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Effects of glycine and alanine on short-term storage and cryopreservation of striped bass (*Morone saxatilis*) spermatozoa[☆]

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Abstract

Three experiments were designed to examine the effects of the amino acids glycine and alanine on short-term storage and cryopreservation of striped bass spermatozoa. In the first experiment, the effect of glycine on post-equilibration motility was evaluated. In the presence of 2.5 or 5.0% Me₂SO, glycine treatments (25, 50, and 75 mM) yielded higher ($P < 0.05$) post-equilibration motility at all equilibration times examined compared to the control. There was no difference ($P > 0.05$) among these three glycine treatments. In the second experiment, glycine and alanine at concentrations of 25, 50, 75, or 100 mM were evaluated for post-thaw motility in the presence of 2.5 or 5% Me₂SO. When compared to the control, both the glycine and alanine treatments showed positive effects on post-thaw motility at all concentrations tested. The highest ($P < 0.05$) post-thaw motility was achieved with 50 mM glycine or 75 mM alanine using 5% Me₂SO. No interaction ($P > 0.05$) between Me₂SO and glycine or alanine was observed, indicating that the effect of glycine or alanine was independent of the concentrations of Me₂SO. In the third experiment, glycine was evaluated for sperm motility, after short-term refrigerated storage and after cryopreservation of the same refrigerated semen. Sperm motility decreased after 24 h of refrigerated storage in 50 mM glycine treatment and the control, when compared to fresh sperm motility. However, 50 mM glycine treatment yielded higher ($P < 0.01$) sperm motility after both 24 and 48 h of storage as well as higher ($P < 0.01$) post-thaw motility when compared to the control. An average of $30 \pm 2.9\%$ and $16 \pm 2.4\%$ post-thaw motility was achieved with the 50 mM glycine treatment after 24 and 48 h of refrigerated semen, respectively. © 2002 Elsevier Science (USA). All rights reserved.

Keywords: Cryopreservation; Striped bass; Spermatozoa; Glycine; Alanine

The hybrid striped bass industry is one of the fastest growing segments in US aquaculture with production increasing almost 10-fold from 1986 to

1995 [32]. The hybrid striped bass industry is now the fourth largest finfish species by value in the US [8]. However, rapid growth remains constrained by continued reliance on wild adult striped bass for seedstock, which poses significant risks to the industry [14,41]. Hybrid striped bass hatcheries also face the problem of different spawning times

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between the striped bass male and white bass female used to create the sunshine bass, the industry standard [17,33]. Additionally because the spawning season of the white bass is earlier than that of striped bass, producing hybrids can be logistically difficult. Cryopreservation of striped bass spermatozoa is one approach that could potentially help solve this problem, as well as reducing the cost of hybrid striped bass production [7].

Some studies have been carried out on the cryopreservation of striped bass spermatozoa [6, 16,18–20]. Kerby [19] found very low or no post-thaw sperm motility was achieved with dimethyl sulfoxide (Me_2SO), glycerol, ethylene glycol, and propylene glycol. Jenkins-Keeran and Woods [18] reported as high as 23% post-thaw motility with 5% Me_2SO at a $-40^\circ\text{C min}^{-1}$ freezing rate. Building upon the latter efforts we describe herein our attempts to improve upon the former cryopreservation protocol.

Free amino acids have been detected in seminal plasma of salmonids [23,30], however, the role of free amino acids in sperm physiology is unclear. They may contribute to seminal plasma osmolality [3] and can have a positive role in sperm vitality [23]. Several amino acids (e.g., glutamine, glycine, proline, and histidine) have been used as non-permeating cryoprotectants to cryopreserve spermatozoa from a number of mammalian species including: ram [29], stallion [35], human [26], and goat [21]. Kundu et al. [21] have shown that the combined cryoprotecting action of amino acids with glycerol or Me_2SO increased post-thaw sperm motility. However, there are limited studies where amino acids have been used as cryoprotectants to cryopreserve fish spermatozoa. Lahnsteiner et al. [22] used an extender containing glycine, which had previously been developed by Borchard et al. [5], to cryopreserve grayling (*Thymallus thymallus*) sperm. Billard [2] developed DCS B4 and DCS D2, two extenders containing glycine, to cryopreserve gilt-head seabream (*Sparus aurata*) sperm, however, since all the extenders examined contained the same level of glycine, the effect of glycine could not be detected.

Our study was conducted as part of ongoing efforts to establish a commercially viable protocol for cryopreservation of striped bass spermatozoa.

In the first and second experiments, we examined the effect of glycine or alanine on post-equilibration motility and post-thaw motility of striped bass spermatozoa. The effect of glycine on short-term refrigerated storage and cryopreservation using refrigerated semen was subsequently evaluated in the third experiment.

Materials and methods

Animals

Three experiments were conducted (Fig. 1). For Experiments 1 and 2, three-year-old striped bass males were randomly selected from a population maintained under the previously described [37,40] ambient photothermal conditions of the flow-through, 6 m-diameter tank system at the University of Maryland's Crane Aquaculture Facility. Water temperature ranged from 5 to 30 °C during the year. In the spring, males ($n = 20$) were moved into a 4 m-diameter tank, part of a recirculating water system, and held at $15 \pm 1^\circ\text{C}$ for the remainder of the 5 week study. Each fish was given a cholesterol cellulose implant [31] containing 150 μg of mammalian gonadotropin-releasing hormone [15], inserted into the dorsal lymphatic sinus, as previously described for striped bass [39]. Three days after administering the implant, the fish were anesthetized in a 70 mg/L quinaldine bath [38] and urine was removed by applying gentle pressure around the urogenital vent. Semen was expressed directly into 50 mL sterile conical tubes and placed immediately on ice. For Experiment 3, semen was collected from striped bass males, which were also maintained in a recirculation system under similar environmental conditions at Fins Technology, Turners Falls, MA. In each experiment, only striped bass sperm samples ($n = 5$) exhibiting motility $\geq 90\%$ were used.

Experiment 1

We examined the effect of glycine on sperm motility after various short-term equilibration times (0–30 min), while using 2.5 or 5.0% Me_2SO (final concentration) as the cryoprotectant. Fresh

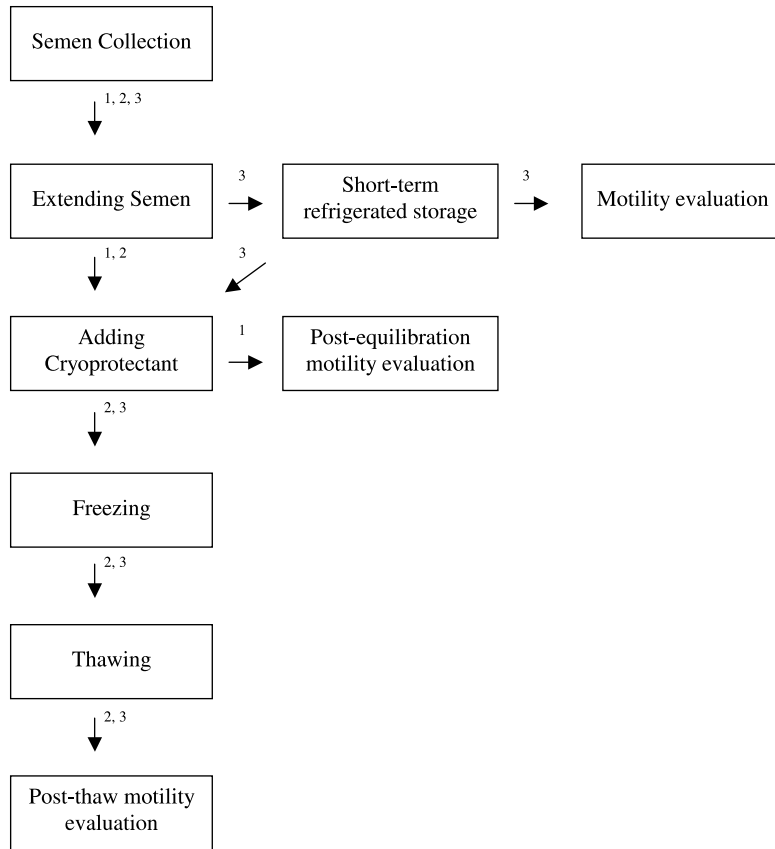


Fig. 1. Flow chart of three experiments. Numbers 1, 2, 3 mean Experiments 1, 2, 3, respectively.

semen was collected from each fish and immediately diluted 1:2 (semen:extender) with Extender 1 as a control (NaCl 14 g/L, KCl 0.4 g/L, NaHCO₃ 0.25 g/L, glucose 1 g/L, pH adjusted to 7.6) or Extender 1 containing various concentrations of glycine (25, 50, 75, or 100 mM) as glycine treatments. Since our isotonic solutions vigorously activated striped bass sperm, the osmolality of the Extender 1 was adjusted to 500 mmol/kg, which was measured using a vapor pressure osmometer (Wescor Model 5400). Although hyperosmotic to striped bass semen and seminal plasma, we have found that this osmotic tension will preclude any striped bass sperm activation without causing damage to the cell membrane for up to 90 min (He and Woods unpublished data). This technique allowed us adequate time to prepare the semen samples for controlled rate freezing without premature con-

sumption of the sperm cells limited energy reserves. Extended sperm were activated using deionized water at 10, 20, and 30 min after sperm were diluted. To estimate the percentage of motile sperm, aliquots of semen (0.01 μ L) were placed into a Makler counting chamber (TS Scientific) and 10 μ L filtered deionized water was added and quickly mixed. The loaded Makler chamber was immediately placed under a compound microscope (Zeiss model D-7082) at 400 \times . The activation of each sample was recorded on videotape using a Hitachi Model KP-140 video camera. The percentage of motile sperm was determined by reviewing videotapes and counting sperm (approximately 200–800 cells), then dividing the number of motile sperm by the total number of sperm. All samples were estimated by the same observer to avoid bias. Spermatozoa that simply vibrated or did not show

progressive forward movement were not considered in the estimates of motility as recommended by Billard and Cosson [4].

Experiment 2

The effects of extenders containing glycine or alanine in various concentrations on post-thaw motility were evaluated. Me₂SO with a concentration of 2.5 or 5.0% (final concentration) was used as the cryoprotectant. Fresh semen was collected from each fish and immediately diluted with Extender 1 containing 0 (control), 25, 50, 75, or 100 mM glycine or alanine in a 1:2 (semen:extender) ratio. Then the extended samples were subsequently diluted 1:1 with Extender 1 containing 5 or 10% Me₂SO. This final sperm mixture was quickly pipetted into 500 µL cryo-straws (TS Scientific) in aliquots of 150 µL. Six cryo-straws were filled and sealed for each treatment and each fish. The total time that the extended semen and cryoprotectant were allowed to equilibrate before freezing was 10 min. Cryo-straws containing sperm samples were frozen together using a programmable freezer (Planer Kryosave-Model KS30) with a selected freezing rate, $-40^{\circ}\text{C min}^{-1}$ until -120°C was achieved [18]. The samples were then immediately placed into liquid nitrogen. Three days later, samples were thawed in a 35°C water bath for 8 s. The thawed semen mixture (0.01 µL) was immediately poured out of each straw into a Makler counting chamber, activated with deionized water, and recorded on videotape. The percentage of sperm that were motile post-thaw was determined as described in above in Experiment 1.

Experiment 3

Fresh semen was collected, extended 1:2 (semen:extender) in Extender 2 (NaCl 8.0 g/L, KCl 0.4 g/L, NaHCO₃ 0.25 g/L, glucose 1 g/L, pH adjusted to 7.6) with defined concentrations of glycine (0, 50 mM), and added into tissue culture tubes. Osmolality of Extender 2 was adjusted to 350 mmol/kg, which was isotonic to the striped bass seminal plasma. The tissue culture tubes were filled with oxygen, shown to be beneficial to

striped bass sperm stored for short periods of time [16], and maintained at 4°C with ice or refrigeration. The samples were packed and shipped overnight from Fins Technology to our laboratory. The percentage of motile sperm was estimated at 24 and 48 h after semen was extended. Sperm motility was estimated using methods previously described in Experiment 1. To estimate the post-thaw motility using refrigerated semen (stored refrigerated for 24 and 48 h), 5% Me₂SO (final concentration) was used. Semen was diluted 1:1 with Extender 1 with defined concentrations of glycine (0, 50 mM). The sperm samples were frozen after 10 min equilibration. The methods used to freeze and thaw sperm samples were identical to those previously described in Experiment 2.

Statistical analysis

Data were subjected to analysis of variance (ANOVA) using mixed-model procedures. When time was involved (Experiments 1 and 3), a repeated measurement design was used in the model. When multiple variables were involved (Experiment 2), the probability of interaction was estimated by mixed-model procedures. The *P* value was preset as 0.05. Data were shown as means \pm standard error of the mean (SEM). All statistical procedures were run using SAS 8.0 software system [28].

Results

Effect of glycine on post-equilibration motility

Glycine in Experiment 1, showed a positive effect on post-equilibration motility when sperm were exposed to cryomedia containing either 2.5% Me₂SO (Table 1) or 5% Me₂SO (Table 2). The glycine treatments with 25, 50, and 75 mM yielded higher ($P < 0.05$) motility at all equilibration times examined compared to the control. The glycine treatment with 100 mM also achieved higher post-equilibration motility than the control, while the difference was not significant at 10 and 20 min in the presence of 2.5% Me₂SO and 10 min when using 5.0% Me₂SO. No difference

Table 1
Post-equilibration motility (mean \pm standard error)

Glycine (mM)	Equilibration time		
	10 min	20 min	30 min
0	68 \pm 2.9 ^a	65 \pm 2.8 ^a	59 \pm 2.0 ^a
25	76 \pm 3.6 ^b	74 \pm 2.6 ^b	71 \pm 2.9 ^b
50	75 \pm 3.6 ^b	75 \pm 2.3 ^b	72 \pm 2.8 ^b
75	74 \pm 3.2 ^b	72 \pm 2.6 ^b	69 \pm 2.5 ^b
100	70 \pm 3.0 ^{a,b}	70 \pm 2.2 ^{a,b}	66 \pm 1.9 ^b

Fresh semen was extended by Extender 1 (control) or Extender 1 containing various glycine concentrations (treatments). Extender 1 contained 2.5% Me₂SO for both the control and treatment samples. Values with different superscript letters are significantly different at 5% level by LSD within one column.

Table 2
Post-equilibration motility (mean \pm standard error)

Glycine (mM)	Equilibration time		
	10 min	20 min	30 min
0	61 \pm 4.4 ^a	54 \pm 3.4 ^a	48 \pm 3.2 ^a
25	70 \pm 5.4 ^b	68 \pm 3.2 ^b	65 \pm 4.2 ^b
50	74 \pm 5.2 ^b	71 \pm 4.2 ^b	68 \pm 3.8 ^b
75	69 \pm 4.0 ^b	65 \pm 3.9 ^b	62 \pm 3.6 ^b
100	66 \pm 3.5 ^{a,b}	61 \pm 3.4 ^b	59 \pm 4.0 ^b

Fresh semen was extended by Extender 1 (control) or Extender 1 containing various glycine concentrations (treatments). Extender 1 contained 5.0% Me₂SO for both the control and treatment samples. Values with different superscript letters are significantly different at 5% level by LSD within one column.

($P > 0.05$) among all four glycine treatments was detected. Both glycine treatments and the control yielded the highest post-equilibration sperm motility at 10 min (Tables 1 and 2). However, the decrease in the percentage of motile sperm from 10 to 30 min using glycine was less than the control ($P < 0.05$).

Effects of glycine and alanine on post-thaw motility

The effects of various concentrations of glycine and alanine with two levels of Me₂SO (2.5 and 5.0%) on post-thaw motility were further examined in the second experiment (Figs. 2a and b). The cryoprotection profiles of glycine and alanine were dosage dependent. Initially, with increasing concentration of glycine or alanine, the post-thaw motility increased and reached an optimum level, but with further increase in concentration the post-thaw motility decreased. The concentration obtaining maximum post-thaw motility varies

between amino acids. Glycine gave the best post-thaw motility (50 \pm 4.3%) at 50 mM. However, alanine obtained the maximum post-thaw motility (48 \pm 3.5%) at 75 mM. No interaction between amino acids and Me₂SO was detected ($P > 0.05$), which suggested that the positive effects of glycine and alanine on post-thaw motility were independent on the concentration of Me₂SO.

Effect of glycine on short-term storage

In our final experiment, sperm motility decreased after 24 h of refrigerated storage in 50 mM glycine treatment and the control, when compared to fresh sperm motility ($P < 0.01$). However, the 50 mM glycine treatment yielded higher ($P < 0.01$) sperm motility compared to the control (Fig. 3a). An average of 65 and 38% sperm motility were achieved with 50 mM glycine after 24 and 48 h storage, respectively. Glycine also demonstrated a positive effect ($P < 0.05$) on post-thaw motility

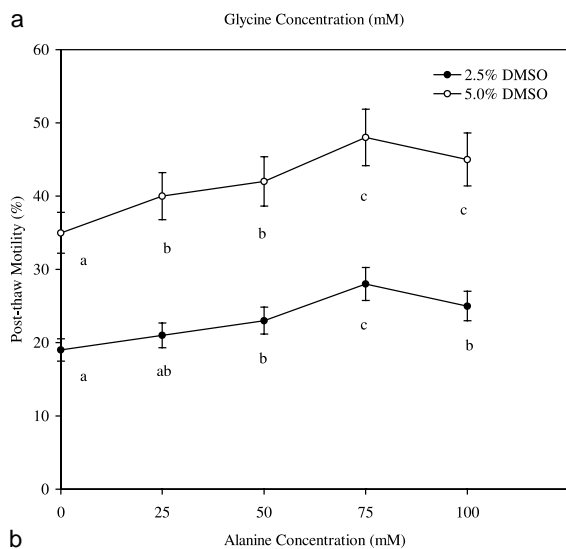
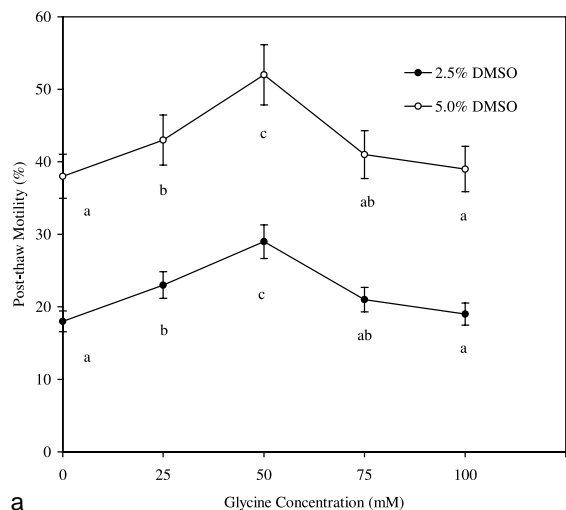


Fig. 2. (a) The percentage of motile post-thaw sperm (mean \pm standard error). Fresh semen was cryopreserved with 2.5 or 5.0% Me₂SO with various concentrations of glycine. Different letters are significantly different at 5% level by LSD within each Me₂SO concentration. (b) The percentage of motile post-thaw sperm (mean \pm standard error). Fresh sperm were cryopreserved with 2.5 or 5.0% Me₂SO with various concentrations of alanine. Different letters are significantly different at 5% level by LSD within each Me₂SO concentration.

compared to the control (Fig. 3b). An average of $30 \pm 2.9\%$ and $16 \pm 2.4\%$ post-thaw motility was achieved with 50 mM glycine treatment when using 24 and 48 h refrigerated storage semen, respectively.

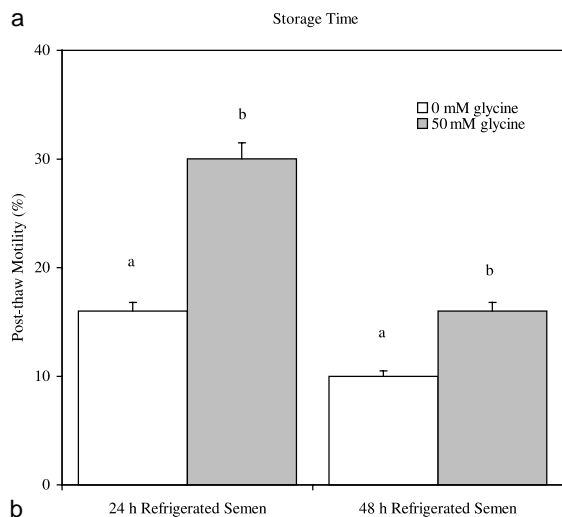
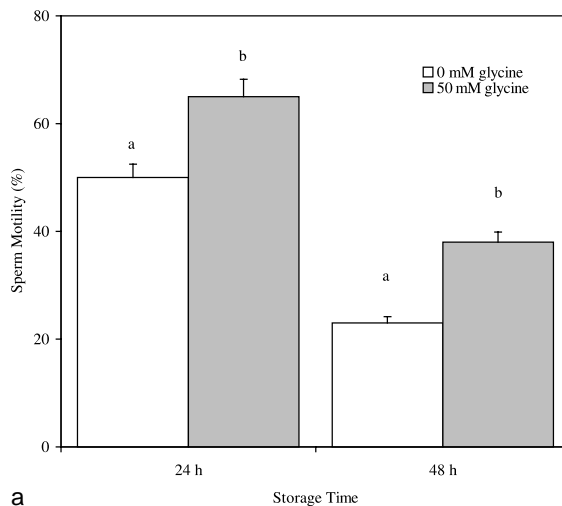


Fig. 3. (a) The percentage of motile sperm (mean \pm standard error). Fresh semen was extended with Extender 2 containing 0 (control) or 50 mM glycine (treatment) and sperm motility was determined after short-term refrigerated storage. Bars with different letters are significantly different at 5% level by LSD within each refrigerated time. (b) The percentage of motile post-thaw sperm (mean \pm standard error). Semen after 24 and 48 h refrigerated storage was cryopreserved with 5% Me₂SO and with (50 mM) or without glycine. Bars with different letters are significantly different at 5% level by LSD within each refrigerated time.

Discussion

The results demonstrated that glycine significantly improved sperm motility after 24 and 48 h of refrigerated storage and post-thaw motility

using stored refrigerated semen. It was reported that the addition of glycine to the extender had a positive effect on sperm motility of sea urchin [36] and bovine [27] when using short-term refrigerated storage. However, the mechanism of how glycine may improve sperm motility is still not clear. Studies with carbon 14-labeled glycine indicated that glycine was metabolized by bovine spermatozoa [10]. They later reported that glycine was beneficial to sperm by reducing lactic acid accumulation in extenders [11]. A more recent study [22] reported that glycine was beneficial for the membrane structural stability of grayling spermatozoa and thus resulted in a higher fertilization rate. In previous studies with striped bass sperm, the best motility after 24 h refrigerated storage was less than 30% [17] and no cryopreservation trial was reported using refrigerated semen. Our results suggested that it is possible to cryopreserve striped bass sperm that were collected at remote farm sites and transported to facilities that are equipped to properly freeze the extended sperm.

Me₂SO has the characteristics of small molecular weight and a high rate to permeate into the cells, which makes Me₂SO a widely used cryoprotectant for cryopreservation sperm cells of various aquatic species including Atlantic croaker [12], yellowfin seabream [13], catfish [25,34], and rainbow trout [9]. However, Me₂SO could also cause damages due to its high osmotic shock and toxicity to cells [24]. Our results showed that the striped bass sperm was very sensitive to Me₂SO. In the absence of glycine, more than 40% sperm lost their motility at 30 min after exposure to 5% Me₂SO due to high osmotic stress (~1250 mmol/kg) and/or toxicity. However, only approximately 20% sperm lost their motility after 30 min equilibration with 50 mM glycine, which showed the characteristic of glycine to protect sperm when exposure to Me₂SO. Within such short equilibration times, the effect of glycine on sperm metabolism should not be shown so apparently. It suggested that glycine may reduce the speed of Me₂SO permeating into the cells and thus minimize the osmotic or toxic stress from Me₂SO. As amino acids are charged molecules, it is possible that they interact electrostatically with the phosphate groups in the sperm plasma membrane phospholipids, thereby forming a layer on the sperm surface [1].

The impact of this layer on sperm plasma membrane may affect the permeability of Me₂SO into the cells and protect sperm plasma membrane from cold shock. The characteristics (i.e., integrity, thickness) of this layer are apparently related to the concentration of amino acids. This explains why the effects of glycine and alanine on post-thaw motility are dosage dependent. Our data demonstrated that the addition of glycine or alanine significantly increased the cryoprotecting efficacy of Me₂SO and there was no interaction between the concentrations of Me₂SO and these two amino acids. These results also suggested that the cryoprotection mechanism of glycine and alanine is different from that of Me₂SO.

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