Chapter 3

Results and discussion

1. Cryoprotective effect of trehalose, sucrose and sorbitol alone at different concentration in NAM with 1 and 2 freeze-thaw cycles.

1.1. Changes in Ca^{2+}-ATPase activity.

The effect of different cryoprotectants including trehalose, sucrose and sorbitol at different concentrations (0, 2, 4, 6 and 8% w/v) on Ca^{2+}-ATPase activity of NAM extracted from bigeye snapper was determined after freeze-thawing for 1 and 2 cycles. Ca^{2+}-ATPase activity of the control decreased by 70% and 85% after 1 and 2 freeze-thawing cycles, respectively compared to initial value (Figure 12a, 12b). The decreasing rate of Ca^{2+}-ATPase activity varied, depending on level of cryoprotectant added. With addition cryoprotectants, Ca^{2+}-ATPase activity was more retained. Higher activity remained as the concentrations increased. The addition of 2% trehalose resulted in higher Ca^{2+}-ATPase activity retained than the addition of 2% sucrose and 2% sorbitol after 1 freeze-thaw cycle. At levels of 4, 6 and 8% cryoprotectants, samples added with trehalose had higher Ca^{2+}-ATPase than sucrose and sorbitol after 1 freeze-thaw cycle (Figure 12a). After 2 freeze-thaw cycles (Figure 12b), the higher Ca^{2+}-ATPase was retained with the addition of cryoprotectants, especially when the concentrations used increased. At level of 8%, all cryoprotectants could retard the decrease in Ca^{2+}-ATPase more effectively than 2, 4, and 6%. The efficacy of all cryoprotectants at every levels decreased to some extent when the samples were subjected to 2 freeze-thaw cycles, compared to 1 cycle.

Ca^{2+}-ATPase activity can be used as an indicator for the integrity of myosin molecules. The globular heads of myosin are responsible for Ca^{2+}-ATPase activity (Benjakul *et al.*, 1997). A decrease in activity after freeze-thaw cycle indicated the

denaturation of myosin, especially in the head region. Based on the decrease in Ca^{2+}-ATPase activity, myosin from NAM underwent denaturation to a higher extent at 2 freeze-thaw cycles, compared to 1 freeze-thaw cycle. The decrease in Ca^{2+}-ATPase activity was possibly associated with the oxidation of sulfhydryl groups on myosin globular head (Hamada *et al.*, 1977; and Jiang *et al.*, 1988) and the tertiary structural changes, which were caused by ice crystals (Suzuki, 1976). From the result, the retardation of denaturation was dependent upon level of cryoprotectant used. Higher level of trehalose, sucrose, and sorbitol could retard protein denaturation more effectively than lower level. This could be explained by the structuring effect on water, which would reduce the mobility of water molecules surrounding the protein and consequently lower the rate of ATP hydrolysis (Auh *et al.*, 1999). From the result, the best stabilization of NAM was observed with cryoprotectants at 8%. Trehalose could retard the decrease in Ca^{2+}-ATPase activity more effectively than sucrose and sorbitol ($p<0.05$). The remaining Ca^{2+}-ATPase activity showed a similar trend after 1 and 2 freeze-thaw cycles. Sussich *et al.* (1998) suggested that trehalose is a more effective protective agent compared to other disaccharides, such as sucrose. Ooizumi *et al.*, (1981) reported that the protective effect of sugars against denaturation of myofibrils monitored by measuring ATPase activity loss was well determined by the number of hydroxyl groups. Sugars can interact with myosin not only at the head portion where the ATPase active site is located but also the tail portion which contributes to filament formation, leading to a less aggregation of protein molecules (Konno *et al.*, 1997).

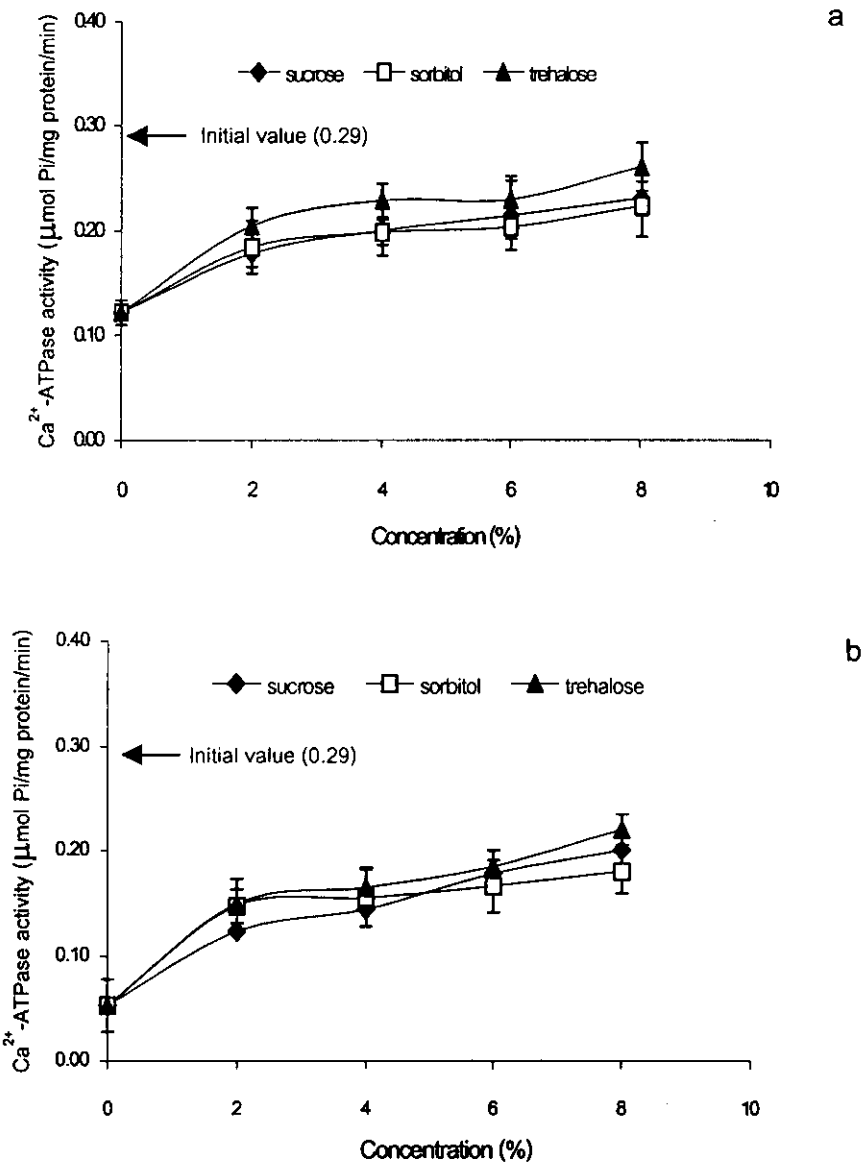

Figure 12 Effect of different cryoprotectants at various concentrations on changes of Ca^{2+}-ATPase activity in NAM (2.5 mg/ml, pH 7.0, 0.6 M KCl) subjected to 1(a), and 2(b) freeze-thaw cycles.

1.2. Changes in sulfhydryl content.

Figure 13 shows the effect of different concentrations of cryoprotectants on the remaining sulfhydryl content of NAM after freeze-thawing for 1 and 2 cycles. The sulfhydryl content of NAM decreased after freeze-thawing process and higher

decrease was observed with increasing freeze-thaw cycles. The decreasing rate of sulfhydryl content was similar to that of Ca^{2+}-ATPase activity (Figure 12), depending on level of cryoprotectant used. Higher level of trehalose, sucrose and sorbitol could retard the decrease in sulfhydryl content effectively than lower level. At a level of 8%, NAM added with trehalose, sucrose and sorbitol contained the sulfhydryl content of 4.87, 4.52 and 4.51 mol/10^5g protein, respectively after 1 freeze-thaw cycle and 3.61, 3.73 and 3.22 mol/10^5g protein, respectively after 2 freeze-thaw cycles. Without cryoprotectants (control), the lowest sulfhydryl content was observed for samples subjected to both 1 (2.09 mol/10^5g protein) and 2 (1.46 mol/10^5g protein) freeze-thaw cycles. No differences in sulfhydryl content between samples added with 2 and 4% cryoprotectant after both 1 and 2 freeze-thaw cycles. No differences in total sulfhydryl content were observed among all cryoprotectants tested at all levels. Therefore, cryoprotectant at a level of 8% could retard the loss of sulfhydryl content most effectively.

From the result, the loss of sulfhydryl content in the control occurred to the highest extent compared to samples added with cryoprotectant. Increased freeze-thaw cycles caused the higher decrease in sulfhydryl group. The decrease in sulfhdryl contents was considered to be due to the formation of disulfide bonds via oxidation of sulfhydryl groups or disulfide interchanges (Benjakul *et al.*, 1997). The result indicated that the freeze–thaw process accelerated the formation of disulfide bonds (Benjakul and Bauer, 2000). The decrease in sulfhydryl content was coincidental with the decrease in Ca^{2+}-ATPase activity. It was presumed that conformational changes of myosin, especially in the head region occurred rapidly after freeze-thawing. From the result, the decrease in sulfhydryl content of NAM was dependent upon type and level of cryoprotectants used.

1.3.Changes in disulfide bonds content

The effect of cryoprotectants at different concentrations on changes of disulfide bonds content in NAM after 1 and 2 freeze-thaw cycles is depicted in figure 14. The highest disulfide bonds content was found in the control and a lower disulfide content was observed as cryoprotectant concentrations added increased. This suggested that the oxidation of sulfhydryl decreased in presence of cryoprotectant, especially at higher concentration. The increase in disulfide bonds content in samples added with 8% trehalose (1.90 mol/10^6g protein) tended to be lower than sample added with 8% sucrose (1.93 mol/10^6g protein) and 8% sorbitol (2.00 mol/10^6g protein) after 1 freeze-thaw cycle. At level of 8% trehalose, sucrose and sorbitol, disulfide bond content of 2.00, 2.06 and 2.24 mol/10^6g protein were observed, respectively, after 2 freeze-thaw cycles (Figure 14b). Sample with 2 freeze-thaw cycles had higher disulfide bonds content than that with 1 cycle at all levels of cryoprotectant tested. Sultanbawa and Li-Chan (2001) found that the NAM and surimi from ling cod without cryoprotectants had increased disulfide bonds after freezing. However, the increased disulfide bond was not observed for treatments with cryoprotectant.

The decrease of sulfhydryl groups with a concomitant disulfide bond formation was generally in accordance with the decreased Ca^{2+}-ATPase activity (Figure 12). The oxidation of sulfhydryl group, especially in the head region caused the decrease Ca^{2+}-ATPase activity (Benjakul *et al.*, 1997). This results indicated that sulfhydryl oxidation and the decrease in Ca^{2+}-ATPase activity can be prevented by addition of cryoprotectant and levels used was found to govern the efficacy in retardation of those changes.

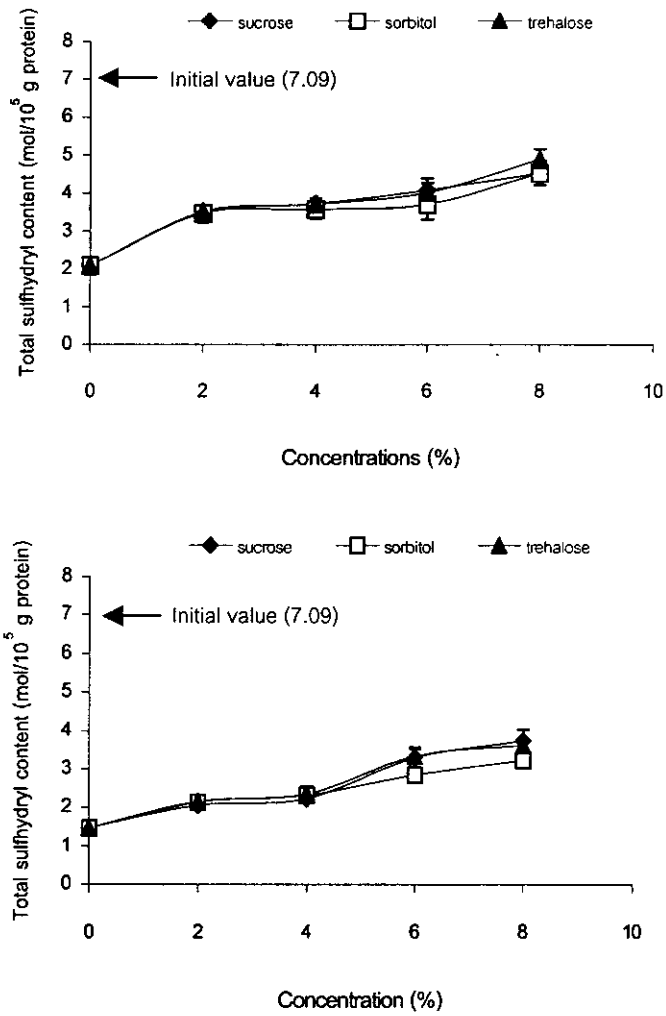

Figure 13 Effect of different cryoprotectants at various concentrations on changes of total sulfhydryl content in NAM (2.5 mg/ml, pH 7.0, 0.6 M KCl) subjected to 1 (a), and 2 (b) freeze-thaw cycles.

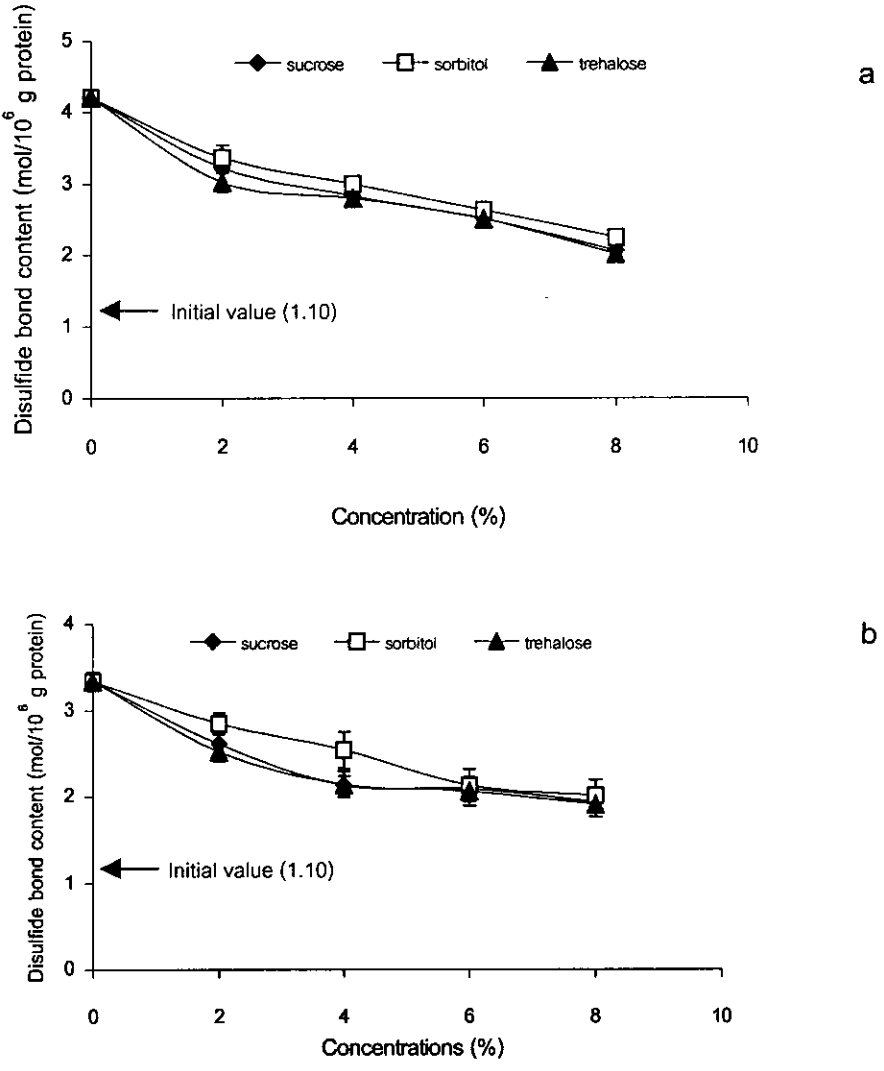

Figure 14 Effect of different cryoprotectants at various concentrations on changes of disulfide bonds content in NAM (2.5 mg/ml, pH 7.0, 0.6 M KCl) subjected to 1 (a), and 2 (b) freeze-thaw cycles.

1.4. Changes in surface hydrophobicity

The changes in surface hydrophobicity were observed after freeze-thawing of 1 and 2 cycles (Figure 15a and 15b). After freeze-thawing, surface hydrophobicity of NAM increased from initial value. Samples subjected to 2 freeze-thaw cycles increased in surface hydrophobicity than those with 1 freeze-thaw cycle. However, such an increased could be retarded in presence of cryoprotectants. Higher concentration of sucrose, sorbitol and trehalose retarded the increase in surface hydrophobicity to a higher extent. At the same level, the differences in surface hydrophobicity were observed among samples added with different cryoprotectants. Efficacy of cryoprotectant to prevent the increase in surface hydrophobicity decreased with increasing freeze-thaw cycles.

From the results, increased surface hydrophobicity suggested that proteins underwent denaturation induced by the freeze-thaw process (Benjakul and Bauer, 2000). Protein denaturation during freezing and frozen storage was mainly caused by formation of disulfide, hydrogen and hydrophobic bonds (Jiang *et al.*, 1988). Freeze-thawing possibly induced the conformational changes of NAM, leading to the exposure of hydrophobic residues. As a consequence, hydrophobic-hydrophobic interaction could be formed with a concomitant aggregation of protein. Benjakul and Bauer (2000) reported that freeze-thaw cycles affected the physico-chemical and enzymatic properties of cod muscle protein. Thus, the increase in surface hydrophobicity was retarded in presence of cryoprotectant, especially with higher level of cryoprotectant.

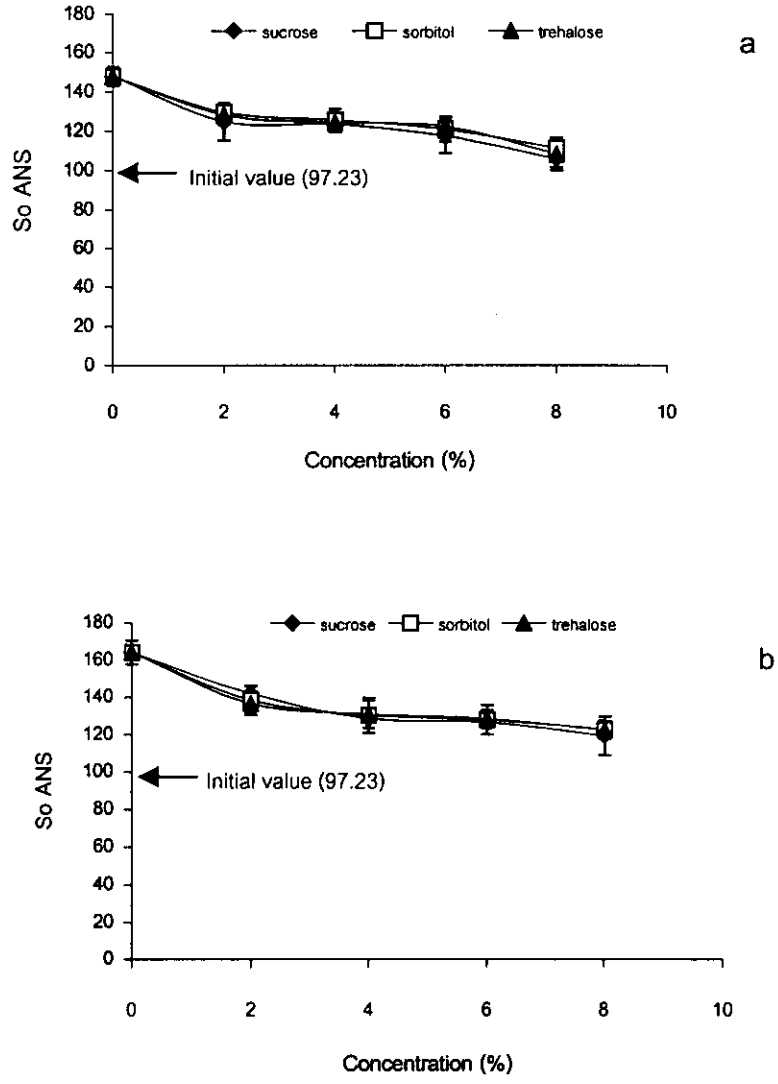

Figure 15 Effect of different cryoprotectants at various concentrations on changes of surface hydrophobicity in NAM (2.5 mg/ml, pH 7.0, 0.6 M KCl) subjected to 1 (a), and 2 (b) freeze-thaw cycles.

1.5. Changes in solubility in 0.6 M KCl

The effect of cryoprotectants at different concentrations on changes in solubility of NAM is shown in Figure 16. The decreased solubility was concomitant with the decreased Ca^{2+}ATPase activity and sulfhydryl content and accompanied

by the increase in disulfide bond and surface hydrophobicity. It has been reported that the decreased protein solubility is primary criterion of protein denaturation during frozen storage caused by intermolecular hydrogen or hydrophobic bonds, as well as disulfide bonds and ionic interaction (Noguchi *et al.*, 1976). Oguni *et al.* (1975) suggested that the solubility of actomyosin decreased with frozen storage and protein aggregation proceeded during frozen storage. From the result, solubility of NAM in 0.6 M KCl decreased with increasing freeze-thaw cycles. The lowest solubility was observed in the control, particularly with 2 freeze-thaw cycles. After 1 freeze-thaw cycle, NAM added with trehalose, sucrose and sorbitol had the solubility of 91.36, 88.88, and 84.80%, respectively. No significant difference in solubility between trehalose and sucrose was observed ($p<0.05$), while sample added with sorbitol had the lowest solubility ($p<0.05$). After 2 freeze-thaw cycles, solubility of samples added with 8% trehalose, sucrose and sorbitol was 79.20, 75.36, and 78.80%, respectively. From the result, the loss in solubility was prevented by addition of cryoprotectant, particularly with higher levels added. Trehalose showed the similar potential in prevention of the loss in solubility, compared to sucrose, but was higher than sorbilol. McGarvey and Craigd (2001) found that trehalose can stabilize chicken egg white lysozyme protein to a greater extent than sucrose.

Srikar and Reddy (1988) reported the decrease in solubility of pink perch protein during frozen storage. Jiang *et al.* (1988) found that solubility of milkfish actomyosin in 0.6 M KCl decreased during frozen. Aggregates formed during frozen storage of minced cod are mostly linked by secondary interaction and disulfide bridges (Tejada *et al.*, 1996). With addition of cryoprotectants, decrease in solubility was retarded. Sugar protects protein from freeze denaturation by increasing the surface tension of water as well as the amount of bound water (Noguchi *et al.*, 1976). This prevents withdrawal of water molecules from the protein, thus stabilizing the protein. Water migrating from proteins to form ice crystals is

caused by disruption of hydrogen bonding and bonds between proteins (Noguchi *et al.*, 1976; Pomeranz,1985). From the result, cryoprotectant at a level of 8% could prevent the physico-chemical changes of muscle protein. As a result, the proteins were more stabilized as shown by the remained solubility.

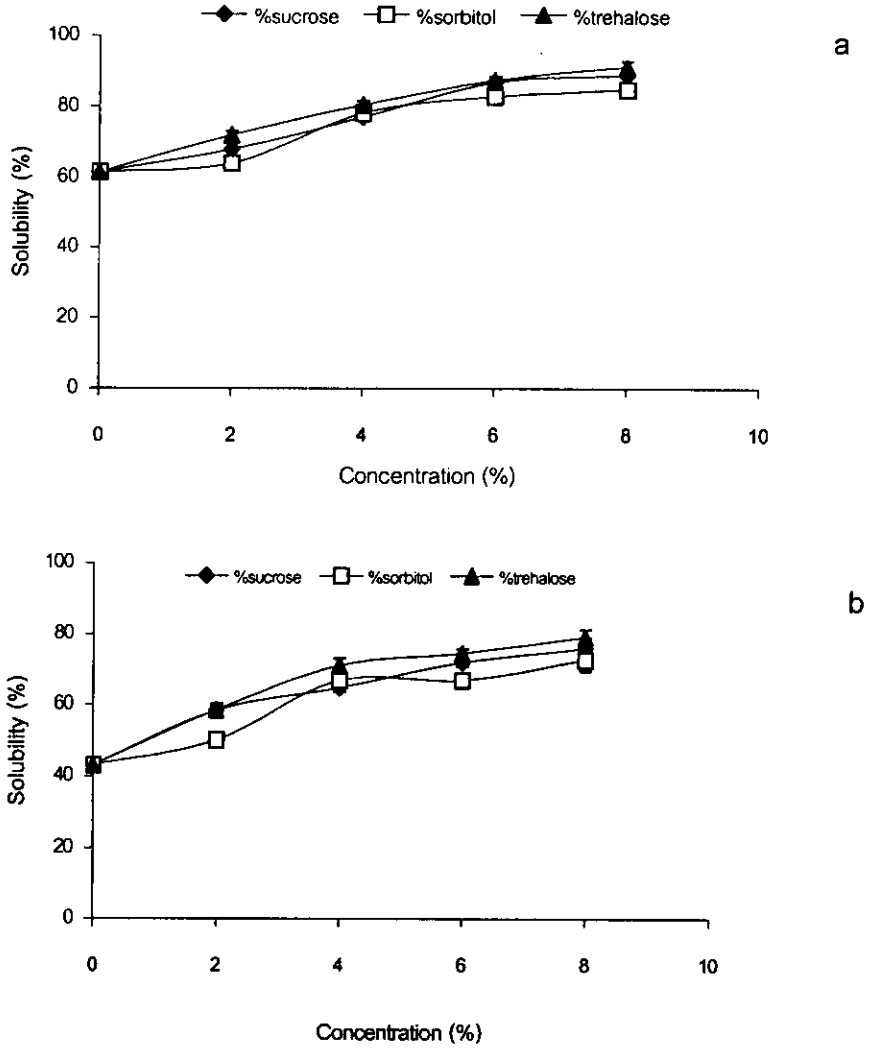

Figure 16 Effect of different cryoprotectants at various concentrations on changes of %solubility in 0.6 M KCl in NAM (2.5 mg/ml, pH 7.0, 0.6 M KCl) subjected to 1 (a), and 2 (b) freeze-thaw cycles.

2. Cryoprotective effect of different formulae of cryoprotectants in NAM with 1 and 2 freeze-thaw cycles.

To optimize the uses of cryoprotectants, the mixture design (Gacula, 1993) was used to formulate the different formulae of cryoprotectants. Cryoprotective efficacy of all formulae was compared with the control.

2.1. Changes in Ca^{2+}-ATPase activity.

Table 4 shows the changes in Ca^{2+}-ATPase activity in NAM added with different cryoprotectant formulae after 1 and 2 freeze-thaw cycles. Ca^{2+}-ATPase activity decreased differently depending on cryoprotectant used. Sompongse *et al.* (1996) found that in presence of cryoprotectant, the decrease in Ca^{2+}-ATPase activity and unfolding of actomyosin were suppressed. Cryoprotectant blends prevented the decrease in Ca^{2+}-ATPase activity more effectively than the control (with no cryoprotectants). Blends including 5.34% trehalose+1.33% sucrose+1.33% sorbitol was the best formula to retain Ca^{2+}-ATPase activity in NAM subjected to 1 and 2 freeze-thaw cycles. After 2 freeze-thaw cycles, cryoprotectant efficacy was decreased to some extent. At level of 8%, cryoprotectant alone showed different efficacy (Table 4). NAM added with 8% sorbitol had lower Ca^{2+}-ATPase than those added with 8% sucrose or 8% trehalose, especially after 2 freeze-thaw cycles. From the result, use of blends could retard the decrease in Ca^{2+}-ATPase activity, indication the higher stabilizing effect of the blends on protein during frozen storage. Thus, the mixture of various cryoprotectants at an appropriate ratio exhibited the enhanced cryoprotective activity.

Table 4. Effect of individual cryoprotectant and cryoprotectant blends on Ca^{2+}-ATPase activity in NAM subjected to 1 and 2 freeze-thaw cycle.

Treatment	Ca^{2+}-ATPase activity (μmol Pi/mg protein/min)	
	1 cycle	2 cycles
Control	0.164±0.011^{a}	0.082±0.008^{a}
8%Sucrose	0.250±0.005^{bc}	0.206±0.011^{cde}
8%Sorbitol	0.243±0.007^{b}	0.174±0.010^{b}
8%Trehalose	0.265±0.011^{cde}	0.217±0.011^{de}
4%%Trehalose + 4%Sucrose	0.276±0.010^{e}	0.204±0.012^{cde}
4%Sucrose + 4%Sorbitol	0.257±0.011^{bc}	0.195±0.010^{bcd}
4%Trehalose + 4%Sorbitol	0.254±0.003^{bcd}	0.192±0.006^{bcd}
2.67%Trehalose+2.67%Sucrose+2.67%Sorbitol	0.266±0.006^{cde}	0.201±0.006^{cde}
5.34%Trehalose+1.33%Sucrose+1.33%Sorbitol	0.277±0.007^{e}	0.221±0.009^{e}
1.33%Trehalose+5.34%Sucrose+1.33%Sorbitol	0.273±0.006^{de}	0.197±0.010^{bcde}
1.33%Trehalose+1.33%Sucrose+5.34%Sorbitol	0.262±0.009^{cde}	0.186±0.011^{bc}

Mean±SD from triplicate determinations.

Different letters in the same column indicate the significant difference ($p \leq 0.05$).

2.2. Sulfhydryl groups and disulfide bonds content

Total sulfhydryl groups and disulfide bonds content of NAM added with different formulae of cryoprotectants after 1 and 2 freeze-thaw cycles are shown in Table 5 and 6. Total sulfhydryl group and disulfide bond content were significantly different among treatments ($p<0.05$).

Sulfhydryl content in the control decreased more extensively, compared to NAM with cryoprotectants after 1 and 2 freeze-thaw cycles (Table 5). No significant difference ($p<0.05$) in sulfhydryl content between NAM added with 8%trehalose and 8% sucrose and blend of 5.34% trehalose, 1.33% sucrose, 1.33% sorbitol was

observed. After 2 freeze-thaw cycles, cryoprotectants both alone and blends, retained sulfhydryl content to a lower extent compared to 1 cycle. In general, the decrease in sulfhydryl group was coincidental with decrease in Ca^{2+}ATPase activity, suggesting oxidation of sulfhydryl groups on active site of myosin (Jiang *et al.*, 1986). Sompongse *et al.*(1996) reported that, in the case of no additives, a decrease in sulfhydryl content and dimer formation of myosin heavy chain (MHC) through disulfide bonding were observed along with a decrease in Ca^{2+}-ATPase activity and increase in surface hydrophobicity.

Table 6 shows the changes of disulfide bonds content in NAM, it was found that disulfide bonds content was highest in the control, compared to other treatments for both 1 and 2 freeze-thaw cycles. However, no marked differences in disulfide bond content were observed among all treatments ($p>0.05$), though disulfide bond content was much lower than the control ($p<0.05$). In general, the increased disulfide bond was coincidental with the decreased sulfhydryl content. Ramirez *et al.* (2000) also provided evidence for the loss of sulfhyldryl group and formation of disulfide bonds during frozen storage of tilapia. However, the extent of involvement of sulfhydryl groups and disulfide bonds in frozen-storage-induced aggregation was suggested to depend on the initial physico-chemical state of myosin. Higher involvement for myosin was found when frozen in solubilized form than when frozen in suspension (Sultanbawa and Li-chan, 2001). From the result, formation of disulfide bonds in the control NAM of control was higher than those added with cyroprotectants. This result indicated that sulfhydryl groups of NAM from control was more prone to disulfide formation. This was possibly facilitated by the structural changes of NAM after freeze-thaw cycles, which made the sulfhydryl groups more susceptible to oxidation. The result suggested that disulfide bonds would play an essential role in actomyosin aggregation. Cryoprotectants added possibly stabilized the protein conformation, in which sulfhydryl groups were not

exposed for oxidation. As a result, disulfide formation was lower in the presence of cryoprotectants.

The result also indicated that number of freeze-thaw cycle affected aggregation of NAM. Proteins underwent changes to a higher extent even in the presence of cryoprotectant.

Table 5. Effect of individual cryoprotectant and cryoprotectant blends on total sulfhydryl group content in NAM subjected to 1 and 2 freeze-thaw cycles.

Treatment	Total sulfhydryl group content (mol /10^5 g protein)	
	1 cycle	2 cycles
Control	4.294±0.155^{a}	1.863±0.114^{a}
8%Sucrose	6.647±0.204^{c}	5.725±0.162^{de}
8%Sorbitol	6.196±0.061^{b}	5.105±0.222^{bcde}
8%Trehalose	6.853±0.241^{c}	5.951±0.098^{e}
4%%Trehalose + 4%Sucrose	6.314±0.191^{b}	5.049±0.103^{bcde}
4%Sucrose + 4%Sorbitol	6.216±0.365^{b}	5.235±0.106^{b}
4%Trehalose + 4%Sorbitol	6.284±0.119^{b}	4.745±0.074^{bcd}
2.67%Trehalose+2.67%Sucrose+2.67%Sorbitol	6.265±0.088^{b}	5.059±0.283^{bcde}
5.34%Trehalose+1.33%Sucrose+1.33%Sorbitol	6.725±0.039^{c}	5.559±0.029^{cde}
1.33%Trehalose+5.34%Sucrose+1.33%Sorbitol	6.127±0.089^{b}	4.863±0.074^{bcd}
1.33%Trehalose+1.33%Sucrose+5.34%Sorbitol	6.010±0.061^{b}	4.578±0.103^{bc}

Mean±SD from triplicate determinations.

Different letters in the same column indicate the significant difference ($p \leq 0.05$).

Table 6. Effect of individual cryoprotectant and cryoprotectant blends on disulfide bonds content in NAM subjected to 1 and 2 freeze-thaw cycles.

Treatment	Disulfide bonds content (mol /10^6 g protein)	
	1 cycle	2 cycles
Control	3.089±0.145^{d}	4.403±0.057^{b}
8%Sucrose	1.986±0.103^{abc}	2.782±0.072^{a}
8%Sorbitol	2.177±0.158^{c}	2.878±0.088^{a}
8%Trehalose	1.832±0.185^{a}	2.657±0.288^{a}
4%%Trehalose + 4%Sucrose	2.034±0.072^{abc}	2.897±0.185^{a}
4%Sucrose + 4%Sorbitol	2.139±0.059^{bc}	2.782±0.251^{a}
4%Trehalose + 4%Sorbitol	1.928±0.057^{ab}	2.954±0.251^{a}
2.67%Trehalose+2.67%Sucrose+2.67%Sorbitol	2.053±0.060^{abc}	2.859±0.101^{a}
5.34%Trehalose+1.33%Sucrose+1.33%Sorbitol	1.976±0.072^{abc}	2.628±0.164^{a}
1.33%Trehalose+5.34%Sucrose+1.33%Sorbitol	2.043±0.175^{abc}	2.763±0.256^{a}
1.33%Trehalose+1.33%Sucrose+5.34%Sorbitol	2.091±0.116^{bc}	2.849±0.187^{a}

Mean±SD from triplicate determinations.

Different letters in the same column indicate the significant difference ($p \leq 0.05$).

2.3 Changes in surface hydrophobicity.

The changes in surface hydrophobicity in NAM added with and without cryoprotectant are shown in Table 7. For the control, surface hydrophobicity increased to higher extent, compared to other treatments. After 1 freeze-thaw cycle, sample added with 8% sucrose had the lowest surface hydrophobicity. After 2 freeze-thaw cycles, no significant differences in surface hydrophobicity among NAM added with 8% sucrose, 8% trehalose and the blend of 5.34% trehalose +1.33% sucrose+1.33% sorbitol were found.

Surface hydrophobicity has been suggested to be a sensitive measure of protein denaturation or alteration in structural conformation (Li-Chen *et al.*, 1986). An increase in the hydrophobicity value generally indicates unfolding of the protein. Protein-protein interactions (aggregates), which would bury hydrophobic patches or apolar amino-acid side chains that had previously been exposed, also occur (Parkington *et al.*, 2000). The cryoprotectants added to prevent the loss of protein functionality from freeze-thaw damages could affect the water structure around the proteins probably in such a way that minimizes the hydrophobic environment that the protein is exposed to. From the result, different cryoprotectants affected surface hydrophobicity differently, possibly due to their varying capability to reduce the exposure of hydrohpobic portion.

Table 7. Effect of individual cryoprotectant and cryoprotectant blends on surface hydrophobicity in NAM subjected to 1 and 2 freeze-thaw cycles.

Treatment	Surface hydrophobicity	
	1 cycle	2 cycles
Control	147.85±0.15^{h}	170.17±4.47^{g}
8%Sucrose	115.86±0.19^{a}	119.62±0.64^{a}
8%Sorbitol	121.36±0.19^{cd}	139.40±1.05^{f}
8%Trehalose	118.41±0.54^{b}	122.63±0.82^{ab}
4%%Trehalose + 4%Sucrose	118.91±0.58^{b}	126.77±0.99^{bc}
4%Sucrose + 4%Sorbitol	122.97±0.92^{cd}	134.30±1.46^{e}
4%Trehalose + 4%Sorbitol	120.86±1.25^{c}	127.80±0.34^{cd}
2.67%Trehalose+2.67%Sucrose+2.67%Sorbitol	120.57±0.18^{c}	128.62±2.29^{cd}
5.34%Trehalose+1.33%Sucrose+1.33%Sorbitol	121.75±0.74^{d}	122.94±2.75^{ab}
1.33%Trehalose+5.34%Sucrose+1.33%Sorbitol	124.71±0.28^{f}	134.92±0.98^{e}
1.33%Trehalose+1.33%Sucrose+5.34%Sorbitol	126.72±0.37^{g}	132.13±2.62^{de}

Mean±SD from triplicate determinations.

Different letters in the same column indicate the significant difference ($p \leq 0.05$).

2.4 Changes in solubility in 0.6 M KCl

Protein solubility of the NAM with and without cryoprotectants after 1 and 2 freeze-thaw cycle is shown in Table 8. After 1 and 2 freeze-thaw cycles, protein solubility decreased in all treatments. The highest decrease was observed in the control after both 1 and 2 freeze-thaw cycles with the solubility of 61.32 and 39.20% respectively. After 1 freeze-thaw cycle, solubility of NAM added with the blend including 5.34% trehalose+1.33% sucrose+1.33% sorbitol (88.49%) was higher than other treatments but was not significantly different from that added with 8% trehalose (87.87%) ($p<0.05$). Treatment including sorbitol and 8% sorbitol alone had

lower solubility than treatment including trehalose or sucrose. Similar trend was found with NAM subjected to 2 freeze-thaw cycles.

With freeze-thawing cycle, proteins underwent aggregation, leading to the loss in solubility and decreased Ca^{2+}-ATPase activity. The decrease of protein solubility is a primary criterion of protein denaturation during frozen storage, resulting from intermolecular hydrogen of hydrophobic bonds, as well as disulfide bonds and ionic interactions (Matsumoto, 1980; Akahane, 1982; Sultanbawa and Li-Chen, 1998). From the result, the denaturation of protein could be prevented by the addition of cryoprotectants. The best cryoprotectant formulae that maintained protein solubility most effectively was 8% trehalose, or blends of 5.34% trehalose, 1.33% sucrose, 1.33% sorbitol.

Table 8. Effect of individual cryoprotectant and cryoprotectant blends on %solubility in 0.6 M KCl in NAM subjected to 1 and 2 freeze-thaw cycles.

Treatment	%Solubility in 0.6 M KCl	
	1 cycle	2 cycles
Control	61.323±0.273^{a}	39.203±0.240^{a}
8%Sucrose	87.288±0.373^{ef}	77.491±0.232^{c}
8%Sorbitol	83.962±0.313^{bc}	73.420±1.859^{b}
8%Trehalose	87.874±0.743^{fg}	80.359±0.355^{d}
4%%Trehalose + 4%Sucrose	86.638±0.576^{e}	72.962±1.288^{b}
4%Sucrose + 4%Sorbitol	83.680±0.320^{bc}	73.282±1.192^{b}
4%Trehalose + 4%Sorbitol	83.725±0.339^{bc}	77.084±0.927^{c}
2.67%Trehalose+2.67%Sucrose+2.67%Sorbitol	84.966±0.448^{d}	71.825±0.437^{b}
5.34%Trehalose+1.33%Sucrose+1.33%Sorbitol	88.493±0.429^{g}	82.190±0.502^{d}
1.33%Trehalose+5.34%Sucrose+1.33%Sorbitol	84.314±0.183^{cd}	73.727±0.440^{b}
1.33%Trehalose+1.33%Sucrose+5.34%Sorbitol	83.235±0.596^{b}	71.733±1.186^{b}

Mean±SD from triplicate determinations.

Different letters in the same column indicate the significant difference ($p \leq 0.05$).

Freeze denaturation of fish proteins was prevented by the use of cryoprotectants (Sych *et al.,* 1990; MacDonald and Lanier, 1991; Park, 1994). Matsumoto (1980) hypothesized that the cryoprotectant molecules may bind or associate with protein molecules at one of the functional groups either by ionic bonds or by hydrogen bonds. Thus each protein molecule is coated with the cryoprotectant. Carpenter and Crowe (1988) suggested that sugars are excluded from the surface of proteins because the sugars raise the surface tension of water, and thereby increase the cohesive forces within the water structure. In this situation, the lower surface tension bulk water tends to hydrate the protein since disruption of the solute-water interactions to form protein-water interactions is energetically unfavorable. Thus, the high surface tension water with its solute is excluded from contact with the protein.

From the study, screening of effective cryoprotectant was carried out using repeated freeze-thaw cycles for the ease and acceleration of its operation. Noguchi and Matsumoto (1971) suggested that *in vitro* model test system is useful in monitoring the denaturation during the frozen storage and in screening the effective substances. From the results, 8% trehalose and the blend of 5.34% trehalose + 1.33% sucrose + 1.33% sorbitol showed the effective cryoprotective property in NAM, as evidenced by maintaining solubility and Ca^{2+}-ATPase activity more effectively than other treatments. The most functional properties of muscle proteins are related to protein solubility (Lin and Park, 1996; Damodaran, 1996). Decrease in solubility during frozen storage and loss of Ca^{2+} -ATPase activity was described for myosin in frozen fish (Li-Chen *et al.*, 1985). Myosin is responsible for surimi gel formation, which is the associated with the quality on surimi-based products (Saeki et al., 1995; Matsumoto, 1979; Grabowska and Sikorski, 1976). Those two formulae were chosen and used in surimi. The changes in physico-chemical property of muscle protein and gel-forming ability of surimi were monitored during frozen storage at –18 ^{0}C up to 12 weeks.

3. Characterization of NAM aggregate with and without cryoprotectants after multiple freeze-thaw cycles.

3.1 Solubility of NAM aggregate in various denaturating solutions

NAM was added with 1) 8%trehalose 2) the blend including 5.34% trehalose+1.33% sucrose+1.33% sorbitol 3) commercial cryoprotectant (4% sucrose+4% sorbitol) and the mixtures were subjected to freeze-thawing for 2 and 4 cycles. The precipitate was collected and dissolved in solution I (1% SDS), solution II (1% SDS+8 M urea) and solution III (1% SDS+8 M urea+2% βME). The solubility of NAM aggregate varied with different solutions used (Figure 17). Aggregate of the control showed the lower solubility in all three solutions, compared to other treatments. In general, solution III tended to render the highest solubility in all treatments. After 1 cycle, solubility of NAM aggregate with 8%trehalose and the blend including 5.34% trehalose+1.33% sucrose +1.33% sorbitol were similar for all solutions tested. However, after 2 cycles, NAM aggregate with 8% trehalose had the highest solubility, compared to other treatments. Solubility in different solutions indicates types of bonds involved in NAM aggregate. Solution I (1% SDS) was considered to solubilize protein aggregated by ionic bond and hydrogen bond. Solution II (1% SDS+8 M urea) was used to solubilize protein aggregated by hydrogen bonds and hydrophobic interaction, and solution III (1% SDS+8 M urea+2% βME) could destroy disulfide bonds involved in protein aggregate (Chawla *et al.*, 1996). Urea is considered as a denaturant and a solubilizing agent and is widely used for solubilization and unfolding of proteins by destabilizing the hydrogen bonding and hydrophobic interactions (Huidobro *et al.*, 1998). Thus, the aggregation of NAM with and without cryoprotectant treatments was caused by hydrogen bonds, hydrophobic bonds and disulfide bonds. The increase in solubility in presence of urea and βME indicated the involvement of hydrophobic interaction and disulfide bond, respectively. The formation of disulfide bond was reconfirmed by the increase in disulfide bonds content (Figure 14 and Table 6), while the

hydrophobic interaction was indicated by the increase in surface hydrophobicity (Figure 15 and Table 7). Tejada *et al.* (1996) reported that treatment with combination of a disulfide bridge reducing agent and SDS produced a substantial increase in the amount of protein extracted during the later stages of frozen storage, thus highlighting the importance of disulfide covalent bonds in aggregation of minced cod. From the result, the control was dissolved in three solutions to the lowest extent, compared to other treatments. After 4 freeze-thaw cycles, the protein aggregate was dissolved slightly lower than those subjected to 2 cycles. It was possibly due to the presence of strong protein-protein interaction through covalent cross-linking, non-polar, or polar bonds (Lian *et al.*, 2000) after freeze-thawing. The aggregates tended to grow in number and size as storage progressed, becoming insoluble in salt solution but still extractable in SDS or SDS plus β-mercaptoethanol (ME) (Huidoro *et al.*, 1998; Lian *et al.*, 2000). Evidence has also been found for the formation of non-disulfide covalent bonds, especially with the control. Insoluble residues were still formed after the solubilizing with solution III. The insoluble residue indicated the protein aggregate cross-linked by non-disulfide covalent bond possibly induced by formaldhyde formed. From the result, after 4 freeze-thaw cycles, aggregate from NAM added with 8% trehalose was dissolved in three solutions more than those added with the blend including 5.34% trehalose+1.33% sucrose+1.33% sorbitol, commercial cyoprotectants and the control. This suggested that 8%trehalose may prevent the strong protein-protein interaction more effectively than other treatments. Once solution I was added, weak aggregate stabilized by mostly hydrogen bond was destroyed, leading to high solubility.

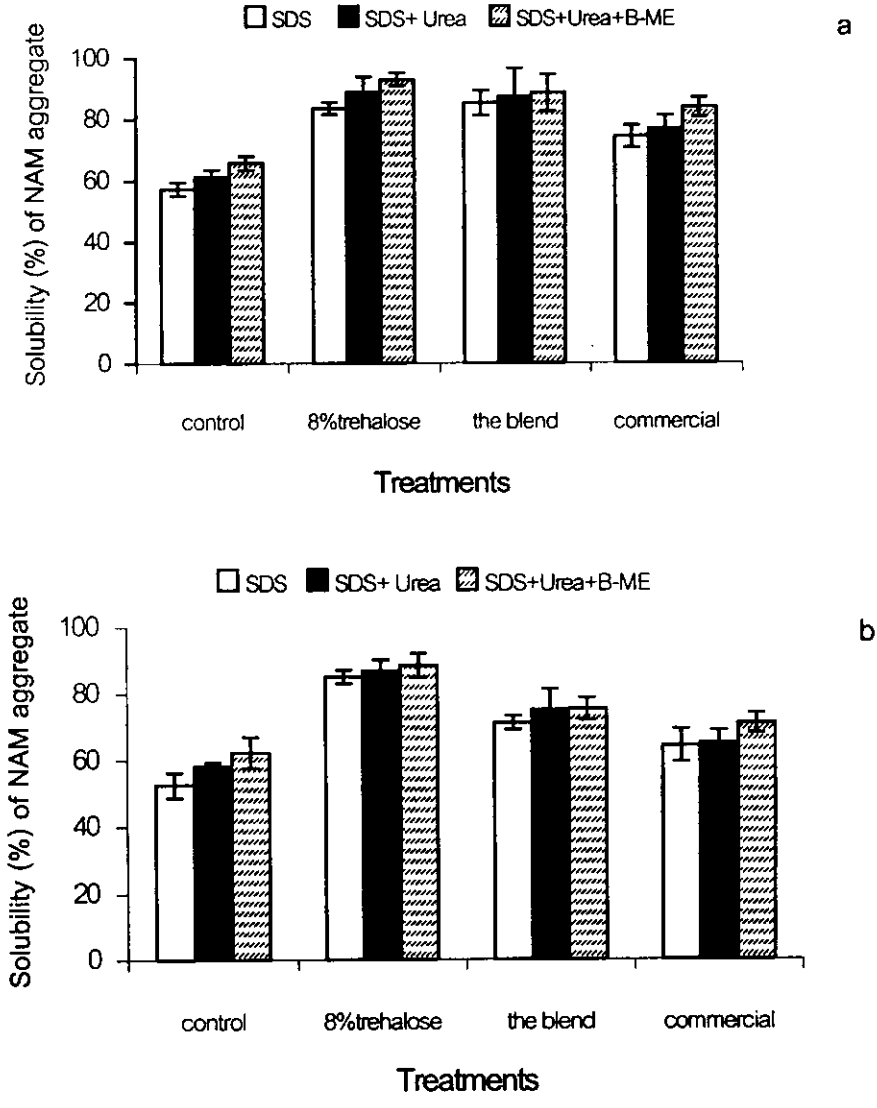

Figure 17 Extractability of protein aggregate from NAM with and without different cryoprotectants in three solution after freeze-thawing for 2 (a) and 4 (b) cycles.

3.2 SDS-PAGE analysis of NAM aggregate in various denaturating solutions.

Protein patterns of NAM aggregate with and without cryoprotectants dissolved with three denaturing solutions are shown in Figure 18. The resulting solution were analyzed with SDS-PAGE under reducing and non-reducing conditions. For non-reducing SDS-PAGE pattern, lower band intensity of MHC were observed in samples dissolved with 1% SDS and 1% SDS+8 M urea of all treatments. However, higher band intensity of MHC in all treatments were observed when dissolved with 1% SDS+8 M urea+2% βME. The similar results were found between samples with 2 and 4 freeze-thaw cycles. β-mercaptoethanol is used to cleave disulfide bonds to increase protein solubility (Lian *et al.*, 2000). The results suggested the important role of disulfide bond in aggregation of NAM, induced by multiple freeze-thaw cycles. For reducing SDS-PAGE pattern of all treatments, after 2 and 4 freeze-thaw cycles, no marked differences in protein pattern were found. MHC band was recovered to a large degree when compared to that band under non-reducing condition. This further confirms that covalent cross-linking in all treatments were predominantly formed by disulfide bonds. Other myofibrillar proteins, such as actin were also changed during freeze-thawing process in all treatment. However, Lian *et al.* (2000) suggested that other myofibrillar protein, such as actin and tropomyosin in red hake were little changed in 17 weeks of frozen storage. Huidobro *et al.* (1998) found that MHC in hake decreased in the 2%SDS extract as storage progressed. Huidobro *et al.* (1998) suggested that another type of protein-protein bond had formed, producing higher molecular weight aggregates, which were not extracted.

From the results, it can be inferred that the type of protein aggregation occurring after freeze-thawing differed from one to another in terms of both percentage of protein and type of aggregates. However, addition of cryoprotectant resulted in the lower amount of aggregate.

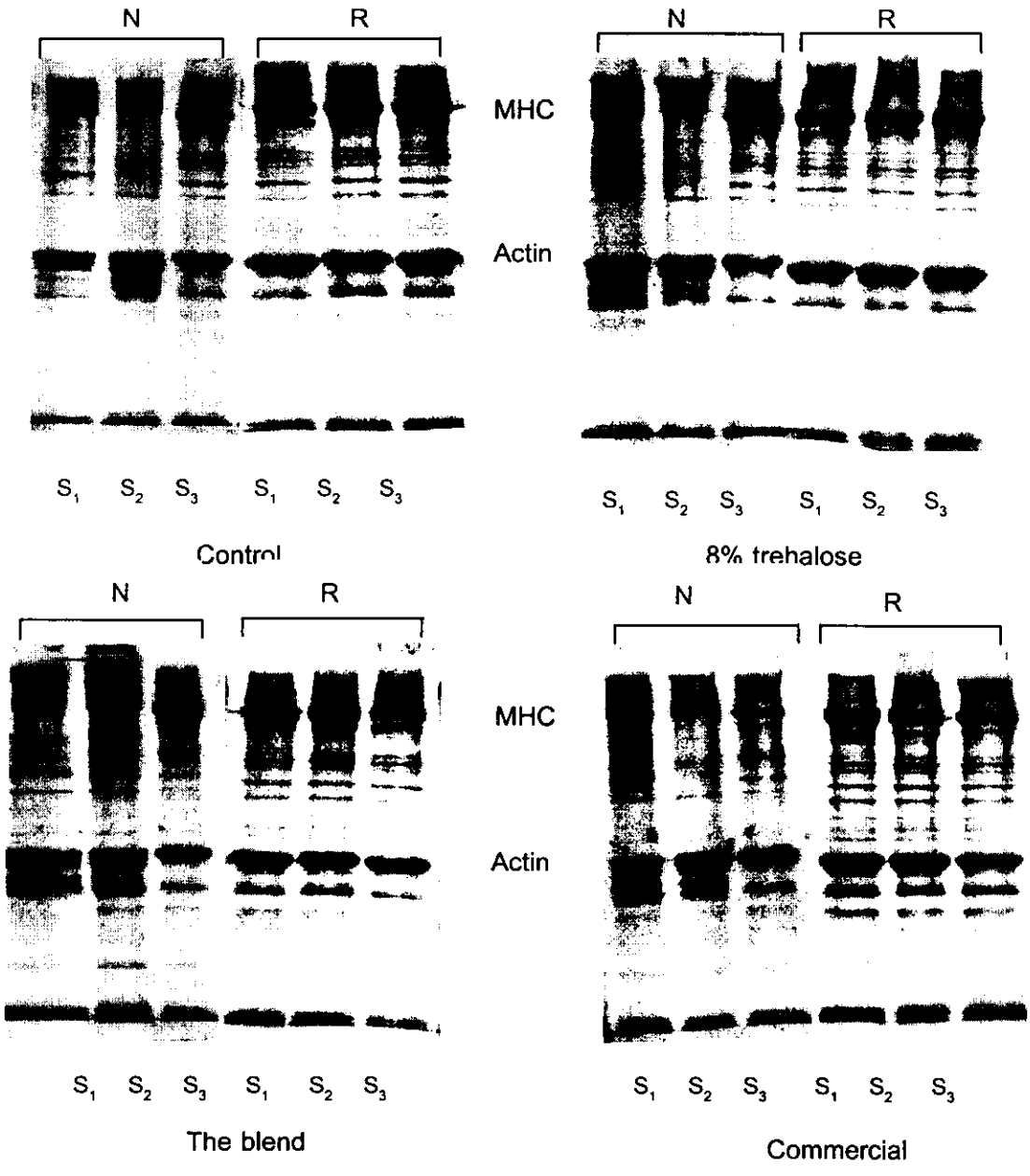

Figure 18a SDS-PAGE pattern of NAM aggregate with and without cryoprotectants after 2 freeze-thaw cycles, dissolved in three denaturing solution, N: non-reducing; R: reducing; S_1: 1% SDS; S_2: 1% SDS+8 M urea; $S_3$1% SDS+8 M urea+β-ME.

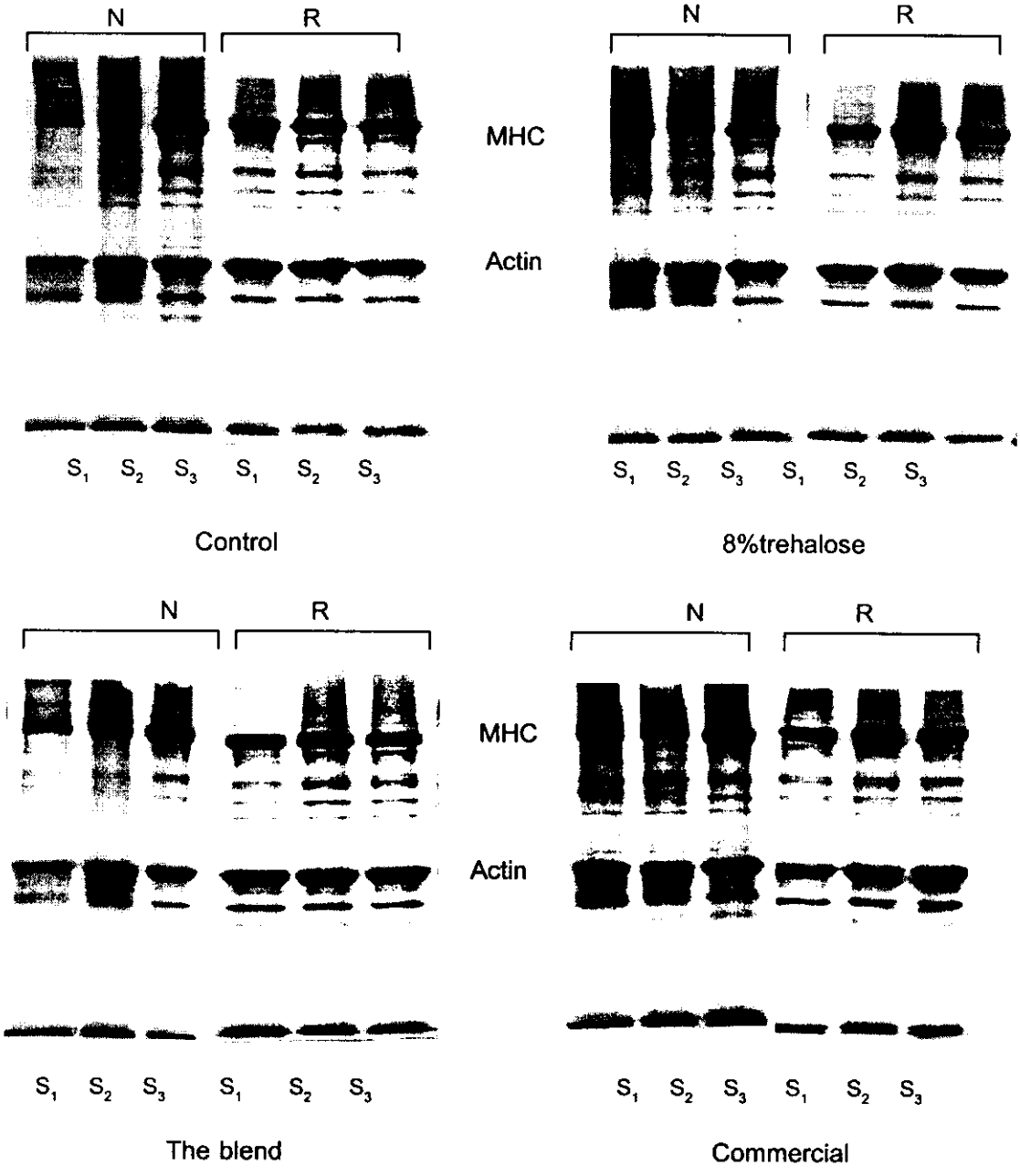

Figure 18b SDS-PAGE pattern of NAM aggregate with and without cryoprotectants after 4 freeze-thaw cycles, dissolved in three denaturing solution, N: non-reducing; R: reducing; S_1: 1% SDS; S_2: 1% SDS+8 M urea; S_3:1% SDS+8 M urea +β-ME.

4. Changes in physicochemical and gelling properties surimi with and without cryoprotectants during frozen storage.

4.1. Changes in pH during frozen storage

No marked changes in pH were observed throughout the frozen storage of 12 weeks. No differences in pH was also found among all samples (Table 9). The pH of all treatments ranged from 6.41 to 6.85. According to Toyoda *et al.* (1992), the pH should be maintained between 6.5 and 7.0 to ensure maximum functional performance of fish protein. The decomposition of nitrogenous compounds causes an increase in pH in fish flesh (Sikorski, *et al.*, 1990). The changes in pH also depend on the liberation of inorganic phosphate and ammonia due to the enzymatic degradation of ATP (Benjakul *et al.*, 2002).

Table 9 Changes in pH of surimi added with different cryoprotectants during frozen storage at –18 ^{0}C for 12 weeks.

Frozen storage (weeks)	pH			
	control	8%trehalose	5.34%trehalose +1.33%sucrose +1.33%sorbitol	4%sucrose +4%sorbitol
0	6.63±0.01[a]	6.64±0.03	6.64±0.01	6.65±0.01
1	6.71±0.03	6.41±0.01	6.69±0.04	6.65±0.03
2	6.60±0.01	6.63±0.03	6.58±0.03	6.63±0.05
4	6.71±0.01	6.42±0.02	6.59±0.01	6.55±0.01
6	6.71±0.04	6.71±0.02	6.56±0.05	6.85±0.10
8	6.60±0.05	6.60±0.03	6.59±0.08	6.63±0.01
10	6.64±0.02	6.60±0.02	6.61±0.02	6.62±0.06
12	6.73±0.02	6.71±0.01	6.64±0.02	6.64±0.06

[a]Mean±SD sample from triplicate determinations.

4.2. Changes in ATPase activity during frozen storage

Ca^{2+}-ATPase activity decreased throughout 12 weeks of frozen storage. (Figure 19a). From the result, after 12 weeks, Ca^{2+}-ATPase activity decreased by 73, 25, 15, and 33% in the control, samples added with 8% trehalose, the blend including 5.34% trehalose+1.33% sucrose+1.33% sorbitol and commercial cryoprotectant, respectively. Among all samples, the control was found to exhibit the lowest activity. Differences in activity among treatments, especially, between treatment with and without cryoprotectant were possibly attributed to different susceptibility to denaturation of surimi protein during frozen storage. The marked decrease in Ca^{2+}-ATPase activity was observed in the control within the first week of frozen storage. After 6 weeks of storage, sample added with commercial cryoprotectant was found to have the lower Ca^{2+}-ATPase activity, compared to those added with 8% trehalose or blends. Ca^{2+}-ATPase activity can be used as an indicator for the integrity of myosin molecules (Benjakul *et al.*, 1997; Auh *et al.*, 1999). The globular heads of myosin are responsible for Ca^{2+}-ATPase activity. A decrease in activity during extended frozen storage indicated the denaturation of myosin, especially, in the head region. Based on the decrease in Ca^{2+}-ATPase activity, myosin in the control underwent denaturation to a highest extent, compared to those from other treatments tested. Auh *et al.* (1999) reported that about 50-55% of activity was lost in sugar-treated Alaska pollock actomyosin. However, more than 90% was lost in the control after 3 weeks of frozen storage. It was apparent that addition of cryoprotectant retarded denaturation rate during frozen storage.

The marked decrease in Mg^{2+}-ATPase and Mg^{2+}-Ca^{2+}-ATPase activities of the control during frozen storage was observed (Figure 19b and 19c). For the samples added with cryoprotectants, both activities decreased slightly as the storage time increased. Mg^{2+}-ATPase and Mg^{2+}-Ca^{2+}-ATPase activities are indicative of the integrity of the actin-myosin complex in the presence of endogenous or exogenous Ca^{2+}ion, respectively (Benjakul *et al.*, 1997). After 12

weeks of storage, Mg^{2+}-ATPase activity decreased by 48, 6, 4, and 10% and Mg^{2+}-Ca^{2+}-ATPase activity decreased by 55, 9, 6 and 16% for the control, samples added with 8%trehalose, the blend and commercial cryoprotectant, respectively.

However, Mg^{2+}-EGTA-ATPase activity increased in all treatments (Figure 19d). Highest increase in Mg^{2+}-EGTA-ATPase activity was found in the control. After week 12 of storage, the increase by 57, 46, 39 and 52% was observed for the control, sample added with 8% trehalose, the blends and commercial cryoprotectant, respectively. From the result, increasing rate of Mg^{2+}-EGTA-ATPase was lowest in sample added with the blends. The result suggested that frozen storage induced the changes in troponin-tropomyosin complex in muscle of all treatments. Therefore, freezing and frozen storage resulted in the denaturation of myosin as shown by the decrease in Ca^{2+}-ATPase activity and denaturation of troponin-tropomyosin complex as indicated by the increase in Mg^{2+}-EGTA-ATPase activity.

Cryoprotectant added could retard the denaturation as indicated by decreasing loss in Ca^{2+}-ATPase, Mg^{2+}-ATPase, and Mg^{2+}-Ca^{2+}-ATPase activities and by decreasing the increase in Mg^{2+}-EGTA-ATPase activity. The changes in ATPase activity was possibly due to the tertiary structural changes, which was caused by ice crystals and the increase in ionic strength of muscle system. Rearrangement of protein via protein-protein interactions was also presumed to contribute to the loss in ATPase activity (Benjakul and Bauar, 2000).

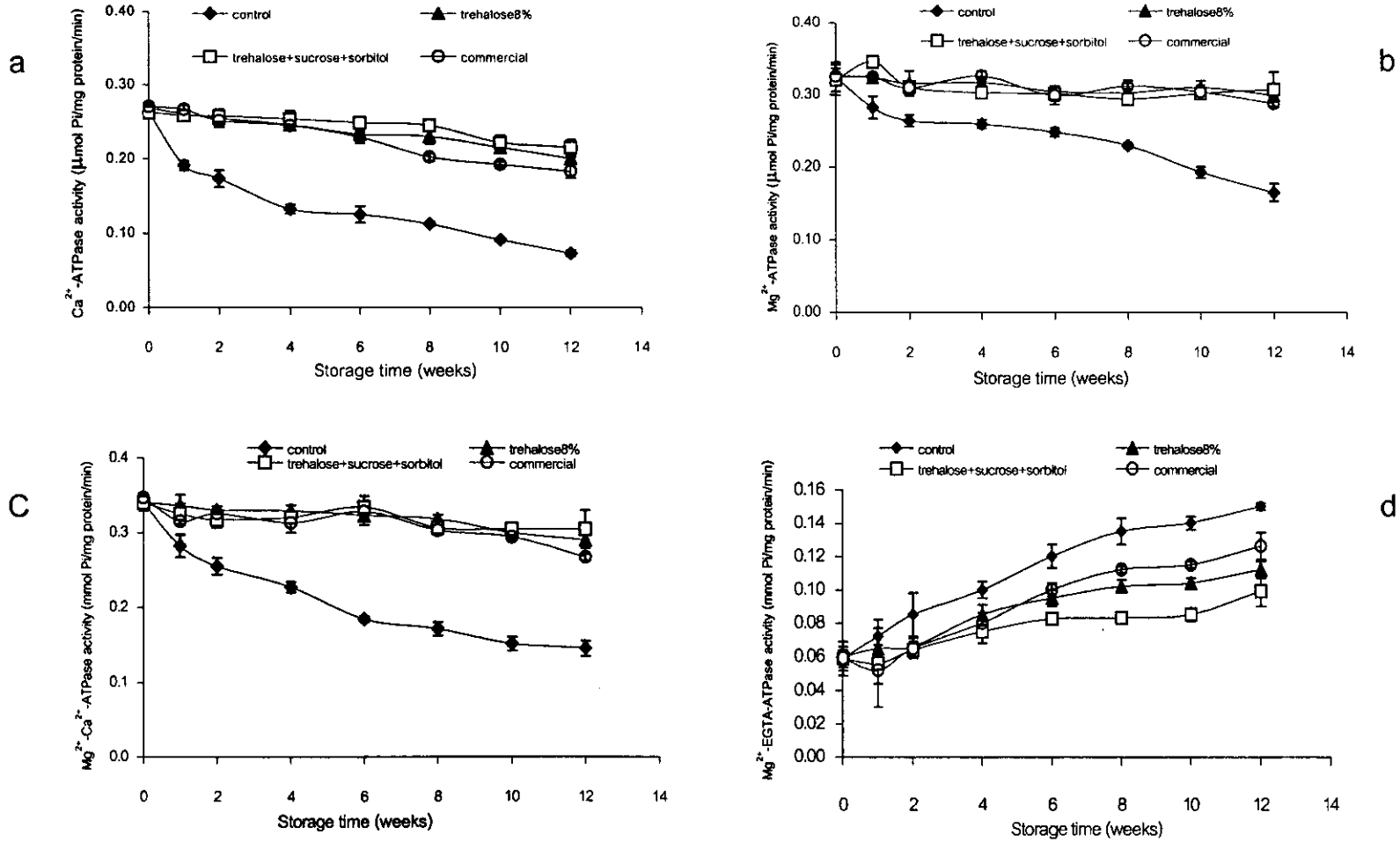

Figure 19 Changes in Ca^{2+} -ATPase (a), Mg^{2+} -ATPase (b), Mg^{2+} -Ca^{2+} -ATPase (c), Mg^{2+} -EGTA-ATPase (d) activity of surimi added with different cryoprotectants during frozen storage at –18 ^{0}C for 12 weeks.

4.3. Changes in Ca^{2+} -sensitivity during frozen storage

Ca^{2+} -sensitivity of NAM extracted from frozen bigeye snapper surimi with and without cryoprotectants is shown in Figure 20. Ca^{2+} -sensitivity was reported to be a good indicator of Ca^{2+}regulation of myofibrillar proteins and was dependent upon the affinity of the troponin molecule for Ca^{2+}ion. Removal of troponin, Ca^{2+} receptive protein of the contractile system, has resulted in a decrease in Ca^{2+}binding capacity (Ebashi *et al.*, 1968; Benjakul *et al.*, 1997). After 12 weeks of storage, decrease in Ca^{2+} -sensitivity by 78% was observed in the control and decrease in Ca^{2+} -sensitivity by 21, 14, and 29% was found in sample added with 8%trehalose, the blends and commercial cryoprotectant, respectively. From the result, the blend including 5.34%trehalose+1.33% sucrose+1.33%sorbitol was the most effective in retardation the loss in Ca^{2+} -sensitivity.

The reduction of Ca^{2+} -sensitivity of myofibrils indicated the denaturation of tropoin or tropomyosin or both. However, the loss in Ca^{2+} -sensitivity during frozen storage was concluded to be the result of the modification of actin-myosin interaction by the oxidation of SH_a in myosin (Sompongse *et al.*, 1996). The decrease in Ca^{2+} -sensitivity was in accordance with the increase in Mg^{2+}EGTA-ATPase activity in all treatments (Figure 16d). From the result, the changes in Mg^{2+}EGTA-ATPase activity of surimi protein during frozen storage could be minimized by addition of cryoprotectant, especially trehalose.

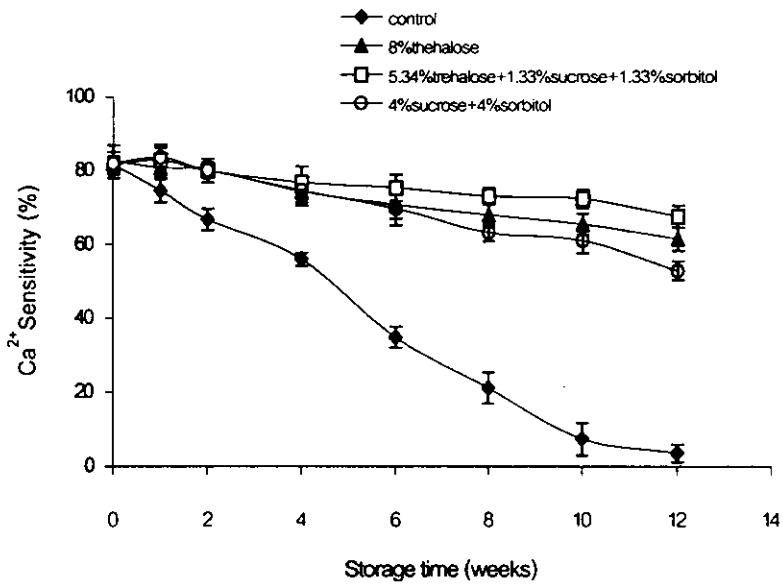

Figure20 Changes in Ca^{2+}-sensitivity in surimi added with different cryoprotectants during frozen storage at $-18\ ^{0}C$ for 12 weeks.

4.4. Changes in total sulfhydryl content during frozen storage

The changes in sulfhydryl content of surimi protein during frozen storage were also monitored (Figure 21). Sulfhydryl content decreased during frozen storage. For the control, the sharp decrease in sulfhydryl content was observed in the first 1 week of storage and continuous decrease was found throughout the storage. Sulfhydryl content in other samples decreased to a lower extent, compared to the control. After 6 weeks of frozen storage, total sulfhydryl content in the control was decreased by 44%. For surimi added with 8% trehalose, the blends or commercial cryoprotectants, total sulfhydryl content was decreased by 26%, 23% and 25%, respectively. However, no differences in total sulfhydryl content among samples added with cryoprotectants. After 12 weeks of storage, total sulfhydryl content was decreased by 65, 33, 30 and 38% for the control, sample added with 8% trehalose, the blends and the commercial cryoprotectant, respectively. Therefore, addition of cryoprotectants could prevent the decrease in total sulfhydryl content.

The decrease in total sulfhydryl contents was considered to be due to the formation of disulfide bonds via oxidation of sulfhydryl groups or disulfide interchange (Benjakul and Bauer, 2000). Oxidation of thiol groups of myosin has been shown to reduce Ca^{2+}-sensitivity and modified actin-myosin interaction (Seki *et al.*, 1979). Buttkus (1970) reported that in freshly killed fish, 42-43 sulfhydryl groups per $5x10^5$g of myosin molecule were found. Two types of sulfhydryl groups on the myosin head portion (SH_1 and SH_2) have been reported to be involved in ATPase activity of myosin (Chen *et al.*, 1995). Another sulfhydryl groups (SH_a) found later was localized in the light meromyosin region of myosin molecule (Yamashita and Itorigome, 1997; Horigome and Yamashita, 1977). Somponges *et al.* (1996) reported that SH_a was responsible for oxidation of MHC and its dimer formation, resulting in an increase in Mg^{2+}-EGTA-ATPase activity of carp actomyosin during iced storage.

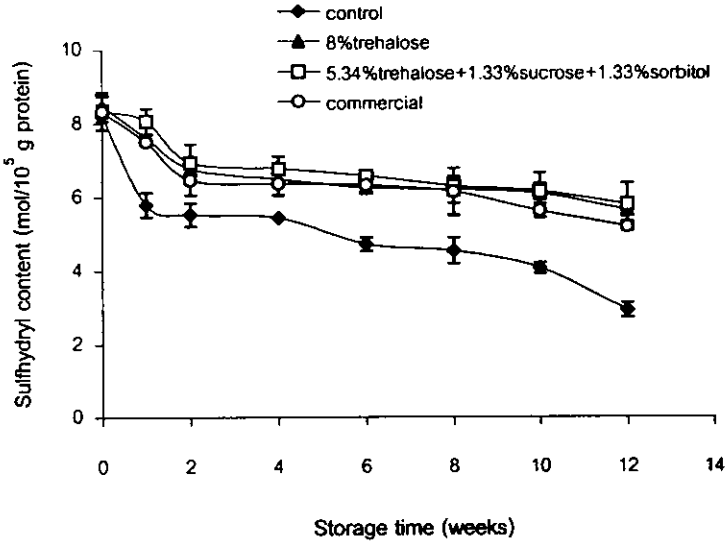

Figure 21 Changes in sulfhydryl group in surimi added with different cryoprotectants during frozen storage at –18 ^{0}C for12 weeks.

4.5. Changes in disulfide bonds content

Changes in disulfide bond content in frozen surimi during storage is depicted in Figure 22. Disulfide bond formation was observed throughout the frozen storage. Highest formation was found in the control. After 12 weeks of storage, disulfide bond in the control, sample added with 8% trehalose, the blends and commercial cryoprotectant increased by 68, 52, 47 and 55%, respectively. Therefore, the addition of cryoprotectant decreased the formation of disulfide bonds. Generally, no marked differences in disulfide bond content were found among samples added with different cryoprotectant formulae. The increase in disulfide bond was in accordance with the decrease in sulfhydryl groups suggesting the oxidation of sulfhydryl group to disulfide of muscle protein, especially during extended storage. However, cryoprotectant could reduce such changes. The result was in agreement with Ramirez *et al.* (2000) who found that myosin from *Tilapia milotica* lossed of sulfhydryl groups with the concomitant formation of disulfide bonds during frozen storage.

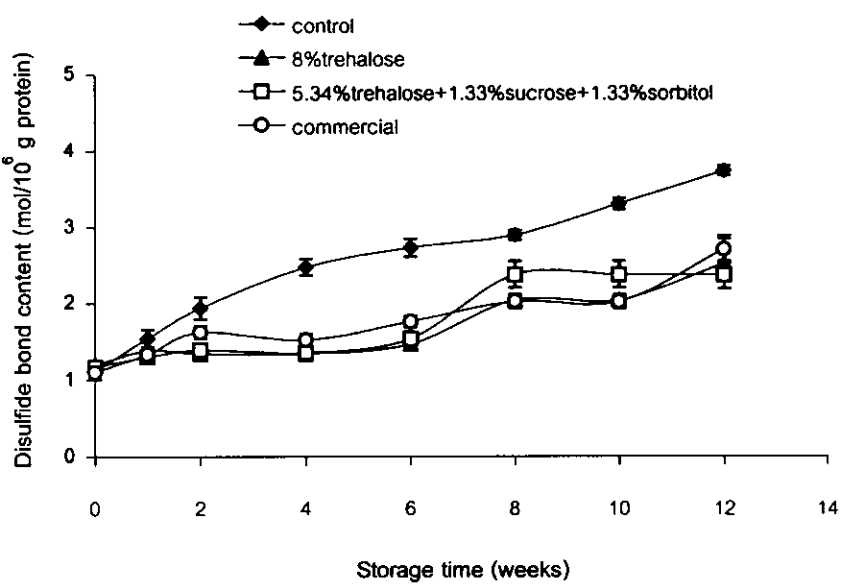

Figure 22 Changes in disulfide bonds content in surimi added with different cryoprotectants during frozen storage at –18 ^{0}C for 12 weeks.

4.6. Changes in surface hydrophobicity during frozen storage

Changes in surface hydrophobicity during frozen storage of surimi from bigeye snapper with and without cryoprotectants are shown in Figure 23. Initial S_0ANS of surimi protein ranged from 84.40 to 87.18, which was comparable to that found in the previous study. During frozen storage, surimi with cryoprotectant showed the lower increase in surface hydrophobicity than control. After 6 weeks of frozen storage, S_0ANS was 158.52, while S_0ANS in samples added with 8% trehalose, the blends or commercial cryoprotectants ranged from 111.77 to 121.16 respectively. Generally, higher S_0ANS was found in the control than other treatments. It suggested that the addition of the cryoprotectant would be a means to retard the exposure of hydrophobic portion of protein molecules during frozen storage. After 12 weeks of frozen storage, S_0ANS of the control was 181.55. S_0ANS samples added with 8%trehalose, the blends or commercial cryoprotectants ranged from 134.90 to 143.59. However, no significant difference in S_0ANS among samples added with three cryoprotectant formulae throughout 12 weeks but was significantly different from the control. The increase in surface hydrophoicity indicates an exposure of the interior of the molecule due to denaturation on degradation (Multilangi *et al.*, 1996). Such changes usually result in decreased water-holding ability, less succulence of intact flesh or lower gelling ability in surimi (Busk, 1984).

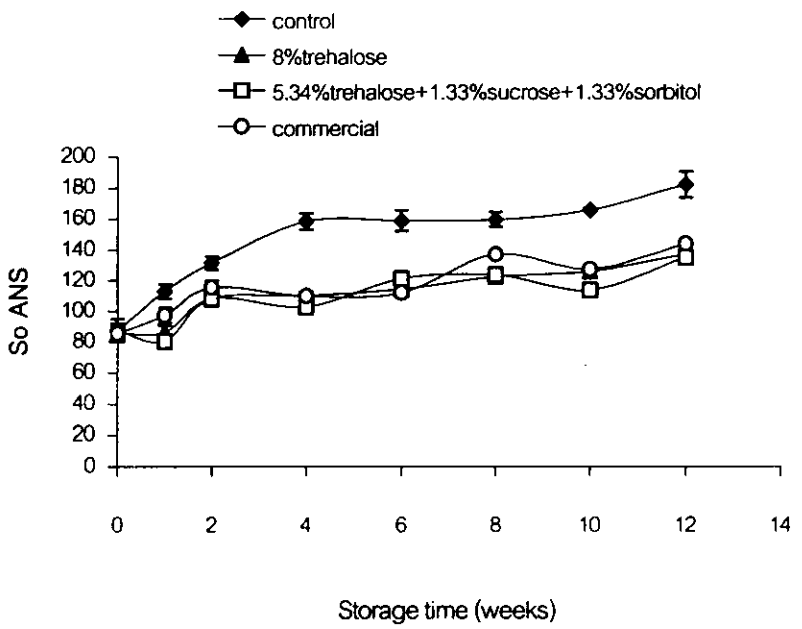

Figure 23 Changes in surface hydrophobicity (S_0ANS) in surimi added with different cryoprotectants during frozen storage at –18 ^{0}C for 12 weeks.

During extended frozen storage, the protein underwent conformational changes, in which the hydrophobic portions were exposed. As a result, hydrophobic interaction might take place, leading to the aggregation and loss in solubility. During denaturation, which is commonly induced by freezing or frozen storage, hydrophobic and hydrogen bonds buried inside the protein molecules become exposed and broken from their native arrangement. As a consequence, conformational change in coiled or helical section of the peptide chain occur and reform in a manner different from those in the native structure (Morawetz,1972; Benjakul *et al.*, 2000). Thus, frozen storage directly affected the conformational changes in protein molecules, leading to the loss in functionality but those changes could be suppressed with cryoprotectants.

4.7. Changes in solubility in 0.6 M KCl during frozen storage

Solubility of surimi protein in 0.6 M KCl decreased during frozen storage (Figure 24). The marked decrease in solubility was observed with the control throughout the storage of 12 weeks. Nevertheless, the decrease in solubility was lowered with the addition of cryoprotectants. After 12 weeks of frozen storage, solubility of the control decreased to 27.07%, while the solubility in sample added with 8%trehalose, the blends and commercial cryoprotectants decreased to 42.92, 46.29, and 42.04%, respectively. Sych *et al.* (1991) found that surimi treated with 4%, 6% and 8% lactitol, and 8%sucrose+sorbitol (1:1) showed only slight losses in protein solubility during frozen storage. Salt extractable protein in surimi without additive and with 2% lactitol was 22% and 40%, respectively.

From the result, the decreased solubility indicated the aggregation as well as denaturation of proteins caused by freezing and frozen storage. The aggregate was markedly formed via the formation of disulfide, hydrogen and hydrophobic bonds during frozen storage (Jiang *et al.*, 1988). Disulfide bond formation contributed to the loss of protein extractability during frozen storage of halibut mince (Lim and Haard, 1984). Benjakul *et al.*, (2003) found the continuous decrease in sulfhydryl group with a concomitant increase in disulfide bond formation in all species including lizardfish, croaker, threadfin bream and bigeye snapper during extended frozen storage. Some researchers have reported that true indication of the cryoprotective action as an ingredient against protein denaturation is maintenance of the extractability of salt-soluble proteins during frozen storage (Park *et al.*, 1988; Scott *et al.*, 1988). As salt-soluble proteins concentrations decreased, strain decreased and the overall gel-forming ability also decreased (Reynolds *et al.*, 2000). Denaturation or aggregation of protein could be minimized with the addition of cryoprotectant.

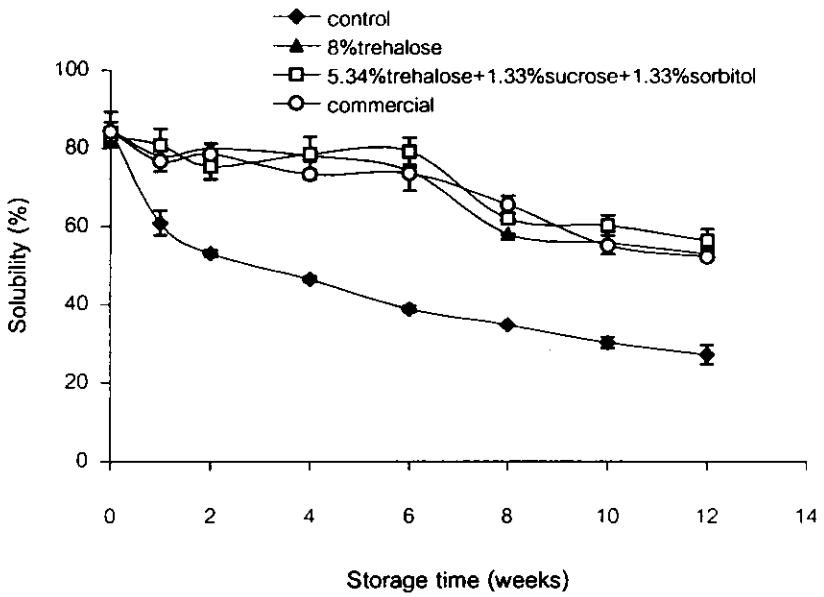

Figure 24 Changes in %solubility in 0.6 M KCl of surimi added with different cryoprotectants during frozen storage at −18 ^{0}C for 12 weeks.

4.8. Changes in protein patterns during frozen storage

Protein patterns of surimi with and without cryoprotectants analyzed by SDS-PAGE profiles under both reducing and non-reducing after 0, 4, 8, and 12 weeks of frozen storage are shown in Figure 25. In general, intensity of MHC band decreased with increasing storage time when analyzed under non-reducing condition. Very low intensity of MHC was observed in the control after 8 weeks of storage. The intensity of actin band also decreased with increasing storage time. Those changes were found in sample without cryoprotectant (Figure 25). More MHC and actin was retained in the sample added with cryoprotectants. Under reducing condition, no marked differences in protein pattern were observed among all samples. This indicated that disulfide bond played an essential role in protein aggregation. Dissociation of actomyosin into F-actin and myosin occurs immediately after freezing, and myosin component primarily undergoes aggregation and

insolubilization (Matsumoto, 1980; Sultanbawa and Li-Chan, 1998). From the result, β-Mercaptoethanol used was able to cleave disulfide bonds and increased protein solubility. The electrophoretic pattern of surimi with and without cryoprotectant during frozen storage had the marked increase intensity of MHC in presence of β-ME.

From the result, it indicates that all cryoprotectant formulae studied were effective in preventing the aggregation or insolubilization of myosin during frozen storage.

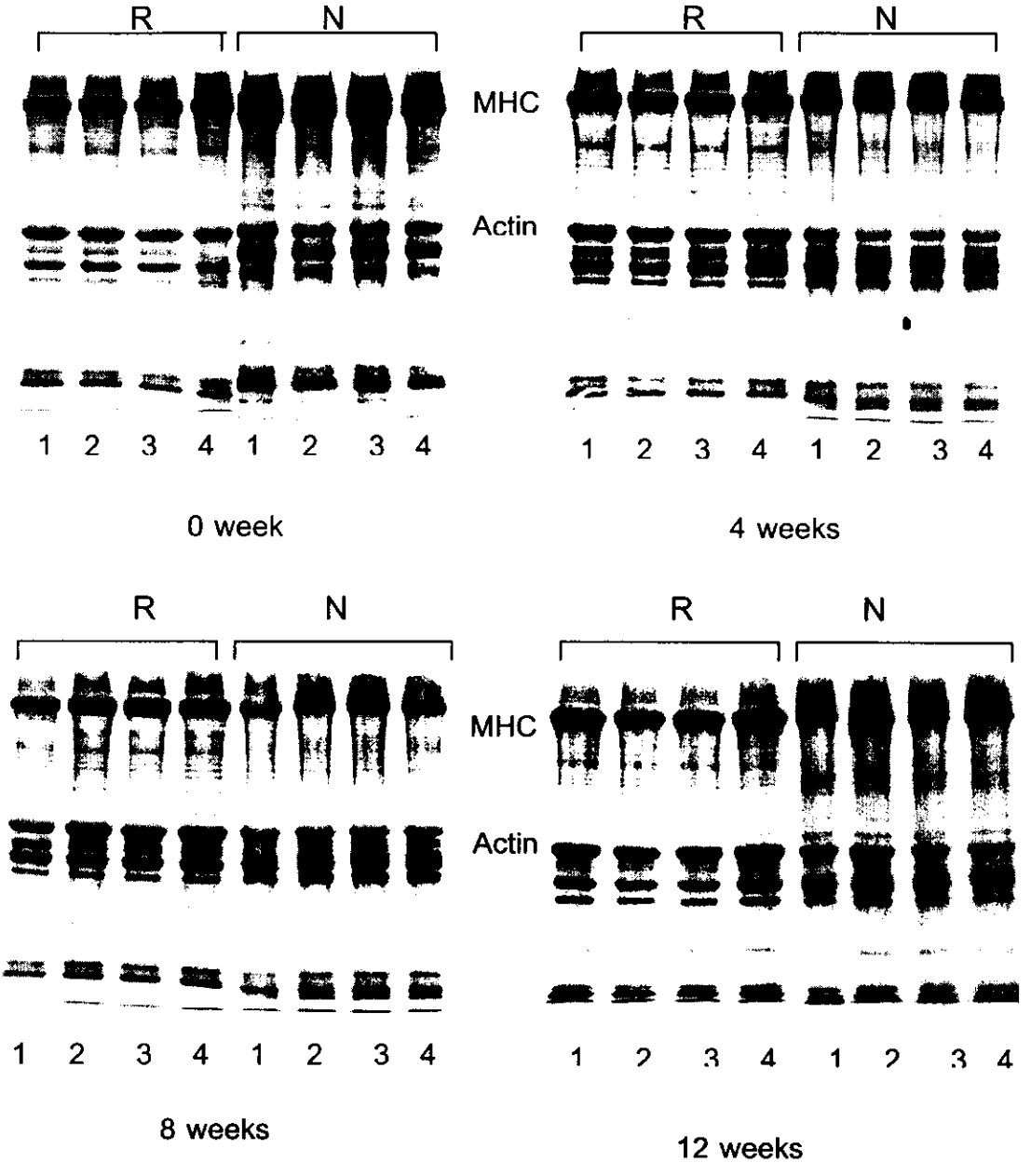

Figure 25 SDS-PAGE of surimi added with different cryoprotectants during frozen storage at –18 ^{0}C for 12 weeks, 1: control; 2: 8%trehalose; 3: the blend including 5.34%trehalose+1.33%sucrose+1.33%sorbitol; and 4: commercial cryoprotectant; R: reducing condition; N: non-reducing condition.

4.9. Changes in breaking force and deformation during frozen storage

Breaking force and deformation of surimi with and without cryoprotectants decreased with the increasing frozen storage time (Figure 26a and Figure 26b, Appendix 1 Table 1 and Table 2). The marked decrease in breaking force was generally observed in surimi without cryoprotectant throughout the storage. Surimi added with 8%trehalose or the blends had the higher breaking force than that added with commercial cryoprotectant after 4 weeks of storage ($p<0.05$). However, no significant differences in breaking force were found among samples added with different cryoprotectant formulae during week 6-12.

The highest decrease in deformation (Figure 26b) was observed in the control and slight decrease was observed in sample added with 8% trehalose, the blends and commercial cryoprotectant. No marked differences in deformation were observed among all samples added with cryoprotectants kept up to 8 weeks. However, sample added with the blends including 5.34% trehalose+1.33% sucrose +1.33% sorbitol had the highest deformation where stored for 10 and 12 weeks. So, in the presence of cryoprotectant, the loss in gel-forming ability was retarded. This result was generally in accordance with the changes in physicochemical property of muscle protein.

At week 0, gel surimi was classified as SA grade (breaking force = 840-850 g; deformation $\simeq$ 12 mm) in all treatments, both with and without cryoprotectants. After 12 weeks, the control was decreased to A grade (breaking force= 492 g; deformation 6.05 mm), while gels of surimi added with 8% trehalose, the blend and commercial cryoprotectants were still with SA grade (breaking force = 762.83, 789.2, and 763.96 g; deformation = 10.27, 11.36, and 10.60 mm, respectively). So, the quality of gel surimi was remained in the presence of cryoprotectant even with prolonged frozen storage.

Breaking force and deformation is a good index for the gel-forming ability of proteins. The higher breaking force and deformation, the greater the gel-forming

ability of the protein system is assuming that the protein gel is, made of a highly elastic network (Park *et al.*, 1988). Gelation is the formation of insoluble protein aggregates and destruction of some functional side chains (Xiong, 1996). According to Lee and Park (1998), gelation of surimi proteins involves both covalent and non-covalent bonds. The major covalent bonds are disulfide and glutamyl-lysine covalent linkage, and the major non-covalent bonds are hydrophobic interactions, hydrogen bonds and ionic bonds (Pomeranz, 1991). Formation of such intermolecular bonds could theoretically be driven by oxidative stresses and hence, affect the gelation process of surimi (Lee and Lanier, 1995). However, a general prevention of myofibrillar proteins from freeze-induced denaturation and aggregation, whether or not oxidation is involved, seems to be most critical for stabilization of the functionality of surimi during frozen storage (Wang and Xiong, 1998). Addition of cryoprotectants was shown to promote retardation of protein conformational changes and denaturation during frozen storage, resulting in higher gelation ability (Park *et al.*,1988).

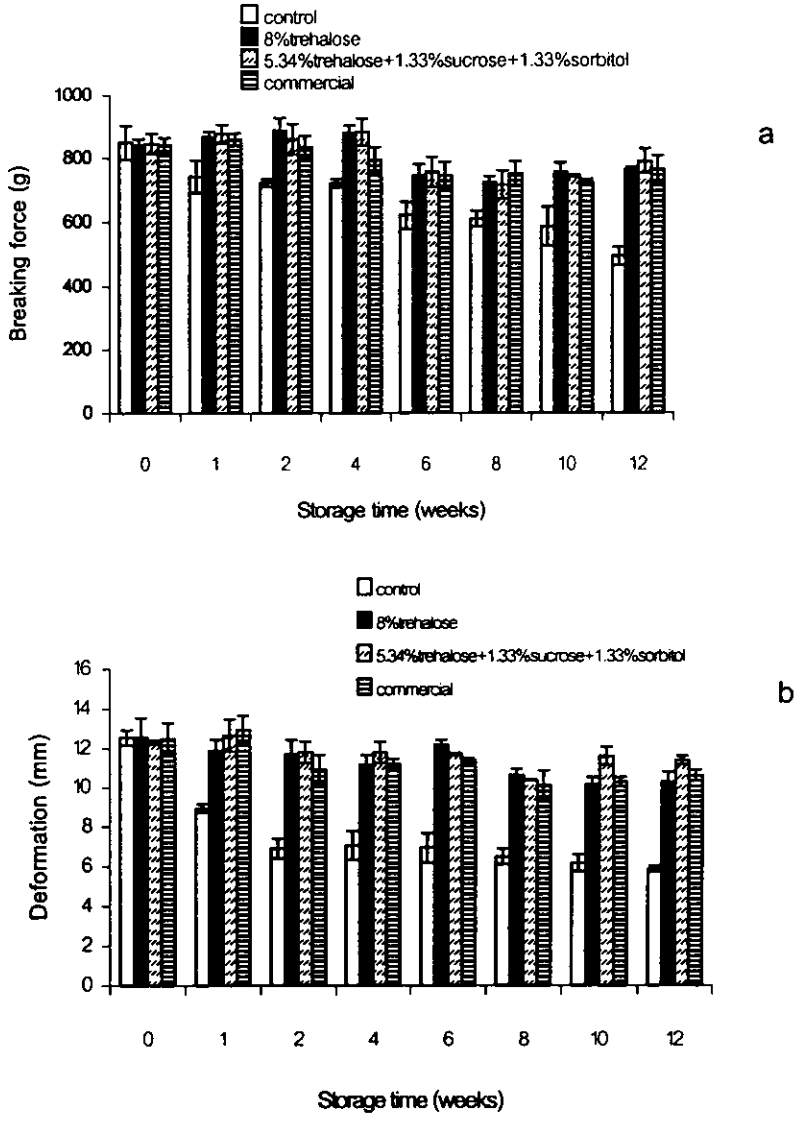

Figure 26 Changes in breaking force (a), deformation (b) of surimi added with different cryoprotectants during frozen storage at –18 ^{0}C for 12 weeks.

4.10. Changes in expressible moisture during frozen storage

Expressible drip in surimi gel is shown in Figure 27. Expressible moisture increased as the storage time increased up to 12 weeks. After 2 weeks of storage, the expressible moisture was increased to approximately 5.13% in the control, while gel prepared from surimi added with 8%trehalose, the blends and commercial cryoprotectant had the expressible moisture of 4.24, 4.17, and 4.28%, respectively.

The expressible moisture in the gels prepared from surimi without cryoprotectant increased markedly as the storage time increased (Figure 27). It indicated that less water was imbibed in the gel matrix. Denaturated protein induced by extended frozen storage had the low affinity to water. Additionally, the gel matrix formed possessed the lower water holding capacity. After 12 weeks of storage, highest increase in the expressible moisture was found in the control (7.95%). However, no differences in expressible moisture were found among all samples added with different cryoprotectant formulae. The expressible moisture increased because the texture deteriorated with the increment of free water during frozen storage (Samson and Regenstein, 1986). Cryoprotectants lessened the amount of expressible moisture of the surimi during storage. Water-holding capacity is directly correlated to myofibrillar protein content (Smith, 1987). The increase in expressible moisture of mince during frozen storage may have been due to a change in microstructure of myofibrillar proteins from a continuous filamentous matrix to a globular matrix (Suvanich *et al.*, 2000). Cheng *et al.* (1979) also stated that the loss of water-holding capacity of tissues during frozen storage was correlated with a decrease in myofibrillar protein solubility. Decrease in water-holding capacity leads to increased expressible moisture and protein denaturation (Reddy and Srikar, 1991). Expressible moisture was found to be an effective measure to determine the textural change of surimi in frozen storage (Jahncke *et al.*, 1992). The usefulness of expressible moisture in monitoring the early changes of textural quality in frozen stored fish or surimi was also reported by other researchers (Hsieh and Regenstein, 1989; Jahncke *et al.*, 1992). Expressible moisture values corresponded well with other textural and sensory parameters (Nowsad *et al.*, 2000).

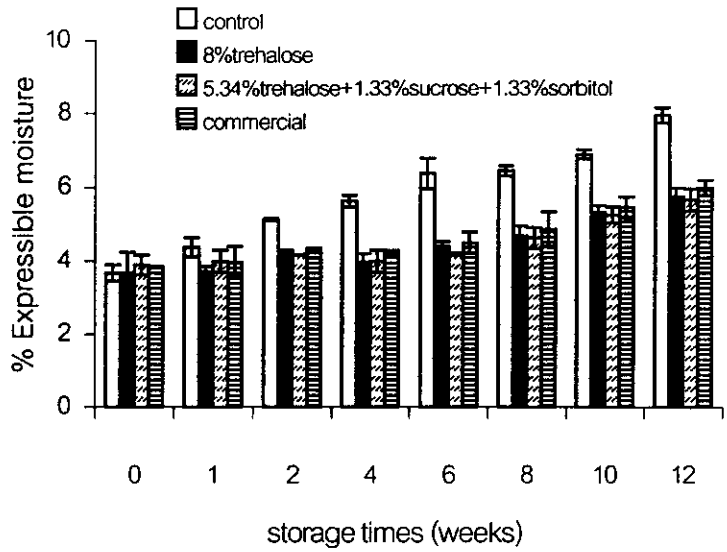

Figure 27 Changes in expressible moisture of surimi added with different cryoprotectants during frozen storage at –18 ^{0}C for 12 weeks.

4.11. Changes in microstructure of surimi gel during frozen storage

Microstructures of gels produced from surimi with and without cryoprotectants at week 0 and week 12 are illustrated in Figure 28. Gel from the control had the similar microstructure to those added with cryoprotectants at week 0. However, after 12 weeks, gel with a denser network and a larger void was found in the control, whereas a regular network was found in gels prepared from surimi added with cryoprotectants, 8%trehalose, the blends or commercial cryoprotectants. Protein gel structure became increasingly fine and continuous, the gel retention of moisture increased (Hermansson, 1982). The same conditions, which increased gel firmness, generally enhanced uniformity of gel microstructure, resulting in improved water-binding characteristics (Woodward and Cotterill, 1986). During frozen storage, surimi proteins in the control were denatured mainly due to the formation of hydrophobic interaction and covalent bonding among protein molecules (Jiang *et al.*, 1986). The slow freezing caused formation of larger crystals, probably due to recrystallization and regrouping of small crystals. This

resulted in the appearance of considerable protein aggregation over large areas of the matrix. The decrease in breaking force and deformation (Figure 26) and the highest change in physicochemical properties in the control after 12 weeks of storage confirmed the result of the changes in microstructure. Gao *et al.* (1999) reported that myofibrillar proteins underwent aggregation through protein-protein interactions during frozen storages. These lumps were condensed by heating and thus reduced the uniformity of the protein dispersion in the gel matrix, resulting in a weakened texture.

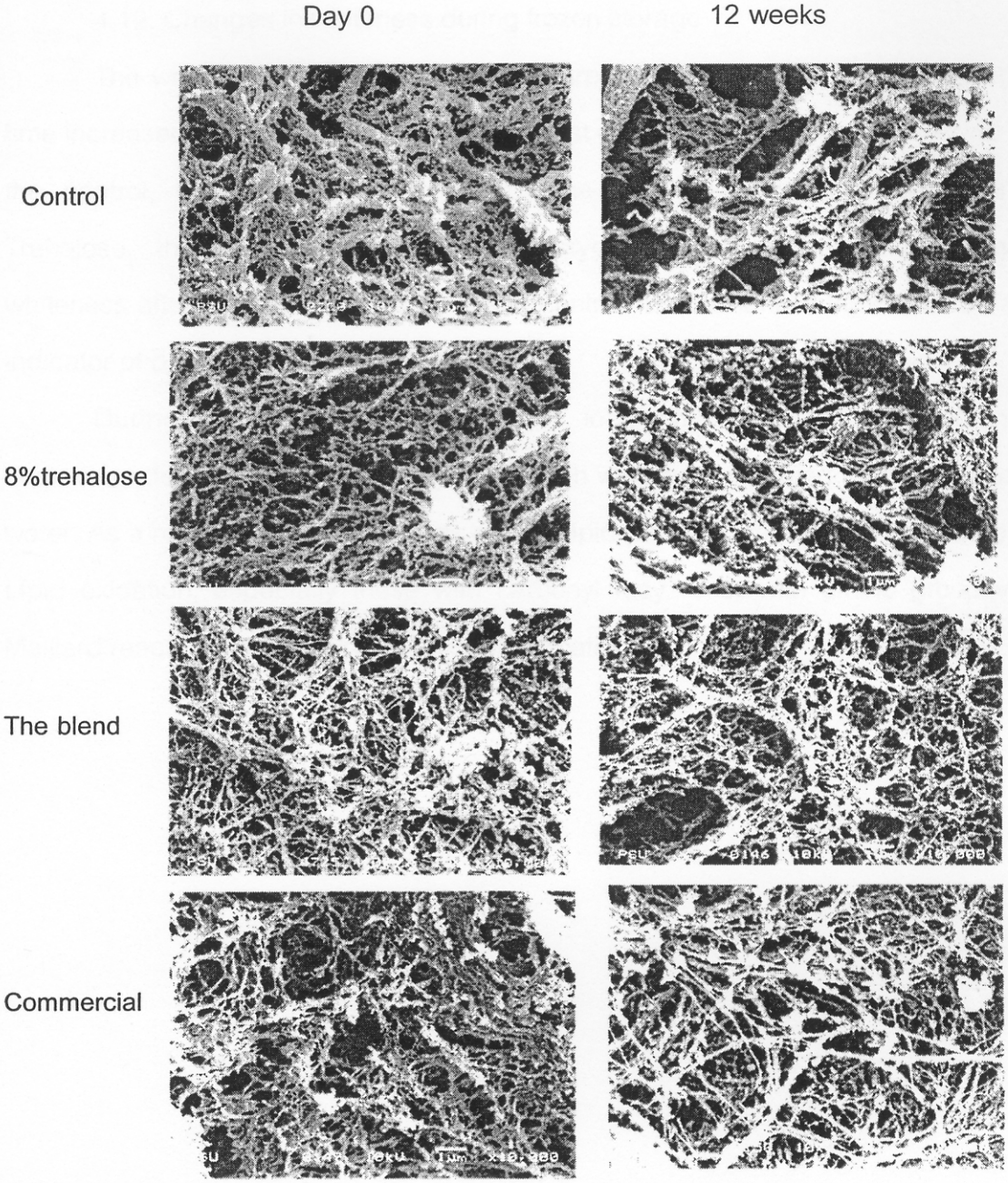

Figure 28 Scanning electron micrographs of bigeye snapper surimi gels with and without cryoprotectants after frozen storage for week 0, and 12, at -18 ^{0}C.

4.12. Changes in whiteness during frozen storage

The whiteness of surimi gels from all treatments decreased as the storage time increased ($p<0.05$) (Table 10). The highest decrease in whiteness was found in the control, especially after 12 weeks. The addition of cryoprotectants, 8% Trehalose, the blends and commercial cryoprotectant, rendered the higher whiteness after 6 weeks, compared to the control. The whiteness index can be an indicator of browning (Auh *et al.*, 1999).

During extended storage, protein in surimi without cryoprotectants underwent denaturaion and aggregation, which were accompanied by the released water. As a result, proteins as well as cellular lipid possibly were prone to oxidation. Lipid oxidation, especially those with carbonyl may react with amino group via Maillard reaction, resulting in brown color formation.

Table 10 Changes in whiteness of surimi added with different cryoprotectants during frozen storage at –18 ^{0}C for 12 weeks.

Frozen storage (weeks)	Whiteness			
	control	8%trehalose	5.34%trehalose +1.33%sucrose +1.33%sorbitol	4%sucrose +4%sorbitol
0	81.33±0.11^{aD}	81.24±0.03^{aD}	81.18±0.16^{aC}	81.24±0.03^{aD}
1	81.09±0.46^{abD}	81.38±0.39^{aD}	81.13±0.59^{abC}	81.05±0.19^{abD}
2	78.46±0.22^{aC}	79.27±0.38^{bB}	79.47±0.44^{bB}	79.48±0.30^{bC}
4	78.13±0.64^{aC}	78.28±0.23^{aA}	78.64±0.27^{abA}	78.97±0.27^{bB}
6	78.31±0.66^{aC}	78.96±0.15^{aAB}	78.60±0.55^{aA}	78.98±0.09^{aB}
8	77.56±0.10^{aB}	79.57±0.33^{bBC}	79.72±0.16^{bB}	79.84±0.49^{bC}
10	77.93±0.17^{aBC}	79.07±0.13^{bB}	79.39±0.58^{bB}	79.02±0.24^{bB}
12	76.24±0.53^{aA}	79.09±0.16^{cB}	78.90±0.22^{bA}	78.20±0.47^{bA}

Mean±SD from triplicate determination.

Different letters (small) in the same row indicate the significant difference ($p<0.05$).

Different letters (capital) in the same column indicate the significant difference ($p<0.05$).

5. Change in physicochemical and gelling properties of surimi with and without cryoprotectants subjected to multiple freeze-thaw cycles.

5.1. Changes in pH

No changes in pH were observed with increasing freeze-thaw cycles (Table 11). With the same freeze-thaw cycle, no marked differences were found among all samples tested. Therefore, addition of cryoprotectant did not affect pH changes after freeze-thawing process.

Table 11 Changes in pH of surimi added with different cryoprotectants subjected to multiple freeze-thaw cycles.

Freeze thaw cycles	pH			
	control	8%trehalose	5.34%trehalose +1.33%sucrose +1.33%sorbitol	4%sucrose +4%sorbitol
0	6.60±0.03[a]	6.58±0.02	6.59±0.03	6.58±0.04
1	6.63±0.01	6.50±0.01	6.54±0.02	6.53±0.01
2	6.58±0.01	6.52±0.02	6.50±0.01	6.47±0.01
4	6.61±0.03	6.56±0.01	6.53±0.02	6.50±0.06
6	6.62±0.02	6.52±0.01	6.52±0.12	6.54±0.01

[a]Mean±SD from triplicate determinations.

5.2. Changes in ATPase activity

Ca^{2+}-ATPase activity of all samples decreased with increasing freeze-thaw cycles (Figure 29a). With the same freeze-thaw cycle, the control was found to exhibit the lowest activity, compared to those added with cryoprotectants. Differences in activity between those with and without cryoprotectants were possibly due to the differences in denaturation of protein. After 6 freeze-thaw cycles, sample added with the blends had the highest Ca^{2+}-ATPase activity. No significant

differences were observed between samples added with 8%trehalose and commercial cryoprotectant ($p<0.05$). After 6 freeze-thaw cycles, Ca^{2+}-ATPase activity was decreased by 69% in the control and 28%, 18% and 30% in samples added with 8%trehalose, the blends and commercial cryoprotectant, respectively when compared to fresh surimi. After freeze-thawing, the loss of activity was due to the tertiary structural changes caused by ice crystallization (Jiang *et al.*, 1988). Hamada *et al.* (1977) concluded that the decrease in Ca^{2+}-ATPase activity was highly related to the oxidation of sulfhydryl groups. The decrease in Ca^{2+}-ATPase activity suggested that proteins rapidly underwent denaturation induced by the freeze-thaw process. However, the addition of cryoprotectant could retard the decrease of Ca^{2+}-ATPase activity induced by freeze-thawing.

The marked decrease in Mg^{2+}-ATPase and Mg^{2+}- Ca^{2+}-ATPase activities of surimi after multiple freeze-thaw cycles was also observed (Figure 29b and 29c). In presence of cryoprotectants, lower decreasing rate was found even with repeated freeze-thawing. After 6 freeze-thaw cycles, Mg^{2+}-ATPase activity was decreased by 57, 29, 21 and 26% and Mg^{2+}- Ca^{2+}-ATPase activities was decreased by 58, 25, 16 and 28% for the control, sample added with 8%trehalose, the blends and commercial cryoprotectants, respectively, compared to the fresh surimi. However, Mg^{2+}-EGTA-ATPase activity increased in all treatments during storage. Mg^{2+}-EGTA-ATPase activity with highest increasing rate was found in the control when compared with other treatments (Figure 29d). No significant differences in Mg^{2+}-EGTA-ATPase activity among samples added with cryoprotectants after 6 freeze-thaw cycles. The increase in Mg^{2+}-EGTA-ATPase activity suggested the loss in Ca^{2+}-sensitivity of myofibrillar proteins (Seki *et al.*, 1979).

The results suggested that cryoprotectants could retard the denaturation induced by freeze-thawing as indicated by decreasing a loss in Ca^{2+}-ATPase Mg^{2+}-ATPase and Mg^{2+}- Ca^{2+}-ATPase activities and by decreasing the increase in Mg^{2+}-

EGTA-ATPase activity. The results also indicated that surimi protein was susceptible to denaturation caused by freeze-thawing. The denaturation of surimi protein could be minimized by the presence of 8%trehalose, the blend including 5.34% trehalose+1.33% sucrose+ 1.33% sorbitol or commercial cryoprotectants (4% sucrose+4% sorbitol).

5.3. Changes in Ca^{2+}-sensitivity after freeze-thaw cycles.

Ca^{2+}-sensitivity of surimi decreased as freeze-thaw cycles increased (Figure 30). Sharp decrease in Ca^{2+}-sensitivity was noticeable in the control. The higher decrease in Ca^{2+}-ATPase sensitivity was found with increase freeze-thaw cycles. After 6 freeze-thaw cycles, Ca^{2+}-sensitivity in the control, sample added with 8%trehalose, the blends or commercial cryoprotectant decreased by 90, 17, 23 and 34%, compared to that of fresh surimi, respectively. Native tropomyosin contributes to the Ca^{2+}-sensitivity of myofibrillar proteins (Ebashi *et al.*, 1968). The loss of Ca^{2+}-sensitivity indicates the denaturation of tropomyosin or troponin/tropomyosin complex, which was induced by freeze-thawing process. From the result, denaturation rate could be decreased by addition of cryoprotectants. Trehalose (8%) or the blends rendered the higher efficacy in maintaining Ca^{2+}-sensitivity, compared to commercial cryoprotectants, especially with increasing freeze-thaw cycles.

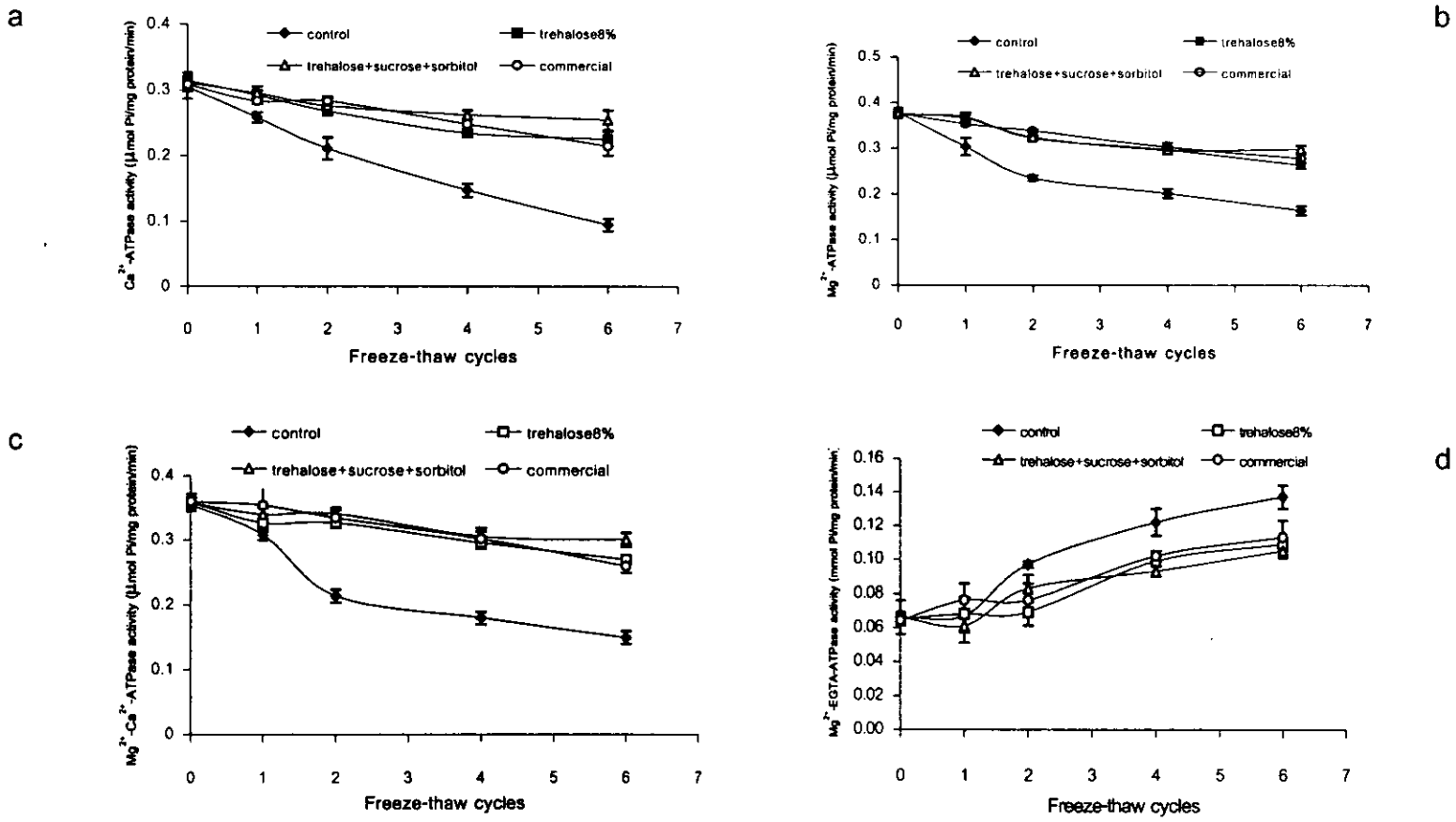

Figure 29 Change in Ca^{2+}-ATPase (a), Mg^{2+}-ATPase (b), Mg^{2+}-Ca^{2+}-ATPase (c), Mg^{2+}-EGTA-ATPase (d) activities of surimi added with and without different cryoprotectants and subjected to multiple freeze-thaw cycles.

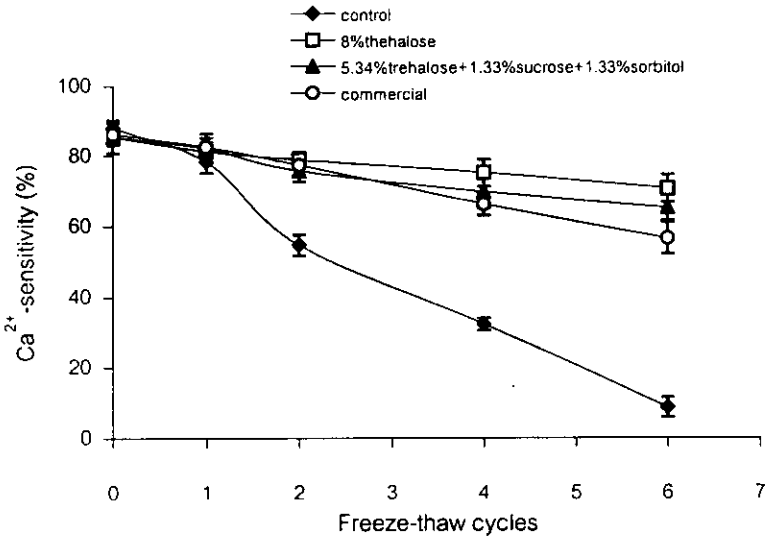

Figure 30 Changes in Ca^{2+}-sensitivity of surimi added with different cryoprotectants and subjected to multiple freeze-thaw cycles.

5.4. Changes in total sulfhydryl contents and disulfide bonds content

The total sulfhydryl contents and disulfide bonds content are important in maintaining structure and functions of native proteins. The total sulfhydryl contents of all treatments continuously decreased when the number of freeze-thaw cycles increased (Figure 31). The total sulfhydryl contents in the control decreased to a higher extent, especially when freeze-thaw cycle increased. The addition of cryoprotectants was found to retard the decrease in total sulfhydryl contents, however no differences in sulfhydryl group were observed among those samples. After 6 freeze-thaw cycle, the total sulfhydryl contents of samples added with 8%trehalose, the blends and commercial cryoprotectant was decreased by 38, 25 and 33%, compared to fresh surimi, while the decrease in the control was about 56%. The reduction in the total sulfhydryl contents might be explained using the following two reasons: the total sulfhydryl contents of inter-or intra-proteins formed cross-linkage (Huidobro *et al.*, 1998); and the exposed sulfhydryl contents in protein

interacted with additives or small molecular compounds (e.g., peptide) (Lian *et al.*, 2000).

The changes in disulfide bonds content after freeze-thawing with different cycles are shown in Figure 32. Disulfide bonds content in samples with and without cryoprotectant increased when freeze-thaw cycles increased ($p<0.05$). The highest increase in disulfide bonds content was found in the control, compared to those added with cryoprotectants. It was postulated that the polymerization of muscle proteins via disulfide bond occurred with increasing freeze-thaw cycles. Corresponding to the formation of disulfide bonds formation, sulfhydryl content simultaneously decreased through freeze-thawing process (Figure 31). The result indicated that freeze-thaw cycles possibly exposed sulfhydryl groups which were further oxidized to disulfide bonds (Benjakul *et al.*, 2001). Structural changes of surimi protein after freeze-thawing made the sulfhydryl groups more susceptible to oxidation. Ramirez *et al.* (2000) also suggested head-to-head interactions with a strong participation of disulfide bonds during frozen storage induced aggregation of myosin. The loss of enzyme activity is found when protein molecules unfold, and ice is formed. Increased protein concentration in the unfrozen phase fosters the formation of intermolecular disulfide bridges (Park, 1994). This aggregation is thought to prevent the enzyme molecules from returning to their native and active conformation after thawing (Carpenter and Crowe, 1988). However, cryoprotectants should prevent the protein from unfolding and also intercalate between the unfolded protein molecules and block the formation of disulfide bridges.

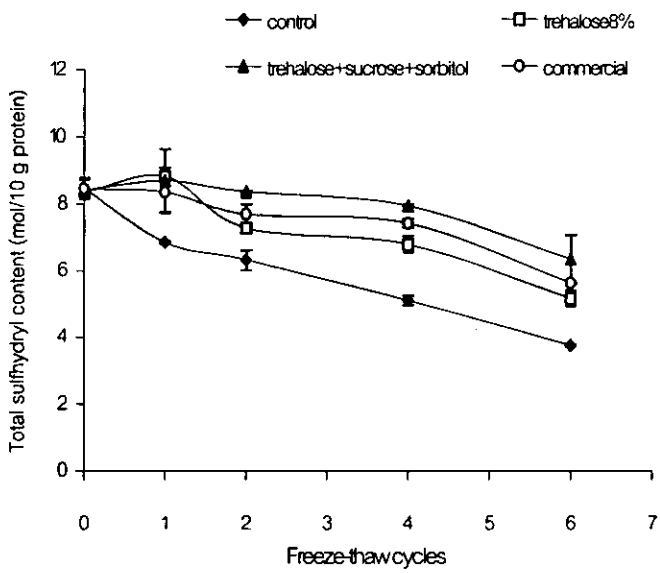

Figure 31 Changes in sulfhydryl content of surimi added with different cryoprotectants and subjected to multiple freeze-thaw cycles.

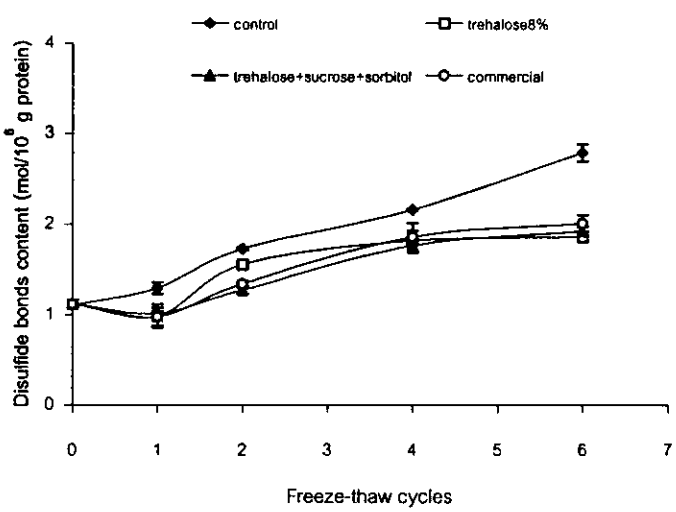

Figure 32 Changes in disulfide bonds content of surimi added with different cryoprotectants and subjected to multiple freeze-thaw cycles.

5.5. Changes in surface hydrophobicity

Changes in surface hydrophobicity of surimi protein with different freeze-thaw cycles are shown in Figure 33. Higher number of freeze-thaw cycles generally increased surface hydrophobicity of surimi protein to a higher extent, compared to a lower number of freeze-thaw cycles. Among all samples tested, the control showed the highest increase in S_0ANS. Addition of cryoprotectant resulted in the retardation of S_0ANS increase at every freeze-thaw cycles studied. No significant differences in surface hydrophobicity were observed among those added with different cryoprotectants at every freeze-thaw cycles. The increased surface hydrophobicity indicated the structural changes of actomyosin after freeze-thawing, causing the hydrophobic groups emerged at the surface of molecule and subsequently formed hydrophobic interaction to reduce free energy (Sano *et al.*, 1990). Cryoprotectant added into surimi could retard the exposure of hydrophobic groups and prevented the reactive group to form hydrophobic interaction. Increased protein hydrophobicity is related to increased exposure of hydrophobic groups on the protein, which, in turn facilitates protein denaturation (LeBlanc and LeBlanc, 1992). LeBlanc and LeBlanc (1992) reported that denaturation process leads to increased hydrophobicity with a concomitant decreased solubility and increased muscle toughness.

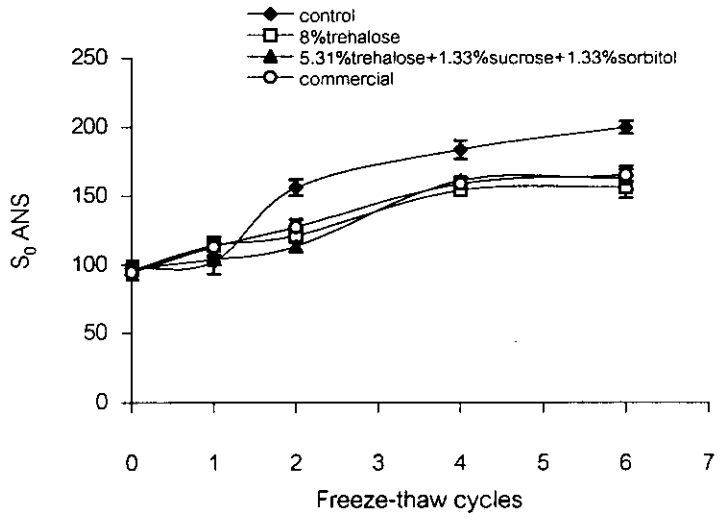

Figure 33 Changes in surface hydrophobicity of surimi added with different cryoprotectants and subjected to multiple freeze-thaw cycles.

5.6. Changes in Solubility in 0.6 M KCl

Solubility in 0.6 M KCl decreased continuously when freeze-thaw cycle increased. After 6 freeze-thaw cycles (Figure 34), solubility in 0.6 M KCl of the control decreased by 70% whereas the solubility was decreased by 29, 21 and 25%, compared to that of fresh surimi, for sample added with of 8% trehalose, the blends and commercial cryoprotectant, respectively. Maldistribution and subsequent aggregation of protein molecules caused by repeated ice crystallization are related to the decrease in protein solubility (Yoshikawa *et al.*, 1995). No differences in solubility among samples added with cryoprotectant treatments were observed with all freeze –thaw cycles studied. Cryoprotectants affected protein solubility and gelation properties of mince beef (Park *et al.*, 1993). Solubility is an important functional characteristic related to protein hydration and is a prerequisite for other functionality.

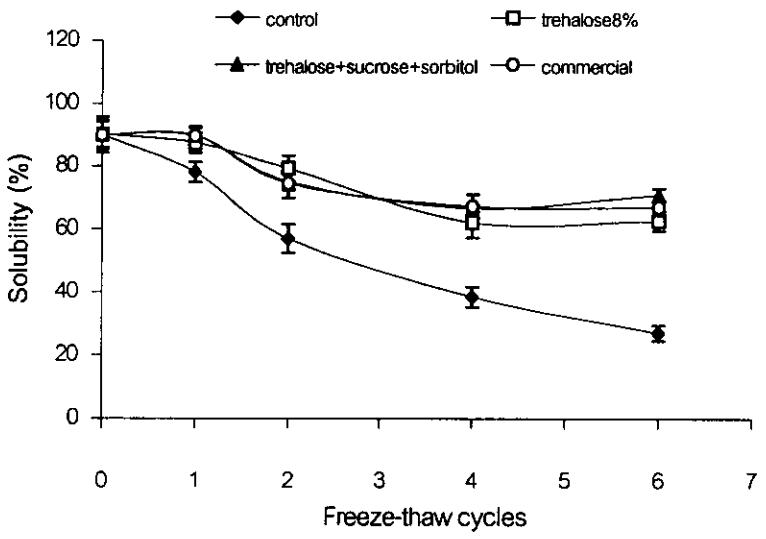

Figure 34 Changes in solubility in 0.6 M KCl of surimi added with different cryoprotectants and subjected to multiple freeze-thaw cycles.

5.7. SDS-PAGE of surimi

Figure 35 shows the SDS-PAGE profiles proteins patterns under both reducing and non-reducing condition of surimi with and without cryoprotectants and subjected to different freeze thaw cycles. Under non-reducing condition, the electrophoretic pattern of surimi showed that MHC band intensity decreased, especially in the control as the freeze-thaw cycles increased up to 6 cycles. Samples added with cryoprotectants had a high MHC band intensity than the control. The result suggested that MHC underwent polymerization and aggregation to a larger extent when subjected to repeated freeze-thawing process. From the result, cryoprotectant effectively reduced the aggregation induced by freeze-thawing. When analyzed under reducing condition, it was noted that similar MHC band intensity was found among all samples with and without cryoprotectants. It indicates that disulfide bond was a major contribution to aggregation particularly in the control. From the result, cryoprotectants added may prevent the interaction of

proteins, leading to the lowered aggregate found. Among all cryoprotectant treatments (8% trehalose, the blend including 5.34% trehalose + 1.33% sucrose + 1.33% sorbitol, and commercial cryoprotectant (4% sucrose + 4% sorbitol), no differences in intensity of MHC were found at every freeze-thaw cycles tested.

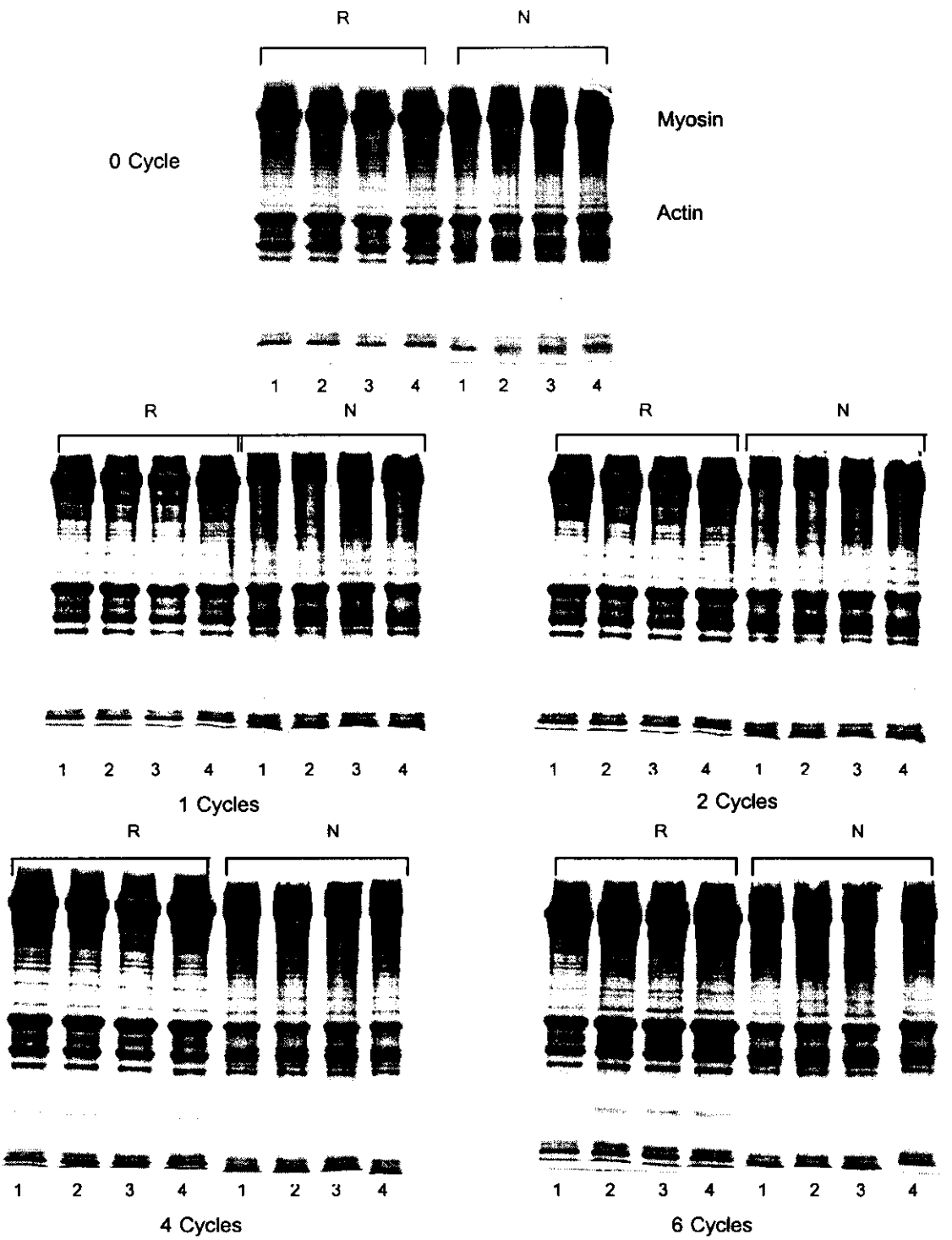

Figure 35 SDS-PAGE of surimi added with and without different cryoprotectants and subjected multiple freeze-thaw cycles, 1: control; 2: 8%trehalose; 3: the blend including 5.34%trehalose+1.33% sucrose+ 1.33%sorbitol; and 4: commercial cryoprotectant; R: reducing; N: non-reducing.

5.8. Changes in breaking force and deformation

Breaking force and deformation of all samples decreased when freeze-thaw cycle increased (Figure 36a and 36b, Appendix 1 Table 1 and Table 2). Breaking force expresses the hardness, and deformation reflects the stickiness or the elasticity of a gel (Lee, 1986). After 6 freeze-thaw cycle, 54, 25, 21 and 28% decrease in breaking force was observed in the control, samples added with 8%trehalose, the blends and commercial cryoprotectant, respectively, compared to that of fresh surimi (Figure 36a). The highest decrease in breaking force was found in the control, compared with other treatments at all freeze-thaw cycles studied. No differences in breaking force were observed among all samples added with different cryoprotectant formulae at all freeze-thaw cycles. Textural properties of gels prepared from surimi with and without cryoprotectant correlated well with the results of physicochemical property and conformational changes of natural actomyosin. Considerable loss of the native conformation of actomyosin observed in this study led to inferior gel network and poor textural properties. From the result, cryoprotectants were shown to reduce the poor gel-forming ability caused by repeated freeze-thawing.

The same results were observed for the changes in deformation (Figure 36b). Highest decrease in deformation was found in the control, when compared to other treatments. The deformation of control, samples added with 8%trehalose, the blends and commercial cryoprotectant was decreased by 65, 39, 32 and 37%, compared to that of fresh surimi, respectively after 6 freeze-thaw cycles. The cryoprotectant addition could retard the decrease in deformation, indicating that the surimi gel with cryoprotectant had more elasticity than the control. In the present study, decreased deformation showed the strong correlation with increasing freeze-thaw cycles. Deformation is influenced mainly by protein quality and is considered to be an important measure of protein functionality (Hamann, 1988). Park *et al.*

(1988) found that deformation was more important than breaking force in explaining kamaboko gel texture.

At 0 freeze-thaw cycle, surimi gel was classified as SSA grade in all treatments (breaking force = 845-872 g; deformation =12.12.24 mm) both with and without cryoprotectant. After 6 freeze-thaw cycles, gel of the control was decreased to B grade (breaking force = 380 g; deformation = 4.28 mm), whereas gels of surimi added with 8% trehalose, the blends and commercial cryoprotectants were decreased to SA grade (breaking force = 633.69, 671.23 and 628.63 g; deformation = 7.42, 8.11 and 7.73 mm., respectively). The lower quality of gel surimi without cryoprotectants was generally observed, compared to those added with cryoprotectants.

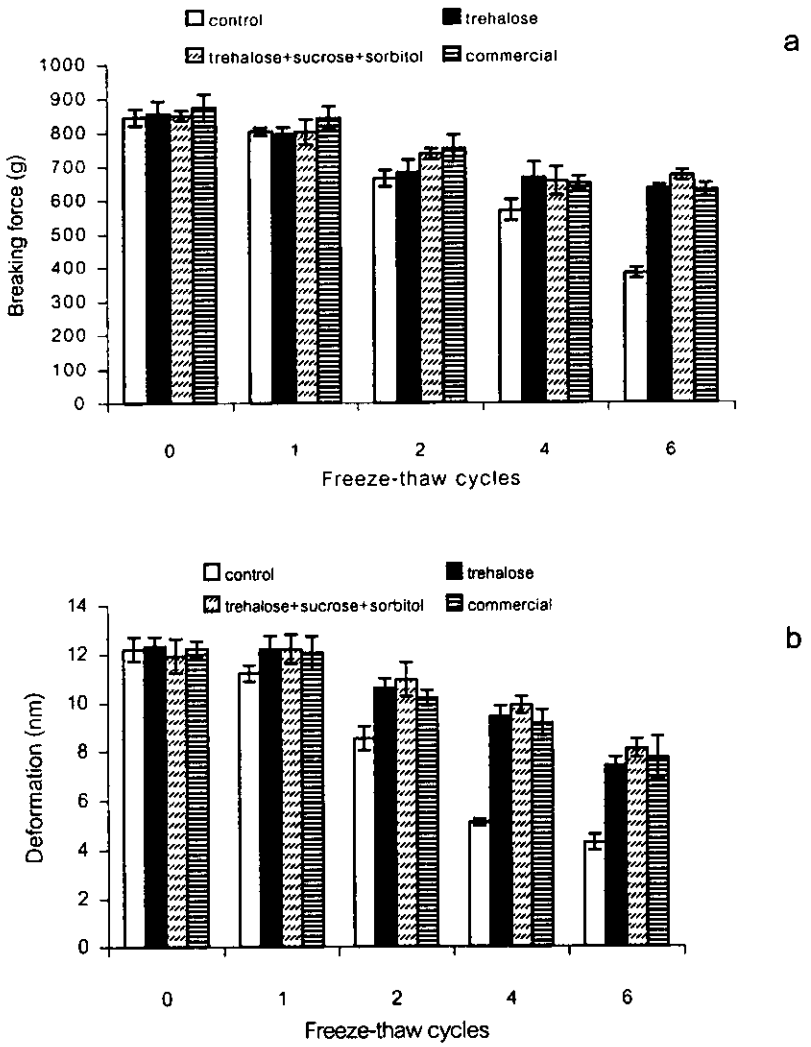

Figure 36 Changes in breaking force (a), deformation (b) of surimi added with different cryoprotectants and subjected to multiple freeze-thaw cycles.

5.9. Changes in expressible moisture of surimi gel

Figure 37 shows the changes in expressible moisture of gel obtained from surimi subjected to different freeze-thaw cycles. From the result, surimi gel without cryoprotectants had the higher expressible moisture, compared to gel from other

treatments, especially after 2 freeze-thaw cycles. For surimi gel with cryoprotectant, lower expressible moisture was found at every freeze-thaw cycles tested. Cryoprotectant addition could improve water-binding abilities and retard myofibrillar proteins denaturation. No differences were found among samples added with 8%trehalose, the blends and commercial cryoprotectant. When surimi was freeze-thawed repeatedly, proteins lost their functionality, including gelation as well as water-holding capacity. As a result, less water was imbibed in the gel network, leading to higher expressible moisture. The increased expressible moisture in surimi gel of all treatments correlated with a decrease in breaking force and deformation (Figure 36). The changes became more intense in surimi without cryoprotectants Yoon and Lee (1990) reported that the increase in rigidity of gels was formed with an increase in expressible moisture. A reduction in water holding probably resulted from contraction of the gel matrix following ice crystal formation (termed "freeze syneresis"). This led to increased rigidity, indicative of rubbery texture. Expressible moisture was found to be an effective measure to determine the textural change of surimi proteins in frozen storage (Nowsad *et al.*, 2000). Iwata and Okada (1971) reported that as the quality of surimi deteriorates, gel forming ability decreases and expressible water increases. This was supported by our experiments showing that gel-forming ability decreased along with increased expressible moisture, especially in the gels prepared from surimi without cryoprotectant addition and subjected to multiple freeze-thaw cycles.

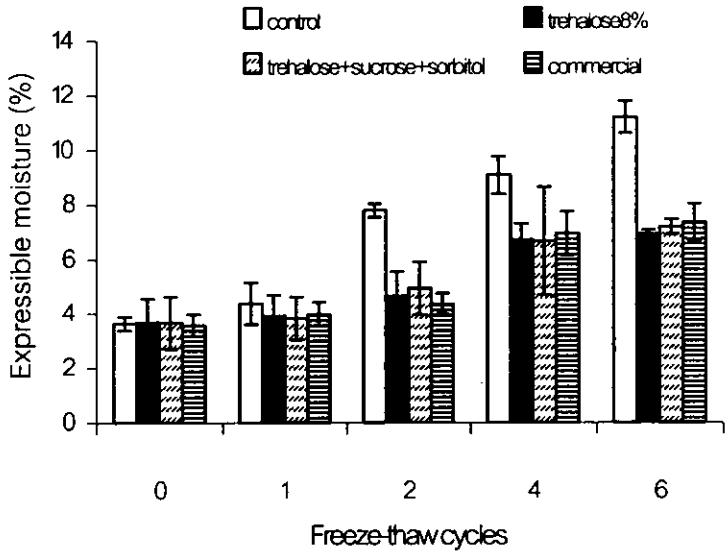

Figure 37 Changes in expressible moisture of gel surimi produced form surimi with different cryoprotectants and subjected to multiple freeze-thaw cycles.

5.10. Changes in whiteness of surimi

Whiteness of gels prepared from surimi with and without cryoprotectants and subjected to different freeze-thaw cycles is shown in Table 12. Decrease in whiteness was observed in the control after 1 freeze-thaw cycle, but no difference in whiteness among samples added with cryoprotectants were observed with increasing freeze-thaw cycles. The whiteness plays an important part in the appearance, presentation and acceptability of fish products. Whiteness changes can occur during frozen storage due to lipid oxidation and pigment degradation processes (Dias *et al.*, 1994).

Table 12 Changes in whiteness of surimi added with different cryoprotectants and subjected to multiple freeze-thaw cycles.

Freeze thaw (cycles)	Whiteness			
	control	8%trehalose	5.34%trehalose +1.33%sucrose +1.33%sorbitol	4%sucrose +4%sorbitol
0	82.56 ± 0.22^{abC}	82.31 ± 0.15^{abAB}	82.68 ± 0.13^{bAB}	82.28 ± 0.20^{aA}
1	82.96 ± 0.21^{cC}	82.11 ± 0.25^{aA}	82.88 ± 0.05^{cB}	82.68 ± 0.12^{bAB}
2	82.27 ± 0.28^{aB}	83.33 ± 0.20^{bB}	82.99 ± 0.15^{bB}	82.96 ± 0.04^{bB}
4	81.01 ± 0.19^{aAB}	82.94 ± 0.07^{dAB}	82.49 ± 0.20^{cA}	82.26 ± 0.13^{bA}
6	80.61 ± 0.17^{aA}	82.99 ± 0.18^{cAB}	82.78 ± 0.17^{cB}	82.35 ± 0.05^{bA}

Mean±SD from triplicate determination

Different letters (small) in the same row indicate the significant difference ($p\leq0.05$)

Different letters (capital) in the same column indicate the significant difference ($p\leq0.05$)

The results demonstrate that 8% trehalose or the blend including 5.34% trehalose+1.33% sucrose+1.33% sorbitol exhibited a beneficial effect on maintaining the physico-chemical properties of NAM and surimi proteins. This was complained by the good water retention properties and gel-forming ability. Textural properties and protein functional properties of frozen surimi can be improved by addition of trehalose or the blends. The addition of 8% trehalose, a non-sweet disaccharide, and the blend of 5.34% trehalose+1.33% sucrose +1.33% sorbitol could reduce the sweetness to same extent. Thus, it can be used to substitute for the commercial cryoprotectants (4% sucrose+4% sorbitol) in surimi to maintain the functionality of muscle proteins during frozen storage.