THE UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

KINETIC AND PHYSICAL PROPERTIES OF ISOPEROXIDASE A_3 FROM TOBACCO TISSUE CULTURE W-38

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the

degree of

DOCTOR OF PHILOSOPHY

BY

DARRYEL LYN REIGH

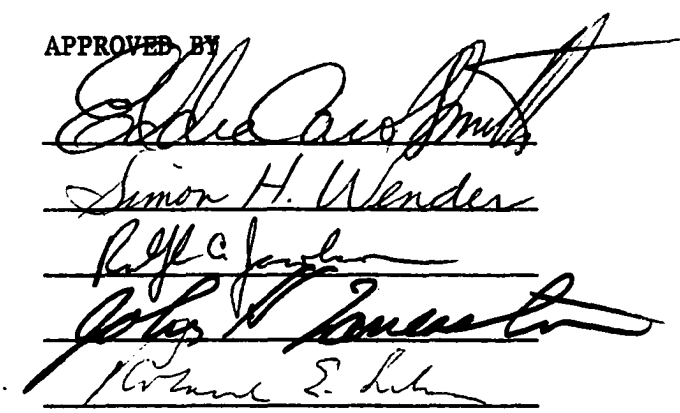
Norman, Oklahoma

1973

KINETIC AND PHYSICAL PROPERTIES OF ISOPEROXIDASE A_3

FROM TOBACCO TISSUE CULTURE W-38

APPROVED BY



DISSERTATION COMMITTEE

KINETIC AND PHYSICAL PROPERTIES OF ISOPEROXIDASE A_3 FROM TOBACCO TISSUE CULTURE W-38

By: Darryel Lyn Reigh

Major Professor: Eddie C. Smith, Ph.D.

The separation of a single anodic isoperoxidase from other isoperoxidases of tobacco tissue culture W-38 was achieved using ammonium sulfate fractionation and DEAE-cellulose chromatography. The molecular weight of this isoperoxidase is about 54,000. It appears to contain disulfide linkages and a heme group is indicated.

In the presence of H_2O_2 this isoperoxidase appears to oxidize several phenolic compounds including scopoletin, ferulic acid, catechol, guaiacol, caffeic acid, esculetin and chlorogenic acid.

The kinetics of scopoletin oxidation were investigated most extensively since this isoperoxidase tends to utilize scopoletin as a substrate much more readily than some other isolated isoperoxidases. Sigmoidal dependence of velocity on scopoletin concentration is observed. A mechanism is postulated to explain this observation.

Several phenolic compounds inhibit the scopoletin activity of this peroxidase as do manganous ions and IAA. Speculations are made concerning the regulation of IAA oxidase through the regulation of scopoletin peroxidase.

ACKNOWLEDGEMENTS

The author wishes to thank Dr. E. C. Smith for his guidance, knowledge and above all else for his special ability to understand and encourage. Without this encouragement and understanding this work could never have been accomplished. Dr. S. H. Wender, whose vast knowledge contributed greatly to this study, is also acknowledged. Appreciation is expressed to members of my advisory committee, Dr. R. A. Jacobson, Dr. R. E. Lehr and Dr. J. H. Lancaster for their advice and time.

Special thanks go to Dr. Ernest Sturch and Dr. Larry Smith for their contribution to my scientific training and for their insight into some critical philosophical problems.

Thanks are also expressed to fellow graduate students, particularly, to Bernie Powell and Jerry Pickering, who furnished some comparative data that was incorporated into this study.

There is no way to adequately express my appreciation to my parents. They wanted only that I have something better and were willing to make any sacrifice to that end.

A special appreciation is expressed to Chris who contributed more than she may ever realize.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF ILLUSTRATIONS

LIST OF ILLUSTRATIONS (cont.)

ABSTRACT

The separation of a single anodic isoperoxidase from other isoperoxidases of tobacco tissue culture W-38 was achieved using ammonium sulfate fractionation and DEAE-cellulose chromatography. The molecular weight of this isoperoxidase is about 54,000. It appears to contain disulfide linkages and a heme group is indicated.

In the presence of H_2O_2 this isoperoxidase appears to oxidize several phenolic compounds including scopoletin, ferulic acid, catechol, guaiacol, caffeic acid, esculetin and chlorogenic acid.

The kinetics of scopoletin oxidation were investigated most extensively since this isoperoxidase tends to utilize scopoletin as a substrate much more readily than some other isolated isoperoxidases. Sigmoidal dependence of velocity on scopoletin concentration is observed. A mechanism is postulated to explain this observation.

Several phenolic compounds inhibit the scopoletin activity of this peroxidase as do manganous ions and IAA. Speculations are made concerning the regulation of IAA oxidase through the regulation of scopoletin peroxidase.

CHAPTER I

INTRODUCTION

Findings of numerous workers in the field indicate the probable importance of peroxidase in plant tissue metabolism. The catalytic activity of peroxidase has been shown to be important in the oxidation of indole-3-acetic acid (IAA) (1); in lignification (2,3); in the oxidation of reduced pyridine nucleotides (4); and in the oxidation of some phenolic compounds (5,6,7,8).

Much of this work has utilized mixtures of isoenzymes of peroxidase. The largest amount of peroxidase research has utilized horseradish peroxidase (HRP). Only a few references pertain to investigations done with single isolated isoperoxidases. It has been demonstrated that isoperoxidases from a single source may vary significantly in physical properties such as molecular weight (9,10), Reinheit Zahl (11) and amino acid sequence (12). Kinetic properties may also vary for isoperoxidases. The rate constants (k_4) for guaiacol oxidation by Japanese radish isoenzymes vary by factors of 2 to 600 (9). Schafer *et al.* (13) demonstrated that the ability of scopoletin to stimulate guaiacol oxidation was different for two isoenzymes from tobacco tissue. Results that will be presented in this study demonstrate significant differences in the scopoletin

oxidizing ability of these same two isoenzymes. Many problems could exist with mixed isoenzyme effects, particularly in evaluating kinetic mechanisms of phenolic inhibition of IAA oxidase activity, such as those mechanisms proposed by Sirois and Miller for scopoletin (6) and by Gelinas for ferulic acid (7).

Much of the kinetic research involving peroxidases has been conducted utilizing synthetic (non-physiological) substrates such as diphenylamine, phenylenediamine, and guaiacol and/or electron acceptors such as dichlorophenol (DCP). Most "physiological" studies have been involved with the IAA oxidase activity of these enzymes and the effect of some naturally occurring phenolic compounds on this activity (6,7,14, 15). The emphasis on this aspect of peroxidase action has been justified by the importance of IAA as a growth regulator. Preoccupation with this function alone, however, has tended to limit severely the overall understanding of peroxidase action. Most peroxidase isolations indicate that from two or three to possibly as many as fifteen isoperoxidases may be elaborated by higher plants. If these observed numbers of isoenzymes are not artifacts of isolation, it seems inconsistent with metabolic economy that all of these isoenzymes would function mainly as IAA oxidases. Raa (16) has utilized a staining technique to show that peroxidase is associated with several cell organelles including the cell wall and the nucleus. Other attempts have been made to show the specific tissue and organelle location of individual isoperoxidases (17,18). Studies of compartmentalization of specific isoperoxidases and of the possible existence of substrate preferences for these isoenzymes (5) could result in the postulation of a more varied function

for peroxidase that would be much more consistent with metabolic economy.

Scopoletin is an example of a physiologically significant compound that has been shown to be involved in peroxidase action. Several workers have investigated the effect of scopoletin on IAA oxidase activity (6,8,15). Scopoletin has been indicated to be involved in growth regulation either directly (19) or indirectly (15) through its effect on IAA oxidase activity. It has also been postulated to be involved in lignification (20). Andreae (8) in investigating the inhibition of IAA oxidase activity by scopoletin first demonstrated that this compound was oxidized by IAA oxidase preparations. Reigh et al. (5) devised an assay procedure for following the kinetics of scopoletin oxidation making it possible to investigate kinetically the effect of peroxidase on this naturally occurring, physiologically significant compound. No doubt, other naturally occurring phenolics may also be oxidized by peroxidase, and the possible physiological importance of these oxidations should be considered.

In summary, the study of the action of isolated, single isoperoxidases on physiological substrates should contribute to the understanding of some of the very important aspects of plant growth, development, and general metabolism.

CHAPTER II

MATERIALS AND METHODS

Tobacco Tissue Culture W-38

The W-38 tissue was grown on the revised medium of Linsmaier and Skoog (21) in subdued light at approximately 25°C. Three pieces about 5mm X 5mm X 3mm were placed in an Erlenmeyer flask containing 50 ml of solid media. The pieces were cut from tissue 5-6 weeks old. The transferring was done in a laminar flow hood (Agnew-Higgins model M-142) using standard aseptic technique.

W-38 Culture Medium	per 1 liter
Skoog's salt solution minus Ca^{++}	100 ml
$CaCl_2$ solution (Skoog's 10x)	100 ml
Fe^{++}-EDTA Solution	5 ml
Sucrose	20 gm
Deionized water	765 ml
Adjust pH to 5.6 with 0.1N NaOH	
Agar	10 gm
Inositol	100 mg
IAA solution	20 ml
Kinetin solution	2 ml

Thiamine solution 10 ml

Melt agar and dispense 50 ml into 125 ml Erlenmeyer flask. Autoclave 15 minutes at 121°C and 18 psi.

Skoog's Salt Solution		1 liter	
NH_4NO_3	16.5 gm	H_3BO_3	62 mg
KNO_3	19.0 gm	$MnSO_4 \cdot H_2O$	169 mg
$MgSO_4 \cdot 7H_2O$	3.7 gm	$ZnSO_4 \cdot 4H_2O$	86 mg
KH_2PO_4	1.7 gm	10 ml Minor Salts	

Minor Salts Solution		1 liter	
KI	830.0 mg	$CuSO_4 \cdot 5H_2O$	25 mg
$Na_2MoO_4 \cdot 2H_2O$	250.0 mg	$CoCl_2 \cdot 6H_2O$	25 mg

Fe^{++} - EDTA Solution		1 liter	
$FeSO_4$	5.52 gm	EDTA	3.24 gm
		Na_4 EDTA	4.22 gm

$CaCl_2$ Solution (Skoog's - 10X)		1 liter
$CaCl_2 \cdot 2H_2O$	4.4 gm	

IAA Solution		100 ml
IAA	10 mg	

Thiamine Solution		100 ml
Thiamine hydrochloride	10 mg	

Kinetin Suspension		100 ml
Kinetin	10 mg	

Disc Gel Electrophoresis

Anodic disc gel electrophoresis was performed using a Buchler Polyanalyst apparatus according to the method of Orstein and Davis (22). Gels were 7.5% acrylamide with 0.2% cross-linker. Runs were made at

a running pH 9.3. Bromphenol blue was used as tracking dye. After completion of electrophoresis, peroxidase bands were visualized by placing gels in a mixture of 2 parts 1% guaiacol, 2 parts 50 mM sodium phosphate buffer (pH 7.0) and 1 part 0.5% H_2O_2.

Starch Gel Electrophoresis

Starch gel electrophoresis was performed using a modified Smithies apparatus (23). The gel was 10% starch in 5 mM histidine. Electrophoresis was at 400 V for approximately four hours at a running pH 7.0. Cathodic and anodic isoperoxidases could be resolved and were visualized by placing in the staining solution used for disc gel electrophoresis. Anodic isoperoxidases showed the same migration pattern as that seen on disc gel electrophoresis.

Thin-layer Chromatography

Avicel SF was suspended in water (22 g/100 ml) and layered 0.375 mm thick onto glass plates. Several solvent mixutres were used to develop the plates in an ascending system. The solvent used to verify formation of scopoletin products was KFW (methylisobutylketone-formic acid-water-14:3:2). Spots were detected in UV light (366 nm, Black-ray B-100).

Scopoletin Assay

A typical assay contained 2.5 ml of 1.5 mM scopoletin, 0.3 ml of 400 mM sodium citrate (pH 5.5) and 0.1 ml of 150 mM H_2O_2. Final concentrations were 1.25 mM scopoletin, 5 mM H_2O_2 and 40 mM sodium citrate. Assays were initiated by adding 0.1 ml of enzyme preparation and increase in absorbance at 450 nm was recorded at 30 second intervals. Lag times of from one to three minutes occur depending on enzyme concentration, H_2O_2 concentration and pH followed by linear rates for several minutes.

Guaiacol Assay

The guaiacol assay was the method of Lance (24). A typical assay contained 15 mM guaiacol, 5 mM H_2O_2 and 40 mM sodium citrate (pH 6.5). Assays were initiated by adding enzyme preparation, and increase in absorbance at 470 nm was recorded at 30 second intervals. Linear rates were observed for up to four to six minutes.

Esculetin Assay

A typical assay contained 1.0 mM esculetin, 5 mM H_2O_2 and 40 mM Tris-HCl (pH 7.5). Assays were initiated by adding enzyme preparation, and increase in absorbance at 469 nm was recorded at 30 second intervals. Rates were linear up to four to six minutes.

Catechol Assay

A typical assay contained 15 mM catechol, 5 mM H_2O_2 and 40 mM sodium citrate (pH 5.0). Assays were initiated by adding enzyme preparation and increase in absorbance at 400 nm was recorded at 30 second intervals. Rates were linear up to three to four minutes.

UV-Visible Spectrophotometry

All enzyme assays and absorption spectra utilized a Varian Techtron UV-Visible Model 635 spectrophotometer equipped with an automatic cell programmer.

Chemicals

Scopoletin was obtained from Sigma Chemical Company and was recrystallized from methanol. Guaiacol was obtained from Eastman Chemical Company and was redistilled before use. Other chemicals were used without special preparatory procedures. Avicel SF was obtained from American Viscose Division of FMC Corporation.

CHAPTER III

ISOLATION AND CHARACTERIZATION

Several isoenzymes of peroxidase from tobacco tissue culture W-38 can be separated by either anodic disc gel electrophoresis or by slab electrophoresis on starch. Four or five anodic isoenzymes are observed on disc gel electrophoresis at a running pH 9.3. Two of these anodic isoperoxidases, which have similar mobilities to those designated A_1 and A_3 by Stafford and Galston (25), were separated by Schafer et al. (13) using an isoelectric focusing technique. The mobilities of these isoperoxidases and other anodic isoperoxidases of tobacco tissue W-38 are shown in Figure 1. It was shown that scopoletin stimulated the guaiacol oxidizing ability of isoperoxidase A_3 but had no effect on the guaiacol oxidizing ability of A_1 (13). Techniques to separate A_3 from other isoperoxidases were evaluated to further investigate these findings. The most successful isolation procedure is summarized in Table 1.

Preparation of Enzyme

Forty grams of tissue, 25 g of Polyclar AT (optional), and 1 g of sodium dodecyl sulfate (SDS) in 100 ml of 50 mM sodium phosphate buffer (pH 6.0) were homogenized for 10 minutes at 8000 rpm in a Sorvall

Figure 1. ANODIC ISOPEROXIDASES FROM TOBACCO TISSUE CULTURE W-38. Bands were visualized with guaiacol and H_2O_2. Mobilities are relative to bromphenol blue.

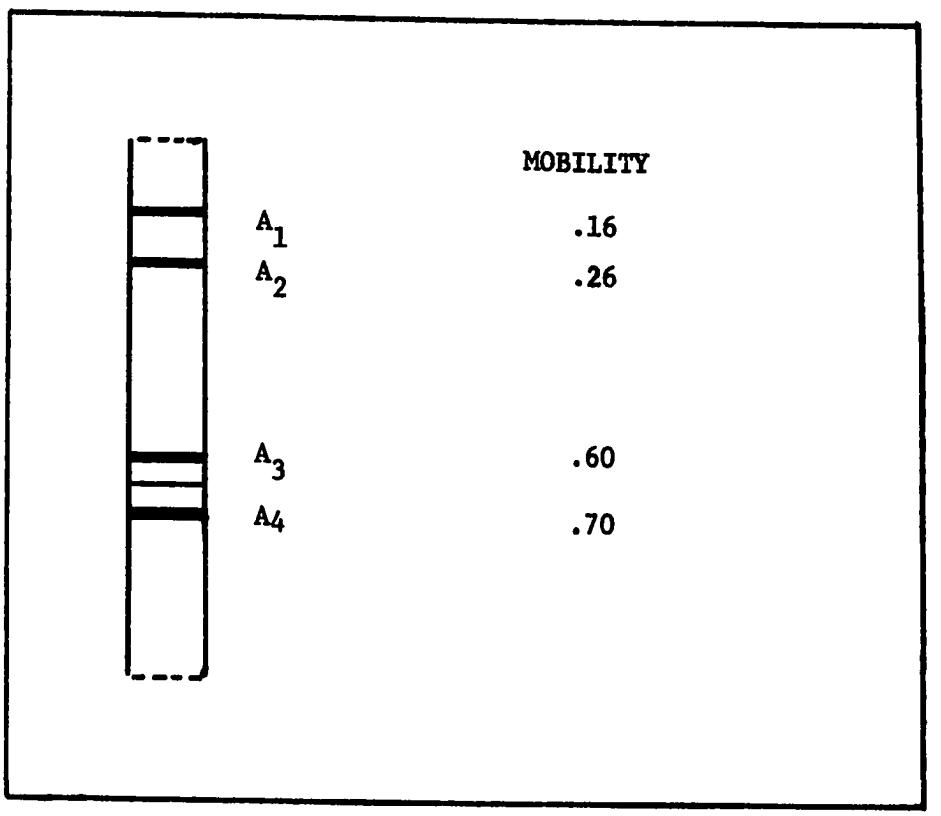

MOBILITY
A_1
.16
A_2
.26
A_3
.60
A_4
.70

TABLE 1

ISOLATION PROCEDURE FOR ISOPEROXIDASE A_3

STEP 1

Homogenize tissue in 50 mM sodium phosphate (pH 6.0) for 10 minutes at 8000 rpm. Centrifuge 20 minutes at 15,000 x g. Save supernatant.

STEP 2

Saturate to 40% with solid $(NH_4)_2SO_4$. Centrifuge 20 minutes at 27,000 x g. Discard pellet.

STEP 3

Saturate to 90% with solid $(NH_4)_2SO_4$. Centrifuge 20 minutes at 27,000 x g. Take up pellet in small volume 5 mM sodium phosphate (pH 6.0).

STEP 4

DEAE-cellulose chromatography. Elute with a stepwise gradient of sodium phosphate (pH 6.0).

Omnimixer. The homogenate was then centrifuged for 20 minutes at 15,000 x g. The resulting supernatant was desalted by dialysis against deionized water or on a Sephadex G-25 column eluted with 5 mM sodium phosphate buffer (pH 6.0). Dialysis helps prevent excessive dilution which may occur on G-25 and is thus probably the method of choice. In some cases before proceeding to ammonium sulfate fractionation the supernatant was concentrated by thin-film perevaporation.

The supernatant was next brought to 40% saturation with $(NH_4)_2SO_4$. The pellet from centrifugation at 27,000 x g for 20 minutes was discarded and the remaining supernatant was then brought to 90% saturation with $(NH_4)_2SO_4$. The pellet remaining after centrifugation again at 27,000 x g for 20 minutes was dissolved in as small a volume of water as possible and dialyzed against 500 volumes of 5 mM phosphate buffer (pH 6.0).

The sample was then applied to a DEAE-cellulose column (pre-equilibrated with 5 mM phosphate buffer). Elution was stepwise with pH 6.0 phosphate buffer from 5 mM to 50 mM (5→15→30→40→50). Fractions were collected and subjected to anodic disc gel electrophoresis. Samples containing only A_3 were concentrated by thin-film perstillation. The concentrate was then rechecked by anodic disc gel and starch gel electrophoresis to verify separation of A_3 from other isoperoxidases. SDS electrophoresis showed A_3 as the only visible protein component of the concentrate. Fold purification is not stated since isoperoxidases which have guaiacol activity are being separated away. A_4 which has considerable scopoletin activity, is also being separated away so that

scopoletin activity cannot be used to show fold purification.

Molecular Weight Determination

The molecular weight of A_3 was estimated to be approximately 54,000 by both SDS electrophoresis and gel permeation chromatography on Sephadex G-150.

SDS electrophoresis was conducted by the method of Weber and Osborn (26). Gels were 10% acrylamide, 0.2% N',N' dimethylbisacrylamide and 0.1% SDS. The following solution was applied to gels that were 6 mm x 8 cm.

5 µl	0.05% Bromphenol blue
5 µl	2-mercaptoethanol
100 µl	protein standards or A_3 in 10 mM sodium phosphate (pH 7.0), 1% SDS and 0.1% 2-mercaptoethanol
1 drop	Glycerin

After electrophoresis at 8 ma per sample, gels were removed and fixed in 20% sulfosalicylic acid for 16 hours at 37° C, washed with deionized water, stained with Coomasie brilliant blue for 4 hours at 37°C and de-stained with 7% (v/v) acetic acid until clear.

Mobilities (M) were calculated according to the method of Weber and Osborn (26) as

$$M = \frac{\text{distance of protein migration}}{\text{gel length after de-staining}} \times \frac{\text{gel length before staining}}{\text{distance of dye migration.}}$$

A semi-logarithmic plot of molecular weight versus mobility is shown

in Figure 2.

Determination of molecular weight by gel permeation chromatography on Sephadex G-150 was accomplished by applying a mixture of standards and isoperoxidase A_3 in 5 mM sodium phosphate (pH 6.0) to a column 15 mm x 600 mm. Flow rate of 5 mM sodium phosphate (pH 6.0) was approximately 5 mls per hour. Standards were located by $O.D._{280}$ and A_3 was located using the guaiacol assay. A semi-logarithmic plot of molecular weight versus elution volume is shown in Figure 3.

The Nature of Iron in Isoperoxidase A_3

Heme iron has been shown to occur in peroxidases. The separation of the heme group from the protein to yield a catalytically inactive (guaiacol activity) enzyme has been accomplished using acetone/HCl mixtures (27). The isolated heme moiety and the holoenzyme show absorption maxima in the range of 400 nm. Isoperoxidase A_3 also has some properties of heme containing proteins in that the scopoletin activity is inhibited 100% by 5 mM cyanide. Isoperoxidase A_3 has been shown to contain iron by boiling the enzyme in the presence of o-phenanthroline. This results in the formation of a red color characteristic of iron phenanthroline complexes. The iron of isoperoxidase A_3 is not removed by simple dialysis against EDTA or o-phenanthroline. The iron is also not removed in the presence of guanidinium hydrochloride and o-phenanthroline. The iron must thus be held very tightly, which would be expected for heme complexes. Isoperoxidase A_3 does not, however, exhibit the typical heme absorption spectrum. No maximum occurs in the 400 nm region although there is considerable absorbance in this region (Figure 4).

Figure 2. DETERMINATION OF MOLECULAR WEIGHT OF ISOPEROXIDASE A_3 BY DODECYL SULFATE-POLYACRYLAMIDE GEL ELECTROPHORESIS.

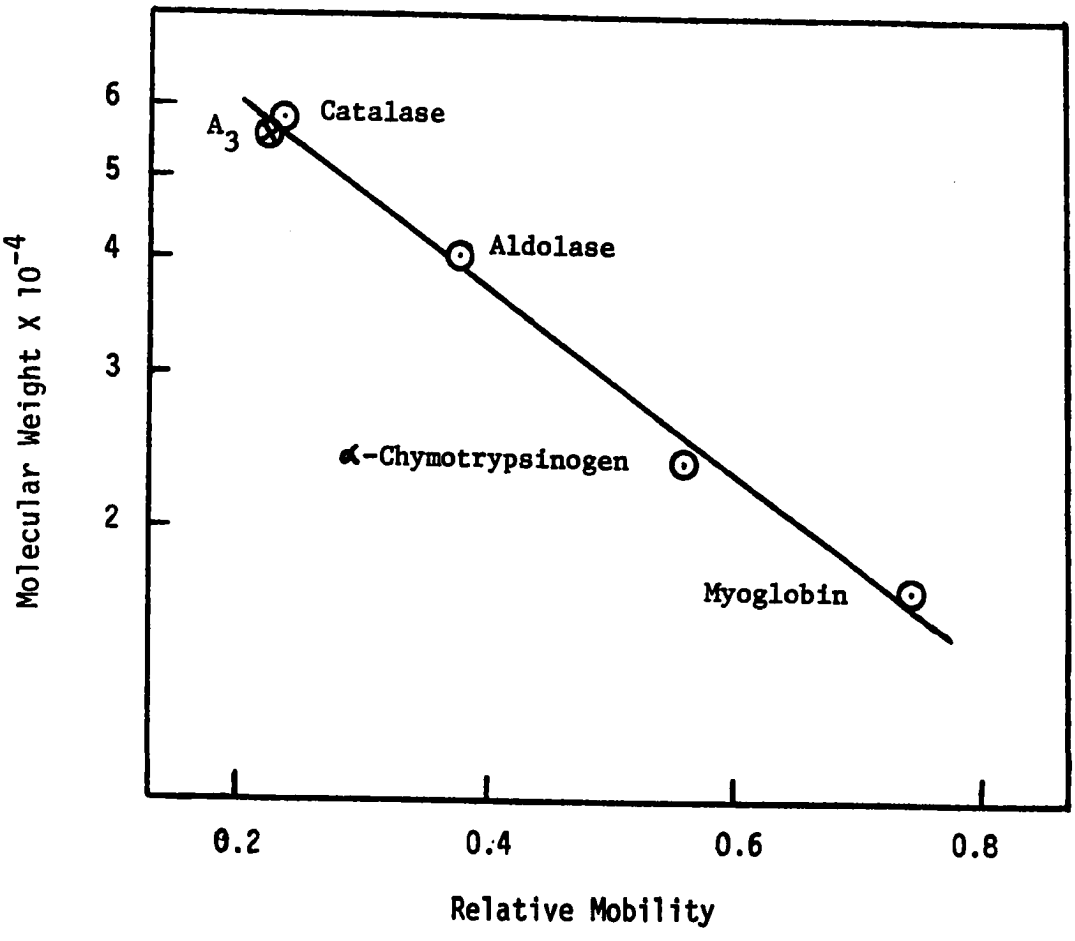

Molecular Weight X 10^{-4}
6
5
4
3
2
A_3
Catalase
Aldolase
α-Chymotrypsinogen
Myoglobin
0.2
0.4
0.6
0.8
Relative Mobility

Figure 3. DETERMINATION OF MOLECULAR WEIGHT OF ISOPEROXIDASE A_3 BY GEL PERMEATION CHROMATOGRAPHY ON SEPHADEX G-150.

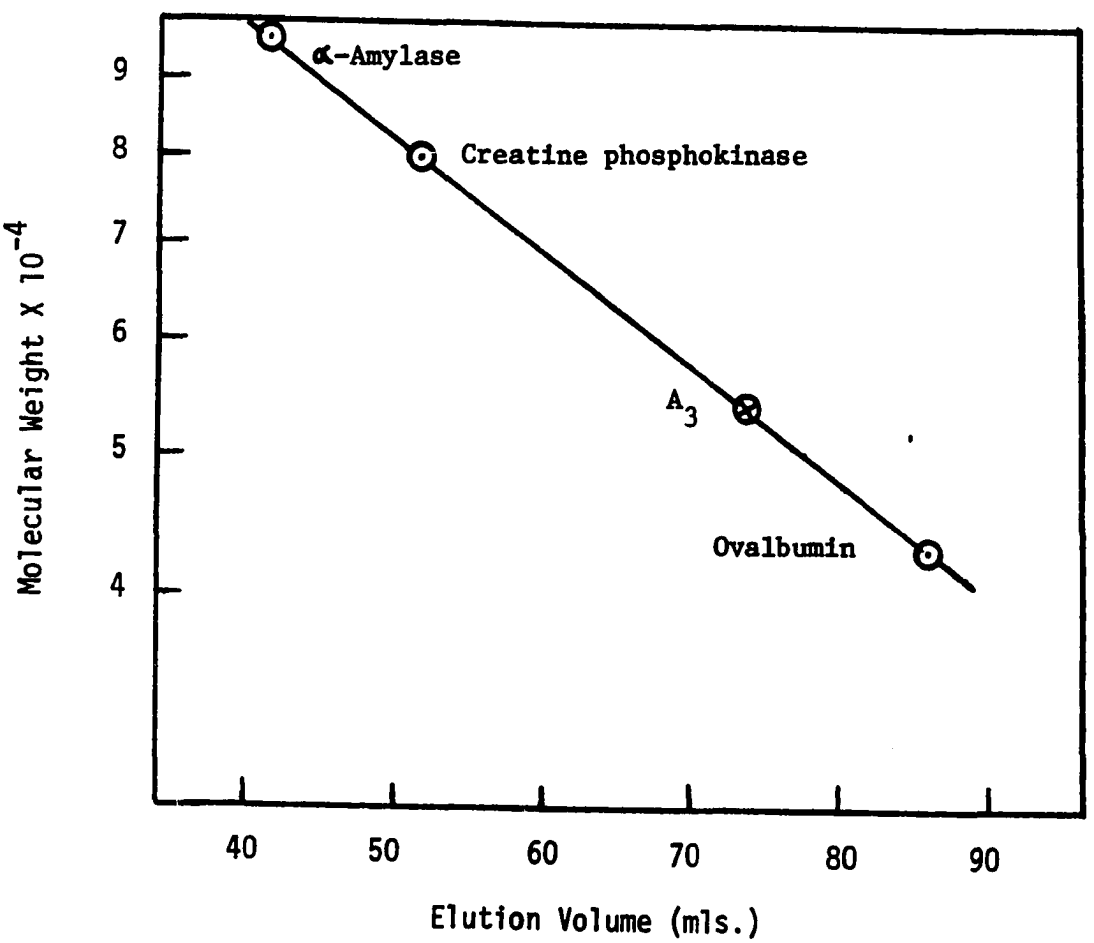
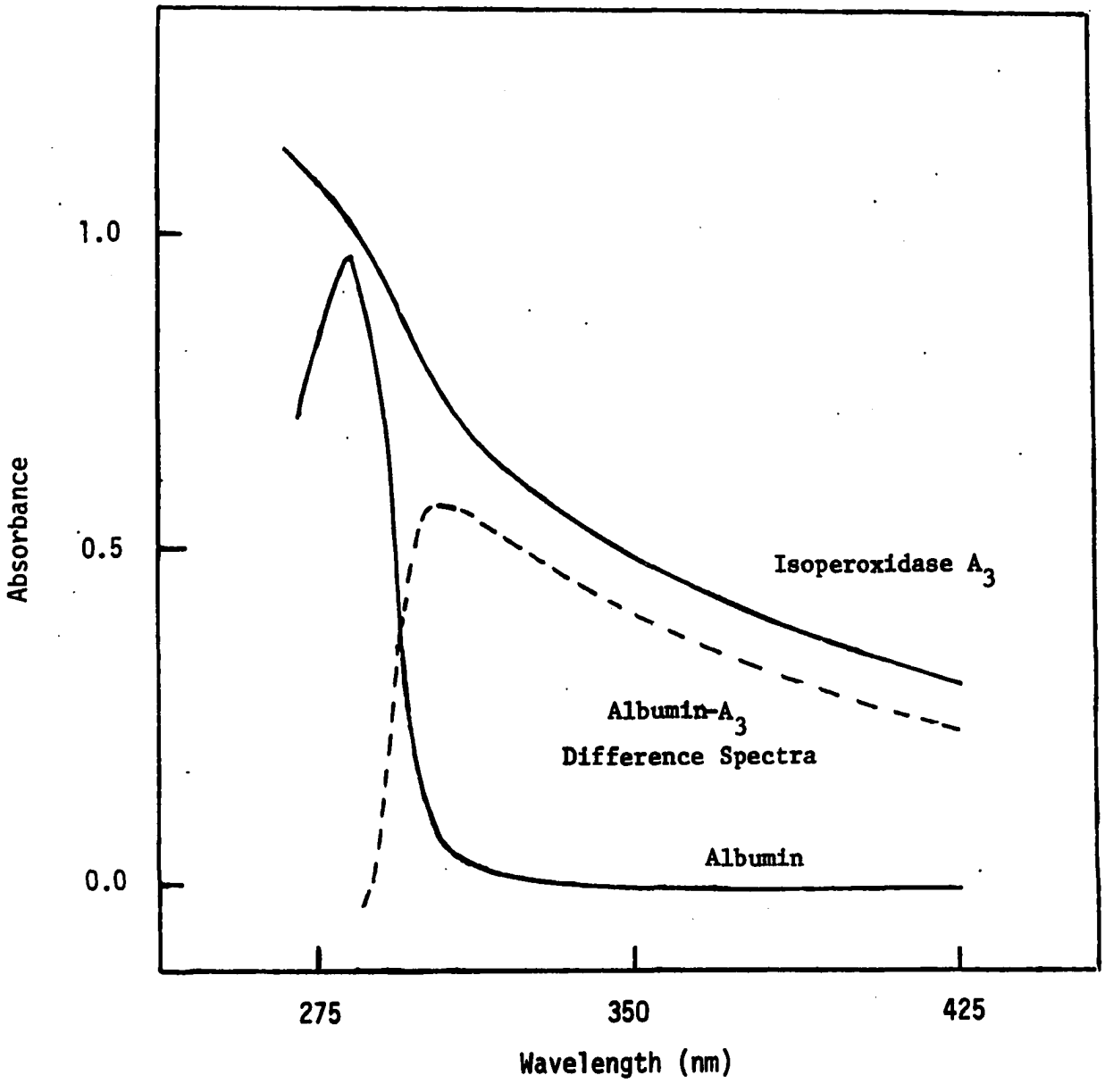

α-Amylase
Creatine phosphokinase
A3
Ovalbumin
9
8
7
6
5
4
Molecular Weight X 10-4
40
50
60
70
80
90
Elution Volume (mls.)

Figure 4. ABSORPTION SPECTRA OF ISOPEROXIDASE A_3 AND ALBUMIN AND THE DIFFERENCE SPECTRUM OF A_3 VS. ALBUMIN. Isoperoxidase A_3 and albumin solutions were adjusted to the same $O.D._{280}$.


Absorbance
1.0
0.5
0.0
Isoperoxidase A_3
Albumin-A_3
Difference Spectra
Albumin
275
350
425
Wavelength (nm)

Isoperoxidase A_3 absorbs much more in this region than a protein such as albumin. A difference spectrum of isoperoxidase A_3 and albumin adjusted to the same $O.D._{280}$ yields a peak at 300 nm (Figure 4). Addition of albumin to horseradish peroxidase results in the disappearance of the HRP absorption maxima at 403 nm (Figure 5).

Certain evidence indicates that isoperoxidase A_3 does indeed include heme iron (inhibitors, chelators), however, the absorption spectrum of A_3 does not correspond with the spectra observed for other heme containing proteins. The experiments with albumin suggest that protein interactions with heme could possibly alter the heme absorption spectra and that not all heme proteins would necessarily show a maximum near 400 nm. The nature of iron in A_3 thus cannot be resolved until quantities of A_3 large enough to attempt heme dissociation experiments can be obtained.

Effect of SH reagents

Several SH reagents including cysteine, 2-mercaptoethanol, dithiothreitol and reduced glutathione inhibited the scopoletin activity of isoperoxidase A_3. Methionine, as well as disulfide compounds such as cystine and oxidized glutathione had no effect on the scopoletin activity (Table 2). These compounds have been implicated in the breaking of disulfide bonds in horseradish peroxidase by Shih *et al.* (11). This group utilized p-chloromercuribenzoic acid to show that lack of inhibition by this compound demonstrated that no free SH groups were present in HRP. Isoperoxidase A_3 is not significantly inhibited by PCMB suggesting that it also contains no free SH groups and that the

Figure 5. EFFECT OF ALBUMIN ON THE HRP ABSORPTION SPECTRUM. The solution was 80% albumin and 20% horseradish peroxidase previously adjusted to the same $O.D._{280}$.

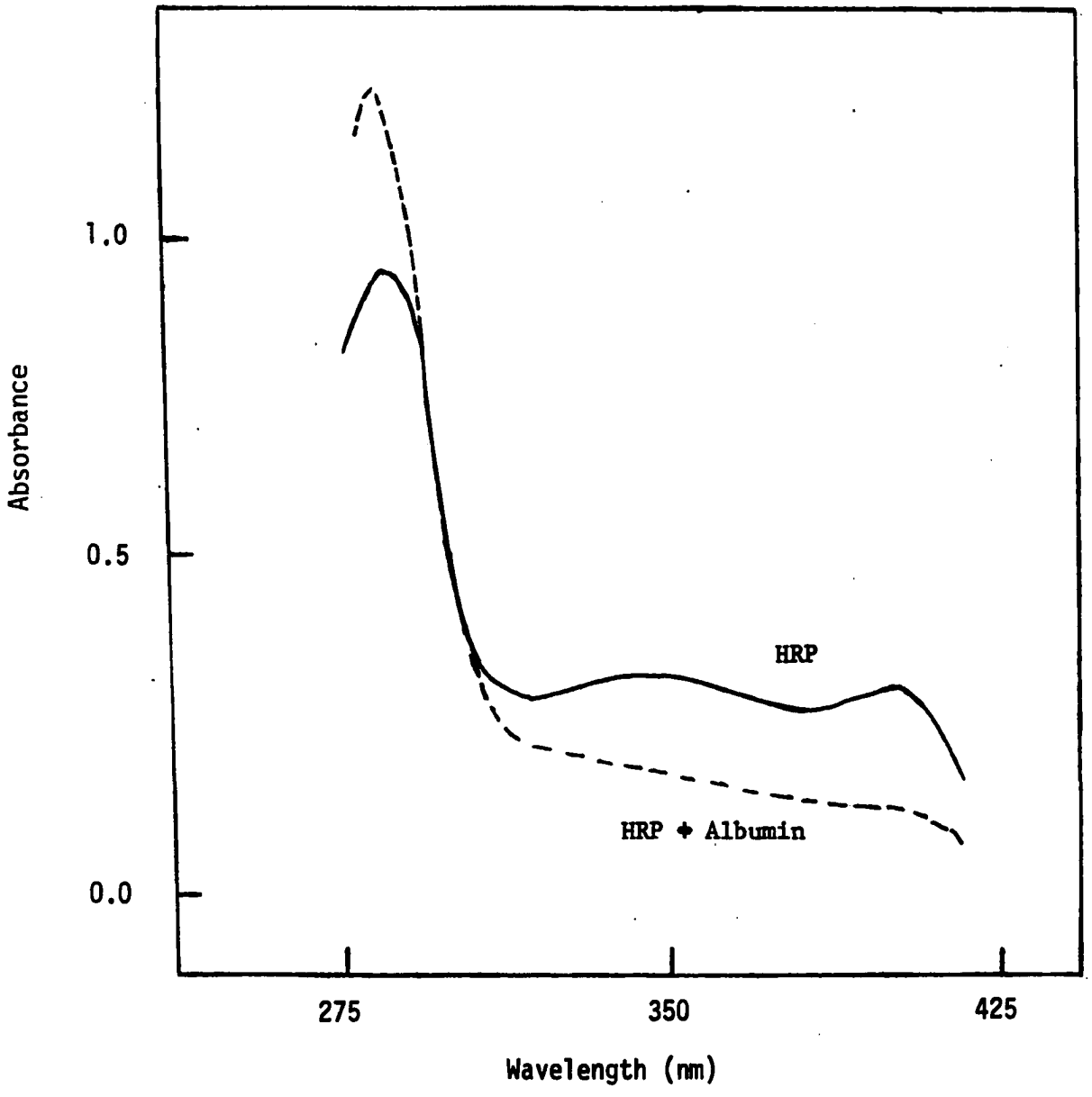

Absorbance
1.0
0.5
0.0
HRP
HRP + Albumin
275
350
425
Wavelength (nm)

TABLE 2

EFFECT OF SULFUR CONTAINING COMPOUNDS

ON THE SCOPOLETIN ACTIVITY OF ISOPEROXIDASE A_3

Assays were run at 1.25 mM scopoletin, 5 mM H_2O_2 and 1 mM effector in 40 mM sodium citrate (pH 5.5).

EFFECTOR	% OF CONTROL ACTIVITY
Cysteine	0
Dithiothreitol	0
2-Mercaptoethanol	0
Reduced glutathione	10
Oxidized glutathione	98
Cystine	100
Methionine	100

inhibition by SH reagents is due to breaking of disulfide bonds. Also after heating at 70°C for 10 minutes, all guaiacol activity is lost, but upon standing for several hours complete recovery of activity is observed. The ability of isoperoxidase A_3 to renature quickly after exposure to heat indicates also that disulfide linkages may be present in this enzyme.

CHAPTER IV

SCOPOLETIN, A SUBSTRATE FOR ISOPEROXIDASE A_3

Scopoletin has been indicated to have some probable significance in overall plant metabolism. Wender (28) has reported that scopoletin accumulates in plants under a wide variety of stress conditions. Winkler (29) listed several diseases that have been reported to cause increases in scopoletin and/or scopolin concentrations. Black rot resistant sweet potatoes have been reported to synthesize more scopoletin than the susceptible variety (70). Synthetic growth regulators such as 2,4 D (31) and maleic hydrazide (32) when sprayed on tobacco plants cause an increase in scopoletin levels.

On a molecular level scopoletin has been shown to inhibit IAA oxidase activity (8,15), but also to stimulate the activity at lower concentrations (15). Kajinami et al. (33) reported that scopoletin inhibits glucose-6-phosphate dehydrogenase, the enzyme involved in the initial reaction of the pentose phosphate pathway. Tryon (34) reported a correlation between scopoletin content and differentiating ability in tobacco tissue cultures. The possible involvement of scopoletin in such important physiological functions as enzyme regulation, cell growth

and differentiation stimulated an interest in scopoletin as a substrate for peroxidase catalyzed reactions.

Andreae (8) demonstrated that crude preparations of IAA oxidase from potatoes were capable of scopoletin oxidation. Schafer et al. (13) demonstrated that scopoletin stimulated the guaiacol oxidizing ability of isoperoxidase A_3 but had no effect on the activity of isoperoxidase A_1. These peroxidases were single isoenzymes from tobacco tissue W-38. The observations of Andreae and Schafer suggested that A_3 might oxidize scopoletin and that this oxidation was responsible for the stimulation of the guaiacol reaction. When isoperoxidase A_3 was added to a mixture of scopoletin and H_2O_2 the rapid formation of a yellow product was observed. Methods were then sought to verify that scopoletin was indeed oxidized by isoperoxidase A_3.

Scopoletin Product Formation and Assay

Upon addition of isoperoxidase A_3 to a mixture of scopoletin, H_2O_2 and 40 mM sodium citrate (pH 5.5) a blue intermediate was first noted followed by the formation of a yellow product which absorbed at 450 nm. After several hours this mixture was spotted on Avicel SF thin layer plates and the chromatogram was developed with a mixture of methylisobutylketone, formic acid and water (14:3:2). Several spots were observed depending on the time allowed for reaction and the amount of mixture spotted. The most consistent observation was a yellow non-migratory spot (R_f=0). Scopoletin has an R_f of about 0.80 in this solvent system. Scopoletin levels upon reaction were diminished as observed by both thin layer chromatography and UV

spectrometry. These observations indicated that scopoletin was indeed converted rapidly to a new substance in the presence of H_2O_2 and isoperoxidase A_3.

The observation that the product formed from scopoletin absorbed at 450 nm while scopoletin absorbs negligibly at this wavelength served as the basis for an assay of this reaction (Figure 6). A typical assay contained 1.25 mM scopoletin (this value is close to the limit of scopoletin solubility in water), 5 mM H_2O_2 and 40 mM sodium citrate (pH 5.5). A_3 was added to initiate the reaction, and the increase in absorbance at 450 nm was observed.

Kinetics of A_3 Catalyzed Scopoletin Oxidation

Figure 7 shows the pH optimum for scopoletin oxidation to be about 5.5. Figure 8 shows the dependence of velocity on initial scopoletin concentration. A Lineweaver-Burk plot (35) of this data is concave upward (Figure 9). A Hill plot (36) of this data yields an n of 1.5. (Figure 10). A modified Lineweaver-Burk plot of 1/v versus $1/S^{1.5}$ is a straight line and was used to determine $S_{0.5}$ (Figure 11). The $S_{0.5}$ for scopoletin is about 0.6 mM. The non-linearity of the Lineweaver-Burk plot and $n > 1$ for the Hill plot are conditions necessary for substrate cooperative effects (37). These results would indicate two or more binding sites for scopoletin. Although this possibility must still be considered, an alternate mechanistic explanation is proposed in Chapter VII.

Figure 12 shows the dependence of velocity on initial H_2O_2 concentration. The $S_{0.5}$ is about 0.9 mM.

Figure 6. ABSORPTION SPECTRA OF SCOPOLETIN REACTION MIXTURES. Mixture of 1.25 mM scopoletin and 5 mM H_2O_2 in 40 mM Tris-HCl (pH 7.5) before and after addition of isoperoxidase A_3.

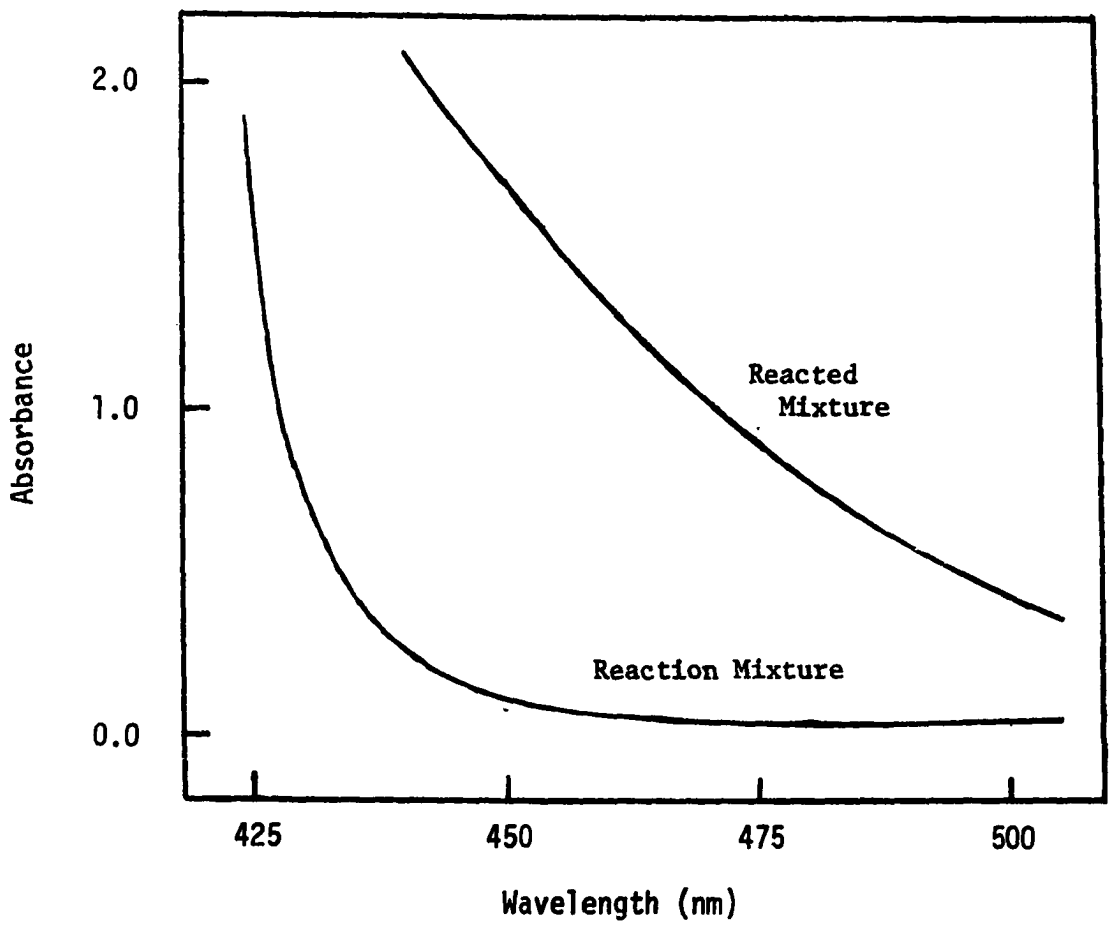

2.0
1.0
0.0
Absorbance
Reacted
Mixture
Reaction Mixture
425
450
475
500
Wavelength (nm)

Figure 7. EFFECT OF PH ON THE SCOPOLETIN ACTIVITY OF ISOPEROXIDASE A_3. Assays were run at 1.25 mM scopoletin, 10 mM H_2O_2 and 40 mM sodium citrate buffer.

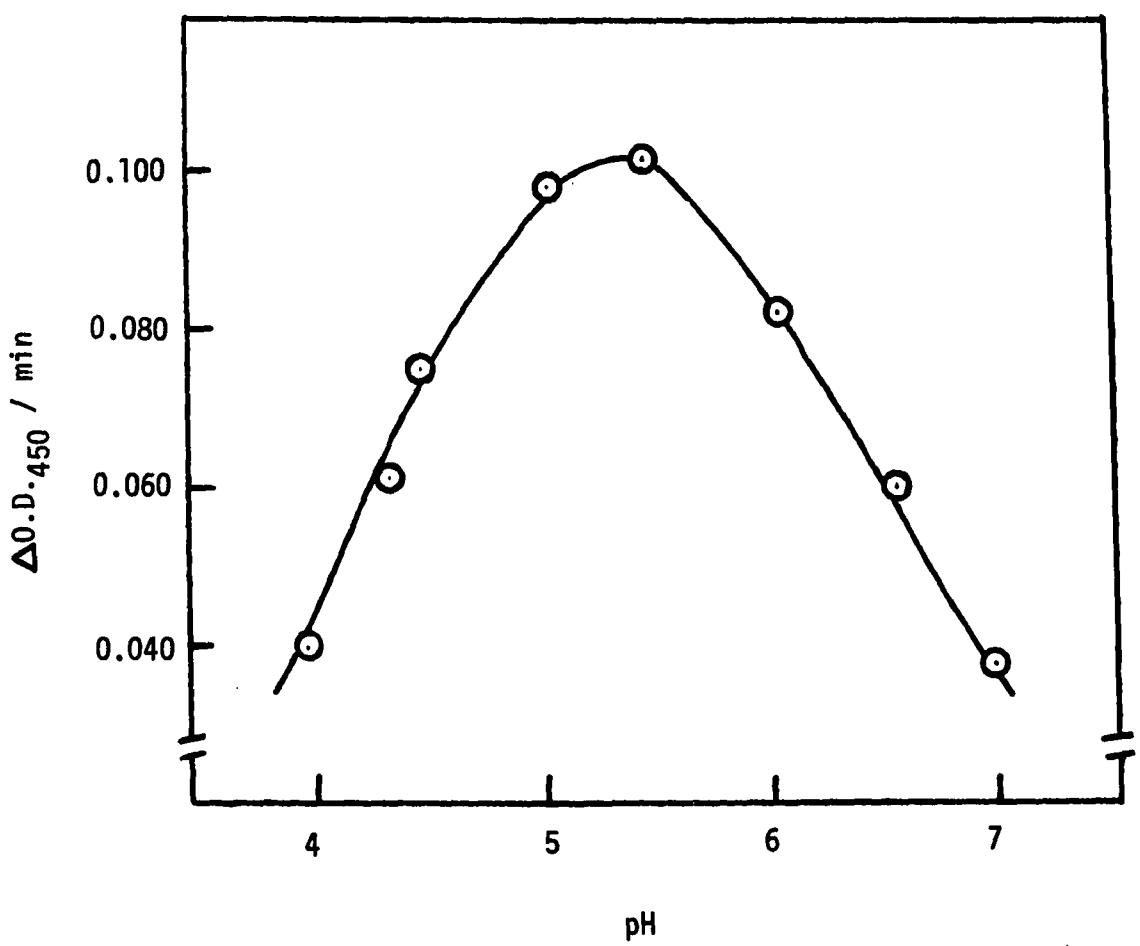

0.100
0.080
0.060
0.040
ΔO.D.450 / min
4
5
6
7
pH

Figure 8. EFFECT OF SCOPOLETIN CONCENTRATION ON THE ACTIVITY OF ISOPEROXIDASE A_3. Assays were run at 10 mM H_2O_2 in 40 mM sodium citrate (pH 5.5).

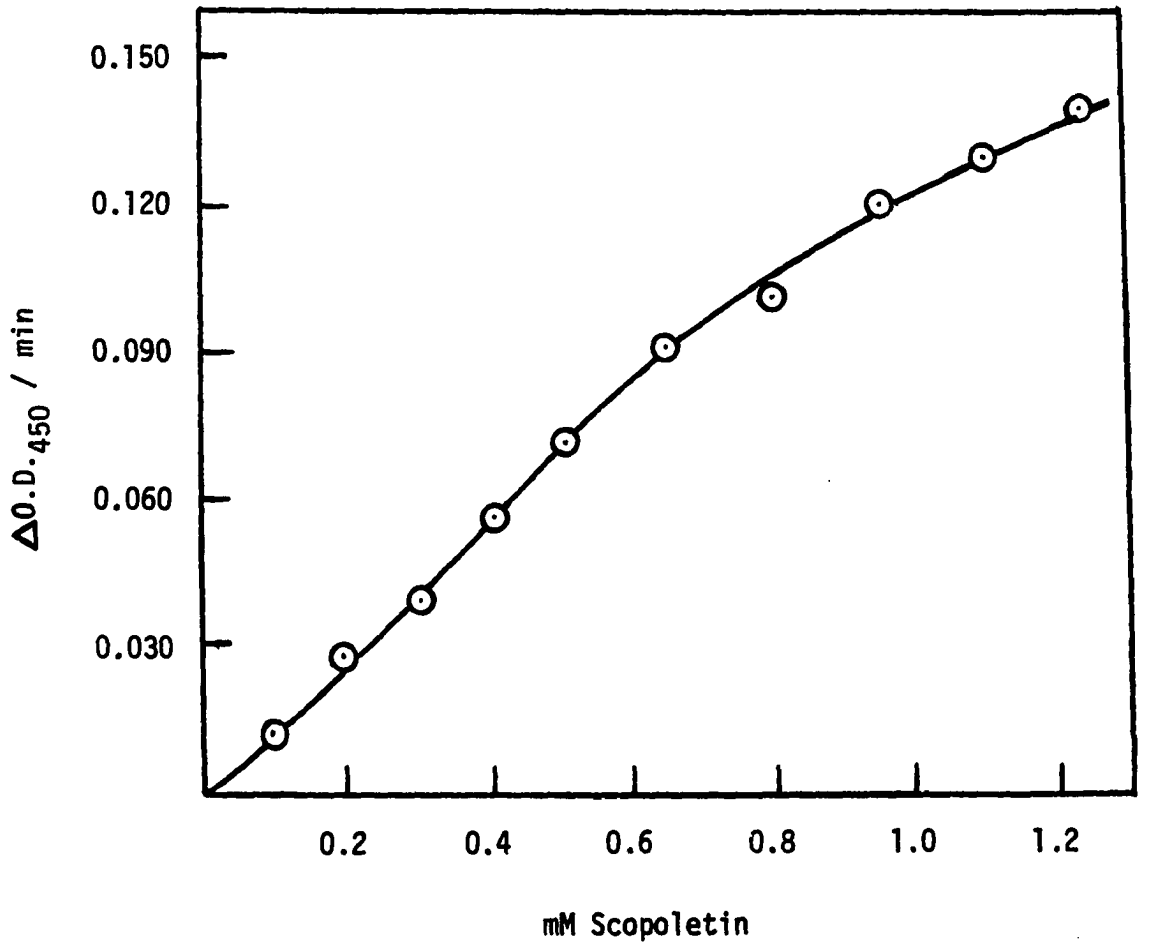

0.150
0.120
0.090
0.060
0.030
ΔO.D.450 / min
0.2
0.4
0.6
0.8
1.0
1.2
mM Scopoletin

Figure 9. DOUBLE RECIPROCAL PLOT OF ACTIVITY VS. SCOPOLETIN CONCENTRATION FOR ISOPEROXIDASE A_3. Assays were run at 10 mM H_2O_2 in 40 mM sodium citrate (pH 5.5).

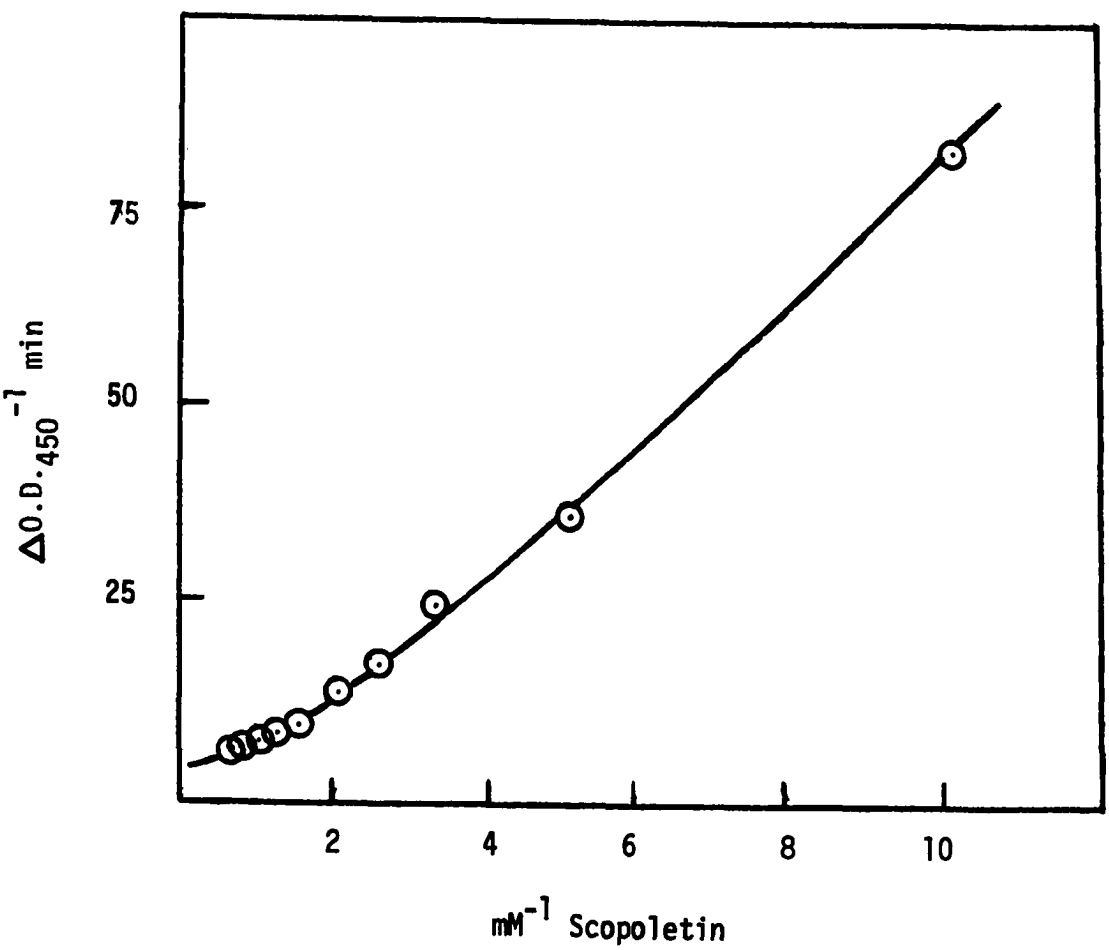

75
50
25
ΔO.D.$_{450}^{-1}$ min
2
4
6
8
10
mM^{-1} Scopoletin

Figure 10. HILL PLOT OF ACTIVITY VS. SCOPOLETIN CONCENTRATION FOR ISOPEROXIDASE A_3. Assays were run at 10 mM H_2O_2 in 40 mM sodium citrate (pH 5.5). $v-\Delta O.D._{450}/$ min.

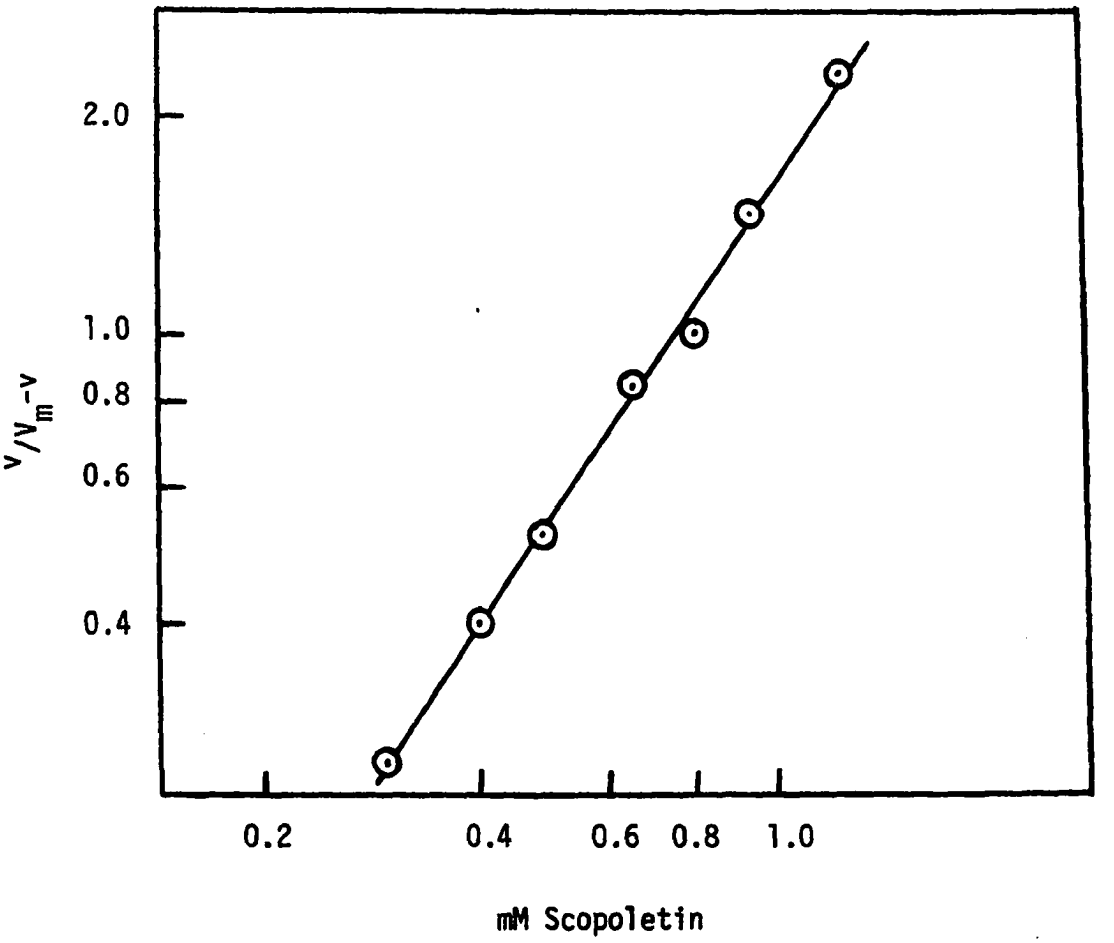

2.0
1.0
0.8
0.6
0.4
v/Vm-v
0.2
0.4
0.6
0.8
1.0
mM Scopoletin

Figure 11. MODIFIED DOUBLE RECIPROCAL PLOT OF ACTIVITY VS. SCOPOLETIN CONCENTRATION FOR ISOPEROXIDASE A_3. Assays were run at 10 mM H_2O_2 in 40 mM sodium citrate (pH 5.5).

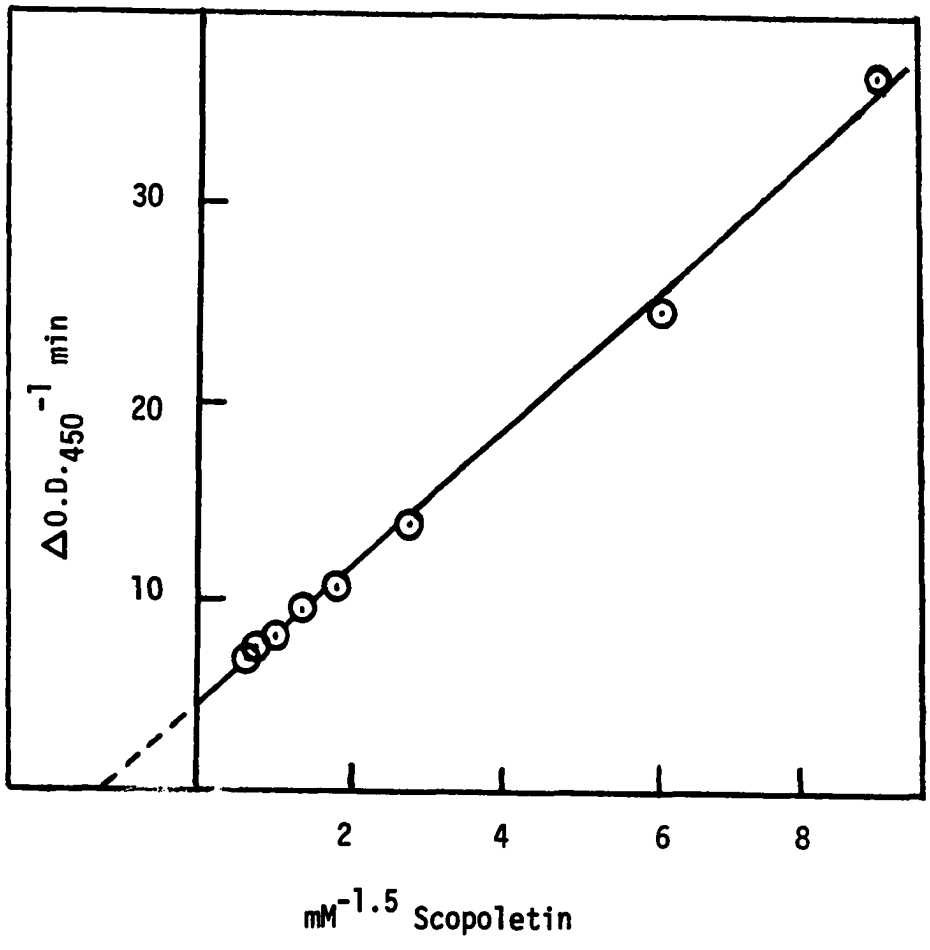
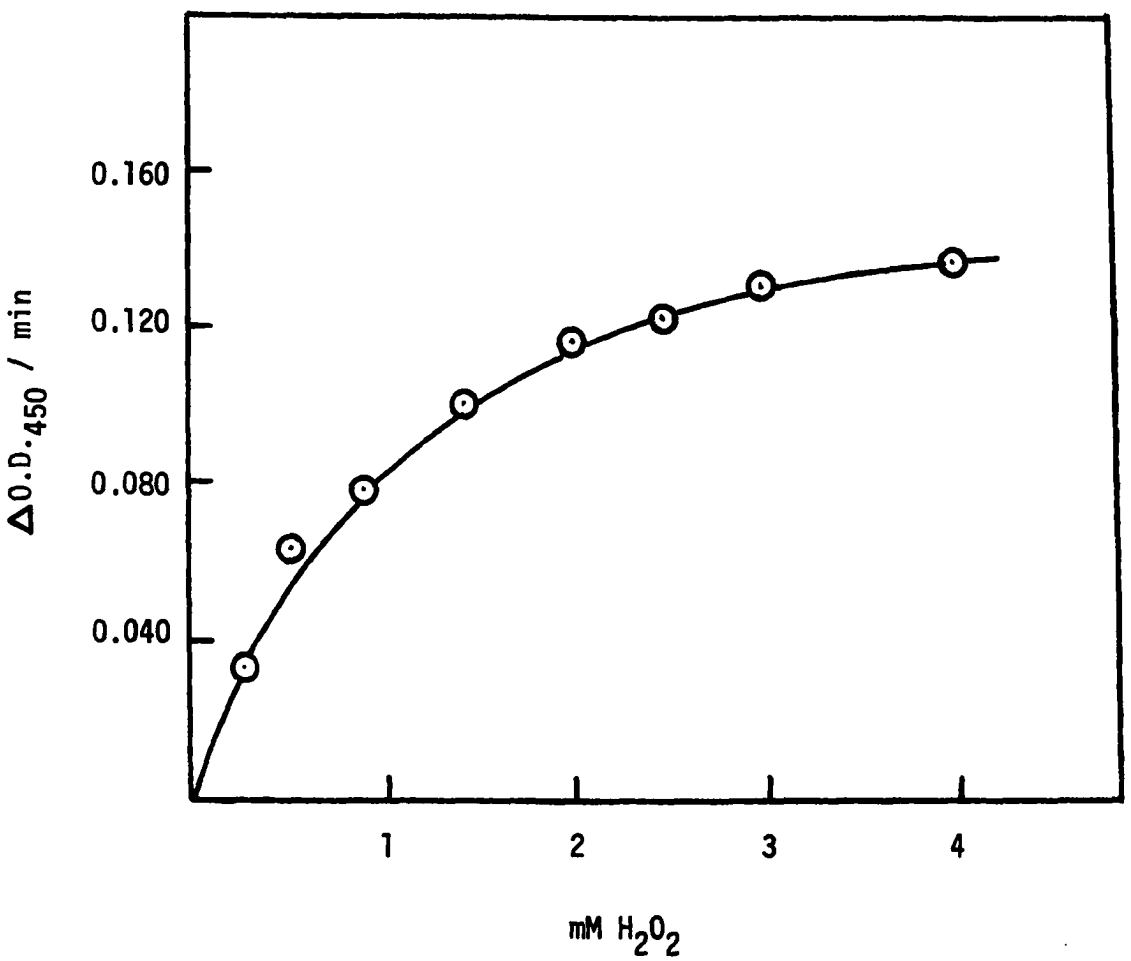

30
20
10
ΔO.D.$_{450}^{-1}$ min
2
4
6
8
$mM^{-1.5}$ Scopoletin

Figure 12. EFFECT OF H_2O_2 CONCENTRATION ON THE SCOPOLETIN ACTIVITY OF ISOPEROXIDASE A_3. Assays were run at 1.25 mM scopoletin in 40 mM sodium citrate (pH 5.5).


0.160
0.120
0.080
0.040
ΔO.D.450 / min
1
2
3
4
mM H2O2

Scopoletin Effect on Guaiacol Oxidation

Based on the observations that scopoletin can be oxidized by A_3, an attempt was made to explain the results of Schafer et al. (13) concerning scopoletin stimulation of A_3 guaiacol activity. Guaiacol oxidation is followed at 470 nm. The oxidation of scopoletin leads to a product that also absorbs in the range of 470 nm. Results presented in Chapter VI demonstrate that relative to guaiacol oxidation scopoletin is oxidized 100-150 times better by isoperoxidase A_3 than by isoperoxidase A_1. Isoperoxidase A_3 has a greater affinity for scopoletin than for guaiacol. The $S_{0.5}$ is 0.6 mM and 4 mM respectively.

It would appear then that stimulation of A_3 by scopoletin was due to the oxidation of scopoletin which would either add to the total amount of $\Delta O.D._{470}$ by product formation or possibly initiate guaiacol oxidation with some scopoletin oxidation intermediate. Scopoletin which is turned over much more slowly by A_1 would then show no observable effect on the measurement of guaiacol activity of that isoperoxidase.

Effectors of Scopoletin Oxidation

Table 3 shows the effect of various compounds on the isoperoxidase A_3 catalyzed oxidation of scopoletin. Assays were 1.25 mM scopoletin, 5 mM H_2O_2, 40 mM sodium citrate (pH 5.5) and effector as listed.

Inhibition by the substrate analog compounds such as the substituted benzoic acids, cinnamic acids and coumarins probably

TABLE 3

EFFECT OF VARIOUS COMPOUNDS

ON THE SCOPOLETIN ACTIVITY OF ISOPEROXIDASE A_3

EFFECTOR	COMMON NAME	% OF CONTROL ACTIVITY
No effector		100
0.4 mM Substituted benzoic acids		
Benzoic acid		100
2,3 di-OH		1
2,5 di-OH		1
3,4 di-OH		4
2,6 di-OH		72
3-OH, 4-Me		90
4-OH, 3-OMe	Vanillic acid	10
3,4 di-Me		91
0.4 mM Substituted cinnamic acids		
Cinnamic acid		91
4-OH	p-Coumaric acid	56
3,4 di-OH	Caffeic acid	0
3-OMe, 4-OH	Ferulic acid	6
0.4 mM Substituted ferulic acids		
Hydroferulic acid		0
Isoferulic		1
3-O-glucosyl ferulic acid		94

TABLE 3 (cont.)

EFFECTOR	COMMON NAME	% OF CONTROL ACTIVITY
0.4 mM Substituted coumarins		
Coumarin		100
6,7 di-OH	Esculetin	7
6-OH, 7-O-glucosyl	Esculin	99
7-OMe, 6-O-glucosyl	Scopolin	87
0.4 mM		
Indole-3-acetic acid	IAA	18
0.4 mM		
Chlorogenic acid		17
$MnCl_2$		
5 mM		3
0.05 mM		73
0.005 mM		86

occurs by binding to the site of scopoletin oxidation. In fact, several of these compounds appear to be oxidized by isoperoxidase A_3. The dihydroxybenzoic acid derivatives that do not have a bulky group between hydroxyls (such as in 2,6 dihydroxybenzoic acid) are strong inhibitors of scopoletin oxidation. The hydroxy-methoxy compound (vanillic acid), which is analagous to scopoletin as to benzene ring substitution also inhibits. However, the hydroxy-methyl compound (3-hydroxy, 4-methylbenzoic acid) does not inhibit strongly.

The dihydroxy or methoxy-hydroxy grouping also inhibits in the case of the naturally occurring phenolics esculetin and ferulic acid. Addition of a glucose group to the hydroxyl group drastically reduces the inhibition as in esculin and 3-O-glucosylferulic acid.

The mono-hydroxy compound (p-coumaric acid) has an intermediate degree of inhibition.

The coumarin portion of the ring appears important in binding for related compounds such as scopoletin ($S_{0.5}$ = 0.6 mM) and guaiacol ($S_{0.5}$ = 4 mM) and esculetin ($S_{0.5}$ = 0.27 mM) and catechol ($S_{0.5}$ = 6 mM); however, coumarin itself has no inhibitory effect. It appears that a dihydroxy or hydroxy-methoxy grouping may be necessary for binding and/or reaction.

The effects of IAA, chlorogenic acid and Mn^{++} on scopoletin appear quite interesting, but at this point only speculations can be made. Scopoletin reportedly inhibits IAA oxidase while IAA inhibits scopoletin peroxidase. Mn^{++} inhibits scopoletin peroxidase, but is thought to serve as a cofactor for IAA oxidase. An interesting growth regulating control

mechanism could possibly be postulated if the fundamental question of the active form of IAA as a growth regulator were resolved. Without the Mn^{++} effect IAA and scopoletin work in the same direction with increased IAA levels resulting in increased scopoletin levels and then in turn increased IAA levels. With Mn^{++} present in the scheme, however, increase in Mn^{++} would result in a decrease in IAA through IAA oxidase action. The antagonism between the increased Mn^{++} and decreased IAA levels should regulate the amount of scopoletin oxidized by peroxidase, which would in turn regulate the amount of IAA oxidized by IAA oxidase. The actual control could even possibly exist on one enzyme since Galston et al. (38) have demonstrated that peroxidase activity and IAA oxidase activity seem to be separate functions which are both found on HRP. The $S_{0.5}$ for scopoletin corresponds rather closely to the levels of scopoletin that were reported by Einhellig et al. (19) to have an intermediate inhibitory effect on the growth of tobacco seedlings. Scopoletin at 1×10^{-4} M showed no inhibition, 1×10^{-3} M showed great inhibition, and 5×10^{-4} M showed intermediate inhibition. The $S_{0.5}$ for scopoletin is 6×10^{-4} M. These figures indicate that scopoletin levels maintained by scopoletin peroxidase could be in the range of effective growth regulation.
Some experiments indicate that IAA may be involved in ion uptake (39). If IAA could effect Mn^{++} uptake, a fine control method for IAA and scopoletin levels in the cell could be established.

An interesting speculation could also be made that coarse control of growth would be regulated by the different levels of individual isoperoxidases which utilize scopoletin at different rates.

Significance of Scopoletin Oxidation

Possible physiological significance of the oxidation of scopoletin other than a possible involvement in growth regulation is still obscure. In vitro studies indicate that oxidation of scopoletin in the presence of light probably results in the polymerization of scopoletin product molecules. This conclusion has been suggested from observations that reaction of scopoletin leads to a product that is insoluble in most organic solvents, that does not migrate in thin layer chromatography systems which readily move scopoletin, and does not demonstrate a parent ion peak by mass spectrometry. Innerarity (21) has indicated that scopoletin may be involved in the formation of lignin-like products. Uptake of radioactive scopoletin into a lignin-like fraction was observed. The possibility that a major portion of scopoletin could be polymerized perhaps with other compounds in the presence of peroxidase to form a "lignin-like" structure seems plausible from the information so far obtained. Large quantities of isoperoxidase A_3, which utilizes scopoletin readily, are found in the filtrate of cells stirred gently in buffer. This indicates that there is probably a large amount of A_3 associated with the cell wall, which is thought to be the site of lignin biosynthesis.

Chromatography of scopoletin products indicates that some nonpolymer compounds are at least formed as intermediates. In vivo these intermediates could possibly be transferred to subsequent enzymes which would function to bring about metabolic interconversions of phenolic compounds.

CHAPTER V

ALTERNATE SUBSTRATES FOR ISOPEROXIDASE A_3

Some of the more widely used substrates for peroxidase such as guaiacol and catechol were used to compare with some of the physiological substrates other than scopoletin. Esculetin, ferulic acid, IAA, chlorogenic acid, and caffeic acid were some of the substrates tested as substrates for isoperoxidase A_3.

Guaiacol

Guaiacol oxidation was followed by the procedure of Lance (24) at 470 nm. The pH optimum for guaiacol oxidation was 6.5 (Figure 13). Figure 14 shows the dependence of velocity on initial guaiacol concentration. A Lineweaver-Burk plot of this data is linear and yields an $S_{0.5}$ for guaiacol equal to 4 mM (Figure 15).

Table 4 shows the effect of some compounds on the guaiacol activity of isoperoxidase A_3. The assay was 4 mM guaiacol, 5 mM H_2O_2, 40 mM sodium citrate (pH 5.5) and 0.4 mM effector. The stimulatory effect of scopoletin has been discussed earlier. Caffeic acid and ferulic acid oxidation does not appear to add significantly to the absorbance at 470 nm and these results would indicate that the oxidation of these

Figure 13. EFFECT OF PH ON THE GUAIACOL ACTIVITY OF ISOPEROXIDASE A_3. Assays were run at 15 mM guaiacol and 5 mM H_2O_2 in 40 mM sodium citrate buffer.

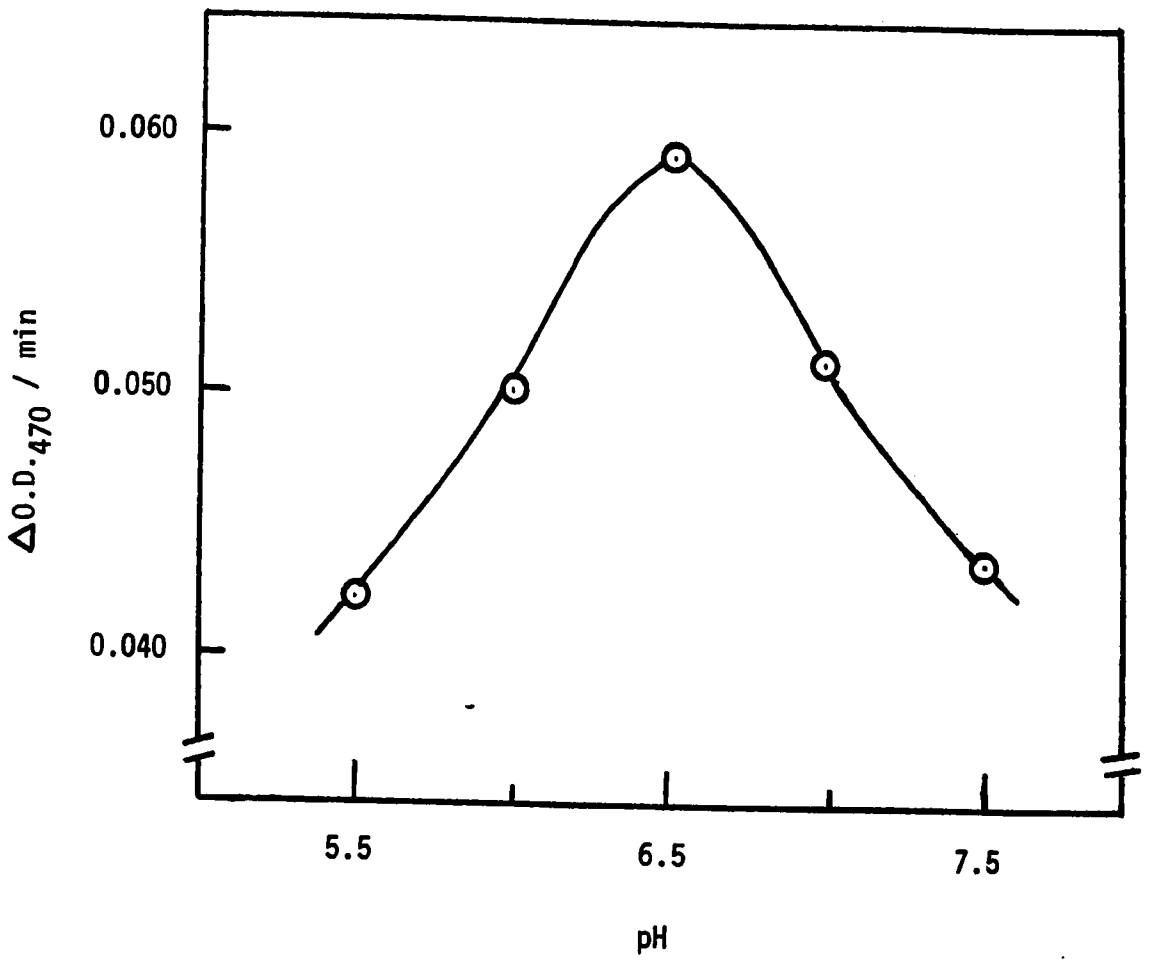

0.060
0.050
0.040
ΔO.D.470 / min
5.5
6.5
7.5
pH

Figure 14. EFFECT OF GUAIACOL CONCENTRATION ON THE ACTIVITY OF ISOPEROXIDASE A_3. Assays were run at 5 mM H_2O_2 in 40 mM sodium citrate (pH 6.5).

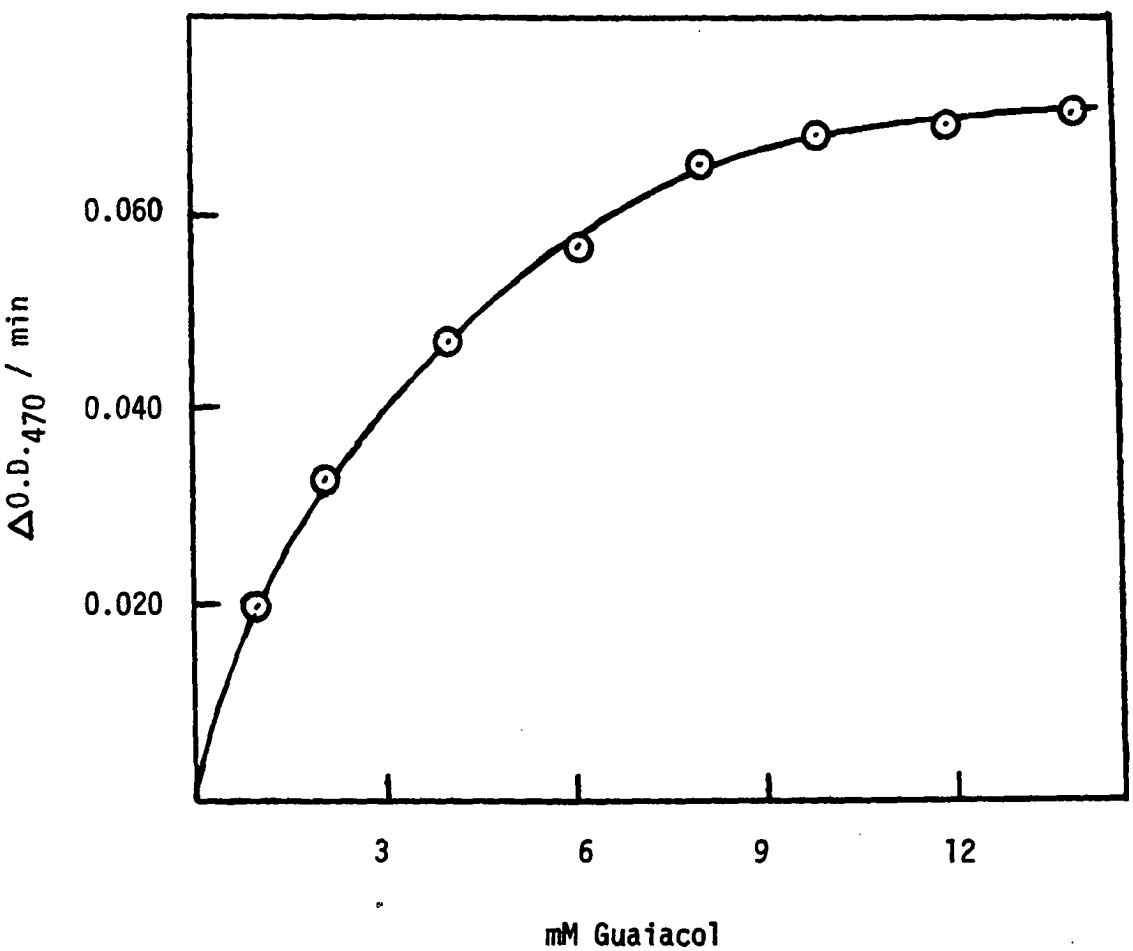

0.060
0.040
0.020
ΔO.D.470 / min
3
6
9
12
mM Guaiacol

Figure 15. DOUBLE RECIPROCAL PLOT OF ACTIVITY VS. GUAIACOL CONCENTRATION FOR ISOPEROXIDASE A_3. Assays were run at 5 mM H_2O_2 in 40 mM sodium citrate (pH 6.5).

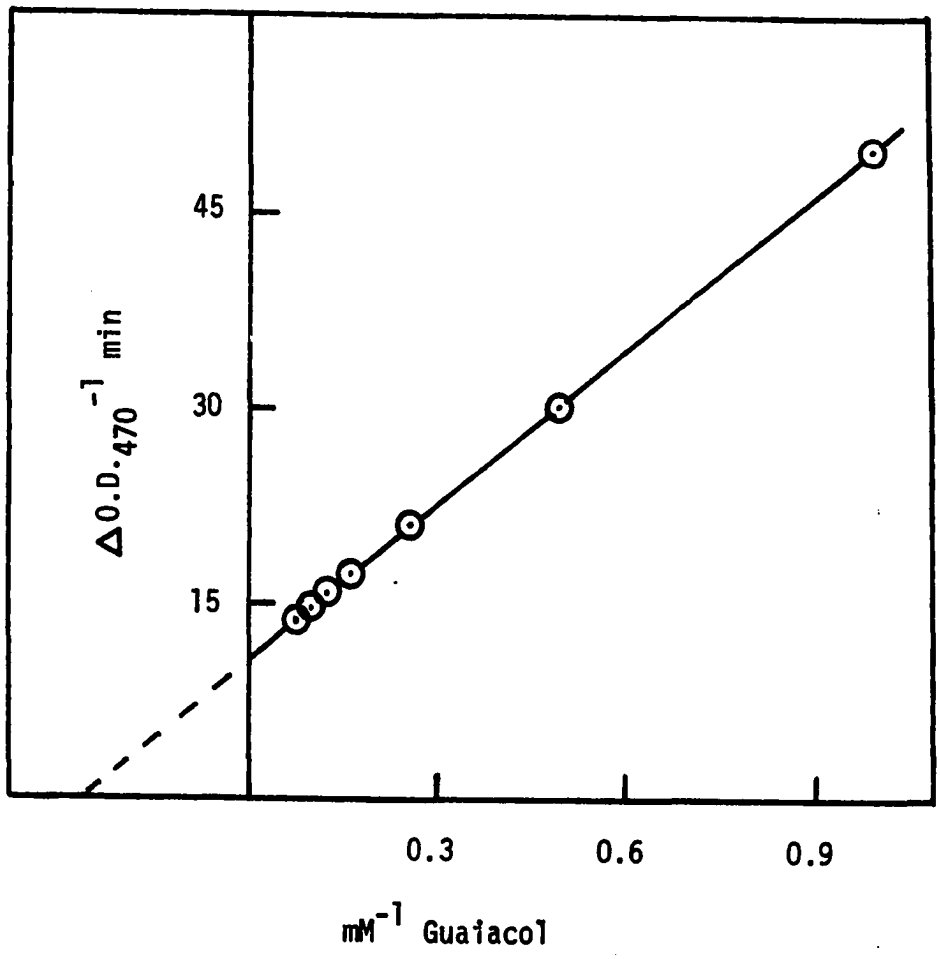

45
30
15
$\Delta O.D._{470}^{-1}$ min
0.3
0.6
0.9
mM^{-1} Guaiacol

TABLE 4

EFFECT OF VARIOUS COMPOUNDS

ON THE GUAIACOL ACTIVITY OF ISOPEROXIDASE A_3

EFFECTOR	% OF CONTROL ACTIVITY	EFFECT
0.4 mM		
Catechol	14	-
Esculetin	10	-
Caffeic acid	190	+
p-Coumaric acid	94	o
Ferulic acid	180	+
Scopoletin	165	+

+ - activation o - no effect - - inhibition

compounds may initiate a rapid, probably non-enzymatic degradation of guaiacol. Esculetin inhibition suggests that although this compound in oxidized, the color is not significant as compared to the guaiacol control and that the esculetin product cannot initiate guaiacol oxidation. This would also apply to catechol. p-Coumaric acid, which does appear to be oxidized, has no effect on the reaction.

The above results could also be explained by a two site enzyme with interaction between sites. The binding of an effector to one substrate site would either activate, inhibit, or possibly have no effect on the oxidation at another site through conformational changes in that site.

Esculetin

When isoperoxidase A_3 was added to a mixture of 1.0 mM esculetin, 5 mM H_2O_2 and 40 mM Tris-HCl (pH 7.5), the formation of a yellow product was noted. The yellow product absorbed at 469 nm while esculetin absorbed negligibly at this wavelength (Figure 16). The increase in absorbance at 469 nm was used to investigate esculetin oxidation catalyzed by isoperoxidase A_3. The pH optimum for esculetin oxidation was 7-7.5 (Figure 17). Figure 18 shows the velocity dependence on initial esculetin concentration. A Lineweaver-Burk plot of this data is linear and yields $S_{0.5}$ for esculetin equal to 0.27 mM (Figure 19).

Catechol

When isoperoxidase A_3 was added to a mixture of 18 mM catechol, 5 mM H_2O_2 and 40 mM sodium citrate (pH 5.0), a yellow-black color

Figure 16. ABSORPTION SPECTRA OF ESCULETIN REACTION MIXTURES. Mixture of 1 mM esculetin and 5 mM H_2O_2 in 40 mM Tris-HCl (pH 7.5) before and after addition of isoperoxidase A_3.

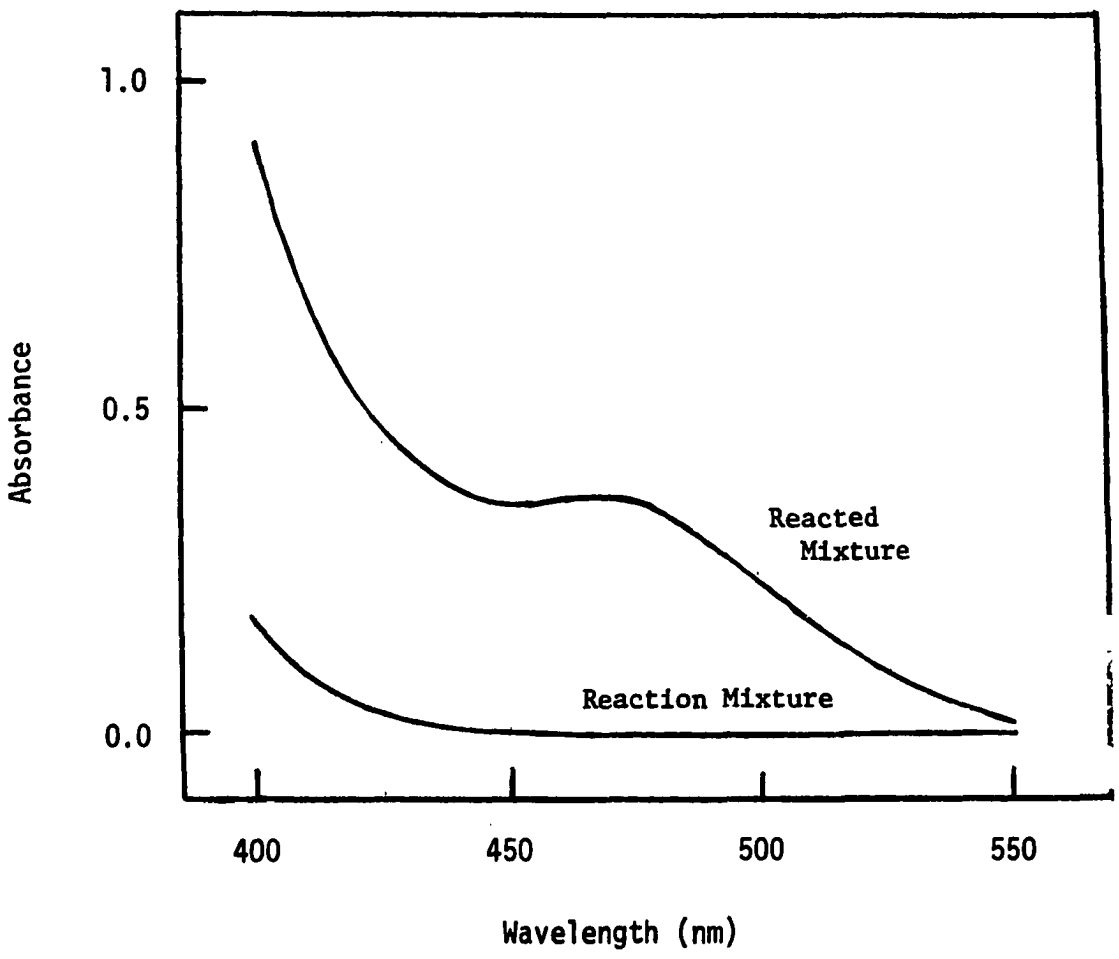

1.0
0.5
0.0
Absorbance
Reacted
Mixture
Reaction Mixture
400
450
500
550
Wavelength (nm)

Figure 17. EFFECT OF PH ON THE ESCULETIN ACTIVITY OF ISOPEROXIDASE A_3. Assays were run at 1.0 mM esculetin, 5 mM H_2O_2 and 40 mM sodium citrate or Tris-HCl buffer. ⊙ -sodium citrate ▲ -Tris-HCl

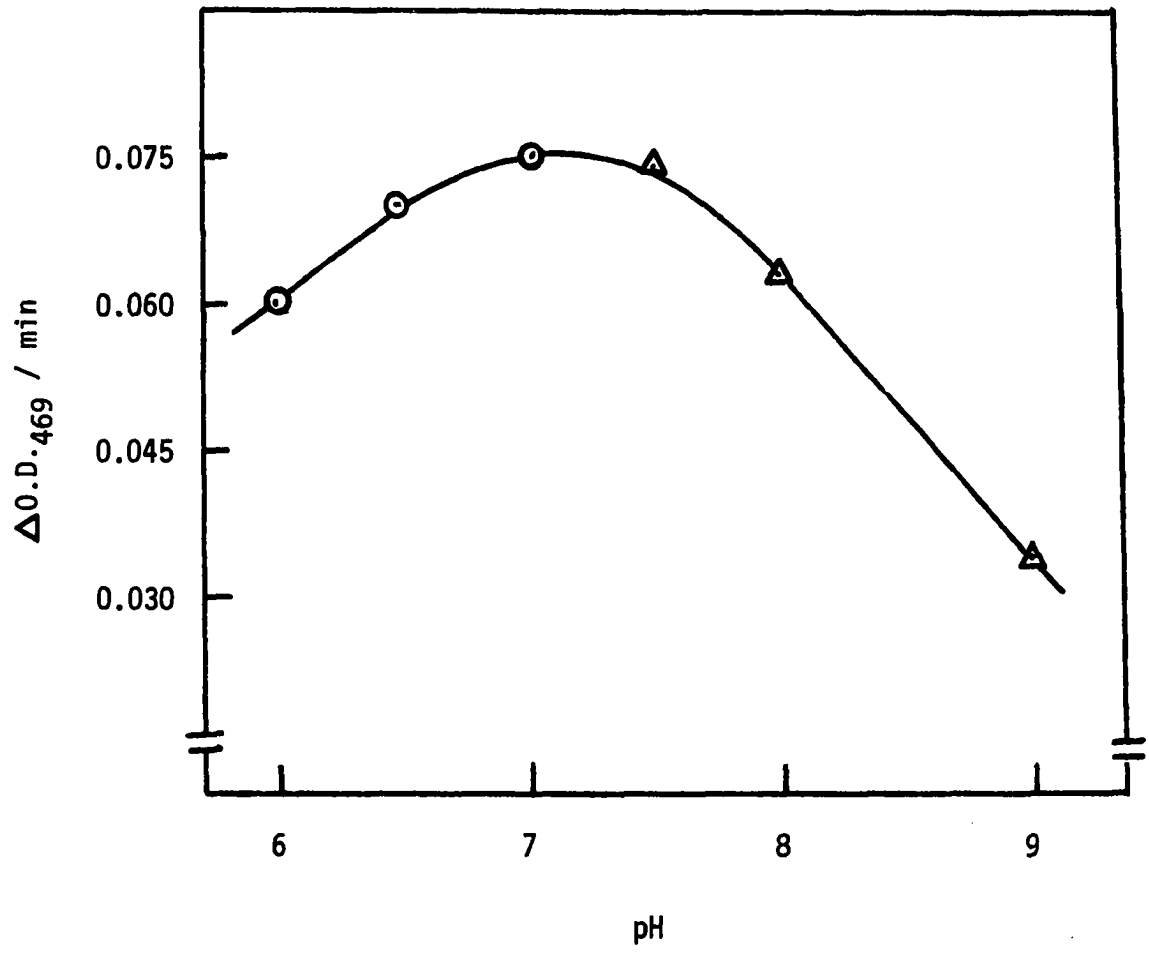

0.075
0.060
0.045
0.030
ΔO.D.469 / min
6
7
8
9
pH

Figure 18. EFFECT OF ESCULETIN CONCENTRATION ON THE ACTIVITY OF ISOPEROXIDASE A_3. Assays were run at 5 mM H_2O_2 in 40 mM Tris-HCl (pH 7.5).

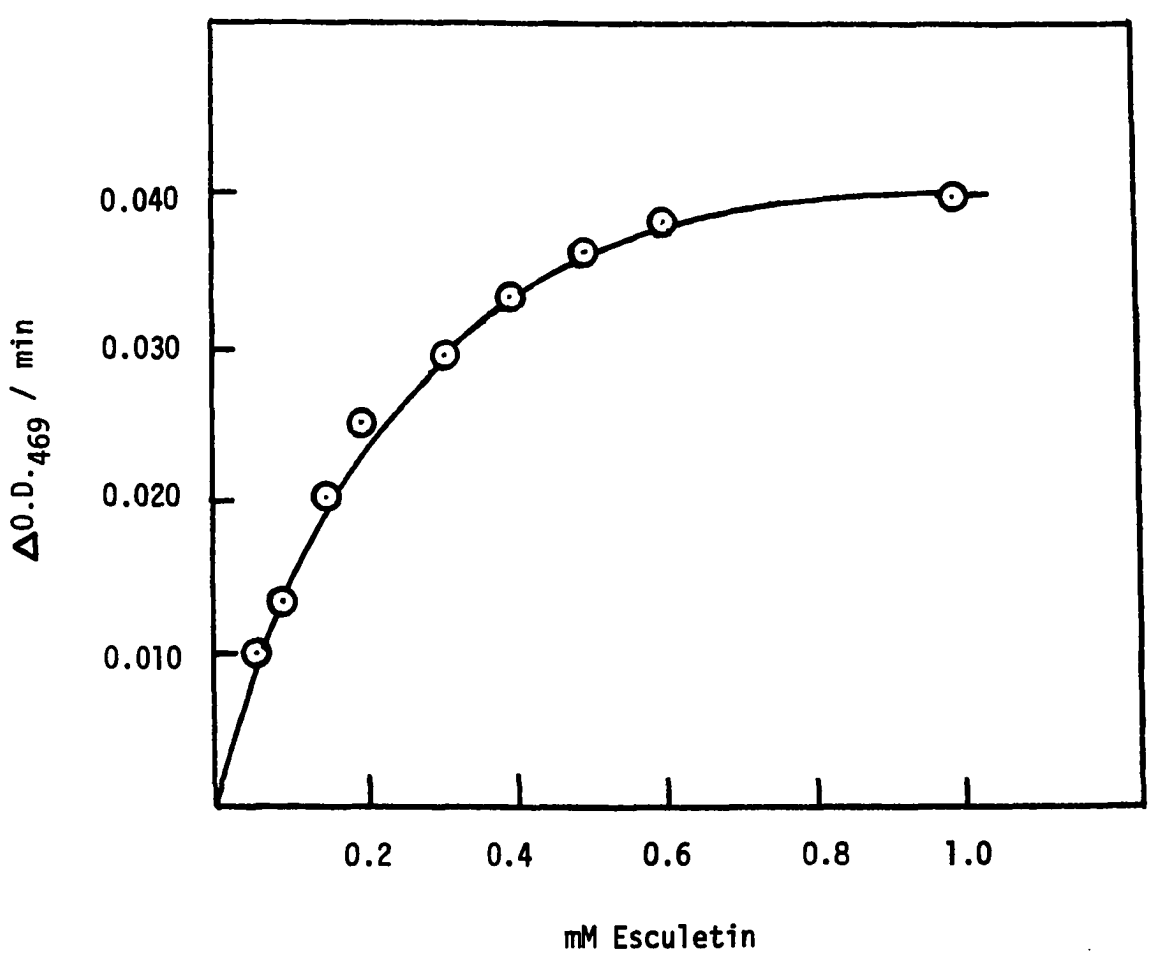
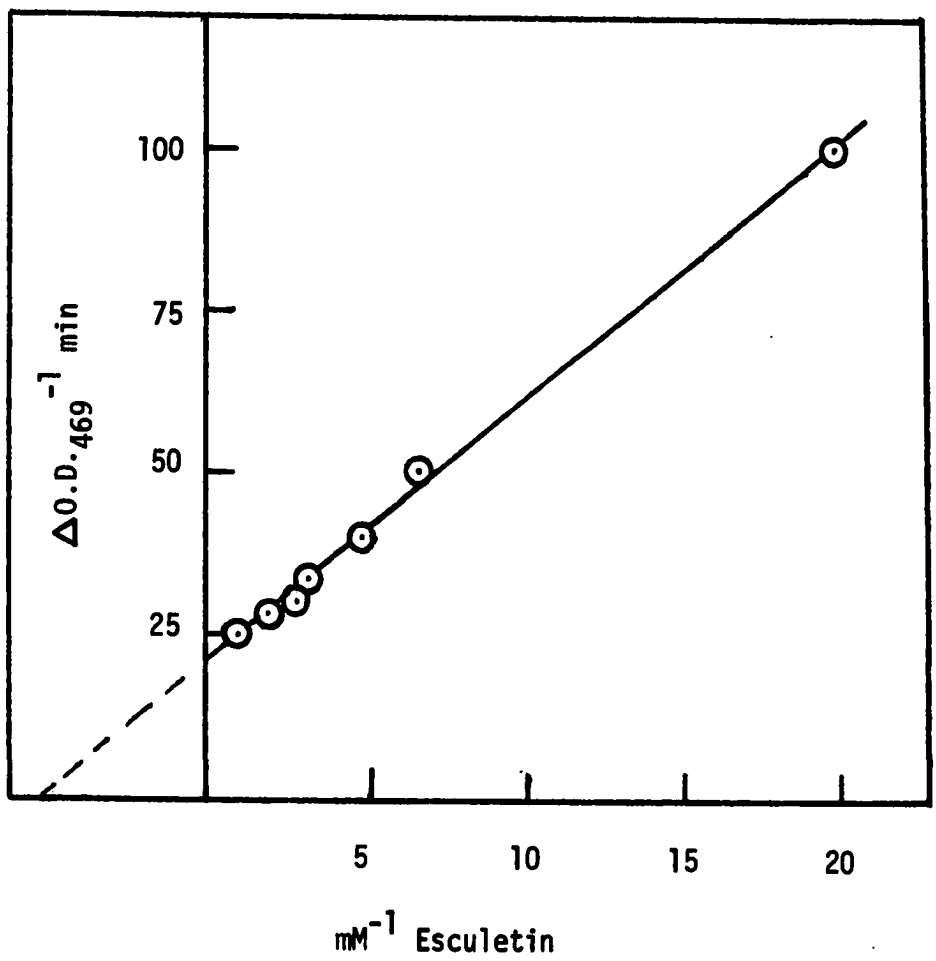

0.040
0.030
0.020
0.010
ΔO.D.469 / min
0.2
0.4
0.6
0.8
1.0
mM Esculetin

Figure 19. DOUBLE RECIPROCAL PLOT OF ACTIVITY VS. ESCULETIN CONCENTRATION FOR ISOPEROXIDASE A_3. Assays were run at 5 mM H_2O_2 in 40 mM Tris-HCl (pH 7.5).


100
75
50
25
$\Delta O.D._{469}^{-1}$ min
5
10
15
20
mM^{-1} Esculetin

formation was observed. This product absorbed at 400 nm while catechol absorbed negligibly at this wavelength (Figure 20). The increase in absorbance at 400 nm was used to investigate catechol oxidation catalyzed by isoperoxidase A_3. The pH optimum for catechol oxidation is 5.0 (Figure 21). Figure 22 shows the dependence of velocity on initial catechol concentration. A Lineweaver-Burk plot of this data is linear and yields $S_{0.5}$ for catechol equal to 6 mM (Figure 23).

IAA Oxidase

IAA oxidase activity has not been observed for isoperoxidase A_3. The method of Ray (40) using IAA and H_2O_2 and observing the change in absorbance at 262 nm showed no activity even when using preparations twenty times more concentrated than those used for guaiacol assay. The method of Gordon and Weber (41) using Mn^{++}, DCP and IAA showed no change in Salkowski color reagent. The UV spectrum of IAA in the presence of H_2O_2; Mn^{++} and DCP; H_2O_2, Mn^{++}, and DCP was not altered upon addition of isoperoxidase A_3.

Additional Substrates

Several other substrates including chlorogenic acid, ferulic acid, and caffeic acid appear to be oxidized by H_2O_2 in the presence of isoperoxidase A_3. Visible color formation and/or changes in the visible and UV spectra of these compounds upon reaction indicate that they are oxidized in the presence of isoperoxidase A_3. Chlorogenic acid which has been implicated in growth and development (42) and in the inhibition of several enzymes (43) is of particular interest

Figure 20. ABSORPTION SPECTRA OF CATECHOL REACTION MIXTURES. Mixture of 15 mM catechol and 5 mM H_2O_2 in 40 mM sodium citrate (pH 5.0) before and after addition of isoperoxidase A_3.

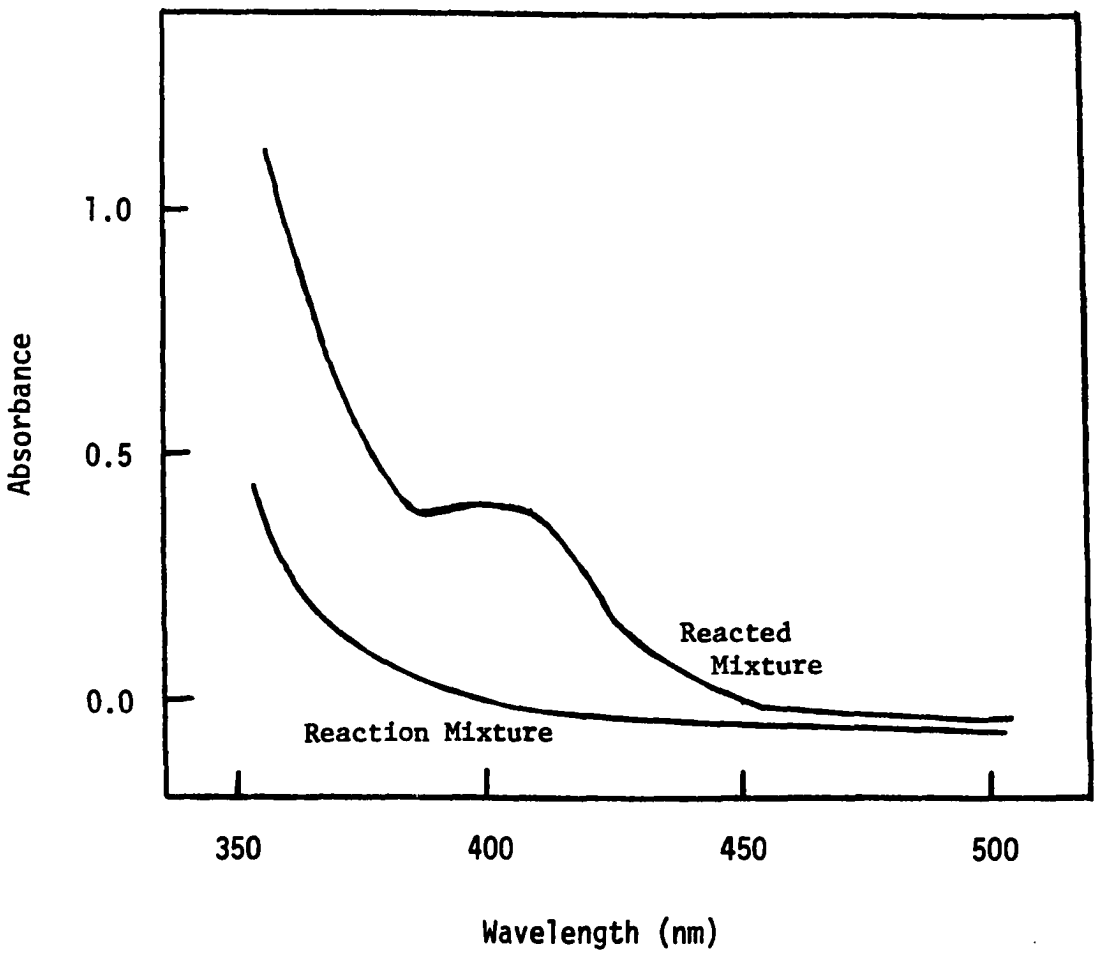

Absorbance
1.0
0.5
0.0
Reacted
Mixture
Reaction Mixture
350
400
450
500
Wavelength (nm)

Figure 21. EFFECT OF PH ON THE CATECHOL ACTIVITY OF ISOPEROXIDASE A_3. Assays were run at 15 mM catechol and 5 mM H_2O_2 in 40 mM sodium citrate buffer.

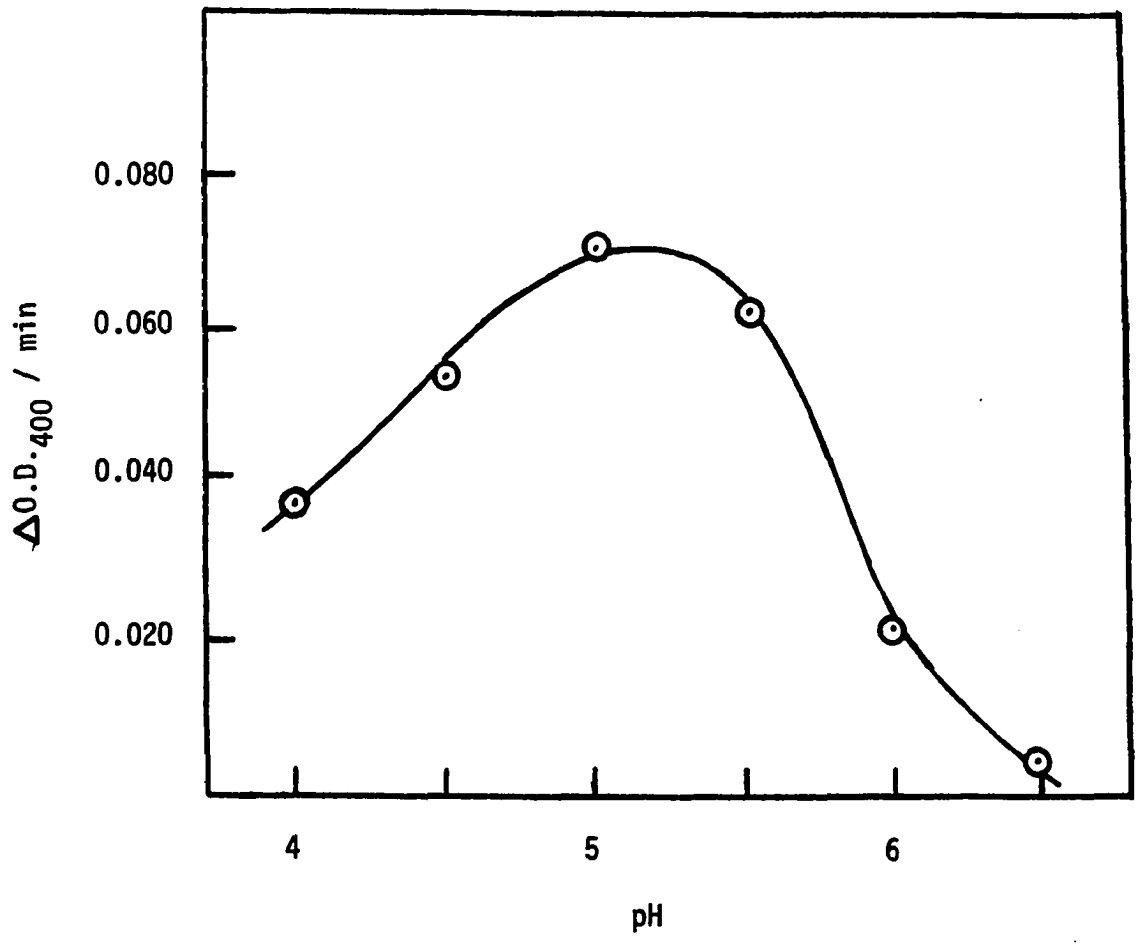

0.080
0.060
0.040
0.020
ΔO.D.400 / min
4
5
6
pH

Figure 22. EFFECT OF CATECHOL CONCENTRATION ON THE ACTIVITY OF ISOPEROXIDASE A_3. Assays were run at 5 mM H_2O_2 in 40 mM sodium citrate (pH 5.0).

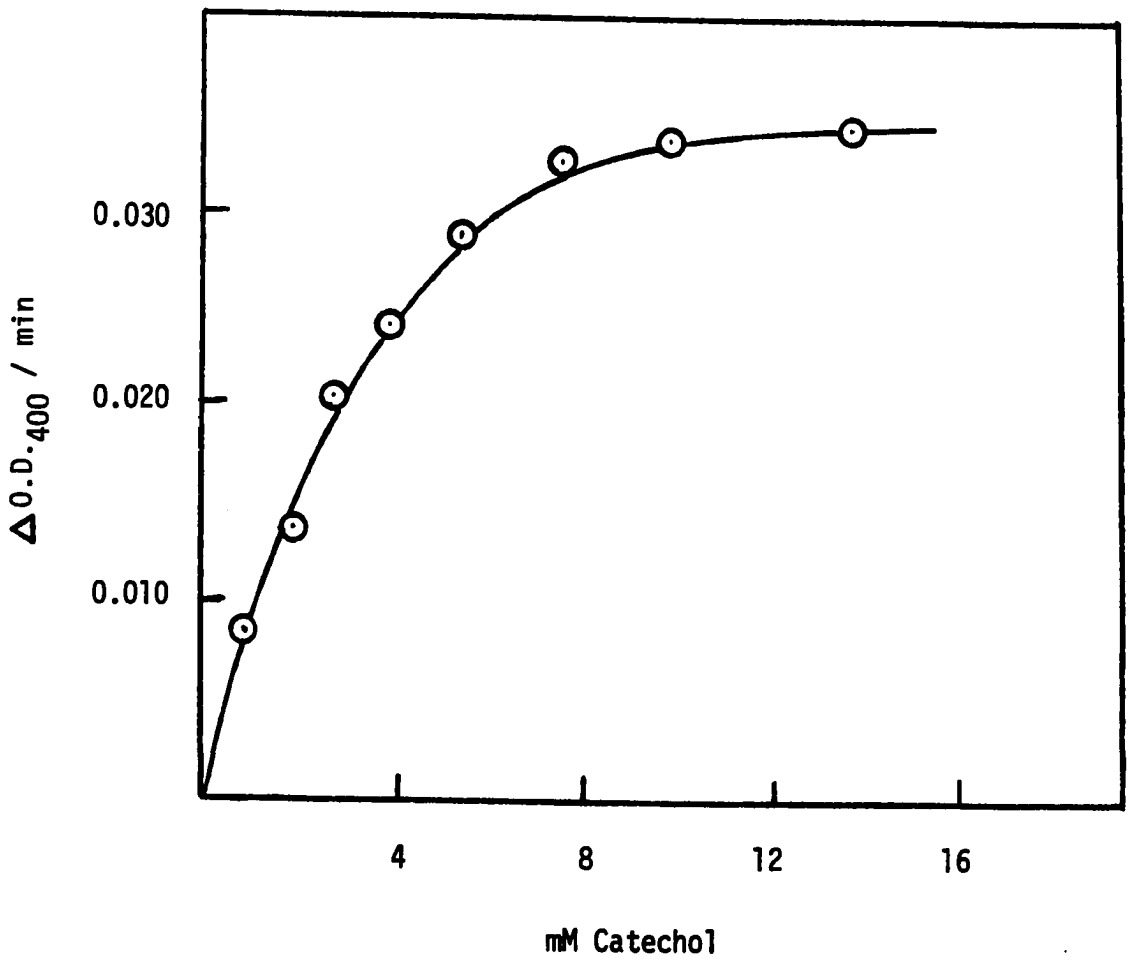
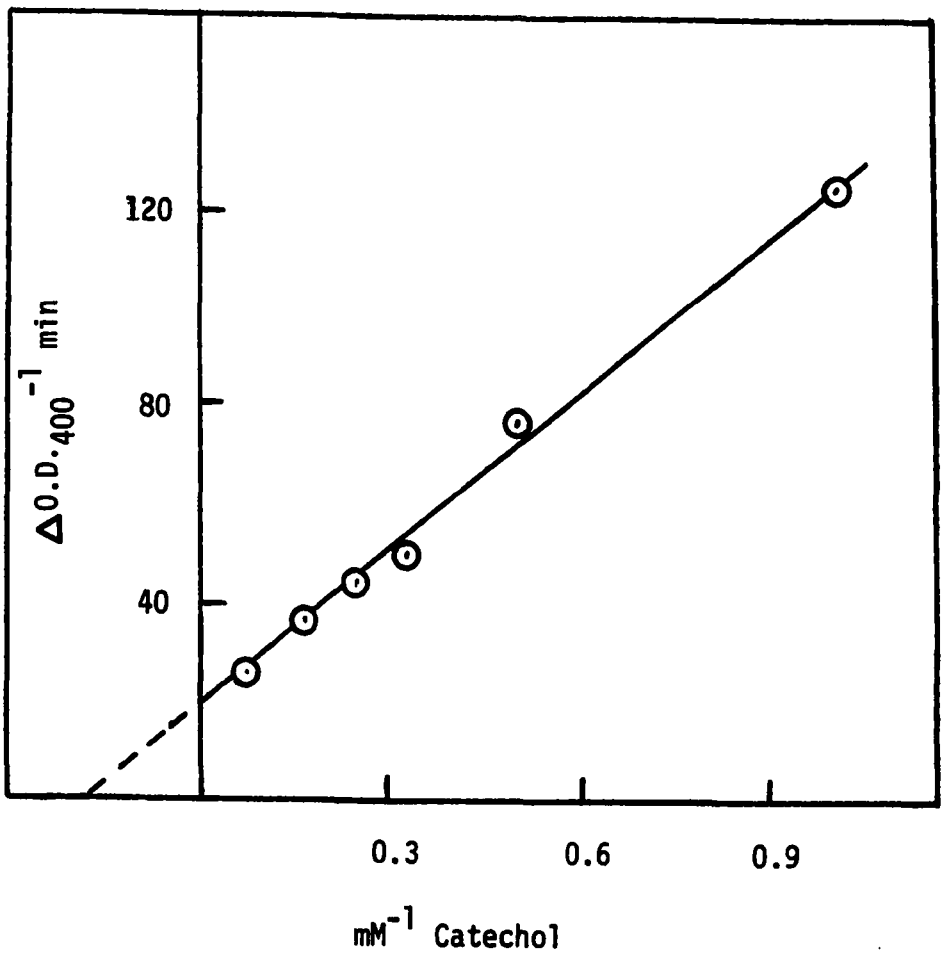

0.030
0.020
0.010
ΔO.D.400 / min
4
8
12
16
mM Catechol

Figure 23. DOUBLE RECIPROCAL PLOT OF ACTIVITY VS. CATECHOL CONCENTRATION FOR ISOPEROXIDASE A_3. Assays were run at 5 mM H_2O_2 in 40 mM sodium citrate (pH 5.0).


120
80
40
ΔO.D.$_{400}^{-1}$ min
0.3
0.6
0.9
mM^{-1} Catechol

as a substrate for isoperoxidase A_3. Kinetic studies of chlorogenic acid were prohibited by the lack of an assay method that could yield linear rates for any adequate time period.

Summary

Table 5 summarizes the data obtained from kinetic studies of scopoletin, catechol, guaiacol and esculetin as isoperoxidase A_3 substrates. The pH optima for scopoletin and esculetin are significantly different. The $S_{0.5}$'s for these two substrates are in the same range; however, the $S_{0.5}$ for catechol is significantly higher. As more information is obtained concerning the physiological role of these compounds in metabolism, the significance of the pH optima and $S_{0.5}$ in the utilization of these substates should become apparent.

TABLE 5

SUMMARY OF PH OPTIMA, $S_{0.5}$, AND HILL SLOPE VALUES FOR SUBSTRATES OF ISOPEROXIDASE A_3

SUBSTRATE	PH OPTIMUM	$S_{0.5}$	HILL SLOPE
Scopoletin	5.5	0.6 mM	1.5
Esculetin	7.0- 7.5	0.27 mM	1.0
Guaiacol	6.5	4 mM	1.1
Catechol	5.0	6 mM	1.0

CHAPTER VI

COMPARATIVE SUBSTRATE UTILIZATION FOR DIFFERENT PEROXIDASE PREPARATIONS

Several of the substrates mentioned previously were used to compare the relative oxidation rates of these substrates as compared to the guaiacol rate for several peroxidase preparations. All peroxidase preparations were diluted to obtain a uniform guaiacol oxidation rate. This same dilution was used to measure the rates of oxidation of other substrates. The ratios for each preparation are defined as the rate of substrate oxidation divided by the rate of guaiacol oxidation

$$\text{ratio} = \frac{\Delta \text{O.D./min (substrate)}}{\Delta \text{O.D./min (guaiacol)}}.$$

Assay conditions and wavelength are listed in Table 6.

Table 7 shows the oxidation ratios for various substrates with various peroxidase preparations. A_1 and A_2 are isolated isoperoxidases from tobacco tissue W-38. C_4 is an isolated isoenzyme from tobacco tissue WR-132. This data was supplied by Powell (44) and Pickering (45), respectively. A_3 is an isolated isoperoxidase also from tobacco tissue W-38. Crude is a crude extract from W-38. HRP is Sigma Type I.

TABLE 6

ASSAY CONDITIONS FOR COMPARISON EXPERIMENTS

OF VARIOUS PEROXIDASE PREPARATIONS

SUBSTRATE	H_2O_2	BUFFER	ACTIVITY
1 mM Esculetin	5 mM	40 mM pH 7.5	ΔO.D.$_{469}$/ min
1.25 mM Scopoletin	5 mM	40 mM pH 5.5	ΔO.D.$_{450}$/ min
1 mM Chlorogenic acid	5 mM	40 mM pH 4.5	ΔO.D.$_{400}$/ min
0.2 mM Ferulic acid	5 mM	40 mM pH 5.0	ΔO.D.$_{310}$/ min
15 mM Guaiacol	5 mM	40 mM pH 6.0	ΔO.D.$_{470}$/ min

TABLE 7

RELATIVE SUBSTRATE OXIDATION RATIOS

FOR VARIOUS PEROXIDASE PREPARATIONS

	SCOPOLETIN	FERULIC ACID	CHLOROGENIC ACID	ESCULETIN
A_1*	0.03	3.0	0.95	0.60
A_2*	0.10	2.8	0.66	0.27
A_3	4.0-5.0	3.5-5.0	1.0-2.0	0.39-0.48
C_4*	0.40	1.1	0.23	0.43
W-38 CRUDE	0.22-0.29	2.5-2.6	0.50-0.55	0.37-0.41
HRP	0.11-0.28	3.7-3.9	1.0-1.1	0.42-0.47

* Data for these isoperoxidases were supplied by B. Powell and J. Pickering.

Although one cannot predict the actual substrate preferences for a single isoperoxidase because turnover numbers are not known, it can be demonstrated that one isoperoxidase probably utilizes a particular substrate better than another isoperoxidase. It appears that A_3 utilizes scopoletin much more readily than other preparations or other isoperoxidases. Ferulic acid also appears to be utilized somewhat better by A_3 than by the other isoperoxidases listed.

The physiological significance of the apparent preference of one isoperoxidase over another for particular substrates cannot be stated until the physiological roles of these phenolic substrates are more clearly established. As possible physiological roles are demonstrated and the in vivo significance of peroxidative reactions is shown, the importance in overall metabolism of factors regulating these preferences, such as, turnover numbers, $S_{0.5}$, and pH optima should be apparent. Oxidation of phenolic compounds would then be regulated by the presence of a specific isoperoxidase, under certain phenolic concentration and pH conditions, in a particular location in the plant tissue or organelle.

CHAPTER VII

PROPOSED MECHANISM OF SCOPOLETIN OXIDATION AND POSSIBLE NATURE OF PRODUCTS

Mechanism of Scopoletin Oxidation

Mechanistic speculations concerning oxidation of substrates by isoperoxidase A_3 must attempt to explain the data available, and the validity of the mechanism can be judged only on the information at hand. Most of the observations with isoperoxidase A_3 involve only spectrophotometric and thin layer chromatography observations. Electron spin resonance techniques would ideally be applied and should add much valuable information to describe more adequately the mechanism of scopoletin oxidation.

Mechanistic speculations were particularly needed to explain the observations made with scopoletin as an isoperoxidase A_3 substrate. Evaluation of the kinetic data yielded results that would indicate substrate cooperative effects for scopoletin. The Hill plot with n=1.5 and the concave upward Lineweaver-Burk plot are conditions necessary for substrate cooperative effects (37). Gel permeation chromatography and SDS electrophoresis both yielded

the same molecular weight for isoperoxidase A_3. These results indicate that A_3 does not contain subunits, and although cooperative effects have been noted in some single chain proteins (46) it seemed necessary also to consider a possible mechanistic explanation for the kinetics observed.

Scopoletin oxidation at pH 5.5 occurs with the formation of a blue intermediate which then results in the formation of yellow end product. At pH 7.0 the blue intermediate is not noted and only the formation of the yellow product is observed. Measurement of product formation is at 450 nm which would be for the yellow product. The blue intermediate absorbs maximally at 615 nm. Based on these observations and the data indicating that a scopoletin product is polymerized by isoperoxidase A_3, the following mechanism could be postulated. This mechanism only attempts to explain the observations made with scopoletin. H_2O_2 may participate at more than one step of the reaction. This reaction is the simplest mechanism that might explain the data available. Alteration would most likely be necessary as more data are collected.

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \qquad (1)$$

$$ES \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E + P_1 \text{ (blue)} \qquad (2)$$

$$2P_1 \xrightarrow{k_5} P_2 \text{ (yellow)} \qquad (3)$$

Subsequent polymerization of P_2 may occur, but this would be assumed

not to be responsible for initial color formation.

If reaction 3 is assumed to be the rate limiting step ($k_3 \gg k_5$) and steady state assumptions are made with $k_3 \gg k_4$ one finds

$$V = \frac{dP_2}{dt} = \frac{k_5 k_3^2 (E)^2 (S)^2}{(K_s + (S))^2} \qquad \text{Eqn. 1}$$

at constant enzyme concentration this equation generates a sigmoidal saturation curve for substrate (S) and the equivalent of a Hill plot would yield an n of 2.

If reaction 2 becomes the rate limiting step ($k_5 \gg k_3$) velocity then depends on the formation of P_1. The dependence of P_1 formation on S concentration is given by the Michaelis-Menten equation

$$V = \frac{dP_1}{dt} = \frac{k_3 (E)(S)}{K_s + S} \qquad \text{Eqn. 2}$$

If k_3 and k_5 are not greatly different one would observe a substrate (S) dependence somewhere between first and second order.

For scopoletin oxidation at pH 5.5, it appears k_3 and k_5 are both significant since blue intermediate formation and yellow end product formation are both observed. At pH 7.5 it appears that $k_5 \gg k_3$ since the blue intermediate is stable in the more acidic solutions (k_5 lower).

At pH 5.5 sigmoidal dependence of velocity on scopoletin concentration with a reaction order between first and second order, would be predicted from this mechanism. At pH 7.5 a nonsigmoidal (first order) dependence of velocity on scopoletin concentration

would be predicted. Figure 24 shows the dependence of velocity on scopoletin concentration as a function of pH. The observed values of n obtained from Hill slope plots of these data are 1.7 at pH 4.0, 1.5 at pH 5.5 and 1.0 at pH 7.5 (Figure 25). A greater than first order dependence on enzyme concentration would also be predicted. Figure 26 demonstrates that the dependence on enzyme concentration is indeed greater than first order at pH 5.5.

Formation of the blue intermediate of scopoletin oxidation can be followed by the change in absorbance at 615 nm. At pH 7.5 no sigmoidal dependence on scopoletin concentration up to 0.6 mM scopoletin is observed. This would be predicted from the first two reactions of the proposed mechanism.

The above results fit the proposed mechanism rather well; however, attempts to prove or disprove substrate cooperative effects for scopoletin binding should be continued.

Nature of the Product of Scopoletin Oxidation

The structure of the products of scopoletin have not been determined. The mobility on thin layer chromatography, mass spectrometry data and solubility suggest that the final product of the *in vitro* reaction in the presence of light is probably a polymer (Chapter IV). NMR data suggest that the vinyl protons (τ 2.8-3.2) of scopoletin disappear upon reaction of scopoletin with peroxide in the presence of A_3. The presence of at least one hydroxyl on the benzene portion of coumarin rings also seems to be necessary for oxidation since coumarin itself does not appear to be oxidized. Although it is

Figure 24. EFFECT OF PH ON THE SCOPOLETIN SATURATION CURVES FOR ISOPEROXIDASE A_3. Assays were run at 10 mM H_2O_2 in 40 mM sodium citrate buffer.

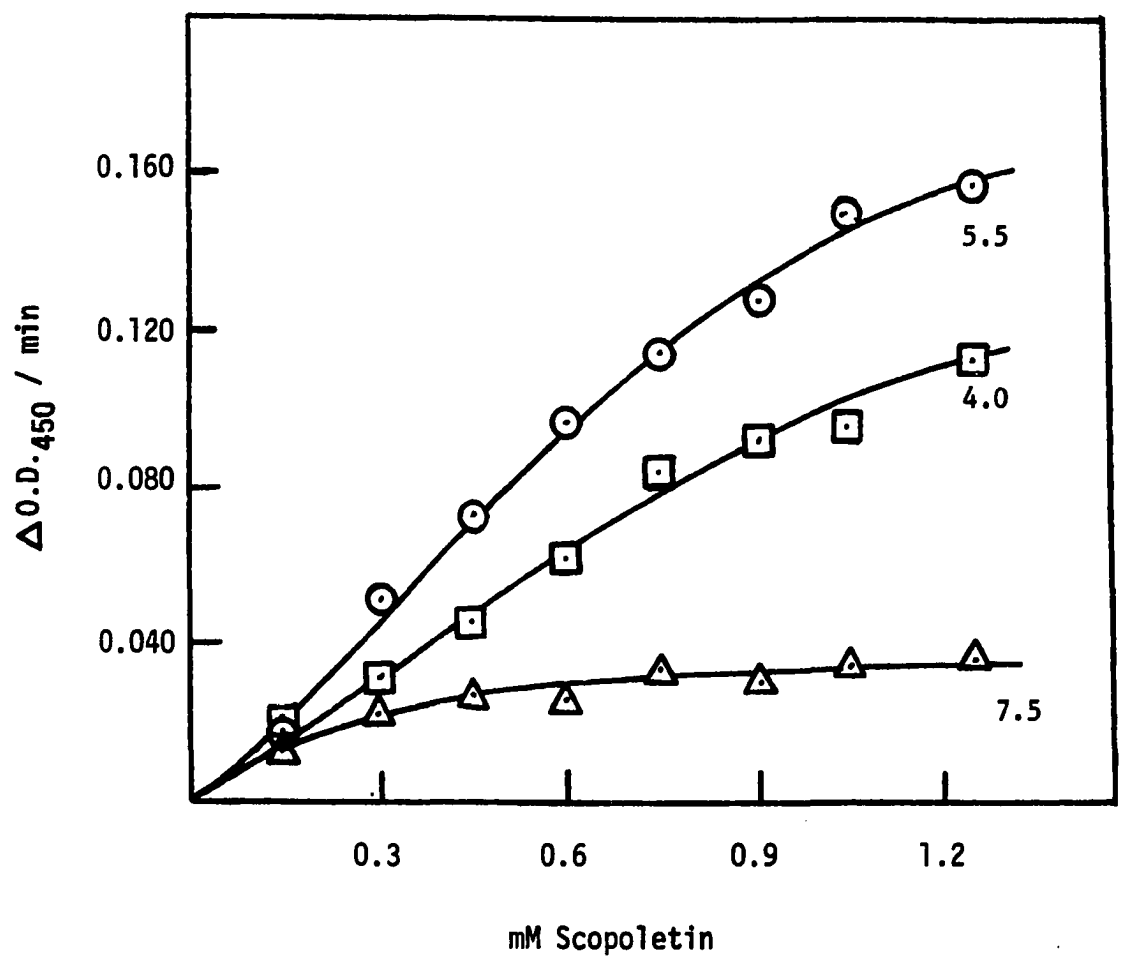

0.160
0.120
0.080
0.040
ΔO.D.450 / min
5.5
4.0
7.5
0.3
0.6
0.9
1.2
mM Scopoletin

Figure 25. HILL PLOT OF ACTIVITY VS. SCOPOLETIN CONCENTRATION AT VARIOUS PHs. Assays were run at 10 mM H_2O_2 in 40 mM sodium citrate buffer. v-ΔO.D.$_{450}$/ min.

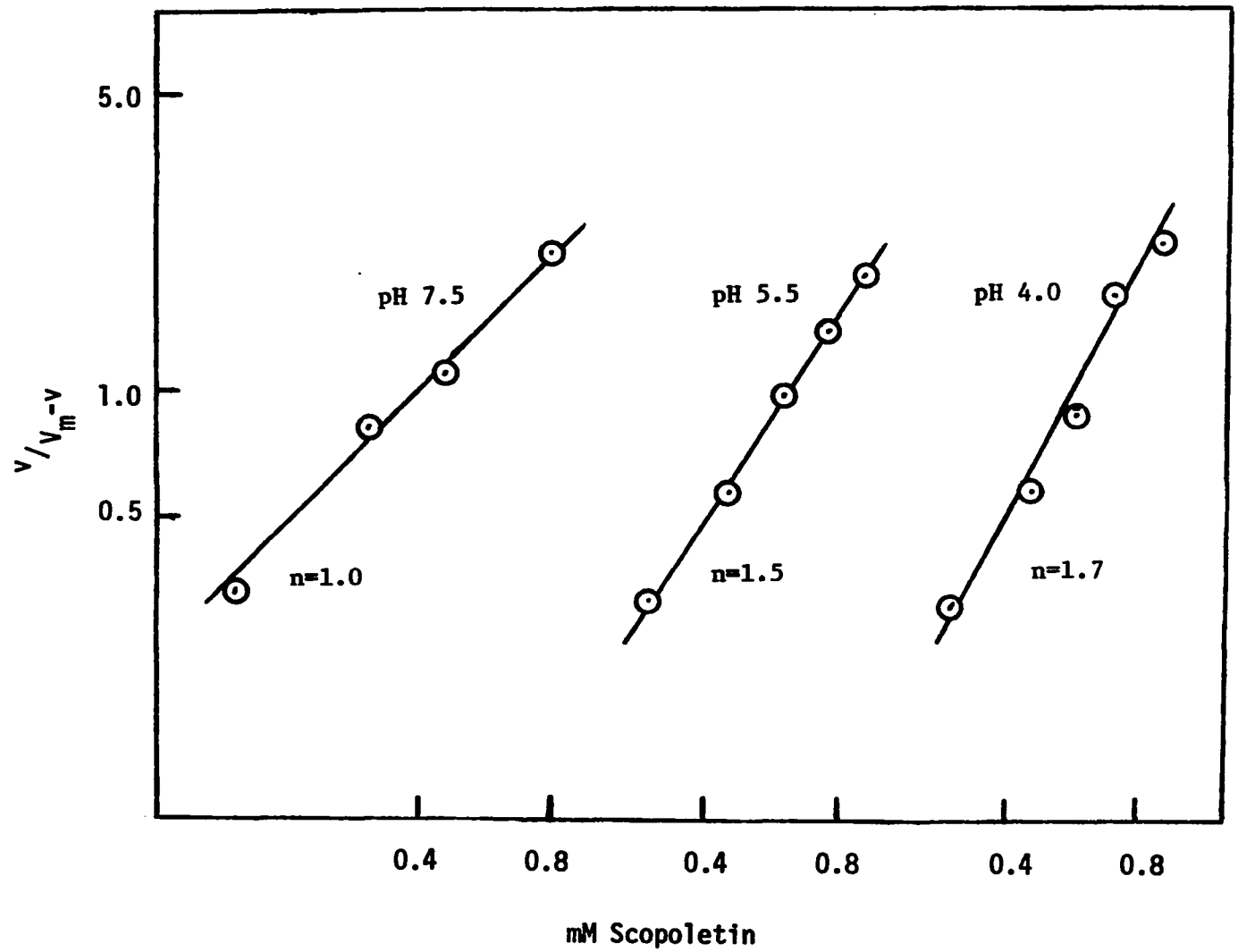
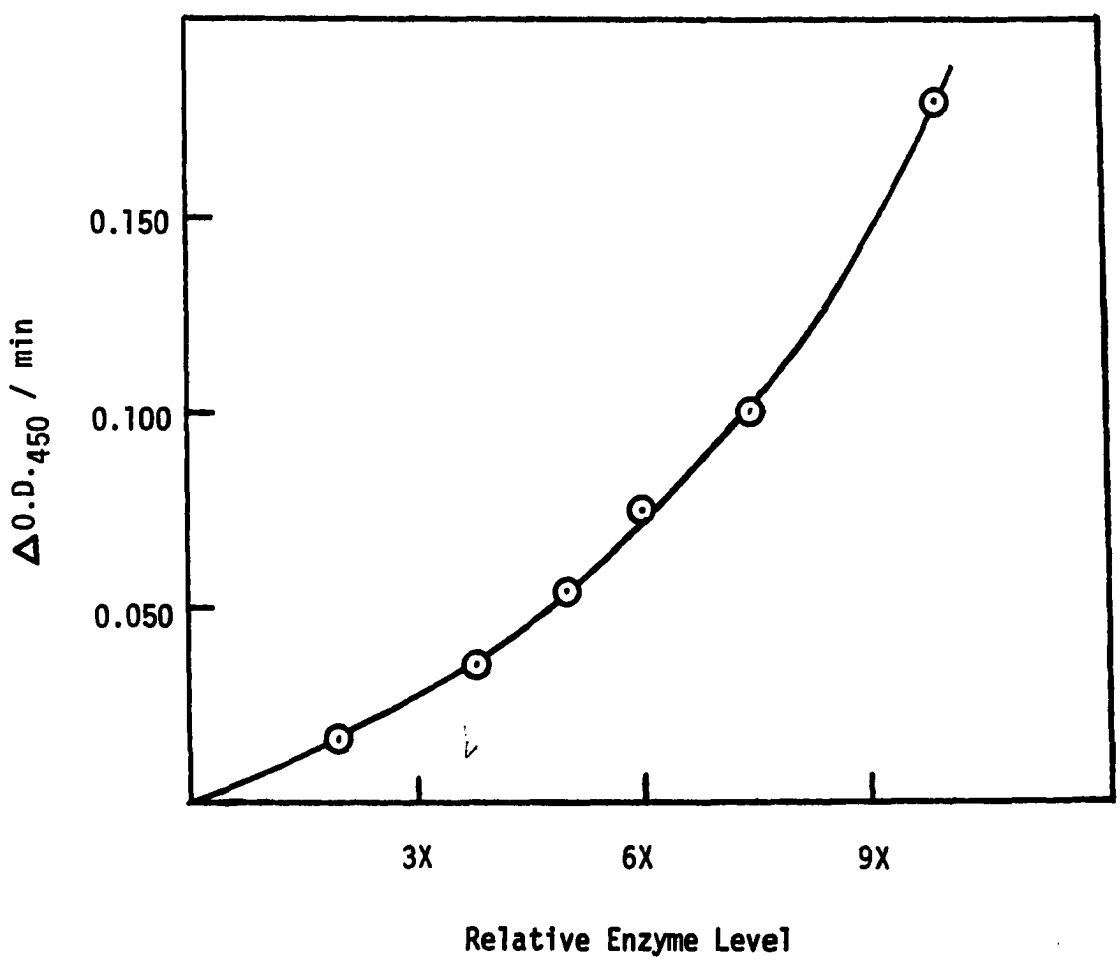
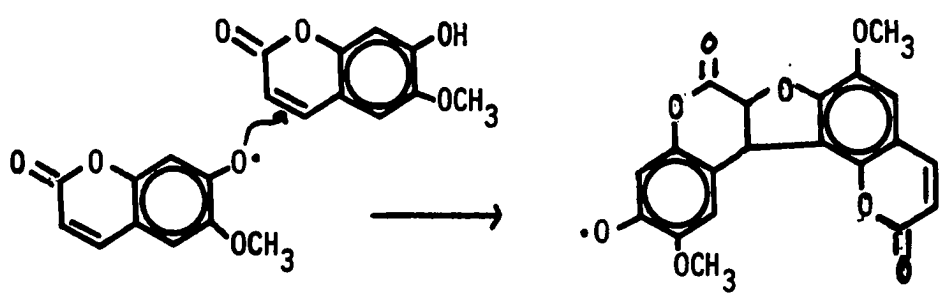

5.0
1.0
0.5
v/Vm-v
pH 7.5
n=1.0
pH 5.5
n=1.5
pH 4.0
n=1.7
0.4
0.8
0.4
0.8
0.4
0.8
mM Scopoletin

Figure 26. EFFECT OF ENZYME CONCENTRATION ON THE SCOPOLETIN ACTIVITY OF ISOPEROXIDASE A_3. Assays were run at 1.25 mM scopoletin, 5 mM H_2O_2 and 40 mM sodium citrate (pH 5.5).


0.150
0.100
0.050
ΔO.D.450 / min
3X
6X
9X
Relative Enzyme Level

possible that this hydroxyl is only necessary for binding, the hydroxyl group of guaiacol is known to be involved in formation of the final product of guaiacol oxidation (47). A plausible reaction involving the hydroxyl group of scopoletin which also would cause loss of vinyl protons upon reaction would be the reaction of the hydroxyl of one molecule of scopoletin with the double bond of the coumarin ring. A radical formed at the 6-hydroxy of scopoletin could attack the double bond leading eventually to a polymerized product.



This type of reaction sequence is similar to the polymerization of isoeugenol which has been used as a model for a lignin-like product (48).

The structural analysis of the scopoletin oxidation product is complicated by the limited solubility of the polymerized product in solvents suitable for NMR analysis. The separation and analysis of intermediates are complicated by the polymerization that occurs with exposure to light.

CHAPTER VIII

SUMMARY

The separation of isoperoxidase A_3 from other isoperoxidases of tobacco tissue culture W-38 was achieved using ammonium sulfate fractionation and DEAE-cellulose chromatography. SDS gel electrophoresis indicates that this preparation does not contain significant amounts of other contaminating proteins. The molecular weight of this isoperoxidase is about 54,000. Isoperoxidase A_3 also appears to contain disulfide linkages and the presence of a heme group is indicated. An explanation for the lack of a characteristic heme visible absorption spectrum, however, has not been obtained.

Isoperoxidase A_3 in the presence of H_2O_2 appears to oxidize several phenolic compounds including scopoletin, ferulic acid, catechol, guaiacol, caffeic acid, esculetin and chlorogenic acid. Evaluation of the kinetics of scopoletin oxidation yields results indicative of substrate cooperative effects for scopoletin. An alternate, mechanistic explanation not involving substrate cooperative effects also seems to be plausible.

The effect of various compounds including phenolics, IAA and

Mn^{++} on the scopoletin activity is shown. Many of these compounds, including IAA and Mn^{++}, inhibit the scopoletin activity of isoperoxidase A_3.

The isolated isoperoxidase A_3 appears to have a greater ability to oxidize scopoletin than does the crude preparation when compared to guaiacol activity. The scopoletin oxidizing ability relative to guaiacol for isoperoxidase A_3 compared to the values obtained for other isoperoxidases indicates that A_3 utilizes scopoletin as a substrate 10-150 times better than some of the other isolated isoperoxidases. A_3 also utilizes scopoletin much more readily than commercial HRP preparations when comparisons similar to those above are made.

It would appear then that different isoperoxidases do indeed have different substrate preferences, and the significance of these preferences should be considered in overall plant metabolism as more information concerning the various functions of peroxidase in plants is obtained.

The involvement of scopoletin in IAA oxidase inhibition and thus plant growth regulation may be controlled by the relative levels of IAA, scopoletin and Mn^{++} since IAA and Mn^{++} inhibit scopoletin oxidation. The $S_{0.5}$ for scopoletin oxidation is also in the range of scopoletin concentration that has growth effects.

All data observed indicate that in many studies individual isoperoxidases should probably be considered separately since they do indeed show distinct differences in substrate preferences. Other

significant characteristics may also differ such as $S_{0.5}$ and inhibition patterns. The ability of these isoperoxidases to utilize physiologically significant compounds should encourage investigations into this area. The investigations of individual isoperoxidases with physiologically significant substrates should yield valuable insight into overall plant metabolism.

REFERENCES

1. Galston, A. W., J. Bonner and R. S. Baker. 1953. Flavoprotein and peroxidase as components of the indoleacetic acid oxidase system of peas. Arch. Biochem. Biophys. 42:456-470.

2. Siegel, S. M.. 1955. The biochemistry of lignin formation. Physiol. Plantarum. 8:20-32.

3. Harkin, J. M. and J. R. Obst. 1973. Lignification in trees: indication of exclusive peroxidase participation. Science. 180:296-297.

4. Akazawa, T. and E. E. Conn. 1958. The oxidation of reduced pyridine nucleotides by peroxidase. J. Biol. Chem. 232:403-415.

5. Reigh, D. L., S. H. Wender and E. C. Smith. 1973. Scopoletin, a substrate for an isoperoxidase from Nicotiana tabacum tissue culture W-38. Phytochemistry 12:1265-1268.

6. Sirois, J. C. and R. W. Miller. 1972. The mechanism of scopoletin-induced inhibition of the peroxidase-catalyzed degradation of indole-3-acetate. Plant. Physiol. 49:1012-1018.

7. Gelinas, D. A. 1973. Proposed model for the peroxidase-catalyzed oxidation of indole-3-acetic acid in the presence of the inhibitor ferulic acid. Plant Physiol. 51:967-972.

8. Andreae, W. A. 1952. Effect of scopoletin on indoleacetic acid metabolism. Nature 170:83-84.

9. Morita, Y., C. Yoshida and Y. Maeda. 1971. Properties and structures of peroxidase isoenzymes of Japanese-radish. Agr. Biol. Chem. 35:1074-1083.

10. Misawa, M. and S. M. Martin. 1972. Two peroxidases isolated from kidney bean suspension cultures. Can. J. Bot. 50:1245-1252.

11. Shih, J. H. C., L. M. Shannon, E. Kay and J. Y. Lew. 1971. Peroxidase isoenzymes from horseradish roots. IV. Stuctural relations. J. Biol. Chem. 246:4546-4551.

12. Shannon, L. M., E. Kay and J. Y. Lew. 1966. Peroxidase isoenzymes from horseradish roots. I. Isolation and physical properties. J. Biol. Chem. 241:2166-2172.

13. Schafer, P., S. H. Wender and E. C. Smith. 1971. Effect of scopoletin on two anodic isoperoxidases isolated from tobacco tissue culture W-38. Plant Physiol. 48:232-233.

14. Goldacre, P. L., A. W. Galston and R. L. Weintraub. 1953. The effect of substituted phenols on the activity of indoleacetic acid oxidase of peas. Arch. Biochem. Biophys. 43:358-373.

15. Imbert, M. P. and L. A. Wilson. 1970. Stimulatory and inhibitory effects of scopoletin on IAA oxidase preparations from sweet potato. Phytochemistry 9:1787-1794.

16. Raa, J. 1973. Cytochemical localization of peroxidase in plant cells. Physiol. Plant. 28:132-133.

17. Gordon, A. R. and N. A. Alldridge. 1971. Cytochemical localization of peroxidase A in developing stem tissues of extreme dwarf tomato. Can. J. Bot. 49:1487-1496.

18. Sheen, S. and G. R. Rebagay. 1970. Localization and tissue difference of peroxidases in Nicotiana tobacum and its progenitor species. Bot. Gaz. 131:297-304.

19. Einhellig, F. A., E. L. Rice, P. G. Risser and S. H. Wender. 1970. Effects of scopoletin on growth, CO_2 exchange rates and concentration of scopoletin, scopolin and chlorogenic acids in tobacco, sunflower and pigweed. Bull. Torrey Bot. Club 97:22-23.

20. Innerarity, L. T. 1970. A study of scopolin and scopoletin metabolism. Ph.D. dissertation. University of Oklahoma.

21. Linsmaier, E. M. and F. Skoog. 1965. Organic growth factor requirements of tobacco tissue cultures. Physiol. Plant. 18:100-127.

22. Orstein, O. and B. J. Davis. 1962. Disc electrophoresis, part II. Distillation Products Industries. Rochester, N. Y.

23. Brewer, G., Jr. 1970. An introduction to isozyme techniques. Academic Press, New York, N. Y.

24. Lance, C. 1955. Sur la détermination de l' activité peroxydase des extraits bruts de tissus végétaux. Rev. Gen. Bot. 62: 609-621.

25. Stafford, H. A. and A. W. Galston. 1970. Ontogeny and hormonal control of polyphenoloxidase isozymes in tobacco pith. Plant Physiol. 46:763-767.

26. Weber, K. and M. Osborn. 1969. The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. J. Biol. Chem. 244:4406-4412.

27. Theorell, H. 1940. Reversible spaltung einer peroxydase. Ark. Kemi. Min. Geol. 14B:1-3.

28. Wender, S. H. 1970. Effects of some environmental stress factors on certain phenolic compounds in tobacco. Recent Advances in Phytochemistry 3:1-35.

29. Winkler, B. C. 1967. Quantitative analysis of coumarins by thin layer chromatography, related chromatographic studies, and the partial identification of a scopoletin glycoside present in tobacco tissue culture. Ph.D. dissertation. University of Oklahoma.

30. Minamikawa, R., T. Akazawa and I. Uritani. 1963. Analytical study of umbelliferone and scopoletin synthesis in sweet potato roots infected with Ceratocystis fimbriata. Plant Physiol. 38:493-497.

31. Dieterman, L. J., L. Rohrbaugh, V. Thiesfeld and S. H. Wender. 1964. Identification and quantitative determination of scopolin and scopoletin in tobacco plants treated with 2,4-dichlorophenoxyacetic acid. Anal. Biochem. 30:139-145.

32. Winkler, B. C., W. J. Dunlap, J. W. Mizelle, L. M. Rohrbaugh and S. H. Wender. 1969. Quantitative analysis of scopolin and scopoletin in tobacco plants treated with maleic hydrazide. Tob. Sci. 13:19-20.

33. Kajinami, S., S. H. Wender and E.C. Smith. 1971. Glucose-6-phosphate dehydrogenase from tobacco tissue culture WR-132. I. Inhibition by scopoletin and scopolin. Phytochemistry 10:1501-1503.

34. Tryon, S. 1956. Scopoletin in differentiating and non-differentiating cultured tobacco tissue. Science 123:590.

35. Lineweaver, H. and D. Burk. 1934. The determination of enzyme dissociation constants. JACS 56:658-665.

36. Hill, A. V. 1913. The combinations of haemoglobin with oxygen and with carbon monoxide I. Biochem. J. 7:471-480.

37. Koshland, D. E., Jr. 1970. The molecular basis for enzyme regulation. The Enzymes Vol. I. 3rd Edition. Academic Press. New York, N. Y.

38. Galston, A. W., S. Lavee and B. Z. Siegel. 1968. The induction and repression of peroxidase isozymes by 3-indoleacetic acid. Biochemistry and Physiology of Plant Growth Substances.

39. Rubinstein, B. 1973. Auxin-induced ion uptake into coleoptile segments. Plant Physiol. Supplement 51:3.

40. Ray, P. M. 1956. The destruction of indoleacetic acid. II. Spectrophotometric study of the enzymatic reaction. Arch. Biochem. Biophys. 64:193-216.

41. Gordon, S. A. and R. P. Weber. 1951. Colorimetric estimation of indoleacetic acid. Plant Physiol. 26:192-195.

42. Kefei, V. I., G. S. Muromtsev, V. N. Agnistikova, S. A. Saidova and T. I. Drakina. 1969. Biosynthesis and physiological activity of indoleacetic acid and gibberellin in the presence of phenolic growth inhibitors and morphactin. Dokl. Akad. Nauk. SSSR 188:1182-1185.

43. Sondheimer, E. 1964. Chlorogenic acid and related depsides. The Botanical Review. 30:667-711.

44. Powell, B. 1973. Personal communication.

45. Pickering, J. 1973. Personal communication.

46. Kolb, D. and G. Weber. 1972. Cooperative binding due to negative interactions of two ligands in single chain proteins. Fed. Proc. 31:423.

47. Whitaker, J. R. 1972. Principles of Enzymology of the Food Sciences. Marcel Dekker, Inc. New York, N. Y. p. 599.

48. Bonner, J. 1950. Plant Biochemistry. Academic Press, New York, N. Y. p. 127.