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THE UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

ISOLATION AND CHARACTERIZATION OF TWO ANODIC ISOPEROXIDASES 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

BERNARD LAWRENCE POWELL

Norman, Oklahoma

1974

ISOLATION AND CHARACTERIZATION OF TWO ANODIC ISOPEROXIDASES

FROM TOBACCO TISSUE CULTURE W-38

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ABSTRACT

Two anodic isozymes of peroxidase from *Nicotiana tabacum* W-38 tobacco tissue cultures have been successfully isolated utilizing a combination of DEAE-cellulose and CM-cellulose chromatography. Molecular weights of the two isoperoxidases as determined by SDS gel electrophoresis were 49,000 for isoperoxidase A_1 and 89,000 for isoperoxidase A_2. Gel filtration chromatography yielded values of 90,000 for A_2 and 103,000 for A_1 indicating that isoperoxidase A_1 might exist as a dimer. These molecular weights are higher than reported from other plant systems.

Kinetic studies with these two isoperoxidases reveal a striking similarity in response to both effectors and substrates. However, small differences in pH optima, substrate specificities, and cellular localization may be significant in defining the roles of these two isoperoxidases within the plant.

A preference for ferulic acid as substrate is indicated for both isoperoxidase A_1 and A_2. This fact, together with the substrate inhibition at high ferulic acid concentrations, may be of consequence in controlling the activity of these two enzymes *in vivo*.

CHAPTER I

INTRODUCTION

Galston and Baker in 1950 reported that indoleacetic acid (IAA) oxidase from peas was a multiple enzyme system consisting of a light-activated flavoprotein which produced hydrogen peroxide from an unknown substrate and a peroxidase which uses H_2O_2 in the oxidation of IAA.[1] Continuing studies revealed evidence that IAA oxidase from several systems was a peroxidase.[2,3] Hinman and Lang later demonstrated complete oxidation of IAA with horseradish peroxidase (HRP).[4] One year later, Bastin concluded, on the basis of kinetic experiments, that the peroxidative and IAA oxidative sites were identical.[5] While these data cannot be interpreted to mean that peroxidases from all higher plant systems are IAA oxidases, the major thrust of the research, on plant peroxidative systems, until recent years, has been in IAA metabolism.

Since the study of peroxidase in higher plants has been largely limited to the use of crude extracts in assessing the IAA oxidase activity present, the oxidation of phenolic compounds such as scopoletin was not investigaged further except in relation to a possible role in controlling the level of auxin in the plant.[6] The effect of several naturally occurring aromatic compounds such as ferulic acid, caffeic acid, and cinnamic acid upon the *in vitro* oxidation of IAA by commercially obtained HRP has been tested both before and after these compounds had been subjected to

reaction with HRP in the presence of H_2O_2.[7] This was done in hopes of correlating the *in vivo* effects of phenolic acids on growth with their effects on IAA oxidation. In a more exhaustive approach, Sirois and Miller[8] discussed, in detail, the inhibition of IAA oxidase by scopoletin. They showed a complex relationship between the concentration of substrate and inhibitor in regulating the enzyme's activity. In a similar vein, Gelinas reported that ferulic acid, which was shown to be a substrate for HRP in the presence of H_2O_2, inhibited the peroxidase-catalyzed oxidation of IAA by HRP.[9]

These reports[7,8,9] firmly established the fact that many naturally occurring phenolic compounds could be oxidized by peroxidase in the presence of H_2O_2. These include syringic acid, eugenol, vanillin, cinnamic acid, caffeic acid, and chlorogenic acid in addition to ferulic acid and scopoletin mentioned above.

The interpretation of these results is rendered extremely complex by the fact that even highly purified crystalline peroxidase can be shown to consist of several isozymic variants.[10,11,12] Studies with the isoperoxidases of peas[13] showed that the relative rates of IAA oxidation and guaiacol peroxidation were not the same among the different isozymes. While attempting to purify these isozymes from tobacco roots, Sequeira and Mineo[14] isolated afraction which possessed IAA oxidase activity but no peroxidase activity.

It is possible, but unlikely, that all of the isozymic components of peroxidase from higher plants are utilized solely for the conversion of IAA. This would be an apparent violation of metabolic economy. Although the physiological significance of multiple forms of peroxidase

remains largely unknown, the evidence revealing the oxidation of several naturally occurring phenolic compounds, in addition to IAA, makes it imperative that individual isoperoxidases be isolated and characterized, not only in respect to their physical properties, but also in regard to possible substrate specificities. The mounting evidence implicating peroxidase in the oxidation of several naturally occurring phenolic substances requires a closer look at the kinetics of such reactions with individual isoperoxidases. Data recently reported by Lee[15] indicated that the activities of IAA oxidase and peroxidase were not equivalent. This fact further emphasizes the importance of considering these activities separately.

Reigh et al.[16] have investigated in detail the kinetics of reaction of an isoperoxidase, termed A_3, from tobacco callus cultures, with scopoletin, a compound known to occur naturally in tobacco as well as many other plants. Such studies with individual isoperoxidases should provide useful information in the course of elucidating the metabolic stream of events involved in the oxidation of IAA and other naturally occurring phenolic substrates.

CHAPTER II

MATERIALS AND METHODS

W-38 Tobacco Tissue Culture

The source material for the enzymes isolated and characterized in this work was *Nicotiana tabacum* W-38 callus tissue culture. The tissue was grown on a revised medium of Linsmaier and Skoog[17] with 2 µg /1 of IAA, 200 µg /1. of kinetin and an increased amount of thiamine hydrochloride (1 mg /1). The tissue was grown at room temperature under continual subdued light. Three pieces of tissue approximately 5x5x3 mm were cut from 5-6 week old stock tissue and placed in 50 mls. of the above medium. All operations were carried out in a laminar flow hood utilizing sterile techniques.

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

Enzyme solutions were analyzed for anodic components by polyacrylamide disc gel electrophoresis utilizing a Buchler Polyanalyst Disc Electrophoresis apparatus according to the method of Ornstein and Davis.[18] Gels were composed of 7.5% acrylamide and 0.2% N,N'-methylenebisacrylamide (crosslinker). Runs were made at pH 9.3. Bromphenol blue was added to the upper buffer to serve as a tracking dye. After completion of electrophoresis, gels were placed in a mixture of 2 parts 1% guaiacol, 2 parts 50 mM sodium phosphate buffer (pH 6.0) and 1 part 0.2% H_2O_2.

Starch gel electrophoresis was accomplished using a modified Smithies apparatus.[19] The gel was 10% starch in 5 mM histidine. A constant voltage of 400 volts was applied to the gel for appoximately 4 hrs at a pH of 7.0. The peroxidase bands, both anodic and cathodic, were visualized in a solution containing 3-amino-9-ethylcarbazole following the procedure of Graham et al.[20]

Thin Layer Chromatography

Avicel SF was suspended in water (22g/100ml) and layered onto glass plates using a Desaga-Brinkmann spreader to a thickness of 0.375 mm. The chromatograms were placed in various solvents and developed in the ascending mode. Spots were detected in UV light (366 mm Blackray B-100). Chromatograms were further analyzed by exposing the plates to NH_4OH vapors before visualizing under UV light.

Enzyme Assays for Isoperoxidases A_1 or A_2 with Various Substrates

Guaiacol

The oxidation of guaiacol in the presence of isoperoxidases A_1

or A_2 was measured according to the method of Lance.[21] Addition of enzyme solution was utilized as the initiating factor. The increase in absorbance at 470 nm was measured as product was formed. The rates of reaction were observed to be linear for 4 to 6 minutes.

Ferulic Acid

A decrease in absorbance at 310 nm (Figure 17) was utilized in the assay for ferulic acid oxidation. A typical assay contained 0.2 mM ferulic acid, 5 mM H_2O_2 and 40 mM phosphate buffer pH 4.5-5.0. The assay was initiated by addition of enzyme. Linear rates were observed for 2 to 4 minutes.

Scopoletin

The assay for scopoletin oxidation was performed according to the method of Reigh et al.[16]

Chlorogenic Acid

A typical assay solution was composed of 1 mM chlorogenic acid, 5 mM H_2O_2 and 40 mM phosphate buffer pH 4.5. The assay was initiated by addition of enzyme solution. A new absorption maximum was observed at 400 nm. The increase in absorbance at this wavelength was measured and a linear rate was observed for 3 to 4 minutes.

Esuletin

A typical assay contained 1 mM esculetin, 5 mM H_2O_2 and 40 mM phosphate buffer pH 7.5. The assay was initiated by addition of enzyme. The increase in absorbance at 469 nm, where a new absorption maximum

appeared, was recorded. The observed rate of reaction was linear for 4 to 6 minutes.

All assays were performed on a Varian Techtron Model 635 UV-Visible spectrophotometer equipped with a two pen recorder and automatic cell programmer.

Chemicals

Ferulic acid was obtained from Sigma Chemical Company. No special preparation was necessary before use. Scopoletin, obtained from Sigma Chemical Company, was recrystallized from methanol. Guaiacol was obtained from Eastman Chemical Company and was redistilled before use. Avicel SF was obtained from American Viscose Division of FMC Corporation. Electrostarch was obtained from Electrostarch Company, Otto Hiller, Madison, Wisconsin. Polyclar AT was obtained from GAF Corporation, Chemical Division. DEAE-cellulose and CM-cellulose were obtained from Reeve Angel Corporation. No other chemicals required any special preparation before use. In all cases, substrate solutions were subjected to thin layer chromatography before use to verify the purity of the compound until a pattern of stability could be established. In general, most substrate solutions were stable for a period of two weeks before it became necessary to prepare new solutions.

CHAPTER III

ISOLATION AND CHARACTERIZATION OF ISOPEROXIDASES A_1 AND A_2

Anodic electrophoresis, on polyacrylamide disc gels, of a crude peroxidase preparation from W-38 tobacco callus tissue indicates the presence of at least four major bands. Based upon their electrophoretic mobilities these isoperoxidases are apparently equivalent to those designated A_1, A_2, A_3, and A_4 by Stafford and Galston.[22] Starch gel electrophoresis enables the identification of at least 4 cathodic bands and corroborates the existence of the 4 anodic bands shown by the disc gel method. Figures 1 and 2 show typical results of these two methods of analysis.

Schafer et al. reported a disparaging response to scopoletin as an effector of the guaiacol oxidizing ability of two partially purified isoperoxidases, A_1 and A_3.[23] While scopoletin stimulated the oxidation of guaiacol by isoperoxidase A_3, it had no effect on the ability of isoperoxidase A_1 to oxidize guaiacol. Later, Reigh showed that scopoletin was oxidized by A_3 in the presence of H_2O_2.[16] These data point to the importance of ascertaining the catalytic properties of individual isoperoxidases.

The isolation of isoperoxidases A_1 and A_2 was accomplished utilizing a combination of DEAE-cellulose and CM-cellulose chromatography.

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

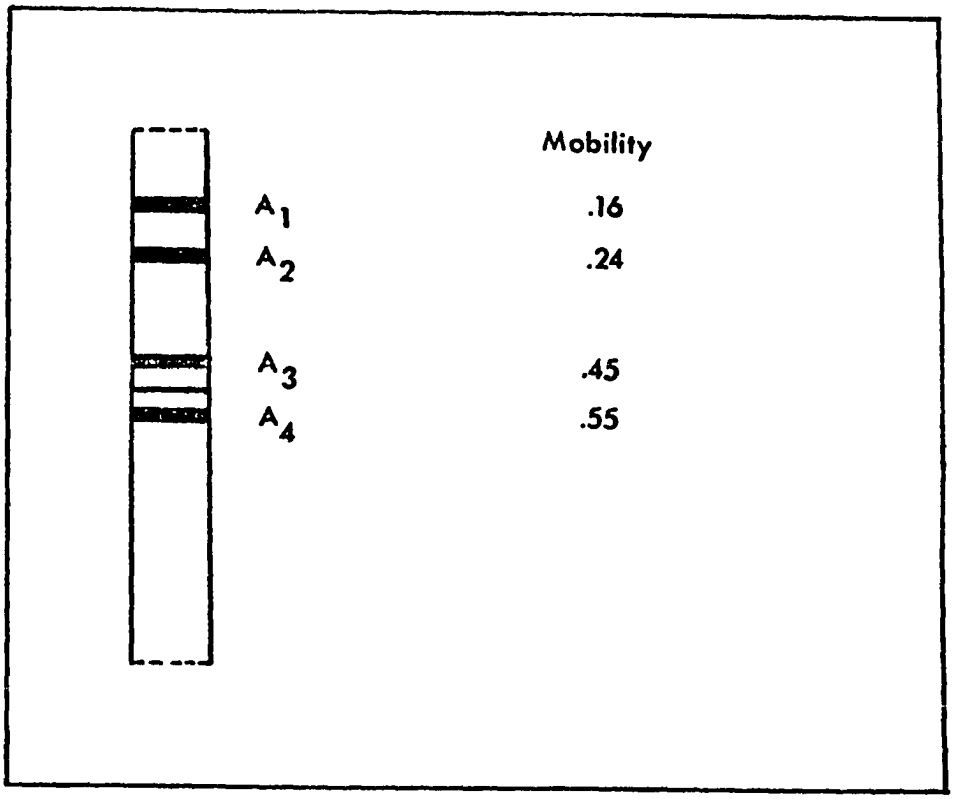
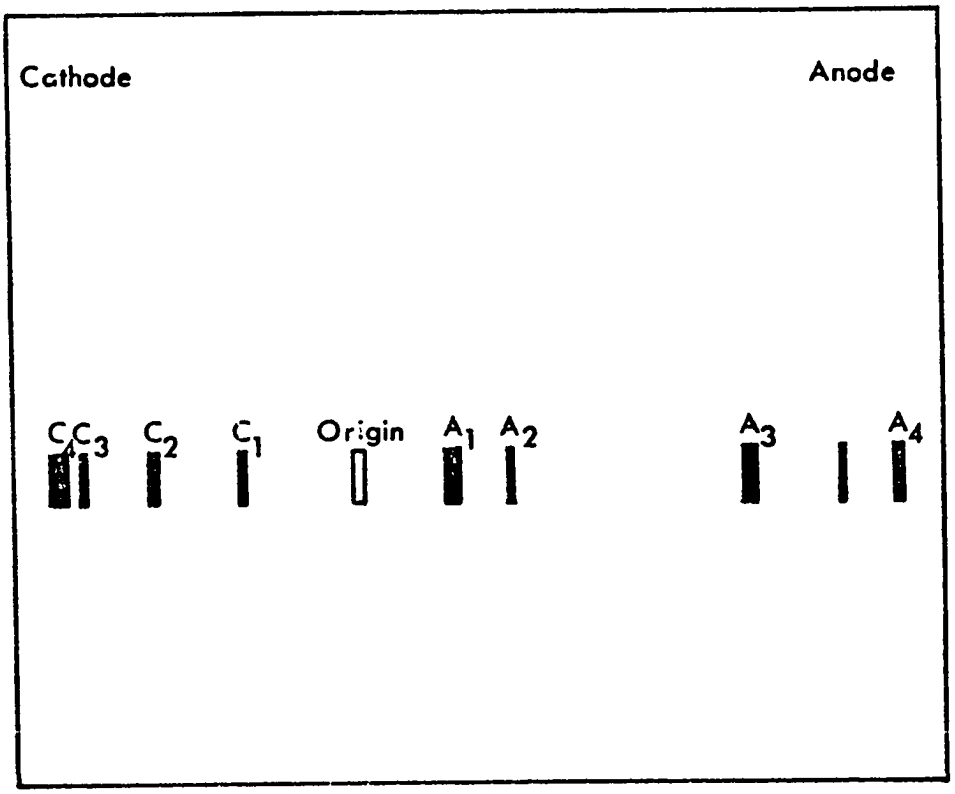

Mobility
A_1
.16
A_2
.24
A_3
.45
A_4
.55

Figure 2. STARCH GEL ELECTROPHORESIS OF ISOPEROXIDASES FROM TOBACCO TISSUE CULTURE W-38. Bands were visualized with 3-amino-9-ethyl-carbazole and H_2O_2.


Cathode
Anode
C_4 C_3
C_2
C_1
Origin
A_1
A_2
A_3
A_4

The experimental conditions were altered slightly with each successive preparation in order to optimize efficiency of separation. The most successful method is summarized in Table 1.

Preparation of Enzymes

Fifty grams of W-38 tobacco callus tissue, 20 grams of Polyclar AT, 100 mls of 50 mM phosphate buffer pH 6.0 and approximately 0.1 gram of sodium dodecyl sulfate (SDS) were homogenized in a Sorvall Omnimixer at 8000 rpm for 12 minutes. The homogenate was then centrifuged at 27,000 X g for 30 minutes. The resulting supernatant was brought to 30% saturation with $(NH_4)_2SO_4$. The solution was centrifuged at 27,000 X g for 30 minutes and the pellet was discarded. The remaining supernatant was then brought to 90% saturation with $(NH_4)_2SO_4$ and the solution centrifuged again at 27,000 X g for 30 minutes. The pellet, which contained more than 90% of the original peroxidase activity as measured by the guaiacol assay, was redissolved in as small a volume of 50 mM phosphate buffer (pH 6.0) as possible. This solution was then dialyzed against 100 volumes of distilled water for 24 hours. The water was changed once during the period of dialysis.

This sample was applied to a DEAE-cellulose column pre-equilibrated with 5 mM phosphate buffer (pH 6.0) or distilled water. Elution was begun with either 5 mM phosphate (pH 6.0) or distilled water depending upon the isoperoxidase desired. Elution with water allowed much better separation of isoperoxidase A_1, whereas initial elution with 5 mM phosphate (pH 6.0) gave significantly larger amounts of A_2. In either case the flow rate was adjusted to approximately 1 ml per

TABLE 1

ISOLATION PROCEDURE FOR ISOPEROXIDASES A_1 AND A_2

STEP 1

Homogenize tissue in 50 mM phosphate buffer (pH 6.0) for 12 minutes at 8000 rpm. Centrifuge for 30 minutes at 27,000 X g. Save supernatant.

STEP 2

Bring supernatant to 30% saturation with solid $(NH_4)_2SO_4$. Centrifuge for 30 minutes at 27,000 X g. Discard pellet.

STEP 3

Bring supernatant to 90% saturation with solid $(NH_4)_2SO_4$. Centrifuge for 30 minutes at 27,000 X g. Redissolve pellet in small volume of 5 mM phosphate buffer (pH 6.0).

STEP 4

DEAE-cellulose chromatography. Elute with 5 mM phosphate buffer (pH 6.0). Substitution of distilled H_2O as initial eluant will preferentially separate isoperoxidase A_1 at this step.

STEP 5

Analyze fractions by disc gel electrophoresis. Combine

TABLE 1 (cont.)

appropriate fractions and lyophilize.

STEP 6

Redissolve lyophilized powder. CM-cellulose chromatography. Batchwise techniques. Filter off cellulose. Save filtrate.

STEP 7

Analyze filtrate by starch gel electrophoresis.

STEP 8

Lyophilize filtrate. For kinetic analysis, redissolve lyophilized powder to appropriate activity levels.

minute and 10 ml fractions were collected. After approximately 1 liter of eluate was collected, the fractions were spot tested for guaiacol activity. The test solution consisted of 2 parts 1% guaiacol, 1 part 0.2% H_2O_2 and 2 parts 50 mM phosphate buffer (pH 6.0). Peaks of activity were analyzed by polyacrylamide disc gel electrophoresis utilizing crude extract as a reference.

Fractions containing band A_1 and A_2 respectively were combined and concentrated separately by thin film perevaporation. (In later purification procedures concentration was accomplished by lyophilization especially in preparation for SDS electrophoresis). After concentration, these samples were applied to separate CM-cellulose columns pre-equilibrated with 5 mM phosphate buffer (pH 6.0). The eluting buffer was again 5 mM phosphate, pH 6.0. As in DEAE-cellulose chromatography, fractions were spot-tested and the most active fractions were analyzed by starch gel electrophoresis to ascertain the extent of contamination, if any, by cathodically migrating isoperoxidases. Those fractions found to be free from such contamination were assayed to determine if a further concentration step was necessary. Those with sufficient activity, as measured by the guaiacol assay, were utilized directly for kinetic studies to be discussed later.

In later purification procedures, CM-cellulose chromatography was replaced with batchwise techniques which were equally successful and much less time-consuming. This practice eliminated the necessity of further concentration steps.

Because all of the isoperoxidases present in a crude extract are capable of utilizing guaiacol as a substrate in the presence of H_2O_2,

perhaps to differing degrees, it is currently impossible to determine the amount of purification achieved.

Molecular Weight Determination

Molecular weights of isoperoxidases A_1 and A_2 were determined by utilizing the SDS electrophoresis procedure of Weber, Pringle and Osborn.[24] Gels 7 cm in length were prepared with a final acrylamide concentration of 7.5%. Protein standards had molecular weights ranging from 25,000 to 130,000.

Standard protein samples and samples of isoperoxidases A_1 and A_2 were prepared, with the indicated differences, as follows:

Two mg of each protein were dissolved in 1.0 ml (0.1 ml for A_1 and A_2) of 0.01 M sodium phosphate, pH 7.0. Nine mls (0.9 ml for A_1 and A_2 sample) of 0.01 M sodium phosphate, pH 7.0, containing 1% SDS and 1% 2-mercaptoethanol were added to each sample and the tubes were capped with aluminum foil and placed in a 100°C water bath. After 2 minutes of incubation the samples were cooled to room temperature. One ml of each protein solution was then used for preparation of the sample prior to loading onto the gel. To each sample was added the following components:

0.04 ml 0.05% Bromphenol Blue

0.04 ml 2-mercaptoethanol

Several crystals of sucrose were added to each sample and 150-200 microliters of each sample (approximately 0.04 mg of protein) were applied to each gel. Electrophoresis was continued for about 4.5 hours at a current of 8 milliamps per gel.

Protein bands were visualized using the general method of Weber

et al.[24] Gels were placed in a solution of 0.25% wt/vol. Coomassie Brilliant Blue R250 (Mann) in 45% vol/vol methanol and 9% acetic acid for 3-4 hours. Gels were then placed in a destaining solution of 15% acetic acid in 10% methanol. This solution was changed several times until the protein bands were visible against the lighter background of the gel. Mobilities (M) were calculated using the following equation:

$$M = \frac{\text{Distance of Protein Migration}}{\text{Gel Length after De-staining}} \times \frac{\text{Gel Length before Staining}}{\text{Distance of Dye Migration}}$$

Semi-logarithmic plots for isoperoxidases A_1 and A_2 of molecular weight versus mobility are shown in Figures 3 and 4, respectively. Determinations were made at least two times on different preparations for both isoperoxidases. Values of 89,000 for isoperoxidase A_2 and 49,000 for isoperoxidase A_1 were consistently reproducible using this method.

Since isozymes of peroxidase from different plant systems have been shown to contain carbohydrate components,[25,26] duplicate SDS polyacrylamide gels were stained for glycoprotein in an attempt to determine if isoperoxidases A_1 and A_2 contain carbohydrate. The general procedure described by Glossmann and Neville[27] was used except the gels were allowed to incubate in Schiff's reagent for approximately 18 hours. Schiff's reagent was prepared according to the method of McGuckin and McKenzie.[28] Neither isoperoxidase A_1 nor A_2 were found to contain carbohydrate. On the other hand, isoperoxidase C_4 purified from WR-132 tobacco suspension culture was shown to contain carbohydrate using the same method.[29]

A Sephadex G-150 column prepared by the method of P. Andrews[30] in a 58 X 1.5 cm Ace glass column was used as a second independent determination of the molecular weights of isoperoxidases A_1 and A_2. The

Figure 3. DETERMINATION OF THE MOLECULAR WEIGHT OF ISOPEROXIDASE A_1 BY ELECTROPHORESIS ON SODIUM DODECYL SULFATE-POLYACRYLAMIDE DISC GELS.

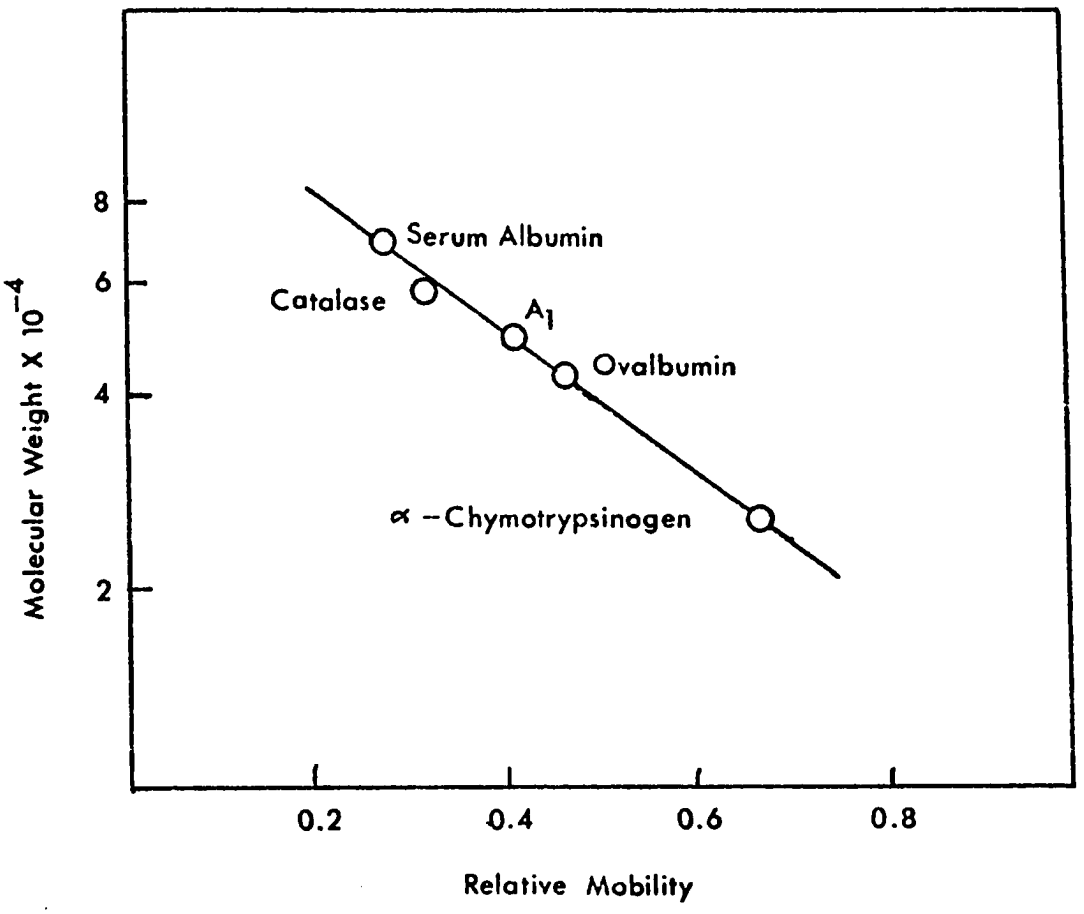
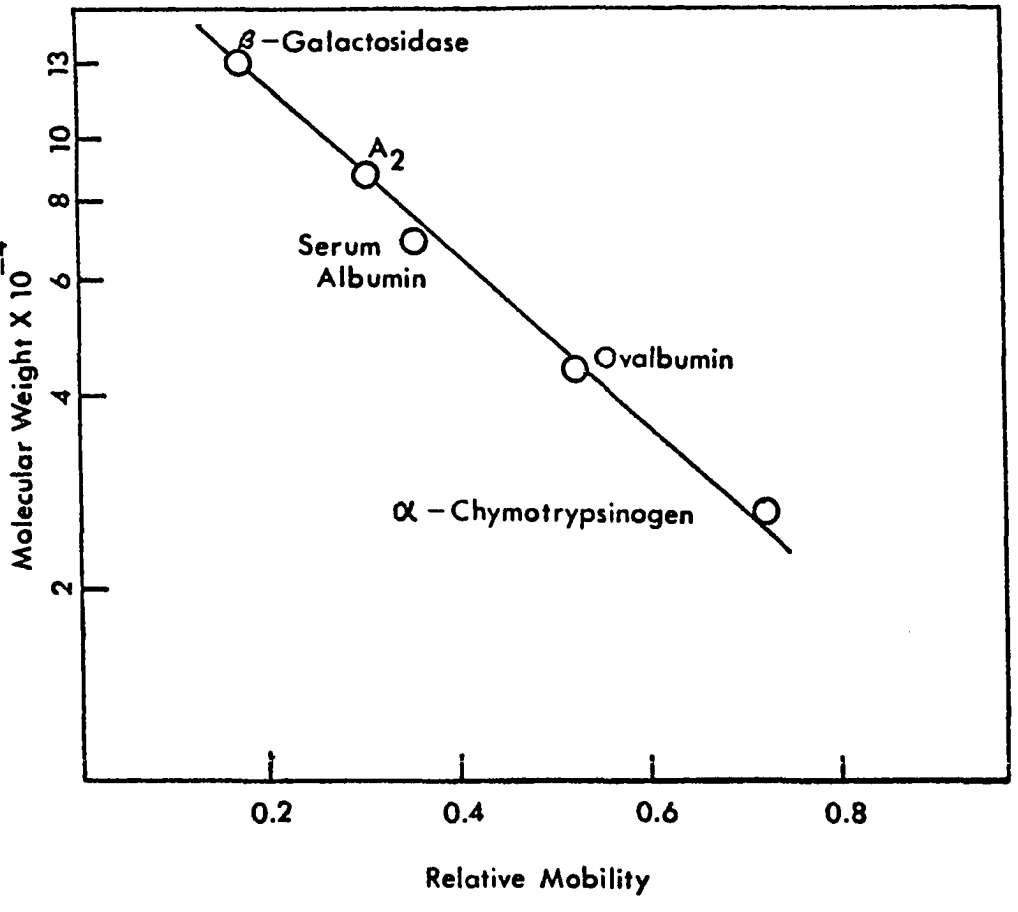

Molecular Weight X 10^{-4}
8
6
4
2
Serum Albumin
Catalase
A_1
Ovalbumin
α -Chymotrypsinogen
0.2
0.4
0.6
0.8
Relative Mobility

Figure 4. DETERMINATION OF THE MOLECULAR WEIGHT OF ISOPEROXIDASE A_2 BY ELECTROPHORESIS ON SODIUM DODECYL SULFATE-POLYACRYLAMIDE DISC GELS.


β-Galactosidase
A2
Serum Albumin
Ovalbumin
α-Chymotrypsinogen
Molecular Weight X 10^-4
13
10
8
6
4
2
0.2
0.4
0.6
0.8
Relative Mobility

equilibrating and eluting buffer was 5 mM phosphate, pH 6.0. Two mg each of solid protein standards were dissolved in 2.0 ml of the appropriate enzyme solution, either A_1 or A_2. A few crystals of sucrose were added to the solution to increase the density and the entire 2 ml sample was layered on the top of the column bed using a Pro pipettor. Separate columns were used for each enzyme. The flow rate was adjusted to approximately 20 ml/hr and 2 ml fractions were collected. The absorbance of each fraction at 280 nm was measured to determine the presence of protein. The peroxidase activity of appropriate fractions was measured to ascertain the elution volume of isoperoxidase A_1 or A_2. The molecular weights of A_1 and A_2 were obtained from semi-logarithimic plots of molecular weight versus elution volume as shown in Figures 5 and 6.

The value of 90,000 for isoperoxidase A_2 obtained by this method is in excellent agreement with that of 89,000 from SDS electrophoresis. However, the molecular weight of A_1 was shown to be 103,000, or approximately twice the value given by SDS electrophoresis. Since SDS is known to disrupt subunit structure in proteins, a possible dimeric structure for isoperoxidase A_1 is suggested.

Table 2 is a comparison of the molecular weights of several isoperoxidases from various species. A cursory look at the values listed reveals one striking point. The values of 103,000 for isoperoxidase A_1 and 90,000 for isoperoxidase A_2 are higher than reported from other plant systems. The values of 54,000 for isoperoxidase A_3 from W-38 reported by Reigh,[31] and 68,000 and 44,000 for isoperoxidase C_3 and C_4, respectively, from WR-132 tobacco suspension culture reported by Pickering,[29] are much smaller than those for A_1 and A_2. The possibility that these large

Figure 5. DETERMINATION OF THE MOLECULAR WEIGHT OF ISOPEROXIDASE A_1 BY GEL FILTRATION CHROMATOGRAPHY ON SEPHADEX G-150.

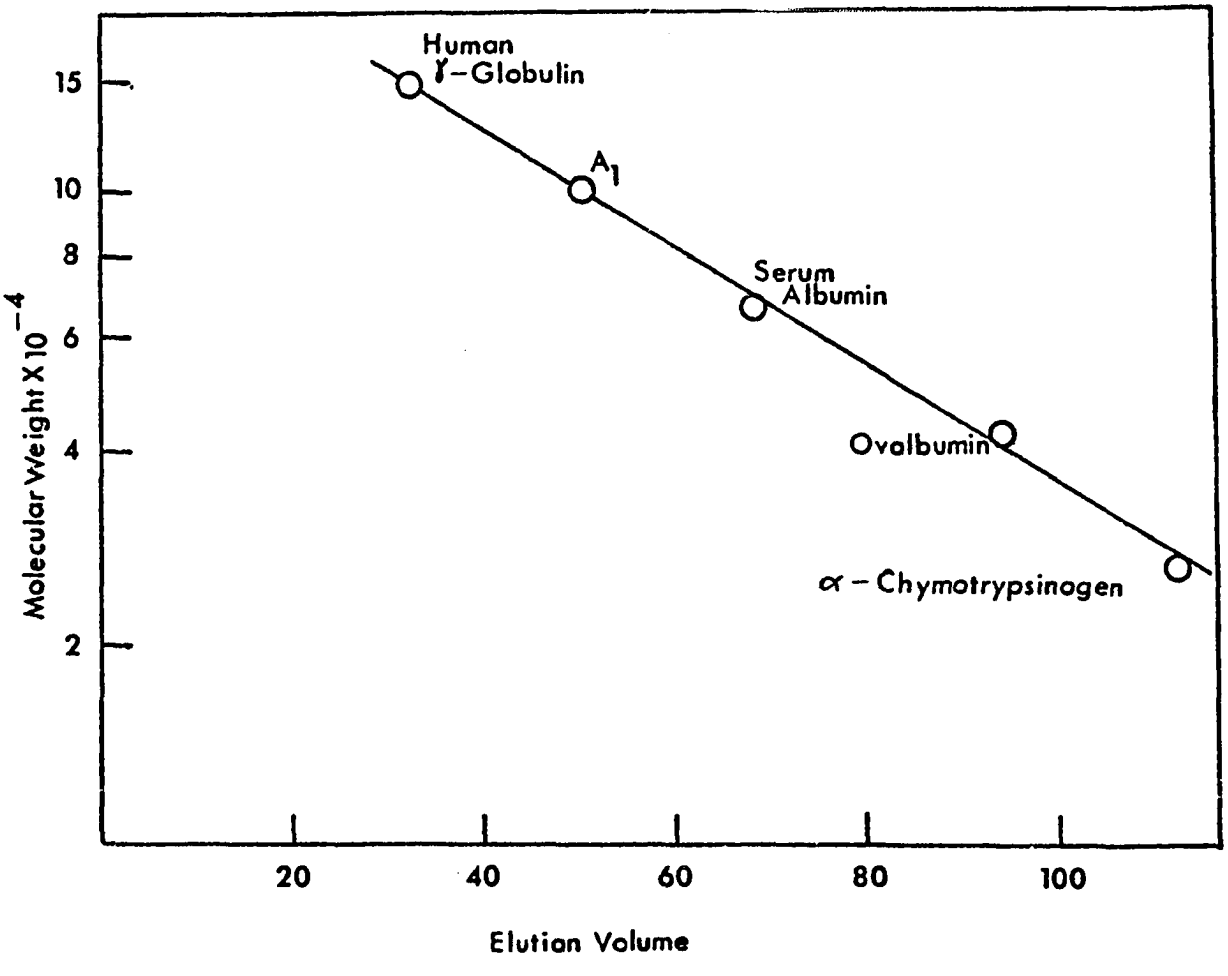

Human
γ-Globulin
A1
Serum
Albumin
Ovalbumin
α-Chymotrypsinogen
15
10
8
6
4
2
Molecular Weight X 10⁻⁴
20
40
60
80
100
Elution Volume

Figure 6. DETERMINATION OF THE MOLECULAR WEIGHT OF ISOPEROXIDASE A_2 BY GEL FILTRATION CHROMATOGRAPHY ON SEPHADEX G-150.

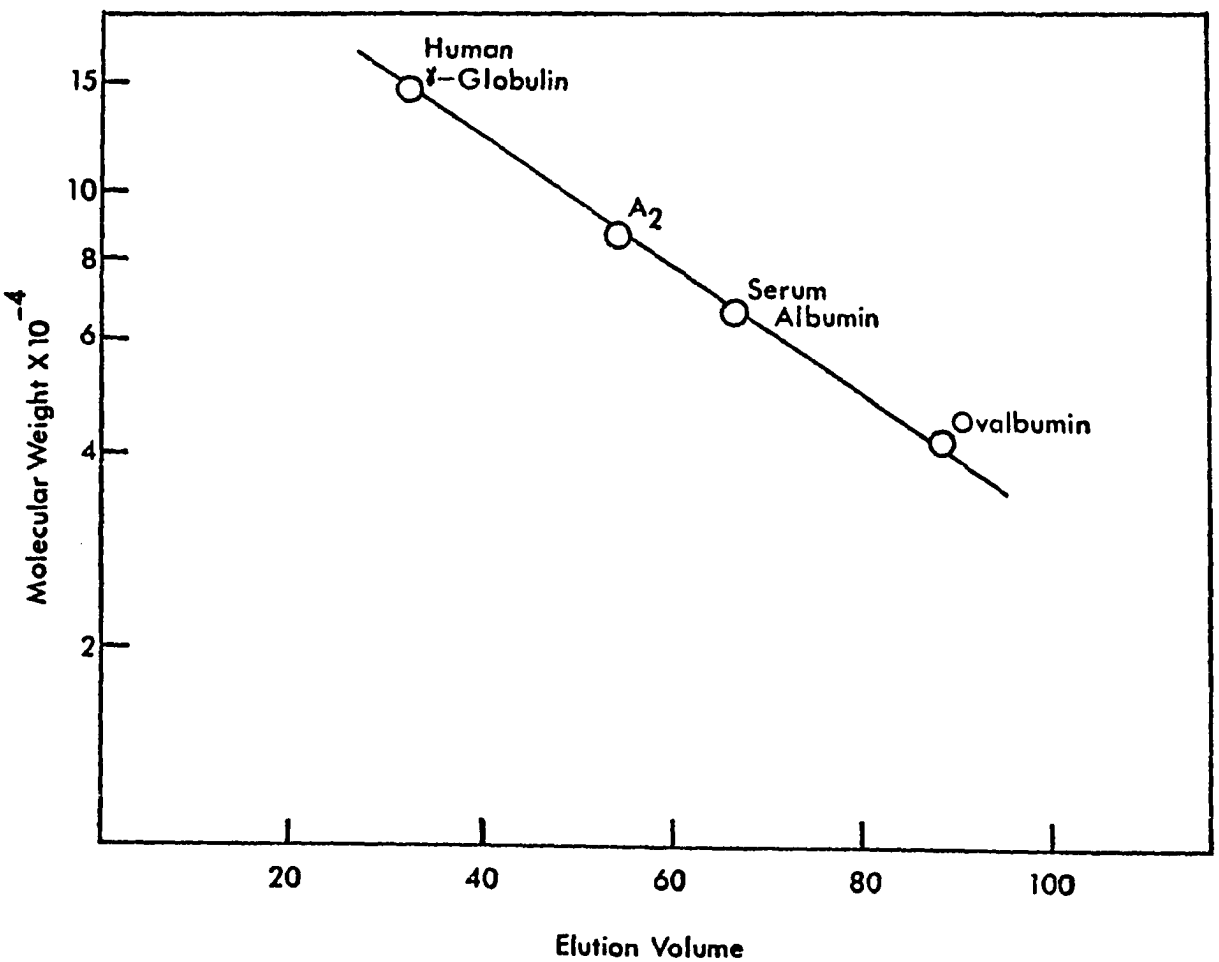

Human
γ-Globulin
A2
Serum
Albumin
Ovalbumin
15
10
8
6
4
2
Molecular Weight X 10^-4
20
40
60
80
100
Elution Volume

TABLE 2

MOLECULAR WEIGHTS OF ISOPEROXIDASES

FROM VARIOUS SOURCES

SOURCE	ISOZYME NOTATION	MOLECULAR WEIGHT	REFERENCE
Tobacco tissue culture W-38	A_1	103,000	—
	A_2	89,000	—
	A_3	54,000	31
Tobacco tissue culture WR-132	C_3	68,000	
	C_4	44,000	29
Horseradish	I, II, III, IV	40,000	32
	A_1, A_2, A_3		
	B, C, D, E	40,000	25
Wheat germ	Peroxidase 556	45,000	
	Peroxidase 566	32,000	33
Turnip	TP-A_1	49,000	
	TP-A_2	45,000	
	TP-D	43,000	34
Japanese radish	JRP-a	54,500	35
	3 and 5	44,000	
	12	40,500	
	15 and 16	30,000	36

TABLE 2 (cont.)

SOURCE	ISOZYME NOTATION	MOLECULAR WEIGHT	REFERENCE
Root of *Vicia lens*	None	50,000	37
Red alga *Cystoclonium purpureim*	None	40-50,000	38
Green alga *Enteromorpha linza*	None	220,000	39

molecular weight isozymes could be precursors for smaller molecular weight isoperoxidases in W-38 tobacco tissue cannot be discounted. Another notable point among peroxidase isozymes from the same species, namely those from horseradish, Japanese radish and turnip is the remarkably similar molecular weights. This trend is not evident in isoperoxidases from W-38 tobacco tissue culture.

To determine if sufficient quantities of isoperoxidase A_1 or A_2 could be isolated from W-38 tobacco tissue to permit amino acid analysis a preparation of isoperoxidase A_1, free from other protein as determined by SDS-gel electrophoresis, was subjected to preliminary amino acid analysis. Although the relative proportions of amino acids are significant, complete analysis was not possible because of some non-protein contaminant present in the A_1 sample analyzed. This contaminant is quite probably CM-cellulose which could have come from CM-cellulose batchwise techniques performed just prior to lyophilization. Extraction of a larger quantity of W-38 tissue combined with an ultracentrifugation step after CM-cellulose batchwise chromatography should increase the yield of enzyme and eliminate the CM-cellulose contamination.

The results of the preliminary analysis shown in Table 3 reveal the expected results for a protein with net negative charge in that relatively large amounts of aspartate and glutamate are indicated. The values presented for each amino acid are relative to that of phenylalanine which was arbitrarily set at one. The presence of large amounts of acidic amino acids is in good agreement with a similar analysis of isoperoxidase A-1 from HRP. Values for the basic amino acids arginine, lysine and histidine are not reported because they were present in amounts so small as

TABLE 3

COMPARISON OF THE AMINO ACID COMPOSITION

OF ISOPEROXIDASE A_1 FROM W-38 AND A-1 FROM HRP*

AMINO ACID	A_1 W-38**	A-1 HRP***
Aspartic acid	3.5	2.5
Threonine	3.5	2.5
Serine	1.0	2.5
Glutamic acid	3.0	1.5
Proline	2.5	1.0
Glycine	3.0	1.5
Alanine	2.5	2.5
Valine	2.5	1.0
Methionine	1.5	0.5
Isoleucine	2.0	1.0
Leucine	3.0	1.5
Tyrosine	1.0	0.5
Phenylalanine	1.0	1.0

*The values presented for each amino acid are relative to that of phenylalanine which was arbitrarily set at 1.0.

**Data was compiled from preliminary amino acid analysis of A_1 from W-38.

***Data was calculated from amino acid analysis presented in reference 25.

to defy detection in the sample which was analyzed. This fact indicates that the protein should exhibit a rather large net negative charge. However, electrophoretic mobilities on disc and starch gel do not support this finding. This apparent discrepancy is resolved if one realizes that the reported amounts of aspartic and glutamic acids include the corresponding amides. No cysteine was found, but this appears to be somewhat suspect in view of the effect of SH reagents, such as 2-mercaptoethanol, upon the activity of the enzyme.

Even though the results of preliminary amino acid analysis were not completely satisfactory, the experiment did prove that such an approach is entirely feasible and should be extended to include all but the most minor isoperoxidases present in W-38 tobacco callus tissue.

CHAPTER IV

KINETICS OF ISOPEROXIDASES A_1 AND A_2 WITH THE SYNTHETIC SUBSTRATE GUAIACOL

Initial studies with preparations of isoperoxidases A_1 and A_2 free from other contaminating isoenzymes were begun with the synthetic substrate guaiacol. The oxidation of guaiacol in the presence of isoperoxidases A_1 and A_2 was followed spectrophotometrically at 470 nm by the method of Lance.[21] The effect of pH on the guaiacol oxidizing ability of the two isoperoxidases is shown in Figures 7 and 8. Figures 9 and 10 illustrate the substrate saturation curves for isoperoxidases A_1 and A_2 respectively, utilizing guaiacol as substrate. Lineweaver-Burk[40] plots of these data are linear and yield $S_{0.5}$ values of 5.0 mM and 5.8 mM for the oxidation of guaiacol by isoperoxidases A_1 and A_2 respectively (Figures 11 and 12). An increase in the concentration of H_2O_2 from 5 mM to 10 mM and finally to 20 mM causes a significant change in the $S_{0.5}$ value for the oxidation of guaiacol in the presence of isoperoxidase A_2 ($S_{0.5}$=8.5 mM at 10 mM H_2O_2; $S_{0.5}$=10.3 mM at 20 mM H_2O_2). In addition, the maximum velocity is also increased as H_2O_2 concentration is increased (V_{max}=.095 at 5 mM H_2O_2; V_{max}=.123 at 10 mM H_2O_2; V_{max}=.125 at 20 mM H_2O_2). On the other hand, isoperoxidase A_1 reveals only slight alterations in the $S_{0.5}$ or V_{max} values when the H_2O_2 concentration is increased. Such results indicate a direct effect on binding of guaiacol

Figure 7. EFFECT OF pH ON THE GUAIACOL OXIDIZING ABILITY OF ISOPEROXIDASE A_1. Assays were run at 20 mM guaiacol and 5 mM H_2O_2 in 50 mM phosphate ⊙ or citrate ○ buffer.

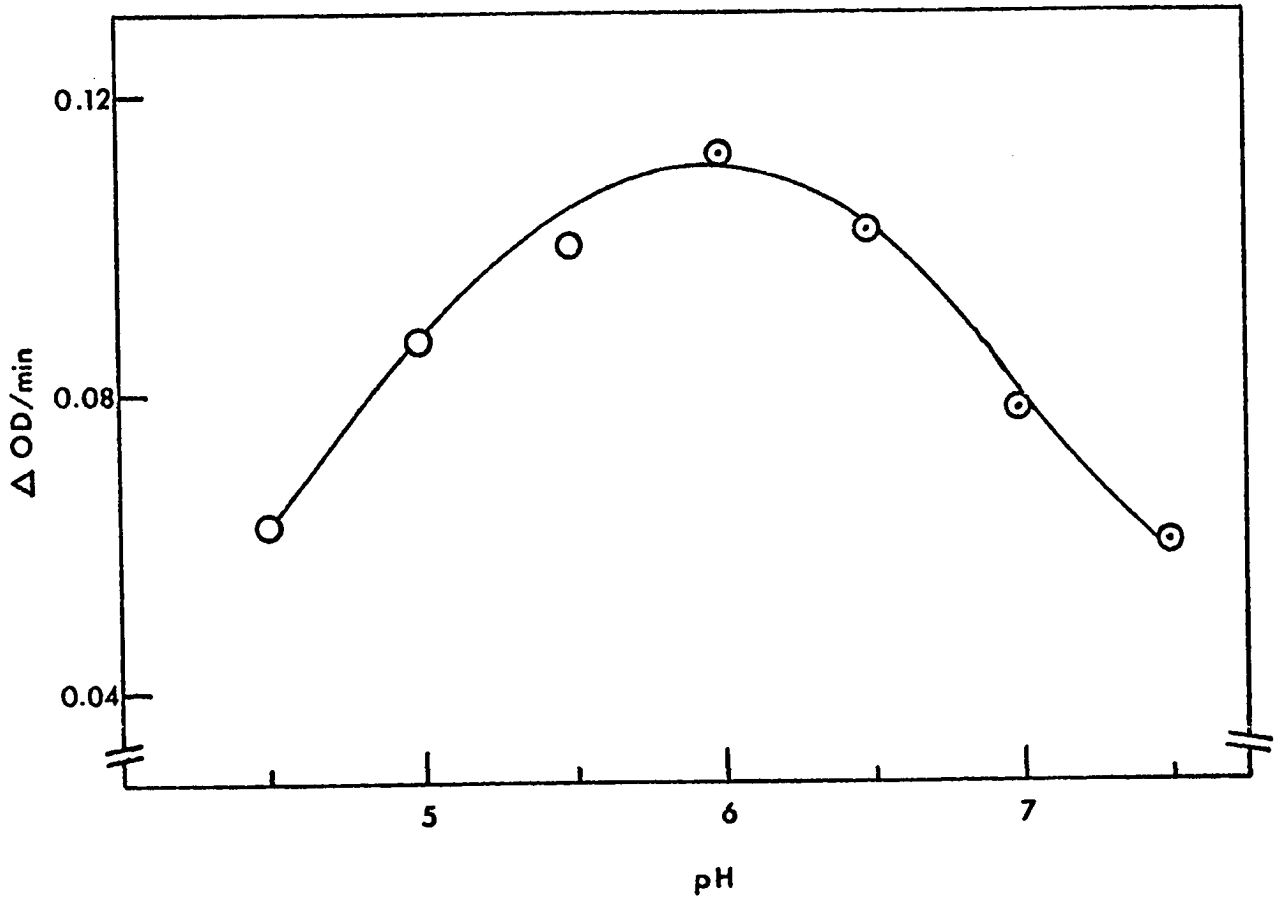

0.12
0.08
0.04
Δ OD/min
5
6
7
pH

Figure 8. EFFECT OF pH ON THE GUAIACOL OXIDIZING ABILITY OF ISOPEROXIDASE A_2. Assays were run at 20 mM guaiacol and 5 mM H_2O_2 in 50 mM phosphate ⊙ or citrate ○ buffer.

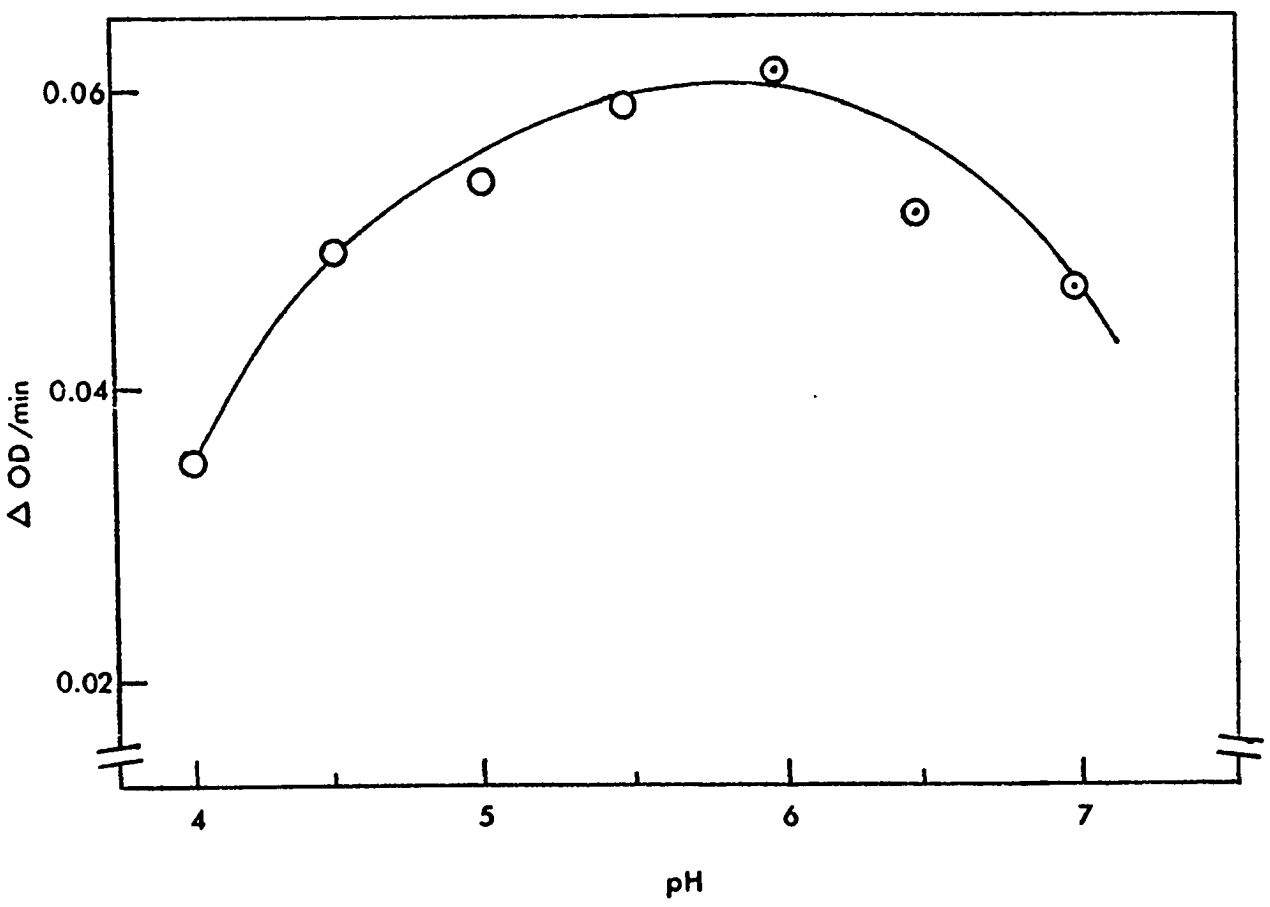

0.06
0.04
0.02
Δ OD/min
4
5
6
7
pH

Figure 9. GUAIACOL SUBSTRATE SATURATION CURVE FOR ISOPEROXIDASE A_1. Assays were run at 5 mM H_2O_2 in 50 mM phosphate buffer, pH 6.0.

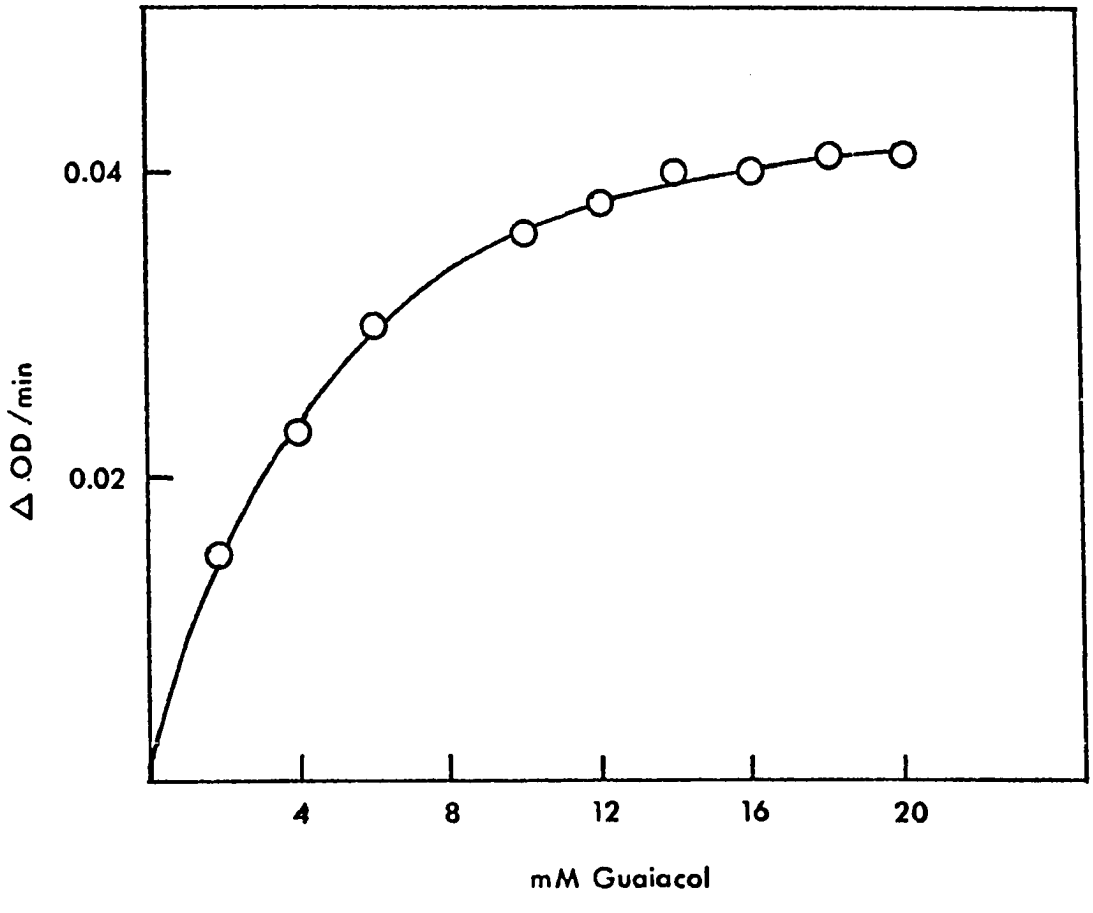

0.04
0.02
Δ OD / min
4
8
12
16
20
mM Guaiacol

Figure 10. GUAIACOL SUBSTRATE SATURATION CURVE FOR ISOPEROXIDASE A_2. Assays were run at 5 mM H_2O_2 in 50 mM phosphate buffer, pH 6.0

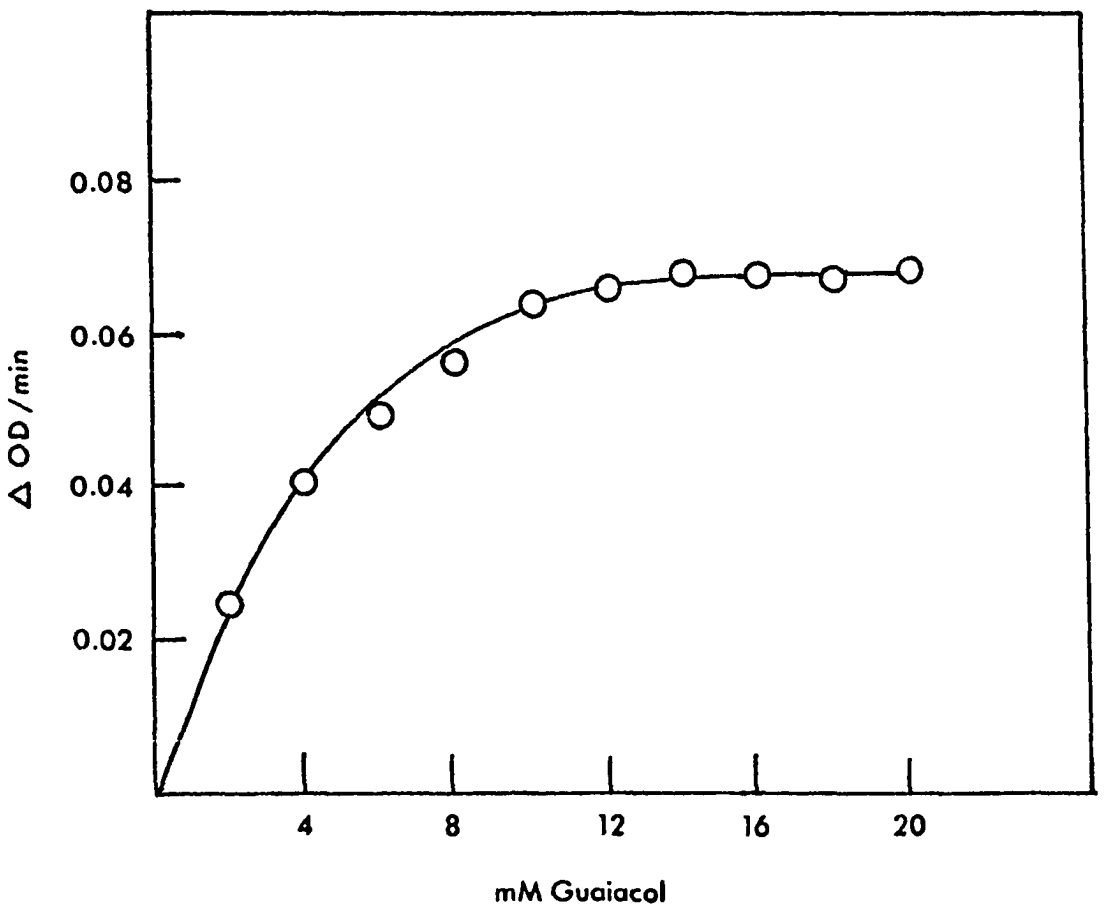

0.08
0.06
0.04
0.02
Δ OD / min
4
8
12
16
20
mM Guaiacol

Figure 11. DOUBLE RECIPROCAL PLOT OF VELOCITY VERSUS GUAIACOL CONCENTRATION FOR ISOPEROXIDASE A_1. Assays were run at 5 mM H_2O_2 in 50 mM phosphate buffer, pH 6.0.

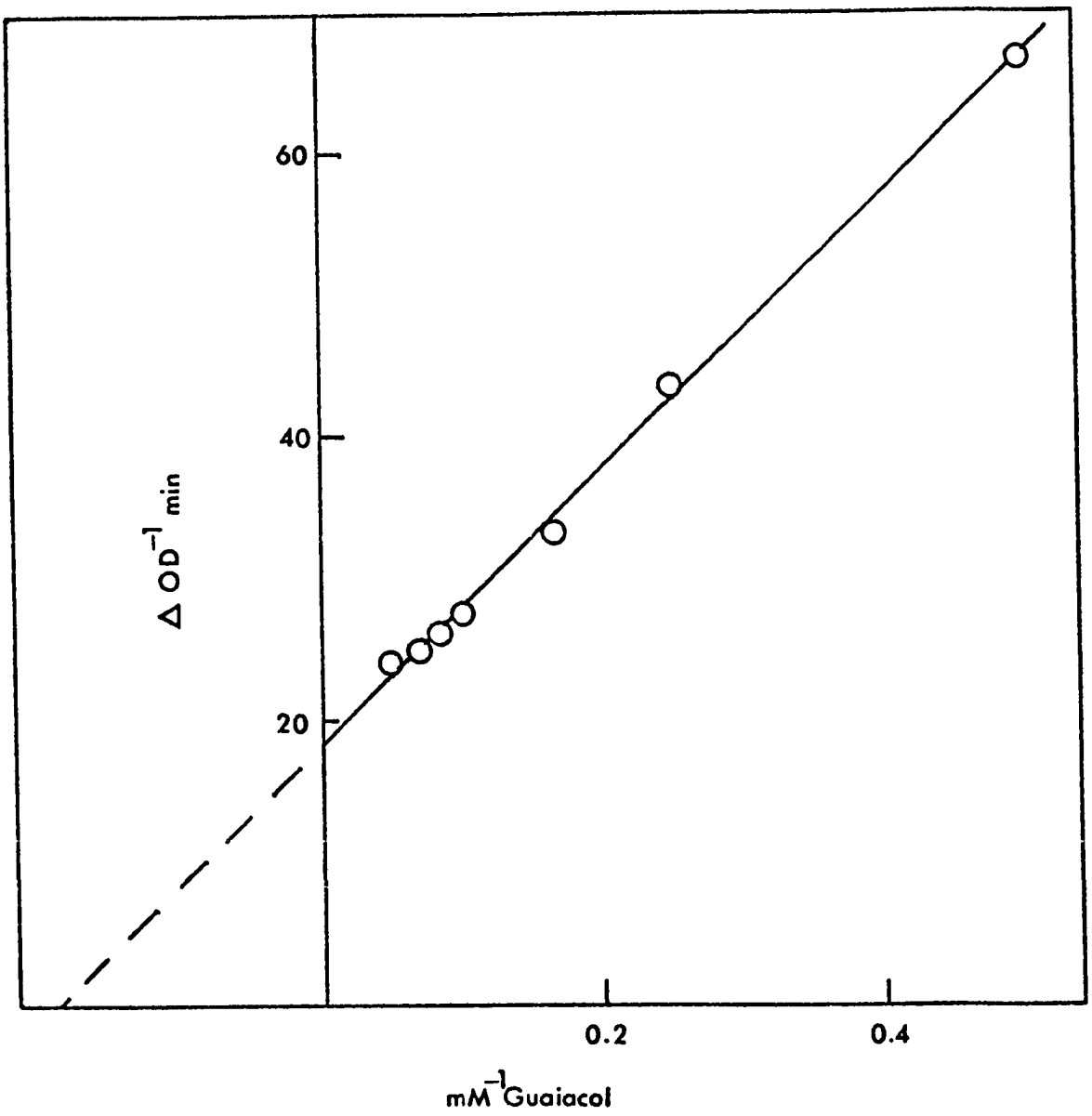
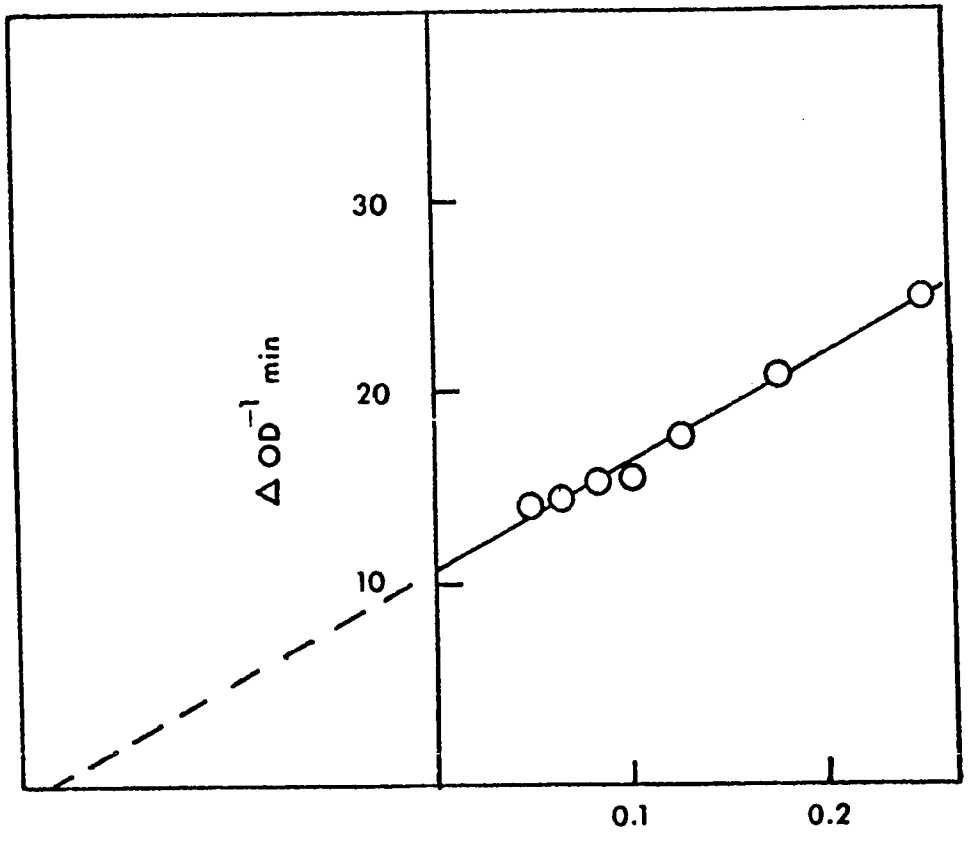

60
40
20
Δ OD^{-1} min
0.2
0.4
mM^{-1} Guaiacol

Figure 12. DOUBLE RECIPROCAL PLOT OF VELOCITY VERSUS GUAIACOL CONCENTRATION FOR ISOPEROXIDASE A_2. Assays were run at 5 mM H_2O_2 in 50 mM phosphate buffer, pH 6.0.


30
20
10
Δ OD^{-1} min
0.1
0.2
mM^{-1} Guaiacol

as a consequence of H_2O_2 binding in the case of isoperoxidase A_2. However, isoperoxidase A_1 doe not seem to be susceptible to such effects in response to increased concentrations of H_2O_2.

The increase in maximum velocity together with the increase in $S_{0.5}$ indicates that the response to increasing H_2O_2 concentrations is a complex one and not describable by any simple model yet devised. The effect may be transmitted through some alteration of a key group or groups on the enzyme molecule by such relatively high H_2O_2 concentrations.

Because of the fact that most IAA oxidases appear to be peroxidases, isoperoxidases A_1 and A_2 were tested to determine if they too could function as IAA oxidases. Using the method of Ray,[41] in which IAA and H_2O_2 were incubated with enzyme, no change in absorbance at 262 nm was observed even when using enzyme preparations 10 times more concentrated than those used for measuring guaiacol activity.

The method of Lee[42] using $MnCl_2$, IAA and 2,4-dichlorophenol failed to show any change in color, after 30 minutes incubation, when combined with Salkowski color reagent. Commercial HRP solutions were shown to oxidize IAA by both of the above methods.

It was reported some time ago[43] that IAA oxidase activity of crude peroxidase is stimulated by moncphenols and inhibited by o- and p-diphenols. Since then, many phenolic compounds have been reported to affect the activity of peroxidase preparations.[7,8,9,44] In an effort to determine the possible physiological role of isoperoxidases A_1 and A_2, several of these phenolic compounds were investigated for their effects on the guaiacol oxidizing capabilities of the enzymes. The reaction mixture contained 6 mM guaiacol, 5 mM H_2O_2 and 0.4 mM effector. The

TABLE 4

EFFECT OF VARIOUS COMPOUNDS

ON THE GUAIACOL OXIDIZING ABILITY OF ISOPEROXIDASES A_1 AND A_2

EFFECTOR	COMMON NAME	% OF CONTROL ACTIVITY A_1	A_2
No effector		100	100
0.4 mM Substituted benzoic acids			
2,3 di-OH		32	27
2,6 di-OH		96	96
3,4 di-OH		15	12
3,4,5 tri-OH		9	10
2,4,6 tri-OH		90	93
3-OH, 4-Me		103	103
3,4 di-Me		111	104
4-OH, 3-OMe	Vanillic acid	107	104
3-OH, 4-OMe	Isovanillic acid	95	86
0.4 mM Substituted cinnamic acids			
4-OH	p-Coumaric acid	126	129
3,4 di-OH	Caffeic acid	201	191
4-OH, 3-OMe	Ferulic acid	324	310
0.4 mM Substituted caffeic acids			
Hydrocaffeic acid		9	8
Caffeic acid, ethyl ester		13	13

TABLE 4 (cont.)

EFFECTOR	COMMON NAME	% OF CONTROL ACTIVITY A_1	A_2
0.4 mM Substituted ferulic acids			
Hydroferulic acid		50	50
Isoferulic acid		95	93
3-O-glucosyl ferulic acid		100	100
0.4 mM Substituted coumarins			
6,7 di-OH	Esculetin	14	15
7-OH, 6-O-glucosyl	Esculin	98	98
6-OMe, 7-OH	Scopoletin	100	100
6-OMe, 7-O-glucosyl	Scopolin	100	100
0.4 mM			
Indole-3-acetic acid	IAA	93	86
0.4 mM			
Chlorogenic acid		5	4
1.0 mM Sulfur-containing compounds			
2-mercaptoethanol		0	0
L-cysteine		0	0
L-methionine		100	100

results of this study are summarized in Table 4. Both enzymes exhibit strikingly similar responses to the effectors utilized in this study. Stimulation is observed with ferulic acid, caffeic acid and p-coumaric acid, with ferulic acid exerting the most pronounced effect at 300% of the control activity. Reigh reported activation of isoperoxidase A_3, isolated from the same tissue culture system, by ferulic acid of only 180% of control.[31] In addition, scopoletin was shown to stimulate isoperoxidase A_3 by 65%, whereas it had no effect on either A_1 or A_2.

Chlorogenic acid and other compounds having an o- diphenol configuration (esculetin, 3,4-dihydroxybenzoic acid, the ethyl ester of caffeic acid, and 2,3-dihydroxybenzoic acid) inhibit in varying degrees the ability of isoperoxidases A_1 and A_2 to oxidize guaiacol. Chlorogenic acid, however, is by far the best inhibitor of those tried. In contrast to the inhibition exhibited by other o- diphenol compounds studied, caffeic acid, at 0.4 mM concentration, strongly stimulated the activity of these two isoperoxidases in their ability to oxidize guaiacol. Esterification of the carboxyl group of caffeic acid or hydrogenation of the side chain double bond, however, resulted in the usual inhibition by o-diphenols.

Of the two unsubstituted monophenol compounds tested, p-coumaric acid stimulated enzyme activity as expected; whereas, p-hydroxybenzoic acid actually exhibited a very slight inhibition. Variations in effect on activities of isoperoxidases A_1 and A_2 were also found among the 4-hydroxy-3-methoxy compounds investigated. Of these, ferulic acid was very highly stimulatory on the guaiacol oxidizing ability of A_1 and A_2; hydroferulic acid was inhibitory; and isoferulic acid was slightly inhibitory. Glucosidoferulic acid, scopolin and scopoletin showed neither stimulatory

nor inhibitory action. Thus, although many 4-hydroxy-3-methoxy-phenyl and unsubstituted monophenolic and diphenolic compounds exert substantial influence in varying degrees on inhibition or stimulation of the guaiacol oxidizing capabilities of isoperoxidases A_1 and A_2, it is evident that these phenolic features alone are not the sole determinants of stimulation or inhibition for these two isoperoxidases.

The effect of 2-mercaptoethanol and cysteine upon the ability of isoperoxidases A_1 and A_2 to oxidize guaiacol is quite noteworthy. Both compounds completely inhibit the activity of these two enzymes, indicating the presence of a critically important SH group at or near the active site. The inhibitory effect of 1 mM cysteine disappears after approximately 10 minutes with 90% of the activity being recovered. Preincubation of cysteine with H_2O_2 for 10 minutes eliminates the inhibition entirely. Reaction of this incubation mixture with p-chloromercuribenzoate at various times reveals that cysteine is being destroyed by H_2O_2. If cysteine concentration is increased to 4 mM, the inhibition is complete, and none of the activity is recovered.

Further investigation of the high degree of stimulation of the guaiacol oxidizing ability of isoperoxidase A_1 and A_2 by ferulic acid, revealed several interesting aspects. The stimulation by ferulic acid disappears on preincubation of the enzyme with ferulic acid and H_2O_2 for 10 minutes, suggesting that the enzyme is capable of the oxidative destruction of ferulic acid in the presence of hydrogen peroxide. Incubation of the enzyme with ferulic acid alone, or H_2O_2 alone, does not have any effect on the observed stimulation. The addition of either isoperoxidase A_1 or A_2 to a solution containing 0.8 mM ferulic acid and H_2O_2

causes rapid conversion of ferulic acid to a yellow product absorbing at 485 nm. When the reaction mixture was subjected to thin layer chromatography, ferulic acid was found to have been converted to an unknown compound which fluoresces green under UV light after exposure to NH_4OH vapors. These data confirm that ferulic acid may act as a substrate for isoperoxidases A_1 and A_2.

Initial attempts to develop an assay based on the ability of these enzymes to oxidize ferulic acid were not completely successful. Therefore, studies were initiated to determine what effect different concentrations of ferulic acid had on the ability of the enzyme A_2 to oxidize guaiacol. Variation of the ferulic acid concentration at constant guaiacol concentration (6 mM) produces a Michaelis-Menten type saturation curve for isoperoxidase A_2 (Figure 13). The same results are obtained with isoperoxidase A_1. The ferulic acid concentration which yields one half of maximal stimulation is 0.06 mM. This value may be related to the $S_{0.5}$ of the enzyme for ferulic acid as a substrate. The experiment was repeated at guaiacol concentrations of 1, 2, 4, 8 and 10 mM. A graphical presentation of these data is shown in Figure 14. Analysis of the figure reveals that the ferulic acid concentration which gives the largest % activation is 0.4 mM at all concentrations, maximum activation is obtained with 0.2 mM ferulic acid. Indeed, the combination of 1 or 2 mM guaiacol with 0.2 mM ferulic acid yields a higher degree of stimulation than any other tested. Higher concentrations of ferulic acid at this low guaiacol concentration actually begin to inhibit the activation of the guaiacol activity.

Since it had already been show that ferulic acid can act as a substrate for isoperoxidase A_2, the above data seem to indicate that, at guaiacol concentrations less than $S_{0.5}$, concentrations of ferulic acid

Figure 13. EFFECT OF FERULIC ACID CONCENTRATION ON THE OXIDATION OF GUAIACOL IN THE PRESENCE OF ISOPEROXIDASE A_2. Assays were run at 6 mM guaiacol and 5 mM H_2O_2 in 50 mM phosphate buffer, pH 6.0.

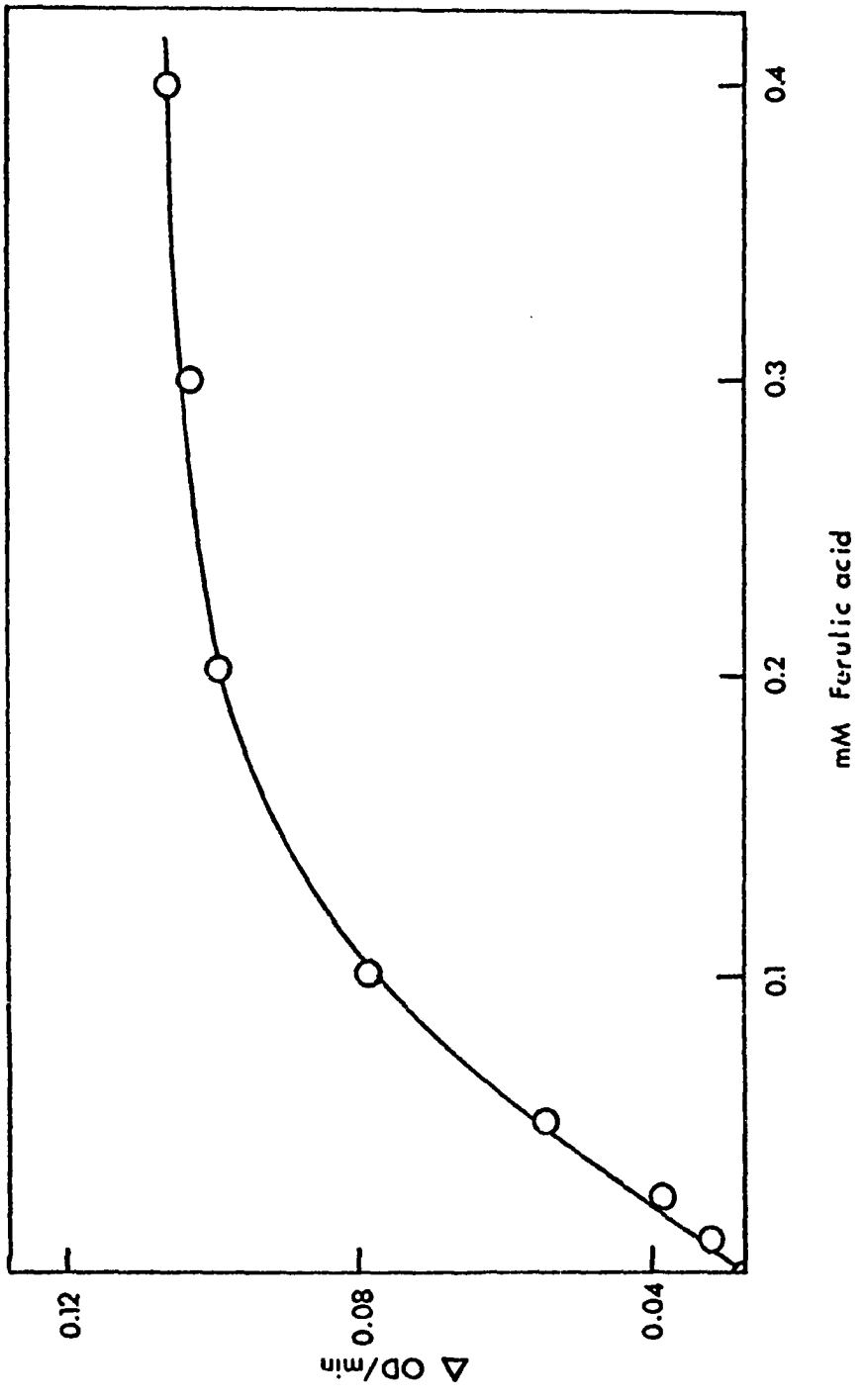
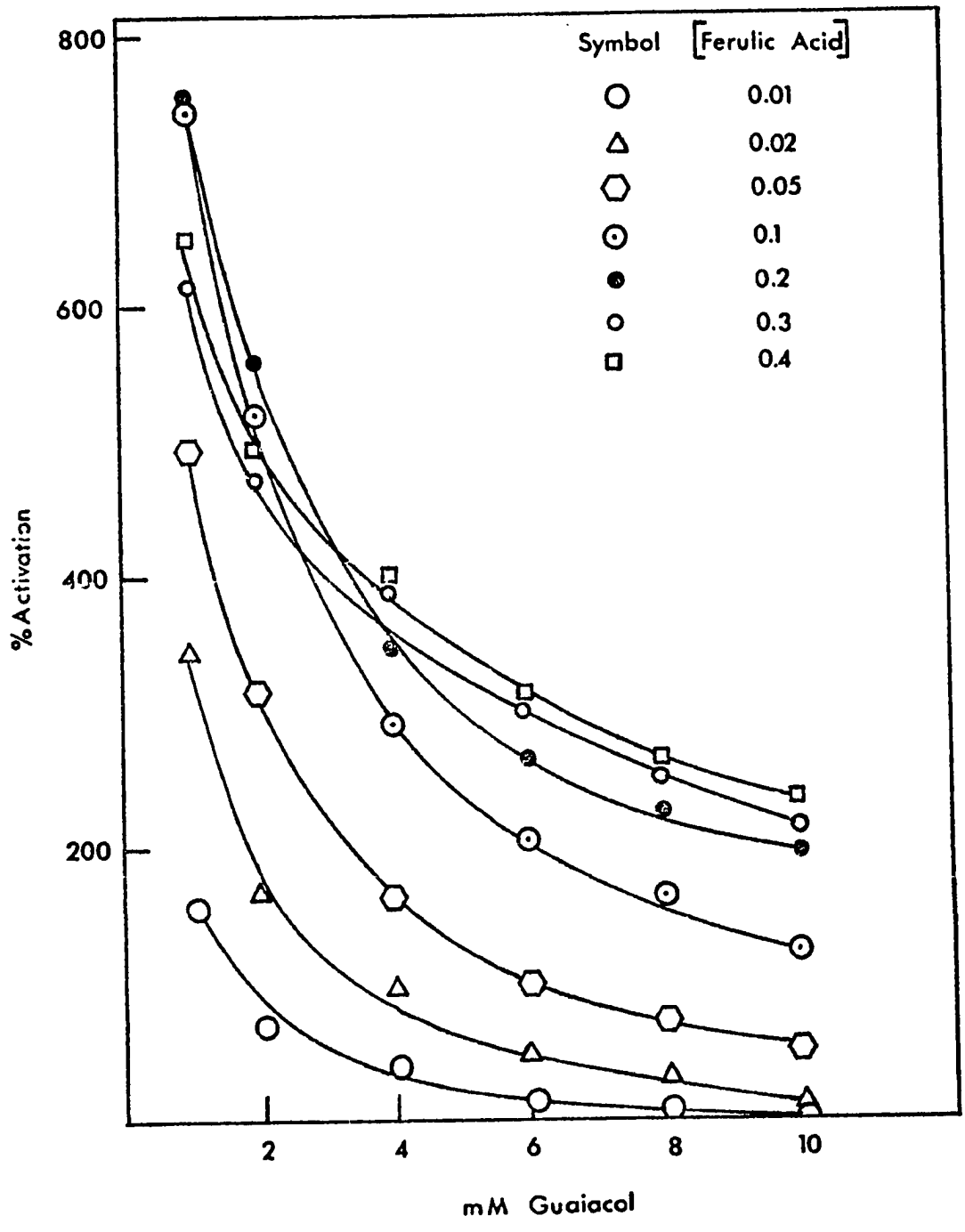

0.12
0.08
0.04
Δ OD/min
0.1
0.2
0.3
0.4
mM Ferulic acid

Figure 14. ACTIVATION OF THE GUAIACOL OXIDIZING ABILITY OF ISOPEROXIDASE A_2 BY FERULIC ACID. Assays were run at 5 mM H_2O_2 in 50 mM phosphate buffer, pH 6.0.


800
600
400
200
% Activation
Symbol
[Ferulic Acid]
0.01
0.02
0.05
0.1
0.2
0.3
0.4
2
4
6
8
10
mM Guaiacol

above 0.2 mM may act as a substrate rather than an activator. Indeed, ferulic acid in combination with guaiacol may produce a product mixture which absorbs at 470 nm, thus adding to the absorption at 470 nm of the guaiacol product alone. The apparent increase in activity, therefore, may not be due to activation in the strict sense of the word. At higher concentrations of ferulic acid in the presence of low guaiacol concentrations, ferulic acid may be the dominant substrate and the product formed would have very little absorbance at 470 nm. In this case, the activation observed would be less than that seen at lower ferulic acid concentrations. At higher concentrations of guaiacol, an increase in the amount of ferulic acid present causes an increase in the observed activation, indicating that the combination of ferulic acid and guaiacol products may be responsible for an apparent increase in absorbance at 470 nm.

CHAPTER V

FERULIC ACID AS A SUBSTRATE FOR ISOPEROXIDASES A_1 AND A_2

The fact that isoperoxidases A_1 and A_2 can catalyze the oxidation of ferulic acid in the presence of H_2O_2, has been established beyond reasonable doubt. Incubation of a solution of 0.9 mM ferulic acid and 5 mM H_2O_2 in 50 mM phosphate buffer, pH 6.0, in the presence of either enzyme is followed by the rapid conversion of ferulic acid to an unknown product. One ml aliquots of an identical reaction mixture were extracted with 0.5 ml of ethyl acetate, which effectively "kills" the reaction, at times from 2 to 15 minutes after initiation of the reaction. These aliquots were then spotted on a thin layer plate spread to a thickness of 0.375 mm with Avicel SF. The chromatogram was developed in a solution of benzene:methanol (3:1) using the ascending mode. The results of this experiment are shown in Figure 15.

A similar reaction mixture allowed to incubate for one hour before extraction with ethyl acetate was spotted in the same manner. Figure 16 indicates the presence of products which do not appear in earlier extracts of the reaction mixture.

Under the same reaction conditions, spectrophotometric analysis discloses the appearance of a transient peak at 500 nm when the solution is scanned repeatedly from 550 to 470 nm. This peak disappears after

Figure 15. THIN LAYER CHROMATOGRAM OF FERULIC ACID REACTION MIXTURE. The reaction mixture contained 0.9 mM ferulic acid and 5 mM H_2O_2 in 50 mM phosphate buffer, pH 6.0. The reaction was initiated by addition of either isoperoxidase A_1 or A_2. One ml aliquots were extracted with ethyl acetate at the times indicated and spotted. The R_f values are: 0.87 for ferulic acid and 0.94 for the product spot. The solvent was benzene:methanol (3:1).

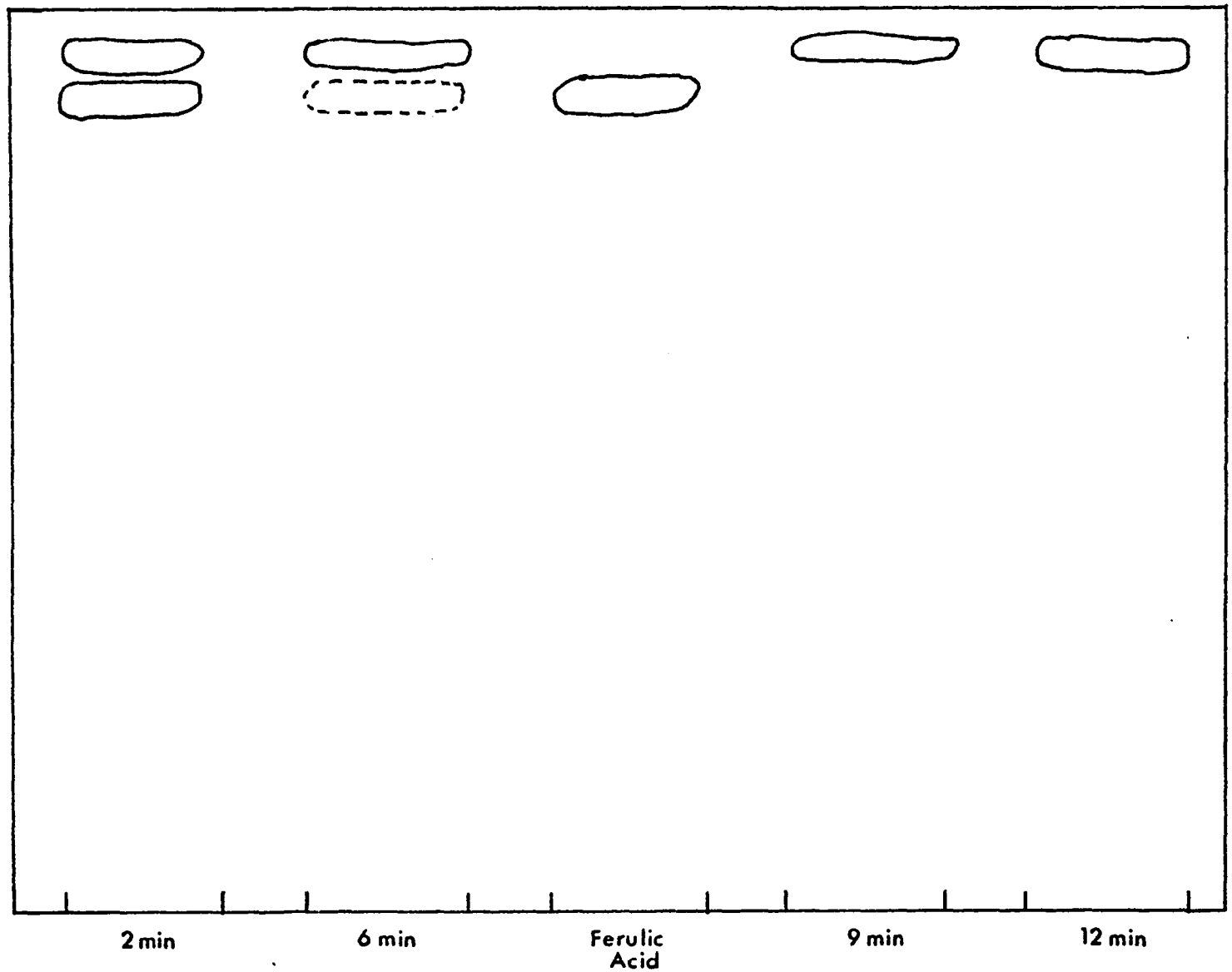
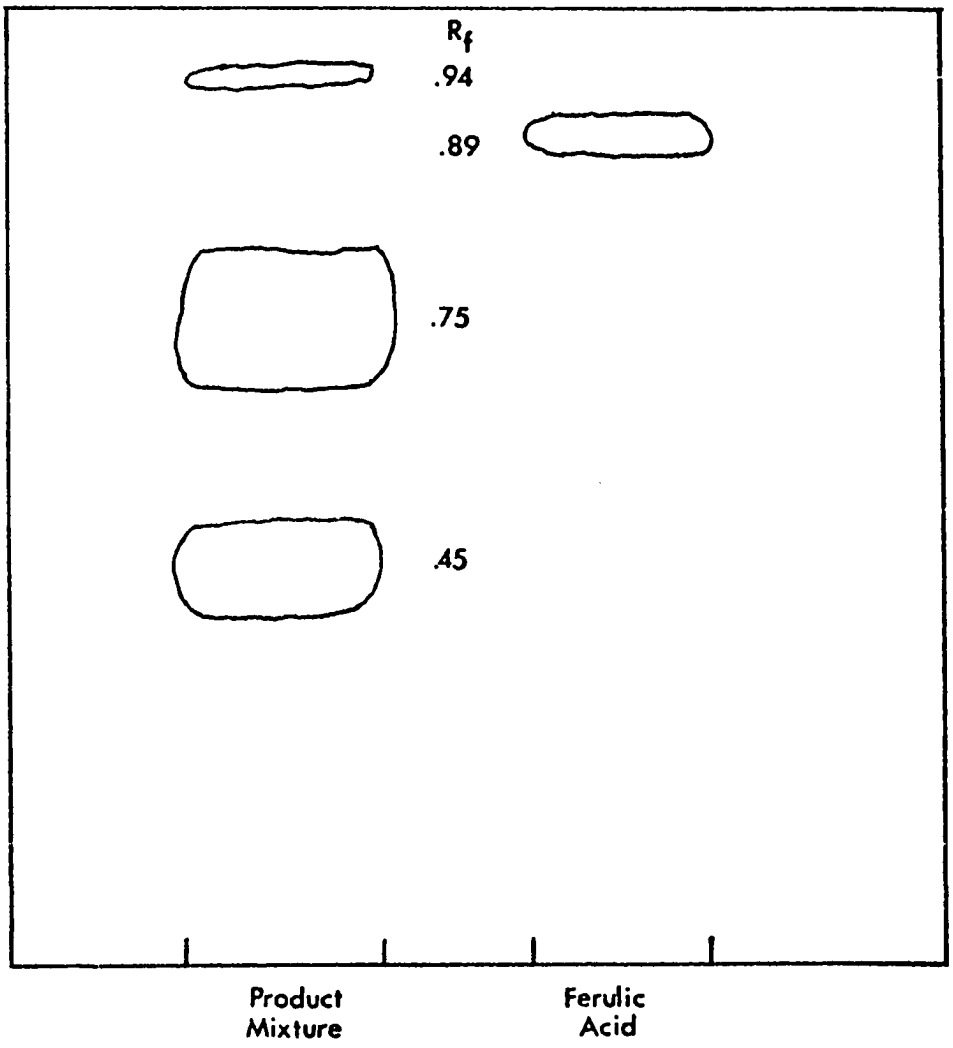

2 min
6 min
Ferulic Acid
9 min
12 min

Figure 16. THIN LAYER CHROMATOGRAM OF FERULIC ACID PRODUCT MIXTURE. The reaction mixture consisted of 0.9 mM ferulic acid and 5 mM H_2O_2 in 50 mM phosphate buffer, pH 6.0. Addition of either isoperoxidase A_1 or A_2 initiated the reaction. The mixture was allowed to stand for 1 hour before extracting and spotting. The solvent was benzene:methanol (3:1).


R_f
.94
.89
.75
.45
Product Mixture
Ferulic Acid

approximately 20 minutes of reaction. This fact may indicate the formation of an initial product or intermediate which, upon further reaction, yields additional products. The data obtained from Figure 16 lend credence to this supposition.

Since the peak observed at 500 nm is of such small intensity, it could not be used for kinetic analysis of isoperoxidases A_1 or A_2. Further inspection of the spectra of the ferulic acid reaction mixture both before and after the addition of either isoperoxidase A_1 or A_2 shows a significant decrease in absorbance in the ultraviolet region at 310 nm (Figure 17). This observation served as the basis for development of an assay procedure for oxidation of ferulic acid by isoperoxidases A_1 and A_2. A typical assay contained 0.2 mM ferulic acid, 5 mM H_2O_2 and 50 mM citrate buffer (pH 4.5 or 5.0). Isoperoxidase A_1 or A_2 was added to initiate the reaction, and the decrease in absorbance at 310 nm was measured.

Figures 18 and 19 show the pH optima for ferulic acid oxidation to be 4.5 for isoperoxidase A_1 and 5.0 for isoperoxidase A_2, respectively. The effect of ferulic acid concentration on the activity of isoperoxidases A_1 and A_2 is shown in Figures 20 and 21. Both enzymes exhibit a marked decrease in activity above 0.24 mM ferulic acid. Spectrophotometric analysis of the product of ferulic acid oxidation by isoperoxidases A_1 and A_2 at 0.2 mM and 0.4 mM ferulic acid reveal no difference in the product formed. Thin layer chromatography verifies this result showing the same products formed at both concentrations with isoperoxidase A_1 or A_2. This substrate inhibition would seem to indicate that the enzyme has two sites for ferulic acid binding. If this is the case, one could surmise that one site is available for normal substrate binding,

Figure 17. ABSORPTION SPECTRA OF FERULIC ACID REACTION MIXTURE BEFORE AND AFTER ADDITION OF EITHER ISOPEROXIDASE A_1 OR A_2. The reaction mixture contained 0.2 mM ferulic acid and 5 mM H_2O_2 in 50 mM phosphate buffer, pH 6.0.

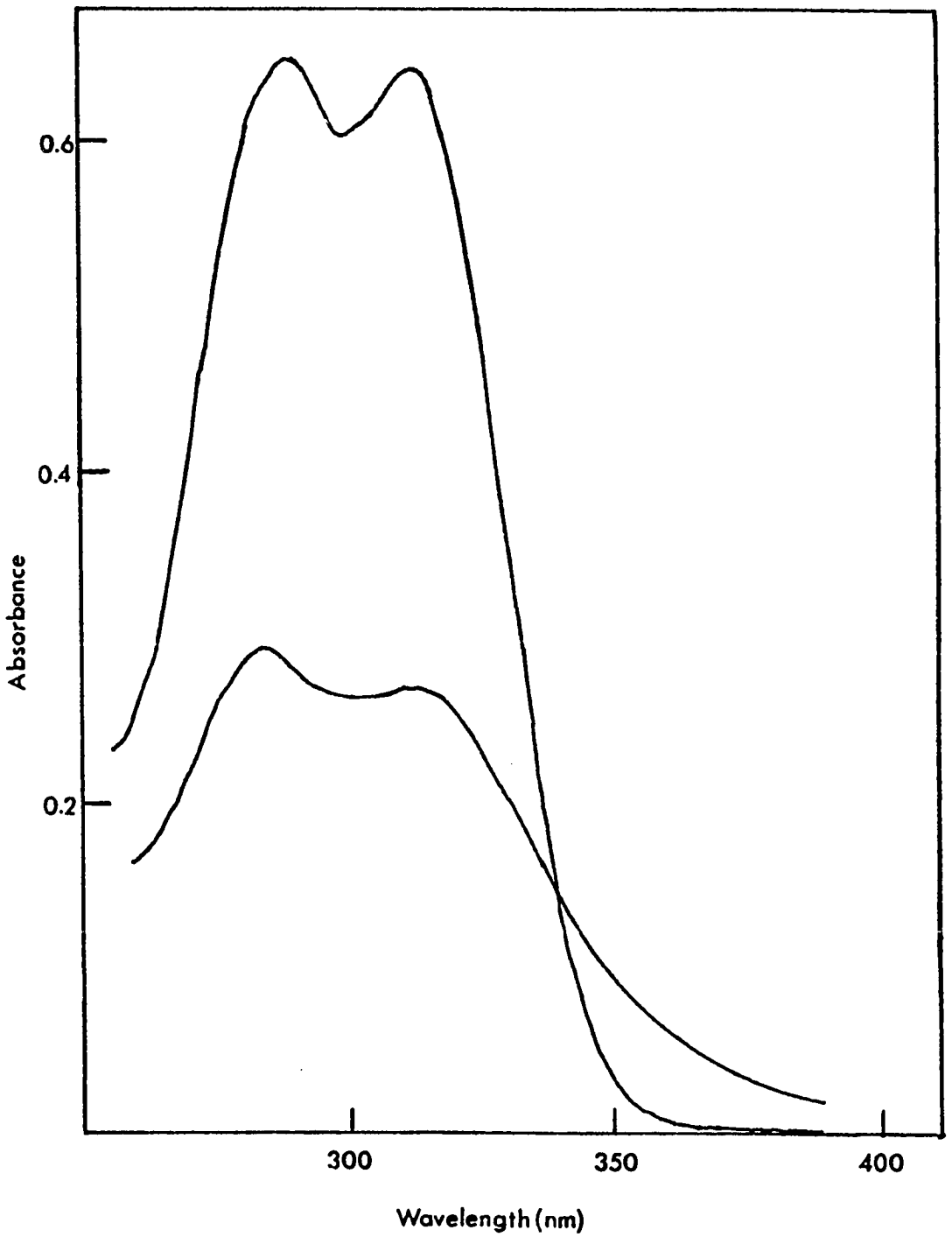

0.6
0.4
0.2
Absorbance
300
350
400
Wavelength (nm)

Figure 18. EFFECT OF pH ON THE FERULIC ACID OXIDIZING ABILITY OF ISOPEROXIDASE A_1. Assays were run at 0.2 mM ferulic acid and 5 mM H_2O_2 in 50 mM phosphate ⊙ or citrate ○ buffer.

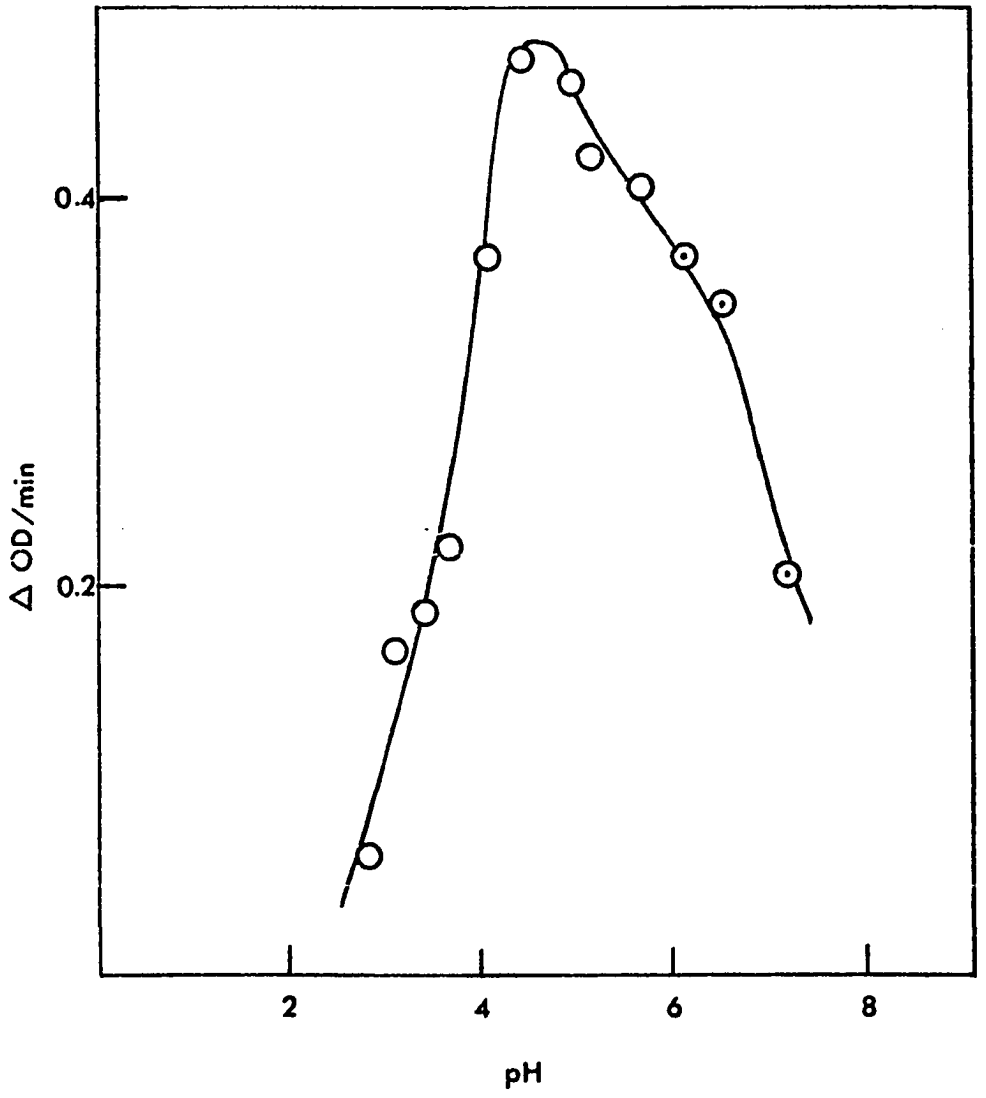

0.4
0.2
Δ OD/min
2
4
6
8
pH

Figure 19. EFFECT OF pH ON THE FERULIC ACID OXIDIZING ABILITY OF ISOPEROXIDASE A_2. Assays were run at 0.2 mM ferulic acid and 5 mM H_2O_2 in 50 mM phosphate ⊙ or citrate ○ buffer.

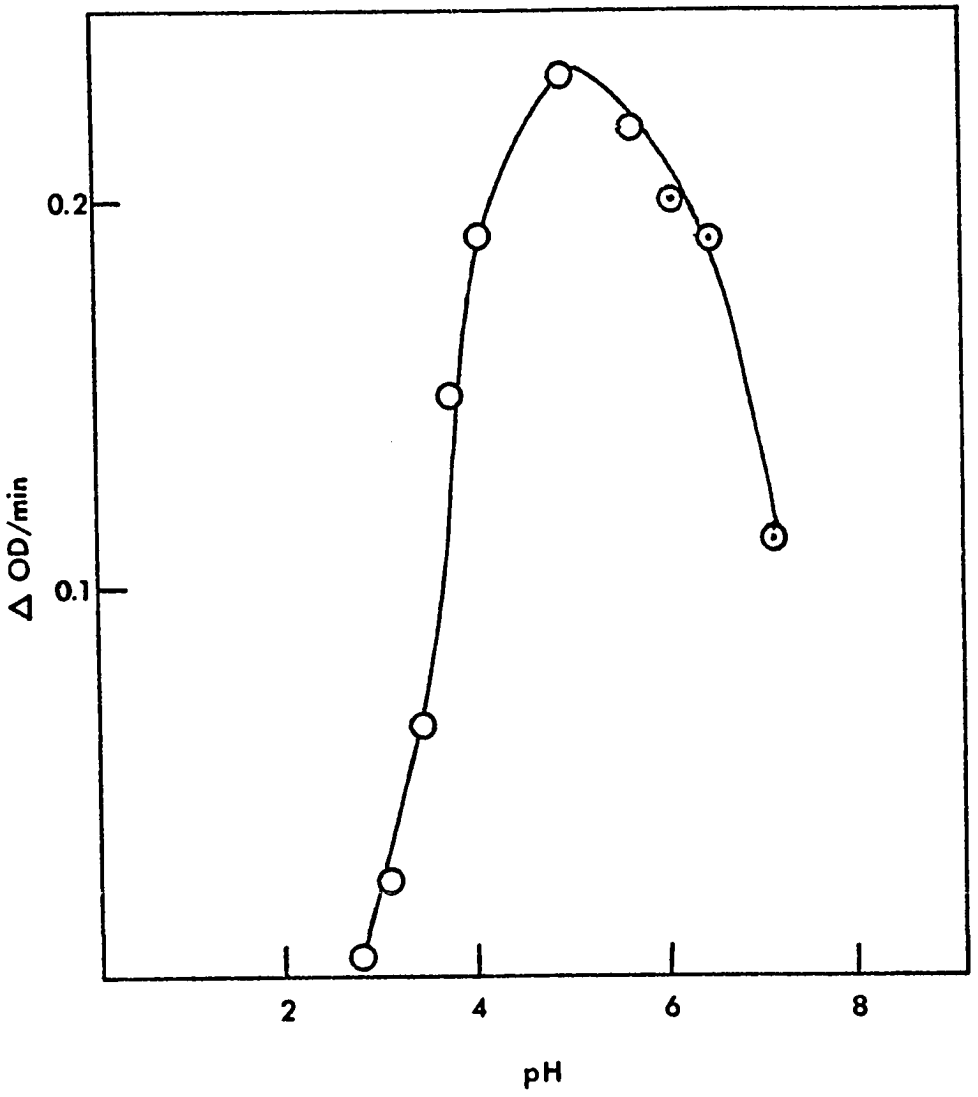

0.2
0.1
Δ OD/min
2
4
6
8
pH

Figure 20. FERULIC ACID SUBSTRATE SATURATION CURVE FOR ISO-PEROXIDASE A_1. Assays were run at 5 mM H_2O_2 in 50 mM citrate buffer, pH 4.5.

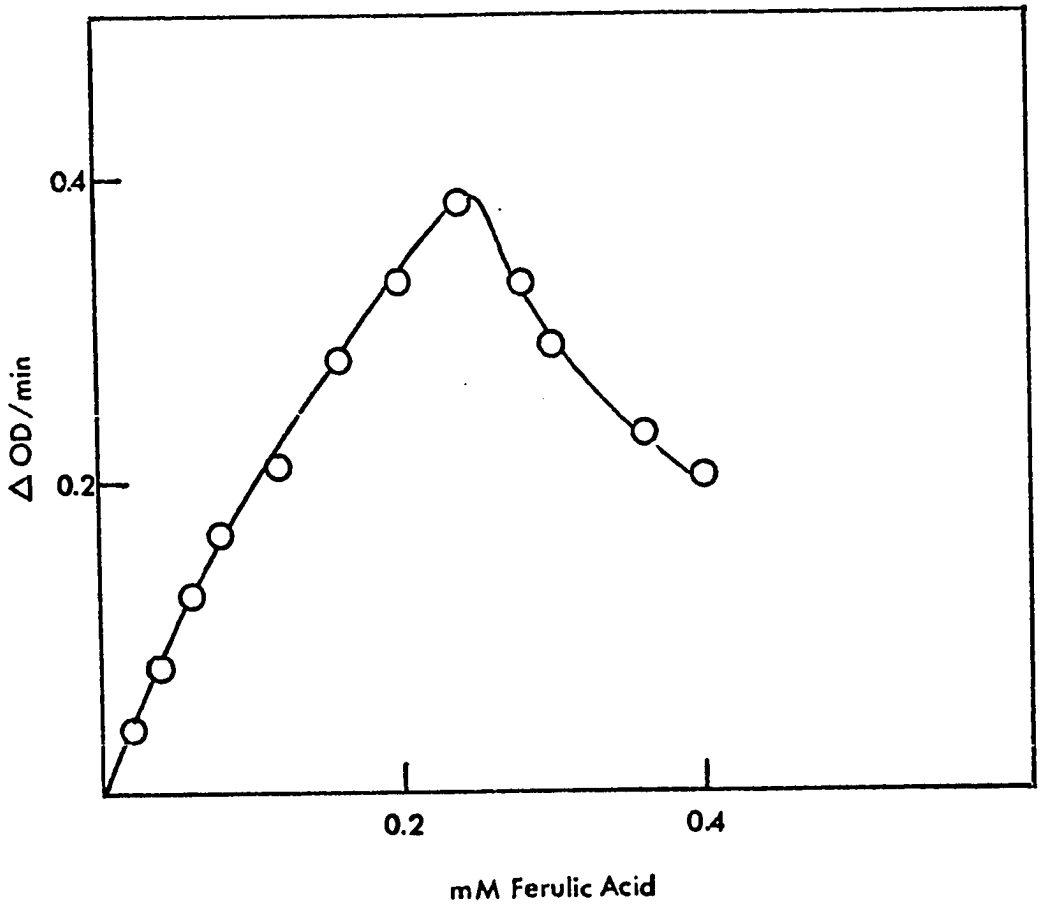
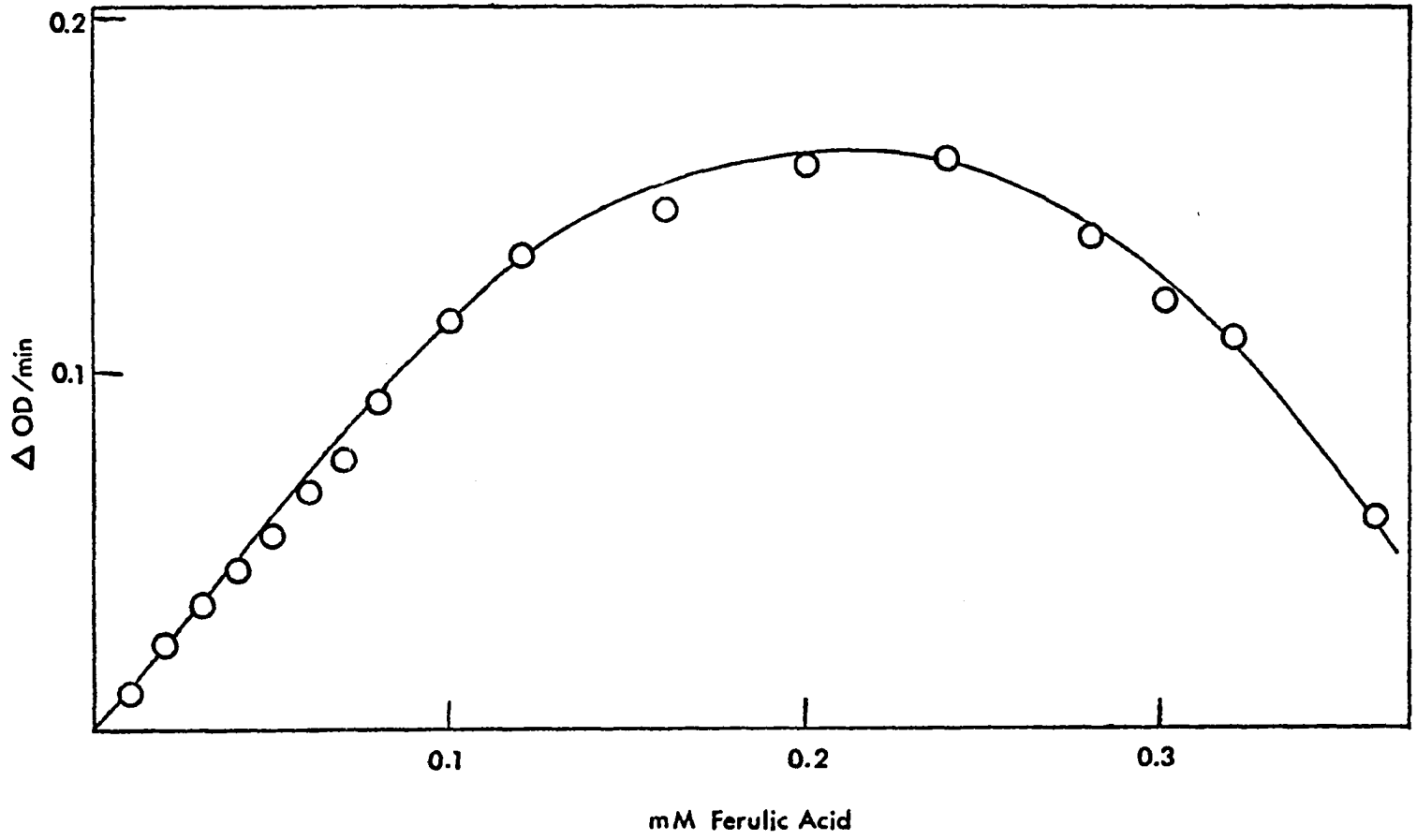

0.4
0.2
Δ OD/min
0.2
0.4
mM Ferulic Acid

Figure 21. FERULIC ACID SUBSTRATE SATURATION CURVE FOR ISO-PEROXIDASE A_2. Assays were run at 5 mM H_2O_2 in 50 mM citrate buffer, pH 5.0.


0.2
0.1
Δ OD/min
0.1
0.2
0.3
mM Ferulic Acid

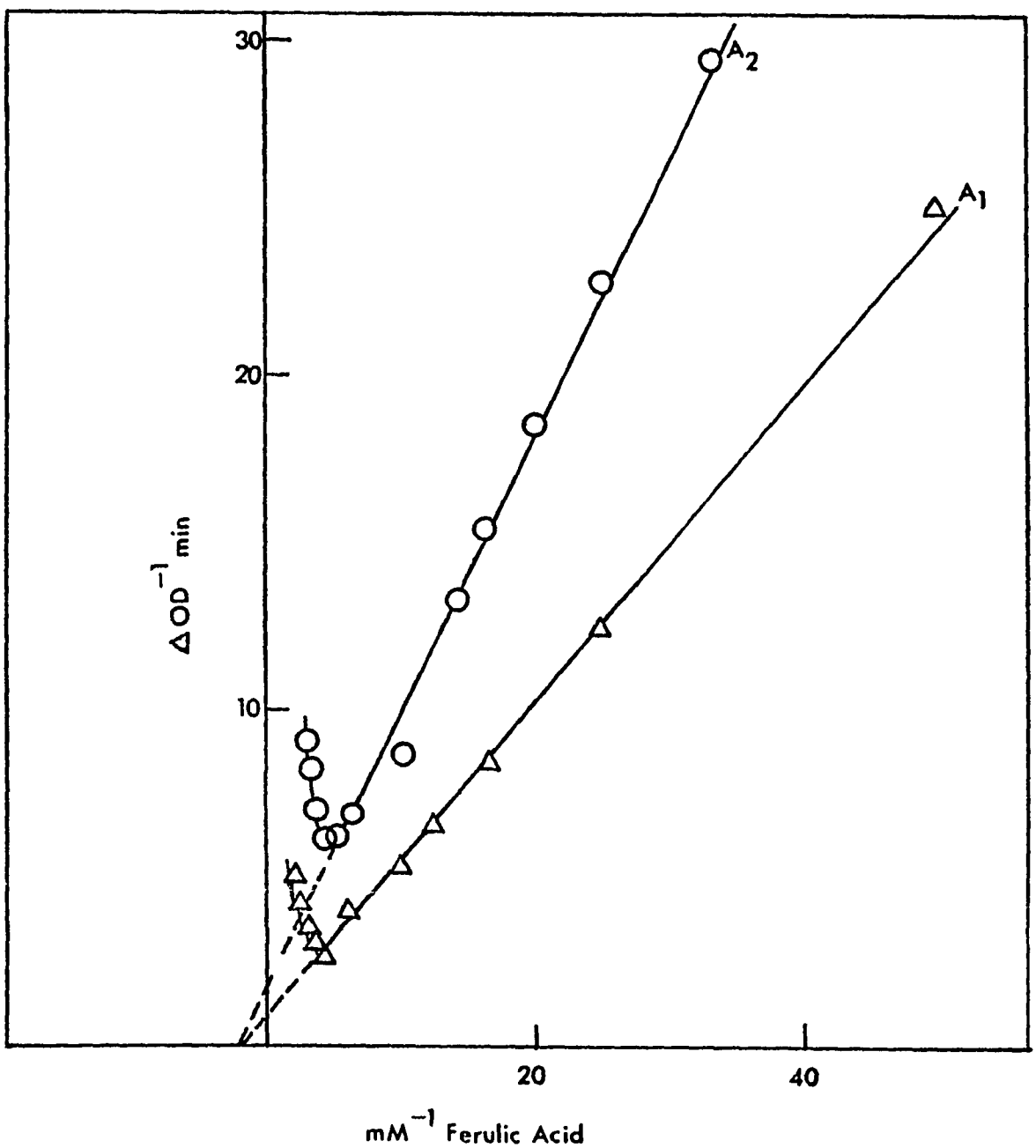
while, at high concentrations of ferulic acid, the second site might bind ferulic acid and, in so doing, it might have an antagonistic effect on binding at the substrate site thus inhibiting the enzyme's catalytic ability.

A Lineweaver-Burk[40] plot of the data from Figures 20 and 21 indicates an apparent $S_{0.5}$ value of 0.4 mM ferulic acid for isoperoxidase A_1 and A_2 (Figure 22). The upward curve of these plots is indicative of an enzyme system exhibiting substrate inhibition.

The apparent $S_{0.5}$ value of 0.4 mM obtained from the respective Lineweaver-burk plots does not compare favorably with the value of 0.06 mM obtained from the ferulic acid activation data for isoperoxidase A_2 discussed in Chapter IV. However, the substrate inhibition observed at concentrations of ferulic acid above 0.24 mM make it difficult to ascertain the accuracy of the value obtained from the Lineweaver-Burk plots. The maximum velocity actually attained does not agree at all with the values calculated from Figure 22 (1.13 for A_1 and 0.51 for A_2). The experimental values are 0.385 for isoperoxidase A_1 and 0.160 for isoperoxidase A_2. This fact makes the value of 0.4 mM even more questionable. If the substrate concentration at half of these velocity values is taken as the $S_{0.5}$ value for ferulic acid, the agreement with the earlier activation data is quite good. The respective concentrations are 0.09-0.1 mM for isoperoxidase A_1 and 0.07-0.08 mM for isoperoxidase A_2. While this application is not strictly correct, it seems a more reasonable approach than the evaluation of the Lineweaver-Burk plot data.

In an effort to determine the amount of ferulic acid destroyed, in units more practical than ΔOD/min, by isoperoxidases A_1 and A_2, it has

Figure 22. DOUBLE RECIPROCAL PLOT OF VELOCITY VERSUS FERULIC ACID CONCENTRATION FOR ISOPEROXIDASES A_1 AND A_2. Assays were run at 0.2 mM ferulic acid and 5 mM H_2O_2 in 50 mM citrate buffer, pH 4.5 or 5.0.


30
20
10
ΔOD^{-1}_{min}
A_2
A_1
20
40
mM^{-1} Ferulic Acid

been assumed that the conversion of ferulic acid to product is in a one to one stoichiometric relationship. The extinction coefficient of ferulic acid at 310 nm is easily calculated to be 1.95×10^4 liters moles^{-1} cm^{-1} (ε_F). Data from Figure 15 reveal that the reaction of ferulic acid and H_2O_2 in the presence of isoperoxidases A_1 or A_2 apparently goes to completion. However, at this point, an appreciable amount of absorbance at 310 nm remains. This absorbance, thus, can be attributed entirely to product. The extinction coefficient of the product at 310 nm is then calculated to be 4×10^3 liters moles^{-1} cm^{-1} (ε_p). The conversion of velocity from units of ΔOD/min to micromoles ferulic acid altered/min is complicated by the presence of ferulic acid and product, both of which absorb at 310 nm. At any given time, the change in optical density (ΔOD) is related to the initial concentration of ferulic acid (C_{F_0}), the concentration of ferulic acid at time t (C_{F_t}) and the concentration of product (C_p) by the following equation:

1) $$\Delta OD = \varepsilon_F C_{F_0} - \varepsilon_F C_{F_t} - \varepsilon_P C_P$$

At any given time the concentration of product, C_p, is given by equation 2:

2) $$C_P = C_{F_0} - C_{F_t}$$

Substituting the value for C_p from equation 2 into equation 1 and solving for C_{F_t} yields equation 3:

3) $$C_{F_t} = \frac{\Delta OD - C_{F_0}(\varepsilon_F - \varepsilon_P)}{(\varepsilon_P - \varepsilon_F)}$$

Using the velocity values of 0.385 for isoperoxidase A_1 and 0.160 for isoperoxidase A_2 obtained upon reaction of 0.24 mM Ferulic acid and

5 mM H_2O_2 in the presence of both of these enzymes, one can easily calculate an initial velocity in terms of micromoles ferulic acid altered per minute utilizing equation 3. The corresponding values are 0.075 and 0.03 micromoles ferulic acid altered per minute for isoperoxidases A_1 and A_2, respectively. These numbers show that the two enzymes are extremely active in catalyzing the conversion of ferulic acid.

CHAPTER VI

NATURE OF THE PRODUCT OF FERULIC ACID OXIDATION BY ISOPEROXIDASES A_1 AND A_2

Incubation of 0.4 mM ferulic acid, 5 mM H_2O_2 and 50 mM citrate buffer (pH 4.5 or 5.0) with isoperoxidase A_1 or A_2 leads to the rapid formation of a pink intermediate which disappears upon exposure to light or prolonged standing (> 30 min). When the reaction mixture is spotted on a thin layer plate after only 15 minutes of reaction, one and only one product appears with an R_f value of 0.94 using benzene:methanol (3:1) as solvent. This seems to correspond with the appearance of a peak in the visible spectra at 500 nm observed when enzyme is added to the reaction mixture. Since this peak disappears approximately 30 minutes after initiation of the reaction, the obvious conclusion cannot be overlooked; that is, the initial product formed and having an R_f value of 0.94 is the pink intermediate first observed upon addition of the enzyme.

As shown in Figure 16, multiple products begin to appear on thin layer chromatograms spotted with reaction mixture allowed to stand for more than 30 minutes. The decreasing R_f values of these products may indicate polymerization products. This type of reaction does not necessarily require mediation by enzyme after the initial conversion is accomplished, if that conversion is characterized by free radical

formation which might be expected with a peroxidative mechanism. Once this free radical is built up to sufficient amounts, the remaining ferulic acid should be quite susceptible to attack at several positions. This would explain the formation of multiple products and the possibility of polymer formation would be reasonable under these circumstances. Such polymer formation is also supported by the proposed involvement of ferulic acid and other naturally occurring phenolic compounds as precursors of lignin in higher plants.[45,46]

CHAPTER VII

RELATIVE SUBSTRATE SPECIFICITIES OF ISOPEROXIDASES A_1 AND A_2

Although isoperoxidases A_1 and A_2 have been shown to differ structurally, little specific information is known about their physiological functions. Earlier references to the oxidation of scopoletin by isoperoxidase A_3[16] and ferulic acid oxidation by isoperoxidases A_1 and A_2 in the presence of H_2O_2, make manifest the importance of discovering the substrate specificities of individual isoperoxidases. It is evident that more than one naturally occurring phenolic compound is capable of acting as substrate for these isoperoxidases *in vitro*, perhaps with differing degrees of effectiveness.

In the investigation of the specificity of isoperoxidases A_1 and A_2 for naturally occurring compounds, numerous phenolic compounds which affected the guaiacol oxidizing ability of the enzymes were examined as possible substrates. In addition to ferulic acid, two of the compounds, chlorogenic acid and esculetin, have been demonstrated to be capable of acting as substrates. This finding was verified using thin layer chromatography on Avicel SF in a solvent of methylisobutylketone: formic acid: water (14:3:2). Both compounds were shown to have disappeared after incubation with either enzyme in the presence of H_2O_2 with concomitant appearance of multiple products in both cases. In

addition, visible spectrophotometry revealed a new absorption maximum appearing for both compounds; 469 nm for esculetin and 400 nm for chlorogenic acid. The R_f values of these products indicate that they too might be polymeric in nature.

Using the procedure described by Reigh et al.[16] for isoperoxidase A_3, scopoletin could also be shown to act as a substrate. However, under the same conditions that led to rapid oxidation of scopoletin by H_2O_2 in the presence of A_3, very little, if any, oxidation could be observed with A_1 or A_2.

The significance of multiple substrates for individual isoperoxidases is currently unknown. Even the physiological reasons for multiple isoperoxidases are not clear. Recently, an attempt to clarify certain physiological effects, such as dehydration, upon isoperoxidases in relation to substrate specificities for nonphysiological compounds has been reported.[47] Also, activity ratios between some of these synthetic substrates (guaiacol and o-dianisidine) have been used to try to clarify the role of isoperoxidases in plants.[48] It seems however, more significant to attempt to relate these effects in terms of probable *in vivo* substrates. This required the development of a reliable assay for each of the four natural substrates (ferulic acid, scopoletin, esculetin and chlorogenic acid) and guaiacol. The assays for ferulic acid and guaiacol were discussed earlier. The scopoletin assay developed by Reigh et al.[16] was utilized for measuring the oxidation of scopoletin by H_2O_2 in the presence of isoperoxidases A_1 and A_2. The rate of chlorogenic acid oxidation was measured by following the increase in absorbance at 400 nm. Esculetin oxidation revealed an increase in absorbance at 469 nm and this was the basis of the assay used. Table 5 lists the pH optima for isoperoxidases

TABLE 5

PH OPTIMA FOR ISOPEROXIDASES A_1 AND A_2 FOR EACH OF FIVE SUBSTRATES

SUBSTRATE	PH OPTIMUM A_1	PH OPTIMUM A_2
Ferulic acid	4.5	5.0
Scopoletin	5.25	4.5
Esculetin	7.5	7.5
Chlorogenic acid	5.0	4.5
Guaiacol	6.0	6.0

A_1 and A_2 for each of the five substrates. The various assay conditions utilized for comparing substrate specificities are listed in Table 6. A comparison of the potential to be a substrate for each isoperoxidase is shown in Table 7. In order to control as many variables as possible, all assays were run with that amount of isoperoxidase A_1 or A_2 which gave the same initial velocity utilizing guaiacol as substrate. The velocity obtained for the other four substrates was then compared to guaiacol according to the following formula:

$$\frac{\Delta OD/min\ Natural\ Compound}{\Delta OD/min\ Guaiacol} = Activity\ Ratio$$

The pH was maintained at the pH optimum for the activity of isoperoxidase A_1 or A_2 with each of the five substrates. In three other cases, the pH was held constant (4.5, 5.25 or 6.0) in reaction with all substrates. Although any attempt to draw quantitative conclusions from these data is premature, it is apparent that neither of these isoperoxidases is capable of catalyzing the oxidation of scopoletin to any significant degree. On the other hand, ferulic acid is apparently the preferred substrate, of those tested, for both isoperoxidases.

As the pH of the assay approaches the pH optimum for the particular substrate in question and moves further from the pH optimum for guaiacol, the ratio predictably increases. This trend holds true in every case tested.

Data reported by Reigh[31] on isoperoxidase A_3 also isolated from W-38 tobacco tissue culture, reveal that A_3 is 160-250 times more active in catalyzing the oxidation of scopoletin than either isoperoxidase A_1 or A_2. Marklund et al.[49] have shown a distinct substrate preference for two peroxidase isoenzymes from horseradish, A2 and C2. In comparing the

TABLE 6

ASSAY CONDITIONS FOR SUBSTRATE

SPECIFICITY COMPARISON EXPERIMENTS

SUBSTRATE CONCENTRATION	H_2O_2 CONCENTRATION	ACTIVITY
15 mM Guaiacol	5 mM	ΔOD_{470}/ min
0.2 mM Ferulic acid	5 mM	ΔOD_{310}/ min
1.25 mM Esculetin	5 mM	ΔOD_{469}/ min
1.0 mM Chlorogenic acid	5 mM	ΔOD_{400}/ min
1.25 mM Scopoletin	5 mM	ΔOD_{450}/ min

TABLE 7

SUBSTRATE SPECIFICITY COMPARISON**

SUBSTRATE	pH OPTIMUM		pH=4.5		pH=5.25		pH=6.0	
	A_1	A_2	A_1	A_2	A_1	A_2	A_1	A_2
Guaiacol	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Esculetin	.35	.33	.10	.05	.14	.17	.20	.27
Scopoletin	.03	.02	0	.02	.03	.01	.03	.02
Chlorogenic acid	.63	.95	1.1	1.2	.77	.57	.58	.39
Ferulic acid	2.8	2.0	5.2	1.9	2.9	2.1	2.8	1.7

**Velocities for each naturally occurring substrate are presented relative to velocity using guaiacol as substrate. Relative oxidation ratios are presented for isoperoxidases A_1 and A_2 with each substrate at the pH indicated.

rate constants for the reaction of various substrates with these isoenzymes, they found that coniferol had a rate constant 30 times greater than any other substrate tested. Others tested included guaiacol, catechol, ascorbic acid and uric acid. Their data also revealed that A2 was twice as active in reaction with coniferol as C?.

The apparent preference of one isoperoxidase over another for a particular substrate cannot be fully understood until the physiological roles of these phenolic compounds in plants is more clearly established. The particular kinetic parameters which describe each isoperoxidase will be responsible for controlling its activity within the plant. Small differences in $S_{0.5}$, pH optimum and substrate specificity may be magnified within the uniquely controlled confines of a specific plant organelle.

CHAPTER VIII

SUMMARY

The success of isolating two individual isoperoxidases from W-38 tobacco tissue has emphasized the importance of similar studies in establishing a possible physiological role for this class of enzymes.

With the exception of the increase in $S_{0.5}$ and V_{max} values for the oxidation of guaiacol in response to increasing concentrations of H_2O_2 in the presence of isoperoxidase A_2, these two isoperoxidases exhibit amazingly similar behavior in response to both effectors and substrates. However, isoperoxidase A_3 does indeed differ from isoperoxidases A_1 and A_2 in regard to these two parameters. The physical properties of these isoperoxidases also differ to a significant degree. Isoperoxidase A_1 appears to be a dimer with a monomer molecular weight of approximately 49,000, whereas isoperoxidases A_2 and A_3 do not possess such characteristics exhibiting molecular weights by both SDS gel electrophoresis and gel filtration of 89,000 and 54,000 respectively.

A recent report by Lee[15] showed that isoenzymes of peroxidase were present in all subcellular fractions of tobacco callus cells. The relative amounts of each isoenzyme, however, were different. In addition, certain isoperoxidases present in one subcellular fraction were found to be totally lacking in another. This study indicates that there are significant differences in peroxidase isoenzyme composition among sub-

cellular loci in the same tissue. Raa reported earlier that peroxidase activity was usually highest in the cell nucleus.[50] In older cells, however, he showed that the cell walls became the most heavily stained structure. Although his work did not include isoenzyme constitution of these subcellular components, it is almost certain that the distribution of the isoperoxidases must vary. An intriguing hypothesis is that subcellular localization may be a major controlling factor in regulating the activity of isoperoxidases A_1 and A_2.

The data compiled on isoperoxidases A_1 and A_2 reveal that there are indeed differences which might be significant in relation to the role of these isoperoxidases *in vivo*. Differences in pH optima, substrate specificities and cellular localization may be extremely sensitive to slight changes in cellular conditions which dictate preference for one isoperoxidase over another. Cellular concentrations of several of these naturally occurring compounds, in particular ferulic acid which was shown to inhibit its own oxidation by isoperoxidases A_1 and A_2 at high concentrations, could be an important controlling factor in regulating their activity.

As more information concerning the *in vivo* characteristics of isoperoxidases is uncovered the entire picture of peroxidative action may become clearer. Identification of specific products of the oxidation of compounds such as ferulic acid, chlorogenic acid and scopoletin should provide a great deal of insight into possible biochemical pathways in which isoperoxidases A_1 and A_2 are involved.

Because of the nearly ubiquitous nature of peroxidases in higher plants, any information gained concerning their mode of action should be invaluable in unraveling the mystery of certain metabolic pathways in plants which still defy complete identification.

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