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Identification of strombine and taurine as cryoprotectants in the intertidal bivalve *Mytilus edulis*

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Cryoprotectants have not been identified in intertidal invertebrates tolerant to freezing, probably due to the lack of a simple, non-correlative assay for the presence of these compounds. Protection of phospholipid vesicles against fusion and leakage and protection of freeze-labile enzymes following a freeze-thaw cycle can be used as an indication of the presence of cryoprotectants in extracts from organisms. The hemolymph of the intertidal bivalve mollusc, *Mytilus edulis* was effective in reducing freeze-induced fusion of phospholipid vesicles and denaturation of lactate dehydrogenase, indicating the presence of a cryoprotectant. The ethanol soluble fraction of the blood protected phospholipid vesicles against freeze-induced fusion and leakage and lactate dehydrogenase against freeze-induced denaturation. The ethanol-soluble fraction was fractionated further with Sephadex G-25 column chromatography, thin-layer chromatography, and paper chromatography. Taurine and strombine were identified as two compounds present in the hemolymph that were capable of cryoprotection.

Introduction

Many intertidal invertebrates are capable of surviving the presence of ice in their extracellular fluids and are thus considered to be freezing tolerant [1]. The mechanisms of freezing tolerance in these organisms, however, are as yet unclear. The typical cryoprotectants that are found in terrestrial organisms tolerant to freezing are either absent in intertidal organisms or are present in such low concentrations that they could not account for all of the freezing tolerance. Calcium has been correlated with an increase in freezing tolerance in one intertidal invertebrate, but its increase could only account for forty percent of the observed freezing tolerance [2]. This finding suggests that other cryoprotectants are present but have not yet been identified. The present method for identifying putative cryoprotectants in organisms is to measure changes in tissue solute concentrations and correlate them with changes in freezing tolerance. This method works well for known cryoprotectants but not for novel cryoprotectants because it would be easy to overlook unknown compounds. It also is limited by the fact that correlation does not imply cause and effect. A non-correlative method for identifying possible cryoprotectants would circumvent these problems. In this paper we describe a new method for identifying possible cryoprotectants using protection of liposomes and freeze-labile enzymes after freeze-

Abbreviations: SUV, small unilamellar vesicle; RET, resonance energy transfer.

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thaw cycles as an 'assay'. Using this method, we have been able to identify strombine and taurine as likely cryoprotectants in the freezing tolerant intertidal bivalve, *Mytilus edulis*.

Materials and Methods

Animals. Specimens of *Mytilus edulis* were collected from Stonington, Connecticut on December 12, 1986 and transported to Connecticut College where hemolymph samples were immediately taken from the sinus in the posterior adductor muscle. The hemolymph samples were frozen and shipped on dry ice to the University of California, Davis where they were stored at −20° C for three weeks.

Resonance energy transfer. Freeze-thaw induced fusion of small unilamellar vesicles (SUVs) was monitored by measuring resonance energy transfer (RET) between fluorescent probes in two populations of 1-palmitoyl-2-oleoylphosphatidylcholine/phosphatidylserine (POPC/PS, mole ratio 85 : 15) SUVs as described previously [3,4]. Vesicles were prepared by sonicating lipids (20 mg/ml) to clarity in the presence of either 2 mole percent of the donar probe (CA9C) or the acceptor probe (NBDPE) and 10 mM Tes (pH 7.0) containing 0.1 mM EDTA (buffer 1). The SUVs (0.2 mg of each population) were frozen in buffer 1 containing various volumes of the extract to be tested. The extracts were all dissolved in buffer 1 and the volume of the samples was held constant (0.2 ml) by addition of buffer 1 when necessary. The samples were placed in liquid nitrogen for 3 min and thawed at room temperature. The volume was then adjusted to 2.5 ml by addition of buffer 1, and decrease in the fluorescence of the donor probe resulting from vesicle fusion was measured on a Perkin-Elmer LS-5 fluorescence spectrophotometer (excitation, 365 nm; emission, 460 nm).

Leakage assay. Freeze-thaw induced leakage of contents of the SUVs was measured as previously described [5]. SUVs were made by sonicating to clarity 20 mg lipids (POPC/PS, 85 : 15 mole ratio) in 0.1 M carboxyfluorescein and buffer 1. The SUVs were separated from the carboxyfluorescein by passing them through a Sephadex G-50 column (1 × 15 cm) and eluting with buffer 1. Samples containing 0.4 mg carboxyfluorescein-loaded SUVs and various volumes of extract were prepared and the volume was adjusted to 0.2 ml with buffer 1. The samples were frozen in liquid nitrogen for 3 min and then thawed at room temperature. After three freeze-thaw cycles, aliquots (5 μl) were placed in 2.5 ml buffer 1 and the fluorescence was measured on a Perkin-Elmer LS-5 fluorescence spectrophotometer (excitation, 460 nm; emission, 500 nm). Since carboxyfluorescein is self-quenching at high concentrations, the carboxyfluorescein-loaded SUV's do not fluoresce. However, when carboxyfluorescein leaks from the vesicles, it is diluted by the buffer and fluoresces. Therefore, the fluorescence value measured here represents the amount of carboxyfluorescein leaked during the freeze-thaw cycle. The total carboxyfluorescein present in the SUVs was measured by lysing the vesicles with 10 μl 1% Triton X-100 and remeasuring the fluorescence. The amount of leakage could then be expressed as a percent of the total carboxyfluorescein present in the vesicles.

Lactate dehydrogenase assay. Freeze-thaw denaturation of lactate dehydrogenase was measured in the presence of extracts of the hemolymph from *Mytilus edulis*. The extracts were dried under nitrogen at 60° C and dissolved in assay buffer containing 40 mM Tris-HCl (pH 7.5), 50 mM KCl, and 50 μg/ml lactate dehydrogenase (buffer 2). Aliquots of this solution were diluted with buffer 2 to produce samples with various concentrations of extract. An aliquot (5 μl) of each sample was placed in 2.0 ml buffer 2 containing 2 mM pyruvate and 0.15 mM NADH. The reaction was followed by measuring a decrease in absorbance at 340 nm. Another aliquot (20 μl) was frozen in liquid nitrogen for 30 s and thawed at room temperature. After thawing, the lactate dehydrogenase activity was measured as described above. Results are reported as percent activity recovered following freezing.

Phosphofructokinase assay. Phosphofructokinase was purified and assayed according to the method of Carpenter et al. [6]. Freeze-thaw experiments were also carried out as outlined by Carpenter et al. except that the final phosphofructokinase concentration used in the assay buffer (buffer 3) was 75 μg/ml.

Fractionation of the hemolymph. Ethanol-soluble fractions of the hemolymph were prepared by adding 100% ethanol to a final concentration of

67%. The precipitate was removed by centrifugation and the resulting supernatant was dried under nitrogen at 60°C. The residue was dissolved in either buffer 1, buffer 2 or distilled water. Freeze-thaw induced fusion and leakage of liposomes were measured using various dilutions of the samples in buffer 1. Freeze-thaw induced denaturation of lactate dehydrogenase was measured using various dilutions of the samples in buffer 2. The sample dissolved in distilled water was placed on a Sephadex G-25 column (2 × 20 cm), and eluted with distilled water. Fractions (2.0 ml) were collected and elution of low molecular weight organic compounds was followed by measuring the absorbance at 215 nm. For measuring freeze-thaw induced fusion of liposomes the fractions were lyophilized and reconstituted in buffer 1. For measuring freeze-thaw induced denaturation of enzymes, the fractions were dissolved in either buffer 2 or buffer 3.

Chromatography. Compounds were identified on silica plates or paper which had been developed with either 95% ethanol/water (5:1, v/v) or 95% ethanol/ammonia/water (80:5:15, v/v), and detected with ninhydrin. Areas on the silica plates corresponding to detected compounds were scraped from the plate and dissolved in 60% ethanol. The ethanol was evaporated from the samples under nitrogen at 60°C and the resulting residue was dissolved in buffer 1 and tested for reduction of freeze-thaw induced fusion of SUVs.

Other assays. Carbohydrates were measured with anthrone [7]. Proteins were measured with the Peterson modification of the Lowry method [8] and total amino acids were measured with ninhydrin [9]. Individual amino acids were measured with a Beckman 121 M amino acid analyzer. Unknown compounds were identified with a Varian T-60 60 MHz ^{1}H-NMR and a Perkin-Elmer 1700 Fourier transform infrared spectrometer.

Results

Cryoprotection by whole hemolymph

Whole hemolymph collected from *Mytilus edulis* reduced probe intermixing in SUVs following one freeze-thaw cycle from 28.9% to 9.1%. The whole hemolymph also completely protected lactate dehydrogenase activity from freeze-thaw damage,

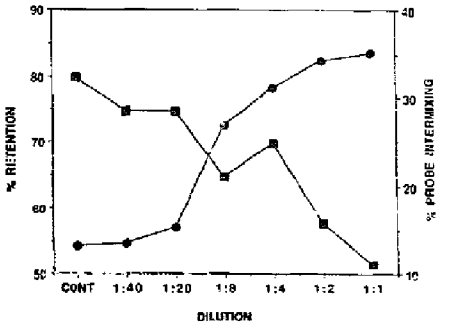


Fig. 1. The effects of dilution of the ethanol-soluble fraction of *Mytilus* hemolymph on freeze-induced fusion (■) and leakage (●) of SUVs.

increasing activity from 26% in the controls to 100% in the presence of the hemolymph.

Cryoprotection by fractions from hemolymph

The ethanol-soluble fraction of the hemolymph reduced freeze-thaw induced probe intermixing, leakage of contents and denaturation of lactate dehydrogenase. Fig. 1 shows the effects of dilution of the ethanol-soluble fraction on freeze-thaw induced probe intermixing and leakage of carboxyfluorescein from SUVs. Note that both probe intermixing and retention of carboxyfluorescein show concentration-dependent responses. Freeze-thaw induced reduction of lactate dehydrogenase

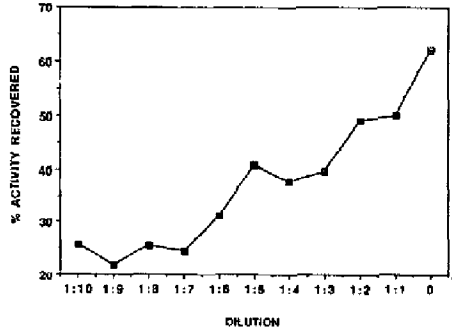


Fig. 2. The effects of dilution of the ethanol-soluble fraction of *Mytilus* hemolymph on freeze-induced denaturation of lactate dehydrogenase.

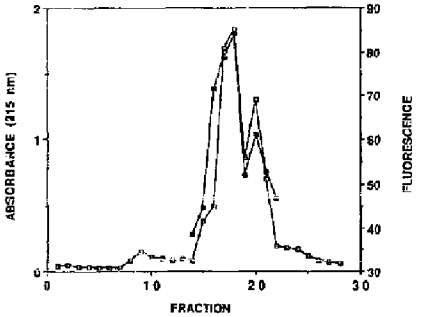


Fig. 3. Elution profile from the Sephadex G-25 column (2 ml/fraction) showing absorbance at 215 nm (□) and an increase in fluorescence indicative of a decrease in fusion (■). See text for explanation.

activity also shows a concentration-dependent response (Fig. 2).

Further fractionation of the extract on Sephadex G-25 column yielded two peaks (Fig. 3). When the fractions were tested for ability to protect liposomes against fusion during freezing, only one of the peaks resulted in a decrease in fusion below control values. Note that the results are presented as fluorescence of the donor probe so that an increase in fluorescence is indicative of a reduction in probe intermixing. The fluorescence for fractions 14, 15, 19, 20, 21 and 22 were equal to or below the control value of 60, indicating that these fractions were either fusogenic or non-protective, whereas the fluorescence for fractions 16, 17 and 18 were greater than the control value, indicating a reduction in fusion. None of the fractions, including those that protected liposomes, reduced freeze-thaw damage of lactate dehydrogenase or phosphofructokinase. Fractions 18 and 19, which also protected liposomes, however, did protect phosphofructokinase when frozen in the presence of 0.6 mM zinc. These results suggest that the cryoprotectants in the fractions might have been too dilute to function in protecting enzymes since most cryoprotectants will stabilize phosphofructokinase at much lower concentrations when zinc is present [27].

Identification of cryoprotectants

The two fractions with the greatest protective capacity (18 and 19) were combined and found to contain only amino acids. No carbohydrate or protein were present in these fractions. Amino acid analysis (Table I) showed that taurine and glycine represent over 80% of the amino acids present. The amino acids present in the combined fractions were separated on silica gel thin-layer plates and resolved into five spots corresponding to the five compounds measured by amino acid analysis. When the spots were eluted from the plates and their ability to reduce probe intermixing in liposomes during freeze-thaw was assayed, we found that only the unknown compound and the one that co-migrated with taurine were effective.

TABLE I

AMINO ACID ANALYSIS OF COMBINED FRACTIONS 18 AND 19 FROM THE SEPHADEX G-25 COLUMN

Amino acid	Concentration (mM)
Taurine	14.8
Glycine	4.3
Alanine	1.7
Ornithine	1.0
Lysine	1.0
Unknown	?

In view of its effectiveness as a cryoprotectant, the unknown compound was further purified by paper chromatography and found to co-migrate with strombine and alanopine. The compound was identified as strombine using ^{1}H-NMR (^2H_2O), showing a doublet at δ 2.1 (3H) $J = 7.0$ Hz, a singlet at δ 4.19 (2H) and a quartet at δ 4.17 (1H) $J = 7.0$ Hz. This identification was verified by comparing spectra of the unknown compound with those of authentic strombine using Fourier transform infrared spectroscopy.

Discussion

This paper presents a new technique for identifying novel cryoprotectants in organisms tolerant to freezing. The technique is based on protection of liposomes and freeze-labile proteins during freeze-thaw cycles. Membranes are a major site of damage during freeze-thaw cycles [10], and there is good evidence that many purified proteins are susceptible to freezing damage [11–16]. Although

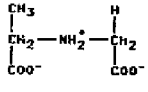
liposomes are not as complex as in vivo membranes and there are conflicting reports on the lability of enzymes to freezing in intact tissues [17–22], these techniques can be used as convenient assays for the presence of cryoprotectants in biological fluids.

The types of damage done to membranes during freezing include fusion [23–25], liquid-crystalline to gel phase transitions which lead to leakage of cell contents [10], and lateral phase separation of membrane constituents [10,23]. The source of damage to labile proteins probably includes denaturation due to alterations in the tertiary structure and/or dissociation of multimeric enzymes into their constituent parts [11,18,26]. Cryoprotectants prevent this damage [3,24], and we show here that stabilization of liposomes and enzymes in vitro provides a rigorous test for the presence of cryoprotectants.

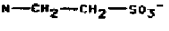
Whole hemolymph of *Mytilus edulis* was effective in protecting model membranes and lactate dehydrogenase against damage resulting from freeze-thaw, indicating the presence of a cryoprotectant. Furthermore, the cryoprotectant was retained in the ethanol-soluble fraction, suggesting that it was a low molecular weight compound. Upon dilution of the ethanol-soluble fraction, its ability to preserve both membranes and enzymes was impaired (Figs. 1 and 2). Fractions 18 and 19 from the Sephadex G-25 column still protected membranes during freezing but protection of lactate dehydrogenase was lost, probably due to the dilution on the column. These fractions also did not protect phosphofructokinase, but protection could be restored by the addition of 0.6 mM zinc. Carpenter et al. [27] have shown that the concentration of cryoprotective agents needed for protection of phosphofructokinase is greatly reduced when zinc is present. Although the mechanism of the effect of zinc is still unclear, use of phosphofructokinase with addition of zinc provides a sensitive test for putative cryoprotectants.

The cryoprotective role of taurine is consistent with reports implicating this molecule in stabilization of membranes against oxidative effects and against other agents that induce leakiness in membranes (see Ref. 28 for a recent review). It is found in high concentrations in *Mytilus* muscle (400 mmol/kg) and other tissues and increases during exposure to high salinities [29,30]. Increased salinity has been correlated with increased freezing tolerance in *Mytilus* [31] and Williams [32] suggested that taurine (along with alanine) might be acting colligatively to increase freezing tolerance in a manner similar to other cryoprotectants. The data presented here suggest that taurine may also function in stabilizing membranes and proteins during freezing. In at least one marine bivalve, taurine is also produced during exposure to anaerobic conditions [33].

Strombine, along with alanopine, octopine, and lysopine, is a common end product of anaerobic glycolysis in marine molluscs [34]. These compounds are produced by dehydrogenases which act as a secondary redox control mechanism [35]. *Mytilus* has been shown to possess strombine, alanopine and octopine dehydrogenases; but, during anaerobic exposure and recovery, strombine is preferentially produced [36] reaching levels as high as 10 nmol/g wet wt. in adductor muscles. Exposure to anaerobic conditions has been shown to increase the freezing tolerance of *Mytilus* [31] and *Modiolus demissus* [37]. The mechanisms by which this increase occurred were largely unknown, but now appear to be at least partially due to the production of strombine. Finally, we propose that taurine and strombine act as cryoprotectants for membranes by direct interaction with them. There is already good evidence that another imino acid, proline, shows such interactions [3,38–40] as do sugars such as sucrose and trehalose [3,40,41]. Examination of the structure of these molecules (Fig. 4) suggest that they may be capable of binding to the polar head groups of phospholipids, where they would presumably affect the fluidity of the bilayer [41] and prevent fusion by



STROMBINE



TAURINE

Fig. 4. Structure of strombine and taurine.

preventing close approach of two bilayers due to increased charge density at the bilayer surface. We are presently engaged in experiments designed to test this hypothesis.

The mechanism of protection of enzymes during freeze-thawing is fundamentally different than that for lipid bilayers. As we have shown elsewhere [42], the effects of the amino acids in the combined fraction follow the rules established by Timasheff and colleagues [43] for stabilization of proteins in solution; the molecules that stabilize in solution are excluded from the solvation shell of the protein. The presence of these solutes in a protein solution creates a thermodynamically unfavorable situation since the chemical potentials of both the protein and the additive are increased [43]. Taurine falls into the category of stabilizing molecules according to Timasheff's definition. No such data are available for strombine.

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