Investigation of cryoprotectants -treated protein deterioration during chilled and frozen storage: Electrophoretic pattern, functional properties and kinetic modeling

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Research Article

Keywords: Surimi protein, Cryoprotectants, Chilled and frozen storage, Functional properties, Modeling studies

Posted Date: August 18th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1960293/v1

Abstract

The relative cryoprotective effects of flaxseed protein and pectin in comparison with conventional cryoprotectant on stability of Capoor (*Cyprinus carpio*) surimi proteins during storage at -20°C for 4 months and at 4°C for 10 days were investigated. The results show that the reaction rate constants of second order kinetic, *k*, in samples stored at -20°C were much smaller than similar samples stored at 4°C, indicating decreased the rate of protein denaturation in frozen samples. Although, pectin caused to improve water-holding capacity (27.8%; 4°C and 21.5%; -20°C) on account of the formation of a separate gel and represent highly more inhibitory impact on the ice crystals growth, during the overnight soaking, protein denaturation may have been occurred. The flaxseed protein -treated sample showed the lowest decrease of SH content and reaction rate constant at both –20°C and 4°C than others. The results exhibited that salt extractable protein and sulfhydryl content changes were in good agreement with the second order kinetic model. Regarding to SDS- PAGE results, no major changes were observed in intensity of myosin heavy chain, actin and myosin light chain bands in flaxseed proteins treated surimi over chilled and frozen storage.

1. Introduction

Surimi, a wet concentrate of proteins, is the deboned, minced, and washed from fish myofibrillar protein, being blended with cryoprotectants to prepare the manufacture of seafood imitation products. Surimi and its derivative are perceived to have wholesome and nutritious attributes (Bashir et al., 2017; Martín-Sánchez et al., 2009).

Myofibrillar proteins with high functional properties are major proteins of fish muscle, contributing as most important proteins in surimi production (Azadian et al., 2012). Although, long-term storage of marine products is through chilling or freezing, the denaturation of myofibrillar proteins may take place which causes loss of functional properties in these proteins (Duangmal & Taluengphol, 2010). The addition of cryoprotectant is required in order to prevent denaturation of proteins and retain functional properties of protein (Monto et al., 2021). Diverse compounds were found to be used as cryoprotectant including low molecular weight sugars and polyols (sucrose, sorbitol, lactitol, palatinit, and maltodextrin), amino acids, carboxylic acids and polyphosphates (Cando et al., 2016; Cao et al., 2022; Iglesias-Otero et al., 2010; Monto et al., 2021). Saccharide as predominant cryoprotectant is usually used to prohibit protein denaturation and quality degradation of surimi. Moreover, surimi containing saccharide has been exhibited to be capable of maintaining a higher amount of protein than surimi without additives (Arpi et al., 2018). However, this commercial cryoprotectant leads to excessive sweetness and calories. Aside from affecting the taste of the surimi, this restricts the number of consumers on the market, indicating a need to find an alternative (Cao et al., 2022). Protein additives are widely used as proteinase inhibitors in surimi processing to improve the physical properties of surimi gels and control the activity of heat stable proteinase (Duangmal & Taluengphol, 2010; Monto et al., 2021). Flaxseed protein has no sweetness and contains low caloric value. It contains different levels of polysaccharide gums and emulsion stabilizing compounds which have been assessed as additives in food systems such as canned-fish sauce. Hydrocolloids such as carrageenan, pectin, carboxymethylcelluslose, and xanthan gums have been used as additives in the development of surimi products (Iglesias-Otero et al., 2010; Monto et al., 2021). Some textural benefits and decrease in production of dimethylamine and formaldehyde were reported for certain hydrocolloids (Martín-Sánchez et al., 2009).

The surimi industry demands from numerous white and underutilized species with some of the so-called “less valuated fish” (Bashir et al., 2017; Martín-Sánchez et al., 2009). Since great availability, low cost and good gel- forming ability of Capoor fish, it was used for surimi production in present study. Although numerous researches have referred the beneficial effect of diverse additives on surimi, the concomitant comparison three categories saccharide, proteinaceous and hydrocolloid cryoprotectants under chilling and freezing during storage time on functional and electrophoretic pattern as well as modeling study have been not demonstrated. The objectives of the presented study are to investigate water binding capacity (%), salt extractable protein (%), sulfhydryl content and electrophoresis pattern of Capoor surimi protein before and after adding different additive categories, namely flaxseed protein hydrolysate (proteinaceous compounds) and pectin (hydrocolloid compounds); to compare their effectiveness with that of the commercial blends (4% sucrose + 4% sorbitol + 0.2% sodium tripolyphosphates); and to study the modeling of protein deterioration during various time of chilling (4 ^{o}C) and freezing (-20 ^{o}C).

2. Materials And Methods

2.1. Materials

Pectin (low methoxyl), chlortetracycline and DTNB (s, s'-dithio-bis (2- nitrobenzoic acid) were purchased from Sigma company. Other chemical compounds were purchased from Merck Company. Capoor (*Cyprinus carpio*) fish was provided from Fars Fisheries Company of Iran and flaxseed protein was isolated from flaxseed meal and found in market.

2.2. Preparation of surimi

Surimi production was based on the method conducted by Moosavi-Nasab et al. (2005) with slight modification (Moosavi-Nasab et al., 2005). Following removing the dark muscle of the filleted Capoor fish, the cleaned filleted fish were weighed, then minced in a slicing machine using a 4 mm screen. The minced flesh was washed three times with cold water (at 4°C) for 15 min each cycle, at a ratio of mince: water 1: 4.

The first wash water contained 0.2% $NaHCO_3$ and 0.1% NaCl. This improvement is a result of a decreased rate of denaturation as the muscle pH is increased and solubility of the sarcoplasmic proteins of dark-flesh species (Capoor fish) is increased in this alkaline saline leaching solution. The enhanced removal of sarcoplosmic proteins also results in a lighter colored surimi due to removal of hem pigments. Additionally, the flavor is improved as the removal of carbonyl compounds is also enhanced. The final wash water contained 0.2% NaCl to facilitate dewatering. After each wash, minced flesh dewatered (Fig. 1). Final surimi (Raw surimi) divided in to 4 portions. One portion was used as control surimi without any additives (group 1). Commercial cryoprotectants including 4% sucrose and 4% sorbitol and 0.2% sodium tripolyphosphates were added to surimi (group 2). 1% flaxseed protein hydrolysate was added to another portion (Group 3) and 1% pectin was mixed with the fourth portion (Group 4).

Surimi (w/w) was directly incorporated with all additives except pectin by mixing at low speed in a Hobart mixer for 1 minute. Hydrocolloid (pectin) treatment was prepared by soaking 600g of surimi in solution (2 L) of 1% LMP. This solution was prepared by rapid agitation and heating to completely dissolve hydrocolloid. Surimi slurry was held at 4 ^{O}C overnight with agitation. The next day dewatering was carried out.

Finally, the various formulations of surimi samples were divided into two groups. One group was stored at -20°C for 4 months and another group was stored at 4°C for 10 days. Chlortetracycline (5PPM) and 1% potassium sorbate were added to the samples stored at 4°C to prevent growth of microorganisms. The frozen surimi samples were taken for analyses at times 0 and after 2- and 4-months storage at -20°C, and also surimi samples were taken for analyses at times 0 and after 5- and 10-days storage at 4°C.

2.3. Determination of water binding capacity (WBC)

WBC was determined according to Azadian et al. (2012) (Azadian et al., 2012). 5 mL of distilled water was added to 2.5 g of surimi. A mixture was mixed thoroughly and equilibrated at 4°C for 24 h, then centrifuged at 1930 g for 10 minutes with a Sorvall Centrifuge model MSE, England, at 4°C. The supernatant was decanted and the sediment weighted. WBC can be calculated by dividing the weight gain for each pellet by the original weight of each surimi.

2.4. Determination of salt extractable protein (SEP)

15 grams of surimi in 60 mL of 5% NaCl in 40 mM Tris-HCl pH 7.0 buffer was homogenized (Silverson homogenizer, England), at 1800 rpm for 1 min on ice. The homogenate was centrifuged at 9770 g for 10 min at 4°C using a Sorvall centrifuge model MSE, England. After centrifugation, the supernatant was decanted and to the pellet 60 mL of the same Tris-HCl buffer was added and mixture was the centrifuged at 9770 g at 4°C for 10 min. Using bovine serum albumin as a standard, the Lowry method was used to determine the protein concentration in the two supernatants from each sample. The amount of protein in the supernatant expressed as a percentage of the total protein content before centrifugation is called the SEP% (Sultanbawa & Li-Chan, 1998).

2.5. Preparation of natural actomyosin (NAM)

NAM was prepared as described by Benjakul et al. (1997) with some modifications (Benjakul et al., 1997). 10 g surimi was homogenized (silverson homegenizer, England) in 100 mL of chilled 0.6 M KCl (pH 7.0) for 4 min. The homogenate was centrifuged (Bio-Dynamic centrifuge, USA) at 8370 g for 30 min at 4°C. The precipitated NAM was mixed with three volumes of chilled distilled water. NAM was centrifugated at 8370 g (20 min at 4 ^{o}C), and the pellet was dissolved by stirring in an equal volume of 0.6 M KCl (pH 7.0). By centrifuging at 8370 g for 30 minutes at 4°C, undissolved debris was removed. NAM was maintained in ice during all analyses.

2.6. Determination of total sulfhydryl (SH) content

A modified method of Duangmal et al. (2010) was used for determining the total sulfhydryl content (Duangmal & Taluengphol, 2010). A 4.5 mL of 0.2M Tris-HCl buffer solution containing 8 M urea, 2% SDS and 10 mM EDTA (pH 6.8) was added to 0.5 mL of NAM suspension (4 mg/mL). Then, 0.4 mL of 0.1% DTNB in distilled water (pH 7.2) was added to 4 mL of the mixture and incubated at 40 ^{o}C for 25 min. In order to determine the absorbance at 412 nm, a spectrophotometer model MSE, England, was used. As a blank, 0.6 M KCl was substituted for the sample. SH content was determined by the extinction coefficient of 13600 M^{-1} Cm^{-1}.

2.7. Determination of drip loss

After 2- and 4-months storage at -20 ^{o}C, frozen surimi samples were thawed overnight at 4 ^{o}C, then the drip loss was determined.

2.8. Electrophoretic analyses

Under denaturing and reducing conditions, polyacrylamide slab-gel electrophoresis was performed as described by Duangmal et al. (2010). SDS-PAGE was performed on surimi samples at times 0 and after 2- and 4-months storage (for samples stored at -20 ^{o}C) and at time 0 and after 5- and 10-days storage (for samples stored at 4 ^{o}C) (Duangmal & Taluengphol, 2010).

2.9. Kinetic modeling

In order to determine the reaction rate of salt extractable protein and sulfhydryl content changes, the experimental data of surimi samples with different cryoprotectants were averaged and fitted to the first order and second order kinetic models. For the first order reaction, the equation can be expressed as follows (M. Van Boekel, 1996; M. A. J. S. Van Boekel, 2008):

$$\frac{dC}{dt} = -kC$$

1

By integrating both sides of the Eq. (1):

$$\ln\frac{C}{C_0} = -kt$$

2

$$C = C_0 e^{-kt}$$

3

where C_0 is the initial concentration, C is the concentration at time t, and k is the reaction rate constant of the first order reaction.

Assuming second order kinetic, the rate of reaction can be determined as follows (M. Van Boekel, 1996; M. A. J. S. Van Boekel, 2008):

$$\frac{dC}{dt} = -kC^2$$

4

Integration leads to:

$$\frac{1}{C} = \frac{1}{C_0} + kt$$

5

$$C = \frac{C_0}{1 + C_0 kt}$$

6

where *k* is the reaction rate constant of the second order reaction.

2.10. Statistical analysis

Each experiment was run in triplicates. Statistical analysis was carried out using SPSS. Analysis of variance (ANOVA) was also performed on all data, for each variable studies.

3. Results And Discussion

3.1. Water binding capacity (WBC) of surimi samples

Surimi gels' water binding capacity is determined by the amount of protein-water interactions, which gives an indication of how the water is linked within the protein matrix. WBC of surimi samples containing different cryoprotectants during storage at -20 ^{o}C and 4 ^{o}C are shown in Tables 1 and 2. As seen in the Table 1, all ingredients tested induced an increase in WBC irrespective of the additive after storage under storage at 4 ^{o}C and − 20 ^{o}C. After 4 months storage at -20 ^{o}C, WBC of the control surimi and surimi mixed with flaxseed protein, sucrose + sorbitol + polyphosphate and pectin, were as 0.36, 0.51, 0.54 and 0.62 (g/g), respectively. WBC decreased as follow, control surimi (58.1%) > surimi + with flaxseed protein (37%) > surimi + sucrose + sorbitol + polyphosphate (30.8%) > surimi + pectin (21.52%).

Table 1

Water binding capacity (g/g) and salt extractable protein (%), Sulfhydryl content (mol/ 10^5 g protein) and Drip loss (%) of surimi samples with different cryoprotectants during storage (0, 2 and 4 months) at -20 ^oC

Additives	Water binding capacity (g/g)			Salt extractable protein (%)			Sulfhydryl content (mol/ 10^5 g protein)			Drip loss (%)	
	0	2 m	4 m	0	2 m	4 m	0	2 m	4 m	2 m	4 m
Control	0.86± 0.04aA	0.42± 0.02bC	0.36± 0.04cC	69.8± 1.3aA	55.3± 0.5bB	46.3± 1.5cB	9.4 ± 0.3aA	4.6± 0.3bC	4.2 ± 0.2bB	15.0 ± 0.2aA	20.6 ± 0.6bA
Flaxseed protein	0.81± 0.04aAB	0.55 ± 0.02bB	0.51± 0.04bB	62.8± 0.1aC	56.9± 1.3bB	55.1± 1.5bA	8.7 ± 0.2aB	6.3± 0.1bA	5.8 ± 0.4bA	12.3 ± 0.3bB	14.6 ± 0.5aB
Sucrose + sorbitol + polyphosphate	0.78± 0.03aB	0.58± 0.04bAB	0.54± 0.03bB	66.2± 1.2aB	63.2± 0.7bA	49.4± 1.4cB	8.8 ± 0.4aB	5.9 ± 0.2bB	5.5 ± 0.4bA	11.5 ± 0.4aC	12.9 ± 0.7aC
Pectin	0.79± 0.03aB	0.64± 0.02bA	0.62± 0.03bA	22.6± 0.8aD	19.3± 0.2bC	15.8± 0.3cC	ND	ND	ND	9.8 ± 0.5aD	10.5 ± 0.5aD

*Data fallowed by different small and capital letters in a row and column are significantly different ($P < 0.05$), respectively.

Table 2

Water binding capacity (g/g) and salt extractable protein (%), Sulfhydryl content (mol/ 10^5 g protein) and Drip loss (%) of surimi samples with different cryoprotectants during storage (0, 5 and 10 days) at 4 ^oC

Additives	Water binding capacity (g/g)			Salt extractable protein (%)			Sulfhydryl content (mol/ 10^5 g protein)		
	0	5 d	10 d	0	5 d	10 d	0	5 d	10 d
Control with antibiotic	0.87± 0.04aA	0.69± 0.02bB	0.26± 0.03cD	69.1± 0.8aA	29.9± 1.2bB	27.3± 0.4cC	9.3 ± 0.2aA	5.9± 0.3bA	4.1 ± 0.3cB
Control without antibiotic	0.86± 0.04aA	0.68± 0.01bB	0.25± 0.02cD	69.6± 1.3aA	29.4± 0.9bB	26.9± 0.9cC	9.4± 0.3aA	5.8± 0.2bA	4.0± 0.5cB
Flaxseed protein	0.81± 0.04aAB	0.74 ± 0.02bA	0.39± 0.01cB	62.8± 0.1aC	48.5± 1.5bA	41.0± 0.5cA	8.7 ± 0.2aB	5.8± 0.1bA	4.6 ± 0.2cA
Sucrose + sorbitol + polyphosphate	0.78± 0.03aB	0.69± 0.02bB	0.35± 0.01bC	66.2± 1.2aB	48.3± 0.7bA	35.4± 0.7cB	8.8 ± 0.2aB	5.4 ± 0.1bB	4.2 ± 0.1cB
Pectin	0.79± 0.03aB	0.76± 0.03aA	0.57± 0.03bA	22.6± 0.8aD	16.9± 0.2bC	11.2± 0.9cD	ND	ND	ND

*Data fallowed by different small and capital letters in a row and column are significantly different ($P < 0.05$), respectively.

As shown in the Table 2, after 10 days storage at 4 ^oC, WBC of the control surimi containing antibiotic + sorbate (0.26 g/g), control surimi without antibiotic + sorbate (0.25 g/g), surimi mixed with flaxseed protein (0.39 g/g), sucrose + sorbitol + polyphosphate (0.35 g/g) and pectin (0.57 g/g), were determined. WBC decreased as order, control surimi ± antibiotic + sorbate (70%) > surimi + sucrose + sorbitol + polyphosphate (55.1%) > surimi + flaxseed protein (51.8%) > surimi + pectin (27.8%). As seen

in the results (Table 2), the decrease of WBC in control surimi containing antibiotic + sorbate was not significantly different from control surimi without antibiotic + sorbate. Therefore, antimicrobial agent didn't show cryoprotective effect in surimi protein.

This is obvious that different ingredients are able to bind water molecules with the various methods. In the case of commercial additive (4% sucrose + 4% sorbitol + 0.2% sodium tripolyphosphates), as phosphate ions are bonded to water, repulsion of protein groups is enhanced due to the predominance of negatively charged protein groups, reducing protein-protein interaction. This allows for more binding sites to be available for water in protein structures (Cando et al., 2016). On the other hand, in sugar alcohols, more OH groups can contribute to a higher likelihood of interactions with protein and water molecules (Abedi & Pourmohammadi, 2021).

Flaxseed protein contains cystine, lysine and other amino acid residues which promotes SH group protein oxidation to form intermolecular disulfide bonds (S-S) and -(γ-glutamyl)lysine crosslinks, respectively (Abedi & Pourmohammadi, 2021; Cando et al., 2016; Pourmohammadi & Abedi, 2021). Transglutaminase is endogenous enzyme which catalyze ε-(γ-glutamyl)lysine crosslinks and promotes a gel network structure to improve the gel quality (Duangmal & Taluengphol, 2010). In addition, Yuan et al. (2021) reported that L-glutamine (L-Glu) increased hydrogen bonds and electrostatic repulsions, modifying the microstructure of surimi, and therefore, promoting the WHC of surimi gels (Yuan et al., 2021). By destroying myofibrils, a three-dimensional gel network is prevented. This action is usually caused by fish's endogenous heat-activated protease (Duangmal & Taluengphol, 2010). Flaxseed protein possibly possesses as functional binders in surimi gels and also contain protease inhibitors (Udenigwe et al., 2009), resulting in increasing WBC.

A large number of hydrocolloids, namely carbohydrates (starch, carrageenan, alginates, xanthan and high methoxyl pectins) and proteins (fish gelatin, egg white, casein, and beef plasma protein) have been commonly used as additives in order to improve the mechanical and functional properties (water holding capacity) of surimi gels (Duangmal & Taluengphol, 2010; Hernández-Briones et al., 2009).

Results showed that during storage at both temperatures, WBC in all samples generally decreased, however, this reduction was significantly lower in surimi containing pectin than other samples and the highest decrease was found in the control surimi. At the end of storage at 4 ^{o}C, decrease of WBC in surimi containing flaxseed protein added surimi was significantly lower than sucrose + sorbitol + polyphosphate, while at the end of storage at -20 ^{o}C, WBC did not show significant difference between sucrose + sorbitol + polyphosphate added surimi + flaxseed protein.

As results conducted by Han et al. (2014), FTIR results showed that three peaks of hydrogen protons can be displayed, implying three water states, including free water (100–1000 ms), immobilized water (10–100 ms) and bound water (< 10 ms) (Han et al., 2014). Immobilized water, accounting over 95%, was depicted as the main water form in surimi, which usually generated between myofibrils and had a high relation with the fish's WHC (Jinjin Liu et al., 2013). Plus, the amount of bound water covered less than 2%, which is usually tightly bound to macromolecules thus having the lowest fluidity (L. Zhang & McCarthy, 2012). Meanwhile, free water occupied about 2%, which is easy to lose under the influence of external forces, reducing the surimi's ability to retain water. In consistent with Cao et al. (2022), compared with control surimi, surimi with additives (polyol, flaxseed protein and pectin) displayed an enhance in the amount of immobilized water and bound water, implying that the three additives could all limit the flow of water (Cao et al., 2022). Furthermore, it may be identified to fact that their interactions with proteins to promote their stabilization and significantly inhibit the deterioration of surimi quality (Juan Liu et al., 2016). Meanwhile, pectin was seen to enhance the most conspicuous restriction on water migration, in turn represent increasing the bound water. It suggested that by increasing the viscosity of the composite and forming a network structure using pectin, water flow can be restricted, in turn preventing ice crystal growth. Cao et al. (2022) examined WHC of surimi gels with different concentrations of inulin (1%, 4%, 8%, and 10% w/w) under various the number of freeze-thaw cycles. When the amount of additive was augmented to 4% and 8%, the WHC of surimi gels was significantly ($p < 0.05$) improved, indicating that 4% and 8% inulin + surimi gels exhibited the strongest WHC among repeated freezing and thawing (Cao et al., 2022).

Gandotra et al. (2012) and Duarte et al. (2020) noted that deterioration of fish quality in chilled storage (4 ^{o}C) have great impact than frozen storage (-20 ^{o}C) which could be attributed to protein denaturation and proteolysis caused by autolysis by cathepsin, calpain, and collagenase and enzymatic activities of psychrotrophic microbial growth (Duarte et al., 2020; Gandotra et al., 2012). In addition, peptides and free amino acids can be formed promoting the microbial growth and production of biogenic amines. In this regard, degradation rate relied on species and storage conditions. The enzymatic actions rate at chilled storage was recorded greater than frozen storage, implying to limit storage time in fatty fish (Duarte et al., 2020).

3.2. Salt extractable protein (SEP) of surimi samples

Myofibrillar proteins are soluble in salt solution. Salt solubility is considered as one of the vital characteristics of myofibrillar protein. Cryoprotective additives reduce protein denaturation by preserving salt-soluble proteins' extractability during cold (4°C) and frozen (-20°C) storage. The extractability of salt-soluble proteins following the various treatments over 4 months frozen storage and 10 days cold storage is shown in Tables 1 and 2.

As shown in Table 1, during 4 months storage at -20 ^{o}C, SEP % represent decrease pattern from 69.8–46.3% (control surimi) to 62.8–55.1% (flaxseed protein), 66.2–49.4% (sucrose + sorbitol + polyphosphate), and 22.6–15.8% (pectin) after adding diverse additives to surimi. The percent reduction in SEP % of different surimi formulations containing various additives was as follow, control surimi, 33.6%; flaxseed protein, 12.3%; sucrose + sorbitol + polyphosphate, 25.3% and pectin, 30.0%.

As shown in Table 2, during 10 days storage at 4°C, SEP % decreased in all surimi samples. The SEP % reduction of different surimi formulations was as order surimi + antibiotic + sorbate (60.4%), control surimi without antibiotic + sorbate (61%) > surimi + pectin (50.4%) > surimi + sucrose + sorbitol + polyphosphate (46.5%) > flaxseed protein hydrolyses (41%). After 10 days storage at 4 ^{o}C decrease of SEP% in control surimi were significantly higher than other samples.

Data analysis depicted that as a function of storage time and temperature, SEP% decreased significantly for control samples ($P < 0.05$). Generally, the samples protein solubilities decreased when the storage time increased and they were significantly different (P 0.05) and also the largest decrease occurred after 2 months (20°C) and 5 days (4°C). Concerning to SEP% data, various additive treatments and storage times had significant effects. The SEP% for control surimi decreases rapidly early during cold and frozen storage, whereas the SEP% for surimi treated with flaxseed protein hydrolyses remains relatively stable during this period. In other word, as measured by SEP%, the greatest stabilizing impact was indicated for flaxseed protein hydrolyses treatment, implying flaxseed protein hydrolyses protected myofibril proteins from freeze denaturation, in turn induce highest WBC among other treatments

Protein denaturation during cold and frozen storage may induced by the formation of hydrogen, disulfide or hydrophobic bonds, as well as ionic interactions (Monto et al., 2021; Nopianti et al., 2012). Protein solubility decreases during chilled and frozen storage as a result of denaturation (Iglesias-Otero et al., 2010; Ismail et al., 2012; Li et al., 2014; Nopianti et al., 2012; Zhou et al., 2006). In other word, surimi shows a slower decrease in solubility when a cryoprotectant is added, suggesting cryoprotectants may prevent denaturation of proteins. As threadfin bream surimi's protein solubility decreased dramatically, it indicates that proteins were denaturated due to frozen storage (Ismail et al., 2012; Nopianti et al., 2012). Polydextrose exhibited a great ability to maintain threadfin bream surimi's protein solubility as same as sucrose. Similarly, In the absence of a cryoprotectant, protein solubility in trehalose-treated tilapia surimi rapidly decreased during frozen storage (Li et al., 2014; Zhou et al., 2006).

In line with Sych et al. (1990), SEP data for LM Pectin hydrocolloid treated surimi was initially low (22 – 15% at – 20°C and 22 – 11% at 4°C) and also remained low throughout cold and frozen storage. Low SEP of pectin -treated surimi possibly implied loss of solubility owing to the incorporate hydrocolloid method. Hydrocolloids (pectin, carrageenan and xanthan) did not show any protective impact on extractable myosin. Although, hydrocolloids cause to improve water-holding capacity on account of the formation of a separate gel in the minced fish matrix and may make improvements to surimi products, during the soaking treatments in hydrocolloids overnight, protein denaturation may have occurred. Thus, their incorporation method is main limiting factor.

3.3. Sulfhydryl (SH) content of surimi samples

Among protein functional groups, SH groups are most reactive. When surimi is frozen, ice crystals increase intercellular osmotic pressure, protein molecules are denatured by salting or heavy metal action, and sulfhydryl groups are exposed to oxidation, causing a decrease in content and a concomitant increase in disulfide bonds. Therefore, the amount of total sulfhydryl in a protein is a good indicator of protein oxidation. As the amount of total sulfhydryl decreases, the more protein oxidation will occur (Cando et al., 2016; Lv et al., 2021; Pan et al., 2010). The produced free amino acid or small peptide due to autolysis or/and during chilling at chilled storage was recorded greater than frozen storage, facilitating to form disulfide linkage (Duarte et al., 2020).

SH content of surimi samples with different cryoprotectant during storage at -20 ^{o}C and 4 ^{o}C are shown in Tables 1 and 2. The SH content of all the samples varied as the function of additive and storage time. As storage time increased, the amount of SH decreased and was significantly different (P 0.05) from month to month in frozen storage (-20°C) and day to day in cold storage (4°C).

For surimi featuring a cryoprotectant, the SH contents showed the slower trend compared to control surimi during frozen storage. The flaxseed protein hydrolyses -treated sample showed the lowest decrease around 33% (-20°C) and 47.1% (4°C), followed by sucrose + sorbitol + polyphosphate 37.5% (-20°C) and 52.2% (4°C). There was not significantly difference between the SH content in surimi without antibiotic + sorbate and surimi containing antibiotic + sorbate stored at 4 ^{o}C. Therefore, antibiotic + sorbate did not show any cryoprotective effect in Capoor surimi. A similar decreasing trend in the SH content was also reported by Zhou et al. (2006), Pan et al. (2010) and Nopianti et aal. (20121) in tilapia, grass carp surimi and threadfin bream surimi treated with different cryoprotectant during frozen storage, respectively (Nopianti et al., 2012; Pan et al., 2010; Zhou et al., 2006). Besides, the same trend was observed by Qian et al. (2021) in beef myofibrillar protein after storage at − 1 to − 18°C for 28 to 168 days (Qian et al., 2021) and Turgut et al. (2016) in refrigerated beef meatballs (Turgut et al., 2016).

At both temperatures of storage, SH content in pectin added surimi was not measurable, probably denaturation of the protein may have occurred during the overnight soaking in pectin. The sharp diminish in the SH content of the raw surimi in present study indicates the denaturation of surimi protein. Furthermore, the lower rates of denaturation in the other samples suggests that cryoprotection is effective at alleviating denaturation (Nopianti et al., 2012; Pan et al., 2010). During frozen storage, the ice crystal formation would lead to structural changes in myofibrillar protein. Due to conformational changes, the reactive SH groups of myosin molecules might be exposed. Thereby, it is believed that the decrease is caused by the formation of disulfide bonds via the oxidation of SH groups or disulfide interchange, which results in the aggregation of proteins during freezing or cold storage (Qian et al., 2021). The decrease in salt extractable protein is in agreement with the reduce in the total SH level. Moreover, previously Cando et al. (2016) stated improved redisposition of the proteins owing to the conversion of α-helical structures to β-sheets (Cando et al., 2016). The formation of a more ordered network was accompanied with a higher density of cross-links due to protein aggregation. All in all, in both storage temperatures, flaxseed protein hydrolyses retarded the oxidation of SH groups to disulfide bonds more than any other cryoprotectant. As the result, flaxseed protein depicted the most cryoprotective effect on Capoor surimi and can be an alternative for sucrose + sorbitol + polyphosphate.

3.4. Drip loss of surimi samples

Frozen surimi's drip loss is considered a key measure of its quality (Cao et al., 2022). Drip loss of surimi samples with different cryoprotectants after 4 months storage at -20^{o}C, are displayed in Table 1. After thawing, water loss rates of all surimi formulations augmented significantly as the time of storage increased ($p < 0.05$). The drip loss % of the control surimi, and surimi containing flaxseed protein, sucrose + sorbitol + polyphosphate and pectin were increased around 37.3%, 15.7%, 10.82% and 6.6%. As shown at the end of storage at -20 ^{o}C, drip loss (%), in control surimi was significantly higher than other samples and in pectin -treated surimi was remarkably lower than other samples.

Possibly there are two reasons for the reduction in water retention capacity of frozen aquatic products: firstly, there are two types of mechanical damage to muscle tissue, caused by ice crystals or internal stresses, which induces cell gaps to widen and

cell membranes to rupture, resulting in the loss of extracellular fluid and part of the internal fluid (Fig. 1) (Lv et al., 2021). It is possible that the crystallization of water during freezing storage could stretch and squeeze the fish muscle, causing deformations that cannot be completely recovered. Surimi could not re-absorb water due to the pores left by ice crystals and the muscle damage, causing drip loss to increase (Leygonie et al., 2012). In other words, proper cryoprotectants can be used to prevent the formation of large extracellular ice crystals, which would reduce the damage and drip loss caused by ice crystal formation. It could assume the water retention capacity of the system will elevate by introducing hydroxyl groups. These results confirmed that the cryopreservation of sucrose + sorbitol + polyphosphate and pectin could prevent drip loss which was actually associated to the appearance the hydroxyl groups to a great extent (Leygonie et al., 2012). Cao et al. (2022) determined growth of ice crystals in the presence and absence of cryoprotectant. There was significant damage to muscle fibers in the absence of cryoprotectant (Fig. 1), because the ice crystals were large and irregular, occupying much of the space and squeezing its structure (Cao et al., 2022). Conversely, the cryoprotectant-surimi mixtures produced small and regular ice crystals that did not damage the muscle tissue. In addition, the inhibition of nucleation and growth of ice crystals is caused by the restriction effect of additive on water by hydrogen bonding and a raise in amount of non-freezing water, implying that cryoprotectant containing hydroxyl group (sucrose + sorbitol + polyphosphate and pectin) represent a more significant inhibitory impact on the ice crystals growth than flaxseed protein hydrolysate. Therefore, inhibiting the growth of ice crystals in an additive-dependent manner. Furthermore, the hydroxyl groups of cryoprotectant embedded into the ice as a result of hydrogen bond interactions may have resulted in damage to the ice crystals (Zhu et al., 2019). The shape and size of ice crystals can also affect the degree of mechanical damage to surimi. Therefore, controlling size, shape and distribution of ice crystals in surimi, positively may cause to prevent freezing damage (Hashimoto et al., 2015; Leygonie et al., 2012). The second major issue is that the structure change of proteins decreases their ability to retain water, and the melted water cannot be reunited with the protein molecules and separated from them (Lv et al., 2021). Meanwhile, regarding the results obtained by SH content and SEP%, pectin might denature the protein structure and vital inhibitory effect on growth of ice crystals was ascribed to its hydroxyl groups.

Similar observation was identified by Cao et al. (2022) after adding different amount of inulin and Jittinandana et al. (2005) by utilizing of sucrose/sorbitol, trehalose, and trehalose/sorbitol as cryoprotectants (Cao et al., 2022; Jittinandana et al., 2005). Cao et al. (2022) proved that aside from binding to water, inulin could also interact with myofibrillar proteins via hydroxyl groups and prohibit the aggregation of myofibrillar proteins following freezing, displaying outstanding cryoprotective performance in cryopreservation of surimi. As a consequence, through conjugating cryoprotectant hydroxyl groups with myofibrillar proteins, they observed a reduction in thawing water loss in reconstructed protein. Similarly, xylooligosaccharides and carrageenan oligosaccharides were found to reduce the area of ice crystals through interaction with the crystals for frozen peeled shrimp, thereby restricting their growth and promoting their solvation of the crystals (B. Zhang et al., 2020).

3.5. Modeling of SH content and SEP of surimi samples

Experimental results of changes of the salt extractable protein and sulfhydryl content with time for surimi samples are presented in Figs. 2 and 3, respectively. For all samples, the amounts of salt extractable protein and sulfhydryl content decreased with time.

The data was fitted to first and second order reaction models by plotting ln (C/C_0) and $1/C$ versus t, respectively. The calculated parameters of k and R^2 are presented in Tables 3 and 4. According to Tables 3 and 4, the R^2 values were greater than 0.9 in most cases for the second order kinetic model, indicating that the changes of salt extractable protein and sulfhydryl content better fit to the second order than first order kinetic model. The results show that the reaction rate constants of second order kinetic, k, in samples stored at -20°C were much smaller than similar samples stored at 4°C, indicating that storage at -20°C decreased the rate of protein denaturation in samples. As the amount of salt extractable protein and sulfhydryl content in different surimi samples after 4 months frozen storage were higher than similar samples stored for 10 days at 4 ºC.

Table 3
Kinetic parameters for salt extractable protein changes in surimi samples during storage at -20 ºC and 4 ºC.

Additives	Temperature (ºC)	first order kinetic		second order kinetic	
		k (d^{-1})	R^2	k ($g.mg^{-1}.d^{-1}$)	R^2
Control with antibiotic	-20	-	-	-	-
	4	0.1197	0.58	0.0011	0.83
Control without antibiotic	-20	0.0036	0.99	0.00003	0.99
	4	0.1228	0.62	0.0011	0.86
Flaxseed protein	-20	0.0013	0.85	0.000009	0.92
	4	0.0462	0.96	0.0004	0.99
Sucrose + sorbitol + polyphosphate	-20	0.0022	0.85	0.00002	0.89
	4	0.0704	0.96	0.0007	0.97
Pectin	-20	0.0030	0.98	0.00009	0.98
	4	0.0727	0.98	0.0023	0.97

Table 4
Kinetic parameters for sulfhydryl content changes in surimi samples during storage at -20 ºC and 4 ºC.

Additives	Temperature (ºC)	first order kinetic		second order kinetic	
		k (d^{-1})	R^2	k ($g.\mu mole^{-1}.d^{-1}$)	R^2
Control with antibiotic	-20	-	-	-	-
	4	0.0889	0.97	0.0014	0.99
Control without antibiotic	-20	0.0081	0.76	0.0001	0.89
	4	0.0936	0.96	0.0015	0.98
Flaxseed protein	-20	0.0039	0.91	0.00006	0.92
	4	0.0777	0.92	0.0011	0.99
Sucrose + sorbitol + polyphosphate	-20	0.0046	0.87	0.00006	0.89
	4	0.0828	0.92	0.0012	0.99

On the other hand, for salt extractable protein at both temperatures of -20°C and 4°C, the pectin sample had the highest reaction rate constant even larger than the control sample, while the reaction rate of flaxseed protein was the lowest. The reaction rate of sucrose + sorbitol + polyphosphate was higher than that of the flaxseed protein, which indicates that the remained amounts of salt extractable protein in flaxseed sample were larger than the sucrose + sorbitol + polyphosphate sample after the same storage time. Moreover, for the sulfhydryl content, the second order reaction rate constants of flaxseed protein sample at both −20°C and 4°C were lower than sucrose + sorbitol + polyphosphate. Results show that modification of surimi with flaxseed protein hydrolyses had a positive effect in preserving salt extractable protein and sulfhydryl content of samples.

3.5. SDS-PAGE of surimi samples

The SDS-PAGE patterns of surimi samples with different cryoprotectants at 4 ^{o}C (time 0, 5 and 10-day storage) and − 20 ^{o}C (time 0, 2 and 4-month storage) are shown in Figs. 4 and 5.

The MHC and actin band intensity of all the samples reduced with storage time. Plus, the intensity changes in MHC and actin bands at -20 ^{o}C storage was greater than 4 ^{o}C. According SDS-PAGE results, control surimi in presence and absence of antibiotic + sorbate stored at 4^{o}C was similar, so the results of control surimi without antibiotic + sorbate have not been shown. At storage at 4 ^{o}C and − 20 ^{o}C, intensity of MHC band in control surimi was higher than cryoprotectant-treated surimi. This increase was most likely due to the polymerization of MHC and the enhanced cross-linking of MHC. Myosin contains 42 SH groups located in the head portion which was oxidized to disulfide bonds, the aggregation and subsequent insolubility of actin and myosin over the frozen storage (Nopianti et al., 2012; Pan et al., 2010). In the postmortem phase of refrigerated fish, cathepsin, calpain, and collagenase are responsible for the autolysis of proteins and collagen. In fact, early postmortem changes in the texture of fish are caused by lysosomal (cathepsins B and L) and cytosolic enzymes (calpain system), which are responsible for the hydrolysis of myosin heavy chains (MHC). The hydrolysis is mainly the result of cathepsins, while calpains are known to enhance the proteases' ability to hydrolyze myofibrillar proteins (Duarte et al., 2020). The result was in line with salt extractable protein which profoundly reduce pattern in control surimi during storage time. No major changes were observed in intensity of MHC, actin and MLC bands in cryoprotectant-treated surimi following storge at 4 ^{o}C and − 20 ^{o}C. On the contrary, the intensity of MHC and MLC band was more affected in pectin-treated surimi (line 5, 9 and 11) than other cryoprotectant-treated surimi and diminished which might be due to denaturation of proteins occurring during surimi soaking in pectin solution. The result was consisted with unmeasurable SH content in pectin-treated surimi. The similar result was reported by (Duangmal & Taluengphol, 2010) who stated that MHC band significantly decreased after adding transglutaminase and formation of ε-(γ-glutamyl)lysine isopeptide. Moosavi-Nasab (2005) performed SDS-PAGE on Alaska Pollock surimi samples and reported that the SDS-PAGE patterns of control surimi and surimi containing whey protein isolate, whey protein concentrate, soy protein isolate and flaxseed protein showed no substantial changes after 6 months storage at -20 ^{o}C and only fading of the MLC bands with MW of 23 KDa and MHC bands with MW of 136 KDa were observed (Moosavi-Nasab et al., 2005).

As the results of Native-PAGE (Fig. S1 and S2) of surimi samples at both temperatures of storage show, during preparation of surimi, in all surimi samples, disappearance of actin band (since time 0) was observed because of actin was converted to aggregates with high MW. These results were similar to findings of Moosavi-Nasab (2005), who reported during preparation of surimi, the disappearance of the actin band was accompanied by the appearance of the bands in the range of 100 to 300 KDa.

4. Conclusions

The results showed that during storage of surimi, functional properties and stability of proteins were reduced at both temperatures. However, this reduction was much lower in surimi containing cryoprotectant in comparison with the control surimi. All the results obtained after 4 months storage at -20 ^{o}C were comparable to those after 10 days storage at 4 ^{o}C. Changes of the salt extractable protein and sulfhydryl content with time were fitted to the first and second order kinetic models for different surimi samples at temperatures of -20°C and 4°C. Values of R^2 showed that the data better fit to the second order kinetic model than first order model. The reaction rate constant of flaxseed treated sample was the lowest in both temperatures. The results confirmed that flaxseed protein with no sweetness and considerable caloric value had a cryoprotective effect similar to sucrose + sorbitol + polyphosphate and even better. Therefore, it can be an alternative for sucrose + sorbitol + polyphosphate, which because of high sweetness and caloric value are undesirable for consumers in surimi.

In this research, results of SDS-PAGE showed no major changes in intensity of MHC, actin and MLC bands in surimi added with flaxseed proteins during storage at 4^{o}C and − 20^{o}C. Therefore, it can be an alternative for sucrose and sorbitol which because of high sweetness and caloric value are undesirable for consumers in Capoor surimi.

Declarations

Author Contribution

EA wrote the manuscript conceived and designed the research and analyzed the data. MM conceived and designed the research and analyzed the data. SM and LM conducted experiments. All the authors read and approved the manuscript.

Data Availability

Research data are not shared.

Funding statement

Not applicable.

Consent to Participate

The present paper has been approved by all named authors.

Consent for Publication

The present paper, which is original, has not been published before and is not currently being considered for publication elsewhere.

Ethical Approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest

The authors declare that they have no conflict of interest.

Competing Interests

The authors declare no competing interests.

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Supplementary Figures

Supplementary Figures S1 and S2 are not available with this version

Figures

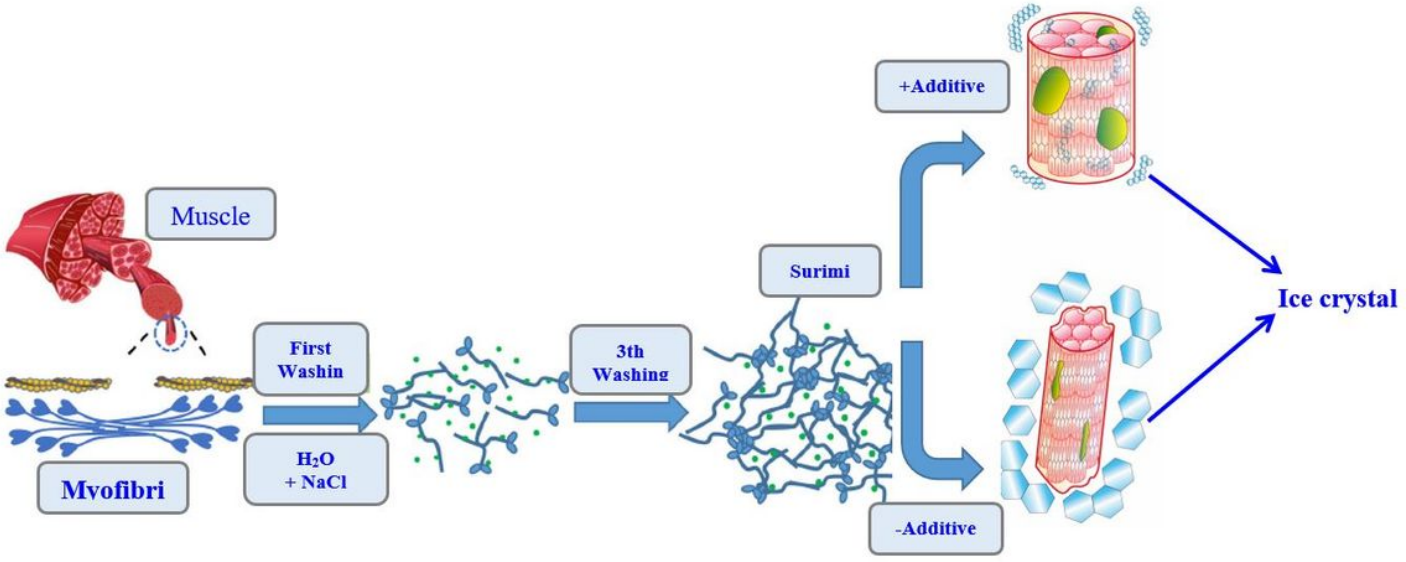

Figure 1

The role of additives in the processing of surimi products under frozen storage.

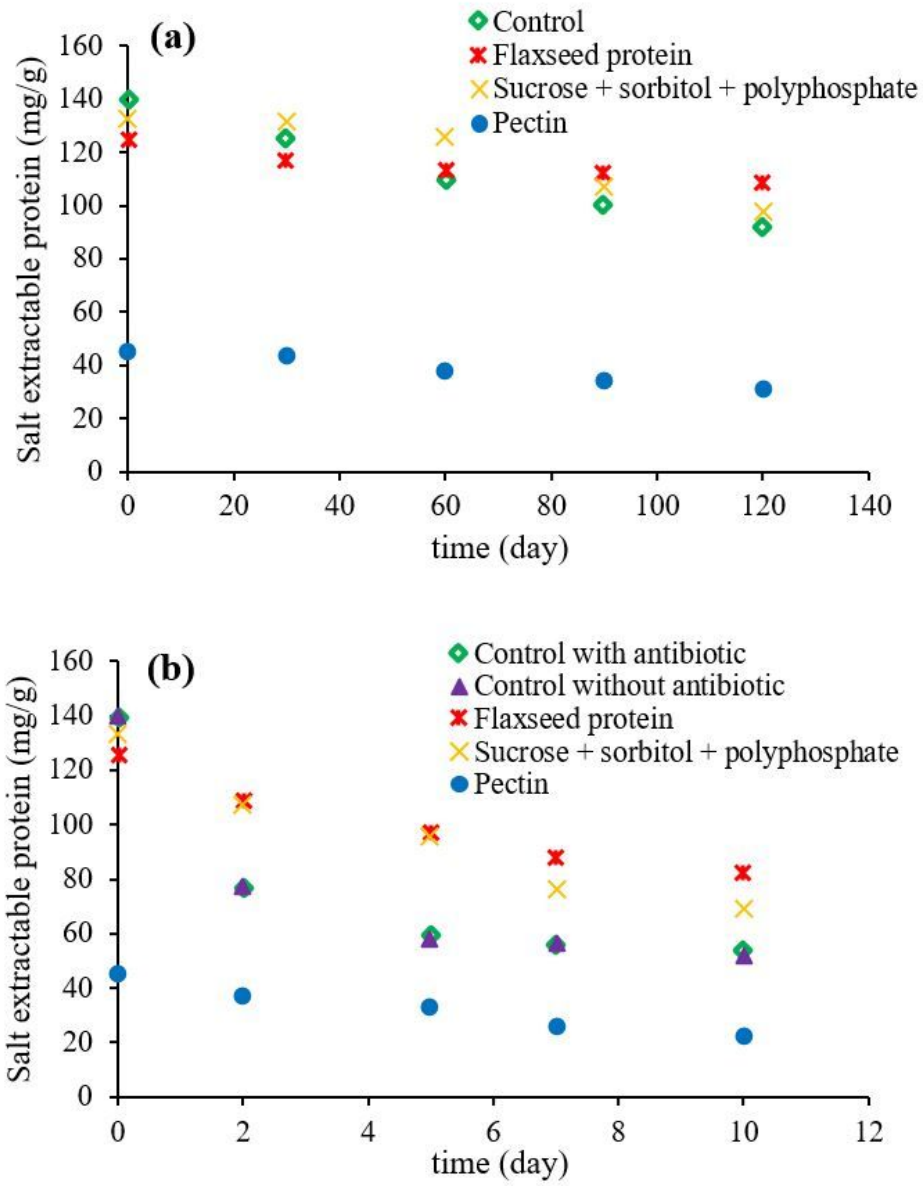

Figure 2

Changes of the salt extractable protein in surimi samples during storage at (a) -20 °C, and (b) 4 °C.

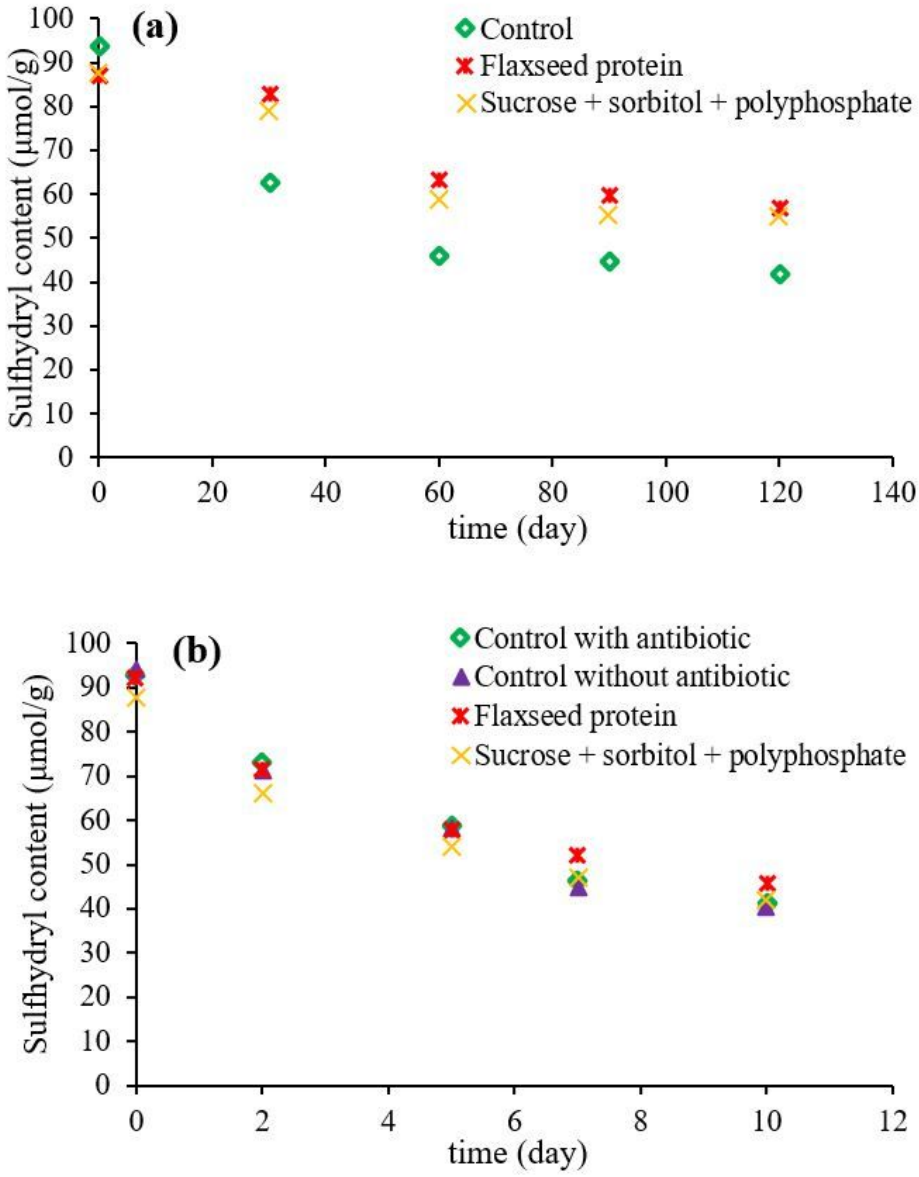

Figure 3

Changes of the sulfhydryl content in surimi samples during storage at (a) -20 °C, and (b) 4 °C.

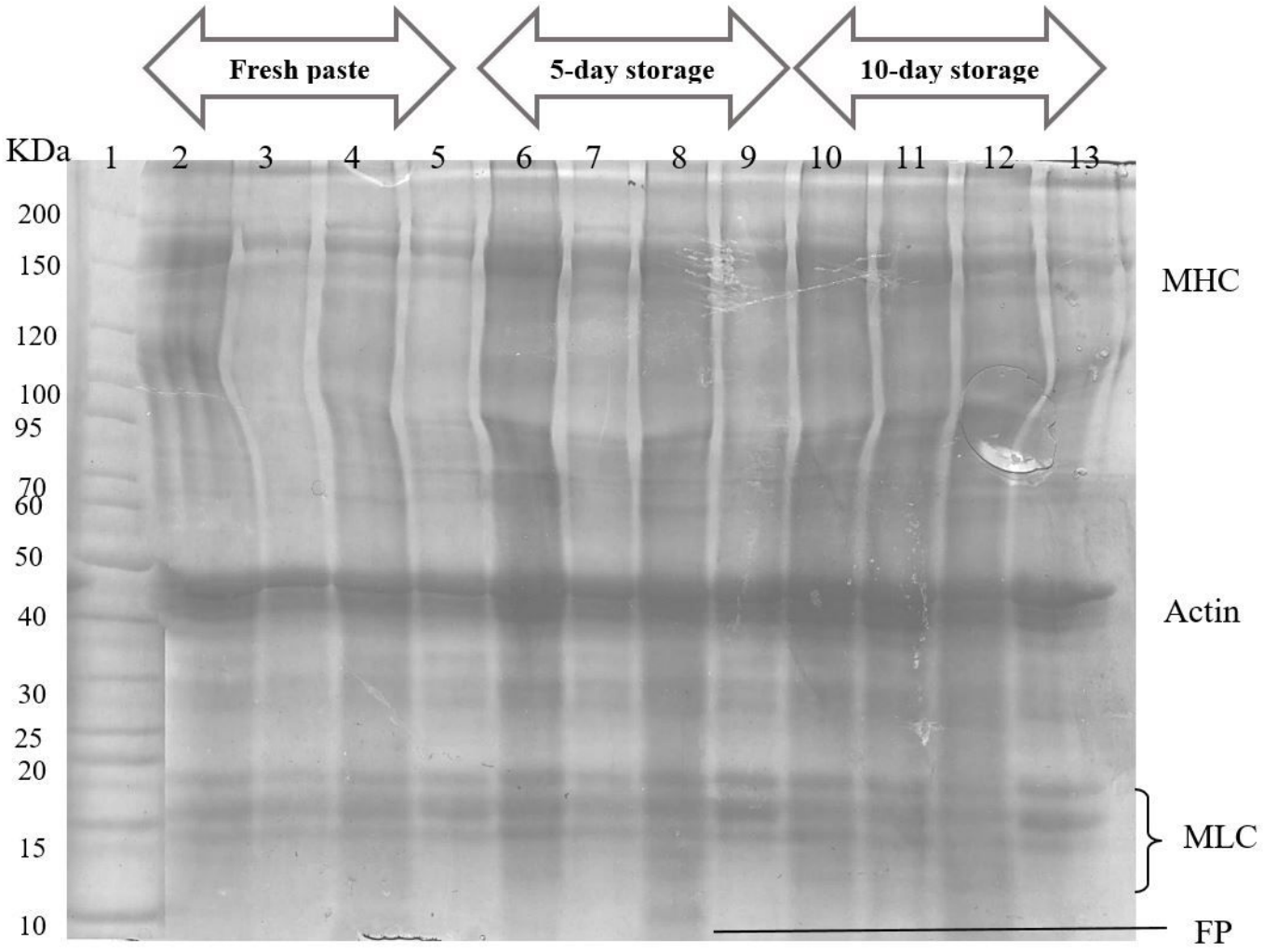

Figure 4

SDS– PAGE patterns of surimi samples with different cryoprotectants during storage at 4°C. Samples in the columns were: standard sample (1), control surimi (2), surimi added with sucrose + sorbitol + polyphosphate (3), surimi added with flaxseed (4), surimi added with pectin (5), control surimi (6), surimi added with sucrose + sorbitol + polyphosphate (7), surimi added with flaxseed (8), surimi added with pectin (9), control surimi (10) surimi added with sucrose + sorbitol + polyphosphate (11), surimi added with flaxseed (12), surimi added with pectin (13), MHC: myosin heavy chain, MLC: myosin light chain, FP: flaxseed protein.

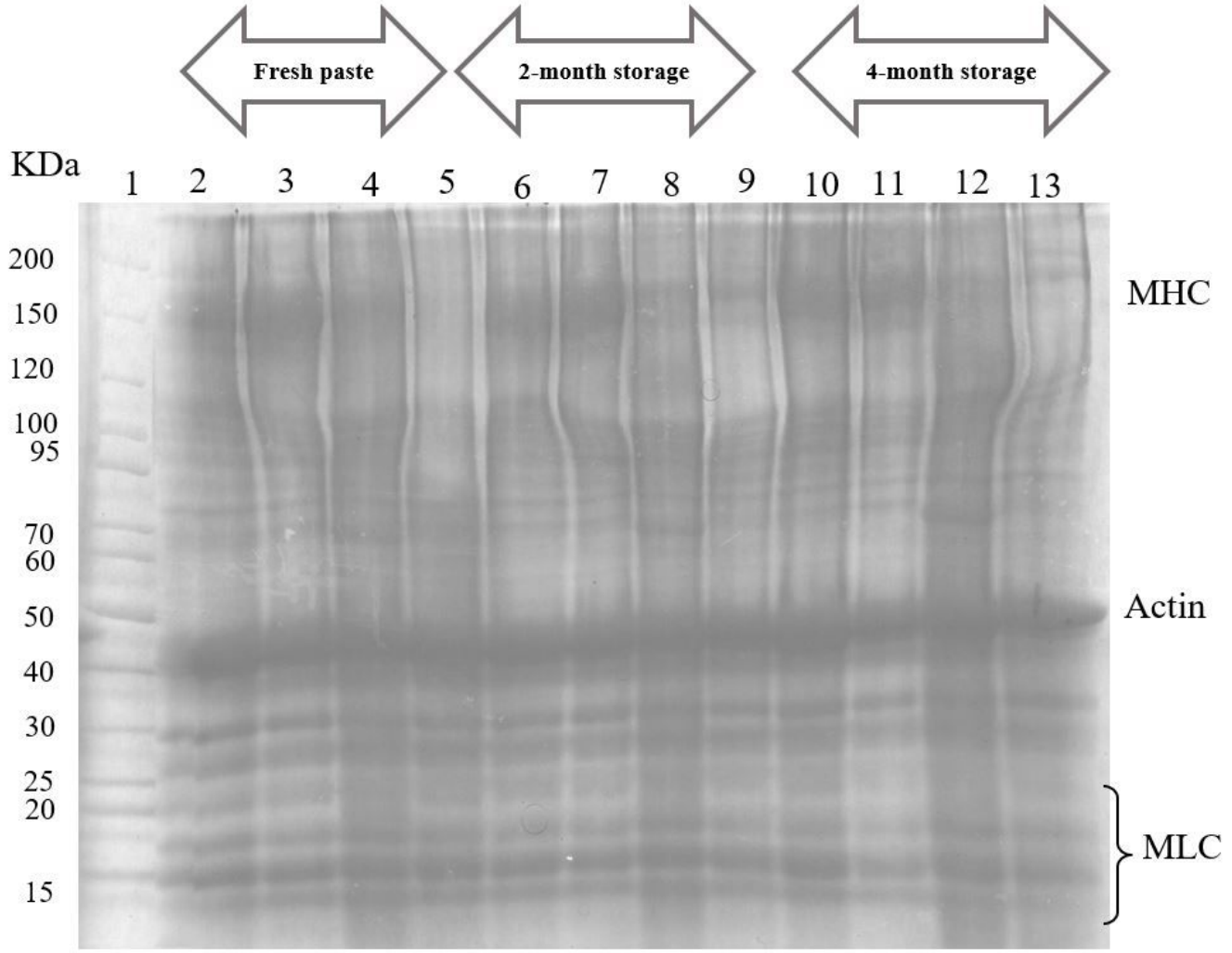

Figure 5

SDS– PAGE patterns of surimi samples with different cryoprotectants during storage at -20 ^{o}C. Samples in the columns were: standard sample (1), control surimi (2), surimi added with sucrose + sorbitol + polyphosphate (3), surimi added with flaxseed (4), surimi added with pectin (5), control surimi (6), surimi added with sucrose + sorbitol + polyphosphate (7), surimi added with flaxseed (8), surimi added with pectin (9), control surimi (10) surimi added with sucrose + sorbitol + polyphosphate (11), surimi added with flaxseed (12), surimi added with pectin (13), MHC: myosin heavy chain, MLC: myosin light chain.