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PICKERING, Jerry Wayne, 1945-
PART I. CHARACTERIZATION OF TWO ISOPEROXIDASES FROM TOBACCO TISSUE CULTURE WR-132. PART II. EFFECT OF SOME PHENOLIC COMPOUNDS ON ENZYME ACTIVITIES IN RAT LUNG.

The University of Oklahoma, Ph.D., 1974
Chemistry, biological

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THE UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

PART I. CHARACTERIZATION OF TWO ISOPEROXIDASES FROM TOBACCO TISSUE CULTURE WR-132

PART II. EFFECT OF SOME PHENOLIC COMPOUNDS ON ENZYME ACTIVITIES IN RAT LUNG

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the

degree of

DOCTOR OF PHILOSOPHY

BY

JERRY WAYNE PICKERING

Norman, Oklahoma

1974

PART I. CHARACTERIZATION OF TWO ISOPEROXIDASES FROM TOBACCO TISSUE CULTURE WR-132

PART II. EFFECT OF SOME PHENOLIC COMPOUNDS ON ENZYME ACTIVITIES IN RAT LUNG

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ACKNOWLEDGEMENTS

I would like to express my appreciation to Professor Eddie C. Smith for his assistance to me throughout my graduate career and also to Professor Simon H. Wender for his counsel and guidance during the course of this study. It was through their knowledge, patience and encouragement that this study was completed.

I wish to thank Dr. Ralph A. Jacobson, Dr. Leonard Beevers and Dr. Stanley C. Neely for their helpful suggestions in the preparation of this manuscript.

I owe a debt of gratitude to Dr. Bernard L. Powell who collaborated with me on some of these studies and also to John D. Hoover for his assistance and advice. I express a special thanks to Susan Walker for typing this manuscript and for maintenance of tissue cultures.

I wish to acknowledge my mother for her many sacrifices so that I might reach this goal. I also thank Judy for her encouragement, faith and assistance throughout my graduate work.

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ABSTRACT

Using fractional ammonium sulfate precipitation and carboxymethyl cellulose chromatography, two cathodic migrating isoenzymes of peroxidase, which were designated isoperoxidases C_3 and C_4, were isolated from tobacco suspension culture WR-132. Various kinetic and physical properties were determined for each of the two isoperoxidases.

Molecular weights, determined by SDS-electrophoresis and gel filtration in guanidinium chloride, were 44,000-46,000 for isoperoxidase C_4 and 67,000-68,000 for isoperoxidase C_3. Of the two isoperoxidases, only C_4 stained for carbohydrate on SDS-gels.

Using guaiacol as substrate, Michaelis constants were 7.5 and 4 mM for C_3 and C_4, respectively, and the pH optima were 6.0 for both enzymes. A number of naturally occurring compounds were shown to affect, in an almost identical fashion, the ability of both isoperoxidases to oxidize guaiacol. p-Hydroxycinnamic acids, notably ferulic acid, stimulated, while o-diphenols inhibited, the guaiacol activity of both enzymes. Isoperoxidase C_4 was inhibited by sulfhydryl compounds and, therefore, may contain a cysteine residue at the active site. C_4 was stable at 60°C for 30 min, and freezing and thawing of a dilute solution of C_4 resulted in only a 23% reduction in activity (guaiacol).

Ferulic acid was shown to be converted, by both isoperoxidases C_3 and C_4 in the presence of hydrogen peroxide, to an intermediate, light

sensitive product. This initial product readily formed other unknown oxidation products. Using thin-layer chromatography and UV difference spectra as criteria, three other naturally occurring phenolic compounds, namely scopoletin, esculetin and chlorogenic acid, were also found to be substrates for both C_3 and C_4. Assay procedures were developed to monitor the peroxidase catalyzed oxidations of ferulic acid, esculetin and chlorogenic acid. Using direct assay procedures for each substrate, the pH optima for both isoperoxidases C_3 and C_4 were determined to be 4.5, 5.5, 5.5 and 7.5 mM for scopoletin, ferulic acid, chlorogenic acid and esculetin, respectively, as substrates. K_m's for ferulic acid, esculetin and scopoletin as substrates for C_4 were calculated to be 0.3, 0.45 and 0.53 mM, respectively.

Scopoletin and esculetin saturation curves for isoperoxidase C_4 were observed to have a sigmoidal dependence upon substrate concentrations, whereas the oxidations of ferulic acid and guaiacol by C_4 followed simple Michaelis-Menten kinetics. Evidence was presented that the sigmoidal dependence of velocity upon scopoletin concentration was apparently not the result of multiple substrate binding sites on the enzyme. The data were discussed in relation to a previously preposed mechanism for oxidation of scopoletin by horseradish peroxidase.

Activities for isoperoxidases C_3 and C_4 with respect to each of the above natural substrates were compared to that for guaiacol. The resulting activity ratios were nearly uniform for the two isoperoxidases. Therefore, it was concluded that isoperoxidases C_3 and C_4 adhered to the strict definition of isoenzymes in that, although they were different in molecular structure, they had virtually identical catalytic ability with respect to substrates utilized in this study. Both isoperoxidases C_3 and C_4

appeared to prefer ferulic acid as substrate of those tested. A possible involvement of ferulic acid and isoperoxidases C_3 and C_4 in lignification was discussed.

In another study, several phenolic compounds, namely scopoletin, esculetin, scopolin, esculin and chlorogenic acid, known to be present in either cigarette smoke or tobacco, substantially inhibited the activity of rat lung glutathione (GSH) peroxidase, GSH reductase and glucose-6-phoaphate dehydrogenase, although the results with GSH peroxidase were inconclusive. The data were discussed in relation to a previously proposed protective mechanism against lipid peroxidation in which these enzymes are involved and also in relation to previous data concerning the effects of cigarette smoke on other enzymes.

I. CHARACTERIZATION OF TWO ISOPEROXIDASES FROM TOBACCO TISSUE CULTURE WR-132

CHAPTER I

INTRODUCTION

Peroxidase was first isolated from horseradish roots in 1918 by Willstätter and Stoll (87). Since then, peroxidases have been detected in many different plants and have been studied extensively (72). Due largely to their broad substrate specificity, the exact physiological function of peroxidase in higher plants remains to be defined. However, peroxidase has been implicated in several different physiological processes. Among these are peroxidases involved in the biosynthesis of ethylene (51,53); in lignification (19,25,77); in the oxidation of reduced pyridine nucleotides (1); and in the conversion of oxaloacetate to malonate (61). However, since Van Offenbeck first implicated peroxidase in the enzymatic destruction of indole-3-acetic acid (IAA) in 1935 (77), the majority of research on peroxidase has focused on a characterization of their role as IAA oxidases (24). The *in vivo* catalytic function of peroxidase is further confused by the existence of multiple forms of the enzyme in every plant examined (72). The peroxidase isoenzymes of tobacco tissue culture have been established (41,79), and as many as twelve different isoenzymes, which are called isoperoxidases in this dissertation, are present (68).

The ability of peroxidase to oxidize phenolic compounds, utilizing either peroxide or oxygen as electron acceptor, is well documented. Peroxidases are thus known to be polyphenol oxidases (5), although not all peroxidases function in this capacity (79). In the presence of hydrogen peroxide, peroxidase converts monomeric and dimeric model compounds *in vitro* to lignin-like structures (19). Numerous studies have involved the effect of various naturally occurring phenolic compounds on different IAA oxidase preparations. Andreae (3) first noticed inhibition of IAA oxidase in oat roots by scopoletin, which was destroyed in the process. According to Imbert and Wilson, scopoletin (31), as well as chlorogenic and caffeic acids (32), inhibits IAA oxidase when present at high concentrations but stimulates the IAA oxidase activity at low concentrations. Ferulic acid produced similar results with IAA oxidase from pineapple (22). Therefore, phenolic compounds may be involved in regulation of growth by virtue of their ability to affect IAA oxidase activity (18,76,89). Conversely, IAA has been suggested to inhibit lignification (78).

Variations in the number or intensity of isoperoxidases, as visualized by electrophoresis, occur in tobacco tissue cultures in response to changes in environmental conditions. IAA at high concentrations induces the formation of new isoenzymes of IAA oxidase (42). Subsequently, Lee (43) has observed different IAA oxidase to peroxidase activity ratios for isoperoxidases in different subcellular fractions, and that kinetin drastically increased these ratios. New isoperoxidases were also elaborated by cultured tobacco cells in response to variations in the amount of exposure to light (73). Variation in the Ca^{++} concentration of the culture medium, which is directly related to the amount of lignification, causes

variations in the intensity of different isoperoxidases (70). It is apparent from these studies that different isoenzymes of peroxidase may have different functions *in vivo* due either to differences in their cellular location or their specificities toward different physiological substrates.

In an effort to understand more clearly the role of peroxidases within the plant and the physiological reason for the existence of multiple forms of peroxidase, isoperoxidases from a number of different plants have been isolated and studied individually (28,29,54,56,57,66). Isoperoxidases from several sources, notably horseradish (9,34,37,45, 52,74), have been purified and such physical properties as molecular weight and carbohydrate and amino acid compositions have been determined. Different isoperoxidases from a single source in some instances exhibit quite different physical and kinetic properties while others appear to be remarkably similar, either in catalytic ability or physical properties or both. A summary of some kinetic properties for different isoperoxidases is shown in Table I-1.

In the present study, two isoperoxidases have been isolated from tobacco suspension culture, WR-132. A comparison of some physical and kinetic properties of the two isoperoxidases was undertaken. Kinetic behavior of the two enzymes was ascertained not only in relation to guaiacol but to several naturally occurring phenolic compounds, namely ferulic acid, scopoletin, esculetin, and chlorogenic acid, which were shown to be substrates for the two isoperoxidases. Much of the data presented here was the subject of two recent communications (62,63).

TABLE I-1

SUMMARY OF SOME KINETIC PROPERTIES OF ISOPEROXIDASES FROM DIFFERENT PLANTS

Source	Isoperoxidase Designation	Substrate Utilized	pH Optima	Specific Activity	k_{1/k_4} Ratios+	Activity Ratios (o-dianisidine/ guaiacol)	Ref.
Egg plant leaf	A	guaiacol	7.0	*	40.0	2.32	36
	B_1		6.0		5.4	7.57	
	B_2		6.0		7.45	0.08	
Alaska pea leaf	C_1	guaiacol	7.7	76	23,18.2	8.60	49
	C_2		7.7	17.3	25	-	
	C_3		7.7	-	13.5,11.9	6.10	
	N		7.7	0.33	8.4	-	
Tomato	A	guaiacol	7.5	500	*	*	15
	B		5.5	1300			
Horseradish	A-1	o-dianisidine	5.8	2.2	*	*	37
	A-2		5.6	2.3			
	A-3		5.6	0.9			
	B		5.0	0.1			
	C		5.0	0.1			
	D		5.0	0.2			
	E		5.0	0.3			

* not reported

+ k_{1/k_4} are rate constants

CHAPTER II

MATERIALS AND METHODS

Tissue Cultures

Tobacco tissue culture WR-132 (Nicotiana tabacum L., var. Xanthi), a line of suspension culture, was the source of isoenzymes of peroxidase used for the following studies. The cell line was originally obtained from Dr. A. C. Olson, U. S. Department of Agriculture, Albany, California. Approximately 2 g of cells were aseptically transferred, with the use of a laminar flow hood (Agnew-Higgins), into 50 ml of culture medium in a 125 ml flask. Cells were grown with constant agitation on a rotating shaker (180 rpm), and were harvested after an optimum growth period of 10-13 days in subdued light at room temperature.

The culture medium was that of Murashige and Skoog (58), except 2,4-dichlorophenoxyacetic acid was used instead of indole-3-acetic acid, and kinetic was deleted.

Electrophoresis

Starch gel electrophoresis was accomplished using the apparatus and procedure described by Brewer (6). Gels were prepared at a starch (Electrostarch Co.) concentration of 10% in 5 mM histidine (pH 7.0). Electrophoresis was continued for 4½ hours at a constant voltage of 400 volts. The electrode buffer was 0.41 M sodium citrate (pH 7.0).

Revised WR-132 Culture Medium

Compound	mg/l	Compound	mg/l
NH_4NO_3	1650	$Na_2MoO_4 \cdot 2H_2O$	0.25
KNO_3	1900	$CuSO_4 \cdot 5H_2O$	0.025
$CaCl_2 \cdot 2H_2O$	440	$CoCl_2 \cdot 6H_2O$	0.025
$MgSO_4 \cdot 7H_2O$	370	Sucrose	3000
KH_2PO_4	170	myo-Inositol	100
Na_2EDTA	37.3	Glycine	20
$FeSO_4 \cdot 7H_2O$	27.8	Thiamine·HCl	1
H_3BO_3	6.2	Niacin	5
$MnSO_4 \cdot 4H_2O$	22.3	Pyridoxine·HCl	5
$ZnSO_4 \cdot 4H_2O$	8.6	2,4-Dichlorophenoxy-acetic acid	0.5
KI	0.83		

Peroxidase bands were visualized by placing the gels in a solution of 0.1 g 3-amino-9-ethyl carbazole in 10 ml of dimethylformamide, 185 ml of 50 mM acetate buffer (pH 5.0), 10 ml $CaCl_2$ and 0.2 ml of 30% H_2O_2 (23).

Peroxidase Assays

Guaiacol

Peroxidase assays with respect to guaiacol as substrate were accomplished using a procedure based upon that of Lance (38) which was modified by Schafer (70). Assays contained guaiacol, 5 mM H_2O_2 and 40 mM phosphate buffer (pH 6.0). The reaction was initiated by the addition of 0.1 ml of enzyme and the velocity of the reaction was monitored by measuring the increase in absorbance at 470 nm. Guaiacol concentration was 6 mM

for inhibitor studies and the preincubation experiment and 15 mM for the substrate comparison study and pH profiles.

Scopoletin

Assays with scopoletin as substrate were monitored by an increase in absorbance of oxidation product(s) at 450 nm. An assay solution contained 1.25 mM scopoletin, 50 mM citrate buffer (pH 4.5) and 5 mM H_2O_2. The reaction was started by the addition of 0.1 ml of a peroxidase preparation.

Ferulic Acid

A typical assay contained 0.2 mM ferulic acid, 5 mM H_2O_2, and 50 mM phosphate buffer (pH 6.0). The reaction was initiated by the addition of 0.1 ml of the enzyme preparation, and the reaction rate was monitored by the disappearance of ferulic acid absorbance at 320 nm (see Figure 10).

Esculetin

Esculetin assays were determined by monitoring the absorbance of product(s) at 469 nm (see Figure 15). Assay solutions contained 50 mM Tris-HCl buffer (pH 7.5), 1.0 mM esculetin and 5 mM H_2O_2. The reaction was initiated by the addition of 0.1 ml of enzyme solution.

Chlorogenic Acid

The peroxidase catalyzed oxidation of chlorogenic acid was monitored by measuring the absorbance of product(s) of the reaction at 400 nm, since products absorb more appreciably at that wavelength. An assay solution contained 1.0 mM chlorogenic acid, 5 mM H_2O_2 and 50 mM citrate buffer (pH 5.5). The reaction was initiated by the addition of 0.1 ml of enzyme preparation.

Enzyme assays were accomplished using a Hitachi-Perkin Elmer Model 139 spectrophotometer or a Varian Techtron Model 635 UV-visible recording spectrophotometer.

Thin Layer Chromatography

Avicel SF (FMC Corporation) was suspended in distilled water, (22 g/100 ml) homogenized for 45 seconds in a Waring Blender and layered at a thickness of 0.375 nm onto glass plates. Ferulic acid reaction products were developed with ascending chromatography using benzene: methanol (3:1) as solvent. Reaction products and ferulic acid were visualized under ammonia with a UV lamp (366 nm, Black Ray, B-100).

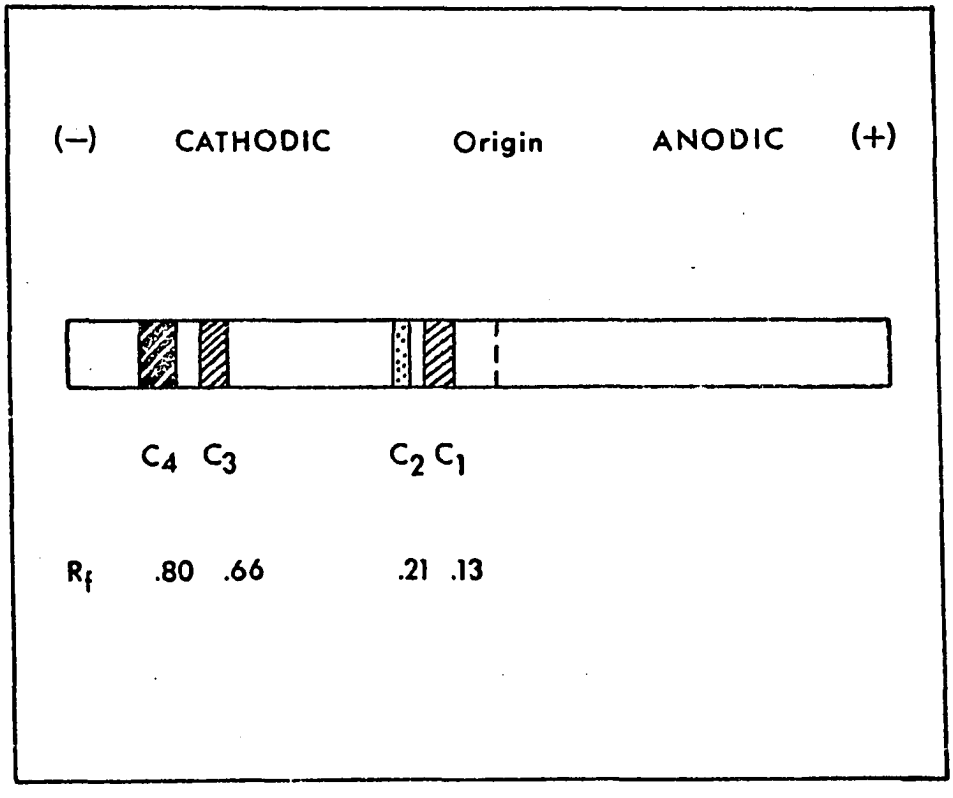
Other Methods and Chemicals

Protein concentrations were determined by the method of Lowry and coworkers (48).

Phenolic compounds were obtained from Sigma Chemical Co. or Fluka AG or had been previously synthesized in this laboratory. Ferulic acid and scopoletin were recrystallized in 95% ethanol before use.

Nuclear magnetic resonance (nmr) spectra of ferulic acid and its oxidation products were taken on a Varian A-60 spectrophotometer. Samples were run in deuteroacetone using tetramethylsilane (TMS) as internal standard. Chemical shifts were reported in δ values (PPM from TMS).

Figure 1. STARCH GEL ELECTROPHORETIC PATTERN AT pH 7.0
OF CATHODIC ISOPEROXIDASES FROM TOBACCO SUSPENSION CULTURE WR-132


(−)
CATHODIC
Origin
ANODIC
(+)
C4 C3
C2 C1
Rf
.80 .66
.21 .13

CHAPTER III

ISOLATION AND PHYSICAL PROPERTIES

When subjected to starch gel electrophoresis at pH 7.0, crude WR-132 cell homogenates revealed the pattern of isoperoxidases shown in Figure 1. No anodic migrating isoperoxidases were evident in the cell extracts, although anodic bands are observed in the cell medium (73). The pattern of cathodic isoperoxidases was similar to that reported by Stafford and Galston (79), and their nomenclature was adopted. In some cell extracts an additional minor band was seen between C_3 and C_4, and another which migrated more rapidly than C_4 sometimes appeared. However, C_4 was the most active band with respect to guaiacol or 3-amino-9-ethyl carbazole. The isoperoxidases designated C_3 and C_4 have been isolated, and studies on these two isoperoxidases are reported here.

Preparation of Enzymes

WR-132 culture cells were harvested using a Buchner funnel and washed thoroughly with 50 mM phosphate buffer (pH 6.0). One hundred grams of cells were homogenized in a Sorvall Omnimixer at 6000 rpm for 10 min with 100 ml of 0.9 M KCl in 50 mM phosphate buffer (pH 6.0), 10 g glass beads, 0.1 g sodium dodecyl sulfate (SDS) and 0.25 ml of β-mercaptoethanol (BME). The homogenate was centrifuged for 10 min at 30,000 x g, and the resulting supernatant was subjected to ammonium sulfate precipitation. The protein which precipitated between 40 and 90% saturation with respect

to ammonium sulfate was collected by centrifugation (15,000 x g for 10 min) and redissolved in a minimum volume (5 ml per 100 g cells) of 5 mM phosphate buffer (pH 6.0). The concentrated protein solution was dialyzed against approximately 500 volumes of 5 mM phosphate buffer (pH 6.0), and then centrifuged (30,000 x g for 10 min) to remove any insoluble material. Ten ml of the resulting clear golden supernatant containing 20-25 mg protein was applied to a carboxymethyl cellulose column (60 x 2.5 cm, Ace Glass Co.), which had previously been equilibrated in 5 mM phosphate buffer (pH 6.0). Approximately 2 liters of 5 mM buffer were passed through the column to elute any proteins which were anodic at pH 6.0. The column was then washed with an additional 2 liters of 10 mM phosphate buffer. Isoperoxidase C_4 was eluted from the column with 20 mM buffer. Isoperoxidase C_3 was obtained by subsequent elution with 50 mM phosphate buffer (pH 6.0).

Fractions were spot checked for peroxidase using a solution of 2 parts 1% guaiacol, 2 parts 50 mM phosphate buffer (pH 6.0) and 1 part 0.5% H_2O_2. Peroxidase active fractions were subjected to starch-gel electrophoresis, and fractions containing only C_3 and C_4 were pooled. The pooled fractions were concentrated by lyophilization. The dry samples were redissolved and adjusted to the proper dilution for enzyme assays. Isoperoxidases C_3 and C_4 were prepared for molecular weight studies by dialysis of the concentrated samples against distilled water, followed by lyophilization, to yield dry protein samples free of buffer salts.

Molecular Weight Determinations

Molecular weights for isoperoxidases C_3 and C_4 were determined by gel filtration in guanidinium chloride according to the method of Mann and Fish (50). A 90 x 1.5 cm column (Pharmacia) equipped with a Mariotte

flask was packed with 4% agarose (Sepharose 4B, Pharmacia) which had previously been equilibrated in a solution of 6 M guanidinium chloride in 50 mM acetate buffer (pH 4.75). Protein samples were allowed to stand overnight in 6 M guanidinium chloride containing 0.1 M BME (pH 8.6) and were then acetylated by the addition of solid iodoacetate. Samples applied to the column contained approximately 2 mg of each protein, 0.2% blue dextran, 0.1% DNP-alanine and 20% sucrose in a volume which did not exceed 0.5 ml.

An optimum flow rate at 15 cm head pressure was used, and fractions of 0.8-1.0 g were collected. Elution positions were determined by monitoring blue dextran at 630 nm, DNP-alanine at 360 nm and protein at 280 nm. The distribution coefficient, K_d, for each protein was calculated according to the equation:

$$K_d = \frac{Ve - V_{BD}}{V_{DNP} - V_{BD}}$$

where: Ve is the elution weight of the protein sample

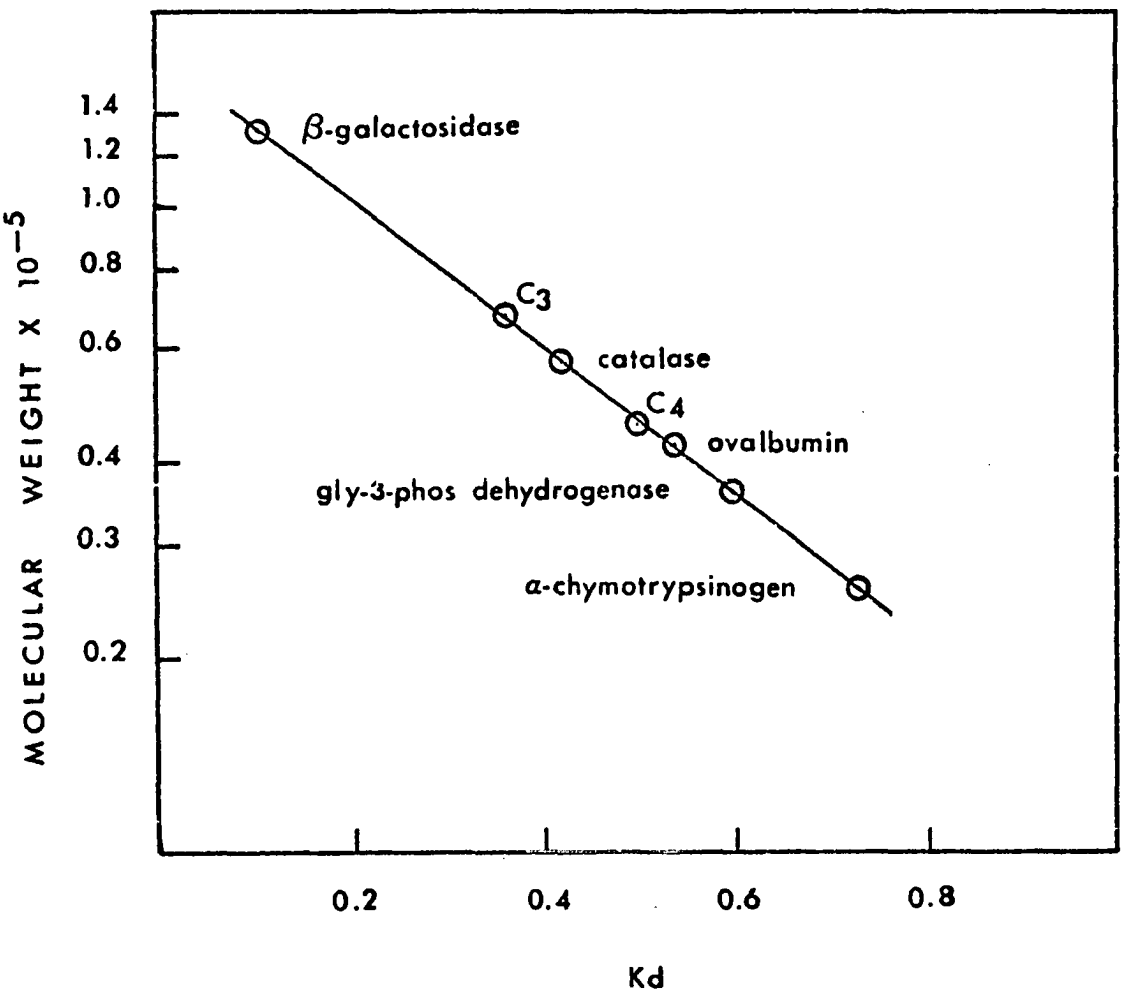
V_{BD} is the elution weight of blue dextran

V_{DNP} is the elution weight of DNP-alanine

Molecular weights for C_3 and C_4 were obtained from a standard plot of K_d against log molecular weight (Figure 2).

Molecular weights for isoperoxidases C_3 and C_4 were also determined using the SDS gel electrophoresis procedure of Weber, Pringle and Osborn (85). Gels were prepared 7 cm in length and at an acrylamide concentration of 7.5%. Individual protein standards and samples of C_3 and C_4 were dissolved in 10 mM sodium phosphate (pH 7.0), and one part of this solution was mixed with nine parts of 10 mM sodium phosphate (pH 7.0) containing 1%

Figure 2. DETERMINATION OF MOLECULAR WEIGHTS OF ISOPEROXIDASES C_3 AND C_4 BY GEL FILTRATION IN GUANIDINIUM-HYDROCHLORIDE.


MOLECULAR WEIGHT X 10^{-5}
1.4
1.2
1.0
0.8
0.6
0.4
0.3
0.2
β-galactosidase
C_3
catalase
C_4
ovalbumin
gly-3-phos dehydrogenase
α-chymotrypsinogen
0.2
0.4
0.6
0.8
Kd

SDS and 1% BME. The samples were then incubated in a 100°C water bath for 2 minutes and cooled to room temperature. Sucrose was added to facilitate layering and 0.045 ml each of 0.05% bromophenol blue and BME were added. Approximately 0.05 mg of protein was applied to each gel.

After electrophoresis for 4½ hours at 8 ma per tube, gels were removed and placed in a staining solution of 0.25% Coomassie brilliant blue in 45% (v/v) methanol in 9% (v/v) acetic acid for 4-5 hours, according to the procedure of Weber and coworkers (85). Gels were de-stained in 7.5% acetic acid in 5% methanol over a period of several days. Mobilities were calculated for each protein according to the equation

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

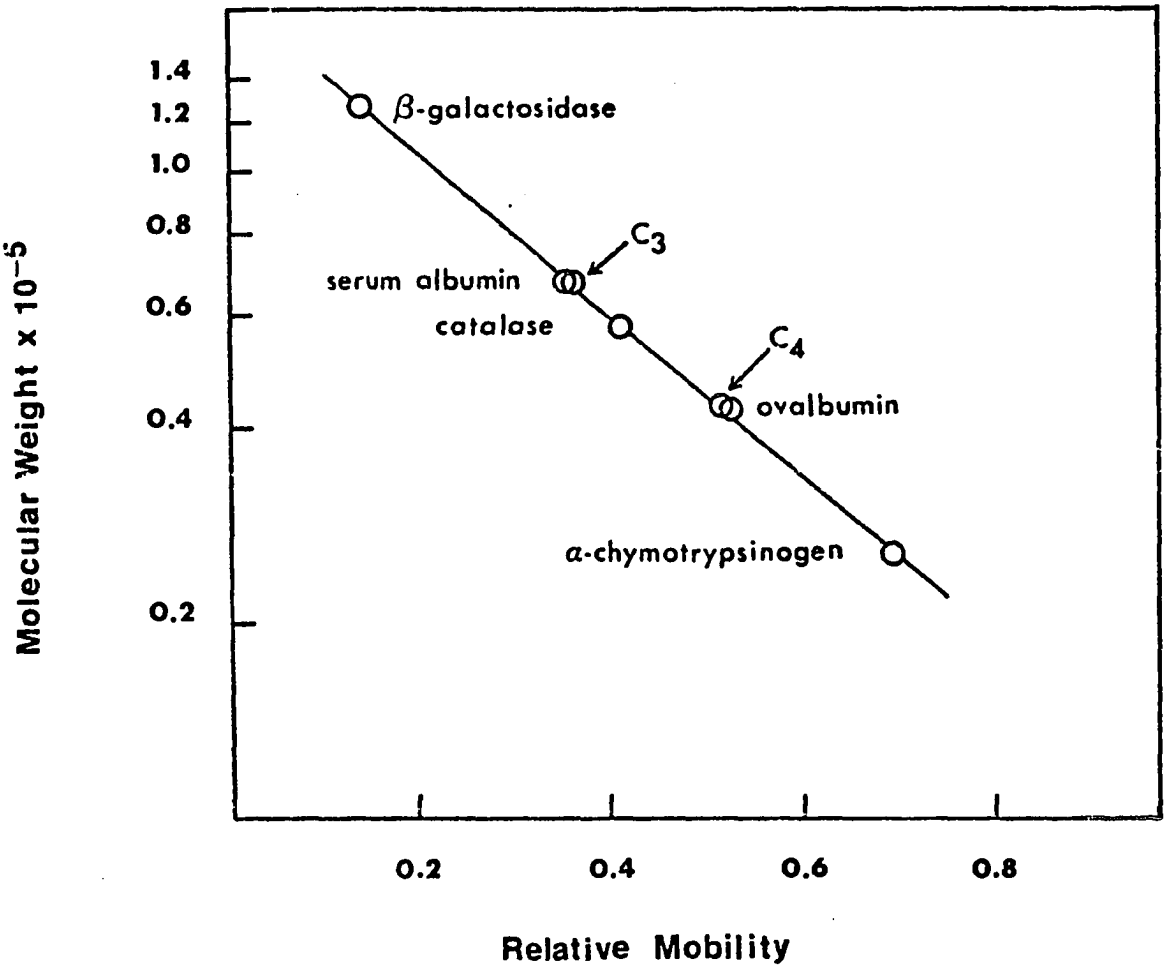
Molecular weights for C_3 and C_4 were determined from a plot of log molecular weight versus mobilities for protein standards as represented in Figure 3.

When subjected to SDS polyacrylamide gel electrophoresis, crude WR-132 cell homogenates revealed up to ten protein bands. Preparations of C_3 and C_4 after carboxymethyl cellulose chromatography in some cases contained minor traces of the other isoperoxidase, as well as other minor contaminating protein bands. These were not visible on starch gels stained with 3-amino-9-ethyl carbazole. However, one preparation of C_3 was apparently homogeneous, since no other protein bands were visible. A typical preparation of isoperoxidase C_4 was contaminated with traces (faintly visible bands) of C_3 and a glycoprotein having a molecular weight of 28,000.

Glycoprotein Determination

Following electrophoresis, SDS-gels were selectively stained for glycoproteins using the method of Glossman and Neville (20). Gels were

Figure 3. DETERMINATION OF MOLECULAR WEIGHTS OF ISOPEROXIDASES C_3 AND C_4 BY DODECYL SULFATE-POLYACRYLAMIDE GEL ELECTROPHORESIS.


Molecular Weight x 10^{-5}
1.4
1.2
1.0
0.8
0.6
0.4
0.2
β-galactosidase
C_3
serum albumin
catalase
C_4
ovalbumin
α-chymotrypsinogen
0.2
0.4
0.6
0.8
Relative Mobility

washed for 48 hours with 40% (v/v) methanol in 7% (v/v) acetic acid and placed in 1% periodic acid in 7% acetic acid for 1 hour at 4°C in the dark. The gels were then placed in Schiff's reagent for approximately 18 hours, also at 4°C in the dark. After Schiff's reagent, the gels were washed several times with 1% sodium bisulfite in 0.1 N HCl and stored in this solution. Glycoprotein bands increased in intensity after several hours.

To reduce the possibility of artifactual staining of non-glycoprotein bands, a control was run in which the periodic acid oxidation step above was deleted (20). Ovalbumin, which is a well characterized glycoprotein, was also run as a control. Under these conditions, isoperoxidase C_4 revealed an intensely stained band, while C_3 apparently was not a glycoprotein.

Results and Discussion

Molecular weights for isoperoxidases C_3 and C_4, determined by the two methods described above, are summarized in Table I-2. The significant difference in molecular weight between C_3 and C_4 is inconsistent with the conclusion of Rücker and Radola (68) that all isoperoxidases from tobacco tissue cultures have similar molecular weights. However, the molecular weights for C_3 and C_4 agree well with molecular weights of isoperoxidases from other sources. For example, six isoperoxidases from Japanese radish (57) possess molecular weights which range from 30,000-54,000. Three isoperoxidases from turnip have molecular weights of 43,000-49,000, while a fourth is approximately equal in molecular weight (65,000) to isoperoxidase C_4 (28). In view of the fact that all seven isoperoxidases of horseradish are glycoproteins (45,86), it is interesting that only one

of the isoperoxidases in this study, namely C_4, which has the smaller molecular weight, is evidently a glycoprotein.

TABLE I-2

SUMMARY OF PHYSICAL PROPERTIES
OF ISOPEROXIDASES C_3 AND C_4.

Isoperoxidase	Molecular Weight SDS-electrophoresis	Molecular Weight Gel Filtration	Presence of Carbohydrate
C_4	44,000	46,000	yes
C_3	68,000	67,000	no

CHAPTER IV

KINETIC STUDIES

Introduction

Isoperoxidases from several different plants have been characterized with respect to guaiacol as substrate. As shown in Table I-1, two major cathodic migrating isoperoxidases from Alaska pea (36) and three isoperoxidases from egg plant leaves (49) displayed different ratios of rate constants and pH optima with guaiacol as substrate. Two major isoperoxidases from tomato plants exhibited different pH optima for oxidation of guaiacol, and different concentrations of guaiacol and H_2O_2 were required for maximum activity (15). However, four isoperoxidases of horseradish have identical pH optima and specific activities with o-dianisidine.

Results and Discussion

Isoperoxidases C_3 and C_4 appear to have similar catalytic ability with respect to guaiacol. The pH optimum for the oxidation of guaiacol by both isoperoxidases was found to be 6.0 (Figure 4). Both isoperoxidases appear to follow simple Michaelis-Menten kinetics with respect to guaiacol as substrate (Figure 5 and 6), and K_m's for isoperoxidases C_3 and C_4 were found to be 7.5 mM and 4 mM respectively.

Figure 4. EFFECT OF pH ON THE OXIDATION OF GUAIACOL BY ISOPEROXIDASE C_4. Assay solutions contained 12 mM guaiacol, 40 mM buffer and 5 mM H_2O_2. Δ-citrate, O-phosphate.

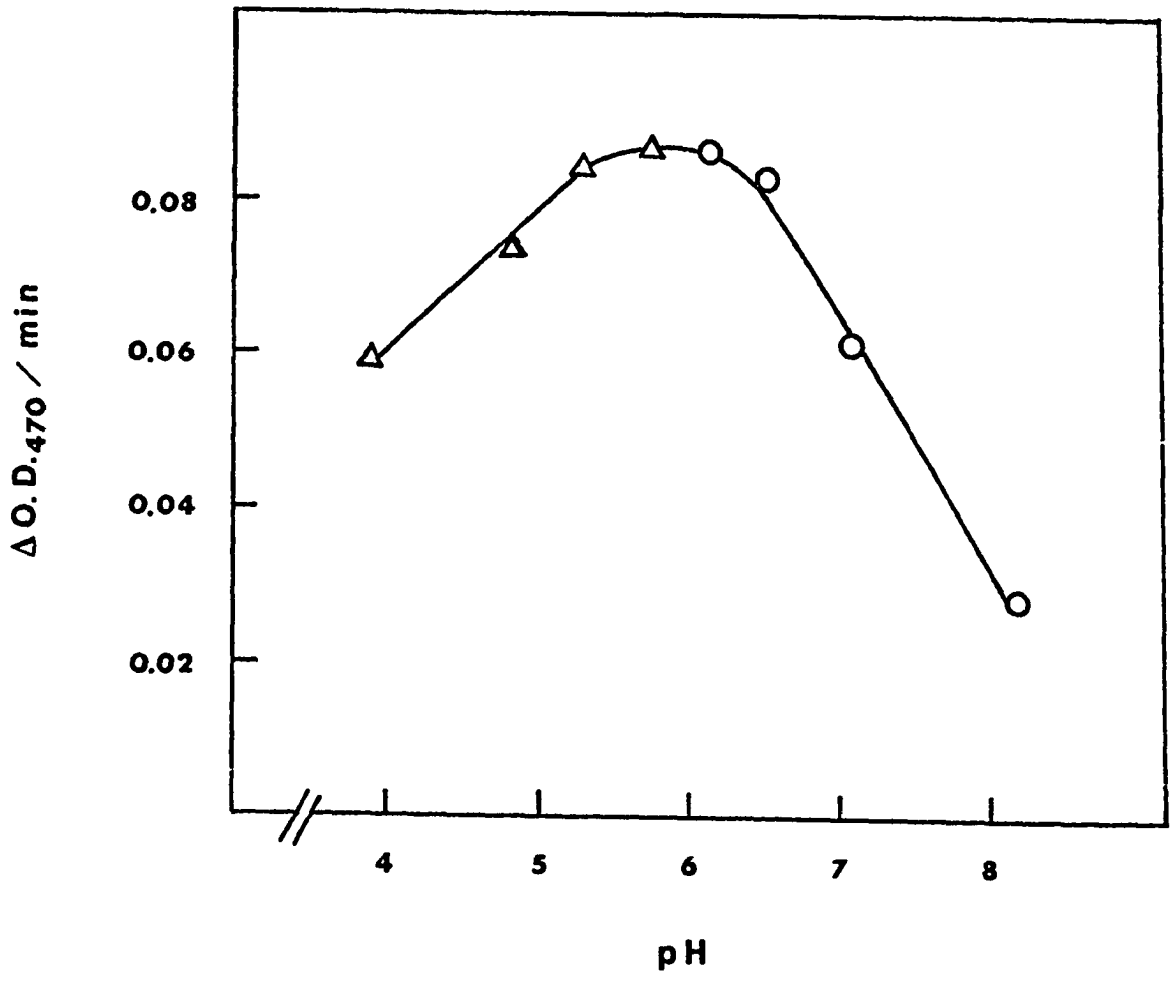

0.08
0.06
0.04
0.02
ΔO.D.470 / min
4
5
6
7
8
pH

Figure 5. GUAIACOL SATURATION CURVE FOR ISOPEROXIDASE C_3. Assay solutions contained 5 mM phosphate buffer (pH 6.0), and 5 mM H_2O_2.

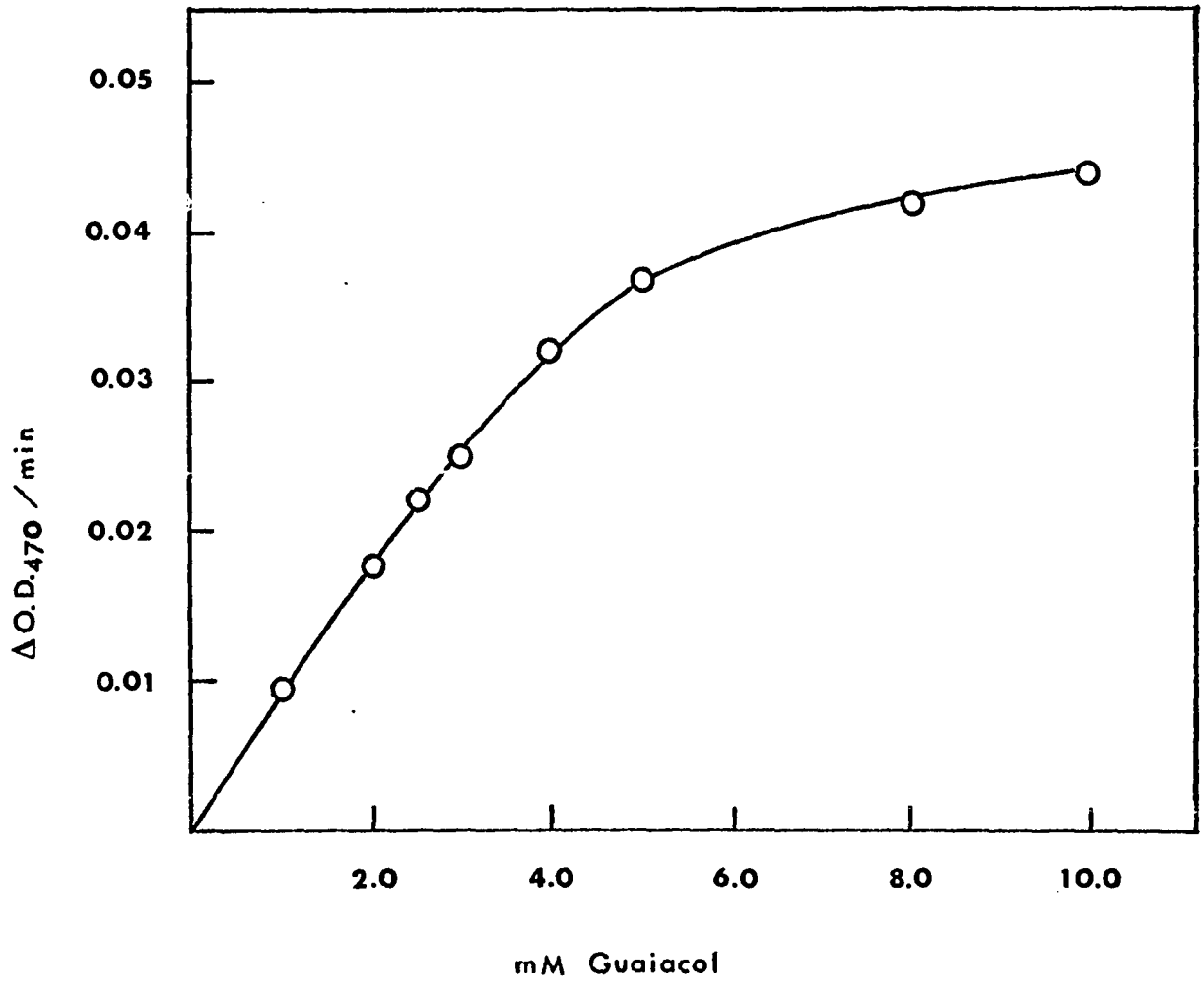
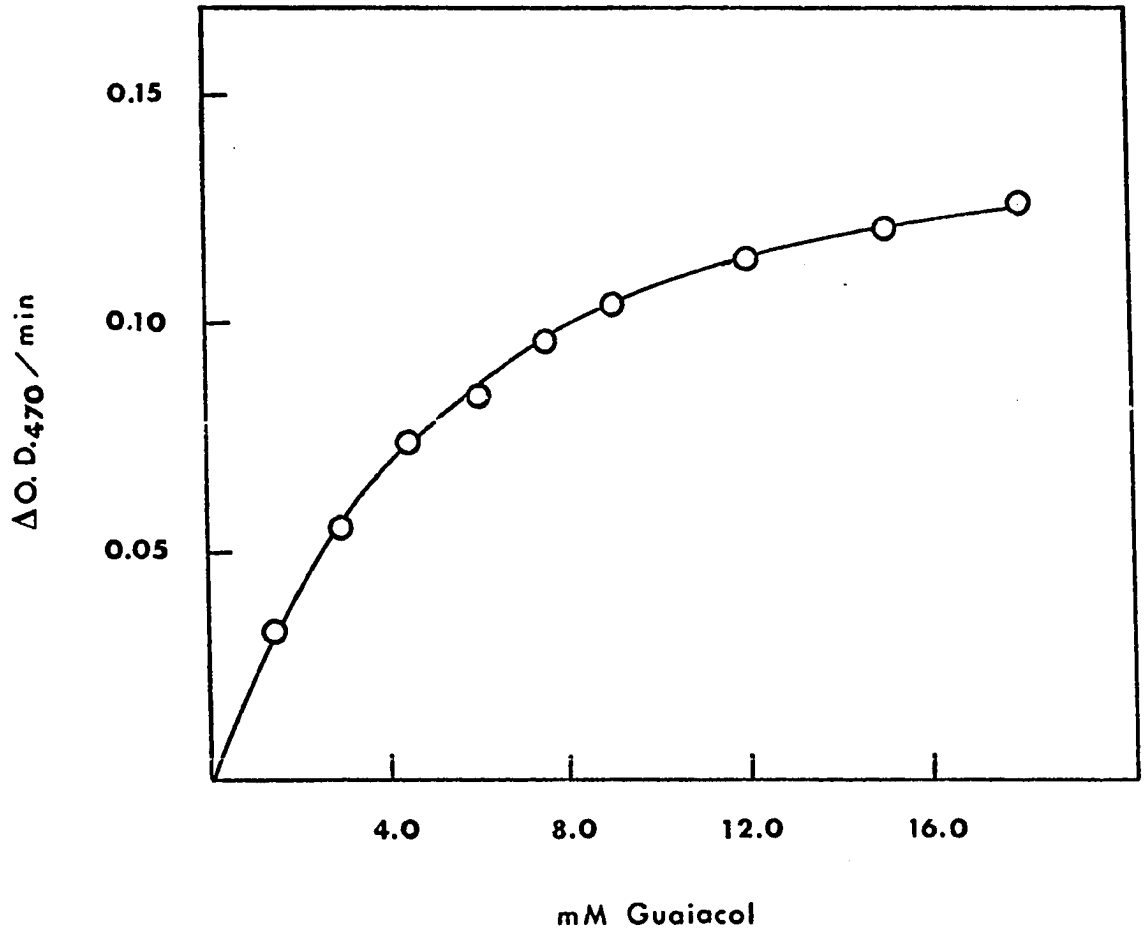

0.05
0.04
0.03
0.02
0.01
ΔO.D.470 / min
2.0
4.0
6.0
8.0
10.0
mM Guaiacol

Figure 6. GUAIACOL SATURATION CURVE FOR ISOPEROXIDASE C_4. Assay solutions contained 5 mM H_2O_2 and 40 mM phosphate buffer (pH 6.0).


0.15
0.10
0.05
ΔO. D.470 / min
4.0
8.0
12.0
16.0
mM Guaiacol

At a concentration of 1 mM, β-mercaptoethanol completely inhibited the ability of isoperoxidase C_4 to oxidize guaiacol, and total inhibition was also observed with cysteine for 17 min, after which time, 61% of control activity was restored, as indicated in Table I-3. When cysteine was incubated with hydrogen peroxide for 20 min before the addition of the enzyme, the lag period was decreased to only 3 min, and 96% of control activity was re-established. Serine which differs from cysteine structurally in that it contains an OH instead of an SH group, had no effect on guaiacol activity when present at the same concentration as cysteine. A reasonable interpretation of the above phenomena is that there could be a sulfhydryl group on the enzyme molecule necessary for catalytic activity. The restoration of activity after an initial lag period in the presence of cysteine could be the result of the non-enzymatic oxidation of the exogenous cysteine to cystine by hydrogen peroxide. Some of the original activity was lost due to the formation of disulfide linkages between cysteine and a sulfydryl group at or near the enzyme active site.

Isoperoxidase C_4 appeared to be very stable over a wide temperature range. No loss in activity with respect to guaiacol was detected after incubation of the enzyme at 60° for one hour. Freezing of a dilute solution of the enzyme and thawing at room temperature resulted in a loss of 38% of the activity present before freezing. However, if the same enzyme solution was thawed slowly at $4^{\circ}C$, 77% of the original activity was retained.

The effect of a number of naturally occurring phenolic compounds on the guaiacol activity of isoperoxidases C_3 and C_4 was determined in an effort to establish a possible involvement of these compounds in the

TABLE I-3

EFFECT OF VARIOUS COMPOUNDS ON THE

GUAIACOL OXIDATION BY ISOPEROXIDASES C_3 AND C_4

EFFECTOR	COMMON NAME	% OF CONTROL ACTIVITY	
		C_3	C_4
No effector		100	100
0.4 mM Substituted benzoic acids			
Benzoic acid			
2,3 di-OH		22	22
3,4 di-OH		-	15
3,4 di-OMe		100	100
p-OH		117	117
4-OH, 3-OMe	Vanillic acid	110	110
3-OH, 4-OMe		100	100
0.4 mM Substituted cinnamic acids			
Cinnamic acids			
4-OH	p-Coumaric acid	126	124
3,4 di-OH	Caffeic acid	365	175
4-OH, 3-OMe	Ferulic acid	535	395
3,4 di-OMe		-	100
3-OH, 4-OMe	Isoferulic acid	84	86
0.4 mM Ferulic acid derivatives			
Ferulic acid			
3-O-glucosyl		100	100
Hydroferulic acid		-	58

TABLE I-3 (cont.)

EFFECTOR	COMMON NAME	% OF CONTROL ACTIVITY	
		C_3	C_4
0.4 mM Caffeic acid derivatives			
Caffeic acid			
Ethyl ester		13	11
Hydrocaffeic acid		14	14
0.4 mM Substituted coumarins			
Coumarin			
6,7 di-OH	Esculetin	16	16
6-OMe, 7-OH	Scopoletin	105	102
7-OH, 6-O-glucosyl	Esculin	60	51
6-OMe, 7-O-glucosyl	Scopolin	100	100
0.4 mM Substituted quinic acids			
Quinic acid			
3-O-feruloyl		-	40
3-O-caffeoyl	Chlorogenic acid	8	8
0.05 mM Quercetin		-	50
0.4 mM Cinnamic alcohol	Coniferyl alcohol	4	4
0.4 mM Indole-3-acetic acid		96	100

TABLE I-3 (cont.)

EFFECTOR	COMMON NAME	% OF CONTROL ACTIVITY C_3	% OF CONTROL ACTIVITY C_4
1.0 mM Amino acids			
Serine		-	100
Methionine		-	100
Cysteine		-	61*
1.0 mM β-mercaptoethanol		-	0

- not determined
* zero for 17 min
Assay solutions contained 5 mM H_2O_2 and 50 mM phosphate buffer (pH 6.0)

physiological function of the two isoperoxidases. As shown in Table I-3, the effect of all compounds tested on the guaiacol activity was virtually identical for both isoperoxidases, except the greater stimulation by ferulic and caffeic acids observed with C_3. Most of the compounds listed in Table I-3 were also tested by Powell *et al.* (63) as possible effectors of the oxidizing ability of two anodic isoperoxidases, designated A_1 and A_2, from tobacco tissue culture. Each of these compounds affected the rate of oxidation of guaiacol by A_1 and A_2 in an almost identical fashion as that reported here for C_3 and C_4.

Goldacre and coworkers (21) reported some time ago that the IAA oxidase activity of crude peroxidase from peas was stimulated by monophenols and inhibited by o and p-diphenols. Lee and Skoog (44) found that 3,4-dihydroxybenzoic acid inhibited, while monohydroxy and 2,4-dihydroxybenzoic acids stimulated IAA oxidase activity in tobacco. With the notable exception of caffeic acid, all compounds which have an o-diphenol substitution and which were investigated here show varying degrees of inhibition of the guaiacol activity of both isoperoxidases. The most pronounced inhibitory effect was evident with coniferyl alcohol and chlorogenic acid. In sharp contrast to the inhibition observed with other o-diphenolic compounds tested, caffeic acid stimulated the guaiacol oxidizing ability of both isoperoxidases. However, if the free carboxyl group of caffeic acid was blocked by the formation of the ethyl ester, strong inhibition was observed. This carboxyl group, therefore, is a necessary requirement for the stimulation.

The unsubstituted monophenolic compounds tested, namely p-coumaric and p-hydroxybenzoic acids, stimulate the guaiacol oxidizing ability of

both enzymes as expected from previous literature results (21,44). Ferulic acid, which has a 4-OH, 3-OMe substitution, stimulated the guaiacol oxidation by the greatest amount. However, any variation in the structure of ferulic acid eliminated the observed stimulation. Hydrogenation of the vinyl carbons of ferulic acid to yield hydroferulic acid resulted in 42% inhibition, and reversal of the positions of the hydroxy and methoxy groups to yield isoferulic acid, resulted in 14% inhibition. Substitution of the phenolic group resulted in 60% inhibition in the case of 3-O-feruloyl-quinic acid and in no effect with glucosidoferulic acid. Scopoletin, the corresponding lactone of ferulic acid, was reported by Schafer (71) to stimulate by 80% the guaiacol activity of an anodic isoperoxidase, designated A_3, from tobacco tissue cultures. Scopoletin had no significant effect on the guaiacol acitivity of either isoperoxidase in this study.

As noted above, caffeic acid, which differs from ferulic acid structurally by possessing an OH rather than an OMe at position 3, also greatly stimulated the guaiacol activity of both isoperoxidases but to a lesser degree than ferulic acid. p-Coumaric acid also stimulated the guaiacol activity, but to a lesser degree than caffeic acid. Therefore, the OH at position 4 was required for the stimulatory effect and substitution at position 3, preferably a methoxy group, enhanced the stimulatory effect. The carboxylic group and unsaturation in the side chain are also essential for the stimulatory effect. Therefore, it appears that ferulic acid is structurally unique in its ability to stimulate oxidation of guaiacol by the two isoperoxidases.

To determine if ferulic acid could be a substrate for the two isoperoxidases, a preincubation experiment was devised. As shown in Table I-4,

isoperoxidase C_4, H_2O_2 and guaiacol were each added in turn to a reaction mixture in which all other components had incubated for 5 min. Addition of enzyme or hydrogen peroxide after 5 min did not reduce the observed stimulation by ferulic acid. However, incubation of ferulic acid, hydrogen peroxide and isoperoxidase C_4, before the addition of guaiacol, reduced the stimulation after 5 min and completely eliminated the stimulation after 10 min. This indicated that isoperoxidase C_4 catalyzed the oxidation of ferulic acid in the presence of H_2O_2. Gelinas (18) has proposed a mechanism for the oxidation of ferulic acid by horseradish peroxidase.

When isoperoxidase C_4 was added to a solution containing 0.8 mM ferulic acid and 5 mM H_2O_2, a salmon pink color rapidly formed but gradually was replaced by a yellow color. The initial oxidation product of the

TABLE I-4

PREINCUBATION EXPERIMENT

Assay Condition	ΔO.D./min/0.1 ml
Normal assay with ferulic acid	202
Enzyme added after 5 min	202
H_2O_2 added after 5 min	187
Guaiacol added after 5 min	128
Guaiacol added after 10 min	71
Normal assay without ferulic acid	71

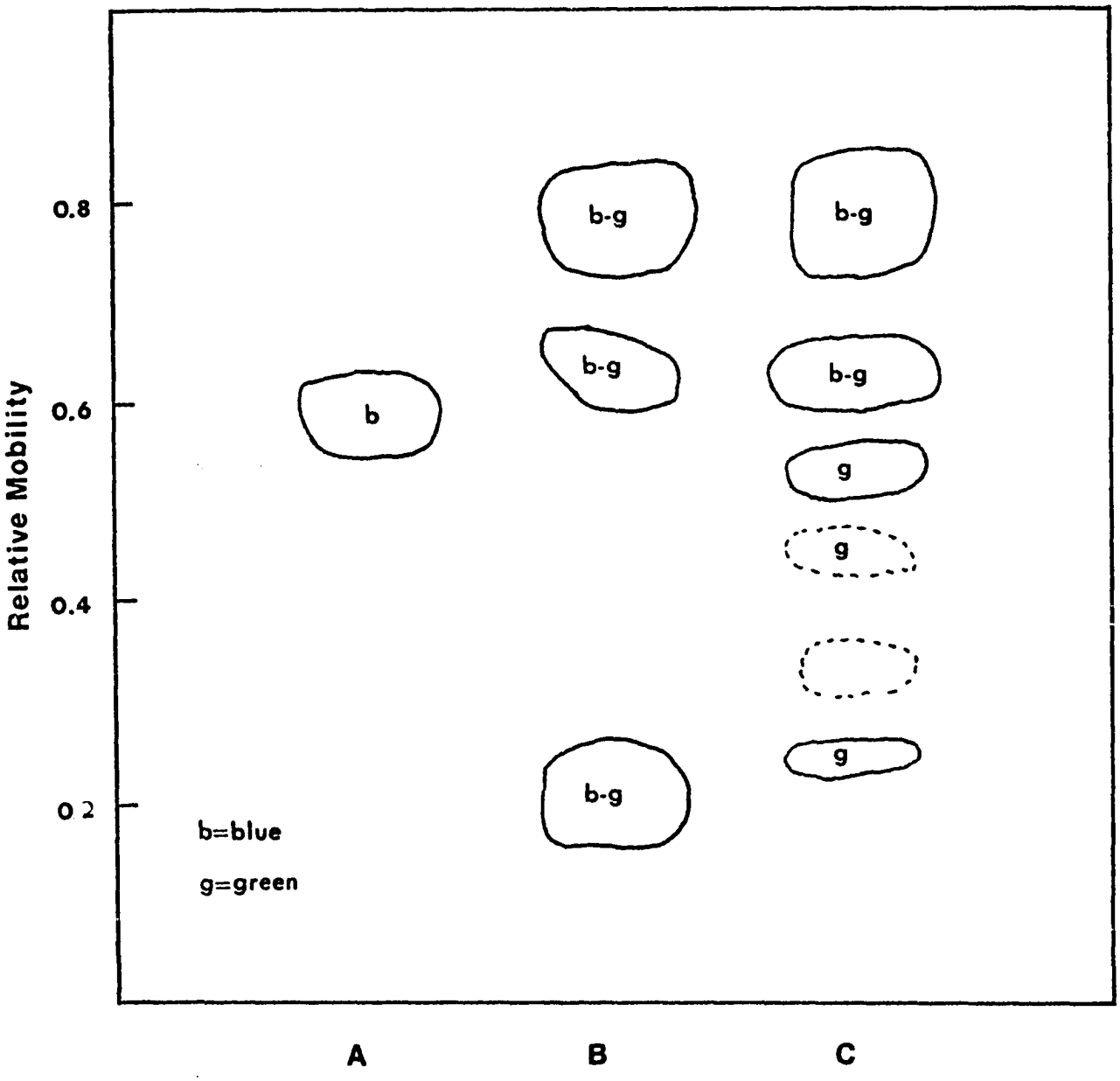
Assay solutions contained 5 mM H_2O_2, 0.4 mM ferulic acid, 50 mM phosphate buffer (pH 6.0) and 0.1 ml of isoperoxidase C_4 enzyme solution.

reaction exhibited a small transient absorption peak at 540 nm not seen with ferulic acid. This absorption maximum at 540 nm disappeared within 5 min, but exposure of the reaction mixture to sunlight or fluorescent light hastened the disappearance. A reaction product appeared after 30 sec, as revealed by thin-layer chromatography, and this product had a relative mobility of 0.64 and fluoresced blue-green under ammonia, compared to ferulic acid which had a relative mobility of 0.59 and fluoresced blue. Ferulic acid was completely destroyed within 10 min, and if the reaction mixture had been exposed to light, multiple products were visible on the thin-layer plates (Figure 7). Extraction of this reaction mixture with ethyl acetate revealed additional products in the extract.

An attempt was made to isolate the initial major product in pure form by carrying out the reaction in the dark. However, the product was unstable and readily formed additional products. The initial major impure product turned blood-red in basic solution at pH 9.0, a property displayed by natural lignin (27). This blood-red solution was acidified and extracted with ethyl acetate to yield a product which had a relative mobility of 0.32 compared to a relative mobility of 0.94 of the major product before the basic treatment. In view of the fact that ferulic acid is converted to polymeric products by other peroxidase preparations (77), it seems reasonable to assume a much higher molecular weight for this product than that of ferulic acid. Also, formation of the initial unstable product is consistent with the generally accepted free radical type mechanism by which coniferyl alcohol and other lignin monomers are polymerized by peroxidase to lignin (8).

Comparison of the nmr spectrum of ferulic acid with that of its oxidation

Figure 7. THIN LAYER CHROMATOGRAPH OF FERULIC ACID OXIDATION PRODUCTS. A-ferulic acid, B-reacted mixture after 15 min, light exposed, C-ethyl acetate extract of reacted mixture. The reaction mixture contained 0.4 mM ferulic acid, 5 mM H_2O_2 and 0.1 ml of isoperoxidase C_4. Spots were visualized by their fluorescence under ammonia at 360 nm.


Relative Mobility
0.8
0.6
0.4
0 2
b
b-g
b-g
b-g
b-g
g
g
g
b-g
b=blue
g=green
A
B
C

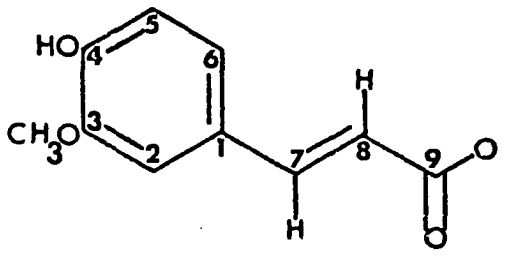
products revealed proton absorptions which were unique to each (Table I-5). The most conspicuous difference between ferulic acid and the products is the disappearance of the vinyl protons (H-7, δ6.42 and H-8, δ7.70) in ferulic acid and the appearance of a signal (δ1.40) in the products which can be assigned to protons on saturated carbons. The methoxy group of ferulic acid (H-3, δ4.00) remains intact and the products also apparently contain phenolic groups. More definitive statements concerning the possible structure of the reaction products must await a procedure for their separation and more detailed spectral analysis.

In lieu of a reliable assay procedure for the enzymatic oxidation of ferulic acid, a K_m for ferulic acid for isoperoxidase C_4 was determined by monitoring stimulation of the guaiacol activity by ferulic acid at varying concentrations of ferulic acid. A plot of relative guaiacol activity vs ferulic acid concentration yeilded a Michaelis-Menten type saturation curve (Figure 8), and a tentative K_m for ferulic acid, estimated by the method of Lineweaver and Burk (47), was 0.023 mM. When compared to a K_m for guaiacol of 6 mM, isoperoxidase C_4 obviously had a much higher affinity for ferulic acid than guaiacol as substrate. The stimulation effect of ferulic acid was overcome at high concentrations of guaiacol (Figure 9). A plausible explanation for this is that ferulic acid is a competitive activator of the guaiacol oxidation by C_4. Since ferulic acid contains a guaiacyl moiety, ferulic acid and guaiacol probably compete for the same enzyme active site. However, the remaining portion of the ferulic acid structure, other than the guaiacyl group, is evidently involved in binding to the enzyme active site, since C_4 prefers ferulic acid over guaiacol as substrate. The pH optimum for the isoperoxidase C_4

TABLE I-5

COMPARISON OF CHEMICAL SHIFTS OF PROTONS IN FERULIC ACID AND ITS OXIDATION PRODUCTS

Proton position	Ferulic acid,	Products,
3	4.00	3.95
4 and 9	6.15	7.30
2,5 and 6	6.8-7.4	6.5-7.8
7	6.42	-
8	7.70	-
unknown	-	5.82
unknown	-	1.40



Ferulic Acid

catalyzed oxidation of guaiacol in the presence of ferulic acid was 6.0, which was identical to the value obtained in the absence of ferulic acid.

The products of the isoperoxidase C_4 catalyzed oxidation of ferulic acid were observed to have negligible absorbance at 320 nm compared to ferulic acid (Figure 10). This differential absorbance at 320 nm was utilized to monitor the C_3 and C_4 catalyzed oxidation of ferulic acid. Using this assay procedure, both isoperoxidases revealed a broad pH optimum at 5.5 (Figure 11). This value agrees well with the value of 6.0 for guaiacol in the presence of ferulic acid.

A plot of velocity versus ferulic acid concentration for isoperoxidase

Figure 8. EFFECT OF FERULIC ACID CONCENTRATION ON GUAIACOL OXIDATION BY ISOPEROXIDASE C_4. Assay solutions contained 6 mM guaiacol, 5 mM H_2O_2 and 50 mM phosphate buffer (pH 6.0).

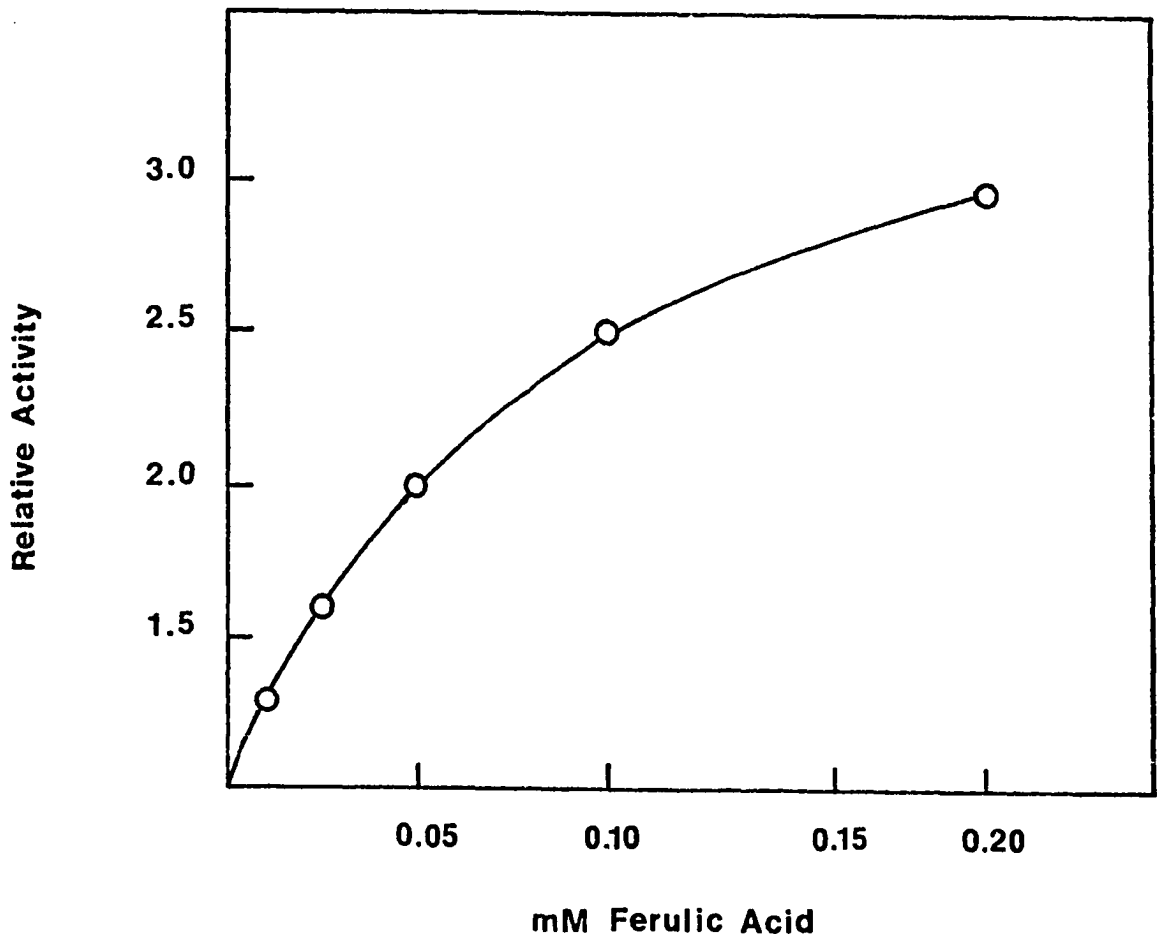

Relative Activity
3.0
2.5
2.0
1.5
0.05
0.10
0.15
0.20
mM Ferulic Acid

Figure 9. DOUBLE RECIPROCAL PLOTS OF GUAIACOL CONCENTRATION VS. ACTIVITY FOR ISOPEROXIDASE C_4. Curve A-normal assay, curve B-assay with 0.01 mM ferulic acid. Assay solutions contained 50 mM phosphate buffer (pH 6.0) and 5 mM H_2O_2.

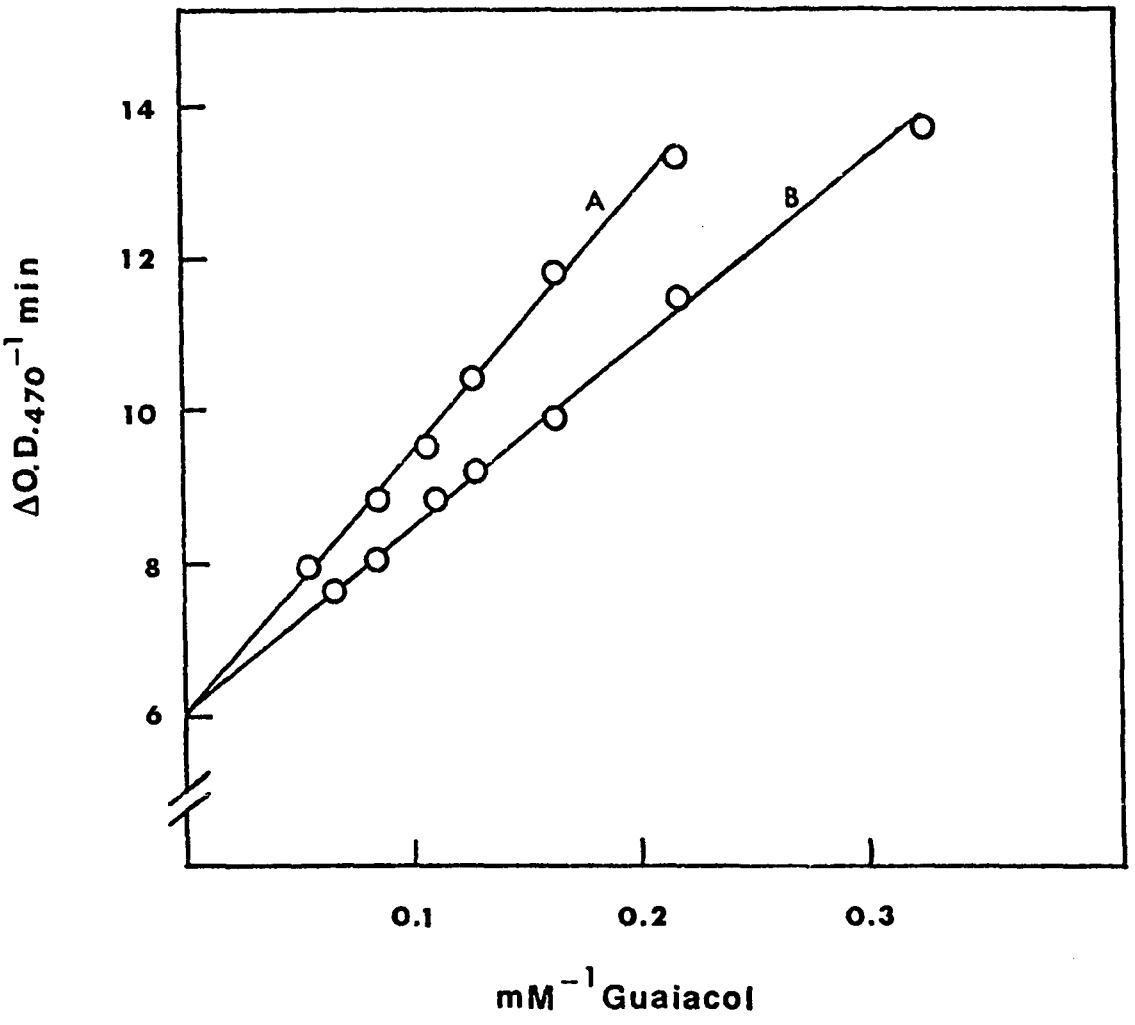

14
12
10
8
6
A
B
0.1
0.2
0.3
ΔO.D.470⁻¹ min
mM⁻¹ Guaiacol

Figure 10. SPECTRA OF FERULIC ACID AND ITS OXIDATION PRODUCTS. Curve A-before addition of C_4, curve B-after addition of C_4. Assay solutions contained 0.1 mM ferulic acid, 5 mM H_2O_2 and 50 mM phosphate buffer (pH 6.0).

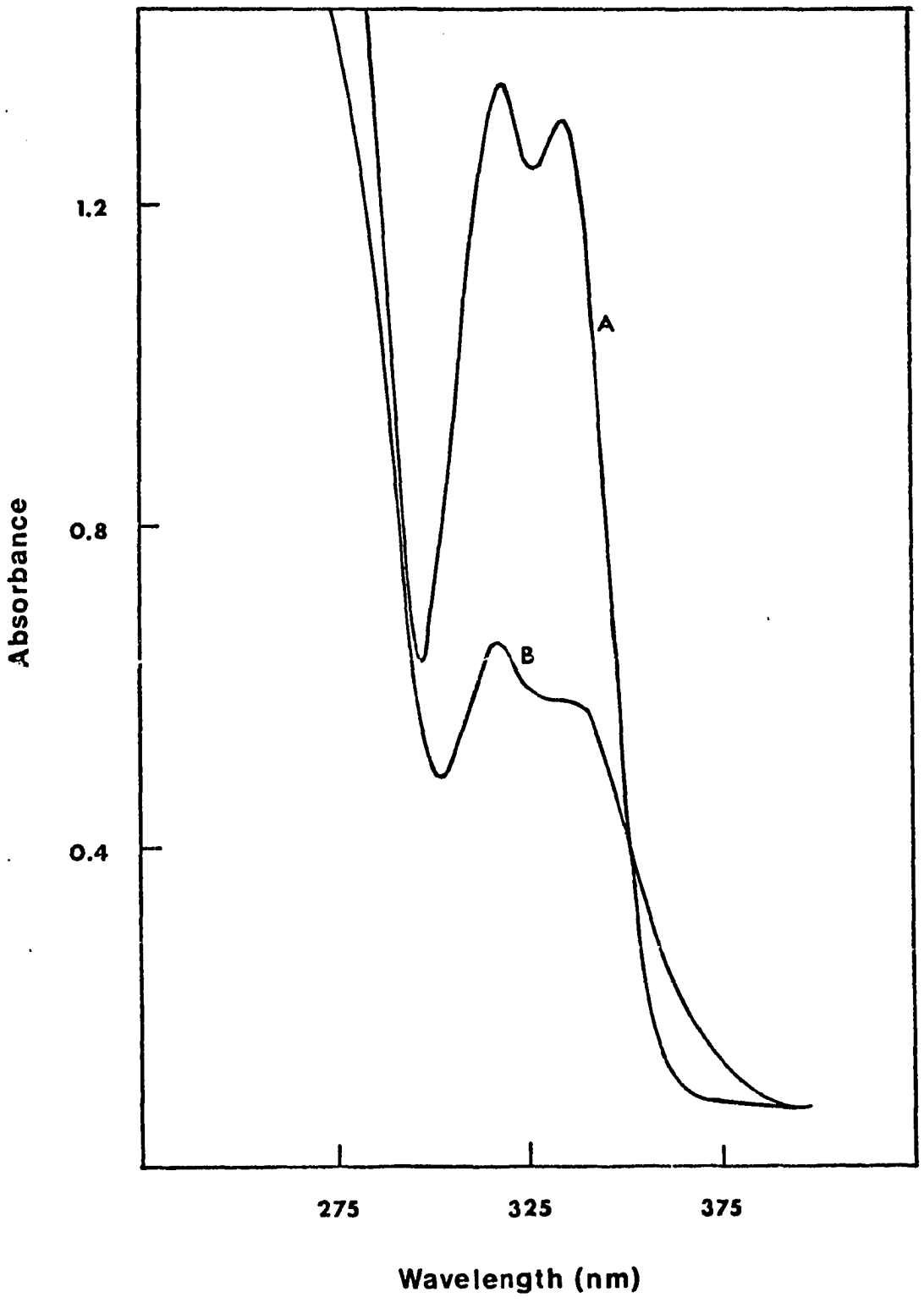
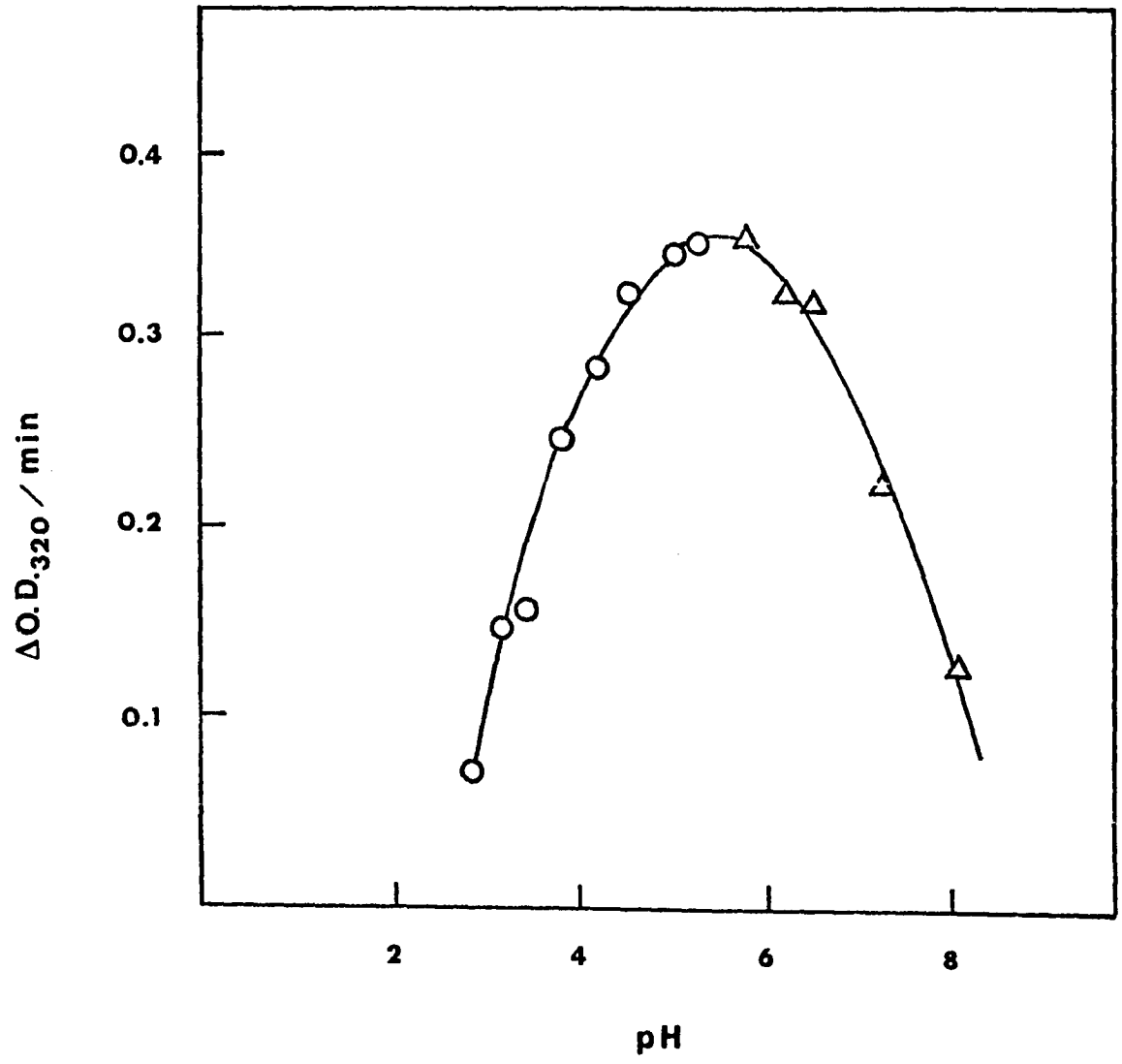

1.2
0.8
0.4
Absorbance
A
B
275
325
375
Wavelength (nm)

Figure 11. EFFECT OF pH ON THE FERULIC ACID OXIDATION BY ISOPEROXIDASE C_4. Assay solutions contained 0.2 mM ferulic acid, 5 mM H_2O_2 and 80 mM buffer O-citrate, Δ-phosphate.


0.4
0.3
0.2
0.1
ΔO.D.320 / min
2
4
6
8
pH

C_4 appeared to follow simple Michaelis-Menten kinetics up to a ferulic acid concentration of about 0.2 mM (Figure 12). At this point the velocity decreased with an increase in ferulic acid concentration. However, if the reaction was monitored at 360 nm, where products absorb more strongly than ferulic acid, the reaction velocity did not diminish at ferulic acid concentrations above 0.2 mM (Figure 13). The K_m for ferulic acid was calculated to be 0.3 mM by both assay procedures. This value does not agree with the above K_m of 0.023 mM for ferulic acid estimated by monitoring the ferulic acid activation of guaiacol. But, clearly the K_m determined by a direct assay for ferulic acid oxidation should reflect the more accurate value.

Using the 320 nm assay procedure, the hydrogen peroxide concentration was varied while ferulic acid was maintained at 0.2 mM. A plot of hydrogen peroxide concentration versus velocity is shown in Figure 14. The K_m for hydrogen peroxide for isoperoxidase C_4 was 2 mM.

In addition to ferulic acid, several other naturally occurring phenolic compounds have been found to be oxidized by peroxidase preparations, including scopoletin (76) and chlorogenic acid (79). A few of the phenolic compounds which either stimulated or inhibited the guaiacol oxidation by isoperoxidases C_3 and C_4 were investigated as potential substrates for the two enzymes. Using difference spectra and thin-layer chromatography as criteria, several of the phenolic compounds, namely scopoletin, chlorogenic acid, caffeic acid, esculetin and coniferyl alcohol, were demonstrated to be oxidized by both isoperoxidases in the presence of hydrogen peroxide. The difference spectra of esculetin and its oxidation product(s) are shown in Figure 15.

Figure 12. FERULIC ACID SATURATION CURVE FOR ISOPEROXIDASE C_4. Velocities (Δ OD/min) were measured at 320 nm. Assay solutions contained 50 mM phosphate buffer (pH 6.0), and 5 mM H_2O_2.

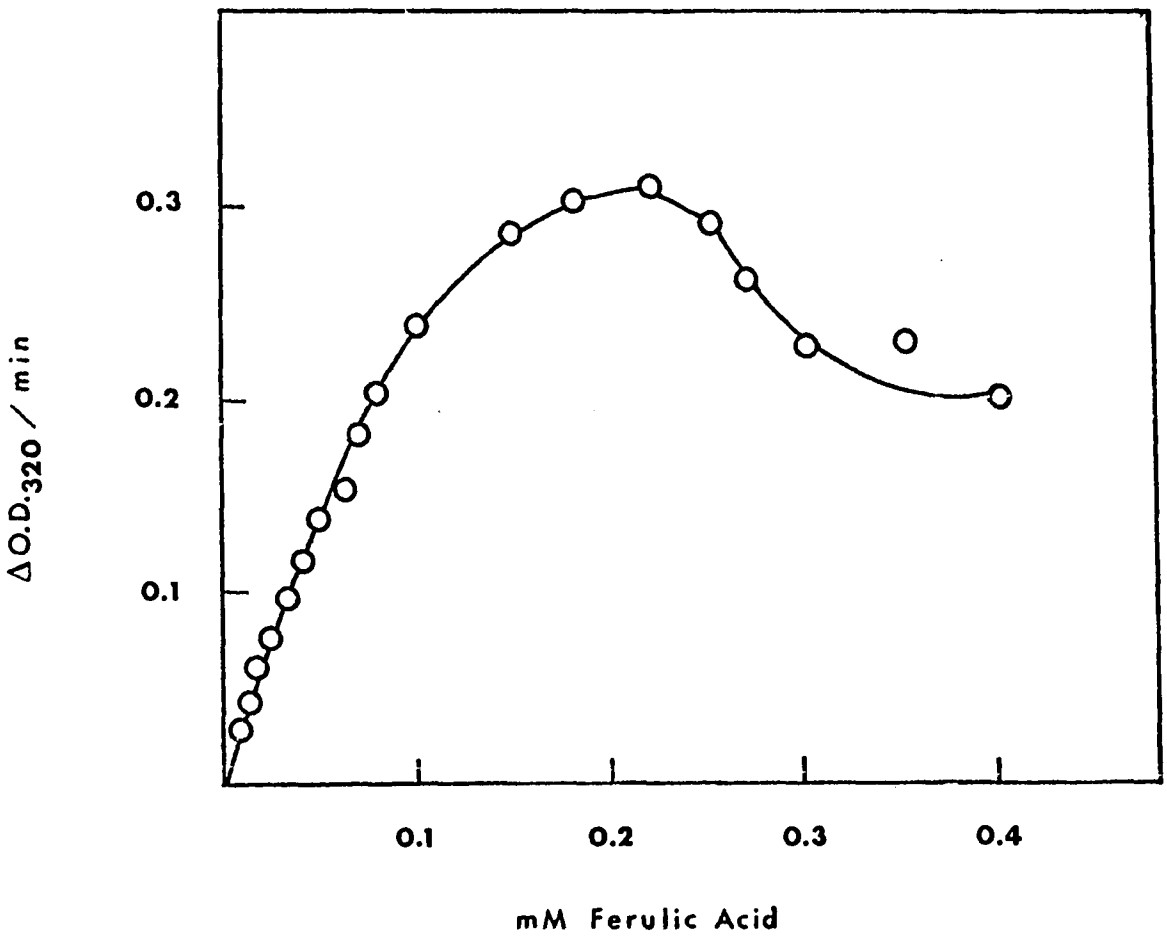

0.3
0.2
0.1
ΔO.D.320 / min
0.1
0.2
0.3
0.4
mM Ferulic Acid

Figure 13. FERULIC ACID SATURATION CURVE FOR ISOPEROXIDASE C_4. Velocities (Δ OD/min) were measured at 360 nm. Assay solutions contained 50 mM phosphate buffer (pH 6.0) and 5 mM H_2O_2.

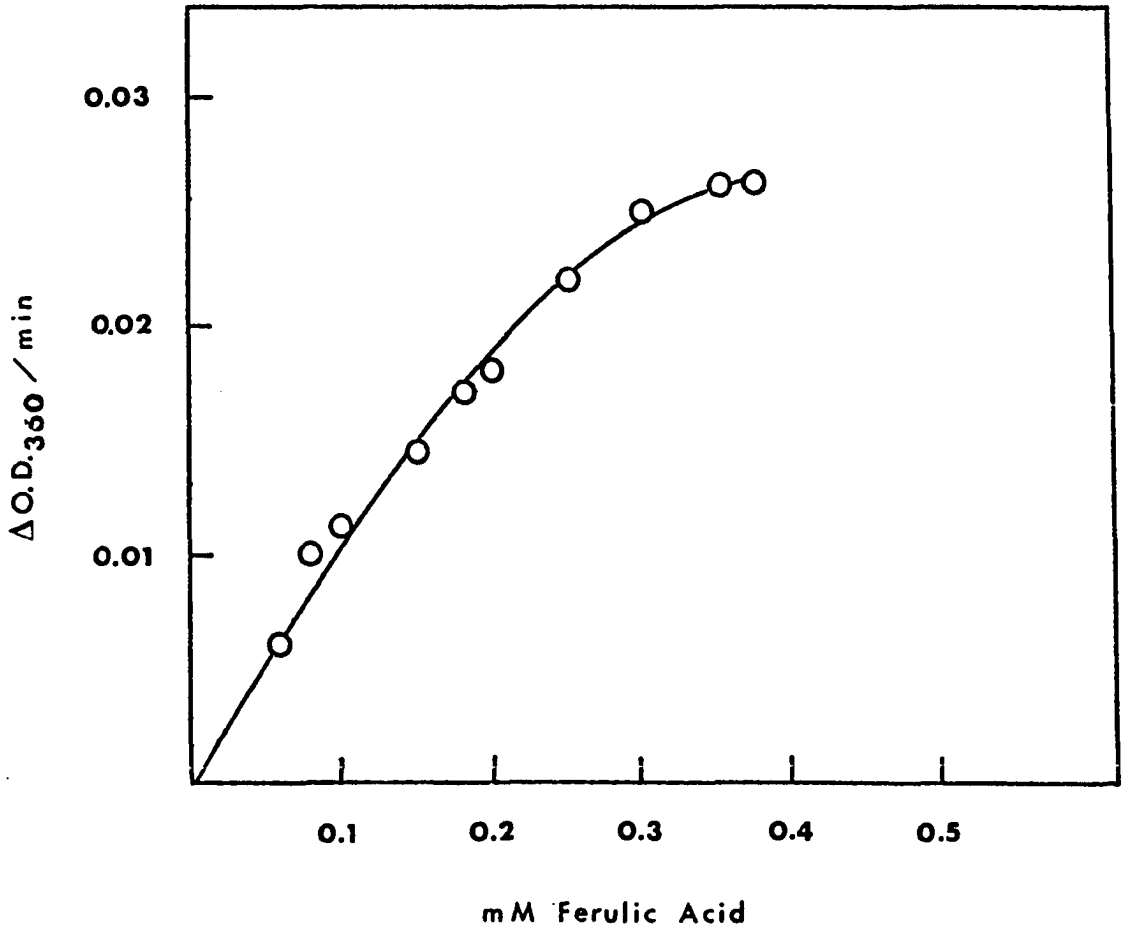

0.03
0.02
0.01
ΔO.D.360 / min
0.1
0.2
0.3
0.4
0.5
mM Ferulic Acid

Figure 14. HYDROGEN PEROXIDE SATURATION CURVE FOR ISOPEROXIDASE C_4. Assay solutions contained 0.2 mM ferulic acid, and 0.15 M citrate buffer (pH 5.5).

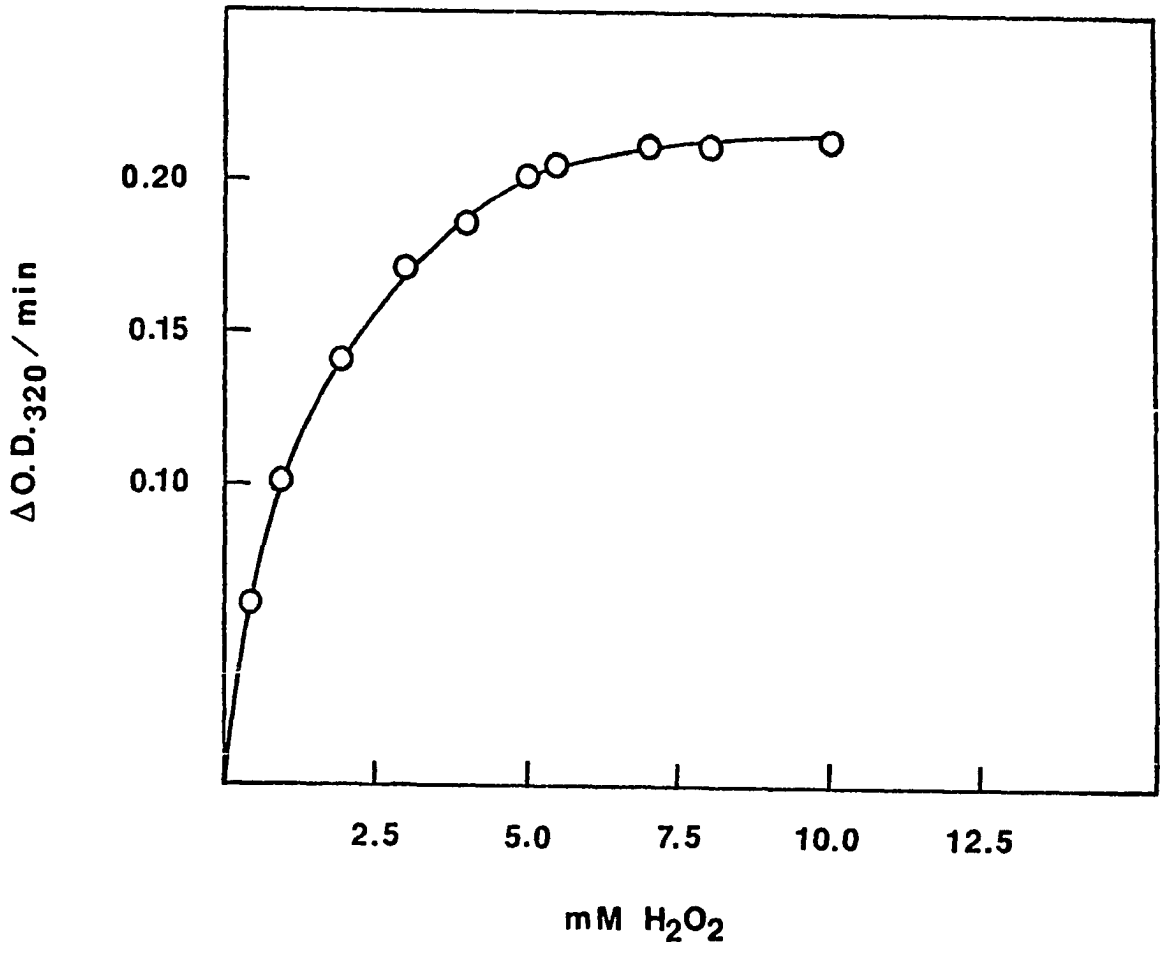
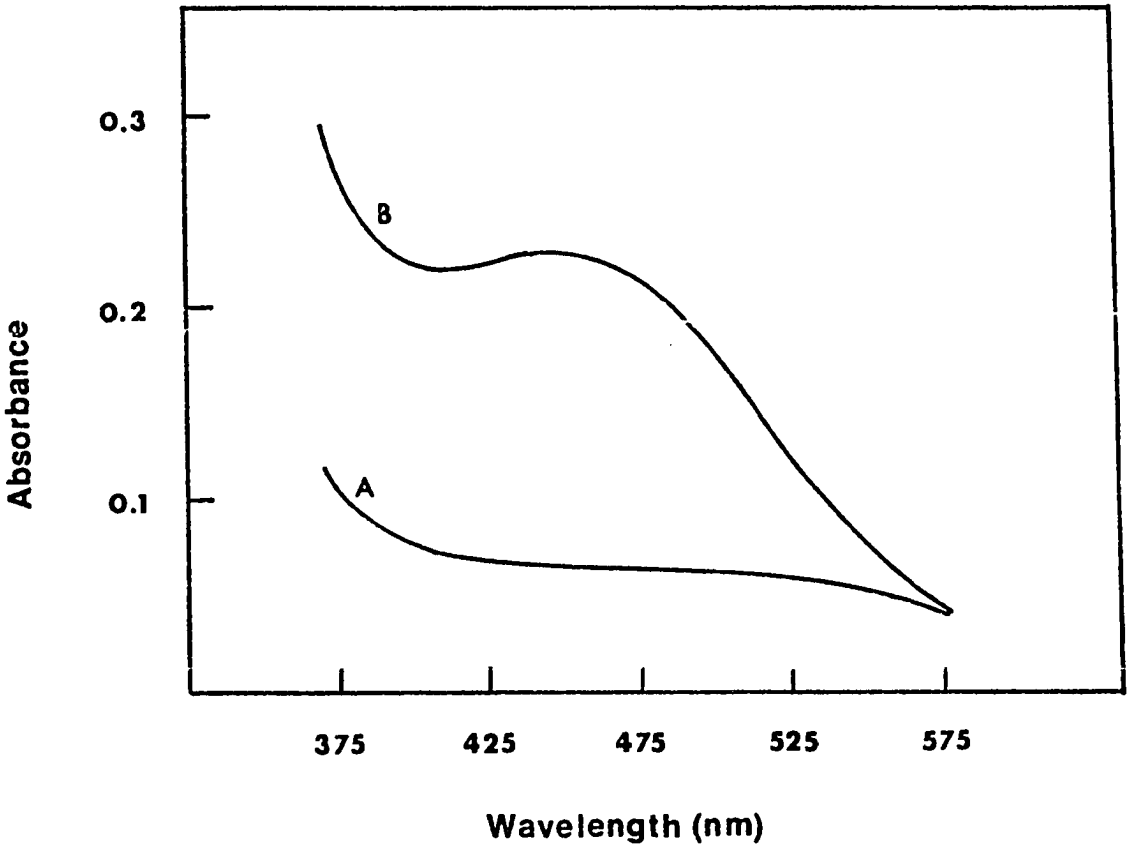

0.20
0.15
0.10
ΔO.D.320 / min
2.5
5.0
7.5
10.0
12.5
mM H2O2

Figure 15. SPECTRA OF ESCULETIN AND ITS OXIDATION PRODUCTS. A-before addition of isoperoxidase C_4, B-after addition of isoperoxidase C_4. Reaction mixture contained 0.35 mM esculetin, 5 mM H_2O_2 and 50 mM phosphate buffer (pH 7.5).


0.3
0.2
0.1
Absorbance
B
A
375
425
475
525
575
Wavelength (nm)

Using assay procedures previously described, the pH optima for both isoperoxidase C_3 and C_4 were 7.5 and 5.5 respectively for esculetin and chlorogenic acid as substrate. The effects of esculetin concentration and pH on the oxidation of esculetin by C_4 are displayed in Figure 16 and 17. A summary of pH optima, K_m's and Hill slopes (26) for the different substrates of isoperoxidase C_4 appears in Table I-6. The pH optima for C_3 with respect to all the substrates tested were identical to those for C_4. Of all the substrates investigated, ferulic acid appeared to be the best substrate for C_4, since it had the lowest K_m value.

The isoperoxidase C_3 and C_4-catalyzed oxidation of scopoletin was monitored at 450 nm as previously described by Reigh (67). At enzyme concentrations in which ferulic acid was completely destroyed within 10 min, the velocity (ΔO.D./min) of the scopoletin reaction was negligible.

TABLE I-6

SUMMARY OF MICHAELIS CONSTANTS, HILL SLOPES AND pH OPTIMA FOR SUBSTRATES OF ISOPEROXIDASE C_4

Substrate	K_m	Hill Slope	pH Optima
Guaiacol	4 mM	1.0	6.0
Scopoletin	0.53 mM	1.75	4.5
Esculetin	0.45 mM	1.4	7.5
Ferulic acid	0.30 mM	1.0	5.5
Chlorogenic acid	-	-	5.5

Figure 16. EFFECT OF pH ON THE ESCULETIN OXIDATION BY ISOPEROXIDASE C_4. Assay solutions contained 0.4 mM esculetin, 5 mM H_2O_2 and 50 mM buffer ○-citrate-phosphate, △-phosphate, □-Tris-HCl, ♂-glycine-NaOH.

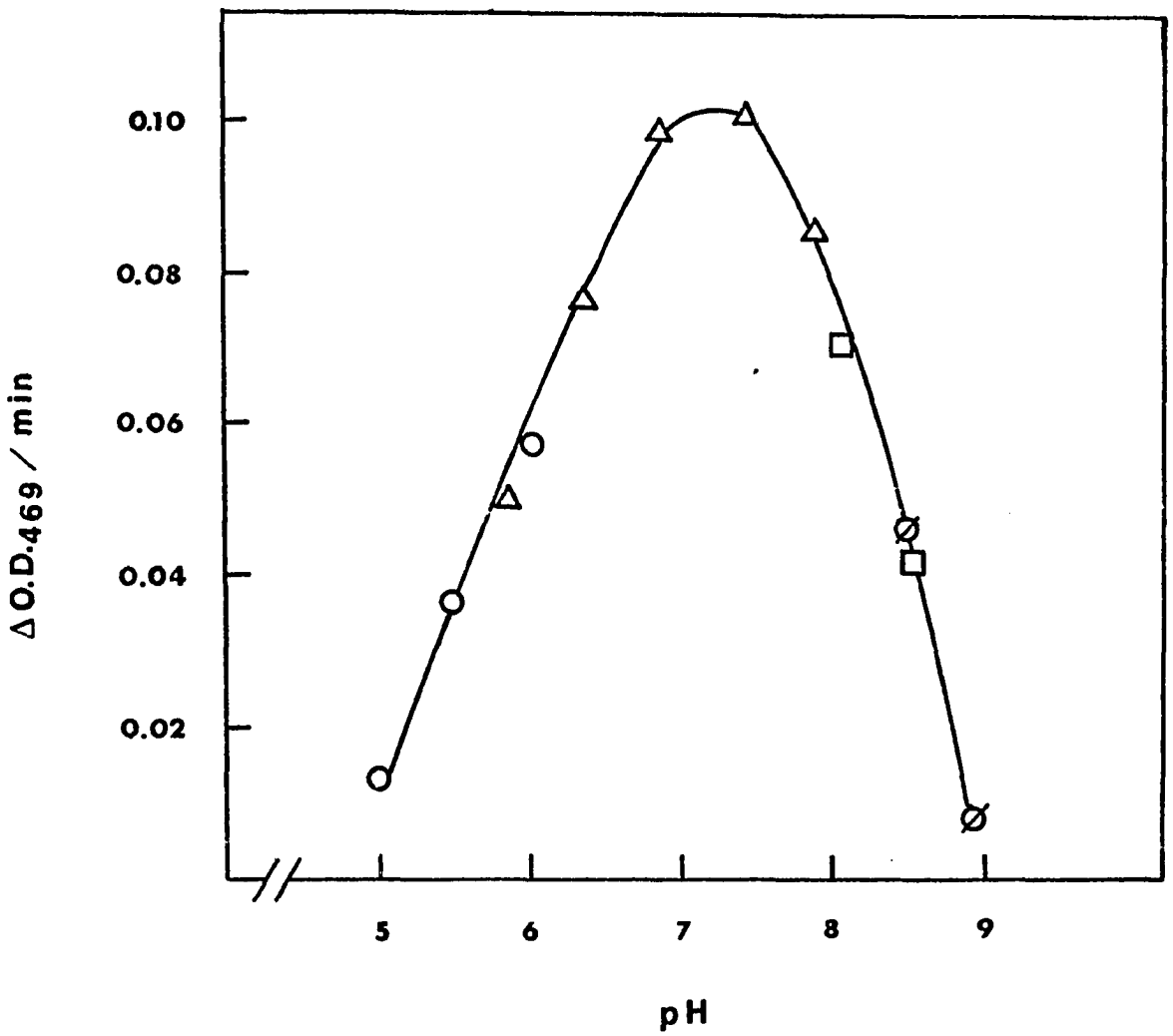
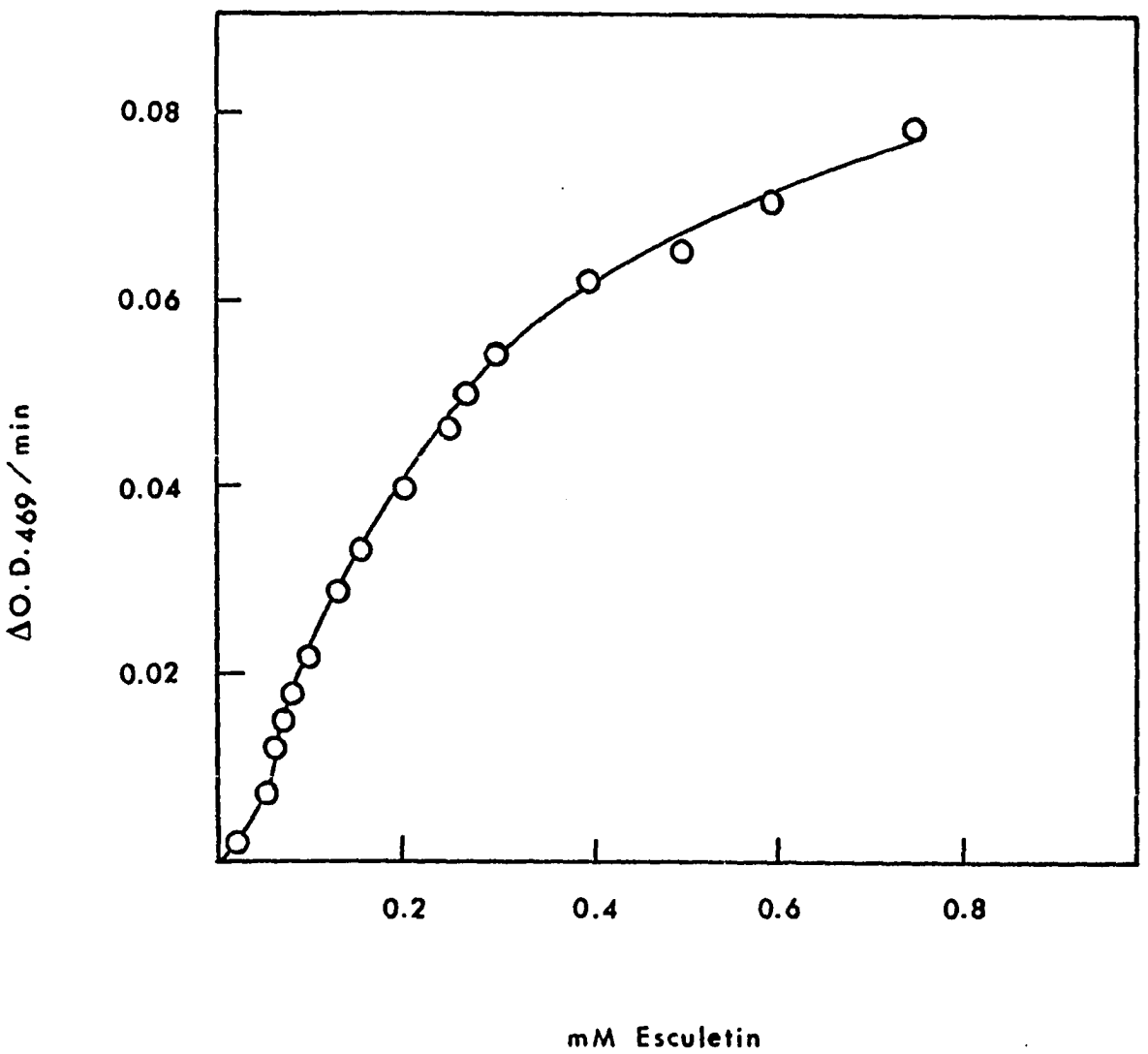

0.10
0.08
0.06
0.04
0.02
ΔO.D.469 / min
5
6
7
8
9
pH

Figure 17. ESCULETIN SATURATION CURVE FOR ISOPEROXIDASE C_4. Assay solutions contained 5 mM H_2O_2, and 50 mM phosphate buffer (pH 7.5).


0.08
0.06
0.04
0.02
ΔO.D.469/min
0.2
0.4
0.6
0.8
mM Esculetin

However, at higher enzyme concentrations, scopoletin was oxidized by the two isoperoxidase preparations. Saturation curves for scopoletin and ferulic acid as substrates for C_4 are compared in Figure 18. The scopoletin curve was run at higher enzyme concentrations and normalized to the ferulic acid curve by adjusting the V_{max} of both assays to the same value. The velocity at each scopoletin concentration was multiplied by a conversion factor of 1.6.

The rate of oxidation of scopoletin by isoperoxidase C_4 shows a sigmoidal dependence on scopoletin concentration, whereas the ferulic acid curve is non-sigmoidal. Hill slopes (26) for scopoletin and ferulic acid as substrates are 1.75 and 1.0 respectively (Figure 19). Hill slopes reflect the number of substrate binding sites present on an enzyme molecule (26). However, multiple substrate binding sites are only observed with allosteric enzymes having molecular weights much larger than those reported for peroxidases (28,50,57,63,74). If isoperoxidase C_4 contained more than one substrate binding site for scopoletin but not for ferulic acid, ferulic acid should inhibit scopoletin oxidation non-competively and vice versa. Attempts to resolve this were unsuccessful, since neither substrate could be assayed in the presence of the other. Mixed products were evidently formed, because the wavelengths used for both assay procedures were shifted. Ferulic acid inhibition of esculetin oxidation by C_4, though, was overcome at high esculetin concentrations, as evidenced by the Lineweaver-Burk (47) plots shown in Figure 20. This type of behavior is indicative of competition between esculetin and ferulic acid for the same enzyme active site. Therefore, if esculetin and ferulic acid bind to the same site, it seems reasonable to speculate that scopoletin

Figure 18. COMPARISON OF FERULIC ACID AND SCOPOLETIN SATURATION CURVES FOR ISOPEROXIDASE C_4. Activity was measured at 320 nm for ferulic acid and 450 nm for scopoletin. Ferulic acid assays contained 50 mM phosphate buffer (pH 4.5), 5 mM H_2O_2 and 0.1 ml of enzyme solutions (19 µg protein/ml). Scopoletin assay solutions contained 50 mM phosphate buffer (pH 4.5), 5 mM H_2O_2 and 0.1 ml of enzyme solution (73 µg protein/ml).

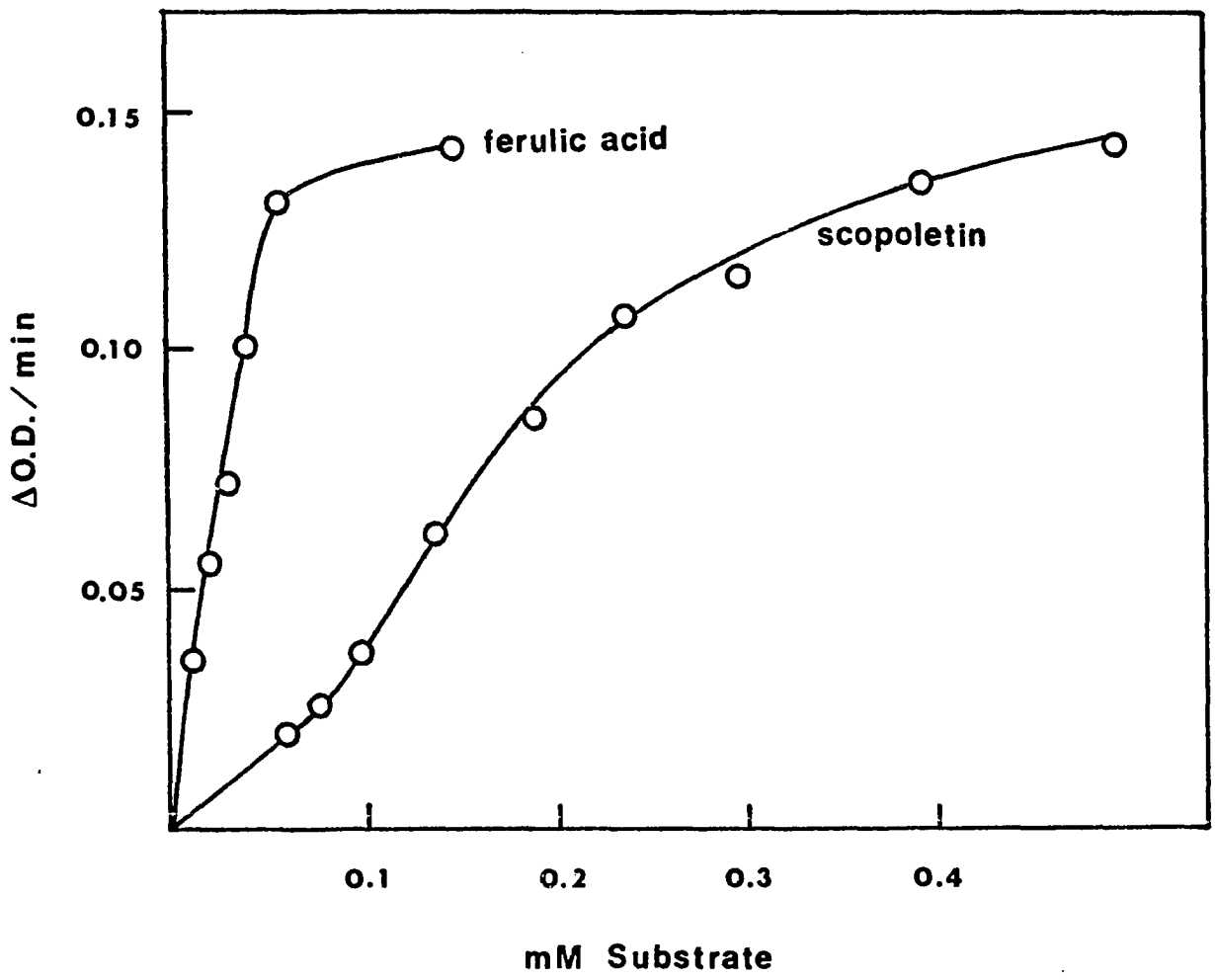

0.15
0.10
0.05
ΔO.D./min
ferulic acid
scopoletin
0.1
0.2
0.3
0.4
mM Substrate

Figure 19. COMPARISON OF HILL PLOTS FOR FERULIC ACID AND SCOPOLETIN OXIDATION BY ISOPEROXIDASE C_4. Velocities were ΔOD/min at 450 nm for scopoletin and 320 nm for ferulic acid. Assay solutions for scopoletin contained 50 mM citrate buffer (pH 4.5) and 5 mM H_2O_2. Ferulic acid assay solutions contained 50 mM citrate buffer (pH 5.5) and 5 mM H_2O_2.

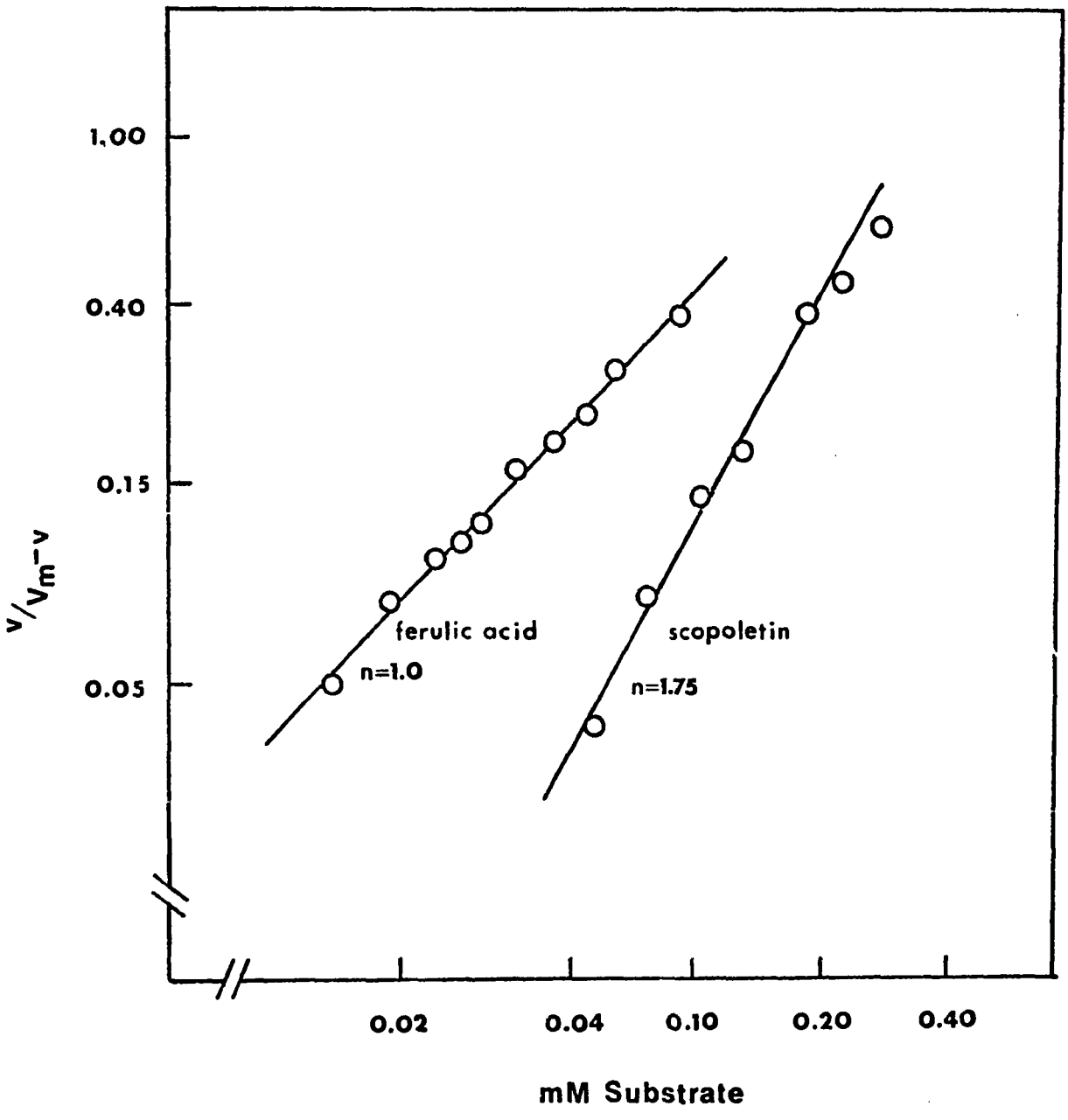
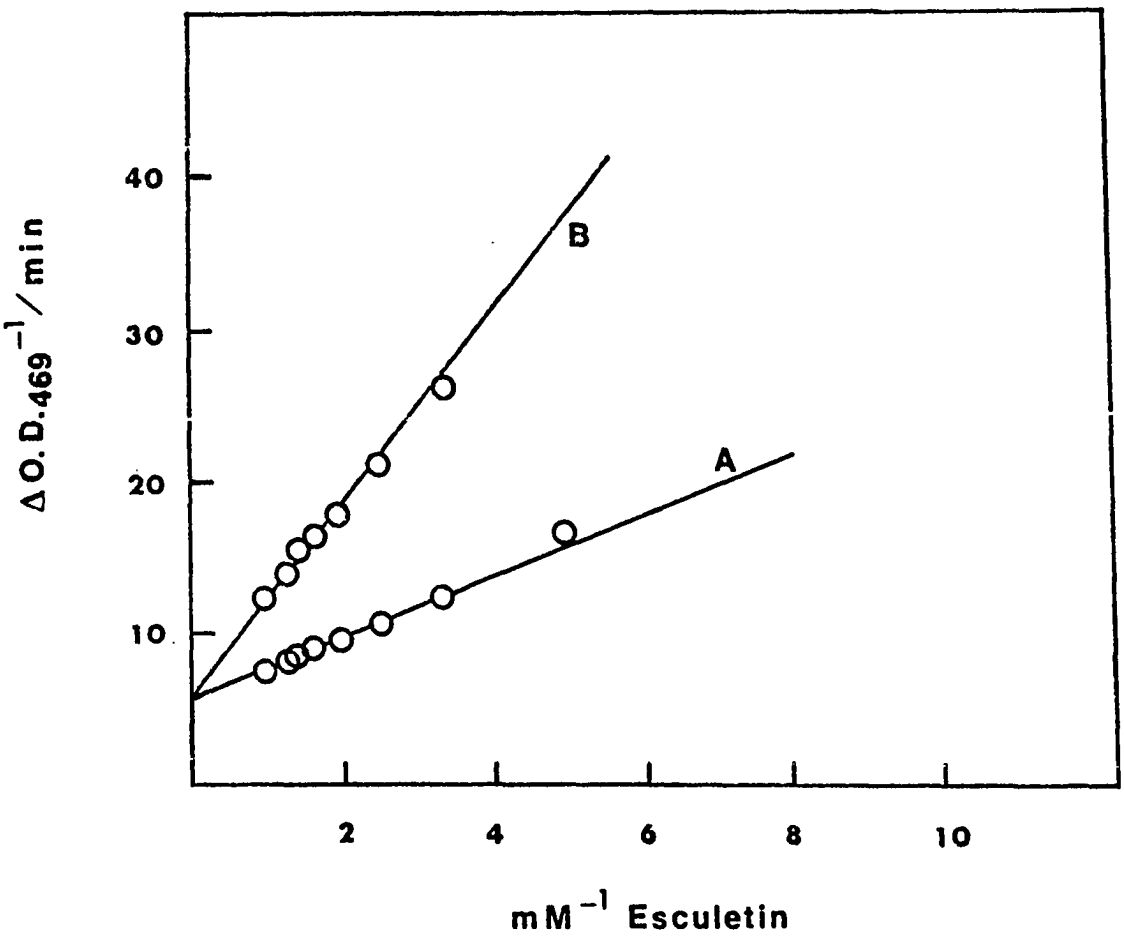

1.00
0.40
0.15
0.05
v/Vm-v
ferulic acid
n=1.0
scopoletin
n=1.75
0.02
0.04
0.10
0.20
0.40
mM Substrate

Figure 20. DOUBLE RECIPROCAL PLOTS OF ESCULETIN CONCENTRATION VS. ACTIVITY FOR ISOPEROXIDASE C_4. Curve A-normal assays, curve B-assays with 0.15 mM ferulic acid. Assay solutions contained 50 mM phosphate buffer (pH 7.5), and 5 mM H_2O_2.


40
30
20
10
B
A
2
4
6
8
10
ΔO.D.469⁻¹/min
mM⁻¹ Esculetin

and ferulic acid may also compete for the same site.

Sirois and Miller (76) and Gelinas (18) have proposed reaction mechanism for the horseradish peroxidase (IAA oxidase) catalyzed oxidations of scopoletin and ferulic acid, respectively, in the presence of IAA. In both schemes, neither the oxidation of scopoletin or ferulic acid occurred in the absence of IAA, since hydrogen peroxide was generated by the initial reaction of IAA with the enzyme. A sigmoidal dependence on IAA concentration, in the presence of both scopoletin (76) and ferulic acid (18), was explained as a result of the formation of scopoletin-peroxidase or ferulic acid-peroxidase complexes which do not react as readily with IAA. It appears, though, that the mechanism proposed by Sirois and Miller (76) for the sigmoidal effect of scopoletin on IAA oxidase activity in the presence of IAA is insufficient to explain the sigmoidal scopoletin saturation curve for isoperoxidase C_4 in the absence of IAA reported here, since IAA is an integral part of that mechanism.

It would be premature to propose reaction mechanisms for the peroxidase catalyzed conversions of scopoletin and ferulic acid based on the available evidence. However, Reigh (67) has found that oxidation of scopoletin concentration at pH 4.0, but not at pH 7.5. Scopoletin and ferulic acid are structurally very similar except that ferulic acid contains a free carboxl group and is capable of donating a proton more readily than does scopoletin. Any reaction mechanism intended to explain the sigmoidal nature of the scopoletin saturation curves must account for the above observations.

The specificities of isoperoxidases from various sources toward

TABLE I-7

COMPARISON OF SUBSTRATE ACTIVITY RATIOS

Substrate	pH Optima		pH of Assay pH=4.5		pH=5.5		pH=6.0		pH=7.5	
	C_3	C_4	C_3	C_4	C_3	C_4	C_3	C_4	C_3	C_4
Guaiacol	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Esculetin	0.68	0.63	0.37	0.26	0.49	0.32	0.32	0.34	1.10	1.40
Scopoletin	0.02	0.01	0.03	0.02	0.05	0	0	0.04	0.06	0.03
Chlorogenic acid	0.53	0.46	1.00	0.86	0.68	0.66	0.60	0.38	0.67	0.61
Ferulic acid	2.10	2.20	2.70	2.90	2.70	3.20	1.90	1.90	1.70	2.40

different substrates have been resolved (2,9,34,53,54,57). Several workers have determined substrate activity ratios for individual isoperoxidases from the same source. As summarized in Table I-1, three isoperoxidases from egg plant, two of which were cathodic, exhibited activity ratios (o-dianisidine/guaiacol) which ranged from 0.08 to 7.57, and two cathodic isoperoxidases from Alaska pea also differed in their activity ratios of o-dianisidine to guaiacol (48). Since scopoletin, and chlorogenic and ferulic acids have been identified in tobacco tissue cultures (17), and esculetin has been reported to be present in tobacco leaf (81), it seemed more meaningful in the present study to compare catalytic properties of isoperoxidases C_3 and C_4 to these probable *in vivo* substrates.

Activity of C_3 and C_4 was determined for each of the natural substrates (ferulic acid, scopoletin, esculetin and chlorogenic acid) and compared to that for guaiacol. Concentrations of the preparations of C_3 and C_4 were adjusted to that concentration of each which gave the same activity with guaiacol as substrate. As indicated in Table I-7, the activity ratios are nearly identical for the two enzymes. Powell *et al.* (63) have demonstrated that activity ratios for two isoperoxidases, A_1 and A_2, from tobacco tissue culture, toward these same substrates were analogous to those reported here for C_3 and C_4. Based upon these results, and the inhibitor studies described earlier, it appears that all four of these isoperoxidases from tobacco tissue culture, namely A_1, A_2, C_3 and C_4, adhere to the strict definition of isoenzymes. That is, they are different structurally, but they have nearly uniform catalytic ability, at least with respect to all the substrates investigated in this report.

Since extinction coefficients for the various products are not known, it is impossible to determine the rates of oxidation of the different substrates quantitatively. But, scopoletin evidently is not oxidized to any significant degree at the enzyme concentrations used. Reigh (67) has described much higher scopoletin to guaiacol activity ratios, using the same assay procedures as employed here, for an anodic migrating isoperoxidase, A_3 from tobacco tissue culture. Apparently A_3 has a different affinity for scopoletin than do any of the four isoperoxidases A_1, A_2, C_3, or C_4, and, therefore, must be placed into a different group of isoperoxidases than A_1, A_2, C_3 and C_4.

Isoperoxidase C_4 was assayed according to the procedure of Lee (42) to determine if it could function to oxidize IAA. Under conditions in which commercially available horseradish peroxidase readily oxidized IAA, no activity could be demonstrated for C_4 (54). Also IAA had no effect on the guaiacol oxidizing ability of either C_3 or C_4. In view of the recent suggestion of Lee (44) that IAA oxidase and peroxidase activities in tobacco are not equivalent and should be considered separately, it appears that oxidation of IAA may not be the primary function of isoperoxidases C_3 or C_4 within the plant.

One factor in support of the involvement of peroxidase in lignification is its association with the cell membrane where lignification occurs (25). Schafer (70) has found that an isoperoxidase from tobacco tissue culture W-38, designated C_4, which is electrophoretically identical to isoperoxidase C_4 in this study, was present in higher relative proportions in membrane fractions. Based on these results, he concluded that C_4 was probably associated with the cell membrane and thus could possibly be

involved in lignification.

Ferulic acid and other p-hydroxycinnamic acids have been isolated from the natural lignin of various grasses, although no ferulic acid was found in the lignin of tobacco (78). Ferulic acid was converted *in vivo* by peroxidase, a polymer which had properties similar to natural lignin from grasses. These observations led Stafford (78) to propose that two forms of lignin exist in grasses. One, a conventional type lignin, if formed by the action of peroxidase on coniferyl alcohol and coniferyl aldehyde, and another "acid lignin" is formed by polymerization of ferulic acid and other p-hydroxycinnamic acids. Innerarity (33) found that when radioactive scopoletin was fed to tobacco tissue cultures, the label appeared in lignin isolated from these cultures.

Based on numerous studies with radioactive tracers, a pathway for the formation of lignin was constructed some time ago (8). In this scheme, ferulic acid is not an immediate precursor of lignin but must first be reduced to coniferyl alcohol, which is then polymerized to form lignin. But, the structure of lignin is varied and complex. The fact that ferulic acid has not been shown *in vivo* to be converted directly into lignin might be a consequence of the cellular location of ferulic acid in relation to peroxidases.

CHAPTER V

SUMMARY AND CONCLUSIONS

In summary, the two isoperoxidases, namely C_3 and C_4, from tobacco suspension culture WR-132 have significantly different molecular weights, and only C_4 contains a carbohydrate component. However, the two isoperoxidases were found to be virtually identical in their catalytic ability with respect to several different substrates, namely guaiacol, ferulic acid, scopoletin, chlorogenic acid and esculetin. Although all of the phenolic compounds tested were evidently oxidized by isoperoxidases C_3 and C_4 in the presence of hydrogen peroxide, ferulic acid was the preferred substrate of those investigated, based on K_m values, substrate activity ratios and the unique ability of ferulic acid to stimulate the guaiacol oxidation by C_3 and C_4. In view of the fact that both isoperoxidases utilized ferulic acid as a substrate *in vitro*, it seems probable that ferulic acid could also function as a substrate for the two enzymes *in vivo*. Although suspension cultures do not form lignin in significant amounts, one might venture the speculation that the two isoperoxidases in this study likely may be involved in one or more steps in the conversion of ferulic acid into a lignin.

II. EFFECT OF SOME PHENOLIC COMPOUNDS ON ENZYME ACTIVITIES IN RAT LUNG

CHAPTER I

INTRODUCTION

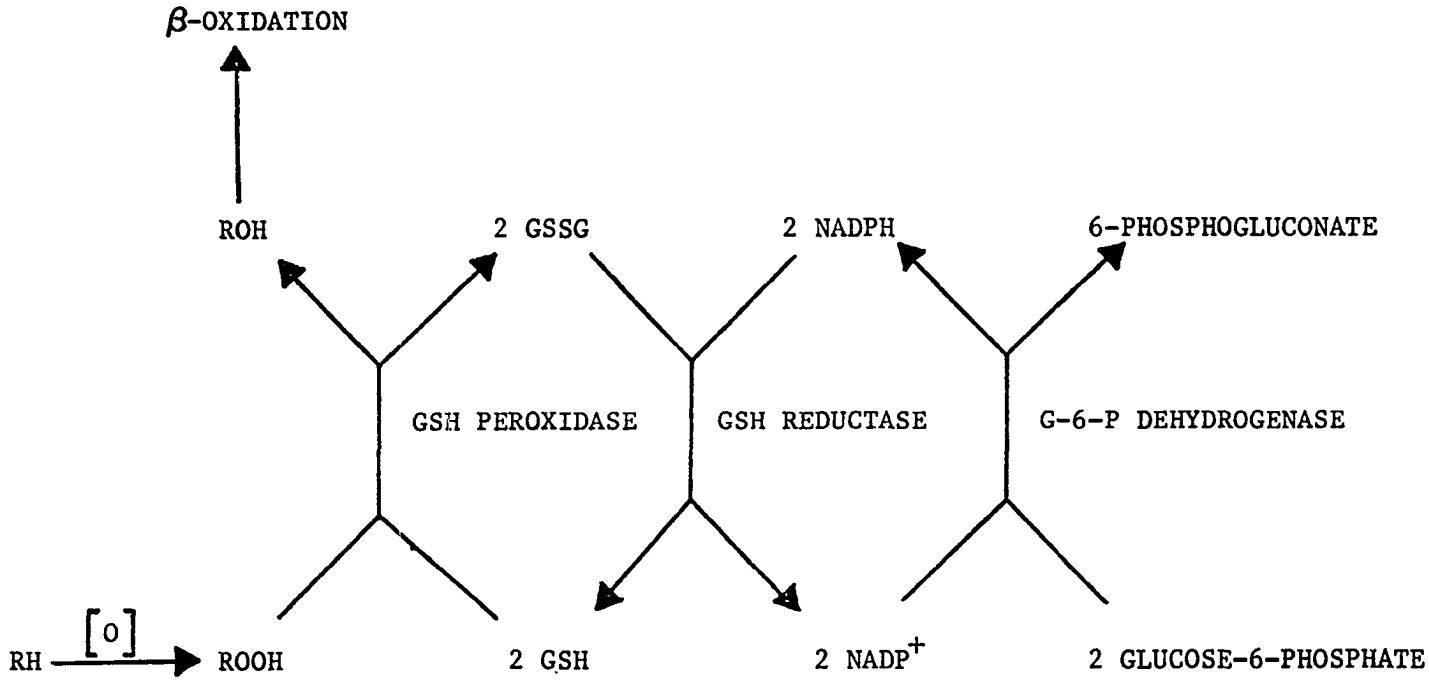
Laboratory mice are protected from lethal doses of ozone and nitrogen dioxide by prior injection of compounds containing either sulfhydryl (SH) or disulfide groups (16). DeLucia and coworkers (13) have suggested that ozone can damage lung tissue by either direct oxidation of SH groups or generation of lipid hydroperoxides by reaction with unsaturated fatty acids. These workers have discovered that acute exposure to ozone caused a reduction in the SH group concentration in rat lung and a loss in activity of enzymes such as glucose-6-phosphate (G6P) dehydrogenase and glutathione (GSH) reductase, which have SH groups at functional sites. However, prolonged exposure to ozone resulted in a 32% rise in the activity of G6P dehydrogenase. These workers further proposed that increased levels of reduced nicotine adenine dinucleotide phosphate (NADPH) by G6P dehydrogenase is involved in a cellular adaptation to maintain normal SH levels.

Glutathione has been demonstrated to reduce potentially damaging lipid peroxides in rat liver homogenates (10) and GSH peroxidase catalyzes the involved reaction (11,46). Christophersen (11) has postulated that

glutathione could thus function *in vivo* to counteract lipid peroxide damage to SH groups. Subsequently, Chow and Tappel (12) have confirmed the presence of lipid peroxides in the lungs of ozone exposed rats and they also discovered that the activities of GSH peroxidase, GSH reductase and G6P dehydrogenase are significantly increased in rat lung homogenates after prolonged ozone exposure (5-7 days). Furthermore, they proposed that these enzymes may comprise a protective mechanism to detoxify lipid peroxides which result from exposure to ozone, and other air oxidants such as nitrogen dioxide. A reproduction of this scheme is shown in Figure 21. Potentially damaging lipid peroxides are reduced by GSH peroxidase to harmless hydroxy fatty acids which are metabolized via the β-keto oxidation pathway. Increased demand for GSH and, in turn, NADPH lead to an increase in the activity of GSH reductase and G6P dehydrogenase.

In the present study a number of phenolic compounds, as well as nicotine, which are known to be present in cigarette smoke, or tobacco, or both, were investigated to determine what possible effects they might have on the activities of the above enzymes.

Figure 21



SCHEME OF LIPID PEROXIDE METABOLISM. RH, polyunsaturated fatty acid; ROOH, fatty acid hydroperoxide; ROH, hydroxy fatty acid; GSH, reduced glutathione; GSSG, oxidized glutathione.

Chow, C. K. and Tappel, A. L., Lipids, 1, 518-524. (1972).

CHAPTER II

MATERIALS AND METHODS

Enzyme Preparation

Lung homogenates were prepared according to a slight modification of the procedure of Chow and Tappel (12). Mature rats, which had been fed a normal diet, were sacrificed. Lungs were removed immediately and washed with isotonic KCl. A 20% suspension of the rat lung tissue in 0.25M sucrose containing 1 mM EDTA was homogenized at 6,000 rpm for 10 min in a Sorvall Omnimixer. The homogenate was centrifuged first at 1000 x g for 10 min and then at 105,000 x g for 90 min. The resulting clear red supernatant was used for enzyme assays.

Enzyme Assays

The assay for glutathione peroxidase was based upon the procedure of Paglia and Valentine (60). The assay solution contained 0.1 ml of 0.0084 M NADPH, 0.1 ml of 1.125 M NaN_3, 0.1 ml of 0.15 M GSH, 1.49 ml of 0.1 M phosphate buffer (pH 7.0) containing 0.01 N EDTA, 0.1 ml of 0.0022 M H_2O_2 and 0.055 ml of glutathione reductase (200 U/ml). The reaction was initiated by the addition of 0.1 ml of the enzyme preparation.

Purified glutathione reductase (Sigma) was assayed by the following procedure. The assay solution contained 0.02 M phosphate buffer (pH 7.0),

0.2% oxidized glutathione, 0.1% bovine serum albumin and 0.1 ml of 0.0084 M NADPH. The reaction was initiated by addition of the enzyme preparation, which had been diluted to give a velocity of approximately 0.1 ΔO.D./min.

The assay procedure for GSH reductase in rat lung homogenates was based upon that of Racker (65). The assay solution contained 0.05 M phosphate buffer (pH 7.6), 0.1 mM NADPH, 0.1% BSA and 0.2% oxidized glutathione in a total volume of 3.0 ml, and the reaction was initiated with 0.1 ml of enzyme preparation. Glucose-6-phosphate dehydrogenase was assayed according to the method of Brown and Wray (7), except the pH of the assay solution was 7.6 as described by Langdon (30) for erythrocytes. The assay solution contained 0.02 M Tris-HCl buffer (pH 7.5), 0.3 mM $NADP^+$, 5 mM D-glucose-6-phosphate and 5 mM $MgCl_2$, and 0.2-0.3 ml of the enzyme preparation in a total volume of 3.0 ml.

All effector compounds were prepared at a concentration of 1.2 mM in 2% (v/v) ethanol and 1.0 ml was added to each assay solution to give a final concentration of 0.4 mM. Control assay solutions contained 1.0 ml of 2% (v/v) ethanol.

Electrophoresis

Electrophoresis was accomplished by the method of Orstein and Davis using a Buchler Polyanalyst Disc Electrophoresis apparatus (59). Gels were stained for peroxidase with a solution of 2 parts 1% guaiacol, 2 parts 50 mM phosphate buffer (pH 6.0) and 1 part 0.5% H_2O_2. G6P dehydrogenase bands were visualized by the procedure of Barnes and coworkers (4) which was modified by Hoover (30). The staining solution contained 20 mM Tris-HCl buffer (pH 8.0), 1 ml of 15 mM D-glucose-6-

phosphate, 0.2 ml of 75 mM $MgCl_2$, 0.1 ml of 15 mM $NADP^+$, 0.2 ml of 0.52 mM nitroblue tetrazolium and 0.24 mM phenazine methyl sulfate in a total volume of 2.7 ml per gel. Gels were stained in the dark for 30-60 min.

CHAPTER III

RESULTS

Nicotine, as well as various phenolic compounds, known to be components of either cigarette smoke, or tobacco, or both were investigated as possible effectors of the activities of rat lung GSH peroxidase, GSH reductase and G6P dehydrogenase. As indicated in Table II-1, at a concentration of 0.4 mM, five phenolic compounds, namely scopoletin, esculetin, scopolin, esculin and chlorogenic acid, show marked inhibition of all three of the above enzymes, while none of the other compounds tested had any significant effect upon any of these enzymes. Of those phenolic compounds which demonstrated an inhibitory effect, scopoletin is the most abundant in cigarette smoke. In 1958, scopoletin was identified in the main stream smoke of 29 brands of cigarettes in common use at that time (88). Chlorogenic acid (80) and esculetin (14) have also been identified in cigarette smoke in trace amounts. Esculin and scopolin, the glucosides of esculetin and scopoletin, respectively, have not been found in cigarette smoke, though they are present in tobacco leaves (81).

The determination of GSH peroxidase activity in the rat lung homogenates involved a coupled enzyme assay procedure which utilized commerical GSH reductase. Since rat lung GSH reductase is subject to inhibition by the same five compounds which inhibit GSH peroxidase, but to a lesser degree in each case, the effects of these inhibitors on commerical GSH

TABLE II-1

EFFECT OF PHENOLIC COMPOUNDS AND NICOTINE ON ACTIVITY OF THREE ENZYMES FROM RAT LUNG

0.4 mM Effector	% of Control Activity		
	Glutathione Peroxidase	Glutathione Reductase	Glucose-6-phosphate Dehydrogenase
None	100	100	100
Scopoletin	14	37	43
Esculetin	12	45	68
Chlorogenic acid	24	33	13
Scopolin	17	55	27
Esculin	16	45	49
Salicylic acid	102	96	100
Vanillic acid	100	102	100
p-Coumaric acid	102	102	100
Nicotine	102	102	100
Caffeic acid	100	102	99
Ferulic acid	101	100	100

reductase were determined and found to be similar to those observed with GSH peroxidase. However, commercial GSH reductase could not be assayed at enzyme concentrations used in the coupled assay procedure for GSH peroxidase, since the rate of turnover of NADPH was so rapid that spectrophotometric assays were not possible. In view of these results, it is impossible to ascertain the degree of inhibition of GSH peroxidase by the five phenolic compounds. However, it appears that the inhibition observed for GSH peroxidase may be largely due to inhibition of commercial GSH reductase. More definitive results concerning inhibition of GSH peroxidase must await a direct assay procedure for this enzyme.

Using the assay procedure described here for glutathione peroxidase, isoperoxidase C_4, isolated from tobacco tissue culture WR-132, did not appear to catalyze the oxidation of glutathione.

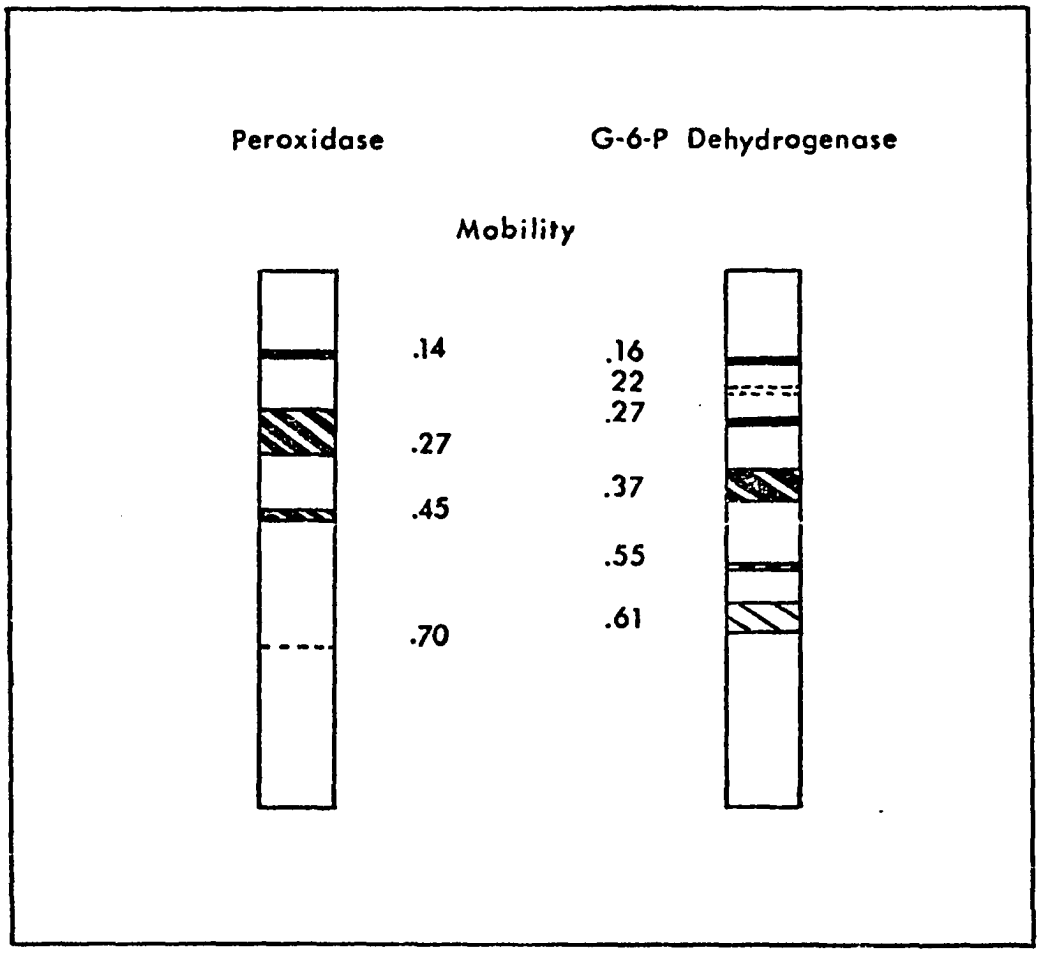
CHAPTER IV

DISCUSSION

Scopoletin, as well as scopolin, at a concentration of 0.1 mM, has been reported to substantially inhibit four $NADP^+$ specific enzymes, namely G6P dehydrogenase, isocitrate dehydrogenase, 6-phosphogluconate dehydrogenase, and malate dehydrogenase, from tobacco suspension cultures (35). Inhibition of G6P dehydrogenase was also observed with ferulic acid (15%) and esculetin (30%). More recently, Hoover (30) has found that one isoenzyme of G6P dehydrogenase was inhibited by scopoletin while another isoenzyme from the same source was stimulated by the same concentration of scopoletin. When subjected to polyacrylamide gel electrophoresis, the rat lung homogenates reveal the presence of up to 6 isoenzymes of G6P dehydrogenase as shown in Figure 22. At least two isoenzymes of GSH peroxidase are also indicated, though it is difficult to distinguish peroxidase from hemoglobin strictly on the basis of electrophoresis.

Cigarette smoke has been shown to inhibit several dehydrogenases. Sato and coworkers (69) have presented evidence that inhibition of succinic dehydrogenase by cigarette smoke was eliminated by cysteine or glutathione. Cigarette smoke also caused a reduction in activities of purified rabbit muscle glyceraldehyde-3-phosphate dehydrogenase and yeast alcohol dehydrogenase (40). The inhibition of both of these

Figure 22. ANODIC ISOENZYMES OF PEROXIDASE AND GLUCOSE-6-PHOSPHATE DEHYDROGENASE.


Peroxidase
G-6-P Dehydrogenase
Mobility
.14
.27
.45
.70
.16
22
.27
.37
.55
.61

enzymes was partially overcome by prior inhalation of the smoke. Lange (39) suggested that this observed inhibition might be due to oxidation of SH groups by peroxides which might be present in the smoke. Subsequently, peroxides have been identified in cigarette smoke (82). Tonge (83) has advanced a mechanism by which free radicals in cigarette smoke can initiate oxidation of cysteine, and presumably sulfhydryl containing proteins.

Cigarette smoke, therefore, according to the above reports, contributes to the destruction of sulfhydryl groups. In addition to this, the data presented here indicate that scopoletin, a component of cigarette smoke, significantly inhibits GSH reductase, G6P dehydrogenase and possibly GSH peroxidase in rat lung. These enzymes are involved in a protective mechanism in animals against damage to sulfhydryl groups by lipid peroxides (Figure 20). Therefore, scopoletin, as well as esculetin and chlorogenic acid, could interfere with this protective mechanism should they gain entry into the lung via cigarette smoke.

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