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ORIGINAL ARTICLE

Suitability of chitosan as cryoprotectant on croaker fish (*Johnius gangeticus*) surimi during frozen storage

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Abstract Effect of chitosan on physicochemical attributes of croaker fish surimi during storage at −20±2 °C for 180 days was evaluated. The quality of croaker surimi added with 1% (w/w) chitosan was examined in terms of muscle protein, thaw drip, gel strength and Ca^{2+} ATPase activity comparing with those surimi samples added with 4% sucrose and 4% sorbitol. Surimi without any cryoprotectant was treated as control. Chitosan showed cryoprotective effect similar to commercial cryoprotectants as both of them minimized the negative effects of frozen storage on physico-chemical attributes of myofibrillar proteins. The residual Ca^{2+} ATPase activity and gel strength of surimi with chitosan were higher than those of control throughout the storage period. Ca^{2+} ATPase activity and gel strength had high positive correlation. It is concluded that chitosan was effective in preservation of croaker muscle protein native structure during 6 months of frozen storage and is comparable to other commercial cryoprotectants.

Keywords Chitosan · Cryoprotectant · Frozen storage · Ca^{2+} ATPase activity · Gel forming ability

Introduction

Surimi is an important intermediate product containing stabilized myofibrillar proteins obtained from mechanically deboned fish flesh that is water- washed for removal of sarcoplasmic protein, blended with cryoprotectants and then used in the production of fabricated seafood products. It has become the intermediate material for variety of sea food analogues, which have a growing consumer demand in India. Croaker fish (*Johnius gangeticus*) dominate the catch along coast of West Bengal State round the year. This is lean fish with low cost and low consumer demand. Cryoprotectants are used to extend the shelf life of frozen foods by preventing deleterious changes in myofibrillar proteins caused by freezing, frozen storage and thawing (MacDonald et al. 1997). Although cryoprotectants such as sucrose, sorbitol and phosphates have been used in surimi industry (Pigott 1986), but the problem of sweetness and high calorific content are of growing concern (Park and Morrissey 2000), which made various researchers to probe for alternative natural cryoprotectants. Chitosan is a low acetyl substituted form of chitin, composed of glucosamine, 2 amino- 2- deoxy-glucose (Shahidi et al. 1999), which has been used as an active material for its functional properties like antimicrobial (Cuero 1999), texturizing (Benjakul et al. 2001), binding (No et al. 2000), emulsifying (Cho et al. 1998) and antioxidant activities (Kamil et al. 2002). Chitosan improved the gel strength of surimi which is considered as single most important parameter for surimi quality and price (Kataoka et al. 1998). However, fundamental and comparative studies on chitosan as a natural cryoprotectant on surimi have not been conducted yet. In the present study, an attempt has been made to produce acceptable surimi from Croaker fish with an objective to investigate the effectiveness of chitosan, as an alternate cryoprotectant on surimi protein during frozen storage in comparison to traditionally used commercial cryoprotectants like sucrose (4%) and sorbitol (4%).

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Materials and methods

Croaker fish (*Johnius gangeticus*), average weight and length of 402.5 g and 31.5 cm respectively, caught along Kakdwip coast of West Bengal were iced on board and brought to the processing centre in an insulated box. They were washed in chilled water and dressed. Meat was picked using roll type fish meat picker and minced using a mincer (Stadler, Mumbai). The minced meat was washed with chilled water and dewatered to a moisture level of about 80% (Gopakumar et al. 1992). Surimi was prepared from mince meat following the method of Muraleedharan et al. (1996) and divided into 3 lots, which were subjected to different treatments viz., T_1 (4% sucrose and 4% sorbitol) and T_2 (1% (w/w) chitosan). Food grade chitosan of MW 300 kDa and 80% degree of deacetylation supplied by Fish Processing Division, Central Institute of Fishery Technology, Cochin and analytical grade reagents supplied by Sigma (St. Louis, USA). The control sample 'C' was without any cryoprotectant. The samples were separately packed in LDPE pouches and frozen at −35 °C in a horizontal plate freezer. Samples were stored in a cold store (−20±2 °C) and drawn at fortnightly interval for different quality analysis.

Proximate composition was determined following the method of AOAC (1995). Salt soluble nitrogen (SSN), water soluble nitrogen (WSN) and non-protein nitrogen (NPN) were estimated following the method of Dyer et al. (1950), AOAC (1995) and Srikar and Chandru (1983), respectively. pH was measured by weighing 5 g of surimi, homogenized in 45 ml distilled water and measured by Cyberscan 510 pH meter (Cyberscan 510, India). Alpha amino nitrogen (AAN) and total volatile base nitrogen (TVB-N) were estimated by the method of Beatty and Gibbons (1937). Minced meat was steam cooked with 2% salt, cooled and assessed for sensory quality (overall acceptability) by 8 panelists using 10-point Hedonic scale (Reddy and Srikar 1991). Standard method recommended by APHA (1984) was followed to estimate the total plate count (TPC) of the sample. Thaw drip (TD) in the frozen mince and surimi was determined following the method of Mishra and Srikar (1989).

Preparation of natural actomyosin and measurement of CA^{2+}- ATPase activity Natural actomyosin (NAM) was prepared according to the method of MacDonald and Lanier (1994). Four g of each surimi sample were homogenized in 40 ml chilled (4 °C) 0.6 M KCl, pH 7.0 for 4 min using a homogenizer. Actomyosin was collected by centrifuging at 5,000 x***g*** for 20 min at 0 °C, and the pellet was dissolved by stirring for 30 min at 0 °C in an equal volume of chilled 1.2 M KCI, pH 7.0. Natural actomyosin was kept in ice during the entire analysis. Ca^{2+}-ATPase activity was determined using modified method of MacDonald and Lanier (1994). Diluted NAM solution (1 ml) was added to 0.6 ml of 0.5 M Tris-maleate, pH 7.0., 10 mM $CaCl_2$, 0.5 ml of 20 mM ATP to initiate the reaction. The reaction was conducted for 5 min at 27 °C, at pH 8 and was terminated by adding 5 ml of chilled 15% (w/v) trichloro-acetic acid. The reaction mixture was centrifuged at 3,500×g for 5 min and the inorganic phosphate liberated in the supernatant was measured by the method of Fiske and Subbarow (1925). Specific Ca^{2+}- ATPase activity of the NAM was expressed as μmoles of inorganic phosphate released/mg protein/min. A blank solution was prepared by adding chilled trichloroacetic acid prior to addition of ATP. The relative total Ca^{2+}-ATPase activity of surimi sample was expressed as the ratio of the activity before and after frozen storage.

Preparation of kamaboko gel Heat induced kamaboko gels were prepared from each surimi sample according to the method of Ian et al. (1995). The frozen surimi sample was thawed at 4 °C for 2 h and placed in a mortar. NaCl (3% w/w) was sprinkled on to the surimi, and the fish-salt mixture was ground at 5 °C for 30 min. The surimi paste was then stuffed into a stainless steel cylindrical mold with a diameter and length of 3 cm each. Both ends of mold were sealed tightly using polyvinylchloride film and rubber bands. Two-step heated gels were carried out by setting at 40 °C for 1 h, followed by heating at 90 °C for 30 min in a water bath. These gels referred to as kamaboko gels were cooled in iced water and stored at 4 °C overnight. Kamaboko gels were equilibrated at room temperature for 30 min before analyzing its textural qualities.

Texture profile analysis (TPA) Texture profile attributes were determined using a TA-XT2i Stable Micro Systems texturometer (Viena Court, England) with software XTRA™ dimension (XTRAD) following the method of Chung and Merritt (1991). Gel samples of 2×2 cm size left to equilibrate at room temperature for 60 min. Then gel samples were placed on a flat platform and were double compressed (25% of original height speed of 12 mm/min using 50N load cell) in a direction perpendicular to the orientation of the muscle by a cylindrical probe of 50 mm diameter. The gel strength (g.cm) was calculated by multiplying breaking strength (g) with deformation distance (cm). The relative gel strength was defined as the ratio of gel strength before and after frozen storage. The samples were allowed for a double compression of 40% with a trigger force of 0.5 kg during which various textural parameters like hardness −1, hardness −2, cohesiveness, springiness, gumminess and chewiness were recorded from a typical force deformation curve (Fig. 1). Data were recorded and analyzed automatically by software provided with the machine. All measurements were made in three

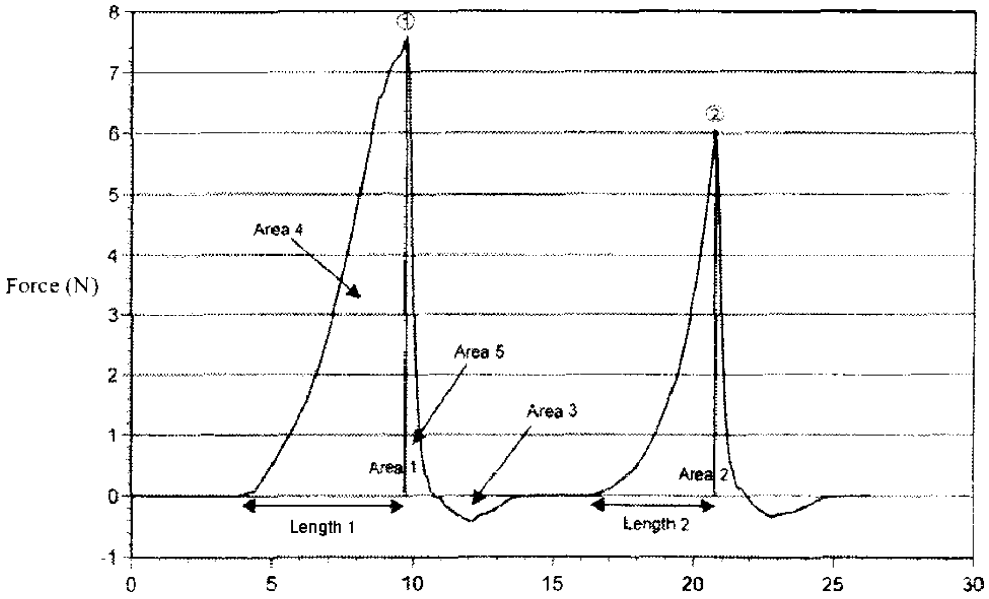

Fig. 1 Typical force by time curve plot to determine texture profile analysis parameters. Peak force 1 and 2 is Hardness 1 and 2; Cohesiveness = (Area 2/Area 1); Gumminess = (Hardness 1×cohesiveness); Springiness = (Length 2/Length 1); Chewiness = (Hardness×springiness×cohesiveness)

replicates with two samples for a total of six measurements. The shear force test was done to measure the force required to shear a piece of cylindrical sample (10 mm in diameter and 10–15 mm long) which was done with 50 N load cell fitted with Warner –Bratzler shear attachment with a shearing speed of 50 mm/min and the shearing direction was set perpendicular to the orientation of the sample. The peak shear force, expressed in gram-force (kgf/g) required to cut the cylinder into 2 pieces was read from the control panel of the analyzer.

All experiments were done in triplicate. Significant differences among means of experimental results were evaluated by one-way analysis of variance (ANOVA) using the EXCEL programme (Microsoft, USA). The least significant difference test was used to find out significant differences between sample means. *P* value of less than 0.05 was considered to be significant.

Results and discussion

Table 1 shows the composition of raw mince and surimi, where protein, fat and ash content reduced by 16, 61 and 32% respectively. The surimi produced with 32% yield was colourless with neutral pH and had a moisture content of 80.0%, which is ideal for commercial surimi production. Muraleedharan et al. (1996) working with surimi preparation from different nonconventional fishes reported similar results.

Table 1 Raw material characteristics of mince and surimi

Composition/characteristics	Raw mince meat	Surimi
Moisture (%)	76.3±0.08	80.0±0.04
Fat (%)	1.9±0.12	0.7±0.09
Ash (%)	0.9±0.02	0.6±0.02
Protein (%)	16.6±0.09	13.8±0.03
Yield (%)	36.3±0.11	32.9±007
pH	7.0±0.04	7.2±0.06
TPC (log cfu/g) (n=3)	12.7±0.01	13.5±0.04

The decrease in TN control sample was 44.9% ($p<0.05$) but in T_1 and T_2 samples, it decreased to 23.7% and 14.9%, respectively (Table 2). This could be expected due to loss of some WSP and other NPN constituents in the free drip after thawing. The decrease is much less in T_1 and T_2 samples than in control, which may be due to their respective cryoprotective effect. Similar results have also been observed by Garg et al. (1982). SSN is considered as an index of protein denaturation in fish (Shyamsunder and Prakash 1994). SSN content of all samples decreased during storage, which can be attributed to the aggregation leading to insolubilization of myofibrillar fraction of proteins. In the present study, decreasing rate of SSN in sample treated with chitosan was lower than in sample treated with sucrose, sorbitol and sodium tripolyphosphate ($p<0.05$), which might be due to higher cryoprotective effect of chitosan. Similar decreasing trend of SSN in surimi prepared from Indian major carps has also been reported by Chakraborty (1984). There was a decrease of 8.4%, 5.6% and 4.6% in WSP content in the Croaker mince and surimi treated by cryoprotectants (T_1 and T_2), respectively. This decrease could be due to loss of water extractable protein in the free drip. Borderias et al. (1985) attributed the decrease in WSP content to the denaturation of sarcoplasmic protein during frozen storage.

The NPN content of control, T_1 and T_2 samples showed decreasing trend during frozen storage which might be due to the drip loss. Similar trend was observed by Mishra and Srikar (1989) in clam meat. The AAN content increased in

Table 2 Changes in bio-chemical characteristics of Croaker fish surimi samples treated with and without additives during storage at −20±2 °C

Storage days	Sample	TN,%	SSN,% of TN	WSP,%	NPN mg/100 gm	TVBN mg/100 gm	AAN (mg/100 gm)	pH	Overall acceptability
0	C	3.3±0.68[a]	82.3±0.23 [a]	2.6±0.63 [a]	364.5±0.25 [a]	4.8±0.03 [a]	11.1±0.15 [a]	7.2±0.10 [a]	7.5±0.05 [a]
	T_1	3.4±0.94 [a]	82.7±0.09 [a]	2.7±0.53 [a]	368.6±0.25 [a]	4.3±0.06 [a]	11.1±0.02 [a]	7.3±0.06 [a]	8.0±0.09 [a]
	T_2	3.4±0.36 [a]	83.4±0.64 [a]	2.6±0.78 [a]	366.3±0.21 [a]	4.4±0.34 [a]	11.0±0.08 [a]	7.3±0.35 [a]	7.8±0.07 [a]
30	C	3.3±0.03 [a]	73.1±0.01 [b]	2.6±0.02 [a]	358.4±0.46 [a]	5.1±0.01 [a]	13.2±0.11 [a]	7.2±0.14 [a]	7.0±0.06 [a]
	T_1	3.3±0.09 [a]	80.9±0.61 [a]	2.7±0.06 [a]	361.9±0.34 [a]	5.1±0.21 [a]	12.1±0.16 [a]	7.3±0.22 [a]	7.7±0.07 [a]
	T_2	3.4±0.56 [a]	81.9±0.06 [a]	2.6±0.09 [a]	362.1±0.06 [a]	5.0±0.05 [a]	12.1±0.07 [a]	7.3±0.05 [a]	7.6±0.02 [a]
60	C	3.3±0.07 [a]	67.4±0.94 [c]	2.7±0.07 [a]	352.6±0.08 [b]	5.9±0.56 [a]	14.9±0.05 [a]	7.1±0.08 [a]	6.5±0.07 [a]
	T_1	3.3±0.52 [a]	79.0±0.03 [a]	2.6±0.12 [a]	358.9±0.06 [b]	5.6±0.37 [a]	12.9±0.07 [a]	7.2±0.07 [a]	7.1±0.09 [a]
	T_2	3.3±0.07 [a]	79.6±0.32 [a]	2.6±0.4 [a]	360.1±0.01 [b]	5.7±0.01 [a]	12.7±0.06 [a]	7.3±0.06 [a]	7.3±0.17 [a]
90	C	2.6±0.61 [b]	63.3±0.56 [d]	2.7±0.35 [a]	343.1±0.43 [b]	6.8±0.49 [b]	15.6±0.12 [a]	7.1±0.15 [a]	6.2±0.12 [a]
	T_1	3.1±0.36 [a]	76.3±0.01 [b]	2.6±0.07 [a]	356.2±0.13 [b]	6.7±0.04 [b]	13.3±0.04 [a]	7.1±0.1 [a]	6.6±0.25 [a]
	T_2	3.2±0.01 [a]	78.3±0.07 [b]	2.5±0.39 [a]	357.8±0.53 [b]	6.4±0.67 [b]	13.3±0.08 [a]	7.2±0.04 [a]	7.0±0.06 [a]
120	C	2.2±0.08 [b]	58.3±0.07 [e]	2.6±0.21 [a]	335.9±0.7 [c]	8.6±0.01 [c]	16.2±0.07 [a]	7.1±0.08 [a]	5.6±0.04 [a]
	T_1	2.9±0.04[bc]	70.6±0.42 [c]	2.6±0.36 [a]	351.0±0.08[ca]	8.1±0.23 [c]	14.3±0.04 [a]	7.1±0.06 [a]	6.3±0.08 [a]
	T_2	3.1±0.58 [ac]	75.6±0.51 [c]	2.5±0.06 [a]	355.5±0.4 [c]	7.8±0.38 [c]	14.2±0.06 [a]	7.2±0.08 [a]	6.9±0.07 [a]
150	C	2.0±0.01 [b]	52.1±0.06 [f]	2.5±0.07 [a]	328.3±0.06 [c]	12.1±0.08 [d]	17.5±0.02 [b]	7.0±0.15 [a]	5.1±0.06 [a]
	T_1	2.6±0.08 [bc]	65.5±0.67 [d]	2.5±0.13 [a]	346.5±0.64 [c]	11.5±0.07 [d]	15.9±0.09 [a]	7.0±0.06 [a]	5.8±0.07 [a]
	T_2	3.0±0.72 [cc]	71.2±0.75 [c]	2.5±0.04 [a]	352.2±0.07 [c]	10.9±0.43 [d]	15.3±0.16 [a]	7.2±0.04 [a]	6.2±0.08 [a]
180	C	1.8±0.52 [b]	48.3±0.05 [g]	2.4±0.25 [a]	316.1±0.45[d]	18.3±0.32 [e]	18.8±0.02 [b]	7.0±0.04 [a]	4.7±0.06 [a]
	T_1	2.6±0.01 [bc]	63.3±0.08 [d]	2.5±0.08 [a]	342.3±0.07 [d]	14.6±0.28 [e]	16.4±0.11 [a]	7.0±0.08 [a]	5.5±0.04 [a]
	T_2	2.9±0.34 [ca]	68.8±0.04 [c]	2.5±0.09 [a]	349.8±0.01 [d]	13.8±0.06 [e]	15.8±0.21 [a]	7.1±0.10 [a]	5.6±0.05 [a]

C Control, T_1 Surimi sample treated with 4% sucrose and 4% sorbitol, T_2 Surimi sample treated with 1% (w/w) Chitosan, *TN* Total nitrogen, $n=3$
Different alphabets in the superscript in same column are significantly different ($p \leq 0.05$)

all the samples during frozen storage, which was significant in case of control sample ($p<0.05$). There was no significant difference in AAN content of T_1 and T_2 samples during storage of 180 days. Freshness parameters viz. NPN, AAN and TVB-N increase ($p<0.05$) during frozen storage. Further, the rate of increase was maximum in control sample while it was not significant for T_1 and T_2 samples, which might be due to the cryoprotective treatment in surimi reducing protein denaturation. pH of all samples showed a slight decrease during storage. Similar observation was made by Rajalekshmi and Mathew (2007) in threadfin bream, where they suggested the decrease of pH, due to precipitation of alkaline Ca, Mg and sodium phosphates. However, the pH values remained near to neutral (6.5–7.5) throughout storage and difference in pH between treatments was of little significance.

A significant decrease in overall acceptability sensory scores ($p<0.05$) was noticed throughout the period of storage. Sensory evaluation data showed highly negative correlation between mean panel scores for overall acceptability and storage period. A linear regression equation $Y=-0.0163\ X+4.1308$ with a correlation coefficient of $r=-0.9907$ was obtained. The decrease in AAN also reflected in loss of flavour (Chakraborty 1984).

Thaw drip loss continuously increased in control, T_1 and T_2 samples during frozen storage (Fig. 2). Similar increase in thaw drip during frozen storage was also observed with sodium lactate as cryoprotectant on Croaker fish surimi (Dey et al. 2007). Addition of tripolyphosphate in to white fish and Burbot fish fillets improved texture properties (Krivchenia and Fennema 1986), which increased the protein solubility, and thus water retention capacity in muscle protein got increased. Prolonged storage results in decreased water retention capacity due to denaturation of proteins because of surface dehydration, ice crystal formation and cell rupture. In the present investigation, chitosan effectively increased the water holding capacity of muscle protein. Also a negative correlation was observed between thaw drip and overall acceptability score ($p<0.05$). The reduction of thaw drip percentage in T_1 and T_2 from that of control during the frozen storage indicated the effect of cryoprotectant in preventing the protein denaturation. Further, thaw drip percentage in T_1 and T_2 samples did not vary significantly.

Ca^{2+}ATPase activity of natural actomyosin extracted from Croaker muscle showed a continuous decrease during the storage period (Fig. 2). For the control sample, relative Ca^{2+}ATPase activity decreased to 35.6% during 180 days

of storage ($p<0.05$). In case of surimi prepared by T_1 and T_2, relative Ca^{2+}ATPase activity decreased to 61.1% and 59.3%, respectively ($p<0.05$). The decrease in Ca^{2+}ATPase activity with the length of frozen storage was possibly due to the conformational changes of myosin globular head as well as the aggregation in this portion. According to Benjakul and Bauer (2000), myosin head possesses the ATPase activities and the rearrangement of protein via protein-protein interactions in myosin during frozen storage was also presumed to contribute to loss in ATPase activity. Cryoprotectant has the ability to recover some myosin ATPase activity and chitosan prevented loss of Ca^{2+}ATPase activity almost to same extent as that of sucrose/sorbitol during isothermal storage at −20±2 °C. Rajalekshmi and Mathew (2007) showed similar effects of chitosan on ATPase activity of thread fin bream surimi during frozen storage.

The gel strength before freezing of kamaboko gels in control, T_1 and T_2 samples were 342, 371 and 473 g.cm, respectively. The initial gel strength values of Croaker fish kamaboko gels with and without additives were different, which may be due to immediate effect of addition of cryoprotectants. Significant reduction in gel strength of all the samples was observed during freezing and frozen storage (Fig. 2). Treatment with standard cryoprotectants improved the gel strength compared to control up to 60 days of frozen storage though it decreased towards the end of storage whereas, chitosan treatment gave higher ($p<0.05$) gel strength than the commercial cryoprotectant blend towards the end of frozen storage period. Specifically, the relative gel strength of the control dropped dramatically to 42.5% of the initial value within first 30 days of frozen storage, whereas kamaboko gels of T_1 and T_2 samples were 83.5% and 85.8% respectively. The result indicated that freezing and frozen storage had a strong effect on the gel forming ability of croaker fish surimi even without additive (control). Kim et al. (1986) elucidated that repeated freezing and thawing of surimi made from Alaska Pollock and sandtrout, accelerated the myosin denaturation, resulting in the substantial decrease in gel strength. After 120 days of frozen storage, residual gel strength of T_1 and T_2 samples were 52.8% and 56.6% while the control had the lowest value of 23.4% ($p\leq0.05$). A high positive correlation ($r=0.894$, $p<0.001$) was also found between relative Ca^{2+}-ATPase activity and relative gel strength of surimi with or without additives. This result corresponds to MacDonald et al. (1996).

Typical force by time curve plot obtained (Fig. 1) was used to calculate different textural parameters of kamaboko gel given in Table 3. Hardness 1 and 2 were estimated as the resistance at maximum compression during 1st and 2nd compression respectively of the gel prepared from the surimi samples. The hardness 1 value was always higher

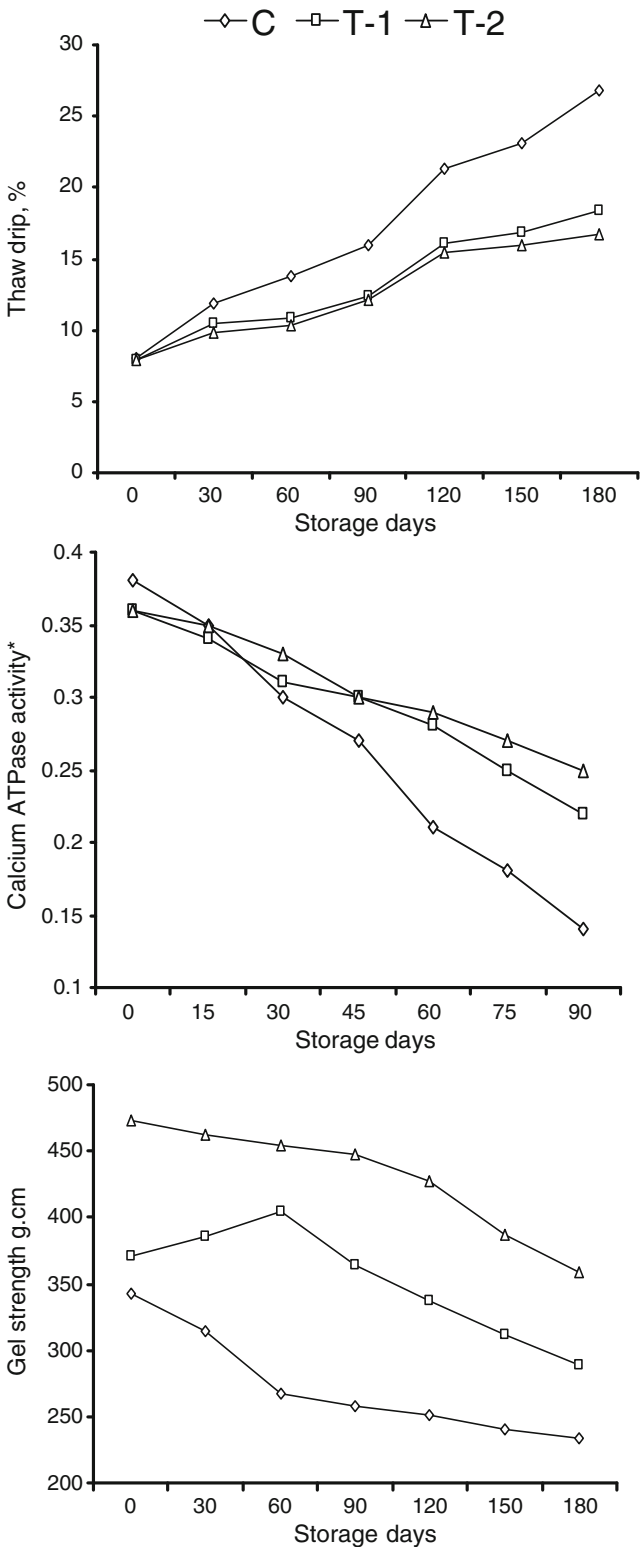

Fig. 2 Changes in thaw drip, Ca^{2+} ATPase activity and gel strength values of dewatered minced meat with no additive (prepared by 4% sucrose, C), surimi 4% sorbitol (T_1) and 1% (w/w) chitosan (T_2) during frozen storage at −20±2 °C for 180 days. * μmoles inorganic phosphate released/mg protein/min

than hardness 2 as the compressed sample had a firm texture compared to an already compressed one. Though, hardness of the samples showed reduction during frozen storage, addition of chitosan controlled the reduction better than sucrose and sorbitol especially towards the end of frozen storage. Cohesiveness, the ratio of positive force area during the 2nd compression to that of 1st compression of the samples also got reduced during frozen storage. It is the capacity of the sample to maintain the intactness after the first compression cycle of the TPA. Cohesiveness values close to 1 indicate tendency of recovery to its original structure after the first compression is high (Munizaga and Canovas 2004). In the present study, T_2 was able to give ideal cohesiveness value during frozen storage. Gumminess which is the amount of energy required to disintegrate a semisolid food product to a state ready for swallowing, decreased with the length of storage though the reduction was less for T_1 and T_2 samples compared to control. Springiness refers to the height that a food recovers during the time that elapses between the end of 1st compression and the start of 2nd compression. Gel enhancing agents such as potato starch and egg white increase the springiness of surimi gels (Munizaga and Canovas 2004). In the present study, chitosan gave higher springiness compared to commercial cryoprotectant which showed the evidence for its gel enhancing property. Chewiness of the surimi gel samples were reduced during frozen storage. The treatment with commercial cryoprotectant blend and chitosan improved the chewiness of the samples and the latter was more effective especially during the initial months of frozen storage. The shear force values decreased with the frozen storage (Table 3), which agrees with the hardness values of TPA, indicating that the product was getting soft with the extention of frozen storage. However, factors related to tissue softening, e.g. release of proteases from lysosomes and cell disruption by ice crystals formation, may also exist in frozen and thawed seafoods and counteract the tissue-toughening factors (Srinivasan et al. 1997).

Conclusion

Cryoprotectants used in the study minimized the negative effects of frozen storage on physico-chemical, bio-chemical and sensory parameters studied. Addition of chitosan improved the gel strength without having any adverse effect on overall acceptability during 6 months of frozen

Table 3 Changes in textural parameters of kamaboko gel prepared from Croaker surimi during storage at −20±2 °C

Storage days	Samples	Hardness 1, kgf.	Hardness 2, kgf.	Cohesiveness	Chewiness, kgf./mm	Springiness,mm.	Gumminess, kgf	Shear force kgf./g
0	C	1.9±0.18 ^{a}	1.1±0.08 ^{a}	1.00±0.13 ^{a}	2.9±0.13 ^{a}	1.6±0.25 ^{a}	1.8±0.12 ^{a}	1.7±0.08 ^{a}
	T_1	1.8±0.14 ^{a}	1.1±0.03 ^{a}	0.9±0.09 ^{a}	2.3±0.16 ^{a}	1.3±0.15 ^{a}	1.7±0.09 ^{a}	1.7±0.15 ^{a}
	T_2	1.8±0.16 ^{a}	1.0±0.01 ^{a}	0.9±0.04 ^{a}	2.4±0.18 ^{a}	1.5±0.21 ^{a}	1.6±0.13 ^{a}	1.7±0.13 ^{a}
30	C	1.7±0.03 ^{a}	1.0±0.09 ^{a}	0.9±0.1 ^{a}	1.6±0.12 ^{a}	1.1±0.06 ^{a}	1.4±0.03 ^{a}	1.6±0.14 ^{a}
	T_1	1.7±0.09 ^{a}	1.0±0.12 ^{a}	0.9±0.11 ^{a}	1.9±0.06 ^{a}	1.2±0.04 ^{a}	1.6±0.08 ^{a}	1.7±0.16 ^{a}
	T_2	1.7±0.13 ^{a}	0.9±0.04 ^{a}	0.9±0.06 ^{a}	2.1±0.09 ^{a}	1.4±0.06 ^{a}	1.5±0.15 ^{a}	1.6±0.07 ^{a}
60	C	1.4±0.07 ^{a}	0.9±0.03 ^{a}	0.7±0.04 ^{a}	1.0±0.07 ^{b}	0.9±0.08 ^{a}	1.0±0.04 ^{a}	1.4±0.06 ^{a}
	T_1	1.5±0.12 ^{a}	0.9±0.07 ^{a}	0.8±0.03 ^{a}	1.3±0.12 ^{bb}	1.1±0.06 ^{a}	1.2±0.06 ^{a}	1.5±0.11 ^{a}
	T_2	1.46±0.07 ^{a}	0.8±0.05 ^{a}	0.8±0.02 ^{a}	1.3±0.04 ^{bb}	1.2±0.01 ^{a}	1.2±0.08 ^{a}	1.6±0.12 ^{a}
90	C	0.8±0.01 ^{b}	0.5±0.02 ^{a}	0.6±0.16 ^{a}	0.4±0.15 ^{c}	0.9±0.03 ^{a}	0.5±0.07 ^{b}	1.3±0.14 ^{a}
	T_1	1.0±0.06 ^{a}	0.6±0.01 ^{a}	0.7±0.01 ^{a}	0.7±0.07 ^{b}	1.0±0.03 ^{a}	0.7±0.0 6 ^{b}	1.4±0.07 ^{a}
	T_2	1.1±0.01 ^{a}	0.7±0.08 ^{a}	0.7±0.07 ^{a}	0.8±0.09 ^{b}	1.0±0.06 ^{a}	0.8±0.01 ^{b}	1.4±0.05 ^{a}
120	C	0.7±0.08 ^{b}	0.4±0.11 ^{b}	0.5±0.04 ^{b}	0.3±0.01 ^{c}	0.8±0.07 ^{a}	0.4±0.08 ^{b}	1.0±0.07 ^{b}
	T_1	0.9±0.04 ^{bb}	0.5±0.03 ^{ab}	0.6±0.02 ^{a}	0.5±0.06 ^{b}	0.9±0.08 ^{a}	0.6±0.02 ^{bb}	1.2±0.11 ^{a}
	T_2	0.9±0.08 ^{bb}	0.6±0.04 ^{ab}	0.7±0.01 ^{a}	0.6±0.06 ^{bc}	1.00±0.04 ^{a}	0.6±0.03 ^{bb}	1.2±0.02 ^{a}
150	C	0.4±0.01 ^{c}	0.3±0.02 ^{b}	0.5±0.06 ^{a}	0.1±0.07 ^{d}	0.6±0.06 ^{b}	0.2±0.04 ^{c}	0.9±0.04 ^{b}
	T_1	0.7±0.08 ^{b}	0.4±0.06 ^{b}	0.5±0.07 ^{a}	0.3±0.03 ^{b}	0.9±0.04 ^{a}	0.4±0.07 ^{bc}	1.0±0.01 ^{b}
	T_2	0.9±0.02 ^{b}	0.5±0.03 ^{b}	0.6±0.15 ^{a}	0.5±0.04 ^{b}	0.9±0.07 ^{a}	0.5±0.03 ^{bc}	1.1±0.04 ^{b}
180	C	0.4±0.03 ^{c}	0.3±0.01 ^{c}	0.4±0.05 ^{c}	0.1±0.05 ^{d}	0.5±0.05 ^{b}	0.2±0.07 ^{c}	0.8±0.02 ^{b}
	T_1	0.6±0.01 ^{b}	0.4±0.08 ^{c}	0.5±0.08 ^{a}	0.2±0.08 ^{bc}	0.8±0.07 ^{a}	0.3±0.02^{cd}	1.00±0.07 ^{b}
	T_2	0.7±0.34 ^{b}	0.4±0.04 ^{cc}	0.6±0.04 ^{a}	0.3±0.09 ^{bd}	0.8±0.01 ^{a}	0.4±0.04^{cd}	1.0±0.03 ^{b}

C, T_1 and T_2, as in Table 2

Different alphabets in the superscript in same column are significantly different ($p \leq 0.05$) ($n=3$)

storage. Thus, it may be inferred that chitosan can effectively be used as an alternative cryoprotectant to sucrose and sorbitol for stabilization of croaker muscle protein native structure during frozen storage.

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