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W. S. ZAUGG

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A Simplified Preparation for Adenosine Triphosphatase Determination in Gill Tissue

W. S. ZAUGG¹

National Oceanic and Atmospheric Administration, National Marine Fisheries Service, Coastal Zone and Estuarine Studies Division, Northwest and Alaska Fisheries Center, 2725 Montlake Blvd. East, Seattle, WA 98112, USA

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A simplified preparation from gill tissue that yields adenosine triphosphatase activities comparable to some microsomal fractions is described. The procedure uses two short (6 and 7 min), low speed (2000 Relative Centrifugal Force) centrifugations at room temperature in contrast to long, high speed, refrigerated centrifugations usually associated with microsomal preparations.

Key words: gill ATPase, ATPase, fish

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L'article qui suit contient une description de préparation simplifiée de tissu branchial dont les activités d'adenosine triphosphatase sont comparables à celles de certaines fractions microsomiques. On utilise à cette fin deux centrifugations courtes (6 et 7 min) et à basse vitesse (2 000 forces centrifuges relatives) à la température ambiante au lieu de centrifugations longues, à haute vitesse et sous réfrigération, ordinairement associées avec les préparations microsomiques.

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INTEREST in biochemical and physiological events occurring in gills of teleosts adapting to salt water has greatly intensified since the appearance of reports by Utida et al. (1966) and Epstein et al. (1967) on relationships between $\text{Na}^+ - \text{K}^+$ adenosine triphosphatase activity ($\text{Na}^+ - \text{K}^+$ ATPase) and salt-water acclimation of eels (*Anguilla japonica*) and killifish (*Fundulus heteroclitus*). During the past several years, numerous publications on several species of fish have demonstrated important relationships between gill $\text{Na}^+ - \text{K}^+$ ATPase activities and such factors as seasons of the year, age, size, environmental conditions, and husbandry practices (Giles and Vanstone 1976; Hoar 1976; McCartney 1976; Johnson et al. 1977; Boeuf et al. 1978; Lasserre et al. 1978; Wedemeyer et al. 1980; Zaugg, Pacific N.W. Reg. Comm. Prog. Rep. 817, 1979).

Most studies employed some form of enzyme purification to eliminate interfering substances and to increase specific activity of gill $\text{Na}^+ - \text{K}^+$ ATPase in the final preparation. Generally, this approach requires prolonged ultracentrifugation (Epstein et al. 1967) and/or salt treatment (Kimiya and Utida 1968) to isolate the more highly active microsomal fractions from gill filaments. Consequently, application of these methods to studies involving large numbers of samples is difficult, time consuming, and in many instances impractical.

¹Present address: National Marine Fisheries Service, Cook Field Station, Cook, WA 98605, USA.

A simplified gill $\text{Na}^+ - \text{K}^+$ ATPase enzyme preparation was described by Johnson et al. (1977) for use in sampling large numbers of fish. In this method $\text{Na}^+ - \text{K}^+$ ATPase activities are determined in crude suspensions of ground gill tissue from which no material is discarded. Although rapid and conveniently applicable to large numbers of fish, this method results in an enzyme preparation with low specific activity when compared to partially purified preparations. Special precautions must be taken with crude preparations to ensure uniform sampling from a nonhomogenous mixture and to prevent settling out of larger particles during reactions.

This note describes a procedure for preparing from gill filaments a homogenate that gives specific $\text{Na}^+ - \text{K}^+$ ATPase activities comparable to purified microsomal fractions but eliminates most of the complex and lengthy procedural steps. The method has been used in determining enzyme activities in several thousand samples of gills from Pacific salmon and trout and has been found to produce consistent, reliable data.

Methodology — Gill filaments (0.05–0.2 g wet weight) are trimmed from supporting arches of killed or anesthetized fish (salmonids were used in developing the method) and immersed in 1 mL of a solution (SEI) containing 0.3 mol/L reagent grade sucrose (102.7 g/L), 0.02 mol/L disodium ethylenediamine tetraacetate (Na_2EDTA , 7.44 g/L), and 0.1 mol/L imidazole (6.8 g/L), all adjusted to a final pH 7.1 with HCl. Inclusion of arches with filaments has resulted in decreased specific activity. Filaments from salmonids in SEI can be left at room temperature (20–24°C) for at least 30 min or on ice for at least 3 h without loss of activity. Tubes or vials containing immersed filaments are then placed on Dry

Ice² or in a freezer at -23°C or colder where they remain stable with no loss of activity for at least 6 wk.

Enzyme preparations are made by manually homogenizing thawed samples (cold) in a conical glass homogenizer until all filaments are just disintegrated (about 7 to 10 strokes). When using gills from large fish, most of the cartilagenous supports can be pressed to the bottom of the homogenizer with the pestle and eliminated in this step. The resulting homogenate is poured into a conical centrifuge tube and placed in ice or ice water. Distilled water (1 mL) is added to the homogenate and mixed. When only very small amounts of filament are available (<50 mg), volumes of SEI and water can be reduced proportionately.

All diluted homogenates are then centrifuged for 7 min in a clinical centrifuge (room temperature) at about 2000 Relative Centrifugal Force (3500–3900 rpm). Supernatant solutions are decanted and discarded, and the tubes are inverted for a short time to drain. Pellets are then thoroughly suspended in 0.5–1 mL (depending on pellet size, giving a final protein concentration between 1 and 3 mg/mL) of SEID [SEI containing sodium deoxycholate (0.1 g/100mL)] using the conical glass homogenizer (30 strokes). SEID should be kept in a glass container at room temperature, as storage in plastic containers or in the cold results in formation of a crystalline precipitate (may be redissolved by warming). Resulting homogenates are centrifuged as before, but for 6 min, and an aliquot of sufficient quantity (about 0.2 mL), to provide enzyme preparation for reactions and protein determinations, is carefully withdrawn.

Several factors such as water temperature, photoperiod, diet, extent of parr–smolt transformation, and species affect the absolute values of specific $\text{Na}^{+}-\text{K}^{+}$ ATPase activities obtained by this or any other method of enzyme preparation. It is therefore important to establish proper baseline activities in each experimental situation before determining any effects of natural changes in physiology or administered treatments. With enzyme suspensions prepared by the method reported here we have generally observed specific activities ranging from 5 to 10 $\mu\text{mol P}_i \cdot \text{mg protein}^{-1} \cdot \text{h}^{-1}$ for parr salmonids and from 12 to 65 $\mu\text{mol P}_i \cdot \text{mg protein}^{-1} \cdot \text{h}^{-1}$ for smolts. Specific activities of microsomal preparations generally fall in about the same range (Lasserre et al. 1978; Lorz and McPherson 1976, 1977; Zaugg and McLain 1972), whereas specific activities of crude preparations are from about 0.5 to 3 $\mu\text{mol P}_i \cdot \text{mg protein}^{-1} \cdot \text{h}^{-1}$ in parr and from 4 to 10 $\mu\text{mol P}_i \cdot \text{mg protein}^{-1} \cdot \text{h}^{-1}$ in smolts (Johnson et al. 1977; Lorz et al. 1978; Ewing et al. 1979).

Freezing of gill filaments facilitates field sampling and allows later laboratory analysis without loss of final enzyme activity. Freezing also ruptures red blood cells which allows soluble cell material, including hemoglobin, to be discarded in the supernatant liquid of the first centrifugation. About 25 to 30% of the total $\text{Na}^{+}-\text{K}^{+}$ ATPase activity is also discarded in this step. When the pellet is suspended in SEID nearly all of the remaining enzyme activity is found in the supernatant liquid, with little or none located in the residual pellet of the second centrifugation. This procedure removes much of the extraneous soluble protein in the first supernatant liquid and

the inactive particulate matter in the second pellet.

We have determined $\text{Na}^{+}-\text{K}^{+}$ ATPase activity in enzyme preparations using the following reaction mixtures. Stock Solution A is prepared by dissolving 4.68 g $\text{MgCl}_2 \cdot 6 \text{H}_2\text{O}$, 9.07 g NaCl, 5.6 g KCl, and 7.83 g imidazole in a final volume of 1 L (including final adjustment of pH to 7.0 with HCl). Stock Solution B is prepared by adding 0.42 g ouabain to 1 L of Solution A. Each enzyme preparation is allowed to hydrolyze ATP in Solution B (ouabain insensitive ATPase activity) and Solution A (ouabain sensitive plus ouabain insensitive ATPase activity), with the difference between the two determined activities (A minus B) being the calculated $\text{Na}^{+}-\text{K}^{+}$ ATPase activity.

One 10- μL aliquot of each enzyme preparation is placed into a 16 \times 100 mm test tube (tube rack in ice water) containing 0.65 mL of Solution A and another 10- μL aliquot into a tube containing the same volume of Solution B. To each of these tubes and appropriate reagent blanks is added 0.1 mL of 0.03 mol/L Na_2ATP (1.84 g/100 mL, adjusted to pH 7.0 with NaOH). The rack containing all tubes with enzyme and ATP is removed from the ice bath, shaken to mix contents of the tubes thoroughly, and then placed in a constant temperature water bath (37°C) where it is gently shaken for the first minute of a 10-min reaction period. After 10 min, the rack of tubes is again immersed in ice water and shaken for 1 min to cool. At this point any of a number of published methods may be used to determine the amount of phosphate that has been hydrolyzed from ATP. We have routinely used an extraction method developed earlier for assay by atomic absorption (Zaugg and Knox 1966, 1967) but modified and adapted to ultraviolet spectrophotometry. Because the final extract to be assayed for phosphate is very stable, this method is particularly suited for analysis of large numbers of samples and is described below.

After terminating the reaction by cooling the rack of tubes is removed from ice water and 1.85 mL of 0.95% (v/v) HClO_4 (room temperature) is dispensed with moderate force (to cause mixing) down the side of each tube, followed immediately by addition of 3.0 mL of purified grade (Baker's) 2-octanol. After addition of 2-octanol, all tubes are immediately transferred to a rack especially designed for the extraction process. The basic requirements of such a rack are that the tubes be supported upright rather tightly, so that very little leaning to one side or another is possible, and that the bottoms of the tubes rest on a 1–2 cm cushion of Styrofoam. Once all tubes are in place, 0.25 mL of ammonium molybdate reagent (58.4 g $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4 \text{H}_2\text{O}$ dissolved in 200 mL of concentrated HCl, diluted to 1 L with distilled water) is immediately dispensed into each. A sheet of plastic film (Saran Wrap, Handi-Wrap, etc.) is then placed over the tops of the tubes and a lid (plywood) to which has been glued a piece of Styrofoam (about 2 cm thick) is placed on top of the tubes (Styrofoam down to form a cushion in contact with the plastic film). The lid and rack are grasped firmly and held tightly together while shaking the tube contents vigorously for 30 s. Following the extraction of phosphomolybdate into the octanol phase, the lid and plastic film are removed and 0.5 mL citrate reagent (143 g citric acid $\cdot \text{H}_2\text{O}$ in 1 L final volume including adjustment to pH 2.9 with approximately 14 g NaOH) is dispensed down the side of each tube [citrate prevents further phosphomolybdate formation in the aqueous phase (Zaugg and

²Reference to trade names does not imply endorsement by the National Marine Fisheries Service, NOAA.

TABLE 1. Comparison of specific $\text{Na}^+ - \text{K}^+$ ATPase activities from varying concentrations of enzyme preparation.^a

Protein concentration in enzyme preparation (mg/mL)	μg protein in the reaction	Specific $\text{Na}^+ - \text{K}^+$ ATPase activity ^b
3.1	31	16.3
2.8	28	15.8
2.5	25	17.0
2.2	22	15.8
1.8	18	16.7
1.7	17	15.1
1.4	14	15.9
1.3	13	15.9
1.2	12	16.4
1.1	11	13.8
0.9	9	14.9
	Mean	15.8
	SD	0.9
	SE	0.3

^aGill filaments from 10 yearling coho salmon (*Oncorhynchus kisutch*) were frozen separately in SEI for 6 d, then thawed and homogenized as described. The homogenate was divided into 11 equal portions from which enzyme preparations were made as outlined in the procedure using varying amounts of SEID to regulate final protein concentrations.

^b $\mu\text{mol P}_i \cdot \text{mg protein}^{-1} \cdot \text{h}^{-1}$.

Knox 1967)]. A new sheet of plastic film is placed over the tops of the tubes, the lid secured, and the rack shaken again for 30 s.

Following extraction in the presence of citrate, all tubes are very briefly centrifuged to accelerate complete separation of the aqueous and organic phases. Absorbance of the octanol layer at 312 nm is determined with a UV spectrophotometer (visible light at 340 nm can also be used but sensitivity is decreased). Recently purchased 2-octanol has had unusually high absorbance at 312 nm and samples have been read at 322 nm with a slight decrease in sensitivity. The amount of phosphate hydrolyzed in unknowns is determined by comparing values to standards using known amounts of phosphate. The ATPase activities are expressed as $\mu\text{mol ATP hydrolyzed} \cdot \text{mg protein}^{-1} \cdot \text{h}^{-1}$. Protein concentrations in the enzyme preparations can be determined by the method of Lowry et al. (1951) as modified by Miller (1959) using bovine serum albumin as a standard.

Table 1 shows the results of varying protein concentration in a representative experiment using the method I described here for enzyme preparation and ATPase assay.

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