MULTIPLE FORMS OF PLANT PHENOLOXIDASE

Thesis for the Degree of Ph. D.

MICHIGAN STATE UNIVERSITY

Spiros Minas Constantinides

1966

This is to certify that the

thesis entitled

MULTIPLE FORMS OF PLANT PHENOLOXIDASE

presented by

Spiros Minas Constantinides

has been accepted towards fulfillment
of the requirements for

Ph. D. degree in Food Science

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Major professor

Date July 19, 1966

O-169

ABSTRACT

MULTIPLE FORMS OF PLANT PHENOLOXIDASE

by Spiros Minas Constantinides

The phenoloxidase (PO) system present in the tissues of mushrooms, potatoes and apples was shown to exhibit the phenomenon of multiple forms. Using an electrophoretic method which employs polyacrylamide gel, distinct multiple forms of PO were obtained.

Cathodical and anodical enzyme forms were separated. These were specific for each species or variety studied. Elution, recovery and other tests ruled out the possibility of artifacts. Substrate specificity was clearly shown to exist. Two tyrosine specific multiple forms were separated. Other substrates showed distinct variations in the mode of reactions with each multiple form. The lag period for a given substrate was different for the different forms of PO. Room temperature up to five hours had no effect on the multiple molecular form pattern, but higher temperatures of up to 60 C caused fragmentation of the dihydroxyphenylalanine (DOPA) specific forms. A group of closely related DOPA specific multiple forms had the unique ability to withstand the temperatures of 70 C for sixty minutes. Sulfite incubated with the enzyme extract caused inactivation of certain forms.

The tyrosine specific multiple forms were little affected by relatively high concentrations of sulfite. Short periods of incubation of the multiple forms with sulfite on the gels followed by washing with water showed no permanent inactivation of the multiple forms. High doses of 2×10^6 rads of γ-irradiation caused very slight inactivation, the pattern of multiple forms being similar to those held for seven hours at 22 C. Urea (4.0 M) inactivated completely the tyrosine specific forms while the DOPA specific forms were gradually inactivated as the urea concentration was increased. Ethylenediamine tetra acetic acid (EDTA) at low concentrations had no effect on the DOPA specific multiple forms, while at extremely low concentration of 0.05% the tyrosine specific bands were inactivated. Trypsin degradation inactivated most of the forms and also gave rise to a new form. Mercaptoethanol and cysteine-HCl completely destroyed all forms. Ascorbic acid had no effect on the multiple forms. Incubation with excess of DOPA caused inactivation of all the multiple forms except for one group. Drastic purification treatments tended to inactivate some of the multiple forms. Fewer multiple forms were separated from commercial mushroom PO than from the Agaricus campestris preparations used in this study.

The PO system seems to be installed in the plant tissues at the very early stages of development. No tissue

specificity of multiple forms was apparent. The intracellular distribution of PO seemed to be specific for each species. In potatoes most of the activity was present in the microsomal and the final supernatant fractions, while in mushrooms and apples most of the activity was in the mitochondrial fractions. Intracellular compartmentalization of the multiple forms of PO was also shown to exist. In mushrooms the tyrosine specific form existed only in the final supernatant, and not in the mitochondria, while the mitochondria exhibited two DOPA specific bands only. Also in apples the pattern of the mitochondrial fraction was different from that of the supernatant.

MULTIPLE FORMS OF PLANT PHENOLOXIDASE

By

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A THESIS

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

DOCTOR OF PHILOSOPHY

Department of Food Science

1966

DEDICATION

To my Mother and my Wife

ACKNOWLEDGMENTS

The author wishes to express his sincere appreciation to Professor Clifford L. Bedford for his guidance and continuous encouragement throughout the course of this work and during the preparation of this manuscript.

The author is also deeply indebted to Professor P. Markakis for his beneficial suggestions on the multiple forms of enzymes, and to Professor J. R. Brunner who provided laboratory facilities and contributed valuable discussions.

Thanks are also extended to the members of the guidance committee, Dr. Geo. Borgstrom, Dr. W. Deal, Dr. L. G. Harmon, Dr. P. Markakis and Dr. S. Schanderl.

Sincere gratitude is expressed to Mr. G. Birk for his assistance in obtaining specimens of mushrooms through the invaluable cooperation of the Great Lakes Mushroom Coop., Warren, Michigan.

The excellent photographic job performed by Mr. P. Coleman of the Experimental Station Photo Laboratory of Michigan State University is greatly appreciated.

The author most gratefully acknowledges the financial assistance for the scholarship granted for the initial part of his studies by the Greek Foundation of State Scholarships

(I.K.Y) and for the financial support by the Michigan State Agricultural Experiment Station.

The patience, understanding and encouragement of the author's wife, Niovi, throughout the course of this graduate program are gratefully acknowledged.

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INTRODUCTION

Although the first observation of enzyme multiplicity was made with lactic dehydrogenase, it is now known to be a widespread, perhaps general phenomenon. More than one hundred enzymes have been shown to exist in unicellular and multicellular species of plants and animals (Furness 1961). Studies over the past seven years have shown that specific types of enzymatic activity may be associated with more than one protein. Differences were found between comparable enzymes in different tissues of the same organism. In addition, a single tissue may yield several enzymes catalyzing the same reaction but having differences in their physical, chemical and kinetic properties (Markert and Moller 1959). At the present time there is no unanimity on the method of identification of a particular form. The multiple molecular forms have been distinguished from one another by electrophoresis, chromatography, salt fractionation ultracentrifugation, immunoelectrophoresis and reaction kinetics. The electrophoretic method is the most commonly employed, resolving macromolecules on the basis of both charge and size (Whipple 1964).

The following terms have been used by different authors for the multiple forms: isoenzymes, isozymes, iso (enzyme's name) as isoamylases, electrophoretic variants,

multiple forms, polymorphs, electrophoretic components, and multimolecular forms. Webb (1964) recommended that multiple molecular forms of an enzyme in a single species should be known as isoenzymes, although this recommendation is not to be interpreted as excluding the use of isozyme if any individual author prefers it.

We do not know how or why enzymatic heterogeneity exists, what advantage it offers to the cell, or how the multiple forms differ chemically or structurally from each other. Multiple enzyme forms could be products of single genes, by modification in the tertiary structure of a gene product or by alterations of a basic protein structure, depending on the site of attachment within the cell.

The relationship of this phenomenon to the one-gene one-polypeptide theory and to the problem of cellular differentiation poses important biological questions which remain to be solved. Their study promises to expand our knowledge in a variety of fields ranging from embryology and the studies of evolution, to physiology and pathology. The study of them has already proved that it has clinical diagnostic application (Kaplan et al. 1960).

Phenoloxidase (PO), classified as an o-diphenol: O_2-oxidoreductase EC 1.10.3.1, by the Commission on Enzymes of the International Union of Biochemistry (1961), and referred to as tyrosinase, polyphenoloxidase, phenoloxidase, phenolase,

catechol oxidase, laccase and cresolase, is widely distributed in plants, invertebrates and higher animals.

Although phenoloxidases are widely distributed throughout the plant kingdom, their role in plant metabolism is not clearly understood. It has been proposed that plant PO acts as a hydroxylating enzyme in vivo, catalyzing hydroxylations which are believed to occur during the biosynthesis of o-diphenolic compounds in the potato tuber (Patil and Zucker 1965).

PO is characterized by two different catalytic activities, both involving molecular oxygen. The oxidation of monohydric phenols such as tyrosine, phenol or p-cresol involving the insertion of a hydroxy group ortho to the one already present is referred to as cresolase activity. The removal of two hydrogen atoms from a dihydric phenol as catechol to yield the corresponding o-quinone is referred to as catecholase activity. Mallegg and Dawson (1949) and Mason 1955a, b) have indicated the reaction as follows:

$$\text{monophenol} + 2e + O_2 \longrightarrow \text{o-diphenol} + O^{=}$$

$$2\ \text{o-diphenol} + O_2 \longrightarrow 2\ \text{o-quinone} + 2\ H_2O$$

However Dressler and Dawson (1960a, b) have indicated that oxidation of monophenol does not proceed via an o-dihydric phenol.

In the past few years data have been presented indicating that plants contain several different phenoloxidases that exhibit unique catalytic and physical properties. However the information is very confusing and the results are contradictory. The present study was therefore undertaken in an attempt to elucidate the nature and properties of the phenoloxidase system from plant tissues and from within the cell structures.

REVIEW OF LITERATURE

In recent years molecular heterogeneity of proteins has often been observed, particularly for enzymes, where the existence of multiple molecular forms has been extensively documented. Markert and Moller (1959) introduced the concept of an isoenzyme, which they defined as one of the molecular forms in which protein may exist with the same enzymatic specificity within a single organism. This term has gained wide popularity and has been used in an operational sense to cover any series of enzymes with roughly similar properties regardless of genetic relationship. In nearly all cases the genetic relationships are not known. A review of the work up to 1961 on multiple forms of different enzymes is presented by Furness (1961).

The first electrophoretic method used to demonstrate the multiplicity of enzyme forms was starch gel electrophoresis (Furness 1961). Later other gel electrophoretic methods, especially polyacrylamide gel (disk), were used (Whipple 1964).

The heterogeneity of lactic dehydrogenase was first tested by Wieland and Pfleiderer (1957). Markert and Appella (1961) investigated the physicochemical properties of two purified isozymes from beef heart lactate dehyrogenases (LDH) and found a remarkable similarity between the two. Markert

(1963) later found that LDH from beef tissue may be resolved electrophoretically into five isozymes, each being a tetramer. These tetramers can be dissociated into 2 monomers by freezing in 1 M NaCl, namely LDH-1 and LDH-5, that are differentiated by charge and aminoacid composition. On thawing, reassociation into functional tetramers occurs. A mixture of equal quantities of the two isozymes after dissociation and reassociation produces all five isozymes in the expected proportions of 1:4:6:4:1. Kaplan and Ciotti (1961) suggested that there has been considerable evolutionary change in the lactic and malic dehydrogenases and that this enzyme change is not without significance and is of importance in the survival and perpetuation of new species.

Laufer (1961) found the existence of isozymes among the hydrolytic dehydrogenating enzymes that occur in cell free blood preparations. He also proposed that the insect hormones act on specific sites of the chromosomes, the isozymes being a particular expression of these developmental interactions. Riggins and Kiser (1964) discovered that urine from tract inflamations gave a different isozyme pattern than normal. Withycombe and Wilkinson (1964) found that spermatozoal extract exhibited greater dehydrogenase activity with 2-oxobutyrate as substrate than with pyruvate as the substrate. Mahy and Rowson (1965) reported that of the five electrophoretically distinct forms normally present, only the slowest migrating LDH-5 is

increased in amount during Riley virus infection. Brody (1964) reported that the individual isozymes of LDH differs not only in electrophoretic mobility but also in chemical composition, Michaelis constant, activity against substrate analogues, reaction rate with coenzyme analogues, immunological characteristics, thermal stability and susceptibility to inactivation by excess substrates and by certain chemical inhibitors. Nine forms of LDH have been reported to be present in various tissues of the brook trout (Goldberg 1965). This was an indication that three polypeptide subunits take part in the lactate dehydrogenase composition of this species. Goldberg also observed that the sperm specific LDH contains polypeptide subunits that differ from those in LDH 1, 2, 3, 4 and 5. The recognition of the molecular heterogeneity of enzymes in organisms has offered a new dimension for studying the genetic control of enzyme synthesis (Vesell 1965). In the case of lactate dehydrogenase five distinct types can be identified in most mammalian and avian tissues by the method of starch electrophoresis.

Katz and Kalow (1965) reported several isozymes of lactate, malate and isocitrate dehydrogenase in human skeletal muscle, heart and liver.

The multiple forms of acid phosphatase found in animal organs are distinct molecular forms. There is probably a genetic basis for the multiple forms of the enzyme since they

appear to be species specific and not organ specific (Moore and Angelletti 1961).

Alcohol dehydrogenases in liver were studied by McKinley-McKee and Moss (1965). They found that horse liver alcohol dehydrogenases are heterogeneous on starch gel electrophoresis and that this heterogeneity is due to coenzyme-enzyme complexes.

Carter et al. (1961) separated five forms of ribonuclease from crystallyzed bovine pancreatic ribonuclease.

Three electrophoretic variants of catalase were found in maize endosperm and these were under genetic control (Scandalios 1965 and Beckman et al. 1964b). Catalases purified from human and rat livers showed significant differences when compared to purified erythrocyte catalases of these species by immunoelectrophoresis (Nishimura et al. 1964).

Starch block zone electrophoresis was used to isolate five electrophoretically distinct active cellulolytic components in the crude extracellular cellulase system of Streptomyces antibioticus (Enger and Sleeper 1965).

Starch gel electrophoresis showed three distinct molecular forms of enolase in different species on Salmonidae (Tsuyuki and Wold 1964).

Augustinsson (1961) studied the multiple forms of esterases in vertebrate blood plasma and found each animal species had its own typical set of plasma esterases. The

pattern of protein and of esterases found in Streptococcus faecalis differed from those in Streptococcus durans. Other investigators studied the substrate specificity and inhibiting effects of esterases (Allen 1961 and Ecobichon 1965). Secchi and Dioguardi (1965) found four esterase fractions in serum and six in the hepatic tissue of man.

Two multiple forms of fumarase isolated from Candida utilis showed marked differences in kinetic properties from that of pork heart fumarase (Hayman and Alberty 1961).

The presence of multiple forms of β-galactosidase were reported by Furth and Robinson (1965) in rat tissues, and in Escherichia coli by Appel and Alpers (1965). In Neurospora crassa two β-galactosidases were found by salt and column fractionation, and they differed markedly in their pH optima, heat susceptibility and affinity for substrates (Lester and Byers 1965).

Kinetic and electrophoretic evidence has indicated the presence of only two forms in human cell cultures of glucose adenosine triphosphate phosfotransferase. In rat liver four distinct electrophoretic forms were found (Katzen et al. 1965).

Three major components of glutamate-aspartate transaminase were found in the heart of pig when analyzed with starch gel (Martinez-Carrion et al. 1965).

Yeast hexokinase has been separated into several

fractions by diethylaminocellulose column chromatography (DEAE) (Kaji et al. 1961). In rat tissues at least four types of hexokinases were distinguished by starch gel electrophoresis and chromatography on DEAE cellulose or calcium hydrozylapatite. The liver contained all four types while the kidney contained three, epidydimal fat pad, skeletal muscle, heart and brain, each contained two. Each type of this family of hexokinases was shown to be different from the other (Katzen and Schimke 1965). Schulze et al. (1965) found hexokinases to be susceptible to proteases.

Tissue specific variants of leucine aminopeptidase have been shown to exist in maize (Scandalios 1964). Four different molecular forms of leucine aminopeptidase were found in maize endosperm by means of starch gel electrophoresis (Beckman et al. 1964a).

Malate dehydrogenase activity is a property of a number of different proteins present in tissues of sea urchins and star fish. These proteins differ in electrophoretic mobility, in rates of reaction with pyridine nucleotide analogues and d-malate, in thermal inactivation, and solubility in ammonium sulfate. Of the five l-malate dehydrogenases present in Arbacia eggs only three are found in very young embryos and four after 12 hours of development (Moore and Villee 1963). Kitto et al. (1966) reported five components of the mitochondrial malate dehydrogenase obtained from chicken heart tissue.

Evidence was presented based on differential extraction procedures, pH dependence studies, activation or inactivation by sodium dodecyl sulfate, and differential temperature inactivation, that the pulp of the banana contains at least three molecular forms of pectinesterase (Hultin and Levine 1963).

McCune (1961) obtained six peroxidase active fractions from corn leaf sheath preparations by starch gel electrophoresis. The four major bands differed in their substrate specificity. Paul and Fottrell (1961) demonstrated the existence of differences in the isozyme pattern between species, while Klapper and Hackett (1964) showed that commercial horseradish peroxidase contains multiple components. Yu and Hampton (1964) reported that pathogens can influence the peroxidase isozyme content of plant tissues. Three protein components with peroxidase activity were separated from Ficus glabrata latex by chromatography on DEAE cellulose at pH 7.0. The peroxidase differed slightly from that of horseradish peroxidase (Kon and Whitaker 1965).

Multiple Forms of Phenoloxidase

In the past few years experiments showed that crude phenoloxidase preparations actually are mixtures of several PO proteins each exhibiting unique catalytic and physical properties.

Mushrooms: Mallette and Dawson (1949) first reported the presence of multiple forms of mushroom tyrosinase. Smith and Krueger (1962) using a column of hydroxylapatite, chromatographically separated the crude extract of mushrooms into a series of purified fractions, that included the classically catecholase and cresolase enzymes as well as other enzyme types. Bouchilloux et al. (1963) obtained four active proteins using a column of hydroxylapatite. Jolley and Mason (1965) concluded that the existence of two unlike subunits, one largely cresolase active and the other catecholase active, combining in several proportions may explain the differences observed among the multiple forms of tyrosinase toward mono and diphenols. They also found that the mushroom isozymes were to a certain degree interconvertible, depending on pH, ionic strength and protein concentration.

Neurospora crassa: Horowitz and Fling (1953) showed the presence of several forms of tyrosinase in N. crassa. Initially they were distinguished on the basis of their heat resistance and later they were shown to differ in their electrophoretic properties (Horowitz et al. 1961). They also suggested that the different forms of tyrosinase were alike functionally but differed in structure since the preparations appeared to have similar substrate specificity, Km values and pH optima. Sussman (1961) found that a thermostable and thermolabile form of tyrosinase existed. Fox and Burnett

(1962) were able to separate the enzyme into three components by continuous flow paper electrophoresis. These components differed in their electrophoretic and immunochemical properties as well as thermostabilities, and interconversion of the three forms was observed. Fling et al. (1963) isolated two forms by diatomaceous earth and found that a rapid association-dissociation between molecular species (monomer and tetramer) of tyrosinase occurred.

Mammalian: Brown and Ward (1958) and Shimao (1962) indicated the presence of multiple forms of tyrosinase. Pomerantz (1963) separated and partially purified two tyrosinases from hamster melanoma. They were distinguished by DEAE cellulose chromatography and starch gel electrophoresis.

Potatoes: The PO system of Kennebec potatoes had two components Patil et al. (1963). Uritani (1963) using starch gel electrophoresis, separated crude extracts of sweet potatoes into three distinct bands, each showing a capacity to catalyze the oxidation of chlorogenic acid.

Broad beans: Robb et al. (1965) showed the presence of four multiple forms of tyrosinase. He was not able to distinguish their heterogeneity by their copper content or substrate specificity.

Tobacco: Sisler and Evans (1958) reported that chlorogenic acid is a better substrate for the crude PO from tobacco than for a comparable preparation from mushrooms.

Apples: Walker (1964) found apple PO to be associated with the mitochondria. Harel et al. (1965) found the enzyme to exist also in the chloroplasts.

Other sources: Two forms of laccase have been purified from the culture medium of Polyporus versicolor using ammonium sulfate precipitation, chromatography on hydroxylapatite and DEAE chromatography (Mosbach 1963). The PO system of Drosophila melanogaster was shown to come from at least four protein components. Two components had tyrosinase activity while the other two had dihydroxyphenylalanine activity (Mitchell and Weber 1965).

Phenoloxidase: Preparation and General Properties

The phenoloxidase system of enzymes is widely distributed in nature, and highly purified PO from microbial, plant and mammalian sources have been studied (Kubowitz 1937, Keilin and Mann 1938, Dawson and Magee 1955, Sussman 1949, Mallette 1950, Joslyn and Ponting 1951, Schwimmer 1953, Mason 1955a and b, Bonner 1957, Kassab 1961, Hayaishi 1962, and Mason 1965).

Yasunobu (1959) tested the substrate specificity of a number of PO from various sources, both plant and animal. He concluded that these enzymes catalyze the oxidation of a wide variety of substrates, but that each individual enzyme tends to catalyze the oxidation of one particular phenol or a particular type of phenolic compound more readily than others.

He also suggested that true tyrosinases are those enzymes which catalyze the oxidation of both mono and diphenols.

Different molecular weights of phenoloxidases have been reported. Dawson and Magee (1955) in their review indicate that as the enzyme occurs in nature it is likely to have a molecular weight of 200,000 to 400,000 and that purification results in fragmentation. Other different molecular weights have been reported by Mallette and Dawson (1949), Yasunobu (1959), Frieden and Ottesen (1959), Krueger (1959) and Kertesz and Zito (1957).

The tyrosinase of N. crassa was found to be a crystallizable copper containing enzyme with a molecular weight of 32,000 - 34,000. It is normally produced during the sexual phase of the life cycle, but not during vegetative growth except when growth is inhibited by starvation or by aminoacid analogues or d-aromatic amino acids (Horowitz et al. 1964). Tyrosinase of N. crassa is synthesized under conditions which are unfavorable for growth and general protein synthesis, i.e. after exhaustion of culture medium (Fox et al. 1963). This implies some difference between the mechanism of tyrosinase synthesis and that of general protein synthesis and it seemed likely that the steps involved are those concerned with the synthesis of the polypeptide chain of the enzyme. The polypeptide as originally released from the sites of synthesis lacks enzymatic activity. It therefore constitutes a

kind of proenzyme (protyrosinase) which requires activation. The presence of one or more additional macromolecular "activating" factors, not tyrosinase itself, apparently is required for protyrosinase activation. Rearrangement of tertiary structures seemed to be responsible for activation, either with or without the splitting off of a terminal peptide segment.

Dressler and Dawson (1960a, b) suggested that the enzymatic sites for phenol and catechol are different, also that the oxidation of a monohydric phenol does not proceed via an o-dihydric phenol.

Aerts and Vercauteren (1964) also reported that the oxidation of a monohydric phenol does proceed via the corresponding o-dihydric phenol and that phenoloxidase bears two types of active centers, one for the cresoloxidase activity and one for the catecholoxidase activity.

Kertesz and Zito (1962) reported that the oxidation of a monohydric phenol by PO in the presence of hydroquinone is preceded by an induction period. The induction period is increased rapidly by increasing the hydroquinone concentration and decreased by the addition of small amounts of catechol. When the phenol or enzyme concentration was increased the induction period decreased and the rate of the oxygen consumption increased. Osaki (1963) stated that prolonged induction periods are to be expected when the concentration

of the substrate such as tyrosine is high. The induction period can be decreased by increasing the enzyme concentration.

Pomerantz (1964, 1966) found that the rate of tritium release as H_2O in the tyrosinase l-Tyrosine-3, 5 T reaction was directly proportional to the rate of hydroxylation. Dihydroxyphenylalanime (DOPA) was the most efficient hydrogen donor for hydroxylation. Tyrosine was found to exhibit an apparent substrate inhibition. Pomerantz suggested that reaction was prevented by the combination of excess tyrosine at the DOPA site and excess DOPA at the tyrosine site. Kean (1964) indicated that monophenols could inhibit the oxidation of o-diphenols by occupying sites on the enzyme that would be available to oxygen or to o-diphenols.

Nelson and Dawson (1944) found that phenol inhibited the action of tyrosinase on catechol. Kendal (1949) also reported that phenol competitively inhibits the oxidation of catechol. Karkhanis and Frieden (1961) found a protein inhibitor in the crude mushroom tyrosinase preparations. Lerner (1953) has shown that tyrosinase was inhibited by compounds which complex with copper, by analogues which competitively inhibit its action and by metals that compete with copper. Mayberry and Mallette (1962) reported that excess catechol inhibits its own oxidation by a competitive process, thus accounting for an observed optimum in substrate concentration.

Added phenol, although itself a substrate, inhibits the enzymatic oxidation of catechol by a mixture of two processes, competitive and non-competitive.

Walker (1964) reported that diethylthiocarbamate, and dimercatoethanol were powerful inhibitors of the chlorogenic acid oxidation. He also reported that caffeic acid was formed when apple PO was incubated with p-coumaric acid for long periods of time (4 hours). The thermal inactivation of apple PO was investigated by Walker (1964) who found that inactivation first became marked at 70 C and that activity was destroyed at 80 C.

Sulfur dioxide is known to inhibit the browning caused by PO (Ponting 1960). Sulfite was shown to prevent browning in the systems by combining with the enzymatically produced o-quinones and stopping their condensation to melanins (Embs and Markakis 1965).

Lyr and Luthardt (1965) found that by adding metabolic inhibitors to the culture fluid of fungi a strong induction could be obtained and the synthesis of a new tyrosinase protein occurred. This mode of induction of tyrosinase is probably not restricted to fungi but may also occur in higher plants where tyrosine activity often increases in the region of sublethal injuries as a part of a defense mechanism.

Intracellular Distribution of Enzymes and Their Multiple Forms

Much has been written about the intracellular distribution

of enzymes and their isolation. Some of the early classical papers are those of Hogeboom et al. (1947), Schneider and Hogeboom (1950), deDuve et al. (1955), Schneider and Hogeboom (1956), and Novikoff and Podber (1957). Many reviews have also been published.

Allen (1964) found two groups of six isozymes in the esterases of Tetrahymena. One isozyme appeared to be localized in the microsomes, another in somewhat larger particles, while the remaining isozymes appeared to be localized in fractions that sediment with low centrifugal force. Hsu and Tappel (1964) found six intracellular hydrolases in the rat intestinal mucosa that were associated with lysosomes. Metzger et al. (1965) reported that hepatic glucose dehydrogenase exists predominantly in the heavy microsomal fraction of homogenates. Kun and Volfin (1966) reported kinetic differences between malate dehydrogenase activities of cytoplasmic and mitochondrial extracts of the same tissue, and marked differences in catalytic activities between homologous enzyme preparations obtained from different tissues.

Very little work has been done on the intracellular distribution of PO or its multiple forms within the cell particles. Goldfish tyrosinase was found to exist both in the particulate and in the soluble fraction of skin homgenate (Kim and Chen 1962). Catechol oxidases were shown to exist in several subcellular fractions of apples (Harel et al.

1965). Starch gel electrophoresis separated three components from the choloroplasts of apples and one component from the mitochondria. In the ink gland of the squid, tyrosinase was found to be present in the particulate form within the mitochondria (Vogel and McGregor 1964). It was suggested that this enzyme is synthesized or assembled in some measure in this site, remains latent, and is transmitted through the mitochondrial membrane into the cytoplasm to participate in melanogenesis. Chloroplasts of sugar beets have PO activity (Mayer 1965).

MATERIALS AND METHODS

Enzyme Source

The following plant materials were used to determine enzyme activity, the presence of multiple forms and the intracellular distribution of the enzyme system and its multiple forms.

Apples: (Pyrus malus). The following varieties at harvest maturity were used: Cortland, Jonathan, Northern Spy, Red and Golden Delicious, and Rhode Island Greening. In addition representative samples of Northern Spy at 30, 46, 63, 87, and 130 days after full bloom were used to determine the effect of maturity on the multiple form pattern.

Mushrooms: Agaricus compestris, Agaricus placomyces, Amanita rubenscens, Coprinus comatus, Coprinus micaceus, Suillus grevillei, and Tricholoma venenata.

Potatoes: (Solanum tuberosum). The following varieties were used: Aranac, Katahdin, Kennebec, Ontario, Rural Russett and Sebago.

Representative samples used for enzyme preparations and the determination of multiple forms were packaged in moisture vapor proof polyethylene bags, frozen and stored at -22 C until used. For the determination of the intracellular distribution of the enzyme, the samples were stored at 2 C. All samples were prepared for analysis in a 2 C room.

Separation of Multiple Forms

The least drastic procedures were used to homogenize the tissue and to retain the integrity of the different multiple forms present in the plant tissues.

One part by weight of plant material was homogenized by hand in an all glass tissue grinder (Kontes Glass Corp.) with three to six parts of 0.25M sucrose in 0.05 M phosfate buffer at pH 7.0. The homogenate was immediately centrifuged at 20,000 X g for 20 minutes in a superspeed Serval Angle Centrifuge SS-1, and the supernatant recentrifuged at 100,000 X g for 2 hours (Beckman, Ultracentrifuge, Preparative, Model L-2). The final supernatant was used for electrophoresis.

The method of Davis (1964) and Ornstein (1964) developed for serum proteins using polyacrylamide gel was modified for the separation of the multiple forms of phenoloxidase.

Stock solutions for cathodical proteins:*

A. 1 N HCl 48 ml, TRIS (Tris hydroxy methyl aminomethane) 36.3 gr, TEMED (N, N, N, N', Tetramethylenediamine) 0.23 ml, and H_2O to make 100 ml (pH 8.8 - 9.0).

B. 1 N HCl 48 ml, TRIS 5.98 gr, TEMED 0.46 ml, and H_2O to make 100 ml (pH 6.6 - 6.8).

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*All regents used were Eastman Chemicals products, Rochester 3, N. Y.

C. Acrylamide 60.0 gr, BIS (N. N-Methylenebiscrylamide monomer) 0.4 gr, and H_2O to make 135 ml.

D. Acrylamide 10 gr, BIS 2.5 gr, and H_2O to make 100 ml.

E. Riboflavin 4.0 mg and H_2O to make 100 ml.

F. Catalyst: Ammonium persulfate 0.14 gr and H_2O to make 100 ml.

G. Buffer (dilute to 1/10): Tris 6.0 gr, Glycine 28.8 gr, and H_2O to make 1 liter (pH 8.3).

H. Protein stain: Aniline black 1 gr and 7% acetic acid to make 200 ml.

I. Tracking dye: 0.005% bromphenol blue solution.

Working solutions:

Lower gel: 7% A= 1.0 part, C= 1.4 parts, and H_2O = 2.1 parts.
8% A= 1.0 " C= 1.6 " H_2O = 1.9 " .

In order to form gel the lower gel is combined with the catalyst F 1:1.

Upper gel: B= 1 part, D= 2 parts, E= 1 part, and H_2O = 2 parts.

Stock solutions for anodical proteins: (pH 4.3)*

A. 1N KOH 48 ml, Glacial acetic acid 17.2 ml. TEMED 4.0 ml, and H_2O to make 100 ml (pH 4.3).

B. 1N KOH 48 ml, Glacial acetic acid 2.87 ml, TEMED 0.46 ml and H_2O to make 100 ml (pH 6.7).

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*All reagents used were Eastman Chemicals products, Rochester 3, N. Y.

F. Catalyst: Ammonium persulfate 0.28 gr and H_2) to make 100 ml.

G. Buffer (dilute to 1/10): Beta alanine 31.2 gr. Glacial acetic acid 8 ml, and H_2O to make 1000 ml (pH 5.0).

The rest of the stock solutions and working solutions were identical to the cathodical proteins stocks solutions.

The enzyme preparation was not incorporated in the sample gel, but was layered directly on the top of the already polymerized upper gel. About 0.4 ml of enzyme preparation was used with 2% sucrose added to increase the specific gravity to facilitate layering and to prevent diffusion into the upper liquid. The gel tubes used were 3" x 0.5 mm o. d. From bottom to top, 2 inches of lower gel was introduced, 3/8 inches upper gel, and 6/8 inch length of tube was left for the sample to be introduced.

The current used for the inactive protein separation was 5 milliamperes (MA) per tube. For the separation of the mushroom multiple forms 2 1/4 MA per tube was found to be optimum, for the potatoes 4 MA and for the apples 3 MA per tube were used. Higher currents resulted in band distortions. With mushroom and apple multiple forms the front was allowed to migrate 1 inch toward the cathode, but with potatoes the migration was extended to 1 3/4 inch. Total time of the "run" was 1 1/2 hours.

Preliminary studies were made to determine the optimum concentration of substrate for color development of the multiple forms. A concentration of 1.5×10^{-3} M for all substrates used gave good resolution. Higher concentrations resulted in a dark background. The gels were introduced in the substrate solution and left there until multiple form color bands developed. Ethyl alcohol facilitated the development of the bands. The gels were finally stored in 30% ethyl alcohol. Inactive proteins were stained with stock solution H, according to Davis (1964). The gel was destained by repeated washings with 7% acetic acid. Electrophoresis was carried out at room temperature (22 - 24 C).

Some of the preparations such as those from mushrooms seemed to have slow moving components as well as very fast components. To get the whole pattern within the limits of the gel tube with highest possible resolution and sharpness and to be able to separate some components according to size only, "two-phase or multiphase" gel electrophoresis was introduced. The gel tube was divided into two or more portions, each portion having different concentrations of acrylamide. Usually the concentration ran from low to high (5% - 10%) anode to cathode, from top to bottom of the gel tube. The length of each gel concentration was based on the size of the molecules to be separated.

For recovery studies, the portion of the gel incorporating the multiple forms or form to be recovered was cut off from the rest of the gel with a sharp razor blade and cut into pieces in a small beaker containing 0.5 to 1.0 ml 0.05 M phosphate buffer pH 7.0. After allowing the gel to stand for 30 minutes at room temperature it was frozen. When the gel was needed it was thawed and the drip with 2% sucrose added was pipetted on to the polymerized upper gel of the electrophoretic tube and electrophoresis was started.

Studies made using the method of Fling et al. (1963) involving acetone precipitation and column chromatography were found unsatisfactory because of destruction of enzyme activity and the failure to resolve the enzyme into its possible multiple forms. Paper electrophoresis using the Spinco Durrum Type cell, continuous flow paper electrophoresis (Model CP Spinco) and starch gell electrophoresis did not give good separations of the multiple forms of PO.

Intracellular Distribution of Phenoloxidase

One part of weight of the plant sample was homogenized in an all glass tissue grinder with three to six parts of 0.25M sucrose. The homogenate was centrifuged at 300 x g in a clinical centrifuge for ten minutes, the supernatant removed, the precipitate resuspended twice in 0.25M sucrose and recentrifuged. The supernatants were combined and centrifuged at 300 x g for ten minutes to remove the cell debris. The

supernatant was centrifuged at 10,000 X g for 20 minutes to obtain the mitochondrial fraction. The mitochondrial fractions were centrifuged twice in 0.25 M sucrose. The combined mitochondrial supernatants were centrifuged at 100,000 X g for 2 hours to obtain the microsomal fraction and the final supernatant (Fig. 1). Enzyme activity and protein of the fractions was determined immediately.

Extraction of Mitochondrial Phenoloxidase

The mitochondrial fraction was suspended in a solution of 0.1% Triton X - 100 (Rohm and Haas), in 0.25M phosphate buffer Ph 7.0. The suspension was blended in a Waring blender for 10 minutes, allowed to stand for 30 minutes and centrifuged at 100,000 X g for 2 hours.

Protein Determination

The Lowry method was used (Lowry et al. 1951) with crystalline bovine serum albumin used as the standard.

Enzyme Activity Determinations

Phenoloxidase activity was determined by measuring the rate of oxidation of D L DOPA to dopachrome at 475 mμ. Reactions were carried out in Beckman DU spectrophotometric cells 1 cm diameter at room temperature. The following were introduced in the cell: 3 ml of buffered substrate (0 .1 M phosphate at pH 6.0), 0.00 - 0.15 ml H_2O, and 0.05 - 0.20 ml

enzyme solution. The mixture (3.2 ml) consisted after dilution of 1.41×10^{-2}M DOPA. The enzyme solution was added with plastic plunger. Absorbancy readings were made at 30 second intervals for 2 minutes after introduction of the enzyme. Under these conditions the initial rate of change of absorbancy is an adequate measure of enzyme concentration. Enzyme unit = $\frac{\text{Absorbancy}}{\text{Minutes}} \times 10^{-3}$. Range of absorbancy units used were from 20×10^{-3} to 80×10^{-3}. Specific activity was expressed as units of enzyme per milligram of protein.

RESULTS AND DISCUSSION

Multiple Forms of Phenoloxidase

Polyacrylamide gel electrophoresis was first successfully used by Davis and Ornstein for the separation of serum proteins. These gels are thermostable, transparent, strong, relatively inert chemically, and non-ionic. They can be prepared with a large range of average pore size as indicated by Davis (1964) and Ornstein (1964).

The concentration of the polyacrylamide gel played a very important role in the resolution. Different concentrations of gel were used ranging from 5% up to 10%. With mushrooms, gel concentrations of 6%, 7%, 8%, and 9% were used (Fig. 2). The multiple form pattern of the mushroom PO consisted of three major groups from the anode (top) to the cathode (bottom) of the gel tube. These will be referred to as A, B, and C groups, respectively. With the 6% gel A=2 bands, B=3 and C=1; with 7% gel A=2, B=3, and C=2; with 8% gel A=2, B=3, and C=4; with 9% gel A=2, B=2 and C=3.

The inactive protein showed a different separation pattern depending on the gel concentration (Fig. 3).

Apple and potato PO was best resolved using 8% gel (Fig. 4).

Anodical multiple forms of PO were also found. Four weak bands of activity were evident migrating toward the anode at pH 4.3 (Fig. 5).

Using the two-phase electrophoresis with 7% and 8% gel a new pattern was obtained where the single band of group C (last band toward the cathode) could be split into four bands, indicating the molecular sieving effect of the gel (Fig. 6). Elution of the four bands from the 8% gel and re-running on 7% and 8% gels, resulted in the appearance of a single band on the 7% and the same four bands on the 8% gel.

The results of these studies showed that using different gel strengths and obtaining different pore sizes, permitted the separation of protein molecules that differed in size and charge, including the multiple forms of enzymes.

The current played an important role in obtaining good band separations. A current of 1.25 MA per tube was found to be optimum for mushroom PO (Fig. 7).

The different multiple forms could not be easily liberated from within the pores of the gel. Only 65% of the total activity could be eluted. Repeated separation and elution showed no change in the multiple form pattern or position (Fig. 8). This would indicate that the multiple forms separated by polyacrylamide electrophoresis cannot be considered artifacts of preparation.

To eliminate the possibility of enzyme adsorption on

inactive proteins, protein from potato or apple was mixed with the mushroom enzyme extract. The multiple forms were developed. The pattern obtained was identical to the control. Inactive proteins or other substances present did not bring rise to pseudo-multiple forms.

The pattern of multiple molecular forms was the same whether the enzyme extract was sonicated before electrophoresis, frozen and thawed rapidly and slowly many times, or run on polyacrylamide repeatedly.

Examination of several fruit tissues showed that all tissues have the same pattern of PO multiplicity. The skin, the flesh and the core of apples were tested. The cap and the stalk of mushrooms were examined for multiple forms. In all cases no difference was evident. In the apple the core seemed to have the highest concentration of activity.

During the development of the apple and mushroom the PO multiple form pattern was common throughout all the stages of development. Apples at different stages of development ranging from thirty days after full bloom to one hundred and thirty days were used. The PO system of enzymes seems to be installed in all the fruit tissues at the very early stages of fruit setting.

Substrate Specificy of the Multiple Forms

The mushroom PO system (A. campestris) clearly exhibited substrate specificity. With 7% gel, DOPA, catechol, phenol,

p-cresol, catechine, dopamine, chlorogenic acid, caffeic acid, and p-coumaric acid reacted with all seven bands, while only two bands showed tyrosine activity (Fig. 9 and 10). The color development was characteristic for each substrate and was the same for each reacting band. The intensity of color development showed considerable differences between the various bands (Table I).

Different lag periods of activity for each multiple form were observed. These lag periods depended on the substrate used, the species, and the particular multiple form. For A. campestris, catechol gave the fastest reaction for almost all the bands. The DOPA specific multiple forms of A. placomyces developed faster than the DOPA specific forms of T. venenata. In the case of A. campestris and using DOPA as a substrate band No. 2 of group B, developed first, then the following bands appeared in order: band 2 of group A, band 1 of group B, band 3 of group B, band 1 of group A and finally after twenty minutes band 1 of group C.

The existence of different lag periods showed that the different multiple forms have different affinity for the substrate, or were present at different concentrations. The periodical initiation of color development could bring about irregular reaction rates over a period of time, so initial velocities would not be enough to determine the true activity of all the PO system. Reaction kinetics of each multiple

Table I.--Substrate specificity of multiple forms of mushroom.

Group	Band	DOPA	Tyrosine	Catechol	Catechine	Cafeic Acid	Chlorogenic Acid
A	1	intense	none	light	intense	intense	light
	2	"	"	"	"	"	"
B	1	intense	none	intense	light	faint	faint
	2	"	intense	"	intense	intense	light
	3	"	"	"	"	"	"
	4	"	none	"	faint	light	faint
C	1	intense	none	light	intense	intense	intense
Color		dark brown	Black	Red-brown	Orange	Blue-green	Light yellow

form should be studied individually. The same could be true for inhibition studies.

The mushroom genera and species showed their own individual pattern of separation and in all instances the forms were also substrate specific (Fig. 11). In potatoes differences in the enzyme multiplicity pattern developed with DOPA were evident between the varieties of Aranac, Kennebec, Rural Russett, Sebago, Katahdim and Ontario. The apple varieties (Fig. 12) showed a common major migrating band toward the cathode and two to four other slow moving bands. The multiple form pattern was different for each variety, based on color development with DOPA and Catechol. Sugar beets and Freestone peaches both had at least three bands of DOPA activity. No detailed study was made on them.

The electrophoretic pattern of the inactive protein was different for each variety, species or genus studied in all the above plant sources.

These results led to the conclusion that each multiple form was an entity of its own, with specific characteristics concerning affinity and general behavior toward the substrate.

Effects of Various Treatments on the Multiple Form Pattern of Mushroom Phenoloxidase

Acetone

The classical acetone powder technique has been used

for the study of many proteins and the preparations of enzymes. As acetone is capable of dissolving certain proteins and of denaturing others, although to what extent has not been determined (Keller and Block 1960), it would seem desirable to determine whether acetone purification of the enzyme would alter the pattern of the multiple forms. An acetone powder preparation seemed to destroy the activity of bands 1 of group A and bands 1 and 2 of group B (Fig. 13).

The preparation of commercial mushroom PO (A. campestris) probably involving long and exhausting steps of purification, apparently resulted in the destruction or loss of some of the multiple forms (Fig. 14).

Temperature

Electrophoretic "runs" on mushroom samples stored for 2, 8, 20, and 72 hours at 5 C, showed no change in the polymorphic pattern as indicated in Fig. 15. Samples stored at room temperature (22 C) showed significant changes after 8 hours (Fig. 16). All forms withstood 40 C for 3 minutes as shown in Fig. 17, but after 60 minutes some of the forms were destroyed and others were broken into smaller distinct fragments having DOPA activity as seen in Fig. 17. Similar results were obtained at 50 C and 60 C (Figs. 17, 18). Tyrosine activity showed no fragmentation, but its activity decreased at temperatures of 50 and 60 C. Group C bands were stable for 60 minutes at 70 C (Fig. 18). All forms were

inactivated after 1 minute at 100 C.

The fragmentation occurring at high temperature could lead to the assumption that multiple forms have more than one site for activity, or that one site of activity exists, but the non-active moiety is fragmented causing different sized macromolecules to appear. The unique property of band C to withstand the temperature of 70 C for 60 minutes suggests extreme stability of the active site or sites of this molecule and probably a different configuration.

Sulfite

The gels, after the separation of the multiple forms, were removed from the tubes and incubated for 10 minutes in sodium bisulfite solutions containing 20, 100, 250, 500, 1000 ppm SO_2. The gels were then removed, allowed to drain for 20 seconds, and placed into a 1.5×10^{-3}M DOPA solution. Concentrations of 20 and 100 ppm SO_2 had very little effect on the multiple form pattern. There was a progressive decrease in color development with increased concentration of SO_2 and at 1000 ppm only slight activity was observed in group C (Fig. 19). Doubling the DOPA concentration showed activity in all except two bands (Fig. 20). Thus studying the effect of SO_2 on enzyme activity, it is essential that the substrate concentration be considered as it plays an important role in showing the presence or absence of enzyme activity (Fig. 21).

Gels were also thoroughly rinsed with fresh water for at least twenty minutes after treatment with 250 and 500 ppm SO_2 solution to remove free SO_2. They were then treated with DOPA. The washed gels showed enzyme activity while the unwashed gels showed little or no activity (Fig. 22).

The PO enzyme system was not inactivated when the original enzyme extract was incubated with concentrations of SO_2 in the range of 20 to 500 ppm for 5 hours. However, at 1000 and 2000 ppm the DOPA active bands 1 and 2 of group B were inactivated and at 4000 ppm only band 3 of group B was still active (Fig. 23). The tyrosine specific band was still active after exposure to 1000 ppm (Fig. 24).

These results indicated that sulfite apparently interfered with the DOPA specific site of the enzyme but did not interfere strongly with the tyrosine specific site.

The degree of color formation depended upon the amount of substrate present. These results supported the theory of Embs and Markakis (1965) that no color or melanin formation occurred when sufficient sulfite was present to react with the non-colored intermediate products of o-diphenols (pyrocatechol, chlorogenic acid and caffeic acid).

Higher concentrations of SO_2 are necessary to inactivate the multiple forms when the enzyme system is in the extract form than in the separated form on the gel. Foreign materials in the extract may bind with the SO_2 so that the

total free SO_2 decreases. Bedford and Mayak (1965) have shown that a certain percentage of the SO_2 is actually not recovered from the cherry extract to which it was added.

Irradiation

Irradiation at 250 kilorads from a source of 10,000 curies of Cobalt 60, resulted in the inactivation of the multiple forms of group A. Two thousand kilorads were needed to inactivate band 1 of group B. The other multiple forms were not inactivated, however there was an indication of a slight reduction in their activity as the amount of irradiation was increased. The effect of irradiation was similar to that obtained during the storage of the enzyme at 22 C (Fig. 16).

Ethylene-Diamine Tetra Acetic Acid (EDTA)

EDTA apparently acts exclusively on the tyrosine bands as seen in Fig. 25. A concentration of 0.05% EDTA inhibited tyrosine activity, whereas 4.0% EDTA did not inactivate the same band with DOPA activity. Increasing the EDTA concentration from 0.01% up to 4.0% caused the DOPA specific multiple forms to be inactivated differentially (Fig. 26).

Copper could be the very important moiety for the enzyme active site function. It may affect the tertiary structure of the multiple forms of the enzyme as far as the stereochemistry of the active sites of the molecule is concerned, and the removal or binding of copper may cause a

change in tertiary structure which no longer favors enzyme activity. The copper may also bind the substrate forming a complex.

The DOPA specific forms were not inactivated by concentration of less than 4.0% EDTA. It could be postulated that the copper is deep within the three dimensional configuration of the protein or is tightly bound to the protein of the DOPA specific multiple forms. The absence of competition between the chelating agent and DOPA could also imply that the metal is not primarily engaged in binding any of the components to the enzyme.

Urea

The enzyme extract was incubated for six hours with urea 1.5 M, 4.0 M, 6.0 M and 8.0 M all at pH 7.0. After development of the multiple forms with DOPA, total inactivation of most of the bands occurred at 1.5 M urea except for band 3 of group B. Complete inactivation of this band occurred at 8.0 M urea (Fig. 27). However 4.0 M urea inactivated completely the tyrosine specific bands as may be seen in Fig. 28.

The fact that urea affected the multiple forms preferentially may suggest that the active site of the enzyme is different in each form. Unfolding the molecule had a greater effect on the tyrosine specific forms than on the DOPA specific forms of group B. The tertiary structure may contribute exclusively to the tyrosine specific site on the molecule.

The hypothesis that the bands are distinct species and not artifacts arising one from the other is strengthened by the fact that when treated with denaturing agents no interconversion occurred. This finding contradicts the results of Jolley and Mason (1965) where interconversion of PO was observed in mushrooms.

Sodium Chloride

Different concentrations of NaCL, o.1%, 0.5%, 1.0% and 2.0% were incubated with the enzyme extract for six hours. At the concentration of 1.0% or higher all forms were inactivated except for band No. 3 of group B, and also the same band with tyrosine specificity remained active.

Effect of DOPA on the Tyrosine Specificity and Vice Versa

The gels after multiple form separation were incubated for 15 minutes with DOPA or Tyrosine. The gels were then removed and allowed to drain for 5 seconds. The gel treated with DOPA was then treated with tyrosine and the gel treated with tyrosine was then treated with DOPA. There was no effect of one substrate on the multiple form pattern of the other. Active sites apparently are not affected by the presence of different substrates.

Incubation with Excess DOPA

The enzyme was incubated with DOPA for 5 hours. The multiple form pattern showed that the group C band remained

very active while 2 bands of group B still had weak activity. It is assumed that all products formed must competitively inhibit the enzymes that produce them. Monod and Jacob (1961) stated that, "the products of an enzyme necessarily are analogues of the substrate, and competitive inhibition is expected in any case, whether it is physiologically significant or not depends on the specific construction of the enzyme site."

The resitance of the fast moving multiple form group C to the inhibitory effects of excess products due to the reaction with excess DOPA may indicate that one of the biological roles of these multiple forms is to maintain activity in the presence of excess of products, a kind of defense mechanism against product inhibition. What role this may play in cell metabolism is still to be determined.

Trypsin Digestion

Trypsin (2 X crystallized, Nutritional Biochemicals), 1:50 w/v at pH 8.0 was incubated for 10 hours with the enzyme extract. Electrophoresis showed that only band 3 of group B still had DOPA activity. However a new slow moving band appeared with DOPA activity at a new position. Arginine and lysine seem to be indispensable for the active sites of most of the PO multiple forms. They may take part directly in the active site or indirectly as maintaining the tertiary structure of the protein on which the active site may depend.

Other Treatments

The inactivation of all DOPA and tyrosine bands was complete at even extremely low concentrations of 0.001 M mercaptoethanol. Sulfur may be involved directly or indirectly in the formation of the active sites.

The multiple form pattern was not affected by 2% concentration of ascorbic acid.

Intracellular Distribution of Phenoloxidase

The differential centrifugation scheme used to determine the possibility of intracellular localization of PO activity is shown in Fig. 1.

The first indication of intracellular localization of the PO enzyme suggested that most of the enzyme activity was found in the mitrochondria and microsomal fractions as well as in the supernatant of all species and varieties (Fig. 29).

In potatoes, the microsomal fraction and the final supernatant contained 40% and 46%, respectively, of the total activity. The specific activity of the microsomal fraction was about 15 times that of the supernatant. From a balance scheme of activity and inactive protein the recovery of both was 90-95%. In mushrooms the mitochondrial fraction contained 37% and the supernatant 50% of the total enzyme activity, with the mitochondrial fractions having the highest specific activity, about four times that of the supernatant. Most of the

total activity was found in the mitochondrial fractions of the apples. The specific activity was ten times that of the supernatant, which only had 15% of the total activity. All varieties of apples and potatoes tested had the same intracellular distribution pattern.

Intracellular Compartmentalization of the Multiple Forms of Phenoloxidase

Multiple forms of phenoloxidase have been found to exist in different parts of the cell having different characteristics. All members of the same species or varieties exhibited the same characteristics.

Mushrooms: Fig. 30 depicts the difference in patterns of multiple forms between the final supernatant, mitochondrial solubilized enzyme. Tyrosine specificity seems to belong exclusively to the final supernatant. The soluble enzyme of the mitochondria has two DOPA specific band of Group B and two other weak bands.

Apples: The final supernatant of the apple (Red Delicious) showed three bands, while the mitochondrial soluble enzyme fraction had four bands.

One approach that will lead to understanding the function of an enzyme is to determine precisely the location in the living system in which the enzyme operates. Literature resulting from this work has provided us with information about the topographical distribution of PO in some plant tissues, but remarkably little concerning their function.

The distribution pattern of phenoloxidase activity within the cell was found to be a varietal characteristic. All apple varieties tested had the same distribution pattern; the same was true with potatoes and mushrooms. Also the distribution of the multiple molecular forms was a characteristic belonging to the species or variety. The enzyme was shown to be a particulate as well as a supernatant enzyme. Various multiple forms were situated at different sites within the cell. Such topographical heterogeneity might be expected to have important implications in terms of the activity of the enzymes within the cell. A different situation could result in the exposure of an enzyme to different microenvironmental influences that would be expected to affect its function. Also specific localization of the enzyme could result in channeling biochemical reaction sequences. Indeed a great deal of present day cytological research demonstrates the restrictions of enzymatic capacities to this or that cell structure. The specific situation of macromolecules and the importance of this, for the completion of a biochemical reaction sequence (electron transport and oxidative phosphorylation) is very important. The mitochondria of all plant tissues tested did contain large amounts of PO. The intracellular studies reported here indicate that one of the sites of activity, specifically that of tyrosinase, is restricted to a specific cellular region, in this case the final supernatant. Restriction of a particular member or members to a

specific cellular region could lead to the physiological importance of multiplicity of forms. It is possible that many of the multiple forms of other enzymes separable by electrophoresis may represent enzymes with specific cellular localizations.

The multiple forms found to be located in different parts of the cell should function under different physical or chemical conditions and they should be serving different functions in vivo. This suggests that multiple forms of these cases represent components of alternative metabolic pathways some of which are always functioning and others operative only during specific active phases of development. They could be subject to different feedback control which would have the effect of maintaining useful concentration of enzymatic activity to provide products for two quite distinct metabolic pathways. Recently various multiple forms have been demonstrated to be subject to feedback control by compounds in divergent metabolic pathways (Stadtman et al. 1961). The multiple forms associated with the microsomes may represent the molecular forms of the newly synthesized PO and other multiple forms may be derived from this form.

The phenomenon of enzyme compartmentalization is evident. Enzymes cannot exist in a disorganized manner within the cell. Since they have a role to perform, they should exist at the right place and at the right time to help coordinate the complex system of life within the cell.

CONCLUSIONS

The excellent resolution of disk or polyacrylamide electrophoresis was employed for the first time in the investigations of the phenoloxidase system for multiple forms. The fourteen multiple forms of PO found in the common mushroom, the eleven found in the potato variety Rural Russett and the three forms in the Golden Delicious apple may not be the only forms present. The stability and reproducibility of the enzyme pattern of PO suggested that this multiplicity is not the result of random changes or experimental manipulations, but represents the intracellular state of the enzyme system. The present study shows that the phenomenon of multiplicity of forms is of considerable value in phylogenetic, taxonomic and genetic studies. Comparative enzyme structure also appears to be of importance in classification studies and in evaluation of changes associated with evolution. Future studies on some phenoloxidases may help us ascertain which part of the enzyme molecule undergoes change during evolution.

The multiple forms of PO from mushrooms exhibited the phenomenon of substrate specificity. Of the fourteen DOPA specific bands only two of these were active toward tyrosine, while the rest of the bands showed different reactions toward different phenolic substrates.

The different multiple forms showed different degrees of tolerance toward temperature, sulfite, γ - irradiation, urea, EDTA, NaCL, and trypsin degradation.

A temperature of 60 C caused fragmentation of the DOPA specific forms while the tyrosine specific forms remained intact, with some loss of activity. Two different molecules, one DOPA specific and the other tyrosine specific are postulated, although both have similar electrophoretic properties. The DOPA specific molecule may have many active sites, or it may have only one active site and high temperatures may tend to break this molecule at different points thus changing the original electrophoretic properties of the molecule. The high degree of heat tolerance which one group of multiple forms showed points out the importance of blanching to accomplish enzyme inactivation in the area of food processing.

The inhibition by sulfite on the multiple forms was different for each form, and the tyrosine specific multiple forms withstood sulfite inhibition while DOPA specific forms did not. Sulfite probably blocks the active sites of the forms thus causing inactivation. It is speculated that sulfite may be tied up by different substances present in the enzyme extract thereby decreasing its effect on the enzyme.

Unfolding of the molecule by urea had a greater adverse effect on the tyrosine specific form than on the DOPA

specific form. The tertiary structure may contribute to the tyrosine specific site of the molecule.

EDTA inactivated the tyrosine specific forms at extremely low concentrations while much higher concentrations were needed to inactivate the DOPA specific forms. Copper may affect the tertiary structure of the molecule, in so far as the stereochemistry of the active sites is concerned, so tyrosine specificity which may depend on the tertiary structure is inactivated when the copper is removed. The little competition between the chelating agent and DOPA could also imply that the metal is not primarily engaged in binding any of the components to the enzyme.

Contrary to the hypothesis of Pomerantz (1966) that excess tyrosine combines at the DOPA **site** thus preventing DOPA from acting and vise versa, it was found that the effect of excess tyrosine or DOPA did not affect the active sites for DOPA and tyrosine activity.

Products formed when excess DOPA was incubated with the enzyme extract failed to inhibit one group of multiple forms, while all other DOPA specific forms were inactivated. A defense mechanism against product inhibition is postulated.

Trypsin degradation partially inactivated most of the multiple forms, indicating the significance of arginine and lysine as contributing directly or indirectly to the active site formation. Mercaptoethanol and cysteine-HCL caused

complete inactivation of all forms, while ascorbic acid had no effect on the multiple form pattern.

The PO system seems to be installed in the plant tissues at the very early stages of development, and no tissue specificity of multiple forms is apparent. The intracellular distribution pattern of the PO system is specific for each species. Intracellular compartmentalization of the multiple forms of PO was shown to exist. In mushroom the tyrosine specific form exists only in the final supernatant, while the mitochondrial fraction has only two DOPA specific bands.

The phenomenon of enzyme compartmentalization and multiple form compartmentalization is evident. Enzymes cannot exist in a disorganized manner within the cell. However, it is already clear that enzymatic browning reactions must be very complex, interacting with phenolic substances which are presumably compartmentalized within the mysterious unit of life, the cell.

LITERATURE CITED

Aerts, F. E., and R. E. Vercauteren. 1964. Specificity and mode of action of phenoloxidase from larvae of Tenebrio molitor. Enzymologia 28:1.

Allen, S. L. 1961. Genetic control of the esterase in Protozoan Tetrahymena pyriformis. Annals N. Y. Acad. Sci. 94:753.

Allen, S. L. 1964. The esterase isozymes of Tetrahymena: their distribution in isolated cellular components and their behaviour during the growth cycle. J. Exptl. Zool. 155:349.

Appel, S. H., and D. H. Alpers. 1965. Multiple molecular forms of β-galactosidase. J. Mol. Biol. 11:12.

Augustinsson, K. B. 1961. Multiple forms of esterase in Vertebrate blood plasma. Annals N. Y. Acad. Sci. 94:844.

Beckman, L., J. G. Scandalios, and J. L. Brewbaker. 1964a. Genetics of Leucine aminopeptidase isozymes in maize. Genetics 50:899.

Beckman, L., J. G. Scandalios, and J. L. Brewbaker. 1964b. Catalase Hybrid enzymes in maize. Science 146:1174.

Bedford, C. L., and S. Mayak. 1965. (Unpublished data).

Bonner, W. D. 1957. Soluble oxidases and their functions. Annual Review of Plant Physiology 8:427.

Bouchilloux, S., P. McMahill, and H. S. Mason. 1963. The multiple forms of mushroom tyrosinase. Purification and molecular properties of the enzymes. J. Biol. Chem. 238:1699.

Brody, I. A. 1964. Isozyme Histochemistry: A new method for the display of selective lactate dehydrogenase isozymes on an electrophoretic pattern. Nature 201:685.

Brown, F. C. and D. N. Ward. 1958. Studies on mammalian tyrosinase. I. Chromatography on cellulose Ion exchange agents. J. Biol. Chem. 233:77.

Carter, B. G., B. Cinader, and C. A. Ross. 1961. Immunochemical analysis of the multiple forms of bovine ribonuclease. Annals N. Y. Acad. Sci. 94:1004.

Chen, Y. M., and W. Chavin. 1965. Radiometric assay of tyrosinase and thoretical considerations of melanin formation. Anal. Biochem. 13:234.

Davis, B. J. 1964. Disk Electrophoresis I. Annals N. Y. Acad. Sci. 121:404.

Dawson, C. R., and R. J. Magee. 1955. Plant tyrosinase. Methods in Enzymology 2:817.

de Duve, C., B. C. Pressman, R. Gianetto, R. Wattiaux, and F. Appelman. 1955. Intracellular distribution pattern of enzyme in rat liver tissue. Biochem J. 60:604.

Dressler, H., and C. R. Dawson. 1960a. On the nature and mode of action of the copper protein tyrosinase. I Exchange experiments with radioactive copper and the resting enzyme. Biochim. Biophys. Acta 45:506.

Dressler, H., and C. R. Dawson. 1960b. On the nature and mode of action of the copper protein tyrosinase. II Exchange experiments with radioactive copper and the functioning enzyme. Biochim. Biophys. Acta 45:515.

Ecobichon, D. J. 1965. Multiple forms of human liver esterases. Canad. J. of Biochem. 43:595.

Embs, R. J., and P. Markakis. 1965. The mechanism of sulfite inhibition of browning caused by polyphenol oxidase. J. Food Sci. 30:753.

Enger, M. D., and B. P. Sleeper. 1965. Multiple cellulose system from Str. antibioticus. J. Bacteriology 89:237.

Fling, M., N. H. Horowitz, and S. F. Heinemann. 1963. The isolation and properties of crystalline tyrosinase from Neurospora. J. Biol. Chem. 238:2045.

Fox, A. S., and J. B. Burnett. 1962. Tyrosinase of diverse thermostabilities and their interconversion in Neurospora crassa. Biochim. Biophys. Acta 108:120.

Fox, A. S., J. B. Burnett, and M. S. Fuchs. 1963. Tyrosinase as a model for the genetic control of protein synthesis. Annals N. Y. Acad. Sci. 100:840.

Frieden, E., and M. Ottesen. 1959. A simplified method for the purification of mushroom polyphenol oxidase. Biochim. Biophys. Acta 34:248.

Furness, F. N. 1961. Multiple molecular forms of enzymes. Annals N. Y. Acad. Sci. 94:655.

Furth, A. J. and D. Robinson. 1965. Specificity and multiple forms of β-galactosidase in the rat. Biochem. J. 97:59.

Goldberg, E. 1965. Lactate dehydrogenase in trout: Evidence for a third subunit. Science 148:391.

Harel, E., A. M. Mayer, and Y. Shain. 1965. Purification and multiplicity of catechol oxidase from apple chloroplasts. Phytochem. 4:783.

Hayman, S., and R. A. Alberty. 1961. The isolation and kinetics of two forms of fumarase from Torula yeast. Annals N. Y. Acad. Sci. 94:812.

Hayaishi, O. (ed.) 1962. Oxygenases. Acad. Press. N. Y.

Hogeboom, G. H., W. C. Schneider, and G. E. Pallade. 1947. The isolation of morphological intact mitochondria from rat liver. Soc. for Exptl. Biol. and Medicine. Proceedings. 65:320.

Horowitz, N. H., and M. Fling. 1953. Genetic determination of tyrosinae thermostability in Neurospora. Genetics. 38:360.

Horowitz, N. H., M. Fling, and K. Asano. 1964. The induction of tyrosinase synthesis in Neurospora crassa. Sixth Intern. Congress of Biochem. N. Y.

Horowitz, N. H., M. Fling, and H. Macleod. 1961. Structural and regulative genes controlling tyrosinase synthesis in Neurospora. Cold Spring Harbor Symposium on Quantitative Biology 26:233.

Hsu, L., and A. L. Tappel. 1964. Lysosomal enzymes of rat intestinal mucosa. J. Cell Biol. 23:233.

Hultin, H. O., and A. S. Levine. 1963. On the occurrence of multiple molecular forms of pectinesterase. Arch. Biochem. Biophys. 101:396.

International Union of Biochemistry. 1964. Report on the commission on enzymes. Elsevier. Amsterdam.

Jolley, R. L., and H. S. Mason. 1965. The multiple forms of mushroom tyrosinase. J. Biol. Chem. 240:PC 1489.

Joslyn, M., and J. D. Ponting. 1951. Enzyme catalyzed oxidation browning of fruits products. Adv. in Food Res. 3:1.

Kaji, A., K. A. Trayser, and S. P. Colowick. 1961. Multiple forms of yeast hexokinase. Annals N. Y. Acad. Sci. 94:798.

Kaplan, N. O., M. M. Ciotti. 1961. Evolution and differentiation of dehydrogenases. Annals N. Y. Acad. Sci. 94:701.

Kaplan, N. O., M. M. Ciotti, M. Hamolsky, and R. E. Bieber. 1960. Molecular heterogeneity and evolution of enzyme. Science 131:392.

Karkhanis, Y., and E. Frieden. 1961. Induction period and a protein inhibitor of mushroom tyrosinase. J. Biol. Chem. 236:PC 1-2.

Kassab, R. 1961. Les polyphemoloxidases. Bull. Soc. Pharm. Marseille 10:231.

Katz, A. M., and W. Kalow. 1965. Electrophoretic characteristics of human dehydrogenases. Canad. J. of Biochem. 43:1653.

Katzen, H. M., and R. T. Schimke. 1965. Multiple forms of hexokinase in the rat: tissue distribution, age dependency and properties. Proc. National Acad. Sci. 54:1218.

Katzen, H. M., D. D. Soderman, and H. M. Mitowsky. 1965. Kinetic and electrophoretic evidence for multiple forms of glucose ATP phosphotransferase activity from human cell culture and rat liver. Biochem. Biophys. Res. Comm. 19:377.

Kean, E. A. 1964. A procedure which demonstrates substrate inhibition of tyrosinase. Biochim. Biophys. Acta 92:602.

Keilin, D., and T. Mann. 1938. Polyphenoloxidase. Purification, nature properties. Proc. Roy. Soc. (London) 125B:187.

Kendal, L. P. 1949. The action of tyrosinase on monophenols. Biochem. J. 44:442.

Keller, S., and R. J. Block. 1960. (In Analytical Methods in Protein Chemistry. P. Alexander and R. J. Block, eds. I:2. Pergamon Press).

Kertesz, D., and R. Zito. 1957. Polyphenoloxidase "Tyrosinase" purification and molecular properties. Nature 179:1017.

Kertesz, D., and R. Zito. 1962. Kinetics studies of the polyphenoloxidase action: kinetics in the presence of reducing agents. Biochim. Biophys. Acta 64:153.

Kim, K. H., and T. T. Chen. 1962. Tyrosinase of the goldfish Carassius auratus L. Biochim. Biophys. Acta 59:569.

Kitto, G. B., P. M. Wassarman, J. Michjeda, and N. O. Kaplan. 1966. Multiple forms of mitochondrial malate dehydrogenaes. Biochem. Biophys. Res. Comm. 92:75.

Klapper, M. H., and D. P. Hackett. 1965. Investigations on the multiple components of commercial horseradish peroxidase. Biochim. Biophys. Acta 96:272.

Kon, S., and J. R. Whitaker. 1965. Separation and partial characterization of the peroxidases of Ficus glabrata Latex. J. Food Sci. 30:977.

Koshland, D. E. 1964. The active center in enzymatic action. Bull. de la Soc. de Chimie Biologique 46:1745.

Krueger, R. C. 1959. The nature of the copper in tyrosinase. Feder. Proc. 18:267.

Kubowitz. 1937. Über die chemische zusammensetzung der kartoffeloxydase. Biochem. Zs. 292:221.

Kun, E., and P. Volfin. 1966. Tissue specificity of malate dehydrogenase isozymes. Kinetic discrimination by oxalacetate its mono and difluoro analogues. Biochem. Biophys. Res. Comm. 22:187.

Laufer, H. 1961. Forms of enzymes in insect development. Annals N. Y. Acad. Sci. 94:825.

Lerner, A. B. 1953. Metabolism of phenylalanine and tyrosine. Adv. in Enzymology 14:73.

Lester, G., and A. Byers. 1965. Properties of two β-galactosidases of Neurospora crass. Biochem. Biophys. Res. Comm. 18:725.

Lowry, O. H., N. J. Roebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265.

Lyr, H., and W. Luthardt. 1965. Induction of tyrosinase in higher fungi. Nature 207:753.

Mahy, B. W. J., and K. E. K. Rowson. 1965. Isoenzymic specificity of impaired clearance in mice infected with Riley virus. Science 149:756.

Mallette, M. F. 1950. Tyrosinase. A symposium on copper metabolism. p. 48. McElroy and B. Glass. Baltimore.

Mallette, M. F., and C. R. Dawson. 1949. On the nature of highly purified mushroom tyrosinase preparation. Arch. Biochem. Biophys. 23:29.

Markert, C. L. 1963. Lactate dehydrogenase isozymes. Dissociation and recombination of subunits. Science 140:1329.

Markert, C. L., and E. Appela. 1961. Physicochemical nature of isozymes. Annals N. Y. Acad. Sci. 94:678.

Markert, C. L., and F. Moller. 1959. Multiple forms of enzymes: tissue ontogenic, and species specific pattern. Proc. Natl. Acad. Sci. 45:753.

Martinez-Carrion, M., F. Riva, C. Turano, and P. Fasella. 1965. Multiple forms of supernatant glutamate aspartate transaminase from pig heart. Biochem. Biophy. Res. Comm. 20:206.

Mason, H. S. 1955a. Phenolase. J. Am. Chem. Soc. 77:2914.

Mason, H. S. 1955b. Comparative biochemistry of the phenolase complex. Adv. in Enzymology 16:105.

Mason, H. S. 1965. Oxidases. Ann. Review of Biochem. 34:595.

Mayberry, J. M., and Mallette, M. F. 1962. Inhibition of the tyrosinase oxidation of one substrate by another. J. Gen. Physiol. 45:1239.

Mayer, A. M. 1965. Factors controlling activity of phenolase in chloroplasts from sugar beets. Israel J. of Botany 13:74.

McCune, D. C. 1961. Multiple peroxidases in corn. Annals N. Y. Acad. Sci. 94:723.

McKinley-McKee, J. S., and D. W. Moss. 1965. Heterogeneity of liver alcohol dehydrogenase on starch gel electrophoresis. Biochem. J. 96:583.

Metzger, R. P., S. S. Wilcox, and N. Arne. 1965. Subcellular distribution and properties of hepatic glucose dehydrogenases of selected vertebrates. J. Biol. Chem. 240:2767.

Mitchell, H. K., and U. M. Weber. 1965. Drosophila phenol oxidases. Science 148:964.

Monod, J., and F. Jacob. 1961. General Conclusions: Teleonomic mechanism in cellular metabolism, growth, and differentiation. Cold Spring Harbor Symposium on Quantitative Biol. 26:389.

Moore, B. W., and P. U. Angelletti. 1961. Chromatographic heterogeneity of some enzymes in normal tissues. Annals N. Y. Acad. Sci. 94:659.

Moore, R. O., and C. A. Villee. 1963. Multiple molecular forms of malate dehydrogenases in Echinoderm embryos. Compar. Biochem. Physiol. 9:21.

Mosback, R. 1963. Purification and some properties of laccase from Polyorus versicolor. Biochim. Biophys. Acta 73:204.

Nelson, S. M., and C. R. Dawson. 1944. Tyrosinase. Adv. in Enzymology 4:99.

Nishimura, S., N. Carson, and T. Y. Kobara. 1964. Isozymes of human and rat catalases. Arch. Biochem. Biophys. 108:452.

Novikoff, A. B., and E. Podber. 1957. The contribution of differential contrifugation to the intracellular localization of enzymes. J. Histochem. Cytochem. 5:552.

Ornstein, L. 1964. Disk electrophoresis. I Annals N. Y. Acad. Sci. 121:321.

Osaki, S. 1963. The mechanism of tyrosine oxidation by mushroom tyrosinase. Arch. Biochem. Biophys. 100:378.

Patil, S., H. T. Evans, and P. McMahill. 1963. Electrophoretic separation of the phenolases from potato tubers. Nature 200:1322.

Patil, S., and M. Zucker. 1965. Potato phenolases. Purification and properties. J. Biol. Chem. 240:3938.

Paul, J., and P. F. Fottrell. 1961. Molecular variation in similar enzymes from different species. Annals N. Y. Acad. Sci. 94:668.

Ponting, J. D., 1960. Control of enzymatic browning of fruits. in: Food Enzymes. pp. 105-123. H. W. Schultz ed. AVI. Westport, Conn.

Pomerantz, S. H. 1963. Separation, purification and properties of two tyrosinases from hamster melanoma. J. Biol. Chem. 238:2351.

Pomerantz, S. H. 1964. Tyrosine hydroxylation catalyzed by mammalian tyrosinase: An improved method of assay. Biochem. Biophys. Res. Comm. 16:188.

Pomerantz, S. H. 1966. The tyrosine hydroxylase activity of mammalian tyrosinase. J. Biol. Chem. 241:161.

Riggins, R. S., and W. S. Kiser. 1964. Lactic dehydrogenase isozymes in urine. Invest. Urol. 2:30.

Robb, D. A., L. W. Mapson, and T. Swain. 1965. On the heterogeneity of the tyrosinase of broad beans. Phytochem. 4:731.

Schneider, W. C., and G. H. Hogeboom. 1950. Intracellular distribution of enzymes. J. Biol. Chem. 183:121.

Schneider, W. C., and G. H. Hogeboom. 1956. Biochemistry of cellular particles. Ann. Review of Biochem. 25:201.

Scandalios, J. G. 1964. Tissue specific isozymes variation in maize. J. Heredity 55:281.

Scandalios, J. G. 1965. Subunit dissociation and recombination of catalase isozymes. Proc. of the Natl. Acad. of Sci. 53:1035.

Schulze, I. T., J. Gazith, and S. P. Colowick. 1965. Relative susceptibility of tetramer and dimer forms of hexokinase to modification by proteases. Fed. Proc. 24:224.

Schwimmer, S. 1953. Enzyme system of the white potato. J. Agric. Food Chem. 1:1063.

Secchi, G. C., and N. Dioguardi. 1965. Multiple forms of serum and liver esterases in the normal state and in cirrhosis of the liver. Enzymologia Biologica et clinica 5:29.

Shimao, K. 1962. Partial purification and kinetic studies of mammalian tyrosinase. Biochem. Biophys. Acta 62:205.

Sisler, E. C., and J. J. Evans. 1958. Direct spectrophotometric determination of chlorogenic acid oxidase activity. Biochem. Biophys. Acta 28:638.

Smith, J. L., and R. C. Krueger. 1962. Separation and purification of the phenolases of the common mushroom. J. Biol. Chem. 237:1121.

Stadtman, E. R., G. N. Cohen, and J. Le Bras. 1961. Feedback inhibition and repression of aspartokinase activity in Eschrichia coli. Annals N. Y. Acad. Sci. 94:952.

Sussman, A. S. 1949. Tyrosinase. Quant. Rev. Biol. 24:328.

Sussman, A. S. 1961. A comparison of the properties of two forms of tyrosinases from Neurospora crassa. Arch. Biochem. Biophys. 95:407.

Tsuyuki, H., and F. Wold. 1964. Enolase: multiple molecular forms in fish muscle. Science 146:535.

Uritani, I. 1963. (Paper presented on the 75th anniversary of the Dept. Pathology and Botany, Coonn. Agric. Exptl. Station, New Haven).

Vesell, E. S. 1965. Polymorphism of human lactate dehydrogenase isozymes. Science 148:1103.

Vogel, F. S., and D. H. McGregor. 1964. The fine structure and some biochemical correlates of melanogenesis in the ink gland of the squid. Lab. Invest. 13:767.

Walker, J. R. L. 1964. Studies on the enzymatic browning of apples II. Properties of apple polyphenoloxidase. Austral. J. Biol. Sci. 17:360.

Webb, E. C. 1964. The nomenclature of multiple enzyme forms. Experientia 20:592.

Whipple, H. E. (ed.) 1964. Gel electrophoresis. Annals N. Y. Acad. Sci. 121:305.

Wieland, Th., and G. Pfleiderer. 1957. Nachweis der Heterogenität von Milchsäuredehydrogenasen verschiedenen Ursprungs durch Trägerelktrophorese. Biochem Z. 329:112.

Withycombe, W. A., and J. H. Wilkinson. 1964. Properties of human spermatozoan lactic dehydrogenases isozymes. Biochem. J. 93:11p.

Yasanobu, K. T. 1959. Mode of action of tyrosinase. Pigment Cell Biology, pp. 583-607. ed. M. Gordon. Acad. Press, N. Y.

Yu, M. L., and R. E. Hampton. 1964. Biochemical changes in tobacco infected with Colletotricum destructivum. II. Peroxidases. Phytochem. 3:499.

Figure 1

Differential centrifugation scheme.

Figure 2

Effect of concentration of gel on the resolution of the multiple forms of phenoloxidase (PO) in mushrooms.

Left to right: 6%, 7%, 8%, 9% gel.

Substrate: DL-DOPA

Figure 3

PO active multiple forms and inactive protein in mushrooms.

Left: Multiple molecular forms of phenoloxidase in the common mushroom (Agaricus campestris) separated with 7% and 8% polyacrylamide gel. Sample applied 0.4 ml. Substrate: DL-DOPA.

Right: The corresponding protein pattern, separated in 9% gel. Sample applied 0.1 ml. Developed with 1% Amido Black in 7% acetic acid.

Figure 4

Multiple forms of PO from different genera.

Left: Common Mushroom (Agaricus campestris)

Middle: Potato (Russett var.)

Right: Apple (Yellow delicious var.)

Substrate: DL-DOPA.

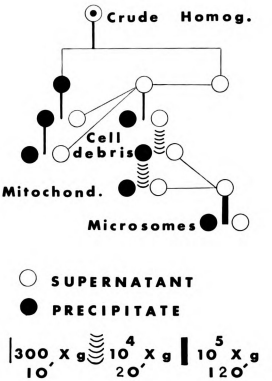

Fig. 1

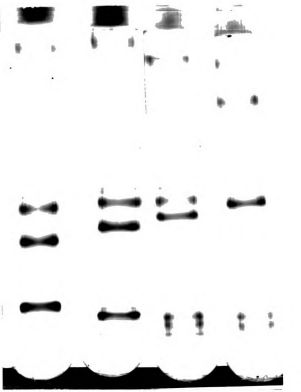

Fig. 2

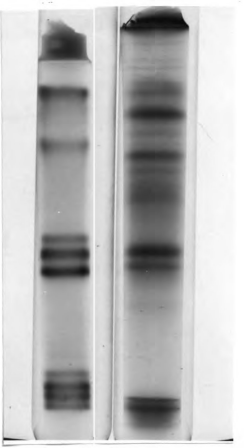

Fig. 3

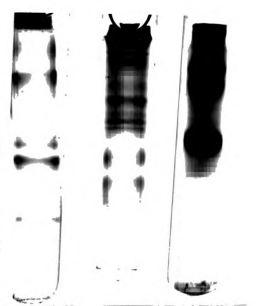

Fig. 4

Figure 5

Cathodical (pH 8.3) and anodical (pH 4.3) multiple forms of PO in mushrooms.

Left: Cathodical multiple forms.

Right: Anodical multiple forms.

Substrate: DL-DOPA

Figure 6

Two-phase polyacrylamide electrophoresis. Separation of the multiple form of PO in mushrooms.

Left: 7% gel.

Right: 7% top half of column, 8% bottom half of column.

Substrate: DL-DOPA.

Figure 7

Effect of current on separation of multiple forms of PO in mushrooms.

Left to Right: 1 MA per tube, 2 MA, 2 1/4 MA, 3 MA.

Substrate: DL-DOPA.

Figure 8

Recovery of multiple forms of PO in mushrooms following elution from the gells.

Left: Recovery of the one band of group C.

Middle: Control

Right: Recovery of the two bands of group B.

Substrate: DL-DOPA.

Fig. 5

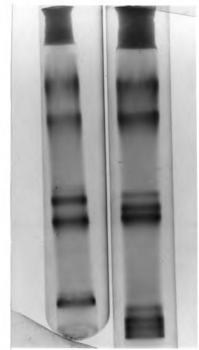

Fig. 6

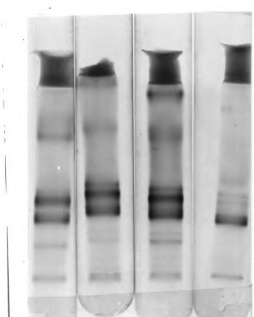

Fig. 7

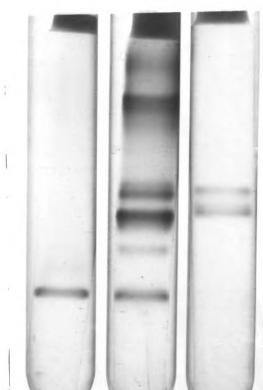

Fig. 8

Figure 9

DL dihydrooxyphenylalamine and L-Tyrosine specificity of multiple forms of PO in mushrooms.

Left: DL-DOPA specific multiple forms.

Right: L-Tyrosine specific multiple forms.

Figure 10

Substrate specificity of multiple forms of PO in mushrooms.

Left to Right: DL-DOPA specific multiple forms, L-Tyrosine, catechol, catechine, cafeic acid, cholorogenic acid. (The fast band is the front).

Figure 11

Species and genus specificity of multiple forms of PO in mushrooms.

Left to Right:	Agaricus campestris	Substrate:	DL-DOPA
	Agaricus placomyces	"	a)DL-DOPA
		"	b)L-Tyrosine
	Tricholoma venenata	"	a)DL-DOPA
		"	b)L-Tyrosine
	Suillus grevillei	"	a)DL-DOPA
		"	b)L-Tyrosine

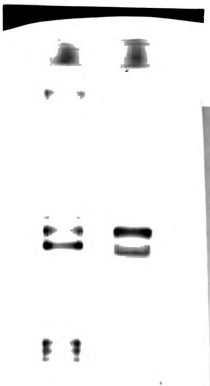

Fig. 9

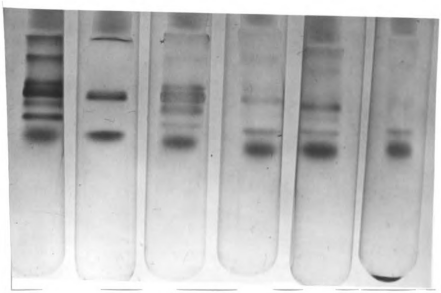

Fig. 10

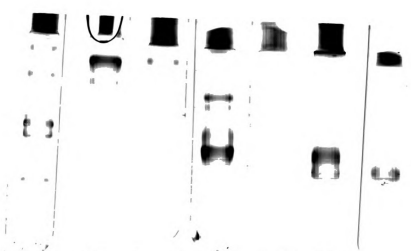

Fig. 11

Figure 12

Varietal specificity of multiple forms of PO in apples.

Left to Right: Spy, Red Delicious, Golden Delicious, Cortland, Jonathan, Grimes Golden.

Substrate: DL-DOPA + Catechol.

Figure 13

Effect of acetone on multiple forms of PO in mushrooms.

Left: Control.

Right: Acetone treated (Acetone powder).

Substrate: DL-DOPA.

Figure 14

Multiple forms of commercial mushroom PO from four different companies.

Left to Right: A, B, C, D.

Substrate: DL-DOPA.

Figure 15

Effect of 5 C with time on the multiple forms of PO in mushrooms.

Left to Right: Hours: 2, 8, 20, 72.

Substrate: DL-DOPA.

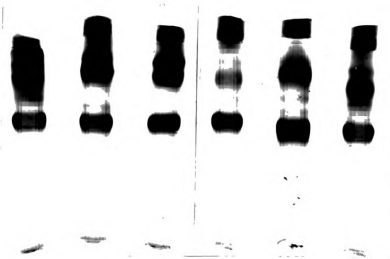

Fig. 12

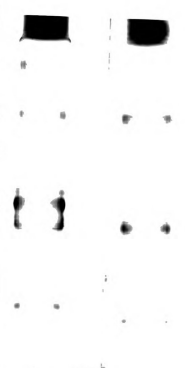

Fig. 13

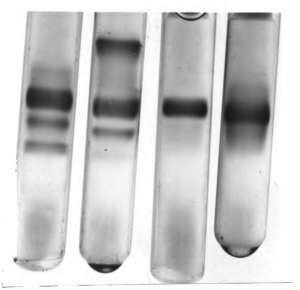

Fig. 14

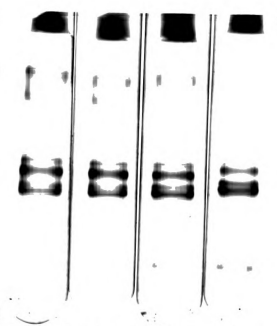

Fig. 15

Figure 16

Effect of room temperature (22 C) with time on the multiple forms of PO in mushrooms.

Left to Right: Hours: 2, 8, 20, 72.

Substrate: DL-DOPA.

Figure 17

Effect of 40 C and 50 C with time on the multiple forms of PO in mushrooms.

Left to Right: Control: 40 C 3 min.; 40 C 60 min.; 50 C 3 min.; 60 C 60 min.

Figure 18

Effect of 60 C, 70 C and 100 C on the multiple forms of PO in mushrooms.

Left to Right: 60 C minutes: 3, 15, 60.

70 C " : 3, 60.

100 C " : 2.

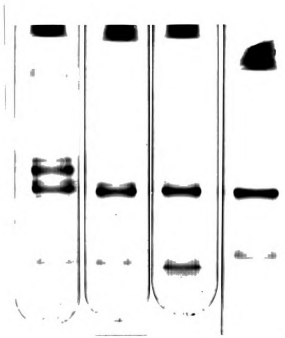

Fig. 16

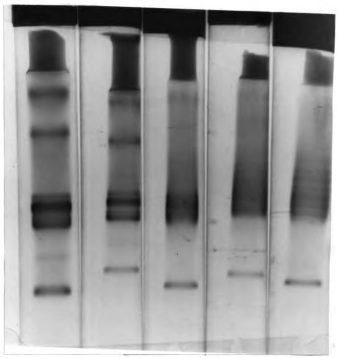

Fig. 17

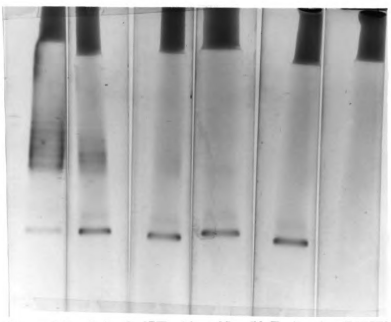

Fig. 18

Figure 19

Effect of sulfite on the multiple forms of PO in mushrooms. Incubation on the gel, 10 minutes.

Right to Left: SO_2 ppm 20, 100, 250, 500, 1000.

Substrate: 1.5×10^{-3}M DL-DOPA

Figure 20

Effect of sulfite on the multiple forms of PO in mushrooms. Incubation on the gel, 10 minutes.

Left to Right: SO_2 p.p.m. 250, 500, 1000.

Substrate: 3.0×10^{-3}M DL-DOPA.

Figure 21

Effect of 1000 p.p.m. SO_2 with different substrate concentrations on the multiple forms of PO in mushrooms. Incubation on the gel, 10 minutes.

Right to Left: DL-DOPA 0.5×10^{-3}M, 3.0×10^{-3}M, 1.2×10^{-2}M.

Figure 22

Effect of sulfite on the multiple forms of PO in mushrooms, following washing off of the sulfite. Incubation on the gel, 30 minutes.

Left to Right:

1) SO_2 p.p.m. 250. Substrate: 3.0×10^{-3}M DL-DOPA Gel not washed.
2) " " " " " " " " " washed.
3) SO_2 p.p.m. 500. Substrate: " " " " " not washed.
4) " " " " " " " " " washed.

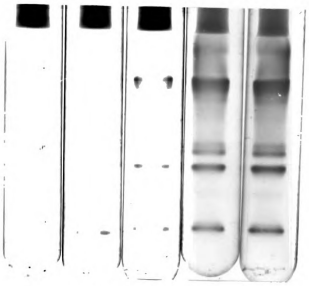

Fig. 19

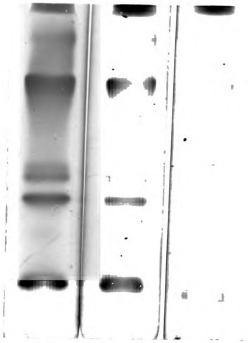

Fig. 20

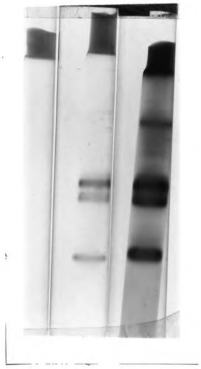

Fig. 21

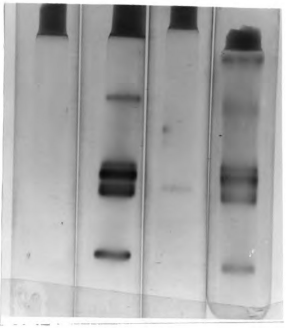

Fig. 22

Figure 23

Effect of incubation of sulfite (6 hrs.) on the multiple forms of PO in mushrooms.

Left to Right: Control: SO_2 p.p.m. 1000 2000, 4000, 8000.

Substrate: DL-DOPA 1.5×10^{-3}M.

Figure 24

Effect of incubation (6 hrs.) of SO_2 on the multiple forms of PO in mushrooms.

Left to Right: Control: SO_2 p.p.m. 1000, 2000, 4000, 8000.

Substrate: L-Tyrosine.

Figure 25

Effect of EDTA on the multiple forms of PO in mushrooms.

Right to Left: Control: EDTA 0.05%; 0.25%; 0.5%; 4.0%.

Substrate: L-Tyrosine.

Figure 26

Effect of EDTA on the multiple forms of phenoloxidase in mushrooms.

Left to Right: 0.01% EDTA 0.5%; 0.25%; 0.50%; 4.0%.

Substrate: DL-DOPA.

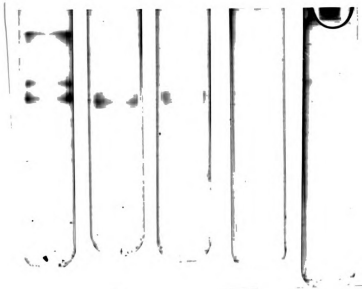

Fig. 23

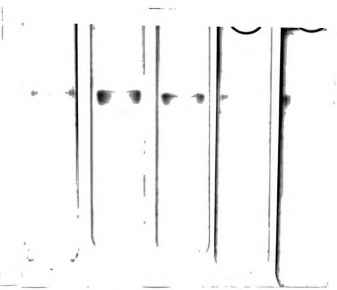

Fig. 24

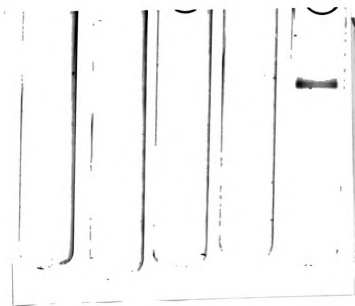

Fig. 25

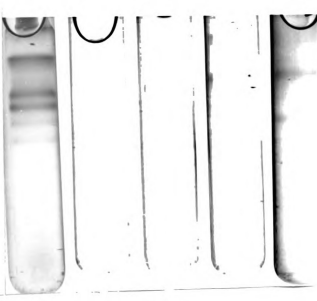

Fig. 26

Figure 27

Effect of Urea on the multiple forms of PO in mushrooms.

Right to Left: Control: Urea 1.5M; 4.0M; 6.0M; 8.0M.

Substrate: DL-DOPA.

Figure 28

Effect of Urea on the multiple forms of PO in mushrooms.

Left to Right: Control: Urea 1.5M; 4.0M; 6.0M; 8.0M.

Substrate: L-Tyrosine.

Figure 29

Intracellular distribution of total PO activity.

Figure 30

Intracellular compartmentalization of multiple forms of PO in mushrooms.

Right to Left: a) Final Supernatant. Substrata: DL-DOPA.

b) " " " : L-Tyrosine.

c) Mitochondrial elution. Substrate: DL-DOPA.

d) " " " : L-Tyrosine.

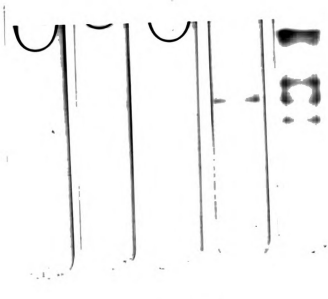

Fig. 27

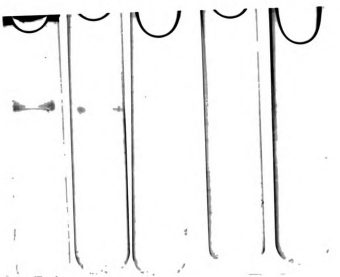

Fig. 28

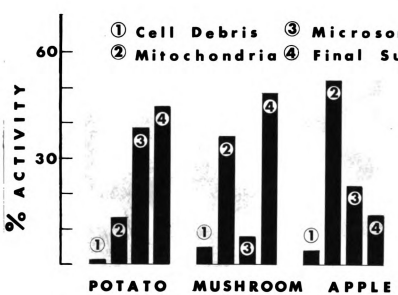

Fig. 29

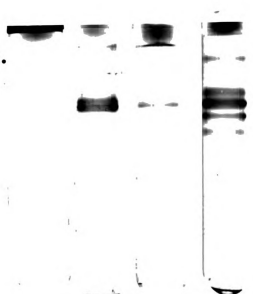

Fig. 30