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Characterization of Branchial Na,K-ATPase from Three Freshwater Fish Species (*Oreochromis niloticus, Cyprinus carpio*, and *Oncorhynchus mykiss*)

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Abstract: Branchial Na,K-ATPase activity was characterized in 3 freshwater fish species (*Oncorhynchus mykiss, Oreochromis niloticus,* and *Cyprinus carpio*) with different ecological needs. Na^+, K^+, and Cl^- concentrations in the gills were also measured. The maximal Na,K-ATPase activity was observed at 100 mM Na^+, 20-40 mM K^+, 3-4 mM Mg^{2+}, and 1 mM ouabain. The maximal velocity (Vmax) of Na,K-ATPase isolated from *O. mykiss* (1.07 µmol Pi/mg prot/h) was lower than that isolated from *O. niloticus* (7.25 µmol Pi/mg prot/h) and *C. carpio* (7.44 µmol Pi/mg prot/h). Nevertheless, Na^+, K^+, and Cl^- levels in *O. mykiss* were higher than the others. The lowest Vmax value can be attributed to the highest ion concentrations in the gills of O. mykiss. However, substrate concentrations, which give half of Vmax (Km) of Na,K-ATPase in the gills of *O. niloticus* and *C. carpio*, were different although they exhibited similar Vmax values. The low Km value in the gills of *O. niloticus* compared to *C. carpio* may be related to high resistance to changing environmental factors.

Key Words: Fish, gill, ion, Na,K-ATPase

Tatlı Su Balıklarındaki (*Oreochromis niloticus, Cyprinus carpio, Oncorhynchus mykiss*) Solungaç Na,K-ATPaz Enzimlerinin Karakterizasyonu

Özet: Bu çalışmada farklı ekolojik gereksinimleri olan üç farklı tatlı su balığında (*O. niloticus*, *C. carpio*, *O. mykiss*) solungaç Na,K-ATPaz enzimlerinin karakterizasyonu yapılmıştır. Aynı zamanda solungaçlarda Na^+, K^+ ve Cl^- düzeyleri de belirlenmiştir. En yüksek Na,K-ATPaz aktivitesi 100 mM Na^+, 20 mM K^+, 3-4 mM Mg^{+2}, 1 mM Ouabain derişimlerinde gözlenmiştir. *O. mykiss*'da (1,07 µmol Pi/mg prot/h), *O. niloticus* (7,25 µmol Pi/mg prot/h) ve *C. carpio* (7,44 µmol Pi/mg prot/h) ile karşılaştırıldığında gözlenen en düşük maximal hız (Vmax) değeri, solungaçlardaki yüksek iyon düzeyleri ile ilişkilendirilebilir. Buna karşılık, *O. niloticus* ve *C. carpio*'da yarı Vmax değerine sahip substrat derişimleri (Km) ise, benzer Vmax değerlerine karşılık farklılık göstermektedir. *O. niloticus*'da *C. carpio*'ya oranla gözlenen düşük Km değerinin, değişen ortam koşullarında *O. niloticus*'un daha dirençli olmasından kaynaklanabileceği bildirilmiştir.

Anahtar Sözcükler: Balık, solungaç, iyon, Na,K-ATPase

Introduction

The fish gill is a key tissue constituting large surfaces to facilitate the gas exchange, acid-base regulation, ionic transport, and excretion of nitrogenous wastes.

Na,K-ATPase plays a pivotal role in the gills of both marine and freshwater teleosts (Heath, 1987; Evans et al., 2005). This enzyme uses the chemical energy from the hydrolysis of ATP for transferring 3 Na^+ ions out of the cell and 2 K^+ ions into the cell to maintain the ionic balance. The regulation of Na^+ and K^+ ion gradients in membranes by Na,K-ATPase is very important for physiological activities including several cellular functions like osmotic balance, Ca^{+2} concentration, membrane potential, cytoplasmic enzyme activity, and muscle contraction (Larsson et al., 1985; Diaz et al., 1998).

Na,K-ATPase is known to be affected by several factors such as salinity and temperature and plays a crucial role in the adaption to changing salinity and

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thermal conditions in different fish species (Inman and Lockwood, 1977; Jagoe et al., 1996). Nile tilapia (*Oreochromis niloticus*) is regarded as one of the most saline tolerant teleosts and the optimum water temperature range is approximately 25-31 °C. Their feeding behavior is based mostly on aquatic invertebrates, detritus, and plankton (Popma and Masser, 1999; Kamal and Mair, 2005). The common carp (*Cyprinus carpio*) is a stenohaline freshwater fish for which the optimal temperature for growth is around 25 °C and it mainly feeds on detritus, plants, and benthic organisms (Maitland and Campbell, 1992; Metz at el., 2003). On the other hand, the natural habitat water temperature for rainbow trout, *Oncorhynchus mykiss,* is around 12 °C, which is relatively low when compared to other species, and it generally feeds on aquatic mollusks, crustaceans, and other small fishes (Gall and Crandell, 1992). It was emphasized that these species, which are mostly cultured freshwater fish, can be also used as important biomarkers in aquatic environments (Sunny and Oommen, 2001; Metz et al., 2003; Veillette and Young, 2004).

Our aim was to characterize the optimum working conditions and performance of the branchial Na,K-ATPase activity from the gills of 3 freshwater fish species that have different characteristics and ecological needs. We also examined the relationship between the Na,K-ATPase activity and Na^+, K^+, and Cl^- concentrations in the gills. Characterization of branchial Na,K-ATPases is a very important aspect of the environmental biology of fishes, especially from the ecotoxicological point of view (Canli and Stagg, 1996).

Materials and Methods

Freshwater fish (*O. mykiss, O. niloticus*, and *C. carpio*) were obtained from fish culturing pools at Çukurova University and Çiftehan, Adana (Turkey). The optimal temperatures of the pools in which the fish were kept were 9 ± 1 °C for *O. mykiss,* and 20 ± 1 °C for *O. niloticus* and *C. carpio*. The fish were killed by a blow to the head and dissected with clean equipment to obtain gill tissues on ice. Filaments of 10 fish were separated from gill arches and pooled for each species.

Gill tissues were homogenized in ice-cold buffer containing 20 mM Tris-HCl, 0.25 M sucrose, and 1 mM EDTA (pH 7.7) with a ratio of 1/10 at 9500 rpm for 2-3 min. Homogenates were centrifuged at 13,000 ×g for 20 min. The supernatants were collected for determination of total protein levels and Na,K-ATPase activity.

The final assay concentrations to measure branchial Na,K-ATPase activity were 40 mM Tris-HCl, 60-140 mM for NaCl, 10-50 mM for KCl, 1-6 mM for $MgCl_2$, 6-9 for pH, and 0.003-3 mM for ouabain. For measuring Na,K-ATPase activity, 50 µl of enzyme suspension (~100 µg protein) was added to 850 µl of incubation media and preincubated for 5 min at 37 °C. The reaction was started by the addition of 100 µl Na_2ATP and incubated for 30 min. The reaction was stopped by adding 500 µl of ice-cold distilled water. Inorganic phosphate was measured as described by Atkinson et al. (1973). Appropriate blanks were included with each assay to correct for non-enzymatic hydrolysis of ATP. A series of KH_2PO_4 (25-250 µM) prepared to use as Pi standard and spectrophotometric analysis was carried out at 390 nm using a Cecil 5000 series spectrometer. Specific Na,K-ATPase activity was calculated from the inorganic phosphate liberated from ATP using the differences between the presence and absence of the ouabain. All assays were carried out in triplicate.

Total protein levels were determined according to Lowry et al. (1951). Specific enzyme activity was expressed as µmol Pi/mg prot/h. Na^+, K^+, and Cl^- levels were also measured in gill homogenates by ion chromatography (Shimadzu LC 10A Liquid Chromatography).

Results

The effects of varying concentrations of Na^+, K^+, Mg^{2+}, ouabain, ATP, and different ranges of pH were tested for characterization of branchial Na,K-ATPase isolated from *O. mykiss, O. niloticus*, and *C. carpio*.

Maximal Na,K-ATPase activity was observed at 100 mM Na^+ concentration in all gill homogenates of the species (Figure 1). However, maximal Na,K-ATPase activity occurred at different K^+ concentrations. Maximal Na,K-ATPase activity was exhibited at 20 mM K^+ in *O. niloticus,* 30 mM K^+ in *C. carpio*, and 40 mM K^+ in *O. mykiss* (Figure 2). Similarly, Mg^{2+} concentrations were also different in different fish species. Maximal Na,K-ATPase activity was measured at 3 mM Mg^{+2} in *C. carpio* and 4 mM Mg^{+2} in *O. niloticus* and *O. mykiss* (Figure 3).

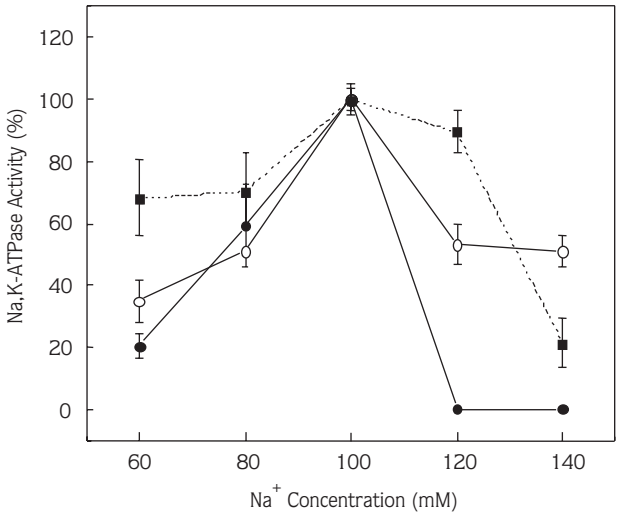

Figure 1. Effect of Na^+ concentration on branchial Na, K-ATPase activity (%) in *O. niloticus*, (—•—), *O. mykiss* (····■····), and *C. carpio* (—o—). Each point corresponds to the average of 3 determinations.

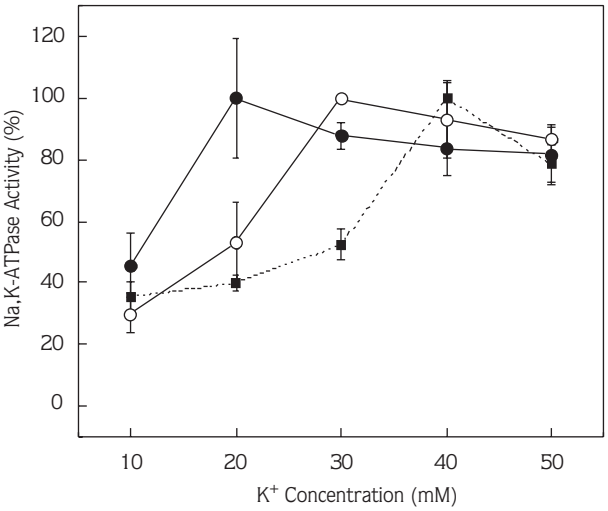

Figure 2. Effect of K^+ concentration on branchial Na,K-ATPase activity (%) in *O. niloticus*,(—•—), *O. mykiss* (····■····), and *C. carpio* (—o—). Each point corresponds to the average of 3 determinations.

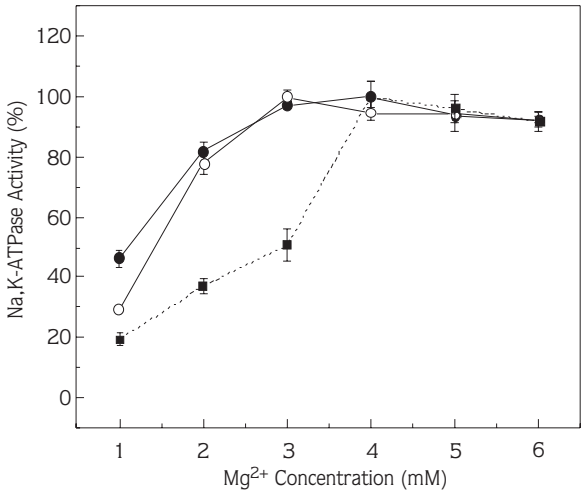

Figure 3. Effect of Mg^{2+} concentration on branchial Na,K-ATPase activity (%) in *O. niloticus*, (—•—), *O. mykiss* (····■····), and *C. carpio* (—o—). Each point corresponds to the average of 3 determinations.

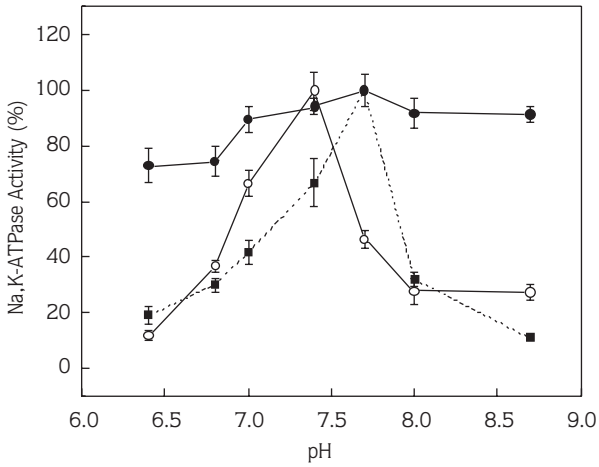

Figure 4. Effect of pH concentration on branchial Na,K-ATPase activity (%) in *O. niloticus*, (—•—), *O. mykiss* (····■····), and *C. carpio* (—o—). Each point corresponds to the average of 3 determinations.

The optimal pH value for Na,K-ATPase was 7.7 in the gills of *O. niloticus* and *O. mykiss*, while it was 7.4 in *C. carpio* (Figure 4).

Inhibition of Na,K-ATPase in different ouabain concentrations is shown in Figure 5. Total (100%) inhibition of Na,K-ATPase occurred in 1 mM ouabain concentrations in all fishes. The effect of ATP on the branchial Na,K-ATPase from *O. niloticus* was assayed using an incubation mixture containing 100 mM Na^+, 20 mM K^+, 4 mM Mg^{+2}, and 1 mM ouabain (pH 7.7). Maximal enzyme activity was observed at 3 mM ATP in *O. niloticus*. In *C. carpio,* the incubation medium contained 100 mM Na^+, 30 mM K^+, 3 mM Mg^{+2}, and 1 mM ouabain, and maximum activity occurred at 4 mM ATP. The incubation medium containing 100 mM Na, 40 mM K, 4 mM Mg, and 1 mM ouabain was assayed for *O. mykiss* and maximum activity was observed at 4 mM ATP (Figure 6).

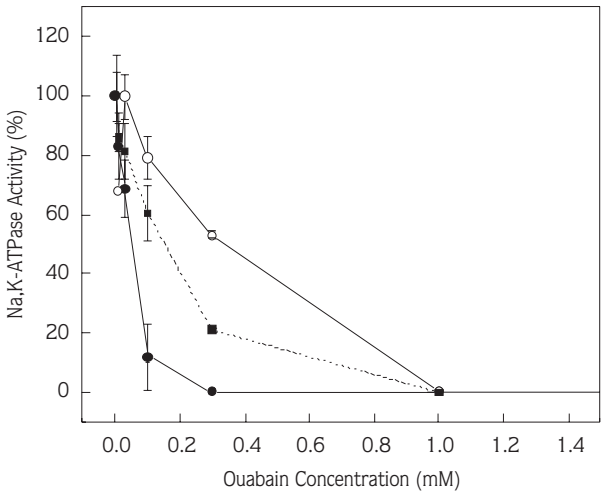

Figure 5. Effect of ouabain on branchial Na,K-ATPase activity (%) in *O. niloticus*, (—●—), *O. mykiss* (····■····), and *C. carpio* (—o—). Each point corresponds to the average of 3 determinations.

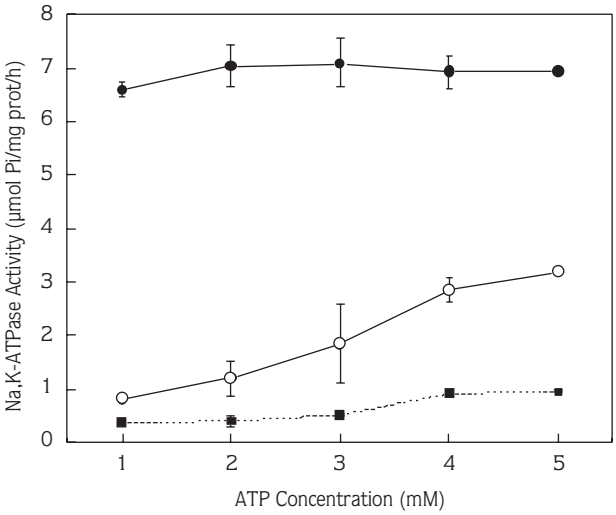

Figure 6. Effect of ATP concentration on branchial Na,K-ATPase activity (%) in *O. niloticus*, (—●—), *O. mykiss* (····■····), and *C. carpio* (—o—)). Each point corresponds to the average of 3 determinations.

According to the kinetic analysis, Vmax and Km in *O. niloticus, C. carpio*, and *O. mykiss* were determined in the following order: 7.25 µmol Pi/mg prot/h – 0.1 mM; 7.44 µmol Pi/mg prot/h – 8.4 mM; 1.07 µmol Pi/mg prot/h – 2.2 mM, respectively (Table).

Na+, Cl^-, and K^+ levels were measured in the gills by ion chromatography (Table). The highest Na^+, Cl^-, and K^+ concentrations were observed in *O. mykiss*. However, in *C. carpio* and *O. niloticus* ion levels were similar but lower than those in *O. mykiss*.

Discussion

The present study shows characteristic behavior of branchial Na,K-ATPase activity from 3 freshwater fish species with different ecological needs. Specific activity of the enzyme and its relationships with Na^+, Cl^-, and K^+ ions in the gill were also correlated. Maximal Na,K-ATPase activity occurred at the same Na^+ concentration (100 mM) in all species. Optimal K^+ concentration for the enzyme was 20 mM in *O. niloticus*, 30 mM in *C. carpio*, and 40 mM in *O. mykiss*. It was shown in sea bass that optimal branchial Na,K-ATPase activity was achieved at 100 mM Na^+ and 20-25 mM K^+ (Diaz et al., 1998). Na^+/K^+ ratios in the literature are usually lower than 6.0 and this is consistent with our findings. On the other hand, pH dependence of Na,K-ATPase activity was maximum at 7.7 in both *O. niloticus* and *O. mykiss*, but at 7.4 in *C. carpio*. These findings are also in agreement with other studies where several species exhibited optimal values ranging from 7.0 to 7.5 (Watson and Beamish, 1981; Webb et al., 2001).

Table. Na^+, K^+, Cl^-, Km, and Vmax values in gill homogenates of *O. niloticus*, *O. mykiss*, and *C. carpio*.

Fish	Na^+	Cl^- (mM)	K^+	Vmax (µmol Pi/mg prot/h)	Km (mM)
O. niloticus	34	45	5	7.25	0.1
C. carpio	29	25	5.7	7.44	8.4
O. mykiss	203	150	6.2	1.07	2.2

In the present study, total inhibition of Na,K-ATPase occurred at 1 mM ouabain in all species. However, it was observed that optimum ouabain concentrations were varied in many studies in the literature. It should be noted that ouabain sensitivity of enzyme can be affected by several factors like temperature, cations, and lipid environment (Diaz et al., 1998). The optimal Mg^{2+} concentration of Na,K-ATPase was 4 mM for *O. mykiss* and *O. niloticus* but 3 mM for *C. carpio*. While maximum enzyme activity was observed at 4 mM ATP in the gills of *O. mykiss* and *C. carpio*, it was 3 mM ATP in *O. niloticus.* The results showed that the Mg^{2+} to ATP ratio is approximately 1.0. This rate has been commonly reported to be optimal in several tissues from poikilothermic animals (Diaz et al., 1998).

The data showed that incubation media for maximal branchial Na,K-ATPase activity were very similar for all fish species. This may indicate general similarities of the enzyme between different fish species despite the differences in their habitat and physiological activities. Nevertheless, kinetic features of Na,K-ATPase varied between *O. mykiss* and other species. Specific activity of Na,K-ATPase of *O. mykiss* was lower than that of *O. niloticus* and *C. carpio*. On the other hand, *O. niloticus* and *C. carpio* showed similar specific activity. Specific activities of these species were also in the range commonly reported for other freshwater teleosts (Diaz et al., 1998). The differences in specific activities may be associated with their osmolalities affected by the temperature of the environment that they inhabit. Although *C. carpio* and *O. niloticus* live in the same thermal conditions, *O. mykiss* lives in much lower temperate waters. It was demonstrated that the decrease in osmolality was accompanied by an increase in Na,K-ATPase activity in osmoregulatory tissues (Guynn et al., 2002). The measurement of Na^+, Cl^-, and K^+ levels showed that the highest levels were observed in the gills of *O. mykiss*. Data from the literature indicated an increase in monovalent ion concentrations due to enhanced serum osmolality to decrease the freezing point of the blood and energy necessary to maintain the ionic gradient and a decrease in Na,K-ATPase activity in osmoregulatory tissues (Guynn et al., 2002). At this point, one may assume that the highest Na^+, Cl^-, and K^+ concentrations in *O. mykiss* can be correlated to the lowest Vmax value of branchial Na,K-ATPase. Sardella et al. (2004) also showed that there was a decrease (86%) in Na,K-ATPase activity in the gills of juvenile Mozambique tilapia hybrids when they were transferred to cold water. They attributed this to a significant increase in plasma osmolality during water change.

It is well known that Na,K-ATPase, which is key in osmoregulatory functions of teleosts, can be affected by different factors such as salinity, temperature, and other factors that affect ion levels. Temperature has a significant effect on the osmoregulatory process by increasing water permeability and drinking rates (Imsland et al., 2003), which might lead to an increase in the excretion of excess ions. It was also demonstrated that a gradual increase in gill Na,K-ATPase was observed at higher temperatures, whereas these effects were absent at lower temperatures (Packer and Garvin, 1998; Handeland et al., 2000).

At the same time, the similarity of the Vmax value of the branchial Na,K-ATPase isolated from *O. niloticus* and *C. carpio* may be attributed to the same thermal conditions in which they live. Nevertheless, the Km value of *O. niloticus* was very low when compared to that of *C. carpio*. This affinity of Na,K-ATPase to its substrate in *O. niloticus* may be related to its high resistance to varying environmental conditions (Ueng et al., 1996). High enzyme activity occurring at the wide pH range in this study is also evidence of its resistance. It was indicated that maximum Na,K-ATPase activity in the gills of both summer and winter acclimatized fish (*Perca flavescens*) and the Km for ATP from winter fish increased as assay temperature increased (Packer and Garvin, 1998). It was indicated that half maximal activity for ATP and specific activity varies not only between species but also between tissues (Diaz et al., 1998). Na,K-ATPase shows different kinetic parameters depending upon the particular physiological function of the tissue within which it is located (Bansal et al., 1985).

In conclusion, although branchial Na,K-ATPase activity from the 3 freshwater species exhibits similarities considering their incubation media, the kinetic features of the enzyme were considerably different among the species, possibly due to differences in physiological functions and environmental conditions. The results of this research may help to predict the sensitivity of branchial Na,K-ATPase to different environmental

conditions. As branchial Na,K-ATPase is a significant osmoregulatory molecule in fish metabolism, it is important to know its natural characteristics, especially for those who want to study the effects of water pollution (Canli and Stagg, 1996).

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