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Osmotic adjustment and requirement for sodium in marine protist thraustochytrid

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Summary

A non-invasive ion-selective microelectrode technique was used to elucidate the ionic mechanisms of osmotic adjustment in a marine protist thraustochytrid. Hypoosmotic stress caused significant efflux of Na^+, Cl^- and K^+ from thraustochytrid cells. Model calculations showed that almost complete osmotic adjustment was achieved within the first 30 min after stress onset. Of these, sodium was the major contributor (more than half of the total osmotic adjustment), with chloride being the second major contributor. The role of K^+ in the process of osmotic adjustment was relatively small. Changes in Ca^{2+} and H^+ flux were attributed to intracellular signalling. Ion flux data were confirmed by growth experiments. *Thraustochytrium* cells showed normal growth patterns even when grown in a sodium-free solution provided the medium osmolality was adjusted by mannitol to one of the seawater. That suggests that the requirement of sodium for thraustochytrid growth cycle is due to its role in cell osmotic adjustment rather than because of the direct Na^+ involvement in cell metabolism. Altogether, these data demonstrate the evidence for turgor regulation in thraustochytrids and suggest that these cells may be grown in the absence of sodium providing that cell turgor is adjusted by some other means.

Introduction

Thraustochytrids are often referred to as the lower fungi (Jennings, 1995) and were shown to require macroamounts of sodium for growth (Jennings, 1983; Garrill *et al*., 1992). However, the specific functions of sodium in this organism remain unknown. At least two options should be considered. First, requirement for Na may be associated with the role of this element in cell osmotic adjustment. Second, sodium might be directly involved in cell metabolism.

Goldstein (1963) proposed that *Thraustochytrium roseum* is an obligatory marine organism. However, it was shown later that thraustochytrids may inhabit a wide range of osmolarities and ion compositions (Booth, 1971). Being inhabitants of terrestrial waters, thraustochytrids are exposed to frequent variations in osmolarity during their life cycle (Damare and Raghukumar, 2008). Consequently, they are expected to have developed an adaptive mechanism for osmotic adjustment to the changing environment.

Cell osmotic adjustment to stress may be achieved either by regulation of uptake of inorganic ions from an external solution, or by changes in the cytosolic concentration of compatible solutes acting as osmolytes (Jones and Pritchard, 1989). The first option has an advantage of being rapid enough to provide the fast osmotic adjustment under the sudden changes in medium osmolarity, while compatible solutes are likely to be involved in a 'fine tuning' of cell turgor (Shabala and Lew, 2002).

The important role of transport processes across the plasma membrane (PM) in cell osmotic adjustment was shown for mammalian (Lang *et al*., 1998 and references within), bacterial (Blount and Moe, 1999; Shabala *et al*., 2009) and algal (Bisson and Gutknecht, 1975; Okazaki and Tazawa, 1990; Beilby *et al*., 1999; Shepherd and Beilby, 1999) cells. Evidence for fungi is not that numerous (Burgstaller, 1997; Lew *et al*., 2006) and, to the best of our knowledge, marine fungi remain essentially unexplored.

In the present study, the non-invasive ion-selective microelectrode (MIFE) technique was used to elucidate the ionic mechanisms of osmotic adjustment in a marine protist traustochytrid ACEM C. The relevant contribution of each ion in the process of cell osmotic adjustment was quantified, and the possible signalling mechanisms were elucidated. The MIFE experiments were supplemented by measurements of cell growth in medium of different osmolarities. Altogether, these data demonstrate the evidence for turgor regulation in thraustochytrid and suggest that these cells may be grown in the absence of sodium providing that cell turgor is adjusted by some other means (such as the use of compatible solutes).

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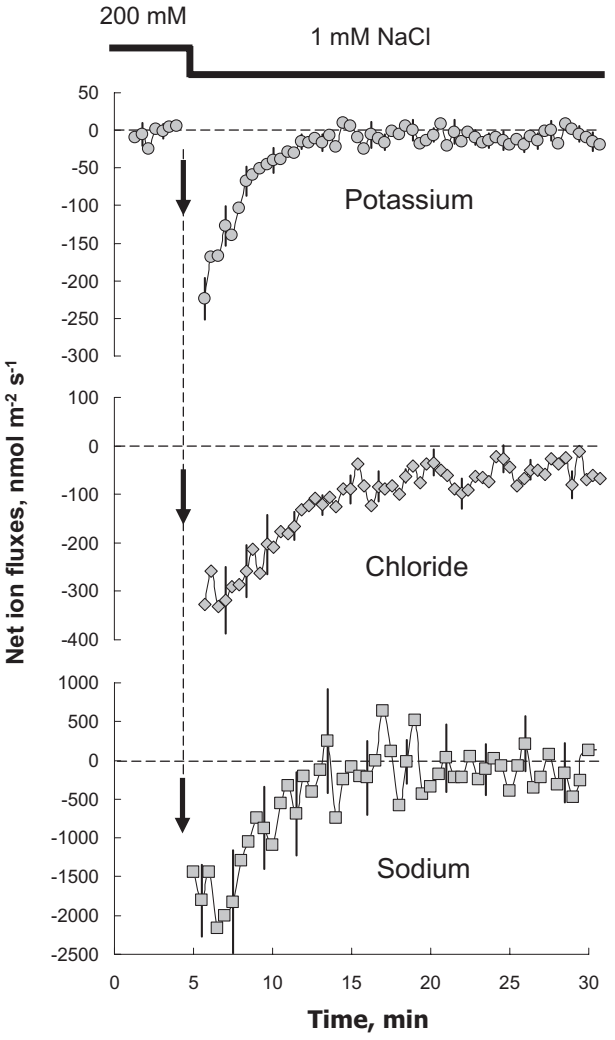

Fig. 1. Kinetics of net K^+, Na^+ and Cl^- flux in response to hypoosmotic stress in thraustochytrid ACEM C. Cells were adapted to 200 mM NaCl for 2 h, and solution was then changed to 1 mM NaCl at time zero. Error bars are SEM ($n = 8$). Due to low signal-to-noise ratio, steady Cl^- and Na^+ flux values at 200 mM NaCl concentration in the experimental solution are not shown (statistically not different from zero).

Results

Transient ion flux kinetics

In steady conditions (cells adapted to 200 mM NaCl for 1–2 h), net fluxes of K^+, Na^+ and Cl^- measured from the cell surface were around zero. Onset of hypoosmotic stress (changes in NaCl concentration in the medium from 200 to 1 mM) caused significant transient net efflux of these ions (Fig. 1). This efflux gradually decreased, and returned to the original values 15–20 min after the hypoosmotic stress onset. At the same time, a significant (~150 nmol m^{-2} s^{-1}) net H^+ influx into the thraustochytrid cell was measured, peaking 5–7 min after the treatment, followed by a gradual decline in net H^+ flux back to the initial value (Fig. 2A).

In steady-state experiments, net H^+ fluxes were measured from the thraustochytrid surface after cell preincubation in an experimental solution containing variable NaCl concentration (0, 1, 10, 50, 100 mM) for 1 h. Net H^+ extrusion increased progressively with an increase in NaCl concentration in the solution. Protons were taken up at NaCl concentrations below 10 mM and actively extruded above this concentration (Fig. 2B).

At 200 mM NaCl in the medium thraustochytrid ACEM C had net Ca^{2+} flux close to zero. The shift in medium osmolality from 200 to 1 mM NaCl resulted in net Ca^{2+} influx of up to ~80 nmol m^{-2} s^{-1} followed by decrease to the original value in ~15 min (Fig. 3, open symbols). The presence of 200 μM $LaCl_3$ suppressed transient calcium flux responses indicating that the observed Ca^{2+} influx was via Ca^{2+} channels (Fig. 3, closed symbols). Interest-

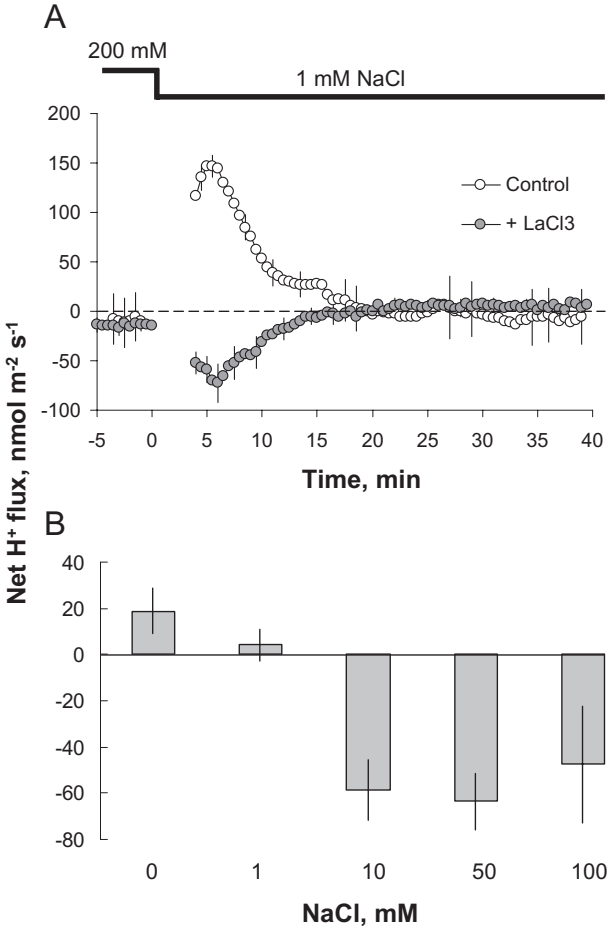

Fig. 2. A. Kinetics of net H^+ flux in response to hypoosmotic stress. Cells were adapted to 200 mM NaCl for 2 h, and solution was then changed to 1 mM NaCl at 3 min. Error bars are SEM ($n = 8$).
B. Net H^+ flux as a function of NaCl concentration. Net H^+ flux was measured in steady-state conditions after 1 h of adaptation at indicated salinities. Error bars are SEM ($n = 15$–30 cells).

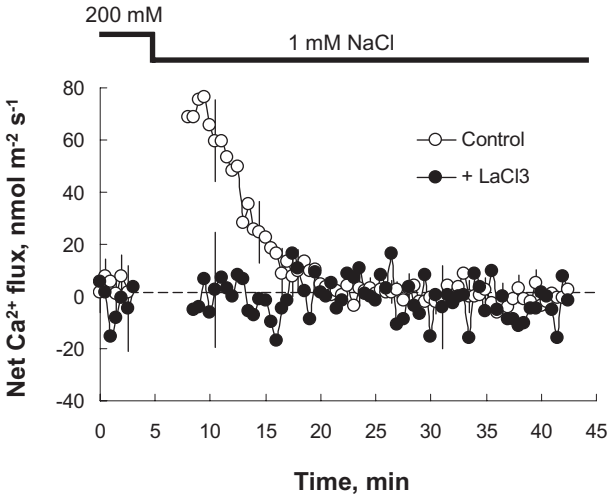

Fig. 3. Kinetics of net Ca^{2+} flux in response to hypoosmotic stress. Cells were adapted to 200 mM NaCl for 2 h, and solution was then changed to 1 mM NaCl at 3 min. Open symbols – control; closed symbols – cells pre-treated with 200 μM $LaCl_3$. Error bars are SEM ($n = 8$).

ingly, pre-treatment with La^{3+} have dramatically altered kinetics of hypoosmotic-induced H^+ flux responses (closed circles in Fig. 2A) while no significant ($P < 0.05$) impact on K^+ flux kinetics was observed (data not shown). Effects of La^{3+} on Cl^- and Na^+ fluxes were not examined.

Quantitative assessment of ion flux contribution to osmotic adjustment

High spatial resolution and a unique ability of the MIFE technique to measure concurrently fluxes of three ions in the same experiment and from the same cell enabled the relative contribution of efflux of each specific ion (Na^+, K^+ and Cl^-) in the osmotic adjustment of thraustochytrid cells to be quantified.

Assuming average cell radius $R = 10$ μm, the cell volume (V) is

$$V = 4/3\pi R^3 = 4.18 \times 10^{-15}\ \mathrm{m}^3,$$

and the cell surface area (S)

$$S = 4\pi R^2 = 1.25 \times 10^{-9}\ \mathrm{m}^2.$$

From Fig. 1, the average net efflux of ions over the 30 min interval, observed during the transient response, is

$$K^+ \text{ efflux} = 25\ \mathrm{nmol\ m^{-2}\ s^{-1}},$$

$$Na^+ \text{ efflux} = 390\ \mathrm{nmol\ m^{-2}\ s^{-1}},$$

$$Cl^- \text{ efflux} = 150\ \mathrm{nmol\ m^{-2}\ s^{-1}}.$$

Assuming surface area of the thraustochytrid cell $S = 1.25 \times 10^{-9}$ m^2 (see above), and the flux being uniform over the cell surface, then in 30 min, the total loss of ions from the cell will be

$$\Delta N = \text{Flux} \times \text{Surface area} \times \text{Time},$$

$$\Delta N = 5.65 \times 10^{-14}\ \text{mol of } K^+,$$

$$\Delta N = 8.79 \times 10^{-13}\ \text{mol of } Na^+$$

$$\Delta N = 3.39 \times 10^{-13}\ \text{mol of } Cl^-.$$

According to van't Hoff's law, the loss of each of these ions will cause a drop $\Delta\Psi$ in the cell turgor as we showed previously (Shabala, 2000)

$$\Delta\Psi = \Delta N * R * T/V,$$

where $R = 8.31$ is the universal gas constant; $T = 296$ K is the ambient temperature; and $V = 4.18\ 10^{-15}$ m^3 is an average cell volume (see above).

Then

$$\Delta\Psi = 0.04\ \text{mPa for } K^+,$$

$$\Delta\Psi = 0.51\ \text{mPa for } Na^+,$$

$$\Delta\Psi = 0.20\ \text{mPa for } Cl^-.$$

Overall, efflux of these three ions will result in the drop of cell turgor by about 0.75 mPa. At the same time, transition from 200 to 1 mM NaCl is expected to cause the change in osmotic potential of about 0.8 mPa (West, 1974). Therefore, almost complete (> 95%) osmotic adjustment is achieved within the first 30 min by efflux of Na^+, K^+ and Cl^- ions in thraustochytrid cells. Of these, sodium is a major contributor (more than half of the total osmotic adjustment), with chloride being the second major contributor. The role of K^+ in the process of osmotic adjustment appears to be relatively small.

Changes in growth parameters

Growth experiments suggested that the optimal growth of thraustochytrium ACEM C does not necessarily require full-strength seawater solution. At 200 mM NaCl, cell growth rate was identical to the control (500 mM) and the yield was even slightly higher (Fig. 4A). When the concentration of NaCl was below 200 mM, both the growth rate and cell yield (Fig. 4A) decreased dramatically. The data were further confirmed by counting the cell numbers after growth for 5 days (Fig. 4B). There was a clear relationship between the total cell number and yield and the amount of sodium present in the medium. At low Na concentrations (0–1 mM), these parameters were between 4% and 7% from control (500 mM NaCl) (Fig. 4). However, when the medium osmolality was adjusted to one equivalent of the 500 mM NaCl (0.98 Os kg^{-1}) with mannitol, neither the rate of cell growth nor cell yield was different from control even in the absence of sodium (Fig. 5). Regardless the amount of sodium in the growth medium, cell growth patterns were similar to those in

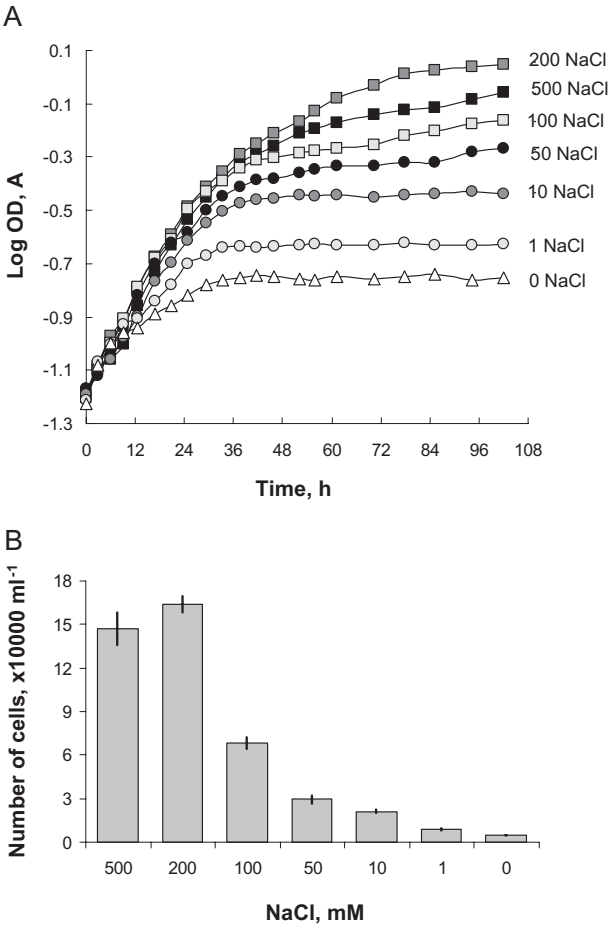

Fig. 4. Kinetics of *Thraustochytrium* sp. growth.
A. Typical examples of growth curves for different NaCl concentrations.
B. The number of cells in the culture after 5-day growth at different NaCl concentrations. Data are average of two experiments with four to six replicates in each treatment. Error bars are SEM ($n = 8–12$).

control for all the variants with isotonic mannitol solutions (Fig. 5B).

Discussion

Our data provide the evidence for osmotic adjustment in thraustochytrid ACEM C cells and demonstrate the importance of PM ion transport activity to this process under hypoosmotic conditions.

In their natural habitats, microorganisms are often exposed to significant osmotic changes. When cells are affected by hypoosmotic stress they are subject to substantial inward flows of water. A decrease in the external solute concentration from 250 mM to near zero would increase the intracellular pressure by more than six atmospheres (Blount and Moe, 1999). Unless steps are taken, these drastic forces will cause the cell to rupture.

Among the cytoplasmic solutes that may be released upon hypoosmotic stress are inorganic ions and solutes of organic origin (proline, trehalose, glycine betaine and even small proteins) (Sukharev *et al*., 1993; Cui and Adler, 1996; Blount and Moe, 1999; Jones *et al*., 2000). Wethered and Jennings (1985) suggested that inorganic ions made the major contribution to solute potential of *Thraustochytrium* spp. sporangia, and estimated the contribution of organic solutes to be insignificant. Our data are consistent with these observations (Fig. 1). Up to 95%

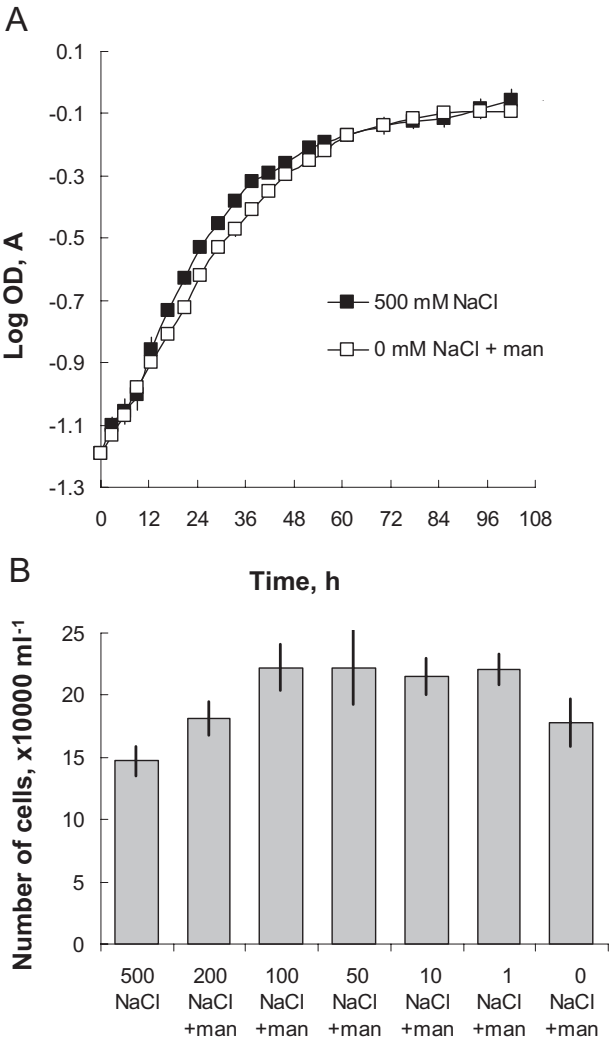

Fig. 5. Sodium is not essential for *Thraustochytrium* sp. growth.
A. Growth curves at isoosmotic conditions. Closed symbols – 500 mM NaCl; open symbols – isotonic (to 500 mM NaCl) sodium-free solution whose osmolality was adjusted by mannitol. Error bars are SEM ($n = 4–6$). Experiments were conducted twice with similar results.
B. The number of cells in the culture after 5-day growth at different NaCl/mannitol ratios. Osmolality of each solution was adjusted to the same value (0.98 Os kg^{-1}; corresponds to 500 mM NaCl) by combining appropriate amounts of NaCl and mannitol. Data are average of two experiments with four to six replicates in each variant. Error bars are SEM ($n = 8–12$).

of cell turgor was adjusted within first 30 min by the efflux of three inorganic ions (Na^+, Cl^- and K^+). These ions are usually attributed to osmotic adjustment of cells of different origin including animal (Ikehara *et al*., 1992; Orlov *et al*., 1993; Hoffmann and Dunham, 1995), yeast (Thome-Ortiz *et al*., 1998), algae (Bisson and Krist, 1995; Beilby and Shepherd, 1996; Beilby *et al*., 1999; Shepherd *et al*., 1999), marine fungi (Rauferova *et al*., 1997) and plant cells (Jones and Pritchard, 1989).

The overall contribution of each of above three ions, however, might be different in various organisms. There is a still a debate in the literature about the relative contribution of each of these ions in turgor regulation in thraustochytrids. As mentioned above, Wethered and Jennings (1985) reported that inorganic ions contributed about 90% of the osmotic potential of solutes within the protoplast of *Thraustochytrium aureum*. In contrast, Garrill and colleagues (1992) showed that sodium and potassium contributed less than 25% of the osmotic potential of *T. roseum* required for osmotic balance at seawater salinity. Our data estimate the relative contribution of the latter two ions as 65% and 5% respectively. Strain specificity might explain this difference. The remaining 25% in our experiments are attributed to Cl^- efflux, the ion that was not measured by Garrill and colleagues (1992).

The overall osmotic adjustment was very rapid. Thirty minutes after the stress onset, 95% of the cell turgor was adjusted (Fig. 1 and model calculations), and fluxes of all ions were levelled around net zero (Figs 1–3). Such a quick response may be explained by relatively small cell volume of thraustochytrid cells (15–30 μm diameter). This is in accord with findings of Bisson and colleagues (1995) who showed that turgor regulation in *Chara longifolia* occurs in two distinct phases: (i) the fast phase, lasting *c*. 5 min, during which about 25% of initial turgor is recovered, and (ii) the slow phase, when the remaining balance of the turgor pressure is achieved. The authors showed that the duration of the second phase was strikingly different between different cells, and showed very strong dependence on cell size (volume). In small cells, its duration was about 40 min, while in larger cells full turgor restoration required up to several days. The calculations presented in our study suggest that almost complete cell turgor was restored within 30 min of stress onset in our experimental conditions. Due to small cell size, the efficacy of turgor regulation in thraustochytrids should be much higher than in giant algae.

The second group of ions studied here, namely H^+ and Ca^{2+}, does not directly contribute to the osmotic adjustment of an organism. The fact that H^+ influx was more pronounced at lower NaCl concentrations (Fig. 2B) suggests that this observation is the consequence of osmotic imbalances. Our data are in accord with observations of cytosolic acidification in response to hypoosmotic stress using animal cells (Lang *et al*., 1998), bacteria (Berrier *et al*., 1992; Schleyer *et al*., 1993), fungi (Luard, 1982) and plants (Li and Delrot, 1987). Interestingly, hypoosmotic-induced H^+ flux kinetics was significantly affected by cells pre-treatment in 200 μM La^{3+}, a known Ca^{2+} channel blocker. This might suggest that effect of changing osmolality on H^+ flux may be not direct but mediated by changes in cytosolic-free Ca^{2+}. It have been previously reported that hypotonic treatment has caused a significant elevation in cytosolic-free Ca^{2+} in a brackish water charophyte *Lamprothamnium succinctum* (Okazaki *et al*., 2002). At the same time, inhibitory phosphorylation of H^+-ATPase is known to be Ca^{2+}-dependent (Palmgren, 2001). Also, hypoosmotic-induced Ca^{2+} uptake (Fig. 3) is expected to cause significant membrane depolarization (similar to one reported by Okazaki *et al*., 2002 for *Lamprothamnium*), and H^+-ATPase pumps in both higher plants and algae are strongly voltage-dependent (Beilby and Walker, 1996; Buschmann and Gradmann, 1997; Gradmann, 2001; Shabala *et al*., 2006b). Further experiments using H^+-ATPase inhibitors are needed to fully address this issue.

At 200 mM NaCl in the medium thraustochytrid cells had net Ca^{2+} flux close to zero. This is a common feature for all living organisms (Norris *et al*., 1996; Sanders and Bethke, 2000). An increase in cytosolic-free calcium ion concentration in response to hypoosmotic stress similar to one observed in the present study was reported for a number of cells of different origin: in plant cells (Takahashi *et al*., 1997a,b; Cessna and Low, 2001), marine organisms (nematocytes and algae respectively) (La Spada *et al*., 1999; Stento *et al*., 2000), yeast (Lichko *et al*., 1980; Eilam, 1982) and mammalian cells (Mongin *et al*., 1997; Lang *et al*., 1998). In certain cell types, extracellular Ca^{2+} was shown to be essential for cell volume regulation (Wong *et al*., 1995; Cessna and Low, 2001). The nature of the observed elevation in cytosolic-free Ca^{2+} is unclear, and both Ca^{2+} influx from the extracellular medium and Ca^{2+} release from internal organelles were suggested (Shepherd and Beilby, 1999; Cessna and Low, 2001). In this study, significant net Ca^{2+} influx (Fig. 3) supports the idea of Ca^{2+} entry into the hypoosmotically stressed cell from the external solution.

Due to Donnan exchange in the cell wall (Arif *et al*., 1995), the observed Ca^{2+} uptake might be a result of increased H^+ uptake in response to hypoosmotic stress (Fig. 2A). However, $LaCl_3$, a known blocker of PM Ca^{2+} channels, completely inhibited stress-induced Ca^{2+} influx into thraustochytrid cells, indicating that the observed Ca^{2+} influx occurred via Ca^{2+} channels (Fig. 3, closed symbols). This is consistent with literature reports, when other Ca^{2+} channel blockers were used (Stento *et al*., 2000).

The physiological role of the observed hypoosmotically induced Ca^{2+} uptake remains to be elucidated. Most likely, Ca^{2+} plays a regulatory role for other channels as was reported for a number of organisms (Ikehara *et al*., 1992; Lang *et al*., 1998; Shepherd and Beilby, 1999). It was reported that influx of Ca^{2+} is essential for activation of the Ca^{2+}-dependent Cl^- channels in turgor-regulating charophytes (Beilby and Shepherd, 2006). It might be interesting to investigate in the future whether hypotonic-induced Cl^- flux kinetics will be altered (prevented) in the presence of Ca^{2+} channel blockers. Interestingly, however, no effect of La^{3+} was found on kinetics of K^+ flux upon the shift in external osmolality from 200 to 1 mM NaCl (data not shown). This may suggest that voltage-gated K^+ channels do not contribute to observed K^+ efflux, pointing out to the possible involvement of stretch-activated channels. This issue has also to be studied in more details in the future.

The observed Ca^{2+} and H^+ influx in thraustochytrid cells might also provide the charge balance. Compensatory ion fluxes are necessary for repolarization of the PM after depolarization due to hypoosmotic stress similar to ones reported by other authors (Hunter and Segel, 1973; Slayman, 1977; Eilam and Chernichovsky, 1987).

According to the literature, sodium has always been considered as an essential nutrient for the growth of thraustochytrid cells. Various studies have shown that thraustochytrids can grow in salinities ranging from 10% to 100% seawater, but cannot grow at zero salinity (Goldstein, 1963; Bahnweg, 1979; Garrill *et al*., 1992). In our experiments, both cell growth rate and yield were severely affected by decrease in NaCl concentration in growth solution (Fig. 4A). Only 4% of the total cell numbers were yielded in sodium-free growth solution (Fig. 4B). It was shown that the requirement for Na^+ can be met only partly by potassium (Jennings, 1983; Garrill *et al*., 1992). Based on this information, it has been suggested that Na^+ plays a crucial role in normal thraustochytrid cell growth and development. However, it was unclear if sodium is directly involved in cell metabolism. Here we demonstrate that sodium appears to be not an essential nutrient in a thraustochytrid studied. In isotonic mannitol solutions, both cell growth rate and yield were unaffected in a wide range of sodium concentrations, ranging from 200 to 0 mM. This observation indicates that decreased yield of thraustochytrid cells at low NaCl concentration (Fig. 4) was due to low osmolality of the growth medium rather than to lack of NaCl *per se*. Therefore, it is likely that thraustochytrid requirements for sodium is due to the role of this ion in cell osmotic adjustment, rather than because of its direct involvement in cell metabolism. One can expect that, under natural conditions, the osmotic role of sodium could be easily assumed by potassium, or by some other means (such as the use of compatible solutes). On a practical note, the finding that cell growth characteristics appear to be unaffected when solution osmotic potential is maintained at the optimal level, similar to those for the seawater (Fig. 5), may be of significant importance for biotechnology industry, where thraustochytrids are grown on a large scale. The use of high concentration of NaCl in the growth medium causes rapid corrosion of fermentation tanks, resulting in significant economic penalties. The use of mannitol (or other compatible solutes) to adjust the medium osmolality may be a possible alternative to existing practices. Findings presented here may lead to large savings for companies involved in growth of thraustochytrids and possibly other organisms of marine origin.

Experimental procedures

Culture preparation and experimental conditions

A culture of thraustochytrid ACEM C (kindly provided by Dr T. Lewis, University of Tasmania) was grown for 4 days essentially as described by Shabala and colleagues (2001a). For MIFE experiments, cells were harvested by centrifugation of 1 ml of culture at 3000 *g* for 10 min; the pellet was washed twice with 2 ml of measuring solution (0.1 mM $CaCl_2$, 0.2 mM KCl, 5% glucose, and 200 mM NaCl if not stated otherwise) and re-suspended in 1 ml of the same solution. Thraustochytrid cells were immobilized on a glass slip inserted into a measuring chamber of 5 ml volume. Poly-L-lysine was used to attach cells to the glass surface essentially as described by Shabala and colleagues (2001a,b; 2006a) followed by cell adaptation to the experimental solution for at least 1 h.

For growth experiments, 4-day-old culture was washed in a modified sodium-free medium (25 mM $MgCl_2$; 10 mM $MgSO_4$; 35 mM $CaCl_2$; 30 mM glutamic acid; 15 mM KCl; 0.1 mM KH_2PO_4; 5 g l^{-1} bacteriological peptone; 5 g l^{-1} glucose; 3 g l^{-1} yeast extract; vitamins and trace metals were provided in amounts stated by Shabala *et al*., 2001a). The culture was used as an inoculum. Growth studies were performed in L-shaped tubes containing 10 ml of above growth medium plus various concentrations of NaCl (0, 1, 10, 50, 100, 200, 500 mM) and mannitol. Half of the cells grew at different levels of NaCl in the absence of mannitol (and therefore, in solutions with different osmolality), while the other half had been grown in isotonic solutions of ~0.98 Os kg^{-1} (equivalent of the seawater, e.g. 500 mM NaCl). The osmolality of growth medium for each treatment was measured and adjusted using 5520 Vapro Vapor Pressure osmometer (Wescor, USA) by adding an appropriate amount of mannitol.

Tubes were inoculated with prepared thraustochytrid culture to give starting $OD_{650} = 0.063$. Culture was grown in thermostatic conditions at 20°C with shaking by the oscillatory motion of the incubator through an arc of 60° at a rate of 60 oscillations min^{-1} (Toyo Kagaku Sangyo, Tokyo, Japan). Kinetics of the growth was monitored spectophotometrically at 650 nm (Spectronic 20+, Milton Roy, USA). The sample size was 4–6 for each treatment, and experiment was repeated twice. Cell number in 1 ml of the culture was counted using the Neubauer Haemacytometer.

Microelectrode ion flux measuring technique (MIFE)

The theory of non-invasive ion flux measurements and detailed description of the MIFE system used in these experiments were given previously (Newman, 2001; Shabala *et al*., 2001a,b; 2006a). Briefly, oven-dried and silanized microelectrodes with external tip diameter of 3–5 μm were first back filled with the appropriate solution (500 mM $CaCl_2$ for calcium; 15 mM NaCl + 40 mM KH_2PO_4 adjusted to pH 6 using NaOH for hydrogen; 200 mM KCl for potassium and chloride; 500 mM NaCl for sodium microelectrodes). Immediately after back filling, the electrodes were front filled with commercially available ionophore cocktails (Fluke catalogue No. 95297 for H^+ 21048 for Ca^{2+} 60031 for K^+ 24902 for Cl^-; 71178 for Na^+). The electrodes were calibrated in a known set of standards before and after use. Electrodes with a response of less than 50 mV $pIon^{-1}$ for monovalent ions or 25 mV pCa^{-1} were discarded. The reference electrode was a flattened glass microelectrode with tip diameter of about 50 μm containing 1 M KCl in 2% agar.

Ion selective electrodes were mounted on a manipulator providing three-dimensional positioning. The experimental chamber containing thraustochytrid cells was placed on a three-way hydraulic micromanipulator, and electrodes were positioned in a line parallel to the chosen cell 10 μm above the surface. In most experiments, three different ions were measured simultaneously from each cell at each time. The chamber was slowly moved up and down by a computer-controlled stepper motor providing electrode movement between 10 and 60 μm from the cell surface at a frequency of 0.1 Hz. The recorded voltages were converted into concentration differences using the calibrated Nernst slopes of the electrodes. Ion fluxes were then calculated using the MIFE software assuming spherical diffusion geometry (Newman, 2001).

Experimental protocol

Two types of MIFE experiments, namely kinetic and steady-state experiments, were performed. In kinetic experiments, a 200 mM NaCl experimental solution in a measuring chamber was replaced with one containing 1 mM NaCl. A peristaltic pump (rate of solution flow up to 6 ml min^{-1}) was used. At least six chamber volumes were pumped through the measuring chamber. Net ion fluxes were measured for 10 min prior to solution change (at high osmolality) followed by 60 min of measurements after the solution was replaced.

In steady-state experiments fluxes were measured from individual cells adapted to new conditions (0, 1, 10, 50 and 100 mM NaCl) for 60 min Fifteen to 30 cells were measured in each treatment.

Lanthanum chloride (a Ca^{2+} channel blocker) was used at 200 μM concentration. The kinetics of Ca^{2+} flux were measured from the surface of thraustochytrid cells pre-treated in an experimental solution containing 200 μM $LaCl_3$ at two levels of NaCl in the solution: at 200 mM NaCl (for 10–15 min) and after a shift to 1 mM NaCl concentration (for 40–50 min). All the measurements were conducted at room temperature (20 ± 2°C).

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References

Arif, I., Newman, I.A., and Keenlyside, N. (1995) Proton flux measurements from tissues in buffered solution. *Plant Cell Environ* **18:** 1319–1324.

Bahnweg, G. (1979) Studies on the physiology of Thraustochytriales. I. Growth requirements and nitrogen nutrition of *Thraustochytrium* spp., *Schizochytrium* sp., *Japonochytrium* sp., *Ulkenia* spp., and *Labyrinthuloides* spp. *Veroff Inst Meeresforsch Bremerhaven* **17:** 245–268.

Beilby, M.J., and Shepherd, V.A. (1996) Turgor regulation in *Lamprothamnium papulosum*. 1. I/V analysis and pharmacological dissection of the hypotonic effect. *Plant Cell Environ* **19:** 837–847.

Beilby, M.J., and Shepherd, V.A. (2006) The characteristics of Ca^{2+}-activated Cl^- channels of the salt-tolerant charophyte *Lamprothamnium*. *Plant Cell Environ* **29:** 764–777.

Beilby, M.J., and Walker, N.A. (1996) Modeling the current-voltage characteristics of *Chara* membranes. 1. The effect of ATP removal and zero turgor. *J Membr Biol* **149:** 89–101.

Beilby, M.J., Cherry, C.A., and Shepherd, V.A. (1999) Dual turgor regulation response to hypotonic stress in *Lamprothamnium papulosum*. *Plant Cell Environ* **22:** 347–359.

Berrier, C., Coulombe, A., Szabo, I., Zoratti, M., and Ghazi, A. (1992) Gadolinium ion inhibits loss of metabolites induced by osmotic shock and large stretch-activated channels in bacteria. *Eur J Biochem* **206:** 559–565.

Bisson, M.A., and Gutknecht, J. (1975) Osmotic regulation in the marine alga, *Codium decorticatum*. I. Regulation of turgor pressure by control of ionic composition. *J Membr Biol* **24:** 183–200.

Bisson, M.A., and Krist, G.O. (1995) Osmotic acclimation and turgor pressure regulation in algae. *Naturwissenschaften* **82:** 461–471.

Bisson, M.A., Kiegle, E., Black, D., Kiyosawa, K., and Gerber, N. (1995) The role of calcium in turgor regulation in *Chara longifolia*. *Plant Cell Environ* **18:** 129–137.

Blount, P., and Moe, P.C. (1999) Bacterial mechanosensitive channels: integrating physiology, structure and function. *Trends Microbiol* **7:** 420–424.

Booth, T. (1971) Distribution of certain soil inhabiting chytrid and chytridiaceous species related to some physical and chemical factors. *Can J Bot* **49:** 1743–1755.

Burgstaller, W. (1997) Transport of small ions and molecules through the plasma membrane of filamentous fungi. *Crit Rev Microbiol* **23:** 1–46.

Buschmann, P., and Gradmann, D. (1997) Minimal model for oscillations of membrane voltage in plant cells. *J Theor Biol* **188:** 323–332.

Cessna, S.G., and Low, P.S. (2001) An apoplastic Ca^{2+} sensor regulates internal Ca^{2+} release in aequorin-transformed tobacco cells. *J Biol Chem* **276:** 10655–10662.

Cui, C., and Adler, J. (1996) Effect of mutation of potassium-efflux system, KefA, on mechanosensitive channels in the

cytoplasmic membrane of *Escherichia coli*. *J Membr Biol* **150:** 143–152.

Damare, V., and Raghukumar, S. (2008) Abundance of thraustochytrids and bacteria in the equatorial Indian Ocean, in relation to transparent exopolymeric particles (TEPs). *FEMS Microbiol Ecol* **65:** 40–49.

Eilam, Y. (1982) The effect of monovalent cations on calcium efflux in yeasts. *Biochim Biophys Acta* **687:** 8–16.

Eilam, Y., and Chernichovsky, D. (1987) Uptake of Ca^{2+} driven by membrane potential in energy-depleated yeast cells. *J Gen Microbiol* **133:** 1641–1649.

Garrill, A., Clipson, N.J.W., and Jennings, D.H. (1992) Preliminary observations on the monovalent cation relations of *Thraustochytrium aureum*, a fungus requiring sodium for growth. *Mycol Res* **96:** 295–304.

Goldstein, S. (1963) Development and nutrition of new species of *Thraustochytrium*. *Am J Bot* **50:** 271–279.

Gradmann, D. (2001) Models for oscillations in plants. *Aust J Plant Physiol* **28:** 577–590.

Hoffmann, E.K., and Dunham, P.B. (1995) Membrane mechanisms and intracellular signalling in cell volume regulation. *Int Rev Cytol* **161:** 173–262.

Hunter, D.R., and Segel, I.H. (1973) Effect of weak acids on amino acid transport by *Penicillium chrysogenum*: evidence for a proton or charge gradient as the driving force. *J Bacteriol* **113:** 1184–1192.

Ikehara, T., Yamaguchi, H., Hosokawa, K., Takahashi, A., Masuya, T., and Miyamoto, H. (1992) Different patterns of cell volume regulation in hypoosmotic media between attached and suspended HeLa cells. *Biochim Biophys Acta* **1111:** 151–158.

Jennings, D.H. (1983) Some aspects of the physiology and biochemistry of marine fungi. *Biol Rev* **58:** 423–459.

Jennings, D.H. (1995) Water relations and salinity. In *The Physiology of Fungal Nutrition.* Jennings, D.H. (ed.). Cambridge, UK: University Press, pp. 398–443.

Jones, R.G.W., and Pritchard, J. (1989) Stresses, membranes and cell walls. In *Plants under Stress: Biochemistry, Physiology, and Ecology and their Application to Plant Improvement*. Jones, H.G., Flowers, T.J., and Jones, M.B. (eds). Cambridge, UK: University Press, pp. 95–114.

Jones, S.E., Naik, R.R., and Stone, M.O. (2000) Use of small fluorescent molecules to monitor channel activity. *Biochem Biophys Res Commun* **279:** 208–212.

La Spada, G., Biundo, T., Nardella, R., and Meli, S. (1999) Regulatory Volume decrease in nematocytes isolated from acontia of *Aiptasia diaphana*. *Cell Mol Biol* **45:** 249–258.

Lang, F., Busch, G.L., Ritter, M., Volkl, H., Waldegger, S., Gulbins, E., and Haussinger, D. (1998) Functional significance of cell volume regulatory mechanisms. *Physiol Rev* **78:** 247–306.

Lew, R.R., Levina, N.N., Shabala, L., Anderca, M.I., and Shabala, S. (2006) Role of a mitogen-activated protein kinase cascade in ion flux-mediated turgor regulation in fungi. *Eukaryotic Cell* **5:** 480–487.

Li, Z.-S., and Delrot, S. (1987) Osmotic dependence of the transmembrane potential difference of broadbean mesocarp cells. *Plant Physiol* **84:** 895–899.

Lichko, L.P., Okorokov, L.A., and Kulaev, I.S. (1980) Role of vacuolar ion pool in *Saccharomyces carlsbergensis*: potassium efflux from vacuoles is coupled with manganese or magnesium influx. *J Bacteriol* **144:** 666–671.

Luard, E.J. (1982) Growth and accumulation of solutes by *Phytophthora cinnamoni* and other fungi in response to changes in external osmotic potential. *J Gen Microbiol* **128:** 2583–2590.

Mongin, A.A., Aksentsev, S.L., Orlov, S.N., and Konev, S.V. (1997) Hypoosmotic shock activates Ca^{2+} channels in isolated nerve terminals. *Neurochem Int* **31:** 835–843.

Newman, I.A. (2001) Ion transport in roots: measurement of fluxes using ion-selective microelectrodes to characterize transporter function. *Plant Cell Environ* **24:** 1–14.

Norris, V., Grant, S., Freestone, P., Canvin, J., Sheikh, F.N., Toth, I., *et al.* (1996) Calcium signalling in bacteria. *J Bacteriol* **178:** 3677–3682.

Okazaki, Y., and Tazawa, M. (1990) Calcium ion and turgor regulation in plant cells. *J Membr Biol* **114:** 189–194.

Okazaki, Y., Ishigami, M., and Iwasaki, N. (2002) Temporal relationship between cytosolic free Ca^{2+} and membrane potential during hypotonic turgor regulation in a brackish water charophyte *Lamprothamnium succinctum*. *Plant Cell Physiol* **43:** 1027–1035.

Orlov, S.N., Kolosova, I.A., Cragoe, E.J., Gurlo, T.G., Mongin, A.A., Aksentsev, S.L., and Konev, S.V. (1993) Kinetics and peculiarities of thermal inactivation of volume-induced Na^+/H^+ exchange, $Na^+,K^+,2Cl^-$ cotransport and K^+,Cl^- cotransport in rat erythrocytes. *Biochim Biophys Acta* **1151:** 186–192.

Palmgren, M.G. (2001) Plant plasma membrane H^+-ATPases: powerhouses for nutrient uptake. *Ann Rev Plant Physiol Plant Mol Biol* **52:** 817–845.

Rauferova, L., Metlicka, R., Benes, I., Kotyk, A., and Janacek, K. (1997) Cell volume regulation in *Claviceps fusiformis*. An animal-type Na, K-ATPase operating in a fungus? *Biochem Mol Biol Int* **41:** 153–160.

Sanders, D., and Bethke, P. (2000) Membrane transport. In *Biochemistry and Molecular Biology of Plants.* Buchanan, B.B., Gruissem, W., and Jones, R.L. (eds). Rockville, MD, USA: American Society of Plant Physiologists, pp. 110–158.

Schleyer, M., Schmid, R., and Bakker, E.P. (1993) Transient, specific and extremely rapid release of osmolytes from growing cells of *Escherichia coli* K-12 exposed to hypoosmotic shock. *Arch Microbiol* **160:** 424–431.

Shabala, L., Shabala, S., Ross, T., and McMeekin, T. (2001a) Membrane transport activity and ultradian ion flux oscillations associated with cell cycle of *Thraustochytrium* sp. *Aust J Plant Physiol* **28:** 87–99.

Shabala, L., Ross, T., Newman, I., McMeekin, T., and Shabala, S. (2001b) Measurements of net fluxes and extracellular changes of H^+, Ca^{2+}, K^+, and NH_4^+ in *Escherichia coli* using ion-selective microelectrodes. *J Microbiol Methods* **46:** 119–129.

Shabala, L., Ross, T., McMeekin, T., and Shabala, S. (2006a) Non-invasive microelectrode ion flux measurements to study adaptive responses of microorganisms to the environment. *FEMS Microbiol Rev* **30:** 472–486.

Shabala, L., Bowman, J., Brown, J., Ross, T., McMeekin, T., and Shabala, S. (2009) Ion transport and osmotic adjustment in *Escherichia coli* in response to ionic and non-ionic osmotica. *Environ Microbiol* **11:** 137–148.

Shabala, S., Shabala, L., Gradmann, D., Chen, Z.H., Newman, I., and Mancuso, S. (2006b) Oscillations in plant membrane transport: model predictions, experimental validation, and physiological implications. *J Exp Bot* **57:** 171–184.

Shabala, S.N. (2000) Ionic and osmotic components of salt stress specifically modulate net ion fluxes from bean leaf mesophyll. *Plant Cell Environ* **23:** 825–837.

Shabala, S.N., and Lew, R.R. (2002) Turgor regulation in osmotically stressed *Arabidopsis* epidermal root cells. Direct support for the role of inorganic ion uptake as revealed by concurrent flux and cell turgor measurements. *Plant Physiol* **129:** 290–299.

Shepherd, V.A., and Beilby, M.J. (1999) The effect of an extracellular mucilage on the response to osmotic shock in the charophyte alga *Lamprothamnium papulosum*. *J Membr Biol* **170:** 229–242.

Shepherd, V.A., Beilby, M.J., and Heslop, D.J. (1999) Ecophysiology of the hypotonic response in the salt-tolerant charophyte alga *Lamprothamnium papulosum*. *Plant Cell Environ* **22:** 333–346.

Slayman, C.L. (1977) Energetics and control of transport in *Neurospora*. In *Water Relations in Membrane Transport in Plants and Animals.* Jungreis, A.M., Hodges, T.K., Kleinzeller, A., and Schultz, S.G. (eds). New York, USA: Academic Press, p. 69.

Stento, N.A., Ryba, N.G., Kiegle, E.A., and Bisson, M.A. (2000) Turgor regulation in the salt-tolerant alga *Chara logifolia*. *Plant Cell Environ* **23:** 629–637.

Sukharev, S., Martinac, B., Arshavsky, V.Y., and Kung, C. (1993) Two types of mechanosensitive channels in the *Escherichia coli* cell envelope: solubilization and functional reconstitution. *Biophys J* **65:** 177–183.

Takahashi, K., Isobe, M., and Muto, S. (1997a) An increase in cytosolic calcium ion concentration precedes hypoosmotic shock-induced activation of protein kinases in tobacco suspention culture cells. *FEBS Let* **401:** 202–206.

Takahashi, K., Isobe, M., Knight, M.R., Trewavas, A.J., and Muto, S. (1997b) Hypoosmotic shock induces increases in cytosolic Ca^{2+} in tobacco suspention-culture cells. *Plant Physiol* **113:** 587–594.

Thome-Ortiz, P.E., Pena, A., and Ramirez, J. (1998) Monovalent cation fluxes and physiological changes of *Debaryomyces hansenii* grown at high concentrations of KCl and NaCl. *Yeast* **14:** 1355–1371.

West, R.C. (ed.) (1974) *CRC Handbook of Chemistry and Physics.* Cleveland, OH, USA: CRC Press.

Wethered, J.M., and Jennings, D.H. (1985) Major solutes contributing to solute potential of *Thraustochytrium aureum* and *T. roseum* after growth in media of different salinities. *Trans Br Mycol Soc* **85:** 439–446.

Wong, P.C., Hau, K.P., and Fong, W.F. (1995) Membrane Ca^{2+} fluxes in rat hepatoma cells exposed to a supraphysiological concentration of asparagines. *Biochem Mol Biol Int* **35:** 1161–1167.