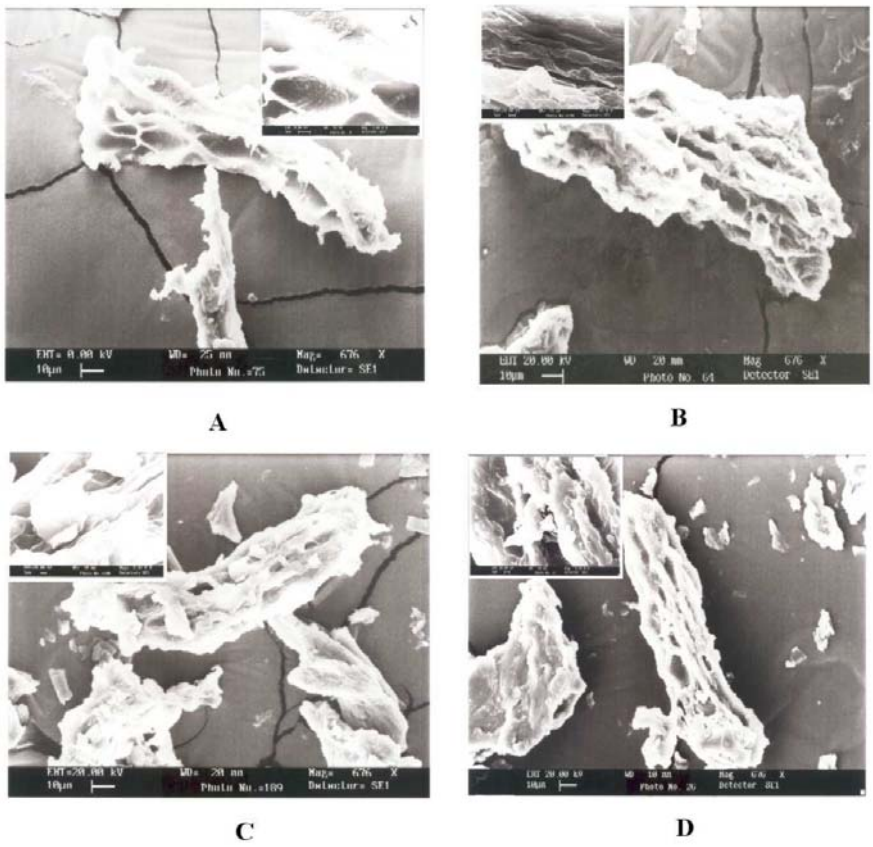


Fig. 50: Scanning electron micrographs of sardine mince treated with 6% mannitol during frozen storage. Micrographs were recorded at a magnification of 500. Inset: Micrographs at a magnification of 4000. Samples were drawn from frozen storage and thawed to room temperature gradually. A) Immediately after freezing (control); B) 30 days; C) 70 days and D) 120 days of storage.

Table 18: Emulsion capacity of proteins extracted in 0.05 M phosphate buffer of pH 7.5 containing 1.0 M NaCl from sardine mince treated with different concentrations of mannitol during frozen storage. (Emulsion capacity is expressed as ml oil/mg protein).

Days of frozen storage	Cosolvent concentration			
	Control (without mannitol)	**2% mannitol**	**4% mannitol**	**6% mannitol**
0	0.13 ± 0.02	0.12 ± 0.01	0.12 ± 0.01	0.14 ± 0.02
14	0.13 ± 0.02	0.12 ± 0.01	0.12 ± 0.01	0.14 ± 0.02
30	0.14 ± 0.02	0.12 ± 0.02	0.14 ± 0.02	0.14 ± 0.01
42	0.12 ± 0.01	0.11 ± 0.01	0.15 ± 0.01	0.14 ± 0.02
51	0.12 ± 0.01	0.12 ± 0.02	0.12 ± 0.01	0.12 ± 0.01
75	0.13 ± 0.01	0.11 ± 0.01	0.12 ± 0.01	0.12 ± 0.02
100	0.13 ± 0.01	0.12 ± 0.01	0.13 ± 0.01	0.10 ± 0.01

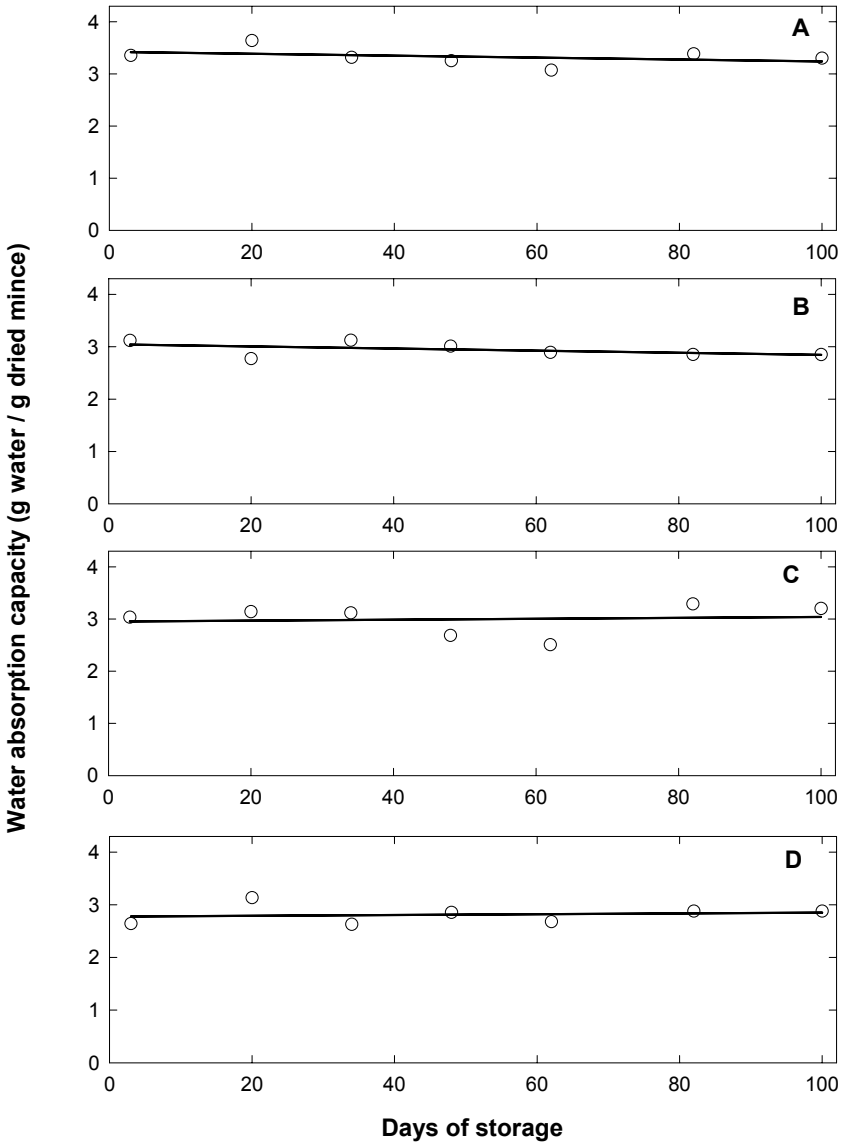


Fig. 51: Water absorption capacity of sardine mince treated with different concentrations of mannitol during frozen storage. Mince were freeze dried before estimating the water absorption capacity and which is expressed as grams of water absorbed per gram of freeze dried mince. A) Control; B) 2% mannitol; C) 4% mannitol and D) 6% mannitol.

storage.

extract from sardine mince was insignificant during frozen storage (Table 18). But for the samples treated with 6% mannitol there was considerable decrease in emulsion capacity after 50 days of storage and reached to values less than the control samples. There was no significant difference in the emulsion capacity of proteins in presence of various concentrations of mannitol. This reduction in emulsion capacity values during frozen storage could be due to formation of aggregates. Three dimensional structure of proteins can be stabilized by both covalent and non covalent interactions. Cryoprotectants reduced the exposure of hydrophobic residues on the surface of actomyosin and consequently the tendency to cluster through intermolecular hydrophobic interactions and to form aggregates (Herrera and Mackie, 2004).

The influence of cosolvents in the exposure of various residues will affect the water structure around proteins. The changes in microenvironment of proteins can be well understood by monitoring the water absorption capacity of freezedried samples. The changes in the water absorption capacity of proteins in presence of varying concentrations of mannitol during frozen storage is shown in Fig.51A-D. The water absorption capacity of the samples treated with mannitol recorded lower values than the control samples indicating the presence of the additive reduced this functionality of mince. The reduction was more for the samples with higher concentration of mannitol. There is a non random distribution of water and stabilizer molecules surrounding the solute; stabilizer molecules are preferentially in the vicinity of the solute since the solute-modifier interactions are energetically favored over the solute-water ones. Water absorption capacity decreased with increase in storage time. A decrease in water binding capacity indicates denaturation of myofibrillar proteins (Sultanbawa and Li-Chan, 1998). Water absorption capacity of untreated samples remained higher than treated samples during the entire period of storage. The reduction in water absorption capacity was similar in case of control samples and samples in presence of 2% and 4% mannitol treated. In the case of samples treated with 6% mannitol water absorption capacity remained almost same during the storage. Mannitol treated samples retained the ability to hold water better than untreated samples.

The detailed study of various properties of proteins during frozen storage of sardine revealed the protective effect of polyhydric alcohols. The hydrodynamic property gel filtration gave a detailed understanding of the composition and properties of proteins of mince. The single peak shown immediately after the void volume in the gel filtration profile of extracted proteins can be attributed as the actomyosin complex (Chan *et al*., 1995). The peaks in the chromatographic pattern of proteins extracted from fish contributed by peak 1 consist of myosin and actin, peak 2 of myosin and peak 3 of actin, tropomyosin and troponin (Mazo *et al*., 1999). The gel filtration profile of mannitol treated samples after 120 days of storage there was a decrease in the height of actomyosin peak. The decrease in peak height has shown that the percentage contribution of the particular fraction reduced in the extract. The decreased extractability of proteins may be a major reason for this. The decrease in the peak height of major protein fraction implies that the molecular species increased in size and shifted to the fraction not extracted in salt solution. Many attempts have been made to compare the gel filtration pattern of fish and shellfish during frozen storage (Chan *et al*., 1995, Shamasunder and Prakash, 1994c). The process of aggregation and conformational changes are able to bring changes in the elution pattern of proteins. The gel filtration profile of fishes like cod indicates dissociation process of high molecular weight components during frozen storage (Ohinishi and Rodeger, 1980). The cleavage of high molecular weight protein to smaller ones are also possible during storage. The smaller molecular weight fractions will be leached from the extract during dialysis. The cleavage of high molecular weight proteins may not increase the peak height of low molecular weight fractions. This also predicts the formation of aggregates during storage under low temperature. The protein profiles by SDS-PAGE helps to understand the process of aggregation during storage.

The protein profile assessed by SDS-PAGE showed a difference in pattern of proteins due to the storage under low temperature. The presence of mannitol did not alter the protein profile. The presence of mannitol did not result in any change in the protein composition or formation of bonds between the protein molecules. During frozen storage the aggregation of proteins was observed, especially the high molecular weight myosin heavy chain. During freezing and frozen storage the protein – protein

interaction favours the formation of protein dimers. From the literature it is evident that during freezing, the MHC was polymerised mostly as dimers (Sultanbawa and Li-Chan, 2001). The formation of aggregates involves various kinds of interaction between protein molecules and different kinds of linkages are possible between the proteins. The covalent cross-linking between the MHC molecules is predominantly formed by disulfide bonds (Lian, *et al*., 2000). Studies by Le Blanc and Le Blanc (1992) and Ramirez *et al.* (2000) revealed a reduction in the SH content and thus a possible formation of disulfide bonds during frozen storage of fish. The increase in number of hidden sulfhydryl groups during frozen storage indicates the aggregation of proteins (Sultanbawa and Li-Chan, 2001). During frozen storage high molecular weight salt soluble aggregates formed in which the proteins are partially linked by non disulfide covalent bonds, but it does not exclude the possible involvement of other kinds of bonding (Mazo *et al*., 1999). Since the low molecular weight protein bands did not show any change in the pattern it could be concluded that the proteolytic activity was considerably low in the minced meat. The formation of aggregates is mainly contributed by the protein – protein interaction of myosin heavy chain. The presence of mannitol at and above 4% effected in decreasing the aggregation of proteins.

The changes in structure-function of proteins will result in changes in the thermal properties of mince. Thermal transition of sardine mince during frozen storage may be influenced by the presence of additives. Since proteins are the major constituents of mince, thermal properties are mainly depends on the properties of proteins present in mince. The transition temperature, which is measured as apparent thermal transition temperature of extracted protein, was 57 ± 1.5ºC. Thermal transition of extracted proteins from mince was mainly contributed by myosin molecules presented in the mince. DSC thermogram of muscle depleted of sarcoplasmic protein fractions, a clear endothermic peak (T_m = 60.1ºC) was observed in case of shark muscle (Chen, 1995). Sano *et al*. (1990a) suggested that the transformation of myosin in the temperature range of 51ºC – 80ºC is due to the interaction among the head portion of myosin molecules. Apparent T_m of proteins from sardine mince increased by 1.9ºC in presence of mannitol. Sugars increase the

thermal denaturation temperature of globular proteins in aqueous solutions. Sugars have also been shown to reduce the extent of unfolding of globular proteins absorbed to interfaces. The presence of manitol are not able to extend the complete protection of proteins during storage. There was decrease in thermal transition temperature even in presence of this additive. Polyhydric alcohol additives are able to reduce the extent of denaturation during storage at low temperature.

Functionality of proteins from sardine mince decreased during various treatments and storage. Emulsion capacity showed a decreasing trend with increase in storage period. Three dimensional structure of proteins can be stabilized by both covalent and non covalent interactions. Protein flexibility is also affected by noncovalent interactions such as hydrogen bonding, van der Waals forces, electrostatic links and hydropohobic interactions. The freeze induced denaturation of proteins in mince resulted in the reduction of emulsion capacity of extract. Presence of different concentrations of polyhydric alcohol did not affect the emulsion capacity significantly. The reason for this might be that the exposures of residues are more important in the formation of stable emulsions than that of the structure of proteins. The presence of polyhydric alcohols did not bring considerable change in the exposure of side chains of actin and myosin molecules of mince. To stabilize an emulsion, the hydrophobic domain of the proteins should ideally be oriented towards the oil phase.

Water absorption capacity is the other important functionality of proteins from mince. The retention of water between the protein molecules reduced during the storage. The water holding capacity depends on the nature of muscle structure and on the nature of additives present in between the protein molecules. The cohesion between water molecules is much larger than that between stabilizer molecules and it remains to be the dominant cause of the hydrophobic behavior of the non-polar solutes (Carrillo-Nava *et al*., 2004). Mannitol treated samples retained the ability to hold water better than untreated samples. Myofibrillar proteins such as myosin partially unfolds when the temperature falls below a particular level and the exposed nonpolar groups can interact with similar groups on the neighboring protein leading to

aggregation that lead to adverse change in the texture and water holding capacity of muscle (McClements, 2002). Hydrophilic interaction of both C = O and N - H groups of proteins molecules occurred during frozen storage and caused protein aggregation-denaturation (Jiang *et al*., 1987). The reduction in water absorption capacity during storage can be attributed to the formation of aggregates. The presence of mannitol inhibited the formation of aggregates so that resulted in better water retention during storage. Also the changes were brought about by the physical structure of muscle in the mince that was recorded by observing the structure at high magnification by SEM.

The surface properties changed due to the presence of mannitol in the mince. This might be either due to the change in water structure around the protein molecules or either by the solubilization of proteins by the presence of these additives. The addition of different additives resulted in microstructure differences on thermally induced fish protein gels (Gao *et al.*, 1999). No significant change in the microstructure of mince was noticed during storage. The retention of porosity of surface of muscle might have been due to the higher water content present in the microenvironment of proteins. This indicates the reduced aggregation of proteins during storage in presence of mannitol compared to control samples. The changes in structure may bring changes in enzymatic properties of mince. The reduction in the ATPase activity during frozen storage indicates that the integrity of actin and myosin molecules was lost during the process of freezing and frozen storage. The loss of ATPase activity is not necessarily synonymous with frozen storage induced aggregation of myosin (Ramirez *et al*., 2000). The loss of ATPase activity does not necessarily imply that the myosin head has been unfolded; it only indicates that myosin head undergo some conformational changes (Sultanbawa and Li-Chan, 2001). The globular head of myosin are responsible for its enzymatic (ATPase) activity, which is sensitive to changes in the configuration of the molecule around the enzymatic site (Mackie, 1993). The change in microstructure clearly indicated the change in the structure of myosin head, which is responsible for the ATPase activity. This also indicates the formation of aggregates during frozen storage that decreases the functionality of mince. In the presence of mannitol as cryoprotectant additive the aggregation of protein were inhibited.

The effect of mannitol on the structure, function and stability of proteins from fish mince both in the control and frozen stored suggests the role played by both covalent and noncovalent interactions in the myosin heavy chain of the proteins. The results also indicate that to a certain extent mannitol prevent aggregation of proteins in fish mince during storage. It also stabilizes the proteins against denaturation through an indirect effect of altering the bulk solvent structure perhaps around the microenvironment of these proteins. These results substantiates that the cosolvents which are known to be preferentially excluded from the vicinity of the protein surface perhaps contribute to the stability and protection of proteins through the steric exclusion effect.

CHAPTER - 5

EFFECT OF CALCIUM SALTS ON THE STRUCTURE AND FUNCTION OF PROTEINS FROM SARDINE MINCE DURING FROZEN STORAGE

EFFECT OF CALCIUM SALTS ON THE STRUCTURE AND FUNCTION OF PROTEINS FROM SARDINE MINCE DURING FROZEN STORAGE

The three dimensional structure of proteins can be stabilized by both covalent and noncovalent interactions. The effect of certain salts from Hoefmeister series on proteins is known to be protective agents from denaturing proteins (Jencks, 1969). Acetate ions favour dissociation of the prawn proteins initially and the dissociated proteins reaggregate (Shamasunder and Prakash, 1994a). Calcium compounds are helpful to improve the gelation characteristics of fish proteins and dependent on specific species and their inherent characteristics (Lee and Park, 1998). The divalent metal ions, calcium and magnesium, at the same concentration, bring about the different extents of association-dissociation in prawn proteins and is a salt dependant phenomenon (Shamasunder and Prakash, 1994b).

Ca^{2+} ions when added as calcium chloride are particularly effective in lowering the water holding capacity of proteins and therefore the viscosity of muscle dispersion. Calcium sulfate is also allowed in the food as an additive. Calcium sulfate is relatively cheap and imparts a white colour to the mince. Sulfates are also known to stabilize the proteins from denaturation. Calcium chloride has been of much interest to meat scientists as well as meat processors due to its ability to tenderize meat (Nurmahmudi and Sams, 1997). In this chapter, an attempt has been made to find out the effect of calcium salts on the properties of proteins from an underutilized low value fish of seasonal abundance. Two calcium salts *viz.* calcium chloride and calcium sulfate were selected to study the effect on the physicochemical and functional properties of proteins during the period of storage at –20°C.

Enzymatic and hydrodynamic properties of fish mince proteins during storage

Enzymatic and structural changes occur to the mince during storage at low temperature in presence of low concentration of salts. Adenosine triphosphatase enzyme (ATPase) is one of the important enzymatic activities shown by the proteins from fish mince. This activity is influenced by the changes in structure of actin and

myosin molecules and is a good tool to understand the integrity of protein molecules. ATPase activity of extract from the calcium treated samples were analysed during storage at low temperature is shown in Fig.52 & 53. The ATPase activity decreased during storage. The decrease was rapid in the initial period of storage and at later part it decreased gradually till it reached a constant value after 60 days of storage. ATPase activity of extract from the samples treated with calcium chloride was more than that of control samples (Fig.52). In presence of $1x10^{-2}$ M calcium chloride activity was slightly more than control samples. ATPase activity of extract from samples treated with $1x10^{-4}$ M calcium chloride was significantly higher than control samples. The decrease in ATPase activity was rapid for samples treated with $1x10^{-4}$ M calcium chloride during frozen storage. Sharp decrease in ATPase activity occurred up to 30 days of storage and it further decreased and recorded 70% reduction after 60 days of storage. After that there was no significant change in the activity of all the samples till the end of the study.

In Fig.53 ATPase activity of extract during storage of calcium sulfate treated samples are shown. ATPase activity of extract from mince samples treated with calcium sulfate decreased during storage. Activity of the samples from calcium treated samples was more than control samples. Low concentration of calcium sulfate i.e. $1x10^{-4}$ M showed highest values in the activity. The difference in activity between $1x10^{-4}$ M and $1x10^{-2}$ M calcium sulfate treated samples were not significant. The activity decreased rapidly up to 30 days of storage. Further reduction was gradual and reached constant values after 60 days of storage. Wagner and Anon (1986) reported that myosin head was affected during first week of storage leading to slight alterations occurring subsequently. Frozen storage of rainbow trout actomyosin resulted in the loss of 90% ATPase activity after 4 weeks of storage (Herrera and Mackie, 2004). Difference in the activity of actomyosin was due to the changes in protein structure and function influenced by the microenvironment. The changes in protein structure may alter the other properties of muscle proteins that will decide the keeping quality of mince during frozen storage.

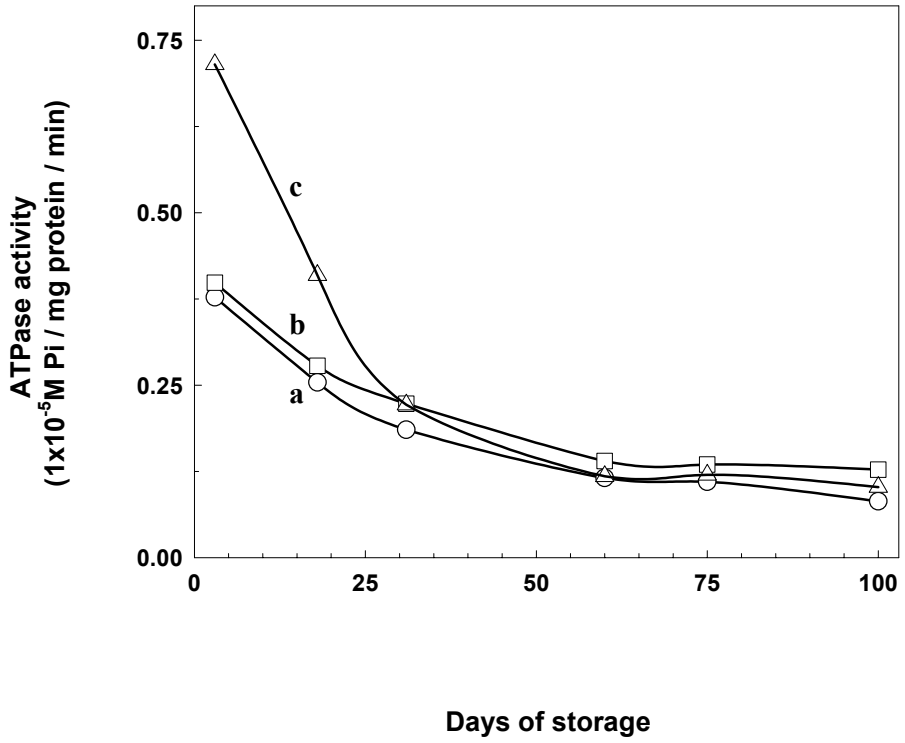


Fig. 52: ATPase activity of proteins extracted in glycine-NaOH pH 9.0 (0.2 M) containing 0.5 M KCl from sardine mince treated with different concentrations of calcium chloride during frozen storage as a function of storage period. a) Control; b) $CaCl_2$ $1x10^{-2}$ M and c) $CaCl_2$ $1x10^{-4}$ M. The enzyme activity described as micromoles of inorganic phosphate released per mg of protein in the extract per minute.

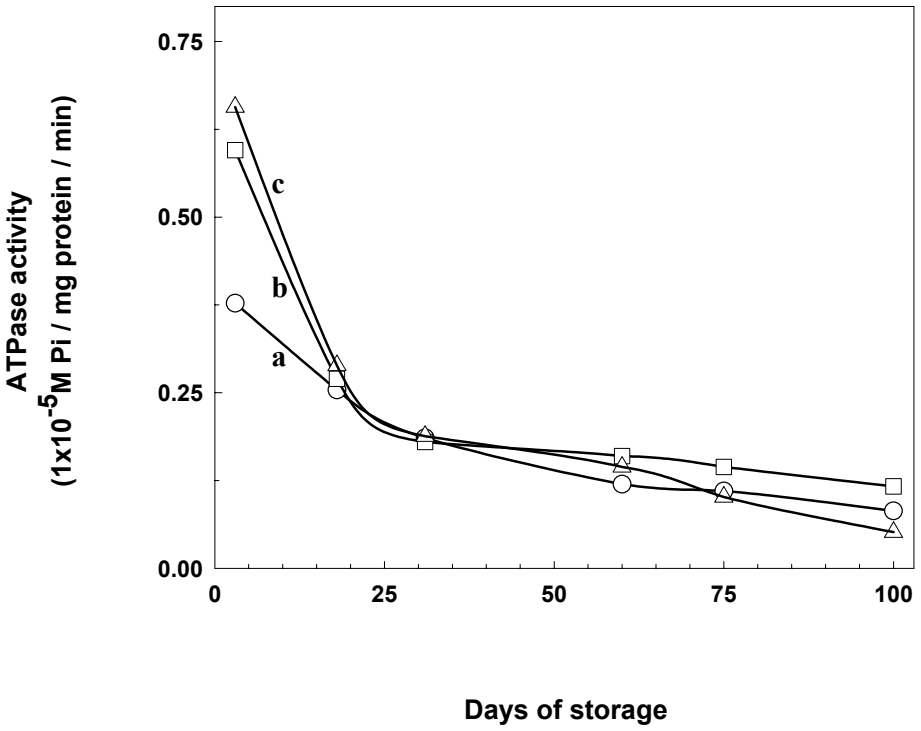


Fig. 53: ATPase activity of total proteins extracted in glycine-NaOH pH 9.0 (0.20 M) containing 0.5 M KCl from sardine mince treated with different concentrations of calcium sulfate during frozen storage as a function of storage period. a) Control; b) $CaSO_4$ $1x10^{-2}$ M and c) $CaSO_4$ $1x10^{-4}$ M. The enzyme activity described as micromoles of inorganic phosphate released per mg of protein in the extract per minute.

Solubility of proteins is one of the major properties influenced by various additives and treatment. Metal ions are known to precipitate the proteins from solutions and thus decrease the solubility of proteins. This property has a direct influence on other functionality of proteins also. Extractability of proteins from the sardine mince in the given buffer during the storage period in presence of calcium chloride is shown in Fig.54 and in presence of calcium sulfate is shown in Fig.55. Solubility/extractability decreased with storage time. The decrease in solubility likely due to changes occurring in the protein microenvironment associated with freezing and thawing (Franks, 1985; Shenouda, 1980). Initial extractability of proteins from the calcium chloride treated samples was lesser than the control samples. Samples treated with $1x10^{-4}$ M calcium chloride have shown the lowest extractability (Fig.54). Extractability of control samples decreased rapidly up to 80 days of storage. The decrease in extractability of $1x10^{-2}$ M calcium chloride treated samples was gradual and was lesser than control samples throughout the storage period. The samples treated with $1x10^{-4}$ M calcium chloride could retain the initial solubility even after 120 days of storage. The extractability was more for these samples than control samples after 80 days of storage. The percentage reduction in extractability was lesser for calcium chloride treated samples compared to the control samples.

When the mince was treated with calcium sulfate instead of calcium chloride it showed totally a different pattern. Samples treated with $1x10^{-4}$ M calcium sulfate showed same extractability as that of control samples and those samples with $1x10^{-2}$ M calcium sulfate were having very less extractability (Fig.55). The reduction in the extractability of the proteins from these samples followed the same pattern as that of control samples. The reduction in extractability was 40% of the initial value after 80 days of storage. The decrease in extractability might have contributed to the changes in hydrodynamic properties of proteins by the presence of salts and storage.

The changes brought in hydrodynamic properties by the presence of salts due to change in shape and size of protein molecules. Elution profile of extracted proteins in selected gel columns will give an indication regarding the changes in the properties of protein molecules. The gel filtration profile of extracted proteins from samples

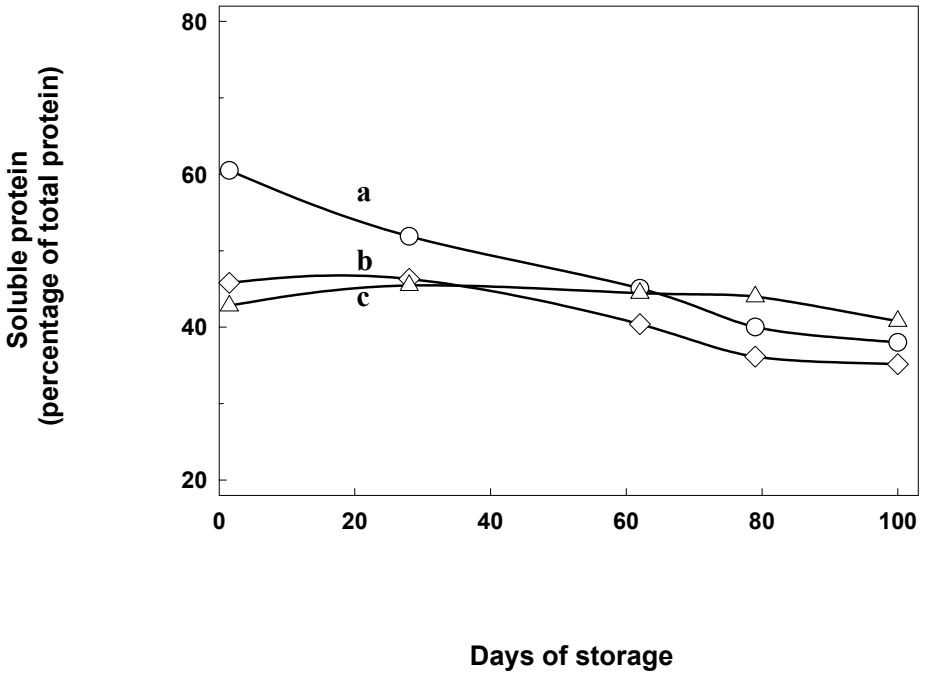


Fig. 54: Extractability of proteins from sardine mince treated with different concentrations of calcium chloride and frozen stored. Proteins were extracted to phosphate buffer (0.05 M) pH 7.5 containing 1.0 M NaCl and the protein content in the supernatant was determined by micro-kjeldahl nitrogen estimation and expressed as percentage of protein solubilized to buffer from total protein content of mince. a) Control; b) $CaCl_2$ $1x10^{-2}$ M and c) $CaCl_2$ $1x10^{-4}$ M.

treated with different concentrations of calcium compounds are shown in Fig.56-57. The change in elution volume of proteins was not significant to show any aggregation of proteins in presence of calcium compounds. The peak height of actomyosin fraction in the extract from samples treated with $1x10^{-2}$ M calcium chloride and elution volume remained same during storage. The second peak which eluted out at 120 ml corresponds to low molecular weight proteins showed decrease in peak height and eluted out at an elution volume 115 ml indicate aggregation of these proteins. Elution profile of protein extracted from samples treated with $1x10^{-4}$ M calcium chloride showed a reduction in the peak height of actomyosin fraction during storage with a corresponding increase in the low molecular weight fraction.

Calcium sulfate in the mince samples resulted in the reduction of actomyosin peak in gel filtration profile (Fig.57). There was an increase in the height of small molecular weight components associated with increase in peak area clearly indicates the degradation of high molecular weight fractions during storage. The change in the profile was more in samples treated with $1x10^{-2}$ M calcium sulfate than the samples with $1x10^{-4}$ M calcium sulfate. In case of samples treated with $1x10^{-4}$ M calcium sulfate elution volume of actomyosin peak remained unchanged but low molecular weight fraction the elution volume shifted from 115 ml to 130 ml during storage. There was a reduction in the peaks of major protein fraction (actomyosin complex) from samples treated with $1x10^{-4}$ M calcium compounds. Protein samples extracted from samples treated with $1x10^{-2}$ M calcium sulfate also showed reduction in peak height of actomyosin peak. The increase in height of low molecular weight fraction was more significant than samples with low concentration of calcium sulfate. These differences in the hydrodynamic properties may influence the functionality of proteins. The changes in functional properties are of utmost importance to determine the various uses of stored sardine mince.

Functional properties of fish mince proteins during storage

Emulsion capacity is one of the important functional properties of proteins. Proteins act as surfactant during the emulsification process. The orientation of

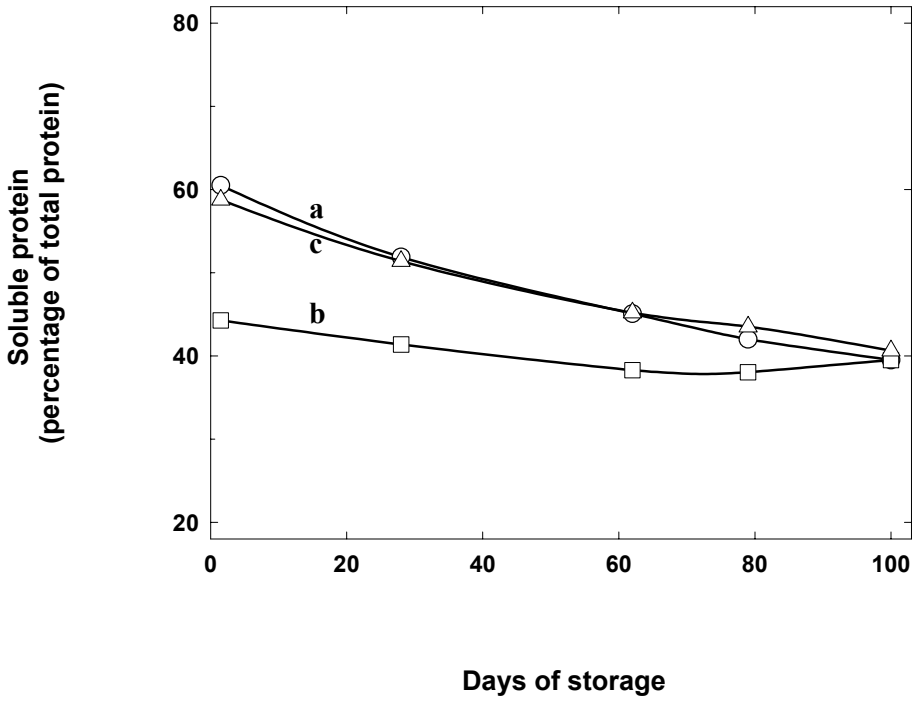


Fig. 55: Extractability of proteins from sardine mince treated with different concentrations of calcium sulfate and frozen stored. Proteins were extracted to phosphate buffer (0.05 M) pH 7.5 containing 1.0 M NaCl and the protein content in the supernatant was determined by micro kjeldahl nitrogen estimation and expressed as percentage of protein solubilized to buffer from total protein content of mince. a) Control; b) $CaSO_4$ $1x10^{-2}$ M and c) $CaSO_4$ $1x10^{-4}$ M.

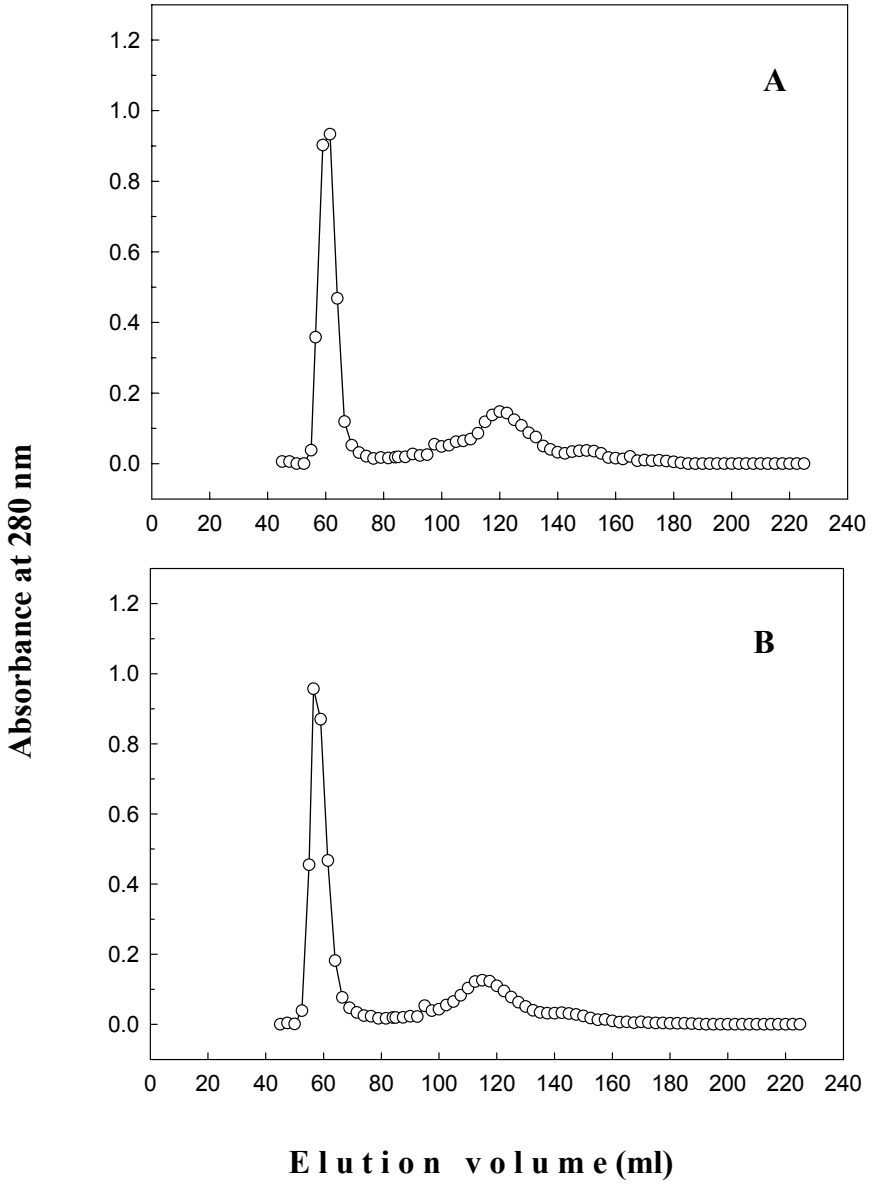


Fig. 56 A, B: Gel filtration profile of proteins extracted with phosphate buffer of pH 7.5, containing 1.0 M NaCl from sardine mince treated with $1x10^{-2}$ M $CaCl_2$ and frozen stored. Extracted proteins were loaded on to Sepharose 6B column and eluted with the above buffer. A) immediately after freezing and B) 120 days of storage.

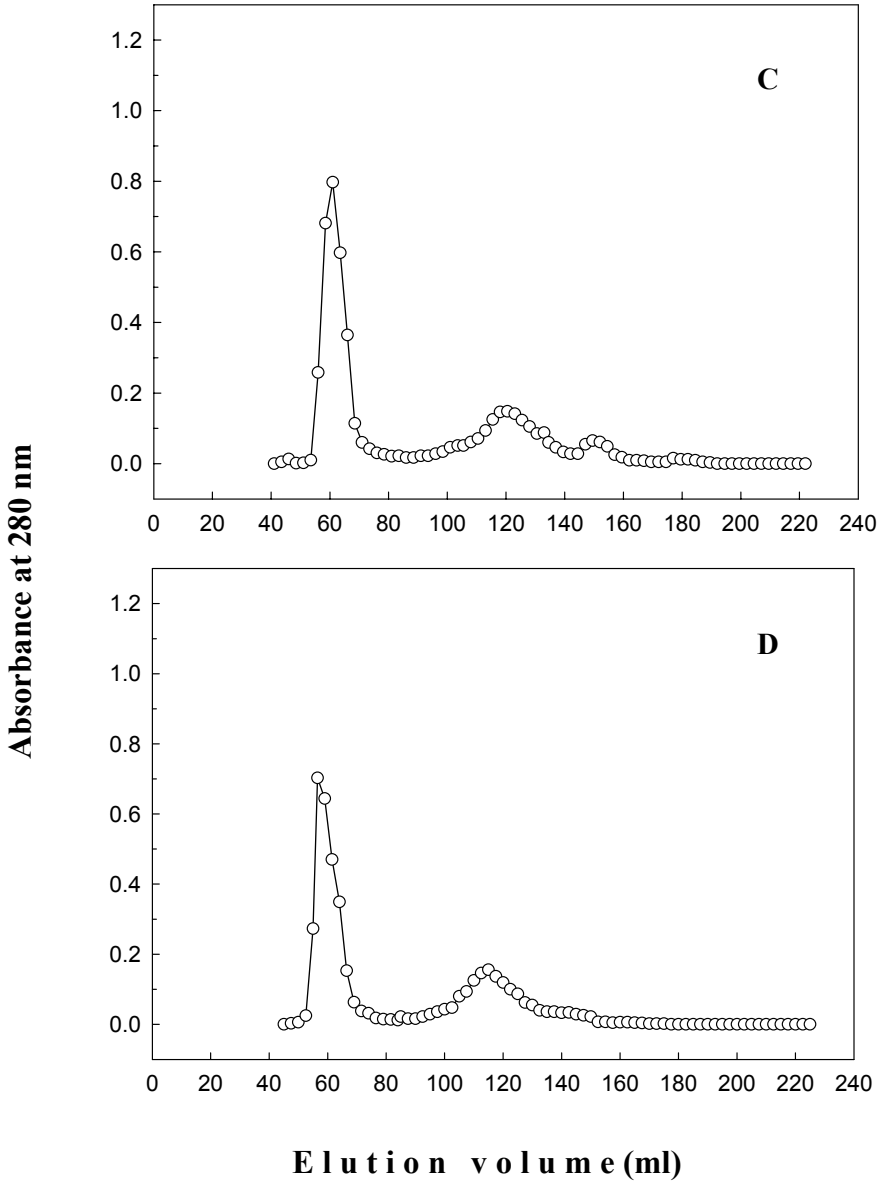


Fig. 56 C,D: Gel filtration profile of proteins extracted with phosphate buffer of pH 7.5, containing 1.0 M NaCl from sardine mince treated with $1x10^{-4}$ M $CaCl_2$ and frozen stored. Extracted proteins were loaded on to Sepharose 6B column and eluted with the above buffer. C) immediately after freezing and D) 120 days of storage.

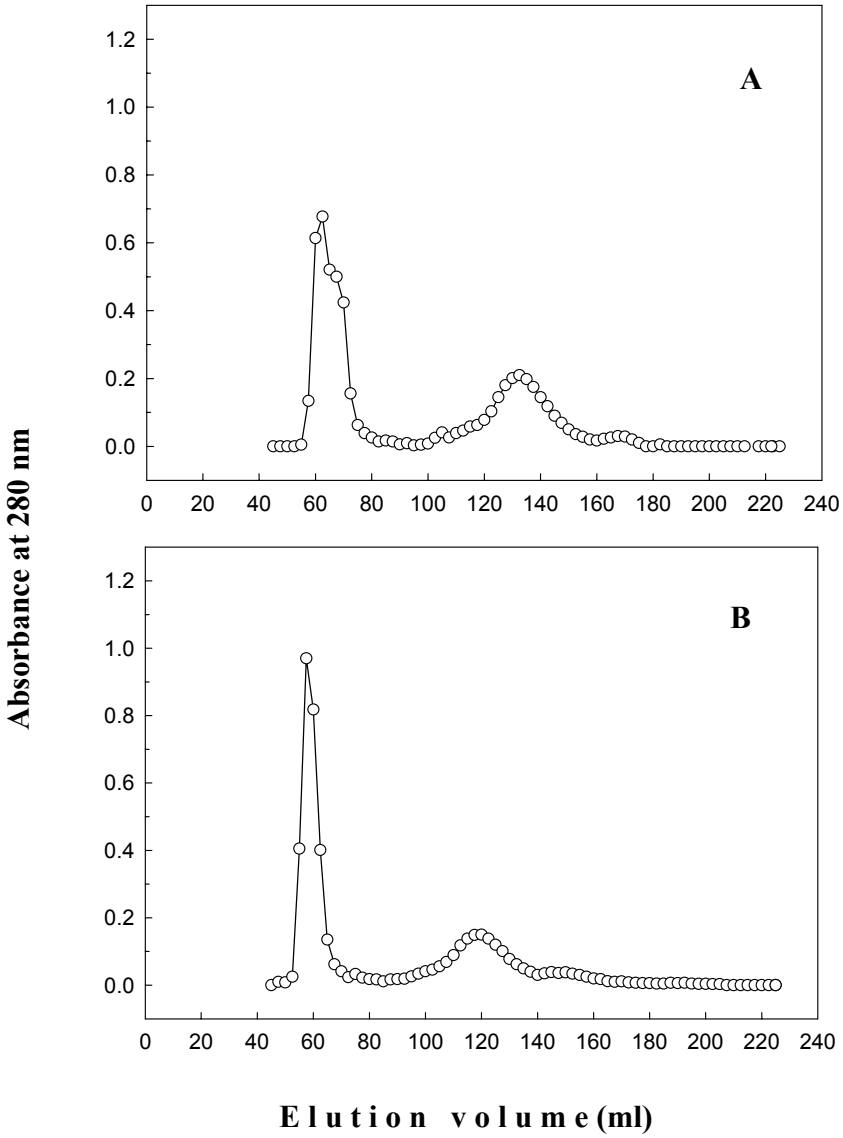


Fig. 57 A, B: Gel filtration profile of proteins extracted with phosphate buffer of pH 7.5, containing 1.0 M NaCl from sardine mince treated with $1x10^{-2}$ M $CaSO_4$ and frozen stored. Extracted proteins were loaded on to Sepharose 6B column and eluted with the above buffer. A) immediately after freezing and B) 120 days of storage.

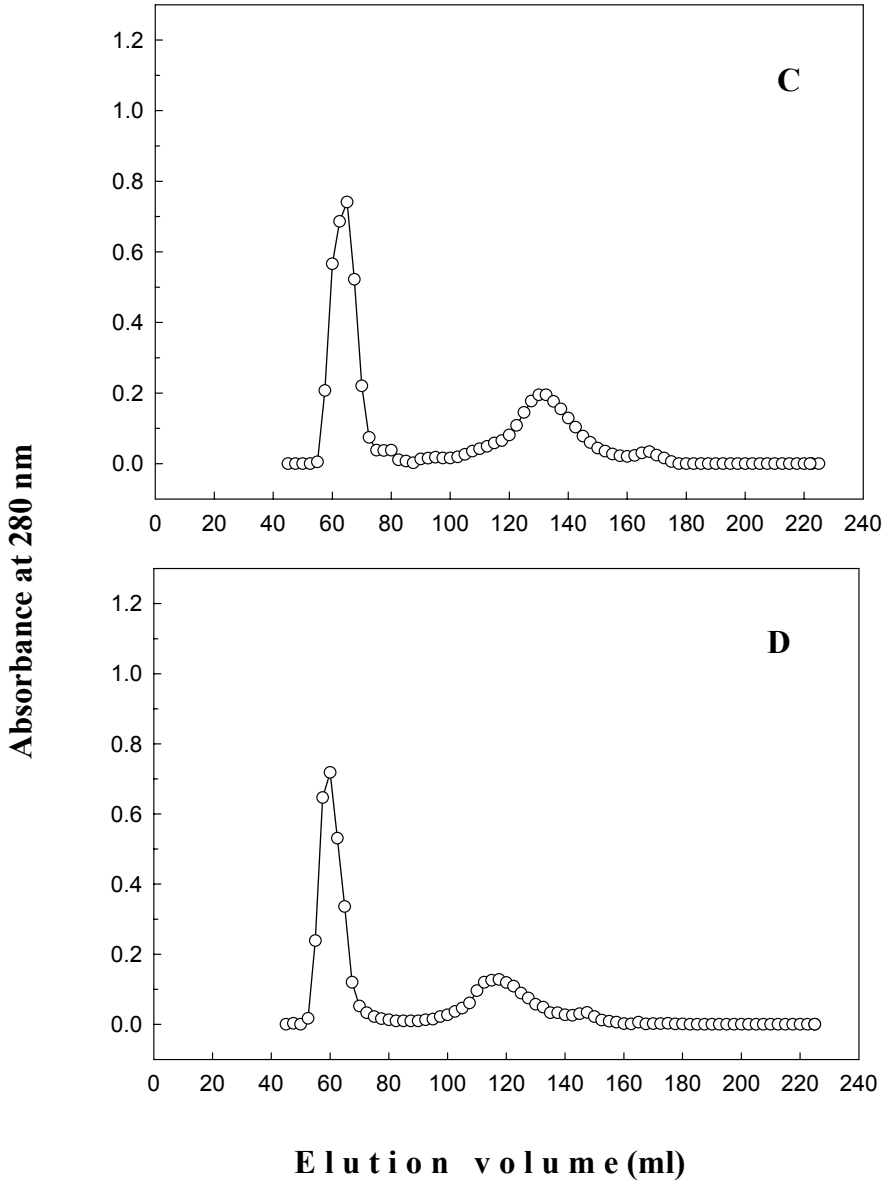


Fig. 57 C,D: Gel filtration profile of proteins extracted with phosphate buffer of pH 7.5, containing 1.0 M NaCl from sardine mince treated with $1x10^{-4}$ M $CaSO_4$ and frozen stored. Extracted proteins were loaded on to Sepharose 6B column and eluted with the above buffer. C) immediately after freezing and D) 120 days of storage.

residues and the extent of exposure of these residues will influence the emulsification capacity of mince. The emulsion capacity of extract from sardine mince samples treated with different calcium salts were measured during storage. The proteins present in the extract mainly contribute to the emulsifying activity. The emulsion capacity of proteins extracted from calcium treated samples are tabulated in Table 19. Emulsion capacity of proteins was more for samples treated with calcium chloride than untreated samples. The higher emulsion capacity was observed in mince samples treated with $1x10^{-4}$ M calcium chloride. Control samples followed a linear relation in reduction of emulsion capacity with increase in storage period. The reduction was relatively small. The decrease in emulsion capacity in presence of calcium salts was more compared to control samples. Emulsion capacity increased after 25 days of storage in case of all the samples. This increase in emulsion capacity could be due to the formation of altered structures by interaction of proteins mediated by the metal ions present. But the emulsion capacity showed a general trend of decrease during storage. After 100 days of storage the capacity to form emulsion by extracts from treated samples decreased to values corresponded to that of control samples.

In presence of $1x10^{-2}$ M calcium sulfate the emulsion capacity was more as compared to control samples (Table 19). This increase in emulsion capacity was not significant compared to control samples. Samples treated with $1x10^{-4}$ M calcium sulfate showed lesser values in emulsification capacity compared to control samples. In all these cases it increased after 25 days of storage. Emulsion capacity showed a general trend of decrease with storage period. To stabilize an emulsion the hydrophobic domain of the proteins should ideally be oriented towards the oil phase. Proteins undergo continuous conformational changes during frozen storage and as a result the location of nonpolar residues varies (Herrera and Mackie, 2004). In presence of calcium sulfate, there was no significant change in emulsion capacity of proteins compared to untreated samples. The reduction in emulsion capacity was more pronounced for samples treated with $1x10^{-2}$ M calcium chloride. Since the formation of emulsion depends on the oil and water phase the water present mince and the capacity to hold water by the mince is an important parameter to be considered.

Table 19: Emulsion capacity of proteins extracted in 0.05 M phosphate buffer of pH 7.5 containing 1.0 M NaCl from sardine mince treated with different concentrations of calcium salts during frozen storage. (Emulsion capacity is expressed as ml oil/mg protein).

Days of frozen storage	Control (without mannitol	Calcium chloride (M)		Calcium sulfate (M)	
		$1x10^{-2}$	$1x10^{-4}$	$1x10^{-2}$	$1x10^{-4}$
0	0.13 ± 0.02	0.17 ± 0.04	0.21 ± 0.02	0.14 ± 0.02	0.12 ± 0.01
22	0.15 ± 0.03	0.19 ± 0.03	0.25 ± 0.04	0.15 ± 0.01	0.16 ± 0.02
52	0.10 ± 0.01	0.07 ± 0.02	0.09 ± 0.02	0.09 ± 0.01	0.08 ± 0.02
75	0.12 ± 0.01	0.13 ± 0.01	0.13 ± 0.02	0.08 ± 0.01	0.12 ± 0.01
87	0.13 ± 0.02	0.13 ± 0.02	0.09 ± 0.01	0.11 ± 0.02	0.07 ± 0.01
100	0.14 ± 0.02	0.13 ± 0.01	0.14 ± 0.01	0.13 ± 0.02	0.15 ± 0.02

The capacity of mince to keep the water in the immediate vicinity is influenced by various parameters and the presence of other compounds around the proteins. Proteins are the major components contribute to the water holding capacity of mince. Water absorption capacity is a major functional property being influenced by the changes in the microstructure and environment of proteins. Fig.58 shows that in presence of calcium chloride the water absorption capacity of mince decreased than that of control samples. The values of water absorption capacity was 9% less for samples treated with $1x10^{-4}$ M calcium chloride than the control samples. Water absorption capacity of mince decreased after an initial increase during storage. The maximum values were recorded after 30 days of storage in case of control samples and after 45 days in case of calcium chloride treated samples. The water absorption capacity decreased gradually with increase in storage time. Rate of decrease in water absorption capacity was more in case of control samples compared to calcium chloride treated samples. This reduction might have been attributed by the aggregation of proteins.

The water absorption capacity of mince in presence of calcium sulfate during frozen storage is shown in Fig.59. The water absorption capacity decreased in presence of calcium sulfate also. Samples with $1x10^{-4}$ M calcium sulfates showed the minimum values. In presence of calcium sulfate also the water absorption capacity decreased after an initial increase during storage. The untreated samples maintained higher values than treated samples during the study. There was an initial increase in water absorption capacity of all samples. The maximum reduction was observed in case of samples treated with calcium sulfate at higher concentration. Changes in water absorption capacity indicate the changes in the structure of protein, which is responsible for maintaining the water molecules in the system. Freezing modifies the structural organization of water, and therefore the network of hydrogen bonds so that many buried hydrophobic residues in the native protein become exposed (Ang and Hultin, 1989; Franks, 1985). Since the major properties of mince which contributed by the presence of proteins, the composition of proteins in the mince is very important to understand these properties. The profile of proteins under reduced conditions will help to explain the influence of the various protein components and its subunits for the maintenance of various properties of mince.

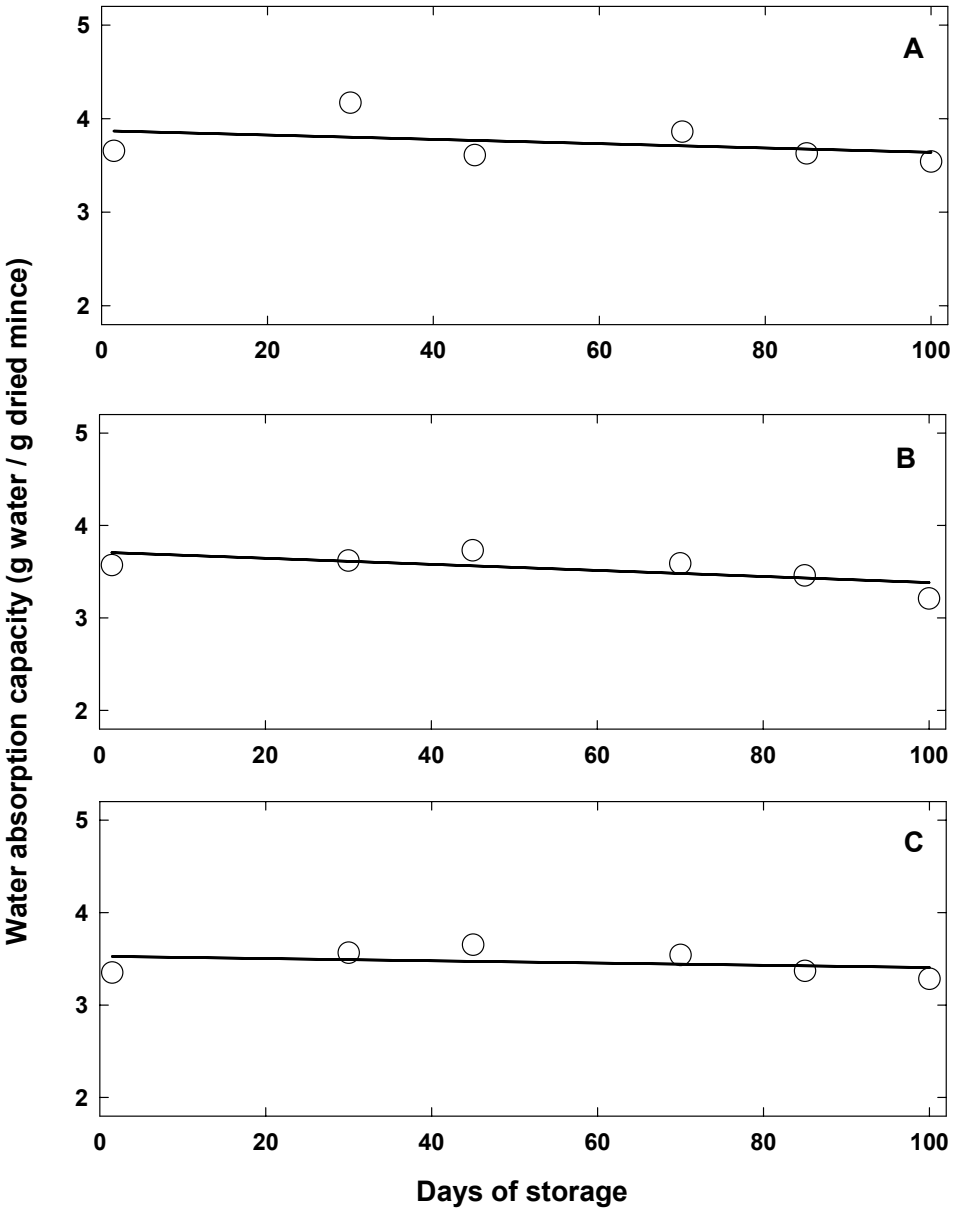


Fig. 58: Water absorption capacity of sardine mince treated with different concentrations of calcium chloride and frozen stored during storage as a function of time. Water absorption capacity determined after freeze drying the samples and expressed as g of water absorbed per g of freeze dried mince. A) Control; B) $CaCl_2$ $1x10^{-2}$ M and C) $CaCl_2$ $1x10^{-4}$ M.

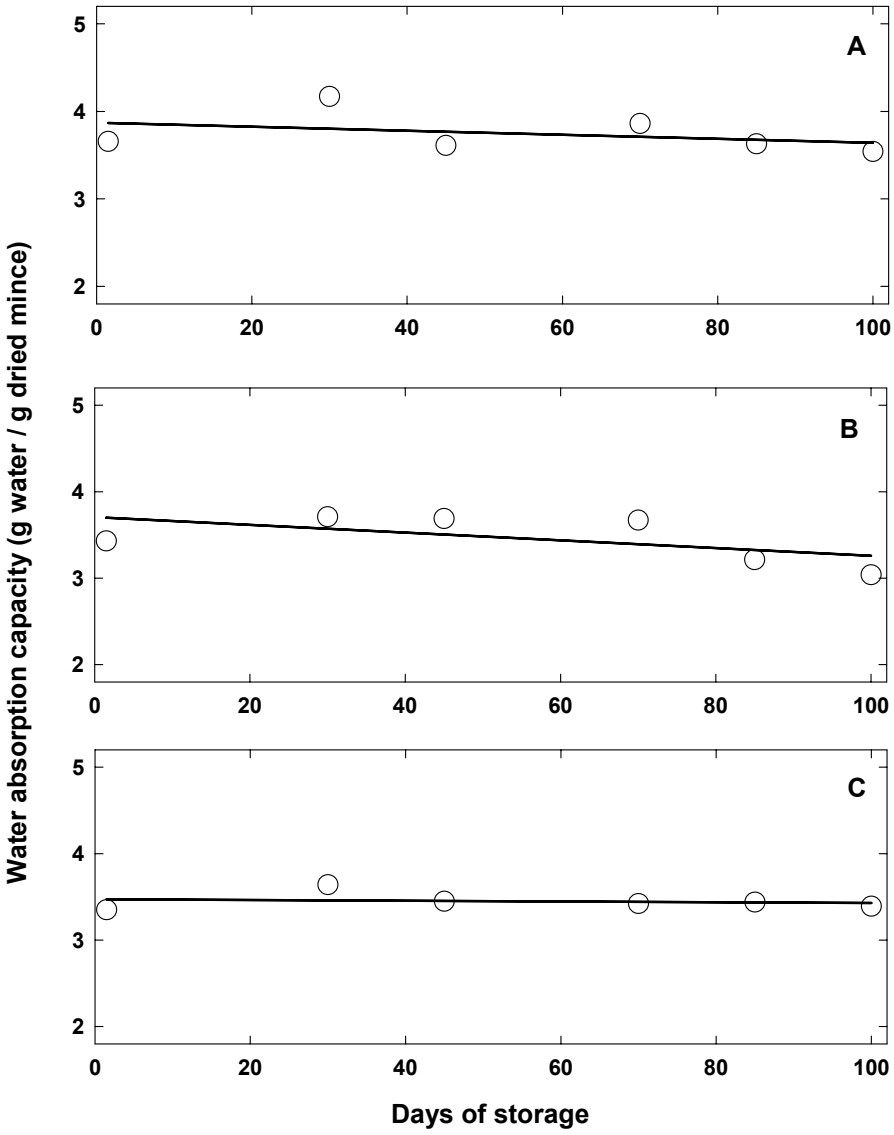


Fig. 59: Water absorption capacity of sardine mince treated with different concentrations of calcium sulfate and frozen stored during storage as a function of time. Water absorption capacities determined after freeze drying the samples and expressed as g of water absorbed per g of freeze dried mince. A) Control; B) $CaSO_4$1x10^{-2} M and C) $CaSO_4$ 1x10^{-4} M.

Physicochemical properties of fish mince proteins during storage

In order to determine the number of protein molecules present typical in fish mince and changes that can occur during frozen storage SDS-PAGE of protein were carried out which will be able to detect any degradation of proteins or complex formation by covalent linkages of proteins and at the same time indicate any association/dissociation (mostly by contributions by SH and disulfides) during frozen storage. The results of SDS-PAGE indicated three major bands (Fig.60) myosin heavy chain (205 kD), actin complex (45 kD) and myosin light chain (20 kD). The SDS-PAGE profile of protein in the presence of two different concentrations of calcium sulfate and calcium chloride is shown in Fig.60A. SDS-PAGE did not reveal any major changes in the protein profile during storage as well as in presence of different concentration of calcium compounds (Fig.60B-F). During storage the bands of myosin heavy chain and actin showed aggregation. This aggregation was more predominant in case of control samples as compared to calcium salt treated samples. However there was no change in the pattern of low molecular weight protein bands due to the presence of calcium salts or due to storage. This showed that the proteolytic activity was not significant enough to cleave the major proteins. The aggregation of proteins occurred during frozen storage as the bands corresponds to actin and myosin showed higher intensity.

From the results of various physicochemical, enzymatic, hydrodynamic and functional properties of mince in presence of calcium salts showed that calcium ions at lower concentration imparts a protective effect on proteins during storage at low temperature. The presence of calcium salts in mince increased the ATPase activity of extract from the mince after freezing. The activity was more for calcium treated samples than control samples during storage. ATPase activity is a good indicator of the integrity of myosin molecules and its ability to bind to other muscle components (Shamasundar and Prakash, 1994c). ATPase activity of frozen mince reduced during storage. The higher activity of extract from mince showed that the presence of low concentration of calcium salts reduced the freeze induced conformational changes of proteins.

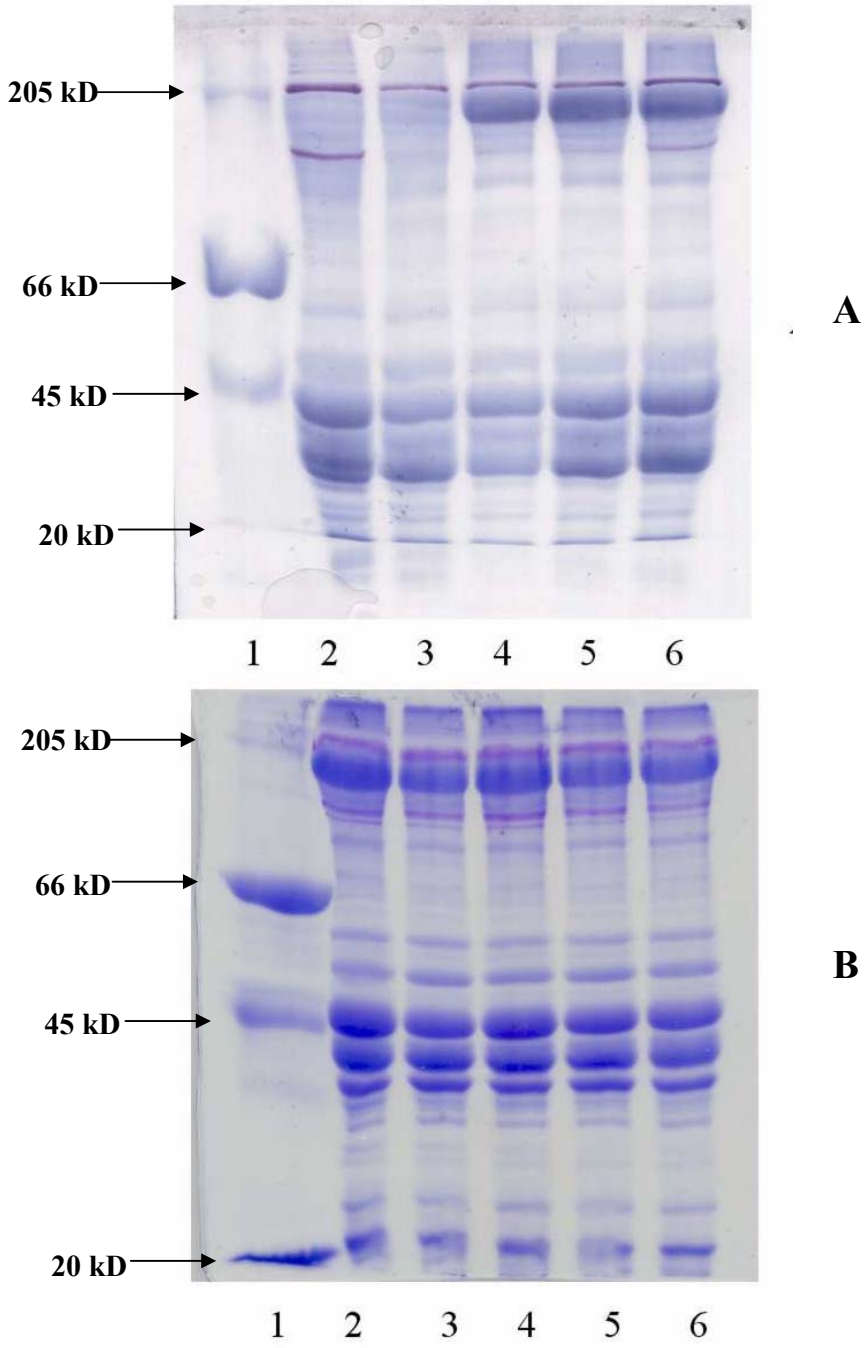


Fig. 60 A,B: SDS-PAGE pattern of proteins extracted from sardine mince treated with calcium salts during frozen storage. A) Immediately after freezing; B) 24 days of storage. 1) Standard proteins - Myosin (205 kD), BSA (66 kD), Ovalbumin (45 kD) and Trypsin inhibitor (20 kD); 2) Control; 3) $1x10^{-2}$ M $CaSO_4$; 4) $1x10^{-4}$ M $CaSO_4$; 5) $1x10^{-2}$ M $CaCl_2$; 6) $1x10^{-4}$ M $CaCl_2$.

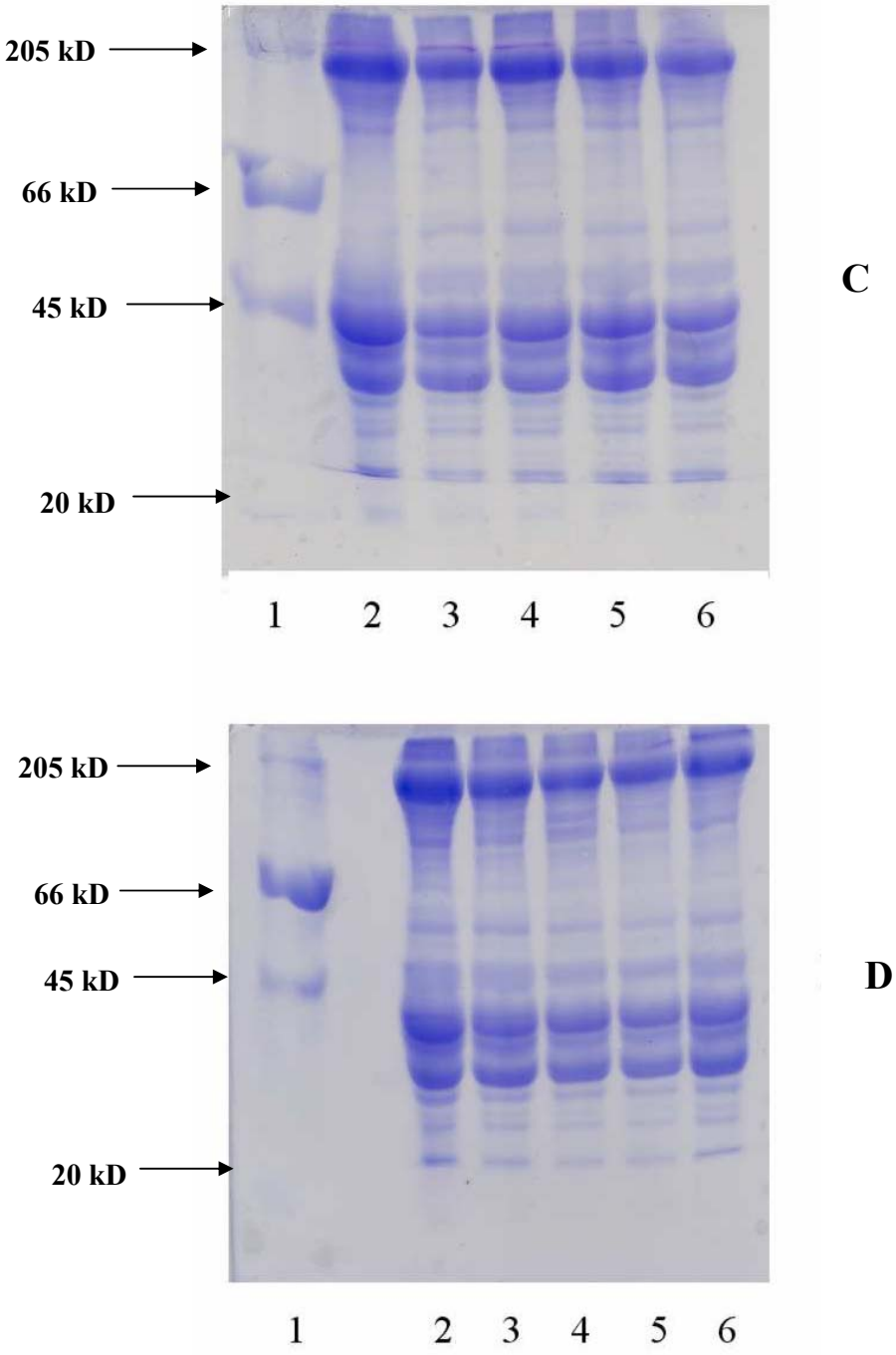


Fig. 60 C, D: SDS-PAGE pattern of proteins extracted from sardine mince treated with calcium salts during frozen storage. C) 52 days; D) 84 days of storage. 1) Standard proteins - Myosin (205 kD), BSA (66 kD), Ovalbumin (45 kD) and Trypsin inhibitor (20 kD); 2) Control; 3) $1x10^{-2}$ M $CaSO_4$; 4) $1x10^{-4}$ M $CaSO_4$; 5) $1x10^{-2}$ M $CaCl_2$; 6) $1x10^{-4}$ M $CaCl_2$.

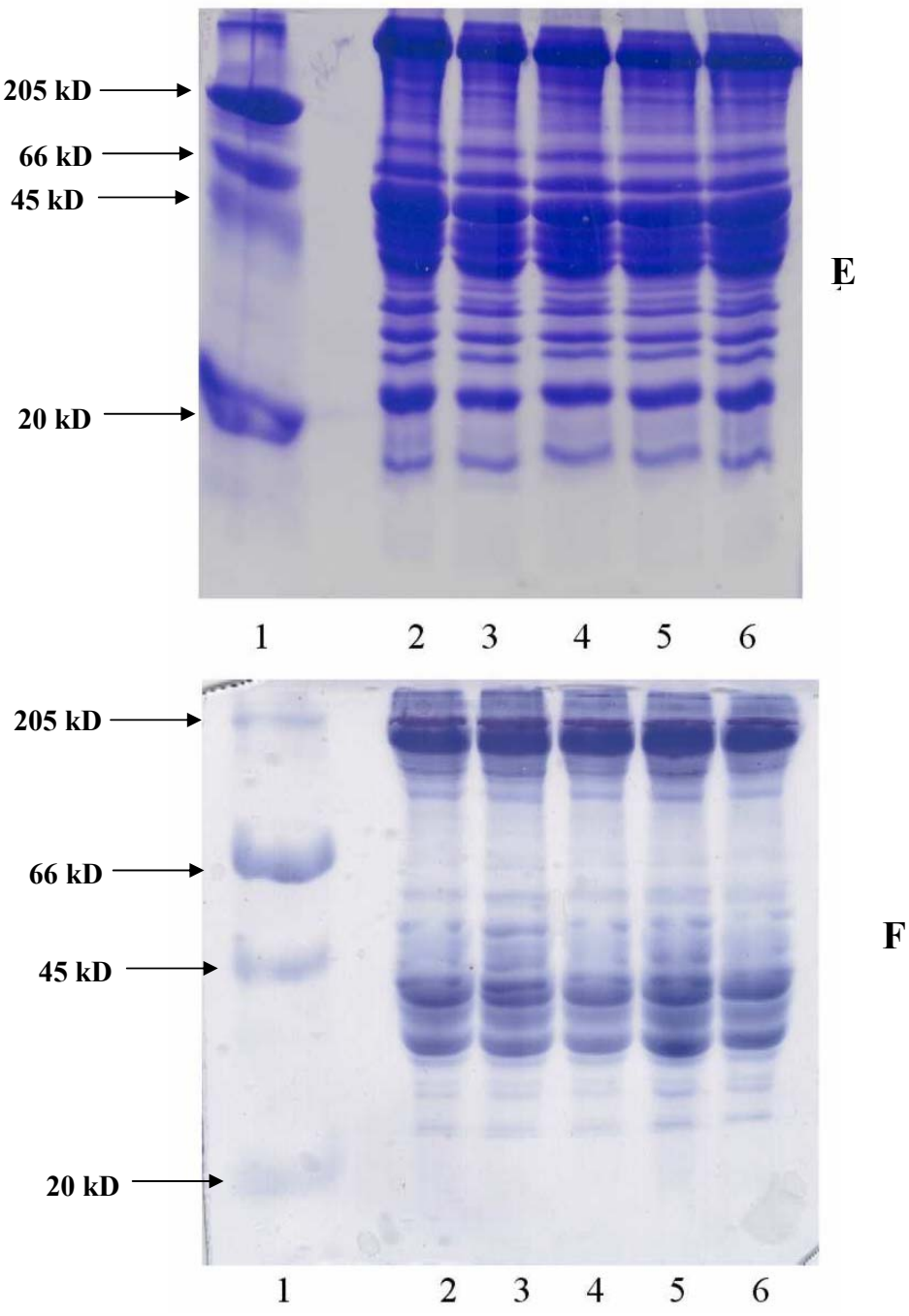


Fig. 60 E, F: SDS-PAGE pattern of proteins extracted from sardine mince treated with calcium salts during frozen storage. E) 110 days; F) 140 days of storage. 1) Standard proteins - Myosin (205 kD), BSA (66 kD), Ovalbumin (45 kD) and Trypsin inhibitor (20 kD); 2) Control; 3) $1x10^{-2}$ M $CaSO_4$; 4) $1x10^{-4}$ M $CaSO_4$; 5) $1x10^{-2}$ M $CaCl_2$; 6) $1x10^{-4}$ M $CaCl_2$.

Extractability of proteins to the high ionic strength buffer is considered as a good indicator of integrity muscle protein. Higher extractability of muscle proteins is required for the better functionality of mince during various product formulations. The functionality of proteins from fish is dependant on the solubility of proteins and any alteration in this will be reflected in the physicochemical and functional properties. The solubility in high ionic strength buffer is taken as index of denaturation of the muscle protein (Lin and Park, 1998; Owassu and Hultin, 1992). Extractability of proteins to the high ionic strength buffer decreased with increase in storage time. The extent of reduction in extractability was less for samples treated with calcium salts than the control samples. Decrease in solubility of proteins accompanies freezing and frozen storage of fish proteins, which could be due to the aggregation of proteins primarily by the formation of dimers of myosin heavy chain (Badii and Howell, 2002; Chan *et al*., 1995; Herrera and Mackie, 2004). Decrease in solubility observed with increase in concentration of calcium chloride was reported by Benjakul *et al*. (2004a). The alteration of protein extractability is a useful factor, which may be used to determine the textural quality of frozen fish muscle, as protein aggregation is accompanied by a significant decrease in their solubility. In the presence of cryoprotectant compounds the decrease in solubility was less because these compounds prevented the drastic changes of proteins associated with freezing and frozen storage. During the frozen storage of fish mince the extractability of proteins to the buffer decreased. The extent of reduction in extractability is less for calcium treated samples compared to the control samples during storage. Reduction in extractability/solubility of the proteins resulted from the aggregation of proteins results in changes in other functional properties also.

The aggregation of proteins can be monitored by gel filtration technique. The gel filtration profile of extracted proteins showed two peaks. The major peak eluting ~63 ml from the column is identified as actomyosin as substantiated from the gel filtration profile of isolated actomyosin in Fig. 15. The percentage peak area of small molecular weight proteins decreased during frozen storage. This may be due to the aggregation of small molecular weight compounds. During frozen storage various conformational changes occurred to protein molecules, which resulted in the exposure of free SH groups and subsequently resulted in disulfide bond formation lead to

aggregation of protein molecules. Ions interact with oppositely charged groups on protein molecules to form a double layer of ionic groups, which decreases electrostatic interaction between protein molecules (Vojdani, 1996). The reduction in extractability, which is directly related to the structure and conformation of proteins in mince, showed that the native structure of protein is altered during frozen storage. This alteration in the protein structure results in the alteration in functionality of mince. Functional properties like emulsion capacity and water absorption capacity also decreased during storage.

The stability or retention of functional properties of proteins such as emulsification properties during freezing and frozen storage is an important criteria that demands an in depth study and evaluation under different conditions (Shamasundar and Prakash, 1994a). Proteins have unique surface properties due to their large molecular weight and tertiary structure, and their amphiphilic properties make them suitable as potent surfactant (Kato *et al*., 1993). Emulsion capacity of proteins reduced during frozen storage. To stabilize an emulsion the hydrophobic domain of the proteins should ideally be oriented towards the oil phase. Kato *et al*. (1985), Kinsella (982) and Xiong (1997) indicated that the change in emulsion capacity is contributed by the changes in structure of protein molecules. Herrero *et al*. (2004) reported that the most pronounced changes occurred during storage of hake at –10°C mainly involving an increase in β-sheet at the expense of α-helices as revealed by Raman spectra. The structural changes in protein molecules is directly related to the water molecules around proteins and its ability to retain water.

The other important functional property is water absorption capacity of mince that may be influenced by the presence of calcium salts. Initially the samples with calcium salts showed lesser water absorption capacity than control samples. The major part of the water in the meat must therefore be held physically within sub cellular (myofibrillar) structures, and is reasonable to postulate that the difference in muscle structure will affect the water distribution and thereby the WHC of the meat (Bertram *et al*., 2002). The water absorption capacity decreased gradually with increase in storage time. Rate of decrease in water absorption capacity was more in case of control samples compared to samples with calcium salts. The presence of salts

and alteration in the structure might have resulted in differential exposure of residues of proteins in mince. The altered exposure of side chains results in the behavior of protein and as a result it change the functional properties also. Some of the changes either structural, physicochemical or enzymatic properties of proteins may be the events occurring during frozen storage, but may not be directly related to functionality loss and cannot therefore be yet another attribute for functional property losses of specific protein (Herrero *et al*., 2004). The changes in the protein composition and its major structural alterations were monitored by the mobility of proteins in poly acrylamide gel under reduced conditions.

The electrophoretic mobility of proteins from mince during storage showed aggregation of major bands during the period. The bands correspond to myosin heavy chain (205 kD) and actin complex (45 kD) are the major ones that under went aggregation during storage. The profile of minor bands remained same during storage period in presence/absence of calcium compounds. Reduction in myosin heavy chain and formation of higher molecular weight components (>205 kD) were observed in presence of calcium compounds (Lee and Park, 1998).

From the results of various physicochemical, enzymatic, hydrodynamic and functional properties of mince in presence of calcium salts showed that calcium ions at lower concentration imparts a protective effect on proteins during storage at low temperature. The physicochemical and functional properties of fish mince in the presence of selected concentrations of calcium salts does indicate that calcium ions has a significant effect during the frozen storage of the fish mince. The ATPase activity, gel filtration studies and solubility profile does indicate the role played by calcium in protein-protein interactions and thus influences the functionality of the proteins. Thus the use of calcium salts in the fish mince perhaps as a cryoprotective additive at low concentrations can be utilized with synergistic effect of may be with other additives such as selected cosolvents and other structure stabilizing agents which can bring in such a stabilization process during the frozen storage of fish mince. These results can be utilized for enhancement of shelf life of fish mince during frozen storage firmly based on physicochemical, enzymatic, hydrodynamic and functional properties in presence of selected metal ions and cosolvents.

SUMMARY AND CONCLUSIONS

SUMMARY AND CONCLUSIONS

Utilization of low economic value fishes for better human consumption is one of the important strategies to be considered for the development of fishery industry and better health management. Indian oil sardine is a pelagic shoaling fish of seasonal abundance. This is a good source of proteins. The composition and characterization of these muscle proteins are very important to decide its technological use and the procedures to follow in order to make a shelf stable product with high quality products. Preparation of unconventional product like surimi (separated meat washed and mixed with cryoprotectants) from Indian oil sardine is a viable alternative to preserve and utilize this underutilized protein source. During freezing and frozen storage of mince various changes will occur to proteins and alters its properties. The freeze-induced denaturation is mainly attributed by the aggregation of proteins, which is reflected in the loss of functional properties of proteins. Various additives have been tried to protect the proteins from denaturation during low temperature preservation.

Metal ions play essential roles in the structure and function of various proteins. Some times these are essential compounds which act as prosthetic groups to the proteins/enzymes. These compounds can induce denaturation of protein molecules and subsequent loss of structure activity of proteins. Zinc is considered as very essential micronutrient in the well being of animals and human beings. The use of zinc to the structural proteins can reveal a considerable amount of information, which is very helpful to understand the exact mechanism of interactions of these compounds with the proteins of interest. Calcium salts can be used as an additive to mince. Calcium ions impart firm texture to mince and may help to impart the protection of quality during storage.

Various sugars and sugar alcohols were used as a protective agents during processing and storage of fish proteins. The influence of polyhydric alcohols on the structure and functionality of fish proteins are not yet understood properly. The stability of fish actomyosin in presence of various cosolvents varies depending on the

nature of cosolvents. The influence of various polyhydric alcohols sorbitol and mannitol on the physicochemical, functional and enzymatic properties of proteins from fish mince will help to understand the mechanism of action of these molecules on fish actomyosin at different conditions.

The influence of various metal on the functionality of proteins during low temperature storage is an important area of research to be explored. Various divalent cations can act as a synergist to cryoprotectant during freezing and frozen storage. The effect of these cations on the various physicochemical, functional and enzymatic properties helps to understand the behavior of the system during the storage under these conditions. This also may give an added advantage of fortification of fish mince with these essential compounds at a very low concentration levels.

Chapter 1 describes the characterization of total extractable proteins from the mince prepared from Indian oil sardine. It describes in detail the physicochemical, functional and structural properties of these proteins. The characterization and properties of isolated actomyosin from sardine have been detailed. The ATPase activity actomyosin was studied as a function of temperature, pH and substrate concentration. Actomyosin was investigated with various biophysical techniques to elucidate the structural characterization of the system.

The mince prepared from sardine has moisture content 78 ± 3% and total protein content of 16.12 ± 1.8 %. Gel filtration profile of proteins from fresh sardine mince showed three major peaks the first one being actomyosin complex and the other two peaks are of low molecular weight in nature. The total protein extract of fish mince was predominantly contributed by myosin and actin as revealed by SDS-PAGE pattern. The secondary structural examination indicated that the proteins had higher content of α-helix. The fluorescence emission spectra showed typical tryptophan fluorescence emission with a fluorescence emission maximum of 335 nm from the proteins. Thermal denaturation profile of proteins showed apparent T_m of 57 ± 1.5°C. The emulsion capacity of fish proteins isolated from prepared mince was 0.13 ± 0.02 ml oil/mg protein. Extractability of proteins from the mince in high ionic strength

buffer was 64 ± 3%. The water absorption capacity of proteins from the mince prepared of oil sardine was 3.65 ± 0.25 g water/g dried mince. The dynamic viscoelastic behaviour of proteins clearly indicated the gel forming ability of proteins during thermal treatment. The maximum storage modulus, considered as the elastic component, value recorded at 76.5°C.

The actomyosin isolated from fresh sardine meat had moisture content of 94.2 ± 1% and rest 5.81 ± 0.7 % protein. The SDS-PAGE of isolated proteins showed four distinct bands corresponding to myosin heavy chain, two light chains and an actin band. FPLC pattern of protein showed a single peak eluted after 12.5 minutes with a flow rate of 0.5ml/min using superose 6 HR column at pH 7.0. The gel permeation chromatography showed a prominent peak eluted at elution volume of 63 ml and a minor peak at elution volume of 134.5 ml where the height of the second peak was negligible compared to first actomyosin complex peak. Free SH content of actomyosin was estimated to be 122 ± 12 μM SH/g of actomyosin. Fluorescence spectra of actomyosin showed typical tryptophan fluorescence with emission maximum of 335 nm. Secondary structural content of actomyosin estimated by far UV-CD spectra showed that the protein is a prominently α-helical which contributes 65% and aperiodic contributes 32% of the total structural content. The effect of pH on the ATPase activity of actomyosin was assessed and recorded the maximum activity at pH 9.0 with a small peak of almost half of maximum activity at pH 7.0. Ca^{2+} activated ATPase activity of fish actomyosin decreased with increase in temperature. The ATPase activity reached the maximum activity at a substrate concentration of 800 μM of ATP. ATPase activity of isolated actomyosin was 0.26 ± 0.03 μM Pi/mg protein/min at 25°C and pH 9.0.

From the above results and discussion it is evident that sardine mince prepared from fresh sardine has functional properties which can be utilized for product development using these properties. The advantage of higher solubility and an elevated emulsion capacity are some of the indicators in this direction. The understanding of the physicochemical, functional and the various enzymatic parameters of both the total proteins and isolated actomyosin would further help in

deriving the rationale and for better understanding of the mechanism of interaction of selected salts and metal ions with the proteins especially actomyosin during frozen storage of fish mince.

Chapter 2 presents detailed study of the interaction of various metal ions with isolated actomyosin from sardine from a biotechnological perspective. The influence of zinc ions on the structure and enzyme activity of actomyosin isolated from Indian oil sardine have been described. The influence of these compounds at varied concentrations was studied under a different temperature and pH conditions to understand the mechanism of influence of these compounds on the structure and enzyme activity of actomyosin. Various changes in the actomyosin in presence of zinc sulfate and zinc chloride were studied using enzymatic and biophysical techniques such as ATPase activity, solubility profile, fluorescence emission spectra and circular dichroic spectra. The effect of calcium salts on the structure, enzyme activity of actomyosin is also dealt in this chapter.

Zinc ions influenced the ATPase activity of fish actomyosin. Very low concentrations of zinc did not change the activity considerably. But in presence of higher concentration in the order of $1x10^{-2}$ M resulted in complete inhibition of activity. The effect was prevalent at different temperatures and pH conditions. Free SH groups decreased with increase in zinc ion concentration followed the decrease in ATPase activity. Free SH content decreased from 122 ± 12 μM SH/g to 75 ± 6 μM SH/g in presence of $1x10^{-3}$ M zinc chloride and to 80 ± 7μM SH/g in presence of $1x10^{-3}$ M zinc sulfate. Relative fluorescence intensity of fish actomyosin decreased with increase in concentration of zinc compounds without much change in wavelength of emission maximum as revealed from fluorescence spectra. Hydrophobicity of actomyosin decreased with increase in zinc concentration. The surface hydrophobicity of actomyosin decreased from 422 ± 20 to 378 ± 23 in presence of $1x10^{-4}$ M zinc chloride and to 289 ± 17 in presence of $1x10^{-4}$ M zinc sulfate. The reduction in the hydrophobicity of actomyosin followed the inhibitory pattern of enzyme activity was due to the presence of zinc ions. Zinc sulfate brought out more reduction in the hydrophobicity than zinc chloride in solutions. The

secondary structural content estimation by far UV-CD spectra of actomyosin in presence of zinc compounds showed that the α- helicity of actomyosin molecules reduced from 65 ± 3% to 39% by the presence of $1x10^{-3}$ M zinc sulfate and to 21 ± 2% in presence of $1x10^{-3}$ M zinc chloride concentrations. Low concentrations of zinc sulfate did not bring considerable changes in the solubility value of actomyosin as revealed from precipitation studies. But with increase in concentration of zinc ions the actomyosin molecules precipitated. The precipitation profile of actomyosin in presence of zinc chloride also followed same pattern as that of zinc sulfate.

The influence of calcium salts on the structure and ATPase enzyme activity of actomyosin from sardine clearly shows that the structure of proteins is not getting affected by the presence of low concentrations of calcium. The ATPase Enzyme activity was low at reduced concentrations. The ATPase activity was activated by calcium concentration of $1x10^{-2}$ M. The intrinsic fluorescence emission spectra of actomyosin in presence of calcium sulfate and calcium chloride did not record any change in emission maximum. The secondary structure as estimated by far UV-CD spectra in presence of calcium sulfate and calcium chloride did not show any considerable change in α- helical content of actomyosin.

It is evident from the fluorescence and CD spectra that the addition of calcium ions have not altered the structure of fish actomyosin. Activity of ATPase enzyme is increased in the presence of higher concentrations of calcium chloride. Lower concentrations of calcium ions is found to be not effective in increasing the enzyme activity of actomyosin. Hence lower concentrations of calcium salts can be used as a stabilizing agent during frozen storage. Addition of zinc salts were found to decrease the enzyme activity. It also alters structure of actomyosin. The utility of these results in the keeping quality of fish mince during storage at low temperature where one desires the structural integrity to be retained for ultimately having a product which is also of functionally stable. These results would thus have wide application in incorporating such additives in the enhancement of shelf life of fish mince and its quality.

Chapter 3 explains the specific interactions of polyhydric alcohols such as sorbitol and mannitol on the enzymatic, structural and physicochemical properties of actomyosin isolated from Indian oil sardine. The interaction studies were carried out using various biophysical techniques. The influence of these cosolvents at various concentrations and different temperatures and pH conditions were investigated thoroughly and presented in this chapter.

The ATPase activity decreased with increase in sorbitol and mannitol concentration. The inhibition of ATPase followed a sigmoid pattern with concentration of these cosolvents. Mannitol was more potent in decreasing the ATPase activity of actomyosin. Polyhydric alcohols resulted in the inactivation of ATPase activity at various pH conditions. The inhibitory activity was prevalent at various temperatures and pH conditions. Intrinsic fluorescence emission spectra of fish actomyosin decreased marginally in presence of polyhydric alcohols. Hydrophobicity of actomyosin decreased with increase in concentrations of polyhydric alcohols. The secondary structural analysis of actomyosin did not show much difference due to the presence of cosolvents. There is a very small difference in the secondary structure by the presence of sorbitol in solution and in presence of mannitol did not make significant difference in the structure of proteins.

The results of these interaction studies clearly reveal that cosolvents (sorbitol and mannitol) could be used to improve the functionality and keeping quality of fish mince during frozen storage. Both the polyhydric alcohols can be used as additive at low concentrations to improve the quality of fish mince. Mannitol perhaps is a better option than sorbitol as mannitol inhibits ATPase enzyme activity at lower concentrations than sorbitol and also it will not affect the structure of actomyosin at this low concentration. Such biotechnological approaches of addition of small quantities of cosolvents would improve the enhancement of quality and shelf life of fish mince.

Chapter 4 deals with biotechnological use of selected polyhydric alcohol in fish protein system. Detailed study of the effect of polyhydric alcohol, mannitol, on physicochemical and functional changes induced to the fish proteins kept under low

temperature storage have been presented. The investigation is carried out under different concentrations of these compounds and its effect to protect the protein molecules during storage under these conditions were detailed and discussed. The enzymatic and structural changes induced to the muscle tissues due to the presence of this additive is also detailed in this chapter.

ATPase activity of proteins from frozen mince was monitored during storage in presence of mannitol. Immediately after freezing, ATPase activity was less in the case of untreated samples than the samples treated with mannitol. Mannitol concentration above 4% is more effective in protecting the enzyme activity of mince than control samples. ATPase activity of frozen mince reduced during storage. Sharp decrease in ATPase activity occurred up to 50 days of storage. Mannitol treated samples have shown a higher activity up to 60 days of storage. After 75 days of storage it attained an asymptotic value. There was no change in the elution volume of the major protein fraction due to the presence of different concentrations of mannitol in the mince immediately after freezing. During storage also there was no change in the profile of proteins from samples treated with mannitol. Slight change in the elution volume of the untreated samples implies the process of aggregation during storage. There was no change in the elution volume of mannitol treated samples, which indicates the ability of mannitol to protect the proteins from aggregation. Apparent T_m values of proteins from mince during frozen storage decreased. A concentration of 4% mannitol was able to protect the structure of actomyosin, the major protein fraction during frozen storage to some extent as revealed by apparent T_m values. There was a reduction in the emulsion capacity of proteins during frozen storage. This reduction in emulsion capacity values during frozen storage could be due to formation of aggregates. The water absorption capacity of the samples treated with mannitol recorded lower values than the control samples indicating the presence of this additive reduced this functionality of mince. The reduction was more for the samples with higher concentration of mannitol. Mince treated with 6% mannitol showed more porous structure and during frozen storage no significant change in the micro structure of mince was noticed.

The effect of mannitol on the structure, function and stability of proteins from fish mince both in the control and frozen stored suggests the role played by both covalent and noncovalent interactions in the myosin heavy chain of the proteins. The results also indicate that to a certain extent mannitol prevent aggregation of proteins in fish mince during storage. It also stabilizes the proteins against denaturation through an indirect effect of altering the bulk solvent structure perhaps around the microenvironment of these proteins. These results substantiates that the cosolvents which are known to be preferentially excluded from the vicinity of the protein surface perhaps contribute to the stability and protection of proteins through the steric exclusion effect.

Chapter 5 deals with use of selected cation on the proteins from fish mince. This gives a detailed study of the effect of calcium compounds on physicochemical, enzymatic and functional changes to the fish proteins kept under low temperature storage have been presented. Calcium chloride and calcium sulfate were incorporated to the mince before freezing and storage. The investigation is carried out under different concentrations of these salts and its effect to protect the proteins molecules during storage under these conditions were investigated and discussed.

The influence of calcium salts on the mince during frozen storage was studied. Initial extractability of proteins from the calcium treated samples was lesser than the untreated samples except the samples treated with $1x10^{-4}$ M calcium sulfate. Samples treated with $1x10^{-4}$ M calcium sulfate showed same extractability as control samples. The elution volume remained unaltered in the gel filtration profile during storage, but there was a slight reduction in the area of peak noticed. Emulsion capacity of proteins was more for samples treated with calcium chloride than untreated samples. The highest emulsion capacity was recorded by samples treated with $1x10^{-4}$ M calcium chloride, but in presence of calcium sulfate there was no significant change in emulsion capacity of proteins compared to untreated samples. The untreated samples maintained higher values than treated samples during the study. There was an initial increase in water absorption capacity of all samples with increase in frozen storage period. The maximum reduction is observed in case of samples treated with calcium

sulfate at higher concentration. SDS-PAGE pattern did not show any alteration in the profile during storage. Ca^{2+}ATPase activity of proteins from the samples were also more for samples treated with $1x10^{-4}$ M calcium chloride and for calcium sulfate treated samples at both the concentrations ($1x10^{-2}$ M, $1x10^{-4}$ M) than that of control samples.

From the results of various physicochemical, enzymatic, hydrodynamic and functional properties of mince in presence of calcium salts showed that calcium ions at lower concentration imparts a protective effect on proteins during storage at low temperature. The physicochemical and functional properties of fish mince in the presence of selected concentrations of calcium salts does indicate that calcium ions has a significant effect during the frozen storage of the fish mince. The ATPase activity, gel filtration studies and solubility profile does indicate the role played by calcium in protein-protein interactions and thus influences the functionality of the proteins. Thus the use of calcium salts in the fish mince perhaps as a cryoprotective additive at low concentrations can be utilized with synergistic effect of may be with other additives such as selected cosolvents and other structure stabilizing agents which can bring in such a stabilization process during the frozen storage of fish mince. These results can be utilized for enhancement of shelf life of fish mince during frozen storage firmly based on physicochemical, enzymatic, hydrodynamic and functional properties in presence of selected metal ions and cosolvents.

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