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Polyphenol Oxidase Inhibition by Glucose Oxidase in Pink Shrimp (*Pandalus borealis*)

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**POLYPHENOL OXIDASE INHIBITION BY GLUCOSE OXIDASE
IN PINK SHRIMP (Pandalus borealis).**

BY

MOHAMMED S. H. AL-JASSIR

**A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENT FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
IN
FOOD SCIENCE AND NUTRITION**

UNIVERSITY OF RHODE ISLAND

1987

THESIS ABSTRACT

DOCTOR OF PHILOSOPHY DISSERTATION

BY

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UNIVERSITY OF RHODE ISLAND

1987

THESIS ABSTRACT

The glucose oxidase and catalase (GOX/CAT) enzyme system was studied as an inhibitor of polyphenol oxidase (PPO) in extracts of whole pink shrimp (Pandalus borealis).

PPO was isolated from shrimp heads and purified by acetone extraction, ultrafiltration, and gel filtration chromatography. A purification of 7.74-fold was obtained with a recovery of 54.3% activity. The shrimp PPO activity was compared to that of mushroom PPO activity. The shrimp PPO was found to have an optimum pH of 6.0-6.5 and to be stable at 25-35 °C. Below freezing, the shrimp PPO was damaged completely and no activity was obtained.

Several phenolic compounds were tested as the substrate of shrimp PPO. L-DOPA and DL-DOPA yielded the highest activity. The K_m of shrimp and mushroom PPO were 4.54 and 0.5 mM, respectively. Varying the concentration of D-glucose, a substrate of GOX/CAT, over the range of 0.5 to 4% had no significant effect on the activity of both mushroom and shrimp PPO. GOX/CAT at a concentration of 2 units/ml inhibited 95.2% and 97% of shrimp and mushroom PPO, respectively, with 0.5% D-glucose. The PPO was completely inhibited by 6 units/ml of GOX/CAT.

The substrates and end products of GOX/CAT were studied for their potential to inhibit mushroom and shrimp PPO. Mushroom and shrimp PPO activity were inhibited completely

by the GOX/CAT end products hydrogen peroxide and gluconic acid at concentrations of 2.0 mg/ml, while glucono-lactone showed a weak inhibition of less than 7.6% and 9.0%, respectively.

Oxygen consumption by mushroom and shrimp PPO was measured during the 2 min duration of the experiment. Oxygen removal reached 2.05 ppm with mushroom PPO and 1.0 ppm with shrimp PPO. However, when GOX/CAT was added, oxygen removal increased rapidly to 7.5 and 7.48 ppm, with 96.2% and 95.9% of the oxygen consumed during the 2 min period for mushroom and shrimp PPO, respectively. D-glucose showed no inhibitory effect on the activity of mushroom and shrimp PPO even at a concentration of 5.0 mg/ml.

The potential of the glucose oxidase/catalase (GOX/CAT) enzyme system for delaying melanosis in iced shrimp was studied. Fresh, pink shrimp (Pandalus borealis) with heads on were treated with various dip solutions. The dip solutions were : firstly, GOX/CAT at concentrations of 2, 4, 6, and 10 units/ml, each with 0.5% D-glucose as a substrate; secondly, sodium tripolyphosphate (STPP) at a concentration of 9% alone and at concentrations of 3%, 6%, and 9% in combination with GOX/CAT at a concentration of 6 units/ml. Finally, sodium metabisulfite at a concentration of 1.25% alone and at concentrations of 0.32%, 0.625%, and 1.25% in combination of GOX/CAT at a concentration of 6 units/ml.

In general, all treated shrimp showed a delay in melanosis development compared to untreated shrimp. Based on the melanosis scores, GOX/CAT at concentrations of 2 and 4 units/ml delayed the melanosis formation by 2 days compared to controls, and by 4 days with GOX/CAT at concentration of 6 and 10 units/ml. The dip solution of 9% STPP delayed melanosis by 2 days compared to the controls, and by 4 days with 3% and 6% STPP, each with GOX/CAT, and by 6 days with the combination of 9% STPP and GOX/CAT. Shrimp treated with sodium metabisulfite, at all concentrations, exhibited no melanosis for the duration of the study compared to the control shrimp. GOX/CAT in combination with sodium metabisulfite did not increase the melanosis inhibition compared to sulfite alone. This was due to the sulfite inhibition effect on GOX/CAT activity.

Finally, the metabisulfite dip treatment was most effective in preventing melanosis development. The combination dip of 9% STPP and GOX/CAT at concentrations of 6 units/ml appeared to be a promising alternative as a second choice. The GOX/CAT concentration of 6 units/ml and 0.5% D-glucose as a dip had a slight effect on preventing black spot in iced shrimp.

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Finally, I would like to thank my family and especially my wife for their faith and patience in me to continue my education.

PREFACE

This dissertation is written to conform with the Graduate School Manuscript Thesis Plan. The text consists of three manuscripts which are written according to the guidelines for publication in the Journal of Food Science.

TABLE OF CONTENTS	
LIST OF TABLES	10
LIST OF FIGURES	10
REVISIONS TO THE MANUSCRIPTS BY GRADUATE THESIS PLAN	
REVISIONS TO THE MANUSCRIPTS BY GRADUATE THESIS PLAN	
1. INTRODUCTION	1
2. MATERIALS AND METHODS	4
3. RESULTS AND DISCUSSION	10
4. CONCLUSIONS	10
5. REFERENCES	10
APPENDIX A. MATERIALS OF TESTING AND ANALYSIS	
APPENDIX B. MATERIALS OF TESTING AND ANALYSIS	
APPENDIX C	
1. INTRODUCTION	10
2. MATERIALS AND METHODS	10
3. RESULTS AND DISCUSSION	10
4. CONCLUSIONS	10
5. REFERENCES	10

TABLE OF CONTENTS

Thesis Abstract	ii
Acknowledgment	v
Preface	vi
Table of Contents	vii
List of Tables	ix
List of Figures	xi

Manuscript I - Polyphenol Oxidase Inhibition By Glucose Oxidase In Pink Shrimp (Pandalus borealis).

I. Introduction	2
II. Material and Methods	5
III. Results and Discussion	14
IV. Conclusion	20
V. References	37

Manuscript II. Inhibition of Mushroom and Shrimp PPO By Substrates and End Products of the GOX/CAT Enzyme System.

I. Introduction	42
II. Material and Methods	45
III. Results and Discussion	51
IV. Conclusion	56
V. References	71

Manuscript III. Inhibition of Melanosis in Pink
Shrimp (Pandalus borealis) By Different Dip
Solutions.

I. Introduction	77
II. Material and Methods	80
III. Results and Discussion	84
IV. Conclusion	89
V. References	96
Appendix I. Literature Review	99
Bibliography	118

LIST OF TABLES

MANUSCRIPT I.

1 - Summary of purification procedure of shrimp PPO	21
2 - The effect of different substrates on shrimp PPO activity	22
3 - The GOX/CAT activity at different concentrations by using ABTS/POD assay	23
4 - The Km and Vmax values of shrimp PPO activity in the presence and absence of GOX/CAT as inhibitor	24

MANUSCRIPT II.

1 - The effect of hydrogen peroxide concentrations on mushroom and shrimp PPO activity	57
2 - The effect of gluconic acid concentrations on mushroom and shrimp PPO activity	58
3 - Oxygen removal by mushroom and shrimp PPO without GOX/CAT	59
4 - Oxygen removal by mushroom and shrimp PPO in presence of GOX/CAT at concentration of 2 units/ml with 0.5% D-glucose as substrate	60
5 - The effect of glucono-lactone on mushroom and shrimp PPO activity	61
6 - The effect of D-glucose concentration on mushroom and shrimp PPO activity	62

MANUSCRIPT III.

1 - Work sheet used for evaluating melanosis development in shrimp	91
2 - The average melanosis rating on iced pink shrimp	92
3 - Effect of GOX/CAT concentration on melanosis development in iced shrimp	93
4 - The effect of sodium tripolyphosphate (STPP) concentrations alone and in combination with GOX/CAT (6 units/ml) on melanosis of ice shrmpr	94
5 - The effect of sodium metabisulfite (SMBS) concentrations alone and in combination with GOX/CAT (6 units/ml) on melanosis of iced shrimp	95

LIST OF FIGURES

MANUSCRIPT I.

1 - Gel filtration chromatography for isolation of PPO from pink shrimp (<u>Pandalus borealis</u>)	26
2 - Effect of pH on shrimp PPO activity	28
3 - Effect of temperature on shrimp PPO activity	30
4 - Lineweaver-Burk plot of shrimp PPO activity in presence of GOX as inhibitor	32
5 - Effect of glucose concentration on activity of mushroom and shrimp PPO (GOX/CAT is 2 units/ml)	34
6 - Effect of GOX/CAT concentration on mushroom and shrimp PPO activity	36

MANUSCRIPT II.

1 - Mushroom PPO activity in presence of hydrogen peroxide or gluconic acid	64
2 - Shrimp PPO activity in presence of hydrogen peroxide or gluconic acid	66
3 - Oxygen removal by mushroom PPO with and without GOX/CAT (0.5% glucose)	68
4 - Oxygen removal by shrimp PPO with and without GOX/CAT (0.5% glucose)	70

ABSTRACT

The glucose oxidase (GOD) enzyme system was used to inhibit polyphenol oxidase (PPO) activity in pink shrimp (*Pandalus borealis*).

POD was found to be highly active in shrimp tissue and was inhibited by GOD. The inhibition was dose-dependent and reversible. The inhibition was more pronounced at 25°C than at 15°C. The inhibition was also more pronounced in shrimp tissue than in shrimp hemolymph.

MANUSCRIPT I.

**POLYPHENOL OXIDASE INHIBITION BY GLUCOSE OXIDASE
IN PINK SHRIMP (*Pandalus borealis*).**

Several polyphenol oxidases were found in pink shrimp. The highest activity was found in the shrimp hemolymph. The activity of polyphenol oxidase was inhibited by glucose oxidase. The inhibition was dose-dependent and reversible. The inhibition was more pronounced at 25°C than at 15°C. The inhibition was also more pronounced in shrimp tissue than in shrimp hemolymph.

ABSTRACT

The glucose oxidase and catalase (GOX/CAT) enzyme system was studied as an inhibitor of polyphenol oxidase (PPO) in extracts of whole pink shrimp (Pandalus borealis).

PPO was isolated from shrimp heads and purified by acetone extraction, ultrafiltration, and gel filtration chromatography. A purification of 7.74-fold was obtained with a recovery of 54.3% activity. The shrimp PPO activity was compared to that of mushroom PPO activity. The shrimp PPO was found to have an optimum pH of 6.0-6.5 and to be stable at 25-35 °C. Below freezing, the shrimp PPO was damaged completely and no activity was obtained.

Several phenolic compounds were tested as the substrate of shrimp PPO. L-DOPA and DL-DOPA yielded the highest activity. The K_m of shrimp and mushroom PPO were 4.54 and 0.5 mM, respectively. Varying the concentration of D-glucose, a substrate of GOX/CAT; over the range of 0.5 to 4% had no significant effect on the activity of both mushroom and shrimp PPO. GOX/CAT at a concentration of 2 units/ml inhibited 95.2% and 97% of shrimp and mushroom PPO, respectively, with 0.5% D-glucose. The PPO was completely inhibited by 6 units/ml of GOX/CAT.

INTRODUCTION

Polyphenol oxidase (PPO) enzyme is widely distributed in nature and easily detected in plant and animal tissues. The melanosis (black spot) on raw shrimp is produced when PPO oxidizes the amino acid tyrosine to the dark pigment melanin. The discoloration is characterized by a darkening of the head, antennae, abdominal shell segments and tailfin (Finne and Migget, 1985). This undesirable blackening phenomenon on shrimp causes serious problems to the seafood industry. Although melanosis formation is not related to the eating quality, safety or decomposition of shrimp, it is visually objectionable to most consumers and therefore less acceptable.

Many investigators have tried to prevent black spot in shrimp. One of the first observations on the prevention and delay of black spot in shrimp involved removal of the shrimp head immediately after the catch was brought on board (Alford and Fieger, 1952). Other methods of inhibiting melanosis include the use of chemicals which interfere with black spot formation, such as ascorbic acid (Faulkner et al., 1954), and cysteine (Mason, 1957; Loomis and Battaile, 1966). Many food

additives have been used as metal chelators, such as sodium tripolyphosphate (STP) and sodium hexametaphosphate (SHP) (Lindsay, 1976). The effectiveness of these compounds is known to vary with pH. Sulfiting agents have been found to be the most powerful substance demonstrated to strongly inhibit shrimp melanosis, especially sodium bisulfite or metabisulfite. Sodium bisulfite is a strong reducing agent and competes with tyrosine for molecular oxygen. It can take up the oxygen and prevent the formation of black spot (Camber et al., 1956; Nickelson and Cox, 1986).

The safety of sulfite has been questioned. Strong asthmatic reactions to bisulfite and other sulfite agents have been reported (Allen and Collett, 1981; Stevenson and Simon, 1981; Werth, 1982). Thus, work must be initiated to find alternatives to replace or reduce the amount of sulfites required to inhibit shrimp melanosis.

The enzyme system of glucose oxidase and catalase (GOX/CAT) has been used in food as a food additive and is considered Generally Recognized As Safe (GRAS) (Searle Biochemics, 1966).

GOX/CAT has been used to remove oxygen and prevent oxidation in food products and also to remove glucose (Reed, 1975). Therefore, it is possible that GOX/CAT might inhibit PPO activity by removing oxygen, which is

essential for PPO to produce melanosis.

The purpose of this study is to determine the shrimp PPO activity in vitro and compare it with mushroom PPO activity, to study the physical properties of shrimp PPO, and finally to determine if GOX/CAT inhibits the activity of shrimp and mushroom PPO.

Abstract

A shrimp PPO activity was determined in vitro and compared with mushroom PPO activity. The shrimp PPO activity was found to be higher than mushroom PPO activity. The shrimp PPO activity was also found to be more stable than mushroom PPO activity. The shrimp PPO activity was also found to be more sensitive to GOX/CAT than mushroom PPO activity.

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MATERIALS AND METHODS

Samples

Fresh, untreated pink shrimp (Pandalus borealis) obtained from West Boothbay Harbor, Maine arrived in the laboratory one day after iced harvest. They were frozen and stored at -80°C until used.

Reagents

A commercial enzyme preparation containing glucose oxidase (EC 1.1.3.4.; β -D-glucose oxidoreductase) and catalase (EC 1.11.1.6; hydrogen peroxide: hydrogen oxidoreductase) (GOX/CAT), OVAZYME (1500 units/ml), was provided by Finnsugar Biochemical, Inc., Elk Grove Village, IL and used throughout this study.

Mushroom PPO (EC 1.14.18.1; monophenol, dihydroxyphenyl-alanine: O_2 oxidoreductase; 4800 units/mg), and D-glucose (anhydrous; reagent grade) were obtained from Sigma Chemical Co., St. Louis, MO.

Sodium phosphate [monobasic (pH 4.3) and dibasic (pH 9.5)] was used as a buffer for the enzyme assay and was obtained from Fisher Scientific Co.

Shrimp PPO extraction procedure

The procedure was a modification of that of Farias (1982). The shrimp heads were separated from the shrimp while they were frozen in order to reduce the loss of the head liquid which contains the PPO. Frozen shrimp heads were blended with 0.05 M phosphate buffer (pH 7.2) at a ratio of 1:2 (w/v) in an electric blender for one minute, strained through four layers of cheesecloth and pressed. The rest of the procedure was carried out at 0-4°C unless otherwise indicated. The thick liquid and suspended materials that passed through the cheesecloth were centrifuged at 12,500 x g for 30 min. The fat particles on the liquid surface of the sample were removed and the precipitate was discarded.

Several solvents were examined for purification of shrimp PPO, including ammonium sulfate (in concentrations ranging up to 80% saturation), polyethylene glycol (PEG; M.W. 8000; 2-10%) and acetone. Acetone produced the highest final enzyme activity and was therefore used in the extractions.

Two volumes of cold acetone (-20°C) were added to the crude sample and the mixture was stirred thoroughly for 2 min. The suspension was placed in a freezer at -20°C for 2 hours to allow the protein precipitate to settle in the bottom of the container, and then the supernatant was discarded; the precipitate was resuspended in cold

acetone and centrifuged at 18,000 x g for 30 min. The precipitate was washed twice with cold acetone (-20 C) and centrifuged, and the supernatant was discarded. The protein precipitate was flushed with nitrogen gas to remove the acetone residue. The dry precipitate (an acetone powder) was stored in a sealed colored bottle at -80 °C until used, and served as the shrimp PPO source for the enzyme assay.

As needed, the acetone powder was dissolved in cold 0.05M phosphate buffer (pH 7.2) at a ratio of 1:50 (w/v) with 2% polyvinylpyrrolidone (added as a phenolic scavenger) and stirred slowly for 2 hours at 2-5 °C. At this point the PPO enzyme activity was determined. The suspension was centrifuged at 23,000 x g for 30 min and the precipitate was discarded. The supernatant was immediately placed in an Amicon DC2 Ultrafiltration chamber with an H1 P100 hollow-fiber cartridge membrane (MW cutoff 100,000). The eluent rate was 18 ml/min. The PPO enzyme activity of the eluent was determined and the solution was concentrated to one fifth of the original volume. The extract was divided into 2-4 ml fractions, and chromatographed on a 2.5 x 10 cm Sephacryl S-200 gel filtration column, using 0.02M phosphate buffer (pH 6.2) as the eluent (Figure 1). The rate of elution was adjusted to 16 ml/hr and 3-ml fractions were collected. The collected fractions were measured immediately for PPO

activity.

Substrate specificity

The following substrates were used to study shrimp PPO activity: L-DOPA (L- β -3,4-dihydroxyphenylalanine), DL-DOPA (DL- β -3,4-dihydroxyphenylalanine), L-tyrosine, catechol (1,2-benzenediol; pyrocatechol), and dopamine (3-hydroxy-tyramine), all purchased from Sigma Chemical Co.

Optimum pH and thermal stability

The optimum pH for shrimp PPO activity was determined by assaying PPO activity at different pH values ranging from 3 to 8 (in 0.5 pH unit increments) by using 0.02M sodium phosphate buffer and shrimp PPO solutions (0.38 mg/ml) at room temperature (25°C).

The thermal stability was tested in aliquots of shrimp PPO solution (0.38 mg/ml) in 0.02M phosphate buffer (pH 6.2) which were held at various temperatures ranging from 25 to 70°C (in 5°C increments) for 10-min. periods prior to cooling and assaying at 25°C.

Assay of GOX/CAT enzyme system activity

The activity of GOX/CAT enzyme system was measured in International Units according to the IUB (1972): one unit of glucose oxidase activity is that amount of enzyme liberating one micromole of hydrogen peroxide per minute

at standard conditions (Worthington, 1982). The activity of GOX/CAT was measured by an assay using 2,2'-Azino-di(3-ethylbenzthiazoline)-6-sulphonate (ABTS) and peroxidase (POD). The ABTS/POD assay was developed from a glucose oxidase assay using O-dianisidine and POD enzyme (Bergmeyer, 1974). ABTS was substituted for O-dianisidine because of the carcinogenic effect of O-dianisidine, in addition, ABTS has greater sensitivity, is very soluble in water, and virtually insensitive to light (Bergmeyer, 1974).

The solutions for the assay were prepared as follows: The buffer was prepared by mixing 0.1M of monobasic and 0.1M of dibasic of sodium phosphate until the pH of 7.0 was reached. D-glucose was dissolved in distilled water to a concentration of 10% and then left overnight to allow equilibrium of mutarotation to be attained. ABTS was prepared by dissolving 18 mg in 100 ml of 0.1M phosphate buffer and then the solution was oxygenated by air bubbles for 3-5 minutes. Peroxidase (90 units/mg) was dissolved in distilled water and diluted to a final concentration of 1 unit/ml. GOX/CAT (1500 units/ml) was diluted in distilled water to final concentrations of 0.2, 0.4, 0.6, 0.8, and 1.0 units/ml.

The spectrophotometer was adjusted to 420 nm and the temperature in the sample chamber was maintained at 25°C. The reaction mixture in each cuvette contained 2.5 ml of

ABTS-buffer mixture (oxygenated) and 0.3 ml of 18% D-glucose solution and 0.1 ml of peroxide. The cuvettes in triplicate were transferred to the spectrophotometer and monitored at 420 nm for 3-5 min to achieve temperature equilibrium and to establish the blank rate, if any. After stabilization, the cuvettes were immediately covered with fitted fluropolymer covers, inverted slowly several times and returned to the spectrophotometer. The absorbance/min was measured from the initial linear portion of the curve.

Assay of mushroom and shrimp PPO

Polyphenol oxidase (PPO) activity was measured directly by spectrophotometry using a Beckman DU-8B UV-visible spectrophotometer. The substrate L-DOPA was dissolved in 0.02M phosphate buffer (pH 6.2) to obtain a final concentration of 10 mM. The mushroom PPO was prepared by dissolving 1 mg of the enzyme (4800 units/mg) in 15 ml of the same buffer and mixed gently to obtain a final concentration of 320 units/ml.

The spectrophotometer was adjusted to 475 nm and the temperature in the sample chamber was maintained at 25 °C. The reaction mixture in each cuvette was 1.45 ml of 0.02M phosphate buffer (pH 6.2) and 1.45 ml of 0.01M L-DOPA solution for mushroom PPO, while for shrimp PPO extract the reaction mixture was 1.35 ml of 0.02M phosphate buffer (pH 6.2) and 1.45 ml of 0.01M L-DOPA solution.

This mixture was oxygenated by bubbling air into the cuvettes through a capillary tube for 3-5 minutes. The cuvettes were transferred to the spectrophotometer and monitored at 475 nm for 3-5 min to achieve temperature equilibrium and to establish the blank rate, if any. After stabilization, the cuvettes were removed and 0.1 ml of the mushroom PPO solution or 0.2 ml of the shrimp PPO solution was added. The cuvettes were immediately covered with fitted fluoropolymer covers, inverted slowly a few times and returned to the spectrophotometer. The absorbance/min was measured from the linear slope of the graph during the first three minutes.

Enzyme activity is reported in International Units (IU) according to the International Union of Biochemistry Committee on Enzymes [1972]. One IU of enzyme corresponds to the amount that catalyzes the transformation of one micromole of substrate to product per minute under specific conditions of pH, temperature, ionic strength, and substrate concentration. Since dopachrome, the compound being measured, has a molar extinction coefficient of $3600 \text{ M}^{-1} \text{ cm}^{-1}$ at 475 nm (Fling et al., 1963), the enzyme activity can be calculated easily. The conversion is straightforward when Beer's law is used, as follows:

$$C = \frac{\Delta A}{E \cdot L}$$

where C is the concentration of product (to be calculated), A is the absorbance change that occurs per minute, E is the extinction coefficient for the product dopachrome ($3600 \text{ M}^{-1} \text{ cm}^{-1}$), and L is the light path length through the cuvette (1 cm). According to this relationship, the absorbance change during 1 minute for the production of 1 mole of dopachrome is 3600. In order to convert the raw data to moles of product formed per minute, each absorbance/min value is divided by 3600.

Assay of GOX/CAT as inhibitor for mushroom and shrimp PPO activity

The GOX/CAT enzyme system was examined for its effect as an inhibitor of mushroom and shrimp PPO in vitro. The concentrations of D-glucose, a substrate for GOX/CAT, and of GOX/CAT (in units/ml) were varied, in order to find the best conditions for inhibiting mushroom and shrimp PPO.

D-glucose was dissolved in 0.02M phosphate buffer (pH 6.2) at different concentrations: 0.5, 1.0, 2.0, 3.0, and 4%. GOX/CAT (1500 units/ml) was dissolved in 14 ml of 0.02M phosphate buffer (pH 6.2) to obtain a concentration of 100 units/ml. Further dilutions were made to obtain concentrations between 0.1 and 6 units/ml.

Each reaction mixture was composed of 1.0 ml of D-glucose solution, 0.9 ml of 0.01M L-DOPA solution, 0.9 ml 0.02M phosphate buffer (pH 6.2), and 0.1 ml the GOX/CAT solution for mushroom PPO, while for shrimp PPO extract the reaction mixture was 1.0 ml of D-glucose solution, 0.9 ml of 0.01M L-DOPA, 0.8 ml 0.02M phosphate buffer (6.2), and 0.1 ml the GOX/CAT solution. The rest of the spectrophotometric procedure was the same as for the PPO assays above.

The reaction kinetics were measured when the concentrations of L-DOPA and the buffer were varied while the concentrations of D-glucose, GOX/CAT, and PPO of shrimp or mushroom were held constant. The K_m and V_{max} were determined.

Protein determination

The method of Lowry et al. (1951) was used to determine the protein concentration in each step of the shrimp PPO purification. A standard curve was prepared with bovine serum albumin, and the concentrations of unknown protein solutions were determined from the graph.

RESULTS AND DISCUSSION

Extraction of shrimp PPO

Most of the shrimp PPO is located in the head area (Bailey et al., 1960); therefore, only shrimp heads were extracted. The first supernatant was turbid due to the presence of proteins and other insoluble materials. Therefore, further separation was needed to purify the shrimp PPO. Different treatments were evaluated for further purification of the shrimp PPO: ammonium sulfate, polyethylene glycol, and acetone. Among these treatments, acetone produced the highest enzyme activity. This result agreed with the reports of Fairs, 1982; Thomas and Jnave, 1973; Chubey and Donell, 1972; and Pefferi et al, 1974.

A summary of the purification procedure developed is given in Table 1. The PPO enzyme activity of the crude acetone powder which was tested directly in a spectrophotometer at 25 °C and wavelength of 475 nm, was low, possibly due to the presence of inhibitors such as proteins or metals. To maintain enzyme activity, the extract was introduced immediately to an ultrafiltration chamber with an H1 P100 hollow fiber cartridge membrane (MW cut off 100,000). There were two reason for ultrafiltration: 1) the volume of the crude sample was

reduced to a desired size and 2) the membrane excluded any proteins with a molecular weight less than 100,000 daltons. As a result the protein concentration in the sample was reduced to 30% and the enzyme activity was increased to more than 4.4 fold. Further purification was attempted by Celite 545 column chromatography and gel filtration such as Sephadex G 50, G 75 and G 100, and finally by gel filtration with Sephacryl S-200. Among these treatments, Sephacryl S-200 column chromatography yielded the highest activity of shrimp PPO. The protein concentration was reduced to more than 64% of original concentration, while the PPO activity was increased 7.74 fold. It is common in enzyme purification techniques to lose some of the enzyme activity, therefore the final recovery was 54.3% of the original enzyme activity. This result was close to the 56.6% recovery by Fairs (1982).

In the fractionation of the sample by Sephacryl S-200 gel filtration chromatography, there were three protein peaks (Figure 1). The shrimp PPO activity was found in the first peak (fraction numbers 5, 6, 7, and 8). These fractions were pooled and used for the enzyme kinetics study.

Various phenolic compounds were tested as a substrate for shrimp PPO activity. These substrates were L-DOPA, DL-DOPA, catechol, dopamine, and L-tyrosine. Table 2 shows the values of K_m and V_{max} of phenolic compounds on the

shrimp PPO activity. The shrimp PPO activity reacted differently with these compounds. The poorest substrate was L-tyrosine because the oxidation of L-tyrosine was very slow in the presence of shrimp PPO and as a result the colour development was slow too. Therefore, it was difficult to determine the K_m and V_{max} values. Dopamine and catechol gave better results because they reacted rapidly and developed colour with shrimp PPO, and as a result the K_m and V_{max} were determined more easily. The shrimp PPO had higher affinity for catechol than for dopamine demonstrated by lower K_m for catechol and higher V_{max} . The best activity of shrimp PPO was obtained by L-DOPA and DL-DOPA respectively. Both yielded similar results but L-DOPA had a lower K_m and a higher V_{max} , indicating that L-DOPA was the best substrate and therefore was selected as the substrate utilized in this study.

The relative activity of shrimp PPO as a function of pH (Figure 2) showed that maximum enzyme activity was reached in pH range of 6.0-6.5. The activity declined slightly at pH 7.0 and significantly at pH values above 7.5 and below 5.5. The shrimp PPO activity was not tested above pH 8.0 because of the rapid auto-oxidation of the substrate L-DOPA in an alkaline environment. Under alkaline conditions, a dark color due to non-enzymatic oxidation occurred with the L-DOPA before the shrimp PPO enzyme

extract was added.

Figure 3 shows the stability of shrimp PPO activity after 10 min of exposure to a different temperature at PH 6.2. The results showed that the enzyme remained fully active between 25 °C and 35 °C. The enzyme activity slowly decreased between 35 °C and 40 °C, dropped sharply above 40 °C, and was completely inactivated at 70 °C .

Table 3 shows the activity of GOX/CAT at different concentrations and the absorbance/min for each of these concentrations by using the ABTS/POD assay. As the GOX/CAT concentration was increased there was an increase in the absorbance/min and in color development. Therefore, the GOX/CAT enzyme system was demonstrated to be effective and was as an inhibitor fro mushroom and shrimp PPO activity.

Lineweaver-Burk plot of shrimp PPO activity (Figure 4 and Table 4) show the shrimp PPO activity with various concentrations of the substrate L-DOPA in the absence of GOX/CAT as inhibitor. When GOX/CAT was absent, the shrimp PPO showed a full activity with a Vmax value of 9.8 uM/min and Km of 4.17 mM. When GOX/CAT was added at a concentration of 0.167 units/ml, the shrimp PPO activity was reduced to more than 64% of its original activity and Km was reduced to 3.33 mM. When the GOX/CAT concentration was doubled (0.33 units/ml), the shrimp PPO activity was decreased to 74% of original activity and Km was reduced

to 2.5 mM. The GOX/CAT enzyme system thus had an inhibiting effect on shrimp PPO. The inhibition was not competitive because a constant V_{max} was not reached and was noncompetitive because the K_m value was not constant. Also, the inhibition was not considered uncompetitive because the plot lines were not parallel. The result may indicate a mixed inhibition of noncompetitive and uncompetitive of shrimp PPO with GOX/CAT enzyme system as inhibitor.

The effect of D-glucose concentration on the activity of GOX as an inhibitor for shrimp and mushroom PPO was studied. The effect of concentrations of 0.5, 1.0, 2.0, 3.0, and 4.0% with GOX activity of 2 units/ml are shown Figure 5. Without the use of GOX/CAT enzyme as inhibitor, the shrimp and mushroom PPO were at full activity. When GOX/CAT was added, the activities of shrimp and mushroom PPO were reduced to the minimum at 0.5% glucose, and the inhibition was almost the same for all concentrations up to 4%. There was essentially no difference in the effect of D-glucose concentration on the activity of shrimp or mushroom PPO. Therefore, there was no need to use higher concentrations of D-glucose to obtain greater inhibition. When GOX/CAT was added to the PPO assay without substrate (D-glucose), PPO activity did not decrease. Therefore, the D-glucose was required as a substrate for GOX/CAT enzyme system to give the inhibitory

effect on shrimp and mushroom PPO activity.

The effect of different concentrations of the GOX/CAT enzyme system on shrimp and mushroom PPO is shown in Figure 6; the D-glucose concentration was constant at 0.5%. At the lowest concentration of GOX/CAT, 0.2 units/ml, the inhibition was more than 40% and 50% for shrimp and mushroom PPO, respectively. At 2.0 units/ml, the inhibition increased to more than 90% and complete inhibition was reached at a concentration of 6.0 units/ml.

CONCLUSION

This is the first investigation of the GOX/CAT enzyme system as an inhibitor of shrimp PPO in extracts. The extraction, purification, ultrafiltration, and gel column chromatography of shrimp PPO yielded a 7.74-fold purification. The shrimp PPO showed a higher activity with L-DOPA than with DL-DOPA as substrate. The optimum pH for shrimp PPO activity was 6.0-6.5 and the thermal stability was between 25 and 35 °C.

Lineweaver-Burk plots of L-DOPA concentration versus PPO activity showed that the Km values were 4.17 mM for shrimp PPO. Varying the concentration of D-glucose substrate for GOX/CAT over the range of 0.5 to 4.0% had no significant effect on the activity of either shrimp or mushroom PPO. GOX/CAT at a concentration of 2 units/ml with 0.5% D-glucose inhibited more than 90% of the PPO activity, while at 6 GOX/CAT units/ml the shrimp and mushroom PPO activity were completely inhibited.

Finally, the GOX/CAT enzyme system strongly inhibited shrimp PPO extract. Since this enzyme is considered Generally Recognized As Safe (GRAS) by the FDA, it may be used with foods instead of sulfite agents, which can cause asthmatic effects in some people.

Table 1

Summary of purification procedures for shrimp PP0.

<u>Extraction steps</u>	<u>Volume (ml)</u>	<u>Conc. (mg/ml)</u>	<u>Total (mg)</u>	<u>Conc. (u/ml)</u>	<u>Specific activity (u/mg)</u>	<u>Total (units)</u>	<u>Recovery (%)</u>	<u>Purification fold</u>
Crude (acetone powder)	500	1.08	540	0.67	0.62	335	100	1.0
* UF	100	0.75	75	2.05	2.73	205	61.19	4.41
Sephacryl S-200	10	0.38	38	18.2	4.49	182	54.32	7.74

* Amicon DC ultrafiltration chamber with an H1 P100 hollow-fiber cartridge membrane (MW cut off 100,000).

Table 2

The effect of different substrates on shrimp PPO activity *

<u>Substrate</u>	<u>Km (mM)</u>	<u>Vmax (uM/min)</u>
L-DOPA	4.17	9.80
DL-DOPA	4.60	9.20
Catechol	5.20	4.70
Dopamine	6.10	3.80
L-tyrosine	0.0	0.0

*Assay condition:

In each cuvette: 1.35 ml 0.02M phosphate buffer, pH 6.2; 1.45 ml 0.01M L-DOPA; and 0.2 ml shrimp PPO extract. Total volume of 3.0 ml, at 25 °C and 475 nm.

Table 3

The activity of GOX/CAT at different concentrations vs. the absorbance/min by using ABTS/POD Assay.*

<u>GOX/CAT</u> <u>(units/ml)</u>	<u>Absorbance/min</u> <u>at 475 nm</u>
0.2	0.15
0.4	0.34
0.6	0.48
0.8	0.56
1.0	0.68

* Assay condition:

In each cuvette: 2.5 ml of ABTS/phosphate buffer mixture, pH 7.0, 0.3 ml of 10% D-glucose, 0.1 ml peroxidase, and 0.1 ml GOX/CAT, at 25°C, the total volume of 3.0 ml.

Table 4

The Km and Vmax values of shrimp PPO activity in the presence and absence of GOX/CAT as inhibitor.*

	<u>Without GOX/CAT</u>	<u>With GOX/CAT</u>	
	<u>Substrate (only)</u>	<u>0.167 units/ml</u>	<u>0.33 units/ml</u>
<u>Km (mM)</u>	4.17	3.3	2.5
<u>Vmax (uM/min)</u>	9.8	3.47	2.5

* Assay condition:

In each cuvette: 1.35 ml of 0.02M phosphate buffer, pH 6.2; 1.45 ml of 0.01M of L-DOPA; and 0.2 ml shrimp PPO extract. The total volume was 3.0 ml, at 25°C and 475 nm.

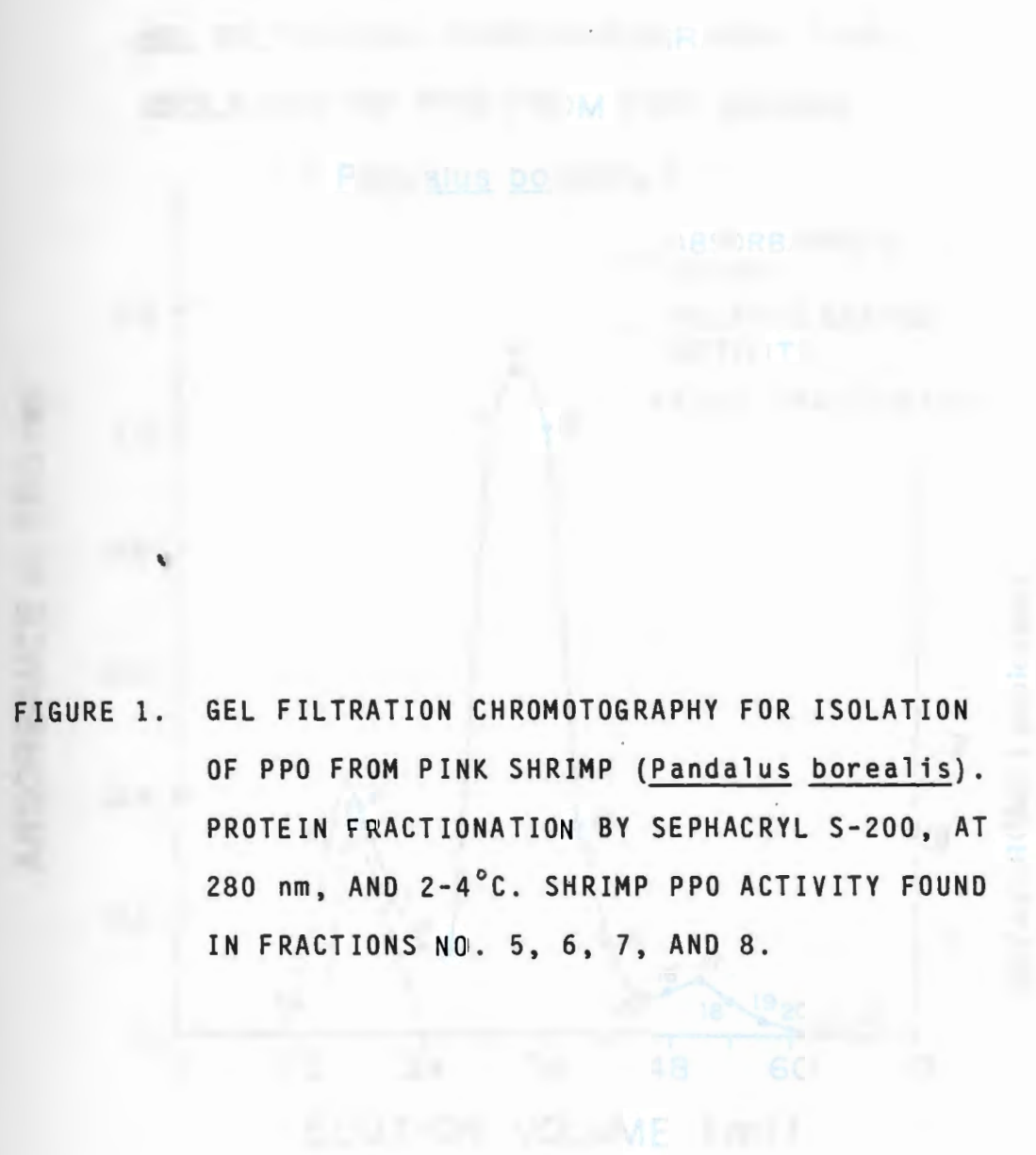
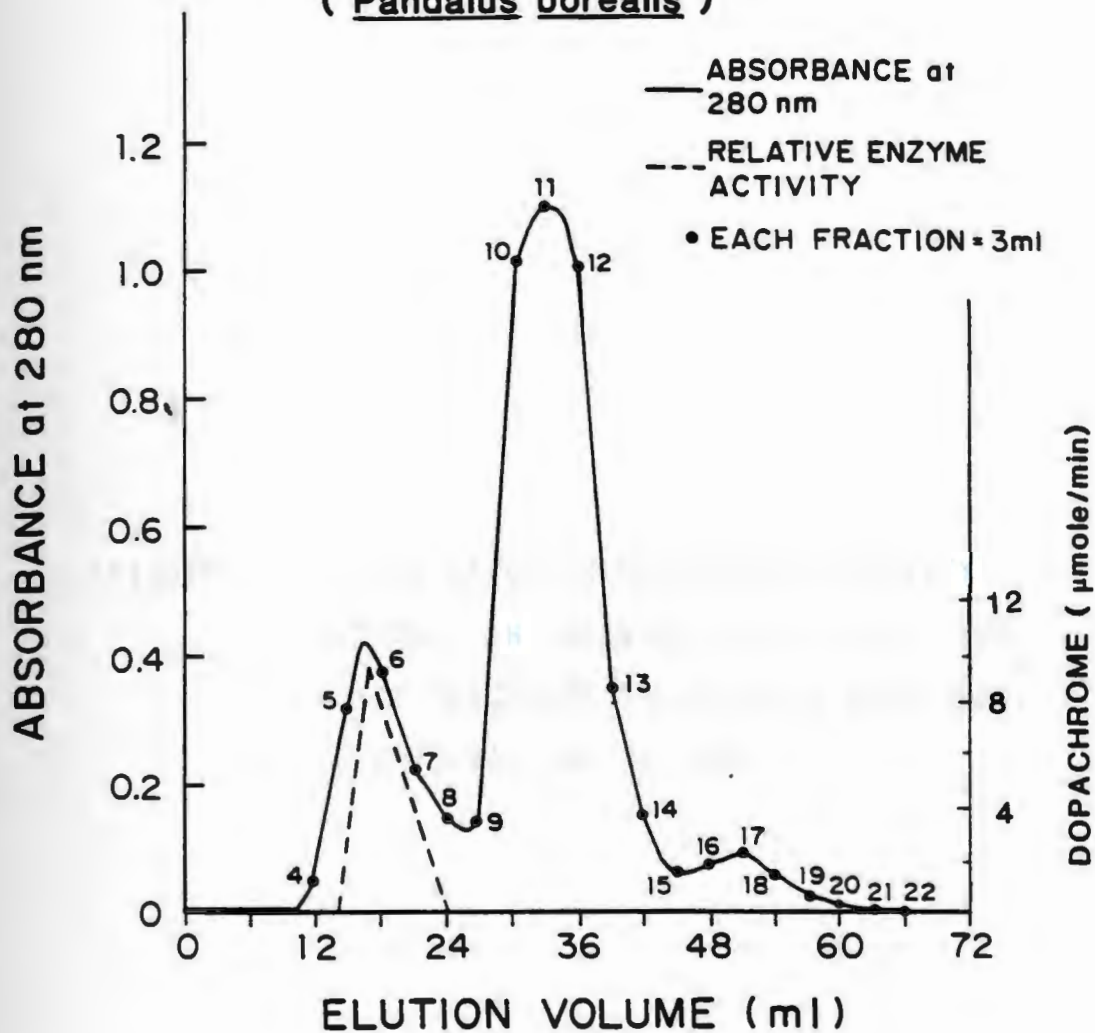


FIGURE 1. GEL FILTRATION CHROMATOGRAPHY FOR ISOLATION OF PPO FROM PINK SHRIMP (Pandalus borealis). PROTEIN FRACTIONATION BY SEPHACRYL S-200, AT 280 nm, AND 2-4°C. SHRIMP PPO ACTIVITY FOUND IN FRACTIONS NO. 5, 6, 7, AND 8.

GEL FILTRATION CHROMATOGRAPHY FOR ISOLATION OF PPO FROM PINK SHRIMP

(Pandalus borealis)



EFFECT OF pH ON SHRIMP PPO ACTIVITY

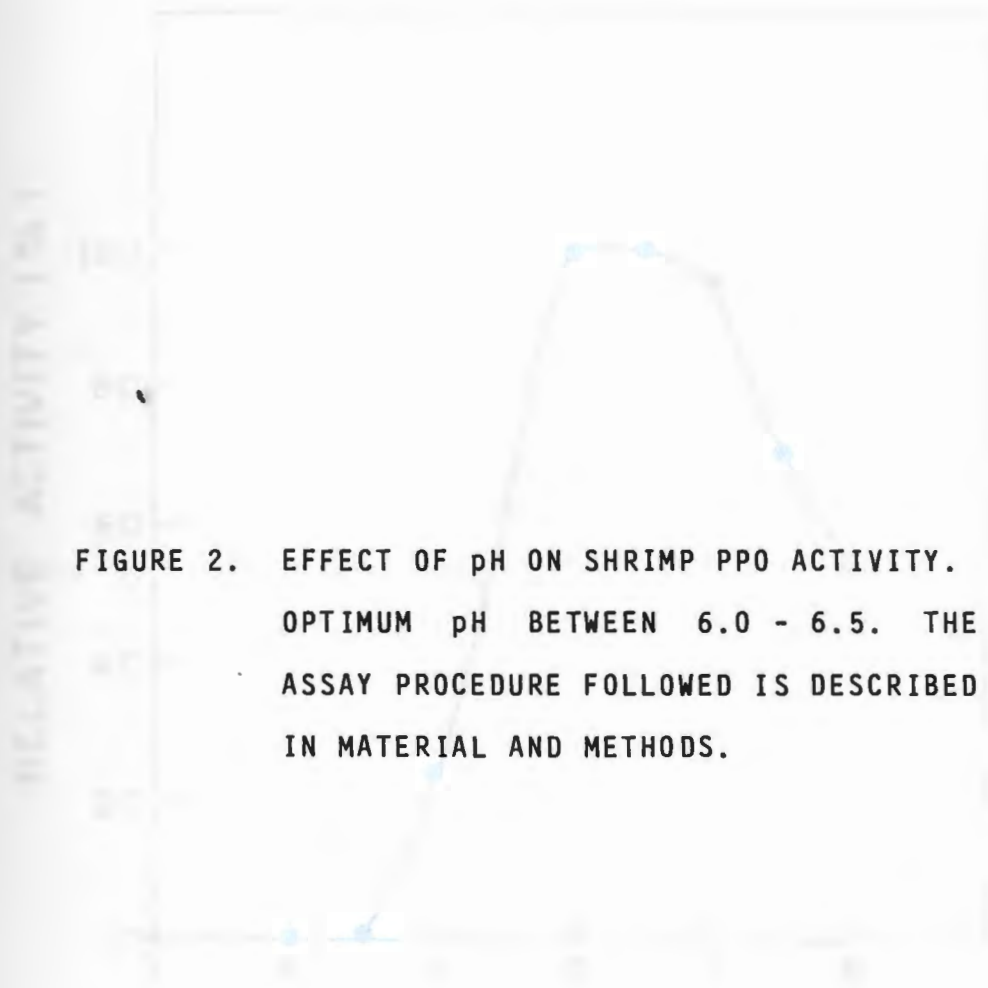
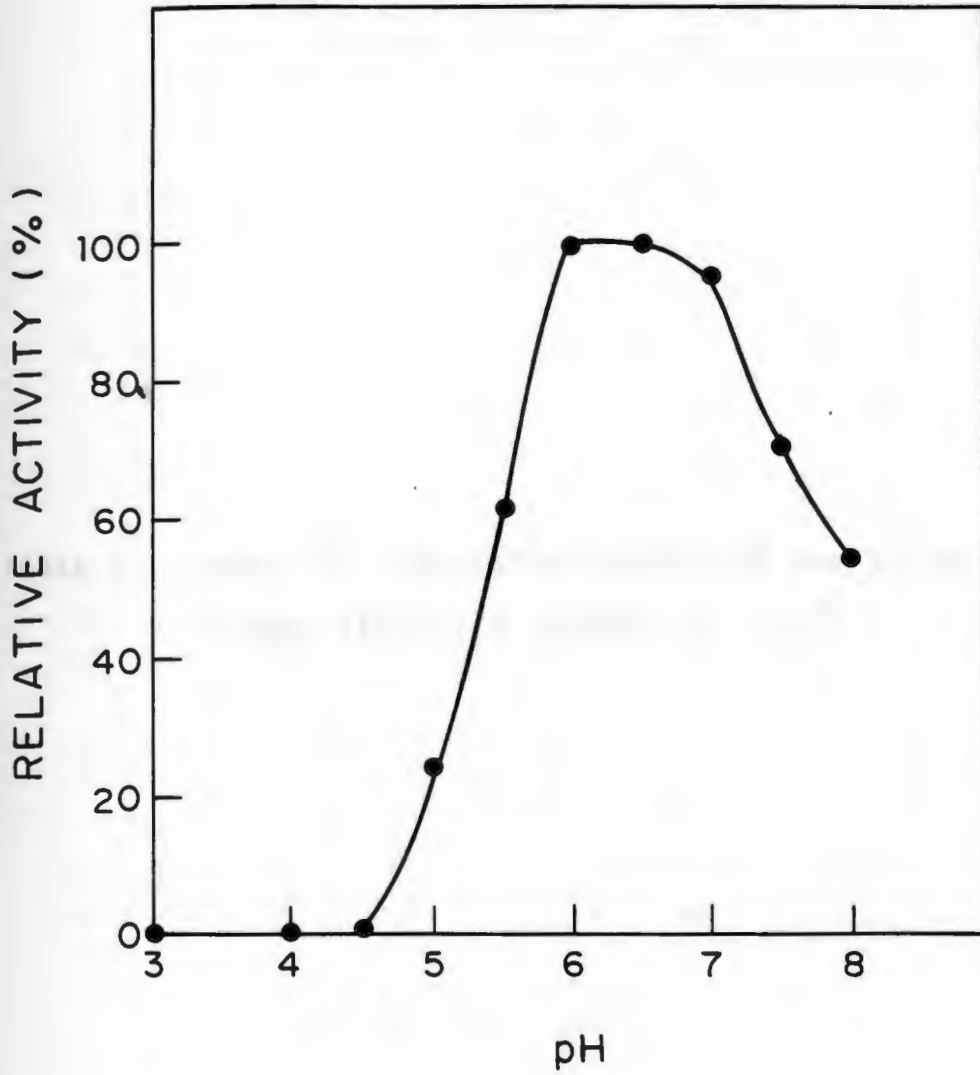


FIGURE 2. EFFECT OF pH ON SHRIMP PPO ACTIVITY. OPTIMUM pH BETWEEN 6.0 - 6.5. THE ASSAY PROCEDURE FOLLOWED IS DESCRIBED IN MATERIAL AND METHODS.

EFFECT OF pH ON SHRIMP PPO ACTIVITY



EFFECT OF TEMPERATURE
ON SHRIMP PPO ACTIVITY

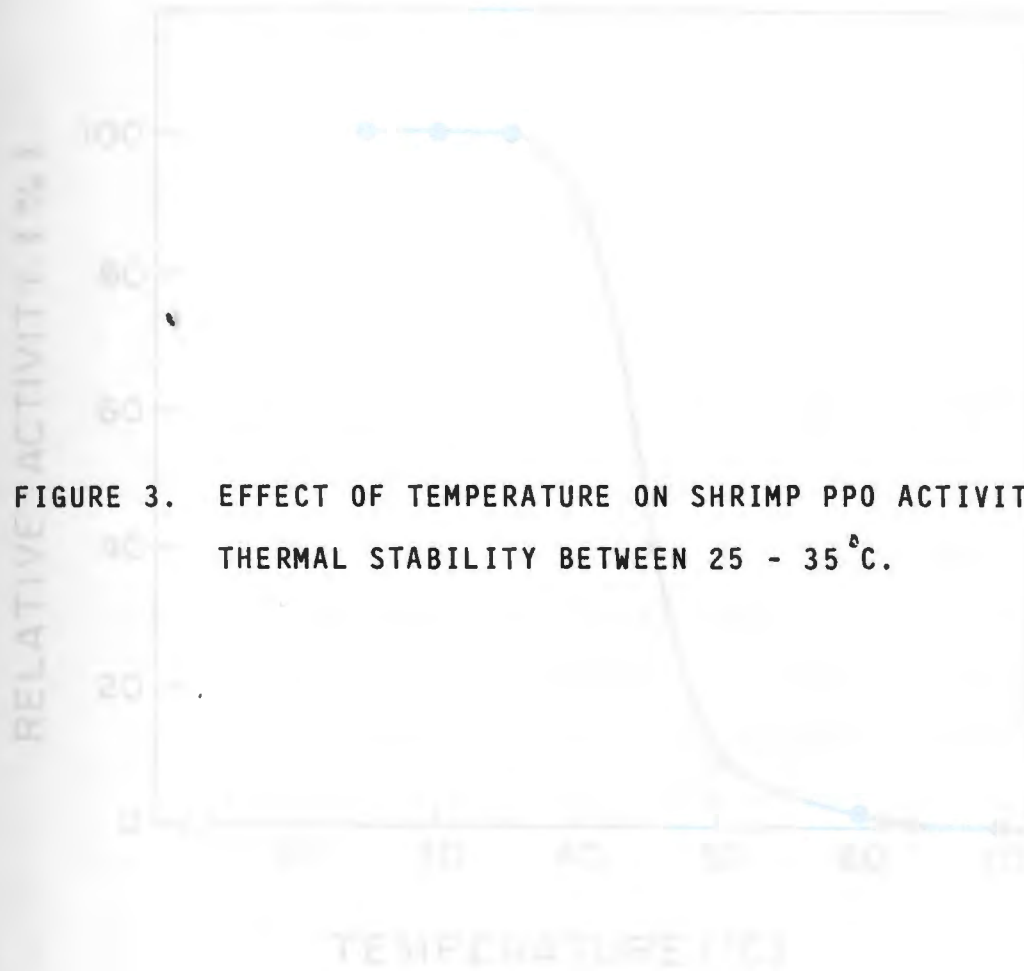
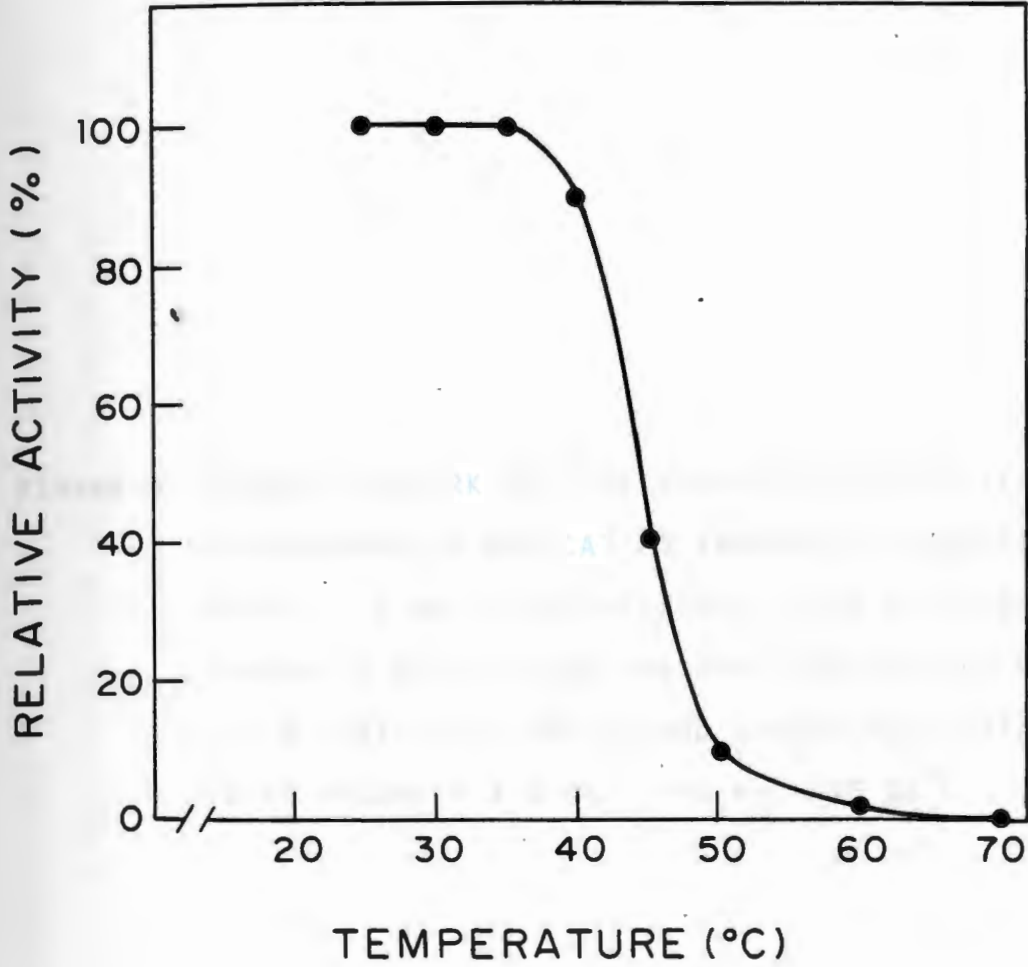


FIGURE 3. EFFECT OF TEMPERATURE ON SHRIMP PPO ACTIVITY.
THERMAL STABILITY BETWEEN 25 - 35 °C.

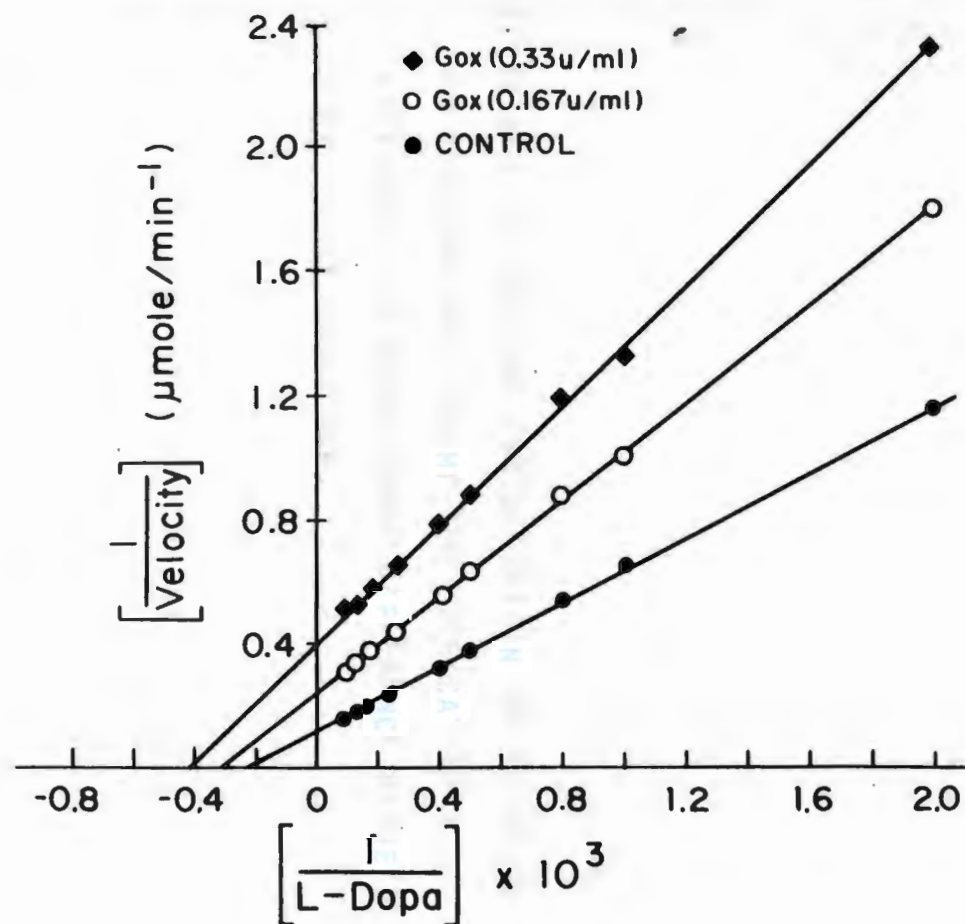
EFFECT OF TEMPERATURE ON SHRIMP PPO ACTIVITY



LINEWEAVER-BURK PLOT OF SHRIMP PPO ACTIVITY
IN PRESENCE OF GOX/CAT AS INHIBITOR

FIGURE 4. LINEWEAVER-BURK PLOT OF SHRIMP PPO ACTIVITY
IN PRESENCE OF GOX/CAT AS INHIBITOR. GOX/CAT
ASSAY: 1.0 ML (0.5%) D-GLUCOSE, 0.9 ML OF 0.01 M
L-DOPA, 0.8 ML 0.02M PHOSPHATE BUFFER (pH 6.2),
0.1 ML GOX/CAT, AND 0.2 ML SHRIMP PPO EXTRACT.
TOTAL VOLUME = 3.0 ML , 475 nm, AND 25 °C.

LINWEAVER-BURK PLOT OF SHRIMP PPO ACTIVITY IN PRESENCE OF GOX AS INHIBITOR



EFFECT OF GLUCOSE CONCENTRATION ON ACTIVITY
OF MUSHROOM AND SHRIMP PPO
(GOX/CAT IS 2 UNITS/ML)

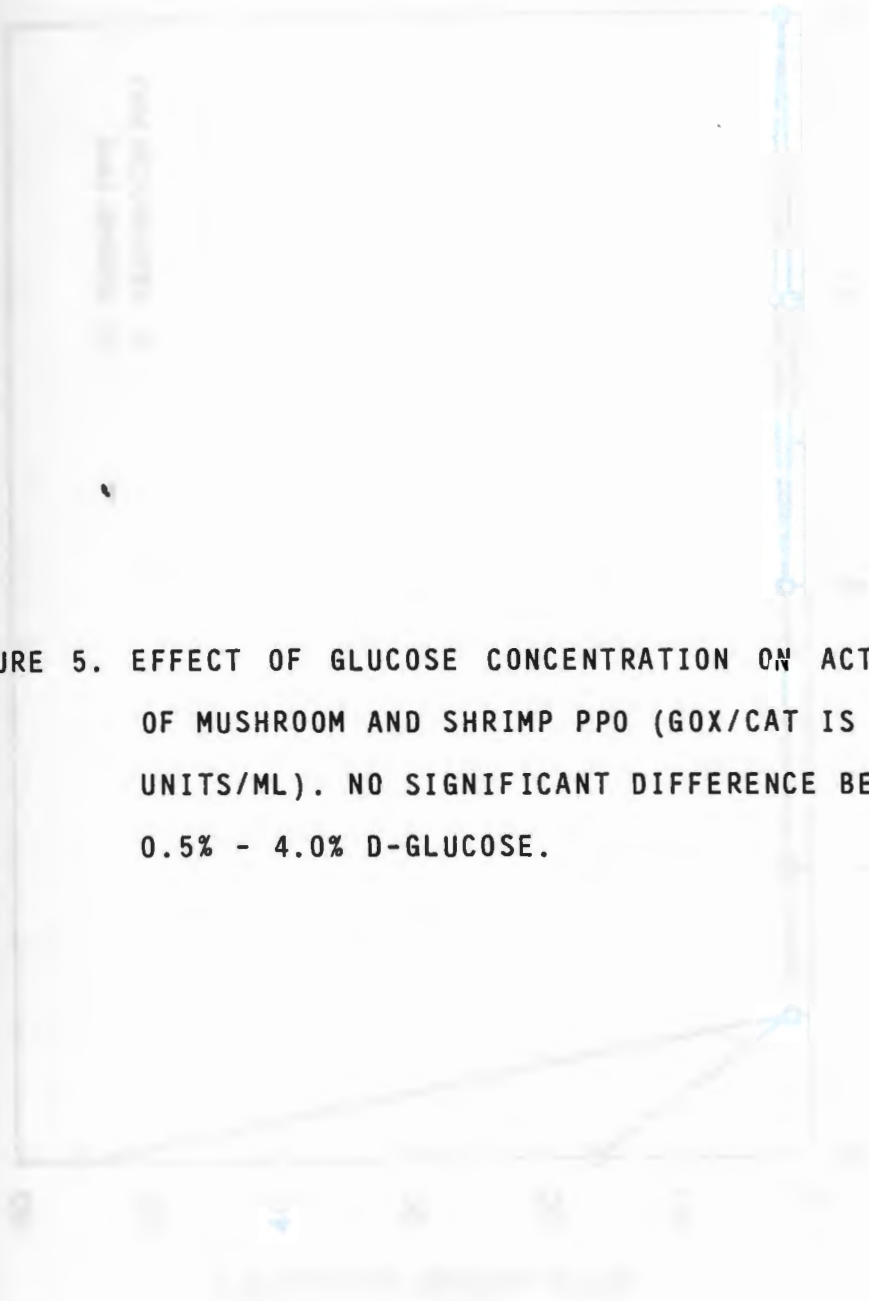
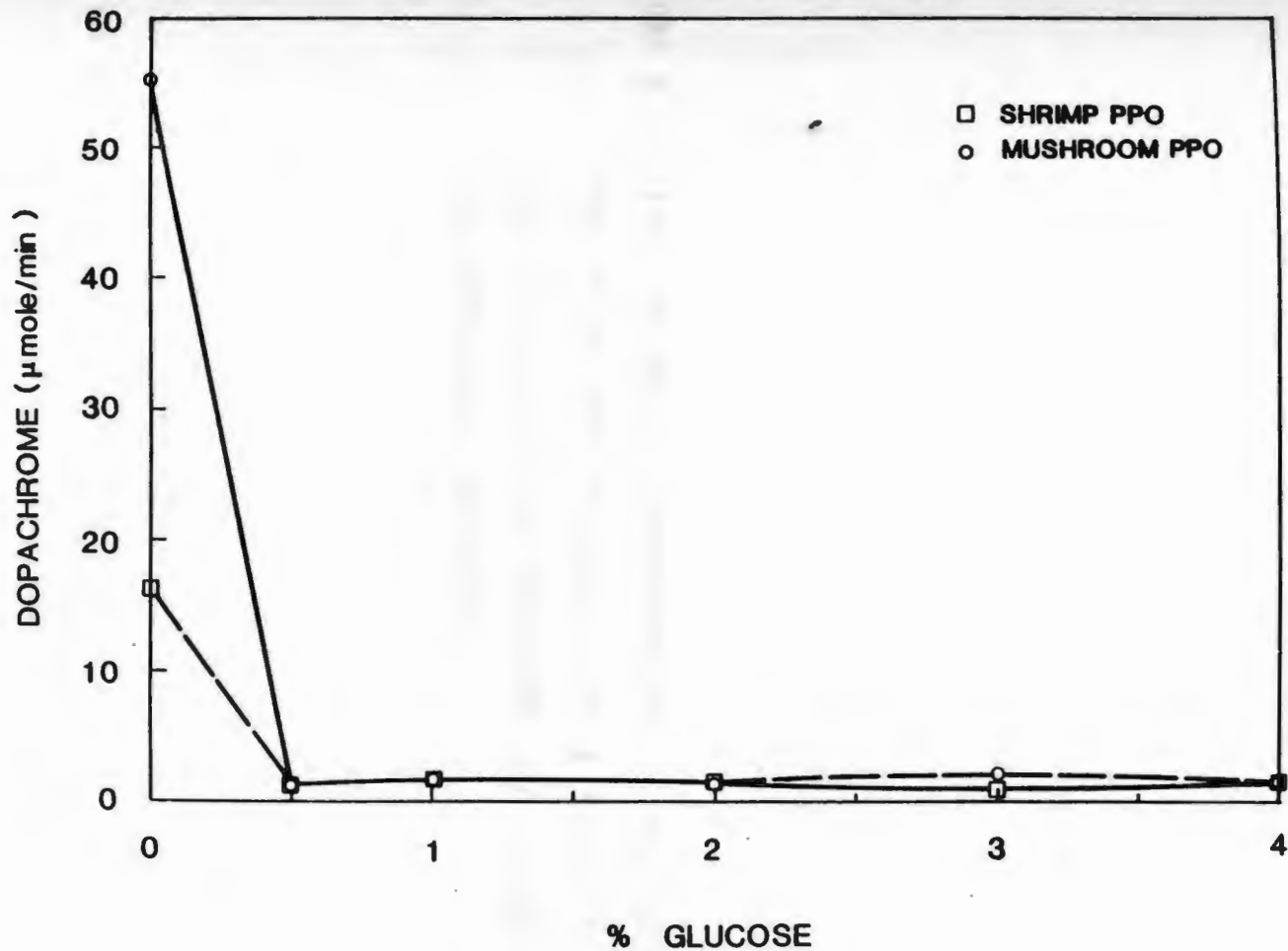


FIGURE 5. EFFECT OF GLUCOSE CONCENTRATION ON ACTIVITY OF MUSHROOM AND SHRIMP PPO (GOX/CAT IS 2 UNITS/ML). NO SIGNIFICANT DIFFERENCE BETWEEN 0.5% - 4.0% D-GLUCOSE.

EFFECT OF GLUCOSE CONCENTRATION ON ACTIVITY
OF MUSHROOM AND SHRIMP PPO
(GOX/CAT IS 2 UNITS/mL)



EFFECT OF GOX/CAT CONCENTRATION ON
MUSHROOM AND SHRIMP PPO ACTIVITY

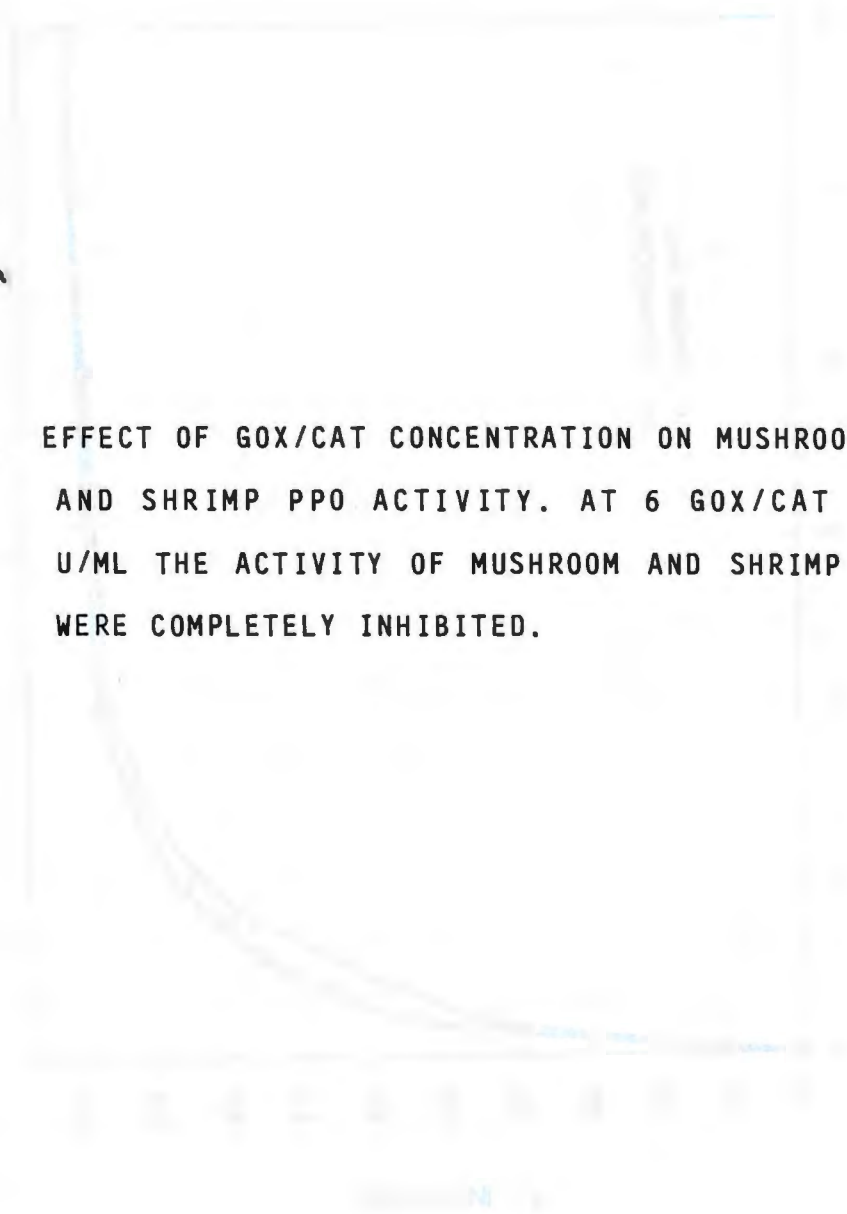
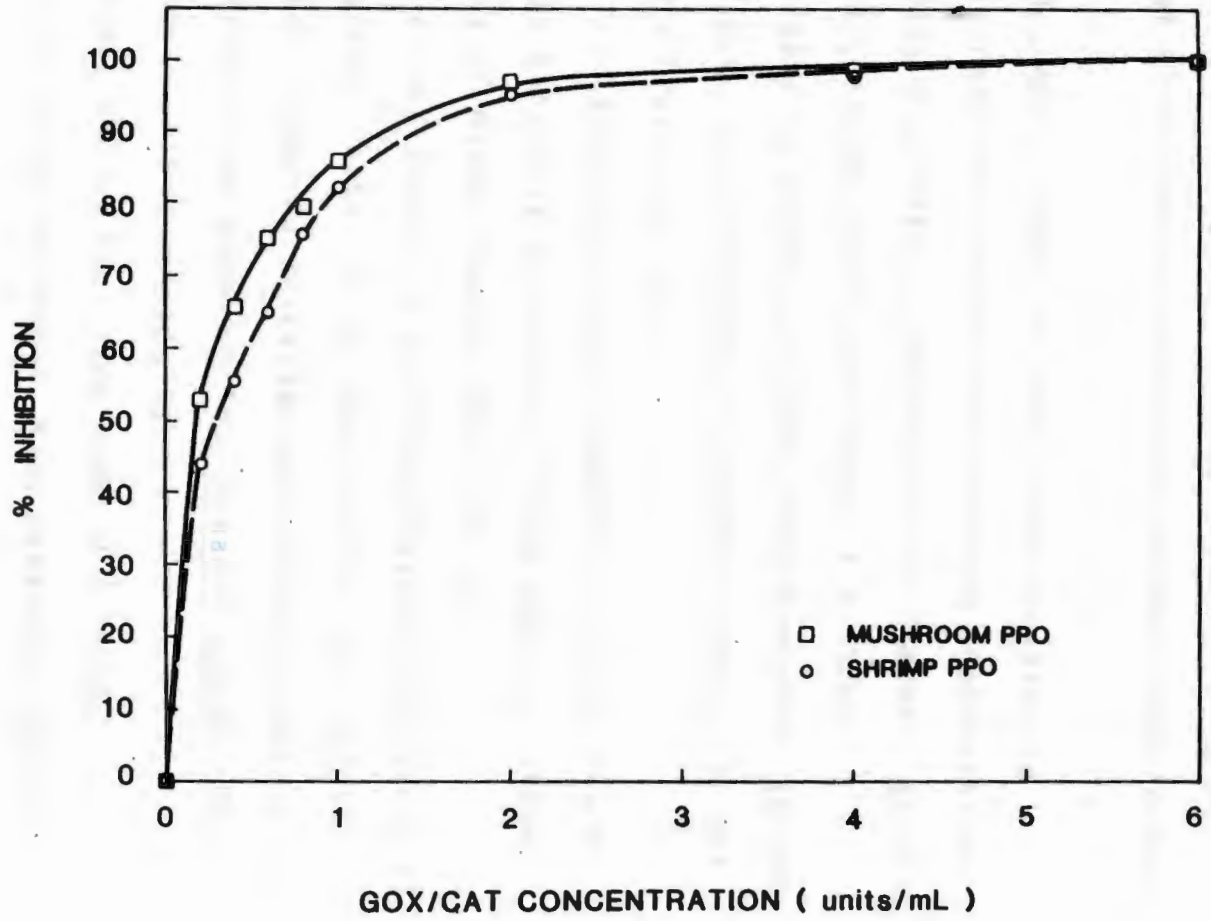


FIGURE 6. EFFECT OF GOX/CAT CONCENTRATION ON MUSHROOM AND SHRIMP PPO ACTIVITY. AT 6 GOX/CAT U/ML THE ACTIVITY OF MUSHROOM AND SHRIMP PPO WERE COMPLETELY INHIBITED.

EFFECT OF GOX/CAT CONCENTRATION ON MUSHROOM AND SHRIMP PPO ACTIVITY



REFERENCES

- Alford, J.A., and Fieger, E.A. 1952. The nonmicrobial nature of black spot on ice-packed shrimp. Food Technol. 6:217.
- Allen, D.H., and Collett, P. 1981. Life-threatening asthmatic reactions to the food and drug preservative, sodium meta-bisulfite. J. Allergy Clin. Immunol. 67:71.
- Bailey, M.E., Fieger, E.A., and Novak, A.F. 1960. Phenoloxidase in shrimp and crab. Food Research. 25:565.
- Bergmeyer, H.U. 1974. Methods of Enzymatic Analysis. Vol 3. Academic Press. Inc. N.Y.
- Camber, C.I., Vance, N.H, and Alexander, J. 1956. How to use sodium bisulfite to control "black spot" on shrimp. University of Miami. Special Bull. No. 12.
- Chubey, B. B. and Dorell, G.D. 1972. Enzymatic browning of stored parsnip roots. J. Am. Soc. Hortic. Sci. 97:107.
- Farias, C.F.M. 1982. Purification and characterization of phenol oxidase from brown shrimp (Penacus azeus). Ph.D. Thesis, Texas A & M University.
- Faulkner, M.B., Watts, B.M., and Humm, H.J. 1954. Enzymatic darkening of shrimp. Food Research. 19:302-310.

- Finne, G., and Migget, R. 1985. The use of sulfiting agents in shrimp and crab. *Food Research* 25:565.
- Fling, M., Horowitz, N.H., and Heineman, S.F. 1963. The isolation and properties of crystalline tyrosinase from Neurospora. *J. Biol. Chem.* 238:2045.
- International Union of Biochemistry, 1972 Standing Committee on Enzymes, Enzyme Nomenclature Recommendations. Elsevier, Amsterdam.
- Lindsay, R.C. 1976. Other desirable constituents of food. In Principles of Food Science. Ed. by Fennema, O.R. Marcel Dekker Inc. New York.
- Loomis, W.D., and Battaile, J. 1966. Removal of phenolic compounds and the isolation of plant enzymes. *Phytochemistry* 5:324.
- Lowry, O.H., Rosenbrough, N.J., Farr, A.L., and Randall, R.J. 1951. Protein measurement with folin phenol reagent. *J. Biol. Chem.* 193:265.
- Mason, H.S. 1957. Mechanism of oxygen metabolism. *Advan. Enzymol.* 19:128.
- Nickelson, R., and Cox, B., 1986. Stop shrimp "black spot". *Marine Advisory Bull., Fisheries. Texas Agric. Extension Service. Sea Grant Program. Texas A & M University.*
- Pefferi, P. G., Baldassari, L., and Cultera, R. 1974. Inhibition by carboxylic acids of an o-diphenol oxidase from *prunus aviam furits*, *J. Sci. Food Agric.* 25:263.

- Reed, G. 1975. Oxidoreductases In Enzymes in Food Processing. 2nd edition. Academic Press, New York.
- Searle Biochemics. 1966. Glucose oxidase: Properties and application to foods. Technical Bulletin No. 2.
- Stevenson, D.D., and Simon, R.A. 1981. Sensitivity to ingested metabisulfites in asthmatic subjects. J. Allergy Clin. Immunol. 68:26.
- Thomas, P. and Janave, M.T. 1973. Polyphenol oxidase activity and browning of mango fruits induced by gamma irradiation. J. Food Sci. 38:1149.
- Werth, G.R. 1982. Inhaled metabisulfite sensitivity. J. Allergy Clin. Immunol. 70:143.
- Worthington Enzymes and related biochemical. 1982. Biochemical Products Division. Worthington Diagnostic Systems Inc. Freehold, N.J.

GENERAL

The enzyme system of mushroom and shrimp peroxidase (GOX/CAT) was studied in the presence of mushroom peroxidase (MPO) from mushroom and shrimp peroxidase (SPO).

The substrates used were hydrogen peroxide and various substrates studied for 1000 minutes. The substrates used were mushroom peroxidase (MPO), shrimp peroxidase (SPO), and mushroom peroxidase (MPO).

MANUSCRIPT II.

INHIBITION OF MUSHROOM AND SHRIMP PPO BY SUBSTRATES AND END PRODUCTS OF THE GOX/CAT ENZYME SYSTEM.

The effect of mushroom and shrimp peroxidase on the activity of GOX/CAT was studied. The substrates used were hydrogen peroxide and various substrates.

Mushroom and shrimp peroxidase activity was studied in the presence of hydrogen peroxide and various substrates. The substrates used were hydrogen peroxide and various substrates.

The effect of mushroom and shrimp peroxidase on the activity of GOX/CAT was studied. The substrates used were hydrogen peroxide and various substrates. The substrates used were hydrogen peroxide and various substrates.

ABSTRACT

The enzyme system of glucose oxidase and catalase (GOX/CAT) was studied as an inhibitor of polyphenol oxidase (PPO) from mushroom and shrimp (Al-Jassir, 1987).

The substrates and end products of GOX/CAT were studied for their potential to inhibit mushroom and shrimp PPO. The substrates were D-glucose and oxygen. The end products were glucono-lactone, gluconic acid, and hydrogen peroxide. Each of these substances was applied in vitro to the enzyme assay of mushroom and shrimp PPO, without using GOX/CAT, except for oxygen, for which GOX/CAT was used at a concentration of 2 units/ml with 0.5% D-glucose as the substrate.

Mushroom and shrimp PPO activity were inhibited completely by the GOX/CAT end products hydrogen peroxide and gluconic acid at concentrations of 2.0 mg/ml, while glucono-lactone showed a weak inhibition of less than 7.6% and 9.0%, respectively.

Oxygen consumption by mushroom and shrimp PPO was measured during the 2 min of the experiment. Oxygen removal reached 2.05 ppm with mushroom PPO and 1.0 ppm with shrimp PPO. However, when GOX/CAT was added, oxygen removal increased rapidly to 7.5 and 7.48 ppm, with 96.2% and 95.9% of the oxygen consumed during the 2 min for mushroom and shrimp PPO, respectively. D-glucose showed no inhibitory effect on the activity of mushroom and shrimp PPO even at a concentration of 5.0 mg/ml.

INTRODUCTION

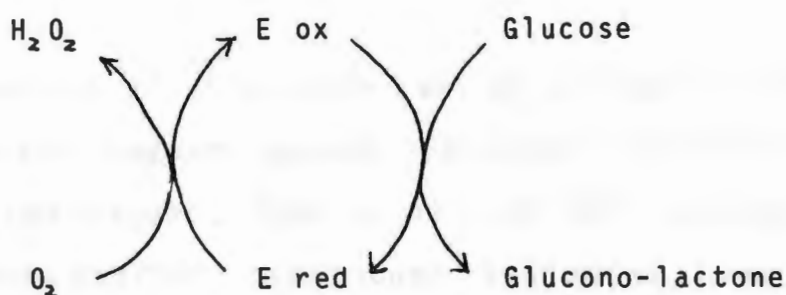
Shrimp melanosis (black spot) is a harmless but objectionable surface discoloration of the shrimp shell caused by the enzyme polyphenol oxidase (PPO), which remains active during refrigeration or ice storage (Otwell and Marshall, 1986). To inhibit melanosis, the United States Food and Drug Administration (US-FDA) permits a one-minute dip in a 1.25% solution of sodium sulfite as Current Good Manufacturing Practice (CGMP) (Finne et al., 1986).

Recently, however, the safety of sulfites has been questioned. Strong asthmatic reactions to bisulfite and other sulfite agents have been reported (Simon et al., 1982; Werth, 1982). Therefore, alternatives must be found to replace or reduce the amount of sulfites required to inhibit shrimp melanosis.

The enzyme system of glucose oxidase and catalase (GOX/CAT) has been used as a food additive and is considered Generally Recognized As Safe (GRAS) (Priputina, 1974; Searle Biochemics, 1966). GOX/CAT has been used to remove oxygen and prevent oxidation in food products, such as beer (Ohlmeyer, 1957), apple wine (Yang, 1955), and canned soda (Underkofler, 1961); to

protect animal fat from oxidation (Reed, 1975); and to protect water and oil emulsions such as mayonnaise (Bloom et al., 1956). GOX/CAT, has also been used to remove glucose from egg albumin and whole egg prior to drying (Baldwin et al., 1953; Scott, 1953). GOX/CAT has been found to extended the shelf life of fresh fish by inhibiting spoilage (Field, 1986).

The general reaction of GOX/CAT as proposed by Rand and Hourigan (1974) is as follows:



with glucose as the substrate, the oxidized form of the enzyme reacts with the sugar molecule. This produces a molecule of glucono-lactone (which spontaneously hydrolyzes in water to gluconic acid) and a reduced form of the enzyme. For the enzyme to continue to function as a catalyst, the oxidized form must be regenerated. This can be achieved when the enzyme reacts with molecular oxygen forming hydrogen peroxide. The activity of an oxidoreductase enzyme is normally dependent on the oxygen solubility and rate of diffusion within the system. This

relationship can be extended one step further to assure a source of oxygen to regenerate continuously the GOX enzyme by incorporating catalase and hydrogen peroxide into the system. Since catalase catalyzes decomposition of hydrogen peroxide into molecular oxygen, this will assure immediate regeneration of the oxidoreductase enzyme (Rand and Hourigan, 1974). The reactions of GOX/CAT continue to recycle until glucose is entirely consumed. The GOX/CAT enzyme system could theoretically be inhibited by its end products glucono-lactone, gluconic acid, and hydrogen peroxide.

The objective of this study was an attempt to learn how the GOX/CAT enzyme system inhibited mushroom and shrimp PPO (Al-Jassir, 1987). All of the substances involved with GOX/CAT reactions: D-glucose, oxygen, glucono-lactone, gluconic acid, and hydrogen peroxide, were tested for their inhibitory effect on PPO.

MATERIALS AND METHODS

Shrimp samples

Fresh, untreated pink shrimp (Pandalus borealis) were obtained from West Boothbay Harbor, Maine; and arrived in the laboratory one day after harvest. The extraction of shrimp PPO was performed as previously described (Al-Jassir, 1987).

Reagents

A commercial enzyme preparation GOX/CAT containing glucose oxidase (EC 1.1.3.4; β -D-glucose oxidoreductase) and catalase (EC 1.11.1.6; hydrogen peroxide: hydrogen oxidoreductase) Ovazyme, containing 1500 units/ml was provided by Finnsugar Biochemical, Inc. (Elk Grove Village, IL) and used throughout this study. D-glucose (anhydrous; reagent grade) was the substrate for GOX/CAT.

Mushroom PPO (EC 1.14.18.1; monophenol, dihydroxyphenyl-alanine: O₂ oxidoreductase; 4800 units/mg) was obtained from Sigma Co. (St. Louis, MO). L-DOPA (L- β -3,4- dihydroxyphenylalanine) was used as a substrate for mushroom and shrimp PPO.

Glucono-lactone (purified; Delta) and hydrogen peroxide (50%) were used in this study. Sodium phosphate

[monobasic (pH 4.3) and diabasic (pH 9.5)] was used as a buffer for the enzyme assay. These substances were obtained from Fisher Scientific (Fairlawn, N.J).

Assay of mushroom and shrimp PPO

Polyphenol oxidase (PPO) activity was measured spectrophotometrically using a Beckman DU-8B UV-visible spectrophotometer. Details of the enzyme assay procedure were described by Al-Jassir (1987).

PPO assay with GOX/CAT

The GOX/CAT enzyme system was examined in vitro for its effect as an inhibitor of mushroom and shrimp PPO. GOX/CAT was used at a concentration of 2 units/ml with 0.5% D-glucose as the substrate. The procedure was described in detail by Al-Jassir (1987).

PPO assay with D-glucose, glucono-lactone, gluconic acid, or hydrogen peroxide

D-glucose and glucono-lactone solutions were prepared by dissolving the powder in 0.02M phosphate buffer (pH 6.2) with gentle mixing to obtain final concentrations of 1.0, 2.0, 3.0, 4.0, and 5.0 mg/ml. These solutions were tested directly in the assay of mushroom and shrimp PPO. Gluconic acid solutions were prepared from the glucono-lactone powder in the same buffer and at

concentrations between 0.2 and 2.0 mg/ml, but these solutions were allowed to stand for 4 hours before they were added to the enzyme assay to permit conversion to gluconic acid and to establish pH stability. The pH was tested for each of these solutions. Hydrogen peroxide (50%) was prepared in the same buffer and concentrations (0.2 to 2.0 mg/ml) were obtained.

The spectrophotometer was adjusted to 475 nm and the temperature in the sample chamber was controlled at 25 °C. The reaction mixture in each cuvette was 1.0 ml of 0.01M L-DOPA solution, 0.9 ml of 0.02 M phosphate buffer (pH 6.2) and 1.0 ml of glucono-lactone (1.0 to 5.0 mg/ml) or gluconic acid or hydrogen peroxide solutions (0.2 to 2.0 mg/ml) for mushroom PPO, while for shrimp PPO extract the reaction mixture was 1.0 ml of 0.01M L-DOPA solution, 0.8 ml of phosphate buffer (pH 6.2), and 1.0 ml of glucono-lactone or gluconic acid or hydrogen peroxide solutions (0.2 to 5.0 mg/ml).

The mixture was oxygenated by bubbling air into the cuvettes through a capillary tube for 3-5 min. The cuvettes were transferred to the spectrophotometer and monitored at 475 nm for 3-5 min to achieve temperature equilibrium and to establish the blank rate, if any. After stabilization, the cuvettes were removed and 0.1 ml of mushroom PPO solution or 0.2 ml of shrimp PPO solution was added. The cuvettes were immediately covered with fitted

fluoropolymer covers, inverted slowly a few times and returned to the spectrophotometer. The absorbance was recorded every 20 sec for 120 sec.

Oxygen consumption during PPO assay

Oxygen consumption was measured by a YSI Model 51B oxygen meter, obtained from YSI Scientific (Yellow Springs Instruments Co., Yellow Springs, Ohio). This instrument was calibrated according to manufacture's instructions as follows: (The mechanical zero was adjusted while the instrument was turned off. The instrument was turned on and the zero switch was adjusted to zero on the concentration scale. The full scale was adjusted to 15. The probe or sensor, was then plugged into the instrument allowed to polarize for 10 to 15 min. The oxygen calibration switch was adjusted to the local altitude).

A beaker filled with distilled water was mixed with a magnetic stirrer so that the water was continually agitated but without breaking the liquid surface, for about 10 min to attain complete aeration. The stirring was continued and the sensor was immersed in the water so that the stainless steel portion containing the temperature-associated elements was submerged. The sensor was allowed to stabilize for a few minutes and then was switched on the junction switch to percentage saturation. The instrument automatically converted the reading from percentage

saturation to concentration (in ppm), making appropriate corrections for solution temperature via the thermistors in the sensor housing. The switch was then turned to read the oxygen concentration and the oxygen concentration was measured in ppm.

The solutions assayed for oxygen were prepared in a beaker as follows: At room temperature ($25 \pm 2^\circ\text{C}$), the mushroom PPO oxygen assay, 29.0 ml of 0.01M L-DOPA was mixed with 29.0 ml of 0.02M phosphate buffer (pH 6.2). For the shrimp PPO oxygen assay, the solution was 29.0 ml of 0.01M L-DOPA and 27.0 ml of 0.02M phosphate buffer (pH 6.2). Each mixture was stirred gently by a magnetic stirrer until a stable reading of dissolved oxygen was obtained (about 10 min). After stabilization, 2.0 ml of mushroom PPO solution or 4.0 ml of shrimp PPO solution was added. At the same time, the consumption of oxygen was recorded every 20 sec for 120 sec, while each solution was stirred continuously.

The mixtures for the effects of GOX/CAT were prepared as follows: 0.5% D-glucose was dissolved in 0.02M phosphate buffer, pH 6.2, for the mushroom PPO assay, 28.4 ml of 0.01M L-DOPA was mixed with 28.4 ml of 0.02M phosphate buffer-0.5% D-glucose (pH 6.2). For shrimp PPO assay, the mixture was 28.4 ml of 0.01M L-DOPA and 26.4 ml of 0.02M phosphate buffer-0.5% D-glucose (pH 6.2). Each mixture was stirred continuously until a stable reading of

oxygen was reached. After stabilization, 1.2 ml of GOX/CAT (2 units/ml) was added and then either 2.0 ml of mushroom PPO solution or 4.0 ml of shrimp PPO solution. The oxygen concentration was recorded every 20 sec for 120 sec.

RESULTS

The results of the oxygen consumption measurements are shown in Figure 1. The oxygen concentration decreased rapidly in the first 20 sec after the addition of the GOX/CAT solution. This initial decrease was due to the consumption of oxygen by the GOX/CAT solution. After 20 sec the oxygen concentration decreased more slowly and reached a steady state after 120 sec. The oxygen concentration was higher in the shrimp PPO solution than in the mushroom PPO solution. This indicates that the shrimp PPO solution has a higher oxygen consumption rate than the mushroom PPO solution.

RESULTS AND DISCUSSION

Hydrogen peroxide

The effect of hydrogen peroxide on mushroom and shrimp PPO activity is shown in Figures 1, 2, and Table 1. When hydrogen peroxide was added to the mushroom PPO assay, at concentration of 0.2 mg/ml, the enzyme activity (measured as dopachrome concentration) increased from 56.67 to 78.33 $\mu\text{M}/\text{min}$, a 38.22% increase (Figure 1 and Table 1). At the same concentration, hydrogen peroxide increased the shrimp PPO activity from 6.2 to 8.2 $\mu\text{M}/\text{min}$ a 32.2% increase. The PPO activity was probably increased due to liberation of oxygen from thermal decomposition of hydrogen peroxide (Kleppe, 1966); more oxygen was available for the PPO enzyme to use and as a result the activity increased. When the hydrogen peroxide concentration was increased to 0.4 mg/ml, the mushroom PPO activity decreased from 78.33 to 51.11 $\mu\text{M}/\text{min}$, a 9.2% inhibition relative to the control; shrimp PPO activity decreased from 8.2 to 5.2 $\mu\text{M}/\text{min}$ (16.1% inhibition). At higher hydrogen peroxide concentrations, the PPO activity decreased progressively, reaching 71.1% and 79.0% inhibition, respectively, for mushroom and shrimp PPO, at a hydrogen peroxide concentration of 1.0 mg/ml. Complete

inhibition of both mushroom and shrimp PPO activity was achieved at a hydrogen peroxide concentration of 2.0 mg/ml. This inhibition may be due to the ability of hydrogen peroxide to attack certain methionine residues located at or near the active site of the enzyme (Kleppe, 1966). If so, then a change in the configuration of the enzyme active site might have occurred and the substrate L-DOPA could not attach to the active site. Since the GOX/CAT enzyme system has a catalase for removing the hydrogen peroxide by breaking it down to water and oxygen, the hydrogen peroxide has no chance to accumulate in the system. As a result, the hydrogen peroxide will be eliminated rapidly by catalase.

Gluconic acid

Plots of mushroom and shrimp PPO activity at increasing concentrations of gluconic acid (Figures 1, 2, and Table 2) show that the enzyme activity decreased slowly and with increasing concentrations of gluconic acid. At the same time, the pH of the reaction mixture decreased in a somewhat similar pattern. For example, at a gluconic acid concentration of 1.0 mg/ml, the pH decreased from 6.20 to 4.85 and the activity of mushroom and shrimp PPO was inhibited by 32.8% and 42.9%, respectively. A complete inhibition of mushroom and shrimp PPO activity was reached at a gluconic acid

concentration of 2.0 mg/ml and pH 3.75. Gluconic acid apparently inhibited the activity of mushroom and shrimp PPO due to the reduction in the pH, which was shifted away from the optimum range of pH 6.0 to 6.5. The pH was maintained constant at 6.2 before and after the inhibition of mushroom and shrimp PPO activity by GOX/CAT (Al-Jassir, 1987). Therefore, the effect of gluconic acid as an inhibitor was due to the decrease of pH.

Oxygen consumption

Oxygen consumption by mushroom and shrimp PPO was plotted in Figures 3 and 4. Before the PPO enzyme was added, the solution of substrate (0.01M L-DOPA) and 0.02M phosphate buffer (pH 6.2) were stirred until oxygen saturation which was 7.8 ppm. When the mushroom or shrimp PPO solution was added, the oxygen reading decreased slowly during the 2 min of the experiment. Only 2.05 and 0.32 ppm were consumed by mushroom and shrimp PPO, respectively, with only 26.3% and 4.1% of the oxygen used by the PPO enzyme (Table 3). Figures 3 and 4, also show a comparison between the mushroom and shrimp PPO activity with and without the GOX/CAT as inhibitor and 0.5% D-glucose as a substrate, respectively.

The GOX/CAT enzyme system was added to solutions at a concentration of 2 units/ml with 0.5% D-glucose as a substrate (Figures 3 and 4). GOX/CAT was added before the

PP0 enzyme was added. The oxygen concentration decreased rapidly in the first min of the reaction. The affinity of GOX/CAT for oxygen was very high; after 20 sec 69% and 46% of the oxygen was removed by GOX/CAT (Table 4), while at the same time only 3.85% and 1% of the oxygen was removed by the PP0 enzyme of mushroom or shrimp, respectively. After 60 sec the oxygen removal by GOX/CAT reached about 90% in both PP0 enzyme systems. Mushroom PP0 had a higher affinity for oxygen than shrimp PP0 did, but when GOX/CAT was added, the oxygen consumption removal was almost the same for both mushroom and shrimp PP0. Therefore, it appears that the affinity of GOX/CAT for oxygen was very high and that mushroom and shrimp PP0 could not compete with GOX/CAT. Finally, it seems that the oxygen removed by GOX/CAT is the main cause of inhibition to the mushroom and shrimp PP0 activity.

Glucono-lactone

Table 5 shows the effect of different concentrations of glucono-lactone on mushroom and shrimp PP0 activity. Before the glucono-lactone was added, the activity was 56.6 and 6.2 $\mu\text{M}/\text{min}$, respectively. When glucono-lactone was added at a concentration of 2.0 mg/ml , the PP0 activity was reduced slightly to 56.10 and 6.12 $\mu\text{M}/\text{min}$ with only 0.9% and 1.3% inhibition, respectively. At glucono-lactone concentrations of 5.0 mg/ml , the PP0

activity was reduced further to 52.3 and 5.64 $\mu\text{M}/\text{min}$, or 7.6% and 9.0% inhibition, respectively. The pH was measured at all concentrations of glucono-lactone before and after the enzyme assay and it was constant at 6.2. This was expected, since there were only 2 min between preparation and assay of a solution for any inhibition of PPO activity. Therefore, it seems that glucono-lactone was a weak inhibitor of mushroom and shrimp PPO activity, even when it was used in a relatively high concentration (5.0 mg/ml). A similar result was reported by Gibson (1964). He found glucono-lactone to be a weak inhibitor for glucose oxidation in the GOX assay, and only 30% inhibition was obtained.

D-glucose

The effect of D-glucose as an inhibitor of mushroom and shrimp PPO activity is shown in Table 6. At concentrations of 1.0, 2.0, 3.0, 4.0, and 5.0 mg/ml, no inhibition occurred. Therefore, D-glucose had no effect on the activity of mushroom and shrimp PPO at the concentrations studied.

CONCLUSIONS

The substrates (D-glucose and oxygen) and the end products (hydrogen peroxide, glucono-lactone, and gluconic acid) of the GOX/CAT enzyme system were studied for their potential inhibitory effect on mushroom and shrimp PPO activity. No inhibition by D-glucose was found even at a concentration of 5.0 mg/ml. Glucono-lactone showed a limited inhibition of the activity of mushroom and shrimp PPO; it was considered a weak inhibitor because only 7.6% and 9.0% inhibition was obtained, respectively. However, the activity of mushroom and shrimp PPO was inhibited entirely by hydrogen peroxide and gluconic acid at concentrations of 2.0 mg/ml. Oxygen consumption by mushroom and shrimp PPO was slow and reached only 26.3% and 4.0%, respectively. However, oxygen consumption was very rapid with GOX/CAT at a concentration of 2 units/ml and 0.5% D-glucose as a substrate; oxygen consumption reached almost 96.0% at the end of 120 sec.

Finally, it seems that GOX/CAT inhibited mushroom and shrimp PPO activity due to the high affinity of GOX/CAT for oxygen. It removed a greater percentage of oxygen in a shorter period of time than either PPO enzyme. As a result, little oxygen was available to be used by the PPO and the activity of the enzyme was reduced with limited conversion of the substrate to product.

Table 1

The effect of hydrogen peroxide concentration on mushroom and shrimp PPO activity.*

Hydrogen Peroxide (mg/ml)	Mushroom		Shrimp	
	Dopachrome (uM/min)	Inhibition (%)	Dopachrome (uM/min)	Inhibition (%)
0.0	56.67	0.0	6.2	0.0
0.2	78.33	0.0 ^{**}	8.2	0.0 ^{**}
0.4	51.11	9.8	5.2	16.1
0.6	37.22	34.3	3.65	41.1
0.8	22.5	60.3	1.92	69.0
1.0	16.39	71.1	1.30	79.0
2.0	0.0	100.0	0.00	100.0

* The pH was held constant at 6.2.

** The mushroom and shrimp PPO activity were increased at 0.2 mg/ml to 38.22% and 32.22%, respectively, with respect to the control activity.

Table 2

The effect of gluconic acid concentration on mushroom and shrimp PPO activity.*

Gluconic acid (mg/ml)	Mushroom		Shrimp	
	Dopachrome (μ m/min)	Inhibition (%)	Dopachrome (μ M/min)	Inhibition (%)
0.0	56.67	0.0	6.2	0.0
0.2	47.78	15.68	4.92	20.64
0.4	46.11	18.62	4.62	25.16
0.6	43.89	22.55	4.41	28.87
0.8	40.56	28.43	4.10	33.87
1.0	38.06	32.84	3.54	42.90
2.0	0.00	100.0	0.00	100.0

* For the concentration of 0.2, 0.4, 0.6, 0.8, 1.0, and 2.0 mg/ml, the pH values are 6.0, 5.85, 5.65, 5.35, 4.85, and 3.75, respectively.

Table 3

Oxygen removal by mushroom and shrimp PPO
without GOX/CAT (0.5% D-glucose)*

Time (sec.)	Mushroom		Shrimp	
	Oxygen (ppm)	Oxygen removal (%)	Oxygen (ppm)	Oxygen removal (%)
0.0	7.8	0.0	7.8	0.0
20.0	7.5	3.85	7.72	1.0
40.0	7.1	9.0	7.66	1.8
60.0	6.7	14.1	7.60	2.56
80.0	6.4	18.0	7.56	3.07
100.0	6.05	22.4	7.52	3.60
120.0	5.75	26.30	7.48	4.10

* pH was held constant at 6.2.

Table 4

Oxygen removal by mushroom and shrimp PPO in presence of GOX/CAT at concentration of 2 units/ml with 0.5% D-glucose as the substrate.*

Time (sec)	Mushroom		Shrimp	
	Oxygen (ppm)	Oxygen removal (%)	Oxygen (ppm)	Oxygen removal (%)
0.0	7.8	0.0	7.8	0.0
20.0	2.4	69.2	4.2	46.15
40.0	1.2	86.6	1.7	78.20
60.0	0.8	89.7	0.9	88.46
80.0	0.7	91.0	0.6	92.30
100.0	0.5	93.6	0.4	94.90
120.0	0.3	96.2	0.35	95.50

* pH was held constant at 6.2.

Table 5

The effect of glucono-lactone concentration on mushroom and shrimp PPO activity.*

Glucono-lactone (mg/ml) ^a	Mushroom		Shrimp	
	Dopachrome (uM/min)	Inhibition (%)	Dopachrome (uM/min)	Inhibition (%)
0.0	56.6	0.0	6.20	0.0
1.0	56.4	0.35	6.18	0.32
2.0	56.1	0.88	6.12	1.29
3.0	55.4	2.12	6.02	2.90
4.0	53.7	5.12	5.88	5.16
5.0	52.3	7.60	5.64	9.03

* The pH was held constant at 6.2.

Table 6

The effect of D-glucose concentration on mushroom and shrimp PPO activity.*

D-glucose (mg/ml)	Dopachrome (uM/min)	
	Mushroom	Shrimp
0.0	56.60	6.20
1.0	56.60	6.20
2.0	56.62	6.24
3.0	56.65	6.22
4.0	56.60	6.18
5.0	56.58	6.24

* The pH reading was held constant at 6.2.

MUSHROOM PPO ACTIVITY IN THE PRESENCE OF
HYDROGEN PEROXIDE OR GLUCONIC ACID

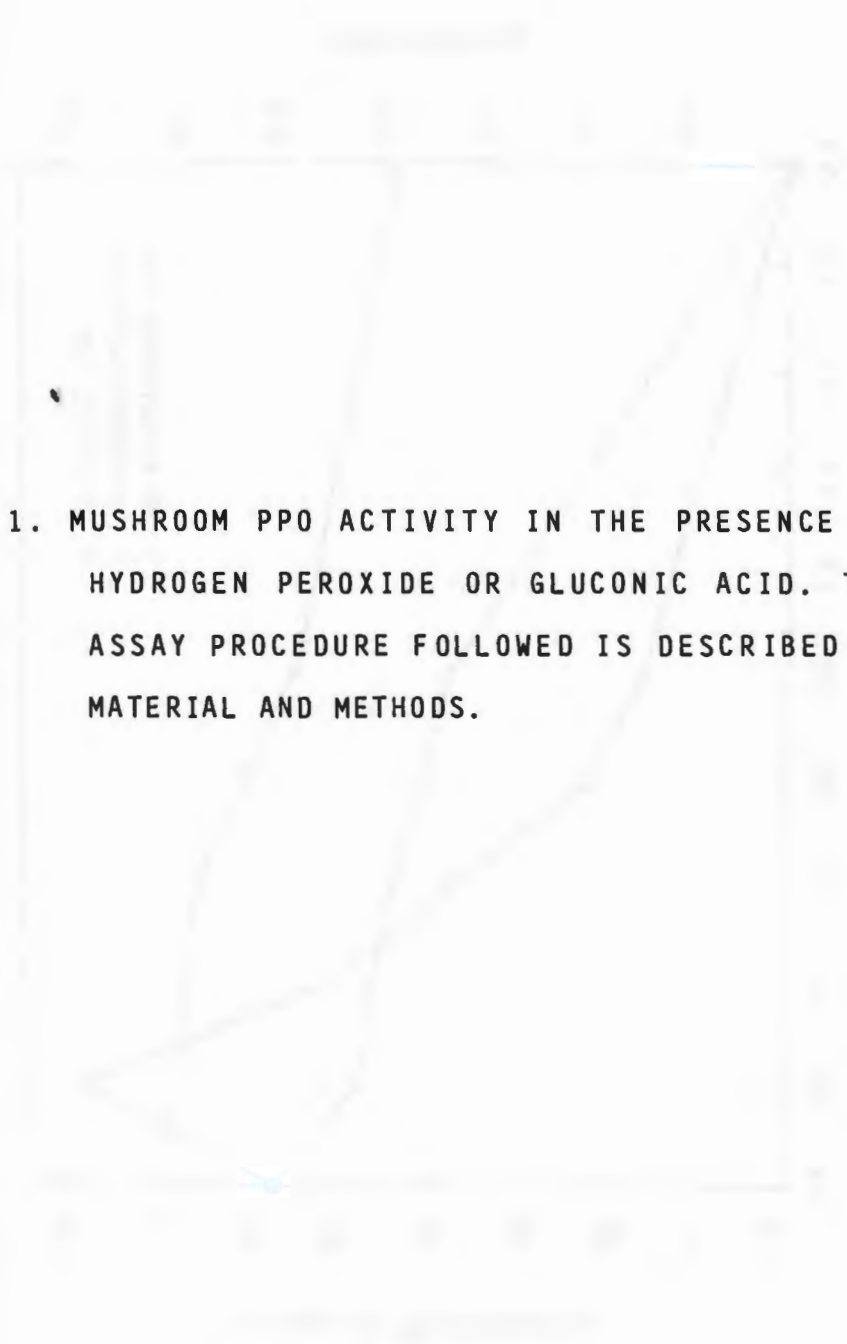
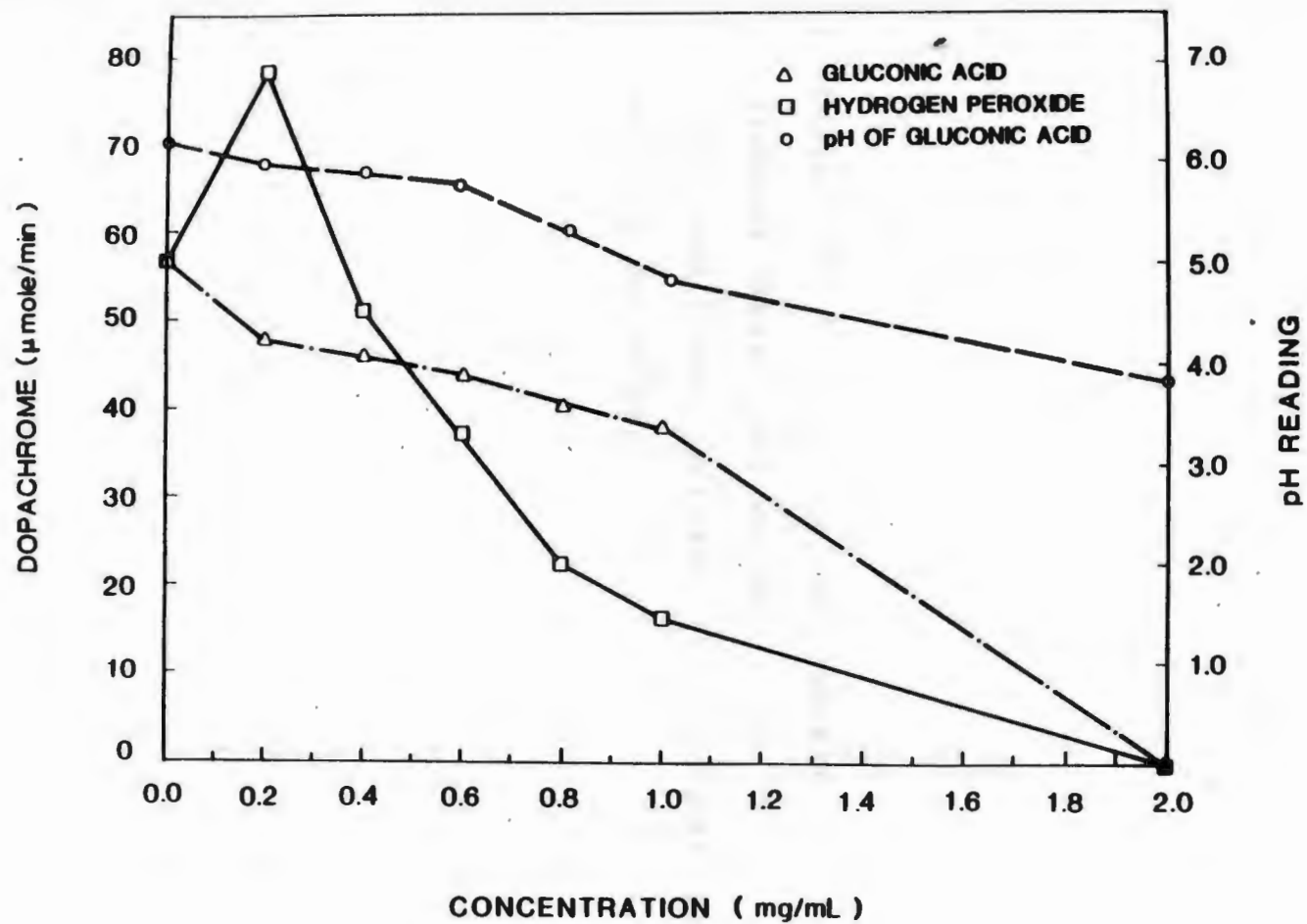


FIGURE 1. MUSHROOM PPO ACTIVITY IN THE PRESENCE OF HYDROGEN PEROXIDE OR GLUCONIC ACID. THE ASSAY PROCEDURE FOLLOWED IS DESCRIBED IN MATERIAL AND METHODS.

MUSHROOM PPO ACTIVITY IN THE PRESENCE OF HYDROGEN PEROXIDE OR GLUCONIC ACID



SHRIMP PPO ACTIVITY IN THE PRESENCE OF
HYDROGEN PEROXIDE & GLUCONIC ACID

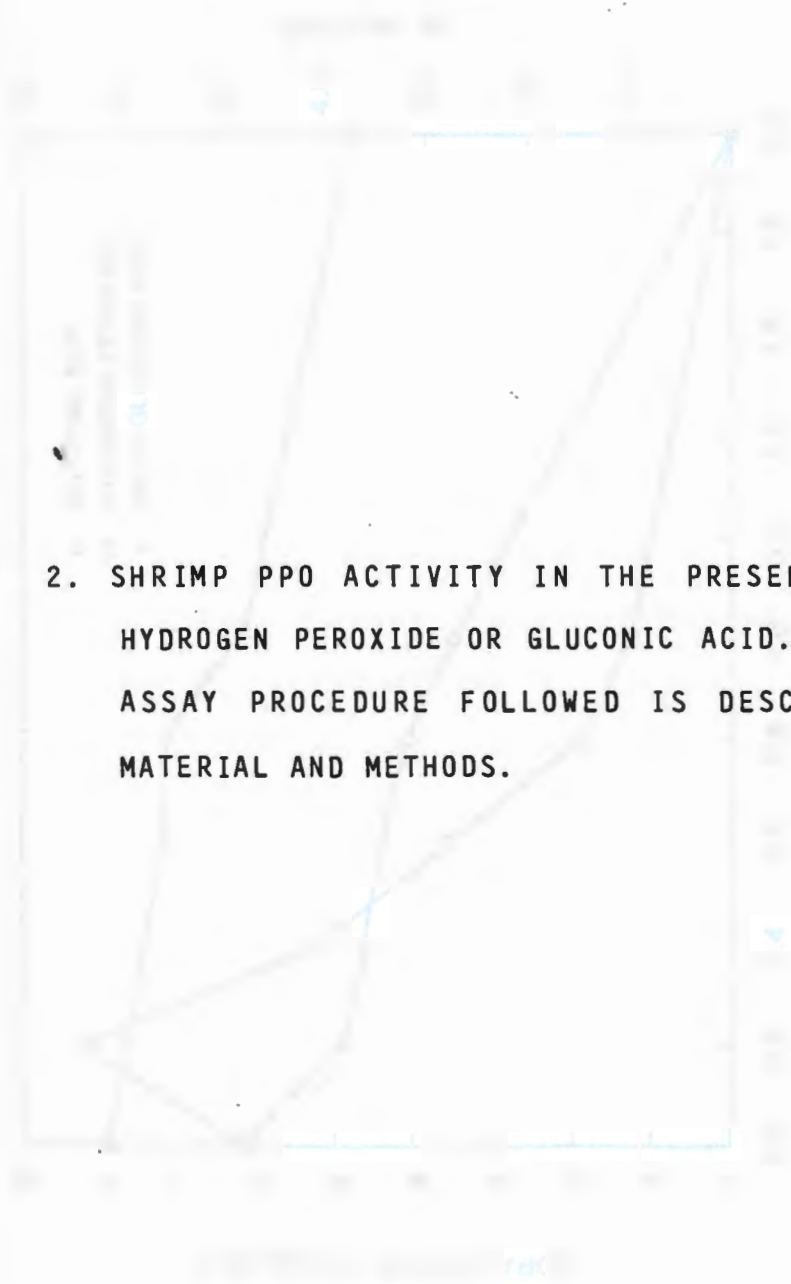


FIGURE 2. SHRIMP PPO ACTIVITY IN THE PRESENCE OF HYDROGEN PEROXIDE OR GLUCONIC ACID. THE ASSAY PROCEDURE FOLLOWED IS DESCRIBED IN MATERIAL AND METHODS.

SHRIMP PPO ACTIVITY IN THE PRESENCE OF HYDROGEN PEROXIDE OR GLUCONIC ACID

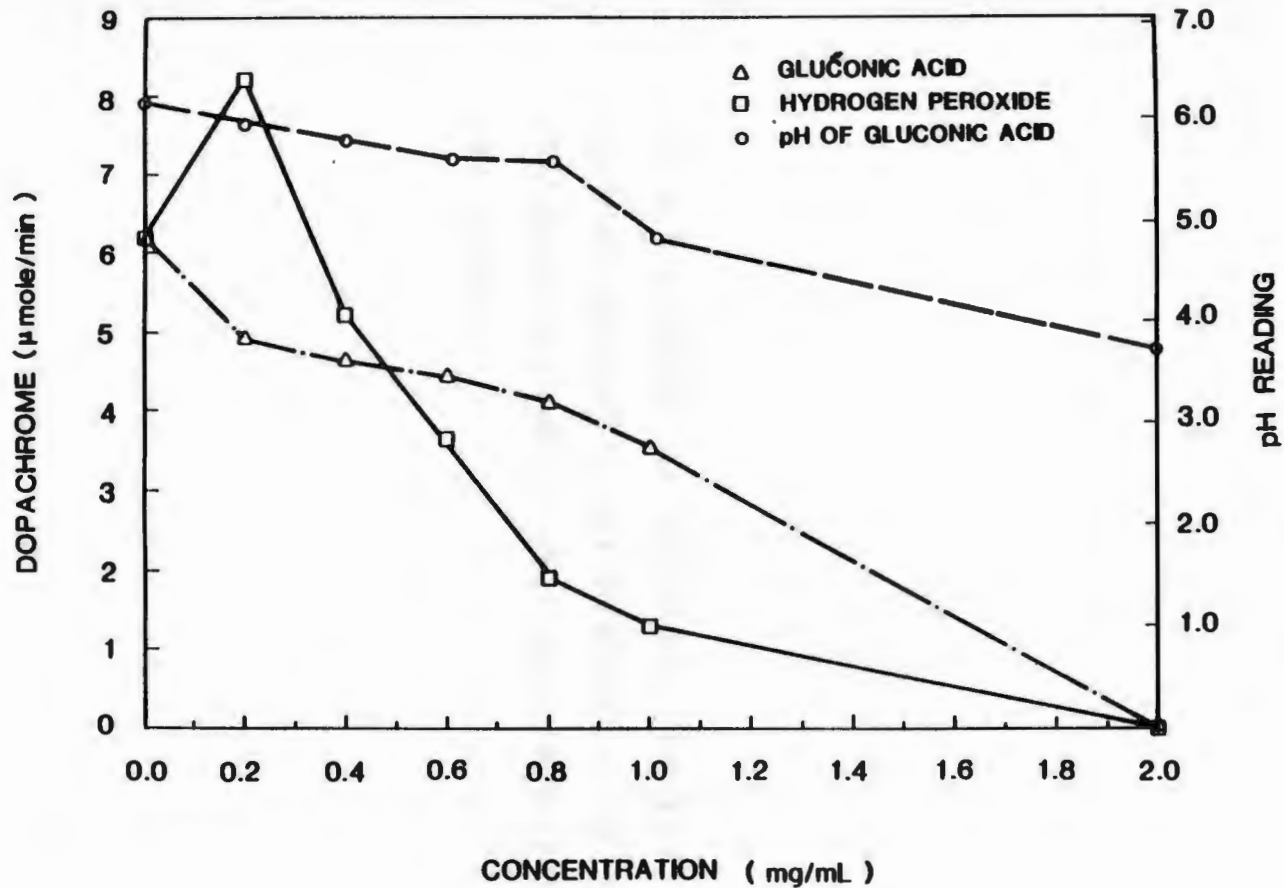
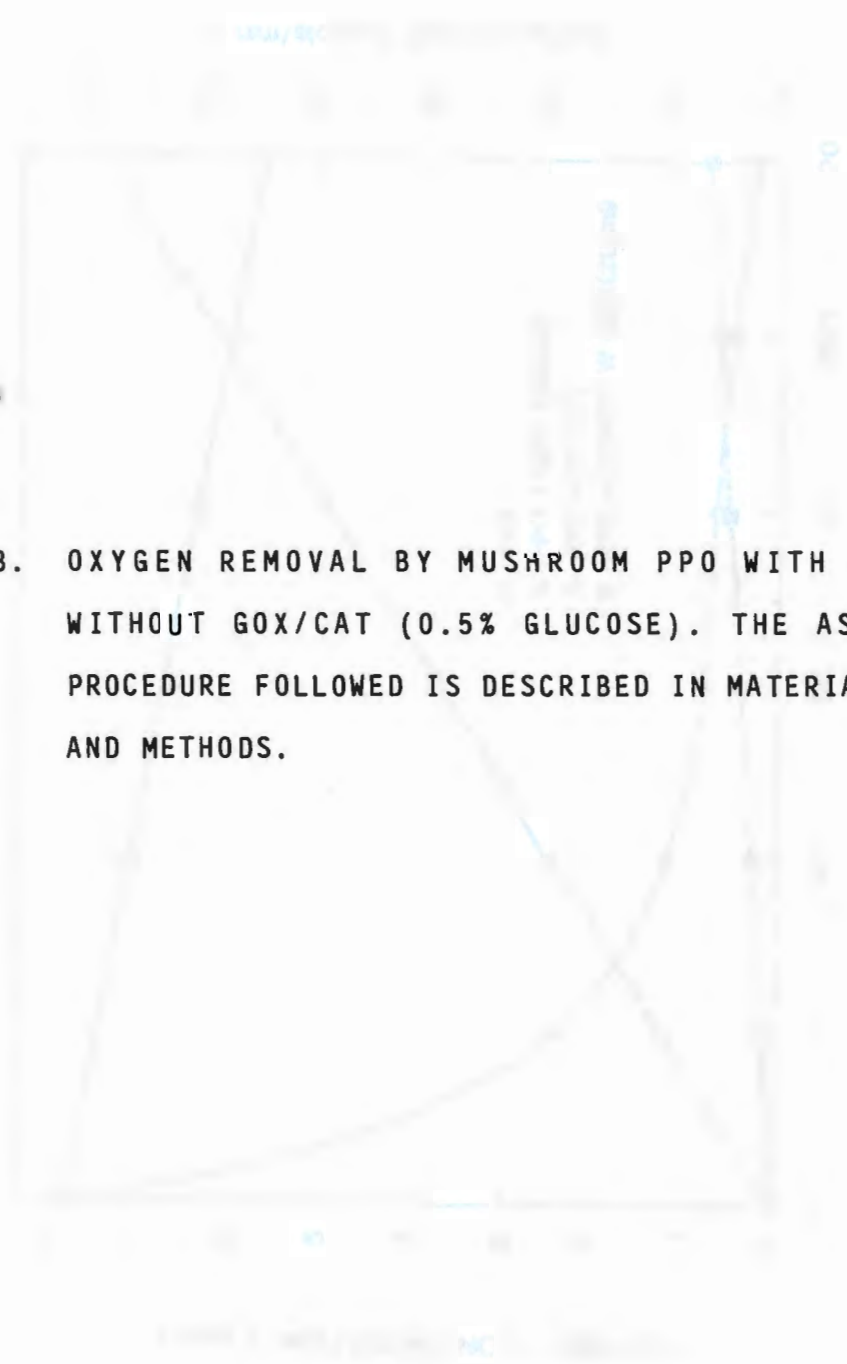
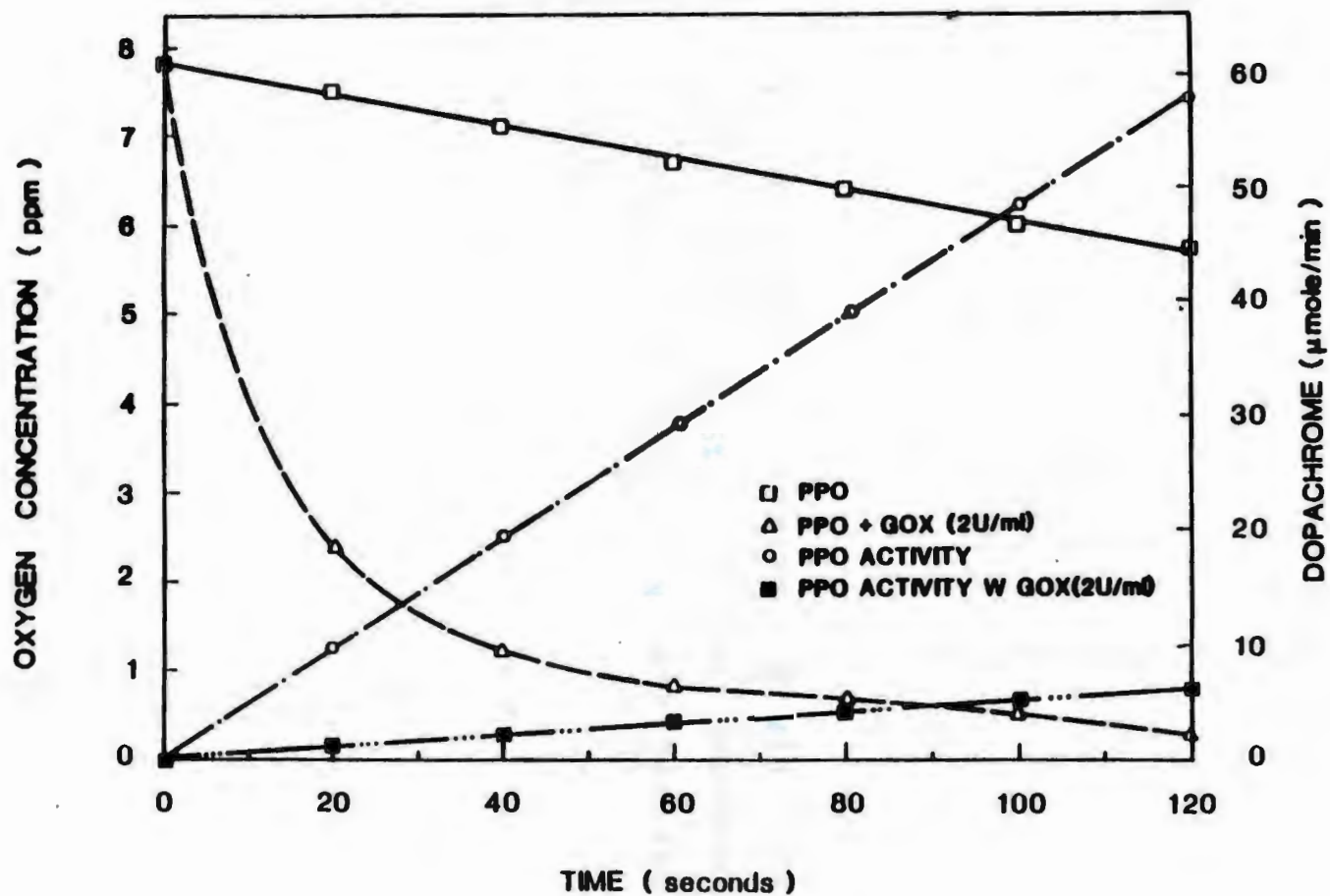


FIGURE 3. OXYGEN REMOVAL BY MUSHROOM PPO WITH AND WITHOUT GOX/CAT (0.5% GLUCOSE). THE ASSAY PROCEDURE FOLLOWED IS DESCRIBED IN MATERIAL AND METHODS.



OXYGEN REMOVAL BY MUSHROOM PPO WITH
AND WITHOUT GOX/CAT (0.5 % GLUCOSE)



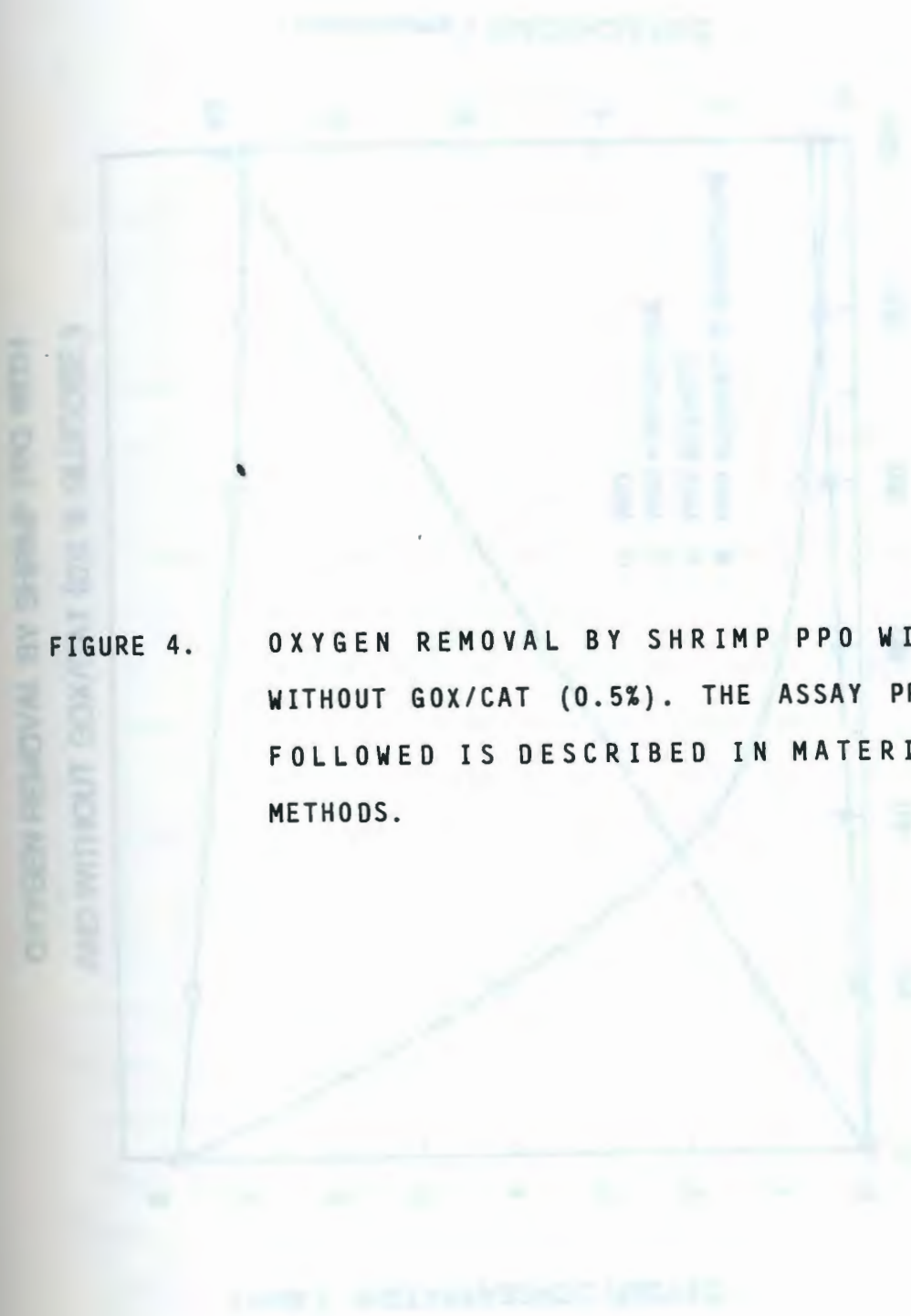
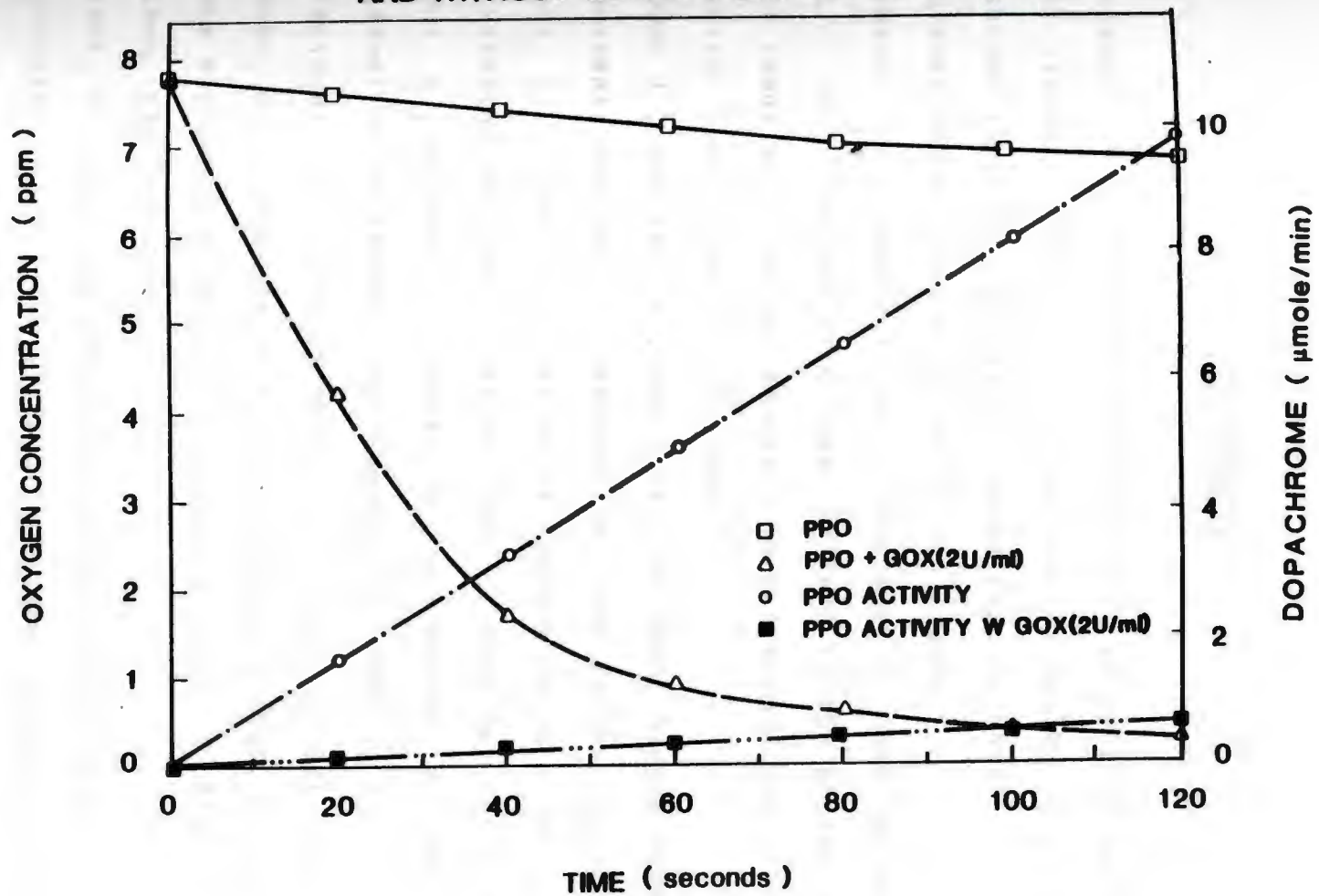


FIGURE 4. OXYGEN REMOVAL BY SHRIMP PPO WITH AND WITHOUT GOX/CAT (0.5%). THE ASSAY PROCEDURE FOLLOWED IS DESCRIBED IN MATERIAL AND METHODS.

OXYGEN REMOVAL BY SHRIMP PPO WITH
AND WITHOUT GOX/CAT (0.5 % GLUCOSE)



REFERENCES

- Al-Jassir, M.S. 1987. The inhibition of polyphenol oxidase by glucose oxidase and catalase enzyme system in pink shrimp (Pandalus borealis). Manuscript 1. Ph.D. Dissertation. University of Rhode Island.
- Baldwin, R.R., Campbell, H.A., Thiessen, R., and Lorant, G.J. 1953. The use of glucose oxidase in the processing of foods with special emphasis on the desugaring of egg white. Food Technol. 7:275-282.
- Bloom, J., Scofield, G., and Scott, D. 1956. Oxygen removal prevents rancidity in mayonnaise. Food Packer. 37:16-17.
- Field, C.E. 1986. Utilization of Glucose Oxidase for Extending the shelf life of fish. J. Food Sci. 51:66-70.
- Finne, G., Wagner, T., DeWitt, B., and Martin, R. 1986. Effect of treatment, ice storage and freezing on residual sulfite in shrimp. J. Food Sci. 51:231-232.
- Gibson, Q.H., Swoboda, B.P., and Massey, V. 1964. Kinetics and Mechanism of Action of Glucose Oxidase. J. Biol. Chem. 239:3927-3934.
- Kleppe, K. 1966. The effect of hydrogen peroxide on glucose oxidase from asperigillus niger. Biochem. J. 5: 139-143.

- Ohlmeyer, D.W. 1957. Use of glucose oxidase to stabilize beer. Food Technol. 11:503-507.
- Otwell, S. and Marshall, M. 1986. Studies on the use of sulfites to control shrimp melanosis (Black spots). Florida Sea Grant Collee, Technical paper 46. University of Florida, Gainesville, FL.
- Priputina, L.S. 1974. Glucose oxidase and catalase as food additives. Chem. Abstract. 81:103, 345.
- Rand, A.G. Jr. and Hourigan, J.A. 1974. Direct Enzymatic Conversion of Lactose in Milk to Acid. J. Dairy Sci. 58:8, 1144- 1150.
- Reed, G. 1975. Application of glucose oxidase. In Enzymes in Food Processing. 2nd edition. Academic Press. New York.
- Scott, D. 1953. Glucose conversion in preparation of Albumin solids by glucose oxidase system. J. Agr. Food Chem. 1:729-730.
- Searle Biochemics. 1966. Glucose oxidase: properties and application to foods. Technical Bulletin No. 2.
- Simon, R.A., Green, L., and Steven, D.D. 1982. The incidence of ingested metabisulfate sensitivity in an asthmatic population. J. Allergy Clin. Immunol. 69:118.
- Underkofler, L.A. 1961. Glucose Oxidase: Production, Properties, and Present and Potential Application. Soc. Chem. Ind. 11:72-86.

- Werth , G.R. 1982. Inhaled metabisulfite sensitivity. J. Allergy Clin. Immunol. 70:143.
- Wiseman, A., ed. 1975. Handbook of Enzyme Biotechnology. Halsted Press, John wiley and sons, Inc. NY.
- Yang, H.Y 1955. Stabilizing apple wine with glucose oxidase. Food Res. 20: 42-46.

ABSTRACT

The objectives of this study were to determine the effect of different dip solutions on the melanization of pink shrimp (*Pandalus borealis*) and to identify the most effective solution for inhibiting melanosis. The study was conducted in a laboratory setting using 24 shrimp per treatment group. The treatments included a control group and four different dip solutions: 1% formalin, 1% potassium permanganate, 1% sodium hypochlorite, and 1% potassium dichromate. The shrimp were dipped for 24 hours and then observed for melanosis over a period of 72 hours. The results showed that the 1% potassium permanganate solution was the most effective in inhibiting melanosis.

MANUSCRIPT III.

**INHIBITION OF MELANOSIS IN PINK SHRIMP
(*Pandalus borealis*) BY DIFFERENT DIP SOLUTIONS.**

The objectives of this study were to determine the effect of different dip solutions on the melanization of pink shrimp (*Pandalus borealis*) and to identify the most effective solution for inhibiting melanosis. The study was conducted in a laboratory setting using 24 shrimp per treatment group. The treatments included a control group and four different dip solutions: 1% formalin, 1% potassium permanganate, 1% sodium hypochlorite, and 1% potassium dichromate. The shrimp were dipped for 24 hours and then observed for melanosis over a period of 72 hours. The results showed that the 1% potassium permanganate solution was the most effective in inhibiting melanosis.

In general, all treated shrimp showed a significant reduction in melanosis compared to the control group. The 1% potassium permanganate solution was the most effective in inhibiting melanosis, followed by the 1% sodium hypochlorite solution. The 1% formalin and 1% potassium dichromate solutions were less effective. The results of this study suggest that the use of potassium permanganate as a dip solution is a promising method for inhibiting melanosis in pink shrimp.

ABSTRACT

The potential of the glucose oxidase/catalase (GOX/CAT) enzyme system for delaying melanosis in iced shrimp was studied. Fresh, pink shrimp (Pandalus borealis) with heads on were treated with various dip solutions. The dip solutions were : firstly, GOX/CAT at concentrations of 2, 4, 6, and 10 units/ml, each with 0.5% D-glucose as a substrate; secondly, sodium tripolyphosphate (STPP) at a concentration of 9% alone and at concentrations of 3%, 6%, and 9% in combination with GOX/CAT at a concentration of 6 units/ml. Finally, sodium metabisulfite at a concentration of 1.25% alone and at concentrations of 0.32%, 0.625%, and 1.25% in combination of GOX/CAT at a concentration of 6 units/ml.

In general, all treated shrimp showed a delay in melanosis development compared to untreated shrimp. Based on the melanosis scores, GOX/CAT at concentrations of 2 and 4 units/ml delayed the melanosis formation by 2 days compared to controls, and by 4 days with GOX/CAT at concentration of 6 and 10 units/ml. The dip solution of 9% STPP delayed melanosis by 2 days compared to the controls, and by 4 days with 3% and 6% STPP, each with GOX/CAT, and by 6 days with the combination of 9% STPP and GOX/CAT. Shrimp treated with sodium metabisulfite, at all concentrations, exhibited no melanosis for the duration of

the study compared to the control shrimp. GOX/CAT in combination with sodium metabisulfite did not increase the melanosis inhibition compared to sulfite alone. This was due to the sulfite inhibition effect on GOX/CAT activity.

Finally, the metabisulfite dip treatment was most effective in preventing melanosis development. The combination dip of 9% STPP and GOX/CAT at concentrations of 6 units/ml appeared to be a promising alternative as a second choice. The GOX/CAT concentration of 6 units/ml and 0.5% D-glucose as a dip had a slight effect on preventing black spot in iced shrimp.

INTRODUCTION

Melanosis, the black discoloration of raw shrimp, called "black spot", is a result of the enzymatically controlled oxidation of the amino acid tyrosine to the dark pigment melanin. The discoloration is characterized by a darkening of the head, antennae, abdominal shell pigments, and tail fin.

Melanin formation is not related to eating quality, safety, or decomposition of shrimp, but it is visually objectionable to most consumers and, therefore, makes the product less acceptable.

The enzyme which causes melanosis is polyphenol oxidase (PPO), and was found to be located in head area, gills, liver, stomach, and blood of shrimp (Bailey et al., 1960).

To control "black spot" on shrimp shells, freshly harvested shrimp have been dipped in dilute solutions of sodium bisulfite immediately after harvest for many years. The U.S. Food and Drug Administration permits a one-minute dip in a 1.25% solution of sodium bisulfite as Current Good Manufacturing Practice (CGMP) (Finne and Miget, 1985). The FDA has determined an acceptable residual sulfite level in shrimp of 100 ppm as SO_2 . Thus, shrimp containing residual sulfite greater than 100 ppm would be

considered adulterated because of an unsafe amount of a food additive (Finne and Migget, 1985). (The 100 ppm level was thought to result from exposure to the 1.25% sodium bisulfite dip for one minute.) Furthermore, the FDA reinstated the requirement to label all foods, including shrimp, which have a sulfite residue in excess of 10 ppm (Marshall and Otwell, 1986).

Recently, the safety of sulfiting agents has been questioned in the clinical literature and by consumer groups. A number of reports have described strong asthmatic reactions to bisulfite and other sulfiting agents (Allen and Collette, 1981 ; Simmon et al., 1982). Therefore, alternatives must be found to inhibit melanosis in shrimp.

The enzyme system of glucose oxidase and catalase (GOX/CAT) has been used in foods as a additive and is considered as Generally Recognized As Safe (GRAS) (Searle Biochemics, 1966). The GOX/CAT enzyme system has been used to remove oxygen and prevent oxidation in food products, and also to remove glucose. GOX/CAT can inhibit PPO activity by removing oxygen (Al-Jassir, 1987) and might also prevent melanosis.

Judokusumo (1985), found that GOX/CAT improved the preservation of iced shrimp according to the sensory evaluation of odor and general acceptability. The shelf life of shrimp was extended to 28-33% with respect to the

control, and depressed biochemical and microbial degradation when GOX/CAT used at concentration of 2 units/ml and 4% D-glucose as a substrate. She reported that melanosis in iced shrimp was not inhibited by the dip solution of GOX/CAT at concentrations of 1 unit/ml and 4% D-glucose solution, because shrimp were 4 days old in ice and had melanosis before treated with GOX/CAT dip solution.

Polyphosphates are known to be effective in preventing oxidation, moisture loss, and texture change in fresh sea food. Polyphosphates function by sequestering or binding a wide variety of metal ions and by directly interacting with proteins. Oxidation is greatly accelerated in the presence of the catalytic metal ions iron and copper. By taking up these ions, phosphates strongly inhibit oxidation (Shimp, 1985). Since melanosis development is due to activity of shrimp PPO in the presence of phenolic compounds and oxygen, STPP could also help to delay the melanosis.

The purpose of this study was to investigate the potential of the GOX/CAT enzyme system for reducing melanosis development when used as a dip for whole shrimp. The effects of different concentrations of GOX/CAT, D-glucose as the substrate, polyphosphates (STPP) in the presence of GOX/CAT, and sodium metabisulfite on whole shrimp melanosis were also studied.

MATERIALS AND METHODS

MATERIALS

Fresh, untreated pink shrimp (Pandalus borealis) obtained from West Boothbay Harbor, Maine were one day old upon arrival in the laboratory. They were frozen and kept at -80°C until used.

A commercial enzyme preparation (GOX/CAT) containing glucose oxidase (EC. 1.1.3.4.; β -D-glucose oxidoreductase) and catalase (EC 1.11.1.6; hydrogen peroxide: hydrogen oxidoreductase), Ovazyme. (1500 units/ml) was provided by Finnsugar Biochemical, Inc., (Elk Grove Village, IL) and used throughout these trials.

D-glucose (dextrose, anhydrous, reagent grade; Sigma Chemical Co., St. Louis, MO) was the substrate. Sodium metabisulfite was obtained from Fisher Scientific Co. (Fair Lawn, NJ). Food grade sodium tripolyphosphate (STPP) was provided by Calgon Corp. (Calgon Center, Pittsburgh, PA).

Dip solutions

The solutions into which whole pink shrimp were dipped included: 1) different concentrations of the GOX/CAT enzyme system at 0.5% D-glucose as substrate

(Table 3), 2) a combination of sodium tripolyphosphate (STPP) and the GOX/CAT enzyme system at a concentration of 6 units/ml (Table 4), and 3) a combination of sodium metabisulfite and the GOX/CAT at a concentration of 6 units/ml (Table 5).

The GOX/CAT dip was prepared as follows: 0.5% D-glucose, the substrate of the GOX/CAT enzyme system, was dissolved in distilled water, mixed gently, and then left overnight at 4 C to allow equilibrium of mutarotation to be attained. GOX/CAT was added to the D-glucose solution at different concentrations and mixed for 5 min. The final concentrations of the GOX/CAT dip solutions were 2, 4, 6, and 10 units/ml.

Dip solutions with STPP concentrations of 3%, 6%, and 9% were used to allow STPP to penetrate the meat under the shell prior to shelling (Shimp, 1985).

In the study of sodium metabisulfite on the rate of melanosis, the concentrations of sodium metabisulfite were 1.25% alone, and concentrations of 0.32%, 0.625%, and 1.25% in combination with GOX/CAT at a concentration of 6 units/ml and 0.5% D-glucose as substrate. The level of 1.25% was the maximum concentration allowable by the FDA for the shrimp dip solutions.

processing, and storage of shrimp

On day 1, whole shrimp (with head on) were thawed and washed in distilled water until they reached room temperature (25 C). The shrimp were soaked for 1 min in one of the dip solutions with intermittent mixing. The whole shrimp were removed from the treatment solutions with stainless steel forceps, drained, and packaged in groups of three shrimp in labelled, sterile Whirlpack bags (Nasco; Cole-Palmer Instrument Co., Chicago, IL). The shrimp were packed in ice and stored at 2-4 °C. Controls were treated similarly except that they were dipped in tap water, packed in sterile bags and stored in ice at 2-4 °C.

Melanosis evaluation

The evaluation of melanosis was performed with a scale similar to one developed by the National Marine Fisheries Service (Otwell, 1986). It was based on a series of color photographs of shrimp showing the normal development of melanosis. Every 2 days for up to two weeks, a bag of 3 shrimp was removed from the iced storage; the shrimp were examined, photographed, and rated for melanosis according to the melanosis score provided by Otwell, (1986). Table 1 shows the melanosis scale with the shrimp melanosis description and the corresponding scores for each condition. A shrimp with no melanosis had a score of 0, while a shrimp with total black spot would be a

score of 10. A rating of 4 or greater represented a measurable defect in product quality and rating of 8 or greater represented a severe defect, approaching an unacceptable product (Otwell, 1986).

Treated shrimp and a control for that trial were then rated on the melanosis scale by a team of 10 panelists. Their ratings were based on the melanosis scale and on the color photographs of the black spot developed in untreated shrimp.

On sampling days, one bag each of treated and control shrimp were removed from iced storage, placed on ice and evaluated for melanosis development. The control and treated values reported represent the means of at least 10 individual ratings. The data was evaluated by using the analysis of variance to determine if significant differences existed between the different concentrations and treatments. Duncan's Multiple Range Test was used. Unless otherwise indicated, "significant" means significant at the 5% level ($\alpha = 0.05$).

RESULTS AND DISCUSSION

Melanosis development in untreated shrimp

Melanosis development was studied as a function of time in untreated pink shrimp (Pandalus borealis) with heads on. The degree of melanosis gradually increased in these control shrimp, which had been dipped in only tap water. Table 2 shows the average of melanosis scores on the control shrimp given by the panelists. The shrimp had no melanosis on day 1, but black spots were noticeable in most shrimp by day 3. By day 5, melanosis due to the full activity of shrimp PPO, had become a severe defect with an average score of 7.8. This score would be considered unacceptable, according to National Marine Fisheries Service (Otwell, 1986). The statistical study showed that there was a significant difference between control shrimp up to day 7, from day 7 on there was no significant difference between the untreated shrimp samples.

Effect of GOX/CAT levels on shrimp melanosis

The effect of the GOX/CAT enzyme concentrations on the delay in melanosis formation was studied (Table 3). Melanosis development was reduced for all the GOX/CAT

treatments. On day 3, the shrimp treated with GOX/CAT at a concentration of 2 units/ml had a melanosis score of 4.2, which was significantly higher than the scores of the other treatments and not significantly different than the score of 5.0 for the control. From day 5 on, there was no significant difference between the treatments with GOX/CAT at concentrations of 2 and 4 units/ml, but it was significantly different than the control shrimp up to day 9. The GOX/CAT treatment at concentrations of 6 and 10 units/ml had no significant difference between them through the period of the study, but but were significantly different than the control shrimp up to day 11.

While the control shrimp reached rejection values on day 5, the GOX/CAT dip solution at concentrations of 2 and 4 units/ml would be not rejected until day 7, 2 days advantage over the control shrimp rating. The GOX/CAT at concentrations of 6 and 10 units/ml would be rejected on day 9, or 4 days advantage over the control shrimp rating.

In general, the dip solutions of GOX/CAT at different concentrations and with 0.5% D-glucose as a substrate, showed a delay in melanosis formation when compared to the control shrimp. The melanosis was reduced further with GOX/CAT at concentrations of 6 and 10 units/ml when compared to the GOX/CAT at concentrations of 2 and 4 units/ml. Therefore, 6 units/ml of GOX/CAT was an

effective concentration, and not significantly different from 10 units/ml. This concentration was not as effective in inhibiting melanosis as indicated by in vitro studies where the GOX/CAT was added directly to the shrimp PPO extract. The available oxygen was apparently removed more rapidly in a short period of time in vitro compared with in vivo. The high molecular weight of the GOX/CAT enzyme system might limit its penetration into the shrimp shell and therefore, the melanosis inhibition was reduced.

Effect of polyphosphate levels on shrimp melanosis

The effect of concentrations of sodium tripolyphosphate (STPP) in combination with the GOX/CAT enzyme system on melanosis formation in iced shrimp was studied (Table 4). Shrimp treated with the 9% STPP solution without GOX/CAT had significantly reduced melanosis scores compared to the control shrimp. By day 7, the STPP treated shrimp would be rejected with a score of 8.2, 2 days advantage over the control. Shrimp treated with 3%, 6%, and 9% STPP in combination with GOX/CAT had delayed melanosis formation when compared with either GOX/CAT or 9% STPP alone. The statistical analysis showed that there was a significant difference between the dip solutions of 3% and 9% STPP, each with GOX/CAT, up to day 11. All the treatments of 3%, 6%, and 9% STPP in combination with GOX/CAT showed a significant difference

compared to the control. The STPP solutions of 3% and 6% STPP, each with GOX/CAT, delayed the development of melanosis 4 days with respect to the control shrimp rating. However, the combination of 9% STPP and GOX/CAT solutions delayed melanosis by 6 days, compared to the control shrimp. This result indicates a synergistic effect of STPP and GOX/CAT on inhibition of melanosis development in iced shrimp.

Effect of metabisulfite on shrimp melanosis

The effect of sodium metabisulfite concentrations in combination with GOX/CAT at concentrations of 6 units/ml and 0.5% D-glucose was studied (Table 5). Melanosis development was delayed in the shrimp treated with a combination of bisulfite and GOX/CAT, and the differences were statistically significant compared to the control. Also, through the period of the study, there was a significant difference between sulfite at concentrations of 0.32% and 0.625%, each with GOX/CAT. No significant difference was obtained between the 1.25% sulfite alone and 1.25% sulfite in combination with GOX/CAT. On day 3, the melanosis was slight in the treated shrimp that had been dipped in solutions of 0.32% and 0.625% bisulfite, each with GOX/CAT, while there was almost no melanosis on shrimp treated with 1.25% sulfite alone or in combination with GOX/CAT. The melanosis developed slowly through the

period of the study and reached scores of 7.0 and 5.4 for the 0.32% and 1.25% sulfite, each with GOX/CAT, respectively. Therefore, based on the melanosis scores, the treated shrimp in this trial were not rejected even at day 15.

A study reported by Hennett et al (1966) indicated that bisulfite was bound to the active site of glucose oxidase which caused an inhibitory effect on the enzyme activity. To verify this finding, the sulfite at concentration of 0.01% was added to GOX/CAT on an enzyme assay and tested spectrophotometrically. The result showed that GOX/CAT was inhibited entirely by this concentration of sulfite. The GOX/CAT which was used in combination with sulfite as a dip probably had little or no effect in reducing the shrimp melanosis due to the inhibitory effect of sulfite. Therefore, it appears that the reduction in shrimp melanosis was due to the sulfite only.

CONCLUSIONS

The GOX/CAT enzyme system as a dip had an effect on delaying melanosis formation in whole iced pink shrimp (Pandalus borealis). Based on melanosis evaluation, GOX/CAT at concentrations of 2 and 4 units/ml could delay melanosis development for 2 days over the control shrimp. Increasing the GOX/CAT concentration to 6 and 10 units/ml effectively doubled the inhibitory effect.

The dip solution of 9% STPP also delayed melanosis development in iced shrimp for 2 days over the control shrimp. Dip solutions which combined 3% and 6% STPP, each with GOX/CAT, could inhibit black spot development for up to 4 days compared to controls. Melanosis was delayed further to 6 days in iced shrimp with the dip combination of 9% STPP and GOX/CAT at a concentration of 6 units/ml.

Dip solutions of sodium metabisulfite at concentrations of 0.32%, 0.625%, and 1.25% proved highly effective and delayed melanosis in iced shrimp through the period of the study with respect to control shrimp. GOX/CAT appeared to have no effect on inhibiting shrimp melanosis when combined with sulfite, probably due to sulfite inhibition on the GOX/CAT enzyme system.

Overall, the comparison between the 3 types of dip solutions showed that the best procedure for shrimp to prevent melanosis was still metabisulfite. However, the combination of 9% STPP and GOX/CAT at a concentration of 6 units/ml appeared to be a promising alternative as a second choice. The GOX/CAT enzyme system at a concentration of 6 units/ml and 0.5% D-glucose as a substrate had a slight effect in preventing black spot in shrimp, as indicated in the "in vitro" studies with shrimp PPO (Al-Jassir, 1987). It was not as effective as indicated in the PPO studies. This may be due to the presence of GOX/CAT enzyme system in the same solution of shrimp PPO extract and as a result the available oxygen was removed rapidly in vitro compared with in vivo. The high molecular weight of the GOX/CAT might limit penetration into the shrimp shell and account for the enzyme being less effective in melanosis inhibition.

TABLE 1.

WORK SHEET USED FOR EVALUATING MELANOSIS
DEVELOPMENT IN SHRIMP (FROM OTWELL, 1986).

Name: _____ Date: _____

<u>Sample</u>	<u>Scores</u>					
A	0	2	4	6	8	10
B	0	2	4	6	8	10
C	0	2	4	6	8	10
D	0	2	4	6	8	10
E	0	2	4	6	8	10
F	0	2	4	6	8	10

Melanosis Scale:

- 0 Absent (no black spot).
- 2 Slight, noticeable on some shrimp.
- 4 Slight, noticeable on most shrimp.
- 6 Moderate, noticeable on most shrimp.
- 8 Heavy, noticeable on most shrimp.
- 10 Heavy, totally unacceptable.

TABLE 2.

THE AVERAGE MELANOSIS RATING ON ICED PINK
SHRIMP GIVEN BY THE 10 PANELSISTS.*

<u>Days</u>	<u>Melanosis score</u>
3	5.0 ^a
5	7.8 ^b
7	9.2 ^c
9	10.0 ^c
11	10.0 ^c
13	10.0 ^c
15	10.0 ^c

* Melanosis scores having the same letter as superscript were not significantly different ($\alpha = 0.05$).

TABLE 3.

EFFECT OF GOX/CAT CONCENTRATIONS ON MELANOSIS
DEVELOPMENT IN ICED SHRIMP (0.5% D-GLUCOSE).

MELANOSIS SCORES *					
DAY	GOX/CAT CONCENTRATIONS, (units/ml)				
	2	4	6	10	CONTROL
3	4.2 ^a	2.8 ^b	2.5 ^b	2.4 ^b	5.0 ^a
5	6.6 ^b	6.2 ^b	5.0 ^c	5.1 ^c	7.8 ^a
7	7.8 ^b	7.4 ^b	6.2 ^c	6.4 ^c	9.2 ^a
9	8.4 ^b	8.0 ^b	8.4 ^b	8.3 ^b	10.0 ^a
11	10.0 ^a	9.6 ^{a,b}	9.2 ^b	9.0 ^b	10.0 ^a
13	10.0 ^a	10.0 ^a	10.0 ^a	10.0 ^a	10.0 ^a
15	10.0 ^a	10.0 ^a	10.0 ^a	10.0 ^a	10.0 ^a

* Each sampling day, melanosis scores having the same letter as superscript were not significantly different ($\alpha = 0.05$).

TABLE 4.

THE EFFECT OF SODIUM TRIPOLYPHOSPHATE (STPP) CONCENTRATIONS ALONE AND IN COMBINATION WITH GOX/CAT (6 UNITS/ML) ON MELANOSIS OF ICE SHRIMP.

DAY	MELANOSIS SCORES *				
	STPP + GOX/CAT			STPP	CONTROL
	3%	6%	9%	9%	
3	2.5 ^{b,c}	2.0 ^c	1.7 ^c	3.2 ^b	5.0 ^a
5	5.7 ^b	3.8 ^c	3.4 ^c	6.6 ^b	7.8 ^a
7	6.2 ^c	6.0 ^c	4.5 ^d	8.2 ^b	9.2 ^a
9	7.6 ^b	7.2 ^b	6.0 ^c	9.2 ^a	10.0 ^a
11	8.8 ^b	8.3 ^{b,c}	7.4 ^c	9.8 ^a	10.0 ^a
13	9.2 ^b	9.2 ^b	8.8 ^b	10.0 ^a	10.0 ^a
15	10.0 ^a	10.0 ^a	9.6 ^a	10.0 ^a	10.0 ^a

* Each sampling day, melanosis scores having the same letter as superscript were not significantly different ($\alpha = 0.05$).

TABLE 5.

THE EFFECT OF SODIUM METABISULFITE (SMBS)
CONCENTRATION ALONE AND IN COMBINATION WITH
GOX/CAT (6 UNITS/ML) ON MELANOSIS OF ICED SHRIMP.

DAY	MELANOSIS SCORES *				
	SMBS + GOX/CAT			SMBS	CONTROL
	0.32%	0.625%	1.25%	1.25%	
3	3.0 ^b	1.9 ^c	0.8 ^d	0.6 ^d	5.0 ^a
5	3.6 ^b	2.2 ^c	1.8 ^c	2.0 ^c	7.8 ^a
7	5.5 ^b	3.7 ^c	2.5 ^d	2.4 ^d	9.2 ^a
9	5.6 ^b	3.8 ^c	2.8 ^d	3.0 ^d	10.0 ^a
11	6.0 ^b	4.0 ^c	3.3 ^c	3.7 ^c	10.0 ^a
13	6.5 ^b	5.5 ^c	5.0 ^c	4.6 ^c	10.0 ^a
15	7.0 ^b	5.8 ^c	5.4 ^c	5.6 ^c	10.0 ^a

* Each sampling day, melanosis scores having the same superscript letter were not significantly different at ($\alpha = 0.05$).

REFERENCE

- Al-Jassir, M. S. 1987. Polyphenol oxidase inhibition by glucose oxidase in pink shrimp (Pandalus borealis).
- Allen, D.H., and Collett, P. 1981. Life-threatening asthmatic reactions to the food and drug preservative, sodium meta bisulfite. J. Allergy Clin. Immunol. 67:71.
- Bailey, M.E., Fieger, E.A., and Novak, A.F. 1960. Phenoloxidase in shrimp and crab. Food Res. 25:565.
- Hennet, E.P., Sowboda, J., and Massey, V. 1966. On The reaction of the Glucose Oxidase from *Asperigulus niger* with Bisulfite. J. Biol. Chem. 241: 3409-3416.
- Finne, G., and Miget, R. 1985. The use of sulfiting agents in shrimp US regulations for domestically produced and imported products. FLorida Sea Grant Program "Monitoring sulfite residuals on shrimp. September 1986.
- Judokusumo, K. 1985. Glucose oxidase treatment to preserve fresh shrimp. Master thesis. University of Rhode Island.
- Otwell, W.S. and Marshall, M. 1986. Studies on the use of sulfiting agents to control shrimp melanosis (black spot). Florida Sea Grant Colloge. Technical paper NO. 46.
- Searle Biochemics. 1966. Glucose oxidase: properties and application to foods. Technical bulletin No. 2.

Shimp, L.A. 1985. Food phosphate in seafood processing.
Seafood leader, Spring. pp 114-119.

Simon, R.A., Green, L. and Stevenson, D.D. 1982. The
incidence of ingested metabisulfite sensitivity in an
asthmatic population. J. Allergy Clin. Immunol. 69:118.

APPENDIX
LITERATURE REVIEW

LITERATURE CITED

APPENDIX I.

LITERATURE REVIEW

LITERATURE REVIEW

Polyphenol oxidase [PPO] is widely distributed in nature and easily detected in plant and animal tissues. PPO is classified as an oxidoreductase and registered in the Enzyme Nomenclature under the number 1.14.18.1 and the systematic name of monophenol, dihydroxyphenylalanine: oxygen oxidoreductase [Enzyme Nomenclature CRC]. It has been called tyrosinase, polyphenolase, phenolase, and catalase at different times. The enzyme participates in two distinct reactions: the hydroxylation of monophenols to o-dihydroxyphenol by insertion of a hydroxyl group in the ortho position of a phenolic compound and the subsequent dehydrogenation of the o-diphenol to the corresponding o-quinone [Duckworth and Coleman, 1970]. For a long time it was not confirmed whether this action was accomplished by two different enzymes or by a dual-function enzyme. Recently, most results suggested that one enzyme performs two functions [Lerner, 1953; Nelson and Dawson, 1944 ; Bailey et al, 1960]. The first function, the conversion of monophenol to diphenol, is referred to as the cresolase activity and the second function, the transformation of the diphenol to quinone, is referred to as the catecholase

activity. The two activities seem to be independent of one another, as indicated by reports of "high-cresolase" and "high-catecholase" enzyme preparations [Nelson and Dawson, 1944 ; Smith and Krugger, 1962]. Figure 1 shows the proposed pathway for the enzymatic oxidation of tyrosine to the dark-colored melanin (Liner and Fitzpatrick, 1950).

The term melanin is applied to the polymer derived from oxidized phenols; no definition has been established regarding the size or precise structure of the polymer [Swan, 1963]. The initial step in the reaction is the oxidation of tyrosine to 3,4-dihydroxyphenylalanine [Dopa]. This reaction is quite slow. The next step in the reaction sequence, the conversion of Dopa to Dopa quinone, is a much faster reaction than the hydroxylation of tyrosine [Nelson and Dawson, 1944]. The remaining steps in the reaction sequence may proceed spontaneously, although the reaction rate is increased in the presence of the enzyme [Mathew and Parpia, 1971]. One of the intermediate reaction products in this sequence is 2-carboxy-2,3-dihydroindole-5,6 quinone [Dopa chrome]. In a cell-free system, this product accumulates in solution and is commonly used to measure enzyme activity [Fling et al, 1963]. In plants this reaction is thought to function as a protective mechanism for areas exposed by tissue damage or by infection. The o-quinones formed by PPO as a

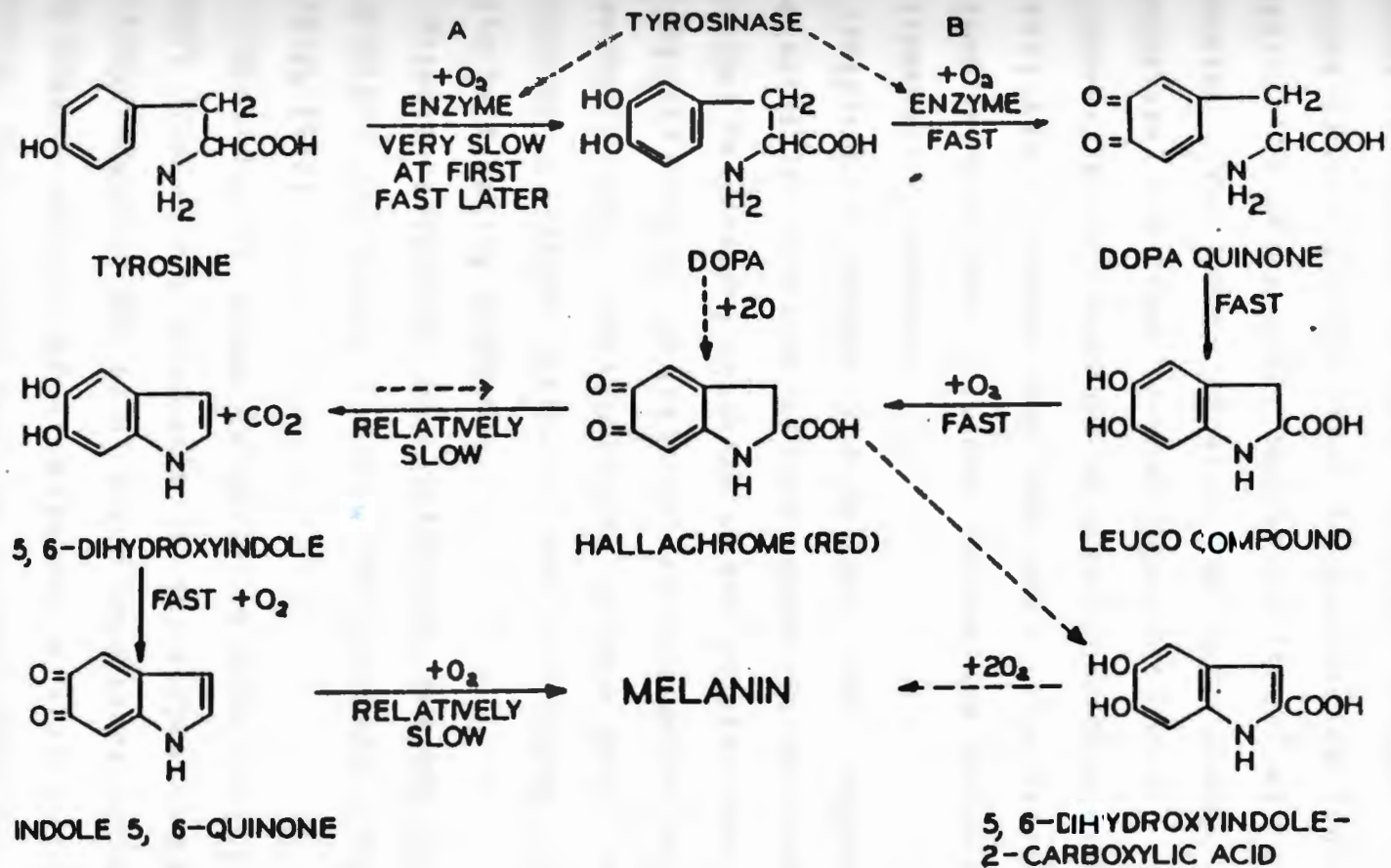


Figure 1. The formation of melanin pigments resulting from the oxidation of tyrosine by polyphenol oxidase (Liner and Fitzpatrick, 1950).

result of a wound are highly reactive with nucleophilic groups (hydroxyl, amino, thiol, or activated methylene groups [Butt, 1980]) tend to polymerize readily, and react with amino acids and thiol functional groups or proteins [Van Sumere et al, 1975; Butt, 1980]. In plant metabolism, PPO plays several important roles:

1. Hydroxylation reactions of phenolic biosynthesis. Mason [1957] has proposed that PPO acts as a hydroxylating enzyme in vivo, for instance, during the biosynthesis of o-diphenolic compounds.

2. Respiration, Mapson and Burton [1962] suggested that the oxidation function of the enzyme is involved in the terminal respiratory sequence of the potato tuber.

3. Resistance of plants to various microorganisms. [Deverall, 1961] concluded that quinone produced by the intensive oxidative action of PPO on injured tissues is toxic to invading pathogens.

4. Disease resistance. Upon infection, the PPO activity in the plant increases and new PPO appears [Frakas and Kiraly, 1962].

Browning is known to occur in many fruits and may result from either enzymatic or non-enzymatic reactions; in these reaction PPO is of major importance in catalysing the brown reaction after cutting, bruising, injury or picking. This browning reaction is desirable, for example in the manufacture of black tea [Takeo, 1966], in grapes

and prunes [Grencarevic and Hawker, 1971]. The browning reaction is undesirable in the handling, processing, or thawing of frozen raw fruits and vegetables and in flours with high levels of PPO [Vamos-vigyazo, 1981]. The reaction of o-quinones may alter the nutritive value of proteins [Szabo, 1979]. In the case of casein this reaction decreases the available lysine and reduces its digestibility. Gross and Coombs [1975] observed high levels of PPO in the manufacture of sugar from beets and cane, causing discoloration and lower yields.

There are many reports on the substrate specificity of PPO. Walker [1964] suggested that chlorogenic acid is a major substrate for PPO in apple and pear fruit. The browning of apple tissue resulting from the action of PPO is very important; it may be involved in the development of physiological disorders of apples, such as "bitter pit", and affects the quality of processed apple products. Palmer [1963] found that dopomine is the main substrate in the banana browning reaction. Mayer et al. [1964] used butanol treatment to activate and solubilize the PPO from sugar beets, a finding that indicates that PPO is bound to lipoprotein structures in the chloroplast; when the complex is broken, the phenolase is activated. In general, the most important natural substrates of PPO in fruits and vegetables are catechol, caffeic acid,

dopamine, quimic acid and chlorogenic acid [Luh and phithakpol, 1972] catechins, [Walker, 1975], and 3,4-dihydroxyphenylalanine [Dopa] and tyrosine [Williams, 1963].

In microorganisms, PPO is normally synthesized under unfavorable conditions [e.g., starvation], but not during vegetative growth. PPO is responsible for the conversion of L-tyrosine to melanin, the main pigment of perithecia and ascospores [Feldman and Thayer, 1974].

It has been demonstrated that PPO plays an important role in the process of sclerotization [Burnet, 1965]. The quinones, which are the oxidation products react with available free amino groups in the cuticle protein, linking adjacent polypeptide chains in a tight network and thereby converting a soft protein into a hard, insoluble material called sclerotin.

Polyphenol oxidase is known to have multiple forms which differ in molecular mass, isomerism and amino acid sequence as has been demonstrated when the enzyme is obtained from different sources. Multiple forms have been found in mushroom tyrosinase [Bouchilloux et al., 1963; Jolley and Mason, 1965], in potatoes and apples [Constantinides and Bedford, 1967], and in chloroplasts from apples [Meyer and Biehl, 1981]. Two enzymes with PPO activity have been purified from the frog Rana pipiens [Mikkelsen and Triplett, 1975]; both enzymes are isolated

in an inactive form that can be activated with trypsin. Burnett,[1965] and Holstein et al. [1971] have isolated three forms of PPO from mouse melanoma and hair follicles designated T1 [80,000 M.W], T2 [62,000 M.W] and T3 [66,000 M.W].

Copper has been found to be present in small amounts in PPO preparations [Keilin and Mann, 1938]. Keilin and Mann [1938] demonstrated that on removal of copper from the enzyme, loss of activity was observed and that activity was regenerated with added copper; therefore, copper is considered the prosthetic group of PPO. Other experiments inhibiting the enzyme with carbon monoxide also gave evidence of the presence of a copper atom in the active site [Keilin and Mann, 1938; Kertesz, 1966, and Nakamura et al. 1966].

Kertesz and Zito [1965] studied mushroom PPO and found that all copper was in the cuprous state. Depending on the mode of preparation and age of the sample, however, they found that the cupric content increased by up to 25%. For The one of their preparations from mushroom copper analysis by electron spin resonance spectroscopy showed that the cupric form was only 7% of the total copper; after two weeks storage, the cupric content was found to be as high as 25%, as determined by 2,2-diquinol.

Solomon [1980] found that tyrosinase contains a

strongly coupled binuclear copper active site, as do other metalloproteins [hemocyanin, laccase, and ascorbic acid oxidase] but is different in its biological function. It is characterized by the lack of an electroparamagnetic resonance [EPR] signal for formally cupric ions. It has only one binuclear copper unit [Gutteridge and Robb, 1975] as does hemocyanin.

The analysis of black spot in shrimp as a post-mortem deteriorative change was first reported by Fieger et al. [1950]. First it was suggested that melanosis was the result of mold growth [Fieger, 1950] Later investigation dismissed the possibility of microbial action, concluding that the black discoloration on the shells of shrimp packed in ice was caused by an enzyme naturally present in the organism [Alford and Fieger, 1952]. In an effort to locate the area of high activity in shrimp, Bailey et al. [1960] found that the most active preparations were obtained by pressing juice from the gill area of shrimp heads. That juice contained not only blood but also cellular fluids from internal organs as liver and stomach. These tissues appeared to be the principal source of enzymes involved in melanogenesis.

There is little information on how environmental factors can accelerate the onset of black spot or increase its intensity. One factor that has proven to affect the amount of active enzyme present in crustacean fluids is

the stage of the molting cycle [Summer, 1967]. In the premolt and molt stages, the activity of phenoloxidase and the levels of phenolic compounds increase [Cobb, 1977]. This results in hardening of the new exoskeleton. After molting, both enzyme activity and the level of substrate drop back to their normal levels. [Massayoshi and Perdigo [1984] showed that discoloration in female shrimp was stronger than that in males. This study showed that injuries did not always induce discoloration in shrimp stored in ice, but when shrimps were subjected to heavy trauma such as a blow, discoloration was found as deep as the superficial muscle. On the other hand most of the common, natural black spots remained in the shell after peeling, and those on the superficial muscle could be scraped off the black membranes.

PP0 is extracted by a large variety of methods, depending on the source and the desired degree of purity. Most of the extraction procedures include all or some of the following steps : fractionation with organic solvents and buffer extraction, use of detergents to solubilize the enzyme, such as Triton x-100 [Harel and Mayer, 1968] or Tween-80 [Ben-Shalom et al.; 1977], precipitation with ammonium sulfate [Scopes, 1978], and presence of a reductant such as ascorbic acid [Constantinides and Bedford, 1967] and a binding agent to avoid the reaction of the enzyme with quinones

[Rhodes, 1977].

The purification of PPO is extremely difficult, because the enzyme is generally present in very low concentrations. Kertesz and Zito, 1965] estimated that even in the best mushroom samples [regarded as the best source of polyphenolase], the concentration of the enzyme is on the order of 0.004% [40 mg/kg].

Another major problem in the isolation of PPO from tissue is the occurrence of phenolic substrate and enzyme side by side. In living tissues these two components are separated within the cells, but on maceration, extraction, and other processing treatments, they come into contact and give rise to quinones and other condensation products. It is very important to prevent the interaction of the enzyme with plant phenolics. Plant phenolics can interact with proteins in any or all of the following four ways :

1. By hydrogen binding, which forms strong bridges between the hydroxyl groups of the phenolics and the oxygen of the peptide bonds.

2. By oxidation to quinone, followed by covalent coupling reactions or by oxidation of protein functional groups by the quinone, yielding a melanoprotein mixed polymer.

3. By ionic interactions since most proteins and phenolics at neutral and acidic pHs have a charge.

4. By hydrophobic interactions, due to interaction between the aromatic ring of the polyphenols and the hydrophobic areas of proteins [Loomis, 1974]. These interactions could yield an inactive enzyme, which would change most of its hydrodynamic properties. In recent years this problem has been solved to some degree by binding the phenol compounds to an insoluble polymer.

The most widely used phenol scavenger is polyvinyl pyrrolidine [PVP], a very strong proton acceptor at neutral or acidic pHs, where phenols are not ionized [Loomis and Battaile, 1966; Anderson and Sower, 1968]. Polyethylene glycol [PEG] is used due to its solubility in acetone [Loomis and Battaile, 1966]. Casein has been used to form hydrogen bonds with the phenol [Thomas and Janavae, 1973].

The initial step in isolating the enzyme is extraction with cold acetone, yielding an acetone powder. This step removes the interfering phenolic substrates from the enzyme. Due to the quick water removal, proteins can be precipitated undamaged; once they are dried they can be stable for prolonged periods of storage. Also, cold acetone has the added benefit of removing lipid materials from the enzyme preparations. Once the interfering substrates are removed, the next important step is obtaining the enzyme in a soluble form. Several liquids have been used for this purpose; however, the aqueous

solutions of moderately high ionic strength and appropriate pH have been found to be very successful [Mathew and Parpia, 1971].

Column chromatography has often been used in purification and separation of PPO. While both adsorption chromatography and ion-exchange chromatography have been employed, the latter has been found to be particularly successful, especially when substituted celluloses such as diethylaminoethyl cellulose [DEAE] and carboxymethyl cellulose [CMC] are used [Peterson and Sober, 1962]. Celite has been found to be selective as an adsorbent for phenolases, but enzyme adsorption was found to be dependent on copper. When copper was removed from phenolases of Neurospora crassa, no adsorption was noted for the apoenzymes [Fling et al, 1963]. Hydrophobic adsorption chromatography has been used to separate phenoloxidases from peaches [Flurkey and Jen, 1980].

PPO activity can be assayed by diverse methods: manometry, polarography, chronometry and spectrophotometry. Mayer et al. [1966] published a critical review of these methods, which measure the PPO activity by monitoring oxygen consumption, product formation, or the coupled reaction by an added reagent. Using diverse assay methods makes comparison of results difficult for two reasons: [1] the units used to express the activity depend on the method used and [2] the

accuracy and sensitivity of the different methods vary.

Spectrophotometric methods were considered the best method to measure the amount of product being formed; o-quinones, when polymerized with phenolics, form a brownish color easily detected at 420 nm. Spectrophotometric methods using coupled reactions have become more popular because : [1] the equipment used is found in most laboratories and [2] the methods are less complicated to perform [Soto,1983].

Inhibition of polyphenol oxidase can be achieved by inactivating the enzyme by a physical or chemical method. Physical methods include the exclusion of oxygen, the storage of raw products at freezing temperatures, and heat inactivation of the enzyme [Yankov, 1963]. Chemical methods are usually preferred over physical methods. Only a few of the substances used to inhibit the activity of PPO in foods are accepted due to restrictive requirements such as non-toxicity, wholesomeness, and effect on taste, flavor, and texture, etc. [Walker, 1975].

Since PPO is a metalloprotein with copper as the prosthetic group, it can be inhibited by metal-chelating agents such as cyanide [Walker, 1975], carbon monoxide [Pierpoint, 1966]; sodium diethyldithiocarbamate [DIECA] [Robb, et al, 1966], mercaptobenzithiazole [Palmer and Roberts, 1967]. Most of these reagents are used by biochemists to avoid the interaction with

polyphenols during the isolation of different molecules from plant tissues [Rhodes, 1977]. Benzoic acid and some substituted cinnamic acids were found to be the most competitive inhibitors of the enzyme from sweet cherries [Piffere, et al. 1974], apples [Walker, 1964], pears [Walker, 1976], and apricots [Soler et al. 1965].

Many investigators have tried to prevent black spot in shrimp. One of the first observations on the prevention and delay of black spot in shrimp involved removal of the shrimp head immediately after the catch is brought on board [Alford and Fieger, 1952]. Other methods of inhibiting melanosis include the use of chemicals which interfere with black spot formation. One of these compounds is ascorbic acid, which has been proven to be useful in delaying black spot formation in shrimp [Flaukner et al., 1954]. Ascorbic acid delays the reaction by competing for available oxygen. Cysteine and glutathione have been used to reduce PPO activity by combining with quinones to form thioethers [Mason, 1955; Loomis and Bataille, 1968]. Many food additives have been used as metal chelators, such as sodium triphosphate [STP], sodium hexametaphosphate [SHP], and sodium acid pyrophosphate [SAPP] [Lindsay, 1976]. The effectiveness of these compounds is known to vary with pH.

Of the many additives suggested as treatment against black spot in raw shrimp, sulfiting agents are by far the

most effective. Six sulfiting agents-sulfur dioxide, sodium and potassium metabisulfite, sodium and potassium bisulfite, and sodium sulfite-have been widely used in foods. Sulfiting agents have been found to be the most powerful and substance demonstrated to strongly inhibit in shrimp melanosis, especially sodium bisulfite or metabisulfite. Sodium bisulfite is a strong reducing agent and competes with tyrosine for molecular oxygen. It can "tie up" the oxygen and prevent the formation of black spot [Camber et al., 1956; Nickelson and Cox, 1986]. Sulfiting agents were first introduced for use by the shrimp industry during the early 1950s. All sulfiting agents are considered GRAS [Generally Recognized As Safe]; however, the fact that a substance is listed as a GRAS additive does not mean, that it can be used safely at any level in foods. Thus, in 1956 the U.S. Food and Drug Administration [FDA] issued letters that gave "prior sanctions" for use of sulfites to control shrimp melanosis. This sanction specifically stated the use of 1.25% sodium bisulfite dips for one minute, and that product labelling was necessary. More recent FDA decisions reaffirmed these practices [Federal Register, 1982], but continuing regulatory scrutiny could restrict or eliminate the application of sulfite on shrimp. This regulatory action is prompted by an increasing concern for adverse "allergic"

reactions most common among hyper-[sulfite-] sensitive asthmatics [Otwell and Marshall, 1986]. In January, 1985 the FDA established 100 ppm [part per million as SO₂] as the action level or maximum allowable level of residual sulfite permissible in the raw edible portion of shrimp. In April, 1985, the FDA reinstated the requirement to label all foods, including shrimp, that have a sulfite residue in excess of 10 ppm [Federal Register, 1985].

Thus, work must be initiated to find alternatives to replace or reduce the amount of sulfites required to inhibit shrimp melanosis.

The enzyme system of glucose oxidase and catalase [GOX/CAT] has been used as a food additive and is considered as GRAS [Searle Biochemic, 1966]. Glucose oxidase have been produced from a number of fungi such as Penicillium notatum (Keilin and Hartee, 1952), Penicillium amagasakiense (Kusai, et al., 1960), and the most popular production form Asperigillus niger (Swoboda and Massay, 1965; Pazure and Kleepe, 1964). The enzymes from each of these organisms have about the same isoelectric point (4.2-4.3), the same pH optimum (5.5-5.8), and contain two flavin adenine dinucleotide units per mole of enzyme (Furia, 1977).

Glucose oxidase shows a high affinity for D-glucose (Gibson and Swoboda, 1964; Adams, et al., 1960). Other

hexoses, pentoses, or disaccharides are not oxidized, or are oxidized only at negligible rates. (Pazur and Kleepe, 1966). For all practical purpose purified glucose oxidase reacts only with glucose, and this was made the enzyme available tool for analysis, as well as for other purposes.

The usual commercial preparations of glucose oxidase contain catalase, which is advantageous in most of the applications. The overall reactions of the commercial glucose oxidase/catalase system (GOX/CAT) are shown in Figure 2. For the industrial application the GOX/CAT enzyme system is used to remove glucose and to remove oxygen.

The most important application of glucose oxidase for the removal of glucose is from the egg albumen and whole eggs prior to drying (Scott, 1953; Baldwin, et al., 1953).

Oxygen is responsible for a wide types of deterioration of foods. It has been demonstrated that addition of glucose oxidase is very effective in removing residual oxygen from beer and stabilized it (Ohlmeyer, 1957; Reinke, et al., 1963). Glucose oxidase found to remove oxygen from unpasteurized apple wine, prevented microorganism growth, and off-flavor development (Yang, 1955). Glucose oxidase have been found to be effective in protecting cans of carbonated beverages against oxidative corrosion, such as canned soda (Underkofler, 1961); to

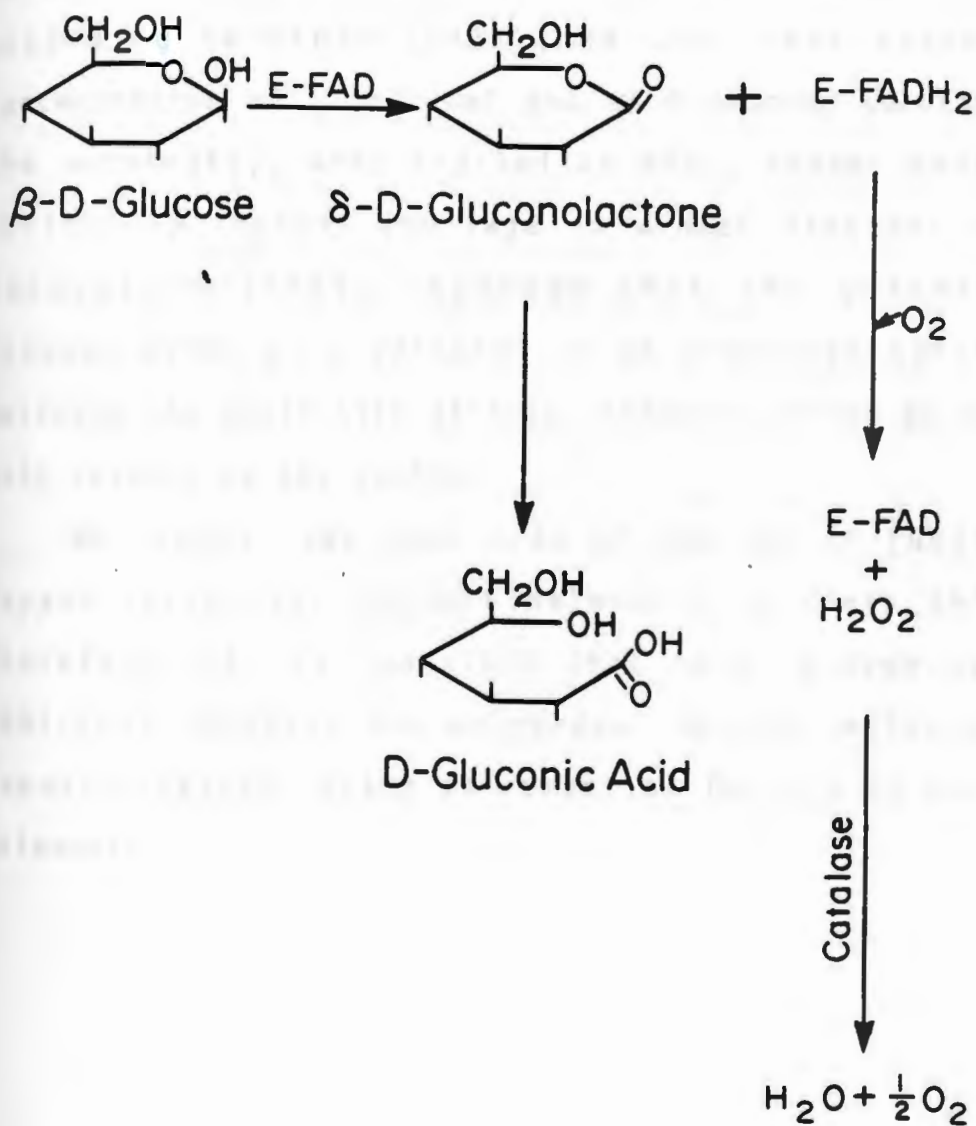


Figure 2. Overall reactions of the commercial glucose oxidase/catalase system. (Furia, 1977).

protect the water and oil emulsion by removing oxygen from mayonnaise (Bloom, et al., 1956). Glucose oxidase has been reported to produce acid (gluconic acid) for milk coagulation (Rand, 1972). Recently, the GOX/CAT enzyme system has been used for its bacterial effect in fish. According to Field (1981), he used this system at concentration of 1 units/ml and 4% D-glucose solution as the substrate, when applied as adip, showed definite ability to inhibit spoilage in winter flounder fish. Judokusumo (1985) reported that the GOX/CAT at concentration of 2 units/ml in 4% D-glucose solution, extended the shelf life of fresh atlantic shrimp by 28-33% with respect to the control.

No report has been made of the use of [GOX/CAT] enzyme system to inhibit melanosis in fresh shrimp. Therefore, it is possible that this enzyme system [GOX/CAT] inhibits the polyphenol oxidase activity by removing oxygen, which is essential for PPO to produce melanosis.

REFERENCES

- Adams, E.C., Mast, R.L. and Free, A.H. 1960. Specificity of glucose oxidase. Arch. Biochem. Biophys. 91:230-234.
- Alford, J.A and Fieger, E.A. 1952. The non-microbial nature of the black spot on ice-packed shrimp. Food Technol. 6:217.
- Al-Jassir, M.S. 1987. The inhibition of polyphenol oxidase by glucose oxidase and catalase enzyme system in pink shrimp (Pandalus borealis). Manuscript 1. Ph.D. Dissertation. University of Rhode Island.
- Allen, D.H., and Collete, P. 1981. Life-threatening asthmatic reaction to the food and drug preservative, sodium metabisulfite. J. Allergy Clin. Immunol. 67:71.
- Anderson, R.A, and Sower, J.A. 1968. Optimum conditions for bonding plant phenols to insoluble polyvinylpyrrolidone. Phytochemistry 7: 293.
- Bailey, M.E, Fieger, E.A., and Novak, A.f. 1960. Phenoloxidase in shrimp and crab. Food Research. 25: 565.
- Baldwin, R.R., Campbell, H.A., Thiessen, R., and Lorant, G.J. 1953. The use of glucose oxidase in the processing of foods with special emphasis on the desugaring in egg white. Food Technol. 11:503-507.

- Ben-Shalom, N., Kahn, V. Harel, E., and Mayer, A.M. 1977. Olive catechol oxidase changes during fruit development. J. Sci. Food Agric. 28: 545-550.
- Bergmeyer, H.U. 1974. Methods of Enzymatic Analysis. Vol 3. Academic press. Inc. NY.
- Bloom, J., Scofield, G., and Scott, D. 1956. Oxygen removal prevents acidity in mayonnaise. Food packer. 37:16-17.
- Bouchilloux, S., McMahill, P., and Masom, H.S. 1963. The multiple forms of mushroom tyrosinase. J. Bio.Chem. 5: 1699-1707.
- Burnett, P.G.J. 1965. The metabolism of aromatic compounds in "Aspect of Insect Biochemistry. (ed. Goodwin) P.49. London Academic Press.
- Butt, V.S. 1980. Direct oxidase and related enzymes. In: The Biochemistry of Plants. Ed. Sarraf, P.K and Conn, E.E Eds. [Academic Press, New York]. 12: 81-121.
- Camber, C.I., Vance, N.H, and Alexander, J.1956. How to use sodium bisulfite to control "black spot" on shrimp. Univ. Miami. special Bull. No. 12.
- Chubey, B. B. and Dorell, G.D. 1972. Enzymatic browning of stored parsnip roots. J. Am. Soc. Hortic. Sci. 97:107.

- Cobb III, B.F. 1977. Biochemistry and physiology of shrimp: effect on use as food. In "Handling, Processing and marketing of tropical fish". Tropical Products Institute, London.
- Constantinides, S.M. and Bedford, C.L. 1967. Multiple forms of phenoloxidase. J. Food Sci. 32: 446-450.
- Deverall, B.J. 1961. Phenolase and pectic enzyme activity in the chocolate spot disease of beans. Nature. 189: 311.
- Duckworth, M.W., and Coleman, J.E. 1970. Physiochemical and Kinetic properties of mushroom tyrosinase. J. Biol. Chem. 245: 1613-1087.
- El-Bayoumi, M.A. and Frieden, E. 1957. A spectrophotometric method for the determination of the catecholase activity of tyrosinase and some applications. J. Am. Chem. Soc. 79: 4854-4858.
- Faulkner, M.B., Watts, B.M., and Humm, H.J. 1954. Enzymatic darkening of shrimp. Food Research, 19: 302-310.
- Federal Register. July,9, 1982. Sulfiting agents : Proposed affirmation of GRAS status with specific limitation. 47 [132]: 29956-29963.
- Federal Register, 1985. Food labeling, proposed rules concerning sulfiting agents; proposed rule. 50, No. 64 : 13306-13307.

- Feldman, J.F. and Thayer, J.P. 1974. Cyclic AMP-Induced Tyrosinase Synthesis. *Biochem. Biophys. Res. Comm.* 61: 977.
- Fieger, E.A., Colmer, A.R., and Alford, J.A. 1950. Black shrimp and black spotted shrimp. *Southern Fisherman.* 10: 12.
- Field, C.E. 1986. Utilization of Glucose Oxidase for Extending the shelf life of fish. *J. Food Sci.* 51: 66-70.
- Finne, G., Wagner, T., DeWitt, B., and Martin, R. 1986. Effect of treatment, ice storage and freezing on residual sulfite in shrimp. *J. Food Sci.* 51: 231-232.
- Fling, M., Horowitz, N.H., and Heinemann, S.F. 1963. The isolation and properties of crystalline tyrosinase from *Neurospora crassa*. *J. Biol. Chem.* 238: 2045.
- Flurkey, W.H. and Jen, J.J. 1980. Purification of peach polyphenol oxidase in the presence of added protiase inhibitors. *J. Food Biochem.* 4: 29-42.
- Finne, G. and Migget, R. 1985. The use of sulfiting agents in shrimp and crab. *Food Research* 25:565.
- Frakas G.L., and Kiraly, Z. 1962. Role of phenolic compounds in the physiology of plant disease and disease resistance. *Phytopathol.* 4: 105.
- Furia, T.E. 1977. Enzymes. Handbook of Food additives. 2nd Ed. Vol.1. Intechmark Corporation, Palo, Alto, California.

- Gibson, Q.H., Swoboda, B.P., and Massey, V. 1964. Kinetics and Mechanism of Action of Glucose Oxidase. *J. Biol. Chem.* 239: 3927-3934.
- Gross, D., and Coombs, J. 1975. Enzymatic color formation in beet and cane juices. Paper presented at the 15th General Assembly of C.I.T.S. Vienna.
- Grencarevic, M. and Hawker, J.S. 1971. Browning of sultana grape berries during drying. *J. Sci. Food Agric.* 22: 270-272.
- Gutteridge, S. and Robb, D. 1975. The catecholase activity of Neurospora tyrosinase. *J. Biolchem.* 54: 107-116.
- Harel, E. and Mayer, A.M., and Shain, Y. 1965. Purification and multiplicity of catechol oxidase from apple chloroplasts. *Phytochem.* 4: 891-893.
- Hennet, E.P., Swoboda, J., and Massey, V. 1966. On the reaction of the Glucose Oxidase from Asperiglus niger with Bisulfite. *J. Biol. Chem.* 241: 3409-3416.
- Holstein, T.J., Quevedo, W.C. Jr. and Burnett, J.B. 1971. Multiple forms of tyrosinase in rodents and lopomorphs with special reference to their genetic control in mice. *J. Exp. Zool.* 177: 173.
- International Union of Biochemistry. 1972. Standing Committee on Enzymes, Enzyme Nomenclature Recommendations. Elsevier, Amsterdam.
- Jolley, R.L. and Mason, H.S. 1965. The multiple forms of mushroom tyrosinase. *J. Biol. Chem.* 240: 1489-1490.

- Judokusumo, K. 1985. Glucose oxidase treatment to preserve fresh shrimp. Master thesis. University of Rhode Island.
- Keilin, D. and Mann, T. 1938. Polyphenol oxidase : Purification, nature, and properties. Proc. Roy. Soc. London B. 125: 187-204.
- Kielin, D., and Hartree, E.F. 1952. Specificity of glucose oxidase (Notation). Biochem. J. 50:3310341.
- Kertesz, D. and Zito, R. 1965. Mushroom polyphenol oxidase : Purification and general properties. Biochem. Biophys. Acta 96: 447-462.
- Kertesz, D. 1966. The copper of polyphenol oxidase. In: The Biochemistry of copper. Peisach, J. and Aisen, P. Eds.[Academic Press, N.Y.], pp. 359-369.
- Kleppe, K. 1966. The effect of hydrogen peroxide on glucose oxidase from Asperigllus niger. Biochem. J. 5 139-143.
- Kusai, K., Sekuzu, I. and Nakai, M. 1960. Crystallization of glucsoe oxidase from Penicillium amagasakeinse. Biochim. Biophys. Acta. 40:555-557.
- Lerner, A.B. 1953. Metabolism of phenylalanine and tyrosine. Advanvce, Enzymol. 14: 73.
- Lindsay, R.C. 1976. Other desirable constituents of food. In "Principles of Food Science". ed. By Fennema, O.R., Marcel Dekker Inc., New York and Basel.
- Linser, A.B. and Fitzpatrick, B. 1950. Biochemsitry of melanin formation. Physiol. Rev. 30: 91-123.

- Loomis, W.D. and Battaile, J. 1966. Removal of phenolic compounds and the isolation of plant enzymes. *Phytochemistry* 5: 324.
- Loomis, W.D. 1974. Overcoming problems of phenolics and quinones in the isolation of plant enzymes and organelles. *Methods of Enzymol.* 31: 528-544.
- Lowry, O.H., Rosenbrough, N.J., Farr, A.L., and Randall, R.J. 1951. Protein measurement with folin phenol reagent. *J. Biol. Chem.* 193:265.
- Luh, B.S. and Phithakpol, B. 1973. Characteristics of polyphenoloxidase related to browning in cling pears. *J. Food Sci.* 37: 264-268.
- Mapson, L.W. and Burton, W.G. 1962. The terminal oxidases of the potato tuber. *Biochem. J.* 82: 19.
- Massayoshi, O., and Perdigao, N.B. 1984. On physiological aspect of black spot appearance in shrimp. *Bulletin of the Japanese Soci. Scien. Fisheriers.* 50 [10]: 1763-1769.
- Mason, H.S. 1955. Comparative biochemistry of the phenolase complex. *Adv. Enzymol. Relat. Subj. Biochem.* 16: 105-184.
- Mason, H.S. 1957. Mechanisms of oxygen metabolism. *Advan. Enzymol.* 19: 128.
- Mathew, A.G. and Parpia, H.A, 1971. Food browning as polyphenol reaction. *Adv. Food Res.* 19: 75.

- Mayer, A.M., Harel, E., and Shain, Y. 1964. 2,3-naphthalenediol, a specific competitive inhibitor of phenolase. *Phytochem.* 3: 447-451.
- Mayer, A.M., Harel, E., and Ben-shaul, J. 1966. Assay of catechol oxidase - A critical comparison of methods. *Phytochem.* 5: 783-789.
- Meyer and Biehl, 1981. Activation of latent phenolase during spinach leaf senescence. *New Phytochem.* 20: 959-995.
- Mikkelsen, R.B. and Triplett, E.L. 1975. Tyrosinase in *Rana pipiens*. *J. Biol. Chem.* 250: 638.
- Nakamura, T., Sho, S. and Ogura, Y. 1966. On the purification and properties of mushroom tyrosinase. *J. Biochem.* 59: 481-486.
- Nelson, J.M. and Dawson, C.R. 1944. Tyrosinase. *Advan. Enzymol.* 4: 99.
- Nicklson and Cox 1986. Stop shrimp "black spot" Marine advisory Bulletin, Fisheries. Texas Agric. Extension Service. Sea Grant program. Texas A & M University.
- Ohlmeyer, D.W. 1957. Use of glucose oxidase to stabilize beer. *Food Technol.* 11:503-507.
- Otwell, W.S., and Marshall, M. 1986. Studies on the use of sulfiting agents to control shrimp melanosis [black spot]. Florida Sea Grant College. Technical Paper No.46.
- Palmer, J.K. 1963. Banana polyphenoloxidase preparation and properties. *Plant physiol.* 38: 508.

- Palmer, J.K. and Roberts, J.K. 1967. Inhibition of banana polyphenol oxidase by 2-mercaptobenzothiazole. *Science*. 157: 200-201.
- Pazur, J.H., and Klee, K. 1964. Oxidation of glucose and related compounds by glucose oxidase from Asperigulus niger. *Biochemistry*. 3:578-583.
- Peterson, E.A. and Sober, H.A. 1962. Column chromatography of proteins. Substituted cellulose. *Methods. Enzymol.* 5: 3.
- Pierpoint, W.S. 1966. The enzymatic oxidation of chlorogenic acid and some reactions of the quinone produced. *Biochem. J.* 98: 567.
- Piffere, P.G., Baldassari, L., and Cultrera, R. 1974. Inhibition by carboxylic acids of an o-diphenol oxidase from Brunus avium fruits. *J. Sci. Fd. Agric.* 25: 263-270.
- Priputina, L.S. 1974. Glucose Oxidase and Catalase as Food Additive. *Chem. Abstract.* 81: 103, 345.
- Rand, A.G. Jr. 1972. Direct enzymatic conversion of lactose to acid: glucose oxidase and hexose oxidase. *J. Food Sci.* 37:698.
- Rand, A.G. Jr. and Hourigan, J.A. 1974. Direct Enzymatic Conversion of Lactose in Milk to Acid. *J. Dairy Sci.* 58:8, 1114-1150.
- Reed, G. 1975. Oxidoreductases In Enzymes in Food Processing. 2nd edition. Academic press. N.Y.

- Reinke, H.G., Hoag, L.E., and Kincaid, C.M. 1963. Effect of antioxidants and oxygen scavengers on the shelf-life of canned beer. Am. Soc. Brewing Chemists Proc. PP 175-180.
- Rhodes, M.J.C. 1977. The extraction and purification of enzymes from plant tissues. In: Regulation of Enzyme Synthesis and activity in Higher Plants. Smith, H., Ed. [Academic Press; New York], 245-269.
- Robb, D.A., Swan, T., and Mapson, L.W. 1966. Substrates and inhibitors of the activated tyrosinase of bread bean [Vicia faba L.] Phytochem. 5: 665-675.
- Scopes, R.K. 1978. Techniques for protein purification. In: Techniques in the life Sciences. Vol. Biol. [Elsevier: Amsterdam]. pp 1-42.
- Scott, D. 1953. Glucose conversion in preparation of alumin solids by glucose oxidase catalase system. J. Agr. Food Chem. 1:727-730.
- Searle Biochemics, 1966. Glucose oxidase: Properties and application to foods. Technical Bulletin No. 2
- Shimp, L.A. 1985. Food phosphate in seafood processing. Seafood leader, spring. pp 114-119.
- Simon, R.A., and Steven, D.D. 1982. The incidence of ingested metabisulfate sensitivity in as asthmatic poputlation. J. Allergy Clin. Immunol. 69: 118.

- Smith, J.L. and Krugger, R.C. 1962. Separation and purification of the phenolases of common mushroom. *J. Biol. Chem.* 237: 1121.
- Soler, A., Garcia, F.S. and Lozono, J.A. 1965. Structure of inhibitor of apricot phenolase. *Chem. Abst.* 65: 2558.
- Solomon, E.I. 1980. In: copper proteins. Spiro, T.G., Ed. [Wiley: New York., in Press.
- Soto, L.A.S. 1983. Inhibition of polyphenol oxidase by sulfur dioxide. Ph.D. thesis. Oregon State University.
- Stevenson, D.D. and Simon, R.A. 1981. Sensitivity to ingested metabisulfites in asthmatic subjects. *J. Allergy Clin. Immunol.* 68:26.
- Summer, N.M. 1976. Cuticle sclerotization and blood phenoloxidase in the fiddler crab, Uca pugnax. *Comp. Biochem. Physiol.* 26: 259.
- Swan, G.A. 1963. Chemical structure of melanins. *Ann. N.Y. Acad. Sci.* 100: 1005.
- Swoboda, B.E., and Massay, V. 1965. Purification and properties of the glucose oxidase from Aspergillus niger. *J. Biol. Chem.* 240:2209-2215.
- Szabo, M.T. 1979. Effect of polyphenols on digestion of proteins *Proc. 19th Hung. Annu. Meet. Biochem.* pp. 223.
- Takeo, T. 1966. Tea leaf polyphenol oxidase. Part III. Studies on the changes of polyphenol oxidase activity during black tea manufacture. *Agric. Biol. Chem.* 30: 529-533.

- Thomas, P. and Janavae, M.T. 1973. Polyphenol oxidase activity and browning of mango fruits induced by gamma irradiation. *J. Food Sci.* 38: 1149.
- Underkofler, L.A. 1961. Glucose oxidase: production, properties, and potential applications. *Soc. Chem. Ind. London, Monograph* 11:72-86.
- Vamos-Vigyazo, L. 1981. Polyphenol oxidase and peroxidase in fruits and vegetables. *CRC. Crit. Rev. Food Sci. Nutr.* 15: 49-127.
- Van Sumere, C.F., Albrecht, J., Dedonder, A., Depooter, H., and Pe, I. 1975. Plant proteins and phenolics. In: The Chemistry and Biochemistry of Plant Proteins. Harborne, J.B., and Van Sumere, C.F., Eds. [Academic Press]: New York, pp. 211-264.
- Walker, J.R.L. 1964. Studies on the enzymatic browning of apples. II. Properties of apple polyphenol oxidase. *Aust. J. Biol. Sci.* 17:360.
- Walker, J.R.L. 1975. Enzymatic browning in foods : A review. *Enzymes Tech. Digest.* 4: 89-100.
- Walker, J.R.L. 1976. The control of enzymic browning in fruit juices by cinnamic acid. *J. Food Technol.* 11: 341.
- Werth, G.R. 1982. Inhaled metabisulfite sensitivity. *J. Allergy Clin. Immunol.* 70:143.
- Williams, A.H. 1963. Enzyme inhibition by phenolic compounds. In:Enzyme Chemistry of Phenolic Compounds. Peridham, J.B., Ed. (Pergamon press. Oxford).

Worthington Enzymes and Related Biochemical. 1982.

Biochemical products Division. Worthington Diagnostic
Systems Inc. Freehold. N.J.

Yang, H.Y. 1955. Stabilizing apple wine with glucose
oxidase. Food Res. 20:42-46.

Yankov, S.I. 1983. Heat inactivation of oxidizing enzymes
in some fruits and vegetables. Chem. Abstr. 59: 7851.