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CHARACTERIZATION OF GILL Na^{+}-K^{+}-ACTIVATED ADENOSINE TRIPHOSPHATASE FROM TILAPIA *OREOCHROMIS MOSSAMBICUS*[1]

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Pung-Pung Hwang, Ching-Mu Sun and Su-Mei Wu (1988) Characterization of Gill Na^{+}-K^{+}-Activated Adenosine Triphosphatase from Tilapia *Oreochromis mossambicus. Bull. Inst. Zool., Academia Sinica* **27**(1): 49-56. The sensitivities of tilapia gill Na^{+}-K^{+}-ATPase to ionic strength, pH, concentrations of substrate and inhibitor, temperature, and salinity were studied. The results were:

1. The optimal temperature appears to be higher than 42°C.
2. The higher enzyme activities occur at 3 to 0.6 of Na/K ratio, however the optimal requirement of Na^{+}/K^{+} ratio for the Na^{+}-K^{+}-ATPase activity is 1.7.
3. The optimal concentrations of ions (Na^{+}, K^{+} and Mg^{+2}) are 125, 75 and 7.5 mM respectively.
4. The optimal pH is 7.5; at lower pH levels, enzyme activity rapidly declines.
5. The optimal requirement of ATP is 5.0 mM.
6. 50% of enzyme activity is inhibited at 6.64×10^{-6} M of ouabain, maximal inhibition occur at 5×10^{-4} M.
7. The gill Na^{+}-K^{+}-ATPase activity from sewater tilapia is 12.85 ± 1.32 μmol Pi/mg protein/hr, greater than that from freshwater tilapia, 7.35 ± 1.12 μmol Pi/mg protein/hr.

Key words: Characterization, Gill N^{+}-K^{+}-ATPase, Tilapia.

Na^{+}-K^{+}-ATPase (sodium potassium activaved adenosine triphosphatase) is ubiquitous in all animal cells, but its activity ranges widely. Highest activities have been noted in excitatory and secretory tissues, such as brain cortex, electric eel electroplax, kidney, salt glands of marine birds, and gills of fishes (Bonting, 1970). Its occurrence in the cell is restricted to the plasma membrane, therefore this enyme has been considered as a marker enzyme for plasma membrane (Schuurmans Stekhoven and Bonting, 1981).

In teleost gills, Na^{+}-K^{+}-ATPase is mainly localized in the tubular system of chloride cells, and has been demonstrated to play a critical role on salt secretion of the cell (Sargent *et al.*, 1980; de Renzis and Bornancin, 1984). The activity of this enzyme is now used to monitor the functional state in chloride cell (de Renzis and Bornancin, 1984). The characterization of gill Na^{+}-K^{+}-ATPase have been reported in *Anguilla japonica* (Kamiya and Utida, 1968; Ho and Chan, 1980), *Fundulus heteroclitus* (Epstein *et al.*,

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1967; Towle *et al.* 1977), *Salmo gairdneri* (Pfeiler and Kirschner, 1972), *Oncorhynchus kisutch* (Giles and Vanstone, 1976), *O. tschawytscha* (Johnoson *et al.*, 1977), and *Carassius auratus* (Busacker and Walter, 1981). However, the characterization of the Na^{+}-K^{+}-ATPase of tilapia *Oreochromis mossambicus* remains unknown despite the extensive use of this species in physiological and environmental research (Assem and Hanke, 1979; Foskett *et al.*, 1981; Hwang, 1987).

In the present paper, we report the optimal requirements and the salinity effects of gill Na^{+}-K^{+}-ATPase activity in tilapia *Oreochromis mossambicus*.

MATERIALS AND METHODS

Tilapia *Oreochromis mossambicus* (Peters) obtained from Tainan Fish Culture Station of Taiwan Fisheries Research Institute were reared for over one month in seawater at 25–30°C. The fish sampled for assay varied from 10 to 18 cm in total length and 23 to 40 g in body weight.

Fish were anesthetized with MS222 and killed immediately. The gills were excised and rinsed in chilled 100 mM imidazole-HCl buffer (pH 7.6). After blotting with filter papers to remove excess solution, gill filaments were immediately removed from gill archs, weighted and suspended in the homogenization solution (100 mM imidazole-HCl buffer, pH 7.6, 5 mM Na_2EDTA, 200 mM sucrose, 0.1% sodium deoxycholate) at the ratio of 50 mg of gill filaments per ml of homogenization solution. Homogenization was performed in a glass Potter-Elvehjem homogenizer with a motorized Teflon pestle at 600 rpm for 20 strokes in ice.

The Na^{+}-K^{+}-ATPase activity was assayed in a reaction mixture containing 0.1 ml filtered homogenate and 5 ml reaction buffer (100 mM imidazole-HCl buffer, pH 7.6, 125 mM NaCl, 75 mM KCl, 7.5 mM $MgCl_2$, 5mM Na_2 ATP). The reaction was run at 37°C for 30 min, and then stopped by addition of 2.0 ml ice cold 30% trichloroacetic acid. The protein was precipitated by centrifugation and the inorganic phosphate concentration in the supernatant was determined by the method of Peterson (1978). Total protein was determined by the method of Lowry *et al.* (1951), using crystalline bovine albumin as stantard. The enzyme activity of Na^{+}-K^{+}-ATPase was defined as the difference between the inorganic phosphate liberated in the presence and the absence of 0.5 mM ouabain in the reaction mixture, and was expressed in μmoles inorganic phosphate released per mg of protein per hour. For comparision, enzymatic activities were converted into relative activities (%) of the highest one in each experiment

The incubation conditions of each experiment, unless described under appropriate legends, were the same as those described perviously. All experiments were run 2–4 times with similar results. Individual points in each figures were means of duplicate samples.

All the chemical used were purchased from Sigma Biochemical Co., St. Louis, Missouri.

RESULTS

Temperature and Time

As shown in Fig. 1, release of phosphate from ATP by Na^{+}-K^{+}-ATPase was linear over a 60 min period at 37°C.

Reaction rates increased slowly as incubation temperature increased up to 15°C. While above 15°C, $Na^{+}K^{+}$-ATPase activity showed evident increase and almost a linear relation to the incubation temperature was observed. Even at 42°C, the highest temperature tested, no apparent enzyme activity was lost after 30 min incubation (Fig. 2). The temperature optima for tilapia gill Na^{+}-K^{+}-ATPase activity appears to be higher than 42°C.

Na^{+}, K^{+} and Mg^{+2}

When concentration ratio of Na^{+} to K^{+}

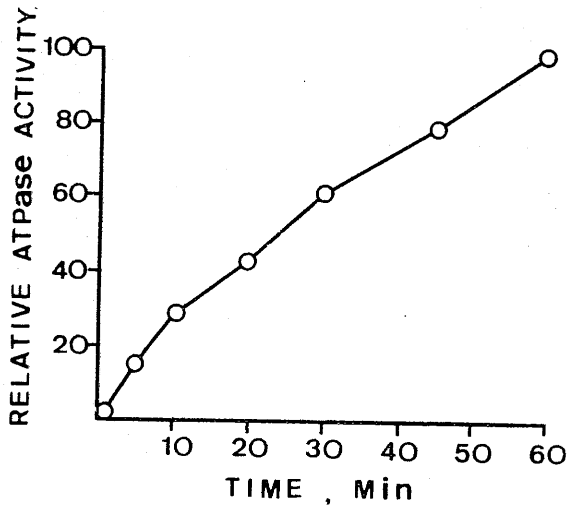

Fig. 1. Tilapia gill Na^+-K^+-ATPase activity as a function of reaction time. 100% activity was 20.35 μmol Pi/mg protein/hr.

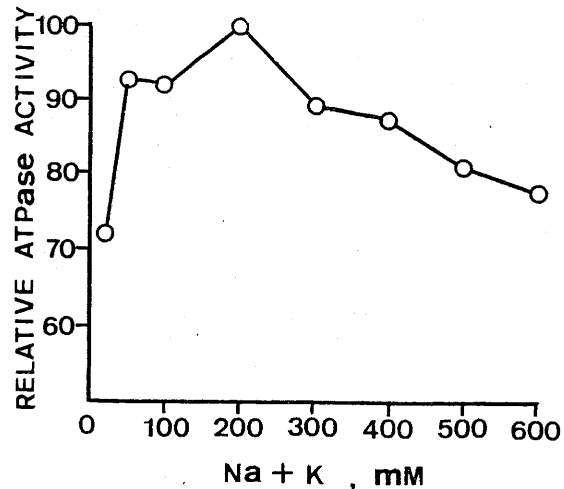

Fig. 3. Tilapia gill Na^+-K^+-ATPase activity as a function of the combined concentrations of Na^+ and K^+. The ratio of Na^+/K^+ was held constant at 1.0. 100% activity was 12.12 μmol Pi/mg protein/hr.

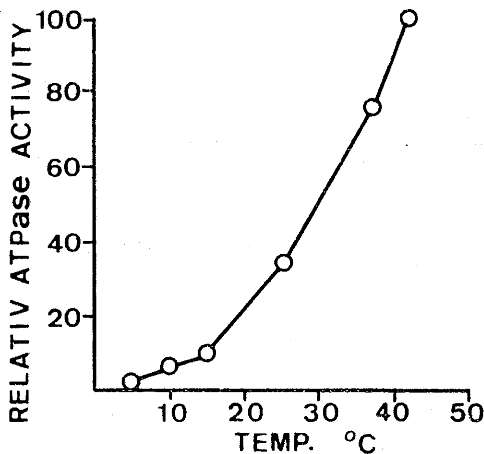

Fig. 2. Tilapia gill Na^+-K^+-ATPase activity as a function of reaction temperature. 100% activity was 12.34 μmol Pi/mg protein/hr.

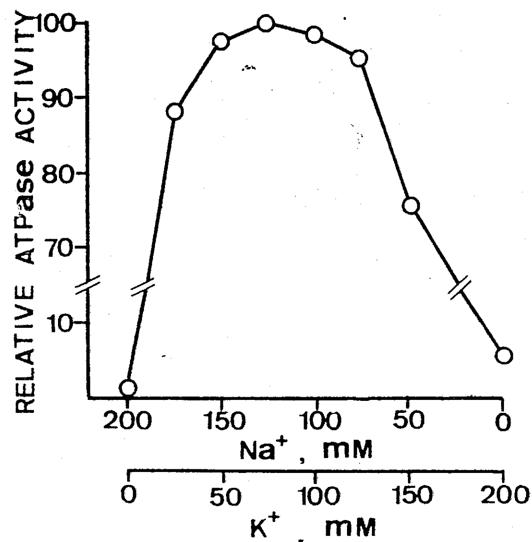

Fig. 4. Tilapia gill Na^+-K^+-ATPase activity as a function of differing Na^+ to K^+ concentrations. The total concentrations of Na^+ and K^+ was constant at 200 mM. 100% activity was 12.68 μmol Pi/mg protein/hr.

was equal, the highest enzyme activity occured at 200 mM of total Na^+ and K^+. However relative enzyme activities were above 85% of the former at 50–300 mM total of Na^+ and K^+ (Fig. 3). As shown in Fig. 4 if total Na^+ and K^+ was 200 mM, relative enzyme activities were similar when the ratio of Na^+/K^+ is ranged from 3 to 0.6. The highest activity was observed at 1.7. Optimal concentration of Na^+ and K^+ for Na^+-K^+-ATPase activity were confirmed in Fig. 5 and 6. It is evident that the highest enzyme activity was obsvered at 125 mM

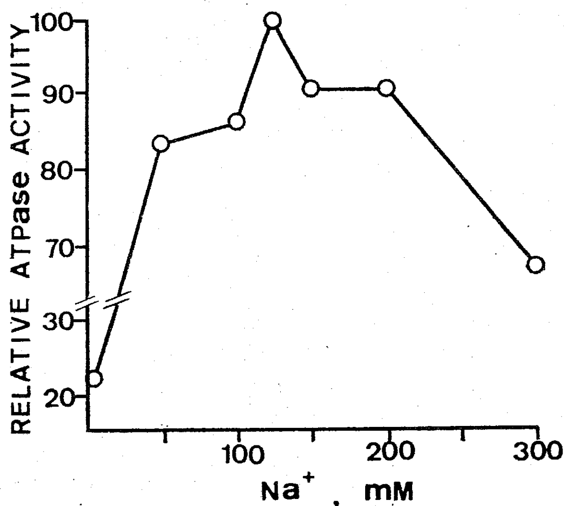

Fig. 5. Tilapial gill Na^+-K^+-ATPase activity as a function of Na^+ concentrations. The K^+ was constant 75 mM. 100% activity was 14.07 μmol Pi/mg protein /hr.

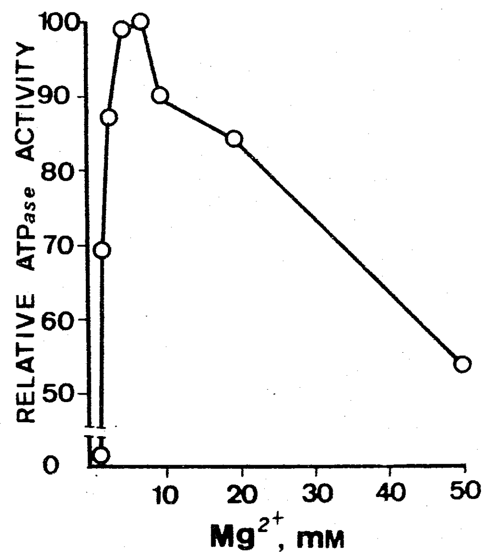

Fig. 7. Tilapia gill Na^+-K^+-ATPase activity as a function of Mg^{+2} concentrations. 100% activity was 11.01 μmol Pi/mg protein/hr.

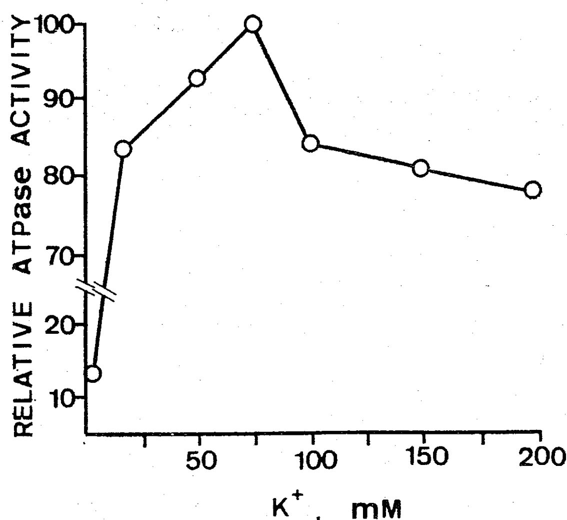

Fig. 6. Tilapia gill Na^+-K^+-ATPase activity as a function of K^+ concentrations. The Na^+ was constant at 125 mM. 100% activity was 13.38 μmol Pi/mg protein/hr.

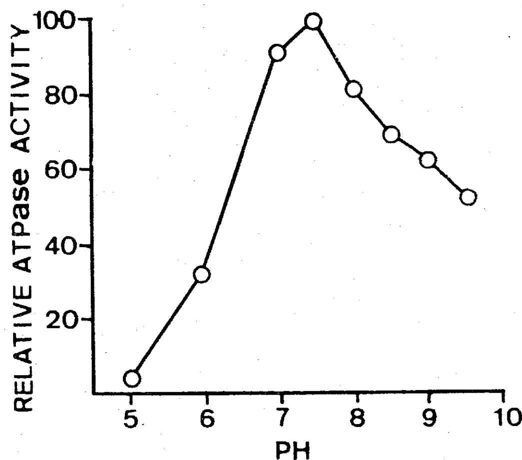

Fig. 8. Tilapia gill Na^+-K^+-ATPase activity as a function of pH. 100% activity was 13.62 μmol Pi/mg protein/hr.

Na^+ when K^+ was kept constant at 75 mM (Fig. 5), and at 75 mM K^+ when Na^+ was kept constant at 125 mM (Fig. 6).

Optimal requirement of Mg^{+2} for the Na^+-K^+-ATPase activity was determined to be 7.5 mM, however the relative enzyme activities between 5 and 7.5 mM of Mg^{+2} showed a difference less than 5% (Fig. 7).

pH

As shown in Fig. 8, it is evident that optimal pH for the Na^+-K^+-ATPase activity was 7.6. This enzyme is more sensitive to acid than to alkaline condition.

Na_2ATP and Ouabain

The maximal reaction rate was found at 5 mM Na_2ATP (Fig. 9). Substrate higher than 5 mM was inhibitory for tilapia gill Na^+-K^+-ATPase activity, and this effect was strengthened by the much higher concentration, 20 to 30 mM, which causes acid condition (pH 6.9 to 6.7). Through Lineweaver-Burk plot, V_{max} and K_m of of the enzyme were 35.71 mol pi/mg protein/hr and 3.39 mM respectively.

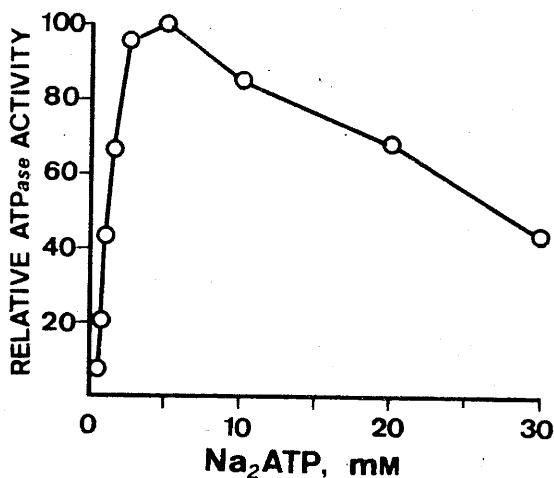

Fig. 9. Tilapia gill Na^+-K^+-ATPase activity as a function of Na_2ATP concentrations. 100% activity was 12.5 μmol Pi/mg protein/hr.

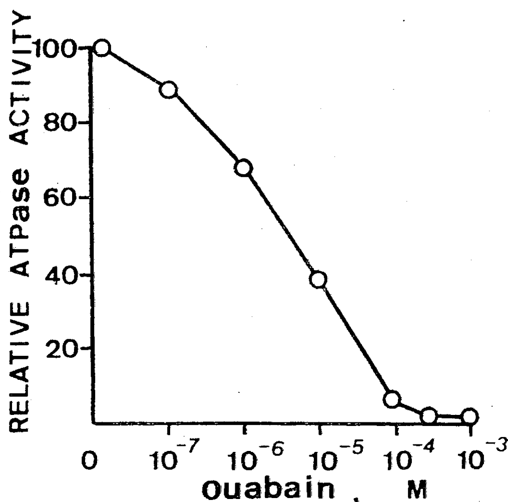

Fig. 10. Tilapia gill Na^+-K^+-ATPase activity as a function of ouabain concentrations. 100% activity was 13.53 μmol Pi/mg protein/hr.

In the primarily experiments, it has been found that the inhibitory effect of ouabain was more effective than the absence of Na^+ and K^+ in reaction medium. As shown in Fig. 10, under the presence of Na^+ and K^+ in reaction medium, 50% of Na^+-K^+-ATPase activity was inhibited at 6.64×10^{-6} M ouabain, and the maximal inhibition occurred at 5×10^{-4} M.

Salinity

Gill Na^+-K^+-ATPase activity from seawater tilapia is 12.85 ± 1.32 μmol Pi/mg protein/hr ($n=12$), much higher than that from freshwater (over one month after transfer from seawater) tilapia, 7.35 ± 1.12 μmol Pi/mg protein/hr ($n=10$).

DISCUSSION

From the present and the previous studies, it is evident that teleostean gill Na^+-K^+-ATPases are sensitive to ionic strength (Epstein *et al.*, 1967; Kamiya and Utida, 1968; Pfeiler and Kirschner, 1972; Giles and Vanstone, 1976; Towle *et al.*, 1977; Johnson *et al.*, 1977; Ho and Chan, 1980; Busacker and walter, 1981). Overall Na^+-K^+-ATPase activity is defined as the Na^+- and K^+-dependent increment of Mg^{+2}-ATPase activity. A typical assay medium for the enzyme from higher vertebrates and most teleost contains 100 mM NaCl and 20 mM KCl, i. e., $Na^+/K^+=5$ (Schuurmans Stekhoven and Bonting, 1981; de Renzis and Bornancin, 1984). However this ratio is not the optimal requirement for tilapia and several other species. The optimal Na^+/K^+ requirement for tilapia is 1.7; chinook salmon, 2.0. (Johnson *et al.*, 1977); goldfish, 0.67 (Busacker and Walter, 1981).

Many preparations of gill tissues utilized the difference between the absence and the presence of Na^+ and K^+ in the reaction mixture to determine the sodium

potassium activated ATPase activity. However, this procedure may lead to problems in the assessment of the enzymatic activity since it is very difficult to remove residual Na^+ and K^+ from reaction mixture (Johnson *et al.*, 1977). Although in rainbow trout (*Salmo gairdneri*), ouabain inhibition is not always a reliable mean to assay Na^+-K^+-ATPase activity (Davis and Wedemeyer, 1971; Pfeiler and Kirschners, 1972). The present results demonstrated that tilapia gill Na^+-K^+-ATPase activity was stimulated by addition of $Na^+$and K^+ and also inhibited by ouabain. These results are consistent with those of *Oncorhynchus tschawytscha* (Johnson *et al.*, 1977), *Anguilla japonica* (Ho and Chan, 1980), and *Carassius auratus* (Busacker and Walter, 1981). Sensitivities of tilapia gill Na^+-K^+-ATPase to Mg^{+2}, ATP, pH and ouabain are similar to those of other species of teleost.

Temperature optima of 35–40°C have been found for gill Na^+-K^+-ATPase preparations from several species of teleost fish (Butler and Carmichael, 1972; Giles and Vanstone, 1976; Johnson *et al.*, 1977), however tilapia gill Na^+-K^+-ATPase activity has optimal temperature higher than 42°C. Rainbow trout (*Salmo gairdneri*) gill Na^+-K^+-ATPase Preparations showed temperature optima of about 45°C (Pfeiler, 1978). High temperature optima (38–50°C) were also found for Na^+-K^+-ATPase from teleost brain (Kohonen *et al.*, 1977) and from tissues of other ectotherms, such as tadpole gills (Bookoom and Alvarado, 1971), crayfish gills (Horiuchi, 1977), shark rectal gland (Ratanabanagkoon *et al.*, 1973) and turtle urinary bladder (Shamoo *et al.*, 1971). As Pfeiler (1978) suggested, relatively high temperature optima appear to be common for Na^+-K^+-ATPase preparations isolated from ectotherms whose normal physiological temperature are much lower.

According to the current models of salt excretion in teleost chloride cell (Silva *et al.*, 1977; Sargent *et al.*, 1980), Na^+-K^+-ATPase actively pumps Na^+ out of chloride cell. Therefore, the extracellular Na^+ reachs a unusually high concentration and diffuses into seawater through the leaky junctions, while Cl^- is actively transported through transcellular (Silva *et al.*, 1977) or paracellular (Sargent *et al.*, 1980) pathways in which the energy required to against the electrochemical gradient could be indirectly supplied by Na^+-K-$^+$-ATPase. These models are supported by the findings concering morphological, biochemical and physiological changes in teleost during seawater adaptation. Upon salt water adaptation of eel, the increase of the activity of gill Na^+-N^+-ATPase was accompanied by a parallel proliferation of the chloride cells and a parallel enchancement of Na^+ effluxs (Utida *et al.*, 1971; Bornancin and de Renzis, 1972; Evans and Mallery, 1975; Sargent *et al.*, 1980). Tilapia (*Oreochromis mossambicus*) probably provides an additional proof for these models, since tilapia developed more and larger chloride cells (Foskett *et al.*, 1981), more leaky junctions between neighboring chloride cells (Hwang, 1987), and much higher gill Na^+-K^+-ATase activity (the present results) following the adaptation to seawater.

Previously, Dharmanba *et al.* (1972) and Dange (1985) also examined the gill Na^+-K^+-ATPase activity in tilapia (*Oreochromis mossambicus*). It is not surprised that their data are much lower than the present results, since the reaction medium and temperature are quite different. In their studies, they did not show whether their assay conditions were optimal for the tilapia gill Na^+K^+-ATPase activity. The present study provides an optimal reaction conditions for the assay of tilapia gill Na^+-K^+-ATPase activity, which is necessary for obtaining more significant data of gill Na^+-K^+-ATPase activity in order to reduce a relative large variances among the individual preparations (Johnson *et al.*, 1977).

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吳郭魚 (*Oreochromis mossambicus*) 鰓經鈉鉀離子活化的腺苷三磷酸酶活性之特性

黃鵬鵬　孫青木　吳淑美

本報告研究吳郭魚鰓經鈉鉀離子活化腺苷三磷酸酶對離子、基質及抑制劑濃度、酸鹼度、溫度與鹽分濃度之敏感度。其結果：

1. 酵素活性於鈉/鉀濃度比在 3～0.6 之間較高，於 1.7 時活性最高。
2. 鈉、鉀、鎂離子之最適濃度分別爲：125、75、7.5 mM。
3. 最適酸鹼度爲 7.5。在酸性時，活性急速下降。
4. 最適腺苷三磷酸濃度是 5.0 mM。
5. 5×10^{-6} M 烏本苷可以抑制50%酵素活性。5×10^{-4} M 烏本苷則可完全抑制酵素活性。
6. 最適溫度高於 42°C。
7. 海水吳郭魚鰓酵素活性爲 12.85±1.32 μmol Pi/mg protein/hr，遠高於淡水吳郭魚之活性，7.35±1.12 μmol Pi/mg protein/hr。