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SURFACE HYDROPHOBICITY AND FUNCTIONAL PROPERTIES OF MYOFIBRILLAR PROTEINS OF MANTLE FROM FROZEN STORED SQUID (*Illex argentinus*) CAUGHT EITHER JIGGING MACHINE OR TRAWLING

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Abstract

The surface hydrophobicity and functional properties of actomyosin from mantle of frozen squid caught either by jigging machines (AME1) or by trawl (AME2) were investigated. Two components of 155 and 55 kDa were present in the gels at zero time of storage. Degradation of the myosin heavy chain and increase in the 155 kDa component occur earlier in AME2. Irrespective of the catch method used no significant ($p>0.05$) changes in protein solubility were observed. The reduced viscosity of both AME1 and AME2 decreased up to months 3 and 5 of frozen storage, respectively. At the beginning of storage, the superficial hydrophobicity of AME2 was 30% higher than that of AME1. SoANS of AME2 significantly increased during 3 to 5 months of storage period and that of AME1 at the end of storage. The emulsion activity index (IAE) of AME2 significantly ($p<0.05$) increased during the first month and decreased after 3 months of storage. IAE of AME1 decreased at month 3 and remained unchanged thereafter. Emulsion stability (ES) of AME2 showed a behavior that was similar to its IAE and that of AME1 remained unchanged.

Key words: squid, catch method, myofibrillar proteins, functional properties, frozen storage

Introduction

Illex argentinus is an ommastrephid squid occurring on the continental shelf and slopes of the Southwestern Atlantic Ocean (Roper, Sweeney & Nauen, 1984). It is the most important species of cephalopods in South American waters, according to its potential yield and exportation volume in recent years. About 141,159 tons of squid were caught during 2003 (Redes, 2005). *Illex argentinus* migrates extensively during its life cycle, moving from a presumed spawning area north of the Patagonian shelf to feeding grounds on the shelf, where it grows and reaches sexual maturation (Rodhouse and Hatfield, 1990). Mature squids then return to the spawning grounds to reproduce and die at the end of one year (Hatakana, 1988).

Squids offer many advantages over other seafood, such as high post-processing yield, very low fat content, bland flavor and very white flesh. In addition, squid meat has shown to have high functionality which is very important in food processing. In fish species the functional properties of the meat, such as water holding capacity, emulsification and gelation capacity, are strongly affected by freezing and frozen storage (Sikorski, 1978; Matsumoto, 1980). These changes are mainly related to modifications in myofibrillar proteins (Matsumoto, 1980; Shenouda, 1980). Several authors have reported some aspects related to handling, processing, and frozen storage of squid (Joseph, Varma & Venketaraman, 1977; Botta, Downey, Lauder & Noonan, 1979; Moral, Tejada & Borderias, 1983). A gradual decrease in protein extractability during frozen storage of whole squid Loligo duvauceli (Joseph et al., 1977) and a decrease in extractability, reduced viscosity, and Mg^{2+}-ATPase activity of actomyosin in frozen stored mantles

of squid (*Illex argentinus*) (Paredi and Crupkin, 1997) were reported. Similar results were obtained when the same species of squid was frozen stored as whole squid (Paredi, Roldán & Crupkin, 2005). Conversely, it was reported that in other species of squid such as *Ommaestrephes sloani pacificus*, extractable actomyosin remains without major changes during frozen storage (Iguchi, Tsuchiya & Matsumoto, 1981).

The effect of frozen storage on the functional properties of muscle from other squid species was reported (Ruiz-Capillas, Moral, Morales & Montero, 2002; Gomez-Guillén, Matinez-Alvarez & Montero. 2003). Ruiz-Capillas et al. (2002) observed a decrease in the viscosity and emulsifying capacity of protein extracts from mantle and arms of frozen stored squid, either whole or eviscerated (*Illex coindetti*). It was also reported that functional properties of mantle proteins from squid (*Loligo vulgaris*), remained very stable during short times of frozen storage (Gómez-Guillén et al., 2003). There are only a few reports on functional properties of myofibrillar proteins from squid (*Illex argentinus*) (Paredi, Davidovich & Crupkin, 1999; Mignino and Paredi, 2006). On the other hand, it is widely accepted that the catch method influences the postmortem biochemical changes in muscle from fish species (Huss, 1995) and it had also been reported that when squid was caught by jigging machines a better quality and yield of products, was obtained (Leta, 1989). However, reports on the possible influence of the catch method and frozen storage on the functional properties of myofibrillar proteins from this squid species, are lacking.

The purpose of the present study was to investigate the behavior of the functional properties of myofibrillar proteins from frozen stored squid harvested by either bottom trawling or jigging machines.

Materials and methods

Squid *Illex argentinus* (de Castellanos) were harvested by commercial vessels on the Patagonian shelf. Captures were done at 45-52º in the Southwestern Atlantic Ocean. Two experiments were performed. In experiment 1 (E1) specimens were caught by jigging machines. In experiment 2 (E2) specimens were caught by trawl. Ten samples of 10 specimens each were packed in polyethylene bags, frozen on board in blocks at -30ºC and stored at this temperature for 9 months. Frozen samples were thawed for 12 h at 10°C and six samples of female squid were taken at zero time (20 days after freezing) and at each period of frozen storage. The specimens were immediately gutted and after separation of tentacles peeled off mantles were used for analysis. Only specimens at stage 4-5 (mature) were analyzed. The sexual maturation stage of the specimens was determined according to Brunetti (1990).

Actomyosin preparation

Actomyosin was obtained from mantles according to the method described by Paredi, De Vido de Mattio & Crupkin (1990). The final pellet of actomyosin was solubilized in 0.01m mol/L phosphate buffer (pH 7) containing 0.6 mol/L Na Cl. All the procedures were performed at 0-4ºC.

Protein determination

Protein concentrations of actomyosin solutions or protein extracts were determined by the Lowry method, with bovine serum albumin (Sigma Chemical Co., USA) as standard. (Lowry, Rosebrough, Farr & Randall, 1951),

Protein Solubility

The total myofibrillar extract was obtained by homogenizing 8g of mantle (cut into small pieces prior to homogenization) in 160mL of 0.6mol/L KCl - 0.003 mol/L $NaHCO_3$ (pH 7.0) solution for 1 min in a Sorvall Omni-Mixer 17106 (Dupont Newton, CT, USA) The homogenate was centrifuged for 20 min at 7500xg in a refrigerated centrifuge Sorvall RC-26 Plus (Sorvall Product, L.P., Newton, CT, USA) at 2-4 ºC. The supernatant was defined as the salt soluble protein fraction. Results were expressed as percentage of salt-soluble protein respect to total protein determined by the Lowry method (Lowry *et al.* 1951).

Reduced viscosity

Reduced viscosity of the actomyosin solution was measured at 20 ± 0,1ºC using an Ubbelodhe viscometer (IVA, Buenos Aires, Argentina), by the procedure described by Crupkin, Barassi, Martone & Trucco (1979). The temperature of the viscometer was maintained by a thermostatic bath

(Thermomix 1480, B. Braun, Germany). Protein concentration covered a range of 0.1-0.4g/100ml.

Hydrophobicity

Protein surface hydrophobicity (So ANS) was determined by the method of Li-Chan, Nakai & Wood (1985). An actomyosin solution (1mg/ml) in 0.010 mol/L phosphate buffer (pH6.0) 0.6mol/L KCl was diluted to 0.01-0.05 g of protein per 100 mL using the same buffer. After the temperature was stabilized at 20ºC, 20µl of 0.008 mol/L1-anilino-8-naphthalene sulfonic acid (ANS) in 0.1 mol/L phosphate buffer (pH 7.0) was added to 2mL of diluted protein. The relative fluorescence intensity (RFI) values of ANS-conjugates were measured on a Shimadzu RF-5301PC spectrofluorometer (Kyoto, Japan) at an excitation wavelength of 370 and an emission wavelength of 470nm. The initial slope (So) of the RFI versus protein concentration (expressed as gram of protein per 100mL) plot, calculated by linear regression analysis, was used as an index of the protein hydrophobicity according to the method of Li-Chan et al. (1985). The initial slope is referred to as So ANS.

Emulsifying activity index (EAI) and emulsion stability (ES)

The emulsions were prepared by the method of Pearce and Kinsella (1978). The actomyosin a 0.1 g/100mL protein solution (w/v, pH 7.0, 3ml) and 1 ml of sunflower oil were homogenized at 5000 rpm for 1 min in a

Sorvall Omni-Mixer 17106 with microattachment assembly. (Sorvall products, Inc, Newton, CT, USA).

EAI and ES were determined by the turbidimetric method of Pearce and Kinsella (1978). The emulsion (50µl) was pipetted from the bottom of the container into 5 ml of 0.1g/100mL sodium dodecyl sulfate (SDS) (w/v) solution, immediately (0min) and 10min after homogenization. Absorbance of the SDS solution was measured at 500nm. Absorbance at 0 time was defined as EAI of protein.

The ES was determined as follows:

$$ES = T/T_0$$

where T_0 and T are turbidities at 0 and 10 min, respectively (Xie & Hettiarachchy, 1997). The analyses were performed in triplicate.

SDS-polyacrylamide electrophoresis (SDS-PAGE)

The SDS-PAGE of actomyosin was performed according to the method of Laemmli (1970) using 10g of polyacrylamide per 100g of solution for separating gel and 4g of polyacrylamide per 100g of solution for the stacking gel in a Minislab gel apparatus (Sigma Chemical Co., St Louis, MO, USA). Thirty micrograms of protein were loaded on the gel for each sample, to obtain a linear response with protein concentration. The mobility-molecular weight curve was calibrated with standards of molecular weights (Broad range, BIO-RAD, Bio-Rad Laboratories Inc, Hercules, CA, USA) and contains: rabbit myosin (205 kDa), *Escherichia coli* β-galactosidase (116 .25kDa), rabbit phosphorylase b (97.4 kDa), bovine albumin (66.2 kDa), egg

albumin (45 kDa), bovine erythrocytes carbonic anhydrase (31 kDa). The voltaje for electrophoresis was set at 90V.

Quantitative actomyosin composition was determined by densitometry of the gels at 600nm with a Shimadzu dual-wavelength chromatogram scanner Model CS 910, equipped with a gel scanning accessory (Kyoto, Japan), and the areas of the bands calculated by the triangulation method, as described by Kates (1975). The relative percentages of each band were calculated as follows: (studied band area/Σ of total bands areas) x 100. Myosin/actin and myosin/paramyosin ratios were calculated by dividing myosin heavy chains plus light chain areas by actin and paramyosin areas, respectively (Paredi et al., 1990).

Statistical analysis

Analysis of variance and the Duncan´s new multiple range test were performed using the Statistica/MAC (Statistica/MAC, 1994) statistical analysis package.

Results and discussion

SDS-polyacrylamide electrophoresis (SDS-PAGE)

SDS-PAGE 10% patterns of actomyosin from mantle of squid harvested by different fishing arts are shown in Fig.1 and Fig. 2. Actomyosin from mantle of squid harvested by either jigging machines (AME1) or trawl shows the

characteristic polypeptidic bands of myosin heavy chain (MHC), paramyosin (PM), actin (A), tropomyosin (TM), and myosin light chains (MLCs). Similar patterns were reported for actomyosin from this and other species of squid (Iguchi et al., 1981; Paredi & Crupkin, 1997; Mignino & Paredi, 2006). As it can also be seen in Fig. 1 two components of 155 and 55 kDa were also present in the gel of AME1 at zero time and these components remained unchanged up to month 5 of frozen storage. After that, a slight increase in the 155 kDa component and the presence of another one of 143 kDa could also be observed in the gels. At zero time of storage the SDS-PAGE 10% pattern of actomyosin from mantle of squid caught by trawl (E2) also showed the presence of both 55 kDa and 155 kDa components (Fig. 2). As it can also be seen in Fig. 2 a decrease in the MHC band and an increase in 155 kDa, 104 kDa and 55 kDa bands occur during frozen storage, probably due to proteolytic activity.

The relative percentages of myosin (M), paramyosin (PM), and actin (A) and the myosin/actin (M/A) and myosin/paramyosin (M/PM) ratios obtained by densitometric analysis of the gels are shown in Table 1. A significant decrease ($p<0.05$) in the relative percentage of myosin and in the M/PM ratio in AME1, was observed during the last month of frozen storage. A significant decrease ($p<0.05$) in the M/A ratio in AME1 occurs since month 5 earlier than the decrease in M/PM. Paredi and Crupkin (1997) reported that frozen stored isolated mantles of the same species of squid produce denaturation-aggregation of myofibrillar proteins, especially myosin. In this way, the decrease in the relative percentage of myosin shown in Table 1 could be attributed to denaturation-aggregation of this protein. Conversely, a

significant decrease ($p<0.05$) in the relative percentage of myosin and a significant increase ($p<0.05$) in that of PM, was observed in AME2 since the first month of storage. As a consequence of that, a decrease in both M/A and M/PM ratios, was also observed. Iguchi et al. (1981) reported a decrease in a relative percentage of myosin with an increase in small proteolytic fragments in frozen stored AM from squid (*Ommaestrephes sloani pacificus*). Cephalopods typically have higher levels of proteolytic activity than most fish species (Kolodziejska & Sikorski; Hurtado, Borderias & Montero, 1999). In addition, it was reported that myosin was the major target protein for proteinases (Nagashima, Ebina, Nakai, Tanaka & Taguchi, 1992; Konno & Fukazawa, 1993) and that the proteolytic activity remained unchanged during the frozen storage (Konno, Young-Je, Yoshioka, Shinho & Seki, 2003). Konno and Fukazawa (1993) reported that myosin was selectively cleaved into two large fragments of 150 and 100 kDa which correspond to heavy and light meromyosin, respectively. In this way, the increase in the relative percentage of PM shown in Table 1 might be due to commigration of this protein with a 104 kDa degradation fragment. Our results suggest that myosin of AME2 denatured in two steps in mantles of frozen stored squid: first myosin is cleaved into 155 and 104 kDa fragments and thereafter the proteolytic fragments aggregate up to the end of storage.

Protein solubility

Irrespective of the catch method used no significant changes ($p>0.05$) in the solubility of protein were observed during frozen storage (Fig. 3). In

agreement with these results, it was reported that soluble proteins from squid mantle *(Loligo vulgaris)* remained unchanged after 1 month of frozen storage (Gomez-Guillén et al., 2003) and that protein extractability in frozen stored squids (*L. duvaucelli)* (Joseph, Perigreen & Nair, 1985) and (*O. sloani pacíficus)* (Iguchi et al, 1981) only decreases slightly even after long frozen storage. Morales (1997) reported that protein solubility is low sensitive to changes in frozen stored cephalopods muscle.

Reduced viscosity and protein surface hydrophobicity

Figure 4 shows the changes in reduced viscosity (VER) and surface hydrophobicity of actomyosin from mantles of frozen stored whole squid. Viscosity is one of the most sensitive functional properties for measuring changes in myofibrillar proteins during frozen storage (Barroso, Careche & Borderias, 1998; Morales, 1997). The reduced viscosity of both AME1 and AME2 shows a similar behavior up to month 3 of frozen storage. At this time of storage a significant ($p<0.05$) decrease in VER could be observed. Thereafter, while reduced viscosity of AME1 remained unchanged that of AME2 significantly ($p<0.05$) decreased at month 5 and thereafter remained unchanged. A similar behavior was observed in the reduced viscosity of AM from frozen stored isolated mantles of the same species of squid (Paredi and Crupkin 1997). In addition, a drastic decrease in viscosity of protein extracts during freezing and frozen storage was reported by different authors in different fish species (Mackie 1993; Ruiz Capillas et al., 2002). Several studies on the structure-function relationships in food proteins emphasized

the importance of protein hydrophobicity on functional properties when different treatments and/or processes were applied (Li-Chan et al, 1985; Nakai, Li-Chan, & Hayakawa, 1986). The aromatic hydrophobicity is widely accepted to monitor changes in the surface hydrophobicity of the proteins (Niwa, Kodha; Kanoh, & Nakayama, 1986; Leblanc & Leblanc, 1992). As it can also be seen in Fig. 4 except for month 3 of frozen storage all the SoANS of AME2 values were higher that those corresponding to AME1. SoANS of AME1 shows a trend to increase between the first and the third month of frozen storage and remained unchanged thereafter up to month 8. A new significant increase ($p<0.05$) was observed in SoANS of AME1 during the last month of storage. SoANS of AME2 remained unchanged up to month 3 and thereafter showed a trend to increase between months 3 and 5 of storage and no significant changes were detected thereafter. Niwa et al. (1986) reported that changes in the hydrophobicity of actomyosin after freezing are due to myosin rather than to actin. Native myosin has hydrophobic residues strongly concentrated in the core of the helix (McLachlan and Karn, 1982) and the surface of the helix is essentially devoid of hydrophobic groups (Boredjo, 1983). In this way, the lower surface hydrophobicity of AME1 could be due to a greater stability of this protein than that of AME2, suggesting some influence of the catch method on the protein stability.

Emulsifying activity index (EAI) and emulsion stability (ES)

The changes in IAE of AME1 and AME2 are shown in Fig. 5. At zero time of storage, similar IAE values were observed in both proteins. The IAE of AME2 significantly ($p<0.05$) increased during the first month of storage and decreased thereafter up to month 7 of storage. No major changes were observed thereafter. The IAE of AME1 significantly ($p<0.05$) decreased at month 3 and remained unchanged thereafter. At month 1 and 3 of frozen storage IAE values of AME2 were significantly ($p<0.05$) higher than those of AME1. The higher IAE values of AME2 could be related to proteolytic activity detected in AME2 since month 1 of frozen storage. In agreement with our results a slight increase in the emulsifying capacity of the proteins from ungutted squid (*Illex coindetti)* muscle at the beginning of frozen storage (Ruiz-Capillas et al., 2002) was reported. In that paper, the authors attributed the increase in the emulsifying capacity to proteolytic activity present in visceral mass components that penetrated the muscle. Endogenous proteolytic activity in mantle of various cephalopods has been described (Hurtado et al., 1999, Konno and Fukazawa, 1993). In this way, the influence of endogenous proteinases of the mantle on the IAE values should not be discarded.

The changes in emulsion stability (ES) of AME1 and AME2 are shown in Fig. 6. The ES of AME2 showed a behavior similar to that of IAE. Conversely, the ES values of AME1 remained unchanged up to month 7 and decreased thereafter up to the end of storage. Except for months 5 and 7 of storage ES values of AME1 were lower than those of AME2. Several factors

have influence on protein stabilized emulsions: rate of diffusion, solubility, viscosity, protein flexibility, net charge, and protein hydrophobicity. In addition, to stabilize an emulsion, a protein must: diffuse to the interface, unfold, expose hydrophobic groups and interact with lipid. In this way, the higher ES values of AME2 respect to AME1 might be due either to a higher unfold and exposition of hydrophobic groups or to a higher content of flexible peptides which can migrate to the interface. In addition, an enhanced emulsion stability of natural actomyosin by apparition of aggregates in the extract was reported (Tejada, Mohamed, Huidobro & Garcia, 2003). Further investigations will be necessary to clarify the mechanism which led to an increase in ES of actomyosin from squid.

Conclusion

Actomyosin from squid caught by trawl shows after a short frozen storage period a higher rate of autolysis, surface hydrophobicity, IAE and ES than actomyosin from squid harvested by jigging machines. These results indicate that the catch method influences the rate of autolysis and the functional properties of myofibrillar proteins from frozen stored squid mantle.

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Legends of figures

Figure 1. SDS-PAGE 10% gels of actomyosin from mantle of frozen squid caught by jigging machines (AME1) MHC, myosin heavy chain (200kDa); PM, paramyosin (103kDa); A, actin (45kDa); TM , tropomyosin (36kDa); MLCs, myosin lights chains (18-20kDa). St: Molecular weight markers. 30 µg of protein (actoymosin) was loaded in each lane of the gel.

Figure 2. SDS-PAGE 10% gels of actomyosin from frozen stored squid caught by trawl (AME2) MHC, myosin heavy chain (200kDa); PM, paramyosin (103kDa); A, actin (45kDa); TM , tropomyosin (36kDa); MLCs, myosin lights chains (18-20kDa). St: Molecular weight markers. 30 µg of protein (actoymosin) was loaded in each lane of the gel.

Figure 3. Changes in solubility of protein of squid mantle during storage at –30ºC. Experiment 1 (■); Experiment 2 (□). Results are expressed as the means of 6 determinations ± SD.

Figure 4. Surface hydrophobicity (SoANS): (□) and Reduced viscosity (VER): (Δ) of actomyosin from squid mantle during storage at –30ºC. Open symbols (AME1), closed symbols indicated (AME2). Results are expressed as the means of 6 determinations ± SD.

Figure 5. IAE of actomyosin from squid mantle during storage at –30ºC. Results are expressed as the means of 4-6 determinations ± SD. AME1 (■) ; AME2 (□). ^{a,b,c,d,e}. It represents a significant difference ($p<0.05$) in data from different months and same experiment.

* Indicate significant differences ($p < 0.05$) between experiments within same month.

Figure 6 . ES of actomyosin from squid mantle during storage at –30ºC. Results are expressed as the means of 4-6 determinations ± SD. AME1(■); Experiment 2 (□).

[a,b,c,d,e] . It represents a significant difference ($p<0.05$) in data from different months and same experiment.

[*] Indicate significant differences ($p < 0.05$) between experiments within same month

Table 1. Relative percentage of myosin (M), actin (A) and paramyosin (PM) and M/A, M/PM ratio of actomyosin from squid mantle during frozen storage.

	Relative percentage(%)[a]			Ratio[a]	
Time	M	**A**	**PM**	**M/A**	**M/PM**
0 (E1)	51.17±8.3^{b,x}	27.78±7.9^{b,x}	10.73±4.3^{b,x}	1.88±0.8^{b,x}	5.06±2.8^{b,x}
0 (E2)	43.35±6.2^{b,x}	23.65±2.5^{b,x}	15.67±2.3^{b,x}	1.86±0.4^{b,x}	3.05±0.7^{b,x}
1 (E1)	49.13±1.3^{b,y}	24.83±4.1^{b,x}	8.97±3.2^{b,y}	2.06±0.73^{b,y}	4.11±0.25^{b,y}
1 (E2)	23.36±2.2^{c,x}	27.89±4.3^{b,x}	22.22±1.8^{c,x}	0.85±0.3^{c,x}	1.03±0.1^{c,x}
3 (E1)	51.30±2.8^{b,y}	30.66±6.3^{b,x}	10.33±2.2^{b,y}	1.73±0.4^{b,y}	5.09±1.0^{b,x}
3 (E2)	16.98±2.8^{c,x}	36.58±3.8^{c,x}	24.31±2.8^{c,x}	0.48±0.1^{c,x}	0.72±0.2^{c,x}
5 (E1)	48.14±4.0^{b,y}	33.60±4.5^{c,x}	9.30±3.0^{b,y}	1.44±0.2^{c,x}	3.98±0.1^{b,y}
5 (E2)	16.93±2.2^{c,x}	37.35±2.0^{c,x}	29.63±1.8^{c,x}	0.45±0.2^{c,x}	0.57±0.2^{c,x}
7 (E1)	42.82±6.8^{b,y}	32.50±8.2^{c,x}	8.20±4.4^{b,y}	1.40±0.4^{c,x}	5.15±1.5^{b,y}
7 (E2)	20.46± 8.2^{c,x}	30.90±1.4^{c,x}	24.30±6.0^{c,x}	0.59±0.2^{c,x}	0.86±0.6^{c,x}
9 (E1)	32.65±6.7^{c,y}	43.77±9.1^{cx}	10.88±3.4^{b,y}	0.87±0.05^{c,x}	2.44±0.3^{b,x}
9 (E2)	5.20±1.6^{d,x}	34.07±3.8^{c,x}	30.32±3.5^{c,x}	0.15±0.5^{c,x}	0.20±0.06^{c,x}

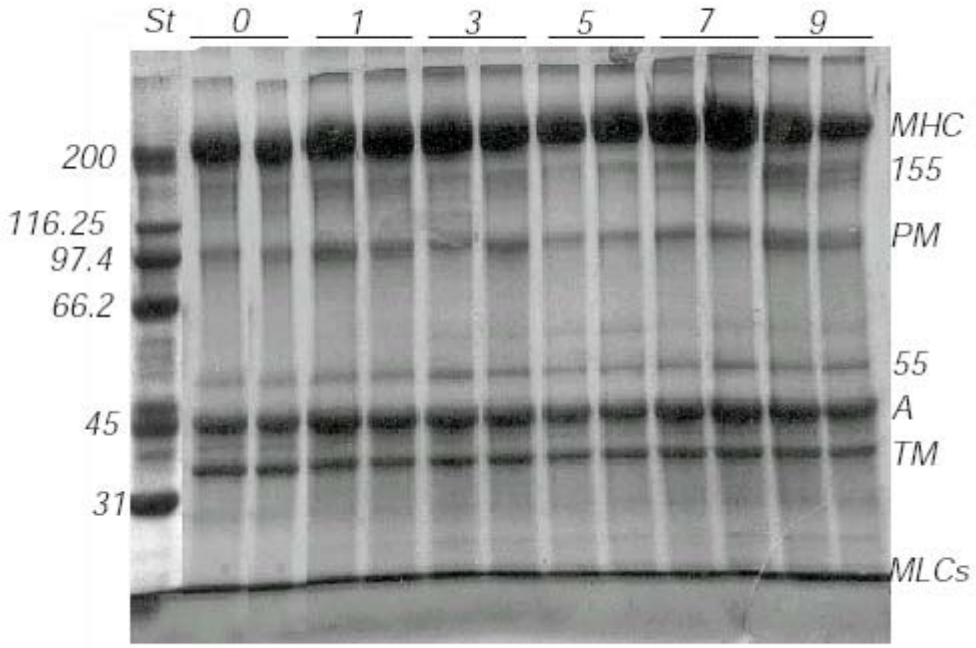
[a] Each value represents the mean ± SD (n=4-6).

^{b,c,d} Means within each column with different superscrips were significantly different ($p<0.05$) within sample during frozen storage.

^{x,y} Means within each column with different superscrips were significantly different ($p<0.05$) within sample different experiment, same time of storage at –30°C.

E1: Experiment with squid cacth by jiggins machine, E2: Experiment with squid cacth by botton trawl.

Fig 1:



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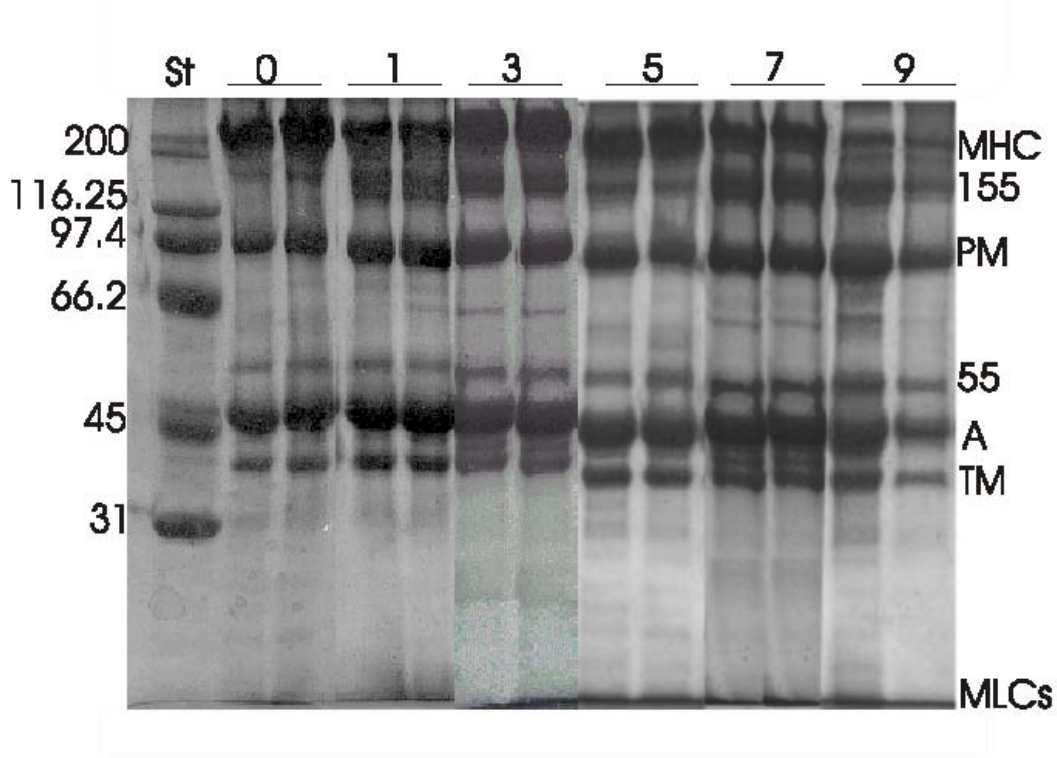

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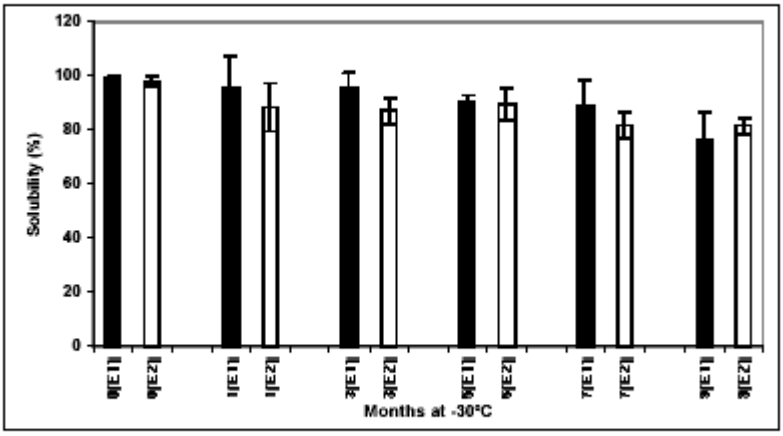

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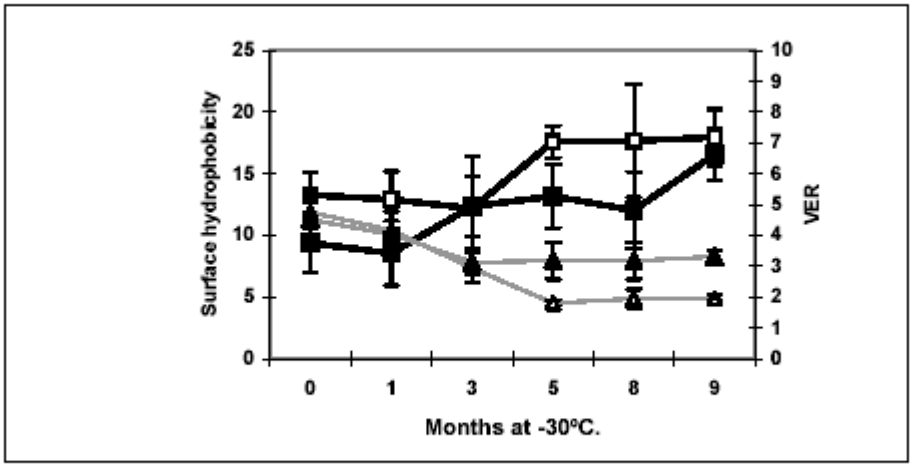

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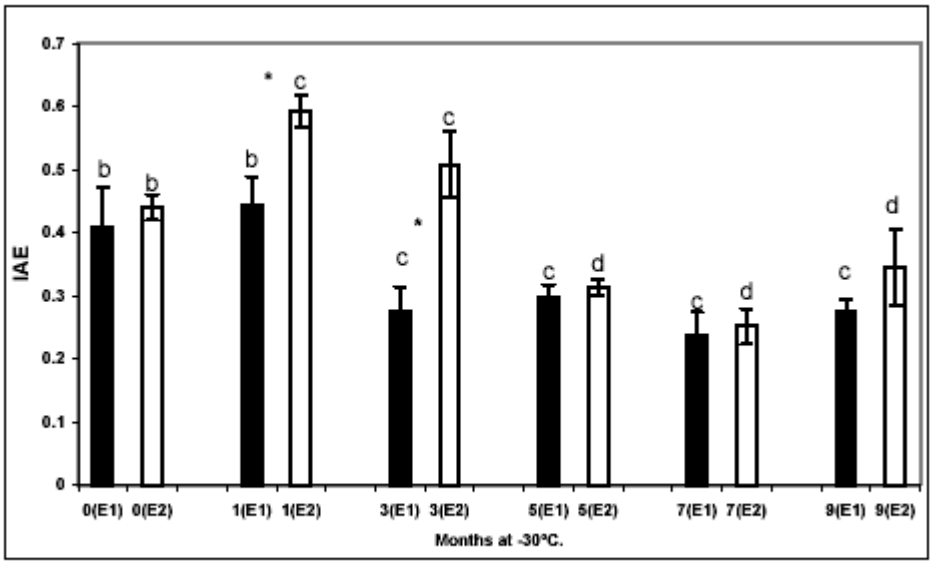

Fig 6: