COMPARATIVE STUDIES ON PEROXIDASE STRUCTURE

by 4589

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A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Biochemistry

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1970

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Acknowledgement:

I wish to express my gratitude to my major professor, Dr. B.A.Cunningham, who made this thesis possible with his help and advice. I wish also to thank my wife, Ellen, for her patience and love.

February, 1970 Sighart Golf

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I. INTRODUCTION

A large number of enzymes are included within the category E.C.1.11.1.7, Donor: H_2O_2 oxidoreductase (hydroperoxidase). They show activity according to the reaction:

$$H_2O_2 + AH_2 \xrightarrow{\text{enzyme}} A + 2H_2O$$

Peroxidase enzymes are found in many species of plants and animals. The hydrogen donor, AH_2, classified by MASON (63), includes halogen acids, aromatic amines such as aniline (38), and phenolics such as guaiacol, pyrogallol and hydroquinone. It also includes uric acid, ascorbic acid, thiols, dihydrofumaric acid, reductic acid and triose reductone.

There is a vast amount of published information about peroxidases from many authors. However, information about chloroperoxidase, an enzyme produced by the fungus Caldariomyces fumago Woron., comes mainly from HAGER et al. (41,10,11). The enzyme chloroperoxidase is a glycoprotein as well as a hemoprotein and has many characteristics comparable to other peroxidases. That fact generates considerable interest in chloroperoxidase from a phylogenetic viewpoint, because it is produced by a lower plant species.

The first objective of this thesis was to improve the fungus growth conditions under which chloroperoxidase is produced. A second objective was to investigate the chloroperoxidase primary structure. As the work progressed, it was successful in improving enzyme yield, but a complex of isoenzymes was found. The second objective was therefore delayed to some extent until the isoenzymes could be resolved. A new isoenzyme was detected, purified by a new procedure, and its properties were studied.

II. LITERATURE REVIEW

A. General Considerations

In 1855 SCHOENBEIN (87) observed that extracts of certain mushrooms and animal tissues produced a blue color in guaiac solutions in the presence of air. The extracts could also utilize hydrogen peroxide or the peroxides in guaiac tincture for this oxidation. Cyanide abolished the reaction and high peroxide concentrations reduced the catalytic power. Schoenbein concluded that the extracts were able to activate atmospheric oxygen. LINOSSIER (55) in 1898 prepared oxidase free peroxidases from leucocytes and showed peroxidases to be separate entities, i.e., they reacted only with peroxides, not O_2. The first link between peroxidase activity and a chemical structure appeared in 1931, when KUHN et al. (52) found a direct relation between activity and light absorption in the Soret region. Peroxidase preparations of high purity were subsequently obtained by BUNER and HOWELL (96) from fig sap, KEILIN and MANN (48) from horseradish, originally discovered by BACH and CHODAT (7), AGNER (3) from pus, DOLIN (28) in Streptococcus faecalis. The latter enzyme is an exception to the heme-protein nature of peroxidase and is instead a flavoprotein.

An extensive compilation of known peroxidase can be found in SAUNDERS' book Peroxidase (86). Despite the vast amount of published information on peroxidase, the physiological role which it plays is by no means clear. It is thought to act as an antibacterial agent, to catalyze biological luminescence and to halogenate, as well as to oxidize indole acetic acid and certain dicarboxylic acids in the presence of manganese

and nucleotides (38,49,4).

Thyroid peroxidase is the best known member whose physiological role, the halogenation of tyrosine for thyroxine biosynthesis, has been quite well accepted. Another peroxidase performing a somewhat similar function has been discovered and purified in crystalline form from the fungus Caldariomyces fumago Woron. (41,84).

B. Caldariomyces fumago and Chloroperoxidase

The fungus Caldariomyces fumago, first mentioned by ZOPF (110) in 1878 as Fumago vagans Persoon, is one of the common "sooty" greenhouse molds. After being renamed by WORONICHIN (107), to its present name, Caldariomyces fumago has been placed into the group of Deuteromycetes.

The first intensive studies on its morphology and some tentative experiments on its physiology were made by ZOPF (110). He hypothesized that in nature the only nutrition for Caldariomyces fumago was probably the honeydew excreted by Aphidae and Coccinae (insects). That would substantiate the saprophytic character of Caldariomyces fumago for its mycelium does not penetrate into living material, such as leaves. In his culturing experiments Zopf found that Caldariomyces fumago is capable of living on sugar (glucose) solution, however too high or too low concentrations inhibit growth. In another study by RAMACHANDRAN and GOTTLIEB (80), on the glucose metabolism of Caldariomyces fumago, both resting cell suspensions (i.e. nongrowing cells), and cell-free extracts of the organism were found capable of oxidizing glucose, gluconolactone, 6-P-gluconate, and 2-keto-

gluconate aerobically, but not anaerobically; glucuronic acid, glucose-6-P or fructose-1,6-di-phosphate were not oxidized. These results as well as supplementary manometric studies employing metabolic poisons, and isotopic glucose, led to the conclusion that this fungus employs a modified ENTNER-DOUDOROFF Pathway (35).

Many compounds, particularly the phosphorylated sugars involved in the EM, HMP, or ED pathways, are usually impermeable to cells. However, phosphatases are often located on the cell surface (82) and hydrolize the phosphate esters, so that the free sugar can be transported and metabolized. In the case of Caldariomyces fumago, suggestive evidence was obtained that an induced enzyme was involved in the oxidation of 2-keto-gluconate.

According to RAMACHANDRAN and GOTTLIEB (80), Caldariomyces fumago uses the ED pathway to an extent of 65% and the HMP pathway 35%. The mycelium lacks hexokinase, and the fungus apparently uses one of the sequences that bypasses hexokinase and leads to the formation of 6-P-gluconate:

Glucose ⟶ Gluconate ⟷ 2-keto-gluconate
↓
6-P-gluconate ↔ 2-keto-6-P-gluconate
↓
2-keto-3-deoxy-6-P-D-gluconate + H_2O
↓
pyruvate + glyceraldehyde-3-P

Another highly significant discovery was made by MORRIS and HAGEN (72), that the fungus releases chloroperoxidase into the growth medium when yeast extract is added to a modified Czapeck-Dox growth medium. The enzyme could therefore be produced under submerged aerobic conditions with much higher yields

in a shorter time. Although structural studies on the exo- and endo-enzymes have not been made, it is reasonable to presume that they are related isoenzymes.

C. Nutritional Factors in Peroxidase Biosynthesis

Part of this thesis describes experiments on the replacement of yeast extract with known vitamins plus anion and cation variations in the modified Hager-Ozapeck-Dox medium, and subsequent effects on the production of chloroperoxidase. A possible role of Mn^{++} in oxidase activity and the essential role of Cl^{-} in halogenating activity led to their inclusion. Other trace metals were also studied.

In strains of Geotrichum, Penicillium, Helimentosporium, Fusarium, and Cladosporium (all fungi imperfecti) peroxidase induction was achieved by the addition of substrate-like compounds: taurin, gallic acid, pyrogallol, ascorbic acid and by oak bark extracts (15). The inducers inhibited growth and did not serve as carbon sources. Similar induction was obtained by the addition of 3×10^{-4} M oxytetracycline. The induction of peroxidase seemed to be a defense against growth inhibition.

Chlorine is a constituent of a number of fungal metabolites such as geodin and mollisin (79,102). Organically bound chlorine is not a necessary precursor in their biosynthesis since these products are readily formed in synthetic media in which the chlorine is present as chloride ion. When the fungus is deprived of chloride it often produces the dechloro analog of the chlorometabolite. In most cases the chlorine is attached to an aromatic ring carbon. Exceptionally, caldariomycin, a dihydroxycyclopentane derivative, carries two chlorine atoms

attached to the same carbon atom.

In studying the synthesis of caldariomycin, SHAW et al. (91) found formation of the carbon chlorine bond to be catalyzed by an enzyme present in Caldariomyces fumago. The enzyme promotes the chlorination of a cyclic β-diketone in the presence of a suitable chloride concentration and hydrogen peroxide to form the natural product caldariomycin.

Chloroperoxidase possesses, moreover, the ability to catalyze oxidation of chloride, iodide, and bromide with subsequent formation of carbon-halogen bonds in the presence of a suitable acceptor molecule. This ability is similar to the synthesis of thyroxine in the mammalian thyroid.

Through intensive research by the Hager group it is suggested that peroxidase mechanisms may be involved in the biosynthesis of other naturally occuring organic halogen compounds, such as chloramphenicol (81) or griseofulvin (40).

The role that Mn^{++} plays in naturally functioning peroxidase systems is not clear. BERTRAND (14) attributed catalytic function to manganese in peroxidase catalyzed reactions and this concept has received some experimental support from BANGA and SZENT-GYÖRGI (3) and THEORELL and SWEDIN (99). The latter authors found trace concentrations of Mn^{++} (less than 5uM) to accelerate oxygen uptake in the system horseradish peroxidase-dihydrofumaric (DHF) acid. If sufficient Mn^{++} was present, two phases could be ditinguished spectrophotometrically with HRP II (19). These were: (a) an oxidase reaction, stimulated by Mn^{++} and (b) an adjacent peroxidatic reaction on which Mn^{++} had no influence. The function of Mn^{++} has not

been completely clear, but it is most likely not the reduction of O_2 to H_2O_2 (44). The stimulation of the DHF oxidation by Mn^{++} occurs under aerobic conditions and much less effect is seen anaerobically with terminal oxidants, such as cytochrome c, Fe^{3+} complexes or dyes (45).

Data on the effect of other metal ions are available only for mixtures of the two types I and II of plant peroxidases, and not for single enzyme species. In the system O_2-turnip peroxidase-triose reductone (44) there was no effect of 10uM Hg^{2+}, Fe^{3+}, or Ag^{+} or of 0.4 uM Zn^{2+}, Ba^{2+}, or Ca^{2+} at pH 4 to 6 or at pH 7 to 8. Stimulation occured at pH 4 to 6 only with Mn^{2+}, but at pH 7 to 8 stimulation was also observed with Co^{2+}, Cu^{2+}, and Hg^{2+}. Trace concentrations of Cu^{2+} have been long known to inhibit DHF oxidase activity of HRP at pH 4 (44).

Another investigation analyzed (75) the effects of peat soil iron deficiency on peroxidase activity in grasses. At low soil iron concentration a decreased peroxidase activity was observed.

There is not much published information on interactions between peroxidase activity and vitamins. Most knowledge comes from Szent-Györgi's early research on the enzymic oxidation of aliphatic and aromatic enediols (9). His interest in ascorbate finally led to the discovery of DHF (63,43). The suggested role of ascorbic or isoascorbic acid was a regulator of peroxidatic hydroxylation of aromatic compounds in the presence of DHF. The oxygen for the hydroxylation is derived from molecular oxygen (63).

Another investigation (53) analyzed the effect of vitamin D and iodine level on peroxidase activity in rat liver, however no definite response was observed.

D. Previous Work on Chloroperoxidase Isolation and Properties

Part of this thesis describes a new procedure for purification of chloroperoxidase, and therefore the method of HAGER and MORRIS (72) and SAE (84) are described in detail for the purpose of comparison. The Hager et al. procedure employs large scale vacuum evaporation, heating the protein to 45°C and subsequent lyophilization of three liters, all of which are difficult. These steps plus subsequent ethanol fractionation seem somewhat harsh, although the reported activity losses were small. One possible effect of that isolation method may have been the loss of isoenzymes, for they were not reported in Hager's work. Sae improved the method of Hager and as a result of new column steps was able to find two chloroperoxidase isoenzymes. Following the dialysis against tap water, Sae adsorbed the enzyme on aluminum gel and chromatographed the gel eluate on DEAE-cellulose at pH 6, and 3.85. Two enzyme peaks from this procedure were subjected to isoelectric focusing and that method confirmed the existence of two chloroperoxidase isoenzymes, isoelectric at pH 3.85 and 3.57, respectively. The major peak with pI 3.85 was called chloroperoxidase A, the minor peak with pI 3.57 was called chloroperoxidase B. The final purification step in both the Sae and Hager procedure is crystallization in ammonium sulfate solution. Some of the properties of the enzymes as determined by Hager and by Sae are listed below:

1. Prosthetic group Ferriprotoporphyrin IX, loosely bound
2. Molecular weight 40,200 (from iron content, Hager)
   42,000 (from sedimentation studies, Hager)
   46,000 chloroperoxidase A (Sephadex G-100 Sae)

40,000 chloroperoxidase B (Sephadex G-100, Sae)

42,000 electron micrograph molecular diameter studies, Sae

3. Amino acid composition, high in aspartic acid, (39 residues), glutamic acid (26 residues) and serine (33 residues) in a total of 126 residues.
4. Carbohydrate content 25-30%, mainly glucosamine and arabinose.
5. Catalytic activity: chloride, bromide and iodide oxidation in presence of H_2O_2, pH optimum about 3, classical peroxidase activity, e.g. guaiacol oxidation at above pH 5.
6. The ability of Hager's enzyme and Sae's isoenzymes A and B to chlorinate monochlorodimedon at pH 2.8 establishes that all the isoenzymes are chloroperoxidases.

E. Peroxidase Isoenzymes

Peroxidases have been isolated in pure or in nearly pure form by conventional methods from sweet potatoe tubers (54), broad bean (Vicia faba L.) leaves (66), turnip (44), Japanese radish (Raphanus sativus L.)(67), wheat germ (97), and many others. Like horseradish peroxidase (100,48), they all contain protohemin as the prosthetic group, and the crude preparations usually contain more than one peroxidase. With improved separation methods for enzymes, there has been an increasing number of isoenzymes discovered in peroxidase systems. Several authors have observed multiple components of HRP (100,46,76,98) with identical spectra or activities. In some preparations of HRP a variable proportion (HRP I)(100) can be electrophoretically separated from the major component, HRP II. The properties of the two hemoproteins are very similar (101). Similar results

are obtained with JRP (69,68,70). Two isoenzymes have been found, one acidic, the other basic.

Other peroxidase isoenzymes have been detected in the extreme dwarf tomato plant (36), and bean leaves (2). In corn leaves (58), up to 21 isoenzymes with peroxidatic activity have been detected.

Isoenzymes were first recognized in connection with the electrophoretic separation of lactic dehydrogenase (LDH)(105). These experiments showed that the classification of enzymes must extend beyond substrate specificity alone (60). Markert and Moeller introduced the term isoenzyme to describe the different molecular forms in which enzymes may exist with the same substrate specificity. The use of the term isoenzyme is now more commonly used to describe multiple enzyme forms. The concept of the isoenzyme was later narrowed to "single enzymes which exist in multiple molecular forms or isoenzymes within the cells of a single organism" by MARKERT and APELLA (61). Genetic control could be monogenic as well as polygenic, where they differ essentially in primary structure.

Although the Markert and Apella definition has not been fully accepted (6,103,43), it is widely used. In most cases the difference between isoenzymes is based on difference in primary structure, which is generally genetically determined. Thus a point mutation leads to a single amino acid substitution and can lead to an isoenzyme pair (sickle cell hemoglobin).

Other chromosomal events, such as crossing over, lead to duplication, or alteration of amino acid sequences or even

entire polypeptide chains, and can generate isoenzyme families which are under polygenic control (106).

Other causes for the occurence of isoenzymes may be conformational differences and hybridization (106), where different subunits associate often in diverse combinations. The LDH system is an example of tetrameric association (62).

It is possible that a differential catalytic role may exist for isoenzymes, but their occurence may also be purely accidental (77). Isoenzymes have also been related to tissue differentiation. DVORAK (29), for example, could detect thirteen different peroxidase isoenzymes in the pumpkin plant (Cucurbita pepo), with different isoenzymic patterns in different parts of the plant. In maize, (17), there are also peroxidase differences in various parts of the plant; four peroxidase isoenzymes were separated from endosperm, six from scutellum and radicula, and five from plemula. In corn leaves, different subcellular particle fractions showed different peroxidase isoenzyme patterns (5). No differences were found between the varieties of corn and their hybrids.

Evidence is accumulating that isoenzymes are used to direct metabolites along specific pathways (106). Such functions include negative feed-back (94), for example in aldolase, where two isoenzymes catalyze different directions of glycolysis. Allosteric control in the case of aspartokinase in E. coli (95) is another example.

Lately the occurence of isoenzymes has been related to environmental changes of the organism, such as changes in temperature, nutritional factors, etc. An example of isoenzyme-

induction by nutritional factors is seen in pumpkin plant growth (30). Pumpkins (Cucurbita pepo) were grown in normal and in Ca^{2+} deficient nutritient solution. After seven days the appearance of certain peroxidase isoenzymes was seen as a function of nutrition.

MCCOWN (65) reported changes of peroxidase isoenzyme patterns with different seasons of the year. Peroxidase of Dianthus leaves grown in summer and winter conditions, showed additional bands in plants grown under winter conditions. Tissue differences were also noted.

ZIEGLER et al. (109) reported peroxidase isoenzyme differences in peas cultivated in light and dark. There were also isoenzyme differences between tall and dwarf peas-plants.

Temperature, too, has a definite effect on induction of peroxidase enzymes. DE JONG et al. (27) reported such effects, after growing tobacco cell cultures for six days at 13°, 24°, and 33°C. At 13°C a peroxidase was detected which was absent at the other temperatures and the major 24°C isoenzyme was not found at 13° or 35°C. A minor isoenzyme was produced at all three temperatures.

One of the most intriguing effects on peroxidase isoenzymes is alteration of the physiological state as, for example, in the aging process. RACUSEN (78) et al. noted age variation of peroxidase isoenzymes in bean leaves that suggested correlation with metabolic events in maturation and senescencence.

Changes in physiological and metabolic states produced by irradiation and infection can also alter enzyme patterns.

GIACOMELLI (39) reported that irradiation causes alteration of barley, which then shows different biosynthetic patterns and isoenzymes. Irradiated barley seeds produced four new peroxidase isoenzymes which were absent from non-irradiated plants. NOVACKY et al. (74) infected various plants (Nicotiana tabaccum, Vigna sinensis, Phaseolus aureus, Phaseolus vulgaris) with various kinds of viruses. Quantitative, but not qualitative changes in the peroxidase isoenzyme patterns were induced by infection and senescence, however the changes by senescence and infection were not identical. No new isoenzymes were induced by either infection or senescence. SOLYMOSY et al. (92) found that virus infected tissue of Phaseolus vulgaris and Nicotiana glutinua showed local lesions and the formation of new peroxidase components. A comparison of the change in the peroxidase isoenzyme-spectrum from various host-virus combinations indicated that the type of change is determined mainly by the host tissue, not by the virus. Lesion formation induced by the treatment of bean leaves with $HgCl_2$ was also accompanied by alterations of the peroxidase zymogram. The changes resembled, but were not the same as changes evoked by virus infection.

F. Peroxidase Molecular Structure

With purified isoenzymes, primary structure analysis of peroxidase will be of greater significance. A peptide map of lactoperoxidase tryptic digest has been published (83), and the separation of peptic digest peptides from performic acid oxidized JRP a has been accomplished (71). The amino acid composition of chloroperoxidase (72), HRP (90), cyto-

chrome c peroxidase (34), myeloperoxidase (88), myoglobin from sperm whale (32), and cytochrome c from Baker's yeast (73), have been investigated and listed in table 3, page 17. The correlation of the amino acid composition among the peroxidases seem to be quite high even without considering alleles (25). Especially close are those of chloroperoxidase and JRP a. The latter fact is supported by a similarity of other chloroperoxidase-JRP properties such as isoelectric point, heat lability, and molecular weight. A connection of these facts with evolutionary hypotheses is quite intriguing since we are dealing with two organisms, one of higher plants, the other of lower fungi. Any hypothesis concerning evolutionary lines in plant peroxidases must be very speculative, however, because no sequences are yet known for these proteins.

The long range goal of this thesis is to begin supplying such information and thus form a basis for better understanding of protein evolution patterns.

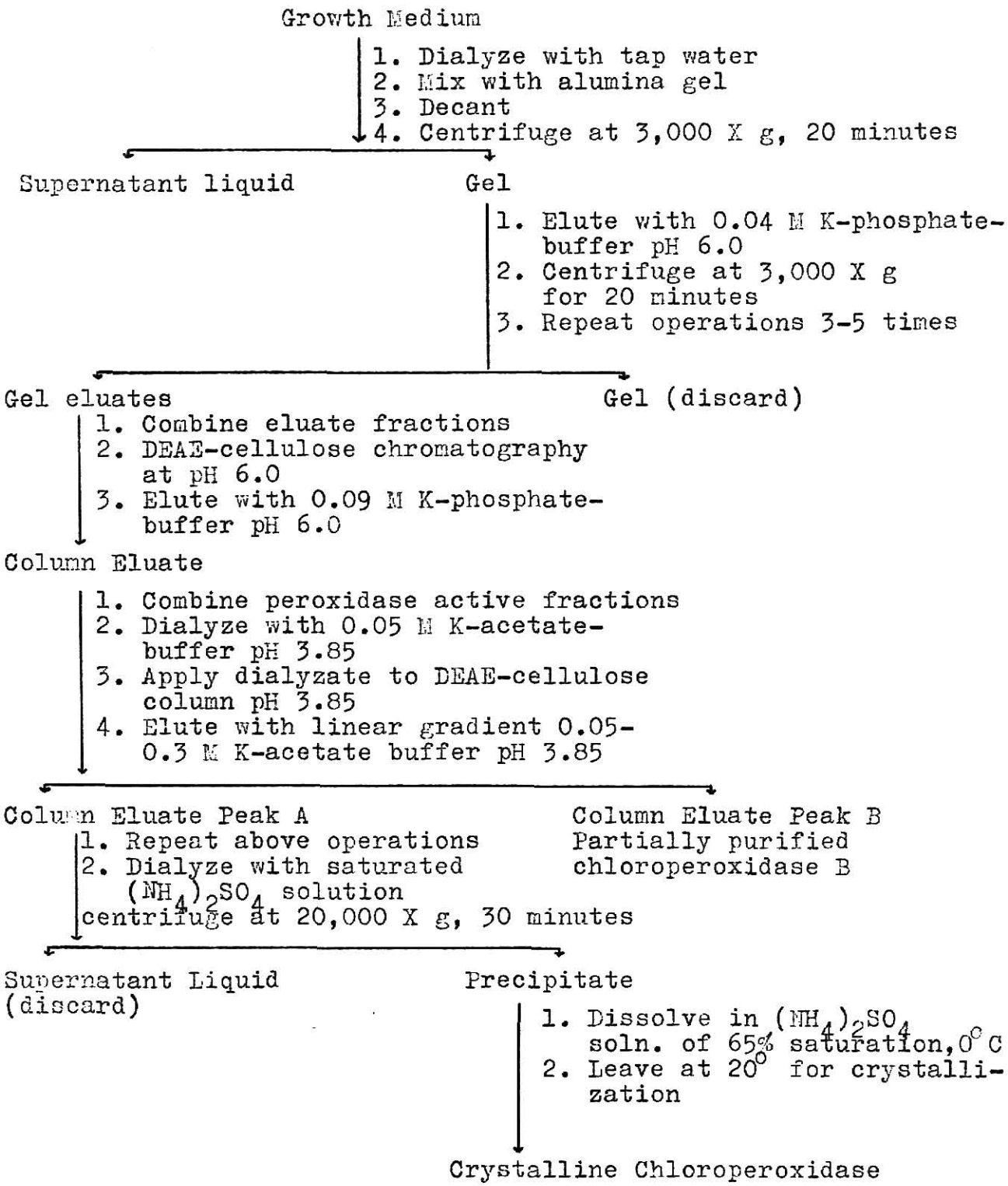


Table 1. Purification Procedure of Chloroperoxidase according to SAE (84). A flow sheet.

30 l Growth Medium
↓ concentrate 10X in vacuum evaporator (45°)
Growth Medium Concentrate
↓
1. Dialyze against 40-liter changes of deionized water at 4°
2. Freeze-dry
↓
Freeze-dried Powder
↓
1. Ethanol fractionation (-10°) 45% centrifuge at 15,000 X g
↓
Precipitate (discard) | Supernatant Liquid
↓
Ethanol fractionation (65%)
↓
Precipitate | Supernatant Liquid (discard)
↓
1. Dissolve in 0.1 M phosphate buffer pH 6.0
2. Column chromatography on DEAE-Sephadex A-50; elute with 0.1-0.2 M linear gradient of phosphate buffer pH 6
↓
Minor Peak of Chloroperoxidase activity | Major Peak of Chloroperoxidase activity
↓
1. Dialyze against 0.01 M phosphate buffer pH 5.0
2. Column chromatography on calcium phosphate gel-cellulos
. elute with 0.01-0.2 M linear gradient of phosphate buffer pH 5
↓
Eluate
↓
1. Precipitate with sat. $(NH_4)_2SO_4$
2. Redissolve in 0.01 M phosphate buffer pH 6 to ca. 8 mg/ml
3. Dialyze against 60% sat. $(NH_4)_2SO_4$
4. Adjust to 70% saturation and allow to crystallyze at 4°
↓
Crystalline Chloroperoxidase
(yield: 3X crystallized, 4 mg)

Table 2. Purification Procedure of Chloroperoxidase according to MORRIS and HAGER (72). A flow-sheet.

Table 3. Comparison of Peroxidases and other Hemoproteins

	CPO	JRP	HRP	CcP	MYP	Myogl	Cyt c	CPO 4.50
AMINO ACID								
met	2	4	3	6	12	2	2	1
cys	2	10	6	1	13	0	3	2
lys	4	7	6	21	11	19	16	6
arg	6	11	18	9	35	4	3	6
his	7	4	1	5	4	12	4	11
ile	9	15	12	8	16	9	4	5
tyr	10	3	3-6	12	8-10	3	5	4
val	12	20	15	12	17	8	3	4
phe	13	18	18	16	17	6	4	8
gly	14	29	18	23	26	11	12	9
thr	17	27	24	14	23	5	8	8
leu	20	36	30	23	43	18	8	11
pro	23	17	18	15	31	4	4	13
ala	24	36	24	16	26	17	7	18
glu	26	26	21	27	37	14	7	11
gln	-	-	-	2	-	5	2	-
ser	33	51	8	14	21	6	4	10
asp	39	51	16	42	51	6	4	18
asn	-	-	-	-	13	2	7	-
trp	-	2	0	6	-	2	5	-
pro-OH	-	12	-	-		-	-	-

- Not determined

CPO=Chloroperoxidase, JRP=Japanese radish peroxidase a
HRP=Horse radish peroxidase, CcP=Cytochrome c peroxidase
MYP=Myeloperoxidase, Myogl=Myoglobin, Cyt c= Cytochrome c from yeast, CPO 4.50= Chloroperoxidase with isoelectric point at pH 4.50

III. EXPERIMENTAL PROCEDURES

A. Guaiacol Assay for Chloroperoxidase

Chloroperoxidase has been determined quantitatively by SAE (84) using a modified MAEHLY and CHANCE (59) method. A similar method, further modified for increased sensitivity and used in the following experiments, is described below. The assay is based on the absorbance increase at 470 mu following peroxidation of guaiacol.

A standard 12mm X 75mm culture tube contained 2 ml of a buffer guaiacol mixture (1 part 0.54 M Na-acetate buffer pH 5.5, 5 parts of 0.027 M guaiacol, 5 parts of distilled water), 0.5 ml of enzyme solution and 0.2 ml of 0.0135 M H_2O_2. The hydrogen peroxide was added to initiate the reaction. One enzyme unit is defined as 200 times the reciprocal seconds to reach an absorbance of 0.2 (470 mu, Coleman Junior spectrophotometer). Dilution factors are multiplied in as required. Reaction time range was 15 to 200 seconds. Typical results are shown in figure 1, page 19.

B. Determination of H_2O_2 Concentration

A standard H_2O_2 stock solution was prepared by titrating H_2O_2 with $Ce(SO_4)_2$ according to FURMAN and WALLACE (37). The reaction is $2Ce(SO_4)_2 + H_2O_2 \longrightarrow Ce_2(SO_4)_3 + H_2SO_4 + O_2$. A Corning Model 12 pH meter , Pt electrode and a saturated KCl-Calomel reference electrode were used to follow the E.M.F. change. Ten ml of approximately 0.03 M H_2O_2 (30% stock diluted 1/300) was added to 25 ml of 3N acetic acid and titrated with 0.02 N $Ce(SO_4)_2$.

In a typical titration (fig. 2, p. 20), the inflection

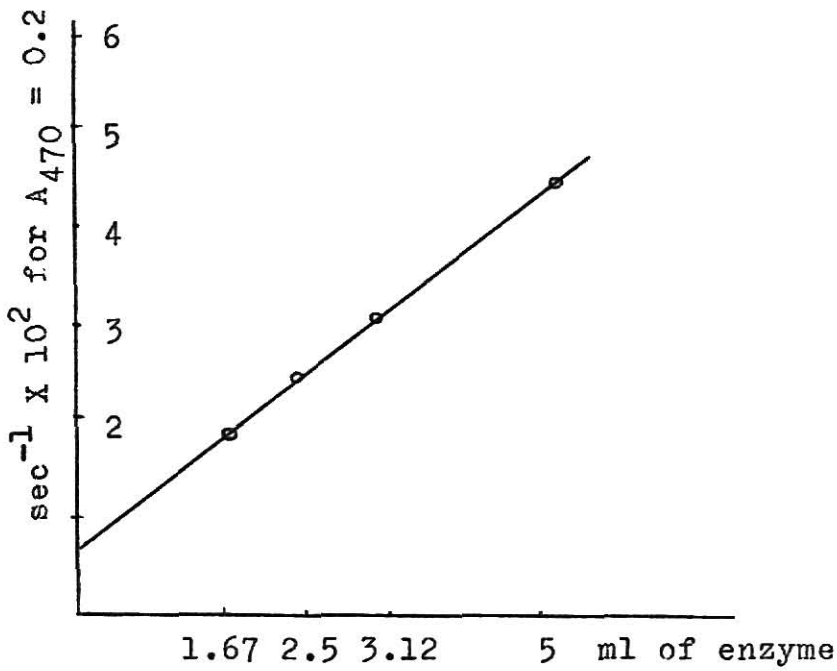


Fig. 1: Guaiacol assay for chloroperoxidase 0.2 M acetate buffer pH 5.5, 2 X 10^{-3} M guaiacol, 1 X 10^{-3} M H_2O_2, 1/t versus ml of enzyme

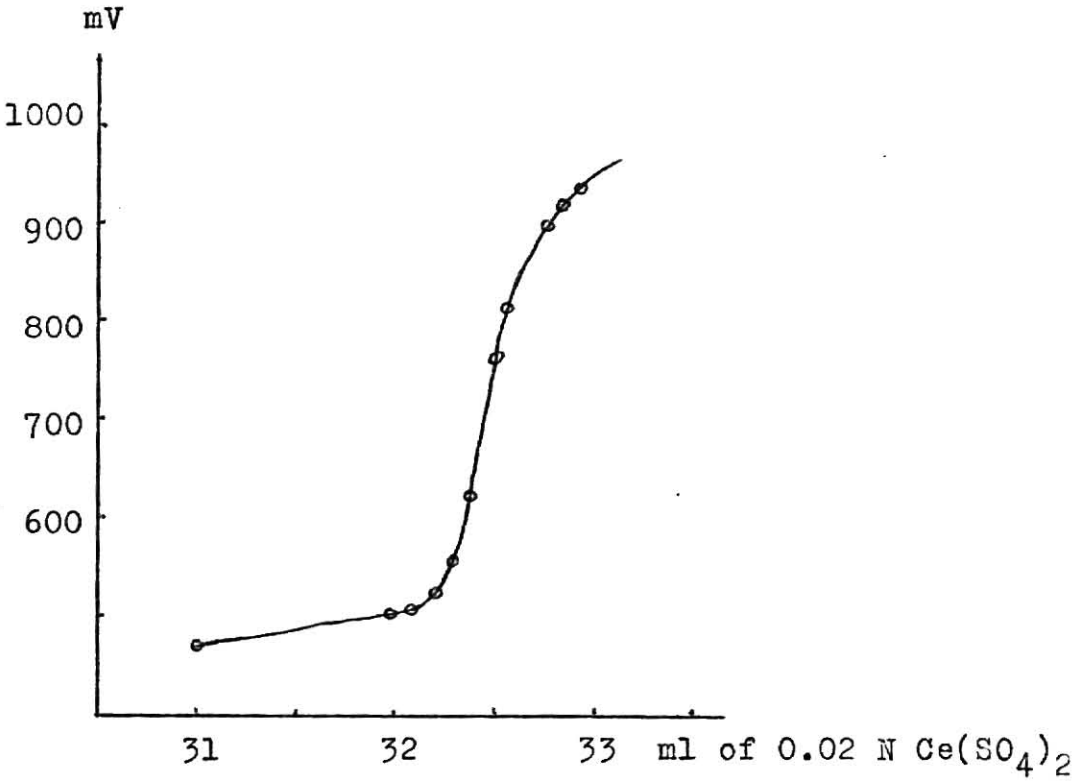


Fig. 2: Titration of H_2O_2 with standard ceric sulfate in 3 N acetic acid

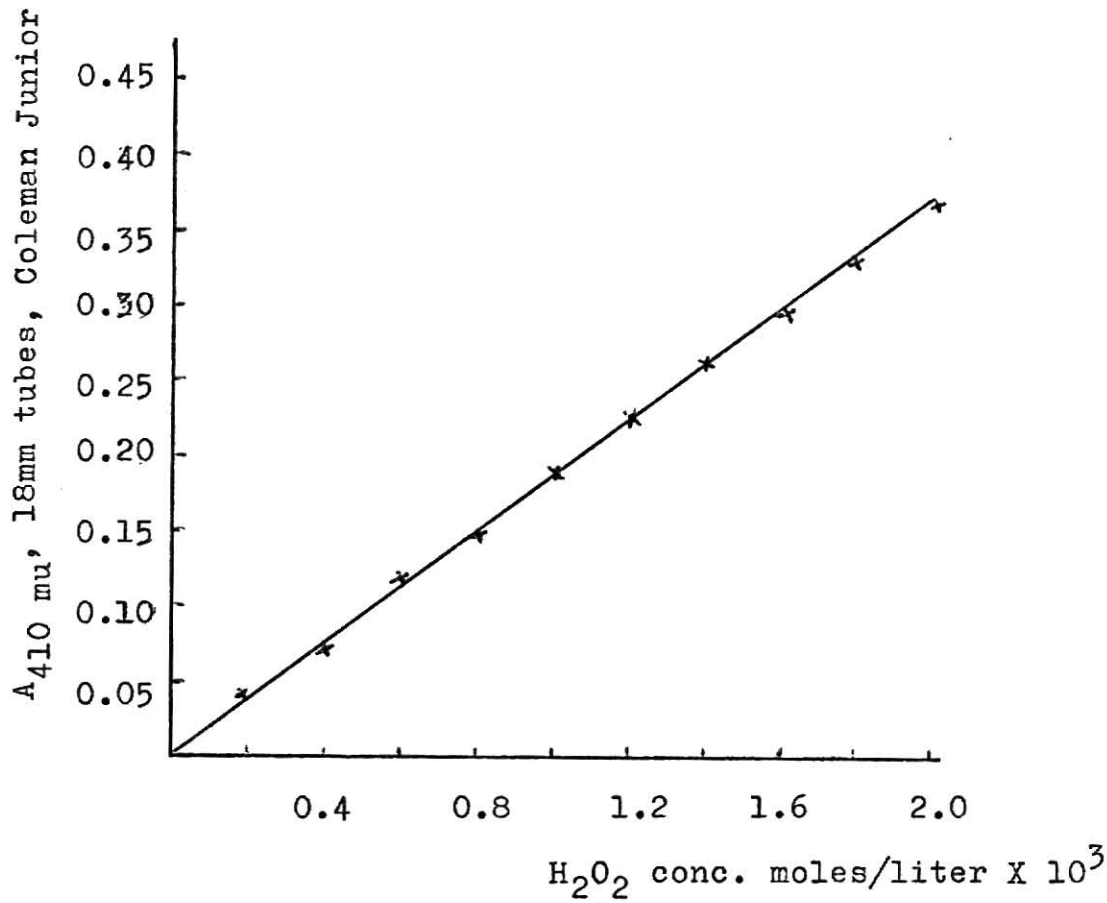


Fig. 3: H_2O_2 colorimetric standard curve, Titanium reagent

point 793 mv. was obtained corresponding to 32.44 ml of 0.02 N $Ce(SO_4)_2$ for a 10.0 ml aliquot of H_2O_2. From the relation 32.44 X 0.02 = 10.0 X H_2O_2 concentration, a normality of 0.06488 is calculated. The H_2O_2 is therefore 0.0324 molar.

The standardized H_2O_2 was used to establish a colorimetric assay using titanium sulfate (33). Titanium reagent was prepared by digesting 1 g reagent TiO_2 in 100 ml conc. H_2SO_4 for 16 hours at 150° C. Cool, dilute to 500 ml and filter through a sintered glass funnel. Dilute 125 ml of that stock solution to one liter with distilled water to prepare the working reagent used below. One ml of H_2O_2 and 5 ml of titanium reagent are added to standard 18 X 150mm tubes. The yellow color (pertitanate) is measured in a Coleman Jr. spectrophotometer at 410 mu (see fig.3, page 21).

C. Determination of Protein Concentration

A modified LOWRY et al. method (57) as employed by LOUIE (56) was used to determine protein. The concentration of crystalline bovine plasma albumin was determined spectrophotometrically from the known extinction coefficient $E^{1\%}_{1cm}$ of 6.60 at 280 mu (23). Using that relationship, protein concentration (mg/ml) = A_{280}, 1 cm X 1.515. To each ml of protein solution (0.02 - 0.2 mg/ml) add 1 ml of alkaline copper reagent, mix and let stand for 10 min. at room temperature. A diluted FOLIN (65a) reagent is then rapidly added with a plunger dispensor and the tubes are heated at 50°C for 10 min., cooled to room temperature and absorbance read at 650 mu in a Coleman Junior spectrophotometer. A typical standard curve is shown in figure 4, page 23.

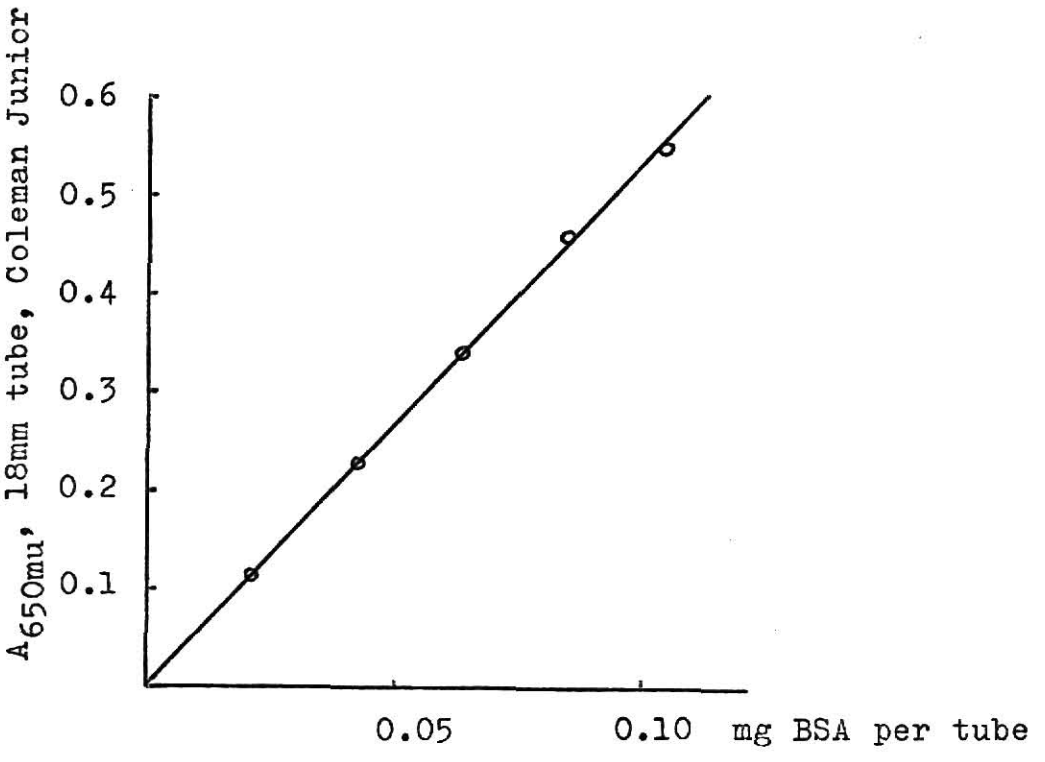


Figure 4: Protein standard curve; modified Lowry et al. method

D. Growth of Caldariomyces fumago

The maintenance of C. fumago cultures used in this laboratory has been described by SAE (84). The same cultures were first propagated on oat meal-agar slants with monthly transfer but were later maintained on a vitamin supplemented agar to be described. In a preparation for large scale submerged cultures, stock mycelia, homogenized in sterile distilled water, were used to innoculate agar slants in 8 or 16 oz. bottles. After 6 - 16 days of growth at room temperature the mycelial pad was scraped off, suspended in sterile distilled water (approx. 50 ml H_2O per 8 oz. bottle equivalent) and homogenized in a Lourdes Multimixer at maximum speed for 30 sec. The final yield of homogenate was usually about 55 ml from one 8 oz. equivalent of mycelia. That volume was used to innoculate 10 - 12 liters of H-CD medium: Hager's modified Czapek-Dox medium (see page 27),or equivalent in a 50 liter roller bottle. Proportionally smaller aliquots of homogenized mycelia were used to innoculate 2.8 liter Fernbach shake flasks, each containing 1 liter of H-CD medium or equivalent. In some cases 1 liter flasks were used, each with 300 ml of medium. The vessels had been autoclaved 30 min. (50 liter bottles) or 20 min. (Fernbach flasks) at 15 psi (121°C.) and cooled to room temperature before innoculation. Numerous supplements to and variations of the basal H-CD medium will be described in the results section.

All growth experiments with submerged cultures were carried out in a constant temperature room at 23°C. Fernbach flasks were shaken on an Eberback Model 6140 rotary platform shaker

usually with tiers of six flasks each. The minimum speed setting was still too fast for a 12 liter - 2 tier load and additional control was provided by voltage reduction through a Variac. A setting of 90 volts gave save, gentle swirling. Large scale culture was achieved by supporting 50 liter carboys horizontally on three pairs of three inch diameter rubber rollers. Power input was at 375 RPM. In either case the gentle agitation prevented large mat formation. A thin layer of liquid adhering to the rotating carboy walls provided effective aeration in those vessels. All vessels were capped with loose fitting aluminum foil to prevent contamination but allow oxygen exchange.

The growth medium pH gradually increases with time. Chloroperoxidase is completely inactivated at pH approximately 7 or greater and therefore the growth medium is best harvested at pH 6 - 6.5. To harvest growth medium the culture is filtered through cheese cloth with squeezing. The enzyme-rich dark colored filtrate can be stored in a cold room at 4^{o}C. for at least one week without loss of activity. After 2-3 months, however, activity will decrease to zero, especially in preparations with high initial activity.

IV. OPTIMIZATION STUDIES, PRODUCTION OF CHLOROPEROXIDASE

A. Introduction

Probably the earliest study of optimal growth conditions for Caldariomyces fumago was by ZOPF (110), who hypothesized that honeydew secreted by aphids provided most of the nutrients for this fungus. He showed that glucose concentration was important and that too little or too much glucose inhibited growth of C. fumago. Later studies by CLUTTERBUCK et al. (21) were concerned mainly with chlorometabolite production and they utilized a simple Czapek-Dox medium. HAGER modified that medium (72) to optimize production of the chloroperoxidase by C. fumago and made the very important discovery that yeast supplementation resulted in chloroperoxidase excretion into the medium. That result has been confirmed by SAE (84) in studies carried out in this laboratory. Some insights gained by Sae's work include the fact that aeration and possible light can influence the production of chloroperoxidase in C. fumago growth medium. In his experiments enzyme first appeared after 80 - 125 hours incubation, increased to 12 - 28 units/ml, and levelled off. On several runs however, Sae observed considerable variation in amount enzyme produced. An average result was 20 units/ml, but in one case 60 units/ml was obtained. That observation was the principle stimulus for my present study of growth conditions affecting chloroperoxidase production by C. fumago. It was desirable to improve both the reproducibility of enzyme yield and the total units of enzyme obtained.

B. Composition of H-CD base medium

The substitution of glucose for sucrose is the principal difference between CD and H-CD base medium. The composition of H-CD base medium is:

glucose	50 g
$NaNO_3$	2 g
KH_2PO_4	1 g
KCl	0.5 g
$FeSO_4$ X $7H_2O$	0.01 g
$MgSO_4$ X $7H_2O$	0.5 g

All values are per liter of water. Subsequent mention of H-CD base medium refers to the above solution.

C. Preliminary Experiments

It was first necessary to reproduce earlier work and this was accomplished. Shake flasks innoculated with oat-agar cultures were supplemented with increasing amounts of yeast extract. (1, 3.3, and 10 g of Fisher Cat. J-2013-C per liter of H-CD base). Age of the cultures was also tested. Peak activity increased with increasing yeast concentration and a subculture 16 days old was 2.2 times more active than a 60 day old culture (fig. 5, page 28). Enzyme yield was similar to that obtained by SAE (84). This experiment firmly established that the enzyme production was controlled by the nutrient level as well as the variability of the innoculum.

Similar experiments tested aeration and a substitution of K_2HPO_4 for KH_2PO_4 in the H-CD base medium. As shown in fig. 6, page 29, aeration is required for enzyme production and a substitution with the dibasic phosphate gives decreased yields.

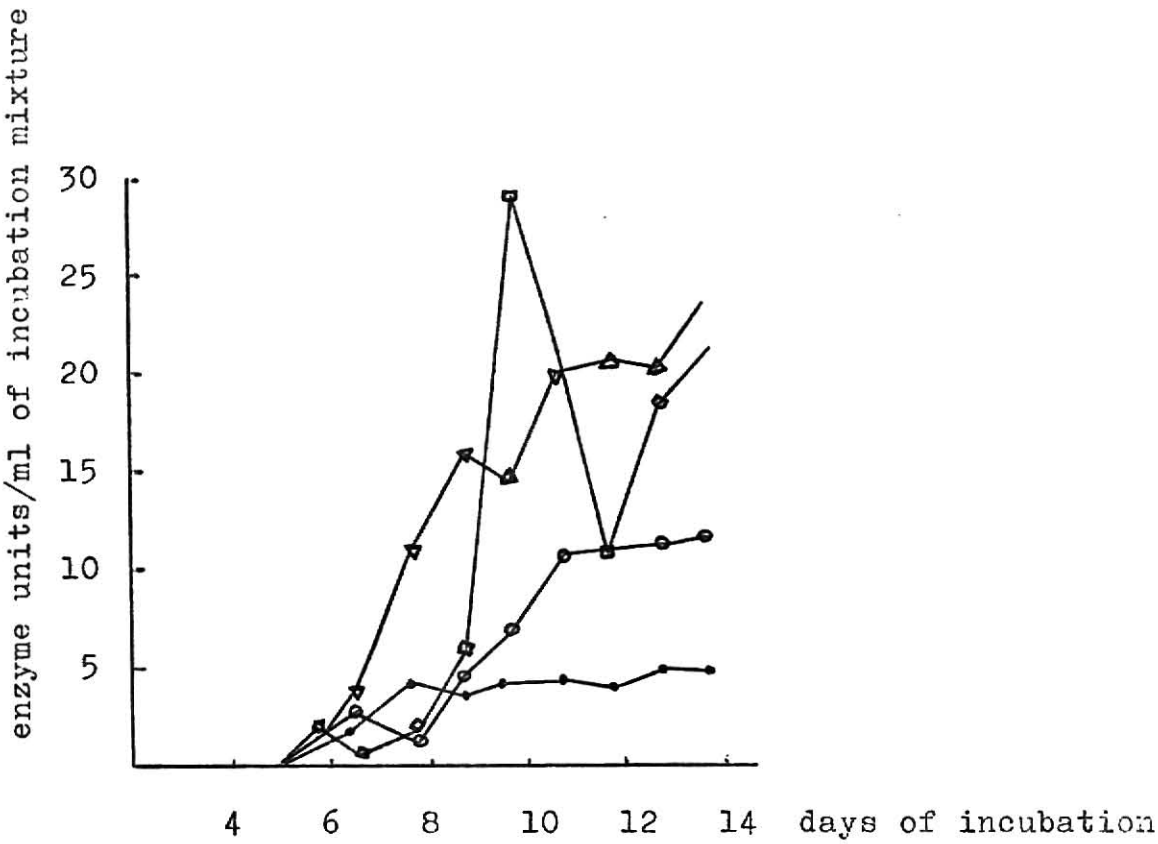


Figure 5: Showing the effects of growth medium on the synthesis of chloroperoxidase. (o—o old culture, □—□10 g yeast, ●—● 1 g yeast, △—△ 3.3 g yeast)

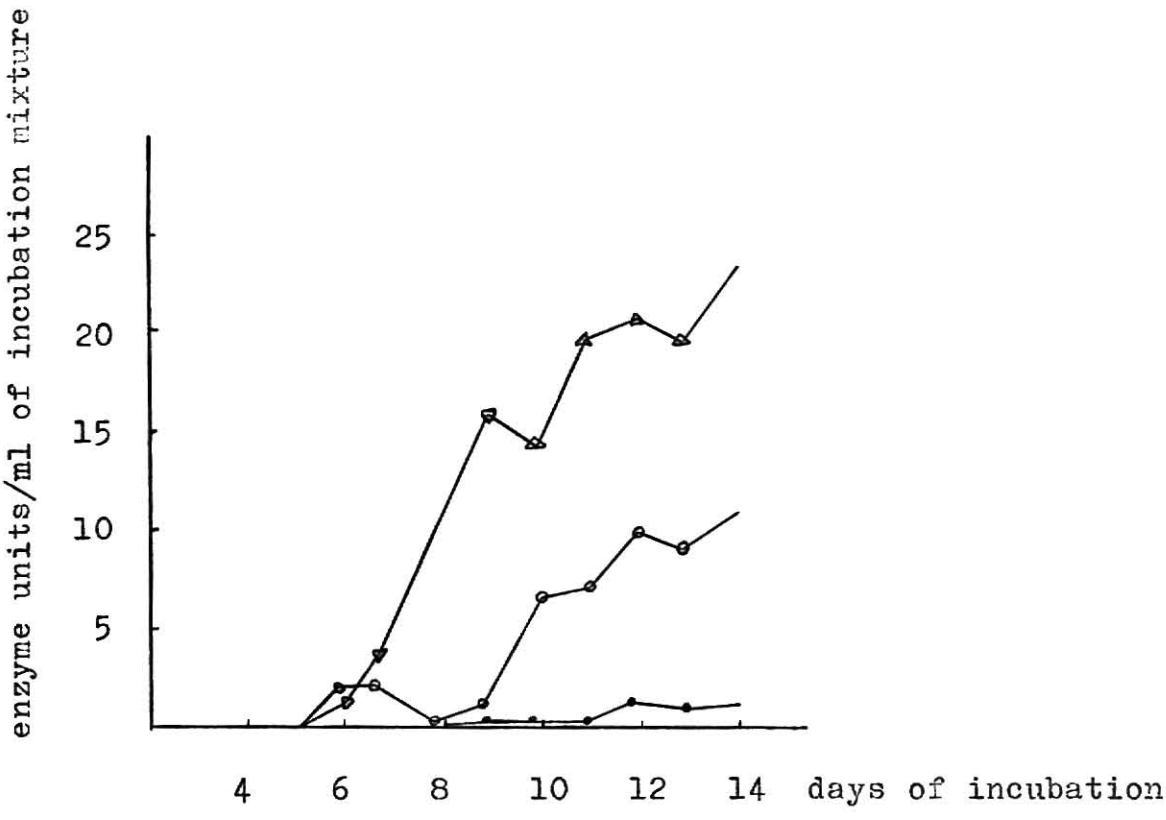


Figure 6: The graph shows enzyme production in relation to growth media variation. (△——△ control, 3.3 g yeast extract, o——o K_2HPO_4 instead of KH_2PO_4 •——• aeration limitation).

D. A Test of some Hypotheses on Chloroperoxidase Production: Honeydew, Procaine, Glutamic acid, DDT, Heme.

Having established that nutritive factors markedly influenced enzyme yields, replacement for yeast extract or modifiers of enzyme production were sought next. ZOPF (110) had shown the saprophytic character of Caldariomyces fumago and demonstrated that the fungus nutrient requirements were completely met by aphid honeydew. CRAIG (22) has analyzed honeydew amino acid and carbohydrate composition and a honeydew substitute was considered as possible replacement for yeast extract. Procaine (89) has been proven to enhance nucleic acid synthesis and may thus indirectly increase enzyme production. Glutamic acid was considered as a possible nutrient because of its central position in amino acid metabolism. DDT has enzyme inducing properties in some systems (1,64). In addition it was of interest to see if the fungus would survive DDT presence and possibly metabolize the chloro-group. The levelling off of enzyme production is typical of control cultures with yeast supplement. It was thought that iron, heme, or heme precursors might overcome such limitations.

Experiments were carried out as in section B using a yeast extract level of 3.3 g per liter and inoculum from oat-agar bottles. A honeydew substitute was prepared by dissolving 1.40 g glutamine, 0.24 g histidine, 1.40 g asparagine, 0.53 g glutamic acid, 0.35 g tryptophan, 0.24 g tyrosine, 0.06 g leucine, 0.05 g isoleucine, 0.08 g phenylalanine, 0.17 g serine, 0.07 g proline, 0.10 g threonine, 0.06 g valine, 0.01 g β-alanine, 0.06 g alanine and 0.01 g aspartic acid in one liter of growth medium. Other

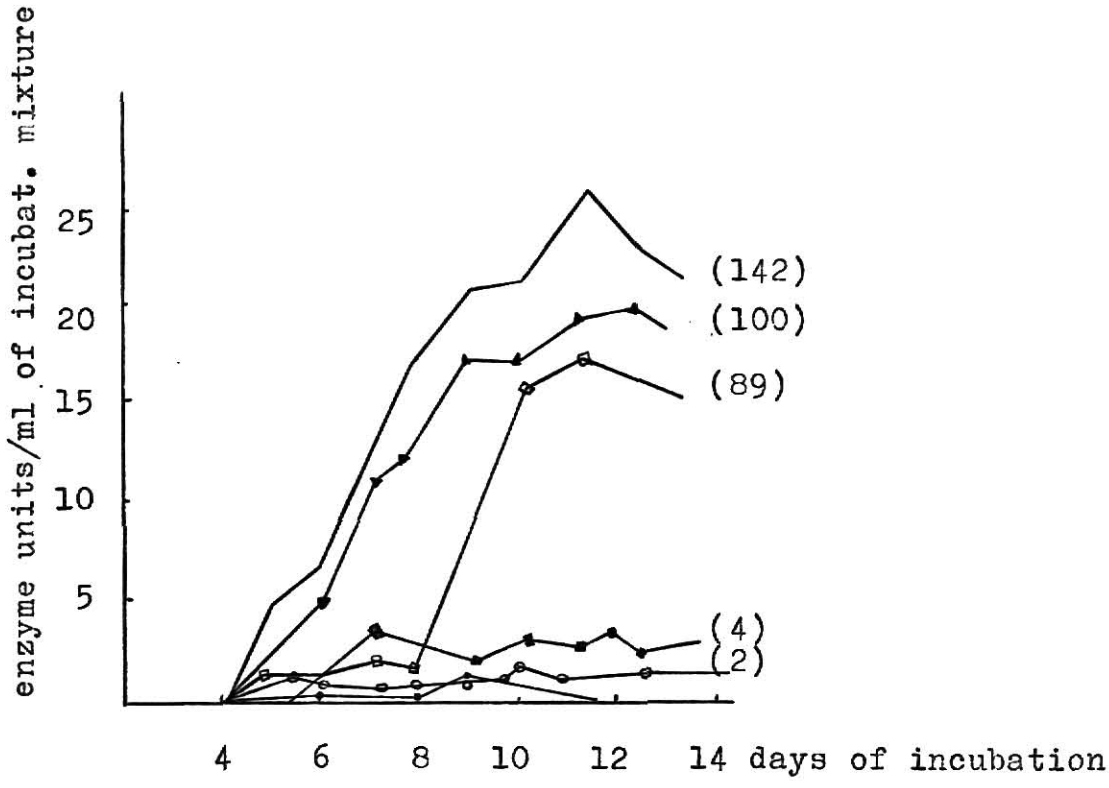


Figure 7: The plot shows enzyme production in relation to growth media variation. Relative values of peak activities are in parentheses.

—— procaine, ▫——▫ DDT, ▲——▲ control,

●——● glutamic acid, ●——● honeydew, ■——■ heme.

variables were added as solids: 0.2 g procaine, 10 g glutamic acid, 1 g DDT, and 0.23 g heme all per liter. All variations except honeydew were in addition to 3.3 g yeast extract per flask. The honeydew flask contained no yeast extract. The results are seen in fig. 7, page 31. Relative values of peak activities are in parentheses.

Conclusions are that procaine stimulates considerably and DDT inhibits slightly. Somewhat surprising, added heme is a strong suppressor of enzyme production in the growth medium. A honeydew substitute (amino acid mixture) gives no activity and cannot replace yeast extract. Glutamic acid inhibits enzyme production completely. It may be that high concentrations of glutamic acid shift the fungal metabolism to alcohol production because a distinct alcohol aroma was detected in the glutamic acid supplemented flasks.

E. Further Studies on Growth Medium Variations

(Glucose concentration, Gluconolactone, Procaine concentration, citrate buffer, light versus dark, agar variations with DDT and procaine).

The experiments were done as in the previous section except one liter flasks were employed. Also, some variation in the agar medium was tried. Effects had been noted with procaine in liquid culture, so some bottle slants were grown in presence of 1 mg procaine per ml of oat-agar. Other slants contained 0.5 mg DDT per ml of oat-agar.

Glucose optimum concentration was an important part of this experiment. SAE (84) had mentioned the possibility of a light effect and this was also investigated. Gluconolactone was in-

cluded to investigate its effects on the Entner-Doudoroff and the Hexose-Monophosphate-Shunt pathway (80). One sample was grown in a 0.01 M Na_2HPO_4-citric acid buffer at pH 3.1 to investigate chloroperoxidase production at constant pH.

Hydrogen peroxide, as an essential hydrogen acceptor in the peroxidatic catalysis was daily added (0.5 ml of 1 M H_2O_2) to another sample.

Finally,a vitamin-metals combination was prepared as a substitute for yeast extract by dissolving 0.07 g of B_{12}, folic acid, pyridoxal, riboflavin, biotin, and ascorbic acid each in 300 ml of growth medium. Additionally, every flask (300 ml total volume) contained 0.033 g of $MnCl_2 \cdot 4H_2O$, $ZnSO_4 \cdot 4H_2O$, $CoCl_2$, $MgCl_2 \cdot 6H_2O$ each. The results are seen in figures 8-13, page 35 - 37. The relative values of peak activities are in parentheses.

The conclusions are that procaine and DDT present in the agar slants inhibit the production of chloroperoxidase; the procaine even more than DDT (see page 35, fig. 8).

In the glucose optimum experiment, concentrations of 5%, 10% and 20% glucose were tried. While the growth of the fungus was not inhibited by either concentration, 10% and 20% glucose almost completely suppressed the enzyme production. Although lower glucose concentrations than 5% have not been tried and referring to ZOPF (110), 50 g of glucose per liter of growth medium seems to be optimal.

Figure 9, page 35 shows the effects of light and darkness on the induction of chloroperoxidase. One flask was darkened after 17.5 days of incubation. Compared to the control (100%)

the darkened sample had only 53% of peak activity, indicating a significant influence of light on the production of enzyme.

The gluconolactone substitute for glucose shows some interesting results as seen in figures 10,12,13, page 36-37. While gluconolactone strongly inhibits peroxidase production when mycelium grown on oat-agar was used for inoculation (fig. 10, page 36), much less inhibition is noted when fungi grown on oat-agar either supplemented with DDT or procaine (concentrations as in the previous experiment) was used (see fig 12,13, page 37).

When the growth medium contained citrate buffer pH 3.1, the mycelium growth was not visibly inhibited, however the chloroperoxidase induction was almost completely suppressed. Again, as in the glutamic acid addition (see page 31), the metabolism seems to be shifted toward alcohol production, because a distinct alcohol aroma was detected in the citrate buffer containing flasks.

The H_2O_2 addition resulted surprisingly in a almost complete (99%) inhibition of chloroperoxidase production.

Vitamins as a substitute for yeast extract resulted in a detectable peroxidase activity, which however was very low if compared to the yeast extract control (see fig. 11, page 36). Again there was a difference in peroxidase activity in the samples containing the mold grown on normal oat-agar, on DDT containing agar and on procaine containing agar. The latter proved to show the highest activity of all three samples, with the DDT sample the lowest. The reason why the samples containing the vitamins show only a small percentage of the control

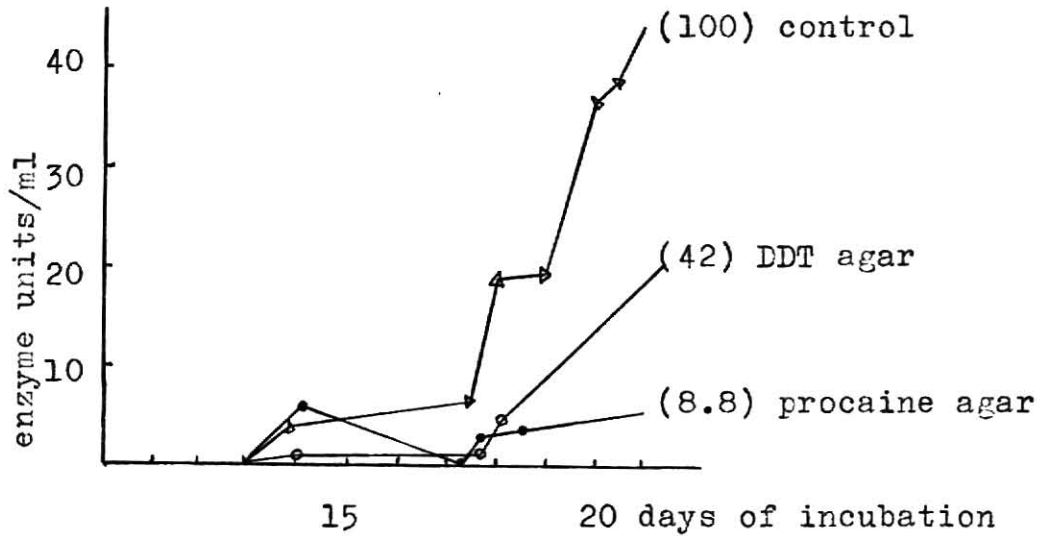


Figure 8.: Activity profile of chloroperoxidase production Mycelium grown on DDT-, procaine-, and oat-meal supplemented agar.

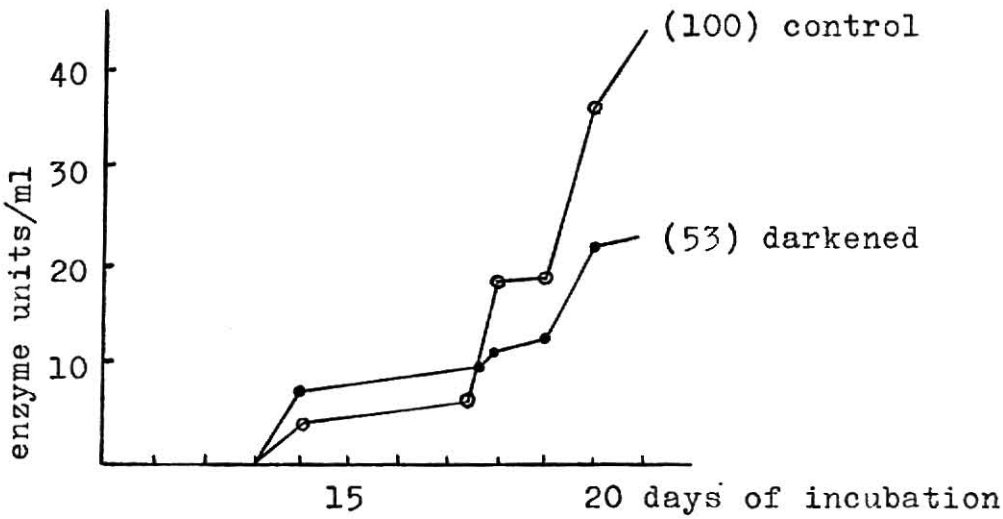


Figure 9.: Activity profile of chloroperoxidase production under darkened and light conditions.

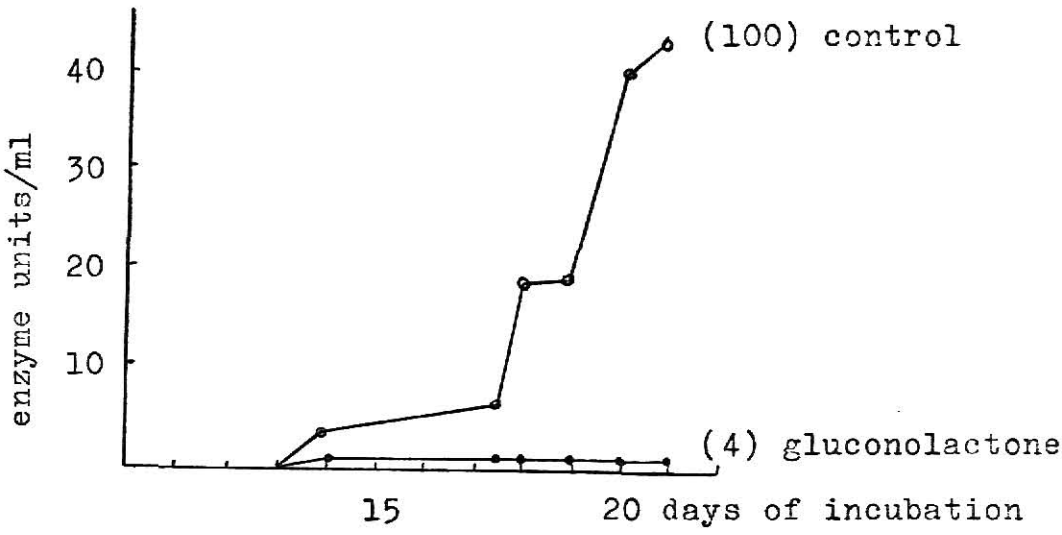


Figure 10.: Activity profile of chloroperoxidase production. Control contains glucose, other sample gluconolactone in the growth medium.

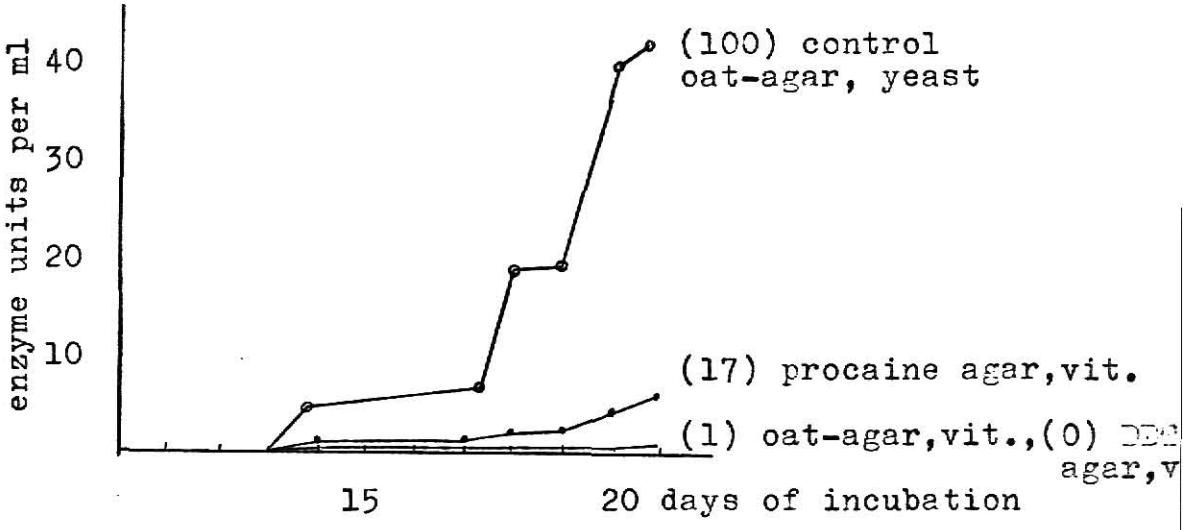


Figure 11.: Activity profile of chloroperoxidase production. Yeast extract replaced by vitamins except in control. Fungus grown on agar as stated (oat, DDT, procaine).

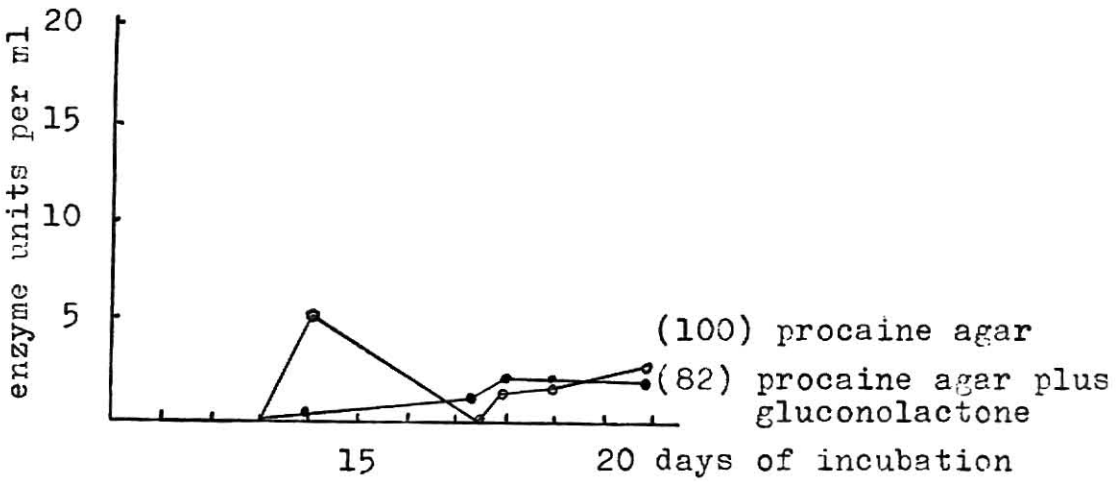


Figure 12.: Time-activity course of chloroperoxidase production, fungus grown on procaine agar was inoculated in glucose (100) and in gluconolactone.

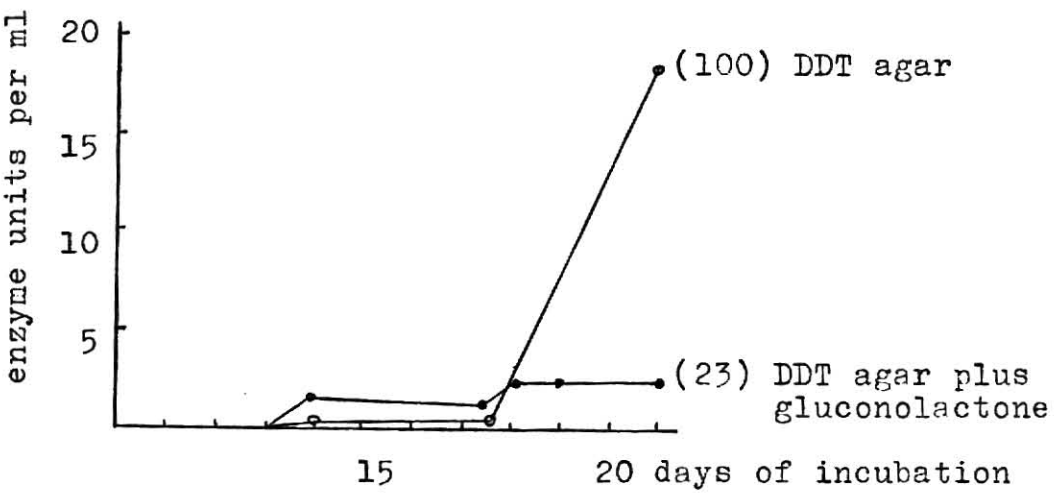


Figure 13.: Time table of chloroperoxidase production. Fungus grown on DDT agar was inoculated in glucose (100) or in gluconolactone supplemented growth medium.

activity could be due to the relatively low initial concentration of vitamins or to the fact that the experiment was stopped while the pH of the samples was still very low (about pH 3.2).

F. Studies on Growth Medium Variation using Vitamin Combinations

The experiments were carried out as in the previous section except that 2.8 liter Fernbach shake flasks were used containing one liter of growth medium. Also the yeast extract was substituted by vitamin combinations except in the control, which contained 3.3 g yeast extract per liter of growth medium. The mycelium used for inoculation was grown on oat-agar slants.

A vitamin substitute was prepared by dissolving 2 vitamin pills (Miles Laboratories) in 1 liter of growth medium. A second vitamin substituted sample was prepared by dissolving 3 mg thiamine, 3 mg riboflavin, 3 mg pyridoxal, 5 mg pantothenate, 20 mg niacinamide, 50 mg vitamin C, 50 ug vitamin B_{12}, 1 pill of Walgreen Wheat Germ Oil and 1 ml of Squibb Cod Liver Oil containing together vitamins D, A, and E, 5 mg biotin, 3 mg folic acid, 0.1 g $MnCl_2 \cdot 4H_2O$, 0.1 g $ZnSO_4 \cdot H_2O$, 0.1 g $CoCl_2 \cdot 6H_2O$, 0.1 g $MgCl_2 \cdot 6H_2O$, 0.1 g $Na_2MoO_4 \cdot 2H_2O$ in one liter of growth medium. A third sample was prepared by using the same vitamins as in the second sample however omitting the metals. Eight more flasks were prepared, the first containing just thiamine, the next containing thiamine, riboflavin, pantothenate, the third containing thiamine, riboflavin, pantothenate, vitamin C, the fourth containing thiamine, riboflavin, pantothenate, vitamin C and niacinamide, the fifth containing thiamine, riboflavin, pantothenate, vitamin C, niacinamide, and folic acid, the sixth containing all the vitamins of the fifth plus additionally pyridoxal, and the seventh containing

all the vitamins of the sixth flask plus additionally biotin, and the last containing all the vitamins from the previous flasks plus additionally the vitamins A, D, and E. The activity was followed daily and the experiment was stopped after 12 days. The flask order stated above is not necessarily the same as shown in fig. 14. In this experiment the influence of each vitamin on the production of chloroperoxidase can be detected by comparing with the corresponding control flask (the one which contains the same vitamin mixture minus the vitamin under question). The results are shown in figures 14-18, page 41-43.

Flask one which contained only thiamine shows considerable activity (see page 43, fig.18). The sample which contained riboflavin and pantothenate in addition to thiamine shows increased activity thus suggesting that either pantothenate or riboflavin or both stimulate production of chloroperoxidase. In a later experiment pantothenate will be assayed again (see page 49). The flask containing thiamine, riboflavin, pantothenate and ascorbic acid proves to have less peroxidase activity than the previous flask which contained the same vitamin combination except ascorbic acid. The conclusion is that ascorbic acid has an inhibiting effect on the production of chloroperoxidase. Samples containing additionally niacinamide and folic acid both show a subsequent reduction of the peroxidase peak activity so that the conclusion is, too, that niacinamide and folic acid cause suppression of enzyme synthesis. The suppression shown from folic acid however is very small (see fig.14), and it will be shown later (see page 52) that folic acid in theabsence of ascorbate and niacinamide can markedly stimulate enzyme production. The sample

containing thiamine, riboflavin, pantothenate, ascorbic acid, niacinamide, folic acid and pyridoxal has a large activity increase if compared with the previous sample and shows that pyridoxal plays an important role in chloroperoxidase production. Biotin, when added to the sample which contains all previously mentioned vitamins, causes a decrease of activity, proving its inhibitory effect. Vitamin A, D, and E, too, seem to suppress the synthesis of chloroperoxidase, as can be seen in fig.14. Vitamin B_{12} causes increased activity (see fig.16, page 42). The addition of metals seem to inhibit the enzyme synthesis (see fig. 15, page 42), although in the early days of incubation a slight stimulation of peroxidase activity could be observed.

The highest activity was shown by the sample containing the two vitamin pills (181 enzyme units/ml of incubation mixture), however this was almost matched by the composite vitamin mixture (flask 9, fig. 16). Most important is the overall conclusion that yeast supplement can be replaced by a known vitamin mixture while simultaneously doubling the yield of soluble chloroperoxidase.

This experiment predicts that yeast extract can be successfully replaced by only a few vitamins, mainly B_{12}, pyridoxal, and thiamine. Vitamin concentration may play a role however, since the sample containing the vitamin pills still produced the highest peroxidase activity (181 enzyme units per ml) and thepresence in the pill of biotin or niacinamide would most likely have some inhibitory effect.

G. Vitamin, Chloride and Iron Concentration Studies

This experiment was carried out as in the previous test.

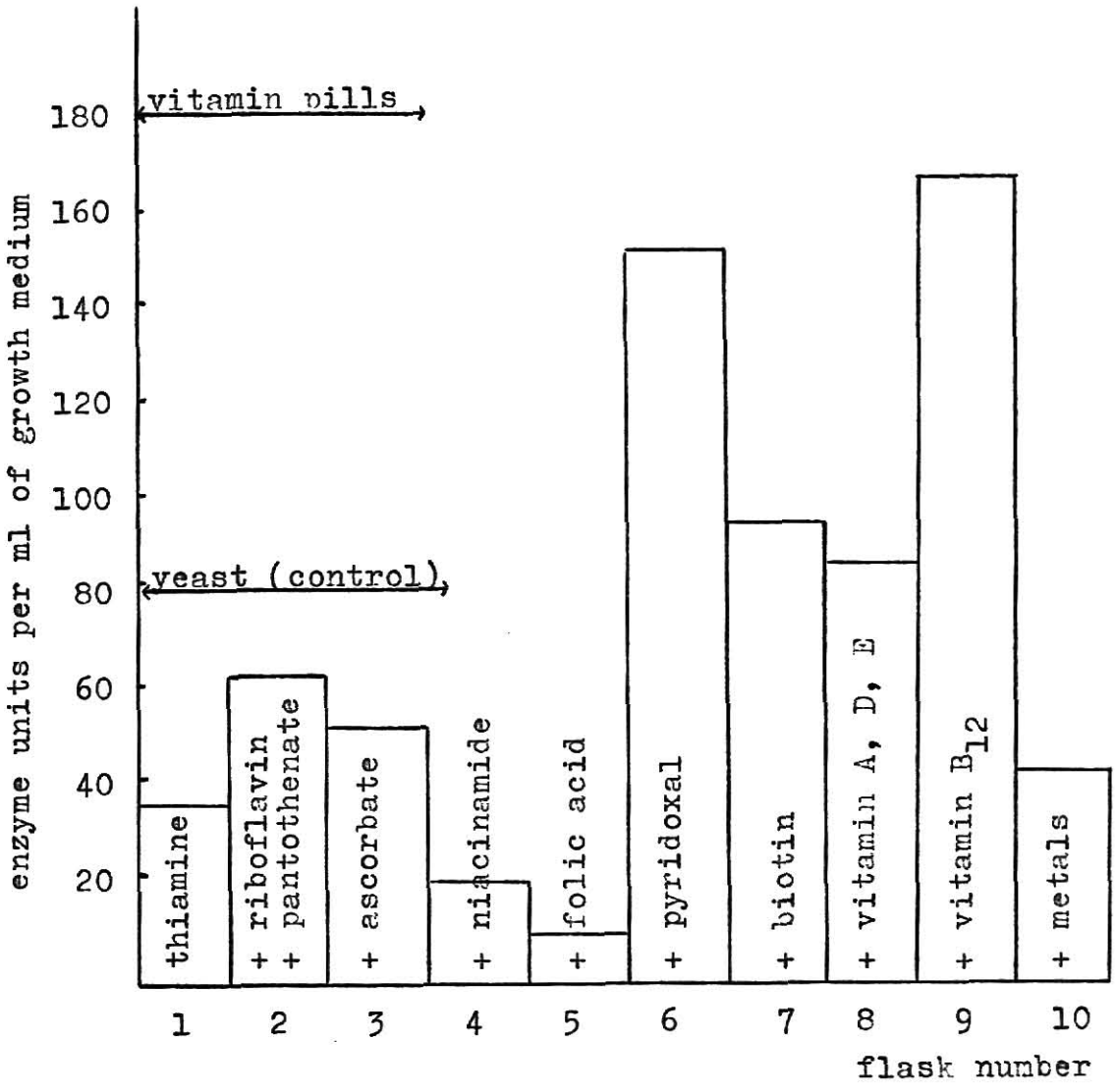


Figure 14: Effects of vitamin combination on chloroperoxidase synthesis. The constituents indicated within the bar segments are cumulative, i.e. each flask contains the named compound plus all the components of all prior flasks.

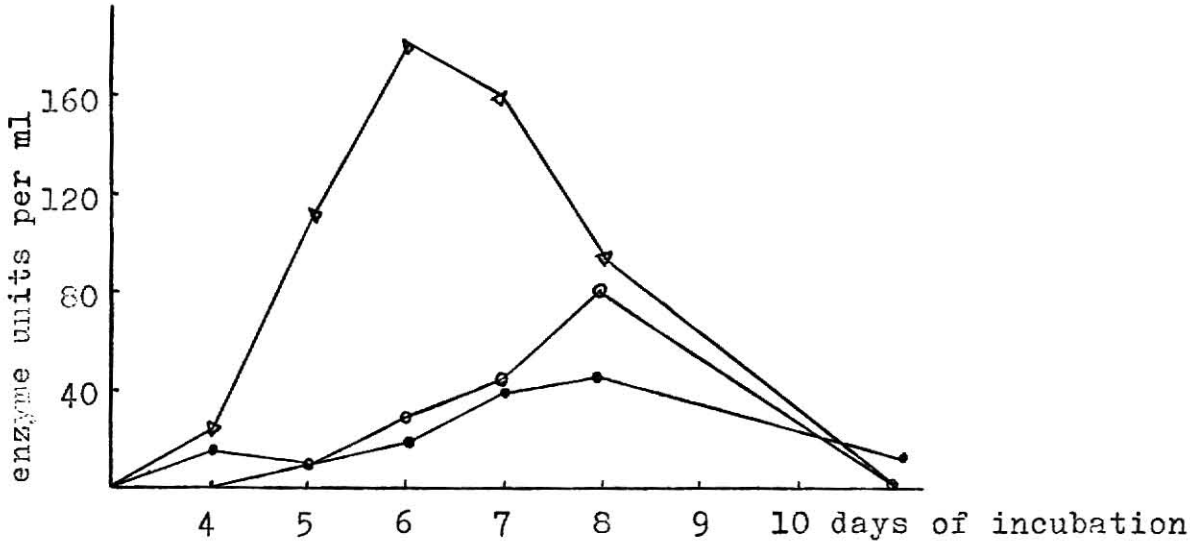


Figure 15.: Effects of vitamin combination on chloroperoxidase production. Δ—Δ contains two vitamin pills, ⊖—⊖ contains yeast extract, ●—● contains metals, B_{12}, vitamin A, D, E, biotin, pyridoxal, folic acid, niacinamide, ascorbate, pantothenate, riboflavin, thiamine.

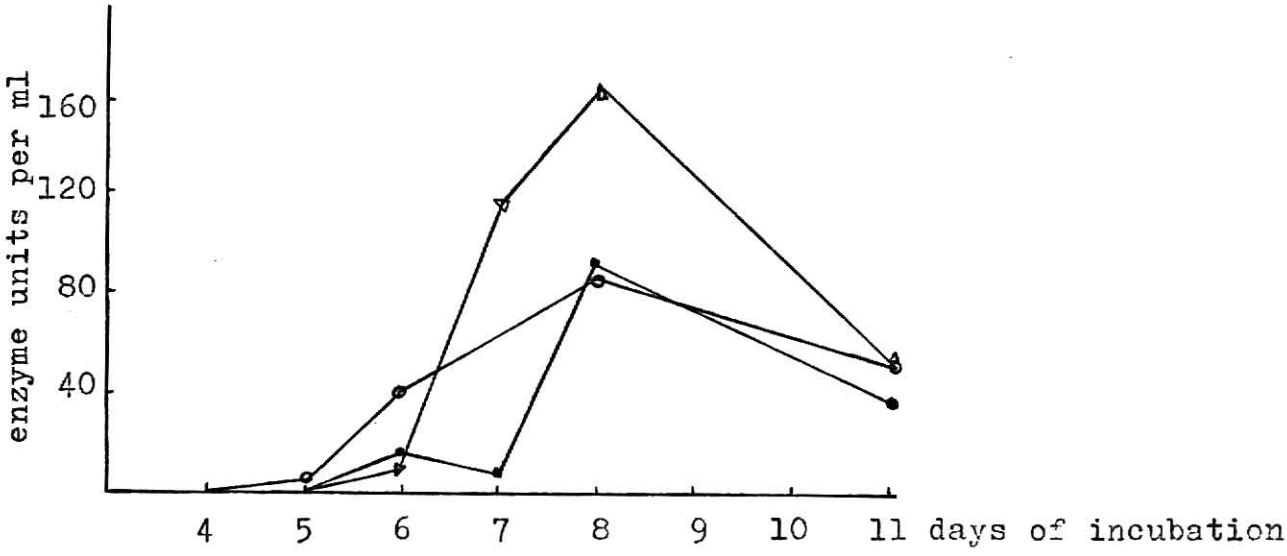


Figure 16.: Effects of vitamin combination on chloroperoxidase production, Δ—Δ (9) contains vitamin A, D, E, biotin, pyridoxal, folic acid, niacinamide, ascorbate, pantothenate, riboflavin, thiamine, B_{12}, ⊖—⊖ (8) contains vitamin A, D, E, biotin, puridoxal, folic acid, niacinamide, ascorbate, pantothenate, riboflavin, thiamin ●—●(7) contains biotin, pyridoxal, folic acid, niacinamide, ascorbate, pantothenate, ribofl., thiamine.

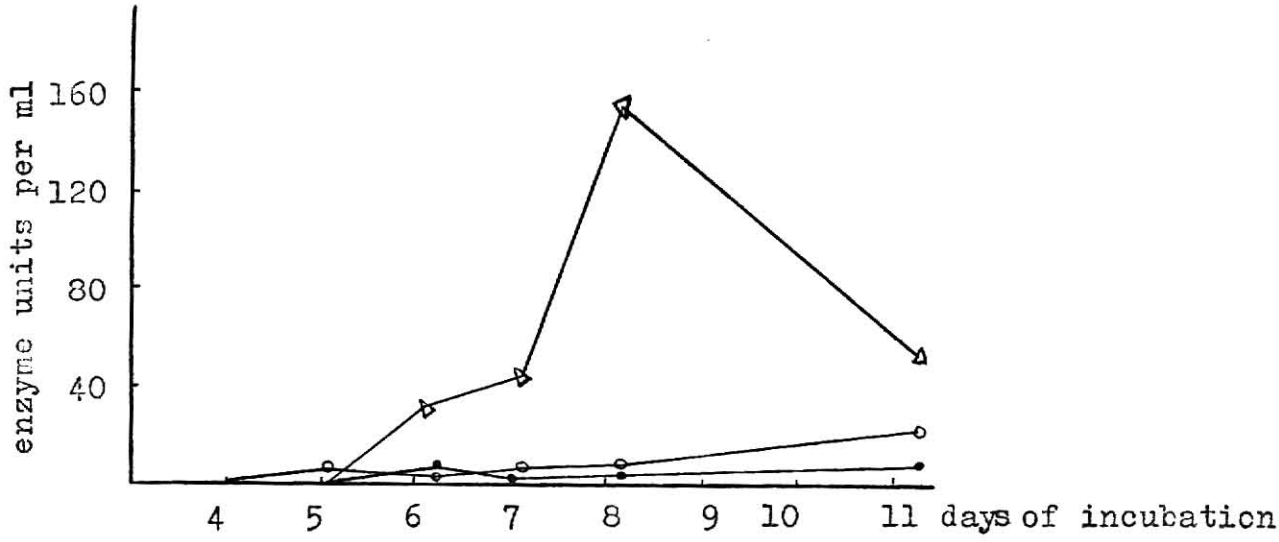


Figure 17.: Effects of vitamin combination on chloroperoxidase production. Δ—Δ (6) pyridoxal, folic acid, niacinamide, ascorbate, pantothenate, riboflavin, thiamine. ●—● (5) folic acid, niacinamide, ascorbate, pantothenate, riboflavin, thiamine, ϴ—ϴ (4) niacinamide, ascorbate, pantothenate, riboflavin, thiamine

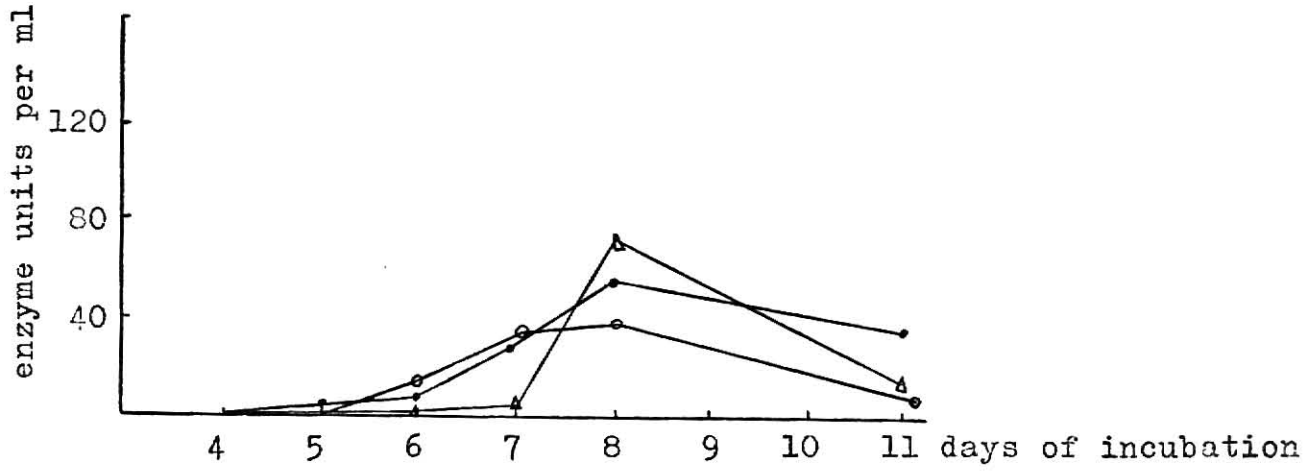


Figure 18.: Effects of vitamin combination on chloroperoxidase production.

●—● (3) ascorbate, pantothenate, riboflavin, thiamine

Δ—Δ (2) pantothenate, riboflavin, thiamine,

ϴ—ϴ (1) thiamine

Yeast extract was present only in the control. The other samples contained a vitamin replacement solution which provided: 50 ug B_{12}, 3 mg pyridoxal, and 3 mg thiamine per liter of growth medium. The vitamin stock solution containing the above mentioned quantity of vitamins per 2 ml of solution, was stored in the freezer, and shortly before use it was thawed and 2 ml aliquots were added to each liter of growth medium. All samples contained the modified H-CD medium except those which were to test chloride and iron concentration.

One sample was prepared by dissolving 50 mg of D(-) Mannitol in one liter of growth medium in addition to the vitamin stock concentration. Since vitamin B_{12} used for the yeast substitute was usedas 0.1% B_{12} in mannitol, this sample was a necessary control for the effect of mannitol on the enzyme production.

The vitamin tests were set up by dissolving in addition to the vitamin stock solution, 150 ug B_{12}/liter of growth medium for one sample, 9 mg thiamine/liter for another sample and 9 mg of pyridoxal for a third sample. A fourth flask contained the vitamin stock solution and additionally 150 ug B_{12}, 9 mg pyridoxal and 9 mg thiamine. These provided tests for three fold additional vitamin concentration either single or combined.

The tests on the chloride and iron concentration were set up by dissolving an additional 0.03 mg $FeSO_4 \cdot 7H_2O$ per liter of the H-CD plus vitamin medium (plus $3XFe^{++}$). Another sample (minus Cl^-) contained no KCl from the modified H-CD medium but contained KNO_3 instead. This sample contained of course vitamin stock concentration. A third sample (minus chloride plus $3XFe^{2+}$) was prepared by dissolving in addition to the vitamin stock

0.03 mg $FeSO_4$/liter of growth medium with KCl of the modified H-CD medium again substituted by KNO_3.

After the mycelium was added to the various growth media and after 18 hours of initial shaking the shaker was stopped for 12 hours, then started again for 6 hours shaking, and stopped again for 6 more hours. From then on the shaking was not interrupted until the end of the experiment (19days). Interruption of shaking decreases the growth medium pH to about 2.5, while in flasks without shaking interruption the pH is never lower than about pH 3. The lower initial pH then allows an increased incubation time, i.e. the time to reach a harvest pH of about 6.5 is extended. The net result, therefore, of a shaking interruption is considerably higher final activity in the growth medium.

The results can be seen in the figures 19-21, page 46,47. Fig. 19, page 46, shows the activity-time curves of yeast extract supplemented growth medium compared to stock vitamin and vitamin plus mannitol supplemented media. Yeast extract supplement produces only 50% activity of that of vitamins. Mannitol inhibits chloroperoxidase production 17%. In the samples containing the vitamin stock solution the highest peak of activity was delayed for 180 hours if compared to the yeast extract containing sample.

Fig. 20, page 47, represents the effects of iron concentration on chloroperoxidase synthesis in the presence or absence of chloride. If in the presence of chloride the iron concentration is increased four fold then the peroxidase activity increases 210%. If in the presence of higher iron Cl^- is now omitted

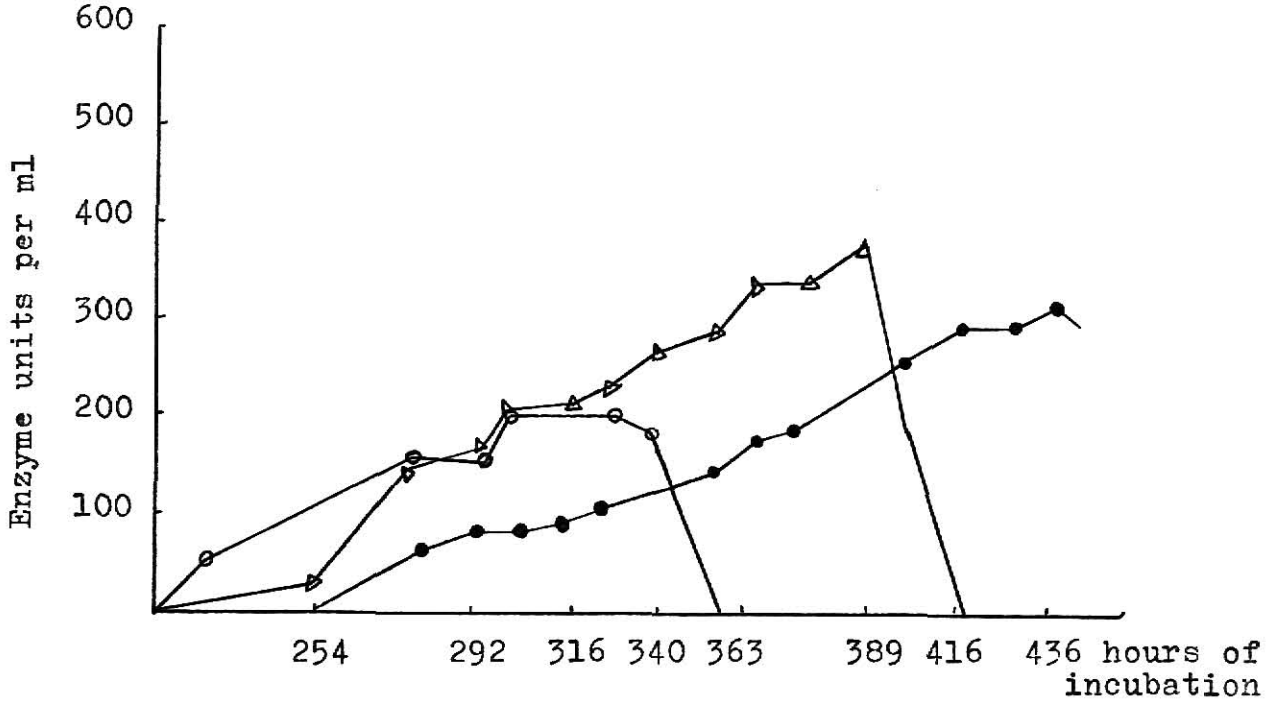


Figure 19: Time course of chloroperoxidase production, samples are containing yeast extract (o——o), a vitamin mixture (Δ——Δ) and a vitamin mixture plus additionally mannitol (●——●).

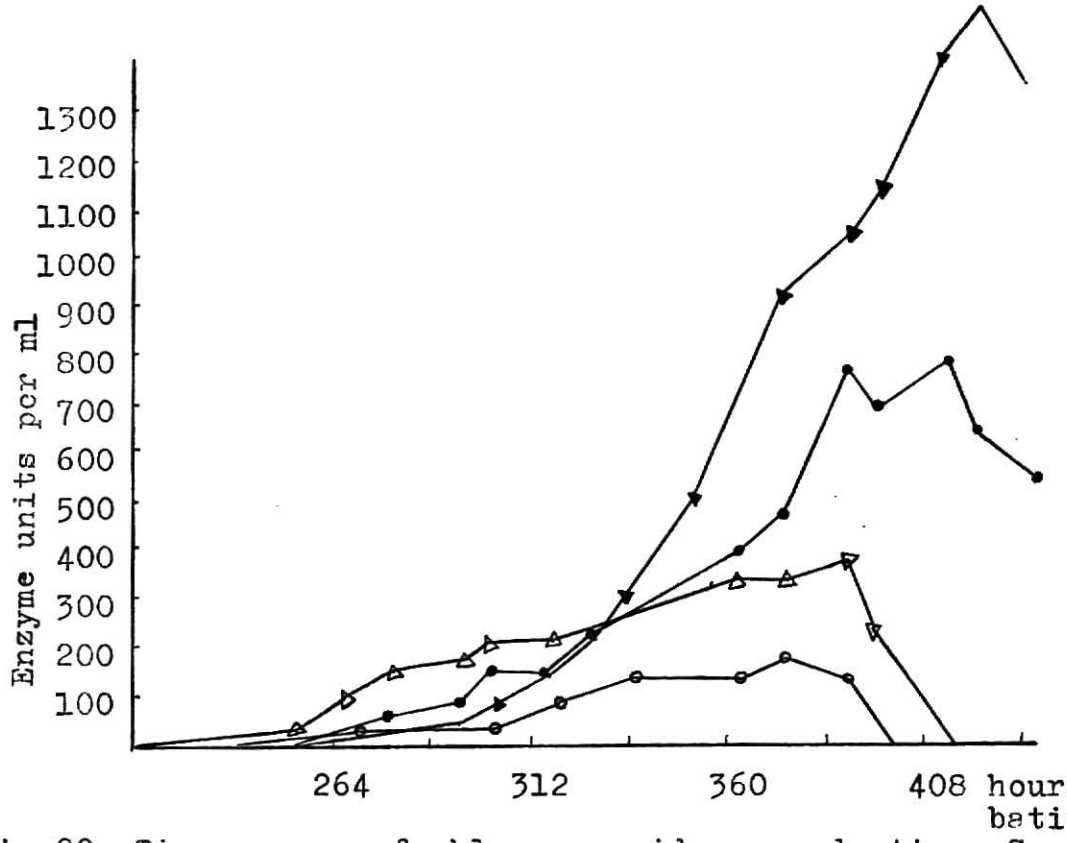


Fig.20: Time course of chloroperoxidase production. Samples with vit.+3XFe^{++} minus chloride (▲——▲), vit.+3XFe^{++} plus chloride (●——●), vit.+Fe^{++} plus chloride (△——△), vit.+Fe^{++} minus chloride (⊖——⊖).

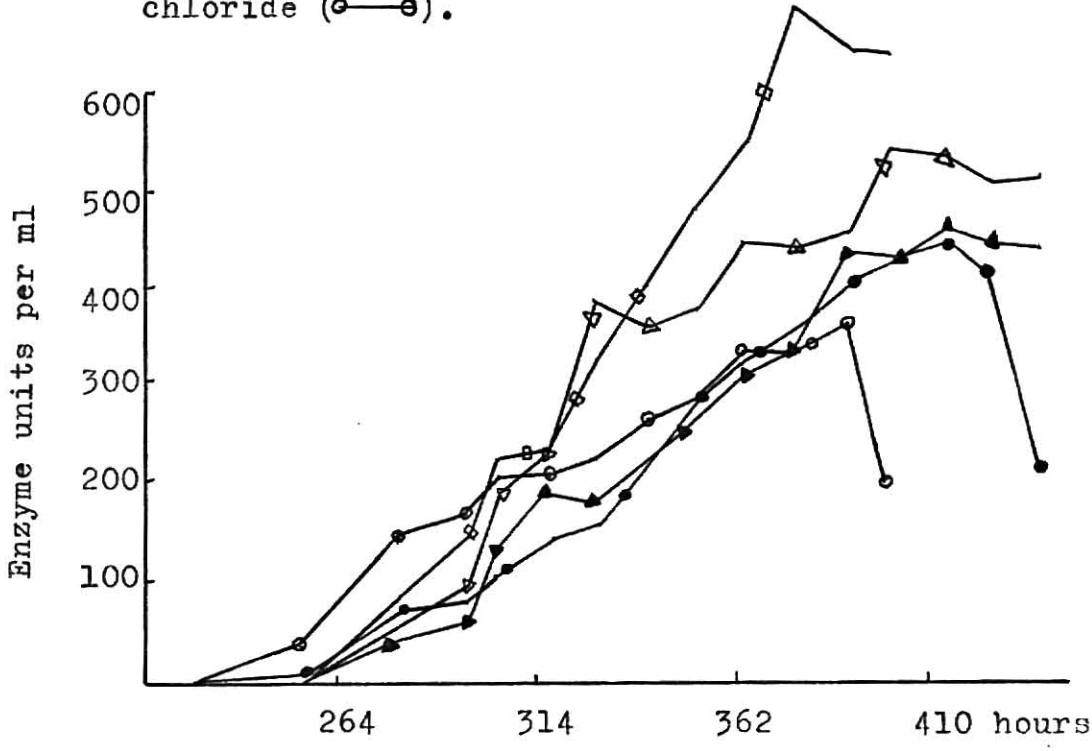


Fig.21: Time course of chloroperoxidase production. Samples with vit. plus chloride (⊖——⊖), vit. plus 3XB_{12} (●——●), vit. plus 3Xpyridoxal (▲——▲), vit. plus 3Xthiamine (△——△), vit. plus three times all vit. (▣——▣). Samples contain chlorid

(replaced by KNO_3) there is a 220% increase over the control which contains, too, high iron concentration and chloride. If the iron concentration of the H-CD medium is not changed however, and chloride is omitted (substituted by NO_3^-), then the peroxidase activity decreases 55%. There is no ready explanation to this. It seems as if chloride enhances the synthesis of chloroperoxidase at lower iron concentrations, however this dependency is overcome at higher iron levels.

Fig. 21, page 47, represents the effects of vitamin concentration on the amount of peroxidase activity produced during inoculation. Addition of three times extra B_{12} results a 17% activity increase, three times pyridoxal results in 19% increase, and three times additional thiamine causes a 31% increase of activity. If three times additional amounts of all three vitamins are simultaneously added to one sample, a 46% activity increase can be observed. These results are compared of course to the activity of a sample containing the stock vitamin concentration.

The results show that increasing vitamin concentration increases enzyme synthesis. The iron concentration and chloride concentration are important factors, too. The conclusion is that a tentative optimal nutrient for the production of chloroperoxidase should contain: 12 mg pyridoxal, 12 mg thiamine, 200 ug vitamin B_{12} and in addition 0.04 mg $FeSO_4 \cdot 7H_2O$ and 0.5 g KNO_3 (instead of 0.01 g $FeSO_4$ and 0.5 g KCl) per liter of H-CD growth medium. In addition, interruption of shaking in the early hours of incubation (stop shaking after 18 hours initial shaking for 12 hours, then shake again for 6 hours and subsequently

stop for 6 more hours) results in a sufficient increase of activity to be done routinely.

H. Effects of Agar Constituents, Age of Cultures, pH of the Growth Medium and some additional Vitamin Studies

The experiments were carried out as in the previoussections, except that shaking was not interrupted but for one sample. The vitamin stock solution mentioned in the previous section replaced yeast extract.

One sample was kept at constant pH by titrating with 0.01 N HNO_3 at first to pH 3.5 and later to pH 2.9. The titrations were carried out daily and were started after the sample had first reached pH 3.7.

Since previous experiments had left some questions about effectiveness of pantothenate and folic acid on the enzyme production, one sample contained in addition to the stock vitamin combination 3 mg folic acid, another 3 mg pantothenate.

In order to test the effects of mycelial age one sample was inoculated with a six days old culture, another with a 16 days old culture (that also served as vitamin stock concentration control for all samples) and a third with a 53 days old mycelium.

One sample was taken from the shaker 222 hours after inoculation and was put back on the shaker 2 days later in order to further investigate effects of shaking interruption.

One sample contained no chloride (Cl^- replaced with NO_3^-). This experiment was necessary in order to verify the high yields of the "minus chloride" flasks reported in the previous section.

Sae had observed that agar slants needed oat-meal extract supplementation in order to give acceptable growth and propa-

gation of chloroperoxidase producing Caldariomyces fumago. The method for oat-meal supplemented agar is: 2 g oat-meal were homogenized in 20 ml water by Waring Blender, then centrifuged at 3,200 X g for 5 minutes. 10 ml of supernatant was used to supplement 1 liter of oat-agar solution.

Success with the vitamin substitute for yeast extract, found in this thesis work, suggested that fungus grown for inoculation might grow just as well on vitamin supplemented agar slants as on oat-agar slants. Thus one sample was prepared by using for inoculation a fungus mycelium grown on vitamin supplemented agar (0.011 mg thiamine, 0.011 mg pyridoxal, 0.45 ug B_{12} per ml of agar solution) instead of oat-meal. Another sample was inoculated with mycelium grown on the same vitamin supplemented agar as above, but which contained no chloride (Cl^- replaced by NO_3^-).

Figures 22-25, page 52, 53, show the results. Figure 22, page 52, depicts additional vitamin tests, principally of folic acid and pantothenate effects. Both vitamins give an increase above the yeast and stock vitamin controls. Addition of folic acid gave a surprising 380% increase and pantothenate a 145% increase of activity. The results, especially from the folic acid were unexpected, because in earlier experiments (see page 41) both vitamins were somewhat undecided and effects on chloroperoxidase production seemed to be small. It is also important to note that folic acid significantly influences the earlier enzyme production in parallel with the yeast extract. If one compares the samples of figure 22, a similar shift to earlier production of enzyme is noted with vitamin pill supplementation.

It may be that the concentration of folic acid is important for general stimulation of enzyme production and also is the factor in vitamin pills and yeast which facilitates more rapid initiation of chloroperoxidase synthesis.

Figure 23, page 52, shows the effects of mycelial age (inoculum) on the time-activity course. The samples with 16 and 52 day old culture contained almost the same activity and were lower than the sample containing younger culture. The 6 day old culture gave 140% increase and a peak activity 3 days earlier.

Figure 24 represents time-activity courses of vitamin control, acid titrated, and shaker interrupted samples. That shaker interruption activity increases (+220%) was again reproduced. As predicted however, the peak of activity was delayed a number of days. The sample had been taken from the shaker just when the activity first could be assayed (222 hours). It remained stationary 2 days (until 268 hours). During that time the activity remained constant. The pH remained at 2.9 to 3.0. When shaking was resumed activity and pH began to increase slowly and activity soon reached a level significantly higher than the control. Daily titration of samples to control the pH value gives results that are somewhat difficult to explain. It was observed that when the growth medium pH was controlled to approximately pH 3.5 the activity increased and then levelled off to a more or less constant value. However, adjusted to a lower pH value the activity decreased to a lower value. When the growth medium was titrated to pH 2.9 a sudden decrease of activity was observed which then levelled off again to a new

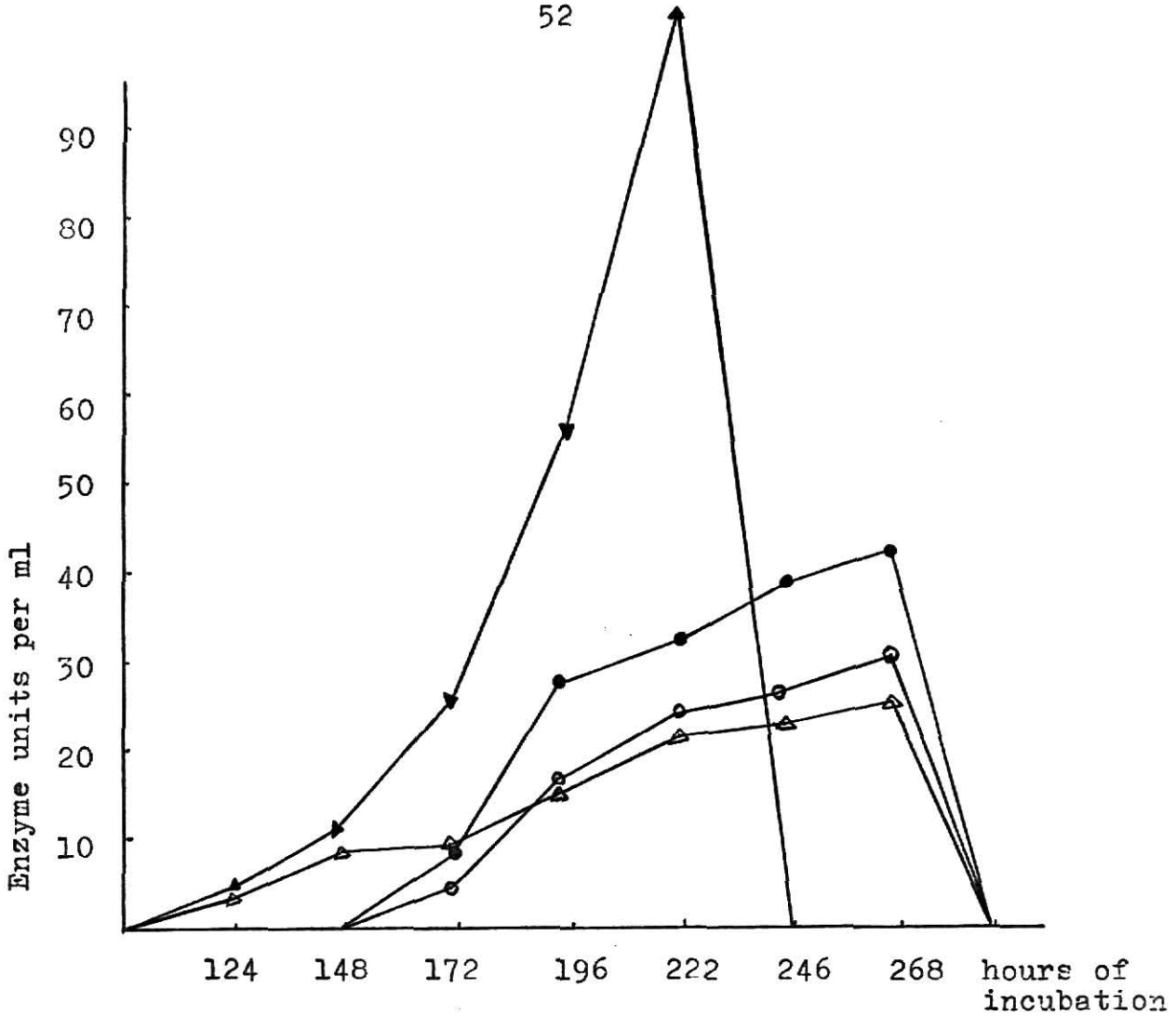


Fig.22: Time activity course of chloroperoxidase production. Samples contain yeast (▲—▲), vitamin stock plus pantothenate (●—●), vitamin stock (⊖—⊖), vitamin stock plus folic acid (▲—▲).

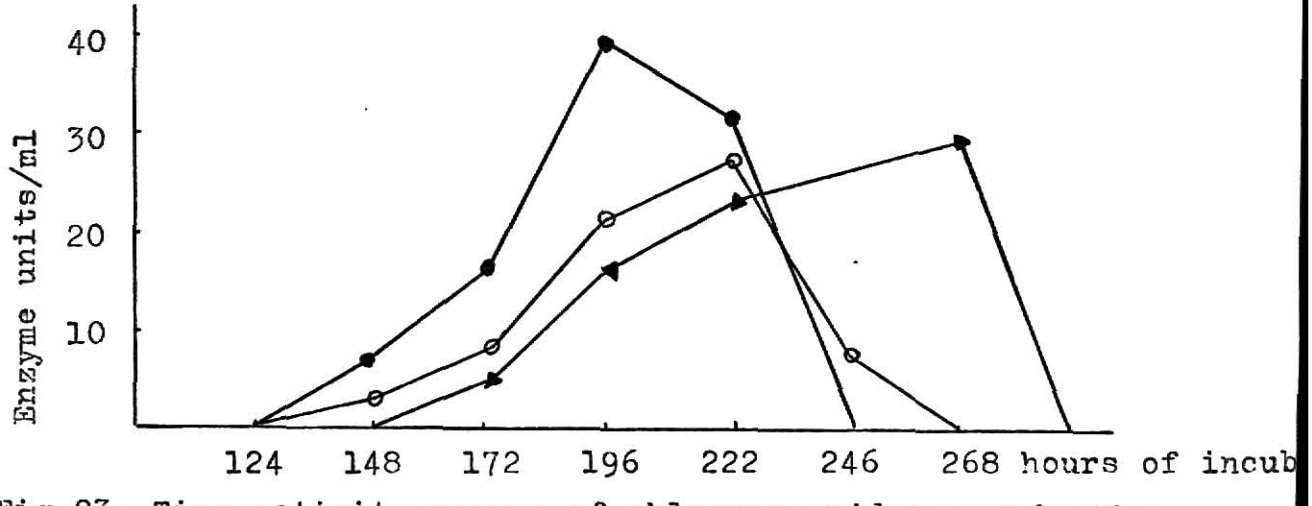


Fig.23: Time activity course of chloroperoxidase production. Samples contain vit. plus 16 day old culture (▲—▲), vit. plus 6 day old culture (●—●), vit. plus 52 day old culture (⊖—⊖).

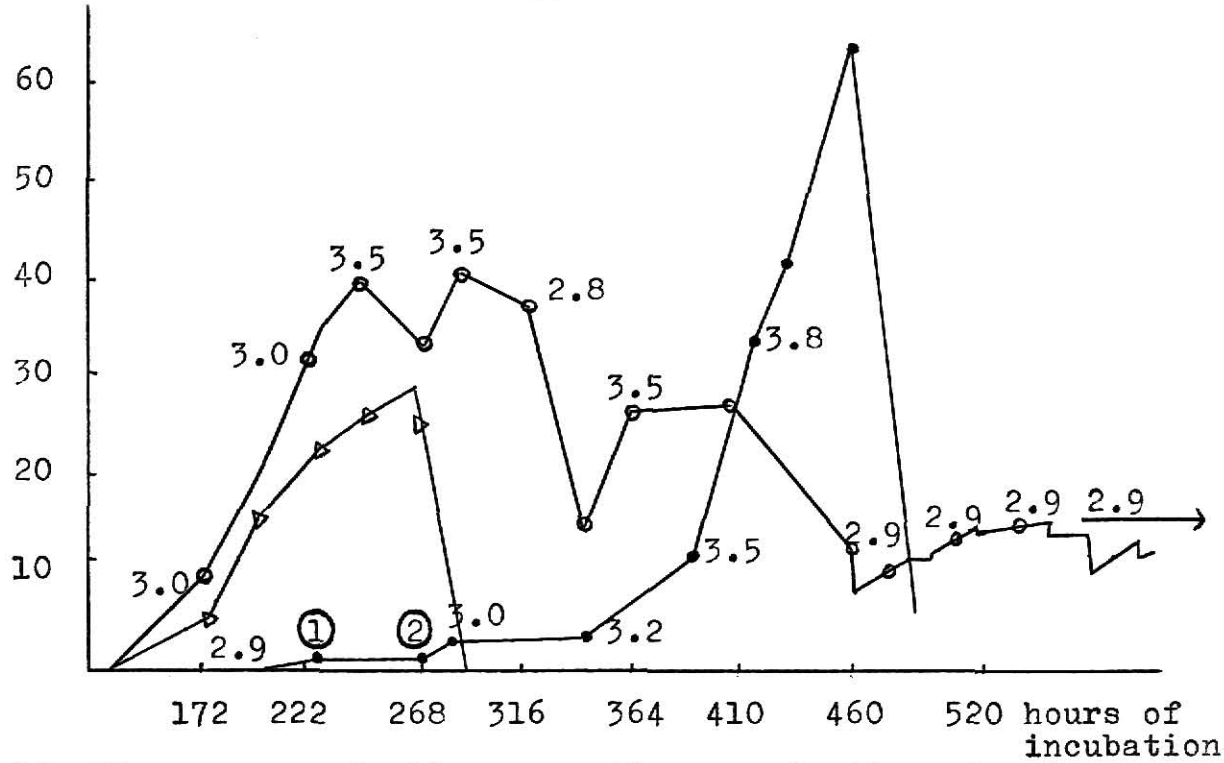


Fig.24: Time course of chloroperoxidase production. Samples contain stock vitamin (▲—▲), vitamin plus standing, shaker interrupted at ①, begin shaking again at ②. Numbers along the graph are pH of growth medium at that point (●—●), vitamin plus acid titration, numbers along the graph are pH to which sample was titrated (⊖—⊖).

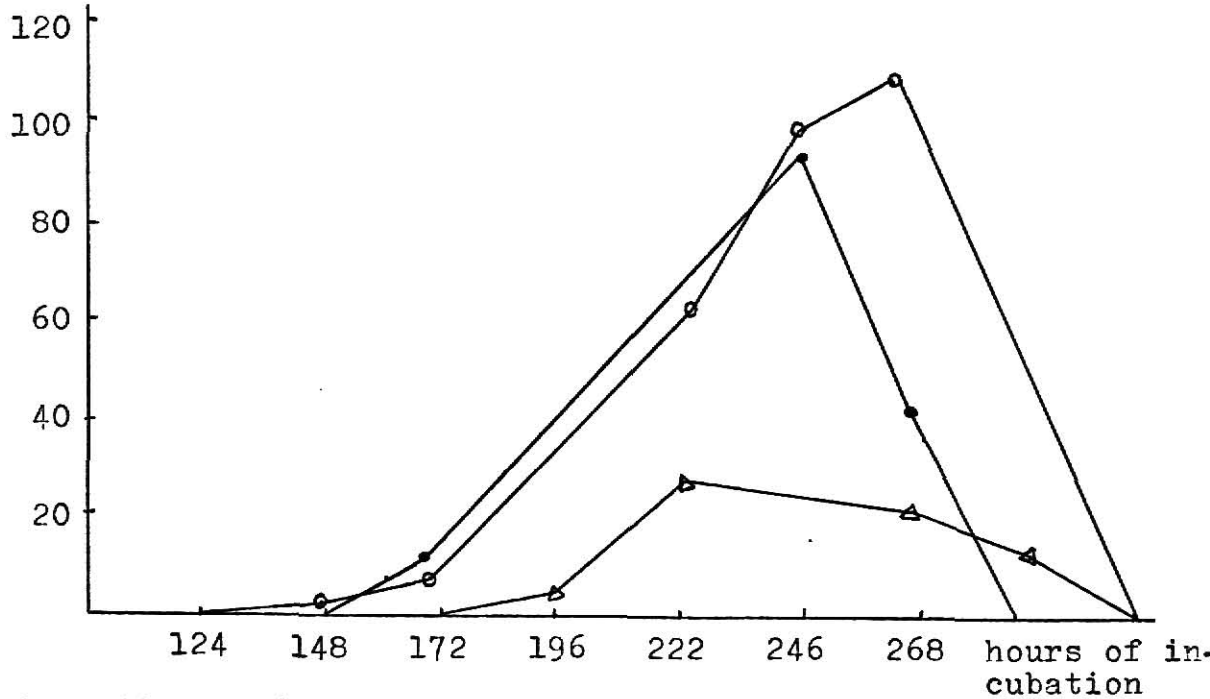


Fig.25: Effects of agar constituents on chloroperoxidase product. 3 x Fe^{++} minus chloride medium is used in all flasks. Oat-meal agar (⊖—⊖), vitamin agar minus chloride (▲—▲), vitamin agar plus chloride (●—●).

smaller value. Just before the daily titration activity was measured and a slight increase was usually observed compared to the previous day. Directly after titration the activity decreased slightly. These data might be explained by the following hypothesis: There is an acid labile enzyme present in the growth medium which is partially inactivated when the 0.1 N HNO_3 is added. Even though the growth medium was stirred during titration, the distribution of the acid might not have been fast enough to prevent inactivation from local acidity. The fact that two different activity levels are reached which titrations to pH 3.5 (about 40 units/ml of inoculation mixture) and pH 2.9 (about 15 units/ml) could be explained if there were two isozymes present in the growth medium: one acid stable and produced at low pH levels, and other acid labile and produced at higher levels. It could be of course that both isoenzymes are produced at the same time and that the acid labile species is deactivated (at least partially) when it is excreted into the growth medium. Later findings in this thesis work which indicate that there are at least two isozymes present in the growth medium and one of these is acid labile.

Figure 25 page 53 represents the time-activity course of samples which were inoculated with mold previously grown on vitamin supplemented agar slants. It shows that agar slants containing the vitamins B_{12}, pyridoxal and thiamine (as replacement for oat-mela supplement) grow fungus which can be used for inoculum in the vitamin modified H-CD medium. Although in this experiment the activity peak was only 87% of the control peak, better results can be achieved with use of a higher

vitamin concentration in the agar.

The figure 25, page 53, also shows that chloride must be present in the agar, because the corresponding chloride free agar sample contained only 26% (peak activity) of the control.

Summary of the Growth Conditions Experiments

It was a major improvement for isolation and purification of chloroperoxidase when it was found by HAGER and MORRIS (72) that the fungus Caldariomyces fumago excretes the enzyme into a yeast extract supplement liquid medium (Czapek-Dox medium with glucose replacing sucrose). They had also observed that the fungus grew well on agar slants only if the agar was supplemented with oat-meal extract. These data suggested complex requirements which were studied further in this thesis to reveal some details of the nutritional requirements.

My experiments show that a complete substitute for yeast extract in the liquid culture medium and for oat-meal in the case of agar slants, has been found. It is composed of a vitamin combination: thiamine, vitamin B_{12}, pyridoxal, folic acid (or tetrahydrofolate) and pantothenate. For substitution of oat-meal in the agar slants only thiamine, pyridoxal and vitamin B_{12} were used but they were adequate for successful propagation. More important than the substitution alone was the finding that chloroperoxidase activity enhancement of up 10 times of the yeast system could be achieved under the proper conditions. These conditions are very complex and will be described below.

1.) The medium has to have good aeration during growth. (see page 29).

2.) Addition of procaine to the growth medium (yeast ex-

tract) results in a significant increase of activity. The possibilities with procaine however were not investigated to a deeper extent (see page 31).

3.) Light seems to have a small positive effect on peroxidase synthesis (see page 35).

4.) A vitamin mixture will substitute for yeast extract in liquid culture or for oat-meal supplement in agar slants. In these experiments it was found useful to use thiamine, pyridoxal, folic acid and pantothenate at a concentration of 12 mg/liter each and vitamin B_{12} at a concentration of 2oo mg/liter of inoculation mixture. Lower vitamin result in less activity. Vitamin concentrations higher than 12 mg/liter have not been tried.

5.) It was found useful to increase the iron concentration of the H-CD medium from 10 mg/liter to 40 mg/liter of growth medium.

6.) Chloride (KCl) omission and substitution by KNO_3 almost doubles the activity when iron is present at 40 mg/liter. (see page 47). However, the agar slants with lower iron concentration have to contain chloride.

7.) The culture used for inoculation should not be older than 7-10 days.

8.) If after shaking is first initiated it is kept on 12 hours, then off 12 hours, then on 6 hours, then off 6 hours and then allowed to remain on continously, a significant activity increase will occur. That result may be due to pH decrease during the motionless period and a subsequent increased incubation time in going from

the lower initial pH (below 3) to the harvest pH (about 6.5).

9.) The liquid should be filtered off from the mycelial mass when the pH of the medium has reached a value of 5.5 to 6.5. As later experiments will show (see page 89), the enzyme is inactivated at pH 6.8 and higher.

Many open questions remain. Although, with the right combination of the above conditions, the results are a highly increased activity (compared to the old method using yeast supplement), variations of peak activity can still occur. In addition to possible contamination by bacteria during the experiment (when opening the vessles for sampling) there is also the possibility that the fungus mycelium might react differently to environmental changes (such as light or temperature). The object of this part of the thesis has been to minimize variations in the growth and maximize production of the enzyme in order to obtain enough chloroperoxidase for primary structure analysis in a short period of time. That objective has been accomplished.

There obviously remain many open questions and their answers could indeed lead to even better results, and a further understanding of metabolic details in C. fumago. Gluconolactone, for example, might be aninteresting glucose substitute. It is possible the 5% gluconolactone tried was too high a concentration and it led to alcohol formation in the same way as 10% glucose did. The efficiency of gluconolactone is doubtless higher during fungal metabolism, because energy can be formed in fewer steps and experiments with lower gluconolactone concentrations could give the answer. The role of metals is another question which deser-

ves further study. Unfortunately all the metals added to the modified H-CD medium were as their chlorides.

Later experiments showed that chloride concentration is very crucial and the metal ion studies (except for iron) should probably be repeated.

A very interesting question is: when the isoenzymes are produced, is the acid stable species still produced at higher pH (from 3.5 on)? If not then it would be possible to stop the inoculation when the pH of the growth medium has reached the proper value and one could perhaps isolate a single chloroperoxidase isoenzyme without risk of contamination by the other.

If, on the other hand, the two chloroperoxidase isoenzymes are produced simultaneously and are excreted, and as soon as the acid labile isoenzyme is dissolved in the growth medium it is denatured due to the low pH, the recovery of this isoenzyme would yield a lower specific activity. Later studies (see page 85) show that chloroperoxidase 4.50 actually does have a lower specific activity. If that observation means the latter hypothesis (simultaneous synthesis of isoenzymes) is the more likely, then perhaps interruption of shaking is not as useful as it first appeared.

V. PURIFICATION AND PROPERTIES OF CHLOROPEROXIDASE

A. Isolation and Purification of Chloroperoxidase-Dialysis of Growth Medium

The filtrate containing chloroperoxidase was dialyzed against distilled water for 36-48 hours at 25°C. Dialysis against tap water (pH 7.5) is easier (a continuous-flow system can be used), however it has to be watched closely. Experience has shown that tap water dialysis beyond 8 hours can cause a pH increase over pH 6.8, which completely inactivates chloroperoxidase.

Distilled water dialysis was performed in a 75 l. plastic container with water changes after 2, 4, 12 and 12 hours. The enzyme solution was filled into dialysis tubes (approximately 1" diameter) which were then floated in the water by means of an air bubble in the tube. The distilled water was stirred with a large magnetic stirring bar at the bottom of the container.

The continuous-flow system described by SAE (84) was set up with size 27 Union Carbide Co. dialysis tubing coiled in the water container (see fig. 26, page 60), and water circulated continuously outside of the dialysis tube in the opposite direction of the enzyme solution flow. The tubing was connected to an open system which contained most of the enzyme solution.

The volune increase after dialysis was negligible (less than 2%).

B. DEAE-Cellulose Capacity Test

A column (5cm x 1.1cm) was gravity packed with a DEAE-cellulose slurry. The DEAE had not been cycled with acid and base, but had been well rinsed with buffer and had the fines

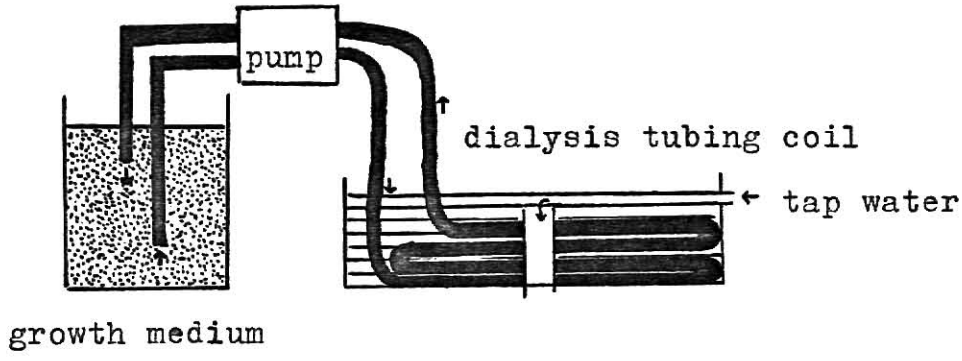


Figure 26: Open continuous dialysis system in tap water

decanted. The column was equilibrated with 0.01 M phosphate buffer to pH 5. The crude enzyme juice which usually has a pH between 4.7 and 5.5 was adjusted to pH 5 with either 0.01 M K_2HPO_4 or 0.01 M KH_2PO_4. In a typical example the crude juice had an activity of 66.6 peroxidase units/ml after pH adjustment. The crude juice was applied to the column continuously and collected in 6ml fractions. The flow rate, controlled by gravity, was at the start 3ml/min.; decreased to 2ml/min. by fraction 28 and to 1.5ml/min. at the end of the experiment (fraction 56). The flow rate decrease is caused by pigment adsorbed from the crude juice.

Fig. 27,page,62 shows a plot of enzyme concentration versus elution volume. In this experiment the DEAE bed volume was 4.3 cm^3 (gravity packed) and it adsorbed 16,000 units of peroxidase (at the break-through volume). That corresponds to a capacity of 3,700 units per cm^3 of bed volume. A capacity test is useful because pigment present in the crude juice makes the DEAE-cellulose unserviceable, and after one run it must be discarded. All regeneration attempts have been unsuccessful. Knowledge of the enzyme adsorbing capacity allows maximum efficiency for utilization of DEAE-cellulose.

C. DEAE-Cellulose Chromatography at pH 5

DEAE-cellulose powder was suspended in distilled water, fine particles decanted, and it was washed with 0.01 M phosphate buffer pH 5 until equilibrated to pH 5. It was then gravity packed into a 10 cm diameter column to a final bed volume of 1740 cm^3 (25 X 10 cm). The column was further washed with three liters of 0.01 M phosphate buffer pH 5. The crude juice (28 l.),

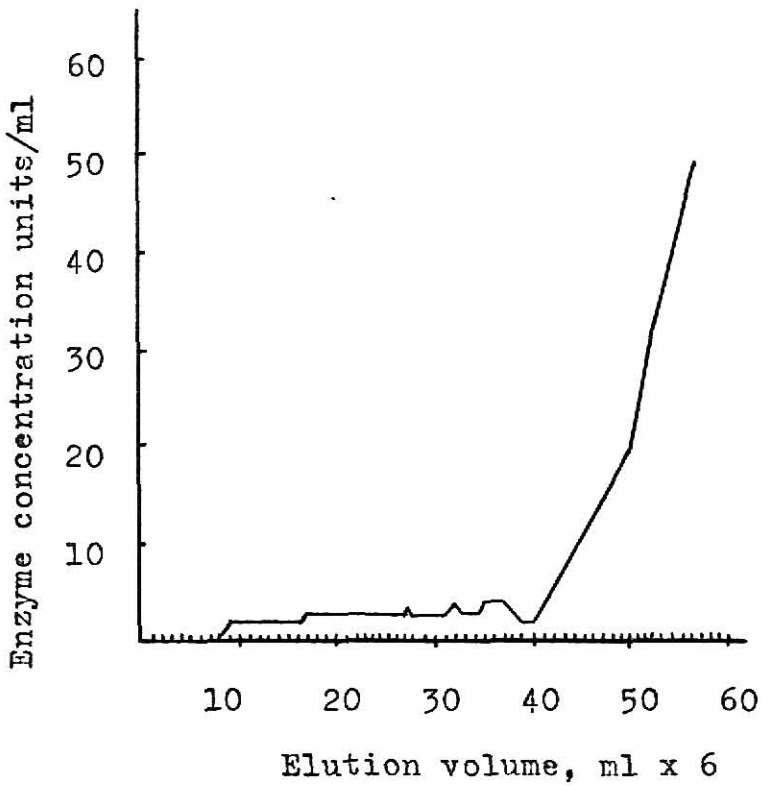


Figure 27: DEAE-cellulose capacity test with the crude juice containing chloroperoxidase.

adjusted to pH 5,was applied to the column by means of a syringe pump at a flow rate of 900 ml per hour. The column was then washed with five liters of 0.01 M phosphate buffer pH 5, and the enzyme eluted with a pH 5 phosphate buffer gradient increasing linearly from 0.01 M to 0.2 M. The gradient was set up with 10 liters of 0.01 M phosphate buffer in the mixing chamber and 10 liters of 0.2 M phosphate buffer in the reservoir.

D. Ammonium sulfate Precipitation

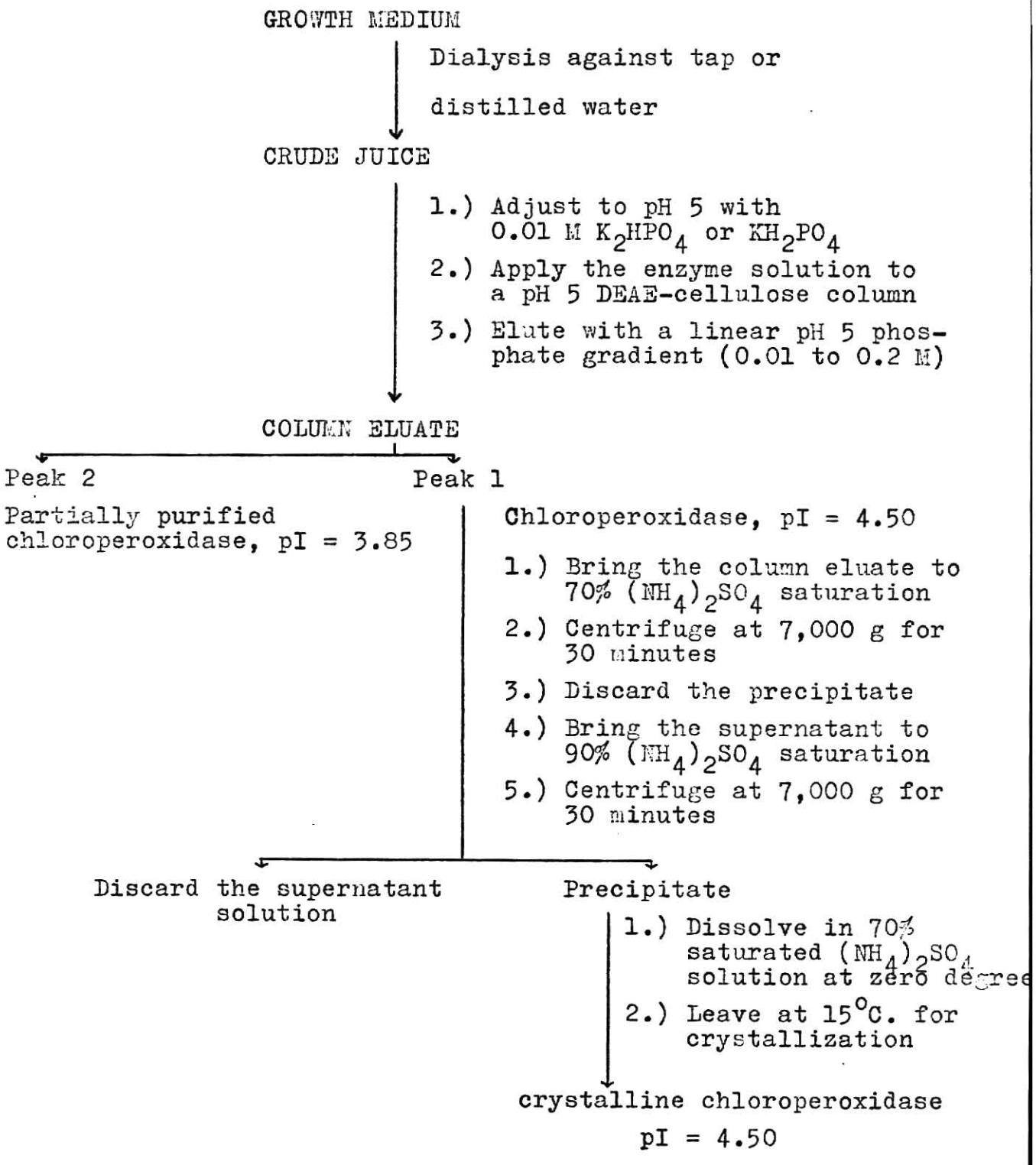
The complete fraction 3 from the pH 5 DEAE-cellulose chromatography (1960 ml, see page 86) was made 70% saturated with ammonium sulfate by adding 920 g of $(NH_4)_2SO_4$. The resulting solution was allowed to stand over night and was then centrifuged at 7000 X g for 30 minutes. The precipitate contained 1% or less of the total activity. The supernatant was then brought to 90% saturated ammonium sulfate by adding 345 g of $(NH_4)_2SO_4$. After 1 hour of standing the solution was centrifuged at 7,000 X g for 30 minutes. The supernatant which contained 7% of the total activity was discarded, the precipitate was used directly for crystallization.

E. Chloroperoxidase Crystallization in $(NH_4)_2SO_4$ Solution

The 90% $(NH_4)_2SO_4$ precipitate was dissolved in a slight excess of 70% ammonium sulfate solution at 0°C. The solution was centrifuged at 4,900 X g, 0°C., for 20 minutes to remove undissolved material. The supernatant solution was then kept at 15°C. in an open plastic container (the top covered with cheesecloth) to allow evaporation. Crystals appeared after app. 10 days; crystallization was complete in about three more weeks.

Crystals were collected by centrifugation and suspended in

Table 4.: FLOW SHEET FOR PURIFICATION PROCEDURE



100% saturated ammonium sulfate solution and stored at 15°C.

For further work the crystals were exhaustively dialyzed against deionized water and the salt free solution was lyophilized.

F. DEAE (pH 5) Chromatography of Fraction 4 (see page 79, 86)

Fraction 4 (1540 ml) was dialyzed against distilled water the pH adjusted to 5 with 0.01 M KH_2PO_4 and applied to a DEAE-cellulose column, previously washed and equilibrated with 0.01 M phosphate buffer pH 5. The column was then washed with 3 liters of 0.01 M phosphate buffer pH 5. The enzyme was eluted with a linear gradient (2 liters of 0.01 M phosphate buffer pH 5 in the mixing chamber and two liters of 0.2 M phosphate buffer pH 5 in the reservoir). The eluate was collected in 15 ml fractions.

G. DEAE (pH 3.85) Chromatography of Fraction 5 (see page 79, 86)

The above pH 5 DEAE-cellulose column was re-equilibrated with 0.05 M acetate buffer pH 3.85. Fraction 5 (see page 86) was adjusted to pH 3.85 with 0.05 M acetic acid and applied to the DEAE-cellulose column. The column was washed with three liters of 0.05 M acetate buffer pH 3.85 and the enzyme was subsequently eluted with a linear gradient (3 liters of 0.05 M acetate buffer pH 3.85 in the mixing chamber and 3 liters of 0.3 M acetate buffer pH 3.85 in the reservoir). The eluate was collected in 20 ml fractions.

H. Isoelectric Focusing

Enzyme obtained from (a) the crude juice, (b) DEAE (pH 5) chromatography, (c) DEAE (pH 3.85) chromatography, and (d) from crystallized and lyophilized material, was applied to a 110 ml LKB-Isoelectric Focusing column. A sucrose density gradient

with pH 3-6 or pH 3-10 ampholyte gradient was prepared and layered into the column with a peristaltic pump as described in the LKB-Electrofocusing Instruction sheet. The protein samples were layered as light density fractions 10 to 15. The column was set up with a bottom anode and a top cathode. The starting voltage of 300 to 400 volts increased to 1000 volts after 6 to 10 hours and was maintained at this voltage for an additional 6 to 39 hours at 15°C. Fractions were collected (2ml) at a rate of 0.4 ml per minute. Protein was measured by absorbance at 280 mu (Coleman Autoset) or by the modified LOWRY et al. method (see page 22); peroxidase activity was assayed by a guaiacol method, and pH was measured with a small Fisher Scientific Combination electrode.

a.) Isoelectric Focusing of the lyophilized and crystallized Chloroperoxidase (see page).

A solution (2.2 ml) containing 5.6 mg of enzyme per ml was layered as the light density part in fraction 12. The flow rate during gradient formation was approximately 1 ml/min. Initial potential was 400 V, and initial current was 5.5 ma. The voltage after 35 hours (end of the run) was 1000 V; the milliamperage was unchanged.

b.) Isoelectric Focusing of Fraction 4 after the second DEAE-Cellulose Chromatography (see page 93).

A 2.2 ml aliquot of combined fractions 96-111 (see page 93) was layered as the light density part of fraction 12, 2.4 ml of combined fractions 112-141 (see page 93) was layered as the light density part of fraction 13, and 2.6 ml of combined fractions 142-186 was layered as the light density part of fraction

14 in the isoelectric focusing column. The gradient forming flow-rate was approximately 1ml/min. Initial potential was 200 V, current 11 ma; at the end of the experiment (after 16 hours) the readings were 810 V and 3 ma.

c.) Isoelectric Focusing of the second Peak obtained from the pH 3.85 DEAE-Cellulose Chromatography (see page 94).

A 4.2ml aliquot of fraction 84 (see page 94) was layered as the light density part of the fractions 11 and 12 into the electrofocusing column. Gradient forming flow-rate was approximately 1ml/min. Initial potential was 400 V, current 6.5 ma.; at the end of the experiment (after 22 hours) the readings were 1000 V and 4 ma.

d.) Isoelectric Focusing of the First Peak obtained from the pH 3.85 DEAE-Cellulose Chromatography (see page 94).

A 2ml aliquot of combined fractions 63-74 (see page 94) was layered as the light density part of fraction 11 into the isoelectric focusing column. Gradient forming flow-rate was approximately 1ml/min. Initial potential voltage was 400 V, the current 4 ma.; at the end of the experiment (after 16 hours) the readings were 1000V, and 4 ma.

e.) Isoelectric Focusing of the Crude Juice (see page 59).

A 13.8ml aliquot of the crude juice was layered as the light density part of combined fractions 10-15 into the isoelectric focusing column. Gradient forming flow-rate was approximately 1ml/min. The initial potential was 600V, the current 7 ma. At the end of the experiment (after 49 hours) the readings were 600V and 2.8 ma. The protein was determined with the modified Folin-Miller assay (see page 22).

I. Iron Determination

Iron was determined by the method of KING et al. (50) from a modification of the method of YONETANI (108). The samples to be assayed were dried in 10ml volumetric flasks at 110°C for 24 hours. The volumetric flasks had been pre-boiled in concentrated HCl in order to remove any iron contamination. The dried samples were digested in one ml of Kjeldahl Digest mixture (1ml SeOCl and 8g of K_2SO_4 in 500ml of 50% H_2SO_4) by heating the digest over the small flame of a Bunsen burner. The digestion was stopped when the solution turned colorless, usually after 6-8 hours of digestion. The flasks were allowed to cool to room temperature. A 25% sodium citrate solution (2ml) was then added and the mixture adjusted to approximately pH 4 with saturated NaOH (pHydrion vivid 3-9 pH paper). To this solution 1ml of a 0.25% o-phenanthroline was added and 0.5 ml of 1% freshly prepared hydroquinone. The flasks were filled to 10ml with distilled water, allowed to stand at room temperature for 60 minutes, the absorbance read at 509 mu and unknowns determined from the standard curve.

Blanks were carried out through the entire procedure including digestion and samples were corrected, if nessecary, for contaminated iron.

The iron standard solution was prepared by boiling 0.3399g of Baker Code 1805 iron wire in 20ml of 6 N HCl and diluting to 1000ml with distilled, deionized water. Aliquots of this solution (0.01-0.07ml) were diluted to 3ml, and to those samples was added o-phenanthroline, hydroquinone and citrate as before. The pH was adjusted, the sample diluted and absorbance

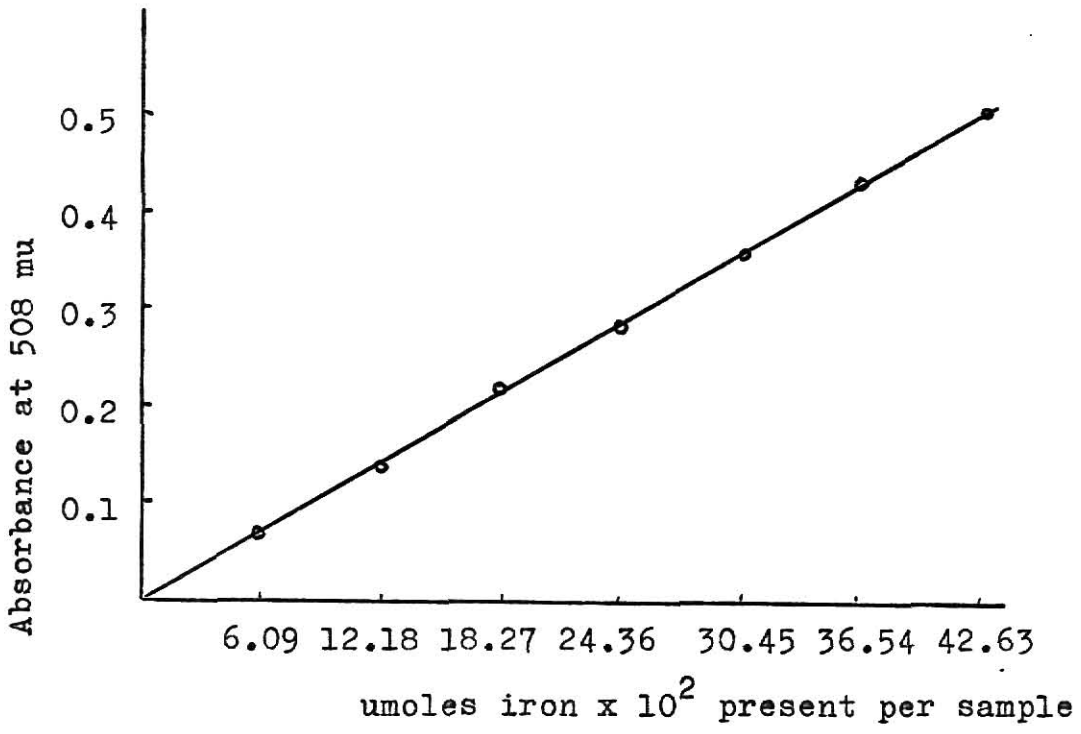


Figure 28: Iron Standard Curve (Coleman Autoset)

determined as before exept pH adjustment required H_2SO_4 instead of NaOH.

J. Chloroperoxidase Molecular Weight

The molecular weight of the crystalliezed and lyophilized chloroperoxidase (see page 65) was determined from iron content (see page 68). The protein (2-5mg) was digested as described above and the percent of iron was calculated after determining the amount of Fe from the standard curve. An aliquot of enzyme was dried in a vacuum oven at 110°C for 24 hours. The ratio of enzyme weight after to enzyme weight before drying was used as a multiplier to correct the enzyme weight to dry basis in the % iron calculation. The minimum molecular weight is the g of enzyme associated with 55.85g Fe.

K. Chloroperoxidase Absorption Spectra

a.) Visible Region (450 mu - 350 mu)

All spectras were obtained by a Cary 14 Recording Spectrophotometer using 1.00cm matched cells. Spectra of crystallized and lyophilized chloroperoxidase (pI at 4.5) were obtained in 0.2 M phosphate buffers at pH 2,4,6,7,8. The millimolar extinction coefficients were obtained using a molecular weight of 26,000. The general approach for pH adjustment was to mix 2ml of aqueous enzyme solution (19.7mg of enzyme in 25 ml of distilled water) with 1ml of the appropriate 0.2 M phosphate buffer.

b.) Visible Region (700 mu - 450 mu)

Equal volumes (0.25ml) of aqueous enzyme solution (12.1mg enzyme in 1.25ml of distilled water) and the appropriate 0.2 M phosphate buffer were mixed in 1ml microcells, and the spectra were determined as described above.

c.) Ultra-violet Region (350 mu - 240 mu)

Enzyme concentration and pH adjustment was as described for (a) visible, 450-350 mu.

In all experiments reference cells contained all components exept enzyme.

L. Titration of the Enzyme with p-chloromercuribenzoate

The method of BENESCH & BENESCH (12) was used. A p-chloromercuribenzoic acid (PCMB) solution was prepared by dissolving 10.56mg of PCMB in 2ml of 0.04 N NaOH, and diluting to 25ml with distilled water. Two ml of that solution was diluted to 25ml with 0.33 M acetate buffer pH 4.6. The acetate solution of PCMB was called stock solution. The stock solution was clarified by centrifugation before use.

Standardization of the PCMB stock was with Glutathione (17.52 mg GSH in 10ml of 0.33 M acatate buffer pH 4.6). Three ml of PCMB stock was placed in a 1ml cell and 3ml of the corresponding buffer in the blank cell. The absorbance is measured at 255 mu and 0.01ml aliquots of GSH solution were then added to both cells. The contents were mixed and the absorbance is measured after each addition. The end point is obtained by extrapolating down from the intersection of the two lines shown in figure 29, page 72. The concentration of PCMB then will be:

$$\frac{[GSH] \times \text{ml GSH at end point}}{3}$$

The enzyme chloroperoxidase was then titrated as follows: Three ml of enzyme (4.87mg of chloroperoxidase dissolved in 10ml of 0.33 M acetate buffer pH 4.6) was placed in a Beckman cell; the blank cell contained 3ml of buffer alone. Standardized

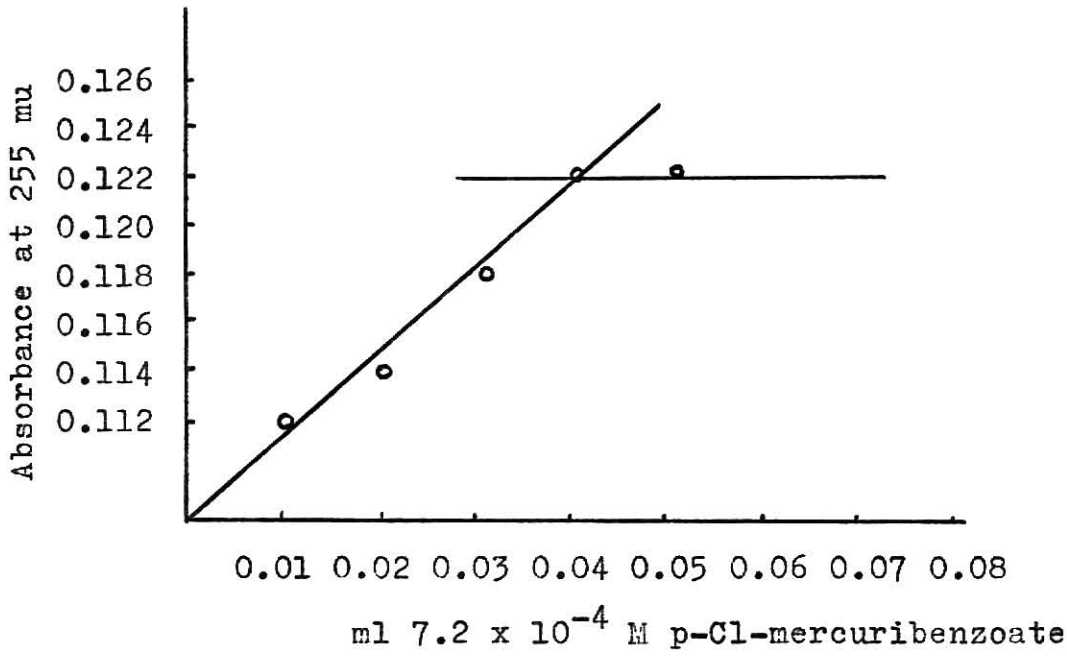


Figure 29: Titration of GSH with p-Cl-mercuribenzoate (Beckman DU)

PCMB (in 0.01ml aliquots) was added to both cells with a micropipette, the contents were mixed and the absorbance measured after each addition.

H. Amino Acid Analysis of pI 4.50 Chloroperoxidase

a.) Hydrolysis of the sample

The method of GUZMAN (26) was used. Crystallized and lyophilized chloroperoxidase (5.55mg) was weighed into a charging tube. The charging tube with the enzyme was introduced into a hydrolysis tube and 2.2ml of 6 N HCl was added (a ratio of 1ml HCl per 2.5mg enzyme). The contents of the tube were frozen in acetone dry-ice, evacuated with an oil pump for 5 minutes, and sealed with a propane torch. Hydrolysis was conducted at 110°C for 21 hours. At the end of that period the tube was cooled to room temperature, the hydrolyzate was filtered through filter paper and the hydrolysis tube and the filter paper rinsed well with water. The filtrate was collected in a 50ml round bottom flask which was then connected to a rotary evaporator, and evaporated (water pump) at 40°C. The residue, containing the equivalent of 5.55mg sample, was dissolved in 0.5ml of water followed by 0.5 ml of 0.2 M potassium phosphate buffer pH 5 and allowed to stand for 4 hours to permit air oxidation of cysteine to cystine. The solution was then brought to pH 2 by adding 0.6ml of freshly prepared 1 N HCl. The resulting solution was quantitatively transferred to a 5ml volumetric flask using 1ml aliquots of pH 2.2 citrate buffer containing 5ml thiadiglycol per liter.

b.) Analysis

The amino acid analysis was carried out using a Beckman Model 120C Amino Acid Analyzer according to the method of SPACK-

MAN, STEIN and MOORE (93). The acidic and neutral amino acids were eluted with pH 2.80 and 4.20 lithium hydroxide buffers, the basic acids with pH 5.25 buffer. The calculations were carried out according to the Beckman Instruction Manual.

N. Cleavage of Chloroperoxidase Peptide Chains

Hydrolysis with PTCK treated (Calbiochem) trypsin was carried out according to the method of CHAU and MARGOLIOSH(20). The digestion was performed in 0.03 M $NaHCO_3$ pH 8.3 or in distilled water pH 6 at 38°C.

5.58mg of chloroperoxidase was dissolved in 0.9ml of buffer or water. At zero time 0.05ml of trypsin solution (5.82mg/ml) was added and the resulting solution was gently stirred with a small magnetic bar. At 3.5 and 6.5 hours of incubation additional 0.05ml aliquots of trypsin were added. The reaction was stopped after 16 hours of digestion by storing the samples at 2°C. Blanks of chloroperoxidase were prepared by dissolving 2.42mg of enzyme in 0.5ml of 0.03 M $NaHCO_3$ pH 8.3 and 2.61mg of enzyme in 0.5ml of distilled water, and a trypsin blank was prepared by incubating the above mentioned trypsin solution at 38°C.

O. Peptide mapping

Descending Paper Chromatography (13) was run on Whatman 3-MM chromatografic paper (18 1/4 x 22 1/2 inches). The sheet was prepared by making a forward fold in the long dimension of the paper along a line one inch from the edge and a backward fold along a line 2.5 inches from the edge, so that the sheet could hang from the buffer reservoir with the second line suspended on the antisiphon rod. The point of origin was 4 inches

from the outer edge of the paper and 2 1/2 inches down from the antisiphon rod. An amount of enzymatic digest equivalent to 3mg chloroperoxidase was applied in small aliquots at the origin with intermittent drying by a hand-style hair dryer. The application spot usually was 0.5 inches in diameter or less.

The solvent system used was 1-butanol:pyridine:glacial acetic acid:water (90:60:18:72). Chromatography was run for 20 hours at room temperature. The cabinet always held two sheets of chromatography paper. After chromatography the papers were clipped to the supporting rods and dried in an oven at 80°C for 20 minutes.

Electrophoresis was carried out in a pyridine-acetate buffer pH 3.6 (100 ml acetic acid, 10 ml pyridine, diluted to 3000 ml) (47). The buffer was applied by spraying. Extra buffer puddles on the paper were blotted with a second sheet of paper. The electrophoresis paper had been extended by approximately 9 cm at its one outer edge by sewing a strip of Whatman 3 MM paper to that side. The extension was necessary in order to fit the paper onto the plate of the high voltage electrophoresis apparatus (Savant FP-22A). The plate was cooled by circulating 15°C water through it. High voltage electrophoresis was carried out for 1 hour at 3000 volts. Under the above conditions 42 ma current was drawn at the start of the electrophoresis increasing to 110 ma at the end.

The papers were taken from the supporting plate and dried in an oven at 80°C for 20 minutes.

Staining the peptide maps was with ninhydrin (31), using the dipping method. Buffered ninhydrin was prepared by adding

1ml pyridine and 1ml glacial acetic acid to 98ml of 0.3% ninhydrine in acetone. Maximum development of ninhydrin color was obtained as follows. A fresh paper without previous staining was dipped through the buffered ninhydrin solution. The dried paper was placed in a cool oven, and the temperature was allowed to increase to 70°C. This gradual heating gave maximal color development with minimum background.

VI. RESULTS AND DISCUSSION

1.) Purification of Chloroperoxidase

The purification procedure developed in this laboratory has simpler operations, milder treatment and a higher yield than the procedure used by MORRIS and HAGER (72) and SAE (84). in addition the recovery of three isoenzymes represents an extension from the isoenzyme pair found by SAE (84) and will have to be recognized in any future work with chloroperoxidase.

The method used by MORRIS and HAGER (72) has several disadvantages (see page 16). Concentrating 30 liters of growth medium with heating the protein to 45°C, and subsequent lyophilization of three liters are cumbersome and time consuming. The heating undoubtedly causes some denaturation (see heat stability curve on page 89). Ethanol fractionation is another harsh treatment of protein, although the reported activity loss has been relatively low. Finally the calcium-phosphate cellulose column is difficult to prepare.

Sae's procedure (84) represents a significant advance (see page 15), however the aluminum gel is difficult to prepare and the timing has to be accurate. An old gel does not release the enzyme to buffer elution as easy as freshly prepared gel. Furthermore due to the improved growth conditions and production of a third isoenzyme, it is desirable to change the DEAE column pH from 6 (used by SAE) to pH 5, where a clean separation of the new isoenzyme could be achieved.

The method developed in this thesis work (see flow-sheet on page 64) starts with a dialysis of the crude juice against running tap water in an open system for not more than 8 hours or

against distilled water for 36 hours. Dialysis is not essential (the ionic strength of the crude juice is less than 0.01 M and does not interfere with enzyme adsorption on DEAE) if one can process the juice within one week. However, if the material has to be stored, dialysis is necessary to preserve activity. Undialyzed material is stable approximately one week and then progressively loses activity, i.e. 50% loss in two months and 100% loss in three months.

Dialysis against tap water is easier to set up since an open system can be applied, however it is generally less efficient because of the pH of the crude juice, which may increase higher than 6.8 where chloroperoxidase is inactivated.

The crude juice, dialyzed or not dialyzed, can be subjected directly to ion-exchange chromatography on DEAE-cellulose at pH 5, and can be eluted with a linear pH 5 buffer gradient (0.01 M phosphate buffer to 0.2 M phosphate buffer). The eluted fractions showed two major activity peaks and one minor activity peak (fig.30, page 79). The isoenzymic character was substantiated by an isoelectric focusing experiment on the crude juice (see page 91). When the crude juice was subjected to an electric field in a natural pH gradient two bands were observed at pH 3.49 and pH 4.42 respectively, proving that two isoenzymes are present in the crude juice. These results are remarkably different fromthose obtained by SAE (84), who showed two crude juice isoenzymes.

Electrofocusing is obviously an excellent method for purification of proteins, however it is still a small scale operation. In order to achieve a maximal separation of the two isoenzymes on

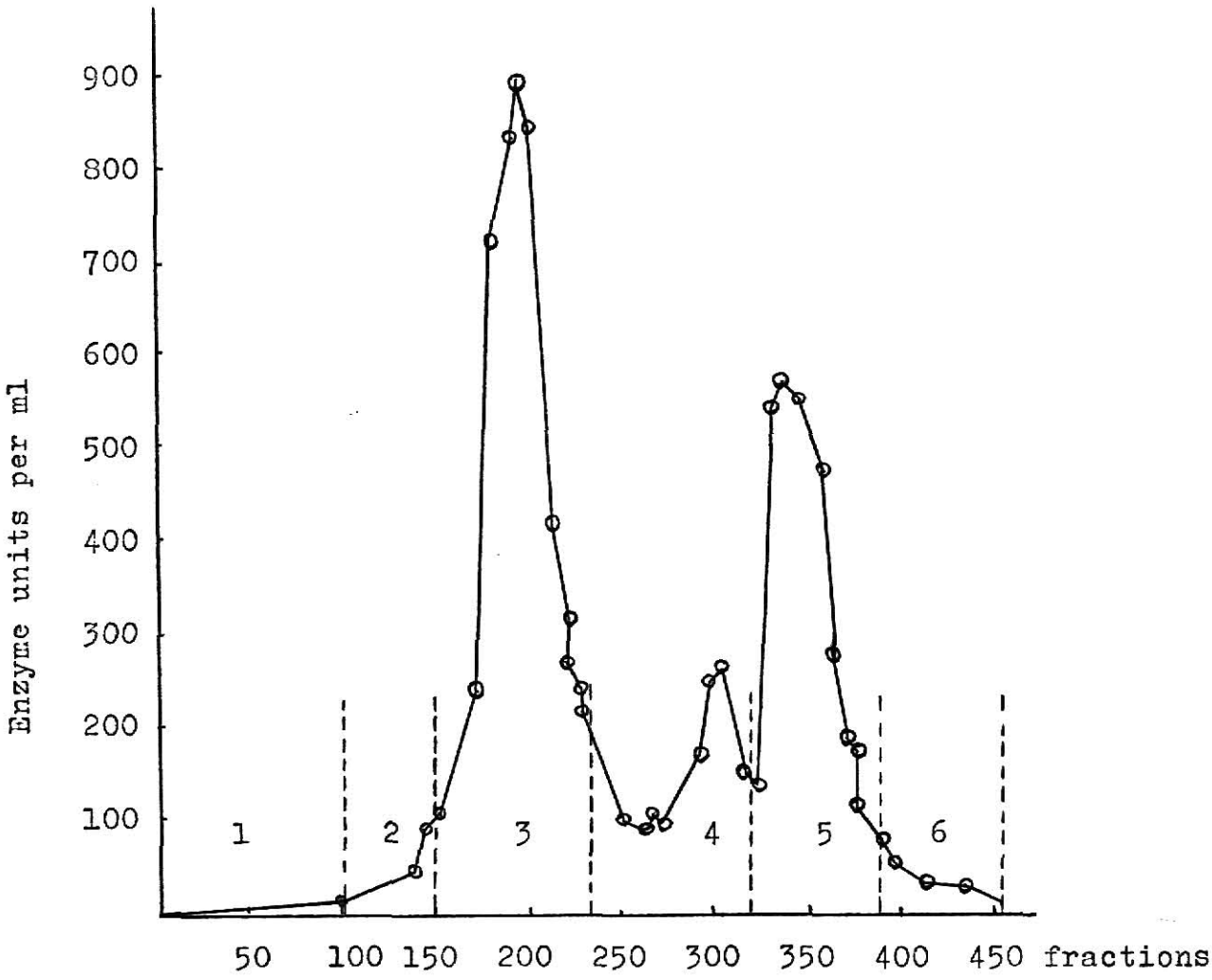


Figure 30: Elution profile of crude juice through DEAE-cellulose at pH 5. The elution gradient is from 0.01 M to 0.2 M phosphate buffer at pH 5. Pooled fractions are indicated by the lined segments above.

Definitions: fraction 1 (test tube 0-100), fraction 2 (test tubes 101-150), fraction 3 (test tubes 151- 230), fraction 4 (test tubes 231-320), fraction 5 (test tubes 321-385, fraction 6 (test tubes 386-450).

a large scale basis the crude juice was adjusted to pH 5 with 0.01 M KH_2PO_4 and applied to a DEAE-cellulose column which was equilibrated with 0.01 M phosphate buffer pH 5. It was found that DEAE-cellulose chromatography at a lower pH (pH 4.5) also separated the two isoenzymes, but at a much lower adsorption efficiency. At pH 4.5, one isoenzyme was adsorbed to the column while the other isoenzyme and all the pigment went through unadsorbed. Although separation was complete and contamination of the adsorbed isoenzyme with the other isoenzyme was negligible, it had poor recovery. In addition, it would leave the second isoenzyme contaminated with pigment.

At pH 5 one would expect positively charged protein to emerge from the column before the major two chloroperoxidase peaks, while those more negatively charged would emerge later. The difference in the isoelectric points of the two isoenzymes is large enough so that a pH 5 elution yields optimal separation of the two isoenzymes. The elution profile, seen in fig.30, page 79, indeed shows acceptable separation.

While the first peak (fraction 3, page 79) of the above fractionation was subjected to ammonium sulfate precipitation, the middle fraction 4 (see page 79) was dialyzed against water and again applied to a DEAE-cellulose chromatography at pH 5. In order to determine whether the minor peaks seen in the middle part of the elution profile of fig. 30, page 79, were artifacts or additional isoenzymes, the adsorbed enzyme was eluted with a linear ionic strength gradient (0.01 - 0.2 M phosphate buffer pH 5) and the elution profile indeed shows three peaks with peroxidatic activity (fig. 41, page 93). To

characterize each peak, isoelectric focusing experiments were performed (see page 91).

Fraction 4 (page 79) was applied to an isoelectric focusing column and the elution at the end of the experiment indeed showed three peaks with peroxidatic activity. The isoelectric points of these peaks were at pH 3.51, 3.89, and 4.55. Very intriguing is the fact that the isoenzyme with pI at 3.89 was not present in the focusing experiment on the crude juice. There also was a slight shift of the pI 4.50 enzyme to pI 4.55. These data will be discussed later (see page 95).

The second major peak of the pH 5 DEAE-cellulose chromatography on the crude juice (fraction 5, page 79) was thought to contain the activity of two isoenzymes which were found by SAE (84) with the pI at 3.82 and 3.50. That fraction was dialyzed against distilled water and the pH adjusted to 3.85 with 0.05 M acetic acid. It was then applied to a DEAE-cellulose column, equilibrated with 0.05 M acetate buffer pH 3.85, and it was eluted with a linear gradient (0.05-0.3 M acetate buffer pH 3.85). The elution yielded two peaks with peroxidase activity, one minor and one major (see fig.42, page 94). Each peak was then subjected to isoelectric focusing and both proved to have isoelectric points at 3.85. That result was somewhat surprising and the situation is discussed further on page 95.

Overall results of isoelectric focusing experiments show that there are three chloroperoxidase isoenzymes. Two of them were named chloroperoxidase A and B by SAE (84). It would be logical to name the third one chloroperoxidase C, however a far more accurate way seems to be to name them according to their

isoelectric point. In this thesis it is thus proposed to name the three isoenzymes chloroperoxidase 4.50, chloroperoxidase 3.85 and chloroperoxidase 3.55.

Fraction 3 from the first pH 5 DEAE-cellulose chromatography (see page 79) was subjected to an ammonium sulfate precipitation. Fig. 31, page 83 shows an $(NH_4)_2SO_4$ fractionation, carried out on an 100 ml aliquot of fraction 3.

The enzyme solution was saturated to 70% with ammonium sulfate and after centrifugation the precipitate which contained 1% of the total activity was discarded. The supernatant was saturated to 90% with $(NH_4)_2SO_4$. After centrifugation the precipitate contained 92% of the total activity. The supernatant solution was discarded.

Ammonium sulfate precipitation has several advantages. It can be carried out very easily and the precipitate can be subjected immediately to crystallization without any further treatment, such as dialysis. The specific activity after this step is 2.5 times greater than the material from the DEAE-cellulose chromatography. The yield from the $(NH_4)_2SO_4$ step was 86%.

2.) <u>Crystallization in $(NH_4)_2SO_4$</u>

A step in purification that is usually held in high regard is crystallization although crystals are no assurance of protein purity. The attempts in this thesis work to crystallize chloroperoxidase 4.50 have been successful. Fig 32.shows a photomicrograph of the first crystals obtained from chloroperoxidase 4.50 solutions. The red crystals were collected and stored in saturated $(NH_4)_2SO_4$ solution. The specific activity of the crystals was the same as after the ammonium sulfate precipitation. The

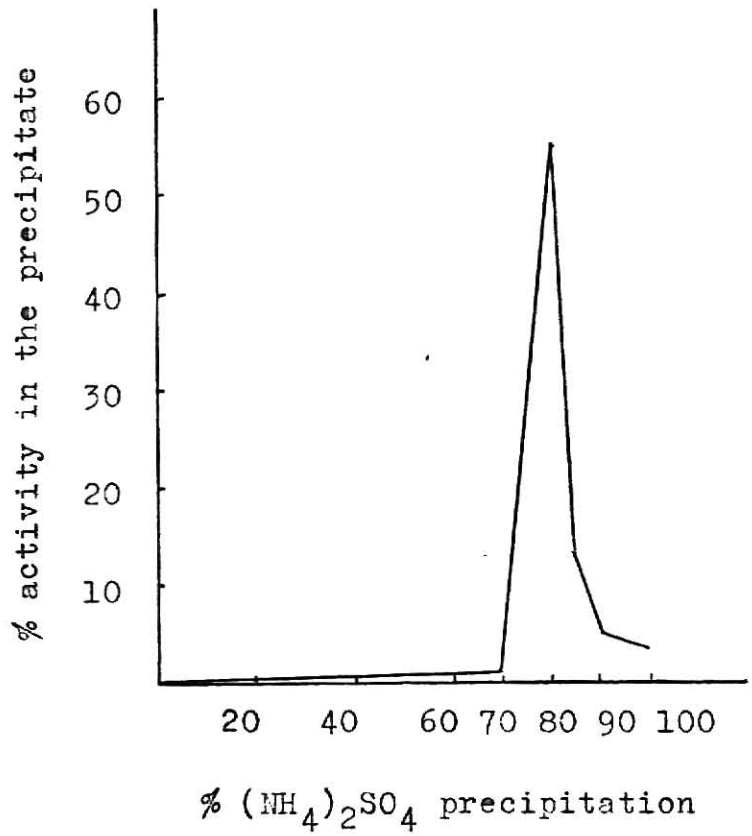


Figure 31: $(NH_4)_2SO_4$ fractionation of chloroperoxidase 4.50

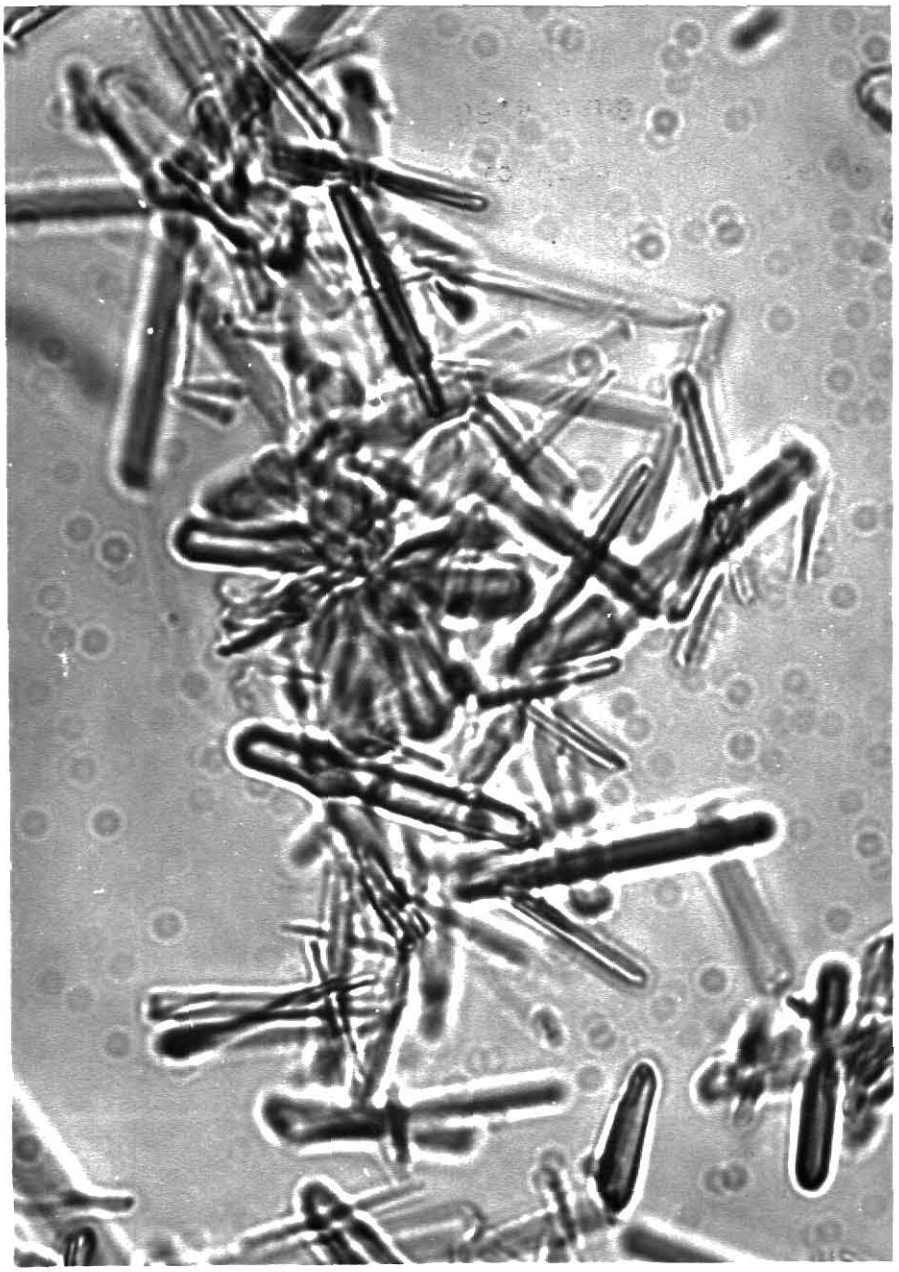


Figure 32: Micrograph of crystalline chloroperoxidase 4.50

crystals obtained are very similar to those obtained by HAGER and MORRIS (72), however they are different from those of SAE (84). Proteins often have different crystal forms. JRPO-a for example has two forms (24,54).

Crystallization of proteins has been looked upon as art rather than science. However if certain rules are obeyed, crystallization is relatively easy. The precipitated and centrifuged protein from the previous $(NH_4)_2SO_4$ fractionation step is dissolved in an $(NH_4)_2SO_4$ solution just short of the necessary saturation to precipitate the enzyme, at 0^oC. After centrifugation to remove any undissolved material the solution is allowed to stand at a constant, higher temperature. The protein crystallizes slowly as a result of the decrease of solubility at the higher temperature and as a result of the slow evaporation of water from the $(NH_4)_2SO_4$ solution. If this evaporation results in a slow enough increase of $(NH_4)_2SO_4$ concentration in the solution, the enzyme will crystallize and not precipitate. Chloroperoxidase even crystallized from solutions where the enzyme was not initially saturated.

Purification steps summarized in table 5 page 86 are general because they can be reproduced with various batches of culture media. The results reported, however, represent one run with 30 liters of crude juice.

The crystallized chloroperoxidase has a somewhat lower specific activity than the one crystallized by SAE (84), however it is close to that reported by Hager and Morris. The purification table on page 86 shows that the second peak from the DEAE-Cellulose chromatography pH 5 (see page 79) has a higher

Table 5: Purification of Chloroperoxidase

Fraction	vol (ml)	total activity units	total protein mg	specific activity units/mg	fold purification	overall yield
Dialyzed crude juice	27500	1.81×10^6	22800	79.5	1	100
DEAE column pH 5 fraction 3 (chloroperoxidase 4.50)	2000	6.8×10^5	660	1030	13	37.6 (+)
DEAE column fraction 5 (chloroperoxidase 3.05)	2090	7.45×10^5	418	1780	22.4	41.0 (+)
DEAE column fraction 4 (mixture)	2200	1.99×10^5	208	960	12.1	11.0 (+)
$(NH_4)_2SO_4$ (chloroperoxidase 4.50) precipitation	281	5.85×10^5	233	2510	31.6	32.3
Crystallization (chloroperoxidase 4.50)	not det.	not det.	not det.	2510	31.6	not det.

(+) Total recovery of principal fractions from DEAE column is 89.6%

specific activity than the first eluted enzyme and could explain with the above mentioned facts: different chloroperoxidases' isoenzymes may have different intrinsic specific activities.

Properties of Chloroperoxidase

SAE (84) already observed in his work that one isoenzyme was very heat labile. Since in the present work the enzyme was sometimes allowed to stand at room temperature for prolonged periods, its stability at 25°C was investigated. Fraction 1, page 79, was held at 25°C over a period of 50 days (see fig. 35). The enzyme was quite stable at this temperature and the loss of activity after 50 days was only about 25%.

Further tests were carried out to find the maximum temperature that chloroperoxidase 4.50 could be subjected without considerable activity loss. The enzyme from fraction 1 (see page79) was incubated at 0°C, 10°C, 20°C, 30°C, 40°C, 50°C for 60 minutes and the activity was plotted versus the temperature (see fig. 34, page 89). The enzyme is fairly stable up to 30°C, at higher temperatures the activity starts to decrease rapidly. The optimum stability is at 10-20°C.

In the experiments on the variation of H-CD growth medium, it was frequently observed that the chloroperoxidase activity rapidly declines at pH values higher than 7. Similar effects were observed when the crude juice was dialized against tap water for prolonged periods (24 hours and longer). Aliquots of crude juice were incubated for 1 hour at 25°C.in 0.2 M phosphate buffers pH 6.0 to pH 7.2 in 0.1 pH increments and were assayed at the incubation pH for guaiacol activity. The plot of peroxi-

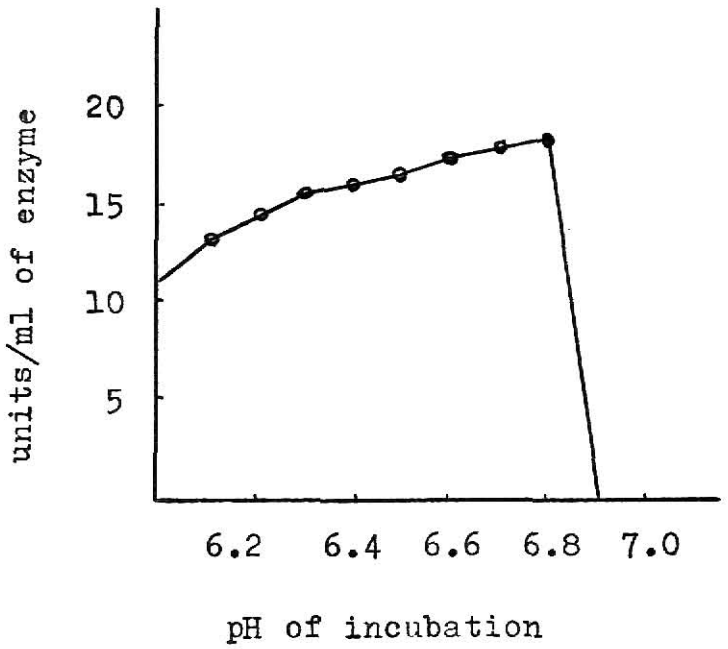


Figure 33: pH-optimum stability curve of chloroperoxidase in crude juice at 25°C (incubation 1 hour)

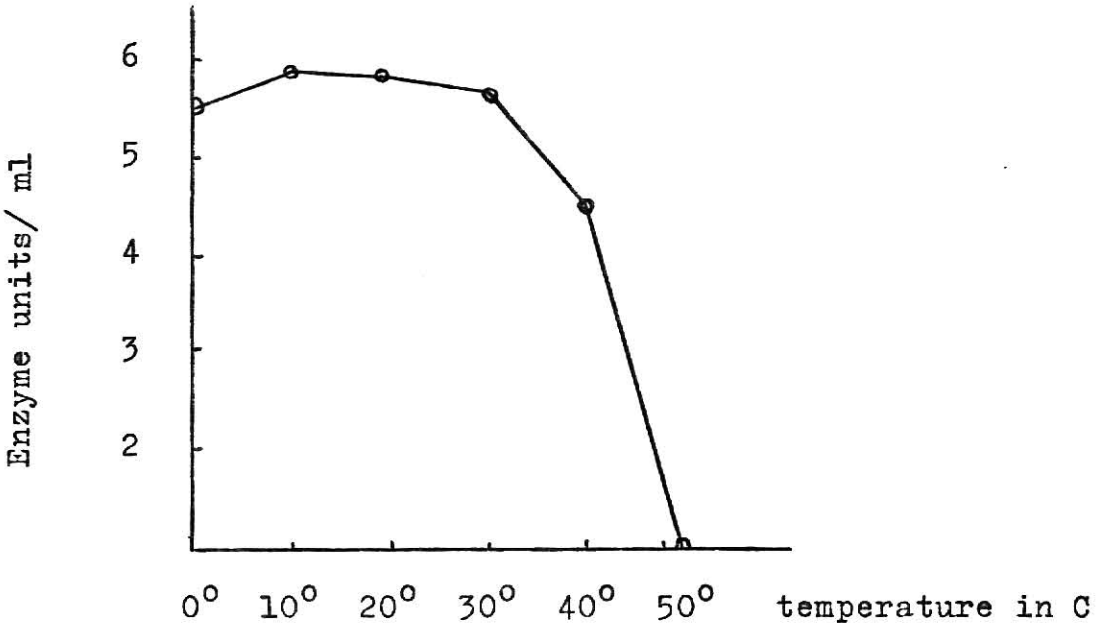


Figure 34: Temperature stability curve of chloroperoxidase 4.50

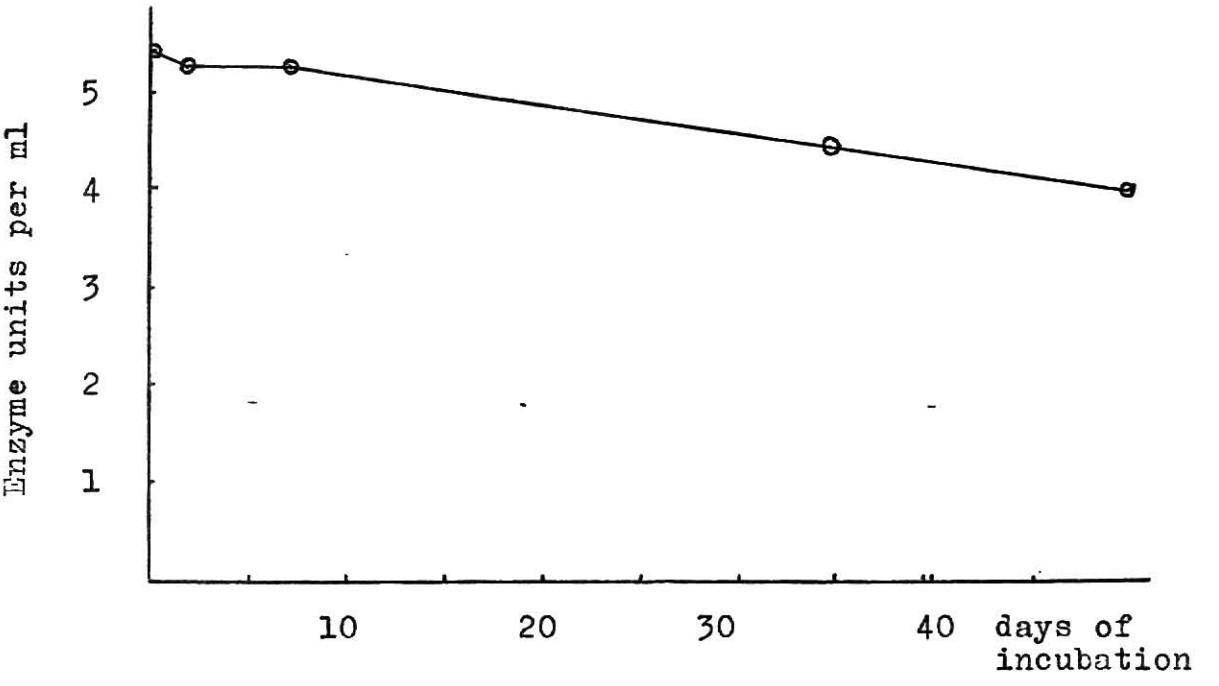


Figure 35: Chloroperoxidase 4.50 stability curve at 25°C

dase activity versus pH of incubation is shown in fig.33, page 88.

Guaiacol activity increases with pH from 6.0 to pH 6.8. At pH 6.9 however the enzyme activity decreases apruptly to zero. The loss is irreversible and the activity cannot be recovered by lowering the pH.

Already SAE (84) had observed a multiple chloroperoxidase protein band after acrylamide gel electrophoresis at pH 8.8. The protein seemed to be cleaved into at least three peptides. In addition to a possible cleavage of primary structure, changes in the tertiary structure of the chloroperoxidase might also occur above pH 6.9, and after the activity decreases. The pH of tryptic digests should probably be below pH 7 to avoid ambiguous peptides from spontaneous breakdown. The pH optimum study clearly shows the reason for enzyme loss during growth experiments where the pH went higher than 7.

Isoelectric Points of Chloroperoxidase

HAGER (72) had reported only one chloroperoxidase, but later SAE (84) proved the existence of two different isoenzymes with electric points at pH 3.57 and 3.85, respectively. Chloroperoxidase 3.85 was the major part of the peroxidase activity in Sae's experiments.

In my thesis work an isoelectric focusing experiment was conducted on the crude juice (see page 91) and, surprisingly, two isoenzymes were found with isoelectric points at pH 3.49 and pH 4.48, respectively. There was no activity at pH 3.85 (see fig. 36). A tentative conclusion was: of the two isoenzymes produced, one was Sae's pI 3.5 enzyme and one was a new isoenzyme (isoelectric point at 4.48) which replaced Sae's pI 3.85 enzyme.

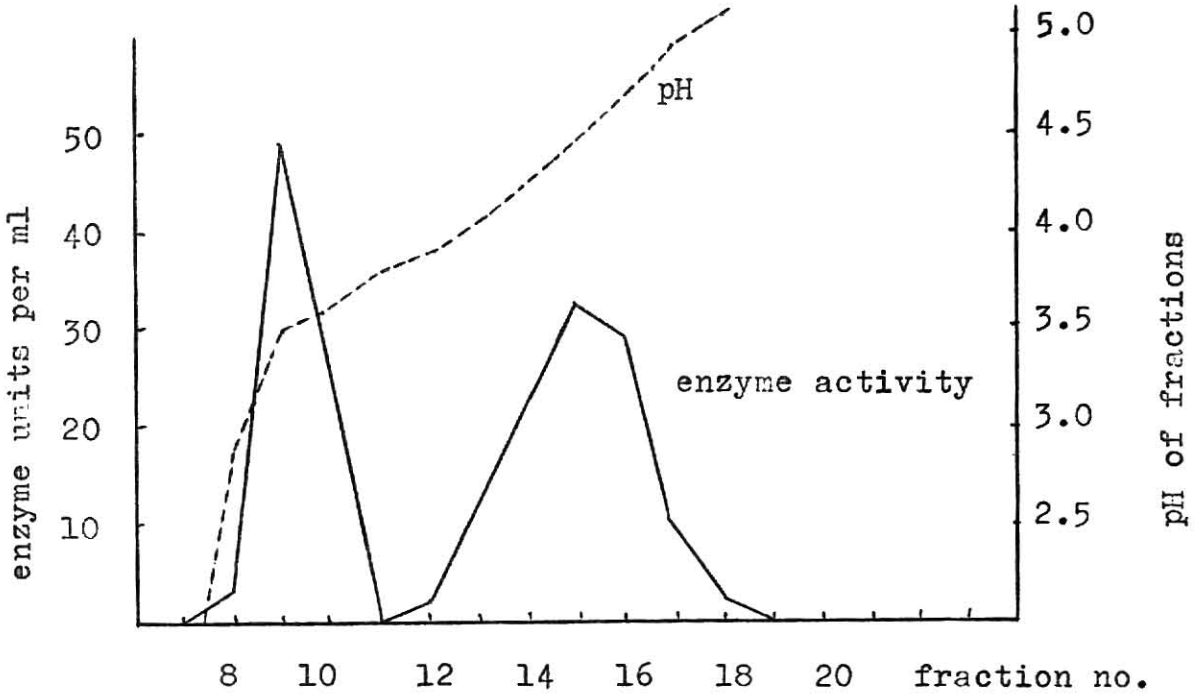


Figure 36: Isoelectric focusing of crude juice. A profile of activity (guaiacol-H_2O_2) versus pH and fraction number.

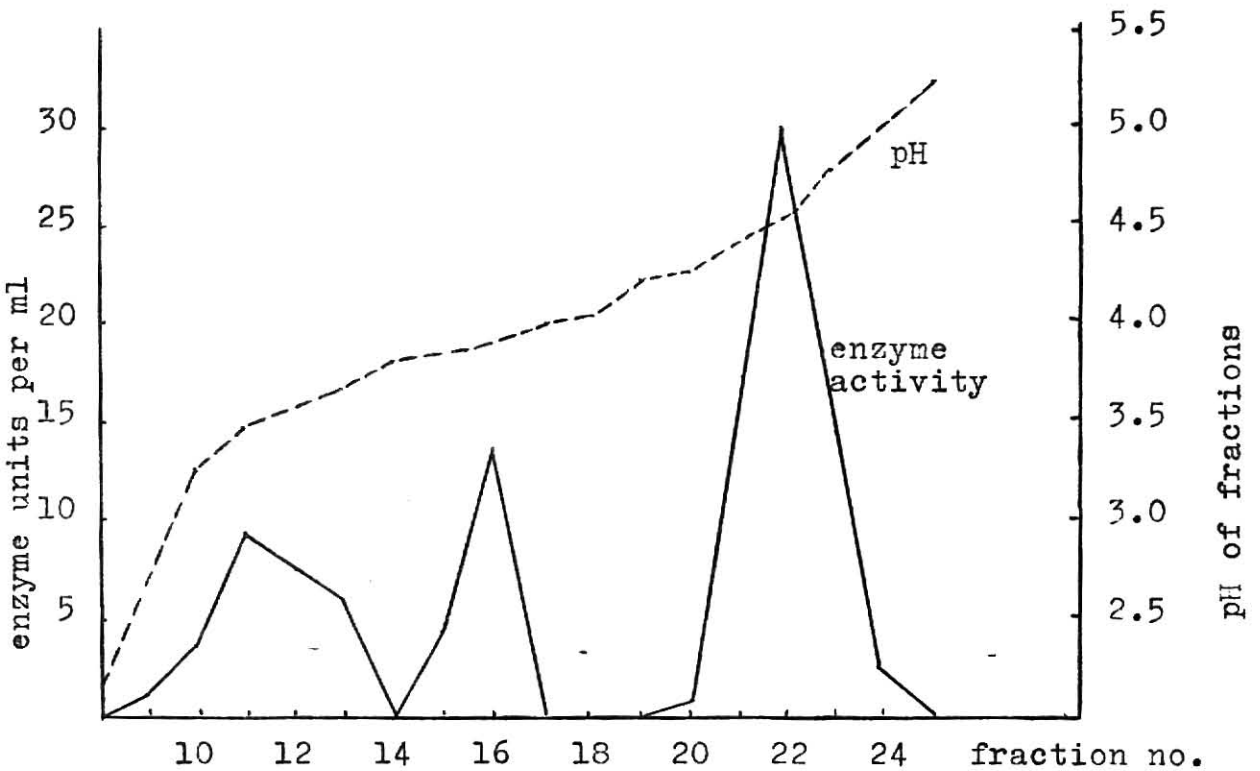


Figure 37: Isoelectric focusing of fraction 4 (see page 94) after the 2nd DEAE pH 5 column.

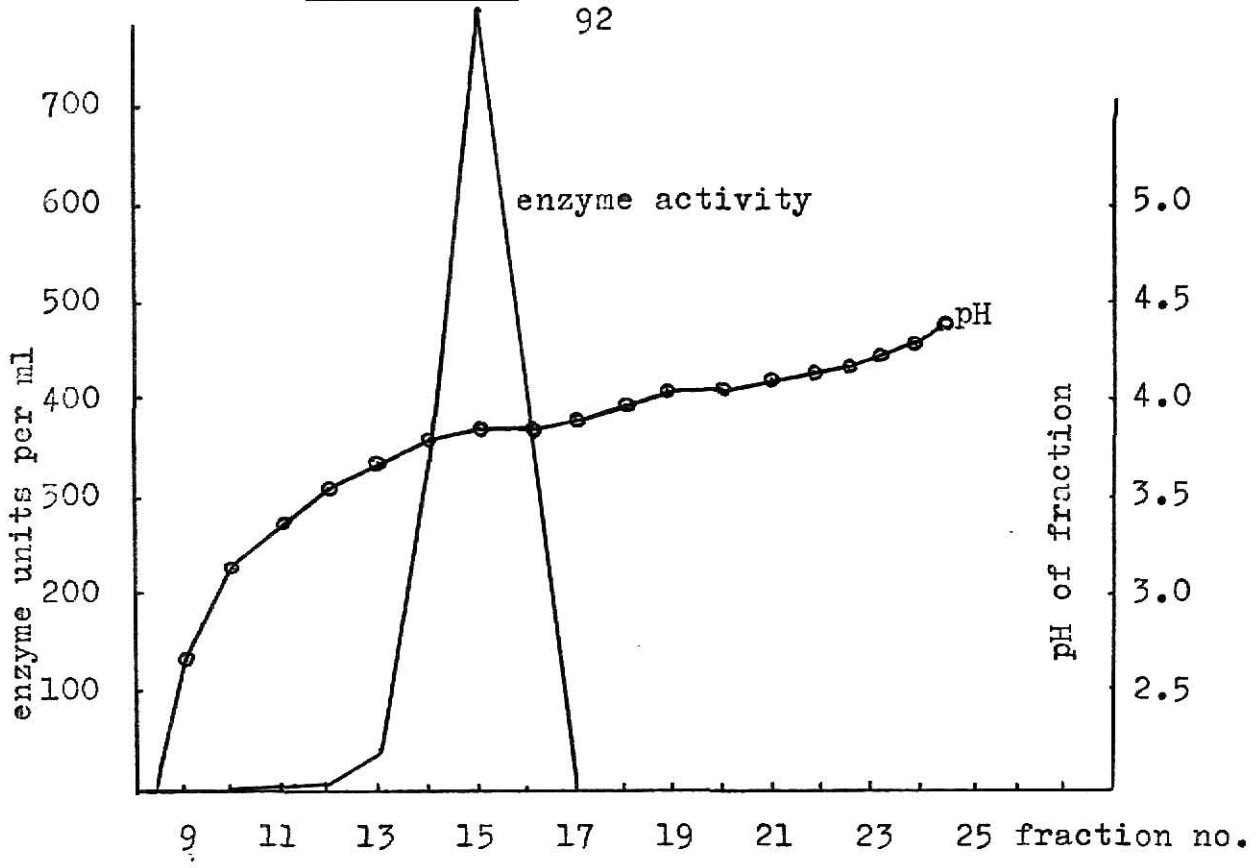


Figure 38: Isoelectric focusing profile of chloroperoxidase from the second peak, pH 3.85 DEAE column (see page 94)

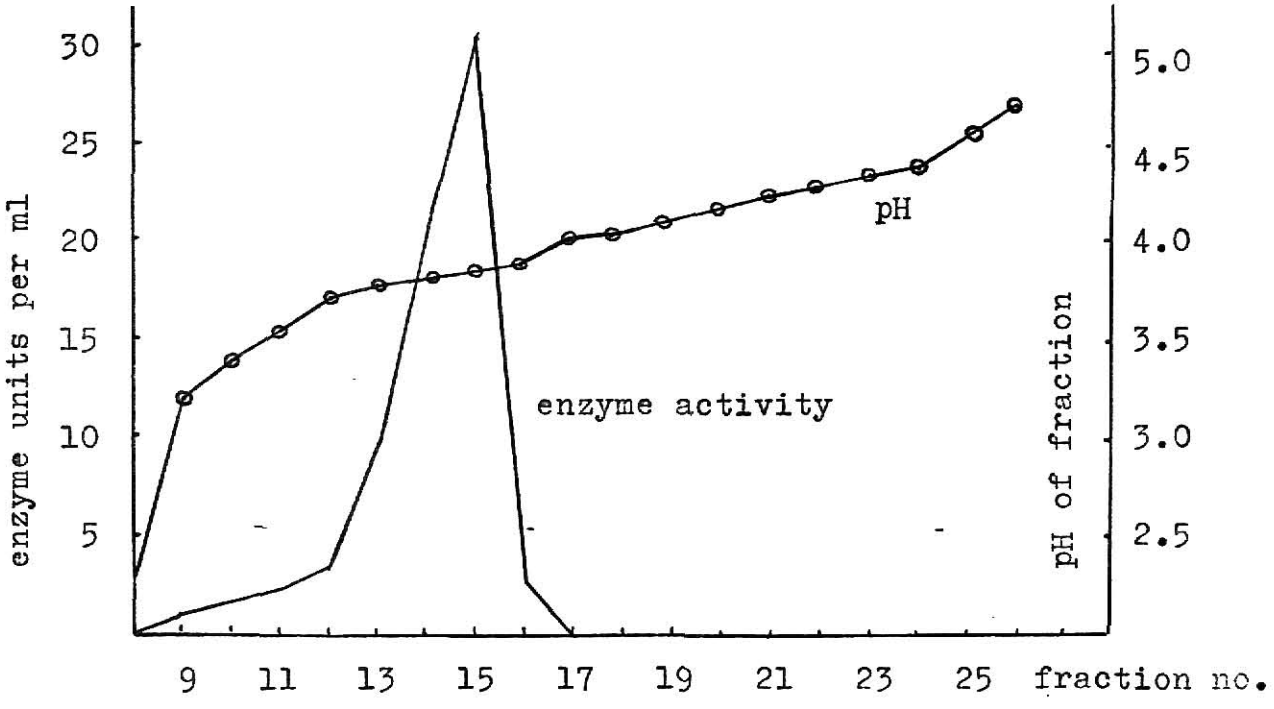


Figure 39: Same as figure 38 except sample is the first peak from the pH 3.85 DEAE column

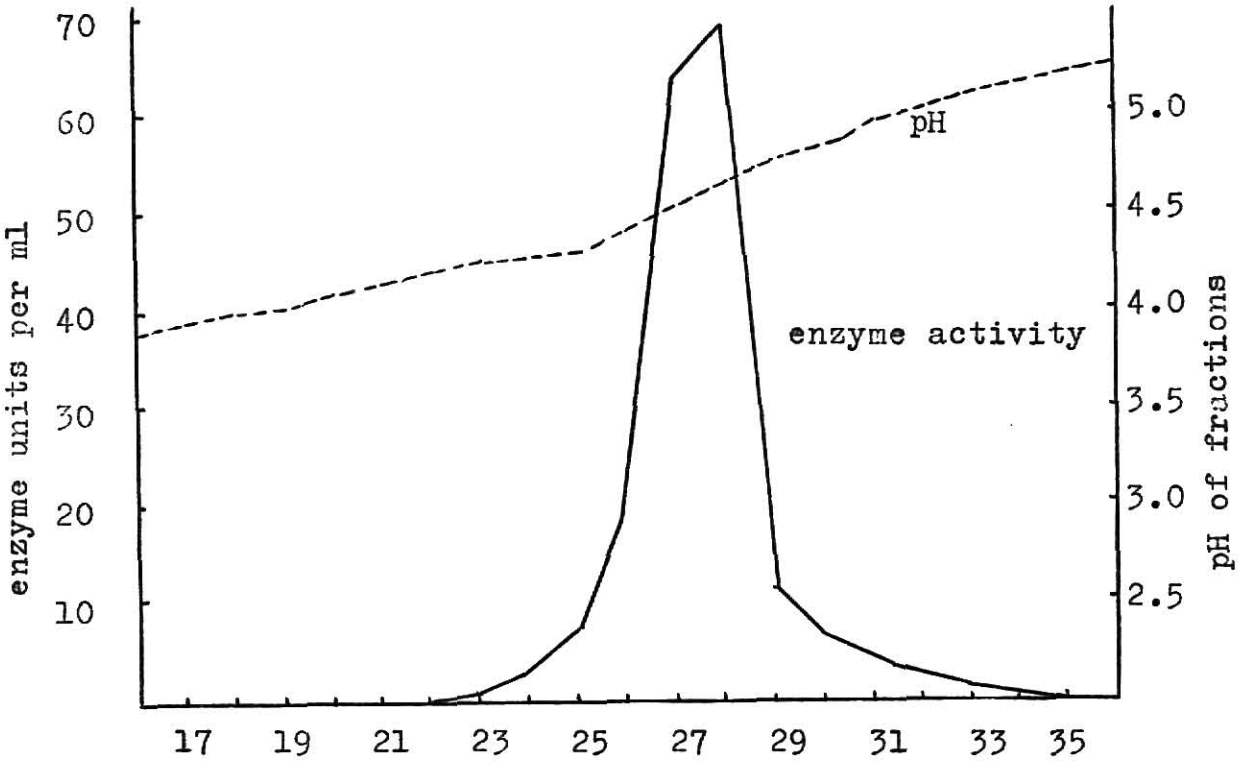


Figure 40: Isoelectric focusing profile of crystallized chloroperoxidase 4.50

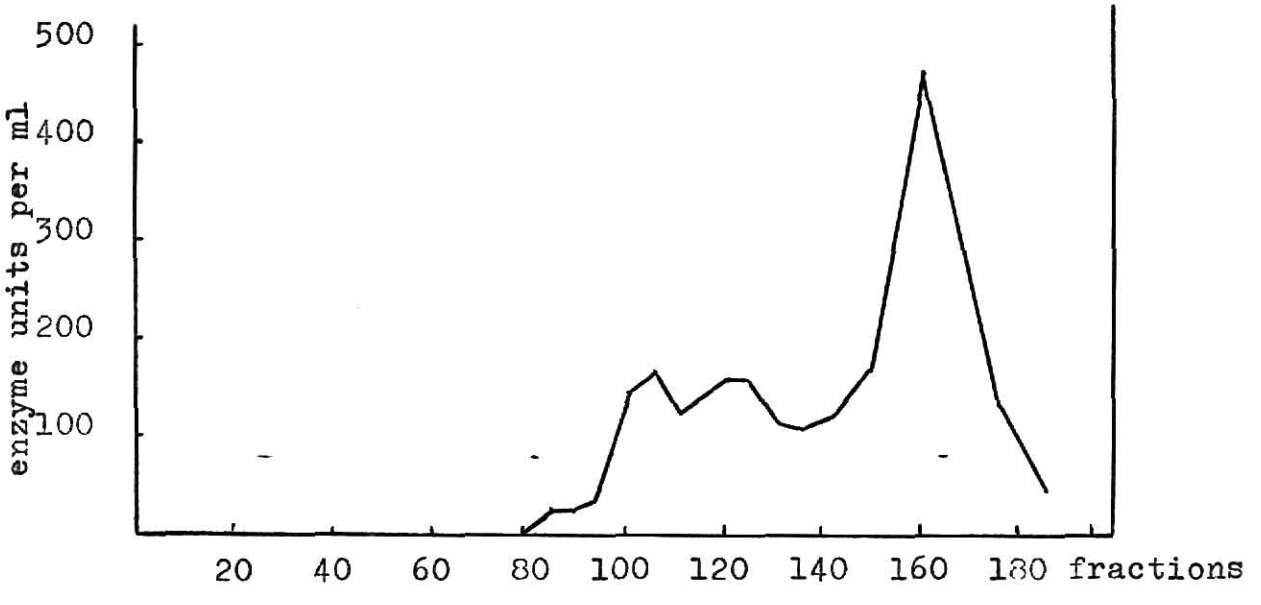


Figure 41: Elution profile of chloroperoxidase fraction 4 (see page 86) re-chromatographed through a pH 5 DEAE column

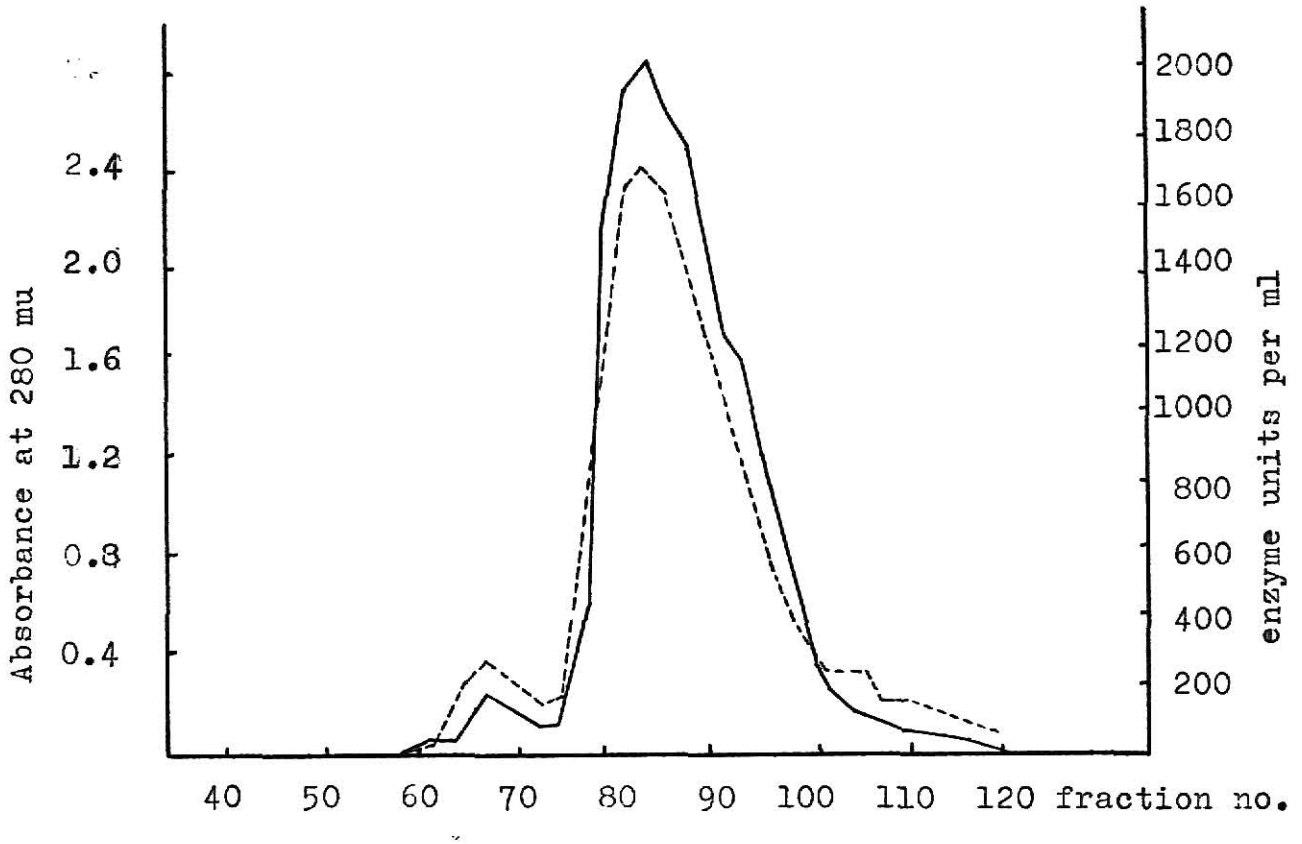


Figure 42: Elution profile of re-chromatographed chloroperoxidase fraction 5 (see page 86) through pH 5 DEAE column.
—— enzyme activity, -----absorbance at 280 mu

Since the elution profile of the pH DEAE-Cellulose column chromatography (see fig. 30, page 79) showed some minor peaks between the two major peroxidase peaks, that middle fraction was re-chromatographed on DEAE-Cellulose at pH 5.

The resulting elution profile (see page 93) gave three peaks, one major and two minor. Representative fractions from these three peaks were applied to an isoelectric focusing column (see page 67) with the result (see fig. 41, page 93) that three isoenzymes with pI at pH 3.48, pH 3.89 and pH 4.56, respectively (see fig. 37, page 91) could be observed.

The second major peak from the first DEAE-Cellulose chromatography elution was applied to a pH 3.85 DEAE-Cellulose column and the elution profile (see fig. 42, page 94) showed one minor and one major peak of peroxidase activity. Samples from both were applied to an isoelectric focusing column and their pI's measured after the elution. Surprisingly, both peaks gave identical pI values at pH 3.85. The conclusion is that the minor peak is an artefact probably caused by an unevenly packed DEAE column.

Finally, the crystallized and lyophilized chloroperoxidase was applied to an isoelectric focusing column. The analysis showed a single component with pI at a pH 4.65.

The variance in pI values is somewhat difficult to explain. The LKB Ampholine Instruction Sheet reports that the isoelectric focusing technique is reproducible to 0.01 pH units. If we assume that in all 5 isoelectric focusing experiments the reproducibility was $\pm$ 0.01 pH units, the explanation for these curious data could be as follows. C. fumago synthesizes two peroxidase iso-

enzymes with pI at pH 3.49 and 4.48, respectively, due to the modified growth conditions. During the isolation and purification procedure the isoelectric points shift to pH 3.85 (from 3.49) and to pH 4.65 (from 4.48). The one isoenzyme with pI at 4.48 in the crude juice, shifts to pI 4.56 after two pH 5 DEAE-cellulose steps and finally the pI shifts to 4.65 after crystallization and lyophilization. The second isoenzyme has its pI at 3.49 in the crude juice. After two DEAE-steps (pH 5 and pH 3.85 column) the pI shifts to 3.85.

These empirical observations of course do not explain the fundamental cause of the variance. Another explanation may be differences in pH measurement from one day to the next, although a variance of 0.1 pH unit would be the maximum error expected from instrumentation or operator error combined.

Molecular Weight of Chloroperoxidase 4.50

The molecular weight of chloroperoxidase 4.50, estimated by iron determination was calculated to 26,500, 26,100, 25,100, 27,600 (on dry weight basis). The average value is 26,400. The values obtained from Sae were 40,000 and 46,000 for his two isoenzymes and the value reported by HAGER and MORRIS (72) was 42,000 from sedimentation experiments. This difference is surprising, however, the chloroperoxidase with MW 26,400 has a very different pI and was grown on a different medium.

A titration of chloroperoxidase 4.50 with p-Cl-mercuribenzoate indicated (see page 71) no free cysteine and an amino acid analysis (see page 97) showed two cysteine residues per molecule of enzyme. A Sephadex G-100 molecular weight estimation was therefore carried out in order to check on the possibility

of a dimeric chloroperoxidase. The VE/VO ratio of HRP (1.82) compared with a VE/VO ratio of 1.92 for chloroperoxidase 4.50 gave a calculated MW from gel filtration of approximately 35,000 or less. This proves that the molecule most be a monomer, i.e. one heme iron for 26,400 MW. The somewhat high value from the Sephadex determination is not readily explained, but may be due to anomalous effects (high values) sometimes observed for glycoproteins (104).

Amino Acid Composition

The amino acids were determined only once and no residues were corrected for decomposition in HCl. The number of residues were estimated as follows (calculated values in parentheses): Lys 6 (5.72), His 11 (10.85), Arg 6 (5.72), Asp 18 (17.5), Thre8(8.13), Ser 10 (9.60), Glu 11 (11.2), Pro 13 (12.6), Gly 9 (9.32), Ala 18 (17.8), Val 4 (3.92), Cyst 2 (2.20), Meth 1 (0.954), Ileu 5 (5.12), Leu 11 (10.6), Try 4 (4.1), Phe 8 (8.1).

The minimum molecular weight of the polyamide chain calculated from these data is 17,400. The additional weight required to make 26,000 MW from the iron content calculation is assumed to be carbohydrate. Hager reported 30% carbohydrate for his chloroperoxidase. The HCl-hydrolysate contained considerable black humin which is characteristic for glycoprotein samples. Although HAGER (72) and BROWN (18) reported no tryptophan present in chloroperoxidase, Sae observed (84) a shoulder region at 290 mu in his spectral studies and suggested that tryptophan was present. My data also suggests the presence of tryptophan, however no specific tryptophan analyses were tried.

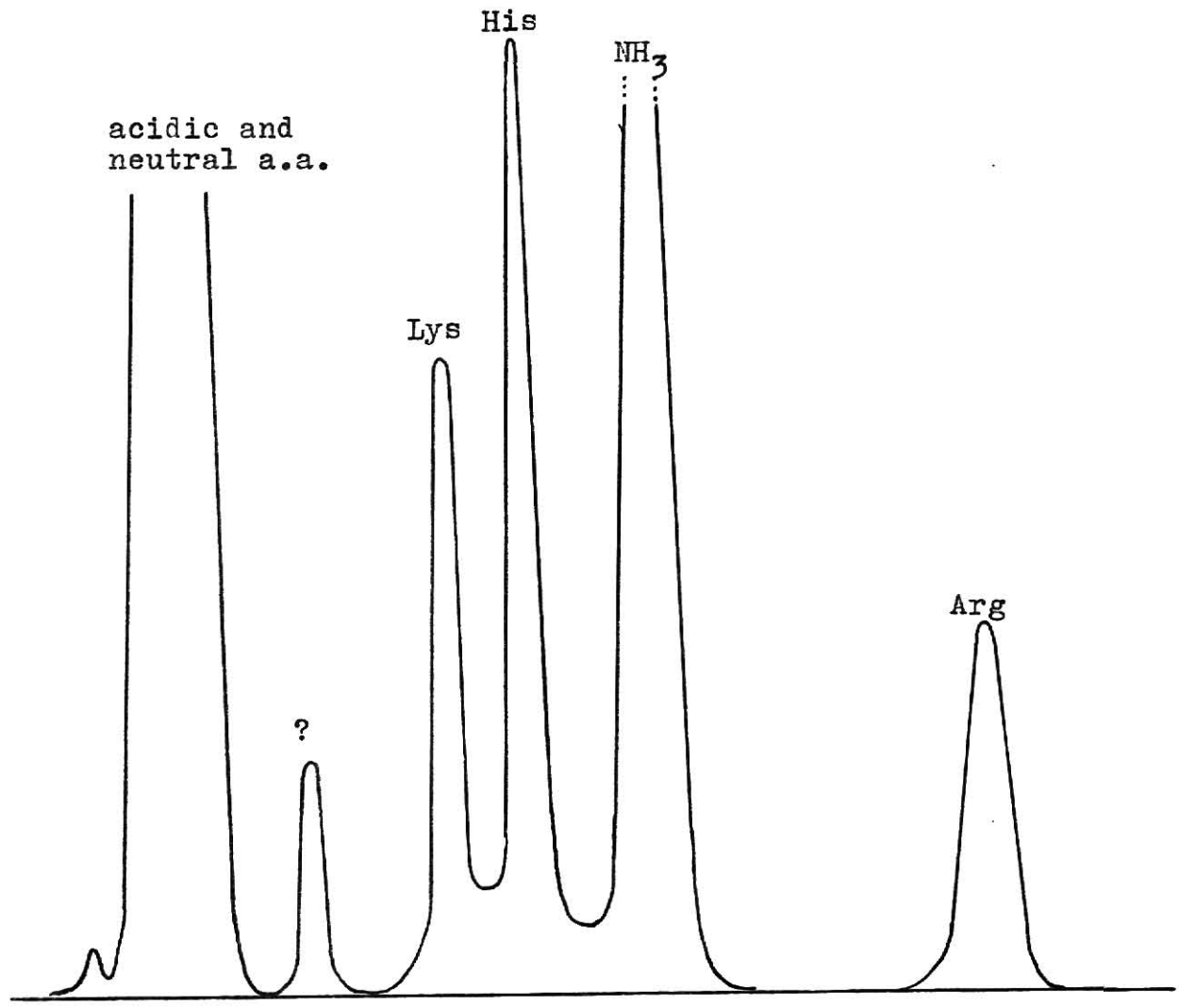


Figure 43: Elution profile of the basic amino acids from Beckman 120C Amino Acid Analyzer (chloroperoxidase 4.50 HCl digest.

When the basic amino acid analysis was carried out (see page 73), a peak was detected (see page 98) near the predicted try position. However, all the try should have been decomposed during hydrolysis and this peak could also represent α-amino butyric acid or Δ-amino valeric acid or some decomposition compound from the heme.

Titration of Chloroperoxidase with p-Cl-mercuribenzoate

This method introduced by BOYER (16), is convenient, rapid and specific for free and accessible SH-groups. SH-groups require several hours for reaction with p-Cl-mercuribenzoate (PCMB) (12).

A GSH-standardization-titration gave the PCMB concentration. Titration of chloroperoxidase with that PCMB did not produce any absorbance increase which proves that chloroperoxidase does not have readily available SH-groups at pH 4.6. The two cysteine residues found in the amino acid determination therefore form a S-S linkage, or loop within a 27,000 MW monomer. BROWN (18) had previously shown that titration of his 40,200 MW chloroperoxidase with PCMB gave no free SH and therefore the two cysteine residues (by amino acid analysis) most likely formed an S-S linkage.

There are striking similarities between the MORRIS and HAGER and BROWN (18) data and the above discussed facts of this thesis work. In all cases two cysteine residues were found by amino acid analysis and no free SH-group could be detected. The amino acids in both cases are quantitatively the same, no one is additionally incorporated. But the isoenzyme discussed in this thesis (26,000 MW) has a much lower number of total

amino acid residues and has a slight change in the ratio of acidic to neutral and basic amino acids. This is reflected in a pI (4.50) which is more basic than Hager's enzyme (probably 3.85).

These isoenzymes are similar in pI, cysteine structure, enzymatic activity, and carbohydrate content, but both are surely different in either shape or size of the molecule due to the distinct difference in molecular weight. This raises many questions about the origin of the isoenzymes and the nature of the events that led to omitting an appreciable number of amino acid residues without omitting any variety of amino acid and without losing peroxidatic activity. It may, however, be significant in this regard that the specific activity and the Soret extinction of chloroperoxidase 4.50 are lower than the other isoenzymes.

Absorption Spectra of Chloroperoxidase 4.50

A.) Visible region from 690 mu to 350 mu

The absorption spectrum of the crystallized chloroperoxidase 4.50 in 0.2 M phosphate buffers of pH 2, 4, 6, 7, 8 are shown in figures 44, 45, page 102, 103. The absorption maxima are listed below.

pH	absorption maxima mu				
2	640	530	505	404	
4	645	536	512	410	
6	645	538	515	412	
7	648	-	535	416	
8	-	-	540	421	362

pH	millimolar absorption coefficient (MW 26000 used)			
2	6.53	9.44	9.98	37.5
4	6.35	10.0	10.65	39.5
6	4.37	8.51	8.85	37.2
7	5.23	-	9.10	37.6
8	-	-	8.25	35.4

The millimolar coefficients closely resemble those obtained by Hager and Sae except for the coefficients of the Soret band (404 mu - 421 mu) which are about 1/2 of those previously reported. Sae had calculated the following maxima for chloroperoxidase 3.85 in distilled water: 398 mu with coefficient 76.3, 513 mu with 13.5, as coefficient, and 648 mu with 5.9 as coefficient, and 543 mu with 12.3 as coefficient.

The above data on the absorption maxima of chloroperoxidase 4.50 in relation to the pH show a definite shift of the maxima toward higher wavelength with increase of pH. At pH 7 the maximum around the wavelength of 535 mu is disappearing and at pH 8 the maxima around 648 mu and 530 mu are disappearing, but a new maximum at 362 mu is appearing.

At all pH values there was a shoulder region around 590 mu which was also observed by Sae but not by Morris and Hager.

The 450 mu to 650 mu region absorption bands are due to electron transitions between the iron atom and its ligands with the peroxidase heme. The Soret band is a $\pi \rightarrow \pi$ transition probably in a region remote from the metal atom (12) and is affected by solvents.

The shift of the maxima with change of pH is probably due to solvent effects. The increase of pH is also accompanied by

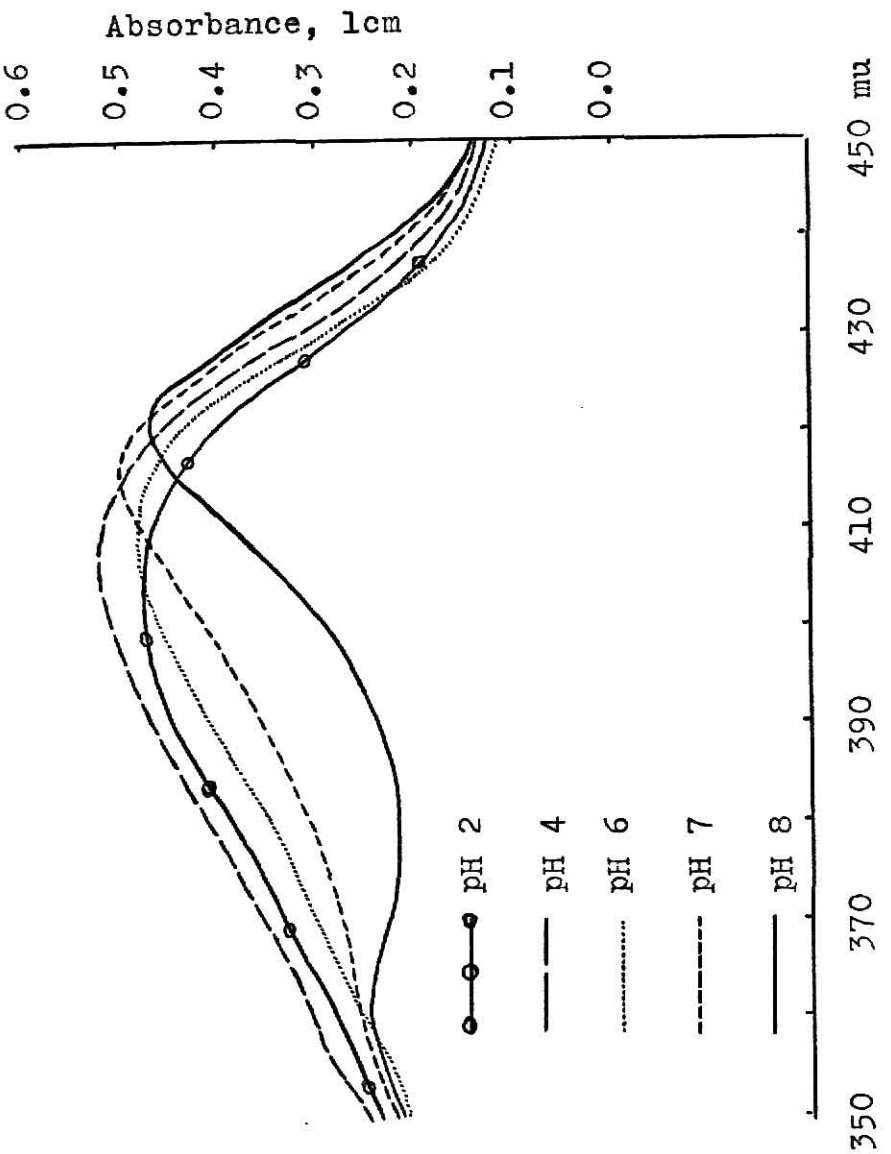


Figure 44:Chloroperoxidase 4.50 absorption spectra in the visible region (450mu-350mu) at different pH in 0.2 M phosphate buffer.

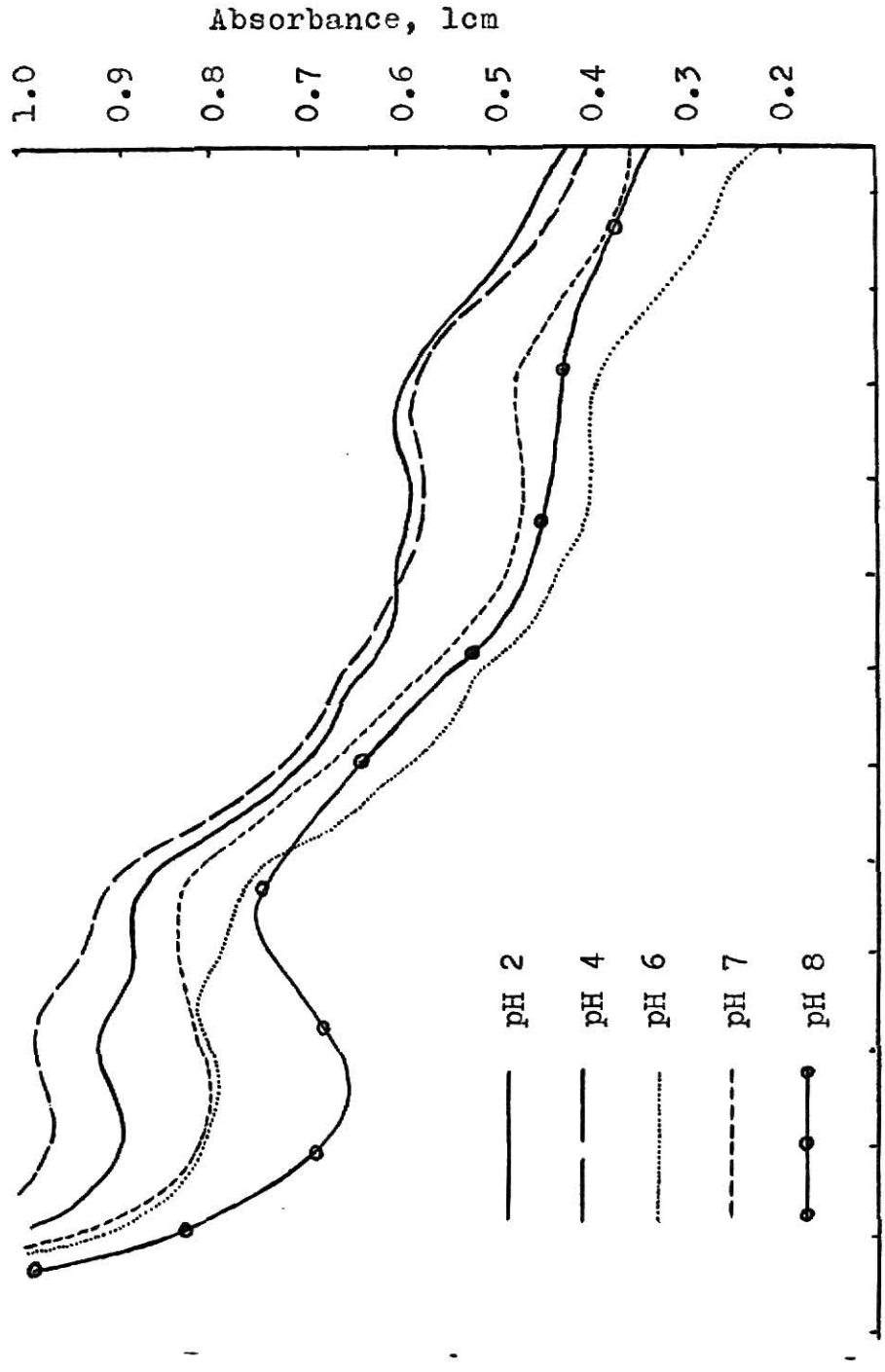


Figure 45: Chloroperoxidase 4.50 absorption spectra (700mu-450mu) at different pH in 0.2 M phosphate buffer.

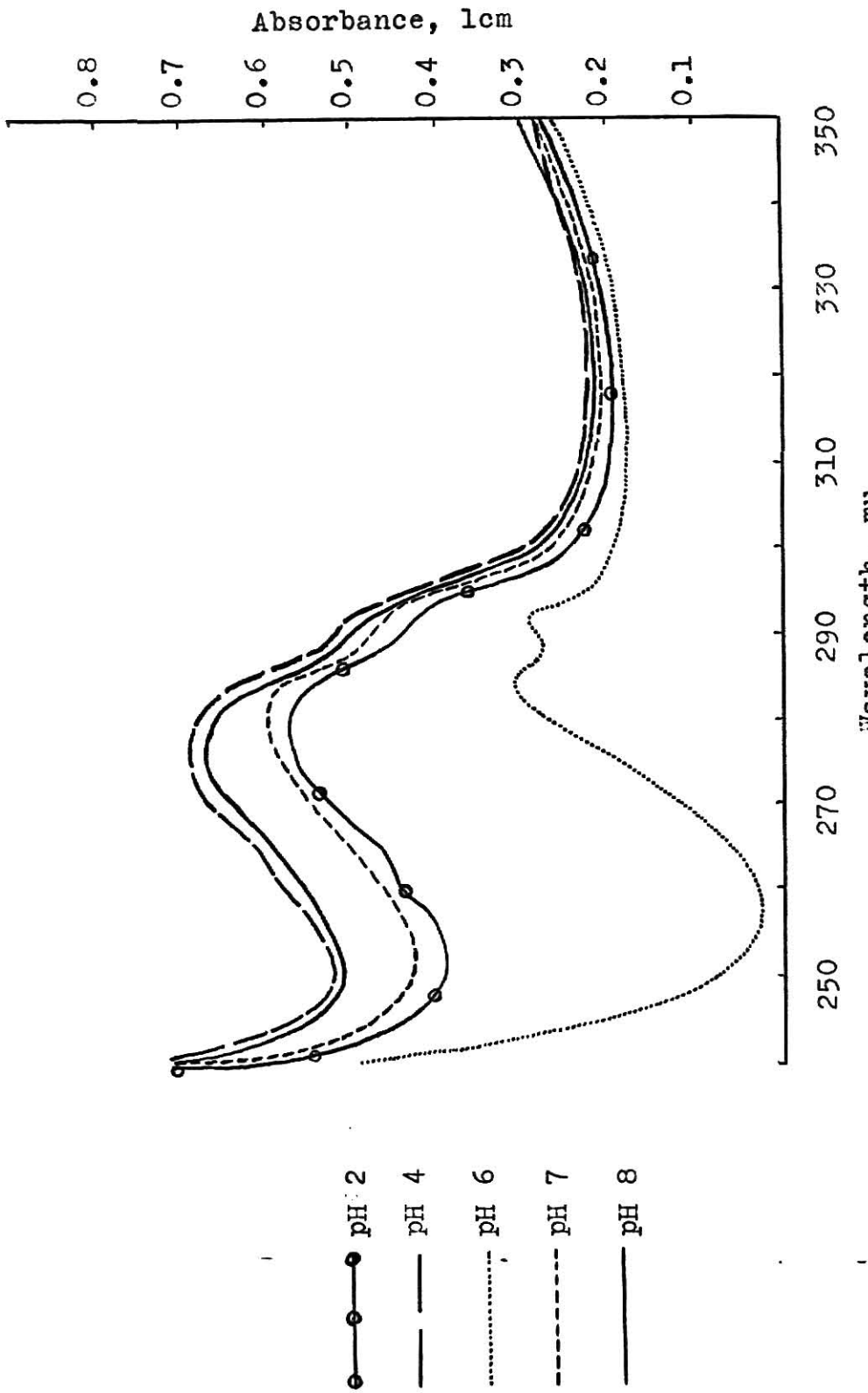


Figure 46: Chloroperoxidase 4.50 absorption spectra (350mu-240mu) at different pH in 0.2 M phosphate buffer.

an increase in K^+ concentration. The disappearance of the maxima at 530 mu and 640 mu at higher pH values (pH 7 and 8) as well as the appearance of the maximum at 362 mu at pH 8 is very likely correlated with significant structural changes that might occur in parallel with the alkaline deactivation of the enzyme. A cleavage of the peptide chain may be involved in such changes (see page 107).

The above studies may be summarized: Chloroperoxidase 4.50 possesses the typical absorption spectrum of hemoprotein similar to chloroperoxidase 3.85 (SAE,84). The spectrum is affected by solvent pH changes (pH 2-8) which may alter tertiary structure or even result in cleavage of peptide chains. All spectra shift to longer wavelength with increase of pH. The maxima at 530 mu disappears at pH 7, and the maxima at 530 mu and 640 mu diappear at pH 8. A new maximum at 362 mu appears at pH 8.

UV - Region (350 mu - 250 mu)

The UV-absorption spectrum of chloroperoxidase has been reported by SAE (84). Figure 46, page 104, shows such a spectrum for chloroperoxidase 4.50 in solution with different pH. It exhibits the properties of many proteins in this region. The absorption shoulder at 290 mu indicates clearly that tryptophan is present among the amino acid residues and in amounts sufficient to give a rather low A_{410}/A_{280} ratio for the hemoprotein. However the amino acid composition reported by Morris and Hager and by Brown does not show tryptophan. No attempt has been made to determine it by a special chemical method.

Rather prominent is the change of absorption spectrum in

the sample containing pH 6 phosphate buffer (0.2 M). There the tryptophan shoulder region contains a prominent peak, while the absorption at 260 mu drops to almost zero.

A possible explanation is that changes in the three-dimensional structure of the enzyme cause the tryptophan residue(s) of the molecule to become more exposed. An additional hypothesis is that increased guaiacol-H_2O_2 affinity of the enzyme with increase of pH results from these structural changes. Indeed, the peroxidatic activity to guaiacol-H_2O_2 increases significantly in the pH range 6.0 to pH 6.8 (see page 88, fig. 33).

Further analysis of conformational changes under these conditions would require studies by far UV-optical rotatory dispersion and circular dichroism and ultimately by X-ray diffraction.

Tryptic Digestion of Chloroperoxidase

Chloroperoxidase 4.50 was subjected to tryptic digestion at 38°C in 0.03 M $NaHCO_3$ pH 8.3 and in distilled water. A peptide map in two dimensions (first direction paper chromatography, second direction high voltage electrophoresis) was prepared. This map shows (see fig. 47, page 107) 12 spots and a band which is difficult to explain but probably represents trypsin. These spots can be correlated with 5 bands which result from separation by high voltage electrophoresis and six bands separated by chromatography. From a single high voltage electrophoresis run 5 bands were detected which confirms the predicted correlation.

The digest (a) in buffer pH 8.3,(b) the digest in distilled water, (c) the blank chloroperoxidase incubated in buffer pH 8.3, (d) blank chloroperoxidase in distilled water, and (e)

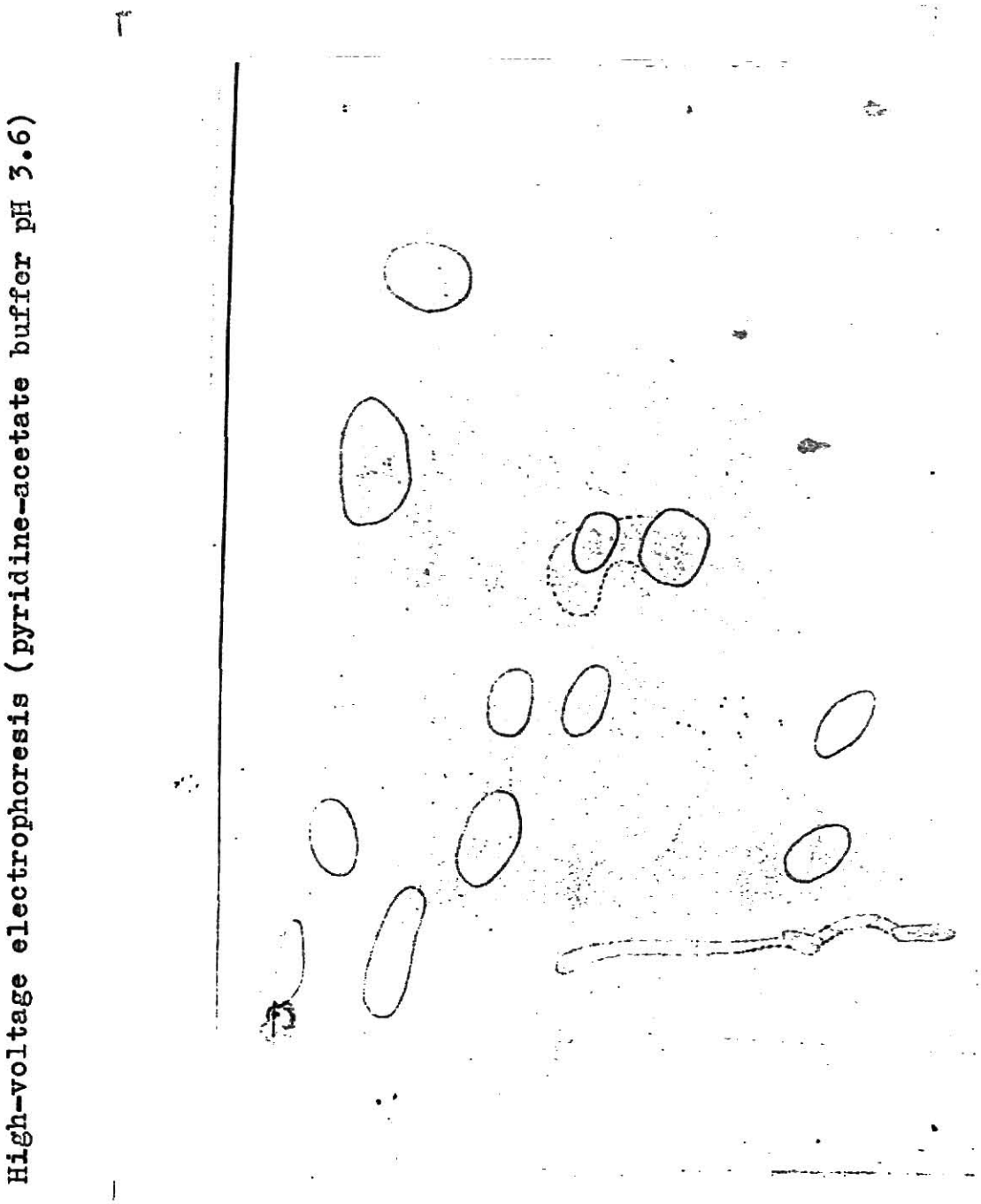
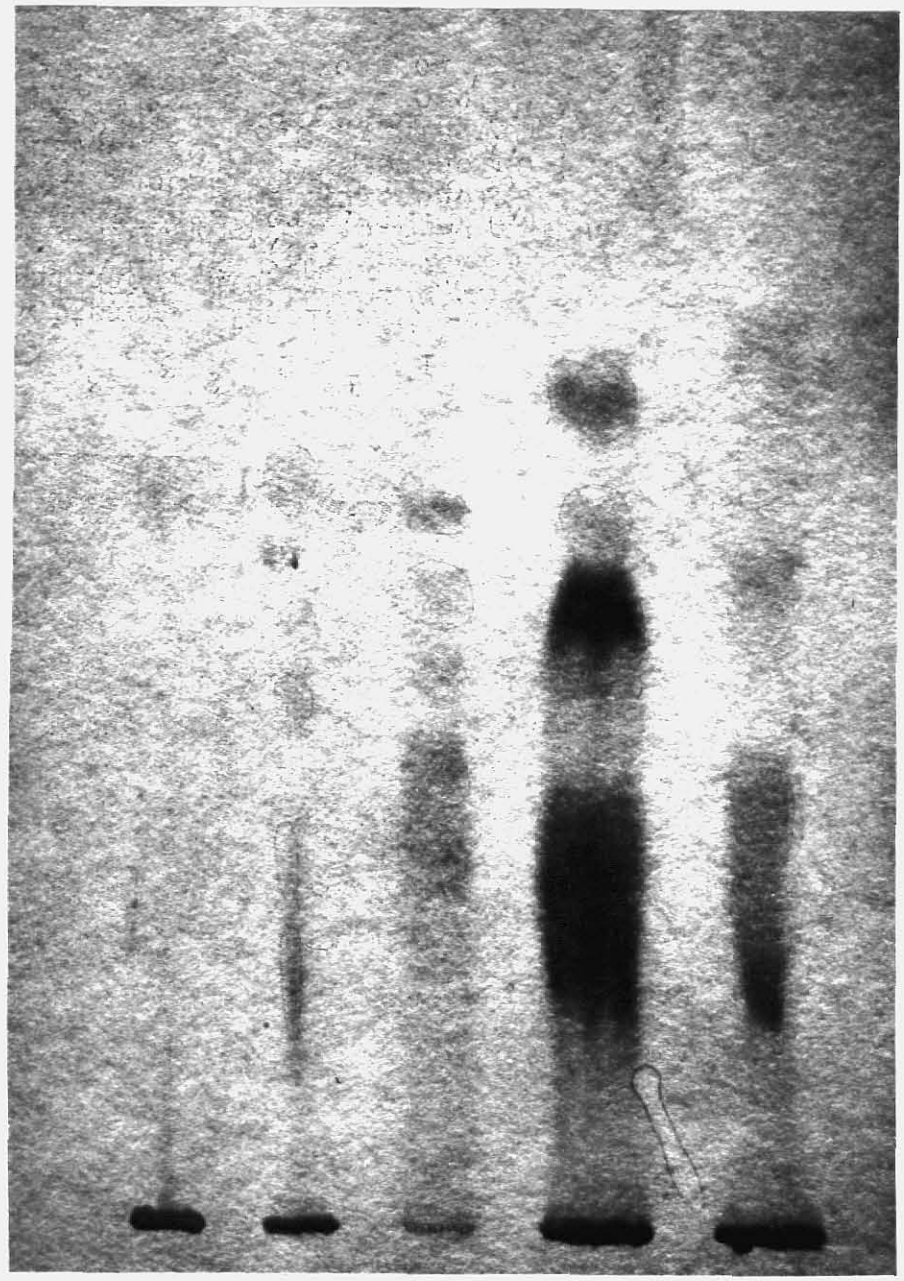


Figure 47: Two-dimensional separation of tryptic digest of chloroperoxidase 4.50



1 2 3 4 5

Figure 48: One-dimensional separation of chloroperoxidase 4.50 (high-voltage electrophoresis in pyridine-acetate buffer pH 3.6). Incubation time was 16 hours.
1.) Chloroperoxidase in 0.03 M $NaHCO_3$ pH 8.3, 38°C
2.) Chloroperoxidase in distilled water at 38°C
3.) Trypsin in 0.03 M $NaHCO_3$ at 38°C
4.) Chloroperox. + trypsin in dist. water at 38°C
5.) Chloroperoxidase + trypsin in 0.03 M $NaHCO_3$, 38°C

a blank trypsin solution in buffer pH 8.3, were subjected to a one-dimensional high voltage electrophoresis (see photo 48, page 108). The results are surprising. A considerable auto-digestion of trypsin is seen such that five different peptide bands are observed. The tryptic digestion of chloroperoxidase in distilled water is more effective if compared to the digestion in pH 8.3 buffer, because the corresponding bands are developed more intensely by ninhydrin. At pH 8.3 buffer and in distilled water chloroperoxidase decomposes to three and four peptides, respectively (incubation temperature 38°C).

These date have to be considered for all future structural work on chloroperoxidase if reproducible peptide maps are to be obtained. The decomposition of chloroperoxidase 4.50 into three and four peptides at high pH (8.3) and high temperature (38°C) reinforces earlier suggestions concerning deactivation at basic pH and related changes in the visible region (see page 100).

SUMMARY

A complete substitute for yeast extract in the H-CD growth medium for production of chloroperoxidase (E.C.1.11.1.7, Donor: H_2O_2 oxidoreductase (hydroperoxidase) has been found. It contains a vitamin combination in addition to some metal and anion changes to further modify the basal medium used by Hager, The supplement composition per liter is: 12mg thiamine, 12mg pyridoxal, 12mg folic acid, 12mg pantothenate, 200ug vitamin B_{12} (instead of 3.3g yeast extract), and 40mg $FeSO_4.7H_2O$ and 0.5g KNO_3 (instead of 10mg $FeSO_4$ and 0.5g KCl). The agar slants used for propagation of the fungus culture have now a supplement with thiamine, pyridoxal and vitamin B_{12} in the above concentrations (instead of 10ml oat meal broth) and equally good enzyme producing cultures are abtained. The average increase of activity is about 10 fold if compared with the yeast extract supplement.

Apparently as a result of the new improved growth medium the same strain of Caldariomyces fumago now produces a unique chloroperoxidase isoenzyme which is more basic than previous forms. Isoelectric focusing studies showed the new isoenzyme to be isoelectric at pH 4.5 and it was named accordingly. Chloroperoxidase 4.5 was purified in crystalline form by a new isolation procedure.(a guaiacol assay was used during the isolation) as described below.

Growth medium was dialized against tap water or distilled water (dialysis was later found not essential) and applied to a DEAE-Cellulose column at pH 5. Elution was with a phosphate buffer gradient (0.01 M - 0.2 M pH 5), and two peaks

were obtained. The first peak representing chloroperoxidase 4.5 was precipitated with 70% $(NH_4)_2SO_4$ and subsequently crystallized in $(NH_4)_2SO_4$ solution.

Iron determination established its molecular weigth to be 26,000. The amino acid composition was determined. No new varieties of amino acids were found, and no known amino acids were omitted (compared to Hager's analysis). However, some amino acids must have occured in smaller numbers because the calculated peptide portion molecular weigth of 17,400 is much smaller than previously observed chloroperoxidase. The balance to MW 26,400 (34%) is carbohydrate.

Some others properties determined were: chloroperoxidase 4.5 has no free SH-groups although two cysteine residues are present in the enzyme molecule. The stability temperature optimum is 10°C-20°C, although it is rapidly decomposed over 30°C. At room temperature (25°C) the decrease of activity after two months was 25%.

Spectral studies on chloroperoxidase 4.5 show marked changes in structure with change of pH (pH 2-8). Especially at pH 6 distinct changes occur in the UV-region which may be correlated with an increase of guaiacol affinity for the enzyme. At pH 7 and pH 8 distinct changes occur in the visible region, possibly due to splitting of peptide chains. The absorption maxima shifted to higher wavelengths with increased pH; two maxima disappear at pH 7 and pH 8 and a new maximum appears at pH 8. The absorption coefficients are similar to those already known exceptthe coefficient for the Soret band which is only half as large as for previously studied chloroperoxidases.

A tryptic peptide map of chloroperoxidase 4.5 was produced which showed 11 spots in the two-dimensional separation (first paper-chromatography, second high-voltage electrophoresis). At pH 8.3 and in distilled water at 38°C, the separation of the incubation solution (only chloroperoxidase) showed 3 or 4 peptide bands if separated with high-voltage electrophoresis.

The isoelectric points of chloroperoxidase isoenzymes present in the crude juice were 3.49 and 4.50. In a later purification step another isoenzyme was found with pI at 3.85, and the more basic isoenzyme was found to have a pI of 4.65. It is possible that the purification steps alter the pI's to higher values, i.e. from 3.49 to 3.85 and from 4.50 to 4.65, however the source of pI variance has not been fully proven.

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COMPARATIVE STUDIES ON PEROXIDASE STRUCTURE

by

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AN ABSTRACT OF A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Biochemistry

KANSAS STATE UNIVERSITY

Manhattan, Kansas

1970

ABSTRACT

A complete substitute for yeast extract in the H-CD growth medium for production of chloroperoxidase (E.C.1.11.1.7, Donor: H_2O_2 oxidoreductase (hydroperoxidase))has been found. It contains per liter of growth medium 12 mg thiamine, 12 mg pyridoxal, 12 mg folic acid, 12 mg pantothenate, 200 ug B_{12} (instead of yeast extract) and 40 mg $FeSO_4 \cdot 7H_2O$ and 0.5 g KNO_3 (instead of 10 mg $FeSO_4$ and 0.5 g KCl). The agar slants used for propagation of the fungus culture are now supplemented with thiamine, pyridoxal and B_{12} in the above concentrations and equally good enzyme producing cultures are obtained. The average increase of activity is about 10 fold.

Apparently as a result of the new improved growth medium the same strain of Caldariomyces fumago now produces a unique chloroperoxidase isoenzyme which is more basic than the previous forms. Isoelectric focusing studies showed the new enzyme to be isoelectric at pH 4.50 and it was named accordingly. Chloroperoxidase was purified in crystalline form by a new isolation procedure as described below.

Growth medium liquid was dialyzed against tap or distilled water and applied to a DEAE-cellulose column at pH 5. Elution was with a phosphate buffer gradient (0.01 M - 0.2 M pH 5),and two peaks were obtained. The first peak representing chloroperoxidase 4.5 was precipitated with 70% $(NH_4)_2SO_4$ and subsequently crystallized in $(NH_4)_2SO_4$ solution.

Iron determination established its molecular weight to be 26,000. The amino acid composition was determined. No new varieties of amino acids were found, no known amino acids were omitted(com-

pared to Hager's analysis). However the acidic amino acids occured in smaller numbers because the calculated peptide portion molecular weight of 17,000 is much smaller than previously obseved chloroperoxidases. The balance to MW 26,000 (34%) is carbohydrate.

Some others properties determined were: chloroperoxidase 4.5 has no free SH-groups although two cysteine residues are present in the enzyme molecule. The stability temperature optimum is 10°-20°C, although it is rapidly decomposed over 30°C.

Spectral studies on chloroperoxidase 4.5 show marked changes in structure with change of pH (pH 2-8). Especially at pH 6 distinct changes occur in the UV-region which may be correlated with an increase of guaiacol affinity for the enzyme. At pH 7 and at pH 8 distinct changes occur in the visible region, possibly due to splitting of peptide chains. The absorption maxima shifted to higher wavelength with increase of pH; two maxima disappear at pH 7 and 8 and a new maximum appears at pH 8. The absorption coefficients for the Soret band are only half as large as for previously studied chloroperoxidases, the other absorption coefficients are similar to those already known.

A tryptic peptide map of chloroperoxidase 4.5 was produced which showed 11 spots in the two dimensional separation (paper chromatography and high voltage electrophoresis). At pH 8.3 and in distilled water at 38°C, the separation of the incubation solution (only chloroperoxidase) shows 3 or 4 peptide bands if separated with high voltage electrophoresis.

The isoelectric points of the two isoenzymes found in the crude juice were proposed to shift from 4.5 to 4.65 and from

3.49 to 3.85 respectively, probably due to the purification steps. The source of isoelectric point variance however has not been fully proven.