THE ISOZYMIC FORMS OF PEROXIDASE FOUND IN THE HORSERADISH PLANT (ARMORACIA LAPATHIFOLIA)

Thesis for the Degree of Ph. D.

MICHIGAN STATE UNIVERSITY
EDWIN H. LIU

1971

This is to certify that the

thesis entitled

The Isozymic Forms of Peroxidase
Found in the Horseradish Plant
(Armoracia lapathifolia)
presented by

Edwin H. Liu

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Biochemistry

Major professor

Date 10-25-1971

ABSTRACT

THE ISOZYMIC FORMS OF PEROXIDASE FOUND IN THE HORSERADISH PLANT (ARMORACIA LAPATHIFOLIA)

By

Edwin H. Liu

The peroxidase system in horseradish (Armoracia lapathifolia) was used to study differences in the catalytic behavior of the individual isozymes, and to determine the association of this enzyme with hydroxyproline containing moieties and with the plant cell wall.

An automatic peroxidase assayer was designed and constructed. A method of quantitatively estimating the relative activities of individual peroxidase isozymes directly on a starch gel zymogram was also developed.

A fluorimetric assay system was used to determine the peroxidase associated with horseradish root cell walls. Twenty percent of the total peroxidase activity found in horseradish roots can be found bound to cell walls, and 98% of this cell wall peroxidase can be released by salt washing and cellulase treatment. The

peroxidase isozymes which are found on the cell wall were identified on starch gel zymograms.

The association of hydroxyproline containing moieties with peroxidase was investigated in a commercially purified enzyme preparation (Worthington HRP-HPOD-6FA), peroxidase found in horseradish root cell sap, peroxidase released from cell walls by cellulase treatment, and in peroxidase found in the incubation medium of aerated root discs.

The apparent enhancement of peroxidase activity by ammonia was used to demonstrate differences in the catalytic activity of two peroxidase isozymes.

A quantitative estimation of the peroxidase activity of cell walls was determined, and a $K_{app}(H_2O_2)$ measured for peroxidase bound to particulate cell walls.

Zymogram stains for peroxidase utilizing eugenol, a lignin precursor, and tyrosine as substrates were developed. These substrates were chosen because they are of possible physiological significance. When horseradish material is subjected to starch gel electrophoresis and stained for peroxidase activity with these substrates, differences in isozyme distribution patterns can be seen.

Storage in slightly alkaline conditions will alter the electrophoretic mobility of peroxidase isozymes, without significantly changing their catalytic activity. Changes in the electrophoretic mobility of peroxidases

can be observed in samples which have been incubated for 38 hours in pH's as low as 7.03. This modification of peroxidase is irreversible; retitration to acidic pH will not alter the electrophoretic mobility of the modified peroxidases.

THE ISOZYMIC FORMS OF PEROXIDASE FOUND IN THE HORSERADISH PLANT (ARMORACIA LAPATHIFOLIA)

By

Edwin H. Liu

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Biochemistry

1971

TABLE OF CONTENTS

Chapter Page

LIST OF TABLES

Table Page

LIST OF FIGURES

Figure Page

CHAPTER I

INTRODUCTION

Review of the Literature

A peroxidase (donor: H_2O_2 oxidoreductase; E.C. 1.11.1.7) is an enzyme which is defined by its ability to utilize hydrogen peroxide to oxidize a wide variety of hydrogen donors, such as phenolic substances, aromatic, primary, secondary and tertiary amines, leuco-dyes of all types, certain heterocyclic compounds such as ascorbic acid and indole, and certain inorganic ions, particularly the iodide ion (Saunders, 1964).

True peroxidases are heme-containing proteins. While they are not particularly specific as to the hydrogen donor, they are very specific in their requirement for hydrogen peroxide. Horseradish peroxidase is a brown enzyme which has a molecular weight of about 40,000 (Keilen, 1951). It consists of a colorless protein, the apo-enzyme, combined with an iron porphyrin which has been identified as protohemin IX. The iron has six coordination positions, four of which are occupied by porphyrin nitrogen atoms and a fifth by a reactive ligand from the apo-enzyme. It is the sixth coordination position of this heme-bound iron

which is reactive in the peroxidase reaction. It acts by combining with hydrogen peroxide. It is thus an obligate characteristic of any true peroxidase that any compound which is known to bind at the sixth coordination position of heme-bound iron, for example cyanide, should inhibit that enzyme's peroxidatic activity. The peroxidases are glycoproteins containing about 18% carbohydrate.

Peroxidases are widely distributed among higher plants; the richest known sources of this enzyme are the sap of the fig tree and the root of horseradish. Peroxidase activity is found in all higher plant tissues which have been investigated. Peroxidases have been located histochemically on the cell walls of plants (DeJong, 1967), but there are no quantitative estimations of the total peroxidase activity on cell walls.

Peroxidases are also commonly found in animals, but are not widely distributed in all tissues. There are also reports of the presence of peroxidase in fungi, bacteria, and algae (Saunders, 1964). The peroxidases found in animals and lower organisms are by no means identical to the peroxidase which is found in higher plants. For example, animal peroxidases are able to catalyze particular reactions such as the halogenation of phenyl compounds. However, all these peroxidases are similar in that they contain protohemin IX as a prosthetic group.

The multicomponent nature of peroxidase

The first observation that peroxidase activity consisted of more than one electrophoretically distinct species was made by Theorell in 1942. By electrophoresis of a pure horseradish peroxidase at pH 7.5 he could resolve two components; the anodically migrating he called "true peroxidase" and the cathodically migrating he called "para-peroxidase." He considered the para-peroxidase to be a derivative of true peroxidase. Keilen and Hartree in 1945 observed that when horseradish peroxidase was stored at 0°C, a new component appeared (Saunders, 1964).

Recognition that peroxidase activity actually exists in multiple forms came from Jermyn (1954), who observed by electrophoresis on filter paper that there were five components of peroxidase isozymes. The number of peroxidase isozymes recognized on a zymogram depends on the tissue, the hydrogen donor employed, and on the imagination of the investigator.

Shannon (1966) reported seven peroxidase isozymes which could be recognized and resolved by ion exchange chromatography. Starch gel electrophoresis of commercial horseradish peroxidase has been reported to show eleven peroxidase isozymes (Klapper and Hackett, 1965). Thin-layer isoelectric focusing is reported to resolve twenty isozymes of horseradish peroxidase (Delincee, 1970).

The multiplicity of peroxidase isozymes visualized by the zymogram technique may represent the products of different genes specifying peroxidase, or modifications of these gene products which serve to alter the electrophoretic mobility of the isozymes.

Peroxidase in plants

The zymogram technique (Hunter and Markert, 1957) which uses histochemical stains to locate enzymes which have been resolved from each other by electrophoresis in supporting gel beds provides an easy and elegant method to determine the isozyme complement of various tissues of a plant throughout development. As already indicated, the peroxidase system shows considerable variation in isozymes, and in any plant system, the peroxidase isozymes expressed in a zymogram depend both on the tissue used for assay and the developmental stage of the plant at the time of assay. Multiple forms of peroxidase have been found to vary with different organs in maize (Scandalios, 1964), petunia (Hess, 1967), peas (Macnichol, 1966), (Siegel, 1967) and barley (Upadhya, 1968; Felder, 1970). Moreover, the peroxidase complement has been shown to be different in a dwarf (d^x) and normal (d^+) tomato which differ by a single gene mutation (Evans and Aldridge, 1965).

Temporal changes in the peroxidase isozyme patterns within a particular organ, commonly known as isozymic

shifts, have been shown to occur in maize (Scandalios, 1969), in the developing pea cotyledons (Siegel and Galston, 1967), in bean leaves (Racusen and Foote, 1966), barley organs (Felder, 1970), and the germinating seeds of wheat (Bhatia and Nilson, 1969), rye (Siegel and Galston, 1966), and barley (Anstine, et al., 1970).

The expression of particular peroxidase isozymes in a developing plant was also shown to depend on the absence or presence of added indoleacetic acid in wheat (Whitmore, 1971) and in peas (Ockerse, Siegel, and Galston, 1966).

It is thus well documented that in all higher plants investigated there exists a multitude of peroxidase isozymes and that the number and relative concentration of peroxidases varies between different tissues and with the developmental stage of an organ. Because there is so much peroxidase activity in a cell and changes in its activity is inversely proportional to growth in plants, this enzyme system has been used as a model to study hormonal control of growth processes in plants (Galston, 1969).

The reports on the tissue distribution of peroxidase in plants and the isozymic shifts which occur with development all lack crucial controls to demonstrate that the enzymic activities which are measured truly represent the enzyme peroxidase and that they account for all the peroxidase isozymes which are present in the cell. For other enzymes, controls would not be necessary; but in the case

of peroxidase, this is required because of the broad range of the artificial dyes used in the assay of peroxidase activity, and the observation that not all isozymes are equally reactive to any one substrate. The dyes used by investigators in the past for the detection of peroxidase in zymograms include guaiacol, benzidine, o-toluidine, and o-dianisidine. None of these reagents or their products resembles natural compounds which can be found in the cell and which could account for peroxidase activity. Guaiacol particularly has several disadvantages as a substrate. Oxidizing reagents other than peroxidase give color reactions with this compound (Saunders, 1964). Therefore, to demonstrate that what one is measuring with these artificial dyes is actually a peroxidase activity, one must (1) demonstrate that it is an enzyme in that it is heat sensitive, (2) demonstrate a requirement for H_2O_2 for color development, and (3) show that a heme-containing protein is responsible for the activity, i.e., that the activity can be reversibly inhibited with low concentrations of cyanide. Unless this is done, the appearance of new bands of peroxidase in a treatment, for example, in response to exogeneously applied hormone, must be regarded with reservations.

The most important reason one must regard zymograms of peroxidatic activities with reservations is that individual peroxidase isozymes have different specific activities with the different substrates used to measure their

activity. The resolved isozymes of horseradish peroxidase exhibit specific activities which differ ten-fold when o-dianisidine was used to assay peroxidase activity. When these same isozymes were assayed for the oxidation of oxaloacetate, the differences in specific activity of individual isozymes did not fit the pattern established with o-dianisidine. In the dwarf tomato plant, an anodically migrating peroxidase isozyme stains very well when benzidine is the hydrogen donor, but reacts very little with guaiacol (Evans, 1968). This same relationship is found for one of the isozymic components of horseradish peroxidase (Jermyn, 1953). In the comparative assay of five isolated isozymes of peroxidase from barley seedlings, using seven different peroxidase substrates, the ratios of specific activity among the five isozymes for any one class of substrate had no correlation to ratios of activity obtained with other substrates (Felder, 1970).

The differences in reactivity for the different substrates among the various isozymes of peroxidase are an indication that the peroxidase isozymes must have subtle differences around the enzyme active site although this has not been demonstrated. One might then wonder whether these peroxidase isozymes might have different functions within the cell. If this is the case, then correlations of total peroxidase activity with any cellular function would serve little purpose.

Hormonal effects on peroxidase expression

Peroxidase is easy to assay with artificial substrates, and this enzyme has been studied extensively as a model for the response of plant tissues to applied hormones. In studies on the growing zone of the sheath in the first leaf in corn, it was observed that a single gene dwarf mutant of maize (dwarf-1) has a greater level of peroxidase activity than the normal variety of this corn. Treatment with giberellic acid stimulates the growth rate of this mutant and lowers the peroxidase activity per unit protein in the rapidly growing section of the plant. The addition of giberellic acid to the normal plant did not lower peroxidase activity (McCune and Galston, 1959). This same effect was noted on a dwarf variety of pea, Progress #9, which also had higher peroxidase activity than the tall variety. Here also, the addition of giberellic acid increased the growth rate of the pea seedling while it lowered the peroxidase activity (McCune and Galston, 1959). In the barley aleurone layer system, the application of giberellic acid to incubating layers results in an increase of peroxidase found in the incubation medium (Harmey, 1969).

In Pelargonium pith cells, peroxidase appears after the tissue is excised from the plant. The formation of peroxidase in this tissue is inhibited by auxin and stimulated by kinetin (Lavee, 1968). In tobacco pith cells,

auxin inhibits the appearance of two cathodically migrating isozymes which usually appear after excision, and kinetin stimulates the appearance of these peroxidase isozymes (Galston, 1969).

In aerated discs from storage tuber tissue such as sweet potato, the inclusion of ethylene in the ambient atmosphere will cause a large increase in peroxidatic activity of the tissue (Imaseki, 1970).

There exists in the literature, then, reports that in various plant tissues the level of peroxidase is modulated by at least four of the five known plant hormones. Abscisic acid could also probably be added to this list, if someone would care to investigate this, since it has already been shown that abscisic acid will shut down the giberellin induced synthesis of enzymes in the barley aleurone layer system (Chrispeels and Varner, 1967).

Among the large numbers of papers describing hormonal effects on peroxidase levels in plants there is only one report of the de novo synthesis of peroxidase in response to hormonal treatment of a tissue. This is in the case of ethylene treatment at a concentration of 1 PPM of aerated sweet potato tissue slices (Shannon, 1971).

Genetic analysis of peroxidase isozymes

The extreme heterogeneity of the peroxidase system in plants is evidenced by the paucity of successful genetic

analysis of the isozymic forms of peroxidase. What genetic analysis that does exist confirms the original notion that peroxidase exists as a monomer with one mole of heme per mole of enzyme (Scandalios, 1969).

Genetic variants of peroxidase isozymes have been found in pollen and liquid endosperm of several inbred maize strains (Scandalios, 1969). One zone of peroxidase activity in corn pollen has been analyzed in detail and evidence shows that the variants in this zone are inherited according to simple Mendelian rules and are determined by co-dominant alleles at one locus.

Genetic analysis of two peroxidase isozymes in developing barley seedlings has shown that the electrophoretic variants are inherited according to Mendelian rules and behave as monomers (Felder, 1970).

A model has been proposed to implicate the expression of peroxidase isozymes in tobacco with the S-incompatibility genes because different peroxidase isozymes are expressed with different combinations of the S-alleles (Pandey, 1967).

The occurrence of hydroxyproline in peroxidase

Maehly and Paleus (1950) analyzed the acid hydrolysate of purified horseradish peroxidase and reported that all the common amino acids were present with the exception of tryptophan and hydroxyproline. Klapper and Hackett

(1965) determined the amino acid composition of three cathodically migrating isozymes of peroxidase from horseradish and did not find any hydroxyproline. However, in an analysis of the seven isozymes of horseradish peroxidase which had been purified to homogeneity by both the criteria of a single peak in sedimentation velocity centrifugation and a single band on disc gel electrophoresis, Shannon (1966) reported the definite presence of hydroxyproline in the three anodically migrating isozymes.

Barnett (1970) has reported that the release of peroxidase from cell walls by treatment with cellulase occurs concomitantly with release of hydroxyproline. Osborne (1970) has reported hydroxyproline containing peroxidases with cellulase treatment of pea cell walls, and furthermore, reports the secretion of an hydroxyproline rich peroxidase from peas in response to treatment with high levels of ethylene (Ridge and Osborne, 1970, 1971).

The question of the presence of hydroxyproline in peroxidase is very important because hydroxyproline is an unusual amino acid in plants and is predominantly found in cell wall protein (Lamport, 1963). It is therefore striking that peroxidase, an enzyme found to be located on the cell walls of plants, should be the only enzyme which is reported to contain hydroxyproline. The speculation immediately arises that peroxidase accounts for the occurrence of hydroxyproline in the cell wall. On the other hand,

it might be argued that the peroxidase contains hydroxyproline because it is attached in some covalent or non-covalent way to a subunit of the hydroxyproline rich protein and that this subunit facilitates the entry of peroxidase into the cell wall (Lamport, 1970).

Use of the Term "Isozyme"

An isozyme system is usually defined as a collection of distinct molecular entities which possess the same enzymatic activity. Practically, isozymes are recognized by subjecting a sample to electrophoresis in a supporting bed of starch or acrylamide gel and visualizing the location of enzymes by staining with substrates which are specific for that enzyme. At this point all that can be said about the various bands of activity visualized is that they represent discrete molecular entities with different charge characteristics. Genetic analysis is required to show the extent to which the various bands of activity represent different gene products.

Unfortunately, although horseradish is the classic source of peroxidase, no genetic analysis has been performed on the peroxidase isozymes. Therefore it is impossible to use a rigorous characterization of peroxidase isozymes since this depends on a knowledge of the genetic origin of the enzyme. An operational definition which recognizes different isozymic forms of an enzymic activity

only on the basis of charge is therefore used in this discussion: peroxidase isozymes are defined as being distinct when they can be resolved by the standard conditions of starch gel electrophoresis at pH 8.3.

Even using the operational definition it is difficult to identify isozymes in the case of peroxidase, because the isozymes observed on starch gel depends on the hydrogen donor used to visualize peroxidase, and the individual peroxidase isozymes have different relative specific activities when assayed with different hydrogen donors. It is therefore necessary to standardize isozyme observations by using a single staining procedure. Benzidine-H_2O_2 has been chosen as the peroxidase substrate by which isozymes are identified. A numbering system for peroxidase isozymes has been devised which is based on the staining pattern obtained when a homogenate of horseradish root tissue is subjected to electrophoresis in 12% starch-gel at pH 8.3 and stained for peroxidase activity with benzidine-H_2O_2 (Figure 1). In this scheme, peroxidase isozymes are numbered sequentially beginning with the most anodically migrating (No. 1) and ending with the most cathodically migrating (No. 7).

Statement of the Problem

Two recent reviews which cover the subject of plant peroxidases (Shannon, 1968; Scandalios, 1969), come

Figure 1. Numbering system for horseradish peroxidase isozymes.

Isozymes are based on starch gel electrophoresis of horseradish root tissue homogenate (pH 8.3) and visualization of peroxidase activity with benzidine-H_2O_2.

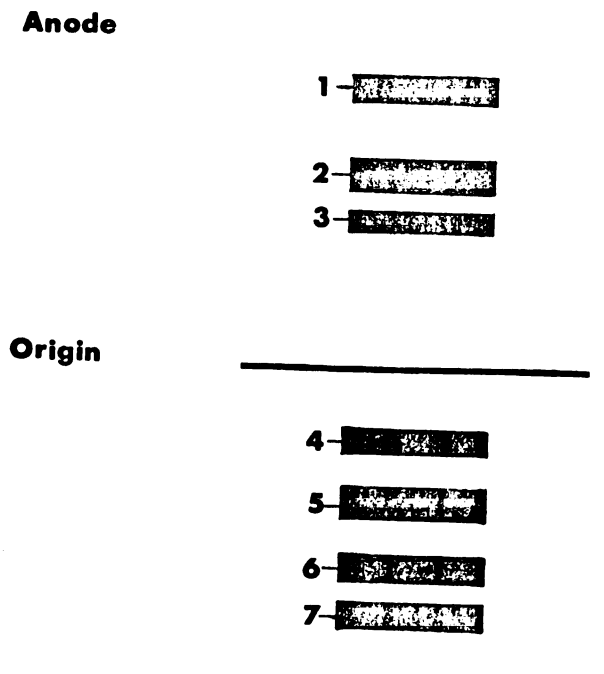


Fig. 1

to the same general conclusions: peroxidase is tremendously diverse and heterogeneous in plants; the physiological role of peroxidase is as yet unknown; before any conclusions as to the biological role of peroxidase may be made, further study is necessary to elucidate the precise biological functioning and chemical structures of peroxidases as particular isozymes rather than as a broad class of enzymes with essentially identical activities.

These comparative investigations on some of the isozymes of horseradish peroxidase are made not with the idea of solving the riddle of the biological functioning of peroxidase and explaining the reasons for the multiplicity of peroxidase isozymes, but merely to learn more about the similarities and differences among some of the isozymes of the horseradish peroxidase system.

The horseradish plant (Amoracia lapathifolia) was chosen as a suitable subject for study because it is the classical plant source for peroxidase. All the kinetic studies of the mechanism of action of peroxidase have been performed with the enzyme from horseradish roots. The wealth of literature on the mechanism of action of peroxidases will be directly applicable to the horseradish system without ambiguity.

It is important to determine whether hydroxyproline is really attached to peroxidase. The relation between

hydroxyproline and peroxidase was determined in a commercially purified preparation of horseradish peroxidase, the supernate of a homogenate of horseradish root tissue, the peroxidase bound to the cell wall, and the peroxidase found external to the cell in the incubation medium of aerated horseradish root discs.

The relation of peroxidase to the horseradish cell wall was thoroughly investigated. A quantitative determination of the peroxidase activity bound to the cell was made and the isozymic forms of peroxidase which are bound to the cell wall were identified. The kinetics of peroxidase bound to the cell wall was also examined.

Although peroxidase isozymes exhibit different specific activities with different substrates, no differences in the construction of the active site of peroxidase isozymes has been reported. We have attempted to demonstrate such a difference between two peroxidase isozymes using the ammonia induced stimulation of peroxidase activity as a criterion.

Differences in the kinetics of two peroxidase isozymes in a common reaction, the formation of fluorescent biphenyls with homovanillic acid, will be discussed.

No one has studied peroxidase from parts of the horseradish plant other than its roots. I suspect that this is because to most investigators the source of

horseradish is the supermarket rather than the field. The distribution of peroxidase isozymes in all tissues of the horseradish plant was determined. We have also determined the distribution of peroxidase isozymes within the cell, bound to the cell wall and external to the cell in the case of horseradish roots.

Attempts were made to modify the electrophoretic mobility of peroxidase isozymes both by enzymic treatment and by incubation at different pH values.

Two zymogram stains for peroxidase which measure reactions of physiological significance were developed. These stains showed large differences in the activity of peroxidase isozymes for these physiological reactions.

CHAPTER II

METHODS AND MATERIALS

Source of Peroxidase

Two sources were used for the peroxidase enzyme. The first source was commercially purified horseradish peroxidase purchased from the G. Worthington Co., Freehold, N. J. This preparation of enzyme contains both anodic and cathodic isozymes of peroxidase, and is labelled by the company HRP-HPOD 6FA. The second source of peroxidase was field and greenhouse grown horseradish plants (Armoracia lapathifolia, Gilib., cv. Maliner Kren) which were originally purchased as root cuttings from the W. Atlee Burpee Co., Philadelphia, Pa.

Analysis of Amino Acids and Sugars

The analysis of protein, amino acids, and sugars was performed in the laboratory of Dr. D. T. A. Lamport using methods which were developed by Dr. Lamport and are routinely used in his laboratory.

Colorimetric hydroxyproline analysis is accomplished by oxidation of hydroxyproline with sodium

hypobromite followed by reaction with Ehrlich's reagent (Kivirikko and Liesma, 1959). Color reaction at 560 nm is then compared to values on a standard curve. Lamport has determined that this reaction will work only on non-peptide linked hydroxyproline, and will cross-react to a small extent with carbohydrate, but the reaction with carbohydrate yields a product with an absorption maximum at 460 nm.

Identification of hydroxyproline on paper following electrophoresis was accomplished by flooding the paper in a solution of Isatin followed by Ehrlich's reagent, and observing the formation of violet spots (Archer, et al., 1950).

Automatic analysis of hydroxyproline in protein fractions was accomplished with the Lamport AutoHyp Analyzer which is an automatic flow-through spectrophotometric system which utilizes a modified hydroxyproline colorimetric reaction which is based on the method of Kivirikko (1959) but which invokes an initial hydrolysis step with 9.5 N. NaOH and neutralization prior to oxidation and reaction with Ehrlich's reagent (Lamport and Miller, 1971).

Identification and separation of hydroxyproline-arabinosides was accomplished by hydrolysis of hydroxyproline containing material under conditions where peptide

bonds are labile and glycosidic bonds are stable, with 0.44 N $Ba(OH)_2$. Determination of bound versus unbound hydroxyproline was accomplished by passing the hydrolysate through a Sephadex G-25 column and assaying the eluate for hydroxyproline. Identification of hydroxyproline-arabinoside species was accomplished either by electrophoresis of the hydrolysate and the observation of retarded electrophoretic mobility of the hydroxyproline-arabinosides compared to free hydroxyproline, or by ion exchange chromatography using an Aminex column (H^+ form) with an HCl gradient. All these methods were developed by Dr. Lamport. The proof that these compounds contain a glycosidic linkage between the hydroxyl group of hydroxyproline and the reducing group of arabinose was performed on material obtained from tomato cells grown in suspension culture (Lamport, 1967). However, since the hydroxyproline-arabinosides from horseradish are chemically and chromatographically identical to those in tomato, and since these compounds are widespread in higher plants (Lamport and Miller, 1971), it was judged that any further characterization of these compounds from horseradish would merely be proving the obvious.

Sugars were resolved chromatographically using an ethyl-acetate : pyridine : H_2O :: 8 : 2 : 1 (v/v) single phase solvent system (Timell, 1960). Sugars were then

detected either with an aniline phthalate dip (Wilson, 1959) or a silver nitrate dip (Trevelyan, et al., 1950).

Arabinose was estimated both manually, using the ferric chloride orcinol method for pentoses, and comparing the color yield at 668 nm to a standard curve (Dische, 1962), or automatically using the Lamport AutoArab Analyzer.

Cell Wall Preparation

Horseradish cell walls were prepared by grinding with 300 micron glass beads in 100% glycerol using an omnimixer. Filtration of the homogenate was accomplished by passing the material over a continually renewable filter of 300 micron glass beads, and the walls were washed free of cytoplasmic material in this way with glycerol. Final filtration and washing of glycerol from the cell wall material was accomplished at -20°C with successive washes of ethanol, acetone and ether. These methods were developed in the laboratory of Dr. R. Bandurski (Kivilaan and Bandurski, 1959) and preparation of horseradish cell walls by this method was done in his laboratory.

Horseradish cell walls were also prepared by grinding horseradish root slices at full speed in a Waring blender for 30 second intervals interspersed with cooling periods for a total grinding time of 10 minutes in a solution of 0.4 M sucrose and 0.1 M phosphate buffer,

pH 7.4. The ground homogenate was then squeezed through six layers of cheesecloth and then centrifuged at 500 g for 15 minutes in 0.1 M phosphate buffer, pH 7.4. The buffer was poured off carefully and the cell wall fragments salt washed by filling the centrifuge bottle with 2 M NaCl, stirring for 10 minutes in the cold room, and then centrifuging for 15 minutes at 2000 g. This is repeated five times. Salts are then removed by centrifuging with water five times and decanting the supernate, and finally allowing cold water to filter over the cell walls in a Büchner funnel.

Separation and Assay of Peroxidase Isozymes

Separation and assay of peroxidase isozymes in horseradish preparations was performed under the guidance of Dr. J. G. Scandalios, in his laboratory. Peroxidase isozymes were visualized with the zymogram technique of electrophoresing samples in starch gel, cutting the gel transversely, and then flooding cut surfaces with a peroxidase specific reaction mixture to visualize isozymes.

The starch is prepared by heating 36.0 g of hydrolyzed potato starch (Connaught Laboratories) in 300 ml of buffer, over an open flame until a gel forms. The gel is then degassed by vacuum, and poured into an 18 x 20 x 0.7 cm perspex mold. The gel is then allowed

to cool to approximately 4°C. Samples are absorbed into 15 x 7 mm wicks of Whatman 3MM filter paper and the wicks inserted into a perpendicular slit in the starch gel. The gel is then subjected to electrophoresis at 4°C across one liter of electrolyte divided into two electrode trays. Electrophoresis is carried out for eight hours at 150 V. Upon completion of the electrophoretic run, the gel is unmolded, cut transversely into three 2 mm slices, and the cut surfaces stained for peroxidase activity. All these methods were developed by Dr. Scandalios, and are in use in his laboratory (Scandalios, 1969).

A discontinuous buffer system is used for starch-gel electrophoresis of horseradish peroxidase. The gel buffer consists of 270 ml of 0.2 M Tris-citrate buffer, pH 8.3 plus 30 ml of 0.2 M Lithium-borate buffer, pH 8.3. The tank electrolyte is 0.2 M Lithium-borate buffer, pH 8.3 (Scandalios, 1969).

The visualization of peroxidase isozymes on a developed starch gel is routinely accomplished with a benzidine-H_2O_2 reaction mixture (Scandalios, 1964). A stock benzidine solution is prepared by gently heating 1 g of benzidine in 9 ml of glacial acetic acid, and then adding 36 ml of H_2O. The reaction mixture is made by mixing equal amounts of the stock benzidine with 0.6% hydrogen peroxide and painting the mixture, with a brush,

onto the cut gel surface. Peroxidase is located by the appearance of bright blue bands.

Horseradish peroxidase is routinely assayed for activity spectrophotometrically with o-dianisidine (3,3-dimethoxybenzidine) as the hydrogen donor. This method was developed by C. S. Worthington, and is found printed in the Worthington Biochemical Catalog (Gregory, 1966). It has been used successfully by numerous investigators, including Shannon, et al. (1966), and Kay, et al. (1967). This reaction follows the peroxidation of o-dianisidine to form a colored product with an absorption maximum at 460 nm. In this report, one unit of enzyme activity will be defined as the change in absorbance at 460 nm of 1 O.D. unit per minute (1 U. $\equiv \Delta 1$ O.D.^{460}/min).

Peroxidase is also assayed by a fluorimetric method using homovanillic acid (Guilbault, et al., 1968). This reaction uses the non-fluorescent homovanillic acid to form the highly fluorescent 2,2'-dihydroxy-3,3'-dimethoxybiphenyl-5,5'-diacetic acid. This reaction was originally developed as a more sensitive peroxidase assay than the usual spectrophotometric ones. However, it is used in this study, not for its sensitivity, but because it uses as the hydrogen donor a compound of a class different from benzidine or o-dianisidine, and what is measured in this reaction is the formation of a condensation product which is a biphenyl. This reaction is also different from

the o-dianisidine reaction because the pH optimum for the homovanillic acid peroxidase assay is 8.5, whereas for the o-dianisidine reaction it is 5.8. Furthermore, we have noticed that below pH 7, no product is formed in the homovanillic acid assay, whereas at pH 8.5 or higher, the o-dianisidine reaction is still active, although at a reduced rate. This reaction is also important because its mechanism is identical to the formation of dityrosine which is catalyzed by peroxidase (Gross and Sizer, 1959) and is known to be physiologically significant in animals.

The homovanillic acid peroxidase assay is monitored by measuring the increase in fluorescence emission at 425 nm with excitation at 315 nm. To obtain standardized results, a 0.1 μg/ml solution of quinine sulfate in 0.1 N H_2SO_4 is used to adjust an Aminco-Bowman spectrophotofluorometer to 0.2 F.U. before each use with excitation at 350 nm and emission at 450 nm (Guilbault, 1968). One unit of enzyme activity by this assay is defined as the change in fluorescence of 1 F.U. per minute (1 U. ≡ 1 F.U./min, excitation at 315 nm, emission at 425 nm).

Isopycnic Equilibrium Sedimentation of Peroxidases

Peroxidase was subjected to cesium chloride isopycnic centrifugation according to methods described by Filner and Varner (1967). 3.0 ml CsCl gradients were employed with an average density from 1.3 to 1.4. 150

enzyme units of beef liver catalase (Worthington) was added to the gradients as a marker. The gradients were formed by centrifuging at 40,000 RPM for 72 hours in a Beckman 65B ultracentrifuge using the SW-65 swinging bucket rotor. At the end of the centrifuge run, 3-drop fractions were collected from the bottom of each tube. The refractive index of every tenth fraction was recorded and plotted.

Hydroxyproline was assayed in every other fraction from the CsCl gradient. Since the hydroxyproline assay is only sensitive to the free amino acid, these fractions were first subjected to acid hydrolysis. It was estimated that the volume of each fraction was 25 µl. 75 µl of 8 N HCl was added to each fraction, making the final concentration 6 N HCl. The tubes were sealed, and incubated for 18 hours at 105°C. The tubes were opened, and placed in a desiccator jar, where HCl was removed by evacuation. The volume of these tubes was then brought up to 1.0 ml with water and hydroxyproline assayed in the usual manner.

Total peroxidase activity was determined in all the fractions which were not used for hydroxyproline determination. A spectrophotometric assay was employed, using o-dianisidine as the hydrogen donor (Shannon, et al., 1966).

Catalase was assayed using an oxygen electrode to monitor the rate of oxygen production (Goldstein, 1968).

In order to resolve and estimate the buoyant densities of the different peroxidase isozymes in this gradient, 5 µl samples of every other fraction in the gradient were placed on paper wicks and these were loaded sequentially in a 12% starch gel. The starch gel was then subjected to electrophoresis and stained for peroxidase activity using benzidine-H_2O_2. This technique allows the resolution of individual peroxidase isozymes in a gradient, and the estimation of their densities, since the peak tube of distribution of any individual isozyme will have the most intense benzidine color reaction (Quail and Varner, 1971). Peak fractions for the individual isozymes was determined by densitometer tracings of photographs of the distribution of each particular isozyme on the starch gel zymogram.

CHAPTER III

NEW METHODS FOR THE ANALYSIS OF PEROXIDASE ISOZYMES

In the course of this research, two original tools for the assay of peroxidase activity were developed. The first was the construction of an automated peroxidase analyzer based on the Technicon system of proportioned pumping. The second was the development of a peroxidase zymogram staining procedure which allows the quantitative estimation of the activity of peroxidase isozymes directly on the supporting gel medium.

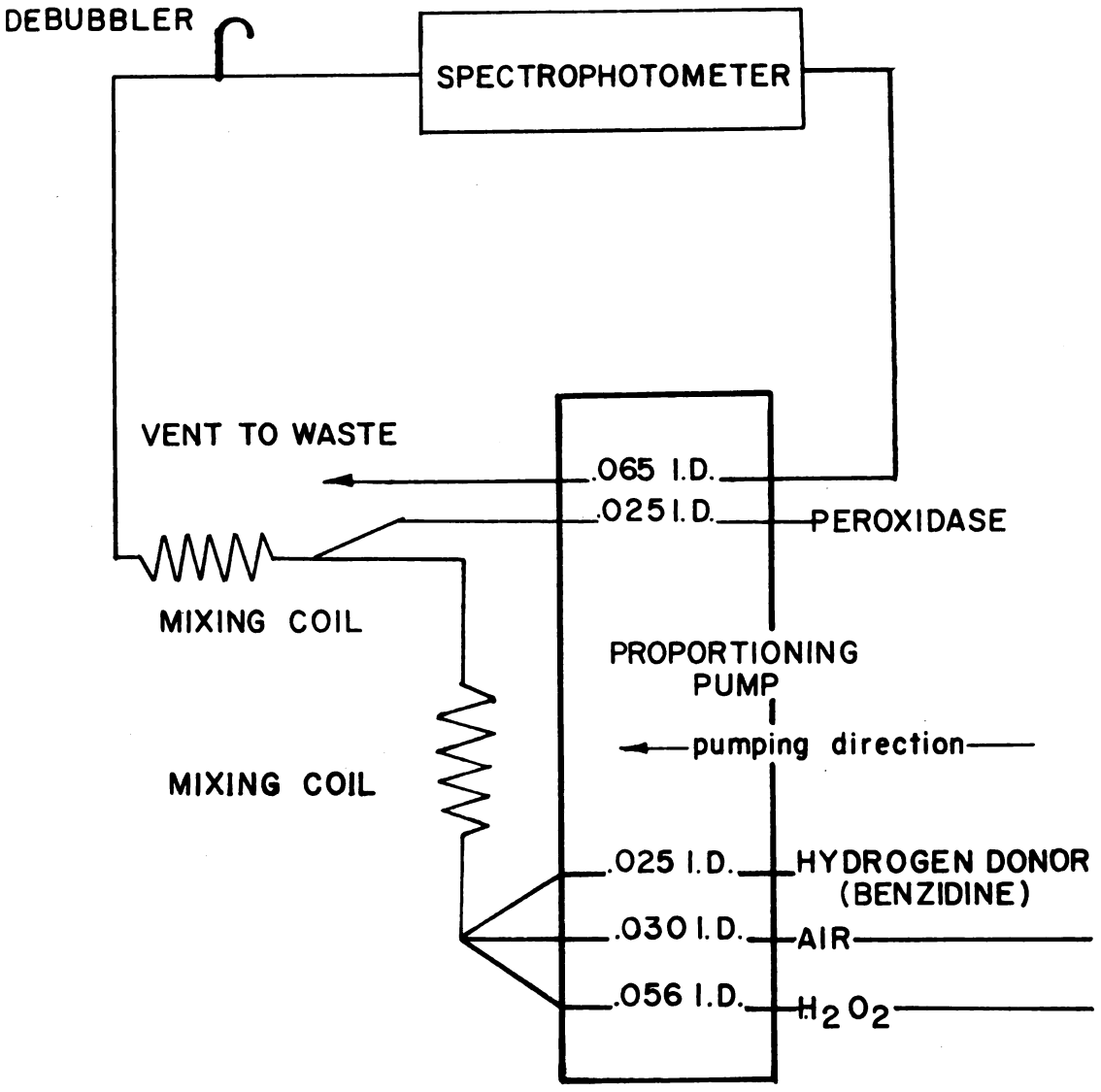
Isoelectric focusing was, of course, not developed in this laboratory; both the isoelectric focuser and the techniques for its use were developed by the LKB Instrument Co., Stockholm, Sweden.

Automated Peroxidase Analyzer

An automatic peroxidase analyzer was constructed using a Technicon proportioning pump and automatic sampler. A schematic diagram of the flow path for this system is shown in Figure 2. The hydrogen donors benzidine, o-dianisidine, and o-toluidine were tried for this reaction, but only benzidine was satisfactory. The other reagents

Figure 2. Schematic flow diagram of automatic peroxidase analyzer.

Tubing internal diameter (ID) values are given in inches.



FLOW DIAGRAM OF AUTOMATIC PEROXIDASE ANALYSER

FIG. 2

left precipitating substances on the glass coils which built up with continuous pumping, and obscured the color reaction within five minutes of pumping.

Basically, this system consists of three lines of reagents, the enzyme, the hydrogen peroxide, and the hydrogen donor, benzidine. In the pumping scheme, the benzidine and H_2O_2 are brought together and mixed, and then the peroxidase is introduced, and these are also mixed. Incubation time can be varied by adjusting the length of tubing between the mixing point of enzyme and reagent, and the flow through cell at a recording spectrophotometer. This time was kept constant at one minute. Color production is read by the spectrophotometer at 610 nm.

The enzyme can come from sampling cups on the automatic sampler, or its concentration can be held constant merely by sampling from a large volume of enzyme solution, while the concentrations of other reagents are varied.

This analyzer shows peaks of color absorption, which represent the color production of the enzyme after one minute's incubation. These peaks were shown to be reproducible when the same concentration of enzyme was presented to the analyzer several times (Figure 3), and color production was shown to be proportional to the concentration of the enzyme assayed (Figure 4).

Figure 3. Automatic peroxidase analysis of multiple samples of 4.1 µg of horseradish peroxidase (Worthington HRP-HPOD-6FC).

Samples of peroxidase solution (8.2 µg/ml) are placed in cups of a Technicon Automatic Sampler. 0.5 ml of solution is withdrawn from each cup in 2 minutes by the sampler.

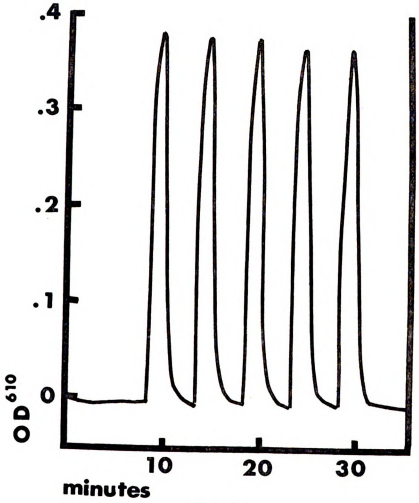


FIG. 3

Figure 4. Automatic peroxidase analysis of varying concentrations of horseradish peroxidase (Worthington HRP-HPOD-6FC).

1. 0.62 µg 2. 1.24 µg 3. 1.86 µg 4. 2.48 µg

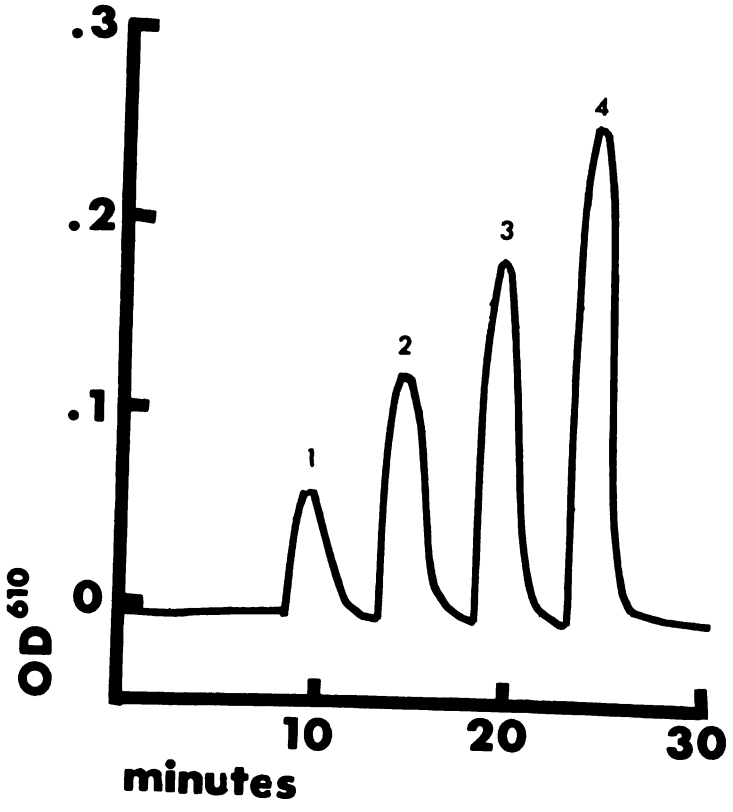


FIG. 4

Ascorbate-benzidine Coupled Peroxidase Zymogram Stain

When ascorbic acid is coupled with the benzidine peroxidase assay, it reduces the blue oxidized form of benzidine, and color development is arrested until the ascorbic acid is completely oxidized, at which time the reaction mixture immediately turns blue (Gregory, 1966). The time taken for blue color to appear is inversely proportional to the enzyme concentration, and directly proportional to the concentration of ascorbate in the reaction mixture. The quantitative estimation of peroxidase was thus made chronometric and a stopwatch could replace a spectrophotometer for peroxidase assay. This idea was transferred to the benzidine reaction mixture for the visualization of peroxidase on starch gel zymograms.

When ascorbate is added to the benzidine reaction mixture and painted on a gel, at first no bands of peroxidase are seen, but as time progresses blue bands which represent peroxidase isozymes suddenly appear on the surface of the gel. The time elapsed before a peroxidase band is visualized on the starch gel is thus inversely proportional to the activity of that particular isozyme.

The following ascorbate-benzidine coupled peroxidase zymogram stain was developed:

70.4 mg ascorbic acid

20.0 ml Stock benzidine (2.5% w/v in 3.5 M acetic acid)

20.0 ml 0.6% hydrogen peroxide

60.0 ml H_2O

Peroxidase is assayed by brushing this solution quickly over the cut face of a starch gel with a clean camel hair brush, and immediately starting a stopwatch. The time required for the appearance of individual peroxidase isozymes is recorded.

The sap from frozen and stored horseradish petioles was used as a source of peroxidase and diluted 2, 4, 8, and 16-fold with water. 40 µl of each of these diluted samples were used to wet individual 7 x 15 mm paper wicks. These were then subjected to electrophoresis and stained with the ascorbate-benzidine coupled zymogram stain. The time elapsed before the appearance of one particular isozyme, the second most anodically migrating, was recorded for each of the dilutions of the petiole sap (Figure 5). The time required for the appearance of this isozyme on the cut surface of the starch gel is shown to be proportional to the concentration of the isozyme applied to the wick.

Isoelectric Focusing of Peroxidase

The supernatant fraction from a homogenate of horseradish roots was subjected to isoelectric focusing over the range of pH 3-10. The focusing run was considered to be complete when the current supplied became constant,

Figure 5. Time required for the visualization of the major anodic peroxidase isozyme of horseradish petiole when a dilution series of fresh horseradish petiole sap is subjected to electrophoresis and peroxidase visualized with a reaction mixture of 4 mM ascorbate-benzidine-H_2O_2.

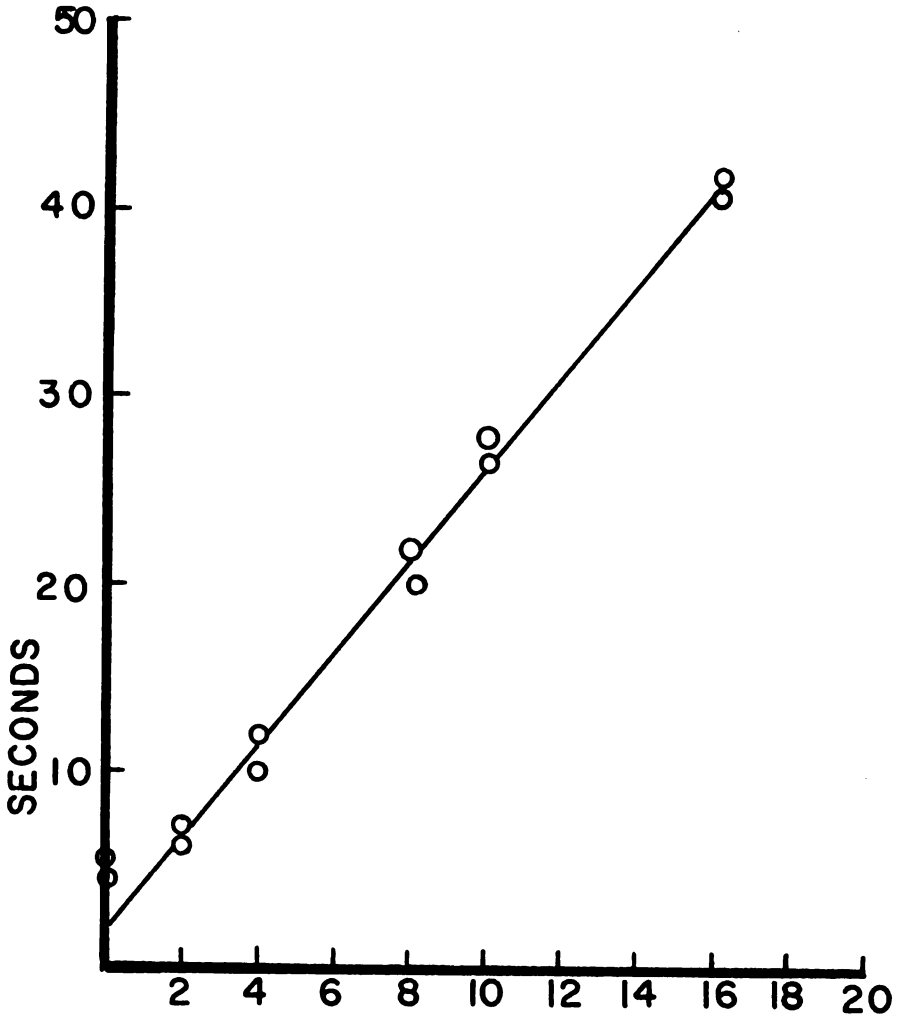

50
40
30
20
10
SECONDS
2
4
6
8
10
12
14
16
18
20
1/DILUTION FACTOR OF FRESH
HORSERADISH PETIOLE JUICE

figure 5

and inspection of the column under ultraviolet light showed the carrier ampholytes to be completely stacked and sharply focused. Fractions were collected from the column, and each was assayed for peroxidase activity spectrophotometrically with o-dianisidine as a hydrogen donor. The pH of each fraction at 0°C was also recorded (Figure 6). Peroxidase is an unusual enzyme system with isoelectric points ranging from pH 3-10. Several peaks of peroxidase activity were noted, and the isoelectric points at these peaks recorded (Table 1). There is considerable overlap

Table 1. Isoelectric points of peaks of peroxidase activity obtained from an isoelectric focusing separation of the supernate from a root homogenate.

pI	Peroxidase Activity ΔOD^{460}/min/ml
2.98	0.70
3.48	4.88
4.28	0.13
5.34	1.05
5.98	7.65
7.25	15.7
8.09	15.5
9.14	51.9
10.00	10.7

pH range used is 3-10. Peroxidase is assayed with o-dianisidine as a hydrogen donor.

Figure 6. Isoelectric focusing run of the supernatant fraction from the homogenate of horseradish roots.

□—□. Peroxidase activity measured with o-dianisidine as hydrogen donor. ○——○. pH.

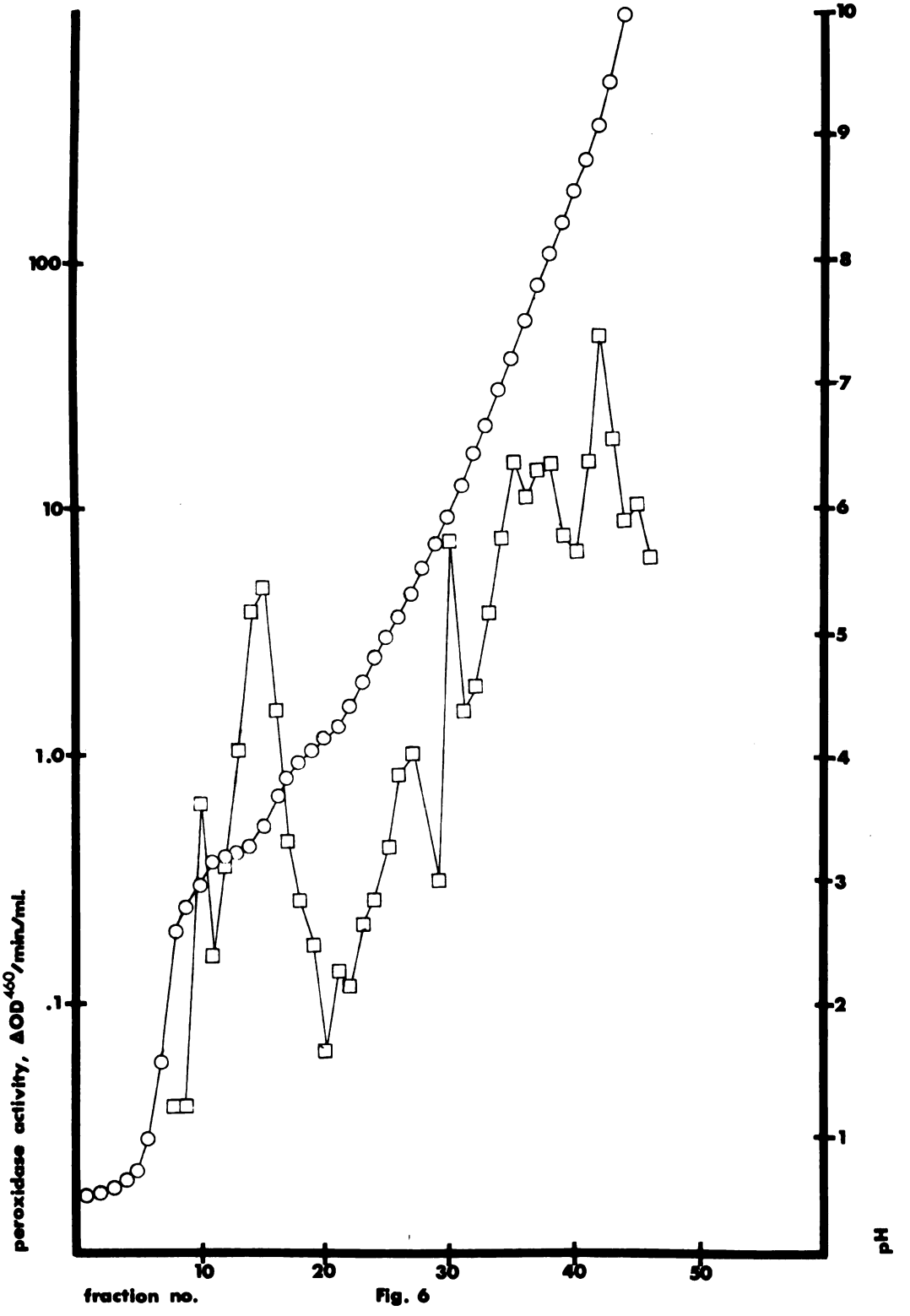

peroxidase activity, ΔOD 460/min/ml.
100
10
1.0
.1
10
20
30
40
50
fraction no.
10
9
8
7
6
5
4
3
2
1
pH

Fig. 6

of isozymes in the fractions collected from the focusing column. For example, the peak of activity at pH 3.48 actually comprises the two major anodic isozymes of horseradish root tissue.

CHAPTER IV

PEROXIDASE ISOZYMES BOUND TO HORSERADISH ROOT CELL WALLS

Three lines of evidence suggest that peroxidase is an extracellular enzyme associated with the cell walls of higher plants. The function of peroxidase in the formation of lignin can be presumed to take place extracellularly and on the cell wall, since lignin is found only in the secondary thickenings of cell walls. Histochemical staining of tissue sections for the location of peroxidase activity shows this enzyme on cell walls. In cultured plant cells, peroxidase is found in the incubation medium, which suggests that it is a secreted enzyme.

It would be valuable then to assay the actual peroxidatic activity of plant cell walls and determine precisely what percentage of the total peroxidase in a tissue is bound to the cell wall. The usual spectrophotometric substrates for peroxidase such as benzidine and o-dianisidine are useful only for assaying the soluble enzyme, and do not produce reliable results when used to measure the peroxidatic activity of particulate, insoluble

cell walls. However, the homovanillic acid assay for peroxidase overcomes the shortcomings of the spectrophotometric substrates in measuring the activity of cell walls. Since this assay is fluorimetric and measures light production at a given wavelength, particulate materials such as cell walls do not interfere greatly with the reaction. The biphenyl reaction product of this reaction also does not appear to bind to the cell walls. This assay allows the continuous monitoring of fluorescence production when cell walls are used as a source of peroxidase. From these data, initial velocities can be calculated, and hence the kinetic properties of peroxidase bound to cell walls can be determined.

The proof that homovanillic acid accurately measures the peroxidatic activities of particulate cell walls can be seen in the calculations of the following sections: when peroxidase is solubilized from cell walls, either by salt washing or by treatment with cellulase, the sum of the peroxidatic activity released and the activity remaining on walls is equal to the peroxidase activity calculated to be on cell walls before the treatment.

Proportion of Total Peroxidase Which is Bound to the Cell Wall

Horseradish root cell walls were obtained by grinding the tissue with distilled water for a total of 15

minutes at full speed in a Waring blender. By blending for one minute periods and allowing for cooling in an ice bath, the temperature of the homogenate was not allowed to rise over 10°C. The resulting homogenate was squeezed through eight layers of cheesecloth. The bulk material which remained in the cheesecloth was regarded as the cell wall fraction. The wet weight of this material was recorded and dry weight estimated by drying and weighing small samples, then calculating a conversion factor. The volume of the homogenate which passed through the cheesecloth was recorded. The peroxidatic activity in this homogenate was presumed not to be associated with cell walls. The wall material was then washed with eight liters of distilled water, and again squeezed through cheesecloth. These walls were labeled "water washed walls." The peroxidatic activity of these walls was determined with the homovanillic assay, and compared to the peroxidase activity found in the supernatant fraction of the homogenate (Table 2). From

Table 2. Peroxidase activity in the supernatant and cell wall fractions of a horseradish root homogenate.

Fraction	Volume	Dry Wt.	Peroxidase Sp. Amt.	Total Peroxidase	% of Total Peroxidase
Supernate	1275 ml	--	6.34 U/ml	8087 Units	80%
Cell Wall	--	41.0 g	49.3×10^{-3} U/mg dry wt	2020 Units	20%

Peroxidase is assayed fluorimetrically with homovanillic acid.

these data, we can calculate that 20% of the total peroxidase found in horseradish root can be found bound to the cell wall. This does not mean that 80% of the total peroxidase activity is cytoplasmic, because the peroxidase found in the supernate also includes peroxidase which is external to the cell but not bound to the wall.

Peroxidase Isozymes Which can be Released from Cell Walls by Salt Washing

The water washed horseradish cell walls were incubated in 750 ml of 2 M NaCl for two hours at 4°C, with stirring. The slurry was squeezed through eight layers of cheesecloth, washed again with eight liters of water, and labeled "salt washed walls." Peroxidase in the salt washed walls and the supernate from salt washing was assayed with homovanillic acid. The specific activity of peroxidase on cell walls dropped from 49.3 to 3.66 x 10^{-3} U/mg dry weight of wall. 92.6% of the activity found in water washed walls can be released by treatment with 2 M NaCl (Table 3). Thus most of the peroxidase found on cell walls is not covalently but rather ionically bound. The nature of this ionic binding is demonstrated by the fact that the cell walls were extensively washed with water and the peroxidase remained attached during this period while 2 M NaCl released the peroxidase to the incubation medium.

Table 3. Peroxidase activity released from horseradish cell walls by treatment with 2 M NaCl.

Fraction	Volume	Dry Weight	Peroxidase Specific Activity	Total Units Peroxidase	% Peroxidase Released	% Recovery
H_2O washed cell walls	--	41.0 g	49.3×10^{-3} U/mg dry wt.	2020	--	--
Salt wash	750 ml	--	2.64 U/ml	1980	93%	--
Salt washed cell walls		41.0 g	3.66×10^{-3} U/mg dry wt.	150	--	106%

Peroxidase is assayed fluorimetrically with homovanillic acid.

Does this peroxidase which can be removed from the cell walls by salt washing represent cytoplasmic peroxidase which has become attached during the homogenization period? Because of the charge characteristics of cell walls which at the extraction pH of 4.6 primarily represents the carboxyl groups of uronic acids, this is quite likely. However, a zymogram of the peroxidases released from cell walls by salt washing (Figure 7) does not have the same relative distribution of isozymes as does an equivalent amount of enzyme from the tissue homogenate. This is an indication that not all the cytoplasmic peroxidase isozymes bind on to cell walls with the same affinity. In fact, there are two isozymes found in the peroxidase released from cell walls that have no counterparts in the cytoplasmic peroxidase isozymes. While salt washing releases 92.6% of the peroxidase bound to the cell wall, it also released 34% of the hydroxyproline found on the cell wall (Table 4).

Horseradish root slices were labeled with ^{14}C-proline by incubation under aerating conditions. After washing, cell walls were prepared from these tissue slices by grinding in a mortar and pestle. The wall fragments were mixed with celite (Hyflo Supercel) and then used to pack a 0.7 x 3 cm column. This cell wall column can be thought of as an analog of an ion exchange resin column. Any peroxidase which was not actually bound on to cell

Figure 7. Peroxidase isozymes associated with horseradish root cell walls.

Samples are adjusted to identical specific activities using the spectrophotometric o-dianisidine assay. Peroxidase activity of samples is 390 U/ml. 1 and 2. Supernatant fraction from a 2.0 M salt washing of horseradish root cell walls. 3 and 4. Supernate from a homogenate of horseradish root tissue slices.

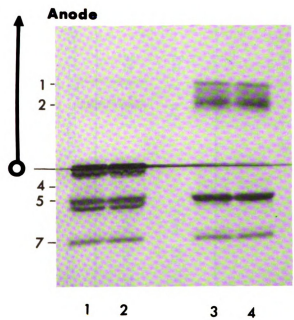


Fig. 7

Table 4. Hydroxyproline released from cell walls by incubation in 2 M NaCl.

Fraction	Dry Weight	Hydroxyproline Content	Total Hydroxyproline	% Hydroxyproline Released
H_2O washed cell walls	41.0 g	2.75 µg/mg wall	112.5 mg	--
Salt washed cell walls	41.0 g	1.81 µg/mg wall	74.2 mg	34%

walls was removed by pumping 100 ml of H_2O across the column. A 40 ml salt gradient from 0-10 M LiCl was then pumped across the cell wall column and 1.0 ml fractions collected. The fractions were then assayed for peroxidase spectrophotometrically using benzidine as a hydrogen donor, and 0.1 ml aliquots from each fraction were removed and ^{14}C counts determined (Figures 8 and 9). The 88% of the peroxidase which is ionically bound to the cell wall is eluted at 1.3 M LiCl. The 50.4% of the radioactivity is also eluted at this molarity.

Chromatography of a hydrolysate from the peak region of counts eluted from the wall (1.3 M) showed that radioactivity appeared in both hydroxyproline and proline, with a hypro/pro ratio of 4.28. Beyond 1.3 M LiCl essentially no peroxidase can be eluted from cell walls even at salt concentrations up to 10 M.

Peroxidase Isozymes Which Can be Released from Cell Walls by Cellulase Treatment

Identification of the peroxidase isozymes which are bound on cell walls and are resistant to salt washing can be obtained by treating the salt washed walls with cellulase. When 11.25 grams (dry weight) of salt washed horseradish cell walls incubated in 400 ml of a 0.5% solution of a cellulase preparation (Trichoderma viride, from Worthington Biochemicals, Freehold, N. J.) peroxidase was

Figure 8. Peroxidase activity released from purified horseradish cell walls with a salt gradient of 0-10 M LiCl.

Peroxidase is assayed spectrophotometrically using o-dianisidine as the hydrogen donor.

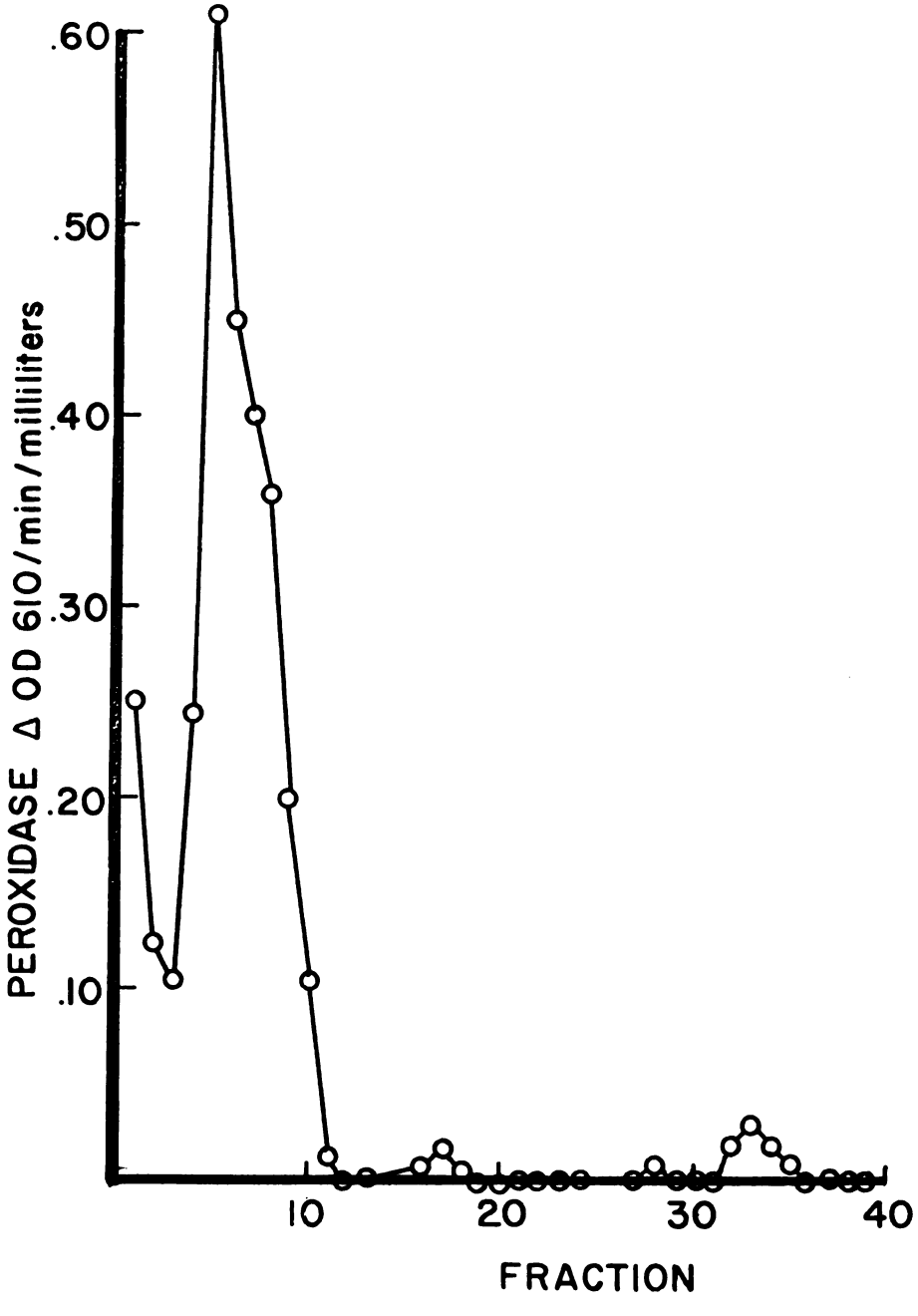

.60
.50
.40
.30
.20
.10
PEROXIDASE Δ OD 610/min/milliliters
10
20
30
40
FRACTION

figure 8

Figure 9. ^{14}C counts released from purified horseradish cell walls with a salt gradient of 0-10 M LiCl. The cells had been incubated for 24 hours in ^{14}C-proline prior to extraction of cell walls.

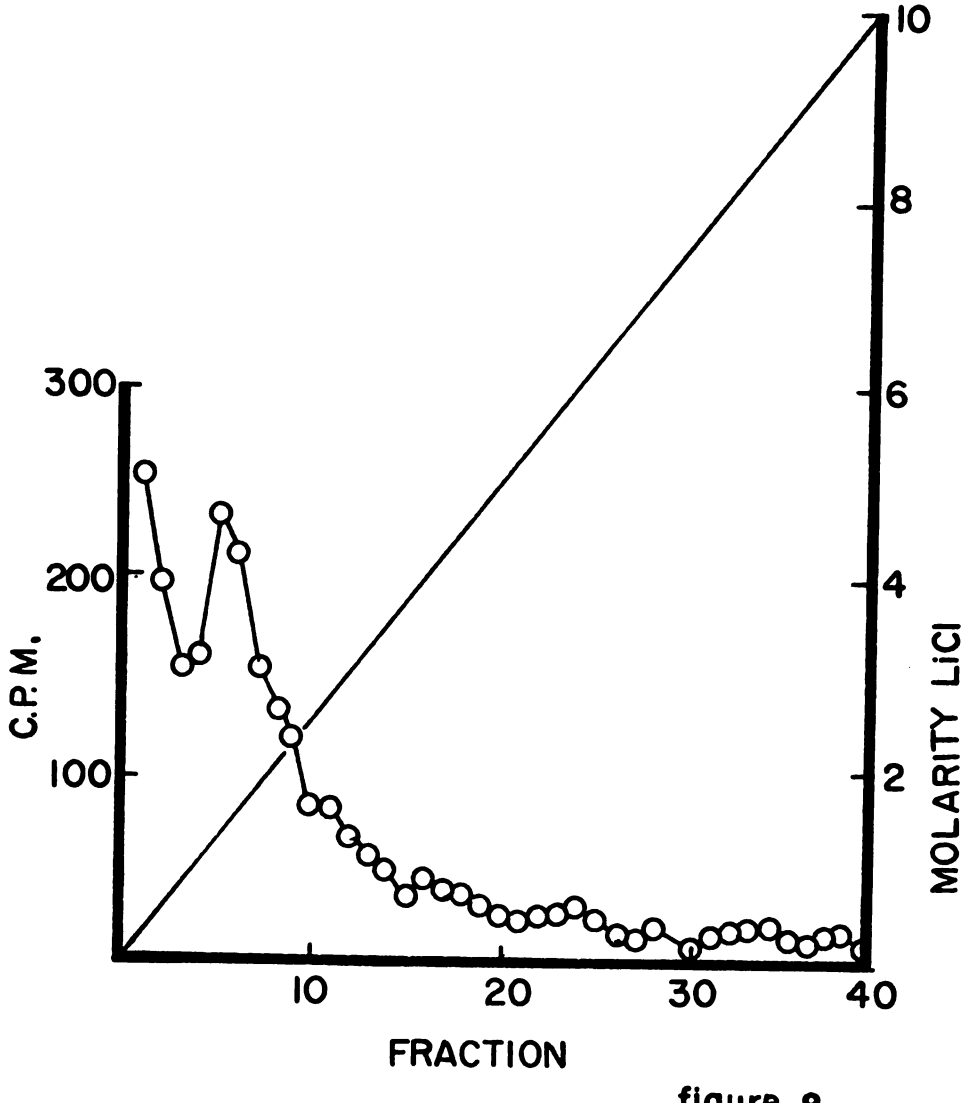


figure 9

solubilized to the incubation medium. After 24 hours incubation at 30°C, the specific activity in the medium of cellulase treated cell walls rose to 37.2 U/ml when assayed spectrophotometrically with o-dianisidine as the hydrogen donor. This compares to a final peroxidase activity of 2.3 U/ml in the medium of a control treatment which did not contain added cellulase (Figure 10). After the cellulase treatment, the specific activity of peroxidase on cell walls drops from 3.66 to 2.88 x 10^{-3} U/mg dry weight when assayed fluorimetrically with homovanillic acid. 75% of the peroxidase activity remaining on salt washed horseradish cell walls can be released by this treatment (Table 5).

The peroxidase which was released from cell walls by the cellulase treatment is genuinely soluble since 93% of the peroxidatic activity in the incubation medium is found in the supernatant fraction after centrifugation for 30 minutes at 100,000 g (Table 6). A zymogram of the peroxidase isozymes which are released from cell walls by cellulase treatment shows only one isozyme which has no counterpart in the horseradish root homogenate (Figure 11).

This cellulase treatment also releases 57% of the hydroxyproline from the cell wall (Table 7).

By a combination of salt washing and enzymic treatment with cellulase we have succeeded in releasing in soluble form 98% of the peroxidase which is associated

Figure 10. Release of peroxidase into the incubation medium of cellulase treated horseradish root cell walls.

○——○. 11.25 g of walls incubated in 400 ml of 0.05 M acetate buffer, pH 5.5, containing 2.0 g of cellulase.
□——□. 11.25 g of walls incubated in 400 ml of 0.05 M acetate buffer, pH 5.5, containing no cellulase.

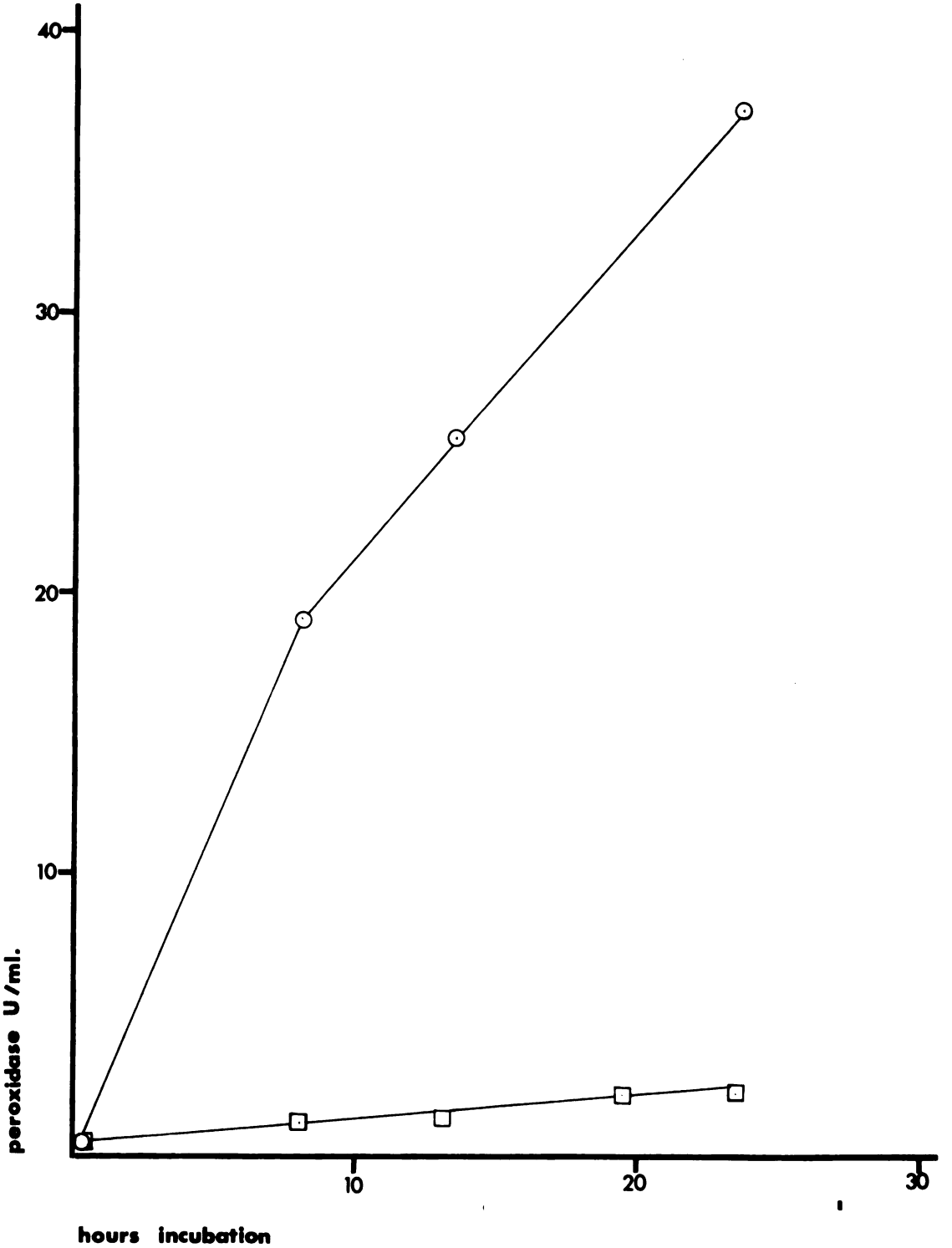


Fig. 10

Table 5. Peroxidase released from 2 M NaCl washed cell walls by treatment with cellulase for 24 hours at 30°C.

Fraction	Volume	Dry Weight	Peroxidase Specific Activity	Total Units Peroxidase	% Peroxidase Released	% Recovery
Salt washed cell walls	--	11.25 g	3.66×10^{-3} U/mg dry wt.	41.3	--	--
Cellulase supernate	400 ml	--	99.5×10^{-3} U/ml	39.8	75.4	--
Cellulase treated cell walls	--	4.52 g	2.88×10^{-3} U/mg dry wt.	13.0	--	128%

Peroxidase activity is assayed spectrophotometrically with homovanillic acid.

Table 6. Peroxidase activity remaining in the supernatant fraction after centrifugation of the incubation medium of cellulase treated horseradish cell walls.

Fraction	Amount Assayed	OD^{460}/min	Peroxidase Units/ml	% Peroxidase in Supernate
Cellulase incubation medium	5 µl	.175	35.6	--
10,000 g supernate	5 µl	.169	33.8	95%
100,000 g supernate	5 µl	.165	33.2	93%

Peroxidase is assayed spectrophotometrically using o-dianisidine as the hydrogen donor.

Figure 11. Peroxidase isozymes released from salt washed horseradish root cell walls by treatment with cellulase.

1. Supernatant fraction from a homogenate of horseradish root tissue. 2. Peroxidase in the incubation medium of a 24 hour cellulase digestion of 2 M NaCl washed horseradish root cell walls.

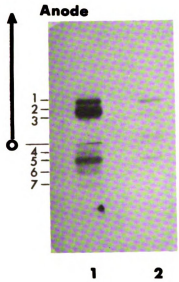


Fig. 11

Table 7. Hydroxyproline released from salt washed horseradish cell walls by cellulase treatment.

Fraction	Dry Weight	Hydroxyproline Content	Total Hydroxyproline	% Hydroxyproline Released
2 M NaCl washed cell walls	11.25 g	1.81 µg/mg dry weight	20.4 mg	--
Cellulase treated cell walls	4.52 g	1.94 µg/mg dry weight	8.81 mg	57%

with horseradish root cell walls, and have identified the peroxidase isozymes which are found on the cell wall. These same combinations of treatments also released 71% of the hydroxyproline from the cell wall fraction (Table 8).

After salt washing the total activity found in the walls and supernatant fraction amounts to 106% of the activity calculated to be on the cell walls before the washing step. After cellulase treatment the sum of peroxidase found in the supernate and remaining on walls is 128% of the peroxidase activity on walls before treatment. These calculations show that the fluorescent homovanillic acid peroxidase assay is a valid method of accurately determining the peroxidatic activity of cell walls.

Table 8. Summary of hydroxyproline and peroxidase released from horseradish cell walls by salt washing and cellulase treatment.

Fraction	Dry Weight	Total Peroxidase Activity	Total Content Hydroxyproline	% Hydroxyproline Released	% Peroxidase Released
H_2O washed cell walls	41.0 g	2020 Units	112.5 mg	--	--
2 M NaCl washed cell walls	41.0 g	150 Units	74.2 mg	34%	93%
Cellulase treated cell walls	16.5 g	47.5 Units	32.2 mg	71%	98%

Peroxidase is assayed fluorimetrically with homovanillic acid as the hydrogen donor.

CHAPTER V

THE ASSOCIATION OF PEROXIDASE AND HYDROXYPROLINE CONTAINING MOIETIES IN HORSERADISH

Shannon, et al. (1966) have reported the presence of hydroxyproline as a constituent amino acid in the three anodically migrating isozymes of horseradish root peroxidase. These three isozymes account for 20% of the total peroxidase activity found in the supernate of a horseradish root homogenate. Hydroxyproline was found in these isozymes after they had been purified to homogeneity by the criteria of chromatography, disc gel electrophoresis and sedimentation velocity centrifugation (Shannon, et al., 1966). In addition to hydroxyproline, these anodic isozymes also contained high amounts of serine, threonine, arabinose and galactose, as compared to the cathodically migrating peroxidases. High levels of these constituents is characteristic of cell wall protein (Lamport, 1965). Since peroxidase is known to be located on the cell walls of plants, it might account for part of the hydroxyproline content of cell walls. In later work, Shannon (1970, private communication) found no hydroxyproline in peroxidases after purification by preparative electrophoresis.

Attempts were made to determine the relation between hydroxyproline and peroxidase in four horseradish systems: a commercially prepared purified horseradish peroxidase, the supernatant fraction from a homogenate of horseradish roots, the supernatant fraction from a cellulase digestion of salt washed horseradish root cell walls, and the incubation medium of aerated horseradish root tissue discs.

Hydroxyproline Associated with Commercially Prepared Peroxidase

Rather than duplicate the work of Shannon in the purification of peroxidase from horseradish root, it seemed more advantageous to start with a purified enzyme preparation, and separate the isozymes of peroxidase chromatographically and assay them for hydroxyproline.

A commercial preparation of purified horseradish peroxidase was chosen (HRP-HPOD-6FA, from the G. Worthington Co., Freehold, N. J.). This preparation contained 1.9% hydroxyproline and 3.14% arabinose on a weight basis (Table 9).

This preparation was assayed for the presence of hydroxyproline glycosides according to methods developed by Lamport (Lamport, 1967). This consists of base hydrolysis of the peroxidase sample by incubation for four hours in 0.44 N $Ba(OH)_2$ at 90°C. After hydrolysis,

Table 9. Hydroxyproline and arabinose found in a commercially purified horseradish peroxidase preparation (Worthington HRP-HPOD-6FA).

Sample	% Weight of Protein	Nanomoles/mg Protein
Hydroxyproline	1.9%	145
Arabinose	3.14%	209

Hydroxyproline was estimated colorimetrically by assaying an acid hydrolysate of the preparation with sodium hypobromite followed by Erlichs reagent according to the method of Kivirriko and comparing the color production at 560 nm to a standard curve. Arabinose was estimated by chromatographing an acid hydrolysate in ethyl acetate : pyridine : water, cutting out the arabinose region, soaking it in water and assaying the eluted arabinose with $FeCl_3$-orcinol pentose reagent and comparing color production at 668 nm to a standard curve.

the sample was neutralized with H_2SO_4 and the precipitating $BaSO_4$ removed by centrifugation. The hydrolyzed sample was then spotted on paper and subjected to electrophoresis in pH 1.9 acetic acid-formic acid buffer. After electrophoresis, the paper was stained for hydroxyproline with the Isatin-Ehrlich's solution. In addition to free hydroxyproline, four spots were visualized which stained as free hydroxyproline, but had retarded electrophoretic mobility compared to free hydroxyproline. The relative electrophoretic mobilities of these compounds compared to free hydroxyproline were 0.72, 0.59, 0.53 and 0.49. These values are similar to those reported for hydroxyproline glycosides

of other species (Lamport, 1967). Resolution of the hydroxyproline glycosides was accomplished by passing the material through an Aminex ion exchange resin column and eluting the hydroxyproline glycosides off the column with an acid gradient (Figure 12). The gradient resolved compounds which stained positively for both hydroxyproline and arabinose. Since the hydroxyproline assay commonly used in Lamport's laboratory (Kivirriko and Liesmaa, 1959) does not react with peptide bound hydroxyproline (Lamport, 1967), it follows that the compounds seen on the aminex column do not have peptide linked hydroxyproline.

Lamport has provided the chemical proof that these compounds, which contain only non-peptide linked hydroxyproline and arabinose are in fact hydroxyproline arabinosides with an o-glycosidic linkage between the hydroxyl group of hydroxyproline and the reducing terminus of arabinose (Lamport, 1967). He has also determined that such hydroxyproline-arabinosides, which typically contain 1-4 arabinose residues per hydroxyproline are distributed throughout the higher plant world (Lamport and Miller, 1971).

A sample of horseradish cell walls was prepared by the non-aqueous method of Kivilaan, et al. (1959). This was subjected to barium hydroxide hydrolysis and analysis for hydroxyproline arabinosides on an aminex ion exchange column (Figure 13). Horseradish cell walls also show hydroxyproline arabinosides.

Figure 12. Hydroxyproline arabinose from horseradish peroxidase (Worthington HRP-HPOD-6FA) resolved by passing a barium hydroxide hydrolysate through an aminex ion-exchange column with an acid gradient.

H_1A_1 = 1 hypro :: 1 arabinose. H_1A_2 = 1 hypro : 2 arabinoses. H_1A_3 = 1 hypro : 3 arabinoses. H_1A_4 = 1 hypro : 4 arabinoses residues. Peaks are recognized by staining for arabinose with orcinol and assaying color production at 668 nm.

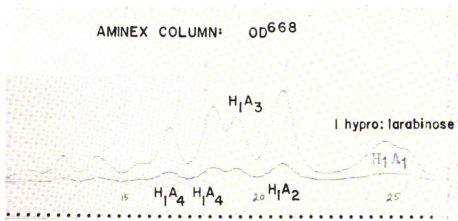

AMINEX COLUMN: OD668
H_1A_3
l hypro: larabinose
H_1A_1
15
H_1A_4 H_1A_4
20
H_1A_2
25

Fig. 12

Figure 13. Hydroxyproline-arabinose from purified horseradish cell walls.

Glycosides are resolved by passage through an aminex ion-exchange column with an acid gradient. Hydroxyproline-arabinose is visualized by reaction for hydroxyproline with an automatic hydroxyproline analyzer constructed by Lamport.

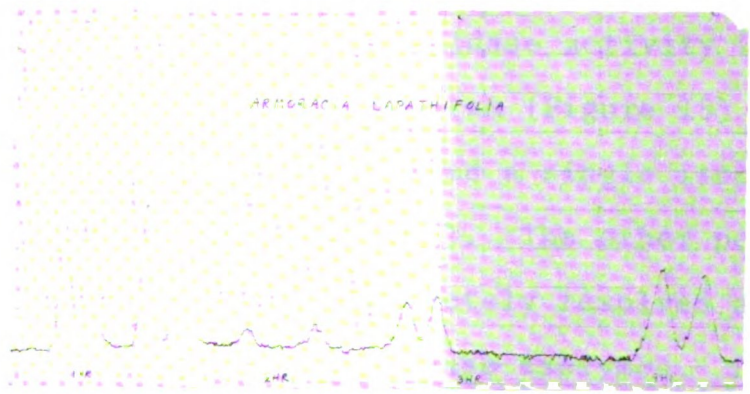


Fig. 13

A 1 x 120 cm sephadex G-75 column was prepared and equilibrated with .01 N acetate buffer, pH 5.2. A sample of commercially purified horseradish peroxidase (Worthington, HRP-HPOD-6FA) was placed on the column, and fractions collected. The eluent fractions were assayed at OD^{275} for protein absorption, and OD^{403} for heme-group absorption (Figure 14). The resulting elution profile contains five peaks of absorbance at 275 nm. Samples from the peak fractions were subjected to electrophoresis on 12% starch gel and stained for peroxidase activity with benzidine-H_2O_2. The resultant zymogram (Figure 15) showed that the first peak (peak I) contained a single isozyme, the most anodically migrating (No. 1). The other peaks of protein (peaks II-V) were incompletely resolved and therefore showed mixtures of peroxidase isozymes. Peak V contained no peroxidatic activity and is a contaminating protein.

Peaks of absorbance at 403 nm were observed to coincide with all the protein absorbance maxima except peak V. The R.Z. (Reinheitzahl) ratio of absorbance at 403 nm to 275 nm was different for each of these peaks. Peak I has an R.Z. value of 0.8, peak II - 3.02, peak III - .26, and peak IV - 0.58. The peak I peroxidase isozyme (No. 1) has chromatographic properties different from all the other peroxidase isozymes, and is clearly separated from them in the elution profile. Disc gel electrophoresis

Figure 14. Resolution of a purified horseradish peroxidase preparation (Worthington HRP-HPOD-6FA) by Sephadex G-75 chromatography.

Peroxidase is eluted with 0.05 M acetate buffer, pH 5.2. Peaks of protein are obtained by reading OD^{275}.

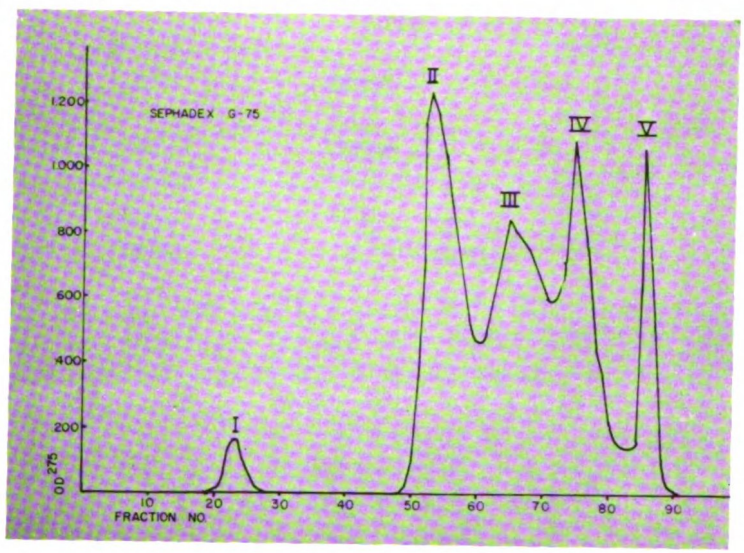
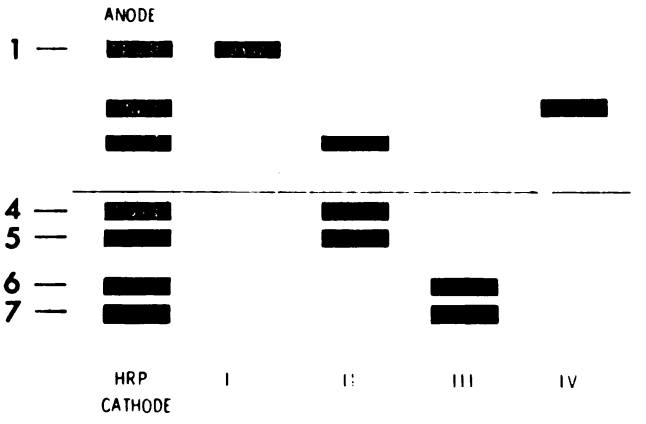

SEPHADEX G-75
I
II
III
IV
V
1200
1000
800
600
400
200
O.D. 275
10
20
30
40
50
60
70
80
90
FRACTION NO.

Fig. 14

Figure 15. Zymogram of peroxidase isozymes from various peaks of activity off a Sephadex G-75 chromatography column.

Peroxidase isozymes are visualized by staining with benzidine-H_2O_2. HRP. Peroxidase isozymes visualized in a zymogram of Worthington horseradish peroxidase HRP-HPOD-6FA. I through IV. Peroxidase isozymes visualized in the peak tubes of protein in a Sephadex G-200 column. These numerals refer to the numbered peaks in figure 19. Peak V in figure 14 has no peroxidase activity.



Peroxidase zymogram of fractions off Sephadex G-75 column

Fig. 15

of this peak of peroxidase activity shows only one band of protein from the Worthington preparation (Figure 16) and it is therefore concluded that peak I represents a single protein species.

The single peroxidase isozyme (No. 1) from peak I was analyzed for hydroxyproline arabinosides by hydrolysis with .44 N $BaOH_2$ at 90°C and electrophoresis at pH 1.9. Four species of hydroxyproline containing compounds were observed. They had relative electrophoretic mobilities of 0.72, 0.59, 0.53 and 0.49 when compared to free hydroxyproline. These correspond to the four hydroxyproline-arabinosides which have a ratio of one hydroxyproline per 1-4 arabinose residues. No free hydroxyproline was detected in this electrophoresis run. It was thus concluded that hydroxyproline glycosidically linked to arabinose is associated with the most anodically migrating isozyme of peroxidase (No. 1). This purified isozyme was analyzed for hydroxyproline by colorimetric analysis and also by automatic amino acid analysis. Both methods showed that hydroxyproline represents about 6.5% of the weight of this protein fraction (Table 10).

Peaks I-IV were assayed for peroxidase activity using the fluorimetric homovanillic acid assay (Guilbault, 1968). Considerable differences in the specific activity of peroxidase isozymes could be detected (Table 11). The specific activity of the most anodically migrating isozyme

Figure 16. Disc gel electrophoresis of the most anodically migrating horseradish peroxidase isozyme (No. 1).

This isozyme was resolved from other peroxidases in a purified enzyme preparation (Worthington HRP-HPOD-6FA) by Sephadex G-75 chromatography. This isozyme corresponds to peak I in the G-75 elution profile.

Fig. 16

Table 10. Amino acid analysis of the most anodically migrating No. 1 isozyme from a commercially purified horseradish peroxidase (Worthington HRP-HPOD-6FA).

Amino Acid	Residues per 40,000 M.W.
Hydroxyproline	38.85
Aspartic	35.61
Threonine	21.39
Serine	40.92
Glutamic	30.00
Proline	?
Glycine	33.00
Alanine	33.00
Valine	40.00
Cysteine	7.00
Methionine	--
Isoleucine	13.00
Leucine	24.00
Tyrosine	1.36
Phenylalanine	8.00
Lysine	16.00
Histidine	7.00
Arginine	11.0

This isozyme shows only one band of protein when subjected to disc gel electrophoresis.

Table 11. Catalytic activities of peroxidase isozymes partially resolved by elution on a Sephadex G-75 column.

Sample	R.Z. (OD^{403}/OD^{275})	Peroxidase Spec. Act. F/min/OD^{275}	Relative Spec. Act.
Peak tube of fraction I	0.80	254	37.6
Peak tube of fraction II	3.02	6.75	1.0
Peak tube of fraction III	0.26	52.2	7.73
Peak tube of fraction IV	0.584	31.6	4.68
Peak tube of fraction V	0.14	0.0	0.0

Peroxidase was assayed fluorimetrically with homovanillic acid, with excitation at 315 nm and emission at 425 nm. Sample numbers refer to peaks of protein labeled in figure 4.

(peak I) is 37.6 times the specific activity of peak II. This difference is significant because the peak II fraction has an R.Z. = 3.0, which means it contains only peroxidase without contamination.

The No. 1 peroxidase isozyme could also be resolved by isoelectric focusing using 1% ampholyte solution with a pH range of 3 to 6 (Figure 17). The No. 1 isozyme of peroxidase was located in a peak of protein which had an isoelectric point pI = 3.0. When a sample of this protein peak was subjected to disc gel electrophoresis and stained

Figure 17. Isoelectric focusing separation of a purified preparation of horseradish peroxidase (Worthington HRP-HPOD-6FA).

Separation is carried out over the range pH 3-6. Points: pH curve. Lines: OD^{275}.

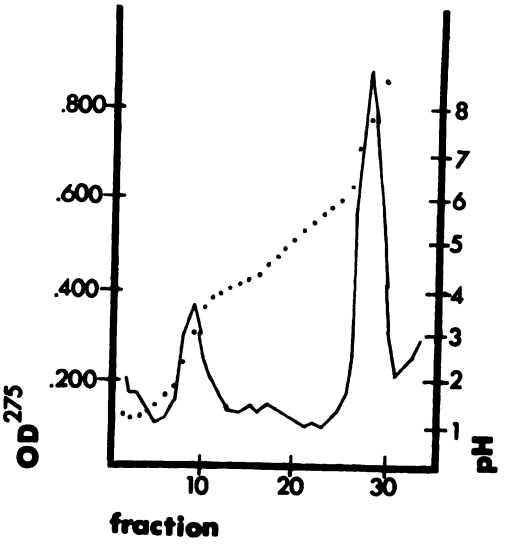


Fig. 17

for protein with buffalo blue-black, a single band of protein was visible.

The Worthington horseradish peroxidase preparation (HRP-HPOD-6FA) thus contains one anodically migrating isozyme which can readily be separated from the other isozymes both by column chromatography with Sephadex G-75, or by isoelectric focusing. This isozyme when separated from the other peroxidase isozymes by these methods can be shown to consist of only one protein by the criteria of disc gel electrophoresis. This seemingly pure isozyme is associated with hydroxyproline, and all of this hydroxyproline is glycosidically linked to arabinose.

A 20 mg sample of the Worthington peroxidase and 150 activity units of beef liver catalase were subjected to isopycnic equilibrium centrifugation with CsCl to form a density gradient with an average $\rho = 1.4$. Three drop fractions were collected in 120 tubes and total hydroxyproline, peroxidase and catalast activity determined across the gradient. Every other fraction was subjected to starch gel electrophoresis. Individual peroxidase isozymes were resolved and the peak tubes of their distribution were estimated by visual inspection of the peroxidase zymogram. All the techniques and assays used in the analysis of the CsCl gradient are described in the Methods section.

Total peroxidase, hydroxyproline and catalase values were determined (Figure 18). The hydroxyproline distribution does not coincide with peroxidase. The density of the hydroxyproline peak fraction is 1.372 while the density of the peak fraction of total peroxidase is 1.342. The calculated density of the beef liver catalase is 1.291. This demonstrates that most of the peroxidase does not contain any hydroxyproline. However, there is some overlap between hydroxyproline and peroxidase distributions. A zymogram of the peroxidase isozymes in this gradient (Figure 19) shows that the peak of peroxidase activity plotted across the gradient (Figure 18) actually represents a composite of several isozymes of different densities. The most anodically migrating isozyme, No. 1, is the densest of all the peroxidase isozymes, and the estimated density of this isozyme, 1.379, is nearly identical to the density determined for hydroxyproline.

However, an inspection of the overlap of peroxidase and hydroxyproline on the CsCl gradient shows that the peak of hydroxyproline begins at tube 34, while no peroxidase can be detected at all until tube 60, at which point the hydroxyproline level is already 70% of maximum. Thus the No. 1 isozyme of peroxidase is fortuitously of the same average density as the hydroxyproline, but if the hydroxyproline profile represents a single species, then

for protein with buffalo blue-black, a single band of protein was visible.

The Worthington horseradish peroxidase preparation (HRP-HPOD-6FA) thus contains one anodically migrating isozyme which can readily be separated from the other isozymes both by column chromatography with Sephadex G-75, or by isoelectric focusing. This isozyme when separated from the other peroxidase isozymes by these methods can be shown to consist of only one protein by the criteria of disc gel electrophoresis. This seemingly pure isozyme is associated with hydroxyproline, and all of this hydroxyproline is glycosidically linked to arabinose.

A 20 mg sample of the Worthington peroxidase and 150 activity units of beef liver catalase were subjected to isopycnic equilibrium centrifugation with CsCl to form a density gradient with an average $\rho = 1.4$. Three drop fractions were collected in 120 tubes and total hydroxyproline, peroxidase and catalast activity determined across the gradient. Every other fraction was subjected to starch gel electrophoresis. Individual peroxidase isozymes were resolved and the peak tubes of their distribution were estimated by visual inspection of the peroxidase zymogram. All the techniques and assays used in the analysis of the CsCl gradient are described in the Methods section.

Figure 18. CsCl isopycnic equilibrium centrifugation of a 20 mg sample of purified horseradish peroxidase (Worthington, HRP-HPOD-6FA).

The gradient is collected from the bottom in 120 three drop fractions. Peroxidase is assayed spectrophotometrically with o-dianisidine as hydrogen donor. 1 peroxidase Unit = 1 OD^{460}/min. Catalase is assayed by monitoring the production of oxygen with an oxygen electrode. 1 catalase Unit = 1 µmole O_2 produced/min. Hydroxyproline is assayed colorimetrically after acid hydrolysis of the fraction. △—△. peroxidase; □—□. catalase; ○—○. hydroxyproline; ●—●. density.

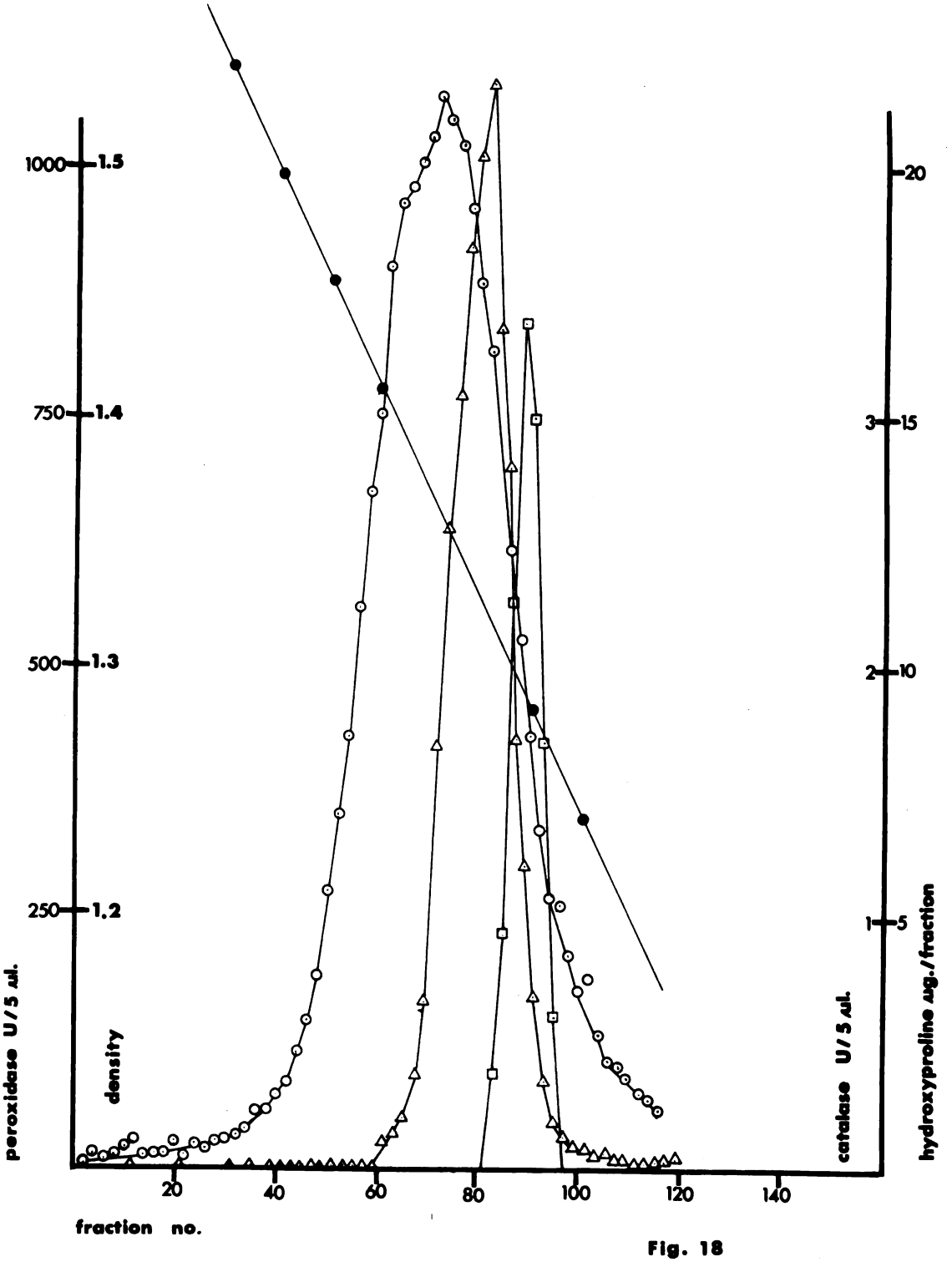

1000
750
500
250
1.5
1.4
1.3
1.2
density
peroxidase U/5 µl.
20
40
60
80
100
120
140
fraction no.
3
2
1
15
10
5
20
catalase U/5 µl.
hydroxyproline µg./fraction

Fig. 18

Figure 19. Resolution of peroxidase isozymes in a CsCl density gradient of purified horseradish peroxidase (Worthington, HRP-HPOD-6FA) by sequential starch gel electrophoresis.

5 µl samples of alternate 3-drop fractions are subjected to starch gel electrophoresis. Odd numbered tubes from 25-119 are assayed in this fashion. Notches are cut in the gel at the position of every fifth sample applied to the gel. Peroxidase isozymes are visualized with benzidine-H_2O_2.

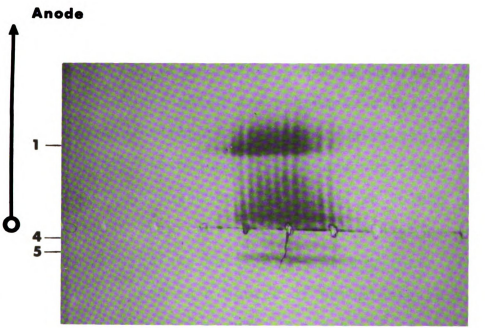


Fig. 19

we can conclude that the hydroxyproline is not attached to peroxidase.

CsCl Gradient Resolution of Hydroxyproline and Peroxidase from the Supernatant Fraction of Horseradish Root Homogenate

Horseradish root tissue was homogenized with water in a Waring blender. The wall material was removed by squeezing through eight layers of cheesecloth. The supernate fraction was collected. This solution contains all the peroxidase isozymes which are presumed to reside within the plasma membrane, although it actually also contains those extra cellular peroxidases which are not bound to the cell wall. The homogenate was reduced in volume by rotary evaporation, then centrifuged at 10,000 g for 30 min. to remove particulate material. The supernate after centrifugation was used as a source of peroxidase in a CsCl gradient centrifugation run. The details of the technique and assay used are described in the Methods section.

A 3.0 ml CsCl gradient with an average density of 1.33 was formed by centrifugation for 72 hours at 40,000 RPM in a Beckman-65B centrifuge, using the SW-65 swinging bucket rotor. At the end of the run, 3-drop fractions were collected in 145 tubes. These tubes were assayed for peroxidase, marker catalase and hydroxyproline.

A plot of peroxidase, catalase and hydroxyproline was constructed across the gradient (Figure 20).

Only one peak of hydroxyproline is seen, and it is near the bottom of the gradient with an estimated density of 1.459. This peak of hydroxyproline does not coincide with any peroxidase activity. The peroxidase activity shows a peak with a density estimated to be 1.305. This is much lighter than the average density of the purified horseradish peroxidase, but is probably because the isozyme makeup of the two preparations is different. A zymogram shows that the buoyant densities of the cytoplasmic peroxidase isozymes differ, and the most anodically migrating isozyme is the densest (Figure 21).

Resolution of Hydroxyproline and Peroxidase from the Supernatant Fraction of Cellulase Treated Horseradish Cell Walls

The supernate of a cellulase digestion of horseradish root cell walls was reduced in volume by vacuum evaporation, and then centrifuged at 10,000 g for 30 min. to pellet particulate matter. The supernate after centrifugation was used as a source of peroxidase in a CsCl gradient centrifugation run. The details of the technique and assays used are described in the Methods section.

A 3.0 ml CsCl gradient with an average density of 1.33 was formed by centrifugation for 72 hours at 40,000 RPM in a Beckman 65B ultracentrifuge using the SW-65

Figure 20. CsCl isopycnic equilibrium centrifugation of the supernate from a horseradish root homogenate.

The gradient is collected from the bottom in 145 three-drop fractions. Peroxidase is assayed spectrophotometrically with o-dianisidine as hydrogen donor. 1 peroxidase Unit = 1 OD^{460}/min. Catalase is assayed by monitoring the production of oxygen with an oxygen electrode. 1 catalase Unit = 1 µmole O_2 produced/min. Hydroxyproline is assayed colorimetrically after acid hydrolysis of the fraction. △—△. peroxidase; □—□. catalase; ○—○. hydroxyproline; ●—●. density.

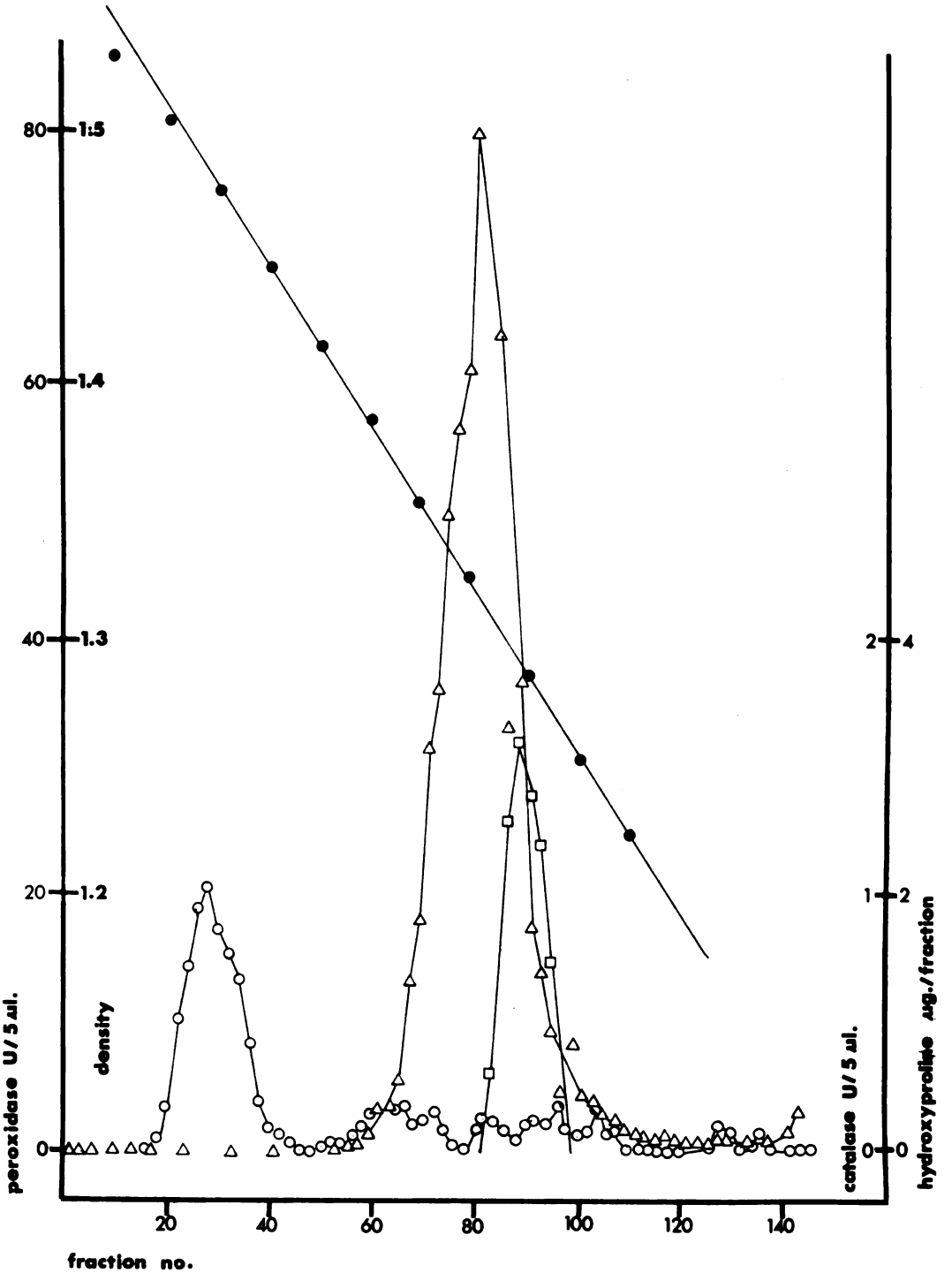

80
60
40
20
0
1.5
1.4
1.3
1.2
density
peroxidase U/5 µl.
2
1
0
4
2
0
catalase U/5 µl.
hydroxyproline µg./fraction
20
40
60
80
100
120
140
fraction no.

Fig. 20

Figure 21. Resolution of peroxidase isozymes in a CsCl density gradient of the supernate of a horseradish root homogenate by sequential starch gel electrophoresis.

5 µl samples of alternate 3-drop fractions are subjected to starch gel electrophoresis. Odd numbered tubes from 45-139 are assayed in this fashion. Notches are cut in the gel at the position of every fifth sample applied to the gel. Peroxidase isozymes are visualized with benzidine-H_2O_2.

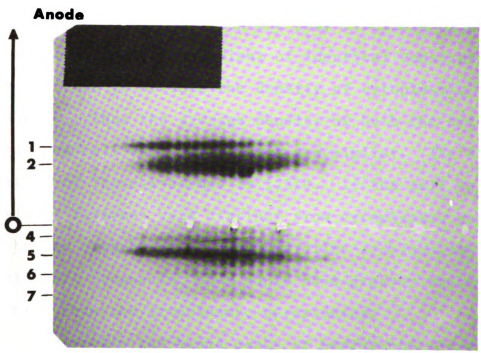


FIG. 21

swinging bucket rotor. At the end of the run, 3-drop fractions were collected in 145 tubes. These tubes were assayed for total peroxidase, marker catalase and hydroxyproline.

Peroxidase, catalase, and hydroxyproline were assayed (Figure 22). The position of the peak of total peroxidase activity is at fraction 81, and the position of the peak of total catalse activity is at fraction 89. These positions coincide with the peak fractions of peroxidase and catalase in a gradient of identical construction which was used to measure the density of cytoplasmic peroxidase. Thus the average density of both the cytoplasmic and cell wall peroxidases are similar. The average densities of these isozymes is 1.310. If the cell wall peroxidase isozymes were distinct from cytoplasmic peroxidase by the attachment of hydroxyproline-arabinosides or by cell wall fragments, they would have greater average densities than the cytoplasmic peroxidases.

There are two broad peaks of hydroxyproline containing components in the cellulase digest of horseradish cell walls, and the densities of these are distinct from the one peak of hydroxyproline found in the cytoplasm. Neither of these two peaks of hydroxyproline containing material coincide with the peak of peroxidase released from cellulase treatment of horseradish walls, nor do they coincide with any of the resolved peroxidase isozymes

Figure 22. CsCl isopycnic equilibrium centrifugation of the incubation medium of a cellulase digestion of salt washed horseradish root cell walls.

The gradient is collected from the bottom in 145 three-drop fractions. Peroxidase is assayed spectrophotometrically with o-dianisidine as hydrogen donor. 1 peroxidase Unit = 1 OD^{460}/min. Catalase is assayed by monitoring the production of oxygen with an oxygen electrode. 1 catalase Unit = 1 µmole O_2 produced/min. Hydroxyproline is assayed colorimetrically after acid hydrolysis of the fraction. △—△. peroxidase; □—□. catalase; ○—○. hydroxyproline; ●—●. density.

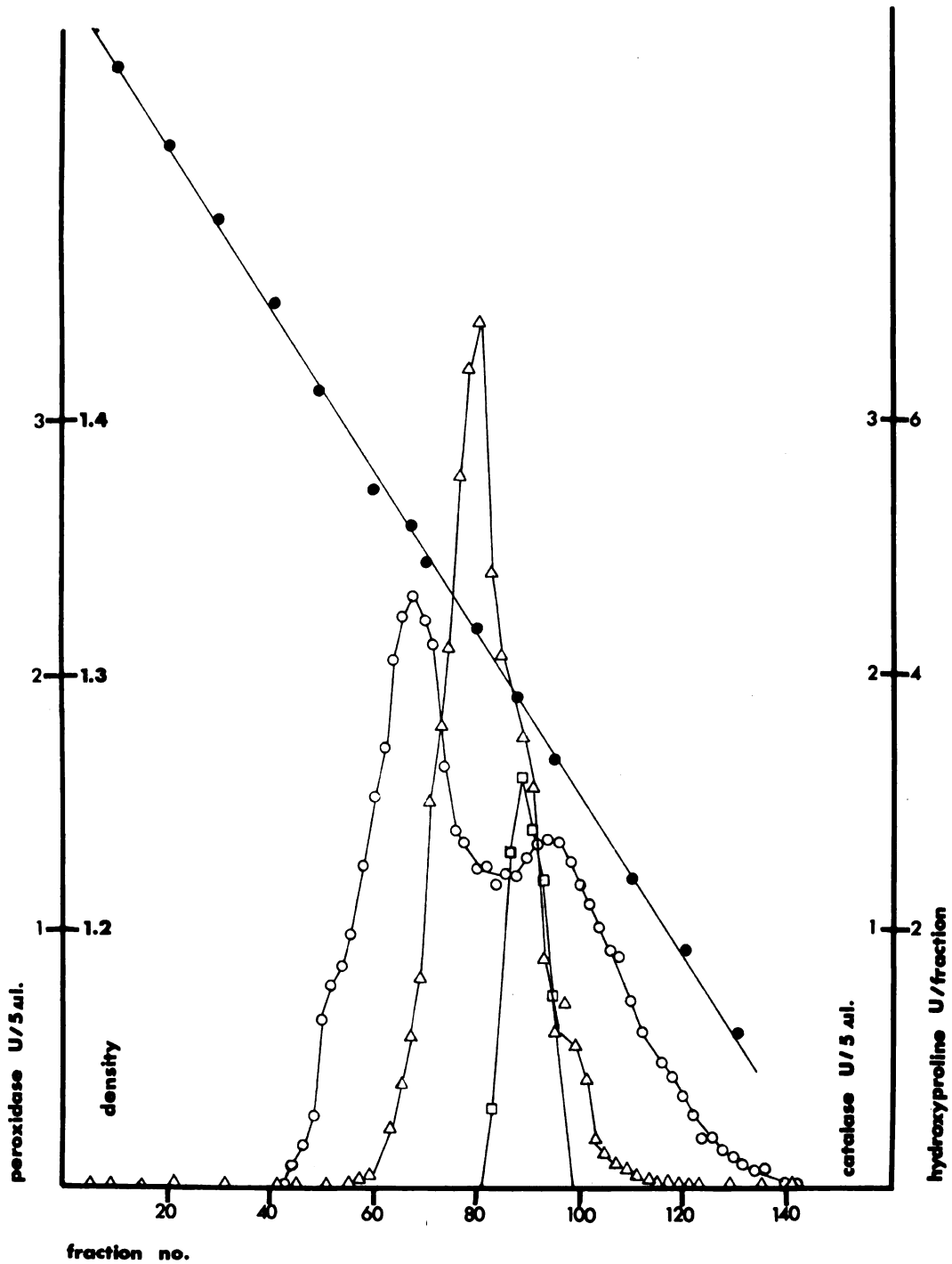

3
2
1
1.4
1.3
1.2
peroxidase U/5 µl.
density
3
2
1
6
4
2
catalase U/5 µl.
hydroxyproline U/fraction
20
40
60
80
100
120
140
fraction no.

Fig. 22

(Figure 23). The densities of these two peaks of hydroxyproline containing material are 1.357, and 1.267.

It is clear that nearly all the hydroxyproline containing material released by cellulase treatment of cell walls is not related to peroxidase. Furthermore, the density of peroxidases from the cell wall is identical to that of cytoplasmic peroxidases, which do not contain hydroxyproline. Therefore, it is unlikely that the peroxidase from cell walls contains any hydroxyproline-arabinosides, or any dense groups which should make them distinct from cytoplasmic peroxidase.

Relations Between Hydroxyproline Containing Components and Peroxidase Found in the Incubation Medium of Aerated Horseradish Discs

In the medium of horseradish root discs incubated under aerating conditions for 40 hours, 83% of the total peroxidase activity is found in the region corresponding to the second most anodically migrating isozyme of peroxidase (No. 2). The possible relation between this isozyme and hydroxyproline was determined by subjecting this medium to Sephadex G-200 chromatography, and assaying the eluent fractions for peroxidase and hydroxyproline (Figure 24). The peroxidase resolved into two fractions. Both fractions contain the No. 2 isozyme.

The hydroxyproline in the incubation medium also separates into two fractions. The first peak of

Figure 23. Resolution of peroxidase isozymes in a CsCl density gradient of the incubation medium of cellulase treated horseradish root cell walls by sequential starch gel electrophoresis.

5 µl samples of alternate 3-drop fractions are subjected to starch gel electrophoresis. Odd numbered tubes from 45-139 are assayed in this fashion. Notches are cut in the gel at the position of every fifth sample applied to the gel. Peroxidase isozymes are visualized with benzidine-H_2O_2.

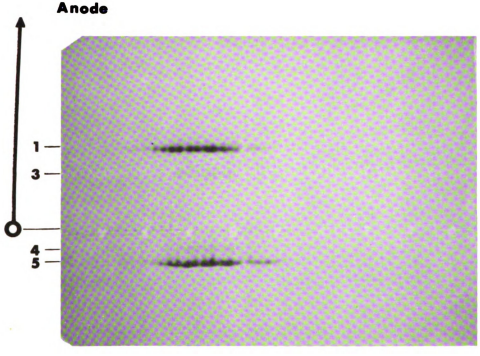


Fig. 23

Figure 24. Sephadex G-200 resolution of peroxidase and hydroxyproline containing components in the incubation medium of horseradish root slices.

Peroxidase is assayed using o-dianisidine as the hydrogen donor. Hydroxyproline is assayed automatically by hydrolysis followed by colorimetric hydroxyproline determination with an automatic hydroxyproline analyzer developed by Lamport. Both peaks of peroxidase activity are eluted behind the void volume.

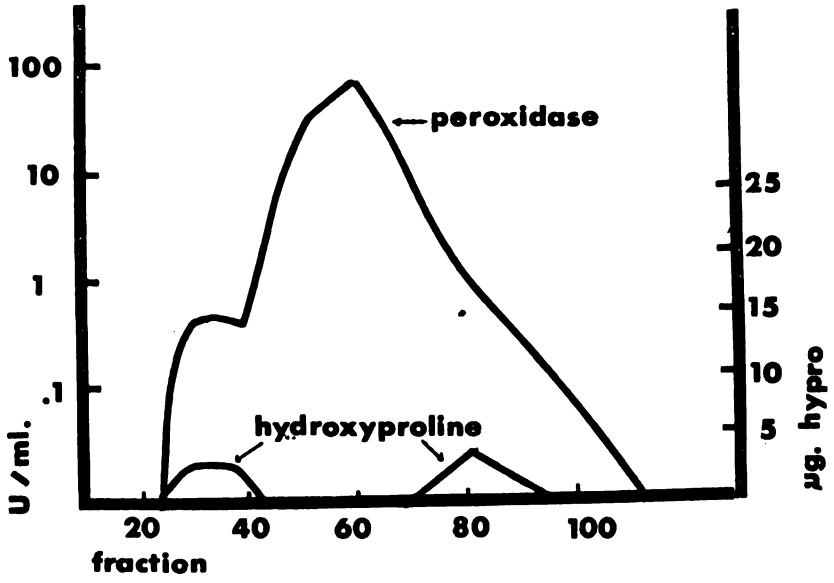

100
10
1
.1
U/ml.
peroxidase
hydroxyproline
25
20
15
10
5
µg. hypro
20
40
60
80
100
fraction

Fig. 24

hydroxyproline co-elutes with fraction-1 of peroxidase. The second peak of hydroxyproline does not co-elute with either peak of peroxidase.

The peroxidase activity of fraction-1 represents less than 0.1% of the total peroxidase found in the incubation medium, yet it co-elutes with 40% of the hydroxyproline. Because of its distinct elution characteristics compared to the rest of the peroxidase in the medium (fraction-2), the fraction-1 peroxidase appears to be of larger size, and may be aggregated with a hydroxyproline containing moiety.

CHAPTER VI

PEROXIDASE FOUND EXTERNAL TO THE CELL

Attempts to identify those peroxidase isozymes which could be found external to the cell membrane but not tightly bound to the cell wall were made in two systems: the exudation fluid from cut horseradish petioles, and the incubation medium of aerated horseradish root discs.

Peroxidase in Horseradish Petiole Exudate

If peroxidase were freely secreted from cells, it might be present in the exudation fluid of cut petioles. A horseradish plant growing in the greenhouse was defoliated at the base of the leaves. The cut surfaces of the petioles were washed extensively with water in order to remove cell sap from broken cells. Rubber tubing was then fitted over each of the petioles, and the connections sealed with modeling clay. In order to reduce evaporation, the plant was thoroughly watered and placed under a bell jar.

Collections of fluid were taken from the cut petioles after 24 and 38 hours. Peroxidase activity in these samples was determined (Table 12) and a zymogram of

Table 12. Peroxidase activities of cut petiole exudation fluid.

Sample	Time Elapsed after Cutting Petiole	Vol. Collected	Peroxidase Activity OD^{460}/min/ml
1	24 hrs	0.8 ml	4.68
2	38 hrs	0.6 ml	0.94

Peroxidase is assayed spectrophotometrically with o-dianisidine as hydrogen donor. Two collections of fluid are obtained from a single plant. The first collection is made 24 hours after cutting petioles. The second collection is made 38 hours after cutting.

the petiole fluid (Figure 25) showed that it contained four peroxidase isozymes, three anodic (corresponding to Nos. 1, 2, and 3) and one cathodic (corresponding to No. 5). These isozymes are compared to a sample of the sap from the freshly cut surface of a horseradish petiole. There are marked differences both in the number of isozymes and their relative concentration. In the petiole cell sap, isozyme No. 1 is more concentrated than No. 2, whereas the relative concentration of these isozymes is reversed in the petiole exudation fluid. It is unlikely that these differences are due to the selective denaturation of peroxidase isozymes over the course of 24 hours at 25°C because of the stability of peroxidase. It therefore seems probable that the peroxidase isozymes observed in the petiole exudation fluid do not represent contamination

Figure 25. Peroxidase isozymes found in horseradish cut petiole exudation fluid.

Peroxidase is visualized with benzidine-H_2O_2.
1. Fluid obtained 24 hours after cutting petiole.
2. Control sample of petiole cell sap obtained by touching a 7 x 15 mm paper wick to the freshly cut surface of a horseradish petiole. 3. Fluid obtained 38 hours after cutting petiole.

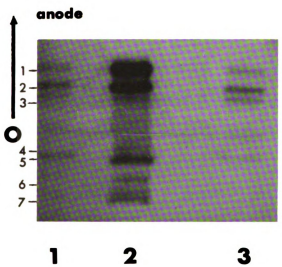


Fig. 25

from broken cells but rather peroxidases which are normally found external to the wounded cell.

Peroxidase Found in the Incubation Medium of Aerated Horseradish Root Tissue

Horseradish root tissue was cut into 3 mm cubes. One-hundred gram portions (fresh weight) of tissue were placed in sterile one liter erlenmeyer flasks. Each flask of tissue was washed with three 500 ml changes of sterile distilled water to remove peroxidase from the cut cell surfaces of the tissue cubes. 200 ml of sterile water containing 20 µg/ml chloramphenicol was then added to each flask. The flasks were stoppered with foam plugs and incubated at 30°C on a rotary shaker in the culture room. One ml aliquots were removed aseptically from each flask after one hour of incubation, and after that at six-hour intervals. One flask was frozen at -20°C for one hour and then thawed and returned to the incubation at 30°C with the other flasks. The freezing step was performed to break the plasma membrane of the horseradish cells and permit the leakage of the cell contents into the incubation medium. Another flask was treated with 100 µg/ml cycloheximide.

A plot of peroxidase activity found in the incubation medium of each flask (Figure 26) shows a sharp increase in activity which reaches a maximum at eight hours in the medium of all the untreated flasks. This characteristic

Figure 26. Peroxidase in the medium of incubated horseradish root tissue as a function of time.

Peroxidase activity is assayed spectrophotometrically using o-dianisidine as the hydrogen donor. ○—○, △—△, □—□. Untreated flasks of 100 g tissue/200 ml. ●—●. Untreated flask of 200 g tissue/200 ml. ▲—▲. Flask of 100 g tissue/200 ml, treated with 100 µl/ml cycloheximide. ■—■. Flask of 100 g tissue/200 ml, frozen and thawed.

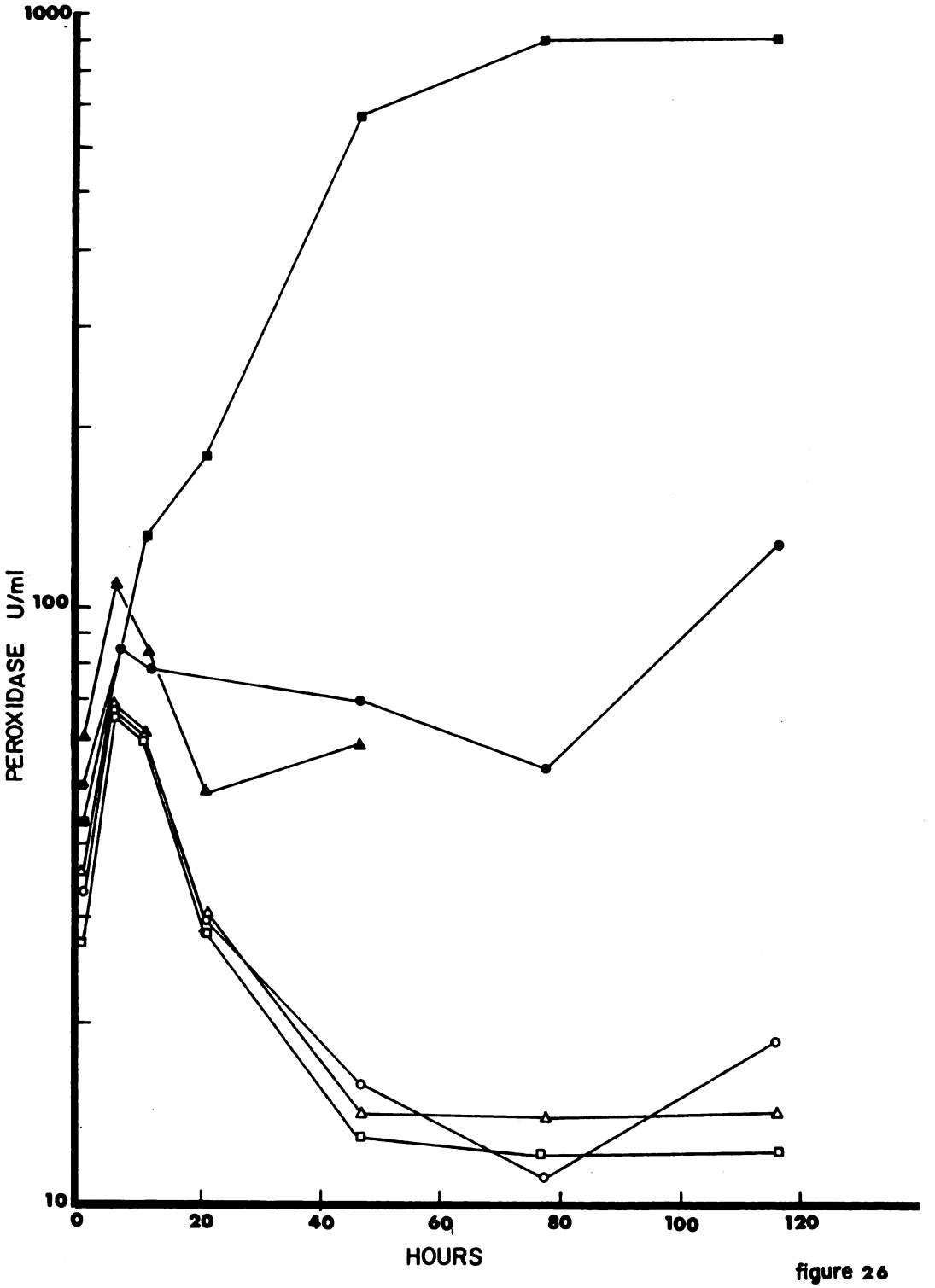


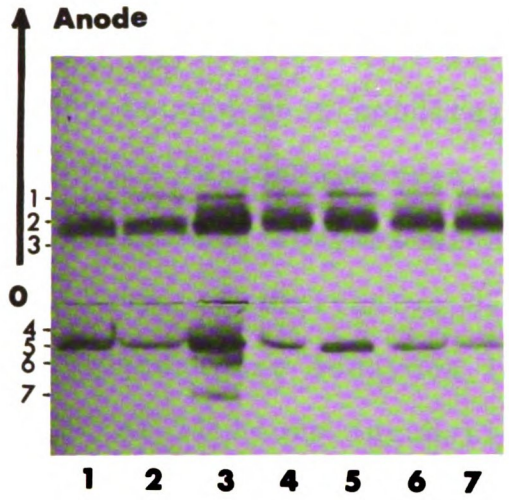
figure 26

increase in activity demonstrates that the peroxidase in the medium does not arise from cut surfaces but is being leacked out of the horseradish root tissue. Beyond eight hours, the peroxidase activity in the medium of untreated flasks decreases. The cycloheximide treated flask shows this same increase in activity up to eight hours, but beyond this time, the activity in the medium does not decrease but remains at a relatively constant level. The frozen and thawed tissue shows a constant increase in peroxidase in the medium until a maximum level is reached at 78 hours. Clearly, horseradish cell contents have spilled out into the medium across the broken plasma membranes.

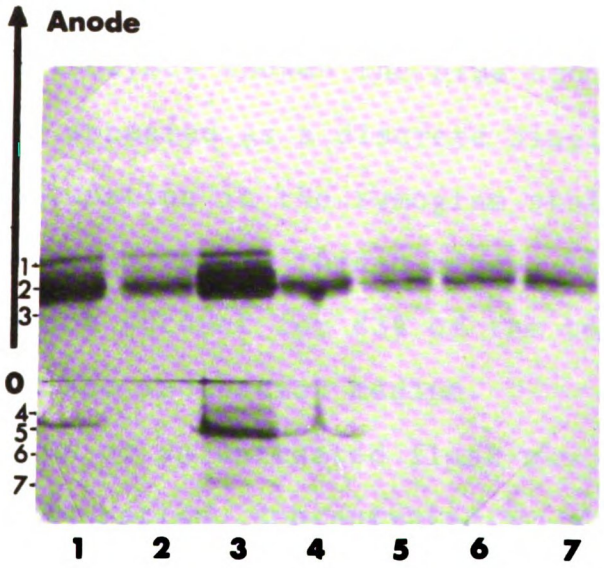
The peroxidase isozymes found in the medium after different periods of incubation (Figure 27) show roughly the same isozymes which were observed in horseradish petiole exudate (Figure 25), but not in the same proportions. After 21 hours incubation, untreated flasks have isozymes corresponding to Nos. 1, 2, 3 and 5 in the medium. After 47 hours incubation, the cathodic No. 5 isozyme is losing activity compared to the anodic isozymes. The frozen and thawed flask shows all the peroxidase isozymes normally found in horseradish root homogenate and this pattern does not change with incubation. The cycloheximide treated flask has a relatively constant total peroxidase activity in the medium after a maximum level is reached at eight hours. However, the isozyme complement changes constantly

Figure 27. Zymogram of peroxidase isozymes found at various times in the media of incubated horseradish root slices.

The samples are taken from the incubation time course of figure 26. 1. 200 g tissue/200 ml, untreated. 2. 100 g tissue/200 ml, untreated. 3. 100 g tissue/200 ml, frozen and thawed. 4. 100 g tissue/200 ml, cycloheximide treated. 5. 100 g tissue/200 ml, untreated. 6. 100 g tissue/200 ml, untreated. 7. 100 g tissue/200 ml, untreated.



21 hr. incubation



47 hr. incubation

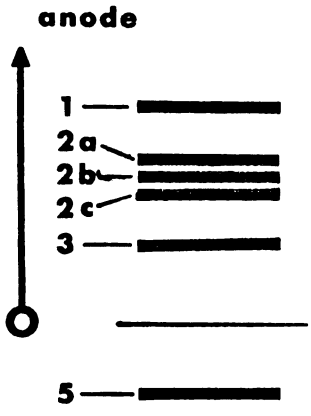
Fig. 27

throughout the incubation, and new isozymes are observed which do not correspond to the peroxidases normally found in tissue homogenates. These may be modifications of previously existing peroxidase isozymes.

In the untreated flasks, the predominant isozyme in the medium was the second most anodically migrating, or No. 2. However, this region appeared to be made up of a large family of peroxidase isozymes with nearly the same electrophoretic mobility. Quantitative estimation of the relative activity of peroxidase isozymes in the medium of an untreated flask after 21 hours incubation was accomplished with the ascorbate-benzidine coupled peroxidase zymogram stain (Table 13). Since the family of isozymes in the No. 2 region had discrete activities, the most active of these isozymes showed the blue benzidine reaction the soonest, and appeared as a discrete single blue band. After further incubation in the zymogram stain the other isozymes in the No. 2 region turned blue. The isozymes in the No. 2 region account for 83% of the total peroxidase activity in the incubation medium.

The medium of an untreated flask was collected after 47 hours incubation, concentrated by air evaporation in a dialysis bag and centrifuged at 10,000 g for 30 minutes. A portion of the supernatant was subjected to Sephadex G-200 chromatography. The eluted fractions were assayed for peroxidase activity and hydroxyproline (Figure

Table 13. The relative activity of peroxidase isozymes using quantitative ascorbate peroxidase assay.



Sample Isozyme	Time for Isozyme Appearance	Relative Peroxidase Activity
1	255 sec	7.5%
2a	120 sec	16.5%
2b	40 sec	50.0%
2c	120 sec	16.5%
3	900 sec	--
5	210 sec	9.5%

The starch gel is flooded with a 4 mM ascorbate-benzidine reaction mixture.

24). The peroxidase eluted from the G-200 column as two peaks. The peak of peroxidase which elutes first from the column (fraction-1) consists of peroxidase molecules of larger apparent size than the bulk peroxidase peak (fraction-2) which elutes from the column less rapidly. It is estimated that the peroxidase activity in fraction-1 is less than 0.1% of the peroxidase in the incubation medium, yet it co-elutes with 40% of the hydroxyproline in the incubation medium. Both fraction-1 and fraction-2 are comprised primarily of the same second most anodically migrating peroxidase isozyme, No. 2. Both fractions of peroxidase eluted after the void volume had passed through the G-200 column.

Horseradish root tissue cubes were incubated in water with 200 µg/ml chloramphenicol and under an atmosphere of 0-, 10-, 100-, and 500-PPM ethylene. The hydroxyproline content increased in the 500-PPM ethylene treatment alone and only after 72 hours incubation. Peroxidase activity in the media of these flasks does not depend on added ethylene (Table 14). The isozymes in the media do not depend on ethylene concentration, and consist primarily of two anodic (Nos. 1 and 2) and one cathodic (No. 5) isozyme.

The incubation medium from the flask treated with 500-PPM ethylene for 72 hours was centrifuged at 10,000 g for 30 minutes, and a portion of the supernatant subjected

Table 14. Hydroxyproline and peroxidase found in the incubation medium of ethylene treated horseradish root slices after 72 hours.

Treatment	Ethylene Conc.	Hydroxyproline Conc.	Peroxidase Activity OD^{460}/min/ml
1	--	6.80 µg/ml	13.7
2	10 ppm	6.80 µg/ml	15.2
3	100 ppm	6.76 µg/ml	14.6
4	500 ppm	16.60 µg/ml	15.8

Peroxidase is assayed spectrophotometrically with o-dianisidine as the hydrogen donor.

to Sephadex G-200 chromatography without dilution. The peroxidase activity was resolved into two peaks (Figure 28), one comprised of peroxidase molecules of apparently larger size (fraction-1) than the other (fraction-2). As was the case in the peroxidase from the incubation medium of horseradish root tissue slices without ethylene treatment (Figure 24), the fraction-1 peroxidase in the ethylene treated flask accounts for less than 0.1% of the total activity in the incubation medium. Both fraction-1 and fraction-2 have the same charge characteristics and are comprised primarily of the same anodic isozyme, No. 2 (Figure 29). Thus, if the peroxidase isozymes in the incubation medium were separated on the basis of charge rather than size, then fraction-1 and fraction-2 would be indistinguishable.

Figure 28. Peroxidase found in the medium of horseradish root tissue incubated in an atmosphere of 500 PPM ethylene.

Horseradish peroxidase peaks are resolved by passing the medium through a Sephadex G-200 column and eluting with 0.1 M phosphate buffer, pH 7.4. Peroxidase is assayed spectrophotometrically using o-dianisidine as the hydrogen donor. Graph is plotted in units of peroxidase activity per ml vs. volume eluted from column.

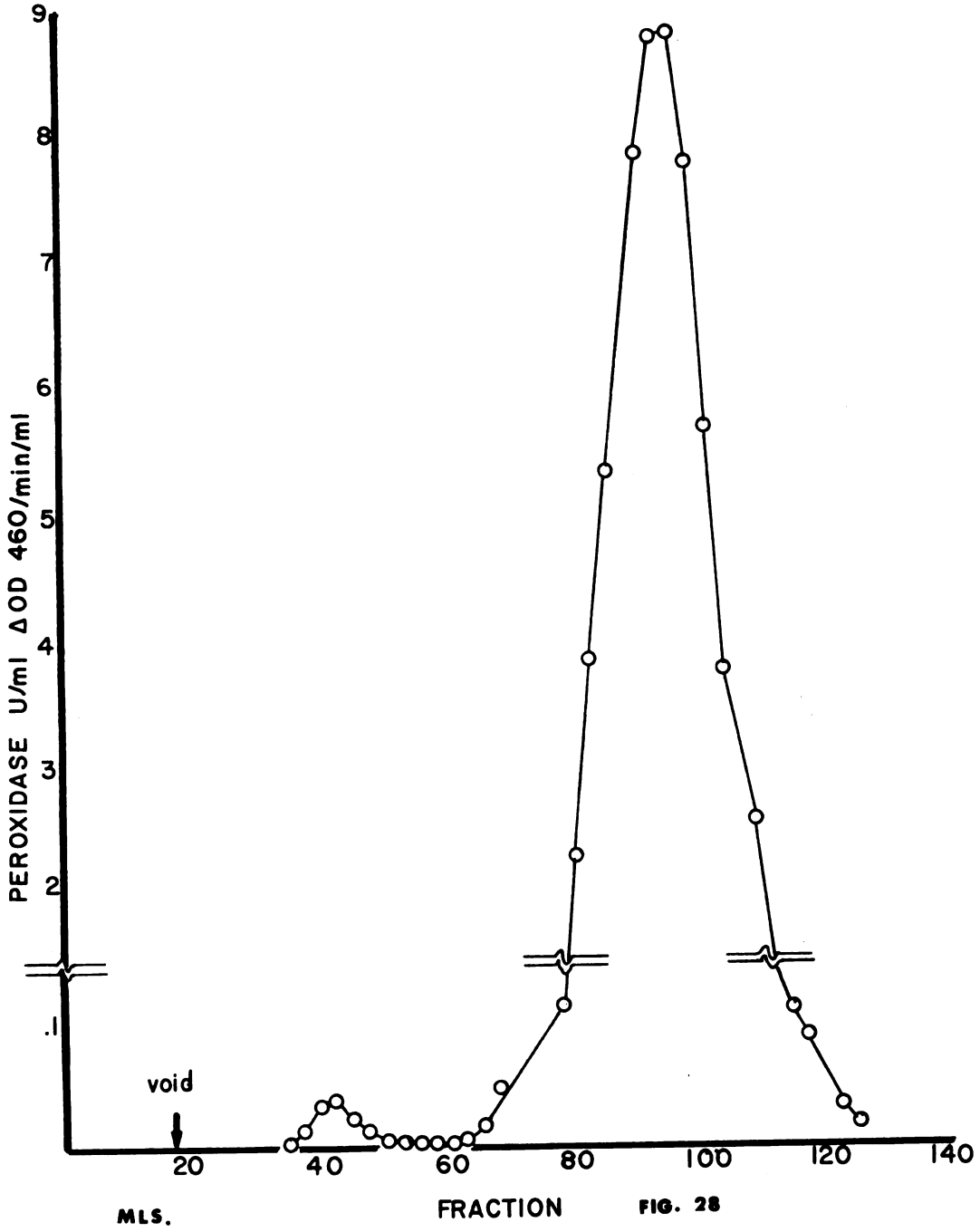

PEROXIDASE U/ml ΔOD 460/min/ml
9
8
7
6
5
4
3
2
.1
void
20
40
60
80
100
120
140
MLS.
FRACTION

FIG. 28

Figure 29. Peroxidase isozymes revealed by electrophoresis of sequentially applied samples of the eluent of Sephadex G-200 column shown in figure 26.

Approximate elution volumes of the column are seen under the photograph. Peroxidase isozymes are observed by staining with benzidine-H_2O_2.

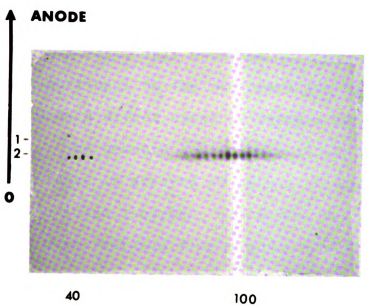


Fig. 29

CHAPTER VII

DIFFERENCES IN THE CATALYTIC ACTIVITIES OF VARIOUS PEROXIDASE ISOZYMES

Ammonia Induced Enhancement of Peroxidase Activity

Ammonia can increase the apparent specific activity of peroxidase. In fact, this observed increase of activity is directly proportional to the concentration of ammonia and can be used as a direct assay of ammonia concentration (Stutts, 1964). The ammonia enhancement effect is observed only when peroxidase is assayed pH values in excess of 7.

Two peroxidase isozymes, the anodic No. 1 and the cathodic No. 5 were isolated from a commercial preparation of horseradish peroxidase (Worthington, HRP-HPOD-6FA) by a combination of starch gel electrophoresis and Sephadex chromatography. These were used to study the effect of ammonia on peroxidase activity using two reactions, the fluorimetric homovanillic acid assay and the spectrophotometric o-dianisidine assay.

When these isozymes were assayed fluorimetrically at pH 9.3 with homovanillic acid as the hydrogen donor,

no increase in peroxidatic activity was observed with the inclusion of ammonia to the reaction mixture.

When the peroxidase reaction was assayed spectrophotometrically with o-dianisidine as the hydrogen donor, the addition of ammonia to the reaction mixture increased the peroxidatic activity of the cathodic No. 5 isozyme, while it had no effect on the activity of the anodic No. 1 peroxidase isozyme (Table 15).

Table 15. Peroxidase activity of isolated isozymes from horseradish in the presence and absence of added ammonia.

Sample	Peroxidase Isozyme	Enzyme Added	NH_4OH Conc.	pH	Peroxidase Act. ΔOD^{460}/min
1	cathodic No. 5	5 µl	--	9.3	.148
2	cathodic No. 5	5 µl	.75 mM	9.3	.269
3	cathodic No. 5	5 µl	1.5 mM	9.3	.435
4	cathodic No. 5	5 µl	3.0 mM	9.3	.628
5	cathodic No. 5	5 µl	6.0 mM	9.3	1.05
6	anodic No. 1	50 µl	--	9.3	.107
7	anodic No. 1	50 µl	.75 mM	9.3	.110
8	anodic No. 1	50 µl	1.5 mM	9.3	.092
9	anodic No. 1	50 µl	3.0 mM	9.3	.105
10	anodic No. 1	50 µl	6.0 mM	9.3	.110

Horseradish peroxidase was obtained from Worthington (HRP-HPOD-6FA). Peroxidase is assayed spectrophotometrically with o-dianisidine as a hydrogen donor.

Both the spectrophotometric and fluorimetric peroxidase reactions performed here were run at pH 9.3 in 0.01 M sodium pyrophosphate buffer.

Superficially, these results indicate that the ammonia of peroxidase activity does not operate for all substrates of the peroxidase reaction, even those such as homovanillic acid whose pH optima for activity are in the range where the ammonia enhancement is most effective. Furthermore, one can conclude that the ammonia enhancement of activity is observed for only one of the two peroxidase isozymes tested, its effect is not on increasing the color production of the product of the peroxidase reaction, because if this were the case, the ammonia effect should be uniformly seen on all isozymes of peroxidase which serve to catalyze the oxidation of o-dianisidine. Ammonia must be bound somewhere in the region of the active site and must actually assist in the turnover of substrate to product molecules. Since all the peroxidase isozymes tested here are shown to be heme-proteins in which the heme-moiety participates in the peroxidatic reaction by binding hydrogen peroxide, it follows that these isozymes of peroxidase differ in the apo-enzyme portion of the protein to such an extent that chemical events which are observed in one isozyme of peroxidase such as the ammonia stimulation of the cathodic No. 5 isozyme are completely absent in another peroxidase such as the anodic No. 1 isozyme.

The observation that the No. 1 isozyme of peroxidase does not participate in the ammonia enhancement effect takes on added significance in the light of a kinetic study of this effect on horseradish peroxidase (Fridovich, 1963). Fridovich shows that ammonia acts to stimulate peroxidase activity in the o-dianisidine reaction at high pH by combining rapidly and reversibly at a saturable site on the catalytic surface of the enzyme, and that this site is not the heme hydrogen peroxide binding site. Since the anodic No. 1 peroxidase isozyme does not demonstrate the ammonia enhancement effect, it follows that the site to which the ammonia binds is either absent in the anodic No. 1 isozyme or blocked sterically from attack by ammonia. Because this isozyme is significantly denser than other peroxidases, it is possible that added carbohydrate provides such steric interference.

This then represents a demonstration that two peroxidase isozymes from the same tissue in the same plant can differ structurally in their active sites.

Homovanillic Acid Peroxidase Assay of Two Peroxidase Isozymes and Purified Horseradish Cell Walls

The 37-fold differences in specific activities isozymes 1 and 5 when measured for biphenyl production using homovanillic acid (Table 11), is much larger than the differences observed when these isozymes are assayed

in the presence of redox dyes such as o-dianisidine (Kay, et al., 1967).

The peroxidase isozyme located anodically (No. 1) is interesting because it can easily be purified to homogeneity on disc electrophoresis. This isozyme has a very high specific activity when assayed for peroxidase using homovanillic acid, and also shows unusual physical characteristics. It elutes off Sephadex columns before the other peroxidases, which indicates that it differs in size.

The activity of this isozyme of peroxidase in producing biphenyls was compared with that of other peroxidase isozymes, in particular the major cathodically migrating No. 5 isozyme and also with the peroxidase which is associated with purified horseradish cell walls.

In this study peroxidase was assayed fluorimetrically with homovanillic acid as the substrate according to the method of Guillbault (1968) at pH 8.0. The biphenyl produced by this reaction does not naturally stick to the cell wall, as is the case in the spectrophotometric redox dye substrates such as benzidine and o-dianisidine, and the quantum yield of fluorescence produced per unit of time when purified cell walls are used as the enzyme source is proportional to the amount of wall material pipetted into the reaction mixture. Furthermore, because this reaction is fluorimetric and measures light production rather than light absorption, the presence of the particulate walls in the reaction mixture does not interfere greatly with the

observation of fluorescence except to increase light scatter. For these reasons, it appears that the fluorimetric homovanillic acid assay for peroxidase is particularly suited for the assay of particulate wall material.

Peroxidase isozymes 1 and 5 from a commercially purified preparation of horseradish peroxidase (Worthington HRP-HPOD-6FA) were used in these studies. These isozymes were resolved from each other by separation on a Sephadex G-75 column or isoelectric focusing column. Since the anodic No. 1 isozyme has a unique pI value (3.0) and a distinct behavior on Sephadex chromatography, it was easy to separate in a form which showed only one band of protein on disc gel electrophoresis. This enzyme was also obtained by running a starch gel of the commercial preparation and by eluting the enzyme from the starch by centrifugation. The enzyme obtained in all these ways behaved identically.

The major cathodic isozyme No. 5 was resolved from the anodic isozyme by Sephadex G-75 chromatography or isoelectric focusing. After these separations the No. 5 isozyme is contaminated with 30% of one other peroxidase isozyme, No. 4, but the R.Z. value is 3.0, indicating that no other contaminating proteins are present. The No. 4 and No. 5 isozymes were then resolved by starch gel electrophoresis and the No. 5 isozyme cut out and eluted from the gel by centrifugation. When the starch gel step was omitted, and the two isozymes were assayed as a mixture

with homovanillic acid, or with o-dianisidine, they behaved identically to the resolved No. 5 isozyme, so it is concluded that starch does not interfere with the expression of the peroxidase reaction.

Once it was established that the enzyme concentration was in a range where fluorescence at 425 mu with excitation at 315 mu was proportional to the concentration of the enzyme, the reaction was studied over a variety of hydrogen peroxide concentrations in order to determine an apparent Michaelis constant with respect to H_2O_2 (K_{app} (H_2O_2)) for the peroxidase reaction. The reaction is not quite linear, but it was possible to draw straight lines through the first 30 seconds of the reaction to estimate an initial velocity. It was shown that high concentrations of hydrogen peroxide inhibit this reaction in a manner which is characteristic of substrate inhibition and which is typical of peroxidase reactions. When the No. 1 isozyme was assayed in this fashion, rate of reaction was proportional to hydrogen peroxide concentration, and one could extrapolate from a Lineweaver-Burk double reciprocal plot an apparent K_m with respect to H_2O_2 of 1.5×10^{-5} M (Figure 30).

Purified cell walls of horseradish root were prepared by the method of Kivilaan (Kivilaan, et al., 1959). These walls were suspended in 0.01 M tris buffer, pH 8.0 and used as a source of enzyme in the homovanillic acid

Figure 30. Peroxidatic activity of the No. 1 anodic isozyme of horseradish peroxidase (Worthington HRP-HPOD-6FA) assayed with homovanillic acid.

Increase in fluorescence with time is recorded at different concentrations of hydrogen peroxide. 1. 3×10^{-6} M H_2O_2. 2. 1.5×10^{-5} M. 3. 1.5×10^{-4} M. 4. $c \times 10^{-4}$ M.

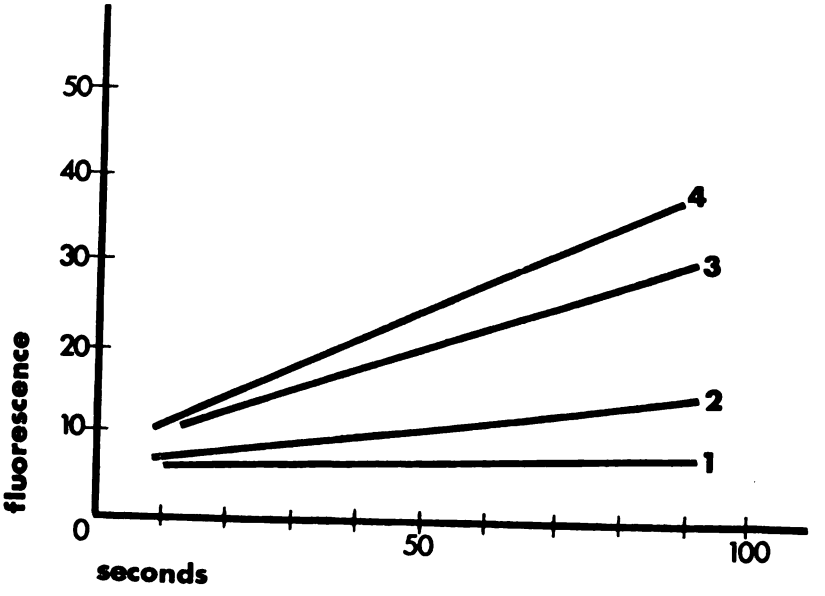


Fig. 30

assay. A plot of peroxidase activity at various hydrogen peroxide concentrations (Figure 31) showed that the rate of the reaction depended on hydrogen peroxide concentration. The extrapolated $K_{app}(H_2O_2)$ for peroxidase associated with purified cell walls was 1.5×10^{-5} M with respect to hydrogen peroxide.

When the cathodic peroxidase isozyme No. 5 was assayed at any given concentration of hydrogen peroxide, the production of fluorescence in the homovanillic acid assay was proportional to the concentration of the enzyme, but when the concentration of hydrogen peroxide was varied over a fixed amount of enzyme, the reaction rate did not vary. However, the reaction stopped abruptly, and no further increase of fluorescence was observed after a certain period of time. The higher the concentration of H_2O_2 supplied to the reaction mixture, the longer it took for the reaction to stop and consequently more biphenyl was produced, but the rate of biphenyl production did not seem to depend on the concentration of hydrogen peroxide (Figure 32). When a boiled control of this sample was assayed, no reaction was observed, so it was concluded that we were dealing with an enzymic reaction. The rate of biphenyl production did vary with changes in enzyme concentration in the reaction mixture (Figure 33).

The $K_{app}(H_2O_2)$ for the anodic No. 1 peroxidase isozyme when assayed fluorimetrically using homovanillic

Figure 31. Peroxidatic activity of the No. 5 cathodic isozyme of horseradish peroxidase (Worthington HRP-HPOD-6FA) assayed with homovanillic acid.

Increase in fluorescence with time is recorded at different concentrations of hydrogen peroxide. 1. $.3 \times 10^{-5}$ M H_2O_2. 2. $.9 \times 10^{-5}$ M. 3. 3×10^{-5} M.

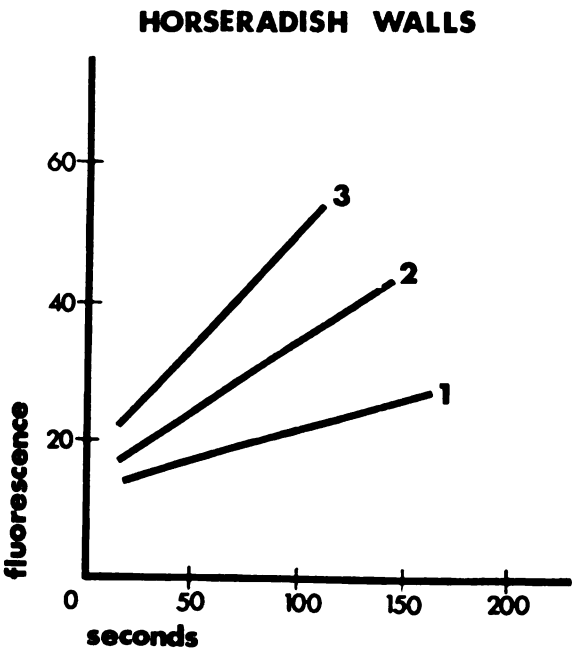

HORSERADISH WALLS
60
40
20
0
fluorescence
50
100
150
200
seconds
3
2
1

Fig. 31

Figure 32. Peroxidatic activity of purified horseradish cell walls assayed with homovanillic acid.

Increase with fluorescence with time is recorded at different concentrations of hydrogen peroxide. 1. 3.3×10^{-6} H_2O_2. 2. 1.65×10^{-5} M. 3. 3×10^{-5} M.

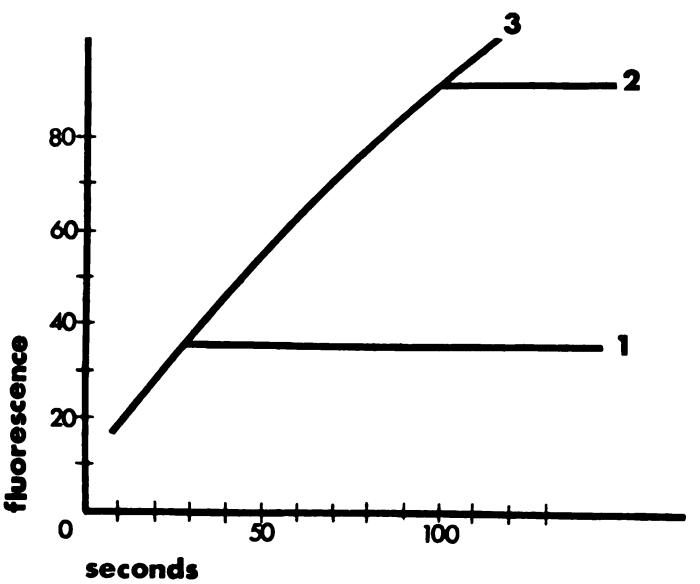


Fig. 32

Figure 33. Peroxidatic activity of the No. 5 cathodic isozyme of horseradish peroxidase (Worthington HRP-HPOD-6FA) assayed with homovanillic acid.

Fluorescence increase over time is noted with different concentrations of enzyme. A. 5 µl of a stock solution of No. 5 enzyme. B. 10 µl of the same stock solution of the No. 5 enzyme. C. 20 µl of the same stock solution of enzyme.

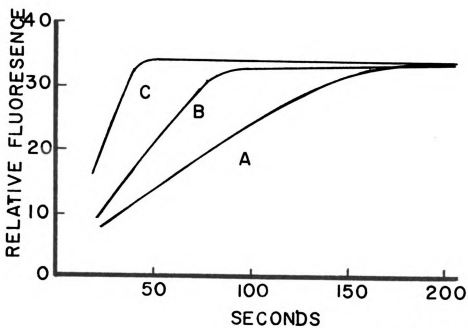


figure **33**

acid is about 100-fold less than the $K_{app}(H_2O_2)$ when the enzyme is assayed spectrophotometrically with o-dianisidine. This seemed odd because the point of attachment of hydrogen peroxide to peroxidase is known to occur at the sixth coordination group of iron in the heme moiety (Saunders, 1964) and therefore if the spectrophotometric and fluorimetric assay of peroxidase are measuring the same peroxidase mechanism, the $K_{app}(H_2O_2)$ should be identical for both assays, yet they differ by two orders of magnitude. The $K_{app}(H_2O_2)$ for the spectrophotometric o-dianisidine reaction was obtained at about pH 6, while the fluorimetric assay was measured at pH 8.0, so the pH difference in the two assay procedures might account for the differing values.

An attempt was made to measure the activity of peroxidase by the fluorimetric assay at pH 6.0, but at pH values lower than 7.0 no peroxidase activity was observed by the fluorescent assay, so it was impossible to determine whether the peroxidase $K_{app}(H_2O_2)$ discrepancy for these two methods is due to the differences in pH in the different reactions.

This two order of magnitude difference in the identical kinetic parameter of two different peroxidase reactions may indicate that the peroxidase enzyme can act mechanistically in different ways for the common function of binding of H_2O_2 to heme.

While bound to the insoluble particles of cell walls, peroxidase is catalytically as active as a soluble form of peroxidase and exhibits an identical dependence on hydrogen peroxide concentration.

CHAPTER VIII

DIFFERENTIAL SENSITIVITY OF HORSERADISH PEROXIDASE ISOZYMES FOR SUBSTRATES OF PROBABLE PHYSIOLOGICAL SIGNIFICANCE

Two substrates are required for peroxidase to act catalytically in a living cell. One of these is hydrogen peroxide; the other substrate required is a suitable hydrogen donor. A wide range of compounds satisfy this requirement. Peroxidase can catalyze the oxidation of amines such as benzidine, o-dianisidine and guaiacol to form colored products, hydroxylation with the absence of the NIH shift, and the formation of free radicals which subsequently condense to form carbon-carbon bonds. Peroxidase derived from animal sources can also catalyze halogenation reactions, but this activity is lacking in peroxidase from horseradish and other plants such as maize.

Since peroxidase does exhibit a wide range of activities, and since in higher plants peroxidase exists in isozymic forms, it is important to know whether all the peroxidase isozymes can utilize the same hydrogen donors with equal efficiency, or whether any differences

exist in the catalytic activity of the various isozymes. If the latter case is true, then it may be speculated that the peroxidase isozymes may have different biological functions in the cell.

In an investigation of the possible substrate specificities of peroxidase isozymes, it would be most meaningful to assay those activities which are definitely known to occur in vivo. There are at least three classes of compounds in organisms whose formation is linked to the enzymic activity of peroxidase. The first are halogenated compounds such as iodotyrosine and diiodotyrosine which are formed after a peroxidase catalyzed oxidation of iodide (Alexander, 1960). However, horseradish peroxidase, either as a purified protein or as an extract of the mascerated tissue, does not catalyze this halogenation reaction (Ljunggren, 1966). The second type of compound formed by peroxidase action is dityrosine, which is the condensation product of tyrosine free radicals. Dityrosine is found as a protein crosslink in resilin (Anderson, 1964), elastin (LaBella, et al., 1967), and in the gel form of collagen (LaBella, et al., 1968). Horseradish peroxidase has the capacity to catalyze the formation of dityrosine at pH's above 7.0; however, this amino acid has not been found in amino acid analyses of horseradish tissue. The third class of compounds whose formation is catalyzed by peroxidase is lignin, which is found in all higher plants. Lignin is

formed by the condensation of phenyl alcohol free radicals.

Because they are known to be physiologically significant, substrates of peroxidase, eugenol (2-methoxy-4-allyl phenol) which is a lignin precursor (Siegel, 1956) and tyrosine were used to develop new zymogram stains which allow the visualization of peroxidase isozymes after starch gel electrophoresis.

Eugenol Stain for Peroxidatic Catalysis of Lignin Formation

Peroxidase in the presence of hydrogen peroxide catalyzes the formation of free radicals of eugenol, and these radicals condense and form a white precipitate. This precipitate is not a lignin compound as such, but when the reaction is performed in the presence of a preformed cellulose matrix such as cellulose powder or paper, the resultant product tests positively for lignin (Siegel, 1956).

Since the eugenol reaction results in a precipitate, and a precipitation reaction is a requisite for good zymogram stains, it appeared that eugenol might be a good choice as a substrate to visualize peroxidase isozymes. Furthermore, since the peroxidase catalyzed eugenol product is essentially a biphenyl compound, it is probable that it would be fluorescent.

The following peroxidase zymogram stain was developed:

75 mg neat eugenol (2-methoxy-4-allyl phenol) (∿60 µl)

100 ml 0.05 M sodium phosphate buffer, pH 6.0

10 µl 30% H_2O_2--final concentration is 9×10^{-4} M

Stir for 30 minutes.

Observe peroxidase isozymes by flooding the cut surface of a starch gel, when viewing under short-wave ultraviolet light. Blue bands of fluorescence appear within one minute.

The sap from frozen and stored horseradish petioles was subjected to starch gel electrophoresis at 150 V for eight hours in the discontinuous tris citrate, lithium borate buffer system, pH 8.3 at 4°C. The developed gel was then cut transversely into three 2 mm thick slices. These slices were stained for peroxidase with either eugenol, benzidine, or tyrosine zymogram reaction mixtures. Peroxidase isozymes were observed and recorded (Figure 34). The peroxidase isozymes visualized with eugenol do not completely correspond to the peroxidase isozymes seen when the benzidine reaction mixture is used. There are two isozymes, one which has migrated 2.1 cm to the anode, and another which migrated 2.8 cm to the cathode, which appear to react with benzidine and not with eugenol. There are also two isozymes, which migrated 1.0 and 3.4 cm to the cathode, which appear to be reactive with eugenol but not with benzidine.

Figure 34. Representation of peroxidase isozymes from frozen and stored horseradish petiole sap.

Samples were subjected to electrophoresis in 12% starch gel. The developed gel was sliced transversely into three 2 mm slices, and each stained for peroxidase activity with either eugenol-H_2O_2, benzidine-H_2O_2, or tyrosine-H_2O_2. Figures to the right of each isozyme are migration distances from the point of insertion of the sample in centimeters.

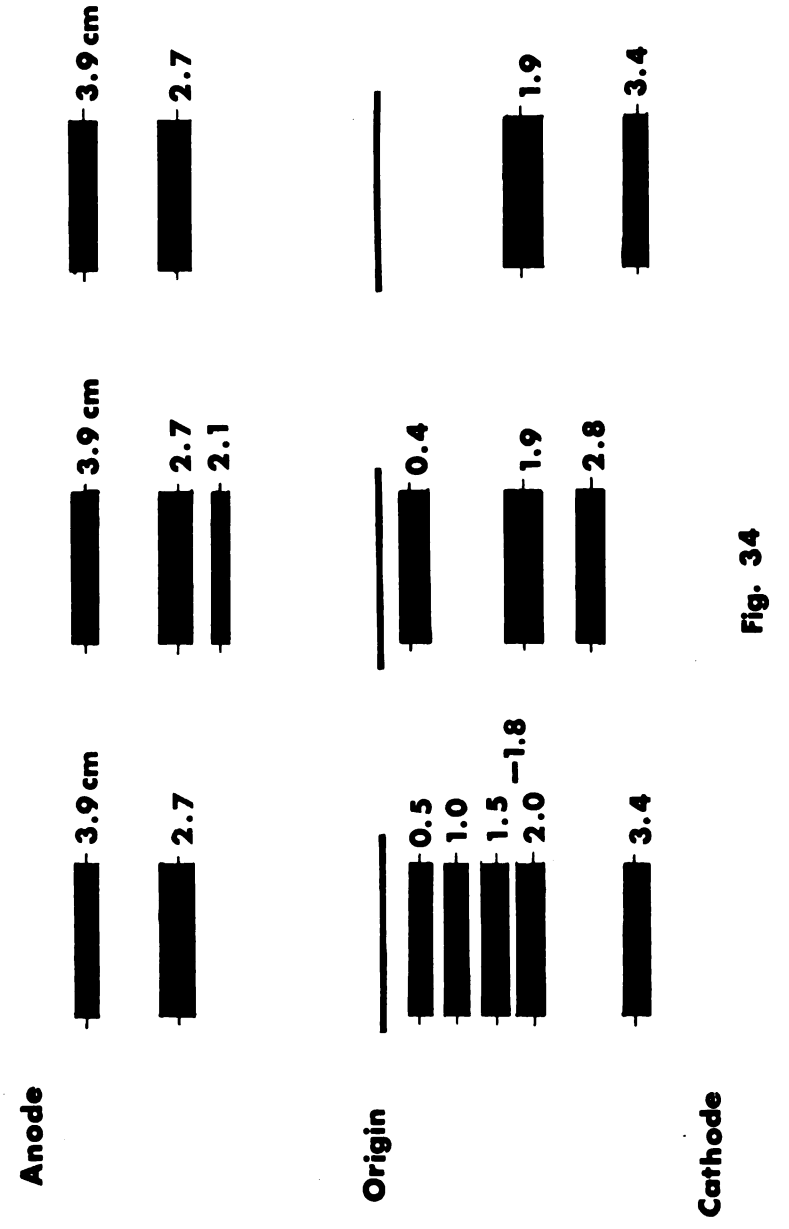

Eugenol
Benzidine
Tyrosine
Anode
3.9 cm
2.7
3.9 cm
2.7
2.1
3.9 cm
2.7
Origin
0.5
1.0
1.5
2.0
1.8
3.4
0.4
1.9
2.8
1.9
3.4
Cathode

Fig. 34

The eugenol stain was judged to be specific for peroxidase for the following reasons: (1) the zones of fluorescence are not recognized on the cut surface of the gel before the application of the eugenol reaction mixture; (2) when the gel is flooded with the eugenol mixture without hydrogen peroxide, no fluorescent bands develop; (3) no fluorescent bands develop when eugenol-H_2O_2 is used to stain a sample of peroxidase which has been previously incubated at 100°C for 30 minutes.

The development of the eugenol-H_2O_2 peroxidase zymogram stain allows us to assess the relative reactivity of peroxidase isozymes for a reaction of probable physiological significance. Comparison of the eugenol stain to the conventional benzidine stain on the same sample leads to the conclusion that the peroxidase isozymes recognized by any zymogram stain depends to a large part on the substrate which is used. Therefore there do exist differences in the reactivity of peroxidase isozymes in a physiologically significant reaction such as the formation of lignin.

Tyrosine Stain for Peroxidase Activity

Perhaps one reason dityrosine has not been identified in horseradish root is because the peroxidase catalyzed formation of this compound does not occur below pH 7.0 and optimum rate of formation is not reached until pH 8.5. This does not mean that peroxidase is unreactive with

tyrosine at pH values below 7.0. A peroxidatic reaction with tyrosine as a substrate has been observed at pH 6.0 (Table 16). The reaction is monitored by measuring the

Table 16. Peroxidase activity of commercially purified horseradish peroxidase (Worthington HRP-HPOD-6FA) using tyrosine as a substrate.

Enzyme	H_2O_2	Tyrosine	OD^{260}/min	OD^{260}/min Per mg Prot.
100 µg	9×10^{-4} M	0.5 mg/ml	0.349	3.49
25 µg	9×10^{-4} M	0.5 mg/ml	0.073	2.92
100 µg	none	0.5 mg/ml	.000	--
none	9×10^{-4} M	0.5 mg/ml	.000	--
100 µg*	9×10^{-4} M	0.5 mg/ml	0.018	0.18

All reactions were run at room temperature, in 0.05 M PO_4 buffer, final pH 6.03.

* Incubated at 90°C for 10 minutes.

increase in absorbance at 260 nm with time when peroxidase is added to a reaction mixture containing 0.5 mg/ml tyrosine and H_2O_2 at a concentration of 9×10^{-4} M. This increase in optical density is roughly proportional to the amount of added enzyme, and does not occur either when hydrogen peroxide is left out of the reaction mixture or when boiled enzyme is introduced. For these reasons, this reaction is judged to be genuinely peroxidatic. The nature of the

product is unknown except that it is nonfluorescent, and absorbs ultraviolet light.

The following peroxidase zymogram stain utilizing tyrosine as a substrate was developed:

1 mg tyrosine

100 ml 0.05 M sodium phosphate buffer, pH 6.0

10 µl 30% H_2O_2--final concentration is 9×10^{-4} M

Stir 30 minutes.

Observe peroxidase isozymes by flooding the cut surface of a starch gel, then viewing under short-wave ultraviolet light. Dark fluorescent absorbing bands appear within one minute.

This reaction mixture was used to stain a starch gel electrophoresis of frozen and stored horseradish petiole sap (Figure 34). The peroxidase isozymes visualized with the tyrosine-H_2O_2 stain do not show a complete correspondence with either the benzidine or eugenol peroxidase stains. One isozyme, which migrated 3.4 cm to the cathode, can be visualized with the eugenol but not with the benzidine stain. The other three peroxidase isozymes visualized with tyrosine-H_2O_2 can also be seen with the benzidine and eugenol stains. However, there are three peroxidase isozymes which stain with benzidine but not tyrosine.

The tyrosine stain was judged to be specific for peroxidase for the following reasons: (1) the zones of fluorescence absorption are not recognized on the cut surface of the gel before the application of the tyrosine reaction mixture; (2) when H_2O_2 is omitted from the reaction

mixture, no fluorescent absorbing bands develop; (3) no fluorescent bands develop when peroxidase has previously been boiled for 30 minutes.

Although the tyrosine product of peroxidase is unknown, it may be of physiological importance since tyrosine is known to be in cells, and peroxidase is now shown to react with tyrosine over wide pH ranges to form either dityrosine or the unknown product, depending on the pH.

An attempt was made to develop a peroxidase zymogram stain which measured the formation of dityrosine. This was done by changing the buffer in the tyrosine reaction mixture to 0.05 M tris, pH 8.5. When this solution was used to flood a starch gel, the development of blue fluorescence was observed in the solution, but no bands appeared on the gel.

If the different isozymes visualized by the eugenol, benzidine and tyrosine stains were due merely to differing sensitivities of the stains themselves to peroxidase, we would have observed that the more sensitive stain, say eugenol, recognized all the isozymes that the less sensitive stain, say benzidine, did, plus others. However, we would never observe that benzidine could recognize isozymes which were insensitive to eugenol and in the same sample find isozymes which were sensitive to eugenol but not benzidine, which is in fact what is actually seen on the gels. We can therefore conclude that the different

isozymes from the horseradish petiole which are recognized by benzidine, eugenol or tyrosine stains represent genuine differences in the reactivity of the peroxidase isozymes with these substrates. Since the tyrosine and eugenol reaction mixtures assay reactions of physiological importance, we can further conclude that these differences in reactivity may be of some biological importance in the living cell.

CHAPTER IX

IN VITRO MODIFICATION OF HORSERADISH PEROXIDASE ISOZYMES

No genetic studies have been made on the peroxidase isozyme system in horseradish. It is not known whether all the peroxidase isozymes which can be found in this plant represent the products of different genes, or whether some of these peroxidase isozymes represent modification products of other peroxidases by the addition or subtraction of charged groups, or by the addition of large blocking groups such as carbohydrates which could serve to alter electrophoretic mobility.

Attempts were made to modify the electrophoretic mobility of peroxidase isozymes by treatments which could conceivably occur either in the cell or during the extraction procedure.

Attempts to Modify Electrophoretic Mobility of Peroxidase Isozymes by Treatment with Carbohydrases

Since horseradish peroxidase isozymes are all glyco-proteins containing from 18-50% carbohydrate, these groups, by adding on to peroxidase and blocking charges,

may have some effect on the electrophoretic mobility of peroxidase isozymes. Commercially purified horseradish peroxidase was incubated with pectinase enzyme preparations which are known to contain many different carbohydrase activities, particularly arabanosidases and galactosidases. The preparations included Pectinol R-10, Pectinol 59-L, and Rhozyme 4A-150, provided by Rohm and Haas, Philadelphia. Peroxidase was incubated in 10% solutions of these enzyme preparations in .05 M PO_4 buffer, pH 6.8 for 24 hours at 25°C. After the incubation period, samples of the treated peroxidase were subjected to starch gel electrophoresis and stained for peroxidase activity with benzidine-H_2O_2. No alteration of the isozyme migration patterns could be detected in any of the treated peroxidase samples.

The most anodically migrating isozyme of horseradish peroxidase (No. 1) may have associated with it hydroxyproline which is glycosidically linked to an araban. A search was conducted for an enzyme preparation which could hydrolyze these hydroxyproline arabinosides in the expectation that these enzymes might also serve to alter the electrophoretic mobility of the most anodically migrating No. 1 peroxidase isozyme.

Such a hydroxyproline-arabinoside hydrolyzing activity was observed in a purified preparation of β-1,3-glucanase prepared from Sclerotium rolfsii, by Prof. Y. Masuda, Osaka, Japan. A sample of a hydroxyproline-

arabinoside with a chain of four arabinose residues (H_1A_4) isolated from tomato cell walls was incubated with the β-1,3-glucanase. The reaction mixture consisted of 0.1 ml of the β-1,3-glucanase (7.4 U/ml), .01 M acetate buffer, .001 M $CaCl_2$ and 740 µg of H_1A_4 in a total volume of 5.0 ml, pH 5.0. After 12 hours incubation at 37°C, the reaction vessel was removed from the water bath, and reduced in volume by vacuum on a rotary evaporator.

Without further treatment, the reaction mixture was spotted on Whatman #4 paper and subjected to electrophoresis for two hours at 5.5 kV in pH 1.9 acetic acid-formic acid buffer. After completion of electrophoresis, the paper was stained for hydroxyproline with Isatin-Ehrlich's reagent (Figure 35). The H_1A_4 which was treated with β-1,3-glucanase showed the presence of hydrolysis products. The H_1A_4 which was incubated for 12 hours in a control vessel which contained the complete reaction mixture but without the β-1,3-glucanase showed no hydrolysis and co-electrophoresed with authentic H_1A_4.

The electrophoretic mobility of the H_1A_4 breakdown products correspond to the 1 hydroxyproline : 3 arabinose (H_1A_3) and the 1 hydroxyproline : 2 arabinose (H_1A_2) glycosides. No free hydroxyproline was detected in the reaction mixture, so the β-1,3-glucanase does not hydrolyze the hydroxyproline arabinose glycosidic linkage.

Figure 35. Electrophoresis of hydroxyproline-arabinosides after 12 hours incubation at 37°C with β-1,3-glucanase preparation from Sclerotium rolfsii.

Electrophoresis is carried out at 5.5 kV for 2 hours at pH 1.9 in acetic-formic acid buffer, and stained for hydroxyproline with Isatin-Ehrlich's reagent. 1. Hypro-1 : arabinose-4 (H_1A_4). 2 and 3. H_1A_4 incubated for 12 hours at 37°C with β-1,3-glucanase preparation. 4 and 5. H_1A_4 incubated for 12 hours without β-1,3-glucanase at 37°C. 6. Hydroxyproline.

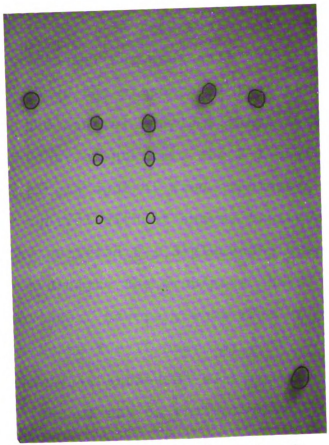


Fig. 35

Samples of the same reaction mixtures were next spotted without further treatment on Whatman #1 paper and chromatographed for 24 hours in a descending solvent system of pyridine : ethyl acetate : water :: 8:2:1. Sugars were then visualized by staining the chromatogram with aniline-phthalate (Figure 36). In reaction samples where laminarin (a β-1,3-linked glucan) was used as a substrate, the enzyme treated sample shows the presence of free glucose, and this was lacking in the control sample without enzyme. Likewise, when H_1A_4 is used as a substrate the β-1,3-glucanase treated sample shows a single spot of free arabinose, and this was lacking in the control without enzyme treatment.

Thus the purified β-1,3-glucanase preparation shows the ability to hydrolyze the arabinose chains in the hydroxyproline arabinosides. However, when this enzyme was incubated with peroxidase for 12 hours at 37°C, a zymogram of the treated peroxidase showed no alterations in the electrophoretic mobilities of any of the peroxidase isozymes.

Therefore, even though β-1,3-glucanase has the ability to hydrolyze the arabinoses in hydroxyproline arabinosides, such an activity does not serve to alter the charge properties of horseradish peroxidase isozymes.

The pH Induced Modification of Peroxidase Isozyme Electrophoretic Mobility

Frahn and Mills (1968) have described the reaction of carbon dioxide with free amino groups in amino acids to

Figure 36. Chromatography of sugars released from samples treated with a β-1,3-glucanase preparation from Sclerotium rolfsii.

Treated and untreated samples are chromatographed without hydrolysis in a pyridine : ethyl acetate : H_2O (8:2:1) solvent for 24 hours and stained for free sugars with analine-phthalate. 1. Laminarin treated with enzyme. 2. Untreated laminarin. 3. Hydroxyproline-1 arabinose-4 (H_1A_4) treated with enzyme. 4. Untreated H_1A_4. 5. Purified peroxidase treated with β-1,3-glucanase enzyme. 6. Standard of known sugars.

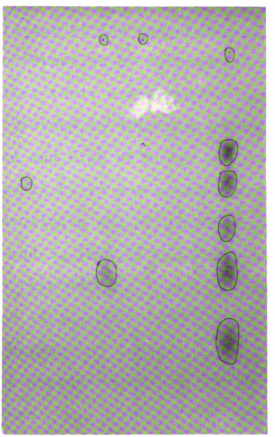


Fig. 36

form carbamates under conditions of low temperature and high pH. The net effect of the addition of CO_2 to the free amino groups of a protein would be to add negative charge to the protein, and these carbamate structures should be stable under the normal handling conditions of enzymes, i.e., storage at low temperatures. It is conceivable that the formation of carbamates on proteins would serve to alter their electrophoretic mobility. An experiment was performed to determine whether the conditions of low temperature and high pH favor the alteration of electrophoretic mobility in peroxidase isozymes. A sample of horseradish peroxidase which contained only two cathodic isozymes, Nos. 4 and 5, in 0.05 M phosphate buffer, pH 7.0 was chosen as the test material. One half of this material was titrated to pH 12.3 with 5 N NaOH. For the control, 0.05 M phosphate buffer, pH 7.0 was added to the remaining half of the enzyme mixture until the two test solutions were of equal volume. 0.5 g of dry ice was dropped in the flask containing the pH 12.3 solution, and both solutions were stored in the cold room at 4°C.

Samples were removed from the test solutions after 12 and 60 hours incubation, and subjected to starch gel electrophoresis (Figure 37). Throughout the incubation period the peroxidase isozymes in the pH 7.0 sample remain constant. In the pH 12.3 sample there is a large change in the electrophoretic mobility of the peroxidase isozymes

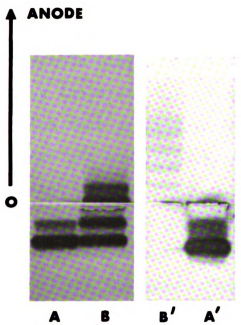


Fig. 37

Figure 37. The incubation of samples of two peroxidase isozymes (Nos. 4 and 5) in the cold room at different pH's.

A. 12 hours incubation at pH 7.0; B. 12 hours incubation at pH 12.3; B'. 60 hours incubation at pH 12.3; A'. 60 hours incubation at pH 7.0.

throughout the incubation period. New isozymes appear which migrate anodically compared to the control, and the trend over time is toward the creation of even more anodically migrating isozymes.

The peroxidase isozymes present after 60 hours incubation in both the pH 7.0 and pH 12.3 samples were subjected to reelectrophoresis in a second dimension (Figure 38). This demonstrates that the new peroxidases observed are discrete molecular entities, and not artifacts generated during the electrophoresis period.

The observation that peroxidase isozymes of increased negative charge are generated by conditions of low temperature and high pH does not in itself demonstrate carbamate formation. Horseradish peroxidase samples were incubated under CO_2 free conditions to determine whether CO_2 was really required for the generation of these anodically migrating isozymes at high pH.

Horseradish roots were homogenized with water in a Waring blender and the cell wall material removed by passage through eight layers of cheesecloth. The pH of the supernate was 4.66. Precautions were taken to remove CO_2 from the solution and ambient atmosphere by bubbling nitrogen through the solution for 60 minutes. CO_2 free NaOH was prepared by making a 50% NaOH solution using distilled water which had been boiled and bubbled with nitrogen, then filtering the solution through glass wool

Figure 38. Reelectrophoresis of peroxidase isozymes in a second dimension.

A'. Peroxidase isozymes (Nos. 4 and 5) incubated for 60 hours at 4°C, pH 7.0; B'. Peroxidase isozymes (Nos. 4 and 5) incubated for 60 hours at 4°C, pH 12.3.

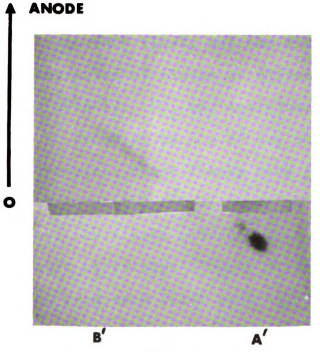


Fig. 38

to remove insoluble carbonates. The NaOH was stored in a bottle previously flushed with nitrogen, and sealed tightly between use. This sodium hydroxide was used to titrate a 20 ml sample of the horseradish root supernate to various pH's and the amount required recorded. Eight 20 ml aliquots of the CO_2 free horseradish root supernate were quickly pipetted into flasks flushing with nitrogen, and immediately sealed. Predetermined amounts of the CO_2 free NaOH was dispensed into each flask, and the flask sealed. This resulted in four pairs of flasks at different pH values ranging from pH 6-12. CO_2 was introduced to one flask of each pair by dropping in 2 g of dry ice. After the dry ice had evaporated, these flasks were also sealed, and all eight flasks incubated for 38 hours at 4°C. At the end of this time, the flasks were unsealed, and pH values and peroxidatic activities of each flask recorded (Table 17). Samples from each flask were subjected to starch gel electrophoresis and peroxidase isozymes visualized with benzidine-H_2O_2 (Figure 39). The CO_2 treated flasks had lower pH values than their untreated duplicates. This is evidence that the CO_2 had gone into solution. Changes in the peroxidase isozyme pattern can be detected at pH values as low as 7.03. The peroxidase isozyme transformations appeared in both the CO_2 treated and CO_2 free flasks. The extent of these transformations depends on the pH rather than the presence or absence of carbon

Table 17. The total peroxidase activity in the supernatant fraction of a horseradish root homogenate after 38 hours incubation at 4°C.

Sample	Initial pH Before CO_2 Addition	CO_2 Added as Dry Ice	pH After 38 Hours Incubation, 4°C	Peroxidase Activity After 38 Hours OD^{460}/min/ml	% Peroxidase Activity Remaining
untreated	4.66	--	4.660	880	100%
1	5.80	--	5.795	840	95.4%
2	5.80	2 g	6.445	860	97.8%
3	7.40	--	7.390	824	93.6%
4	7.40	2 g	7.030	808	91.9%
5	10.00	--	9.940	746	84.8%
6	10.00	2 g	7.605	798	90.8%
7	12.30	--	12.280	212	24.1%
8	12.30	2 g	9.802	668	76.0%

Peroxidase is assayed spectrophotometrically with o-dianisidine as the hydrogen donor.

Figure 39. Peroxidase isozymes in the supernate of horseradish root homogenate incubated for 38 hours in the cold room at various pH values, and with CO_2 treatment of alternate flasks.

1. control sample of supernate, untreated; 2. pH 5.79, CO_2 free; 3. pH 6.44, CO_2 added; 4. pH 7.03, CO_2 added; 5. pH 7.40, CO_2 free; 6. pH 7.60, CO_2 added; 7. pH 9.80, CO_2 added; 8. pH 9.94, CO_2 free; 9. pH 12.28, CO_2 free.

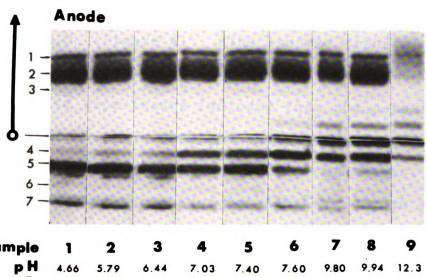

Anode
1
2
3
4
5
6
7
sample 1 2 3 4 5 6 7 8 9
pH 4.66 5.79 6.44 7.03 7.40 7.60 9.80 9.94 12.3

Fig. 39

dioxide. Total peroxidase activity remaining in each flask after 38 hours incubation depends on pH rather than CO_2 addition (Figure 40). Peroxidase activity loss is slight through pH 10, although at pH 12.3, 80% of the peroxidase activity is lost.

Carbamates are unstable at low pH and temperatures around 50°C (Frahn and Mills, 1968). Aliquots from each of the flasks were titrated to pH 4.6 and then incubated at 45°C for 60 minutes. These samples were then subjected to electrophoresis and peroxidase isozymes visualized with benzidine-H_2O_2 (Figure 41). The isozyme transformations were resistant to this treatment.

These experiments indicate that the peroxidase isozyme modifications observed under conditions do not occur because of CO_2 addition to form carbamates. Rather the appearance of these isozymes depends only on pH and incubation time, and once formed, are not sensitive to changes in pH. After 38 hours incubation, isozymes are recognized at pH's as low as 7.03.

Figure 40. Peroxidatic activity remaining after the incubation of samples of the supernate from a horseradish root homogenate for 38 hours at different pH values, 4°C.

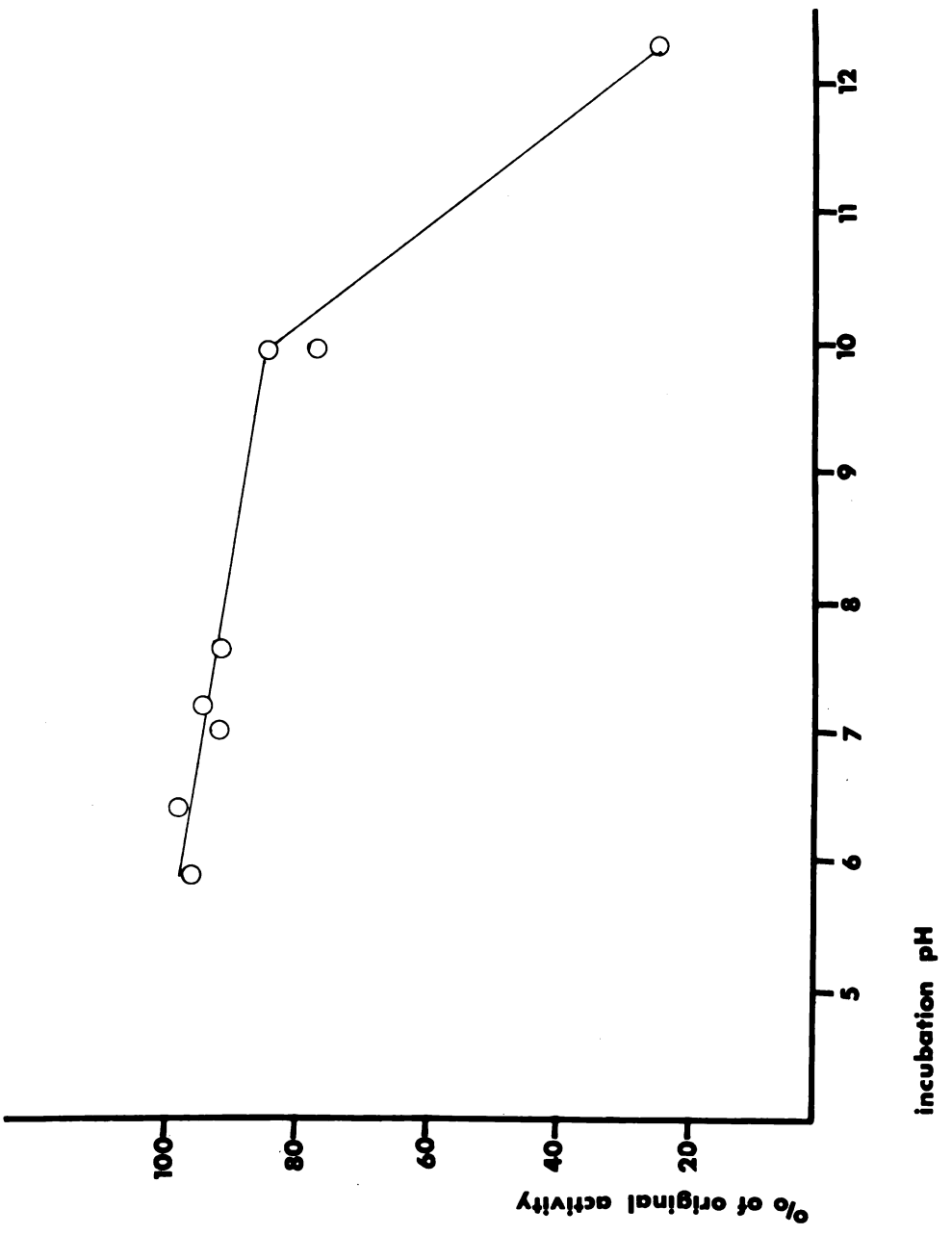

% of original activity
100
80
60
40
20
5
6
7
8
9
10
11
12
incubation pH

Fig. 40

Figure 41. Peroxidase isozymes of horseradish root homogenate which had been incubated for 38 hours at different pH's and then retitrated to pH 4.6 and incubated for 60 minutes at 45°C.

1. untreated homogenate; 2. pH 5.8 incubation; 3. pH 6.4 incubation; 4. pH 7.0 incubation; 5. pH 7.4 incubation; 6. pH 7.6 incubation; 7. pH 9.8 incubation; 8. pH 10.0 incubation; 9. pH 12.3 incubation; 10. untreated homogenate.

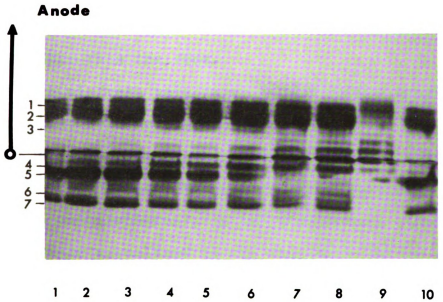


Fig. 41

CHAPTER X

TISSUE DISTRIBUTION OF HORSERADISH PEROXIDASE ISOZYME

Although horseradish peroxidase is the classical system against which all other plant peroxidases are judged, there are no studies of isozyme distribution in organs other than the root.

Equal fresh weights of tissue from a single horseradish plant were mascerated in 1.0 ml of 0.1 M phosphate buffer, pH 7.4. The resulting homogenates were used to wet 7 x 15 mm Whatman 3MM paper wicks; these were subjected to electrophoresis in 12% starch gel. Peroxidase isozymes were visualized in the developed gel by staining with benzidine-H_2O_2. Differences were observed in the relative peroxidase isozyme concentrations among the various plant tissues (Figure 42). It is interesting that even different parts of the root have isozyme distribution differences. The cortex of the root shows a cathodically migrating isozyme which is largely absent in the parenchyma.

When a zymogram of horseradish leaf tissue was stained for peroxidase activity both with eugenol-H_2O_2 and benzidine-H_2O_2, the anodic isozymes could not be

Figure 42. Distribution of peroxidase isozymes in the tissues of the horseradish plant.

Equal fresh weights of tissue were mascerated in 1.0 ml of 0.1 M phosphate buffer, pH 7.4. Without further treatment, a 15 x 7 mm Whatman 3MM paper which was wetted with the homogenate and subjected to starch gel electrophoresis. Samples were obtained from a single field-grown plant, in the spring when the plant was in full flower. 1. Root parenchyma. 2. Root cortex edge. 3. Center of root cortex. 4. Leaf. 5. Petiole. 6. Flowers. 7. Flowering stem. 8. Raceme pedicle.

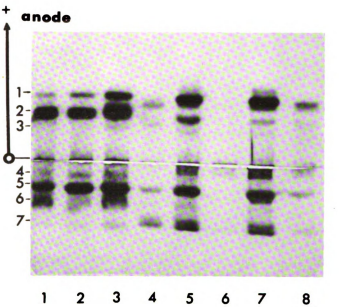


Fig. 42

visualized with the eugenol stain (Figure 43). This contrasts with eugenol staining of petiole tissue where the two major anodic isozymes stain well with eugenol-H_2O_2. The two anodic isozymes in leaves, although they have similar electrophoretic mobilities to the anodic peroxidases in other horseradish tissues, may have slightly different catalytic activities.

Figure 43. Zymogram of peroxidase isozymes from the leaf tissue of a mature horseradish plant.

A homogenate was subjected to electrophoresis on starch gel. Upon completion of electrophoresis, the gel was cut transversely into 2 mm slices. One slice was stained for peroxidase activity with benzidine-H_2O_2, and the other stained for peroxidase with eugenol-H_2O_2. A. Peroxidase isozymes visualized by benzidine. B. Peroxidase isozymes visualized by eugenol.

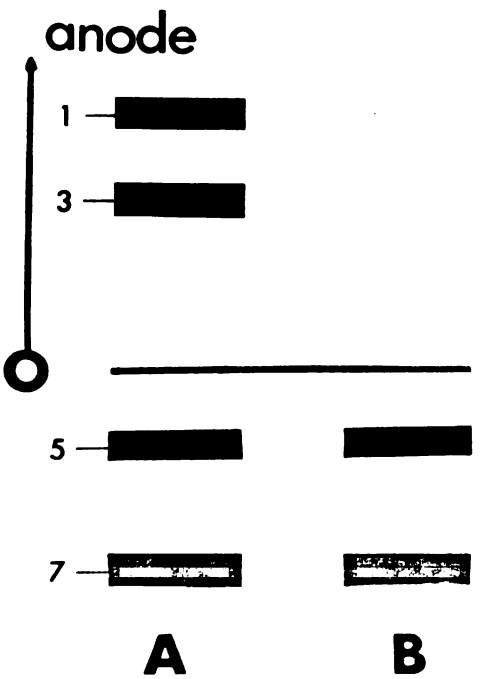


FIG. 43

CHAPTER XI

THE EFFECT OF 2,2'-DIPYRIDYL ON PEROXIDASE ACTIVITY

Holleman (1967) has shown that 2,2'-dipyridyl can serve to inhibit the formation of hydroxyproline from proline in the walls of sycamore cells grown in suspension culture. Since 2,2'-dipyridyl acts to chelate iron, it is likely that the enzyme responsible for the hydroxylation of proline is also iron requiring. A likely candidate for this activity was peroxidase, because of its known ability to function as an oxidase, and because it contains one mole of heme iron per mole of enzyme. If peroxidase hydroxylates proline, then the activity of peroxidase must be inhibited by 2,2'-dipyridyl.

It was decided that this hypothesis would be tested by measuring the activity of a peroxidase sample in the presence and absence of 2,2'-dipyridyl to determine whether the peroxidatic activity of the enzyme was affected. The substrate chosen to measure peroxidase activity was benzidine. This reaction is sufficient to determine whether or not 2,2'-dipyridyl will interfere with peroxidase because the benzidine reaction depends on the active participation of

the heme moiety of peroxidase and thus on the presence of iron. Therefore, even though we are not measuring the actual hydroxylase reaction, if peroxidase is implicated in hydroxylation the redox reaction of peroxidase should also be sensitive to 2,2'-dipyridyl. If peroxidase is not affected by 2,2'-dipyridyl then it can be concluded that the binding constant of iron to heme is greater than the binding constant of iron to 2,2'-dipyridyl, and that peroxidase can definitely be ruled out as the enzyme which hydroxylates proline. If, however, the 2,2'-dipyridyl does inhibit the peroxidase reaction, then it cannot be stated with certainty that peroxidase is responsible for proline hydroxylation.

Peroxidase from two sources, horseradish root and sycamore cells, were assayed with and without 2,2'-dipyridyl. To determine whether 2,2'-dipyridyl had any effect on color production in the peroxidase reaction, it was incubated with benzidine and H_2O_2, and the enzyme added last (Table 18). Color development in the 2,2'-dipyridyl treated cuvettes was about the same as in the untreated enzyme assays. This demonstrated that it was possible to assay peroxidase in the presence of 2,2'-dipyridyl, and that the 2,2'-dipyridyl did not serve to reduce the oxidized product of the benzidine reaction. Samples of both enzymes were then incubated with solutions of 2,2'-dipyridyl at concentrations of 1.0 and 1.72 mM, for 1 hour at 37°C, and the

Table 18. 2,2'-dipyridyl incubated with benzidine and H_2O_2; enzyme added last.

Enzyme Source	2,2'-Dipyridyl Conc.	OD^{610}/min/ml
Horseradish peroxidase	--	.668
Horseradish peroxidase	1 mM	.714
Sycamore peroxidase	--	.394
Sycamore peroxidase	1 mM	.400
Sycamore peroxidase	1.72 mM	.426

enzymes assayed for peroxidase activity after this time. The rate of product formation using the dipyridyl treated enzyme did not vary from the peroxidase activity of the untreated control enzyme (Table 19).

These experiments demonstrate that peroxidase cannot be the enzyme responsible for the hydroxylation of proline in plant tissues.

Table 19. 2,2'-dipyridyl incubated with enzyme; benzidine added last.

Enzyme Source	2,2'-Dipyridyl Conc.	OD^{610}/min/mg Protein
Horseradish peroxidase	--	910
Horseradish peroxidase	1 mM	904
Horseradish peroxidase	1.72 mM	860
Sycamore peroxidase J-1	--	10250
Sycamore peroxidase J-1	1 mM	10600
Sycamore peroxidase J-1	1.72 mM	9940
Sycamore peroxidase J-2	--	13840
Sycamore peroxidase J-2	1 mM	14500
Sycamore peroxidase J-2	1.72 mM	11920

CHAPTER XII

DISCUSSION

The results described here underscore the heterogeneity of the horseradish peroxidase enzyme system. These enzymes have in common the attachment of hematin as a prosthetic group, and the ability to utilize hydrogen peroxide efficiently in the oxidation of suitable hydrogen donors. However, they differ in significant ways in their abilities to oxidize hydrogen donors.

Two aids in the assay of peroxidase have been developed. An automatic peroxidase assayer has been designed and constructed. This machine is basically a continuous flow spectrophotometric assay of peroxidase which is based on the Technicon system of porportioned mixing of reagents. The assayer is able to continuously assay the peroxidase activity in multiple samples as well as automatically determine various simple properties of the peroxidase reaction such as salt tolerance and heat stability. A method for the quantitative determination of peroxidase activity of individual isozymes directly on starch gel zymograms has also been developed. This

method is based on the ability of ascorbic acid to delay the appearance of the colored benzidine-peroxidase reaction product for a time which is directly proportional to the activity of peroxidase. By adding ascorbate to the staining medium for peroxidase zymograms and timing the appearance of the individual isozymes, one can quantitatively estimate the peroxidatic activity of the individual isozymes. This technique can also be used to determine whether one zone of peroxidatic activity is composed of a single molecular species or a collection of isozymes which differ in activity but have very similar electrophoretic mobilities.

Peroxidase is generally thought to be located in the plant cell wall. A fluorimetric peroxidase assay which utilizes homovanillic acid as a hydrogen donor accurately measures the peroxidatic activity of purified cell walls. The reaction product does not bind to cell walls, and because the production of light at a particular wavelength is measured, the presence of particulate fragments of cell walls in the reaction cuvette does not interfere greatly with the assay of peroxidase. Twenty percent of the total peroxidase in horseradish roots is bound to the cell wall. Of the total peroxidase on the cell wall, 93% can be released by washing with 2 M NaCl, and 75% of what remains can be solublized by treatment of the cell walls with a

cellulase preparation. The combination of salt washing and cellulase treatment has allowed the determination of peroxidase isozymes from the cell wall. These techniques solubilize 98.3% of the total peroxidase activity from the horseradish cell wall fraction.

Hydroxyproline has been reported as a constituent amino acid of peroxidase, both in the cytoplasmic peroxidases (Shannon, et al., 1966) and in the peroxidases from the cell wall (Osborne and Ridge, 1971). We have determined that the hydroxyproline found in a commercially purified horseradish peroxidase preparation is glycosidically linked to arabinose. However, a CsCl density gradient of this preparation shows that the buoyant density of peroxidase is distinct from that of the hydroxyproline containing glyopeptide, so the great bulk of peroxidase does not contain any hydroxyproline. There is only one isozyme of peroxidase which has a buoyant density equal to that of the hydroxyproline containing glycopeptide. This is the most anodically migrating peroxidase isozyme, No. 1. Even here there is no clear cut correspondence between peroxidase and hydroxyproline because hydroxyproline can be detected chemically on the CsCl gradient in regions where there is no peroxidase activity. The hydroxyproline in the purified peroxidase preparation probably represents a contaminant. It can therefore be concluded that all the peroxidase isozymes but one (No. 1) definitely do not

contain hydroxyproline. When this isozyme (No. 1) is separated from all the others and shown to have only one band of protein by disc gel electrophoresis, hydroxyproline is found in the amino acid analysis, but this may also represent a contamination.

In a CsCl gradient of the total cytoplasmic fraction of horseradish roots, there is no correspondence at all between peroxidase and hydroxyproline containing macro molecules. It can be calculated from this data that at least 90% of the hydroxyproline in the cell sap of horseradish roots is not associated with peroxidase.

Cellulase treatment of salt-washed horseradish root cell walls solubilizes 57% of the hydroxyproline and 75% of the peroxidase content of the walls. A CsCl gradient of the solubilized material shows no correlation between peroxidase and hydroxyproline containing macro molecules buoyant densities. Two peaks of hydroxyproline are observed, and no isozyme of peroxidase has a buoyant density coincident with that of the hydroxyproline peaks. It can therefore be concluded that the bulk of the hydroxyproline released from cell walls by cellulase treatments is not associated with peroxidase. The buoyant density of peroxidase released from cell walls by cellulase is equal to the buoyant density of peroxidase found in the horseradish root cytoplasm. If the cell wall peroxidases were distinct from cytoplasmic peroxidases by the attachment of

carbohydrate groups such as cell wall fragments or hydroxyproline-arabinosides, this would be reflected in greater densities of the cell wall peroxidases. Since both the cytoplasmic and wall peroxidases have coincident buoyant densities, they probably have the same average chemical composition. This can be taken as further dvidence that the cell wall peroxidases are not associated with hydroxyproline.

An association of hydroxyproline and peroxidase can be seen in the peroxidase which is found in the incubation medium of aerated horseradish root discs. In this medium one isozyme accounts for 83% of the total peroxidase activity. This isozyme can be separated into two fractions by Sephadex G-200 chromatography. The fraction which is of apparently larger size co-elutes with a peak of hydroxyproline containing material. This is not a fortuitous co-elution because the peroxidase has attached to some component to give it the apparently greater size. However, this fraction which is associated with hydroxyproline represents less than 0.1% of the total peroxidase activity in the incubation medium, even though it is associated with 40% of the extracellular hydroxyproline. The two fractions of peroxidase separated by Sephadex G-200 are primarily made up of the same isozyme, therefore they have identical charge characteristics.

One can conclude from these experiments that there is in general no hydroxyproline on horseradish peroxidase, whether it is obtained from the cytoplasm, cell wall, or external to the cell in the incubation medium of aerated root discs. Where an association of hydroxyproline and peroxidase is observed, it is with such a small portion of the total peroxidase activity that it is difficult to imagine it as physiologically significant.

It is important to determine whether the isozymic forms of peroxidase possess the same catalytic mechanism for all substrates, or whether there are important differences in the kinetic behavior of different peroxidase isozymes. If differences do exist, then it is conceivable that the peroxidase isozymes might catalyze different reactions in the cell.

Using a fluorescent assay based on the ability of peroxidase to form biphenyls, the most anodically migrating peroxidase isozyme (No. 1) has a specific activity which is 37.6-fold greater than that of a reference peroxidase isozyme (No. 5). This is the largest difference in activities of peroxidase isozymes reported. A closer look at the peroxidatic activity of these two isozymes using the homovanillic acid fluorescent peroxidase assay shows large differences in the kinetics of these isozymes.

The $K_{app}(H_2O_2)$ for the No. 1 isozyme of peroxidase is 1.5×10^{-5} M H_2O_2 at the optimum pH (8.5) of the homovanillic acid peroxidase assay. The $K_{app}(H_2O_2)$ for this same isozyme (No. 1) is two orders of magnitude greater (1.3×10^{-3} M H_2O_2) when assayed spectrophotometrically at the optimum pH (6.0) of the o-dianisidine peroxidase assay.

Differences in the construction of the active sites of two peroxidase isozymes (Nos. 1 and 5) were observed by assaying the ammonia induced in peroxidase activity at pH values greater than 7.0. When peroxidase is assayed spectrophotometrically with o-dianisidine as the hydrogen donor at high pHs, quite removed from the pH optimum of this reaction (pH 6.0), ammonia increases the rate of the peroxidase reaction. The affect of ammonia on peroxidase activity is striking, and the addition of ammonia to the reaction mixture can serve to increase peroxidase activity as much as 100-fold. The ammonia enhancement effect has an optimum pH of 9.3. Kinetic analysis has determined that ammonia acts in this reaction by binding stoichiometrically at a reactive ligand of peroxidase which is not the site of hydrogen peroxide attachment of heme (Fridovich, 1963). When two peroxidase isozymes were assayed for this effect, a cathodic

isozyme, No. 5, demonstrated the ammonia enhancement, while an anodic isozyme, No. 5, did not.

The lack of the ammonia enhancement effect in one peroxidase isozyme (No. 1) demonstrates an actual structural difference in the active sites of the different horseradish peroxidase isozymes.

One approach to determining a physiological role for peroxidase is to list compounds found in organisms which can only be accounted for by the catalytic action of peroxidase. At least three such classes of compounds exist: the halogenated phenyl compounds such as iodotyrosine which are found exclusively in animal systems; tyrosine condensation products such as dityrosine; and lignin, which is found in the secondary thickenings of cell walls and intercellular spaces of higher plants. We have developed two peroxidase zymogram stains using substrates of physiological significance. One stain measures the peroxidatic lignin formation reaction and uses a lignin precursor, eugenol (2-methoxy-4-allyl phenol), as a substrate. The other stain utilizes tyrosine as a substrate for the peroxidase reaction. Large differences are seen in the isozymes by these two methods and the peroxidase isozymes visualized with an artificial substrate such as benzidine. These results demonstrate that peroxidase isozymes can have different reactivities

with substrates of physiological importance, and these zymogram stains represent the first assay for peroxidase isozymes which are based on a possible physiological function of the enzyme.

Since the horseradish peroxidase system has not been subjected to genetic analysis, it is difficult to determine to what extent the different isozymes represent different gene products or modifications of other isozymes. We have been able to change the electrophoretic mobility of peroxidase isozymes merely by incubating the enzyme in slightly alkaline solutions. These modifications occur with no significant changes in total peroxidase activity. Changes in the peroxidase isozyme pattern can be observed by incubation at 4°C for 38 hours at pH values as low as 7.03. The modified peroxidases all move more anodically than the parent isozymes. They are not, however, the result of CO_2 addition on free amino groups to form carbamates. The transformed peroxidase isozymes are stable, and resist retitration to pH 4.6 and incubation at 45°C for one hour.

The distribution of peroxidase isozymes in all the tissues of the horseradish plant has been determined, as well as the peroxidase isozymes which are found bound to the cell wall. We have also identified the peroxidase isozymes which can be found external to the cell, both in

petiole exudation fluid and in the incubation medium of aerated horseradish root discs.

BIBLIOGRAPHY

BIBLIOGRAPHY

Alexander, N. M., The mechanism of iodination reactions in thyroid glands. Fed. Proc. 19, 173 (1960).

Andersen, S. O., The cross-links in resilin identified as dityrosine and trityrosine. Biochim. Biophys. Acta 93, 213-15 (1964).

Anstine, W., J. V. Jacobsen, J. G. Scandalios, and J. E. Varner, D_2O as a density label of peroxidase in germinating barley embryo. Plant Physiol. 45, 148-52 (1970).

Archer, R., C. Fromageot, and M. Jutisz, Biochim. Biophys. Acta 5, 81 (1950).

Barnett, N. M. and C. R. Curtis, Release of peroxidase and hydroxyproline protein from cell walls by hydrolytic enzymes. Plant Physiol. 45, S-14 (1970).

Bhatia, C. R. and J. P. Nilson, Isoenzyme changes accompanying germination of wheat seeds. Biochem. Gen. 3, 207-14 (1969).

Chrispeels, M. J. and J. E. Varner, Hormonal control of enzyme synthesis: on the mode of action of Gibberellic acid and abscisin. Plant Physiol. 42, 1008-16 (1967).

DeJong, D. W., An investigation of the role of plant peroxidase in cell wall development by the histochemical method. J. Histochem. Cytochem. 15, 335-46 (1967).

DeJong, D. W., E. F. Jansen, and A. C. Olson, Oxidoreductive and hydrolytic enzyme patterns in plant cell suspension cells. Expt. Cell Research 47, 139-56 (1967).

Delincee, H. and B. J. Radola, Thin layer isoelectric focusing on Sephadex layers of horseradish peroxidase. Biochim. Biophys. Acta 200, 404-7 (1970).

Dische, Z., Color reactions of carbohydrates. Methods in Carbohydrate Chemistry 1, 477-514 (1962).

Evans, J. L., Peroxidases from the extreme dwarf tomato plant. Identification, isolation and partial purification. Plant Physiol. 43, 1037-41 (1968).

Evans, J. L. and N. A. Aldridge, The distribution of peroxidase in extreme dwarf and normal tomato. Phytochem. 4, 499-503 (1965).

Felder, Michael R., A comparative genetic, developmental and biochemical study of peroxidases in barley. Ph.D. thesis, University of California at Davis (1970).

Frahn, J. L. and J. A. Mills, Separation of basic amino acids by paper electrophoresis. Anal. Biochem. 23, 546-54 (1968).

Fridovich, Irwin, The stimulation of horseradish peroxidase by nitrogenous ligands. J. Biol. Chem. 238, 3921 (1963).

Fritz, P. J. and K. Bruce Jacobson, Multiple molecular forms of lactate dehydrogenase. Biochemistry 4, 282-9 (1965).

Galston, A. W. and P. J. Davies, Hormonal regulation in higher plants. Science 163, 1288-97 (1969).

Goldstein, D. B., A method for assay of catalase with the oxygen electrode. Analytical Biochem. 24, 431-7 (1968).

Gregory, R. P. F., A rapid assay for peroxidase activity. Biochem. J. 101, 582-3 (1966).

Gross, A. J., and I. W. Sizer, The oxidation of tyramine, tyrosine, and related compounds by peroxidase. J. Biol. Chem. 234, 1611-4 (1959).

Guilbault, George C., P. Brignac, and M. Zimmer, Homovanillic acid as a fluorimetric substrate for oxidative enzymes. Anal. Chem. 40, 190-6 (1968).

Harmey, M. A., Effect of GA on peroxidase levels in barley. Planta 83, 387-89 (1969).

Hess, D., Multiple forms of phenolase and peroxidase in Petunia hybrida. Z. Pflanzen physiol. 56, 295-300 (1967).

Holleman, J., Direct incorporation of hydroxyproline into protein of sycamore incubated at growth-inhibitory levels of hydroxyproline. Proc. Nat. Acad. Sci. 57, 50-4 (1967).

Hunter, R. L. and C. L. Markert, Histochemical demonstration of enzymes separated by zone electrophoresis in starch gels. Science 125, 1294-5 (1957).

Imaseki, H., Induction of peroxidase activity by ethylene in sweet potato. Pl. Physiol. 46, 172-4 (1970).

Jermyn, M. A. and R. Thomas, Multiple components in horseradish peroxidase. Biochem. J. 56, 631-9 (1954).

Kay, E., L. M. Shannon, and J. Y. Lew, Peroxidase isozymes from horseradish roots. II. Catalytic properties. J. Biol. Chem. 242, 2470-3 (1967).

Keilin, D. and E. F. Hartree, Purification of horseradish peroxidase and comparison of its properties with those of catalase and methaemoglobin. Biochem. J. 49, 88-104 (1951).

Kivilaan, A., T. C. Beaman, and R. S. Bandurski, A partial chemical characterization of maize coleoptile cell walls prepared with the aid of a continually renewable filter. Nature 184, 81 (1959).

Kivirikko, K. I. and M. Liesma, A colorimetric method for determination of hydroxyproline in tissue hydrolysates. Scand. J. Clin. Lab. Invest. 11, 128-33 (1959).

Klapper, M. H. and D. P. Hackett, Investigation on the multiple components of commercial horseradish peroxidase. Biochim. Biophys. Acta 96, 272-82 (1965).

Lamport, D. T. A., Oxygen fixation into hydroxyproline of plant cell wall protein. J. Biol. Chem. 238, 1438-40 (1963).

LaBella, F., F. Keeley, S. Vivian, and D. Thornhill, Evidence for dityrosine in elastin. Biochem. Biophys. Res. Comm. 26, 748-53 (1967).

LaBella, F., et al., Formation of insoluble gels and dityrosine by the action of peroxidase on soluble collagens. Biochem. Biophys. Res. Comm. 30, 333-8 (1968).

Lamport, D. T. A., The protein component of primary cell walls. Adv. Bot. Research 2, 151-218 (1965).

Lamport, D. T. A., Hydroxyproline-o-glycosidic linkage of the plant cell wall protein extensin. Nature 216, 1322-4 (1967).

Lamport, D. T. A., The isolation and partial characterization of hydroxyproline-rich glycopeptides obtained by enzymic degradation of primary cell walls. Biochemistry 8, 1955 (1969).

Lamport, D. T. A., Cell wall metabolism. Ann. Rev. Plant Physiol. 21, 235-69 (1970).

Lamport, D. T. A., and D. Miller, Hydroxyproline-arabinosides in the plant kingdom. Manuscript (1971).

LaVee, S., and A. W. Galston, Hormonal control of peroxidase activity in cultured Pelargonium pith. Am. J. Bot. 55, 890-3 (1968).

Ljangrenn, J., Catalytic effect of peroxidases on the iodination of tyrosine in the presence of H_2O_2. Biochim. Biophys. Acta 113, 71-8 (1966).

Macnicol, P. K., Peroxidases of the Alaska pea (Pisium Sativam, L.). Arch. Biochem. Biophys. 117, 347-56 (1966).

Maehley, A. G. and S. Paleus, Acta Chem. Scand. 4, 508 (1950).

Masuda, Y., Role of cell-wall degrading enzymes in cell wall loosening in oat calcoptiles. Planta. 83, 171-84 (1968).

McCune, D. C. and A. W. Galston, Inverse effects of Gibberellin on peroxidase activity and growth in dwarf strains of peas and corn. Plant Physiol. 34, 416 (1959).

Morita, Y. K., K. Shimizu and N. Kada, Studies on phytoperoxidase XX. Peptic hydrolysis of permic-acid oxidized peroxidase of a Japanese radish. Agr. Biol. Chem. 32, 671-7 (1968).

Ockerse, Siegel and Galston, Hormone induced repression of a peroxidase isozyme in plant tissue. Science 151, 452-3 (1966).

Olson, A. C., et al., Tobacco culture media macromolecules-pectic substances, protein and peroxidase. Plant Physiol. 44, 1594-1600 (1969).

Quail, P. H. and J. E. Varner, Combined Gradient-Gel Electrophoresis Procedures for determining Buoyant densities or Sedimentation coefficients of all multiple forms of an enzyme simultaneously. Analytical Biochem. 39, 344-55 (1971).

Racusen, D. and M. Foote, Peroxidase isozymes in bean leaves by preparative disc electrophoresis. Can. J. Bot. 44, 1633-38 (1966).

Ridge, I., and D. Osborne, Hydroxyproline and peroxidases in cell walls of Pisium sativum: regulation by ethylene. J. Experimental Botany 21, 843-56 (1970).

Ridge, I and D. J. Osborne, Role of peroxidase when hydroxyproline-rich protein in plant cell walls is increased by ethylene. Nature, 229, 205-8 (1971).

Saunders, B. C., A. G. Holmes Siedle, and B. P. Stark, Peroxidase--The Properties and Uses of a Versatile Enzyme and Some Related Catalysts. Butterworth, London (1964).

Scandalios, J. G., Tissue-specific isozyme variation in maize. J. Heredity 55, 281-5 (1964).

Scandalios, J. G., Genetic control of multiple molecular forms of enzymes in plants. Biochem. Genet. 3, 37-79 (1969).

Shannon, L. M., E. Kay, and J. Lew, Peroxidase isozymes from horseradish roots. I. Isolation and physical properties. J. Biol. Chem. 241, 2166-72 (1966).

Shannon, L. M., Plant isoenzymes. Ann. Rev. Plant Physiol. 19, 187-210 (1968).

Shannon, L. M., I. Uritani and H. Imaseki, De novo synthesis of peroxidase isozymes in sweet potato slices. Plant Physiol. 47, 493-8 (1971).

Sheehan, J. C. and J. G. Whitney, The synthesis of cis- and trans-3-hydroxyl-L-proline. Two new amino acids from the antibiotic telomycin. J. Amer. Chem. Soc. 85, 3863-65 (1963).

Siegel, B. Z. and A. W. Galston, Biosynthesis of deuterated isoperoxidases in rye plants grown in D_2O. Proc. Nat. Acad. Sci. 56, 1040-2 (1966).

Siegel, B. Z. and A. W. Galston, The isoperoxidases of Pisium sativum. Plant Physiol. 42, 221-6 (1967).

Siegel, S. M., On the biosynthesis of lignins. Physiol. Plant. 6, 134 (1953).

Siegel, S. M., The biochemistry of lignin formation. Physiol. Plant. 8, 20-32 (1955).

Siegel, S. M., Studies on the biosynthesis of lignins. Physiol. Plant. 7, 41-50 (1954).

Siegel, S. M., The biosynthesis of lignin: evidence for celluloses as sites for oxidative polymerization of eugenol. J. Am. Chem. Soc. 78, 1753-5 (1956).

Siegel, S. M., Non-enzymic macromolecules as matrices in biological synthesis: the role of polysaccharides in peroxidase catalyzed lignin polymer formation from eugenol. J. Am. Chem. Soc. 79, 1628-32 (1957).

Stutts, P. and I. Fridovich, A continual spectrophotometric determination of ammonia-producing systems. Anal. Biochem. 8, 70-4 (1964).

Trevelyan, W. E., D. P. Proctor, and J. S. Harrison, Nature 166, 444 (1950).

Upadhya, M. D. and J. Yee, Isoenzyme polymorphism in flowering plants. VII. Isoenzyme variation in tissues of barley seedlings. Phytochemistry 7, 937-43 (1968).

Whitmore, F. W., Effect of Indole acetic acid and hydroxyproline on isoenzymes of peroxidase in wheat coleoptiles. Plant Physiol. 47, 169-71 (1971).

White, A., P. Handler, and E. L. Smith, Principles of Biochemistry, McGraw-Hill, New York (1964).

Wilson, C. M., Quantitative determination of sugars on paper chromatograms. Anal. Chem. 31, 1199 (1959).