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30. This study was supported by the National Science and Technology Authority-Institute-Building Program through a scholarship grant, and by the University of Southern Mindanao through a study leave for E.P. Ocreto.

PROSTAGLANDIN SYNTHETASE FROM PHILIPPINE GORGONIANS

Gisela P. Concepcion* and Lourdes J. Cruz

Optimum extraction and assay conditions for prostaglandin (PG) synthetase from Echinogorgia sp., *a Philippine coelenterate, were studied. The co-oxidation of L-epinephrine with arachidonic acid to adenochrome ($Abs._{max}$ 480 nm) was used to assay PG synthetase activity. Optimum assay temperature was 22°C. Enzyme activity was associated with the microsomal-cytoplasmic fraction. Maximum activity was at pH 7.0. The enzyme was most stable in Tris buffer at pH 8.0 mixed with 0.1% Triton X-100. Optimum extraction temperature was 0-4°C. Purification was done by sequential Sepharose 4B gel filtration, DEAE-cellulose chromatography, and polyacrylamide gel electrofocusing. A 1920-fold purification was achieved. Isoelectric pH was higher than 7.5 but the upper limit is uncertain. Molecular weight was estimated at 160,000 by Sepharose 4B gel filtration. Kinetic studies showed K_m of arachidonic acid to be about 10^{-3}M; V_m about 10^3 µmol epinephrine oxidized per min per mg protein; turnover number about 10^5 per min.*

INTRODUCTION

Prostaglandins (PG) are synthesized from polyunsaturated fatty acids. The first stage of synthesis is mediated by PG endoperoxide synthetase. This enzyme is present in most mammals, other vertebrates, and marine coelenterates (1,2,3). The presence of PG in phylogenetically simple organisms suggests that PG may be involved in the regulation of such processes as ion and water transport, reproduction, and defense against predation or parasitism (1,4,5).

Mammalian PG synthetase has been purified from bovine and sheep vesicular gland microsomes. The bovine enzyme is a membrane-bound complex with

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This article is taken from the Masteral Thesis of GP Concepcion.

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cyclooxygenase and peroxidase activity (6-9). The sheep enzyme is a glycoprotein dimer with an approximate molecular weight of 126,000 (10-11).

Studies on cofactor and activator requirements of the enzyme reveal that iron is probably associated with cyclooxygenase activity in mammals (8) and copper in invertebrates (12). Aromatic compounds like L-epinephrine enhance peroxidase activity in mammals (8) and UV irradiation and O_2 and H_2O_2 exposure, in invertebrates (12). The enzyme from both sources are inhibited by aspirin and related anti-inflammatory drugs(12, 13,14,15,16).

Attempts to purify and characterize PG synthetase from gorgonians like *P. homomalla* have been unsuccessful (17). The present study aims to determine optimum *in vitro* requirements for PG synthetase activity from Philippine Pacific gorgonians, and to purify and characterize PG synthetase from one particular species.

MATERIALS AND METHODS

Specimens were collected from Bolinao, Pangasinan and Anilaw, Batangas at depths of 40-70 feet. Samples were transported in aerated, ice-cooled seawater and later maintained in synthetic aerated seawater at 22-25°C under white light for one month. Feed was freeze-dried, brine shrimp flakes. Air-dried specimens were sent to Dr. Frederick M. Bayer, of the Department of Invertebrate Zoology, Smithsonian Institution for identification.

Extracts were prepared by scraping off the rind of freshly cut specimens. The rind was minced, suspended in 3 vol of 20 mM Tris acetate (pH 8.0)-0.1% Triton X-100 at 0.4°C, and homogenized. The homogenate was centrifuged at 10,500 rpm for 10 min at 0°C in a Sorvall RC2-B Automatic Refrigerated Centrifuge with an SM-24 rotor. The supernate contained the microsomal cytoplasmic fraction.

Unused rinds were lyophilized and stored at 0°C. The PG synthetase assay procedure was modified from Morse *et al.* (12). The assay reaction mixture had a total volume of 1.0 mL with the following effective concentrations: 50 mM phosphate buffer pH 7.0, 1.0 mM arachidonic acid, 1.0 mM L-epinephrine. Reaction was initiated by adding µL quantities of extract, and shaking in a Vortex mixer for 5 sec. Transmittance was read in a Beckman DBG Grating Spectrophotometer at 480 nm, slit setting 0.5-0, 22°C, and monitored every 10 sec for at least 1 min. Specific activity was defined as µmol epinephrine oxidized per min per mg protein.

The Lowry method was used for protein determination (18). Proteins from homogenates and microsomal-cytoplasmic fractions were precipitated with TCA.

Optimum conditions were determined using homogenates of fresh *Echinogorgia sp.* Buffers (20 mM and 100 mM) with pHs ranging from 3-10 were prepared. For assay pH studies, extraction pH was 20 mM phosphate, pH 7.0. For extraction pH studies, assay pH used was 50 mM phosphate, pH 7.0. Homogenates of 3.5-month-old lyophilized *Echinogorgia sp.* were used to determine incubation and assay temperatures at optimum pH.

PG synthetase from 8.5-month-old lyophilized samples of *Echinogorgia sp.* was purified by running the microsomal-cytoplasmic fraction through an equilibrated Sepharose 4B column(27x0.8 cm) maintained at 0-4°C. The eluent was 15 mL of 50 mM Tris acetate (pH 8.0) -0.1% Triton X-100. Flow rate was 0.25 mL per min. One-mL fractions of eluate were assayed for PG synthetase and protein. The active fractions were transferred to a pretreated Fisher No. 8-667A tubing and dialyzed overnight at 0-4°C against 20 mM Tris acetate (pH 8.0)-0.1% Triton X-100.

The dialyzate containing PG synthetase was further purified by running through an equilibrated DEAE-cellulose column (5.5 x 1.3 cm) at 0-4°C. The

column was washed with 10 mL of 20 mM Tris acetate (pH 8.0)-0.1% Triton X-100 followed by 30 mL linear gradient to 100 mM Tris acetate (pH 8.0)-0.1% Triton X-100, and followed further by 5 mL of the 100 mM buffer. Flow rate was 0.3 mL per min. Assays were done on pooled fractions.

The active pool was divided for polyacrylamide gel electrofocusing, reapplication to the Sepharose 4B column, and protein determination. Non-active pools were dialyzed and concentrated with polyethylene glycol and used for protein determination.

Molecular weight was determined by Sepharose 4B filtration. Kinetic studies were done on electrofocused samples at different arachidonic acid concentrations from 0.05 to 1 mM.

RESULTS AND DISCUSSION

Several Philippine gorgonians exhibited a wide range of PG synthetase activity (Table 1). PG synthetase occurrence in diverse taxa makes correlation between PG production and morphological complexity difficult (19). *Echinogorgia sp.* had the highest activity and was used to characterize PG synthetase. Optimum assay pH was about 7.0 (Fig. 1), which is perhaps a compromise of ionic state requirements of the substrate, the cofactor, and the active site residues of the enzyme. The carboxylate in arachidonate, the substrate, at alkaline pH binds ionically with enzyme residues. L-epinephrine, the cofactor, is more water-soluble at low pH where it is protonated. The enzyme itself shows overall conformational stability at high pH with an optimum extraction pH of 8.0. Fig. 1 shows that the assay pH curve is within the pH range where the enzyme is most stable.

PG synthetase activity in lyophilyzed samples stored at 0°C decreased with time. Six-month-old samples and older were used for purification.

Table 1. PG synthetase activity in crude extracts of different gorgonians.

Specimen	PGSase Activity of Crude Extract (μmol epinephrine oxidized / min. mg protein)
Verrucella umbraculum (Ellis & Solander) or *umbella* (Esper)	1.193
Subergorgia mollis (Nutting)	0.0323
Siphonogorgia godeffroyi (Kolliker)	0.312
Muricella sp.	0
Echinogorgia sp.	0.910
Subergorgia reticulata (Ellis & Solander)	0.308
Echinogorgia sp.	3.060
Verrucella umbraculum (Ellis & Solander) or *umbella* (Esper)	0.551
Melithoid, no spicules unidentifiable	0

Sepharose 4B separation was based on size. PG synthetase activity at this stage was 0.0691 mol ep ox min^{-1} $(mg\ prot)^{-1}$ (Fig. 2). On DEAE-cellu-

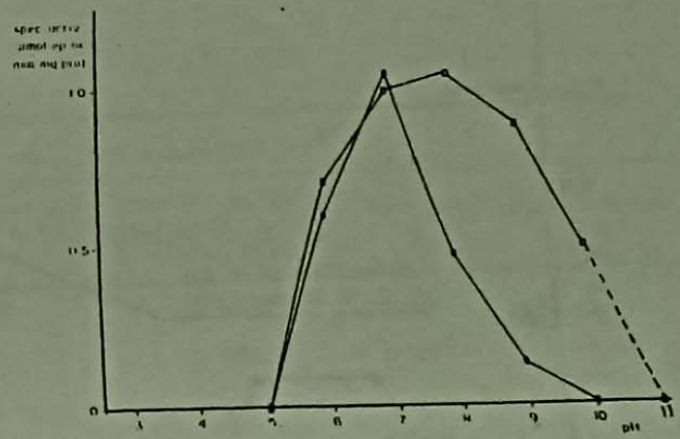

Fig. 1. PG synthetase from *Echinogorgia* sp. (●—●) pH dependence of enzyme activity. A homogenate of fresh specimen, pH 7.0, was used. Assay pH was varied from 3-10 using different buffers (50 mM). (□—□) Enzyme stability at various pH. Homogenates of fresh specimens were prepared at pHs ranging from 3-10 and kept at 0-4°C. The assay was done at pH 7.0.

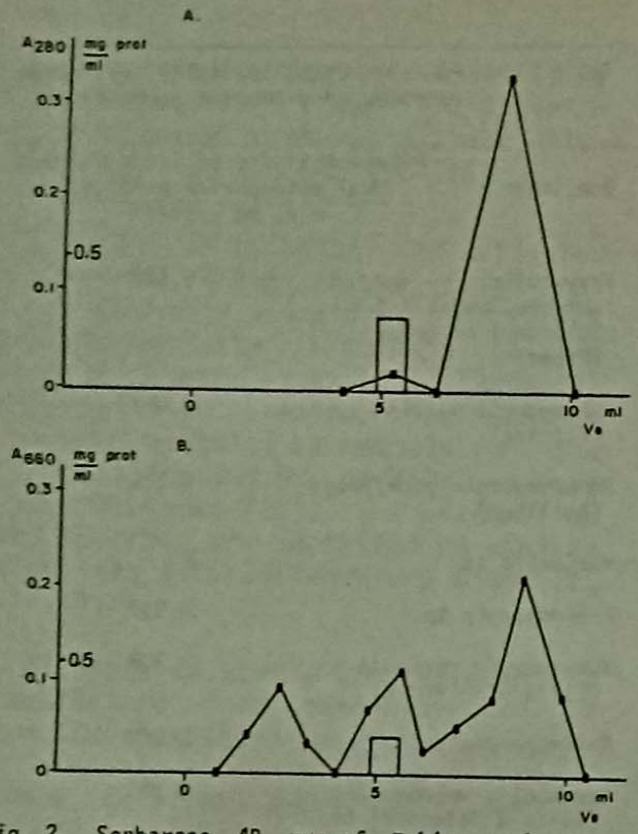

Fig. 2. Sepharose 4B run of *Echinogorgia* sp. microsomal fraction. a. Protein was determined by UV absorption at 280 nm, total specific PGSase activity of the active pool (□) was 0.606 mol ep ox min^{-1} (mg prot)$^{-1}$. b. Protein was determined by the Lowry method, total specific PGAase activity of the active pool (□) was 0.0691 mol ep ox min^{-1} (mg prot)$^{-1}$. The Lowry method is more sensitive; hence, the total specific activity based on Lowry is much lower.

lose, an anion-echanger, the active fraction was eluted immediately, indicating that PG synthetase was either neutral or cationic at pH 8.0. Other protein fractions were eluted much later, indicating they were **strongly** anionic at pH 8.0 (Fig. 3). PG synthetase activity at this stage was 7.4 mol ep ox min^{-1} (mg prot)$^{-1}$.

Partially purified PG synthetase was applied to a Sepharose 4B column to determine its molecular weight (MW). With the use of markers, the MW was determined to be 160,000 (Fig. 4).

Through electrofocusing, the pH was determined to be above 7.5. Near this pH, PG synthetase has a net charge near zero and is most stable, which explains the optimum extraction pH of 8.0. Further purification was achieved at this stage. PG synthetase activity was 29.7 mol ep ox min-1 (mg prot)-1. Table 2 summarizes the degrees of purification at each stage.

Enzyme activity could not be shown to be proportional to concentration. It would seem that anionic inhibitors were removed during the DEAE-cellulose run. The inhibitors are likely present in

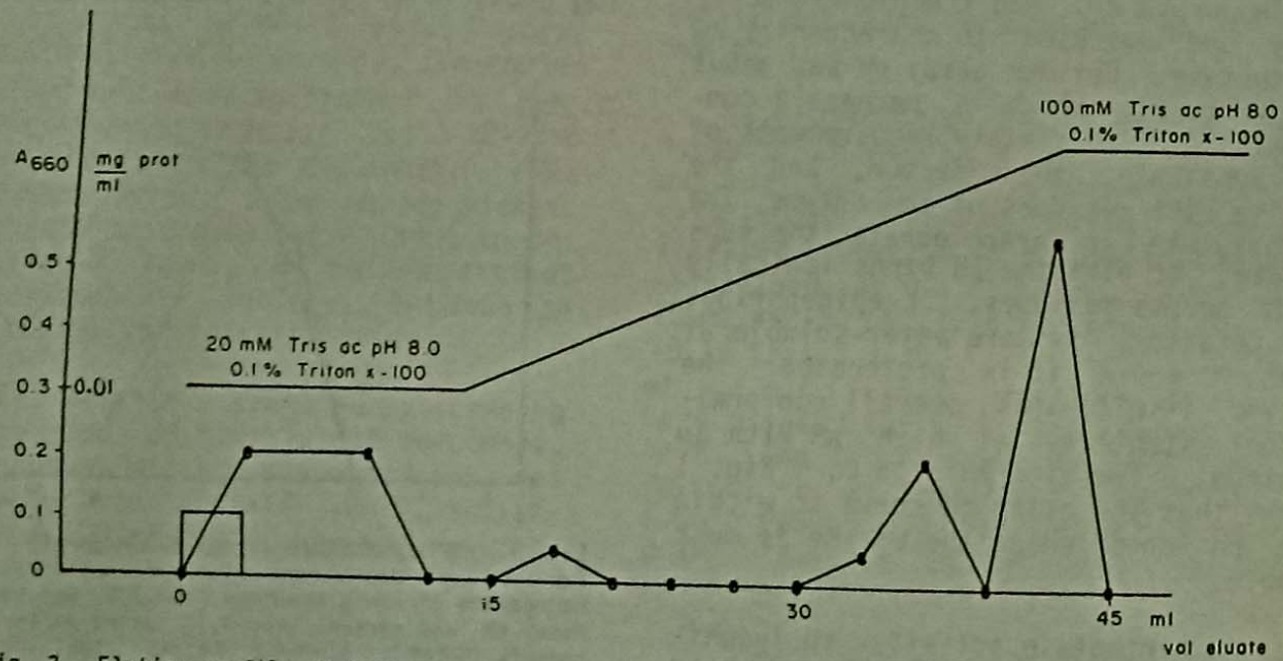

Fig. 3. Elution profile of *Echinogorgia* sp. Sepharose 4B active fraction applied to a DEAE-Cellulose column. Buffer gradient was from 20mM to 100 mM Tris ac, pH 8.0, 0.1% Triton X-100 (——). Protein was determined by the Lowry method (●——●). Total specific PGSase activity of the active pool (□) was 7.40 mol ep ox min^{-1} (mg prot)$^{-1}$.

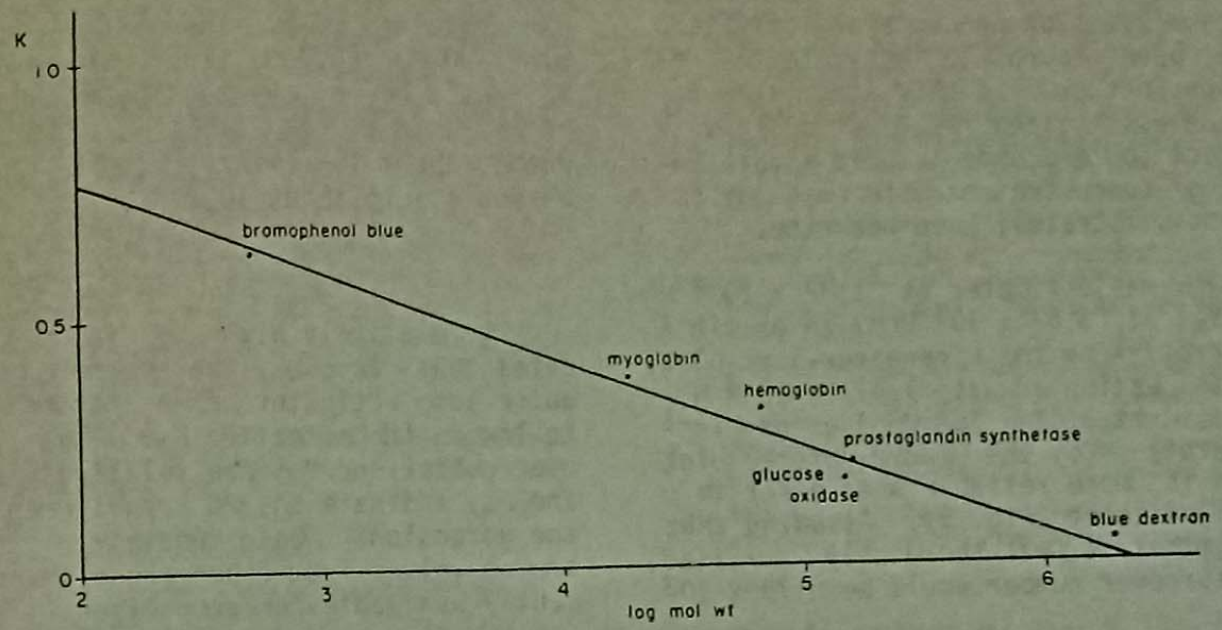

Fig. 4. Estimation of molecular weight of prostaglandin synthetase from *Echinogorgia* sp. by gel filtration on Sepharose 4B. Molecular weight markers are: bromophenol blue--M_r 500, myoglobin-M_r 18,800, hemoglobin-M_r 68,000, glucose oxidase-M_r 150,000, blue dextran-M_r 2,000,000. The M_r of prostaglandin synthetase was interpolated to be 160,000. The least squares method was used to determine the best line.

gorgonian cells but are in compartments separate from PG synthetase; they do not come in contact until the cell is disrupted.

Initial kinetic studies could only yield approximate K_m and V_m values because of the limited amount of electrofocused material, and because the specific activity of the 8-month-old sample was less than 3% of the specific activity of fresh samples. Also, PG synthetase reactions have long been thought to be non-first order, complex, autocatalytic, and autodestructive because of the oxidation and reduction

Table 2. Purification table for sequential run[a]

Active Fraction	Total Volume mL	Total Protein[b] mg	Total Activity $\frac{\mu mol\ epin.\ oxid.}{min}$	Specific Activity $\frac{\mu mol\ epin.\ oxid.}{min\ mg\ prot.}$	Recovery %	Purification fold
Microsomal	0.40	1.22	0.0189	0.0155	100	1
Sepharose 4B	0.80	0.323	0.0223	0.0691	>100[d]	4.46
DEAE-cellulose	4.35	0.0279[c]	0.207	7.40	>100	477
Electrofocusing	1.5	0.00384	0.114	29.7	>100	1920

a - the basis of all values reported here is the original amount of material.
b - protein was measured by the Lowry method.
c - concentration by PEG involved some loss of material.
d - recovery of >100% could indicate the removal of inhibitors.

involved in a series of intermediate steps. Reaction products of activators have been found to activate the reaction further. In this study, the reaction was treated as first order with respect to arachidonic acid at a saturating concentration of cofactor (or second substrate), L-epinephrine.

K_m was estimated at 1.32×10^{-4} M and V_m, at 3.57×10^{1} Umol ep ox min^{-1} (mg prot)$^{-1}$ by the Lineweaver-Burk plot. K_m was estimated at 1.41×10^{-4} M and V_m, at 3.62×10^{1} Umol ep ox min^{-1} (mg prot)$^{-1}$ by the Eadie-Hofstee plot which is more reliable when error in v is significant (Fig. 5). Assuming that PG synthetase was thoroughly purified, the turnover number would be 5.79×10^{3} mmol ep ox min^{-1} (mmol enzyme)$^{-1}$. This was obtained from the Eadie-Hofstee plot and MW 160,000. In fresh samples, V_m would be of the magnitude 10^{3} Umol ep ox min^{-1} (mg prot)$^{-1}$, turnover number about 10^{5} min^{-1}, and K_m of arachidonic acid about 10^{-3} M.

A relatively high K_m value indicates that, *in vivo*, the enzyme may require some activator or a cosubstrate to lower the effective K_m. The turnover number and V_m are relatively high and may indicate the PG requirement of the gorgonian. Unfortunately, there are no values from other organisms presently available for comparison.

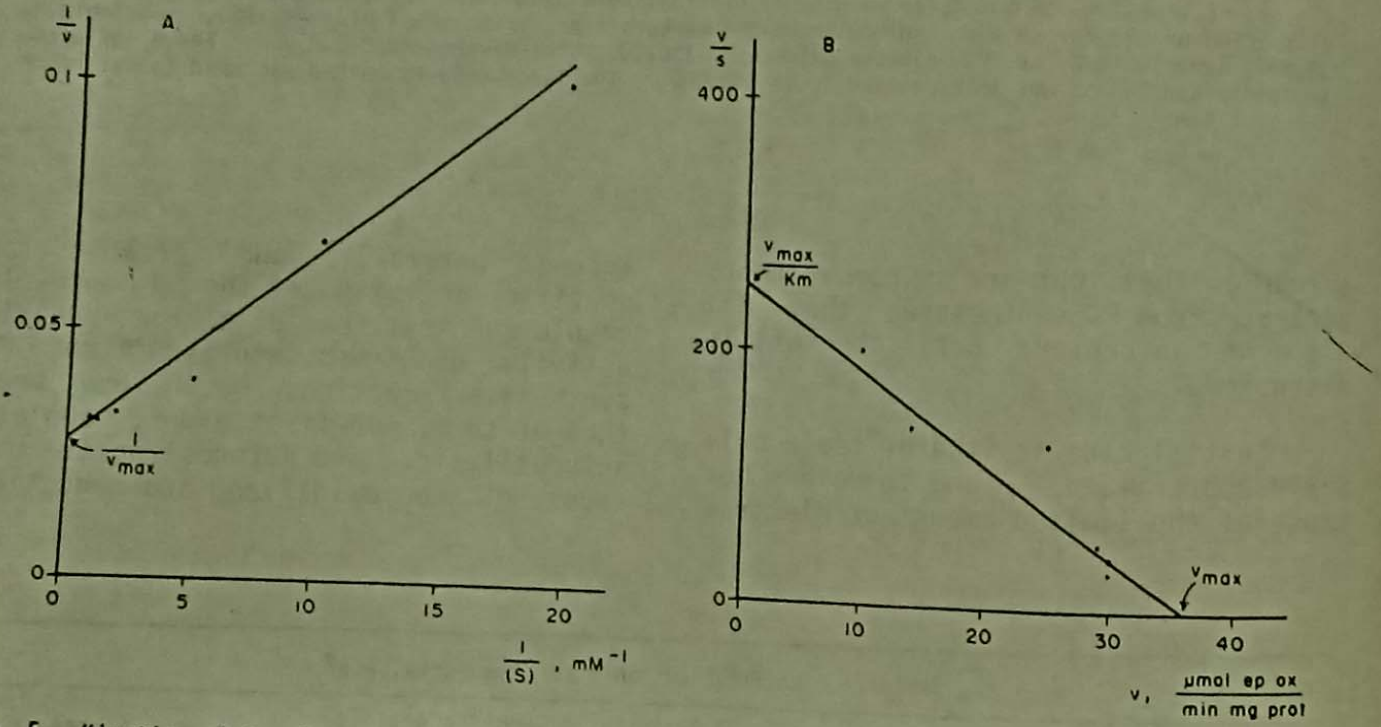

Fig. 5 Kinetic plots for the substrate arachidonic acid using *Echinogorgia* sp. electrofocused sample. a. The Lineweaver-Burk plot gave a $K_m' = 1.32 \times 10^{-4}$ M and $V_m' = 35.7$ mol ep ox min^{-1} (mg prot)$^{-1}$. b. The Eadie-Hofstee plot gave a $K_m' = 1.41 \times 10^{-4}$ M, and $V_m' = 36.2$ mol ep ox min^{-1} (mg prot)$^{-1}$.

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20. This study was supported in part by the National Science Development Board. The author also thanks the Marine Science Center for the procurement of specimens.

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