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The influence of developmental stages and protective additives on cryopreservation of surf clam (*Spisula sachalinensis*) larvae

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Abstract: *This study was performed to find out the optimal larval stage and the most desirable protective additives for cryopreservation of surf clam, Spisula sachalinensis larvae. The survival rates of frozen-thawed larvae increased with post developmental stage. The highest value of 96.1±1.0% was achieved using umbo stage larva as developmental stage and 0.2 M sucrose as protective additive.*

Key words: *Surf clam, Developmental stage, Protective additive, Cryopreservation*
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Introduction

Cryopreservation has been investigated in different organisms at various developmental stages with different kinds and concentrations of cryoprotectants (Chao *et al*., 1997; Choi and Chang, 2003; Tervit *et al*., 2005). In bivalves the cryopreservation of Pacific oyster, *Crassostrea gigas* oocytes and black-lip pearl oyster (*Pinctada margaritifera*) sperm have been recently investigated (Tervit *et al*., 2005; Acosta-Salmon *et al*., 2007). However, cryopreservation studies of developmental stages from trochophore to full-grown umbo stage have not been investigated yet. The optimal cryoprotectant depends on species as well as many other factors; therefore, it is difficult to choose a cryoprotectant for embryo/larva of a species previously not investigated, and also the effect of cryopreservation may differ with protective additives.

In this study, influences of developmental stages, protective additives and cryoprotectants were investigated to develop a suitable cryopreservation method of surf clam, *Spisula sachalinensis*.

Materials and Methods

Broodstock of natural surf clam (*S. sachalinensis*) were collected in June, the natural spawning season, at the coast of Jumunjin, Gangwon-do, Korea.

To investigate effect of developmental stages on cryopreservation, the early trochophore, late trochophore, D-shaped and umbo stage of *S. sachalinensis* larvae were used and they were obtained at 20 hr, 25 hr, 5 days and 16 days after fertilization, respectively. A solution of 0.2 M sucrose in artificial seawater (ASW) was used as the protective additive and 2.0 M ethylene glycol (EG) was used as cryoprotectant.

To investigate effect of protective additives and cryoprotectants on cryopreservation, the D-shaped and umbo stage larvae were used. Protective additives such as 0.2 and 0.5 M monosaccharides (fructose and glucose) and disaccharides (sucrose and trehalose) were used. Cryoprotectants such as 2.0 M dimethyl sulfoxide (DMSO) and EG were used.

All the freezing and thawing protocol (Table 1) used was as outlined by Choi and Chang (2003).

Data from survival rates of larvae in cryopreservation experiments were expressed as means (n=50). The significance of differences among the means of survival rates with each factor was tested by analysis of variance (ANOVA). Differences with a probability value (p) of 0.05 were considered significant.

Results and Discussion

The survival of frozen-thawed surf clam larvae was dependent on developmental stages and was significantly increased with the developing process except for early trochophore which all died after thawing. The survival rate of frozen-thawed umbo stage larvae was the highest (96.1±1.0%, $p<0.05$) among four developmental stages (Fig. 1). Gwo (1995) reported that the trochophore had greater freezing tolerance than other embryonic stages including 2 to 8 cells, morula and gastrula of Pacific oyster. The late embryos and early larvae, which had high tolerance against toxicity of the cryoprotectants, were indicated to be a desirable developmental stage for cryopreservation of Pacific oyster and hard clam, *Meretrix lusoria* (Chao *et al*., 1997). Likewise, Renard (1991) reported the influence of embryo quality on cooling tolerance of Pacific oyster. This difference was probably caused by differences in embryo quality and environmental factors. Also in this study, the increasing of cell numbers of larvae in metamorphosis and the process of exoskeleton formation in the umbo stage might be the reasons for high survival rate.

Table - 1: Protocol for freezing and thawing *Spisula sachalinensis* larvae

Freezing	Thawing
Initial temperature 0°C	Liquid nitrogen
↓	↓
Rate -1°C/min	25°C water bath, 10 min
↓	
-12°C	
↓	
Seeding to initiate ice nucleation	
↓	
Holding 10 min	
↓	
Rate -1°C/min	
↓	
-35°C	
↓	
Liquid nitrogen	
↓	
Holding 1 hr	

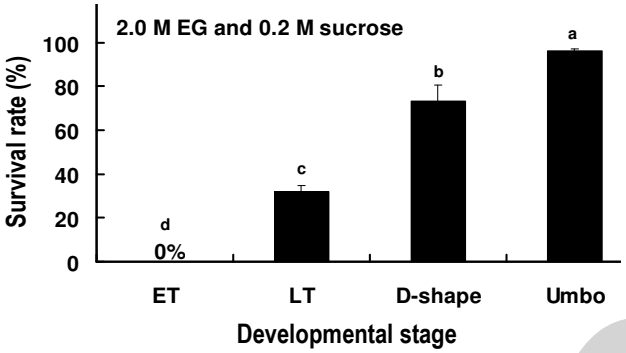

Fig. 1: Effect of developmental stage in larvae of surf clam, *Spisula sachalinensis* cryopreserved with 2.0 M ethylene glycol and 0.2 M sucrose. The larvae were kept for 1 hr in liquid nitrogen. ET: early trochophore, LT: late trochophore (diameter 79.5±4.3 μm), D-shape: D-shaped larva (110.1±6.9 μm of shell length(SL) and 91.7±6.6 μm of shell height (SH), Umbo: umbo stage larva (247.4±17.1 μm of SL and 224.2±18.1 μm of SH). The same indices on each bar indicate absence of a significant difference between treatments (p<0.05)

In the case of 0.2 M protective additives, some of the thawed D-shaped larvae survived in all of the protective additives regardless of their kinds and concentrations. Especially when trehalose with DMSO or EG was used, survival rates were 78.1±1.9% and 90.2±1.0%, respectively. Meanwhile, thawed umbo stage larvae were all dead in fructose and glucose. The survival rate of thawed larvae using the sucrose with EG showed the highest value of 96.1±1.0% (p<0.05) (Fig. 2). In the case of 0.5 M protective additives, the survival of thawed D-shaped larvae was 67.5±5.2% in glucose with DMSO and that of thawed umbo stage larvae was 65.7±4.2% in fructose with DMSO. On occasion of using the thawed umbo stage larvae, they did not survive in glucose with DMSO; whereas, they showed significantly higher survival rates in glucose with EG (p<0.05) (Fig. 3). Therefore on occasion of

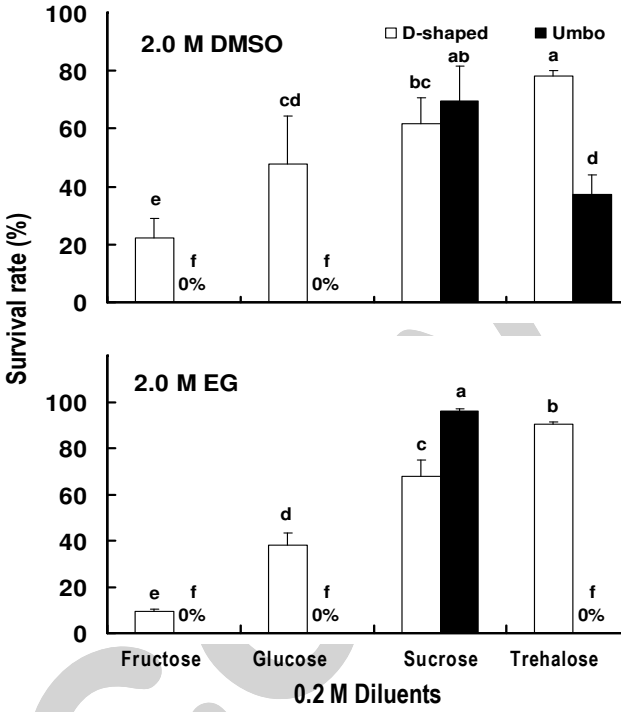

Fig. 2: Effect of 0.2 M protective additives on cryopreserved late D-shaped and umbo stage larvae of surf clam *Spisula sachalinensis*. The larvae were kept for 1 hr in liquid nitrogen. The same indices on each bar indicate absence of a significant difference between treatments (p<0.05)

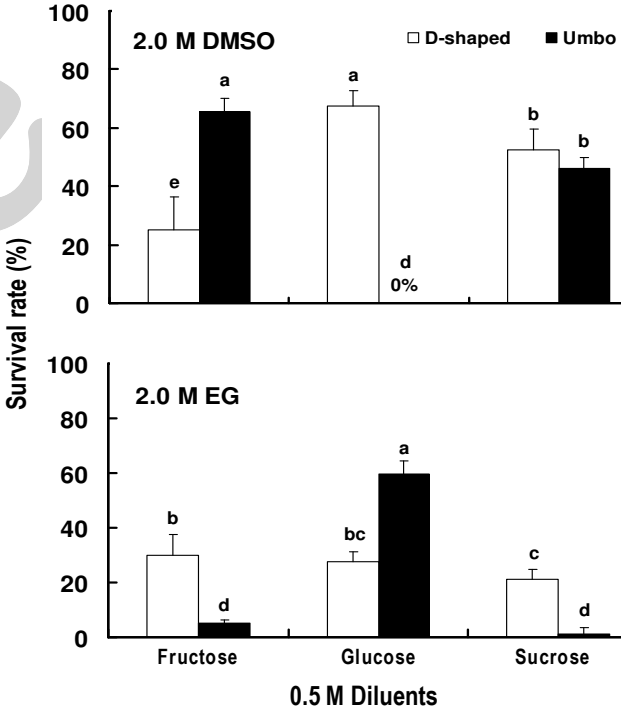

Fig. 3: Effect of 0.5 M protective additives on cryopreserved late D-shaped and umbo stage larvae of surf clam *Spisula sachalinensis*. The larvae were kept for 1 hr in liquid nitrogen. The same indices on each bar indicate absence of a significant difference between treatments (p<0.05)

cryopreservation, monosaccharide and disaccharide must be added at higher and lower concentration, respectively. The use of different concentrations of protective additives may be due to their viscosity. McWilliams *et al.* (1995) demonstrated that osmolality as a function of concentration of monosaccharides was similar to that of disaccharides and also at the same osmolality, the relative viscosity of disaccharide solutions was considerably greater than that of monosaccharides solutions. The high osmolality of cryoprotectant caused biochemical and osmotic injury to embryos (Renard and Cochard, 1989); however, it did not affect survival rate of larvae. The higher survival rate of larvae is owing to addition of sugars and the sucrose has been used as an osmotic buffer to reduce osmotic shock (Choi and Chang, 2003).

In conclusions, the results of our study indicate that the umbo stage larva was the most desirable developmental stage of larvae and 0.2 M sucrose was the most protective additive for cryopreservation of surf clam larvae.

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References

Acosta-Salmon, H., D.R. Jerry and P.C. Southgate: Effects of cryoprotectant agents and freezing protocol on motility of black-lip pearl oyster (*Pinctada margaritifera* L.) spermatozoa. *Cryobiology*, **54**, 13-18 (2007).

Chao, N.H., T.T. Lin, Y.J. Chen, H.W. Hsu and I.C. Liao: Cryopreservation of late embryos and early larvae in the oyster and hard clam. *Aquaculture*, **155**, 31-44 (1997).

Choi, Y.H. and Y.J. Chang: The influence of cooling rate, developmental stage and the addition of sugar on the cryopreservation of larvae of the pearl oyster *Pinctada fucata martensii. Cryobiology*, **46**, 190-193 (2003).

Gwo, J.C.: Cryopreservation of oyster (*Crassostrea gigas*) embryos. *Theriogenology*, **43**, 1163-1174 (1995).

McWilliams, R.B., W.E. Gibbons and S.P. Leibo: Osmotic and physiological responses of mouse zygotes and human oocytes to mono- and disaccharides. *Hum. Reprod.*,**10**, 1163-1171 (1995).

Renard, P. and J.C. Cochard: Effect of various cryoprotectants on Pacific oyster, *Crassostrea gigas* Thunberg, Manila clam, *Ruditapes philippinarum* reeve and king scallop, *Pecten maximus* (L) embryo: Influence of the biochemical and osmotic effects. *Cryo-Letters*, **10**, 169-180 (1989).

Renard, P.: Cooling and freezing tolerances in embryos of the Pacific oyster, *Crassostrea gigas*: Methanol and sucrose effects. *Aquaculture*, **92**, 43-57 (1991).

Tervit, H.R., S.L. Adams, R.D. Roberts, L.T. McGowan, P.A. Pugh, J.F. Smith and A.R. Janke: Successful cryopreservation of Pacific oyster (*Crassostrea gigas*) oocytes. *Cryobiology*, **51**, 142-151 (2005).