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USE OF HIGH PRESSURE FOR IMPROVING THE QUALITY AND SHELF LIFE OF FROZEN FISH

by

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Suggested short title : PRESSURE-SHIFT FREEZING OF CARP FILLETS

Dedicated to

Jean Pierre Brosseau

Gracias por todo!

FOREWORD

This thesis is presented in the form of original papers meant for publication in journals. The first two sections comprise of a general introduction presenting a comprehensive review of the literature on the topic as well as the rationale and objectives for this study. The next six sections constitute the body of the thesis with each chapter representing a complete manuscript, while the last chapter is a summary of the major conclusions. This format has been approved by the Faculty of Graduate Studies and Research McGill University in accordance with the conditions outlined in the Guidelines for Thesis Preparation, Thesis Specification, section 3 entitled Traditional and manuscript-based theses which are as follows :

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While the work reported in this thesis is the responsibility of the candidate, it was supervised by Dr. B. K. Simpson, Department of Food Science and Agricultural Chemistry, Macdonald Campus of McGill University.

PART OF THIS THESIS HAS BEEN PUBLISHED OR SUBMITTTED FOR PUBLICATION AS FOLLOWS:

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Sequeira-Munoz, A., Chevalier, D., B.K. Simpson and Le Bail, A. 2001. Effect of pressure-shift freezing on physico-chemical properties of carp (*Cyprinus carpio*) fillets. J. Food Process Eng.

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ABSTRACT

The spoilage pattern of carp (*Cyprinus carpio*) fillets was investigated. The studies were aimed at evaluating the potential use of pressure-shift freezing to reduce quality deterioration during frozen storage. The effects of pressure treatment at low temperature on fish carp fillets were evaluated and conditions were chosen to reduce any adverse effect on the quality of fish fillet. Pressure-shift freezing treatment was applied to carp fillets and biochemical properties were evaluated and correlated with objective measurement of texture, drip loss and the size of ice crystals formed. Changes in these properties were monitored during frozen storage for a period of 75 days.

Results indicated that proteolityc changes due to endogenous enzymes in fish muscle play an important role in quality deterioration of carp fillets during ice storage. No changes were observed in Ca^{2+}-ATPase, Mg^{2+}-ATPase or Mg^{2+}-EGTA-ATPase activity of actomyosin from carp fillets during iced storage ($p>0.05$). In contrast, Mg^{2+}-Ca^{2+}-ATPase and Ca^{2+} sensitivity of actomyosin decreased during ice storage of fish fillets. No changes were found in the SH content of actomyosin throughout the ice storage of carp fillets ($p>0.05$). The surface hydrophobicity of actomyosin and auto-degradation products increased during the storage period($p<0.05$).

Response surface methodology (RSM) was used to study the effect of high-pressure treatment on some physico-chemical properties (actomyosin extractability, Ca^{2+}-ATPase activity, surface hydrophobicity, TBA value, liquid loss and firmness) of intact fish fillets. Balancing the benefits of low temperature

pressurization with the denaturing effects of pressure on fish proteins, it is evident that there is a region in which the responses of the factors (protein extractability, Ca^{2+}-ATPase activity and protein hydrophobicity) to the processing variables (time and pressure) seemed to be adequate to keep protein denaturation to a minimum. This region lies between 140-175 MPa and 16-18 min. However, it was observed that high-pressure treatment induced changes in colour on fish fillets. The L^*, a^* and b^* values increased as pressure and time treatment increased.

The application of pressure-shift freezing or air-blast freezing resulted in decrease in myofibrillar and sarcoplasmic protein extractability, and reduced actomyosin Ca^{2+}-ATPase activity during frozen storage. However, actomyosin Ca^{2+}-ATPase activity in pressure-shift frozen samples remained relatively higher than that of air-blast frozen samples. On the other hand, levels of thiobarbituric acid and free fatty acids were relatively lower in samples frozen by PSF. The freezing procedure did not seem to have a significant effect ($p>0.05$) on the texture of carp fillets. The ice crystals found in PSF fish samples were mainly intracellular, smaller and more regular shaped than those found in the ABF samples, which were mainly extracellular. Differential scanning calorimetry showed that PSF treatment appeared to be more effective in preventing protein denaturation in post-rigor fish fillets than in the pre-rigor fish fillets.

RÉSUMÉ

Le modèle du processus de détérioration des filets de carpes (*Cyprinus carpio*) a été analyse et ces études avait comme objectif de évaluer la possibilité d'utiliser la congélation par détente haute pression (PSF) a fin de réduire la détérioration de la qualité lors de l'entreposage a l'état congelé. Les effets de la haute pression (HP) a basse température sur les filets de carpes on été évaluer et les paramètres détermine afin de réduire tout effet qui pourrait altérer la qualité des filets de carpes. Les filets de carpe on été soumis a la PSF et leurs propriétés biochimiques on été étudies et mis en corrélation avec des paramètres de texture, de l'exsudation et de la taille des cristaux de glace. Les variations de ces propriétés on été observe pendant le période d'entreposage de une dure de 75 jours.

Les résultats montraient que les changements protéolytique dus aux enzymes endogènes des filets de poisson jouent un rôle important dans la détérioration de filets de carpe lors d'entreposage sur la glace. Aucun changement n'a été observe dans les activités enzymatique : Ca^{2+}-ATPase, Mg^{2+}ATPase or Mg^{2+}-EGTA-ATPase de l'actomyosine extraite des filets de carpes durant la conservation sur la glace ($p>0.05$). Par contre, l'activité enzymatique Mg^{2+}-Ca^{2}-ATPase et la sensibilité de l'actomyosine au ions Ca^{2+} tendent a décroître durant la période d'entreposage de filets de poisson sur la glace. Aucune modification n'a été noter quant a la quantité de groupe SH contenu dans l'actomyosine au long de l'entreposage du filets de carpe sur la glace. L'hydrophobicite en surface de l'actomyosin et la quantité de substances

produites par l'autodegradation ont augmentes pendant la période d'entreposage sur la glace.

Les études statistiques de réponse de surface on été utilise afin de étudier les effets des traitements haute pression sur certaines propriétés physico-chimiques : l'extractabilite, l'activité enzymatique Ca^{2+}-ATPase, l'hydrophobicite en surface de l'actomyosine; les perdes de liquides et la fermeté de filets de poisson intact. En équilibrant les bienfaits de la pressurisation a baise température et ses effets dénaturants sur les protéines de poisson on peut délimiter une zone dans laquelle les réactions des facteurs (l'extractabilite, l'activité enzymatique Ca^{2+}-ATPase, l'hydrophobicite en surface de l'actomyosine; les perdes de liquides et la fermeté de filets) aux variables (temps et pression) semblent appropries afin de réduire au minimum la dénaturation des protéines. Cette région se situe entre 140-175 MPa et 16-18 min. Toutefois, il a été observe que le traitement HP peut provoquer des changements de couleur de filets de poisson. L'augmentation de la pression et de la durée du traitement entraînent un augmentation de L^*, a^* et b^*

L'utilisation de PSF or la congélation par air pulse résulte en un diminution de l'extractabilite des protéines myo-fibrilaires et sarcoplasmiques ainsi que de l'activité Ca^{2+}-ATPase de l'actomyosine durant l'entreposage a l'état congèle. Cependant l'activité enzymatique Ca^{2+}-ATPase de l'actomyosine des échantillons congèle par PSF demeurent relativement plus élève que celle des échantillons congelés par ABF. Par contre, les niveaux de l'acide thiobarbiturique et des acide gras libres étaient relativement plus bas que dans les échantillons congelés par PSF.

La procédure de congélation ne semble pas avoir d'effet significatif sur la texture des filets de carpe. Par contre les cristaux de glaces trouves dans les échantillons de poisson soumis au PSF étaient principalement intracellulaire, plus petits et plus régulier que ceux trouves dans les échantillons traites au ABF qui étaient principalement extracellular. La calorimétrie différentielle a balayage montre que le traitement PSF semble plus efficaces pour prévenir la denaturation de protéines dans le filets de poisson après la rigidité cadavérique plutôt que avant la rigidité cadavérique.

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CONTRIBUTION TO KNOWLEDGE

1. The spoilage pattern of intact carp fillets (freshwater fish) was evaluated. This study showed that proteolityc changes due to endogenous enzymes present in fish muscle play an important role in quality deterioration of intact carp fillets during ice storage. Studies on changes of myofibrillar proteins from carp during ice storage have been reported before. However, these studies are based on proteins systems stored on ice instead of intact fillets.

2. Based on combined treatments of high pressure and time, mathematical models capable of predicting protein denaturation of myofibrillar protein from intact carp muscle were developed. The effect of high pressure on fish muscle may be species dependent due to the great variability on muscle composition among fish species. Thus far, no definitive study on the combined effect of pressure and time on intact carp fillets has been reported.

3. This is the first study of the effect of pressure-shift freezing on physico-chemical properties of intact carp fillets during frozen storage.

4. Pressure-shift freezing treatment was shown to retard lipid degradation (oxidation and hydrolysis) during the first two months of frozen storage of intact carp fillets.

5. Pressure-shift freezing was effective in reducing the size of ice crystal formation during freezing of intact carp fillets. To our knowledge, this is the first study of pressure-shift freezing of freshwater fish fillets.

6. The effect of pressure-shift freezing on pre- and post-rigor carp fillets was evaluated using differential scanning calorimetry. PSF treatment appeared to be more effective in preventing protein denaturation in post-rigor fish fillets than in the pre-rigor fish fillets. This is the first study demonstrating the effects of pressure-shift freezing on post- and pre-rigor carp fillets.

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ΔH_T	Total denaturation enthalpy
ABF	Air-blast freezing
AM	Actomyosin
ANOVA	Analysis of variance
ANS	1-anilinonaphthalene-8-sulphonic acid
ATP	Adenosine triphosphate
°C	Degrees Celsius
Ca^{2+}	Calcium
$CaCl_2$	Calcium chloride
cm	Centimetres
d	Deformation
DSC	Differential scanning calorimetry
DTNB	5,5'-dithiobis (2-nitrobenzoic acid)
EDTA	Ethylenediaminetetraacetic-acid
EGTA	Ethyleneglycol-bis-(beta-aminoethyl ether) N, N'-Tetraacetic acid
F	Load
FAO	United Nations Food and Agriculture Organization
FFA	Free fatty acids
h	Hours
HCl	Chlorhydric acid
HP	High pressure
J	Jules
KCl	Potassium chloride
KDa	kilo Daltons
LSD	Least significant difference
LTHP	Long time/high pressure
LTLP	Long time/low pressure
M	Molar
Mf	Myofibrillar proteins
Mg^{2+}	Magnesium

$MgCl_2$	Magnesium chloride
min	Minutes
ml	Millilitres
MPa	Mega Pascals
MW	Molecular weight
N	Newtons
NaOH	Sodium hydroxyde
nm	Nanometers
P	Pressure
PSF	Pressure-shift freezing
PVC	Polyvinil chloride
RFI	Relative fluorescence intensity
rpm	Revolutions per minute
s	Seconds
SAP	Sarcoplasmic proteins
SDS	Sodium dodecylsulphate
SDS-PAGE electrophoresis	Sodium dodecylsulphate-polyacrylamide gel
SH	Sulfhydryl group
SoANS	Initial slope
SSP	Salt-soluble protein
STHP	Short time/High pressure
STLP	Short time/low pressure
t	time
TBA	Thiobarbituric acid
TCA	Tricloroacetic acid
Tmax	Peak maximun temperature of thermal transition
Tris	
µMPi	Micromoles inorganic ortophosphate
WSP	Water-soluble sarcoplasmic protein

CHAPTER 1

INTRODUCTION

Fish muscle tissue from newly captured animal is a highly desirable food due to its flaky, soft, moist texture, its pleasant mild flavor, and high nutritional value. Fish muscle provides a unique eating experience because of its myotomal structure and collagen that degrades easily on heating, contributing to its desirable texture (Hultin, 1992). However, it is common knowledge that fish are extremely perishable food commodities. Recently the spoilage of fresh fish has been the subject of several reviews (Bonnell, 1994, Davis, 1995, Haard, 1994 and Ashie et al., 1996), and it is evident that interest in endogenous enzymes as a factor responsible for fish spoilage, (along with microbiological activity and oxidative changes) is continuously increasing. As soon as fish is harvested, a series of natural phenomena that eventually cause the spoilage of fish commence. Indeed, like mammalian meats, fish spoils through the combined effect of continuing activity of endogenous enzymes, bacterial growth and chemical reaction (oxidation). There are differences, however in chemical composition, metabolism and environment of the live animal which subsequently reduce the storage life of most fish when compared with meat. Indeed, it is believed that the higher water content, higher free amino acid and the lower content of connective tissue as compared to other flesh food, result in a more rapid spoilage of the fish muscle (Pedroza-Menabrito and Regenstein, 1988)

The initial quality abuse may be avoided by using proper handling procedures. By applying several traditional preservation treatments the high quality shelf-life of many species may be extended for days or weeks, while the

use of procedures and combination treatments based on the application of results of scientific investigations can further retard the quality deterioration of seafood products over several months. There is a continuing search for new approaches founded on better understanding of the effects of different physical and chemical factors on the activity of enzymes and microorganisms (Ward and Baj, 1988).

Preservation in quality of fish products has become an imperative task, as the demand for high quality products has risen. The perceived health benefits of eating more fish is dramatically strengthening the demand in developed countries, where so far, prices have not caused any impediments to sales. In fact, Canadians are including more fish and shellfish products in their diet, although red meat and poultry remain the most popular choices for many consumers. In 1996, total fish consumption reached almost 9 kg per person, up by more than 2 kg from 1991 (Statistics Canada, 1997a,b).

At the beginning of the 1950s, it was generally believed that world marine resources were unlimited. However, nowadays it is the world wide consensus that this idea is far from being true. Indeed FAO (United Nations Food and Agriculture Organization) indicated that no major commercial fish stock remains untouched. Bye the beginning of the 1990s, about 69 percent of the stock for which data were available to FAO were either fully to heavily exploited (44%), overexploited (16%), depleted (6%) or very slowly recovering from overfishing (3%) (Morrow, 1992). As a result, the world catch has fallen in recent years although it now seems to be leveling off at around 100 million tons per year. In fact, experts agree that approximately 100 million tons of fish can be taken from the sea on a sustainable base (FAO, 1997). At the same time, the demand/supply is also

increasing, resulting in a reduction in availability, rising prices, and search for alternative resources to close the gap.

Closing the supply/demand gap requires an increasing, amount of fish available for direct human consumption. During the last years, the percentage of total fish production used for human consumption has stabilized around 70% (FAO, 1997). Three main possibilities for increasing this percentage are:

> Better management to increase yield from existing resources
> Increase production from aquaculture
> Making better use of what is caught and does not end up as human food (Venugopal and Shahidi, 1995).

Without having to increase production, much can be done to improve availability by making better use of what is currently caught. In that sense, reducing post-harvest losses should be one of the first approaches (Morrow, 1992).

Current procedures have proved to be inadequate to preserve the premium quality of fresh seafood for an appreciable period of time (Haard, 1992). Information derived from various studies indicate that high pressure processing may be expected to better preserve seafood quality via controlling endogenous enzymatic activity, less protein denaturation and microbial inactivation (Ashie et al., 1996). Based on this premise, a series of studies on the use of high pressure to control endogenous enzyme activity in fresh seafood were conducted in our lab. These studies proved that high pressure in combination with other techniques such as at low temperature storage or addition of proteases inhibitors could extend the shelf life of sea ice-stored fish (Ashie and Simpson, 1996).

However, the shelf-life extension achieved was still only a few days when compared to conventional freezing. Economic considerations cannot justify the use of high pressure technology when only a few days extended storage occurs. In that sense, this project focused on using high pressure technology combined with frozen storage to achieve the dual purpose of improving quality of the final product and extending its the shelf-life.

Recently pressure-shift freezing was applied in processing tofu and carrots. It was reported that pressure-shift freezing within the range of 200 MPa ~ 400 MPa was effective in improving the texture of frozen tofu. The ice crystals formed in tofu frozen at 200 MPa ~ 400 MPa were smaller than in tofu frozen at 100 MPa or 700 MPa (Fuchigami and Teramoto, 1997).

The use of high pressure during freezing of fish may offer one important advantage over traditional freezing techniques, such as air-blast and plate freezing. This advantage is the increase in freezing rate during freezing that may result in a final product with better quality once thawed (Kalichevsky et al., 1995). The improvement of quality may be a direct result of the reduction of tissue or textural damage and of lipid deterioration and accumulation of final products from lipid oxidation and hydrolysis (Ohshima et al., 1993).

The innovative aspect of this research project lies in its focus on combining high pressure technology with freezing of intact fish fillets. Previous studies on high pressure processing of seafood products mainly concentrated on fresh fish, fresh protein isolates or by-products such as surimi paste. The literature about pressure-shift freezing is indeed sparse, and even more, so is its application to seafood. This research is intended to provide a better

understanding of the deterioration processes during freezing of seafoods, and to use the information obtained to rationalise more effective strategies for preserving the premium quality of "fresh" fish.

Thus the overall objectives of the proposed research are as follows:

(a) To compare the efficacy of pressure-shift freezing of fish fillets with conventional freezing procedures.

(b) To study the effect of pressure-shift freezing on the lipid fraction from fish fillets.

(c) To establish the optimum conditions (time and pressure) for pressure-shift freezing of premium quality fish fillets.

CHAPTER II

LITERATURE REVIEW

Structure and Constituents of Fish Muscle

Fish muscle functions similarly to mammalian muscle, hence there is closeness in the relative amounts of the main proteins of skeletal muscle, myosin, actin and tropomyosin. Structurally, however, there are fundamental differences in the arrangement of muscle cells, and in their attachment to connective tissue. The flesh of fish is constructed of adjacent muscle blocks, called myotomes, separated from each other by sheets of collagenous tissue called myocommata. Within each myotome, the muscle fibers (myomeres or myofibrils) run approximately parallel to each other but at varying angles to the myocommatal sheet to accommodate the juxtapositional rhythmical contractions that occur during swimming. Thus, all the fibres in the myomere contract to a similar extent when the fish bends. This arrangement results in a maximum power output at a given rate of contraction. The myocommata are connected internally to the skin and to the skeletal system, and are also linked to the membrane dividing the fish into epaxial and hypaxial planes and to the median vertical septum (Bremmer, 1992)

Muscle fibers are comprised of bundles of fibrils (myofibrils) arranged longitudinally within the muscle cell and organized within a cytoskeletal framework of desmin-containing intermediate filaments. The basic repeat unit of the myofibrils is the sarcomere (often termed myomere in fish) and each sarcomere is bounded at each end of its long axis by the electron-dense structure known as Z

disc (or Z band, or Z-line). Actin filaments extend from one Z disc to the next and, in cross-section, are arranged in an hexagonal array parallel to and around the myosin rods. The myosin rods themselves do not attach to the Z disc but occur in the mid-portion of the sarcomere. The rod shaped protein tropomyosin occupies the grooves of the actin helix providing a structure for the globular protein troponin to attach at regular intervals. Titin together with nebulin forms "gap filament" which join the thick myosin filaments from their ends to the Z disc and which thus stabilize the myosin in the center of the sarcomere (Bremmer, 1992).

Components proteins

As it was mentioned earlier, water, protein and lipids are the three main components of fish muscle. Approximately 11-27% of fish muscle consist of crude proteins. Fish protein, like those of all other muscle foods, may be classified as:

Sarcoplasmic (albumins), referred to as "myogen", are soluble in the muscle sarcoplasm and may be extracted into water or neutral salt solutions with an ionic strength less than 0.15. They include a large number of proteins such as myoglobin, enzymes and other albumins, as well as antifreeze proteins and glycoproteins. The content of sarcoplasmic proteins is generally higher in pelagic species compared with demersal fish (Shahidi, 1994). Sarcoplasmic enzymes are responsible for quality deterioration of fish after death. Among them are mainly the enzymes of the glycolytic pathway and the hydrolytic enzymes, lysosomes (Sikorski et al., 1990a).

Myofibrillar protein (Globulins) include myosin, actin, tropomyosin and troponins (C, I, and T). These proteins are extracted with a neutral salt solution of ionic strength ranging from 0.3-1.0. Myofibrillar protein undergoes changes during rigor mortis, resolution of rigor mortis and long-term frozen storage. Myosin is the most abundant fraction accounting for 50 to 60% of the total amount in this group while actin, the second most abundant myofibrillar protein, constitutes approximately 20% (Shahidi, 1994).

Stroma proteins (Scleroproteins) include the residue after the extraction of sarcoplasmic and myofibrillar proteins. It is composed of proteins from the connective tissue such as collagen and elastin. This fraction is soluble in HCl or NaOH and represents about 10% of the crude muscle proteins (Shahidi, 1994).

Composition of fish lipids

The total amount of fat in fish is extraordinarily variable. It ranges from 0.2 to 23.7%. Indeed, fish can be classified by their fat content into four categories:

Lean (<2% fat)

Low (2-4 % fat)

Medium (4-8 % fat)

High (8-20 % fat) (Ackman, 1994)

Fatty deposits are frequently found immediately beneath the skin of the fish and along the abdominal wall (Brown, 1986). Fish fat is well know to contain a high level of polyunsaturated fatty acids, and to include, in many cases, significant levels of classes of lipids not ordinarily found in appreciable quantities in terrestrial animals. Marine lipids are composed of phospholipids, sterols, triacylglycerols,

wax esters and minor quantities of metabolic products from these. The unusual lipids include glyceryl esters, glycolipids, sulfolipids and hydrocarbons. Although details on the role of lipids is still not very well understood, it is very clear that they can perform two separate physiological functions in muscle; as a source of energy and as a major components in all cellular membranes. The general structures and cellular locations are illustrated in Fig. 2.1 (German, 1990). Wax esters have been mentioned as a factor to increase buoyancy. (Sikorski et al., 1990a). In most lipids of functional interest, fatty acids are the predominant non-polar portion of lipids molecules typically esterified via their acid terminus to glycerol.

The fatty acid (FA) composition of fish lipids is much more complex than that of lipids for terrestrial plants and animals. The content of different fatty acids in fish lipids depend on numerous factors such as the diet, geographic location, environmental temperature, season, body length, lipid content, etc. The carbon chain length is generally from C_{14} to C_{24}, although C_{12} and C_{26} are found as well. Unsaturated bonds can be found in C_{14} and C_{16}, while C_{20} and C_{22} acids may contain from four to six double bonds. Most of the polyunsaturated fatty acids (PUFAs) of fish lipids occurs as the n-3 type. The distribution of fatty acid in lipids is not uniform. The polyenoic acids occur mainly in phospholipids, while the monounsaturated acids are in triacylglycerols. About 50% of the FA in phospholipids are polyenoic. The triacylglycerols compose 50% of the monoenoic FA, while the rest is more or less equally divided between saturated and polyenoic acids (Sikorski et al., 1990a).

Because of the nature of fish lipids, they may undergo different reactions. Among these reactions, hydrolysis and oxidation are the ones that may have an

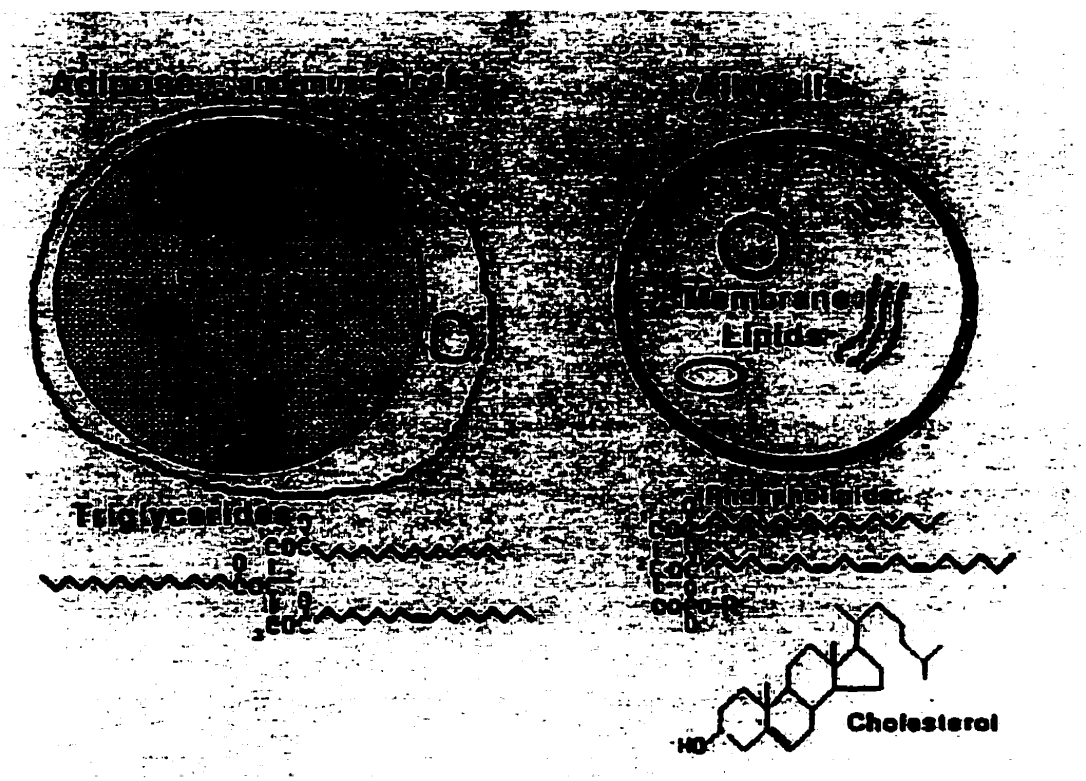

Fig 2.1: Physiological functions of lipids in muscle. (German, 1990)

adverse effect on the quality of fish, being more important during frozen storage. In fatty fish species, lipid oxidation represent a serious problem. These fish contain more free lipids and more dark muscles in which oxidation takes place more rapidly than in white muscle. Particularly susceptible to oxidation, because of a closer contact with lipoxygenase and atmospheric air, are the lipids of the subcutaneous layer and of the skin (Sikorski et al.,1990b).

Spoilage of Fresh Fish

Enzymatic spoilage

The autolytic spoilage is the first type of spoilage that takes place in iced fish and it is due to enzymes contained within the fish itself at the time of death (Haard, 1994). Meanwhile, the flora of the gut cavity, gills and skin acclimatize and adapt to the changed environment. Endogenous enzymes can be distinguished by their source of origin. First those present in the gut of the fish that facilitate its digestion of food. Upon the death of the fish, these enzymes act in an uncontrolled fashion and serve to bring about the breakdown of organs and surrounding tissue causing unwanted texture problems. In some fish such as herring, capelin and mackerel, when harvested during period of heavy feeding, the belly is very susceptible to tissue degradation and may produce the condition known as "belly burst" when the nape area of the fish takes on a brown discoloration (Bonell, 1994). Trypsin-like endopeptidases from pyloric caecae or pancreas along with pepsin like enzymes from the gastric mucosal glands account for most of the important digestive proteases. These enzymes are able to degrade proteins into large-size peptides that can be further split into smaller

peptides and aminoacids by the activity of exopeptidases. The second type of enzymes that cause autolytic spoilage are those present in the flesh of the fish. These enzymes affect changes in seafood quality, particularly flavor, color and texture (Ashie et al., 1996). In the muscle of dead fish, the production of adenosine triphosphate (ATP) stops. During storage, endogenous and microbial enzymes catalyze the conversion of ATP to inosine monophosphate (IMP), a flavor enhancer at quite low concentrations. Then IMP is degraded to inosine and eventually to hypoxanthine, the latter compound has a bitter flavor and is suspected as causing off flavor in stored fish.

Bacterial spoilage

Considered as the most important type of fish spoilage, the action of bacteria creates the undesirable "sour" odors characteristic of spoiled fish. Microorganisms are found on all outer surfaces (skin, gills, surface lime) and in the intestines of live and healthy fish (Huss, 1988). There is evidence that suggests the flora of fish is a function of the environment, for example warm-water fish seem to have more Gram-positive microflora (micrococa, bacilli, coryneforms), while cold water fish carry a predominantly psycrophilic and psychrotrophic Gram-negative population (Moraxella, Acinetobacter, Pseudomonas, flavobacterium and Vibrio) (Shewan, 1977). It is widely accepted that the muscle of a healthy live and freshly caught fish is sterile and that invasion into fish tissue by microorganisms during ice spoilage can takes place through the gill tissue and the vascular system reaching. This invasion can reach the flesh within a few days. Another mode of invasion by the intestines into the body cavity

and belly walls or by the skin into the flesh can also result (Pedroza-Menabrito and Regenstein, 1990). However, Davis (1995) indicated that during ice spoilage the invasion of fish tissue by microorganisms is very limited. Indeed, evisceration seems to be the critical step by which microorganisms are spread about the gut cavity and into the fish flesh, this is because the alimentary canal hosts a large number of microorganisms. For all types of fish, it is the Gram-negative organisms which predominantly cause the fish to become unpleasant to eat (Gibson and Davis, 1995). Of these, the Pseudomonas/Alteromonas/Shewanella group, present on the skin of live cold water fish, eventually comprise over 80% of the bacteria as the fish spoils (Davis, 1993). The metabolic waste-compounds produced from water soluble constituents of tissue are responsible for the characteristic odors and flavors of spoiled fish (Davis, 1993). Metabolic waste compounds include mainly lower fatty acids from carbohydrates, aldehydes and ketones from lipids, ammonia, amines and "biogenic" polyamines from amino acids, and volatile sulphides from sulphur-containing amino acids.

The Pseudomonas-Achromobacter group rapidly metabolize most amino acids, dipeptides and tripeptides found in the non-protein fraction of muscle. Oxidative deamination of amino acids seems to be the primary pathway leading to ammonia and volatile fatty acid accumulation (Liston, 1982). In the early stages of spoilage, the breakdown of protein seems to be minimal because of the high concentration of free amino acids which apparently inhibits proteases (Pedroza-Menabrito and Regenstein, 1988). Hence bacterial production of proteases appears to be repressed at first but then is depressed in the later stages of

spoilage when increased hydrolysis of protein reestablishes the initial levels of free amino acids (Davis, 1995).

Marine fish and shellfish present a special situation because they contain trimethylamine oxide (TMAO) which is thought to have an osmoregulatory function in the live animal by avoiding dehydration in the marine environment (Gibson and Davis, 1995). In general, negligible amounts of TMAO are found in fresh water fish. TMAO can be used by some aerobic spoilage organisms as a terminal electron acceptor, allowing them to grow even in anaerobic conditions which would be otherwise inhibitory. Simultaneously, trymethylamine (TMA) is produce by the reduction of TMAO possibly by endogenous enzymes in muscle fish, but mainly by the enzyme activity of certain bacteria. Further degradation of TMA by fish enzymes produces dimethylamine (DMA) and formaldehyde (FA), however in fresh fish this reaction takes place at a very low speed being more important during the frozen storage of fish. While TMAO is nonodorous, TMA is an ammoniacal substance and when reacts with fat in the muscle of fish, the characteristic fishy odor of low quality fish is produced (Pedroza-Menabrito and Regenstein, 1988).

Chemical changes

Fish fat contains a high proportion of unsaturated fatty acids which are subject to attack by atmospheric oxygen (Pedroza-Menabrito and Regenstein, 1988). Oxidative reactions lead to flavour, color, and possibly textural changes associated with rancidity (Morrison, 1993). The substrates for lipid oxidation in

seafood are molecular oxygen and lipid. Transition metal such as copper and iron are primary activators of molecular oxygen (Hultin, 1992). Lipid hydrolysis is another post-mortem phenomena that takes place in fish and fish products. Hydrolysis is caused by lipolytic activity of fish tissue and forms as major products free fatty acids (FFA) and glycerol. Hydrolysis can also be promoted by bacterial lipases during fish spoilage. It is generally accepted that fatty acid are more susceptible to peroxidation when free than when esterified.

Strategies for controlling spoilage

Until fish is consumed, its quality attributes are prone to change under the impact of the post-harvest, handling, storage and processing, environmental factors, and parameters of applied preservation treatments (Sikorski and Sun Pan, 1994)

The principal aim of fish preservation is to delay, reduce or inhibit microbial spoilage. In the case of fatty fish, the preservation may also aim at reducing or inhibiting oxidation and other undesirable changes in fish lipids, which are highly unsaturated and capable of going rancid at various stages of processing (Hansen, 1988). Fish as a commodity can undergo different treatments to maintain its quality. Some of these treatments are: chilling, freezing, smoking, radiation, drying, salting, fermenting, marinating, and canning. Some fish preservation methods such as canning and smoking change substantially the character of the fish, while the sequence of freezing, storage and thawing, normally aims at retaining the fresh fish character as much as possible.

This is also the aim of chilling and other methods of short-term preservation of wet fish (Hansen, 1988).

The quality preserving effect of chilling fish is generally due to the decrease in the rate of undesirable biochemical and chemical reactions and retardation of the growth and spoilage activity of microorganisms. Generally, the storage life for acceptable sensory quality is only from 5 to 8 days for small fatty fish, and for lean white fish from cold waters between 14 to 21 days. However, the sensory quality decreases significantly due to toughening, excessive drip, and autolytic changes, mainly lipid hydrolysis (Sikorski and Sun Pan, 1994). In fact, chilling represent the major method of fish preservation for a short duration. Chilling may be combined with different methods to extent the shelf life of fresh fish. These methods are: (i) chemical treatments, (ii) low-dose irradiation, (iii) high pressure treatment, and (iv) modified atmosphere storage. The application of two or more sub-optimal environmental conditions simultaneously is considered to be far more effective, since each factor compensates for limitations in the other. This approach is known as the hurdle-barrier concept, which states that several hurdles or inhibitory factors, even if any one of them individually cannot inhibit microorganisms, will nevertheless together reduce or inhibit microbial growth. The multifactorial approach of the hurdle concept is currently the standard for shelf-life extension not only for seafoods, but foods in general. The use of the hurdle-barrier concept has proved in some cases, to extend the shelf life of seafood for up to 1 month. However, this approach results in a final product that is still having a short-term shelf life. Meanwhile freezing represents the best alternative for

long term preservation of fish quality and fresh characteristics (Sikorski et al., 1976).

Frozen foods benefit from two stabilizing factors: reduced temperature and reduced effective moisture content. In general, reactions proceed more rapidly as temperature increases. Therefore, the effect of lowering temperature is to decrease reaction rates or rates of change relating to many important processes. Additionally, the presence of ice in a frozen system implies that the concentration of water in the unfrozen state is reduced. This has a protective effect on the food product making it less susceptible to microbiological deterioration and in several cases enhancing chemical storage stability (Reid, 1997)

Freezing of foods is the result of the combination of two main process. These processes are: (i) lowering of temperature (removal of sensible heat) and (ii) change of phase from liquid to solid (removal of latent heat). Both of these processes are accompanied by a reduction in heat content of the material (Reid, 1993). Depending on species, fish contain 60-80% water, as a consequence, the freezing process mainly concerns the phase transition from liquid to solid water. The larger part of the heat to be removed from the fish is required for the formation of ice (Keizer, 1995).

During the phase transition two processes take place. The formation of ice and, simultaneously the concentration of solutes such as salts and other components in the remaining water increases. These phenomena produce water that becomes frozen over a temperature range between 0-5°C, which is refereed to as the freezing zone or critical zone. The amount of frozen water increases as

temperature decreases, although a temperature of -60°C is required to convert all liquid water, not chemically bound, into ice.

Ideally, there should be no remarkable differences between fresh fish and frozen fish after thawing. If appropriate conditions are used, fish in the frozen state can be stored for several months or more without appreciable changes in quality. However, it is well recognized that deteriorative changes take place in fish during freezing (Santos-Yap, 1995).

It has been reported that freezing and frozen storage can adversely affect the quality of a variety of muscle tissue like beef, pork, fish and chicken. However, it has also been reported that among these commodities particularly fish muscle, this problem of quality deterioration is more severe. Deterioration in texture, flavor, and color is the most serious problem, (Shenouda, 1980).

In general, the modern fishing industry uses temperature of -30 °C for freezing fish, although in some cases temperatures as low as -60°C can be used. Due to the fact that these temperatures are much lower than the most resistant microorganisms, i.e. -12°C, quality deterioration in frozen fish can be attributed mainly to physical, enzymatic and chemical factors. Indeed, the quality of frozen stored fish depends on the: initial quality of the seafood, species, biological factors, preparation before freezing, freshness, rate of freezing, and temperature and time of storage. Additional factors include the effectiveness of the protection against detrimental protein changes, reliability of protection against desiccation and oxidation (Sikorski and Sun Pan, 1994).

Many of the changes in frozen fish muscle are attributed to denaturation of proteins. In fact, during the past 40 years several excellent reviews have summarized the scientific literature in an attend to understand quality deterioration processes on frozen fish as related to protein denaturation (Connell, 1968; Matsumoto, 1979; Dyer and Dingle, 1962; Shenouda, 1980; Sikorki and Kolakowska, 1994).

Changes in texture include alterations in the appearance of the cut surface of the fish, an increasing tendency to lose moisture on thawing, and an increased impression of firmness and dryness when the thawed and cooked fish is eaten (Connell, 1968). Texture deterioration is not the only quality problem associated with storage of frozen fish. Undesirable changes in flavor and color can take place during frozen storage. Deterioration of flavor (off-flavor, rancidity, bitterness, or undesirable fishy taste) is believed to be due to the formation of low-molecular-weight compounds from lipid or protein degradation. Changes in color and appearance (such as loss in intensity of color, loss of surface glossiness, development of freezer burn and surface dehydration, drip, muscle opacity, or chalky appearance) are thought to be due to irreversible changes that occurs in muscle proteins or protein-bound pigments, or to changes in certain pigmented proteins (Shenouda, 1980).

Quality Deterioration on Frozen Fish

As was already mentioned, changes in fish texture are attributed to denaturation of fish proteins. Shenouda (1980) summarized the causes of protein reactions occurring during frozen storage as follow:

Moisture as factor in the denaturation of fish protein

During freezing of fish, the separation of water as pure ice creates an environment that is conducive to protein denaturation. The phenomena responsible for this effect are: (i) Ice crystal formation, (ii) Dehydratation, and (iii) Increase in solute concentration.

Ice crystal formation: This effect was well recognized from early studies. It was observed that freezing at a slow rate causes the formation of inter- and intracellular ice crystals which leads to breakage in the cells, rupturing of membranes, and a disorder of the ultrastructure of the cells tissue. At the same time, a change from liquid to solid phase causes the volume of a specific weight of water to increase, resulting in a continuous pressure from the ice crystals. It is well known that the status of the fish (pre- or post-rigor), freezing rate, storage time, and temperature fluctuations determine the size and location of ice crystals. In pre-rigor fish, ice crystals trend to form mainly intracellularly regardless of the rate of freezing. In on-set rigor mortis fish, inter- and intracellular crystals are formed depending on the freezing rate. At a slow freezing rate, most of the ice crystals formed are located outside the cell and the average size is bigger than those produced using a fast freezing rate. These crystals grow at the expense of

water which diffuses out of the cells, while at high freezing rates, a large number of small crystal are generated both within and outside the cells. During storage, the small ice crystal have a tendency to melt and aggregate to larger ones. This phenomenon is also related to fluctuation in temperature during frozen storage. Even below freezing temperature, if a slight rise in temperature takes place, presumably the small ice crystals melt faster that the larger ones, and when temperature drops again, the melted ice refreezes around the large ice crystal (Shenouda, 1980).

Dehydration: The presence of water is necessary for the structural integrity of some proteins, and the decrease in availability of water as a consequence of freezing may destabilize the sensitive structure of proteins (Connell, 1968). Proteins include both hydrophobic and hydrophilic amino acids. The conformation of most proteins usually follows a general pattern in which a substantial fraction of the hydrophobic side chains are buried inside the molecule. However, some hydrophobic groups remain exposed at the surface, and these seem to be stabilized by a water barrier that mediates hydrophobic-hydrophilic linkages between molecules. In addition, water molecules also adhere to those exposed hydrophobic group side chains in a highly organized water barrier which also mediates the hydrophobic-hydrophilic linkages between molecules. During frozen storage, dehydration of proteins is promoted and what results is the exposure of hydrophobic and hydrophilic groups, that readily interact with similar groups in the same protein molecule or between adjacent protein molecules. These interactions

result in a disruption of the three dimensional structure and consequently into aggregation.

Increase in solute concentration: The water content of biological samples can be divided loosely into free and bound types. During the freezing of fish muscle, part of the water will freeze out, but a considerable portion may remain unfrozen, even at temperatures well below the freezing point. During freezing to -10°C or below, about 10% of the water present in muscle tissue could stay unfrozen. This water is classified as the "bound" fraction. The amount of unfreezable water depends on the temperature and time as well as on the concentration and type of solutes present in the system including various ionic compounds or salts (Xiong, 1997). Thus, as freezing progresses, proteins are exposed to an increased concentration of salts. Freezing out of water can lead to a tenfold increase in the concentration of soluble solutes. An increase in salt concentration is known to affect cell permeability and protein properties. An increase in solute concentration is also the bases for one of the theories of protein denaturation during frozen storage. This theory is based on the effect of salts on the secondary forces (ionic, van der Waals, hydrogen, and hydrophobic), which help to stabilize the tertiary and quaternary structures of protein macromolecules. In the special case of ionic bonding, this take place between appropriately charged groups within protein molecules, between different molecules, or submolecules of proteins, lipids, carbohydrates, nucleotides, etc. Other groups with prominent or inducible dipoles (-OH, -CO) would also be expected to interact with ionic groups. The stability of ionic binding, as well as, all other secondary forces, is dependent on

the dielectric constant, the pH and the ionic strength of the media. Thus, increasing the salt ions presumably will cause competition with the existing electrostatic bonds and the breakdown of some of them, which in turns leads to extensive modification of the protein native structure (Shenouda, 1980). Disruption of energetically and entropically favorable native protein conformation usually results in denaturation, dissociation of subunits, and, ultimately, aggregation of denatured molecules. The extent of protein damage is a function of the type of salts, the freezing rate, the temperature and time of storage, and the characteristics of the protein involved in the interaction.

Most of the studies about the effect of salt concentration on fish myofibrillar proteins as related to freeze-denaturation are centered mainly on two major proteins myosin and actin. It is generally agreed that the susceptibility of muscle proteins to freeze denaturation follows a specific order: myofibrillar proteins to sarcoplasmic proteins to stromal proteins (Xiong, 1997). Studies on myosin systems show that, at high ionic strength, myosin molecules experience a rapid, reversible monomer-dimer equilibrium. With and extended period of exposure to a concentrated solution, the myosin molecules dissociate into subunits: a heavy core, and light components. Furthermore, the light chains undergo irreversible, aggregation during prolonged salt treatment, especially in the absence of thiol protection. The heavy chain core also forms insoluble aggregates, accompanied by conformation changes. Compared to myosin, actin is relatively stable during frozen storage (Shenouda, 1980).

Factor relates to fish lipids and their degradation products

The effect of lipids on protein changes in frozen fish is most significant in lean species. In the lean fish muscle, the lipids are limited to the physiologically necessary membrane lipids that comprise mainly phospholipids and little amount of sterol esters (Sikorski and Kolakowska, 1994). The effects of lipids and their degradation products can be considered from three aspects: whole lipids, free fatty acids, and products of oxidation.

Whole lipids: When studying the effect of whole lipids on fish muscle proteins, one can find that there are two main contradictory theories. One theory believes, that lipiids have a protective effect on proteins while, the second theory believes, they are potentially damaging (Mackie, 1993). Early frozen storage studies on various fish species showed an apparent relationship between their stability and their fat content. Fish such as halibut and rosefish with higher levels of lipids were more stable than those having lower amounts. This protective effect was attributed to the neutral lipid fractions such as triglycerides. It is believed that neutral lipid droplets will dissolve the FFA and diminish their hydrophobic effect on protein (Sikorski et al., 1976). Several reports indicate that the unstable free radical intermediates formed during autoxidation attack the protein molecules, leading to the formation of protein free radicals that may cross-link to form protein-protein aggregates and with lipids form protein-lipid aggregates (Santos-Yap, 1995). Another possible mechanism for the protective effect of lipids is that they reduce the adverse effect caused by the increase in salt concentration due to freezing out water (Shenouda, 1980).

A series of studies carried out by Shenouda and Piggot (1974, 1975a,b, 1976 and 1977), using a model system in which lipids and protein extracted from the same fish were incubated together, suggested that intact lipids may have a detrimental effect on protein stability. The disruption of cellular structure could lead to disorientation or liberation of lipid and protein that under natural conditions do not interact between each other resulting in the formation of lipid-protein complexes affecting the textural quality of the muscle tissue.

Free fatty acids: The damaging effect of lipids on proteins is considered the most acceptable theory as some of the early theories on protein denaturation in frozen storage has been linked to lipid degradation. The two main pathways of lipid degradation are oxidation and hydrolysis.

Hydrolysis of ester bonds in lipids (lipolysis) may occur by enzymatic action or by heat and moisture, resulting in the liberation of free fatty acids (Nawar, 1996). In general, animal tissue does not contain free fatty acids. However, they can form by enzymatic action after the animal is slaughtered. Lipolysis is readily observed in many fish species and is correlated with product quality deterioration of frozen fish. Many studies of fish muscle lipolysis has been with lean fish in which the primary lipid fraction is phospholipid. As a result, interest in the hydrolysis of phospholipids has overshadowed that of triglycerides.

Fatty acids, as products of lipolysis, may interact with myofibrillar protein or may undergo lipid oxidation. Both situations lead to a decrease in fish quality not only by producing rancid flavour and smell, but also by interacting with fish proteins resulting in a product with undesirable texture described as tough, chewy, rubbery, stringy, and fibrous.

There is a general idea that triglyceride hydrolysis stimulates lipid oxidation, in contrast free fatty acids that have been hydrolysed from phospholipids will inhibit oxidation (Erickson, 1997). Some of the possible explanations for this behavior include: (i) the formation or activation of an anti-oxidant during lipolysis of phospholipids, (ii) structural changes in the membrane, (iii) lower availability of free fatty acids to oxidation, or (iv) interference of released fatty acids in the oxidative process (Shewfelt, 1981). On the contrary, Mackie (1993) indicated that phospholipids undergo faster hydrolysis and oxidation than neutral lipids, and that free fatty acids, particularly the polyunsaturated fatty acids, show the greatest susceptibility, indicating the importance of enzymatic hydrolysis in increasing the rate of oxidation

The mechanism of FFA-myofibrillar interaction is likely to be through secondary forces electrostatic, van der Waals, hydrogen and hydrophobic forces, rather than through covalent bond formation. Indeed, it is very probable that free fatty acids will create more hydrophobic regions in the protein and thereby promote greater opportunities for the formation of aggregates through intermolecular bonds (Mackie, 1993)

Lipid oxidation: A free radical process is the basic mechanism upon which lipid oxidation proceeds. The following three stages characterize this process.

Initiation: $In\bullet + RH \rightarrow InH + R\bullet$
$RH + O_2 \rightarrow ROO\bullet + H\bullet$
Propagation: $R\bullet + O_2 \rightarrow ROO\bullet$
$ROO\bullet + RH \rightarrow R\bullet + ROOH$
Termination: $2RO_2\bullet \rightarrow O_2 + RO_2R$
$RO_2\bullet + R\bullet \rightarrow RO_2R$
$2R\bullet \rightarrow R\text{-}R$

During initiation a hydrogen is abstracted from a fatty acid, leaving the fatty acid alkyl radical. Combining quickly with oxygen, this fatty acid alkyl radical is converted to the fatty acid peroxyl as one step of the propagation stage. Additionally, the peroxyl radical abstracts a hydrogen from an adjacent fatty acid, forming a hydroperoxide molecule and a new fatty acid alkyl radical. Breakdown of the hydroperoxide, in turn, is responsible for further propagation of the free radical process (Erickson, 1997). The substrates for lipid oxidation in fish are molecular oxygen and lipid. Transition metals are primary activators of molecular oxygen. Quantitatively, the principal transition metal in fish is iron. Iron occurs as heme pigments, and as nonheme iron complexed to proteins or low molecular weight metabolites (Hultin, 1994). In the nonheme form, iron can participate in the production of the reactive oxygen species, the hydroxyl radical ($OH^{\cdot}$), via the following reaction

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH\bullet + OH^{-}$$

and is particularly effective when Fe^{3+} can be reduced to Fe^{2+} by reducing agents. Along with iron, cooper has also been found to catalyze lipid oxidation in fish muscle.

Extensive autoxidation of fishery products will usually be sufficient enough to prevent them from being marketed or consumed by man. In some species, only one third of the total volume landed is dressed or sold for human consumption. Khayat and Schawall (1983) summarized the factors that play an important role in oxidative reactions in fish tissue.

- Nature of the fat: type of fatty acids, degree of unsaturation, and proportion of phospholipids.

- Distribution of fat in the body: interaction between unsaturated lipids in meat with and aqueous solution containing accelerators or inhibitors of rancidity.
- Presence or absence of other chemical compounds in the tissue which may act as accelerators or inhibitors of rancidity, subject to other influential factors such as pH, chemical environment, etc.
- External factors, such as heat, light, and ultraviolet rays, which tend to change the equilibrium of the tissue.

Fish lipids have been seen as producers of free radicals which will interact with proteins leading to aggregation. The mechanism of the reaction has not been fully established but it is likely that lipid radicals make contact with proteins, extracting hydrogen from labile side chain such as –SH to form protein radicals which could then initiate various reactions with other proteins or lipids to form aggregates. An alternative mechanism is through the end products of lipid oxidation – malonaldehyde, propanal, and hexanal, which react covalently with the side chain groups on proteins primarily histidine, methionine, cysteine, and lysine. Phospholipids are often preferentially hydrolyzed during frozen storage of fish and as they are the main lipid component of the flesh of non-fatty species, it is very likely that malonaldehyde and other products of oxidation will be produced even under conditions recommended for storage.

High Pressure Processing of Food

Consumer demand for minimally processed, microbiologically safe, stable food products that are additive free, has stimulated the interest of food companies in high pressure processing (Hendrickx et al., 1998).

In general, three areas of food processing are potentially interested in high pressure technology:

- Food preservation (microbiology growth and enzymatic activity)
- Food texture
- Phase change processes (Thakur and Nelson, 1998)

Two principles underlie the effect of HP.

(a) Le Chatelier principle: any phenomenon (phase transition, chemical reactivity, change in molecular configuration, chemical reaction) accompanied by a decrease in volume will be enhanced by pressure.

(b) Pressure is instantaneously and uniformly transmitted independent of the size and the geometry of the food. This is known as isostatic pressure (Balny and Masson, 1993).

An advantage of HP technology is that food quality characteristics such as flavour and vitamins are unaffected or only minimally altered by high pressure processing at room temperature (Hendrickx et al., 1998).

Food preservation

Microbiology: The effects of pressure on micro-organisms in food are determined by the effect of pressure on water, temperature during pressure treatment, the food constituent, and the properties and physiological state of the micro-organisms (Hoover, 1993). Vegetative cells, including yeast and moulds, are rather pressure sensitive, i.e. they can be inactivated by pressures of 300 - 600 MPa. Bacterial spores, on the other hand, are highly pressure resistant, since pressures exceeding 1200 MPa may be needed for their inactivation (Hoover et al., 1989). It has been observed that the mode of action of pressure on whole organisms is not necessarily the same. The lethal effect of high pressure on vegetative micro-organisms is thought to be the result of a number of different

process taking place simultaneously, in particular damage to the cell membrane and inactivation of key enzymes including those involved in DNA replication and transcription (Knorr, 1995). The mode of action on bacterial spores is still a matter of speculation. Bacterial spores are killed directly by pressures higher than 1000 MPa. However, spores are sensitive to pressures between 50 and 300 MPa. It is generally agreed that at such pressures, spores germinate followed by death of the germinated spore (Smelt, 1998). Preservation of acid food (pH<4.6) is therefore the most likely application of HP processing. Sterilisation of low acid foods (pH<4.6) on the other hand, will most probably rely on a combination of high pressure processing and other mild treatments. For both pasteurisation and sterilisation, a combined pressure-temperature treatment is frequently regarded as most appropriate (Gould, 1995).

Enzyme activity: Enzymes are a special class of proteins in which biological activity arises from an active site, brought together by the three-dimensional configuration of the molecules. Even small changes in the active site can lead to a loss of enzyme activity (Balny and Masson, 1993). Since protein denaturation is associated with conformational changes, it can change the functionality of the enzyme e.g. increase or loss of biological activity and change in substrates specificity. The pressure needed strongly depends on the enzyme; some enzymes can be deactivated at room temperature by a few hundred MPa, while others can withstand 1000 MPa (Hendrickx et al., 1998). Enzyme inactivation by HP may be divided in two classes. In the first, comparatively low pressures (100 MPa) have been shown to activate some enzymes. Much higher pressures on the

other hand, generally induce enzyme inactivation. For pressure inactivation, there seems to be a minimum pressure below which no or little enzyme inactivation occurs. When pressure exceeds this value, enzyme inactivation (within a specified time interval) increases until completed at a certain pressure (Dornenburg and Knorr, 1998). The pressure inactivation range is strongly dependent on the type of enzyme, pH, medium composition, temperature, etc. For some enzymes, there seems to be a maximum pressure above which no additional inactivation occurs (Heremans, 1995). It has been suggested that the efficiency of high pressure enzyme inactivation is improved by applying pressure cycles. Successive applications of high pressure resulted in higher enzyme inactivation for trypsin, chymotrypsin, and pepsin (Indrawati et al., 1998). Since pressure resistance of enzymes is not related to thermal resistance, knowledge previously amassed for thermal inactivation can not be interpreted for pressure. From an engineering point of view, kinetic data for food quality enzymes are indispensable in the design, evaluation and optimization of the process based on pressure (and temperature). Because of the extreme pressure stability of some food quality enzymes, combined processes (pressure and temperature) might be necessary for enzyme inactivation on an at industriale scale (Gross and Jaenicke, 1994).

Food texture

In addition to food preservation, HP processing opens up the possibility of producing food with novel texture (e.g. meat, fish, dairy products) (Ledward, 1995).

The functional properties of food proteins are those physicochemical properties that affect the behavior of proteins in food systems during preparation, processing storage or consumption (Zayas, 1995). HP can affect protein conformation and can lead to protein denaturation, aggregation, or gelation, depending on the protein system (type protein, pH, ionic strength), the applied pressure and temperature, and the duration of the pressure treatment (Heremans, 1995). HP treatment of food can be used to create new products (new texture or new taste), and to obtain analog products with minimal effect on flavour, colour, and nutritional value and without thermal degradation. HP effects on proteins are primarily related to the rupture of non-covalent interactions within protein molecules and to the subsequent re-formation of intra- and intermolecular bonds within or between protein molecules (Gross and Jaenicke, 1994). Different types of interactions contribute to the stabilization of the secondary, tertiary and quaternary structure of proteins. The first level is the sequence of amino acids in the polypeptide chain. There is no report in the literature on the effect of pressure on covalent bonds. The second level is formed by the hydrogen bonds within and between the peptide chains. In general they are stabilized by pressure, however higher stability is noted for α-helice than β-sheet. The tertiary level is formed by

specific packing of the secondary structure into a more or less globular shape. This level is stabilized by non-covalent interactions. Pressure is expected to affect these interactions. The quaternary structure is mainly held by hydrophobic interactions that are very sensitive to pressure; moderate pressure (<1.5 bar) favors dissociation of olygomeric proteins (Thakur and Nelson, 1998). It has been suggested that pressure denaturation and thermal denaturation proceeds in different ways resulting in products with different texture (Hayakawa et al., 1996). For example, acid-set gel produced from previously high pressure treated milk showed improved rigidity and gel breaking strength and a reduced tendency to syneresis (Hayashi et al., 1987). Electron micrograhs of whey protein gel revealed a higher degree of crosslinking in the heat-induced gels, whereas high pressure generated a more porous network with fewer intermolecular crosslinkages (Hayakawa et al., 1992). High pressure can be used in the tenderization of meat. Tenderization of pre- and post-rigor meat without any additional heating is now possible due to progress in pressurization equipment that permits the application of higher pressures (Johnston, 1995). Apparently, the tenderizing effect of HP (300 MPa) on pre-rigor meat is due to pressure disruption of myofibrillar proteins, specifically the dissociation of actin. Studies have shown that HP may be used to improve water holding capacity and binding of comminuted meats and meat emulsions (Cheftel and Culioli, 1997). The proteins that are believed to be active in binding are myosin and actomyosin. Overall, the pressure treatment could be of interest in maintaining or improving protein functionality, where it is desired to reduce the sodium level of processed meats

(Cheftel and Culioli , 1997). In the case of soy proteins, high pressure produced a softer gel with significantly lower elasticity.

Phase change process

The application of HP reduces the freezing and melting points of water to a minimum of -22°C at 207.5 MPa, as pressure opposes the volume increase occurring on the formation of Type I ice crystals (Kalichevsky et al., 1995). Ice I is unique in having a lower density than liquid water, resulting in a volume increase on freezing at 0°C (Fig. 2.2 and 2.3). The primary application of pressure in relation to the water phase diagram is the increased freezing rates obtained using pressure assisted freezing (the pressure induced melting-point depression enables the samples to be supercooled to –20°C, resulting in rapid and uniform nucleation and growth of ice crystals on releasing the pressure). Additionally, increased thawing rates and also the possibility of non-frozen storage at subzero temperatures can result (Le Bail et al., 1997).

The optimum freezing rate for food products or living cells is highly system-dependent. Slow freezing generally results in larger ice-crystal sizes, which may cause extensive mechanical damage, whereas ultra rapid freezing may cause lethal intracellular ice crystallisation or mechanical cracking. Slow freezing may cause problems due to structural damage, accelerated enzyme and microbiological activities, as well as potentially increased oxidation rates, resulting from the increase in substrate concentration and the insolubility of oxygen in ice (Philippon and Voldrich, 1993).

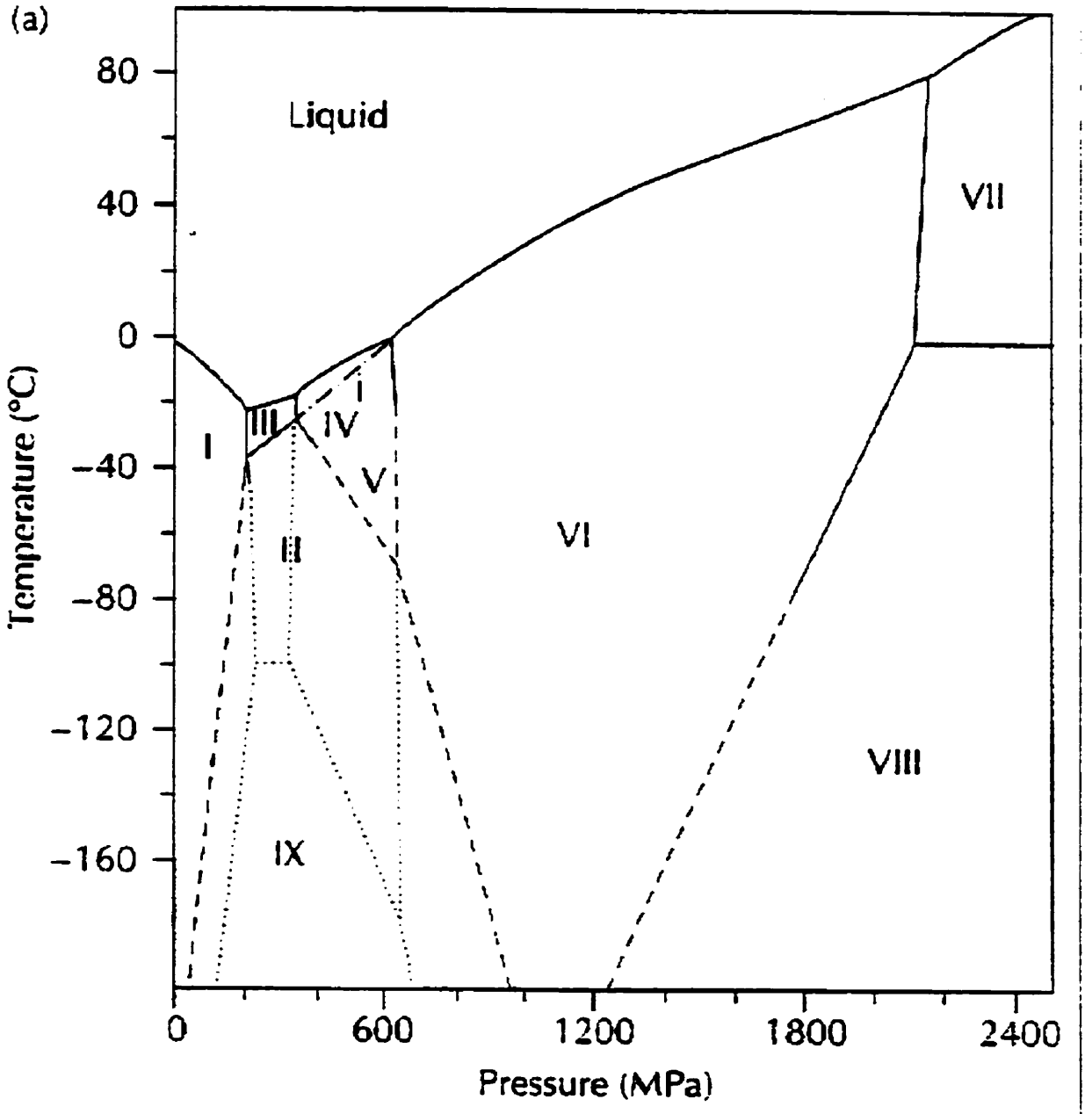

Fig. 2.2: The equilibrium solid-liquid phase diagram of water. (Kalichevsky et al.,1995).

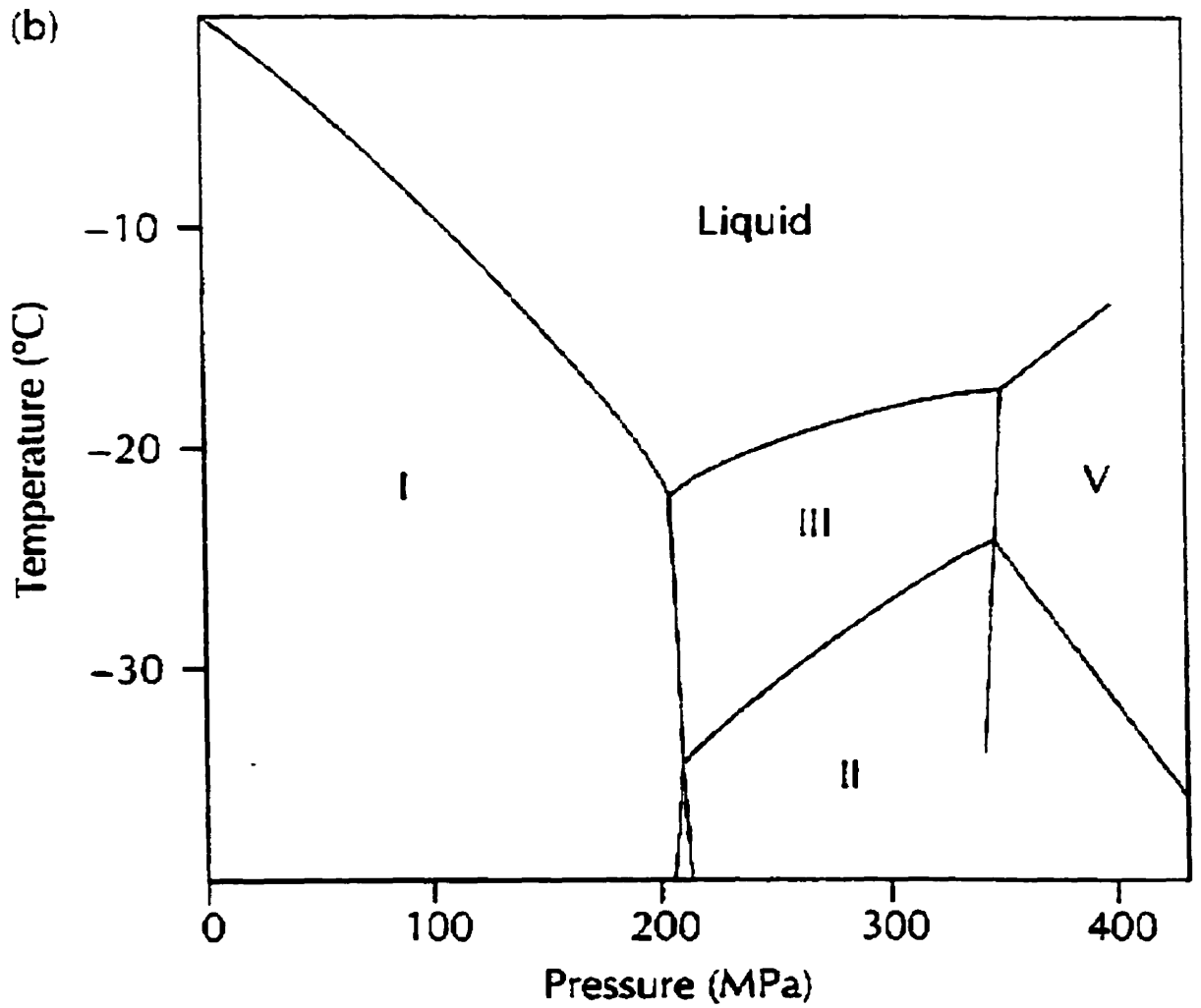

Fig 2.3: The equilibrium solid-liquid phase diagram of water, detail of the region of greatest interest (Kalichevsky, 1995)

For each degree K of supercooling, there is an increase of about tenfold in ice-nucleation. The use of HP facilitates supercooling, and promotes uniform and rapid ice nucleation and growth throughout the sample on pressure release. This produces smaller ice crystals, rather than a stress-inducing ice front moving through the sample (Sanz et al., 1997). The use of high pressure in carrot and in tofu by cooling it to −18°C under 200 MPa yielded a thawed product that had a homogeneous structure very similar to the fresh one (Fuchigami and Teramoto, 1997; Fuchigami et al., 1997a).

Thawing generally occurs more slowly than freezing, potentially allowing further damage to the sample. Faster thawing reduces the loss of liquid retention properties and can improve colour and flavour preservation in fruit. A frozen food at −10 or −18°C may be thawed by pressure treatment at 110 or 200 MPa, respectively. Heating is also required to supply the heat of fusion and also to prevent recrystallization on depressurization. The thawing rate depends only on the conduction of heat, as pressure is transmitted uniformly throughout the sample (Zhao et al., 1998).

HP technology allows for low-temperature non-frozen storage. In this case, the main objective is to use the preservation effect of low temperature avoiding product deterioration due to freezing and thawing effects. The microbial count of most microorganisms maybe reduced by low temperature storage under HP, in some cases, more than by freezing. Enzymes that are inactivated by freezing (catalase, amilase, cathepsin, lactase dehydrogenase) generally had reduced activity after non-frozen storage but were not inactivated (Charm et al., 1977).

High pressure processing of fish and fish products

Fish preservation

Microbial spoilage: High pressure technology has proved to be effective in reducing the total plate counts for tuna meat and squid mantle flesh samples. The total plate counts of both samples decreased with increasing hydrostatic pressure and were lower than 300 colonies per gram of tissue by the end of the pressurisation time (450 MPa, 15 min) (Ohshima et al., 1990).

Control enzymatic activity: Ashie and Simpson (1996) reported that high pressure treatment reduced the residual activity of cathepsin C, collagenase, chymotrypsin-like and trypsin-like enzymes from bluefish and sheephead muscle. The use of protease inhibitor (α_2-macroglobulin), enhanced the capacity of high pressure technology to inactivate endogeneous enzymes in fish gels.

Seafood texture

Fish muscle proteins are typically heat-gelled in the manufacture of crab analog products. Temperatures between 40-60°C are normally required to induce gelation. Proteins are destabilized and may also be induced to gel at low temperature by pressures ranging from 100 - 1000 MPa (Ohshima et al., 1993). Lanier (1996) reported that strong gels can be obtained by using high pressure. It is believed that water soluble proteins contained in fish meat impede gel formation

in Kamaboko. Recently, Okazaki and Fukuda (1996) indicated that pressurisation could be a suitable processing method to give favorable texture to surimi-based products containing high quantity of water soluble protein. On the other hand, Ashie and Simpson (1996) reported that pressure improved tissue firmness or strength of fresh fish up to around 200 MPa for 10 min, beyond which texture generally deteriorated.

CHAPTER III

BIOCHEMICAL PROPERTIES OF ACTOMYOSIN FROM CARP (*Cyprinus carpio*) FILLETS STORED ON ICE

Connecting Statement

The fact that seafoods are different from other muscle protein foods appears to be well established. However, compositional differences associated with species as well as differences associated with the native microflora can result in different spoilage patterns and rates. The work reported in this chapter focuses on the properties of actomyosin extracted from carp fillets previously subjected to ice storage in order to clarify the spoilage patterns.

Contribution of co-authors: Benjamin, K. Simpson, (research/thesis supervisor)

ABSTRACT

Biochemical changes of actomyosin of carp muscle stored on ice were investigated over a period of 12 days by several methods, including ATPase activity, hydrophobicity, total SH groups and autolytic degradation products. No changes were observed in Ca^{2+}-ATPase, Mg^{2+}-ATPase or Mg^{2+}-EGTA-ATPase activity of actomyosin from carp fillets during iced storage ($p>0.05$). In contrast Mg^{2+}-Ca^{2+}-ATPase decreased during the first 6 days of storage and remained almost constant during the rest of the study. Ca^{2+} sensitivity of actomyosin tended to decrease during ice storage of fish fillets. No changes were found in SH content of actomyosin through the ice storage of carp fillets ($p>0.05$). The surface hydrophobicity of actomyosin remain stable during the first 6 days, and then started increasing for the rest of the storage study ($p<0.05$). Autodegradation products increased constantly after the second day of ice storage ($p<0.05$). It is speculated that a decrease in Ca^{2+} sensitivity observed during ice storage of carp fillets may be due to proteinase activity present in the muscle. This study confirms the idea that proteolityc changes due to endogenous enzymes present in fish muscle play an important role in quality deterioration of carp fillets during ice storage.

INTRODUCTION

Fish muscle from newly captured animal is a highly desirable food due to its flaky, soft, moist texture, its pleasant mild flavour, and high nutritional value. However, it is also extremely perishable. Recently, spoilage of fresh fish has been the subject of several reviews (Davis, 1995; Haard, 1994 and Ashie et

al.,1996) which strongly indicate endogenous enzymes as cofactors responsible for fish spoilage, along with microbiological activity and oxidative changes. Once the fish is harvested, a series of natural events begins, that eventually cause the spoilage of fish. Like mammalian meats, fish spoils through the combined effect of continuing activity of endogenous enzymes, bacterial growth and chemical reactions (oxidation). There are differences, however, in chemical composition, metabolism, and environment of the live animal which subsequently reduces the storage life of most fish when compared with meat. Indeed, it is believed that the higher water content, higher free amino acid and the lower content of connective tissue as compared to other flesh food results in a more rapid spoilage of the fish muscle (Pedroza-Menabrito and Regenstein, 1988). It is generally speculated that proteolytic enzymes present as normal tissue constituents, play a role in post-mortem changes in muscle resulting in undesirable flavour and texture alterations (Brown, 1986)

Among the proteins present in fish muscle, the integrity of myofibrillar proteins are of particular interest not only because they influence the texture of fish flesh but also because their functional properties are of prime importance for surimi production and the processing of surimi based products.

Changes in biochemical, physicochemical and functional properties of purified actomyosin and myofibrils from carp stored on ice have been reported (Seki et al., 1979, 1980 and Sompongse et al., 1996 a, b). However, these are model systems and they may not reflect what happens to the protein when the fish is stored whole or as fillets in ice. Studies of myofibrillar proteins extracted from carp muscle after ice storage are scarce. The objective of this study was to

evaluate the biochemical properties of carp myofibrillar proteins during ice storage of fish fillets. This could help to determine and predict the shelf life of ice stored fillets.

MATERIALS AND METHODS

Biological samples and sample preparation

Live carp fish samples (*Cyprinus carpio*) were obtained from a retail market in Montreal, Quebec. Fillets were processed at the retail market, stored in ice and transported to the laboratory at McGill University, Mcdonald Campus. Fillets were packed individually in plastic bags, sealed and stored on ice at -4°C. At days 0, 2, 4, 6, 8,10 and 12 of storage, two fillets from the same fish sample were removed for analysis.

pH determination

Carp muscle was homogenised in 10 volumes water (w/v) and pH was measured using a pH meter (Corning M240, England).

Extraction of actomyosin (AM)

AM was extracted according to the method of McDonald and Lanier (1994), i.e., 7.5 g of fish muscle were homogenised in 75 mL chilled 0.6 M KCl solution (pH 7.0) using a Polytron homogeniser. Excessive heating during the extraction was controlled by placing the blender cup on ice and blending in steps for 20 s followed by a 20 s rest interval for a total extraction time of 4 min. The extract was centrifuged at 5000 g for 30 min at 4°C. AM was precipitated by diluting the supernatant with 3 volumes of chilled distilled water and collected by centrifuging at 5000 g for 20 min at 4°C. AM was then dissolved by gentle stirring with an equal volume of chilled 1.2 M KCl solution (pH 7.0) for 30 min at

4°C. The AM solution was centrifuged at 5000 g for 20 min at 4°C, to remove insoluble material and protein concentration was determined by the Biuret method (Gornall et al., 1949).

Actomyosin ATPase activity

ATP activity was determined using methods of MacDonald and Lanier (1994) and Roura and Crupkin (1995). The prepared actomyosin (AM) was diluted to 2.5 - 4 mg/mL with 0.6 M KCl (pH 7.0) and 1 mL of the diluted solution was added to 0.6 mL of 0.5 M Tris-maleate buffer (pH 7.0). To that mixture, one of the following solutions was added for each ATPase activity assay to a total volume of 9.5 mL: i.e., 10 mM $CaCl_2$ for Ca^{2+}-ATPase, 2mM $MgCl_2$ for Mg^{2+}-ATPase, 0.1 mM $CaCl_2$ and 2 mM $MgCl_2$ for Mg^{2+}-Ca^{2+}-ATPase and 2 mM $MgCl_2$ and 0.5 mM EGTA for Mg^{2+}-EGTA-ATPase. To each assay solution, 0.5 mL of 20 mM ATP was added to initiate the reaction. The reaction was conducted for exactly 8 min at 25°C and terminated by adding 5 mL chilled 15% (w/v) trichloroacetic acid (TCA). The reaction mixture was centrifuged at 3,500 x g for 5 min at 25°C, and the inorganic phosphorus liberated in the supernatant was measured using ascorbic acid as per Chen et al., (1956). Specific activity was expressed as μmoles inorganic phosphorus liberated per milligram of protein in 1 minute (μM Pi/mg protein/min) at 25°C. A blank solution was prepared by adding 5 mL of chilled (4°C) TCA solution (15% w/v) prior to addition of ATP.

Ca^{2+} sensitivity was calculated according to Seki and Narita (1980) as follows:

$$Ca^{2+}\text{ sensitivity} = 1 - \frac{Mg^{2+}\text{-EGTA-ATPase activity}}{Mg^{2+}\text{-}Ca^{2+}\text{-ATPase activity}}$$

Hydrophobicity

Changes in protein hydrophobicity of extracted actomyosin (AM) were determined using 1-anilinonaphthalene-8-sulphonic acid (ANS) as fluorescence probe. Measurements were performed according to the method of Kato and Nakai (1980). Each protein sample (2 mL) was serially diluted with 0.01 M phosphate buffer (pH 7.0) to obtain a protein concentration typically from 0.005 to 0.030 (%w/v). Following this, 10 μl of a ANS solution (8 mM in 0.1 M phosphate buffer, pH 7) was added. Relative fluorescence intensity (RFI) was measured with a spectrofluorometer (Hitachi F-2000, Japan) at excitation and emission wavelengths (ex, em) of 390 and 470 nm, respectively. The net RFI for each protein concentration was computed by subtracting the RFI measured for each solution without the probe from that with the probe. Protein hydrophobicity was calculated from the initial slopes of the plots of RFI vs protein concentration (%, w/v) using linear regression analysis. The initial slope was referred to as SoANS and used as an index of hydrophobicity.

Total SH analysis

Total SH content was measured using 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) according to Ellman (1959) as modified by Sompongse et al. (1996a). Actomyosin (AM) (1mL, 0.4%) was added to 9 mL of 2 M Tris-HCl buffer, pH 6.8 containing 8 M urea, 2% SDS and 10 mM EDTA. A 4 mL aliquot of the mixture was taken and 0.4 mL of 0.1% DTNB solution was added and incubated at 40°C for 25 min. Absorbance was measured at 412 nm with a spectrophotometer (Hitachi U-2000, Japan). A blank was prepared by replacing the sample with 0.5 M KCl (pH 7.0). SH content was calculated from the absorbance using a molar extinction coefficient of 13, 600 M^{-1} cm^{-1} and was expressed as mol/10^5 g protein.

Measurement of autolytic degradation product

Fish muscle (3g) was homogenised in 27 mL of 5% (w/v) TCA. The homogenate was kept on ice for 1 hr and centrifuged at 5000 g for 5 min. Tyrosine in the supernatant was measured as an index of autolytic degradation according to the method described by Morrisey et al., (1993), and expressed as μmol tyrosine/g muscle.

Statistical analyses

Data were analysed by analysis of variance (ANOVA). Mean difference was determined using the least significant difference (LSD) multiple range test. Significance difference was established at $p<0.05$.

RESULTS AND DISCUSSION

Actomyosin ATPase activity

Figure 3.1 shows the ATPase activity of actomyosin extracted from carp fillets after ice storage. ATPase activity has been related to actin/myosin interaction in the presence of endogenous Ca ions, exogenous Ca ions and absence of Ca ions (Roura et al., 1990). No changes were observed in Ca^{2+}-ATPase, Mg^{2+}ATPase or Mg^{2+}-EGTA-ATPase activity of actomyosin from carp fillets during iced storage ($p<0.05$). In contrast Mg^{2+}-Ca^{2}-ATPase decreased during the first 6 days of storage and then remained almost constant during the rest of the study. Mg^{2+} and Mg^{2+}-Ca^{2+}-ATPase activities are indicative of the integrity of the actin-myosin complex, while Mg^{2+}-EGTA ATPase activity indicates

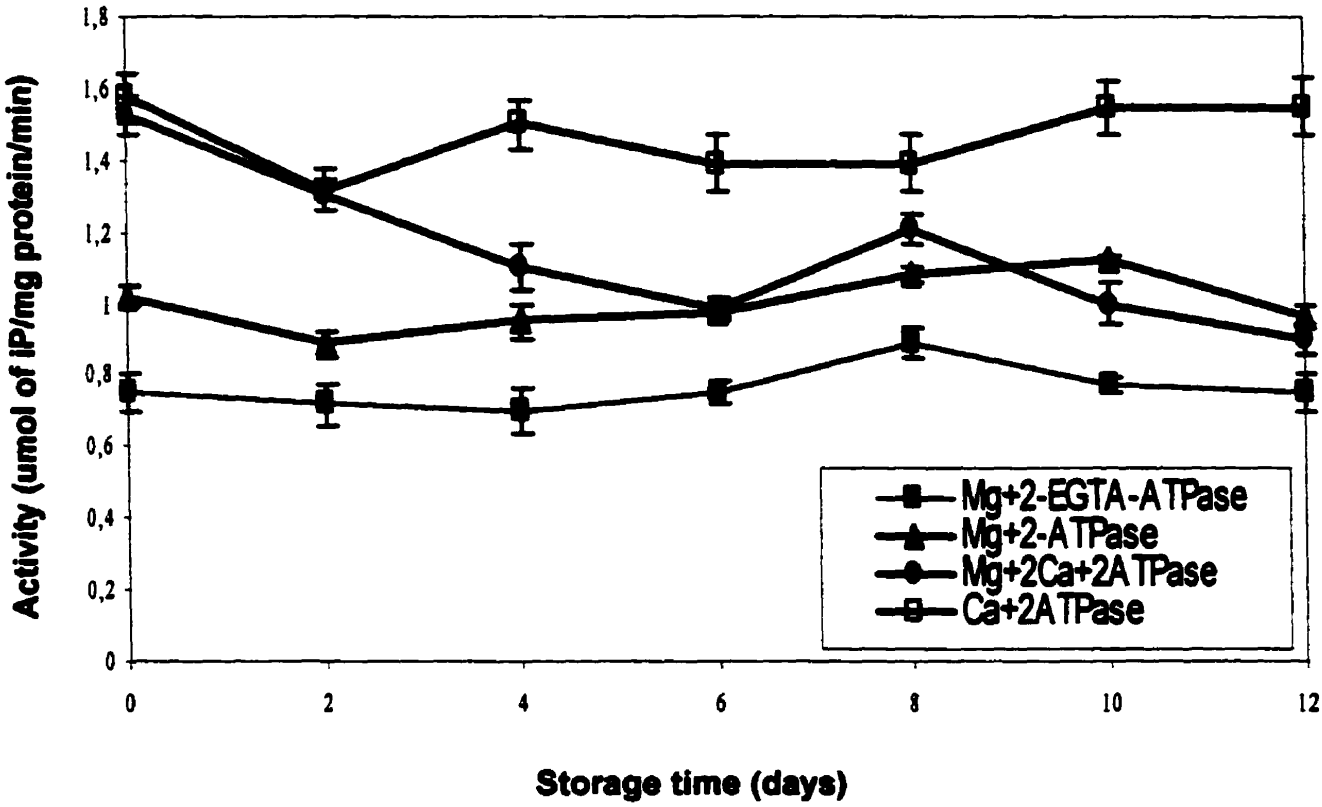

Fig. 3.1: ATPase activities of actomyosin extracted from intact carp fillets stored on ice. Each marker represents the mean of 4 values.

the integrity of the tropomyosin-troponin complex. Changes in ATPase activities of carp myofibrils extracted from minced fish stored on ice were reported by Seki and Narita (1980). When myofibrils were extracted from muscle blocks stored on ice, little changes occurred in ATP activities. However, all the activities tend to decrease during ice storage with the exception of Mg^{2+}-EGTA-ATPase which slightly increased.

The level of pH in fish muscle has been reported to affect ATP activities, in fact all ATPase activities began to decrease when pH declined to 6.4 (Kamal et al., 1991). It was found that pH varies significantly ($p<0.05$) thorough the experiment. However the pH levels were always higher than 6.4 which agree with the behavior observed for ATPase activities. Figure 3.2 shows the Ca^{2+} sensitivity of actomyosin during ice storage. Ca^{2+} sensitivity is a good indicator of Ca^{2+} regulation of myofibrillar proteins. Activity of native tropomyosin has been mentioned as being responsible for Ca^{2+} sensitivity. Loss of Ca^{2+} sensitivity has been attributed to two main factors: (i) filamentation of myofibrils caused by hydrolysis of proteases and (ii) modification of actin-myosin interactions by oxidation of thiol groups on the myosin moiety. The presence of two highly reactive thiol groups on the myosin molecule determines the myosin ATPase activity. These two thiols referred to as SH_1 and SH_2 are located at the head of the myosin molecule. Modification of SH_1 results in an increase in Ca^{2+}-ATPase activity of myosin and a decrease in ATPase activity in the presence of EDTA, whereas modification of SH_2 results in the loss of both ATPase activities (Sekine and Yamaguchi, 1963; Sekine et al., 1962; Reisler et al., 1974). In this study, Ca^{2+} sensitivity of actomyosin tends to decrease during ice storage of

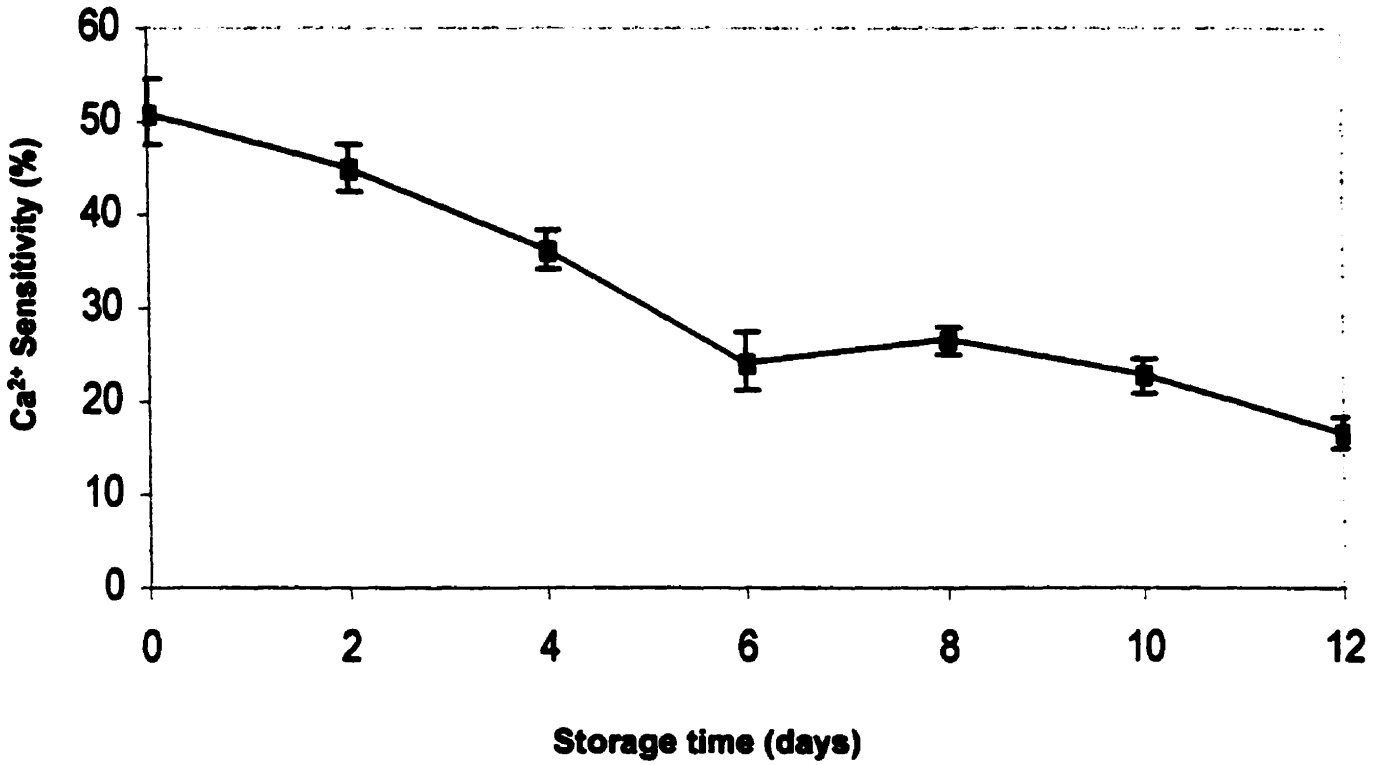

Fig. 3.2: Ca^{2+} sensitivity of actomyosin extracted from intact carp fillets stored on ice. Each marker represents the mean of 4 values.

fish fillets, as was previously reported by Seki and Narita (1980) for both minced fish and muscle blocks during ice storage.

Total SH content

Figure 3.3 shows the SH content of actomyosin through ice storage of carp fillets. In this study, no changes ($p>0.05$) were found for SH content of actomyosin. Sompongse et al., (1996b) using a model system (isolated actomyosin solution) observed a decrease in both total and surface SH. Similar results for model system were reported by Buttkus (1971), Hamada et al., (1977) and Jiang et al., (1986). A decrease in total SH groups was reported to be due to formation of disulfide bonds through oxidation of SH groups or disulfide interchange. Sompongse et al., (1996b) related oxidation of a specific SH group with an increase in Mg^{2+}-EGTA-ATPase activity for carp actomyosin during ice storage. However, in this study no changes were observed for Mg^{2+}-EGTA-ATPase activity. Based on these results, it is speculated that a decrease in Ca^{2+} sensitivity observed during ice storage for carp fillets may be due to proteinase activity present in the muscle rather than oxidation of thiol groups.

Protein Hydrophobicity

Figure 3.4 shows that the surface hydrophobicity of actomyosin remained stable during the first 6 days, and then started increasing for the rest of the storage study. Niwa et al., (1986) suggested that exposure of hydrophobic amino acids in actomyosin occurred in myosin rather than actin. Sompongse et al., (1996b) observed that hydrophobicity of extracted actomyosin stored on ice

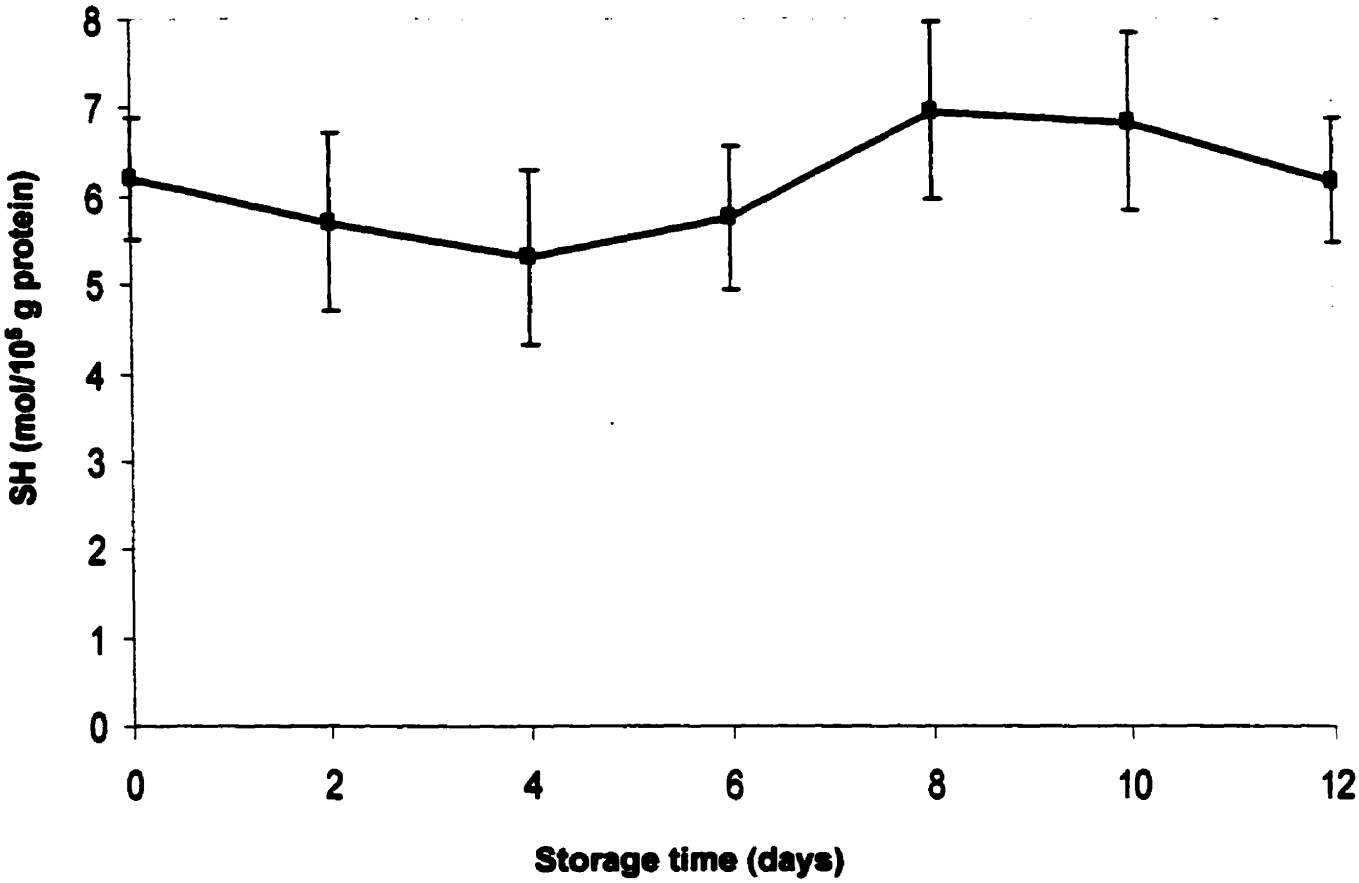

Fig. 3.3: Total SH content of actomyosin extracted from intact carp fillets stored on ice. Each marker represents the mean of 4 values.

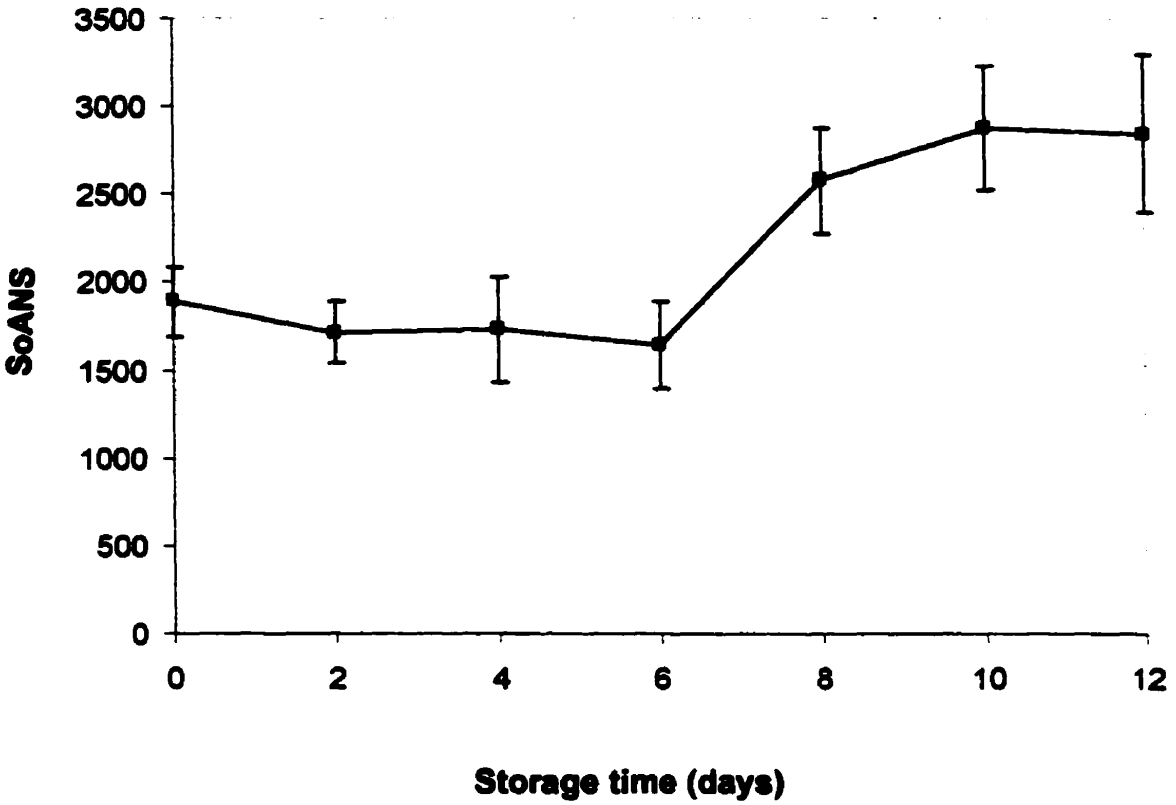

Fig. 3.4: Surface hydrophobicity of actomyosin extracted from intact carp fillets stored on ice. Each marker represents the mean of 4 values.

increased from the beginning of storage. It seems that actomyosin is more stable against unfolding when stored in the intact muscle than once extracted. In other words, intact muscle may have a retarding effect on the unfolding of protein structure. On the other hand, Benjakul et al., 1997 reported an increase in surface hydrophobicity for fish actomyosin from Pacific whiting fillets stored on ice. However, for Pacific whiting fillets hydrophobicity increased after 3 days of storage, while for carp fillets this increased took place only after 6 days of storage. Multilangi et al., (1996) observed that protein hydrophobicity increased with enzymatic hydrolysis. Morrissey et al. (1995) reported that proteolytic activity of Pacific whiting was induced by the infection of Myxosporidea and that the increase of proteolytic activity after 3 days in ice storage resulted in samples with poor textural properties. High levels of enzymatic activity may be responsible for the faster rate of unfolding of myofibrillar protein in Pacific whiting when compared to carp myofibrils.

Autolytic degradation

Degradation products from autolytic activity were monitored during ice storage. Fig. 3.5 shows the increase in proteolytic products reported as tyrosine content. Autodegradation products increased constantly after the second day of ice storage ($p>0.05$). Tokiwa and Matsumiya (1969) as well as Busconi et al., (1989) reported degradation of myofibrils around 0°C and the enzymes thought to be responsible were cathepsins and serine proteases. Proteolytic degradation in Pacific whiting was reported by Benjakul et al., (1997) although the rate seems to

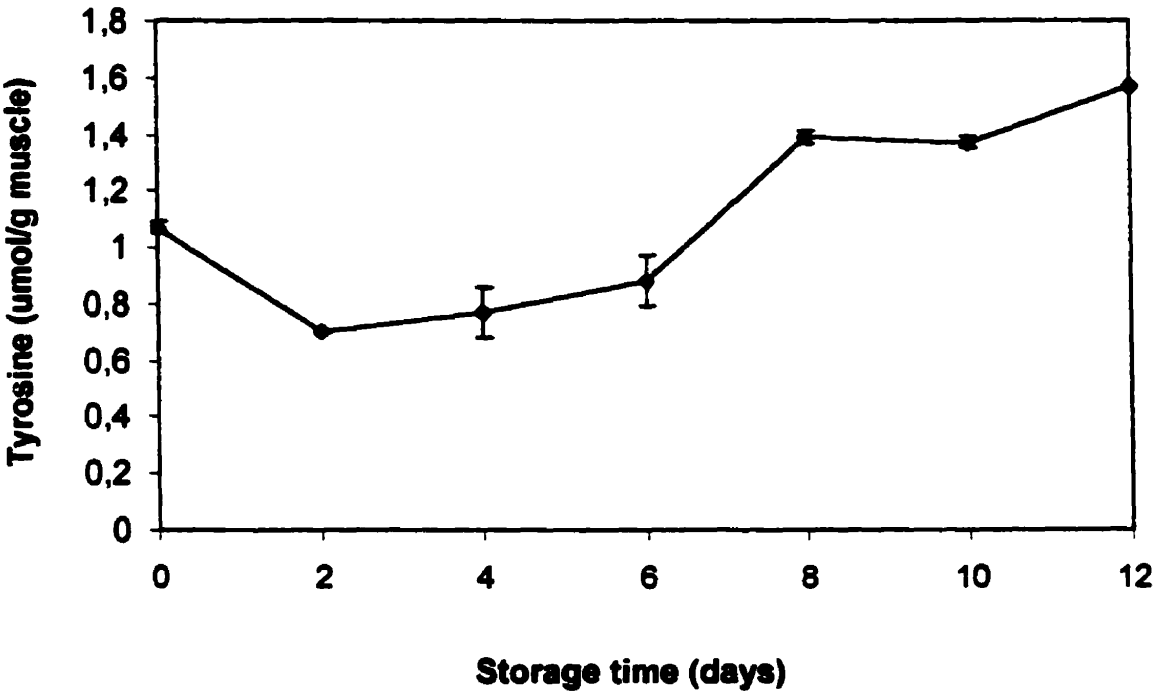

Fig. 3.5: Degradation products from autolytic activity during ice storage of intact carp fillets (proteolytic products reported as tyrosine content). Each marker represents the mean of 4 values.

be higher than that for carp fillets, maybe due to the presence of microbial proteases.

CONCLUSIONS

This study confirms the hypothesis that proteolytic changes due to endogenous enzymes present in fish muscle play an important role in quality deterioration of carp fillets during ice storage. The decrease in Ca^{2+} sensitivity observed during ice storage of carp fillets may be due to proteinase activity present in the muscle. Indeed quality deterioration of seafood material results from the combination of autolytic changes and microbial growth. Ice storage remains a limited procedure in preserving seafoods, therefore it is important to develop procedures that extend the shelf life of fish fillets while at the same time preserving its fresh characteristics.

CHAPTER IV

EFFECT OF LOW TEMPERATURE PRESSURISATION ON PHYSICO-CHEMICAL PROPERTIES OF INTACT CARP (*Cyprinus carpio*) FILLETS

Connecting Statement

In the previous chapter, the spoilage pattern of carp fillets during ice storage was studied. The results indicate that endogenous enzymatic systems in fish fillets play an important role on spoilage. High pressure technology have been shown to be effective in controlling enzymatic activity. However, its use on seafood material may be limited due to adverse effects on colour and texture. This chapter evaluates the combined effect of pressure and time on some physico-chemical characteristics of carp fish fillets.

Contribution of co-authors: Benjamin K. Simpson (research/thesis supervisor)

ABSTRACT

The effect of high pressure treatment on some physico-chemical properties (actomyosin extractability, Ca^{2+}-ATPase activity, surface hydrophobicity, TBA value, liquid loss and firmness) of intact carp fish fillets (*Cyprinus carpio*) were studied using response surface methodology (RSM). The fish samples were pressure treated at low temperature (-5°C), then frozen and stored for 1 month at –20°C. An equation for each of the response parameters listed above was found and the r^2 values ranged from 0.70 to 0.98. Results for TBA value suggested that high pressure treatment may inactivate some enzymes, such as lipoxygenase, that may participate in lipid oxidation. Processing conditions of (140-175 MPa, 16-18 min) seem to result in less protein denaturation, which could be used in a pressure shift freezing process. Application of response surface methodology to analyse combination treatments involving hydrostatic pressure and time could be a very useful tool in studying the effect of high pressure on muscle from different fish species.

INTRODUCTION

Until fish is consumed, its quality attributes are prone to changes due to post-harvest handling, processing, storage, environmental factors, and various other parameters of applied preservation treatments (Sikorski and Sun Pan, 1994). The principal aim of fish preservation is to delay, reduce or inhibit biochemical and microbial spoilage. For example, with fatty fish, preservation

also aims at reducing or inhibiting oxidation and other autolytic changes in fish lipids, which are highly unsaturated and capable of undergoing rancidity at various stages of processing (Hansen, 1980). Chilling represents the major method for short term preservation of fish, while freezing represents the best alternative for long term preservation (Sikorski et al., 1976). Ideally, there should be no major differences between fresh and frozen fish after thawing. If appropriate conditions are used, fish in the frozen state can be stored for several months without appreciable changes in quality (Santos-Yap, 1995). However, freezing and frozen storage can adversely affect the quality of a variety of muscle tissue like beef, pork, fish and chicken (Xiong, 1997). It has also been reported that this problem of quality deterioration during frozen storage was greater in fish muscle compared to other muscle foods (Mackie, 1993) Deterioration in texture, flavour and colour are the most serious problems (Shenouda, 1980).

Many of the changes in frozen fish muscle are attributed to denaturation of proteins. During the past 40 years, several excellent reviews have summarized the scientific knowledge in an effort to better understand the quality deterioration process of frozen fish as related to protein denaturation (Dyer and Dingle, 1961; Connell, 1968; Matsumoto, 1979; Shenouda, 1980; Sikorski and Kolakowska, 1990; Mackie, 1993).

During freezing of fish, separation of water as ice crystals creates an environment that is conducive to protein denaturation (Jiang et al., 1985). The phenomenon and its effect are well known and well studied. Available data indicates that freezing particularly at a slow rate, causes the formation of inter- and intracellular ice crystals which lead to breakage of the cells, rupturing of

membranes, and a disordering of the cell ultrastructure. At the same time, freezing of water causes the volume of a specific weight of water to increase, resulting in a continuous pressure from the forming ice crystals. At slow freezing rate, most ice crystals formed are located outside the cell and their average size is bigger than those produced using a fast freezing rate (Shenouda, 1980).

Some of the quality problems observed in frozen fish have been partly related to cellular disruption that occurs in fish muscle as a result of large size ice crystal formation (Shenouda, 1980). Disruption of cellular structure may induce interactions among enzymes, lipids and proteins that normally do not occur in the intact muscle, and these interactions may lead to protein denaturation and lipid degradation.

As the size of ice crystals formed depends on the freezing rate, it is not surprising that the food industry has developed several freezing processes to induce "quick freezing". These processes include: air-blast freezing, contact plate freezing, spray or immersion freezing.

Presently there is considerable interest in the use of high pressure as a food processing tool (Farr, 1990) which the fishing industry could use. In the specific case of fish freezing, high pressure may become a new alternative method. Kalichevsky et al., (1995) and Le Bail et al., (1997) summarised the effects of high pressure on the solid-liquid phase diagram of water and its potential in food applications. The primary applications of pressure in relation to the water phase changes include increased freezing rates obtained using pressure-assisted freezing, the increased thawing rates and also the possibility of non-frozen storage at subzero temperatures.

The use of high pressure during freezing of fish may offer one important advantage over traditional freezing techniques, such as air blast and plate freezing. The advantage is the increase in freezing rate during the freezing process that may result in a final product with better quality once thawed. The improvement of quality may be a direct result of the reduction of tissue or textural damage, or indirectly by the reduction of lipid deterioration in the final product. However, before applying high pressure as a freezing aid, it is important to understand the effect that high pressure itself has on fish tissue intended for freezing. The objective of this study was to evaluate the effect of high pressure on the physico-chemical properties of fresh intact carp fillets. Also, to establish the appropriate treatment conditions (time and pressure level) for the pressure shift freezing of carp fillets.

MATERIALS AND METHODS

Biological samples and sample preparation

Live carp fish (*Cyprinus carpio*) samples weighing ca. 1020 ± 150 g and 37 ± 5 cm in length were obtained from a retail market in Montreal, Quebec. The fish were slaughtered and filleted at the retail market, placed in plastic bags, stored in ice and transported to the laboratory. Fillets were packed individually in plastic bags, sealed and stored on ice at -4°C for 2 hours before being subjected pressure treated.

Pressure treatment

An isostatic pressure unit (Autoclave Engineers, Columbus, Ohio) was used to pressure treat the samples. Prepared samples were subjected to selected pressure treatments ranging from 80 - 220 MPa with holding times ranging from 8 to 22 min.

A cylindrical container made of polyvinyl chloride (PVC) with some holes in its cover was used as a sample holder. Samples were fixed on a rack with rubber bands and then placed in the PVC container filled with a cold solution (-20°C) of glycol:water (50:50 v:v). Immediately, the PVC container with the sample was placed into the pressure treatment chamber which was at 0-3°C and treated for the preselected pressure and hold time conditions. Treated samples were kept in the whirl-pak bags and frozen in a freezer at -20°C. Samples were stored under frozen condition at -20°C for one month. Packaged frozen samples were totally thawed overnight in a cold room at 4°C. Each experiment and analysis were performed in duplicate on two different batches of fish after one-week interval.

Extraction of actomyosin (AM)

AM was extracted according to the method of MacDonald and Lanier (1994), i.e., 7.5 g of fish muscle were homogenised in 75 mL chilled 0.6 M KCl solution (pH 7.0) using a Polytron homogeniser. Excessive heating during the extraction was controlled by placing the blender cup on ice and blending in steps for 20 s followed by a 20 s resting interval for a total extraction time of 4 min. The extract was centrifuged at 5000 g for 30 min at 4°C. AM was precipitated by

diluting the supernatant with 3 volumes of chilled distilled water and collected by centrifuging at 5000 g for 20 min at 4°C. The recovered AM was then dissolved by gentle stirring with an equal volume of chilled 1.2 M KCl (pH 7.0) for 30 min at 4°C. The AM solution was centrifuged at 5000 g for 20 min at 4°C, to remove insoluble material and protein concentration was determined by the Biuret method (Gornall et al., 1949).

Actomyosin Ca^{2+}-ATPase activity

Ca^{2+}-ATPase activity was determined using the methods of MacDonald and Lanier (1994) and Roura and Crupkin (1995) The prepared actomyosin was diluted to 2.5 - 4 mg/mL with 0.6 M KCl (pH 7.0) and 1 mL of the diluted solution was added to 0.6 mL of 0.5 M Tris-maleate buffer (pH 7.0). To that mixture, a solution of 10 mM $CaCl_2$ was added to a total volume of 9.5 mL , then 0.5 mL of 20 mM adenosine triphosphate ATP was added to initiate the reaction. The reaction was proceeded for exactly 8 min in a temperature controlled incubator at 25°C and terminated by adding of 5 mL chilled 15% trichloroacetic acid solution (TCA) (w/v). The reaction mixture was centrifuged at 3,500 x g for 5 min at 25°C, and the inorganic phosphorus liberated in the supernatant was measured using ascorbic acid as per Chen et al., (1956). Specific activity was expressed as μmoles inorganic phosphorus liberated per milligram of protein in 1 minute (μM Pi/mg protein/min) at 25°C. A blank solution was prepared by adding 5 mL of chilled (4°C) TCA solution (15% w/v) prior to addition of ATP.

Hydrophobicity

Changes in protein hydrophobicity of extracted AM were determined by using 1-anilinonaphthalene-8-sulphonic acid (ANS) as fluorescence probe. Measurements were performed according to the method of Kato and Nakai (1980). Each protein sample (2 mL) was serially diluted with 0.01 M phosphate buffer (pH 7.0) to obtain a protein concentration typically from 0.005 to 0.030% (w/v). Following this, 10 μl ANS solution (8 mM in 0.1 M phosphate buffer, pH 7) was added. Relative fluorescence intensity (RFI) was measured with a spectrofluoremeter (Hitachi F-2000, Japan) at excitation and emission wavelengths (ex, em) of 390 and 470 nm, respectively. The net RFI for each protein concentration was computed by subtracting the RFI measured for each solution without the probe from that with the probe. Protein hydrophobicity was calculated from the initial slopes of the plots of RFI vs protein concentration (%, w/v) using linear regression analysis. The initial slope was referred to as SoANS and used as an index of hydrophobicity.

Thiobarbituric acid (TBA) test

TBA values of fish samples were determined according to the procedure of Vyncke (1970). Exactly 15 g of fish tissue were blended with 30 mL of extracting solution (7.5 % TCA in water, 0.1% propyl gallate and 0.1% EDTA). The homogenate was centrifuged at 1000 g for 15 min at 20°C and the supernatant was used for the TBA reaction. In a screw cap test tube, 5 mL of TBA reagent were mixed with 5 mL of supernatant. The capped tubes were heated in boiling

water for 40 min. The tubes were cooled in running tap water and the optical density was measured at 530 nm against water and 5 mL TBA reagent as a blank.

Liquid Losses

A centrifugation technique was used to measure liquid losses for raw fish fillets. In this technique, 10 g of sample were centrifuged at 5000 g for 10 min. The weight of the released juice was measured and moisture loss calculated as (g of juice/10 g fish) x 100 (Hermmasson and Akesson, 1975).

Objective evaluation of texture

Thawed samples were cut into cubes of dimensions 20 mm x 20 mm x 20 mm and placed in refrigeration for 5 h at 4°C before texture measurement. Objective texture measurements were performed with a texture testing machine (Lloyd Instruments Omnitronix, Mississagua, Ontario). Firmness was measured using a compressive probe at a speed of 20 mm/min. A steadily increasing load (F) was applied to a maximum of 45 N and the corresponding deformation (d) was measured. Values were expressed as firmness (F/d).

Experimental design

Factors and their corresponding levels used in this study were: hydrostatic pressure (80 – 220 MPa) and time (8 – 22 min) as specified in table 4.1. The pressure and time values were chosen based on previous work (Ohshima et al.,

1993; Ashie and Simpson, 1996). To determine the simultaneous effects of these factors on extractability of actomyosin, Ca^{2+}ATPase activity, hydrophobicity, TBA value, liquid losses and Firmness, a 2 x 2 factorial model (Mullen and Ennis, 1979) was used as indicated below:

$$y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{12} X_1 X_2 \quad (1)$$

where X_1 and X_2 correspond to the factors or variables, pressure and time respectively, while ß values represent the corresponding regression coefficients or parameter estimates.

Statistical analysis

Statistical analysis and three-dimensional response surface contour plots were done using Excel (Microsoft, 1997).

RESULTS AND DISCUSSION

Parameter estimates for each response are shown in table 4.2. Those parameters found non-significant were dropped from the model. The final equations that describe the response surface graphics are included in table 4.3. Analysis of variance of the data for the response parameters using the above model showed that the model was highly significant ($p<0.05$) with r^2 values

Table 4.1: Values of coded levels used in the experimental design

Process Variable	Levels				
	$-\alpha$	-1	0	1	α
Hydrostatic Pressure (MPa)	79.3	100	150	200	220.7
Time (min)	8	10	15	20	22

Table 4.2: Parameter estimates for the responses

Parameter	Extractability of actomyosin	Ca^{2+}ATPase activity of actomyosin	Hydrophobicity of actomyosin	Liquid losses	TBA value	Firmness
Intercept	2.651 *	3.511 *	4787.3 *	21.41 *	0.034 *	3.070 *
X_1	0.132 ^{ns}	-0.185 ^{ns}	-1085.4 *	0.34 ^{ns}	0.004 ^{ns}	-0.263 *
X_2	0.016 ^{ns}	0.095 ^{ns}	-115.5 ^{ns}	-1.88 *	-0.035 *	0.102 ^{ns}
X_1^2	0.124 ^{ns}	0.754 ^{ns}	1533.9 *	-1.46 ^{ns}	0.200 *	0.158 ^{ns}
X_2^2	-0.100 ^{ns}	-0.504 *	359.6 *	0.58 ^{ns}	0.027 *	-0.296 *
X_1X_2	-0.359 *	0.830 *	1387.0 *	-1.31 ^{ns}	-0.008 ^{ns}	0.185 ^{ns}

X_1 and X_2 represent hydrostatic pressure and time respectively.
* Signigicant at 5% level, ns = non significant.

Table 4.3: Equation describing the response surface graphics

Parameter	Equation	r^2
Extractability of actomyosin	= 2.65 – 0.359 (P x t)	0.85
Ca^{2+}ATPase Activity of Actomyosin	= 3.51 – 0.50 (t^2) + 0.83 (P x t)	0.88
Hydrophobicity of actomyosin	= 4787.32 – 1085.4(P) + 1535.9 (P^2) + 359.6 (t^2) + 1387 (P x t)	0.98
Liquid loss	= 21.41 – 1.88(t^2)	0.70
TBA value	= 0.034 – 0.035 (t) + 0.01 (P^2) +0.026 (t^2)	0.90
Firmness	= 3.07 – 0.26 (P) – 0.29 (t^2)	0.81

P= pressure, t= time

ranging from 0.70 to 0.98. These indicated that the model adequately explained the responses observed. The effect of low temperature pressurisation on protein extractability of carp muscle is presented in Fig. 4.1. In this study, protein extractability was used as one of the indices of protein denaturation. Several studies have shown that pressure may induce protein denaturation. The extent of denaturation is related not only to the level of pressure used but also to the time of treatment exposure (Okamoto et al.,1990; Hayakawa et al., 1992). The results showed that both short time and low pressure treatments (STLP, t<12 min and P>120 MPa) as well as long time and high pressure treatments (LTHP, t>19 min and P>174 MPa) decreased the amount of soluble protein extracted from thawed fish muscle. On the other hand, short time and high pressure treatments (STHP, t<12 min and P>174 MPa) as well as long time and low pressure treatments (LTLP, t>19 min and P>120 MPa) resulted in a higher amount of soluble protein extracted from thawed fish muscle comparable to that of the control sample (3.27 mg/mL). It is speculated that lower protein extractability after STLP treatment was observed because nuclei ice formation did not take place under these conditions. Once the fish fillets were placed in the freezing chamber, they underwent conventional freezing process that resulted in the formation of large size ice crystals and enhanced protein denaturation. In the case of LTHP, a reduction in protein solubility observed under these extreme processing conditions was probably due to protein denaturation induced by pressure (MacFarlane and Mckenzie, 1976). As indicated, STHP and LTLP treatment resulted in little or no change in protein solubility. Although nuclei ice formation can be hardly induced under these

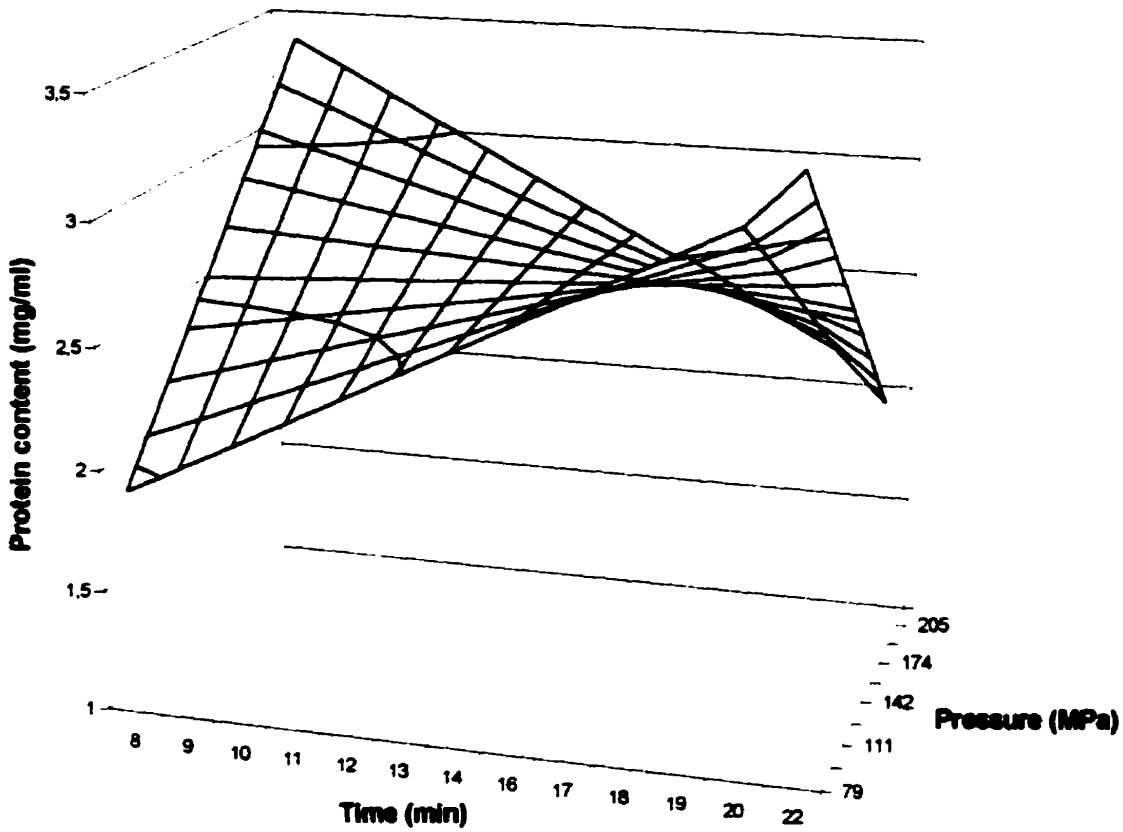

Fig. 4.1: Effect of pressure and time of treatment on extractability of actomyosin from carp muscle.
Control sample: 3.27 ± 0.1 mg/mL.

conditions, protein conformation may have been affected which resulted in an increase in protein solubility. It has been reported that pressure (150 MPa) induced solubilization of meat myofibrillar protein (MacFarlane, 1974) . Although the exact mechanism of pressure-induced protein solubilization is still poorly understood, it is speculated that solubilization results from the depolymerization of actin and myosin. The extent of solubilization depends on pH, temperature and salt concentration (MacFarlane and Mckenzie, 1976). In treatments lasting for more than 5 min, solubilization was higher at low temperature (0°C versus 30°C) (MacFarlane and Mckenzie, 1976).

Biochemical parameters such as changes (decreases) in the enzymatic activity of fish muscle or in the susceptibility of fish proteins to various proteolytic enzymes, are sensitive indicators for monitoring protein denaturation as a consequence of conformational changes that may occur during frozen storage (Shenouda, 1980).

Figure 4.2 shows the effect of time and pressure on the Ca^{2+}-ATPase activity of carp muscle actomyosin. Under pressure treatment, enzymes may undergo (i) activation, (ii) inactivation or (iii) no effect at all (Penniston, 1971; Kurt, 1986). It can be seen that STHP and LTLP induced losses of Ca^{2+}-ATPase activity in carp actomyosin (up to 15% from its original value). As previously mentioned, under these (STHP and LTLP) treatments, nuclei ice formation is not likely to happen. Instead nuclei ice may have started forming once the samples were placed in the chamber at –20°C resulting in big crystals that in combination with the prior applied pressure possibly induced

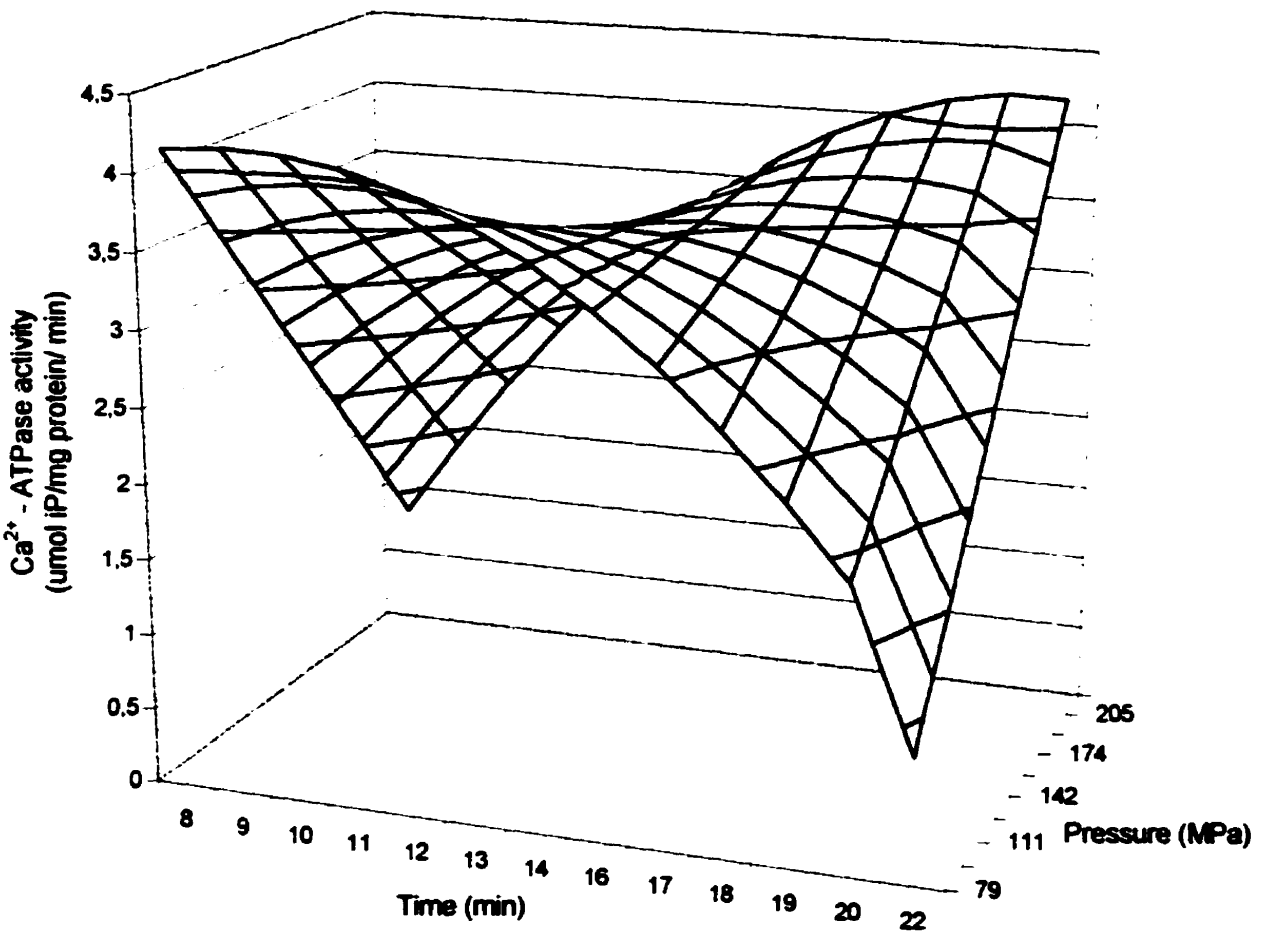

Fig. 4.2: Effect of pressure and time treatment on Ca^{2+}ATPase activity of actomyosin from carp muscle.
Control sample: 3.5 ± 0.3 μmol iP/mg protein/min.

conformational changes to the three-dimensional structure of the enzyme resulting in a loss of enzymatic activity (Penniston, 1971; Shenouda, 1980). On the other hand, STLP and LTHP treatments resulted in little or slight increases in enzymatic activity. Although STLP treatment did not induce nuclei ice formation, enzymatic activity remained higher compared to the STHP and/or LTLP treatments. Yoshikawa et al., (1995) detected Ca^{2+} and EDTA-ATPase activity in insoluble carp myofibrillar proteins and suggested that although protein molecules can get closer together and aggregate, causing a decrease in extractability, their active sites may not be affected and they may retain their enzymatic activity. Therefore, even if STLP and LTHP decreased protein extractability, enzymatic activity remained similar to the control sample. It appears that the absence of nuclei ice formation together with extreme conditions of pressure and time (STHP and LTLP) induced losses in enzymatic activity. In the case of LTHP treatment, formation of nuclei ice during high pressure treatment may have had a protective effect on enzymatic activity of fish actomyosin.

It is generally accepted that hydrophobicity plays an important role in the function of proteins. Interest in hydrophobicity of proteins has long been focused on its contribution to the stabilisation of molecular structure for native proteins. Thus protein hydrophobicity has been used to monitor protein denaturation (LeBlanc et al., 1991). To achieve the minimum free energy in the folding of proteins, non-polar or hydrophobic groups should be restricted to the interior of the folded molecules. Figure 4.3 shows the effect of time and

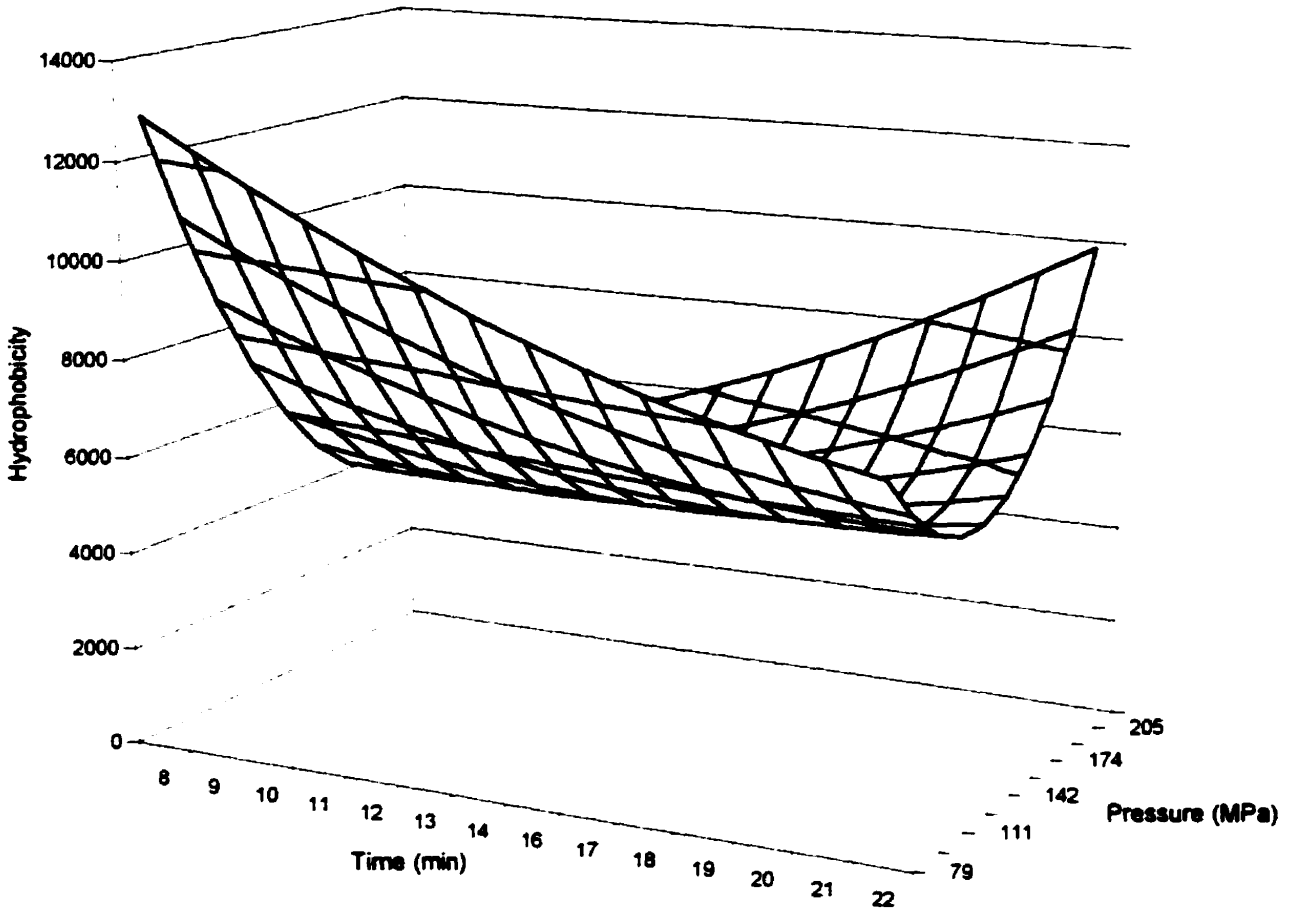

Fig. 4.3: Effect of pressure and time treatment on hydrophobicity of actomyosin from carp muscle. Control value 6400 ± 925

pressure on hydrophobicity of actomyosin from carp muscle. STHP and LTLP treatments resulted in little change in protein hydrophobicity of actomyosin compared to the control sample ($p>0.05$). STLP and LTHP treatment increased protein hydrophobicity. These results are similar to those obtained for extractability of actomyosin (Fig. 4.1). It seems that extreme conditions of pressure and time (LTHP) induced unfolding of the polypeptide chains exposing hydrophobic groups. In the case of STLP as it was mention earlier ice formation did not take place. Conventional freezing resulted in large size ice crystals leading to an increase in protein hydrophobicity and denaturation.

Liquid losses from carp muscle samples varied from 17.5% to 22.5% as illustrated in Figure 4.4. Results showed that only time has a significantly ($p<0.05$) effect on the amount of liquid losses. However, normal variation of moisture content of fish fillets (3.3%) may play an important factor in the results obtained.

It has been reported that high pressure treatment may induce lipid oxidation in fish muscle (Tanaka et al., 1991). Oxidation of sardine lipids was accelerated by the pressure treatment and its extent was dependant on the intensity and duration of the treatment. However in this study, it was observed that lipid oxidation decreased with compression time (Fig. 4.5). The levels of pressure used in the sardine experiments ranged between 50 to 180 MPa and pressurization time ranged between 30 to 60 min compared to the relatively shorter time (22 min) used in this study. One important factor is the temperature at which the experiment was performed. In general the rate of oxidation increases as the temperature is increased (Khayat and Schwall, 1983; Hultin,

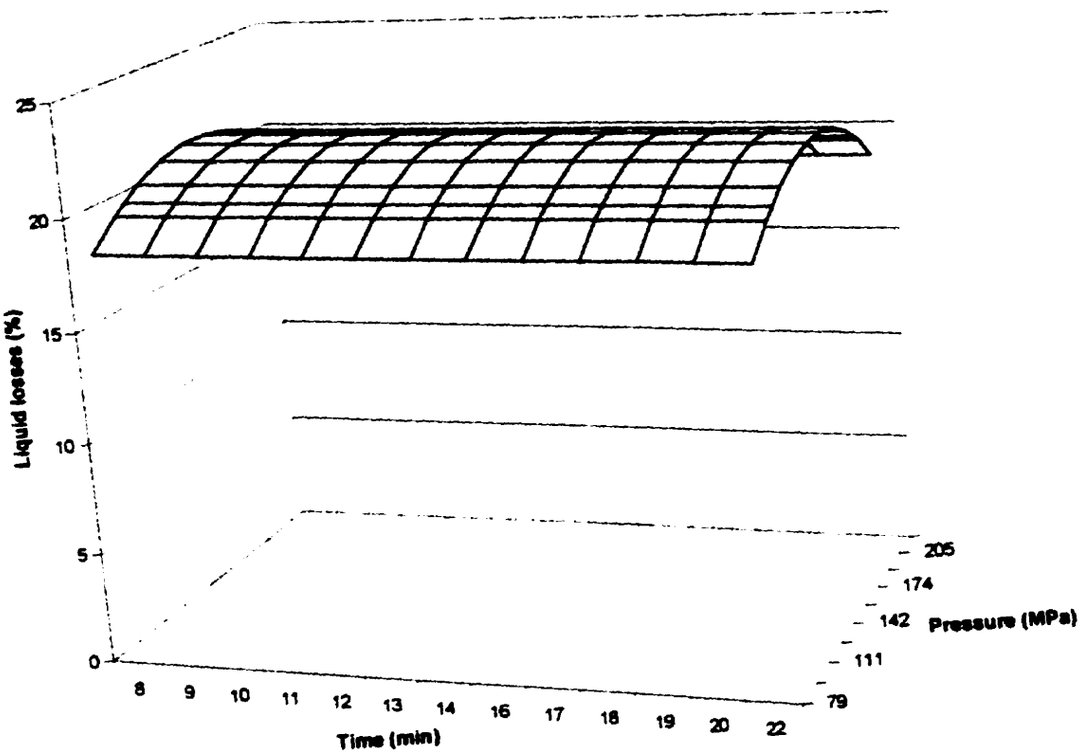

Fig. 4.4: Effect of pressure and time treatment on liquid losses of carp muscle. Control value 21.1 ± 1.9%.

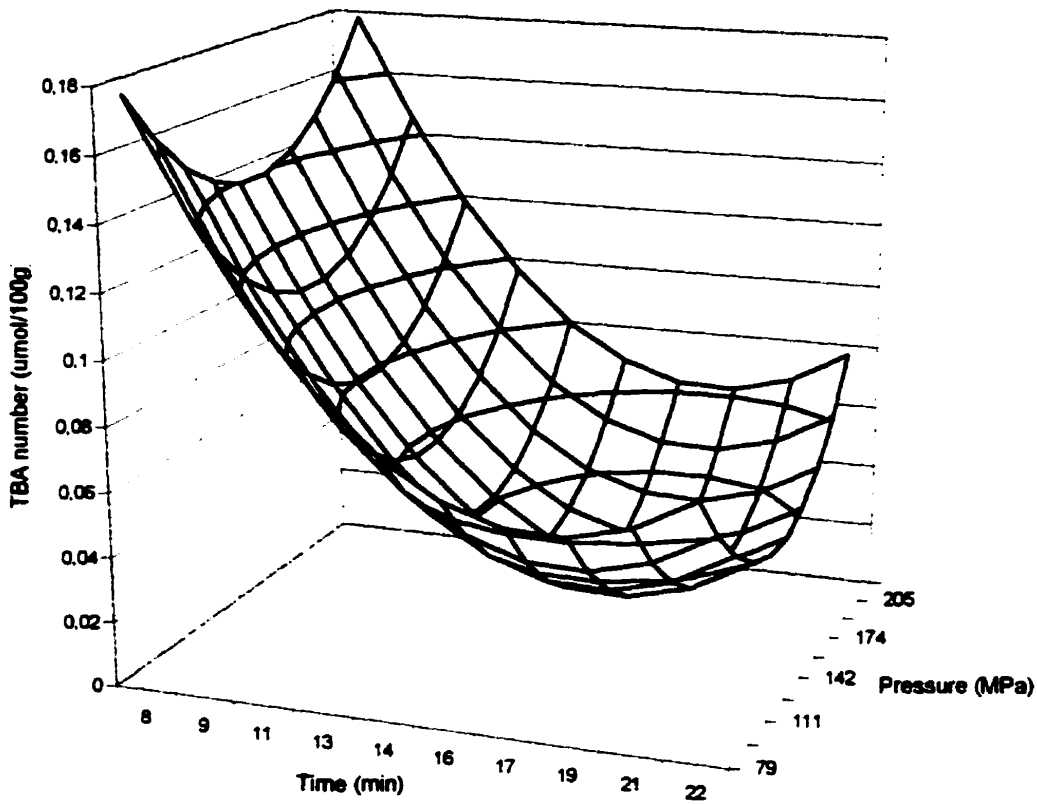

Fig. 4.5: Effect of pressure and time treatment on TBA number of carp muscle. Control value 0.15 ± 0.1 μmol/100 g.

1994). The sardine experiment were carried out at room temperature, while in the present study the temperature range between –10°C to 0°C.

Several enzymatic and nonenzymatic processes, such as autooxidation, photosensitized oxidation, lipoxygenase, peroxidase, and microsomal enzymes, have been suggested as initiators of lipid oxididation in fish tissues (Frankel, 1985; Slabyl and Hultin, 1984; German and Kinsella, 1986; Josephson et al., 1987 and Kanner et al., 1987). Hsieh and Kinsella (1989) indicated that lipoxygenase present in fish tissue can initiate the oxidation of polyunsaturated fatty acids to produce acyl hydroperoxides. As the time of treatment increases, lipoxygenase and other related enzymes may be inactivated which results in a reduction of lipid oxidation. Melton (1983) reported that oxidized flavours were detectable at TBA values 0.3 of 1.0 in beef or pork, 1.0 or 2.0 in chicken, and higher than 3.0 in turkey. In the case of mackerel Ke et al., (1975) established TBA value limit 6.0 for flesh and 12.0 for skin. The values obtained in this experiments are far below these limits, suggesting that they may not represent a quality problem.

Figure 4.6 shows the effect of high pressure-freezing on the firmness and elasticity of fish tissue. As pressureincreased, the tissue firmness gradually decreased. Similar results were reported by Ashie and Simpson (1996), who suggested that pressurisation of fish tissue at 100 MPa enhanced protein-protein interactions resulting in increased firmness. It was further suggested by these authors that beyond 100 MPa, pressurisation caused disruption of such protein-

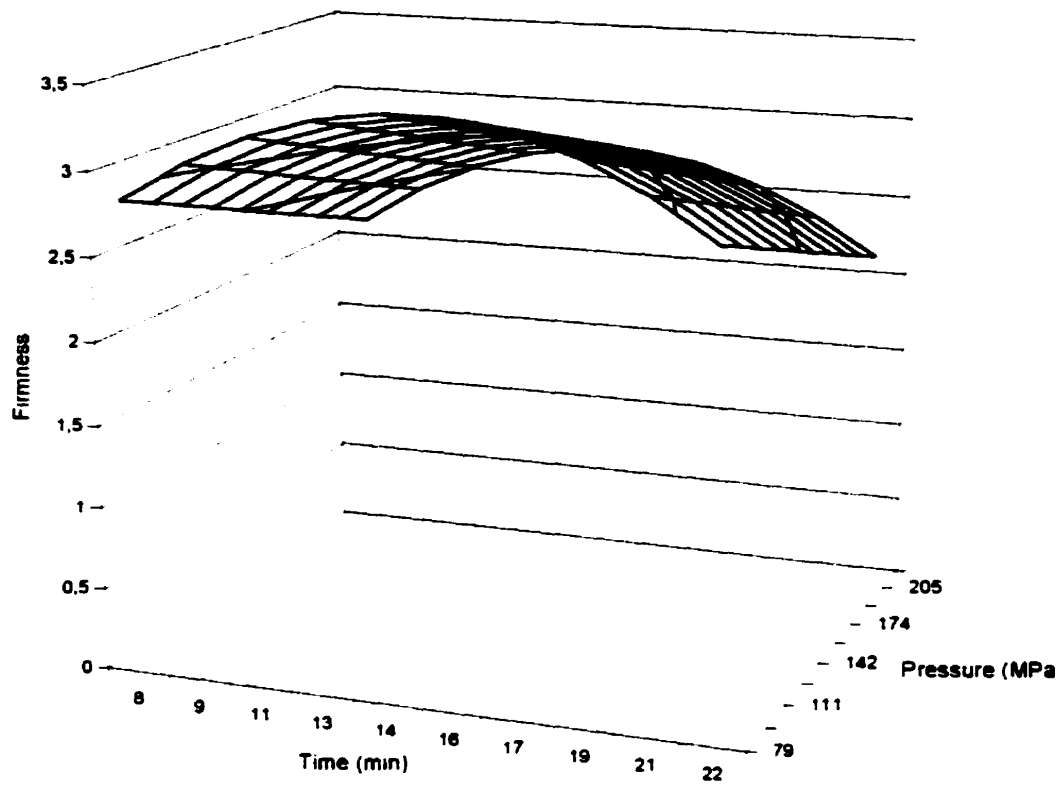

Fig. 4.6: Effect of pressure and time treatment on firmness of carp muscle. Control value 2.6 ± 0.2 N/g.

protein interactions and ruptured the muscle tissue resulting in a reduction in firmness

CONCLUSIONS

Application of response surface methodology to analyse combinations of treatments involving hydrostatic pressure and time could be a very useful tool in studying these parameters, on muscles from different fish species. In this specific case, these results are very useful in defining process conditions for pressure-shift freezing.

Balancing the benefits of low temperature pressurisation with the denaturing effects of pressure on fish proteins, it is evident that there is a region in which the responses of the factors (protein extractability, Ca^{2+} ATPase activity and protein hydrophobicity) to the processing variables (time and pressure) seemed to be adequate in keeping protein denaturation to a minimum. This region lies between (140-175 MPa and 16-18 min) and it is in this region that we suggest HP might be used during pressure shift freezing of carp fillets to induce nuclei ice formation. This information is very valuable because it may help reduce protein denaturation during pressure-shift freezing. Further studies are needed in order to determine the amount of nuclei ice formed during pressure-shift freezing as well as the size and location of the ice crystals.

CHAPTER V

EFFECT OF LOW TEMPERATURE PRESSURIZATION TREATMENT ON FISH FILLETS (*Cyprinus carpio*): CHANGES IN LIPID FRACTION AND COLOR

Connecting Statement

Results presented in chapter 4 indicated that certain processing conditions (140-175 MPa, 16-18 min) seem to result in lower protein denaturation, and thus could be used in the process of pressure-shift freezing. However, it was observed that high pressure treatment may induce changes in color of fish fillets. The information presented in this chapter addresses the effect(s) of low temperature pressurization treatment on the lipid fraction and color of carp fillets. This information provides the basis for the subsequent application of high pressure technology during pressure-shift freezing.

Contribution of co-authors: Benjamin K. Simpson (thesis/research supervisor)

ABSTRACT

Intact carp fillets (*Cyprinus carpio*) were packaged under vacuum and pressurized at (100, 140, 180 and 200 MPa) for 15 and 20 min at 4°C. Changes in the lipid fraction, color and electrophoretic profiles were studied. TBA values increased as pressure and time of treatment increased. Similar results were observed for the amount of FFA released during pressure treatment. The L*, a* and b* values increased as pressure and time of treatment increased. These results provide evidence of the importance in establishing treatment conditions when processing seafood products by reducing changes in appearance and flavor characteristics for these products.

INTRODUCTION

Although the first attempts to apply high pressure (HP) technology to food processing dates back to the late 19th century, the great potential of HP technology for the food industry was first recognized in the 1980s, and HP technology has since become one of the most popular subjects of study in food engineering and technology. The engineering aspects of HP applications in the context of food processing, safety and quality, and the effect of high pressure on ice-water transitions have been recently discussed (Knorr, 1993; Balny and Masson, 1993; Messens et al., 1997; Kalichevsky et al., 1995; Sanz et al., 1997; Thakur and Nelson, 1998). c

Pressure can be effective for low-temperature pasteurization, meat tenderization or inactivation of protelytic enzymes because it alters the intramolecular interactions that stabilize the folded conformation of native proteins, probably by breaking or forming hydrophobic interactions, hydrogen bonds, electrostatic interactions and disulfides linkages (Balny and Masson, 1993). In the particular case of ice-water transition, HP processing offers some potential application such as pressure-shift freezing, pressure-assisted thawing, non-frozen storage under pressure at subzero temperature and formation of different ice polymorphs (Kalichevsky et al., 1995)

Advantages of HP technology include minimal effects on flavor, color and nutritional values of the final products. Some work done on meat and fish has shown that HP may be a useful processing aid for such products (Ohshima et al., 1992). Recently, Ashie and Simpson (1996) applied HP to control enzyme related texture deterioration in seafood. Zhao et al.,(1998) studied the use of HP on thawing of beef. Angsupanich et al., (1999) studied HP effect on myofibrillar proteins, and Ashie et al., (1999) studied the prevention of HP denaturation of muscle proteins by sugars and polyols. However, it is still felt that our understanding of the effects of pressure on muscle tissue characteristics is limited. It is well known that the texture and appearance of fish changes remarkably during heat treatment (Iso et al., 1994). These changes have also been observed in fish meat that was subjected to HP treatment. The response of food products to HP processing is complex, affected by processing parameters, and product characteristics, such as applied pressure, duration of the compression, temperature, product pH and water activity. The purpose of this

work was to study the effect of high pressure (up to 200 MPa), low temperature (4°C) and pressurization time on color of carp fillets, as well as the pressure effect on the lipid fraction of fish flesh. This information would be very useful in defining process conditions for HP treatment of seafoods.

MATERIALS AND METHODS

Fish sampling

Live carp (*Cyprinus carpio*) (weight, ca. 1226 ± 221g and length 40 ± 7 cm), were obtained from an aquaculture farm (Ferme acquacole d'Anjou, Morannes, France), and immediately transported to the pilot plant (Nantes, France). The fish were held in a water tank (10°C) overnight and then slaughtered, cleaned and filleted the next morning. The fresh fish fillets were placed in moisture-impermeable polyethylene bags (La Bovida, France) and vacuum-packaged. Samples were processed between 3-5 h after slaughter.

Pressure treatment

Pressure treatment was carried out in 3L capacity high pressure vessel (ALSTOM, Nantes, France). The stainless steel vessel (12 cm internal diameter and 30 cm internal height) and the pressure transmitting medium (50/50 v/v ethanol/water solution) were maintained at 0°C by circulation of cold ethanol/water solution from an external cryostat through the internal cooling circuit of the high pressure vessel. Pressure was supplied by a high pressure

pump. A K-type thermocouple was installed at the center of the samples. Maximum temperature of the sample during pressure treatment was 4°C. Samples were placed in the high pressure vessel and the pressure was applied at various levels (100, 140, 180, 200 MPa) at a rate of 100 MPa/min. Time treatment refers to the time that the product was subjected to a given pressure (it does not include come up time and release time). After the pressure treatment, pressure was released at a rate of 10 MPa/sec. Directly after pressure treatment, the samples were analyzed for TBA values, free fatty acid content, color and changes in electrophoretic profiles. Each experiment and analysis were performed in duplicate on two different batches of fish, after a one-week interval.

Thiobarbituric acid (TBA) values

TBA number was determined according to the procedure of Vyncke (1970). Fifteen g of homogenized fish muscle tissue were blended with 30 ml of extracting solution (7.5% tricloroacetic acid (TCA) in water, 0.1% propyl gallate and 0.1% EDTA) at 20°C. The homogenate was centrifuged at 1000 g for 15 min at 4°C in a GR 2022 refrigerated centrifuge (Jouan, Saint Herblain, France) and the supernatant was used for the TBA test. Five ml 0.02 M TBA reagent were mixed with 5 ml of the supernatant. The tubes were then capped and heated in boiling water for 1 h. After heating, they were cooled under running tap water to ambient temperature and the absorbance was measured at 530 nm (20°C) with a 1:1 mixture of TBA reagent and water as blank. The TBA value was calculated from a standard curve obtained by reacting known amounts of 1,1,3,3 tetraethoxipropane

with TBA. Triplicate analyses were performed on all samples and the TBA number was expressed in μg of malonaldehyde/g fish tissue.

Free fatty acids

The method described by Kirk and Sawyer (1991) was used to determine free fatty acid content. Diethylether (25 ml), ethanol (70% v/v) (25 ml) and 1% phenolphthalein solution (1ml) were mixed together and then neutralized with 0.1 M NaOH solution. Fish samples (2g) were stirred, and dissolved in the mixed neutral solvent for about 20 min, titrated with 0.1 M NaOH, and constantly shaken until a pink color was formed that persisted for about 15 s. Triplicate analyses were performed on all samples. The fatty acid value was expressed as oleic acid equivalent (Kirk and Sawyer, 1991).

Color measurement

The color of fresh fish fillets and thawed fish fillets were measured by reflectance using a spectrocolorimeter (Vontron instruments, Montigny le Bretonneux, France). Tristimulus L*, a* and b* values were obtained using a white color standard. The measuring head of the spectrocolorimeter had a 20 mm diameter measuring area. Three different fillets were analyzed for each treatment and each fillet was measured in 5 different locations. Averages and standard deviations of the L*, a* and b* values were calculated.

SDS-polyacrylamide gel electrophoresis

Sample (3g) were homogenized with 5% (w/v) SDS and made up to a final volume of 30 ml. The homogenate was incubated at 85°C for 1 hr to dissolve total proteins. The supernatant was collected after centrifuging at 3,500 g for 5 min at 25°C. Protein extracts were subjected to sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Electrophoresis was carried out in 12.5% polyacrylamide gels, and approximatly 30 μg of protein was applied to each slot of the gel. High molecular weight markers (Sigma Chemical Co., USA) were used as standards and each electrophoresis run was carried out in duplicate.

Statistical Analysis

The effect of pressure treatment on parameters such as TBA number, release of free fatty acids and color was analysed by two-way analysis of variance according to the method of Sokal and Rolhf (1995) using Statgraphics plus version 2.1 software (Statistical Graphics Corp., Princeton, NJ, USA). In all cases, the significance level was 5% (α=0.05).

RESULTS AND DISCUSSION

Changes in Lipid fraction

Hydroperoxides are the initial products of oxidation and their measurement is a useful indicator of the stage of oxidation (Shewfelt, 1981; Khayat and Schwall, 1983). Fig. 5.1 shows the effect of HP treatment on TBA values for intact carp fillets. TBA values for HP treated samples increased with pressure. On the other hand, TBA values increased as pressurization time increased for the same pressure level. When cod muscle was exposed to 202, 404 and 608 MPa for 15 to 30 min, the peroxide value of the extracted oils increased with increasing pressure and processing time (Ohshima et al., 1992). Cheah and Ledward (1996) indicated that pressure treatment had little effect on lipid oxidation of minced pork below 300 MPa, but increased proportionally at higher pressures. Our results suggest that fish lipids are more susceptible to pressure treatment than mammalian lipids. A higher percentage of unsaturated fish lipids may be responsible for this behaviour. It has been suggested that water-soluble components such as low molecular weight iron compounds and myoglobin, hemoglobin and ferritin present in meat and fish play a key role during lipid oxidation following high pressure treatment (Hazell, 1982). Pressure treatment may induce the exposure of catalytic heme groups to unsaturated fatty acids or also could induce the release of iron from heme groups to unsaturated fatty acids present in fish (Greene and Price, 1975; Igene et al., 1979). These data show the need to determine the best conditions of HP treatment in order to avoid or reduce the production of oxidation products that may represent a

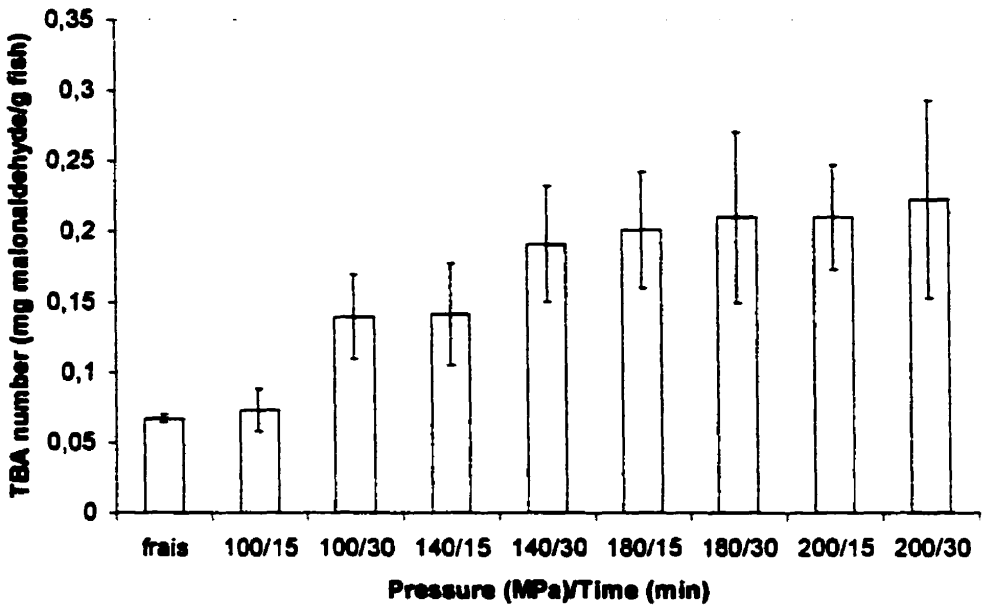

Fig. 5.1: Effect of high pressure treatment on TBA values for intact carp fillets. Each bar represents the mean of 3 values.

problem in products intended for long term storage, and after they have been subjected to HP treatment.

Besides oxidation, lipids may undergo hydrolysis resulting in the accumulation of free fatty acids (FFA). Figure 5.2 shows the effect of HP treatment at low temperature on the amount of free fatty acids present in fish tissue. It is evident that the amount of fatty acids released from HP treated samples increased as pressure increased. Similarly, for a given pressure level, the amount of FFA released increased with processing time. Myofibrillar proteins are considered to be the prime target of FFA and they become largely unextractable in their presence (Shenouda, 1980). It is generally believed that the interactions between proteins and FFA occur primarily through electrostatic, van der Waals, hydrogen and hydrophobic forces. These interactions may create more hydrophobic regions in place of polar or charged groups resulting in a decrease in protein solubility in aqueous buffer, or further intermolecular linkages extensive enough to decrease extractability (Shenouda, 1980).

Color changes

Changes in the color of carp muscle fillets are shown in the Table 5.1. Fresh raw carp muscle appears red and is very similar to beef muscle. The L* value which is an index of visual lightness remains essentially unchanged at 100 MPa regardless of processing time. However at 140 MPa and above, L* values increased with pressure. Similarly for a given pressure level, the L* value

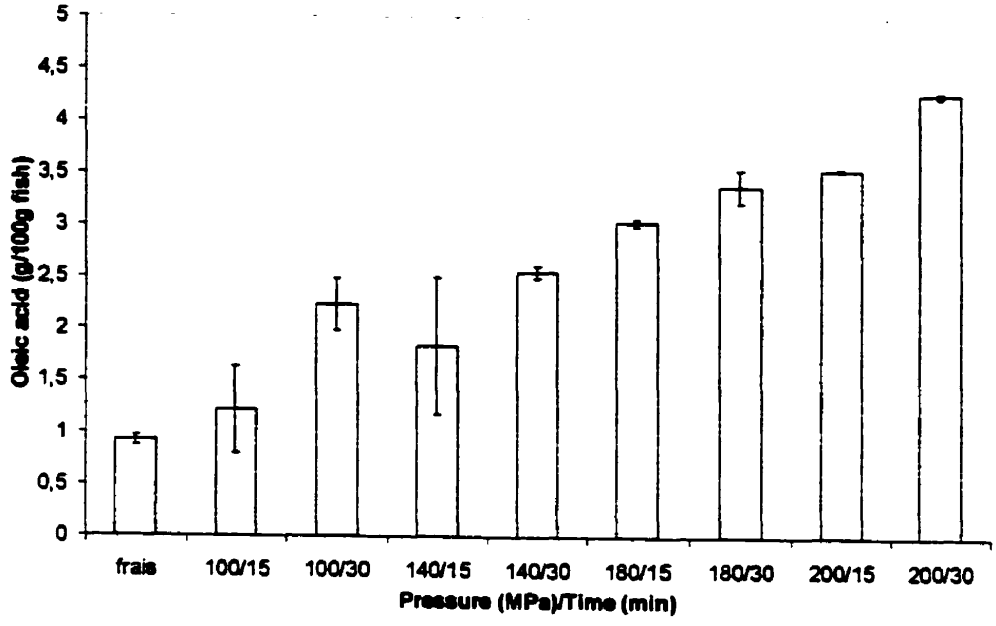

Fig. 5.2: Effect of high pressure treatment on FFA content for intact carp fillets. Each bar represents the mean of 3 values.

Table 5.1: Effect of pressure on color for intact fish fillets

Treatment	*Color parameters*		
	a*	b*	L*
Fresh	1.74 ± 0.14^{a}	-0.13 ± 0.01^{a}	41.7 ± 1.1^{a}
HP 100/15	3.41 ± 0.86^{b}	7.61 ± 0.07^{b}	48.1 ± 2.4^{b}
HP 100/30	9.35 ± 3.76^{b}	3.17 ± 1.51^{c}	40.5 ± 3.5^{ac}
HP 140/15	4.36 ± 2.87^{b}	4.16 ± 0.54^{c}	45.4 ± 2.6^{c}
HP 140/30	7.26 ± 3.39^{b}	4.95 ± 0.66^{c}	45.5 ± 2.9^{c}
HP 180/15	9.29 ± 5.77^{c}	6.61 ± 3.90^{bc}	47.3 ± 1.0^{c}
HP 180/30	13.18 ± 3.27^{d}	12.94 ± 0.78^{d}	54.2 ± 0.3^{c}
HP 200/15	9.38 ± 0.73^{c}	12.23 ± 0.76^{d}	58.3 ± 2.9^{c}
HP 200/30	8.34 ± 3.88^{c}	11.21 ± 3.58^{d}	61.7 ± 3.3^{b}

Means with the same letter in columns are not different ($p>0.05$)

increased as processing time increased. Similar results were reported by Yoshioka et al., (1996) during high pressure thawing of carp muscle and also by Wada (1992) for high pressure treated minced sardine meat. The a* values, normally used as an index of visual redness, increased as pressure increased. It has been reported for mackerel and cod, that the a* value decreased after pressurization (Ohshima et al., 1992). Similarly, Angsupanich et al., (1999) reported that redness of raw cod was lost after treatment at ≥ 200 MPa. Jimenez-Colmenero et al., (1997) observed that when meat emulsions were pressurized at 100 and 300 MPa for 5 min, there was a reduction in the a* value, however, when pressurization time was prolonged for 20 min, the opposite effect took place. They suggested that pressure-induced coagulation of sarcoplasmic and myofibrillar proteins was possibly responsible for this behavior. Finally, the b* values increased as pressure increased, similar to observations made by Yoshioka et al., (1996) during high pressure thawing of carp muscle.

Changes in electrophoretic profile

Figures 5.3 and 5.4 show the electrophorectic profile of fish proteins treated at different of pressures for 15 and 30 min respectively. After 15 min of treatment at any pressure level (100 to 200 MPa), the intensity of bands for the treated samples with MW< 36 kDa decreased. Some of these bands may be sarcoplasmic proteins. It has been suggested that rather than being degraded by high pressure, certain sarcoplasmic proteins become more covalently linked together and are thus resistant to extraction with SDS

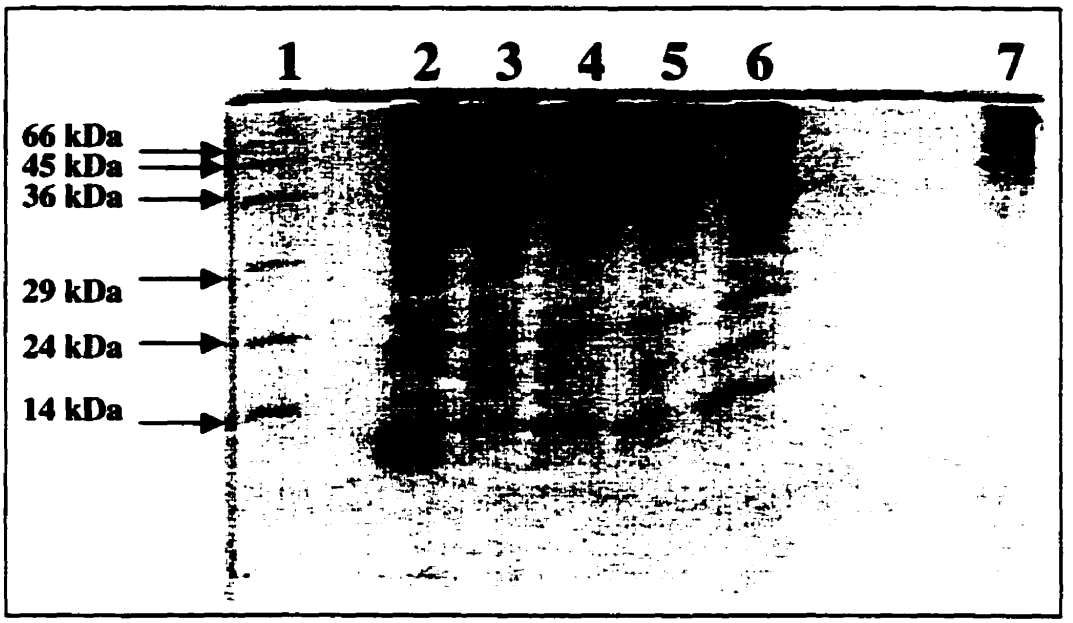

Fig. 5.3: Effect of high pressure on electrophoretic profile of myofibrillar proteins (15 minutes treatment) (1) Low molecular weight markers, (2) fresh sample, (3) 100 MPa, (4) 140 MPa, (5) 180 MPa, (6) 200 MPa, (7) High molecular weight markers.

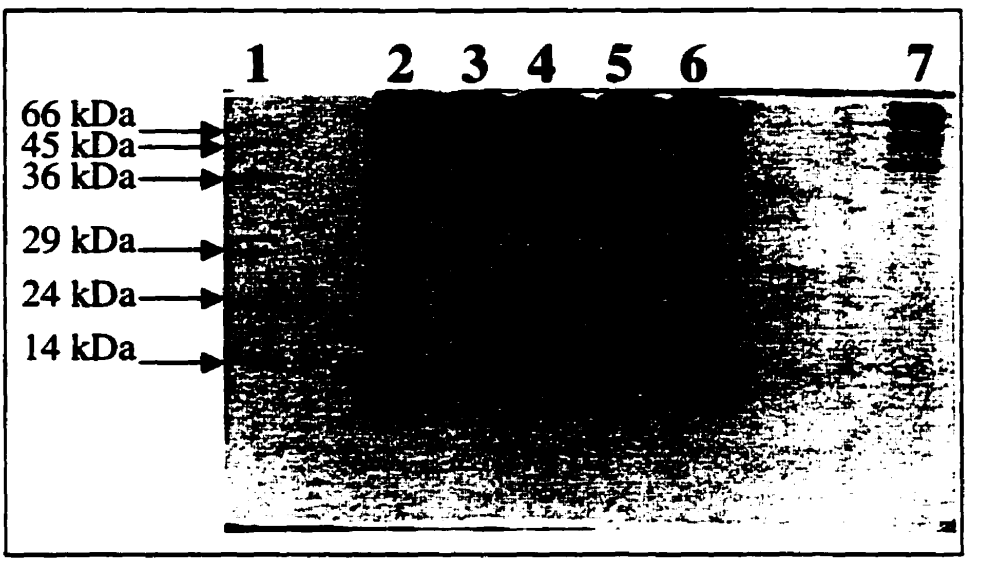

Fig. 5.4: Effect of high pressure on electrophoretic profile of myofibrillar proteins (30 minutes treatment) (1) Low molecular weight markers, (2) fresh sample, (3) 100 MPa, (4) 140 MPa, (5) 180 MPa, (6) 200 MPa, (7) High molecular weight markers.

(Ohshima et al., 1993). In general, after 30 min of treatment at pressures <180 MPa, the intensity of the bands slightly decreased. Pressures <180 MPa may reduce extractability of muscle proteins, however at 200 MPa, the effect seems to be the opposite and the electroporetic profile is very similar to that of the fresh sample with some more intense bands with MW < 24 kDa. These bands could result from depolymerization of myofibrillar proteins, which may lead to an increase in solubilization of myofibrillar proteins. A large increase in solubilization of myofibrillar proteins due to depolymerization was observed between 150-300 MPa (Cheftel and Culioli, 1997).

CONCLUSIONS

These results indicate that two factors changes in color and in the lipid fraction may limit the used of high pressure treatment on carp muscle and fish muscle in general. Changes in color are very important to the consumer acceptability of these products. In some cases, pressure treatment may yield a product with a cooked appearance. On the other hand, changes in the lipid fraction, induced by pressure, may result in odors and flavors related to rancid seafood. The interaction of oxidation products with fish proteins has also been mentioned as a factor for proteins denaturation. These results will be useful in establishing the pressure and time parameters for fish products subjected to high pressure treatments.

CHAPTER VI

EFFECT OF PRESSURE-SHIFT FREEZING ON PHYSICO-CHEMICAL PROPERTIES OF CARP (*Cyprinus carpio*) FILLETS

Connecting Statement

Results presented in chapters 4 and 5 set the basis for establishing the best conditions to carry out pressure-shift freezing of carp fillets. Changes in biochemical properties of actomyosin such as Ca^{2+}-ATPase activity and hydrophobicity, changes in lipid fraction and color were evaluated. This current study was undertaken to evaluate the effect of pressure-shift freezing on physico-chemical properties of carp fillets during frozen storage.

Contribution of co-authors: Benjamin K. Simpson (thesis/research supervisor)

ABSTRACT

Carp (*Cyprinus carpio*) fillets were frozen using either pressure-shift freezing (PSF) or air-blast freezing (ABF) and then stored at -20°C for 75 days. Samples were analyzed for protein solubility, Ca^{2+}-ATPase activity, thiobarbituric acid value, free fatty acid content, texture, color and SDS-PAGE electrophoretic profiles. There was a decrease in extractability for myofibrillar and sarcoplamic proteins as well as actomyosin Ca^{2+}-ATPase activity during frozen storage regardless of the freezing procedure used. However, actomyosin Ca^{2+}-ATPase activity for pressure-shift frozen samples remained relatively higher than the air-blast frozen samples. Levels of thiobarbituric acid and free fatty acids were relatively lower in samples frozen by PSF. The freezing procedure did not seem to have a significant effect ($p>0.05$) on the texture of carp fillets.

INTRODUCTION

It is a world-wide consensus that the world marine resources are limited. For some time now the world marine harvest had declined although it now seems to be levelling off at around 100 million tons per year (FAO, 1995). At the same time, the demand/supply ratio is increasing, resulting in reduced availability of

traditional fish (so called economically important) species, rising prices, and a search for alternative fish species (non-traditional) to bridge the gap. Closing the demand/supply gap requires an increase in the amount of fish available for direct human consumption. The past decade has seen a tremendous growth in variety and quantity of fish raised by aquaculture. Fish farming has expanded greatly since 1984, growing at an average rate of 9% a year. According to Morrow (1992) over half of all freshwater fish production comes from aquaculture. Carp (*Cyprinus carpio*) is a very important fish species for the aquaculture industry. Carp is considered by some ethnic groups as a delicacy usually eaten during the holiday season. In general, however it is still an underutilized species. If exploited, carp may prove to be a significant addition as a source of fish and protein. Without having to increase carp production, much can be done to improve its availability by making better use of what is currently harvested. In that sense, reducing post harvest losses should be one of the first considerations and this can be achieved by the use of improved and more effective preservation techniques. Chilling and freezing are still the most commonly used methods of fish preservation, mainly because they retain the fresh character of fish. Furthermore, consumers associate fresh fish with higher nutritional value than processed fish.

Frozen storage is a long-term preservation method that allows storage of fish under more controlled conditions. Ideally, there should be no difference between fresh fish and frozen fish after thawing. If appropriate conditions are used, fish in the frozen state can be stored for several months without appreciable changes in quality (Santos-Yap, 1995). The reality however is that

freezing and frozen storage can adversely affect the quality of a variety of muscle foods like beef, pork, fish and chicken. This phenomenon of quality deterioration during frozen storage is more pronounced in fish muscle than other muscle foods, with deterioration in texture, flavour, and colour being the most serious problems.

Many of the changes in frozen fish muscle are attributed to denaturation of proteins. During the past 40 years, several excellent reviews have summarised the scientific knowledge concerning fish proteins in an effort to better understand the relationship between protein denaturation and quality deterioration problems in frozen fish (Dyer and Dingle, 1961; Connell, 1968; Matsumoto, 1979; Shenouda, 1980; Sikorski and Kolakowska, 1990).

During freezing of fish, the separation of water as pure ice crystals creates an environment that is conducive to protein denaturation and this phenomenon has been cited as a possible cause for deterioration in frozen fish. Love (1968) summarised the factors that influence the size and location of ice crystals formed during freezing of fish muscle tissue as follows: physiological status of the fish, the freezing rate, the storage time, and temperature fluctuations. It was found that freezing of pre-rigor muscle tissue results mainly in the formation of intracellular ice crystals, regardless of the speed of freezing. In the pre-rigor state, the diffusibility of liquid from the inside to the outside of the cell is limited, because the fluids are tightly bound to intracellular protein. As rigor mortis commences, cellular fluids and released to diffuse into the extracellular spaces. As a result, during freezing of rigor and post-rigor muscle both inter and intracelullar ice crystals are formed (Love, 1966; Shenouda, 1980). Garthwaite (1992) indicated

that freezing particularly at a slow rate, causes the formation of inter- and intracellular ice crystals which lead to breakage of the cells, rupturing of membranes, and a general disordering of the ultrastructure of cells. At the same time, decreasing the temperature causes the volume of a specific weight of water to increase, resulting in a continuous pressure from the ice crystals on cell structure. At slow freezing rates, most of the ice crystals formed are located outside the cell and the average size is bigger than those produced using a faster freezing rate (Shenouda, 1980). Some of the quality problems observed in frozen fish have been partly related to cellular disruption that occurs in fish muscle tissue as a result of large size ice crystal formation (Shenouda, 1980). Disruption of cellular ice crystals may induce interactions among enzymes, lipids and proteins that normally do not occur in intact fish muscle, and which may lead to protein denaturation and lipid degradation. As the size of the ice crystals depends on the freezing rate, it is not surprising that the fish industry has developed several freezing techniques to achieve "quick or fast freezing". Examples of "quick or fast freezing" procedures include: air-blast freezing, plate or contact freezing, spray, or immersion freezing.

There is a lot of interest in the use of high pressure in food processing and the fish industry is one sector taking advantage of this technology (Farr, 1990). In the specific case of fish freezing, high pressure may become a new alternative method. Kalichevsky et al., (1995) and Le Bail et al., (1997) summarised the effects of high pressure on the solid-liquid phase diagram of water and its potential food applications. As pressure increases to 210 MPa, the freezing point of water decreases to -22°C. Increasing the pressure beyond 210 MPa results in

an increase in the freezing point. The unfrozen region of water under high pressure presents a tremendous opportunity for food processing. The primary applications of pressure in relation to the water phase changes are the increased freezing rates obtained using pressure-assisted freezing, the increased thawing rates and also the possibility of non-frozen storage at subzero temperatures.

The use of high pressure during freezing of fish may offer one important advantage over traditional freezing techniques, such as air blast and plate freezing, i.e., the increase in freezing rate during the freezing process could result in a final product with better quality after thawing. The improvement of quality may be a direct result in the reduction of tissue or textural damage, and indirectly by the reduction of lipid deterioration on the final product. Fuchigami et al., (1997a, b) applied high pressure freezing to carrots and reported that pressures of 200, 340 and 400 MPa appeared to be effective in reducing histological damage and improving the texture of thawed carrots. Fuchigami and Teramoto (1997) observed that high pressure freezing between 200 MPa to 400 MPa yielded products with better texture compared to conventional freezing. Similar results were obtained for Chinese cabbage subjected to high pressure freezing (Fuchigami et al., 1998). The behavior of food products under pressure freezing is very complex, and this also is reflective of the variation in food composition. For practical purposes it is not very accurate to extrapolate processing conditions for vegetable material to animal food. Even among muscle tissues, there are differences between beef, pork and fish tissue that call for a better understanding of the effects of high pressure for a specific commodity. Thus, our objective was to compare the biochemical properties of thawed carp fillets subjected to

conventional air-blast freezing (ABF) versus pressure-shift freezing (PSF), and to correlate the observed biochemical properties to some objective measurements of texture.

MATERIALS AND METHODS

Fish sampling

Live carp (*Cyprinus carpio*) (weight, ca. 1226 ± 221g and length 40 ± 7 cm), were obtained from an aquaculture farm (Ferme acquacole d'Anjou, Morannes, France), and immediately transported to the pilot plant (Nantes, France). The fish were held in a water tank (10°C) overnight and then slaughtered, cleaned and filleted the next day. The fresh fish fillets were placed in moisture-impermeable polyethylene bags (La Bovida, France) and vacuum-packaged. Samples were stored in isothermal boxes in a cold room at 4°C and processed between 3-5 h after slaughter. For each pair of fillets obtained, one was subjected to pressure shift-freezing (PSF) and the other to air-blast freezing (ABF).

Freezing processes

Air-blast freezing (Servathin, France) was carried out at -20°C using an air speed of 4 m/s and the samples were designated as ABF. The freezing process was considered to be complete when the temperature at the center of the sample reached -20°C. The temperature at the surface and at the center of the samples

was monitored using a temperature recorder (Model SA 32 AOIP, Evry, France) with K-type thermocouples (1 mm diameter, Omega, Stamford, USA). The nominal freezing time was calculated following the recommendations of the International Institute of Refrigeration (Anon, 1986) as the time required to decrease the temperature in the thermal center by ten degrees below the initial freezing point. The nominal freezing time was about 44 min on average for ABF process. The freezing rate was obtained as the ratio of the distance from the surface to the thermal center of the fish fillet and the freezing period which elapsed from the moment when the surface temperature was 0°C until it reached -10°C at the thermal center. A mean freezing rate of 0.94 cm/h was obtained by ABF at –20°C and an air speed of 4 m/s (3 measurements). The characteristic freezing time was determined according to the definition of Bevilacqua et al., (1979) as the time required to decrease the temperature at the center of the sample from –1°C to –7°C. The characteristic freezing time for ABF process used in this study was 44.4 min.

Pressure shift freezing was carried out in a 3L capacity high pressure vessel (ALSTOM, Nantes, France). The stainless steel vessel (12 cm internal diameter and 30 cm internal height) and the pressure transmitting medium (50/50 v/v ethanol/water solution) were maintained at -14°C by from an external cryostat through the internal cooling circuit of the high pressure vessel. Pressure was supplied by a high pressure pump. A K-type thermocouple was installed at the center of samples and another one in the pressurization medium. A data logger (Model SA 32 AOIP, Evry, France) recorded temperature and pressure data with

an acquisition rate of one measurement per second. Samples were placed in the high pressure vessel and the pressure was increased to 140 MPa at a rate of 100 MPa/min. When the temperature of the samples reached -14°C (≈12 min), pressure was released at a rate of 10 MPa/sec. Samples were immersed in a stirred cooling bath of 50/50 (v/v) ethanol/water solution at -20°C for 7 min to complete freezing. Once frozen, the carp samples were stored in isothermal boxes in a cold room at -20°C for 2, 15, 30, 65 and 75 days. Thawed samples (unpressurized and pressurized samples) after storage at -20°C as well as fresh samples (0 days of storage) were analyzed. Thawing of frozen samples was achieved by placing the samples overnight in a cold room at 4°C. Each experiment and analysis were performed in duplicate on two different batches of fish after one-week interval.

Extraction of actomyosin (AM)

AM was extracted according to the method of MacDonald and Lanier (1994), i.e., 7.5 g of fish muscle were homogenized in 75 mL chilled 0.6 M KCl solution (pH 7.0) using a Waring blender (Waring, New Hartford, CT, USA) . Excessive heating during the extraction was controlled by placing the blender cup on ice and blending in steps for 20 s followed by a 20 s cooling interval for a total extraction time of 4 min. The extract was centrifuged at 5000 g for 30 min at 4°C. AM was precipitated by diluting the supernatant with 3 volumes of chilled distilled water and collected by centrifuging at 5000 g for 20 min at 4°C. The AM was then dissolved by gentle stirring with an equal volume of chilled 1.2 M KCl (pH

7.0) for 30 min at 4°C. The AM solution was centrifuged at 5000 g for 20 min at 4°C to remove insoluble material and protein concentration was determined by the Biuret method (Gornall et al., 1949).

Actomyosin ATPase activity

ATPase activity was determined using the method described by Roura and Crupkin (1995). The prepared actomyosin was diluted to 2.5-4 mg/mL with 0.6 M KCl (pH 7.0), and 1 mL of the diluted solution was added to 0.6 mL of 0.5 M tris-maleate buffer (pH 7.0). The actomyosin solution was brought to a final volume of 9.5 mL with 10 mM $CaCl_2$. The reaction was initiated by adding 0.5 mL 20 mM ATP to the AM solution prepared above, left to proceed for exactly 3 min at 37°C, and then terminated by adding 5 mL chilled 15% (w/v) trichloroacetic acid (TCA). The reaction mixture was centrifuged at 3500 g for 5 min (25°C) and the inorganic phosphorus liberated in the supernatant was measured using ascorbic acid (Chen et al., 1956). The specific activity of actomyosin was expressed as μmoles inorganic phosphorus liberated per milligram protein (μM Pi/mg protein/min) at 37°C.

Thiobarbituric acid (TBA) value

TBA value was determined according to the procedure of Vyncke (1970). Fifteen g of homogenized fish muscle tissue were blended with 30 mL of extracting solution (7.5% TCA in water, containing 0.1% propyl gallate and 0.1% EDTA) at 20°C. The homogenate was centrifuged at 1000 g for 15 min at 4°C in

a GR 2022 refrigerated centrifuge (Jouan, Saint Herblain, France) and the supernatant was used for the TBA test. Five mL 0.02 M TBA reagent were mixed with 5 mL of the supernatant. The tubes with the extract and TBA reagent were capped and then heated in boiling water for 1 h. After heating, they were cooled under running tap water to ambient temperature and the absorbance was measured at 530 nm (20°C) with a 1:1 mixture of the TBA reagent and water as blank. The TBA value was calculated from a standard curve obtained by reacting known amounts of 1,1,3,3 tetraethoxipropane with TBA. Triplicate analyses were performed on all samples and the TBA value was expressed in μg of malonaldehyde/g fish tissue.

Free fatty acids

The method of Kirk and Sawyer (1991) was used to determine the free fatty acid content. Diethylether (25 mL), ethanol (70% v/v) alcohol (25 mL) and 1% phenolphthalein solution (1 mL) were mixed together and then neutralized with 0.1 M NaOH solution. Fish samples (2 g) were stirred and dissolved in the mixed neutral solvent for about 20 min and titrated with 0.1 M NaOH while constantly shaking until a pink color was formed that persisted for about 15 s. Triplicate analyses were performed on all samples. The fatty acid value was expressed as oleic acid equivalent (Kirk and Sawyer, 1991).

Protein extraction

Protein extraction was carried out at 4°C. Minced fish flesh was prepared in duplicate by grinding 50 g of thawed fillet in a food mixer. The water-soluble sarcoplasmic protein content was evaluated by homogenization of 20 g minced samples (in duplicate) with 200 mL of phosphate buffer (0.05 M, pH 7.5) in a Waring blender at 7940 g for 3 intervals of 15 s at 20°C with 5 s cooling interval between each 15-sec run. The homogenates were centrifuged in a GR 2022 refrigerated centrifuge (Jouan, Saint Herblain, France) at 5000 g for 15 min at 4°C. The supernatant was removed and kept as the first part of the water-soluble sarcoplamic protein fraction. The pellet was washed with 200 mL phosphate buffer (0.05 M, pH 7.5) and recentrifuged as described above. The supernatant from the second centrifugation step was added to the first one and the pooled supernatants were designated as water-soluble sarcoplasmic protein fraction (SP). The residual pellet was homogenized with 10-volumes of phosphate buffer (0.05 M, 0.5 M KCl M, pH 7.5) in a Warring blender and centrifuged at 7940 g (20°C) as described above. After removing the supernatant, the pellet was subjected to a second extraction using the same conditions. The two last supernatants were also pooled and designated as myofibrillar protein fraction (MFP). The protein concentrations of SP and MFP fractions were determined using the Biuret method with bovine albumin as standard (Gornall et al., 1949)

Color measurement

The colors of fresh fish fillets and thawed fish fillets were measured by reflectance using a spectrocolorimeter (Vontron instruments, Montigny le Bretonneux, France)). Tristimulus L*, a* and b* values were obtained using a white color standard. The measuring head of the spectrocolorimeter had a 20 mm diameter measuring area. Three different fillets were analyzed for each treatment and each fillet was measured in 5 different locations. Averages and standard deviations of L*, a* and b* values were calculated.

Texture

The cooked samples were kept at 4°C for 12 h to allow them to cool down before measurement. Objective texture measurements were performed with a texture testing machine (Lloyd Instruments LR5K, UK). A Kramer Shear Compression Cell (65 mm x 65 mm x 65 mm) with a 10 blade probe was used. The fillets were cut into approximately 1 cm^3 pieces and 50 g samples were placed in the cell. The texture testing machine was operated with a cross head speed of 10 cm min^{-1}. The return gauge was set at 8.35 cm so that the compression cycle was terminated just after the blades emerged from the bottom of the test cell. Peak heights were registered, and final values are expressed as N/g fish sample.

SDS-polyacrylamide gel electrophoresis

Protein extracts (both sarcoplasmic and myofibrillar fractions) were subjected to sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Electrophoresis was carried out in 12.5% polyacrylamide gels. Approximatly 30 μg of protein was applied to each slot of the gel. High and low molecular weight markers (Sigma Chemical Co., USA) were used as standards and the electrophoresis was carried out in duplicate.

Statistical Analysis

The effect of frozen storage time on parameters such as extraction of actomyosin, actomyosin Ca^{2+}-ATPase activity, TBA number, release of free fatty acids, color and texture was analysed by two-way analysis of variance according to the method of Sokal and Rolhf (1995) using Statgraphics plus version 2.1 software (Statistical Graphics Corp., Princeton, NJ, USA). In all cases, the significant level used was 5% (α=0.05). To test significant differences between means for the freezing processes at the same time of storage, Student T-test was used, whenever the Fisher F-test was not significant ($p \geq 0.05$).

RESULTS AND DISCUSSION

Freezing processes

The time-temperature profiles for the air-blast freezing process of carp fillets are shown in Fig. 6.1 The time elapsed for the center of the fish fillets to

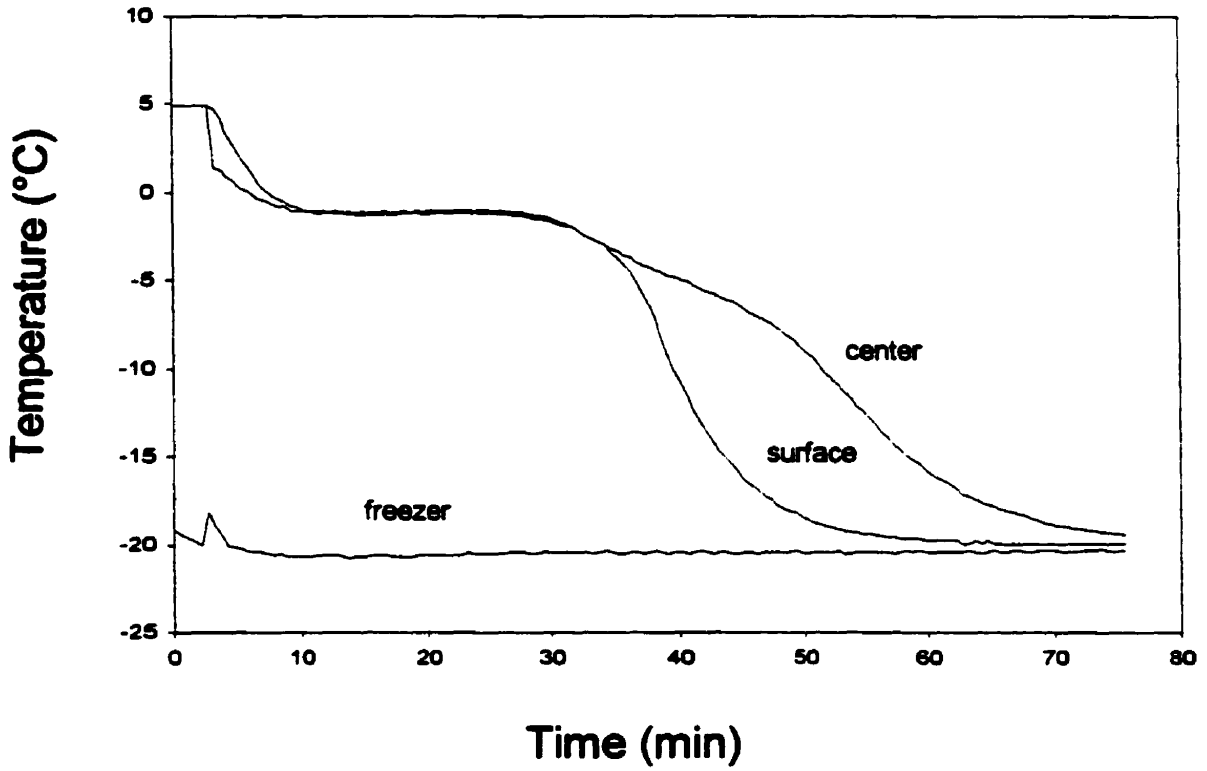

Fig.6.1: Time-temperature profile for carp fillets by air-blast (-20°C, 4m/s) freezing. Temperature was recorded at the centre of the fillet and in the cooling medium.

attain the freezing temperature of -18.3°C was 75.7 min. Carp fillets showed a freezing point of –1.2°C with a freezing plateau of 16.5 min. Pressure and temperature profiles during the pressure shift-freezing process are shown in Fig. 6.2. These indicate that when pressure was increased up to 130 MPa, the temperature of the pressurization medium also increased around 2.2°C. In the case of the fish fillets, no increase in temperature was observed when pressure was increased. However, the cooling process appeared to slow down during pressurization as a result of heat dissipation in the first step (A-B). In the second step of the process curve (B-C), the fish fillets were allowed to cool under pressure until they reached a temperature of -9°C. At this point pressure was suddenly released. During depresurisation, the temperature of the pressurization medium decreased from –13°C to –17°C, and the sample reached a temperature of –1.8°C compared to that of ABF –1.2°C. Finally, in the last step of the process, freezing was completed by using conventional freezing at atmospheric pressure. No freezing plateau was observed due to the ice nucleation induced by the pressure release. The total freezing time for pressure shift freezing was 25 min including the pressurisation step and the final freezing at atmospheric pressure.

Extractability of Fish muscle proteins

Figs. 6.3 and 6.4 show the effects of pressure-shift-freezing (PSF) on the extractability of sarcoplasmic (SP) and myofribrillar proteins (MFP), respectively. The extractability of SP from intact muscle tissue subjected to air-blast freezing (ABF) or (PSF) decreased after 2 days of storage ($p<0.05$).

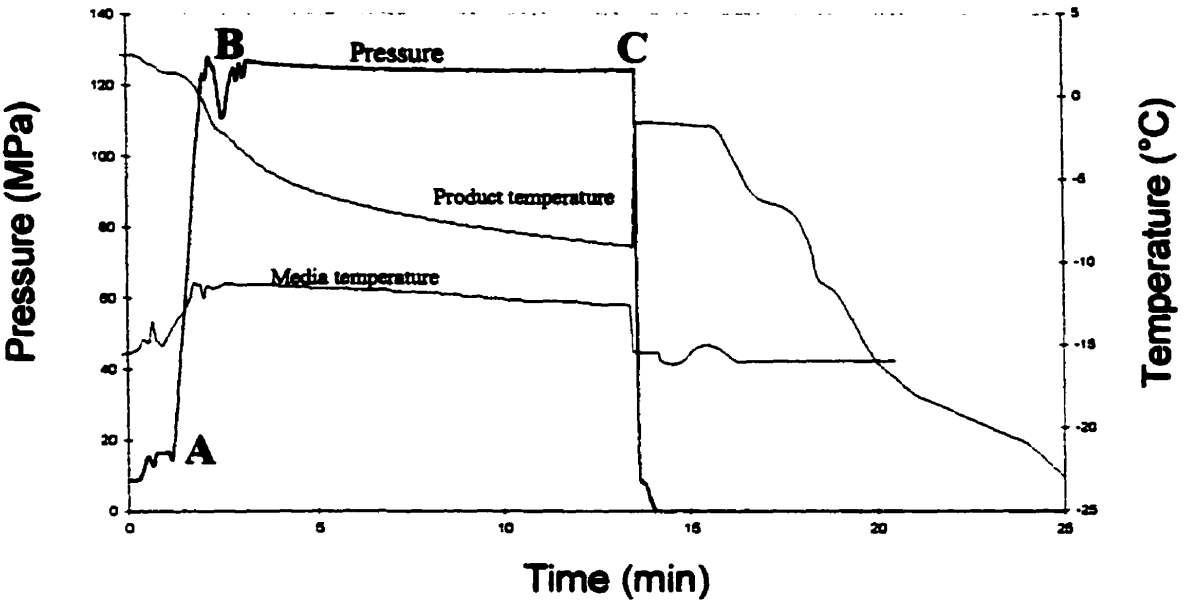

Fig. 6.2: Freezing kinetics of carp fillets frozen by pressure-shift freezing. Temperatures were recorded at the centre of the fillet and in the cooling medium. Pressure was measured with a pressure gauge. Through all the pressurisation process, the temperature of the cooling medium was controlled at -15°C.

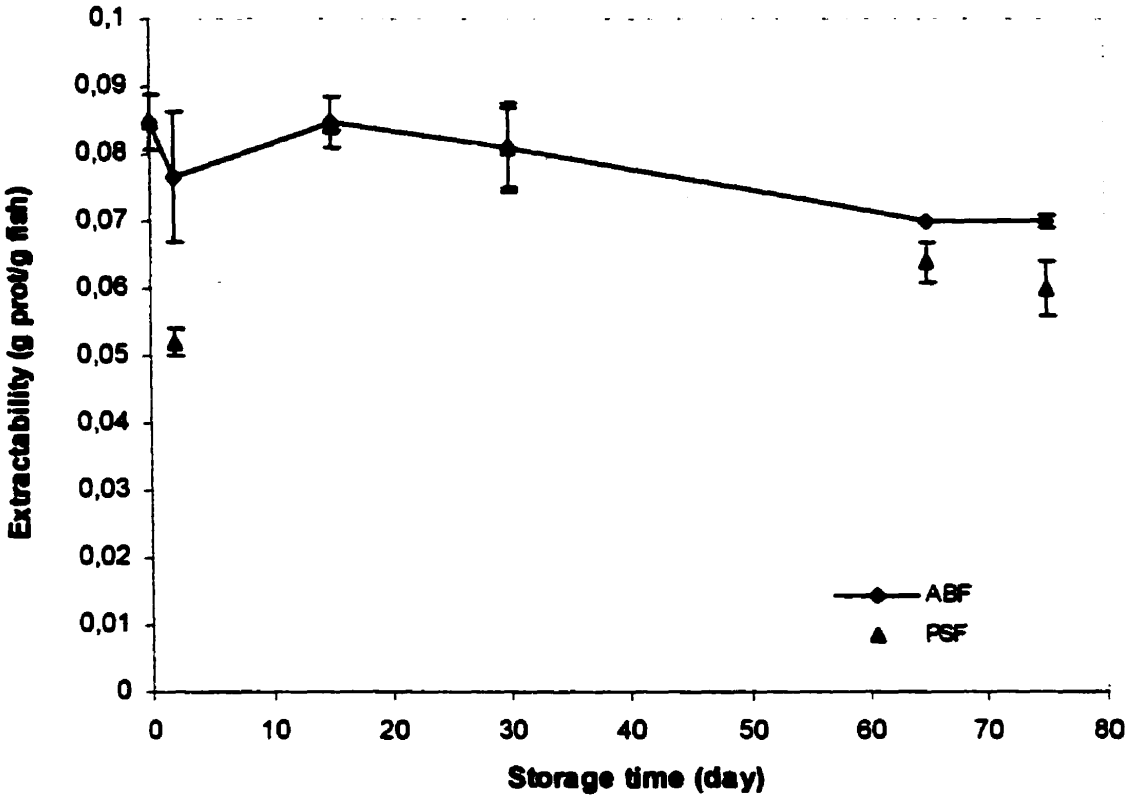

Fig 6.3: Effect of the freezing procedure on the extractability of sarcoplasmic protein from intact carp fillets during frozen storage. Each marker represents the mean of 3 values.

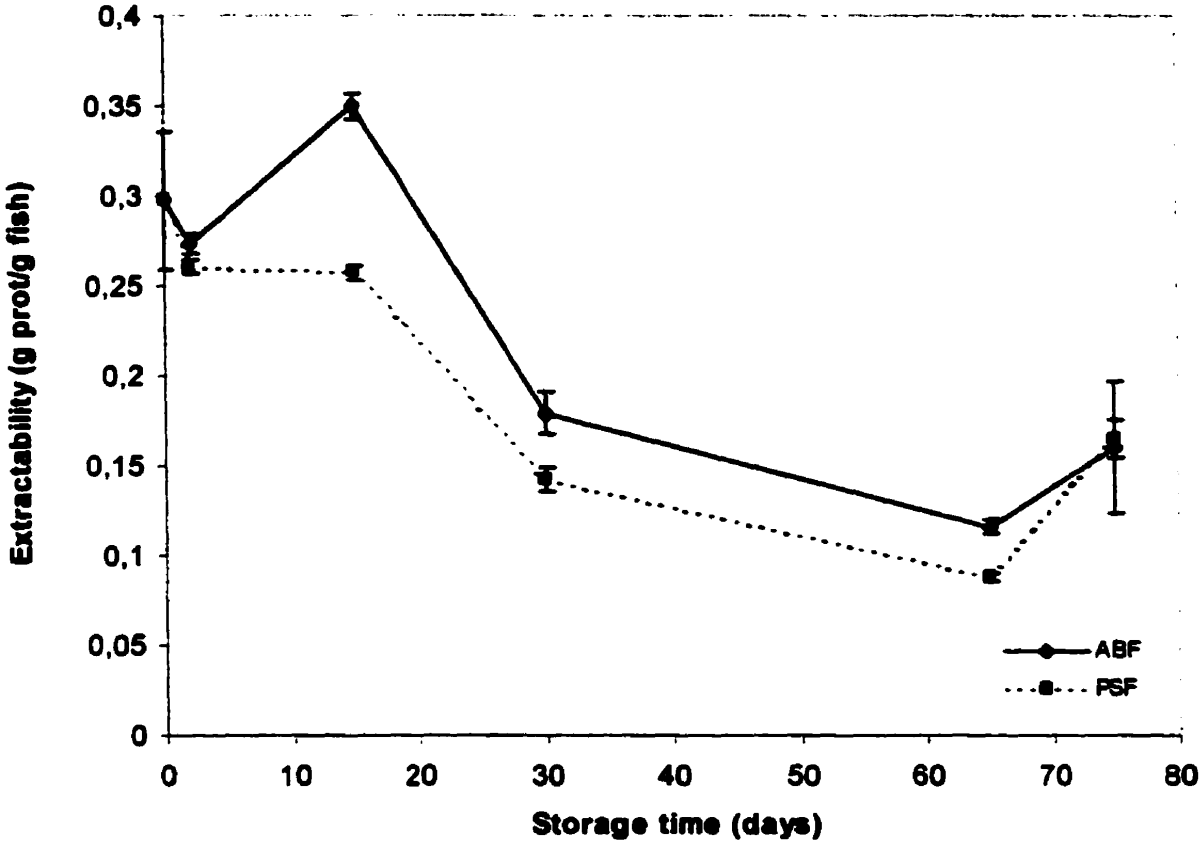

Fig. 6.4: Effect of freezing procedure on the extractability of myofibrillar protein from intact carp fillets during frozen storage. Each marker represents the mean of 3 values.

This behaviour may reflect the effect of freezing on SP regardless of the procedure used to achieve the frozen state. However, after 2 days of storage, extractability of SP subjected to ABF remained constant for the rest of the storage period. The extractability of SP from fish muscle tissue frozen by PSF decreased gradually during the storage time. During the first 65 days of storage there was no significant effect of the freezing method on the extractability of SP ($p>0.05$). However after 75 days of storage, the extractability of SP from the intact muscle tissue subjected to PSF was significantly lower compared to that of SP from intact muscle tissue frozen using (ABF) ($p<0.05$). Dias et al., (1994) reported a decrease in extractability of sarcoplasmic proteins during frozen storage of black and silver scabbardfish. Ohshima et al., (1992) also reported the disappearance of certain bands on the electrophoretic patterns of pressurised cod and mackerel muscle proteins. It was suggested that pressurised sarcoplasmic proteins become covalently linked together resulting in the decrease in protein extractability.

In the case of MFP, their extractability decreased during frozen storage regardless of the freezing procedure used. After 75 days of storage, there was not a significant difference in the extraction of MFP between the two procedures PSF and ABF ($p>0.05$). However, extractability of MFP from fish muscle tissue subjected to PSF was always lower than that of MFP from fish muscle tissue frozen by ABF ($p<0.05$) during the first 65 days of storage. A reduction of MFP extractability during frozen storage of fish was previously reported by Connell (1968) and Zayas (1995). On the other hand, high pressure itself may induce

protein denaturation (Thakur and Nelson, 1998) which would explain the marked loss of extractability of PSF muscle.

Ca^{2+}-ATPase Activity of Actomyosin (AM)

Protein quality is considered to be more reflected by enzymatic activity than by extractability (Shenouda,1980). Small microstructural changes in protein molecules will cause alterations in enzymatic activity. Ca^{2+}-ATPase activity reflects the integrity and functionality of the myosin molecule in the actomyosin complex (Montecchia et al., 1997). The effect of freezing procedure on ATP hydrolytic activity in the presence of Ca^{2+} ions is shown in Fig. 6.5. Enzymatic activity of actomyosin from carp fillet muscle subjected to PSF or ABF decreased during frozen storage ($p<0.05$). Similar observations were made in a model system of carp myofibrils during frozen storage by Kitazawa et al., (1995) and Yoshikawa et al., (1995). Enzymatic activity of AM from carp fillet subjected to PSF was significantly higher than that of carp fillet AM subjected to ABF ($p<0.05$). It seems that although PSF may induce a higher aggregation of myofibril proteins compared to ABF, it does not have a detrimental effect on the enzymatic activity of AM. Yoshikawa et al., (1995) detected Ca^{2+} and EDTA-ATPase activity in insoluble carp MFA proteins and suggested that although protein molecules can get closer together and aggregate, to cause a decrease in extractability, their active sites may not be affected so they may retain their enzymatic activity. Ko et al., (1991) studied the effect of pressure treatment on actomyosin ATPases from flying fish and sardine muscle tissues and observed that at pressures lower than 300 MPa, Ca^{2+}-ATPase activity remained unchanged. As pressure increased to

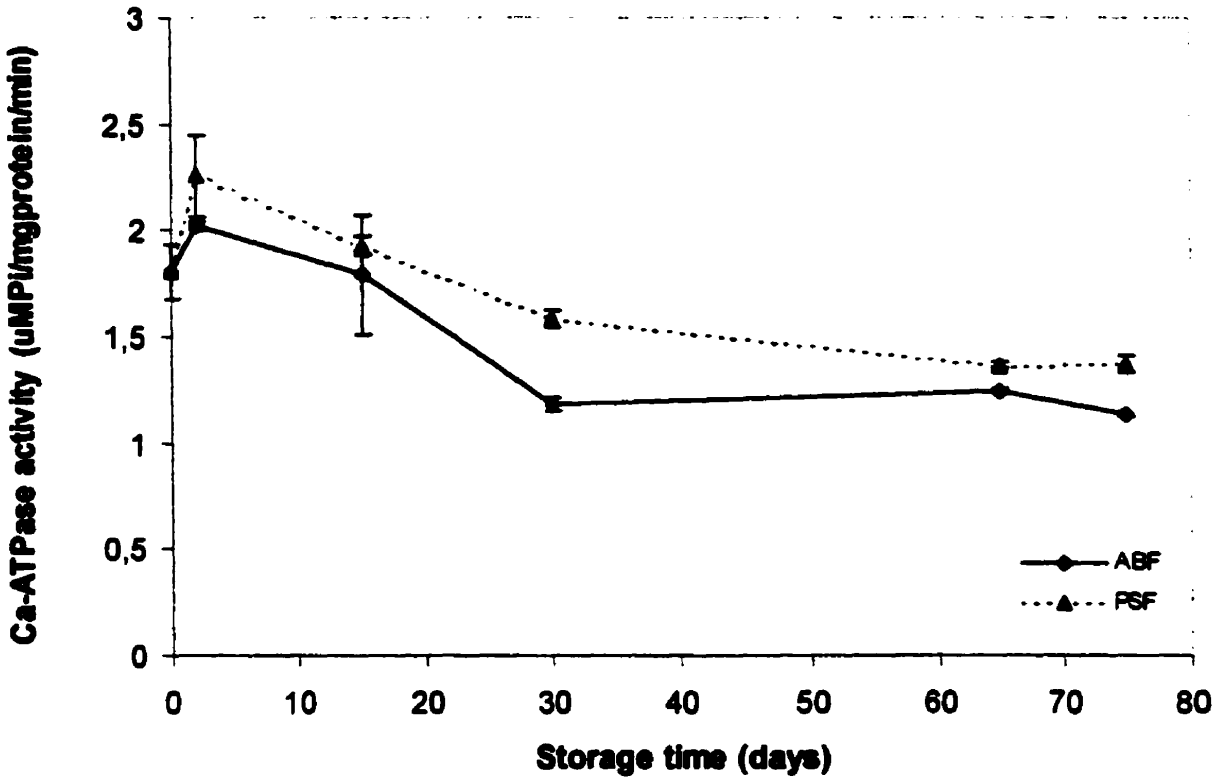

Fig. 6.5: Effect of freezing procedure on Ca^{2+} ATPase activity of carp actomyosin from intact carp fillets during frozen storage. Each marker represents the mean of 3 values.

500 MPa and above, Ca^{2+}-ATPase activity decreased. Similarly, Ashie et al., (1999) reported that Ca^{2+}-ATPase activity of muscle proteins from tilapia was stable under high pressure treatment (0 to 200 MPa, 20 min at 5°C). On the other hand, Ishizaki et al., (1995) noticed a marked activation of Mg-ATPase activity of muscle proteins from yellowfin tuna pressurised at 100 MPa for 10 min.

Lipid Oxidation and Free Fatty Acids Release

It is known that during frozen storage of fish, products of lipids hydrolysis and oxidation can accumulate and cause deterioration of the product (Mackie, 1993). Hydroperoxides are the first products of oxidation and their measurement is a useful indicator of the stage of oxidation (Khayat and Schwall, 1983; Shewfelt, 1981). Fig. 6.6 shows the effect of PSF on the TBA numbers of intact carp fillets compared with those that were frozen using ABF. In general, TBA values of PSF fillets remained constant during frozen storage while those for the ABF fillets increased after the first month of storage. In all cases, the TBA values of the ABF fillets were significantly higher ($p<0.05$) than those of PSF fillets. This data suggests that PSF had a curtailing effect on the oxidation of lipids in carp fillets. Cheah and Ledward (1997) reported an increase in TBA values during a 6 day storage period of minced pork pressurised at 400 and 800 MPa for 20 min at 19°C. However, no significant increased rate in oxidation was observed in muscle samples treated at 300 MPa. In our study, the pressure level used was 190 MPa for only 12 min at -14°C unlike the condition used by Cheah and Ledward

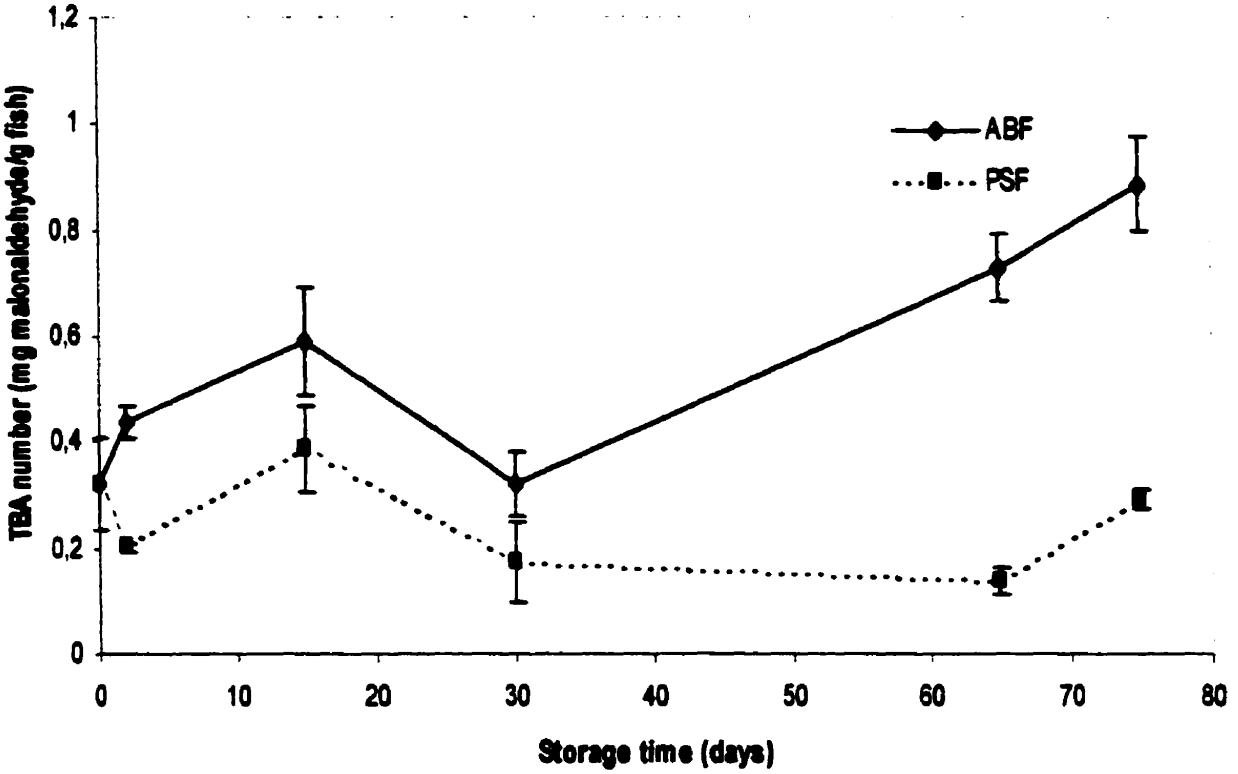

Fig. 6.6: Effect of the freezing procedure on TBA number for carp fillets during frozen storage. Each marker represents the mean of 3 values.

(1997). Ohshima et al., (1993) used pressure levels between 200 to 610 for 15 to 30 min and suggested that isolated extracted marine lipids were more stable against autoxidation than lipids present in intact muscle. On the contrary, Angsupanich and Ledward (1998) observed little changes in TBA values of cod muscle pressurised at 200 MPa for 20 min. However, in the same experiment TBA values increased when samples were treated at 400 MPa or higher for 20 min and continued to increase during the 7 days of storage. Temperature may be a contributing cofactor inducing autoxidation. It has been suggested that accelerated oxidation may be due to the release of iron from haemoglobin and myoglobin which promote auto-oxidation of lipid pressurised fish meat (Tanaka et al., 1991; Ohshima et al., 1992; Cheah and Ledward, 1996). In this study, the processing conditions (pressure level, time and temperature) were probably not severe enough to induce auto-oxidation.

Besides oxidation, lipids may undergo hydrolysis resulting in the accumulation of free fatty acids (FFA). Myofibrillar proteins are considered to be the prime target of FFA and they become largely unextractable in their presence (Shenouda, 1980; Ohshima et al., 1984). It is generally believed that the interaction between proteins and FFA occurs primarily through electrostatic, van der Waals, hydrogen and hydrophobic forces. These interactions may create more hydrophobic regions in place of polar or charged groups resulting in a decrease in protein solubility in aqueous buffer, or further intermolecular linkages extensive enough to decrease extractability (Shenouda, 1980). Fig. 6.7 shows the effects of PSF and ABF on the FFA content of intact carp fillets, which indicates that the level of FFA increased significantly ($p<0.05$) throughout the

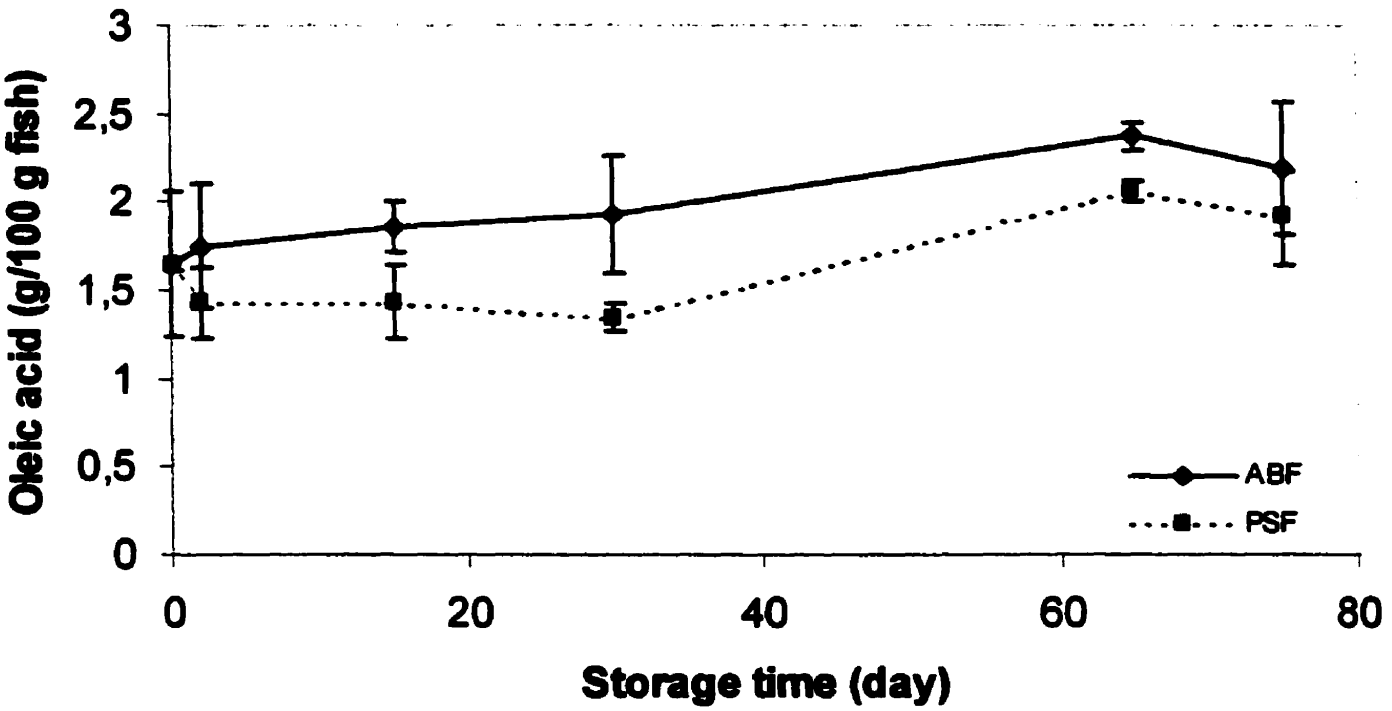

Fig. 6.7: Effect of the freezing procedure on the level of free fatty acids on carp fillets during frozen storage. Each marker represents the mean of 3 values.

storage period for carp fillets frozen using ABF. In the case of the carp fillets frozen by PSF, during the first 30 days of storage the concentration of FFA remained constant, then started increasing during the rest of the study. At the end of the storage study, there were no significant differences ($p>0.05$) between the FFA levels in the PSF samples versus the ABF samples. However it is important to note that between 15 to 65 days of storage, samples treated using PSF had a significantly lower FFA content than the samples frozen by ABF. It seems that PSF had a retardation effect on the release of FFA from lipid hydrolysis. Ohshima et al., (1992) showed that the enzymatic degradation of fish muscle phospholipids was effectively prevented by high hydrostatic pressure treatment. In this study, the pressure level used (140 MPa) may have had an initial inhibitory effect on the activity of phospholipases, but the enzymes may have gradually recovered their activity during the storage period.

Color Changes in Fish Fillets

Changes in color of carp muscle fillets are shown in the Table 6.1. Raw carp muscle appears red when fresh, very similar to beef muscle. The L* value which is an index of visual lightness increased during frozen storage in both ABF samples and PSF samples. Similar results were observed during frozen storage of rainbow trout fillets (No and Storebakken, 1991). Comparisons of the L* values of ABF samples with those of PSF samples did not produce a definite trend. During the first 15 days of storage, the L* values of the ABF samples were higher ($p<0.05$) than those of the PSF samples. However, during the next 50

Table 6.1: Changes in color of carp fillets during frozen storage

Storage time	*Air blast freezing* ABF			*Pressure shift freezing* PSF		
(days)	L*	a*	b*	L*	a*	b*
Fresh	41.9 ± 0.6 a	1.7 ± 1.4 a	1.0 ± 0.4 a	41.9 ± 0.6 a	1.7 ± 1.4 a	1.0 ± 0.4 a
2	49.3 ± 1.4 b	-1.5 ± 0.4 b	3.2 ± 0.8 bc	45.2 ± 2.3 *b	3.0 ± 1.4 *ac	3.1 ± 0.5 ab
15	47.4 ± 1.2 c	-1.3 ± 0.4 b	0.6 ± 0.4 d	43.1 ± 1.0 *ab	5.0 ± 4.6 *b	2.2 ± 0.8 *ab
30	47.5 ± 0.7 c	-1.5 ± 0.6 b	0.4 ± 0.6 d	50.1 ± 4.6 c	2.7 ± 2.1 *ac	3.0 ± 1.7 * b
65	48.1 ± 0.6 bc	-1.7 ± 0.3 b	2.4 ± 1.4 b	48.7 ± 2.3 c	3.4 ± 0.8 c	2.6 ± 2.1 b
75	48.2 ± 1.3 bc	-0.4 ± 0.9 c	4.0 ± 0.9 c	54.5 ± 0.8 *d	-1.0 ± 0.5 d	3.7 ± 1.2 b

+ sign means significant difference between ABF and PSF
Data are expressed as mean ± SD, n = 5.
Means with the same letter in columns are not different (p>0.05)

days there were no significant differences ($p>0.05$) among treatments, although by 75 days of storage, the PSF processed samples showed higher L* values ($p<0.05$) than the ABF samples. This observation may be due to the wide variability of the raw material. In order to process the samples in a prerigor condition, bleeding was not carried out, which may have resulted in fillets with a non uniform coloration. The a* values normally used as an index of visual redness, were negative after the samples had been subjected to the ABF process and tended to decrease during frozen storage. On the other hand, the a* values of fish fillets subjected to PSF did not change during the first 30 days, after which the a* values slightly increased. The negative a* value for PSF at 75 days storage may be due to variation in coloration in the samples due to the bleeding problem mention earlier. There was a significant difference ($p<0.05$) in the a* value between ABF samples and PSF samples. It has been reported that for mackerel and cod, the a* value decreased after pressurization (Ohshima et al., 1992). Similarly, Angsupanich and Leward (1998) reported that the redness of raw cod was lost after treatment at $\geq$ 200 MPa. Overall, the a* values for pressurized carp slightly increased. Cheftel and Culioli (1997), reported that the a* values increased in pressurised beef or pork meat. They suggested that pressure-induced coagulation of sarcoplasmic and myofibrillar proteins was possibly responsible for this behaviour. It is also possible that 140 MPa was not high enough to induce changes in the a* value. No significant changes ($p>0.05$) in the b* values were observed between ABF samples and PSF samples, similar to results obtained by Ohshima et al., (1992) for mackerel and cod.

Changes in Texture

Fig. 6.8 shows the results for texture of cooked carp fillets, no significant differences were observed between ABF and PSF samples. Although it has been reported that toughness of fish flesh increases during frozen storage (Dias et al., 1994; LeBlanc et al., 1988), for carp fillets, the toughness remained stable during frozen storage for both ABF and PSF treatments. It seems that carp fillets were less susceptible to quality deterioration during frozen storage than salt-water fish, probably because of the absence of the trimethylamine system responsible for the formation of formaldehyde, which promotes protein denaturation and deterioration of texture during frozen storage. Angsupunich and Ledward (1998) reported an increase in toughness after pressure treatment (400 and 600 MPa). However, at 200 MPa this effect was almost negligible. In this study, the fact that almost no differences were observed between the two treatments may be due to the low pressure level used and also to the fact the PSF reduces cooking losses in carp fillets (unpublished results). This increase in water content for cooked fish may counteract the effect of pressure denaturation resulting in little or no effect on hardness of the fish fillets.

Electrophoretic Studies

Polyacrylammide gel electrophoresis was carried out to determine the effect of pressure shift freezing on both sarcoplasmic and myofibrillar proteins during frozen storage. The electrophoretic profile presented in Fig. 6.9 for sarcoplasmic proteins for both PSF and ABF treated samples are very similar.

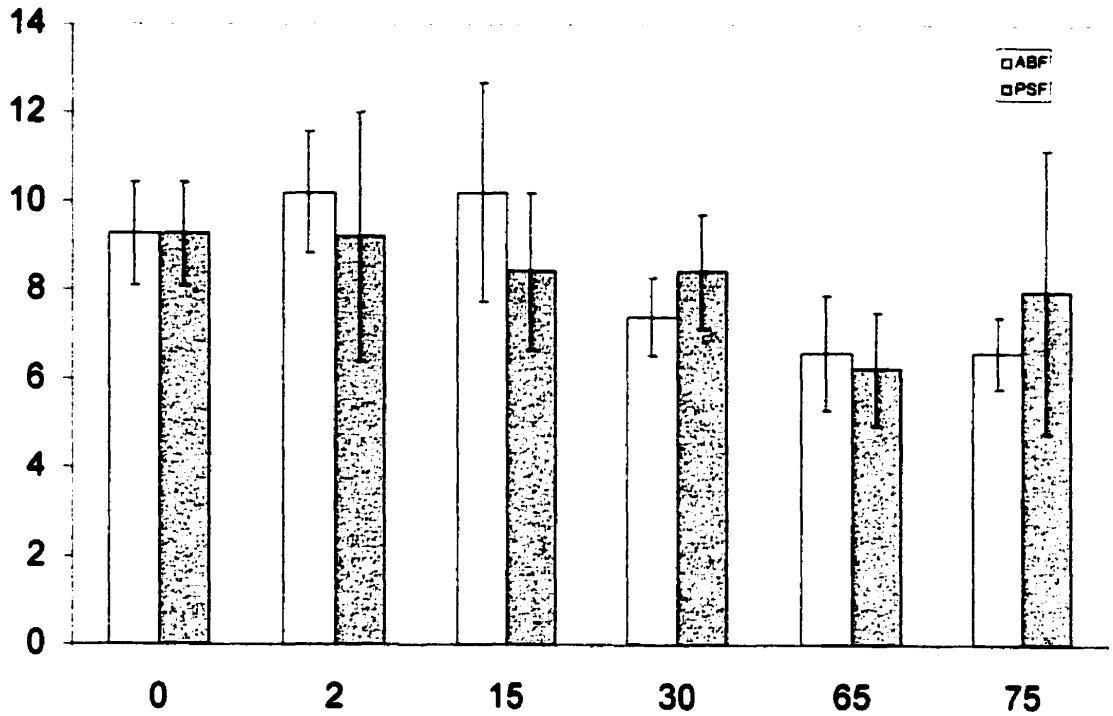

Fig. 6.8: Effect of freezing procedure on the toughness of carp fillets during frozen storage. Each bar represents the mean of 5 values.

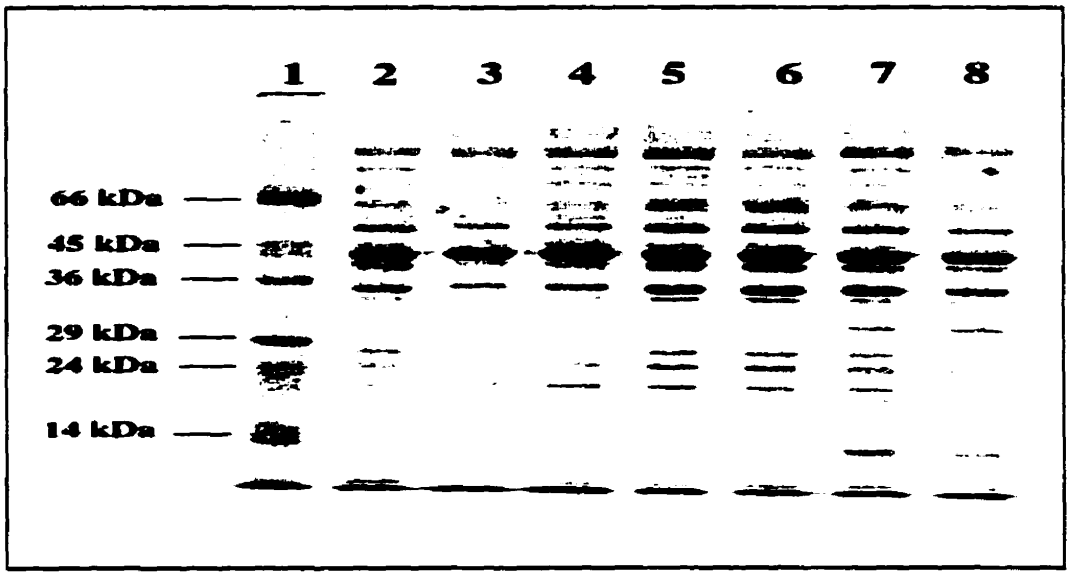

Fig. 6.9: Effect of freezing procedure on the electrophoretic profile of sarcoplasmic proteins. (1) Low molecular weight standard, (2) fresh fish 0 days storage, (3)ABF 2 days storage, (4) PSF 2 days storage, (5) ABF 15 days storage, (6) PSF 15 days storage (7) ABF 65 days storage, (8) PSF 65 days of storage

Both profiles show changes mainly in bands with molecular weights between 36 to 14 kDa at 75 days of storage. There were three new bands with molecular weights around 32, 20 and 14 KDa that appear after 75 days of storage in both ABF and PSF samples. Specifically the intensity of the bands between 29 and 24 kDa tends to decrease regardless of the freezing procedure used. However, the decrease in the bands between 29 to 24 kDa at 75 days of frozen storage seems to be more marked in PSF samples. These results confirm those obtained by measuring the extractable SP and indicate that SP become less extractable after pressure treatment. It has been suggested that, rather than being degraded by high pressure, certain sarcoplasmic proteins become covalently linked together and are thus resistant to extraction with SDS (Ohshima et al., 1993). Fig. 6.10 shows the electrophorectic profile of myofibrillar proteins. There are two bands (40 kDa and 29 kDa) that tend to disappear during frozen storage of carp fillets. On the other hand, there is one band <14 kDa that appears after 75 days of frozen storage for both PSF and ABF samples. Due to the fact that these changes are present in both PSF and ABF samples, it is more likely that they arise as a result of the freezing phenomenon than as a consequence of high pressure treatment. The band with a molecular weight <14 kDa present in the electrophorectic profile of both SP and MFP seems to be the same which would confirm what Ohshima et al., (1992) suggested, that these new components may be fragments of sarcoplasmic proteins degraded by pressurization, either water-soluble fragments derived from myofibrillar protein or soluble fragments of membrane-bound proteins.

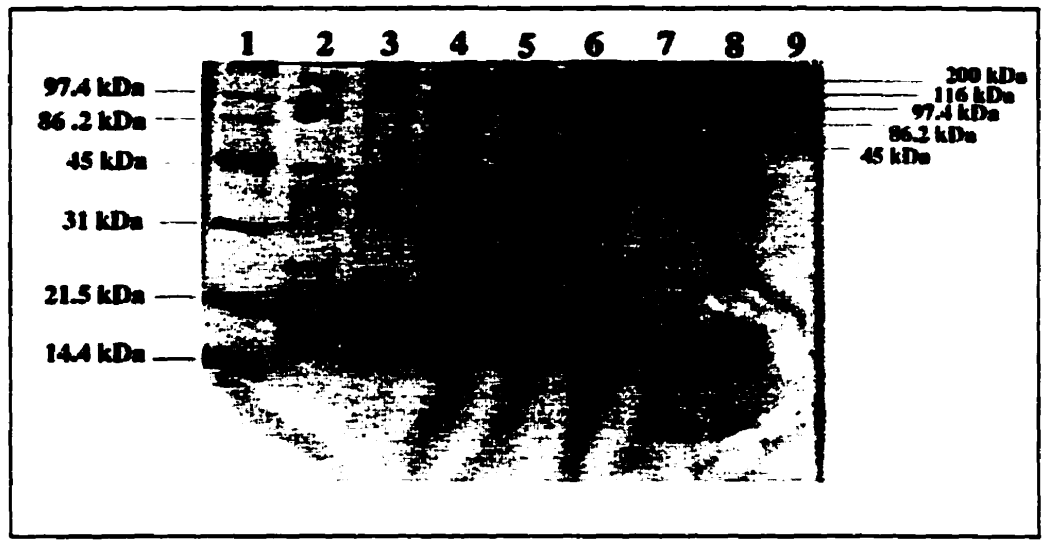

Fig. 6.10: Effect of freezing procedure on the electrophoretic profile of myofibrillar proteins. (1) Low molecular weight standard, (2) fresh fish 0 days storage, (3)ABF 2 days storage, (4) PSF 2 days storage, (5) ABF 15 days storage, (6) PSF 15 days storage (7) ABF 65 days storage, (8) PSF 65 days of storage and (9) High molecular weight standard.

CONCLUSIONS

This study shows the relative advantages and potential use of high pressure technology to increase freezing rates while maintaining a good quality product. Freezing time was reduced by using high pressure technology. However, more important was the reduction in lipid degradation such as oxidation and hydrolysis that may eventually determine the flavor of flesh fillets.

CHAPTER VII

EFFECT OF PRESSURE-SHIFT FREEZING OF CARP (*Cyprinus carpio*) ON THE SIZE OF ICE CRYSTALS AND DRIP VOLUME.

Connecting Statement

In the previous chapters, the effect of pressure-shift freezing on biochemical properties of fish proteins and lipids from carp fillets during frozen storage was addressed. The results indicate that pressure-shift freezing treatment effectively reduced lipid degradation during frozen storage. This chapter addresses the effect of pressure-shift freezing on the size of ice crystals formed during freezing of carp fillets and the implication on drip loss during frozen storage.

Contribution of co-authors: Benjamin K. Simpson (thesis/research supervisor)

ABSTRACT

Carp (*Cyprinus carpio*) fillets were frozen either by pressure-shift freezing (PSF, 140 MPa, -14°C) or by air-blast freezing (ABF), and were stored at –20°C for 65 days. No effect of freezing treatment was observed on the amount of thawing losses for carp fillets. However, PSF treatment was more effective in reducing the amount of cooking losses in thawed carp fillets compared to ABF treatment. Ice crystals found in PSF fish samples were mainly intracellular, smaller and more regular than those found in the ABF samples, which were mainly extracellular. Differential scanning calorimetry showed that when pre-rigor carp fillets were frozen by PSF, there was a reduction (34%) in total enthalpy of denaturation after 65 days of frozen storage compared with the fresh and ABF treated samples. However, when post-rigor carp fillets were frozen by PSF, the total enthalpy of denaturation (12.2 J/g) remained stable after 65 days of storage. The total enthalpy of denaturation for ABF treated samples decreased (~2.5 J/g) after 65 days of frozen storage. Thus, the PSF treatment appeared to be more effective in preventing protein denaturation in post-rigor fish fillets than in the pre-rigor samples.

INTRODUCTION

One of the major developments in the fishing industry during the last 50 years is the application of novel freezing-preservation techniques. Freezing is the only preservation technique that delivers fish in almost the same quality state as

freshly caught fish. Statistics show that in North America and Europe, as much as 60% of all fish consumed, either as fillet or added-value product, had been frozen once or possibly twice (Morrison, 1993). The consumer prefers products that are perceived as fresh, convenient, and most importantly, provide a good sensory experience (Elvevoll et al., 1996). In order to obtain high quality frozen foods, high-quality starting raw materials are necessary; and processing, distribution and storage methods must be carefully controlled. Under frozen conditions, spoilage due to microbial activity, as well as biochemical changes can be slowed down considerably. Although freezing of food provides a "safe" and convenient way of shelf life extension, quality deterioration may take place during storage. The effect of freezing on microbial and biochemical processes are well understood, however physical deterioration as a result of ice formation and changes in ice crystals remains less clear. Sikorski and Kolakowska (1990) and Keizer (1995) suggested some treatment and storage conditions in order to minimize deteriorative changes in frozen fish. The rate of freezing should be high enough to prevent formation of large ice crystals in the extracellular spaces, which concentrates the solutes within the cells. It is recommend to keep the fish at a temperature between –20°C and –30°C if the product is to be stored for a period of more than 4 to 6 months (Shenouda, 1980).

Thus, optimal product quality requires control, not only of the pre-freezing and post-freezing regimes, but also of the freezing process itself. The food industry makes use of several freezing procedures. These procedures may be classified into different types: (i). slow freezing (ii) rapid freezing and (iii) ultrarapid freezing. The classification is based on the rate of movement of the ice front from

the product surface where the initial freezing takes place, to the thermal centre of the food (George, 1993). It is generally accepted that rapid freezing results in smaller ice crystals and leads to better product quality (Denys et al., 1997). The rapid and ultrarapid freezing methods include: fluidized bed, immersion, scraped-surface and cryogenic freezing. The current interest in the application of high pressure to food processing conjures up several potential applications for high pressure on ice water transitions. These application were summarized by Kalichevsky et al., (1995) and include pressure-shift freezing, high pressure thawing and low-temperature non-frozen storage under pressure. The basis of these applications is the effect that high pressure has on ice water transitions. High pressure reduces the freezing and melting points of water to a minimum of –22°C at 207.5 MPa during the formation of type I ice. Type I ice crystals have a lower density than liquid water resulting in a volume increase of about 9% on freezing at 0°C which increases to 13% at –20°C. Pressure opposes the volume increase occurring during the formation of type I ice crystals so the freezing and melting points are decreased. In pressure-shift freezing, a biological sample is cooled under pressure to a temperature just above the melting temperature of ice at this pressure. Upon sudden release of pressure, supercooling of water present in the sample enhances heterogeneous ice nucleation, inducing the formation of a large number of small ice crystals. This obviates damage to tissue and cell structures caused by large crystals that potentially results in excess drip loss and altered texture after thawing (Barry et al., 1998). Research has focused on the mathematical modelling of freezing and thawing for model foods (Chourot et al., 1997; Sanz et al., 1997; Denys et al., 1997; Schluter et al., 1998), and on

different plant and animal food materials. Zhao et al., (1998) studied the effects of high pressure on rapid thawing of frozen beef. Similarly, Chevalier et al., (1999) studied high pressure thawing of whiting fish. In the specific case of high pressure freezing, Fuchigami et al., (1997a) applied high pressure freezing to carrots and reported that pressures of 200, 340 and 400 MPa appeared to be effective in reducing histological damage and improving texture of thawed carrots. Fuchigami and Teramoto (1997) observed that high pressure freezing between 200 MPa to 400 MPa yielded products with better texture than did conventional freezing. Similar results were obtained with Chinese cabbage subjected to high pressure freezing (Fuchigami et al., 1998). The response of food products to high pressure freezing is very complex, and this reflects the variation in food composition. Thus, the objective was to study the effect of pressure-shift freezing on the size of ice crystals and to correlate these results to drip volume and the enthalpy of protein denaturation for fish proteins.

MATERIALS AND METHODS

Fish sampling

Live carp (*Cyprinus carpio*) (weight, ca. 1226 ± 221g and length 40 ± 7 cm), were obtained from an aquaculture farm (Ferme acquacole d'Anjou, Morannes, France), and immediately transported to the pilot plant (Nantes, France). The fish were held in a water tank (10°C) overnight and then

slaughtered, cleaned and filleted the next day. The fresh fish fillets were placed in moisture-impermeable polyethylene bags (La Bovida, France) and vacuum-packaged. Samples were stored in isothermal boxes in a cold room at 4°C and processed between 3-5 h after slaughter. For each pair of fillets obtained, one was subjected to pressure-shift freezing (PSF) and the other to air-blast freezing (ABF). In the case of post-rigor sample, fish were slaughtered and storage on isothermal boxes in a cold room at 4°C for 24 h before being pressurised.

pH determination

Carp muscle was homogenised in 10 volumes water (w/v) and pH was measured using a pH meter (Accument 620 Fisher Scientific, Pittsburgh, PA).

Freezing processes

Air-blast freezing (Servathin, France) was carried out at -20°C using an air speed of 4 m/s and the samples were designated ABF. The freezing process was considered to be complete when the temperature at the center of the sample reached -20°C. The temperature at the surface and at the center of samples were monitored using a temperature recorder (Model SA 32 AOIP, Evry, France) with K-type thermocouples (1 mm diameter, Omega, Stamford, USA). The nominal freezing time was calculated following the recommendations of the International Institute of Refrigeration (Anon, 1986) as the time required to decrease the temperature in the thermal center by ten degrees below the initial freezing point. The nominal freezing time was about 44 min on the average for

ABF process. The freezing rate was obtained as the ratio of the distance from the surface to the thermal center of the fish fillet and the freezing period which elapsed from the moment when the surface temperature was 0°C until it reached -10°C at the thermal center. A mean freezing rate of 0.94 cm/h was obtained by ABF at –20°C and an air speed of 4 m/s (3 measurements). The characteristic freezing time was determined according to the definition of Bevilacqua et al., (1979) as the time required to decrease the temperature at the center of the sample from –1°C to –7°C. The characteristic freezing time for ABF process used in this study was 44.4 min.

Pressure-shift freezing was carried out in a 3L capacity high pressure vessel (ALSTOM, Nantes, France). The stainless steel vessel (12 cm internal diameter and 30 cm internal height) and the pressure transmitting medium (50/50 v/v ethanol/water solution) were maintained at -14°C by circulation from an external cryostat through the internal cooling circuit of the high pressure vessel. Pressure was supplied by a high pressure pump. A K-type thermocouple was installed at the center of samples and another one in the pressurization medium. A data logger (Model SA 32 AOIP, Evry, France) recorded temperature and pressure data with an acquisition rate of one measurement per second. Samples were placed in the high pressure vessel and the pressure was increased to 140 MPa at a rate of 100 MPa/min. When the temperature of the samples reached -14°C (≈12 min), pressure was released at a rate of 10 MPa/sec. Samples were immersed in a stirred cooling bath of 50/50 (v/v) ethanol/water solution at -20°C for 7 min to complete freezing. Once frozen, the carp samples were stored in

isothermal boxes in a cold room at -20°C for 2, 15, 30, 65 and 75 days. Fresh samples and thawed samples (unpressurized and pressurized samples) after storage at -20°C were analysed. Thawing of frozen samples was achieved by placing the packed samples overnight in a cold room at 4°C. Each experiment and analysis were performed in duplicate on two different batches of fish after one-week interval.

Drip Losses

Thawing drip was determined by weighing samples prior to freezing and after thawing. Before weighing, completely thawed fillets were removed from their bags and the surface drip was removed by swabbing with filter paper. Fish samples were then packed in cooking pouches (la Bovida, France) and cooked in a circulating water bath for 20 min at 65°C. Samples were left to cool down at room temperature (20°C) for 1 h. Cooking drip was evaluated as previously described for thawing drip. Drip loss results were presented as the amount of drip per g dry matter of flesh.

Differential Scanning Calorimetry

Four samples, each weighing about 810 mg, were taken from thawed minced fillets and hermetically sealed in aluminium pans. Differential scanning calorimetry (DSC) runs were performed with a differential calorimeter III (Setaram, Caluire, France). Water was used as reference material and indium as calibration material. Samples were scanned from 10°C to 90°C at a heating rate

of 1°C per min. Total denaturation enthalpies (ΔH_T) were estimated as the area under DSC transition line (a baseline was constructed as a straight line from the heat flux at 30°C and at 80°C of the endotherm). Enthalpies were expressed as J per g fish dry matter. The dry matter of fish flesh was determined in triplicate by drying 5 g of fish at 102°C for 24 h, followed by cooling in a dessicator and then weighing.

Histological analysis

Three samples of about 0.5×0.5×0.5 cm were cut from frozen fillets. The samples were placed in flasks containing a fixative solution at -20°C. An indirect technique called isothermal freeze substitution was used to observe the spaces left by ice crystals in the tissue. Samples were fixed with Carnoy fluid (absolute ethyl alcohol 60%, chloroform 30% and glacial acetic acid 10%, v/v) according to the method described by Martino and Zaritzky (1986). Once fixed for 36 h, the samples were brought to room temperature (20°C) and then dehydrated with a series of alcohol solutions (first in absolute ethanol for 2 h then in 1-butanol for 2 h and repeated 3 times). The dehydrated samples were then cleaned in a sequence of 3 baths of bioclear solution (Microm, France) for 25 min and were embedded in paraffin. Samples were cut with a rotary microtome (Model-2030, Reichert-Jung, Vienna, Austria) in 10 μm thin slices. Sections were stained with bright green aqueous solution and finally observed under a microscope (Olympus BH-2, Japan).

Statistical Analysis

The effect of frozen storage time on parameters (drip loss, total denaturation enthalpies (ΔH_T)) was analysed by two-way analysis of variance according to Sokal and Rolhf (1995) using Statgraphics plus software (Version 2.1 Statistical Graphics Corp., Princeton, NJ, USA). In all cases, the significance level used was 5% (α=0.05). To test significant differences between means for freezing processes at the same time of storage, the Student T-test was used, whenever the Fisher F-test was not significant ($p \geq 0.05$).

RESULTS AND DISCUSSION

Freezing processes

Fig. 7.1 shows the time-temperature profile for the air-blast freezing process of carp fillets. The time for the center of the fillet to attain the freezing temperature of -18.3 °C was 75.7 min. Carp fillets showed a freezing point of −1.2°C with a freezing plateau of 16.5 min. Pressure and temperature profiles during the pressure-shift freezing process are shown in Fig. 7.2, and indicate that when pressure was increased up to 130 MPa the temperature of the pressurization medium also increased to about 2.2°C. For the fish fillet itself, no increase in temperature was observed as pressure increased. However, it appeared that the cooling process slowed down during pressurization as a result of heat dissipation in the first step (A-B). In the second step of the process (B-C), the fish fillets were allowed to cool under pressure until they reached a

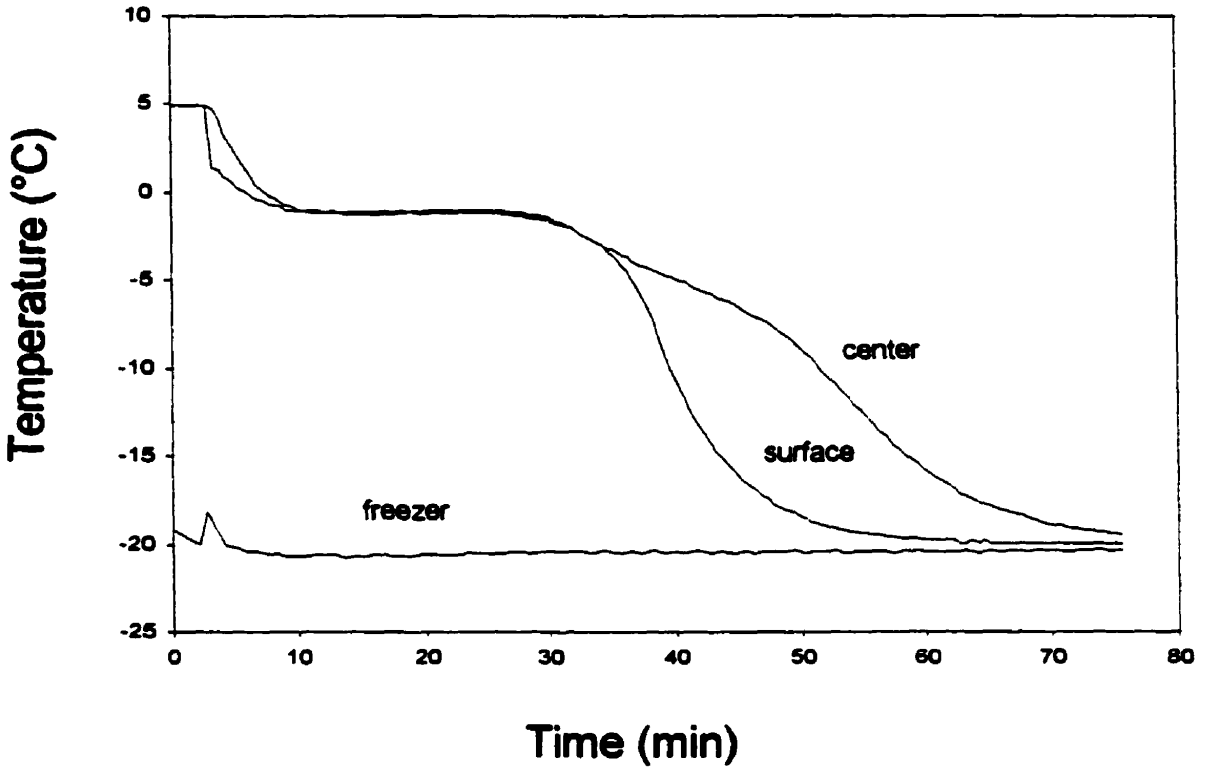

Fig.7.1: Time-temperature profile for carp fillets by air-blast (-20°C, 4m/s) freezing. Temperature was recorded at the centre of the fillet and in the cooling medium.

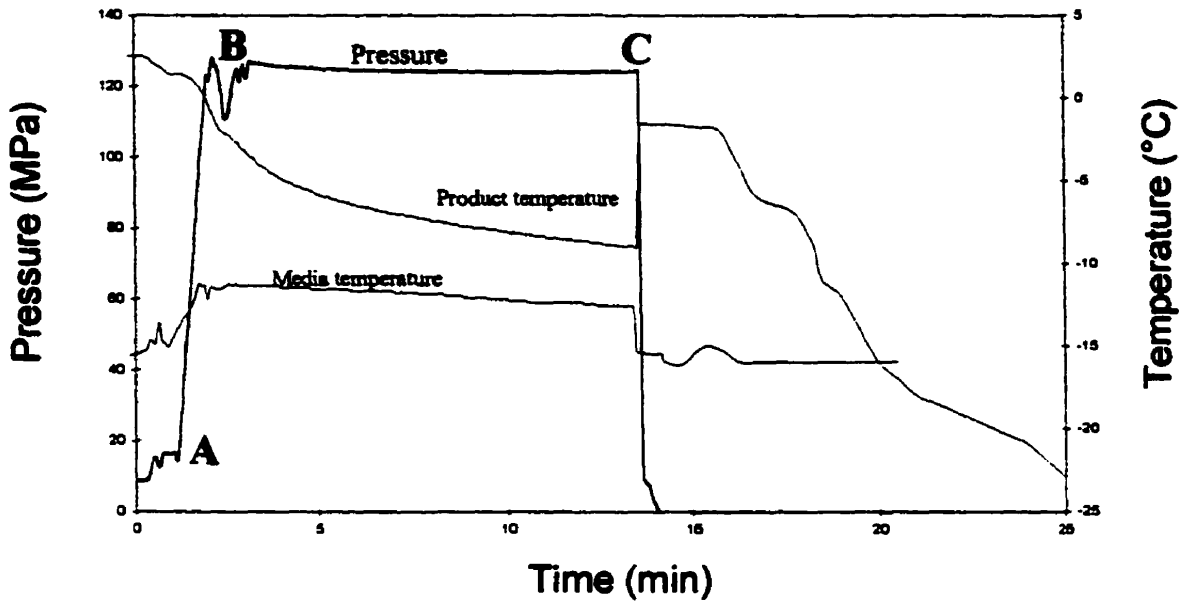

Fig. 7.2: Freezing kinetics of carp fillets frozen by pressure-shift freezing. Temperatures were recorded at the centre of the fillet and in the cooling medium. Pressure was measured with a pressure gauge. Through all the pressurisation process, the temperature of the cooling medium was controlled at -15°C.

temperature of -9°C. At this point pressure was suddenly released. During depressurization, the temperature of the pressurization medium decreased from −13°C to −17°C, and the sample reached a temperature of −1.8°C compared to that of ABF −1.2°C. Finally, in the last step of the process, freezing was completed by using conventional freezing at atmospheric pressure. The total freezing time for pressure-shift freezing was 25 min including the pressurisation step and the final atmospheric freezing.

Drip looses

Acceptability and consumption of frozen seafood is based in part on the overall flavor and textural quality of the cooked product. Loss of muscle fluid as drip may adversely influence the juiciness, tenderness and flavor of the cooked fish (Morrison, 1993). Fig. 7.3 shows the effect of the freezing procedure on thawing losses for carp fillets during frozen storage. Otero et al., (1998) observed that high pressure assisted freezing was effective in reducing drip loss in eggplant. They suggested that massive nucleation obtained by high pressure-assisted freezing had less detrimental effect on cellular structure resulting in less drip loss. Similar observations were made by Koch et al., (1996) with potato cubes processed using pressure-shift freezing. In this study, thawing losses remained almost constant throughout the storage period, regardless of the freezing procedure used. Similarly, there was no significant difference ($p>0.05$) between thawing losses for PSF samples and those for ABF samples.

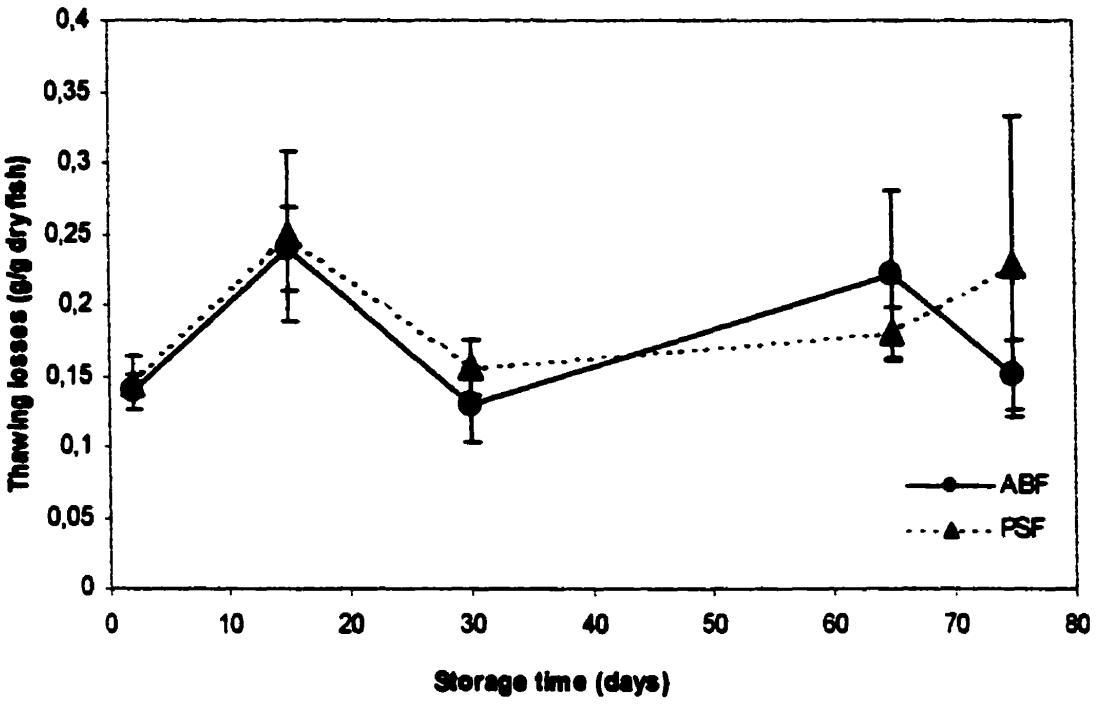

Fig. 7.3: Effect of the freezing procedure on thawing losses for carp fillets during frozen storage. Each marker represents the mean of 3 values.

Ngapo et al., (1999) found no effect of freezing rate on drip losses in pork samples stored for 4 weeks.

In the case of cooking loss (Fig. 7.4), carp fillets frozen by ABF showed significantly higher drip losses ($p<0.05$) compared with the PSF samples. For carp fillets frozen by ABF, drip loss increased over time and became significantly higher ($p<0.05$) than those of fresh fish after 65 days of storage. Similarly, Awad et al., (1969) observed that cooking losses increased during frozen storage of fresh water whitefish muscle. In contrast, cooking loss in PSF samples decreased ($p<0.05$) after 15 days of storage and then remained constant. These results are in agreement with those reported by MacFarlane (1973; 1974) that indicated reduced cooking loss in pre-rigor pressurized beef muscle. It was suggested that pressure treatment increase water holding capacity of meat as a result of a loosening of protein structure leading to increased hydration. An ionic mechanism was proposed and because of electrostriction effects between water molecules and exposed ions, the reaction would be expected to occur with a decrease in volume.

Thermal properties

Figs. 7.5(a,b) to 7.6(a,b) show DSC thermograms of intact carp muscles. The thermograms show three endothermic transitions with T_{max} of 53°C, 68°C and 87°C and a shoulder at about 47°C . Hastings et al., (1985) identified the peaks at 47°C and 53°C as myosin transition, the peak at about 74°C as actin transition, and the peak at 87°C as the sarcoplasmic transition (that is not always present). In our study it seems that the actin transition took place at 68°C. The DSC

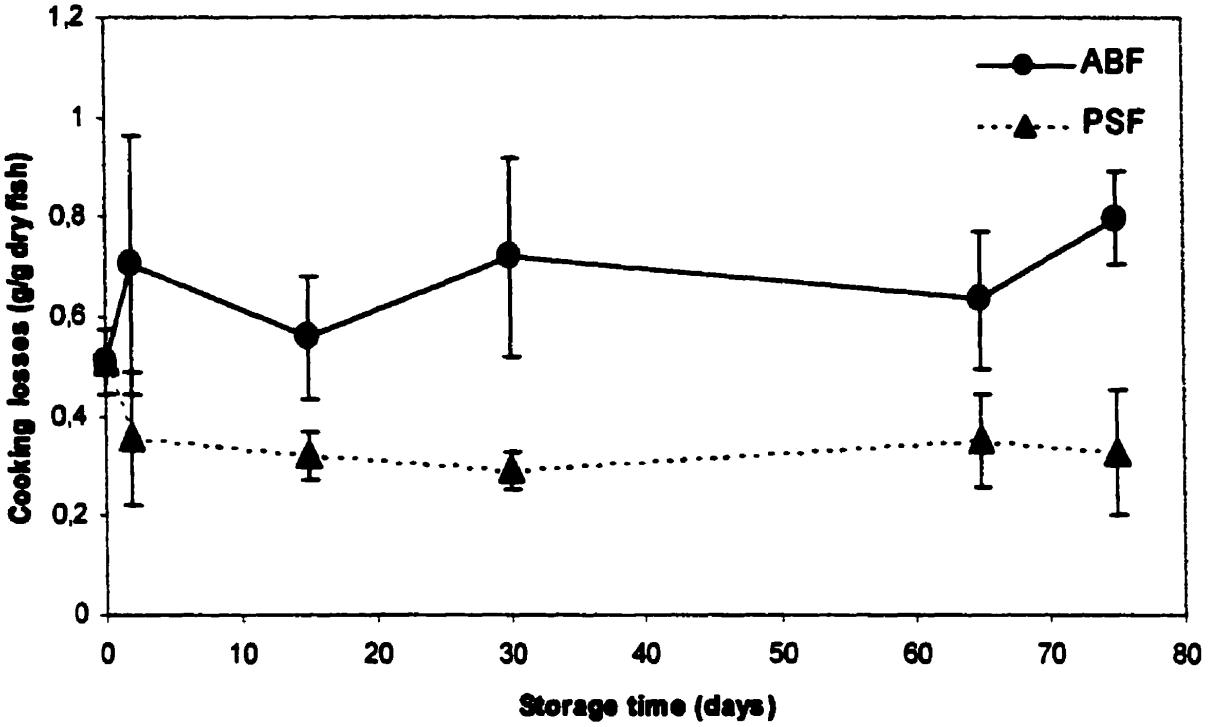

Fig. 7.4: Effect of the freezing procedure on cooking losses for carp fillets during frozen storage. Each marker represents the mean of 3 values.

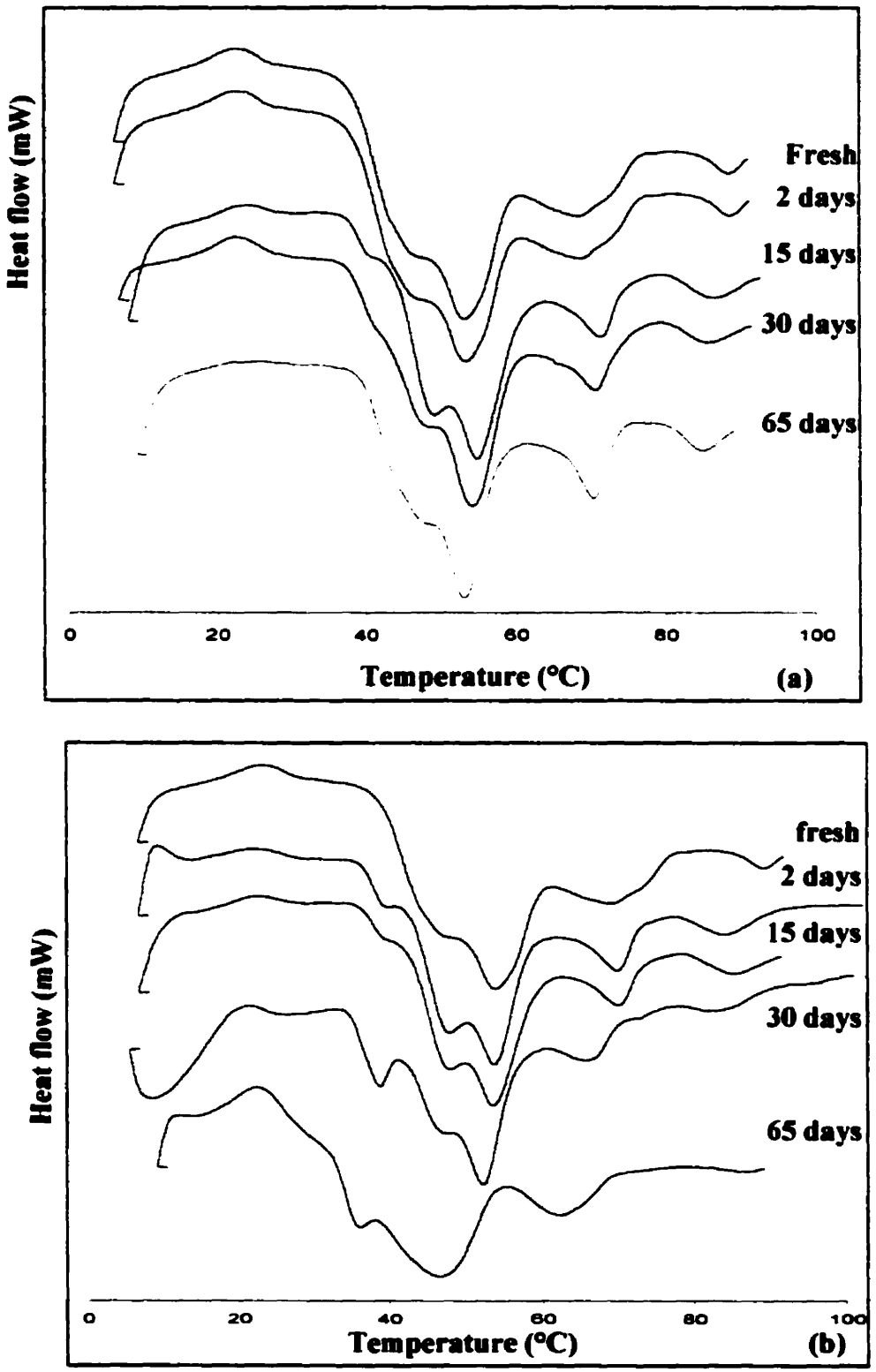

Fig. 7.5: Differential scanning calorimetry of carp fillet frozen by air-blast freezing. (a) pre-rigor fillets, (b) post-rigor fillets.

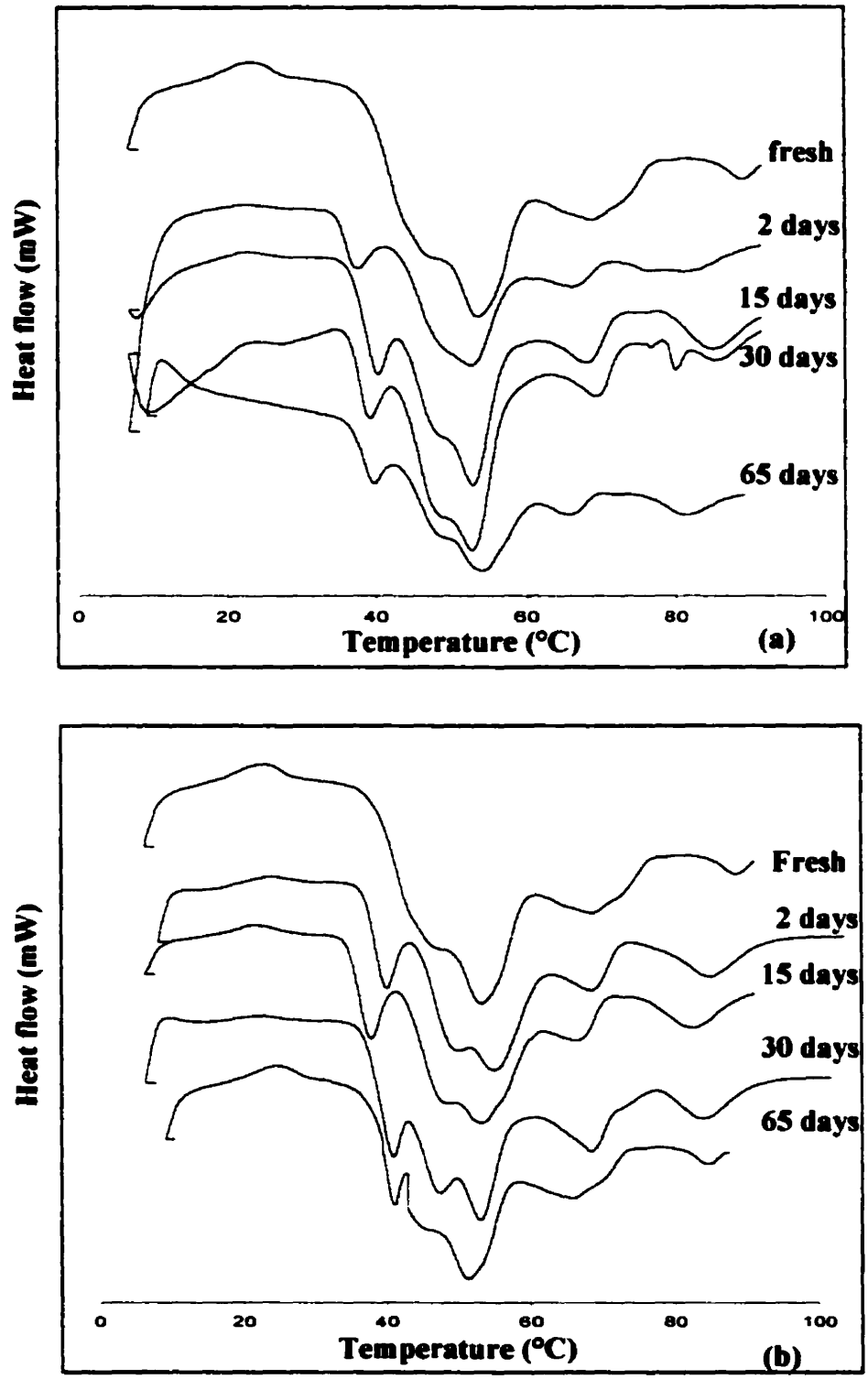

Fig. 7.6: Differential scanning calorimetry of carp fillet frozen by pressure-shift freezing. (a) pre-rigor fillets, (b) post-rigor fillets.

thermograms of mammalian whole muscles showed three transitions with T_{max} values of 57-60°C , 62-67°C, and 74-80°C (Wright et al., 1977; Wagner and Añón, 1986). Some ot the T_{max} values for carp muscle transitions were lower than those of mammalian muscles. Similar results were reported for other fish and mollusc species (Martens and Vold, 1976; Akahane et al., 1985; Howell et al., 1991; Paredi et al., 1994). Oogawa et al., (1993) suggested that the higher lability of fish species proteins compared with that of mammalian muscle proteins may be responsible for the lower temperature transitions of fish proteins. The differences among species in the T_{max} of the transitions seemed to be correlated to the habitat temperature of the fish (Hasting et al., 1985; Davies et al., 1988).

Both the ABF and PSF samples showed thermograms similar to that of fresh fish muscle. Wright et al., (1977) reported the presence of an exothermic peak for thermograms of pre-rigor rabbit muscle at 54°C. Similar results were reported by Park and Lanier (1988) using tilapia which showed a peak near 50°C. However, thermograms for fresh pre- and post-rigor carp muscle showed no peak differences around 50°C. It is thought that the dissapearance of the exothermal peaks begins with the onset of rigor and corresponds to a drop in both ATP and muscle pH. However, Paredi et al., (1995) found no differences between the thermograms of pre-rigor versus post-rigor *Aulacomya* adductor muscle. It was suggested that the exothermal peak was not evident because it coincides with the first endothermal transition at 50°C for fresh *Aulacomya* adductor muscle. In the present study, the endothermal transition of carp muscle is situated in the region between 47 to 53°C.

The combination of conventional freezing and frozen storage at -20°C had little effect on the thermal properties of whole carp muscle. However, after PSF and 2 days of frozen storage, the whole carp muscle showed a new peak at around 40°C that persisted up to 65 days of storage. The rest of the thermal transitions present in fresh fillets showed almost no change. Pressurized pre-rigor sample at 30 days showed a peak at 80°C. This peak could be the sarcoplasmic peak that is not always present, as it was mentioned above. However, further investigation would be needed to identify this peak. Myosin thermal denaturation has been shown to occurs in several structural domains capable of undergoing independent cooperative transitions (Privalov, 1992) depending on the source material and experimental conditions (pH, ionic strength). In Fig. 7.5b, the new peak at 40°C could be assigned to the subfragment S1 of myosin, while the thermal transitions around 47-48°C and 52-53.9°C were attributed to the myosin rod. The transition at 47-48°C may also be due to collagen. However because of the absence of the peak at 40°C in the fresh sample, the assigment of the peak to subfragment S1 is quite unlikely. On the other hand, Angsupanish and Ledward (1998) reported the appearance of a new peak at pressures higher than 100 MPa and suggested that this new thermal transition may reflect the formation of a hydrogen-bonded gel or precipitates. The new peak at 40°C was also present in post-rigor carp muscle that were subjected to PSF and frozen storage as shown in Figs. 7.5b and 7.6b. However, unlike the pre-rigor treated samples whose T_{max} of thermal transitions remained almost constant, the T_{max} for the post-rigor treated samples at 54.5°C and 69.1°C gradually decreased during frozen storage to 51.1°C and 62.2°C, respectively,

after 65 days of storage. Post-rigor freezing by itself is known to induce intercellular ice crystal formation resulting in protein denaturation (Shenouda, 1980). Similarly, pressure treatment may result in protein denaturation by affecting non-covalent bonds that may result in changes in secondary and tertiary protein structure. Thus, it is not surprising that the use of post-rigor pressure treatment produced a shift in some of the T_{max} characteristic of post-rigor whole carp muscle. For the ABF treated samples, the T_{max} of the second peak at 54.5°C and the third peak at 69.1°C gradually decreased during frozen storage, and after 65 days the second peak disappeared and the T_{max} of the third peak shifted to 62.1°C. These results suggest that the prevention of protein denaturation by PSF may be more effective when treatment is applied to post-rigor fish tissue held for a long period of time.

The total entalphy change (ΔH_T) data are shown in Table 7.1. The ΔH_T of ABF pre-rigor samples remained constant during frozen storage, however the ΔH_T of ABF post-rigor samples decreased significantly ($p<0.05$) after 65 days of frozen storage. In the case of the PSF samples, ΔH_T of the pre-rigor samples decreased significantly ($p<0.05$) after 65 days of frozen storage, while the ΔH_T of post-rigor samples remained constant during the frozen storage. These results confirm the suggestion that the application of PSF to post-rigor samples may be more effective in preventing protein denaturation during frozen storage.

Table 7.1: Effect of freezing process (air-blast freezing or pressure-shift freezing) and storage time at –20°C on the enthalpy of protein denaturation for carp fillets.

	Air-blast freezing		Pressure-shift freezing	
	Pre-rigor	Post-rigor	Pre-rigor	Post-rigor
Storage time *(days)*	ΔH_T[1] (J/g)	ΔH_T[1] (J/g)	ΔH_T[1] (J/g)	ΔH_T[1] (J/g)
Fresh	12.3 ± 2.8[a]	12.0 ± 0.3[a]	12.3 ± 2.8[ab]	12.0 ± 0.3[a]
2	11.3 ± 0.7[a+]	8.1 ± 0.9[b]	12.71 ± 0.03[a]	11.9 ± 0.7[a+]
15	13.7 ± 2.5[a]	9.6 ± 0.9[b]	11.9 ± 2.0[ab]	12.8 ± 2.5[a+]
30	11.8 ± 2.8[a]	11.7 ± 0.8[a]	10.6 ± 4.3[abc]	11.8 ± 2.8[a]
65	12.3 ± 0.4[a+]	8.0 ± 0.1[b]	8.1 ± 0.1[c]	12.5 ± 0.4[a+]

[1]Data are expressed as mean ± SD, n = 5.
+ sign means significant difference between (pre-rigor ABF and pre-rigor PSF) or between (post-rigor ABF and post-rigor PSF).
Means with the same letter in columns are not significantly different (p>0.05)

Histological analysis.

Fig. 7.7a shows micrographs of prefrozen carp muscle (control). The cross section of the untreated samples showed an uniform distribution of regular shape fibres. Figs. 7.7b and 7.7c show the micrographs of cross sections from ABF treated samples (pre-rigor and post-rigor, respectively). Ice crystals formed during this freezing process had irregular shapes. The ABF process is considered a slow freezing process (0.9 cm/h) which may cause important shrinkage of the cells and formation of large extracellular ice crystals (Love, 1968; Fennema, 1973). These ice crystals induced deformations of the tissue resulting in pooling the muscle fibres as reported by Bevilacqua and Zaritzky (1979), Grujic et al., (1993) and Martino et al., (1998) for beef and pork meat.

Micrographs of the fish samples frozen by PSF process (pre and post-rigor) and stored 2 days at –20°C are presented in Figs. 7.7d, 7.7e, respectively. Smaller and more uniform (rounded shape) ice crystals were obtained when PSF was used instead of ABF treatment (Table 7.2). It is believed that an uniform and isotopic ice nucleation results in isotropic ice crystal size. Burke et al., (1975) indicated that freezing by pressure release from 150 MPa is theoretically able to achieve supercooling around –14°C, and thereby induce a 140 fold increase in nucleation rate. It was suggested that a large number of small and homogenous ice crystal nuclei formed during depressurization, grew during the second step of the PSF process (atmospheric pressure). The large number of small crystals prevents tissue deformations and shrinkage of cells yielding a good quality

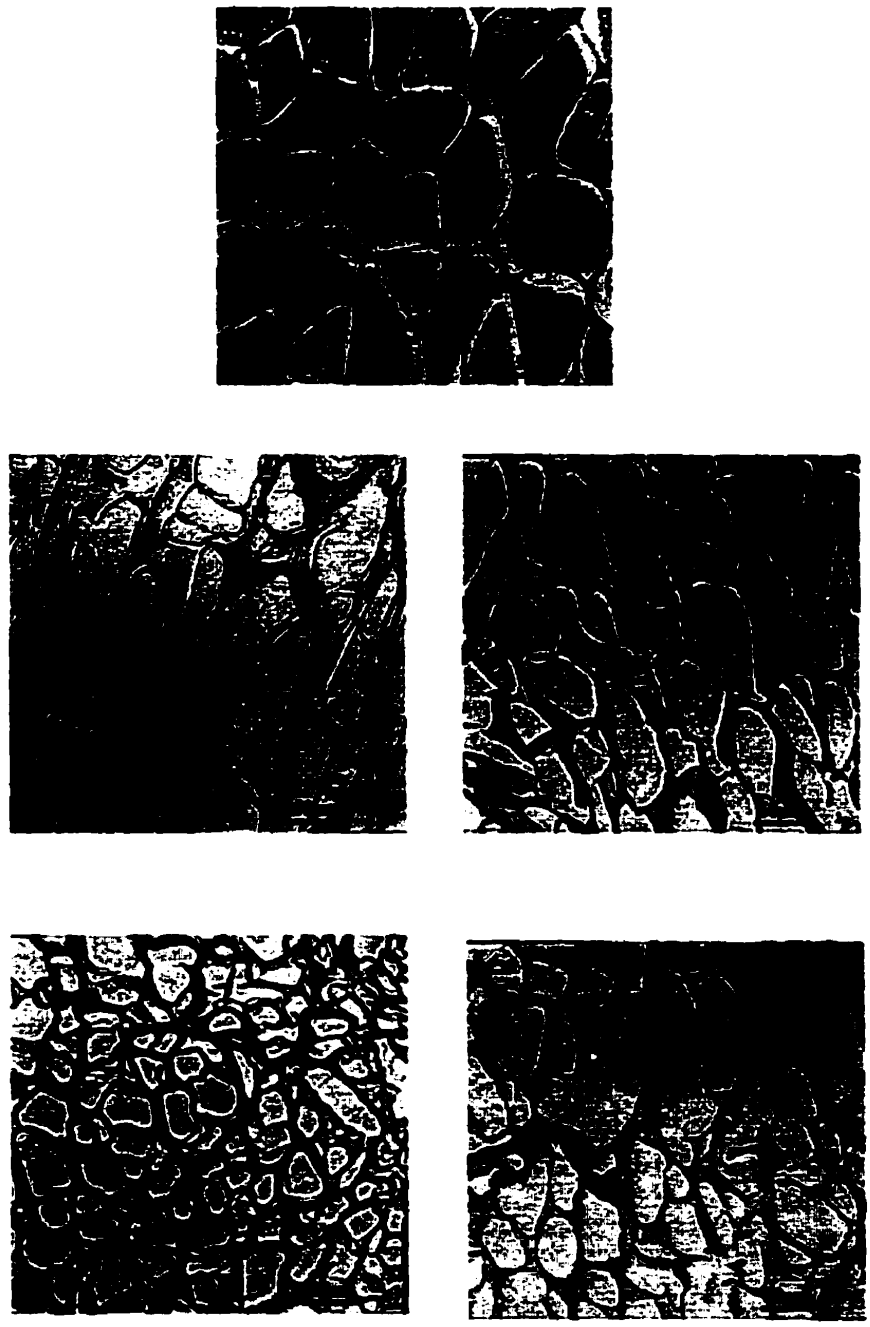

Fig. 7.7: Micrographs of carp fillet tissue. (a) fresh fillet, (b) air-blast frozen pre-rigor carp fillet after 2 days of frozen storage at –20°C; (c) air-blast frozen post-rigor carp fillet after 2 days of frozen storage at –20°C; (d) pressure-shift frozen pre-rigor carp fillet after 2 days of frozen storage at –20°C; (e) pressure-shift frozen post-rigor carp fillet after 2 days of frozen storage at –20°C.

Table 7.2: Ice crystal surface area formed in carp fillets frozen either by air-blast or by pressure-shift freezing.

	Air-Blast Freezing		Pressure-shift freezing	
Storage time (days)	Pre-rigor (μm^2) [1]	Post-rigor (μm^2) [1]	Pre-rigor (μm^2) [1]	Post-rigor (μm^2) [1]
2	2956 ± 524^a	3710 ± 824^a	412 ± 123^a	534 ± 115^a
65	3100 ± 722^a	4032 ± 631^a	452 ± 107^a	587 ± 145^a

[1]Data are expressed as mean ± SD n=40
Means with the same letter in columns are not significantly different ($p>0.05$)

product. Similar results were reported by Martino et al., (1998) on pork meat pieces frozen (-20°C) by pressure release at 200 MPa.

Micrographs of cross sections for carp fillets showed that samples treated in the post-rigor state experienced more tissue disruption than those treated in the pre-rigor state. In the pre-rigor state, the cell fluid is tightly bound to intracellular proteins, which limits its diffusibility from the inside to the outside of the cell. Once frozen, ice crystals are formed mainly intracellularly. On the other hand, in post-rigor muscle tissue, some of the cellular fluids are free to diffuse into the extracellular spaces, in such a case inter- and intra-cellular ice crytals are formed depending on the freezing rate (Shenouda, 1980).

CONCLUSIONS

This study shows the relative advantages and potential use of high pressure technology to increase freezing rates which result in a good quality product. The use of PSF was effective in reducing the size of ice crystals within the fish samples. Furthermore, the ice crystals showed more uniform shape resulting in less disruption of fish tissue. This fact could explain the decrease in cooking losses for PSF samples. In general, total losses of fish fillets were reduced by using PSF treatment. DSC studies suggested that myofibrillar proteins, especially actin and myosin, were affected not only by the freezing treatment but also by the time of frozen storage. The physiological state of the fish fillets plays an important role in the quality of products frozen either by ABF or PSF treatment.

CHAPTER VIII

GENERAL CONCLUSIONS

1. Changes in biochemical properties of actomyosin extracted from intact carp fillets stored on ice showed that proteolityc changes due to endogenous enzymes in fish muscle play an important role in quality deterioration of carp fillets. No changes were observed in Ca^{2+}-ATPase, Mg^{2+}-ATPase or Mg^{2+}-EGTA-ATPase activity for actomyosin from carp fillets during iced storage. In contrast, Mg^{2+}-Ca^{2}-ATPase and Ca^{2+} sensitivity of actomyosin decreased during storage. Autodegradation products increased constantly during ice storage. Ice storage remains a limited procedure in preserving seafoods.

2. Analysis of the combined effect of pressure and time at low temperature by response surface methodology indicated that the mathematical model developed was effective in predicting responses with a relatively good degree of accuracy (r^2 ranges from 0.70 to 0.98). Application of response surface methodology for analyzing combinations of hydrostatic pressure and time could be a very useful tool in studying high pressure effects on muscle from different fish species, and to define processing conditions for pressure-shift freezing. From this work, processing conditions of (140-175 MPa, 16-18 min) seem to result in less protein denaturation, indicating that under these conditions the detrimental effects that pressure-shift freezing may induce on the quality of carp fillets can be reduced.

3. The use of high pressure treatment on seafoods may be limited because of detrimental factors that it may induce on the raw material. Protein denaturation, changes in color and lipid degradation could arise as a result of extreme conditions of pressure and time. In the case of lipid degradation it was observed that TBA values as well as FFA content of HP treated samples increased as pressure increases. On the other hand, TBA values and FFA content increased as time of HP treatment increases at the same pressure level. Components such as myoglobin, hemoglobin and ferritin, present in fish muscle, may play a key role during lipid oxidation caused by pressure treatment. The results reflect the importance in determining the best conditions for HP treatment in order to reduce the production of oxidation products. These products may represent a problem in seafoods that will be stored for long periods of time after HP treatment.

4. Pressure-shift freezing was not effective in preventing a decrease in myofibrillar and sarcoplasmic protein extractability as well as actomyosin Ca^{2+}-ATPase activity during frozen storage. However, actomyosin Ca^{2+}-ATPase activity in pressure-shift frozen samples remained relatively higher than those of air blast frozen samples. On the other hand, pressure-shift freezing was effective in retarding lipid degradation of frozen carp fillets during the first 2 months of storage. No effect of freezing treatment was observed on the amount of thawing losses for carp fillets. Pressure-shift freezing reduced cooking losses for thawed carp fillets; the increase in water content of cooked

fish may counteract the effect of pressure denaturation resulting in no effect on toughness of the fish fillets.

5. Pressure-shift freezing yields a product with smaller ice crystals that are mainly intracellular and more regular than those found in ABF samples, which were mainly extracellular. No changes in size of ice crystals during frozen storage were observed. The total entalphy change (ΔH_T) data obtained from differential scanning calorimetry indicated that the physiological state of the fish fillets plays an important role in the quality of products frozen either by ABF or PSF treatment. The application of PSF to post-rigor samples rather than to pre-rigor samples is more effective in preventing protein denaturation during frozen storage.

6. This study shows the potential advantages of using pressure-shift freezing treatment to improve and extend the quality of fish fillets. The results obtained give some basis for establishing new treatment conditions for the processing seafoods.

BIBLIOGRAPHY

Ackman, R.G. 1994. Seafood Lipids. In *Seafoods: Chemistry, Processing Technology and Quality.* (F. Shahidi and J.R. Botta, Eds.) pp. 34-48. Blackie Academic & Professional. London.

Akahane, T., Chihara, S., Niki, T.P., Santo, T., Tsuchiya, T., Noguchi, S.F., Ookami, H. and Matsumoto, J.J. 1985. Differential scanning calorimetric studies on thermal behavior of myofibrillar proteins. Bull. Jap. Soc. Sci. Fish. 51:1841.

Angsupanich, K. and Ledward, D.A. 1998. High pressure treatment effects on cod (*Gadus morhua*) muscle. Food Chem. 63(1):39-50.

Angsupanich, K., Edde, M. and Ledward, D.A. 1999. Effects of high pressure on the myofibrillar proteins of cod and turkey muscle. J. Agric. Food Chem. 47:92-99.

Anon.(1986) International Institute of Refrigeration. Recommendations for the processing and handling of frozen foods (3th ed.) pp.32-39, Paris: International Institute of Refrigeration

Ashie, I.N.A. and Simpson, B.K. 1996. Application of high hydrostatic pressure to control enzyme related fresh seafood texture deterioration. Food Research Int. 29(5-6):569-575.

Ashie, I.N.A., Lanier, T.C. and MacDonald. G.A. 1999. Pressure-induced denaturation of muscle protein and its prevention by sugars and polyols. J. Food Sci. 64: 818-821.

Ashie, I.N.A., Smith, J.P. and Simpson, B.K. 1996. Spoilage and shelf-life extension of fresh fish and shellfish. Crit. Rev. Food Sci. and Nutr. 36(1-2) :87-121.

Awad, A., Powrie, W.D. and Fennema, O. 1969. Deterioration of fresh-water whitefish muscle during frozen storage at –10 C. J. Food Sci. 34(1):1-9.

Balny, C. and Masson, P. 1993 Effects of high pressure on proteins. Food Rev. Int. 9(4):611-628

Barry, H., Dumay, E.M. and Cheftel, J.C. 1998. Influence of pressure-assisted freezing on the structure, hydratation and mechanical properties of a protein gel. In *High Pressure, Food Science, Bioscience and Chemistry.* (N.S. Isaacs, ed.) pp 343-353, Royal Society of Chemistry, London..

Benjakul, S. Seymour, T., Morrisey, M.T. and An, H. 1997. Physicochemical Changes in Pacific Whiting muscle proteins during iced storage. J. food Sci. 62: 729-733.

Bevilacqua, A., Zaritzky, N. E. and Calvelo, A. 1979. Histological measurements of ice in frozen beef. J. Food Technol. 14(3): 237-251.

Bonnell, A.D. 1994. Raw material quality. In *Quality Assurance in Seafood Processing : A Practical Guide.* (A. D. Bonnell, ed.) pp. 54-76. Chapman & Hall. New York.

Bremmer, A.H. 1992. Fish flesh structure and the role of collagen -its post-morten aspects and implications for fish processing. In *Quality Assurances in the Fish Industry*. (H.H. Huss. ed.) pp. 39-62.Elsevier Science Publishers. Amsterdam.

Brown, W.D. 1986. Fish muscle as food. In *Muscle as food.* (P.J. Betchel, ed.) pp. 406 - 451 Academic Press, Inc, Orlando.

Burke, M.J., George, M.F. and Bryant, R.G. 1975. Water relations of Foods. Food Science and Technology monographs. Duckworth. Academic Press, London.

Busconi, L., Folco, E.J., Martone, C.B., Trucco, R.E. and Sanchez, J.J. 1989. Fish muscle cytoskeletal network: Its spatial orgnization and its degradation by an endogenous serine proteinase. Arch. Biochem. Biophys. 268:203-208.

Buttkus, H. 1971. The sulfhydril content of rabbit and trout myosin in relation to protein stability. Can. J. Biochem. 49:97-107

Charm, S.E., Longmaid, H.E. and Carver, J. 1977. A simple system for extending refrigerated, nonfrozen preservation of biological material using pressure. Criobiology. 14:625-636.

Cheah, P.B. and Ledward, D.A. 1996. High pressure effect on lipid oxidation in minced pork. Meat Sci. 43(2):123-134.

Cheah, P.B. and Ledward, D.A. 1997. Catalytic mechanism of lipid oxidation following high pressure treatment in pork fat and meat. J. Food Sci. 62(6):1135-1141.

Cheftel, J.C. and Culioli, J. 1997. Effects of high pressure on meat: a review. Meat Science 46(3):211-236.

Chen , P.S., Toribara, T. and Wagner, H. 1956. Microderterminations of phosphorus. Anal. Chem. 28(7):1756-1758

Chevalier, D., Le bail, A., Chourot, J.M. and Chantreau, P. 1999. High pressure thawing of fish (*whiting*): Influence of the process parameters on the drip volume. Lebensm.-Wiss.U.-Technol 32(1):25-31.

Chourot, J.M., Boillereaux, L., Havet, M. and Le Bail, A. 1997. Numerical modeling of high pressure thawing: application to water thawing. J. Food Eng. 34(1): 63-75

Connell, J.J. 1968. The effect of freezing and frozen storage on the proteins of fish muscle. In *Low Temperature Biology Foodstuffs*. pp. 333-358. (J. Hawthorn, ed.) Pergamon Press. Oxford.

Davies, J. R., Bardsley, R.G., Ledward, D.A. and Poulter, R.G. 1988. Myosin thermal stability in fish muscle. J. Sci. Food Agric. 45(1):61-68.

Davis, H.K. 1993. Modified atmosphere packaging of fish. In *Principles and Applications of Modified Atmosphere of Food*. (R.T. Parry, ed.) pp. 189-228. Blackie, London.

Davis, H.K. 1995. Quality and deterioration of raw fish. In *Fish and fishery Products : Composition, Nutritive Properties and Stability,* (A. Ruiter, ed.) pp. 215-241, CAB International, Wallingford.

Denys, S., Van Loey, A.M., Hendrickx M.E and Tobback, P. 1997. Modeling heat transfer during high-pressure freezing and thawing. Biotechnology Progress. 13: 416-423.

Dias, J. Nunes, M.L. and Mendes R. 1994. Effect of frozen storage on the chemical and physical properties of black and silver scabbardfish. J. Sci Food Agric. 66(4): 327-335.

Domenburg, H. and Knorr, D. 1998. Monitoring the impact of high-pressure processing on the biosynthesis of plant metabolites using plant cell cultures. Trends Food Sci. Technol. 9(2):355-361.

Dyer, W. J. and Dingle, J.R. 1961. Fish proteins with special references to freezing. In *Fish as Food Vol. 1.* (G. Borgstrom, ed.) pp. 275-327. Academic Press, New York.

Ellman, G.L. 1959. Tissue sulfydryl groups. Arch. Biochem. Biophys. 82:70-77.

Elvevoll, E.O, Sorensen, N.K., Osterud, B., Ofstad, R. and Martinez, I. 1996. Processing of marine foods. Meat Sci. 43:265-275.

Erickson, M.C. 1997. Lipid oxidation: flavor and nutritional quality deterioration in frozen foods. In *Quality in Frozen Food.* (M. C. Erickson and Y.C. Hung eds.) pp. 141-173, Chapman & Hall. New York.

FAO. 1995. Fisheries at the limits? In *Dimensions of Need*. (T. Loftas, ed.) pp 127-128, FAO, Rome, Italy.

FAO. 1997. FAO yearbook – fishery statistics, commodities, 1995. 81: A-1. FAO, Rome, Italy.

Farr, D. 1990. High pressure technology in the food industry. Trends in Food Sci. Technol. 1(1):14-16.

Fennema, O.R. 1973. Nature of freezing process. In *Low Temperature Preservation of Food and Living Matter* (O.R. Fennema, W.D. Powrie and E.H. Marth, eds.) pp. 126-145. Marcel Dekker, New York.

Frankel, E.N. Chemistry of free radical and singlet oxidation of lipids. 1985. Prog. Lipid Res. 24: 197-221.

Fuchigami, M. and Teramoto, A. 1997. Structural and textural changes in kinu-tofu due to high-pressure-freezing. J. Food Sci. 62(3): 828-832, 837.

Fuchigami, M., Kato, N. and Teramoto, A. 1997a. High-pressure-freezing effects on textural quality of carrots. J. Food Sci. 62: 804-808.

Fuchigami, M., Kato, N. and Teramoto, A. 1998. High-pressure-freezing effects on textural quality of Chinese cabbage. J. Food Sci. 63: 122-125.

Fuchigami, M., Miyazaki, K., Kato, N., and Teramoto, A. 1997b. Histological changes in high-pressure-frozen carrots. J. Food Sci. 62(3): 809-812.

Garthwaite, G.A., 1992. Chilling and freezing of fish. *In Fish Processing Technology*. (2nd Ed.) (G.M. Hall, ed.) pp. 93-118, Blackie A & P, London

George, R.M. 1993. Freezing processes used in the food industry. Trends in Food Sci & Technol. 4(5):134-138.

German, J.B. 1990. Muscle lipids. J. Muscle food. 1(4): 339-361.

German, J.B. and Kinsella, J.E. 1986. Hydroperoxide metabolism in trout gill tissue: effect of glutathione on lipoxygenase products generated from arachidonic acid and docosahexaenoic acid. Biochim. Biophys. Acta. 879: 378-387.

Gibson, D.M. and Davis, H.K. 1995. Fish and shellfish products in sous vide and modified atmosphere packs. In *Principles of Modified-Atmosphere and Sous Vide Products Packaging*. (J.M. Farber and K.L. Dodds, eds.) pp. 153-174. Technomic Publisshing Company Book. Pennsylvania.

Gornall, A.G., Bardawill, C.J. and David, M.M. 1949. Determination of serum proteins by means of the biuret reaction. J. Biol. Chem. 177: 751-766.

Gould, G.W. 1995. The microbe as high pressure target. In *High Pressure Processing of Foods*. (Ledward, D.A., Johnston, D.E. Earnshaw, R.G. and Hasting, A.P.M., eds.)pp. 27-35. University Press, Nottingham.

Greene, B.E and Price, L.G. 1975. Oxidation-induced colour and flavour changes in meat. J. Agric. Food Chem, 27:838-842.

Gross, M. and Jaenicke, R. 1994. Proteins under pressure. Eur. J. Biochem. 221(3) :617-630.

Grujic, R., Petrocic, L., Pikula, B. and Amidzic, L. 1993. Definition of the optimun freezing rate –1. Investigacions of structure and ultrastructure of beef *M.*

Longissimus dorsi frozen at different freezing rates. Meat Sci. 33(3):301-318.

Haard, N.F. 1992. Technological aspects of extending prime quality of seafood : a review. J. of Aquatic Food Prod. Technol. 1 (3/4) :1992

Haard, N.F. 1994. Protein hydrolysis in food. In *Seafoods : Chemistry, Processing Technology and Quality*, (F. Shahidi and R. Botta, eds.) pp. 10-33, Chapman & Hall, London.

Hamada, I., Tsuji, K., Nakayama, T., and Niwa, E. 1977. Oxidative denaturation of actomyosin. Nipoon Suisan Gakkaishi. 43: 1105-1109.

Hansen, P. 1980. Fish preservation methods. In Advances in Fish Science and Technology. (J.J. Connell, ed.). pp. 28-34. Fishing News Book. Ltd, Farnham.

Hansen, P. 1988. The reduction of postharvest losses by improved catch handling and storage at sea. In *Postharvest fishery losses*, (M.T. Morrissey, ed.) pp 99-108. University of Rhode Island, Kingston, RI, USA.

Hastings, R.J., Rodger, G.W., Park, R., Matthews, A.D. and Anderson, E. M. 1985. Differential scanning calorimetry of fish mucle: the effect of processing and species variation. J. Food Sci. 50 (3): 503-510

Hayakawa, I., Kajihara, J., Mmorikawa, K., Oda,M and Fujio, Y. 1992. Denaturation of bovine serum albumin (BSA) and ovalbumin by high pressure, heat and chemicals. J. Food Sci. 57(2) 288-292.

Hayakawa, I., Linko, Y.Y. and Linko, P. 1996. Mechanism of high pressure denaturation of proteins. Lebensmittel-Wissenschaftund-Technology. 29(8):756-762.

Hayashi, R., Kawamura, Y. and Kunugi, S. 1987. Introduction of high pressure to food processing: preferential proteolysis of β-lactoglobuin in milk way. J. Food Sci. 52(4):1107-1108.

Hazell, T. 1982. Iron and zinc compounds in the muscle of meats of beef, lamb, pork and chicken. J. Sci. Food Agric. 33:1049-1056.

Hendrickx, M., Ludikhuyze,L., Van den Broeck, I. and Weemaes, C. 1998. Effects of high pressure on enzymes related to food qualilty. Trends in Food Sci. & Technol. 9(1) :197-203.

Heremans, K. 1995. High pressure effects on biomolecules. In *High Pressure Processing of Foods*. (Ledward, D.A., Johnston, D.E. Earnshaw, R.G. and Hasting, A.P.M., eds.)pp. 81-97. University Press, Nottingham

Hermasson, A.M and Akesson, C. 1975. Functional properties of added proteins correlated with properties of meat systems. J. Food Science, 40: 595-602.

Hoover, D. G., Metrick, C., Parineau, A.M., Farkas, D.F. and Knorr, D. 1989. Biological effects of high hydrostatic pressure on food microorganisms. Food technol. 43(3) :99-107.

Hoover, D. G. 1993. Pressure effects on biological systems. Food technol. 47(6) :150-155.

Howell, B.K., Matthews, A.D. and Donelly, A.P. 1991. Thermal stability of fish myofibrils a differentail scaning calorimetric study. Int. J. Food Sci. Technol. 26(3): 283-295.

Hsieh, R. J. and Kinsella, J.E. 1989. Lipoxygenase generation of specific volatile flavor carbonyl compounds in fish tissue. J. Agric. Food Chem. 37: 279-286

Hultin, H.O. 1992. Biochemical deterioration of fish muscle. In *Quality Assurances in the Fish Industry*. (H.H. Huss, ed.) pp. 125- 138, Elsevier Science Publishers. Amsterdam.

Hultin, H.O.1994. Oxidation of lipids in seafood. In *Seafoods- Chemistry, Processing Technology and Quality*. (F. Shahidi and J.R. Botta, eds.)pp. 49-74. Blackie Academic & Professional. London.

Huss, H.H. 1988. Fresh fish-quality and quality changes. pp.10-22. Food and Agricuture Organization, Rome.

Igene, J.O., King, J.A., Pearson, A.M. and Gray, J.I. 1979. Influence of haem pigments, nitrite and non-haem iron on development of warmed over flavour in cooked meat. J. Agric. Food Chem 27:838-842.

Indrawati, A., Van Loey, S., Denys, S. and Hendrickx, M. 1998. Enzyme sensitivity towards high pressure at low temperature. Food Biotech. 12(3) :263-277.

Ishizaki, S., Hayashi, T., Tanaka, M. and Taguchi, T. 1995. High pressure effects on lecithin-added yellowfin tuna actomyosin ATPase activities. Fisheries Sci. 61(4):703-705.

Iso, S., Mizuno, H., Ogawa, H., Mochizuki, Y and Iso, N. 1994. Differential scanning calorimetry of pressurized fish meat. 60: 127-128

Jiang, S.T. Hwang, D.C. and Chen, C.S. 1986. Denaturation and change in SH group ofa ctomyosin from milkfish (*Chanos chanos*) during storage at –20 °C. J. Agric. Food Chem. 36:433-437.

Jiang, S.T., Tsao C.Y. and Ho, M.L. 1985. Effect of the temperature fluctuation on the meat quality of frozen mackerel (*Scomber tapeinocephalus*). In *Storage Lives of Chilled and Frozen Fish and Fish Products*. (Institute International du Froid, ed.) pp. 63-69. Institute International du froid, Paris.

Jimenez-Colmenero, F., Carballo, J., Fernandez, P., Barreto, G., Solas, M.T. 1997. High pressure-induced changes in the characteristics of low-fat and high fat sausages. J. Sci. Food Agric. 75: 61-66.

Johnston, D.E. 1995. High pressure effects on milk and meat. In *High Pressure Processing of Foods*. (Ledward, D.A., Johnston, D.E. Earnshaw, R.G. and Hasting, A.P.M., eds.)pp. 1-5. University Press, Nottingham.

Josephson, D.B., Lindsay , R.C. and Stuiber, D.A. 1987. Enzymic hydroperoxide initiated effects in fresh fish. J. Food Sci. 52: 596-600.

Kalichevsky, M.T., Knorr, D. and Lillford, P.J. 1995. Potential food applications of high-pressure effects on ice-water transitions. Trends Food Sci. Technol. 6(3): 253-258.

Kamal, M., Watabe, S., and Hashimoto, K. 1991. Post-mortem cahanges in myofibrillar ATPase of sardine ordinary and dark muscle. Nippon Suisan Gakkaishi 57:1177-1184.

Kanner, J., German, J.B., Kinsella, J.E. 1987. Initiation of lipid peroxidation in biological system: A review. CRC Crit. Rev. Food Sci. Nutr. 317.

Kato, A and Nakai, S. 1980. Hydrophobicity determined by a fluorescence probe method as its correlation with surface properties of proteins. Biochem. Biophys, Acta. 624: 13-20

Ke, P.J., Ackman, R.G. and Nash, D.M. 1975. Proposed rancidity indexes for assessing the storage life of frozen mackerel. New Series Circular 52 Environment Canada. Fisheries and Marine Service.

Keizer, C. 1995. Freezing and chilling on fish. In *Fish and fishery Products: Composition, nutritive properties and stability*. (A. Ruiter, ed.) pp. 287-313, CAB International Wallingford, UK.

Khayat, A. and Schwall, D. 1983. Lipid oxidation in seafood. Food Technol.37(7):130-140.

Kirk, R.S. and Sawyer, R. 1991. Pearson's composition and analysis of foods. 9th Ed. pp. 640-643. Longman Scientific and Technical, London.

Kitazawa, H., Kawai, Y., Inoue, N., and Shinamo, H. 1995. Influence of KCl on the decrease of Ca^{2+} ATPase activity and solubiltiy of carp myofibrils during frozen storage. Fisheries Sci. 61(6): 1037-1038.

Knorr, D. 1993. Effects of high-hydrostatic-pressure on food safety and quality. Food Technol. 47(6): 156-161.

Knoor, D. 1995. Hydrostatic pressure treatment of food : microbiology. In New Methods of Food Preservation. (G.W. Gould, ed.)pp. 159-175. Chapman & Hall, London

Ko, W.C., Tanaka, M., Nagashima, Y., Taguchi, T. and Amano, K. 1991. Effect of pressure treatment on actomyosin ATPases from flying fish and sardine muscle. J. Food Sci. 56(2):338-340.

Koch, H., Seyderhelm, I., Wille, P., Kalichevsky, M.T. and Knorr, D. 1996. Pressure- shift freezing and its influence on texture, colour, microstructure and rehydration behaviour of potato cubes. Nahrung 40:125-131.

Kurt, L.B. 1986. Effect of pressure-heat treatments on cathepsin B1 activity. J. Food Sci. 51(3):663-667.

Lanier, T.C. 1996. Gelation of surimi pastes treated by high isostatic pressure. In *High Pressure Bioscience and Biotechnology* (R. Hayashi and C. Balny, eds). pp. 357-361. Elsevier Science Publishers. Amsterdam.

Le Bail, A., Chourot, J.M., Barillot, P. and Lebas, J.M. 1997. Congélation-décongélation a haute pression. Rev.Gen.Froid. 972: 51-56.

LeBlanc, E., LeBlanc R. and Blum, I. 1988. Prediction of quality in frozen cod (*Gadus morhua*) fillets. J. Food Sci. 53(3):328-340.

LeBlanc, E.L and LeBlanc, R.J. 1991. Determination of hydrophobicity and reactive groups in proteins of cod (*Gadus morhua*) muscle during frozen storage. Food Chem. 43:3-11.

Ledward, D.A. 1995. High pressure processing – the potential. In *High Pressure Processing of Foods*. (Ledward, D.A., Johnston, D.E. Earnshaw, R.G. and Hasting, A.P.M., eds.)pp. 1-5. University Press, Nottingham

Liston, J, 1982. Recent advances in the chemistry of iced fish spoilage. In Chemistry and Biochemistry of Marine Food Products, (R. Martin, G. Flick, C.E. Hebard and D.R. Ward, eds.) pp. 27-36, AVI Publishing Co., Westport, CT.

Liston, J. 1992. Bacterial spoilage of seafood. In *Quality Assurance in the Fish Industry*. (H.H. Huss, M. Jacobsen, and J. Liston, eds.) pp. 93-105. Elsevier, Amsterdam

Love, M. 1968. Ice formation in frozen muscle, In *Low Temperature Biology of Foodstuffs*. (J. Hawthorn &, E.J. Rolfe, eds.) pp 321- 360 Pergamon, Oxford.

Love, M. 1966. The freezing of animal tissue. In *Cryobiology*. (H.T. Meryman, ed.) pp. 317-405, Academic Press, New York..

MacDonald, G.A. and Lanier, T.C. 1994. Actomyosin stabilization to freeze-thaw and heat denaturation by lactate salts. J. Food Sci. 59(1):101-105.

MacFarlane, J.J and McKenzie, J. 1976. Pressure-induced solubilization of myofibrillar proteins. J. Food Sci. 41: 1442-1446.

MacFarlane, J.J. 1973. Pre-rigor pressurization of muscle: effects on pH, shear value and taste panel assessment. J. Food Sci. 38(2): 294-298.

MacFarlane, J.J. 1974. Pressure-induced solubilization of meat proteins in saline solution. J. Food Sci. 39(3): 542-547.

Mackie, I.M. 1993. The effects of freezing on flesh proteins. Food Rev. Int. 9(4):575-610.

Martens, H. and Vold, E. 1976. DSC studies of muscle protein denaturation. 22nd Eur.Meet. Meat Res. Wor. Malmo, Sweden, J.9

Martino, M. and Zaritzky, N. 1986. Fixing conditions in the freeze substitution technique for light microscopy observation of frozen beef tissue. Food Microstructure. 35: 19-24.

Martino, M.N., Otero, L., Sanz, P.D. and Zaritzky, N.E. 1998. Size and location of ice crystals pork frozen by high-pressure assisted freezing as compared to classical methods. Meat Sci. 50(2): 303-313.

Matsumoto. J.J 1979. Denaturation of fish muscle protein during frozen storage. In *Proteins at Low Temperatures*. (O. Fennema, ed.)pp. 205-224. Advances in Chemistry Series 180. American Chemical Society. Washington, D.C.

MacDonald, G.A. and Lanier, T.C. 1994. Actomyosin stabilization to freeze-thaw and heat denaturation by lactate salts. J. Food Sci. 59:101-105.

Melton, S.L. 1983. Methodology for following lipid oxidation in muscle foods. Food Technol. 37(7) :105-111.

Messens, W., Van Camp, J. and Huyghebaert, A. 1997. The use of high pressure to modify the functionality of food proteins. Trends Food Sci. Technol. 8(1):107-112.

Montecchia, C.L., Roura, S.I., Roldan, H., Perez-Borla, O. and Crupkin, M. 1997. Biochemical and physicochemical properties of actomyosin from frozen pre- and post-spawned hake. J. Food Sci. 62(3):491-495.

Morrison, C.R. 1993. Fish and shellfish. In *Frozen Food Technology*. (C.P. Mallet, ed.)pp.196- 236. Blackie Academic & Professional. London.

Morrissey, M.T., Wu, J.W., Lin, D.D., and An, H. 1993. Protease inhibitor effects on torsion measurements and autolysis of Pacific whiting of surimi. J. Food Sci. 58:1050-1054.

Morrissey, M.T.., Hardley, P.S., and An, H. 1995. Proteolytic activity in Pacific whiting and effects of surimi processing. J. aquatic Food Product Technol. 4: 5-18.

Morrow, J.B. 1992. A changing industry: from internal evolution to external responsiveness. In *Seafood 2000*. (E.G Bligh, ed.) pp 1- 11. Fishing News Books. Oxford.

Mullen, K and Ennis, D.M. 1979. Rotable designs in product development. Food Technol. 33:74-80.

Multilangi, W.A.M., Panyam, D. and Kilara, A. 1996. Funcitonal properties of hydrolysates from proteolysis of heat-denaturated why protein isolate. J. Food Sci. 61: 270-274.

Nawar, W. W. 1996. Lipids. In *Food Chemistry*. (R. Fennema, ed.) pp. 225-320. Marcel Dekker, In. New York.

Ngapo, T.M., Babare, I.H. and Mawson, R.F. 1999. The freezing and thawing rate effects on drip loss from samples of pork. Meat Sci. 53(3):149-158.

Niwa, E., Khoda, S, Kanoh, S., and Nakayama, T. 1986. Exposure of hydrophobic amino acid residues from myosin on freezing. Nippon Suisan Gakkaishi 52:2127-2130.

No, H.K and Storebakken, T. 1991. Color stability of rainbow trout fillets during frozen storage. J. Food Sci. 56(4):969-972, 984.

Ohshima, T., Nakagawa, T. and Koizumi, C. 1992. Effect of high hydrostatic pressure on the enzymatic degradation of phospholipids in fish muscle during storage. In *Seafood Science and Technology*. (E.G Bligh, ed.)pp. 64-75. Fishing News books, Oxford.

Ohshima, T., Ushio, H. and Koizumi, C. 1993. High pressure processing of fish and fish products. Trends in Food Sci. and Tech. 4(5):370-375.

Ohshima, T., Wada, S. and Koizumi, C. 1984. Effect of accumulated free fatty acid on reduction of salt-soluble protein of cod flesh during frozen storage. Bull Japan Soc. Sci. Fish. 50:1567-1572.

Okamoto, M., Mawamura, Y. and Hayashi, R. 1990. Application of high pressure to food processing: textural comparison of pressure and heat induced gels of food proteins. Agric.Biol. Chem. 54:183

Okasaki, E. and Fukuda, Y. 1996. Effect of water-soluble protein on pressure-induced gelation of Alaska pollack surimi. *In High Pressure Bioscience and Biotechnology* (R. Hayashi and C. Balny, eds). pp. 363-368. Elsevier Science Publishers. Amsterdam.

Oogawa, M., Ehara, T., Tamiya, Y. and Tsuchiya, T. 1993. Thermal stabilility of fish myosin. Comp. Biochem. Physiol. 106B: 517-521.

Otero, L., Solas., M.T., Sanz, P.D., de Elvira, C. and Carrasco, J.A. 1988. Contrasting effects of high-pressure-assisted freezing and conventional air-freezing on eggplant tissue microstructure.Zeitschrift-fuer-Lebensmittel-Untersuchung-und-Forschung. 206(2): 338-342.

Paredi, M. E., Tomas. M. C., Crupkin, M. and Añón, M.C. 1994. Thermal denaturation of *Aulacomya ater ater* (Molina) myofibrillar proteins: a differential scanning calorimetric study. J. Agric. Food Chem. 42(5): 873-877.

Paredi, M. E., Tomas. M. C., De Vido de Mattio, N., Crupkin, M. and Añón, M.C. 1995. Postmortem changes in adductor muscles from *Aulacomya ater ater* (Molina) stored at 2-4°C. A differential scanning calorimetric study. J. Agric. Food Chem. 43(7): 1758-1761.

Park, J.W. and Lanier, T.C. 1988. Calorimetric changes during development of rigor mortis. J. Food Sci. 53: 1312-1314.

Pedroza-Menabrito, A. and Regenstein, J.M. 1988. Shelf-life extension of fresh fish- a review. Part I- spoilage of fish. J. Food Qual. 11: 117-127.

Pedroza-Menabrito,A. and Regenstein, J.M. 1990. Shelf-life extension of fresh fish- a review. Part II- preservation of fish. J. Food Qual. 13 :129-146.

Penniston, J.T. 1971. High hydrostatic pressure and enzymatic activity: Inhibition of multimeric enzymes by dissociation. Arch. Bioch. Biophy. 142:322-332.

Philippon, J. and Voldrich, M. 1993. Les hautes pressions: un adjuvant du froid. Rev. Gen. Froid. 968(8):27-32.

Privalov, P.L 1992. Stability of proteins. Adv. Protein Chem. 35, 31-43.

Reid, D.S. 1993. Basic physical phenomena in the freezing and thawing of plant and animal tissues. In *Frozen Food Technology*. (C.P. Mallet, ed.)pp. 1-35. Blackie Academic & Professional. London.

Reid, D.S. 1997. Overview of Physical/Chemical Aspects of Freezing. In *Quality in Frozen Food*. (M. C. Erickson and Y.C. Hung, eds.)pp. 10-28. Chapman & Hall. New York.

Reisler, E., Burke, M., and Harrington, W.F. 1974. Cooperative role of two sulfhydryl groups in myosin adenosin triphosphatase. Biochemistry 13: 2014-2019.

Roura, S.I. and Crupkin, M. 1995. Biochemical and functional properties of myofibrils from post-spawned hake (*Merluccius Hubsi Marini*) stored on ice. J. Food Sci. 60(2): 269-272.

Roura, S.I., Montecchia, C., Goldemberg, A.L., Trucco, R.E., and Crupkin, M. 1990. Biochemical and physicochemical properties of actomyosin ffom pre and post- spawned Hake (*Merluccious hubbsi*) stored on ice. J. Food. Sci: 55: 688-692

Santos-Yap, E. M. 1995 . Fish as Food. In *Freezing Effects on Food Quality*. (L.E. Jeremiah, ed.). pp. 109-133. Marcel Dekker, Inc. New York.

Sanz, P.D., Otero, L., de Elvira, C. and Carrasco, J.A. 1997. Freezing process in high-pressure domains. Int. J. Refrig. 20(5):301-307.

Schluter, O., George, S. Heinz, V. and Knorr. D. 1998. Phase transitions in model foods, induced by pressure-assisted freezing and pressure-assisted thawing. In High Pressure Research in the Biosciences and Biotechnology, (K. Heremans, ed.)pp. 317-324. Leuven University Press, Leuven.

Seki, N. and Narita, N. 1979. Changes in ATPase activities of carp myofibril during ice-storage. Bull. Japan Soc. Sci. Fish. 45:791-799.

Seki, N. and Narita, N. 1980. Changes in ATPase activities and other properties of carp myofibrillar proteins during ice-storage. Bull. Japan Soc. Sci. Fish. 46:207-231.

Sekine, T., and Yamaguchi, M. 1963. Effect of ATP on thebinding of N-ethylmaleimide to SH groups in the active site of myosin. J. Biochem. 54:196-201.

Sekine, T., Barnett, L.M., and Kielley, W.W. 1962. The active site of myosin adenosine triphosphate I. Localization of one of the sulfhydryl groups. J. Biol. Chem. 237: 2769-2772..

Shahidi, F. 1994. Seafood proteins and preparation of proteins concentrates. In *Seafoods: Chemistry, Processing Technology and Quality.* (F. Shahidi and J.R. Botta, eds.) pp. 1-9 . Blackie Academic & Professional. London.

Shenouda, S.Y.K 1980. Theories of protein denaturation during frozen storage of fish flesh. Adv. Food Res. 26:275-311.

Shenouda, S.Y.K., and Pigott, G.M. 1974. Lipid- protein interaction during aqueous extraction of fish protein: Myosin-lipid interaction. J. Food Sci. 39: 726-730.

Shenouda, S.Y.K., and Pigott, G.M. 1975a. Lipid- protein interaction during aqueous extraction of fish protein: Fish actin preparation and purification. J. Food Sci. 40: 520- 527.

Shenouda, S.Y.K., and Pigott, G.M. 1975b. Lipid- protein interaction during aqueous extraction of fish protein: Actin-lipid interaction. J. Food Sci. 40: 723-730.

Shenouda, S.Y.K., and Pigott, G.M. 1976. Electron paramagnetic resonance studies of actin-lipid interaction in aqueous media. J. Agric. Food Chem. 24 (1):11-18.

Shenouda, S.Y.K., and Pigott, G.M. 1977. Fish myofibrillar protein and lipid interaction in aqueous media as detected by isotope labeling, sucrose gradient centrifugation, polyacrylamide electrophoresis and electron paramagnetic resonance. *In Protein Crooslinking: Biochemical and Molecular Aspects.* Adv. Exper. Med. Biol. 86-A, 657.

Shewan, J.M. 1977. The bacteriology of fresh and spoiling fish and the biochemical changes induced by bacterial action. In *Proceeding of the Conference on Handling, Processing and Marketing of Tropical Fish.* p. 51. Tropical Products Inst. London.

Shewfelt, R. 1981. Fish muscle lipolysis - A review. J. Food Biochem. 5 (2): 79-100.

Sikorski, Z.E. and Kolakowska, A 1990. Freezing of marine food. In *Seafood-Resources, Nutritional Composition and Preservation.* (Z.E. Sikorski, ed.) pp. 112-124.,CRC Press, Boca Raton.

Sikorski, Z.E. and Kolakowska, A. 1994. Changes in proteins in frozen stored fish. In *Seafood Proteins.* (Z.E. Sikorski, B. Sun Pan and F. Shahidi, eds.)pp. 99-112. Chapman & Hall, New York.

Sikorski, Z.E., and Sun Pan, B. 1994. Preservation of Seafood Quality. In *Seafoods- Chemistry, Processing Technology and Quality*. (F. Shahidi and J.R. Botta, eds.)pp. 168-195. Blackie Academic & Professional. London.

Sikorski, Z.E., Kolakowska, A., and Burt, J.R. 1990a. Postharvest biochemical and microbial changes In *Seafood- Resources, Nutritional Composition and Preservation*. (Z.E. Sikorski, ed.)pp. 56-75. CRC Press, Boca Raton.

Sikorski, Z.E., Kolakowska, A., and Sun Pan, B. 1990b. The nutritive composition of the major groups of marine food organisms. In *Seafood-Resources, Nutritional Composition and Preservation*. (Z.E. Sikorski, ed.)pp. 29-54. CRC Press, Boca Raton.

Sikorski, Z.E., Olley, J., and Kostuch, S. 1976. Protein changes in frozen fish. Crit. Rev. Food Sci. Nutr. 8: 98-129.

Slabyl, B.M. and Hultin, H.O. 1984. Oxidation of a lipid emulsion by peroxidizing microsomal fraction from herring muscle. J. Food Sci. 49:1392-1393.

Smelt, J.P.P.M . 1998. Recent advances in the microbiology of high pressure processing. Trends in Food Sci. Technol. 9(1) :152-158.

Sokal, R.R. and Rolhf, F.J. 1995. . *Biometry*. 3rd Ed. pp. 328- 367, W.H Freeman & Co., New York.

Sompongse, W., Itoh, Y., and Obatake, A. 1996a. Effect of cryoprotectants and a reducing reagent on the stability of actomyosin during ice storage. Fisheries Sci. 62: 73-79.

Sompongse, W., Itoh, Y., and Obatake, A. 1996b. Role of Sha in the polymerization of myosin Heavy chain during ice storage of Carp actomyosin.. Fisheries Sci. 62: 110-113.

Statistics Canada, 1997a. Food consumption in Canada- Part II 1992. pp. 22-23. Minister of industry, Ottawa.

Statistics Canada, 1997b. Food consumption in Canada- Part II 1996. pp. 44-45. Minister of industry, Ottawa.

Tanaka, M., Xueyi, Z., Nagashima, Y. and Taguchi, T. 1991. Effect of high pressure on the lipid oxidation in sardine meat. Nippon Suisan Gakkaishi. 57(5):957-963.

Thakur, B.R. and Nelson, P.E. 1998. High-pressure processing and preservation of food. Food Rev. Int. 14(4):427-447

Tokiwa, T. and Matsumiya, J. 1969. Fragmentation of fish myofibril. Effect of storage condition and muscle cathepsin. Bull. Japan Soc, Sci. Fish. 35:1099-1109.

Venugopal, V. and Shahidi, F. 1995. Value-added products from underutilized fish species. Crit. Rev. Food Sci. & Nut. 35(5) :431-453.

Vyncke, W. 1970. Direct determination of the thiobarbituric acid value in trichloroacetic acid extracts of fish as a measure of oxidative rancidity. Fette, Seifen, Anstrichmittel, 72,12.

Wada, S. 1992. Quality and lipid changes of sardine meat by high pressure treatment. In *High Pressure and Biotechnology*. (C. Balny, R. Hayashi, K.

Heremans & P. Masson, eds) Vol 224: 253-238.Colloque INSERM/John Libbey Eurotec Ltd. Paris.

Wada, S. and Ogawa, Y. 1996. High pressure effects on fish lipids degradation: Myoglobin change and water holding capacity. *In High Pressure Bioscience and biotechnology*. (R. Hayashi and C. Balny, eds.)pp. 351-356. Elsevier Science. Amsterdam.

Wagner, J.R. and Añón, M.C. 1986. Effect of frozen storage on protein denaturation in bovine muscle 1. Myofibrillar ATPase actitivity and differential scanning calorimetric studies. J. Food Technol. 21(1): 9-18.

Ward, D.R. and Baj, N.J. 1988. Factors affecting microbiological quality of seafoods. Food Technol. 40(3) :85-89.

Wright, D.J., Leach, I.B. and Wilding, P. 1977. Differential scanning calorimetric studies of muscle and its constituents proteins. J. Sci. Food Agric. 28(4): 557-564.

Xiong, Y.L. 1997. Proteins denaturation and functionality Losses. In *Quality in Frozen Food*. (M. C. Erickson and Y.C. Hung, eds.)pp. 111-140. Chapman & Hall. New York.

Yoshikawa, K., Inoue, N., Kawai, Y. and Shinamo, H. 1995. Changes of the solubility and ATPase activity of carp myofibrils during frozen storage at different temperatures. Fisheries Sci. 61(5): 804-812.

Yoshioka, K., Yamada, A. and Maki, T. 1996. Application of high pressurization to fish meat: changes in the physical properties of carp skeletal muscle

resulting from high pressure thawing. In *High Pressure Bioscience and Biotechnology*. (R. Hayashi and C. Balny, eds.)pp. 369-374. Elsevier Science. Amsterdam.

Zayas,, J.F. 1995. Solubility of proteins. In *Functionality of Proteins in Food*. (J.F. Zayas, ed.)pp. 6-75, Springer-Verlag Berlin Heidelberg.

Zhao, Y., Flores, R.A. and Olson, D.G. 1998. High hydrostatic pressure effects on rapid thawing of frozen beef. J. Food Sci. 63(2): 272-275.