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Title: REACTIONS OF HORSERADISH PEROXIDASE WITH THE ISOTHIOCYANATES OF HORSERADISH OIL

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Horseradish peroxidase (HRP) has been investigated extensively and for many years. In spite of this it still exhibits unexplained anomalies. Among these is variation in the reported number of isozymes (from 7 to 42), and reports of instability of the isozyme patterns. Since horseradish is a rich source of organic isothiocyanates (mustard oils), it seemed likely that artifactual chemical modification of the enzyme might occur during or after extraction. The present investigation demonstrates that isothiocyanates can in fact cause extensive modification of HRP without corresponding loss of catalytic activity.

Incubation of HRP with isothiocyanates at alkaline pH and 4°C altered the isozyme pattern. Peroxidase activity was shifted from bands of higher pI to bands of lower pI, some of them preexisting bands and some of them newly generated. With time, the basic "C" band,

initially the principal isozyme, virtually disappeared, and enzyme activity shifted to bands of lower pI. These were discrete bands, presumably formed by stepwise blocking of successive amino groups. Modification of HRP was found to be dependent on pH, reaction time, and isothiocyanate concentration. Amino acid analysis showed loss of 50 % of the lysine residues after reaction of HRP with allylisothiocyanate at pH 10. Reaction of phenyl-(^{14}C)isothiocyanate with HRP at pH 10 resulted in an average of five phenylisothiocyanates incorporated per enzyme molecule. Autoradiography showed four radioactive HRP bands , corresponding to a shift of enzyme activity into these bands.

Incubation of HRP with allylisothiocyanate in the presence of L-lysine at pH 10 showed little protective effect of the lysine, although the concentration of lysine was 800 times the enzyme concentration. This suggested that hydrophobic sites that bind isothiocyanates to the enzyme surface enhance the reaction of the isothiocyanates with the enzyme.

Extraction of HRP from horseradish roots in the presence of adsorbent polystyrene resulted in extracts that were colorless and odorless, in contrast to the brown color and pungent odor of untreated extracts. The initial extracts showed no difference in isozyme pattern

between polystyrene-treated and untreated extracts. After ammonium sulfate precipitation, however, the polystyrene-treated extract retained the original isozyme pattern, while the untreated extract produced new bands of lower pI.

Reactions of Horseradish Peroxidase with the Isothiocyanates of Horseradish Oil

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DEDICATION

To my beautiful mother who left this world the day after I successfully defended this thesis. She always did her best for her children, and always said : "Do your best, and ask the mercy of Allah SWT". She will be much more than a memory forever.

To my father.

To my beloved wife.

To my lovely children, Tika and Zizi.

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ABBREVIATIONS

AllylNCS	allylisothiocyanate
Bis	N,N'-methylene-bis-acrylamide
CD	circular dichroism
CM-cellulose	carboxymethyl cellulose
DEAE-cellulose	diethylaminoethyl cellulose
EDTA	ethylenediamine tetraacetic acid
HRP	horseradish peroxidase
IEF	isoelectric focusing
IUB	International Union of Biochemistry
IUPAC	International Union of Pure and Applied Chemistry
JRP	Japanese radish peroxidase
PAGE	polyacrylamide gel electrophoresis
PhenylNCS	phenylisothiocyanate
PTC	phenylthiocarbamyl
PTH	phenylthiohydantoin
RZ	Reinheitszahl (A_{401}/A_{280})
SDS	sodium dodecyl sulfate
TEMED	N,N,N',N'-tetramethylethylenediamine
TLE	thin layer electrophoresis
TNBS	trinitrobenzenesulfonic acid.

REACTIONS OF HORSERADISH PEROXIDASE WITH THE ISOTHIOCYANATES OF HORSERADISH OIL

INTRODUCTION

Horseradish peroxidase (HRP; I.U.B. No. 1.11.1.7) has perhaps been investigated more extensively than any other enzyme. It was first crystallized by Theorell in 1941 (Saunders *et al.*, 1964). Peroxidases in general have been popular subjects for isozyme studies, partly because the peroxidase assay is easy and sensitive, and partly because of the possible physiological role of peroxidases as indole acetic acid oxidases (Maehly, 1955). Although HRP has been studied intensively, many properties of this enzyme are not completely understood, and its physiological functions remain unclear. In fact, a study of the HRP literature reveals a number of unexplained anomalies. Especially notable is the great variation in isozyme numbers and patterns reported by various authors, and reports that isozyme patterns changed with time after extraction of the enzyme.

In addition to being a rich source of peroxidase, horseradish roots are one of the richest natural sources of organic isothiocyanates or "mustard oils". Isothiocyanates are responsible for the pungency of mustard,

horseradish, and a number of other plant materials. They are also extremely reactive compounds. The reaction of isothiocyanates with amino groups is well known, for example, as the basis of the Edman protein degradation procedure. Isothiocyanates are also widely used for protein modification, and for the production of fluorescence-labeled proteins. Björkman (1973), in an investigation of the nutritional value of rape seed proteins, found that these proteins, as well as human serum albumin, react readily with isothiocyanates at pH values above 6.0.

In this thesis we will review the literature on peroxidases, especially that on the multiple forms of HRP, and the chemistry of protein modification by isothiocyanates. We will then describe experiments that demonstrate modification of HRP by isothiocyanates, and describe extraction procedures in which the horseradish isothiocyanates are removed by using adsorbent polystyrene.

Terminology of "Isozymes" and Multiple Forms of Peroxidase

Two terms, "isozyme" and "isoenzyme", have been used to describe differing molecular forms of proteins which exhibit the same enzymatic specificity in the same

species or the same tissue. The term "isozyme" was introduced by Markert and Moller (1959), and the term "isoenzyme" was introduced by the Standing Commitee on Enzymes of the International Union of Biochemistry (Webb, 1964). The International Union of Pure and Applied Chemistry (IUPAC) and the International Union of Biochemistry (IUB) Commission on Biochemical Nomenclature (IUPAC-IUB, 1977) recommended the term " multiple forms of the enzyme ....." as a broad term covering all proteins catalyzing the same reaction and occuring naturally in a single species (Table I), and recommended that the term "isoenzyme" or "isozyme" should apply only to those forms arising from genetically-determined differences in primary structure of the peptide backbone.

Table I. Classification of "Multiple Forms of Enzymes"[a]

1. Geneticaly independent proteins
2. Heteropolymer (hybrids) of two or more polypeptide chains, noncovalently bound
3. Genetic variants (allelozymes)
4. Conjugated or derived proteins
   a. Proteins conjugated with other groups
   b. Proteins derived from single polypeptide zymogen
5. Polymers of a single subunit
6. Conformationally different forms.

[a]Cited from IUPAC - IUB Commision on Biochemical Nomenclature (IUPAC-IUB, 1977).

Van Huystee and Cairns (1980) stated that the use of the term "isozyme" for the multiple forms of peroxidase has generally diverted attention from the origins of such forms. The term "isozymes" now has been widely interchanged with "multiple forms of enzyme".

We will use the 1964 definition in this work, because the literature on HRP is all based on variable electrophoretic mobility or IEF rather than genetic analysis or sequence analysis. Amino acid analyses suggest the likelihood that there are two distinct HRP proteins, acidic (A) and basic (B, C, D, E) types (Shannon et al., 1966) (Table II). Aibara et al. (1981) isolated six basic HRP isozymes (E1, E2, E3, E4, E5 and E6), and reported that the isozymes contained 0.8 to 14.1 % carbohydrate. They also reported that four (E3 to E6) of the six isozymes had extremely high pI values of over 12. The amino acid and carbohydrate compositions of these basic isozymes are shown in Table III. Based on amino acid compositions, especially the high arginine content, they appear to be similar to B and C. It is noteworthy that E3 to E6, which have unusually high pI values, also have very low carbohydrate content compared to the other HRPs (cf Table II, Table III). One of the isozymes (E4) was crystallized in a form claimed to be suitable for X-ray structural analysis. Apparently this analysis has not yet been done. Only isozyme C has been

Table II. Amino Acid and Carbohydrate Composition of Peroxidase Isozymes.

Amino Acid	Moles of amino acid or sugar per mole enzyme						
	Isozyme						
	A_1[a]	A_2[a]	A_3[a]	B[a]	C[a]	C[b]	C[c]
Asx	42.6	47.0	44.8	48.5	49.4	47	48
Thr	21.1	22.9	22.7	23.4	24.1	23	25
Ser	35.3	38.6	36.9	23.0	23.0	20	25
Glx	18.1	19.3	21.9	19.4	20.0	21	20
Pro	9.6	10.5	11.3	15.8	16.7	19	17
Gly	23.9	24.3	25.9	16.3	16.9	18	17
Ala	26.7	28.7	26.1	22.6	23.4	22	23
Cys	6.0	5.6	5.7	4.7	6.5	8	8
Val	8.5	8.4	9.4	13.0	13.4	19	17
Met	0.4	0.2	1.9	2.3	2.8	3	4
Ile	11.1	9.8	11.3	8.9	9.2	12	13
Leu	30.3	31.3	30.6	31.3	35.3	34	35
Tyr	1.7	1.6	2.65	3.6	4.5	5	5
Phe	16.0	16.7	15.1	15.8	20.0	19	20
Lys	4.2	4.5	4.0	5.8	6.0	6	6
His	2.4	2.8	2.6	2.9	3.0	3	3
Arg	8.4	9.0	7.4	19.6	20.8	21	21
Trp	d	d	d	d	d	1	1
Total	266.3	281.2	280.25	276.9	295.0	301	308
Sugar							
Fucose	5	5	8	9	8	8	d
Arabinose	1	1	2	1	1	d	d
Xylose	4	4	7	8	8	8	d
Mannose	14	16	31	31	33	24	d
Glucosamine	23	24	37	35	35	d	d
N-Acetyl glucosamine	d	d	d	d	d	8	d

[a]Cited from Shih *et al*. (1971) and Whitehead (1969)

[b]Cited from Phelps *et al*. (1971)

[c]Cited from Welinder (1979), based on amino acid sequence analyses

[d]Values not reported.

Table III. Chemical Composition of the Basic Isozymes of Horseradish Peroxidase[a].

Amino Acid	Moles of amino acid or sugar per mole enzyme						
	Isozyme						
	E1	E2	E3	E4	E5	E6	C[b]
Asx	44.7	43.8	38.0	37.5	44.4	41.5	48
Thr	23.1	23.4	23.2	23.9	23.6	27.9	25
Ser	23.2	20.2	22.8	22.8	25.4	29.2	25
Glx	21.6	21.7	20.9	20.9	16.2	16.9	20
Pro	17.5	17.5	14.8	14.4	19.2	11.4	17
Gly	16.8	17.4	30.6	30.4	20.4	23.0	17
Ala	23.8	23.4	23.0	22.7	25.6	32.1	23
Cys	8.4	8.4	8.1	7.9	7.8	8.1	8
Val	19.1	19.1	28.1	28.2	20.0	19.0	17
Met	2.5	3.1	4.1	3.8	3.7	4.7	4
Ile	13.1	12.6	15.4	16.0	14.2	15.7	13
Leu	30.1	29.3	24.4	24.3	34.0	21.5	35
Tyr	5.4	5.9	2.1	2.1	5.3	4.1	5
Phe	16.8	16.6	12.9	13.2	20.9	14.3	20
Lys	6.7	7.3	5.6	5.2	7.3	7.2	6
His	2.9	2.9	4.1	4.0	3.1	3.0	3
Arg	21.3	21.2	24.0	24.5	25.7	22.7	21
Trp	0.8	1.9	1.1	1.2	1.1	1.8	1
Total	297.8	295.7	303.2	303.0	317.9	304.1	308
Sugar							
Glucos-amine	5.4	7.1	0.8	0.7	1.8	1.0	7
Hexose and Pentose	25.7	26.9	0.8	0.8	7.9	2.7	43

[a]Cited from Aibara *et al*. (1981)

[b]Cited from Welinder (1979) and Clarke and Shannon (1976).

sequenced (Welinder, 1979). Horseradish is a sterile hybrid, so conventional genetic analyses are not possible. In the present work, peroxidase isozymes will be shown by isoelectric focusing (IEF) in pH 3 - 10 carrier ampholyte on a 5 % polyacrylamide gel or Sephadex thin layer. The isozymes are visualized by 3-amino-9-ethyl carbazole-H_2O_2 on the polyacrylamide gels, and o-dianisidine-urea hydrogen peroxide on the Sephadex layers. All newly-generated bands will be refered to as "bands" rather than "isozymes". The bands are numbered consecutively according to the Commision on Biochemical Nomenclature (IUPAC-IUB, 1977), the highest mobility towards anode (the lowest pI) is numbered "one". The numbering system will be related to the Shannon groups's isozymes, and the Shannon group's designation will be used in this thesis.

Multiple Forms of Horseradish Peroxidase

The first observation that HRP consisted of more than one component was made by Theorell (1942). He demonstrated two components of his HRP preparation by using electrophoresis techniques. The two components were designated HRP-I and HRP-II. He also refered to HRP I as "paraperoxidase" and to HRP II as "peroxidase". HRP-I occured only in small and variable quantities

compared to HRP-II. Later, Theorell and Akeson (1942) reported that the amino acid compositions of HRP-I ("paraperoxidase") and HRP-II ("peroxidase") were similar. Spectroscopic data for the two peroxidases were also very similar. The authors concluded that HRP-I was a product of HRP-II conversion, possibly an artifact. This is believed to be the first indication of molecular multiplicity in enzymes.

Jermyn and Thomas (1954) reported that electrophoresis on filter paper could demonstrate five forms of HRP from fresh horseradish juice. The ratio of the five forms was seasonally variable. They also reported the apppearance of a new isozyme on the histogram of aged horseradish juice. Keilin and Hartree (1945) observed appearance of a new component when HRP was stored at 0°C.

Klapper and Hackett (1965) investigated several commercial HRP preparations. The commercial peroxidase preparations were subjected to electrophoresis on starch-gels at pH 8.1. By this method they demonstrated 11 HRP isozymes. However, only five of these were always present in appreciable amount. The major isozymes were purified, and each migrated as a single band with the same mobility as the corresponding isozyme in unfractionated enzyme preparations. They concluded that the multiplicity was not the result of modification of a single peroxidase.

Shannon et al. (1966) fractionated HRP by CM- and DEAE-cellulose column chromatography, and purified seven isozymes. The isozymes were designated as isozymes A_1, A_2, A_3, B, C, D and E. The A isozymes were acidic, and the others basic. Since then, Shannon's group has characterized several of the seven isozymes that they detected (Kay et al., 1967; Strickland et al., 1968; Strickland, 1970), and have found differences and similarities in absorption spectra, catalytic properties, circular dichroism spectra, amino acid and carbohydrate composition, and tryptic peptide patterns.

Shannon (1968) reviewed plant isozymes, and reported that various plant peroxidases exist in multiple forms. Morita et al. (1970) demonstrated eighteen Japanese radish peroxidase (JRP) isozyme bands on polyacrylamide gels. Five of the eighteen were purified, and their properties and structures were studied (Morita et al., 1971). The isozymes were found to be quite similar to HRP isozymes. Delincée and Radola (1970) demonstrated 20 isozyme bands, and Hoyle (1977) demonstrated 42 HRP isozyme bands by isoelectric focusing on polyacrylamide gel slabs. Regarding the number of peroxidase isozymes, horseradish and Japanese radish contain more isozymes than any other plant that was reviewed by Shannon (1968). Horseradish and Japanese radish belong to the Cruciferae, a family noted for producing large amounts of glucosino-

lates. When the tissue is injured, as during enzyme extraction, the glucosinolates are hydrolyzed to yield reactive isothiocyanates (mustard oils). The very high, and variable, numbers of isozymes of HRP and JRP might be a result of isothiocyanate modification. Turnip, which is also a member of the Cruciferae, shows only seven peroxidase isozymes using electrophoresis on cellulose acetate film (Mazza, et al., 1968). Perhaps this is because turnip roots contain smaller amounts of glucosinolates than horseradish or Japanese radish.

There is no agreement among scientists on the number of isozymes of HRP. Changes of HRP isozyme patterns have been reported (Theorell and Åkeson, 1942; Keilin and Hartree, 1951; Jermyn and Thomas, 1954; Liu and Lamport, 1973). However none of the authors discussed a possible artifactual alteration of the isozyme pattern by mustard oils. Since horseradish is one of the richest natural sources of organic isothiocyanates (mustard oils), one would anticipate that these reactive isothiocyanates might react with the peroxidase and other proteins in horseradish homogenates during extraction. Maehly (1955) in describing the isolation of crystalline HRP warned that homogenization must be done outdoors, or in a good hood and wearing gas masks, and that crude extracts dissolve cellophane dialysis tubing. He also mentioned that ammonium sulfate precipitates float, and that

purified HRP can be stored in solution for a year or more in the refrigerator. All of these observations suggested the presence of toxic lipid materials (isothiocyanates).

Attempts to cross-link HRP to γ-globulin with a bifunctional reagent specific for amino groups indicated that a substantial percentage of HRP lysines were blocked and not available for the reactions (Modesto and Pesce, 1971). The N-terminal amino acid will not react with Edman's reagent, and tryptic digestion is incomplete (Welinder and Mazza, 1975). All of these observations are exactly what one would expect in a protein modified by isothiocyanates.

All of the facts reviewed above are foundations of the hypothesis that was used in this work, that HRP is contaminated by mustard oils during extraction, resulting in modification of the protein, including production of artifactual isozymes. The purpose of this study was to search for the existence of modified HRP "isozymes" and to isolate unmodified isozymes.

Distribution and Functions of Peroxidase

Peroxidases are heme proteins characterized by the ability to catalyze, at a very rapid rate, the "peroxidatic" oxidation of a number of substrates (Saunders *et al*. 1964) :

$$ROOH + AH_2 \longrightarrow ROH + H_2O + A$$

In addition to hydrogen peroxide, ROOH can be alkyl hydroperoxide and substituted perbenzoic acids (Schonbaum and Lo, 1972; Cotton and Dunford, 1973; Marklund et al., 1974), and oxyhalogen acids (Chance, 1952; George, 1953; Weinryb, 1968). George (1953) discovered that hypochlorite, ClO^-, and chlorite ClO_2^- were capable of forming oxidized HRP intermediates. Hewson and Hager (1979) reported that HRP and chlorite were able to catalyze chlorination of monochlorodimedone to form dichlorodimedone. AH_2 can be any of a number of compounds, including phenolic compounds, aromatic primary, secondary and tertiary amines; leuco dyes; certain heterocyclic compounds such as ascorbic acid and indole; and inorganic ions, particularly the iodide ion. Organic compounds such as guaiacol, pyrogallol, o-dianisidine, p-anisidine, benzidine, toluidine, p-phenylenediamine and many others are commonly used for peroxidase detection (Maehly and Chance, 1954; Delincée and Radola, 1972).

Peroxidases are widely distributed among higher plants. The sap of the fig tree and the root of horseradish are very rich in the enzyme. Horseradish roots are the commonest source of peroxidase. About 70 % of the total peroxidase activity in the horseradish roots is located in vacuoles (Grob and Matile, 1980). Peroxidase has also been located histochemically in the plant cell

wall (De Jong, 1967). Liu (1971) found 20 % of peroxidase activity in horseradish roots is bound to the cell walls. Peroxidases are also commonly found in animals; however they are not distributed in all tissues (Saunders et al., 1964). Saunders et al. also reported that peroxidases are also found in fungi, bacteria and algae. Peroxidases in the thyroid glands are involved in iodination of tyrosine (Saunders et al., 1964). All heme proteins, and even inorganic iron salts, may show peroxidase activity at a much lower rate. According to Maehly and Chance (1954) true peroxidases will show the activity within one minute, while "pseudoperoxidases" typically require an hour or more.

The physiological functions of peroxidase remain unclear, especially in horseradish roots, which are rich in peroxidase. Several functions of peroxidase have been suggested. Plant peroxidases may be involved in indole-3-acetic (IAA) oxidation (Saunders et al. 1964; Hinnman and Lang, 1965), malonate synthesis (Shannon et al. 1963); lignin synthesis (Stafford, 1960). Harkin and Obst (1973) have demonstrated histochemically that peroxidase mediates lignin synthesis, and serves as the major polymerization catalyst. The oxidation of IAA by peroxidase has been studied in detail (Ricard and Job, 1974). Fry (1984) suggested that peroxidase-catalyzed oxidation of polysaccharide-bound phenolic groups of

tyrosine residues in cell walls results in covalent cross-linking of the polysaccharides, thereby increasing the stability of the wall.

Interaction between Proteins and Isothiocyanates

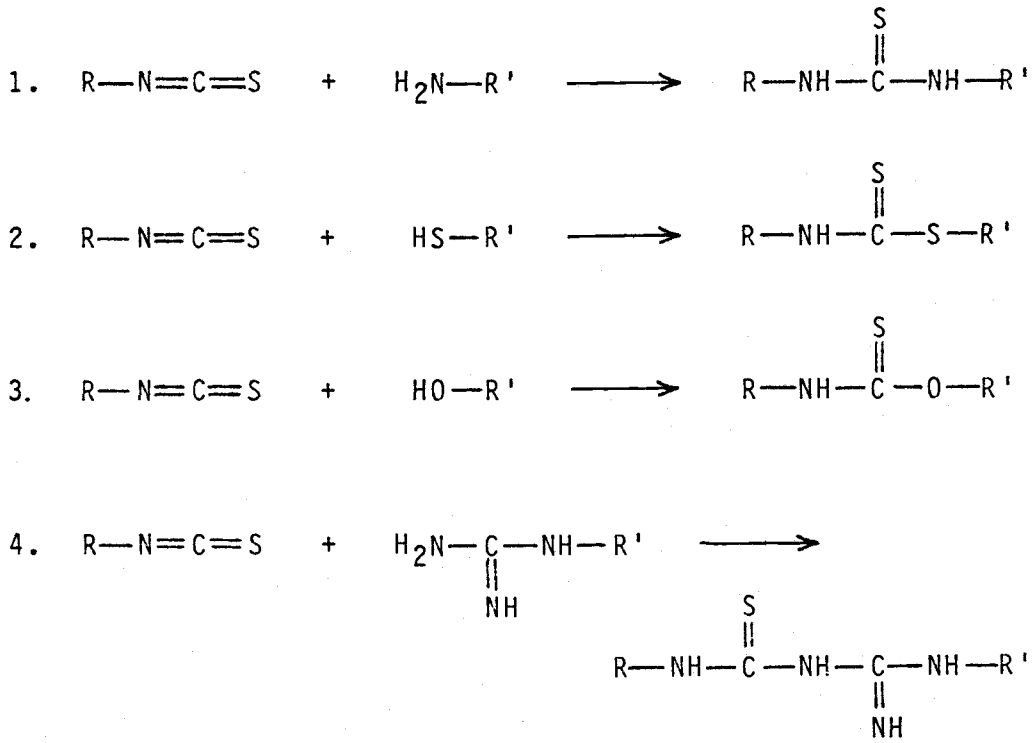
The reactions of isothiocyanates with proteins have been extensively studied, particularly in connection with the Edman protein degradation procedure (Edman, 1956; Ilse and Edman, 1963; Edman and Henschen, 1975; Langdon, 1977). The reaction between phenylisothiocyanate and the N-terminal group of proteins is the first step in the Edman protein degradation procedure (Edman and Henschen, 1975). This reaction is referred to as the coupling reaction, in which a phenylthiocarbamyl (PTC) derivative is formed. In the coupling procedure, the protein is reacted with excess phenylisothiocyanate at alkaline pH (8.6). A mixture of pyridine and water (1 : 1) is used as the solvent, and the reaction is carried out at 37°C for 2 hrs. The next reaction involves the cleavage of PTC-peptide to a phenylthiohydantoin derivative of the N-terminal amino acid, plus a new peptide with one amino acid less than the original. This requires a strongly acidic anhydrous medium. Ilse and Edman (1963) reported that PTC-amino acids show a strong tendency towards oxidative desulfuration with formation of the

phenylcarbamyl derivative. In this reaction sulfur is replaced by oxygen. They mentioned that all PTC-derivatives would show the same tendency to undergo oxidative desulfuration by exposing these derivatives to atmospheric oxygen or other oxidizing agents.

Bjorkman (1973) demonstrated the reaction of isothiocyanates, obtained by hydrolysis of glucosinolates with myrosinase, with human serum albumin (HSA). The reaction was strongly pH dependent. The isothiocyanates reacted readily with HSA at pH values higher than 6.0, whereas reaction was almost zero at pH values lower than 4.25. He also concluded that isothiocyanates mainly reacted with small basic proteins with pI about 11, rich in lysine and terminal amino acids. Horseradish peroxidase is a small protein (MW = 40,000) and about 40 % of total peroxidase activity is due to isozyme C (Shannon system). Although the isozyme C apparently has a blocked N-terminal (Welinder, 1979), there are six lysine residues, as well as sugar amines potentially available for isothiocyanate addition.

Isothiocyanates readily undergo the following addition reactions 1 - 3 under mild conditions (Hughes, 1975). Reaction 4 has been cited from a German patent (Houben and Weyl, 1955), but does not seem to have been studied further. In addition, Ulrich et al. (1968) described the cyclo-addition of isothiocyanate to

amidines and guanidines under mild conditions. In reaction 1, considered to be the most important reaction



of isothiocyanates, the linkage is symmetrical, so that the reverse reaction could yield either the starting materials or a low-molecular weight amine plus a protein-isothiocyanate-capable of undergoing further transformations. This reaction requires a non-protonated-amino group, and thus is favoured by high pH. Terminal amino groups, because of their relatively low pK_a values, are more reactive than ε-NH_2 groups of lysine

residues. However, Konigsberg (1967) reported that $-NH_2$ groups of lysine residues are completely sustituted by phenylisothiocyanate when the pH is kept above 9.0 during the coupling procedure of the Edman degradation. The product of the reaction, N -phenyl-thiocarbamyl lysine (N -PTC lysine) remains intact on hydrolysis with 1N HCl. By hydrolysis with 6N HCl at 110°C for 22 hrs (normal condition of protein hydrolysis), 70 % of the N -PTC was cleaved, liberating free lysine.

In addition to their reactivity, isothiocyanates are very hydrophobic, while the glucosinolates are very water soluble. Thus, the isothiocyanates are released as a unimolecular dispersion, readily available for covalent condensation or for hydrophobic adsorption to proteins. Adsorbed isothiocyanates stick tightly and are available for covalent condensation later. According to Rinderknecht (1960, 1962) the limiting factor in the reaction of isothiocyanates with proteins is the low water solubility of the isothiocyanates. He showed that fluorescein isothiocyanate dispersed on diatomaceous earth reacted with protein within a few seconds.

The reactivity of lysine $-NH_2$ groups in HRP has been studied. The $-NH_2$ groups of lysine residues in HRP isozyme C have been modified with acid anhydrides and trinitrobenzenesulfonic acid (TNBS) (Ugarova _et al_., 1978; Ugarova _et al_., 1979). Anhydrides reacted with

four of six ε-NH_2 groups in isozyme C at 0°C. In comparison, only three of six ε-NH_2 reacted with TNBS at 0°C. However, all six ε-NH_2 reacted with TNBS at 40°C. These modifications of lysine did not affect the catalytic activity of the enzyme. The authors concluded that ε-NH_2 groups of lysine residues are not involved in catalytic activity of the enzyme, and that some of the ε-NH_2 groups are buried.

Chemical Properties of Horseradish Peroxidase

Peroxidase is a glycoprotein comprised of a single polypeptide chain, with about 18 % carbohydrate, and with protohaematin IX as prosthetic group. Peroxidase activity of the enzyme is proportional to the absorbance at 401 nm, the characteristic Soret band of porphyrin rings. The ratio of absorbance at 401 nm to 280 nm, is defined as RZ, commonly used to express the purity of peroxidases. The hemin part of the enzyme can be reversibly dissociated by extraction with cold acid- acetone and the activity is restored when hemin is mixed with the apoprotein (Theorell *et al*., 1943). This finding suggested that the linkage between heme and apoprotein was by coordinate bonding with no covalent bonds. The iron at the center of protoporphyrin IX has six coordination positions, and four of them are occupied by

porphyrin nitrogen atoms. The fifth is occupied by a reactive ligand from the HRP apoenzyme, believed to be a carboxyl group of the protein (Theorell, 1943). According to Keilin and Hartree (1951), the sixth position is occupied by water at neutral pH, and is involved in the peroxidase activity. In addition to iron, calcium also contributes to the structural stability of HRP. Haschke et al. (1978) demonstrated that HRP isozymes A and C contain respectively 1.4 and 2.0 moles calcium per mole enzyme, which can be removed by treatment with guanidine hydrochloride and EDTA. The specific activity of isozyme C falls to about 40 % after calcium removal, and that of isozyme A to 15 % of the initial value. The calcium-free isozyme C regains its activity upon addition of calcium ion; however calcium-free isozyme A remains unchanged during incubation with excess calcium. Welinder (1979) also reported that isozyme C contains two calcium ions per enzyme molecule.

Molecular weights of HRP isozymes range from 30,000 to 50,000 daltons (Conroy et al.,1982). Theorell (1942) reported that his crystalline HRP had a molecular weight of 44,100 daltons. Keilin and Hartree (1951) found that the molecular weight of their HRP preparation was 40,500; however, Cecil and Ogston (1951) reported 39,800 for the Keilin and Hartree HRP preparation. Paul and Stigbrand (1970) isolated four HRP isozymes from pooled horseradish

roots, and found that molecular weights ranged from 33,200 to 41,600 daltons. Welinder (1979) claimed that the molecular weight of isozyme C was about 44,000 including a polypeptide chain of about 33,890 daltons. Meanwhile, Aibara et al. (1981) isolated six basic HRP isozymes, and they described the molecular weights of the isozymes as ranging from 33,700 to 33,900, with the differences in molecular weights being due to differences in carbohydrate content. The polypeptide chain molecular weight ranged from 32,314 to 32,843 daltons. A value of 40,000 is commonly assumed for HRP holoenzyme.

Theorell and Åkeson (1942) carried out elemental analysis of HRP, and obtained the values of : C (47.0 %), H (7.25 %), N (13.2 %), S (0.43 %), O (32.0 %) and Fe (0.127 %). They also found that HRP contained 18 % polysaccharide, and that HRP-II contained two histidine, 18 arginine and 12 lysine residues per molecule enzyme. Since then, the amino acid and carbohydrate compositions of HRP have ben studied extensively (Shannon et al., 1966; Whitehead, 1969; Clarke and Shannon, 1976; Paul and Stigbrand, 1970; Phelps et al., 1971; Shih et al., 1971) (cf. Table II). The complete amino acid sequence of isozyme C was first presented by Welinder (1976). Three years later she published a more detailed report on the structure of HRP isozyme C (Welinder, 1979). The amino acid and carbohydrate compositions of HRP isozymes

obtained by Whitehead (1969), Phelps et al. (1971) and Welinder (1979), are shown in Table II. It was shown that amino acid and carbohydrate compositions of isozyme A_1 and A_2 were similar, but were distinctly different from the amino acid and carbohydrate compositions of isozymes B and C. Isozyme A_3 was similar to A_1 and A_2 in amino acid composition but differed in carbohydrate composition. It was also shown that amino acid and carbohydrate compositions of isozymes B and C were very similar, and their peptide maps appeared indistinguishable (Shih et al., 1971). Clarke and Shannon (1976) reported that the carbohydrate content per molecule of isozyme C was 10 fucose, 9 xylose, 34 mannose and 47 glucosamine residues.

According to elemental analyses of Theorell and Akeson (1942), HRP contained 0.43 % sulfur, corresponding to about six sulfur atoms per molecule HRP. This number is a little lower than the current value of four methionines plus eight half-cystines comprising four disulfide bridges (Welinder, 1979). If isothiocyanates react with HRP during extraction, the number of sulfur atoms per molecule of enzyme obtained by elemental analysis should be higher than the number of cysteine and methionine residues per molecule of enzyme. However, because of the ready desulfuration of PTC-derivatives [see above, Ilse and Edman, (1963)] one would not expect

these extra sulfur atoms to be retained or to show up in the elemental analyses.

Welinder (1976) also reported that there were eight neutral carbohydrate side chains attached to the polypeptide chain of isozyme C, and the N-terminal of the isozyme was blocked as pyrrolidonecarboxylate and buried. However, pyrrolidonecarboxyl peptidase released insignificant amounts of pyrrolidonecarboxylate from soluble "native" peroxidase, and only 6 % of pyrrolidonecarboxylate was released from incompletely dissolved, reduced and S-carboxymethylated HRP apo-peroxidase and 14 % when the isozyme C had been heat-denatured at 90°C before incubation with pyrrolidonecarboxyl peptidase. Dodecylsulfate-denatured peroxidase did not react with phenylisothiocyanate or dansylchloride at alkaline pH; therefore she concluded the N-terminal was blocked and buried. Our understanding of the protein structure is still limited because of the failure of X-ray crystallographic analysis (Braithwaite, 1976).

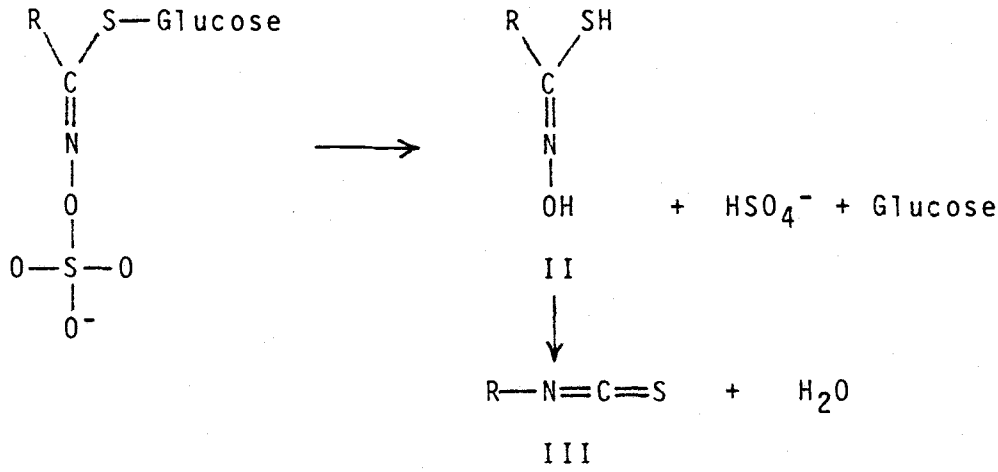
Plant Secondary Product Glucosinolates in Horseradish Roots

Plants contain a great variety of low molecular weight secondary products (Robinson, 1980). Often they are present in large quantities and may be very reactive.

The most ubiquitous plant secondary products are probably the plant phenolic compounds. Phenolic compounds and their oxidation products can interact with proteins and often cause problems in enzyme or other protein isolation from plant tissues (Loomis, 1974). The effects of phenolic compounds on peanut peroxidases have been demonstrated (Srivastava and van Huystee, 1977). Extraction of peanut peroxidases in the presence of Dowex 1-X1 reduced the number of "isozymes" from five to one. Methods for overcoming these problems have also been described elsewhere (Loomis and Battaile, 1966; Lam and Shaw, 1970; Loomis, 1974; Loomis *et al*., 1979).

Glucosinolates are characteristic of the *Cruciferae* family and have value as taxonomic markers at the family level (Richmond, 1973; Kjaer and Larsen, 1973; 1976; 1977). Horseradish is a member of this family, and stores large quantities of glucosinolates in the roots. Beside glucosinolates, the roots also contain the enzyme myrosinase, in a separate compartment (Grob and Matile, 1980). Myrosinase is a β-thioglucosidase, and responsible for the conversion of glucosinolates into mustard oils. During HRP extraction, horseradish roots are crushed or homogenized, the glucosinolates (I) are hydrolyzed by the liberated myrosinase enzyme, and aglycones (II) are produced. The aglycones can undergo rearrangement in several ways. A Lossen rearrangement

results in formation of isothiocyanates (III). Other rearrangements could produce thiocyanates (RSCN), amines (RNH_2), and nitriles (RCN). In the presence of L-ascorbic acid, myrosinase of horseradish root is



activated by a factor of 800 - 1000 fold (Grob and Matile, 1980). According to Grob and Matile the concentration of L-ascorbic acid in horseradish roots is optimal for activating the myrosinase. The mixture of glucosiniolates, myrosinase and L-ascorbic acid in horseradish roots is perfect to produce isothiocyanates in a very short time. Sinigrin, the major glucosinolate of horseradish (Grob and Matile, 1979), is completely converted to allylisothiocyanate in the presence of L-ascorbic acid in 10 minutes (Grob and Matile, 1980). Since myrosinase, glucosinolates, and ascorbic acid are separately compartmentalized in intact cells,

isothiocyanates are not present, as such, in living plants, but they are readily formed as soon as the compartmentalization is broken.

Gilbert and Nursten (1972) identified five of 17 volatile compounds in horseradish oil. They compared the main components of English, Hungarian, Japanese and dried flaked type of horseradish, and concluded the composition of the oil was dependent on the type of horseradish. Allylisothiocyanate and 2-phenethylisothiocyanate were always the major constituents. The other three components were 2-butylisothiocyanate, 4-pentenylisothiocyanate, and allylthiocyanate. It is known that alkylisothiocyanates in general, are unstable and apt to decompose. Kawakishi and Muramatsu (1966), and Kawakishi et al. (1967) reported that p-hydroxybenzylisothiocyanate in H_2O is hydrolyzed, mainly to R-OH and SCN^-. However, Kawakishi and Namiki (1969) reported that allylisothiocyanate in H_2O is mainly decomposed to allyldithiocarbamate, which is further degraded to N,N'-diallylthiourea. It seems reasonable to suggest that these products might also include reactive, hydrophobic, S-free compounds, which could react with proteins.

This laboratory (Loomis et al, 1979; Sandstrom and Loomis, unpublished) has established a new HRP extraction procedure. They used a mixed adsorbent of insoluble polyvinylpyrrolidone (Polyclar AT) and polystyrene

(Amberlite XAD-4) to protect peroxidase from reaction with phenolic and isothiocyanate compounds. Porous polystyrene adsorbs hydrophobic materials from plant homogenates, while polyvinylpyrrolidone adsorbs hydrogen-bonding phenolic compounds. Their enzyme extracts were cleaner, and showed a distinct shoulder at about 400 nm, and low UV-absorbance, odorless and colorless, apparently free of isothiocyanates. In this work their extraction procedure was used.

MATERIALS AND METHODS

General methods are described below. Since details varied from one experiment to another, these details will be described in connection with the experimental results.

Materials

Allylisothiocyanate was a gift from Dr.E.Klein of Dragoco, Holzminden, Germany. Phenylisothiocyanate, 3-amino-9-ethylcarbazole, and o-dianisidine (free base) were purchased from Sigma Chemical Company. [Ring-^{14}C(U)]phenylisothiocyanate in n-heptane (concentration, 0.27 mg/ml; specific activity 51.0 mCi/mmole) and EN^{3}HANCE spray were obtained from New England Nuclear Corporation. Ureahydrogen peroxide was purchased from Pfaltz and Bauer Inc., and 4-amino-antipyrine was from Aldrich Chemical Company. Acrylamide, ammonium persulfate, N,N,N,N'-tetramethylethylenediamine (TEMED), N,N'-methylene-bis-acrylamide (Bis), and Bio-Lyte ampholytes, 40 %, pH 3 - 10, electrophoresis purity reagents, were purchased from Bio-Rad Laboratories. Amberlite XAD-4 was purchased from Rohm and Haas Company, Philadelphia, Pennsylvania, and washed and powdered

according to Loomis et al.(1979). CM-cellulose, 0.85 meq/g, medium mesh; DEAE-cellulose, 0.9 meq/g, medium mesh, and Sephadex G-75-40 and G-100-40 were purchased from Sigma Chemical Company. The mixed bed resin AG 501-X8(D) was purchased from Bio-Rad. All other reagents and solvents were reagent grade and commercially available.

Sources of Peroxidases

Three sources of peroxidases were used. These included two commercial preparations purchased from Sigma Chemical Company, labeled as Sigma Type I HRP, RZ 0.66 and 0.46, and Sigma Type IX HRP, RZ 3.02. The third source of HRP was horseradish plants [*Armoracia rusticana*, Gaertn., Mey., and Scherb. cv.Maliner Kren (Synonyms: *A. lapathifolia*, Gilib.; *Cochlearia armoracia*,L.)-Cruciferae] that were grown in the field or in planting boxes on the roof of the building, from roots purchased from the W.Atlee Burpee Co., Riverside, California. The plants were dug up in early summer, and the washed roots were stored in the cold room at 4°C until needed, up to several months.

Column Chromatography of Horseradish Peroxidase

Sigma Type I HRP, a crude HRP with RZ 0.46, was fractionated according to Shannon *et al*. (1966).

CM- and DEAE-cellulose were washed according to Cooper (1977). The washed CM-cellulose was suspended in a 0.005 M potassium acetate buffer, pH 4.4, and DEAE-cellulose in a 0.005 M Tris-HCl buffer, pH 8.4. Columns (2.5 x 35 cm) were packed with 1 to 2.5 psi N_2 gas pressure on top of the column. The columns were run overnight with the same buffer used for packing.

Sigma Type I HRP (100 mg) was dissolved in 5 ml of the 0.005 M potassium acetate buffer, pH 4.4, and transferred to the CM-cellulose column. The column was eluted with 100 ml of the same potassium acetate buffer. Acidic HRP isozymes were not adsorbed by CM-cellulose column under these conditions and were collected before eluting with a linear gradient system. This fraction was designated Fraction A according to Shannon *et al*. (1966). The column was then eluted with a linear gradient, 0.005 M to 0.1 M potassium acetate, pH 4.4, 500 ml each. Just before the buffer in the first elution system was exhausted, a second elution system was introduced into the column. The second elution system was a linear gradient, 0.1 M potassium acetate, pH 4.4 to 0.25 M potassium acetate, pH 4.9, 500 ml each. Flow rate was

adjusted using gravity to about 0.7 ml/minute and fraction volume to 10 ml per fraction. The fractions were monitored with a spectrophotometer at 280 nm for protein concentration, and at 401 nm for peroxidase concentration. In addition, the fractions were assayed for enzyme activity by a spot test based on the Worthington (1977) assay. Effluent fractions containing the enzyme activity were pooled and designated as fractions (B + C), and (D + E).

Fractions (B + C) were pooled and lyophilized, and then redissolved in 5.0 ml of 0.005 M Tris-HCl, pH 8.4. This solution was transferred to a DEAE-cellulose column for further purification. The column was then eluted with a linear gradient consisting of 500 ml 0.005 M Tris-HCl, pH 8.4, and 500 ml 0.005 M Tris-HCl, pH 8.4 containing 0.1 M NaCl. The basic isozymes were not adsorbed by the column, but negatively-charged contaminating proteins were. The effluent fractions containing peroxidase activity were pooled and lyophilized. The lyophilized enzyme preparation was dissolved in 5 ml 0.06 M potassium acetate buffer, pH 5.6. This solution was applied to a 2.5 x 30 cm Sephadex-100-40 column for further purification according to the procedure of Shih _et al_. (1971). The column was washed with 0.005 M Tris-HCl, pH 8.4, and fractions of 5 ml each were collected. The fractions containing peroxidase activity

were pooled and dialyzed against glass-distilled water, then lyophilized. The enzyme was redissolved in a minimal volume of glass-distilled water. This fraction (B + C) was saved for purification of isozyme C.

Isoelectric Focusing of Peroxidase on Sephadex Thin Layers

Isoelectric focusing on Sephadex thin layers was performed according to Radola (1973). Glass plates, 20 x 20 cm in size, were coated with a 0.6 mm thick layer of Sephadex G-75-40 suspension, containing 1 % of carrier ampholytes in the pH range 3 - 10. Gel suspensions were prepared by adding 2.5 ml of Bio-Lyte (40 %) and 7.5 g Sephadex G-75-40 beads to 100 ml of glass-distilled water. In this work lysine and arginine were not added to the suspensions. The suspensions were deaerated, and 33 ml of the deaerated suspensions were spread evenly on the glass plate by means of a glass rod. The plate was dried in air until the gel layer showed 1 - 3 mm long irregular fissures at the edge. This criterion corresponds to a water loss of about 20 % from the gelslurry (Radola, 1973). Samples (3 µl of 1 mg/ml HRP solution) were taken up in a Hamilton microliter syringe and applied on the plate along a 1 cm line. The plate was then transferred to a precooled (4°C) Desaga, TLE-Double

Chamber and focused. Platinum ribbon electrodes were used, and the focusing was done under N_2 gas flow, and with a filter paper wick presoaked in concentrated KOH solution as CO_2 trap. The electrophoresis was carried out at 200 and 800 V, with varying running times, depending on the resolution required. The pH gradient in the Sephadex layer was measured directly in the gel layer using a microelectrode (Corning) equiped with a flat membrane. Plates were stained for peroxidase activity using a paper print technique with ureahydrogen peroxide and o-dianisidine as primary and secondary substrate according to Delincée and Radola (1972). Photographs were taken and positive transparancies were made by using Polaroid Type 55 film. The positive transparancies were scanned with a Zeineh, Soft Laser Scanning Densitometer Model SL-504-XL from Biomed Instruments Inc.

Incubation of Peroxidases with Isothiocyanates

Peroxidases were incubated with isothiocyanates throughout this study in 0.1 M sodium pyrophosphate ($Na_4P_2O_7.10H_2O$)solution which was adjusted to the desired pH (from 6 to 10) with KOH or HCl solution. Isothiocyanates (0.2 to 1.0 µl) were added to 1 ml peroxidase solution (0.5 to 1.0 mg/ml) with a Hamilton microliter syringe. The mixtures were mixed thoroughly, and allowed

to react at 4°C for specified times. At the end of the reaction time, powdered dry Amberlite XAD-4 (30 mg) was added to each reaction mixture to adsorb the remaining isothiocyanates. The mixtures were then mixed thoroughly and filtered. The supernatants were prepared for further analysis.

Assays

Peroxidase activity was measured spectrophotometrically by following the increase in absorbance at 510 nm due to oxidation of 4-amino-antipyrine (Worthington Enzyme Manual, 1977). Absorbance was followed with a Beckman DB Spectrophotometer. One unit corresponds to the decomposition of one micromole of hydrogen peroxide per minute at 25°C and pH 7.0. Reaction mixtures contained 1.4 ml of 0.0025 M 4-amino-antipyrine with 0.17 M phenol in glass-distilled water, 1.5 ml 0.0017 M hydrogen peroxide in 0.2 M potassium phosphate buffer, pH 7.0, and 0.1 ml of an appropriate concentration of an enzyme preparation.

The enzyme activity was also in some cases determined spectrophotometrically with *o*-dianisidine as the hydrogen donor. This assay has been successfully used by several investigators, including Shannon *et al*. (1966), Kay *et al*. (1967) and Liu (1971). The peroxidase

oxidizes o-dianisidine to form a colored product with an absorption maximum at 460 nm. It was defined (Liu, 1971) that one unit of enzyme corresponds to the change in A_{460} of 1 O.D. unit per minute. Reaction mixtures contained 0.05 ml of 0.5 % o-dianisidine, 0.1 ml of 0.1 M hydrogen peroxide, 0.1 ml of an appropriate concentration of an enzyme preparation, and 2.75 ml of 0.05 M potassium acetate buffer, pH 5.4. Peroxidase concentration was also estimated in some cases by the guaiacol method of Maehly and Chance (1954).

Protein concentrations were determined by a modified Lowry method (Peterson, 1977). Radioactivity was determined with a Beckman liquid scintillation counter (Model LS-320) using Hydrocount fluor, Baker Analyzed Reagent.

Determination of Free Amino Groups

Free amino groups of protein were determined by the trinitrobenzenesulfonic acid (TNBS) method according to Habeeb (1966). To 1 ml of protein solution (0.6 to 1 mg/ml) was added 1 ml of 4 % $NaHCO_3$ (pH 8.5) and 1 ml of 0.1 % TNBS in water. The solution was then incubated at 37° - 40°C in the dark (oven) for 2 hrs; then 1 ml 10 % SDS was added. Finally, 0.5 ml 0.1 M HCl was added to the solution, and the absorbance of the solution at 335

nm was read against a blank in the Beckman DB Spectrophotometer. An extinction coefficient of 1.4×10^4 $M^{-1}cm^{-1}$ (Means and Feeney, 1971) was used to calculate the number of amino groups present.

Amino Acid Analysis

Amino acid analysis was done by Tom Bailey in this Department according to the procedure of Spakman *et al*. (1958). Samples were hydrolyzed *in vacuo* with 6 N HCl for 22 hrs at 110°C and then analyzed on a Beckman 120B Amino Acid Analyzer fitted with a single 6 mm column packed with Dionex DC-6A resin. Quantitative determination of cysteine as cysteic acid was performed according to Spencer and Wold (1969).

Isoelectric Focusing of Peroxidase on Polyacrylamide Slab Gels

Peroxidases were focused on 5 % polyacrylamide slab gels with 3 % of carrier ampholyte in the pH range 3 - 10 according to Hoyle (1978). A gel (165 x 130 x 1 mm) was made with 23.3 ml glass-distilled water, 8.9 ml of 20 % acrylamide with Bis (0.8 %), 30 µl TEMED, 2.6 ml of Bio-Lyte, and 0.6 ml 2 % ammonium persulfate. Samples (3 µl of 1 mg/ml HRP solution) were taken up in a

Hamilton microliter syringe and applied on the plate along a 1 cm line. The gel was transferred to a precooled (4°C) Desaga, TLE-Double Chamber and focused under N_2 gas flow with a filter paper wick presoaked in concentrated KOH solution as CO_2 trap. The electrophoresis was carried out at constant power (1 W) for 13 hrs. Plates were stained for peroxidase activity with H_2O_2 and 3-amino-9-ethylcarbazole, according to Hoyle (1978).

Horseradish Peroxidase Extraction

HRP was extracted from horseradish roots using liquid N_2 homogenization and Amberlite XAD-4 adsorbent polystyrene (Loomis *et al.*, 1979). Horseradish roots (1 - 2 cm diameter) from the field were soaked overnight in tap water at room temperature (Maehly, 1955), cut into 5 - 10 cm sections and frozen in liquid N_2. The frozen roots were ground in liquid N_2 in a 1-gallon Waring Blendor. The root powder in liquid N_2 was poured into a beaker in an ice water bath. Dry Amberlite XAD-4 powder (1.5 g/g frozen roots) was added. The boiling liquid N_2 mixed the Amberlite XAD-4 thoroughly. Polyclar AT (1 g/g frozen roots) was mixed with glass-distilled water (10 ml/g frozen roots) and the thick slurry was stored in a refrigerator at 4°C. The slurry was added to the root

powder and Amberlite XAD-4 mixture just after thawing, and mixed thoroughly in the ice water bath. The mixture was filtered with a basket centrifuge using Whatman GF/B glass fiber filter paper. The supernatant was collected in a beaker in an ice water bath, and ammonium sulfate was added to 50 % saturation. The homogenate was filtered through Whatman No. 1 filter paper in a Buchner funnel in the cold room at 4°C. The supernatant solution was collected and brought to 90 % ammonium sulfate saturation. After standing for about 15 minutes the residue was collected by centrifugation at 10,000 x g for 20 minutes, redissolved in minimal volume of water and dialyzed against glass-distilled water. The dialysate was centrifuged at 23,000 x g for 10 minutes and designated as ammonium sulfate fraction and saved for further purification.

Preparative Isoelectric Focusing

Preparative IEF was done in pH 3 - 10 Bio-Lyte on a 10 x 16 x 0.2 cm polyacrylamide slab gel. The polyacrylamide slab gel was prepared according to Hoyle (1977), but was 2 mm in thickness. The sample containing isozyme C was applied by using a Hamilton microliter syringe as a line about 7 cm from the anode. The IEF was run at constant power of 2 W for 6 hrs. The visible

isozyme C band was cut out from the slab gel, and sliced into pieces. The gel slices were placed in a dialysis bag containing 0.005 M potassium acetate buffer, pH 4.4, for electroelution from the gel. Electroelution was performed at 80 - 100 V overnight (about 14 hrs) at 4°C. The eluate in the dialysis bag was collected and transferred onto a 1 x 25 cm column of mixed bed ion exchanger AG 501-X8(D) to separate the isozyme C from carrier ampholytes according to the procedure of Bauman and Chrumbach (1975). The column was then eluted with glass-distilled water; the flow rate was adjusted to 0.7 ml/minute, and 1.5 ml fractions were collected up to 60 fractions.

Preparative Polyacrylamide Gel Electrophoresis

Preparative polyacrylamide gel electrophoresis was done in a vertical slab apparatus, and performed with acidic and neutral systems according to Gabriel (1971), in the cold room at 4°C.

Acidic System

Running gel. The stock solution (a) was made by mixing 48.0 ml 1 N KOH, 17.2 ml glacial acetic acid (17.4 N), 4.0 ml TEMED, and then the final volume was adjusted

to 100 ml with glass distilled water. The pH of the solution was 4.3. Stock solution (b) was 30 % acrylamide and 0.8 % Bis solution in glass-distilled water, and (c) was 0.28 % ammonium persulfate solution in glass-distilled water. The running gel (13 x 14 x 0.2 cm) was made by mixing 1 part of (a), 2 parts of (b), 4 parts of (c) and 1 part of glass-distilled water. This gives a 7.5 % polyacrylamide gel.

Stacking gel. The stock solution (a) was made by mixing 48.0 ml 1 N KOH, 2.87 ml glacial acetic acid (17.4 N), 0.46 ml TEMED, then the final volume was adjusted to 100 ml with glass-distilled water. The pH of the solution was 6.7. Stock solution (b) was 10 % acrylamide and 2.8 % Bis solution in glass-distilled water, and (c) was 4 % riboflavin solution in glass-distilled water. The stacking gel was made by mixing 1 part of (a), 2 parts of (b), 1 part of (c) and 4 parts of glass-distilled water. Polymerization was carried out by exposing to fluorescent light.

Tray buffer. The tray (electrode) buffer was made by mixing 1.04 g β-alanine and 0.3 ml glacial acetic acid in 1 l glass-distilled water. This gives a buffer with 0.012 M β-alanine and 0.005 M acetate, and a pH of 4.5.

Neutral System

Running gel. The stock solution (a) was made by

mixing 48.0 ml 1 N HCl, 6.85 g Tris, 0.46 ml TEMED; and the volume was adjusted to 100 ml with glass-distilled water. The pH of the solution was adjusted to 7.5. The stock solution (b) was the same as in the acidic system, and (c) was 0.14 % ammonium persulfate solution. The running gel was made by mixing 1 part of (a), 2 parts of (b), 4 parts of (c) and 1 part of glass-distilled water. This gives a 7.5 % polyacrylamide gel.

Stacking gel. The stock solution (a) was made by mixing 39.0 ml concentrated phosphoric acid (44.5 N), 4.95 g Tris, 0.46 TEMED, and glass-distilled water. The total volume of the solution was 100 ml and the pH 5.5. The stock solution (b) was the same as in the acidic system, and (c) was 4.0 % riboflavin solution in glass-distilled water. Polymerization was carried out by exposing to fluorescent light.

Tray buffer. The tray (electrode) buffer was 0.01 M diethylbarbituric acid-Tris, pH 7.0. The buffer was made by dissolving 1.84 g diethylbarbituric acid and 0.33 g Tris in 1 l glass-distilled water.

Electrophoresis Procedure

Electrophoresis was performed in a cold room (4°C) at 100 V for 4 hrs, then the voltage was gradually increased up to 250 V; however the electric current was kept below 30 mA. The total time of run was about 16

hrs. The isozyme C band was cut out from the gel. The gel slice was placed in a dialysis bag containing the tray buffer for electroelution. Electroelution was performed at 100 V overnight (about 24 hrs) at 4°C. The solution in the dialysis bag was collected and subjected to IEF on polyacrylamide gel according to Hoyle (1978).

RESULTS AND DISCUSSION

Fractionation of Sigma Type I HRP

Sigma Type I HRP, RZ 0.46, was fractionated by CM-cellulose column chromatography according to Shannon *et al*. (1966) as described above. Figure 1 shows the profile of HRP elution from the CM-cellulose column. This elution profile agrees with that obtained by Shannon *et al*. (1966). IEF on Sephadex layer confirmed (data not shown) that fractions Nos. 5 to 30 contained acidic isozymes (Fraction A), and Nos. 120 to 160 contained mixed B and C isozymes and designated "B + C", and fractions Nos. 180 to 210 contained mixed D and E isozymes and designated "D + E". Fractions A and (D + E) were not purified further, but fractions (B + C) were pooled and subjected to DEAE-cellulose column chromatography at pH 8.4 as described above. The isozymes were not adsorbed by DEAE-cellulose. Although peroxidase (B and C isozymes) in the fractions did not adsorb to DEAE-cellulose column, this step was necessary in order to remove negatively-charged contaminating proteins (Shannon *et al*., 1966). The effluent containing peroxidase activity was collected, and found to have an RZ of 2.5. This fraction was then applied to a Sephadex G-100-40 column for

Figure 1. CM-cellulose cation exchange elution profile of commercial HRP.

Sigma Type I HRP, RZ 0.46, was fractionated on a CM-cellulose column. Column size : 2.5 x 35 cm. First elution system, linear gradient 0.005 M to 0.1 M potassium acetate buffer, pH 4.4, 500 ml each. Second elution system, linear gradient, 0.1 M potassium acetate, pH 4.4 to 0.25 M potassium acetate, pH 4.9, 500 ml each. Fraction volume, 10 ml. Fractions are designated according to Shannon's system.

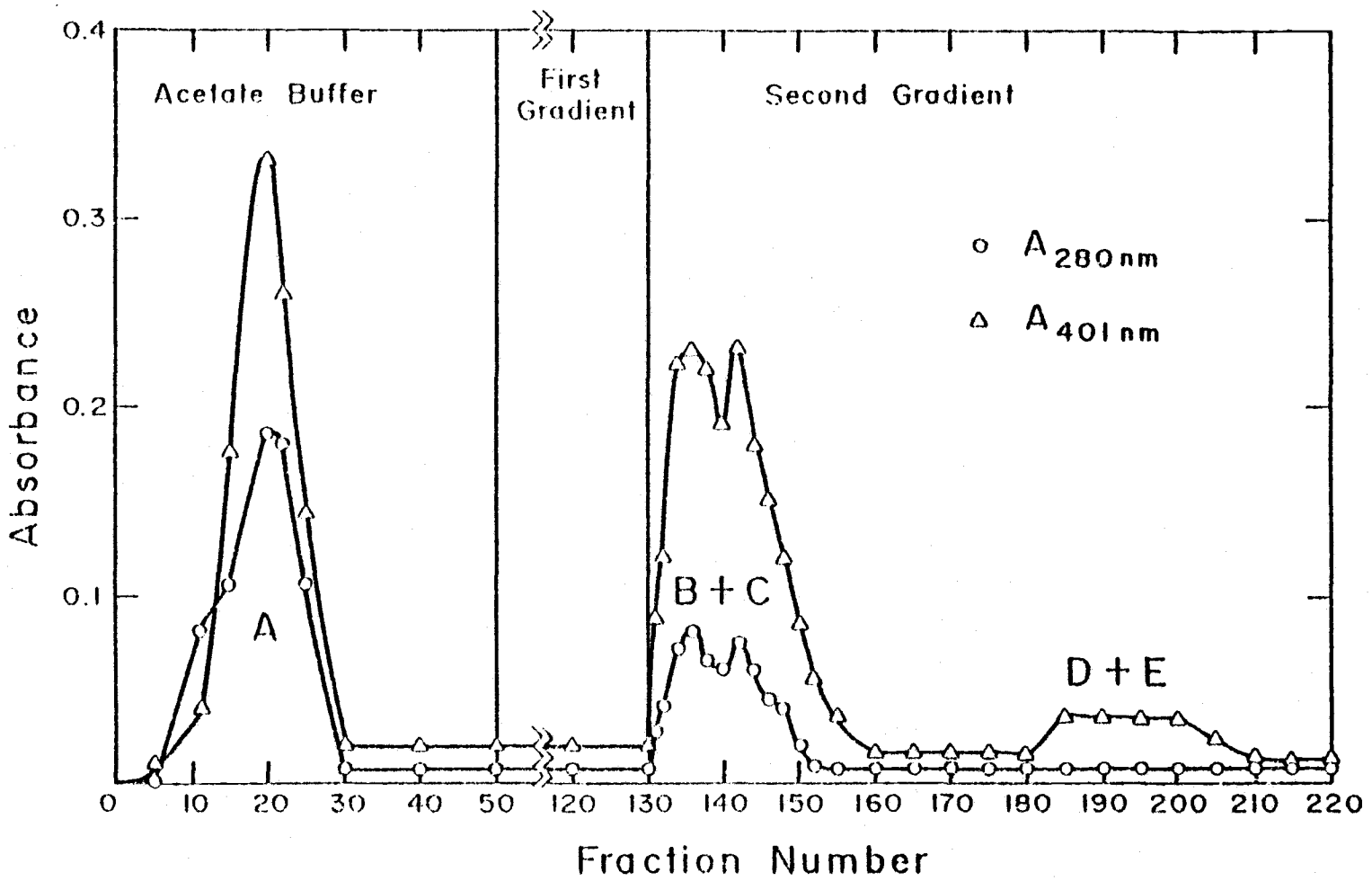

Acetate Buffer
First Gradient
Second Gradient
o A_{280nm}
Δ A_{401nm}
A
B + C
D + E
Absorbance
0.4
0.3
0.2
0.1
0
10
20
30
40
50
120
130
140
150
160
170
180
190
200
210
220
Fraction Number

Figure 1

further purification (Shih et al., 1971), and eluted with 0.005 M Tris-HCl buffer, pH 8.4. Figure 2 shows the elution profile of the fraction from the Sephadex column, as well as the RZs of the fractions. It was found by analytical IEF (data not shown) that there was no separation of isozyme B from isozyme C by gel filtration; but based on the RZs, non-peroxidase protein was removed. Therefore, all of the B and C fractions were pooled.

Figure 3 shows IEF patterns of the column fractions compared to the original Type I HRP. The bands are numbered consecutively according to the Commision on Biochemical Nomenclature (IUPAC-IUB, 1977) (the highest mobility towards anode is numbered "one"), and are also designated by Shannon's A, B, C system. Fraction A from the CM-cellulose column chromatography consists of three bands which were designated by Shannon et al. (1966) as isozymes A_1, A_2 and A_3. Bands Nos. 1, 2 and 3 of Sigma Type I HRP were coincident with isozymes A_3, A_2 and A_1; therefore the bands Nos. 1, 2 and 3 in this study are considered to be identical with A_3, A_2 and A_1. Fraction (B + C) was supposed to contain isozymes B and C (Shannon et al., 1966); however IEF showed the presence of another band (No. 6). It was shown that bands Nos. 5 and 7 were coincident with isozymes B and C. Therefore bands Nos. 5 and 7 are designated as bands B and C, and band No. 6 will be called C'. Band No. 4 was not reported by

Figure 2. Sephadex G-100-40 elution profile of Fraction (B + C).

Fraction (B + C) resulted from CM-cellulose chromatographic column. Column size : 2.5 x 30 cm. Elution system, 0.005 M Tris buffer, pH 8.4. Fraction volume, 5 ml.

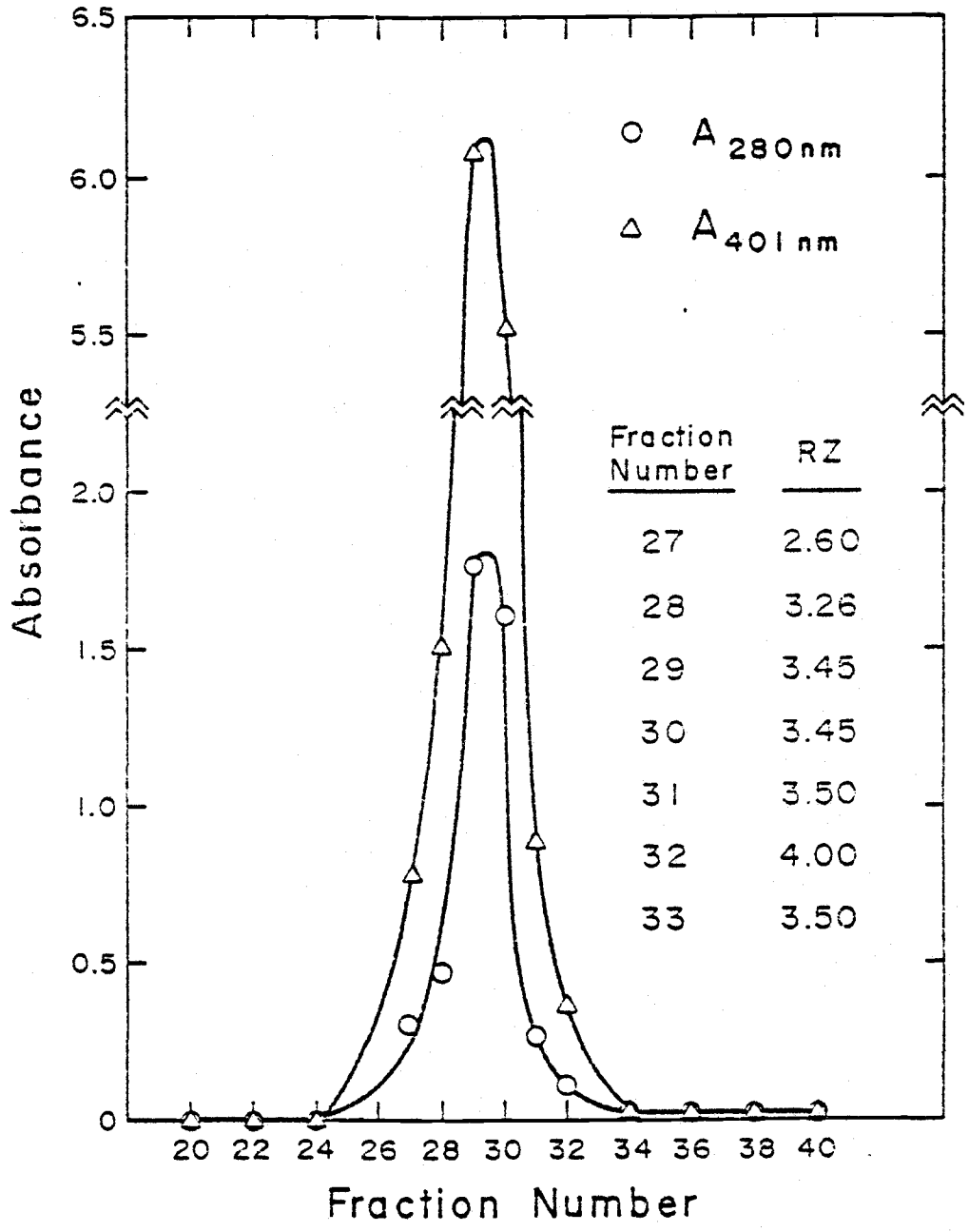
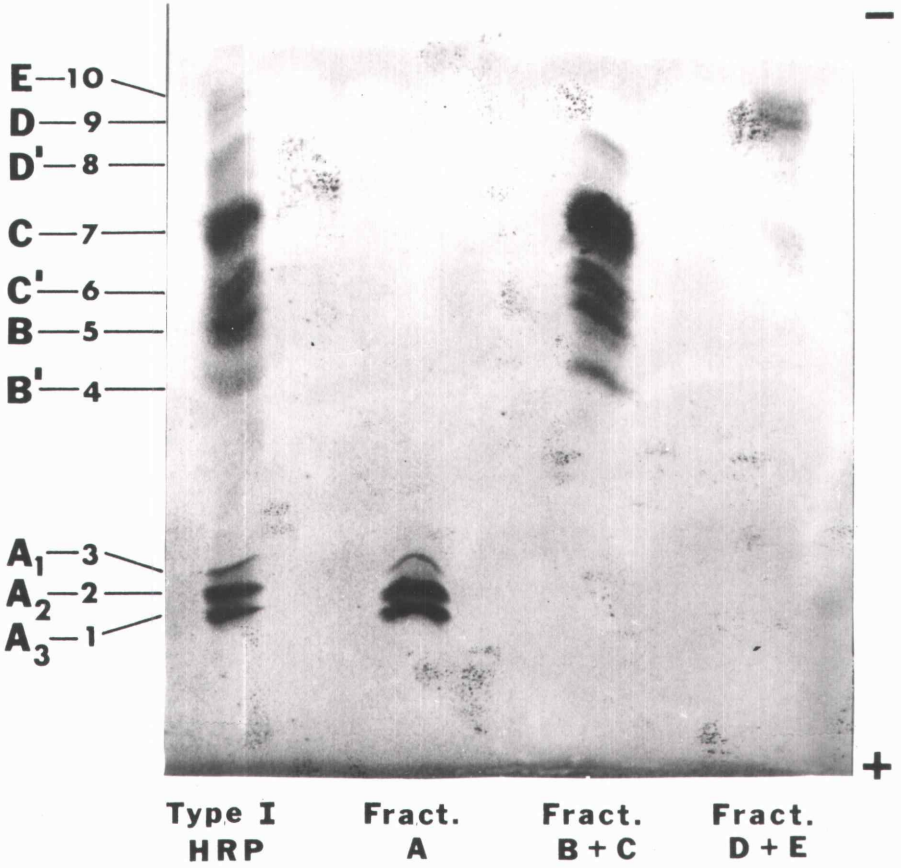

6.5
6.0
5.5
2.0
1.5
1.0
0.5
0
Absorbance
20 22 24 26 28 30 32 34 36 38 40
Fraction Number
○ A 280nm
△ A 401 nm
Fraction Number
RZ
27 2.60
28 3.26
29 3.45
30 3.45
31 3.50
32 4.00
33 3.50

Figure 2

Figure 3. Isoelectric focusing of HRP and HRP CM-cellulose column chromatography fractions.

IEF was done in pH 3 -10 Bio-Lyte on 20 x 20 x 0.06 cm Sephadex G-75-40 layer, and focused at 200 V for 4 hrs, followed by another 3 hrs at 800 V.



Fractions A, (B + C), (D + E) were obtained by fractionation of Type I HRP by CM-cellulose column chromatography

Figure 3

Shannon *et al*. (1966). It is designated here as B'. Bands Nos. 9 and 10 were coincident with isozymes D and E. Band No. 8 was not reported by Shannon *et al*. (1966), and is designated here as D'.

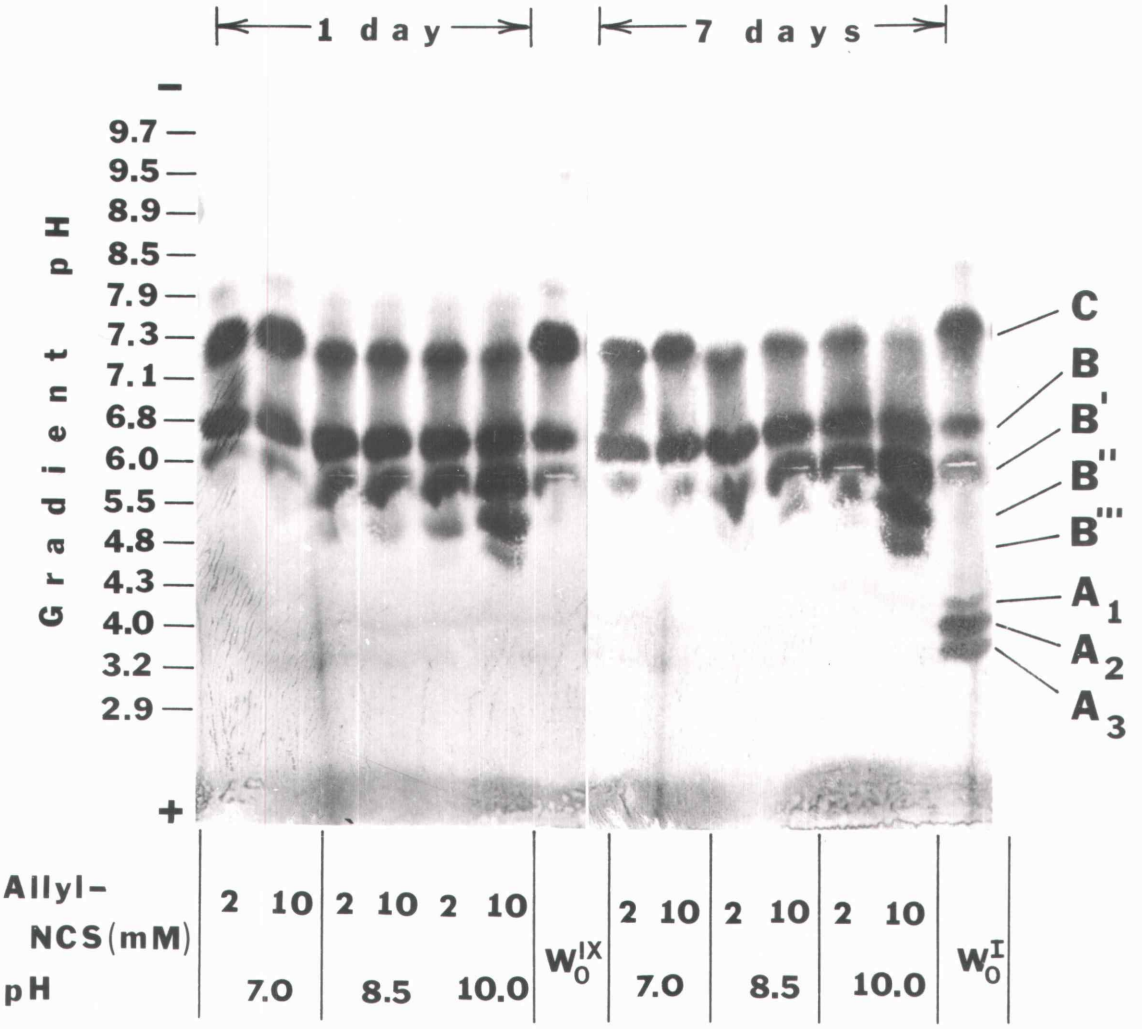
Modification of Basic Isozymes of Horseradish Peroxidase by Allylisothiocyanate

Sigma Type IX HRP (0.50 mg/ml; 0.013 mM), RZ 3.02, and claimed to be pure isozyme C (Sigma Chemical Catalog, 1984), was incubated with 0.2 and 1.0 µl/ml (2 and 10 mM) allylNCS for 1 and 7 days at pH 7.0, 8.5 and 10.0. The band patterns were examined by IEF on Sephadex thin layers (Figure 4), and peroxidase activities of bands were compared by densitometry (Figure 5).

It can be seen from Figure 4 that Sigma Type IX HRP contained three bands, rather than the single isozyme claimed by the supplier. Sigma Chemical Company (Sigma Chemical Catalog, 1984), using disc electrophoresis at pH 4.5 according to Reisfeld *et al*. (1962), claimed that their Type IX HRP was a single basic isozyme. Conroy *et al*. (1982) also claimed that Sigma Type IX HRP was homogeneous and similar to isozyme C of Shannon *et al*. (1966). However, they used sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to Weber and Osborn (1969) to confirm that the enzyme was

Figure 4. Isoelectric focusing of Sigma Type IX HRP after allylisothiocyanate treatments.

Sigma Type IX HRP was incubated with 0.2 and 1.0 µl/ml (2 and 10 mM) allylNCS at 4°C for 1 and 7 days. The IEF was performed in pH 3 - 10 Bio-Lyte on a 20 x 20 x 0.06 cm Sephadex G-75-40 layer. Cathode at the top. Peroxidase bands were stained and designated as explained in Figure 3.



W_0^{IX} : freshly made Sigma Type IX HRP solution in water

W_0^{I} : freshly made Sigma Type I HRP solution in water

Figure 4

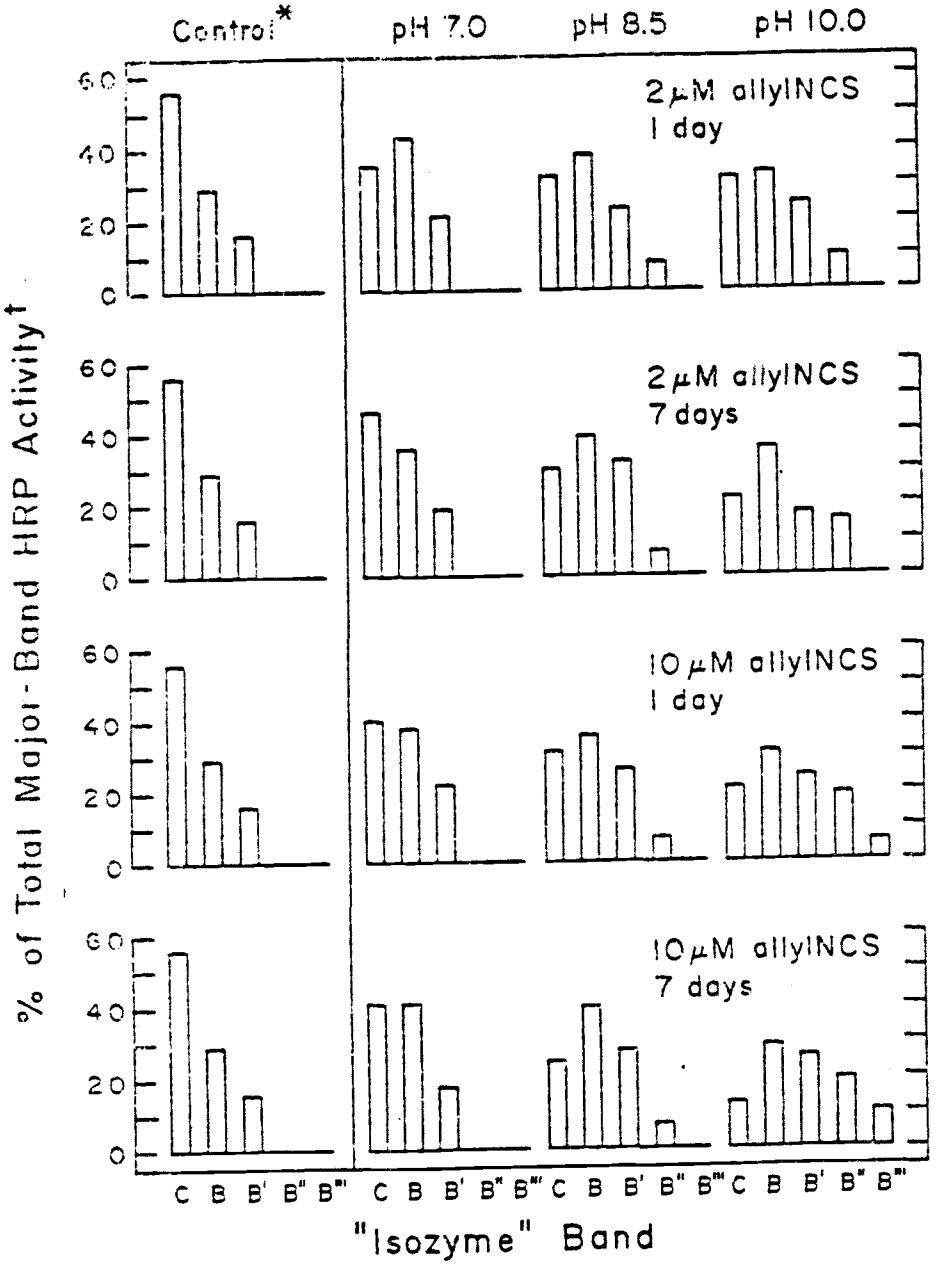
homogeneous. Since the molecular weights of peroxidase isozymes are close to each other, SDS-PAGE can not be used to determine homogeneity of the enzyme.

Figure 4 also shows that in this experiment, allyl-NCS treatment at pH 7.0 or higher altered the banding pattern of the enzyme. The C band enzyme apparently reacted with allylNCS, and its peroxidase activity was shifted to bands of lower pI. Incubation at pH 8.5 and 10.0 resulted in formation of new bands, designated as B" and B". The number of new bands increased, and the proportion of peroxidase activity in the new bands, increased with increasing pH, increasing allylNCS, or increasing time.

A positive transparency of the photograph shown in Figure 4 was subjected to densitometry to obtain quantitative comparisons of peroxidase activities in the different bands. The results are shown in Figure 5. The results shown qualitatively in Figure 4 and quantitatively in Figure 5 suggest that at the higher pHs, incubation of HRP with allylNCS produced a stepwise conversion of isozyme C into discrete "isozymes" of lower pI. The conversion to B represents augmentation of a preexisting band. The B',B" and B"' bands are new. With time, the C band activity virtually disappeared, to be replaced by activity in the bands of lower pI. It is noteworthy that the modified HRP focuses primarily as

Figure 5. Distribution of peroxidase activity among "isozymes" of Sigma Type IX HRP after allylisothiocyanate treatments.

Sigma Type IX HRP (0.50 mg/ml; 0.013 mM) was treated with 0.2 and 1.0 µl/ml (2 and 10 mM) allylNCS at 4°C for 1 and 7 days. The percent peroxidase activities were based on densitometry of Figure 4.



*Control : Freshly-made H_2O solution of Sigma Type IX HRP (W_0^{IX} of Figure 4). For convenience it is repeated with each set of treatments

†The" smear" of activity between B and C was not included in the measurements.

Figure 5

discrete bands, rather than as smears. Thus the modification must involve discrete protein R-group modification, and not just random reactions. The region between the C and B bands shows an unusual amount of "smear" activity which might possibly be due to modification of the C band during IEF, as a result of exposure to high pH within the gradient. This region sometimes appears to resolve into a band which we designate as C'. The results suggest the following stepwise conversions by additions of allylNCS :

$$C \longrightarrow (C') \longrightarrow B \longrightarrow B' \longrightarrow B'' \longrightarrow B'''$$

Reaction of AllylNCS with Free Amino Groups of Sigma Type I HRP

Sigma Type I HRP, RZ 0.66, (0.54 mg/ml; 0.014 mM) was incubated with allylNCS (1 µl/ml; 10 mM) at pH 7.0, 8.5 and 10.0. Sigma Type I HRP is a crude peroxidase containing all of the isozymes that are shown in Figure 3. The number of free amino groups per molecule enzyme was determined by the TNBS method of Habeeb (1966). Table IV shows the number of free amino group per molecule of enzyme (based on MW 40,000). It was found that there were 17 free amino groups per HRP molecule before reaction with allylNCS. Clarke and Shannon (1976) obtained 6 lysine residues and 47 glucosamine residues

Table IV. Number of Free Amino Groups[a] per Molecule of HRP after Incubating with AllylNCS.

Sigma Type I HRP (0.54 mg/ml, 0.014 mM), RZ 0.66, was incubated with allylNCS (1 μl, 10 mM), at 4°C. Control, freshly-made HRP solution in glass-distilled water, contained 17 amino groups per molecule enzyme.

pH	Incubation Time	
	1 Day	7 Days
H_2O, no allylNCS	18	17
7	20	19
8.5	18	16
10	16	12

[a]The number of free amino groups was determined by the TNBS method according to Habeeb (1966).

per molecule of isozyme C, or 53 amino groups per isozyme C molecule. TNBS apparently did not react with all of the amino groups, possibly because some of the amino groups were blocked by isothiocyanates during extraction. It is also likely that some of the glucosamine residues are acetylated, as N-acetyl glucosamine. Incubation of the enzyme with allylNCS at pH 10.0 for 7 days, reduced the number of free amino groups to 12. This indicated that approximately six amino groups reacted with allylNCS. In the case of peroxidase protein, only the six lysine ε-NH_2 groups are available for reaction, since the N-terminal is apparently blocked (Welinder, 1979).

Results of amino acid analysis of Sigma Type IX HRP are shown in Table V. The amino acid composition of the enzyme was similar to literature values for isozyme C (Table II), except for the number of aspartic acid and cystine residues, which we assume to be an error in our analyses.

Incubation of the Sigma Type IX HRP with allylNCS at pH 8.5 for 7 days reduced the number of lysine residues by one. This coincided with loss of C band activity and increase of peroxidase activities of B, B' and B" bands (Figure 4). Incubation at pH 10.0 reduced the recovery of lysine residues by 50 % (3 out of 6), and IEF indicated that simultaneously almost all of C band peroxidase activity was converted to B, B', B" and B'"

Table V. Amino Acid Analysis of Sigma Type IX HRP after Incubating with AllylNCS for 7 Days.

Amino Acid	Number of Residues/Molecule of HRP			
	Untreated[a]	AllylNCS Treated		Literature[b], [C]isozyme
		pH 8.5	pH 10.0	
Asx	57	49	49	48
Thr	24	23	24	25
Ser	23	24	25	25
Glx	20	21	21	20
Pro	17	16	17	17
Gly	17	19	20	17
Ala	23	23	23	23
Cys	5	5	5	8
Val	16	16	16	17
Met	2	3	3	4
Ile	12	13	13	13
Leu	36	37	36	35
Tyr	6	5	6	5
Phe	21	22	21	20
Lys	6	5	3	6
His	3	4	4	3
Arg	21	21	22	21
Trp	--	--	--	1
Total	309	306	308	308

[a]Freshly-prepared solution of HRP in H_2O. Not incubated

[b]Cited from Welinder (1979).

bands (Figure 4). There were no significant changes in other amino acid residues, except that the number of aspartic acid residues per enzyme molecule was now in agreement with literature values for isozyme C. These data suggest that ε-NH_2 groups of lysine residues were reaction sites of allylNCS. Konigsberg (1967) reported that 70% of N^{ε}-phenylthiocarbamyl lysine (N^{ε}-PTC lysine) was recovered as lysine after hydrolysis with 6N HCl at 110°C for 22 hrs. Therefore, it is possible that all of the six lysine residues were blocked by allylNCS, but then some of the blocking groups (amino acid analysis showed 3) were removed by 6 N HCl hydrolysis during amino acid analysis.

Modification of Sigma Type I HRP Isozyme Pattern by Allylisothiocyanate

Sigma Type I HRP, RZ 0.66, (1 mg/ml) was incubated with 0.2 and 1.0 µl (2 and 10 mM) allylNCS at pH 6.0, 7.0, 8.0, 9.0 and 10.0, and in unbuffered water solution. Sigma Type I HRP is a crude peroxidase containing all of the isozymes that are shown in Figure 3. The isozyme patterns were followed with IEF on Sephadex G-75-40.

Table VI shows the effect of allylNCS treatments on peroxidase activity. Although the peroxidase assays are more variable than would be desired, it appears that

Table VI. Peroxidase Activity of HRP after AllylNCS treatments.

Sigma Type I HRP, RZ 0.66 (1 mg/ml, 0.025 mM) was incubated with allylNCS (2 and 10 mM) for 1, 7 and 30 days at 4°C. Enzyme activity assayed by the Worthington procedure (1977).

pH	AllylNCS concentration (mM)	% of Original Activity[a]		
		1 Day	7 Days	30 Days
6	0	75	98	76
	2	78	84	84
	10	78	75	75
7	0	85	76	82
	2	78	95	93
	10	93	78	84
8	0	93	80	75
	2	84	84	75
	10	87	75	78
9	0	93	71	80
	2	115	82	89
	10	93	78	78
10	0	91	93	71
	2	98	98	93
	10	100	91	49
H_2O	0	87	75	69
	2	75	80	60
	10	82	96	38

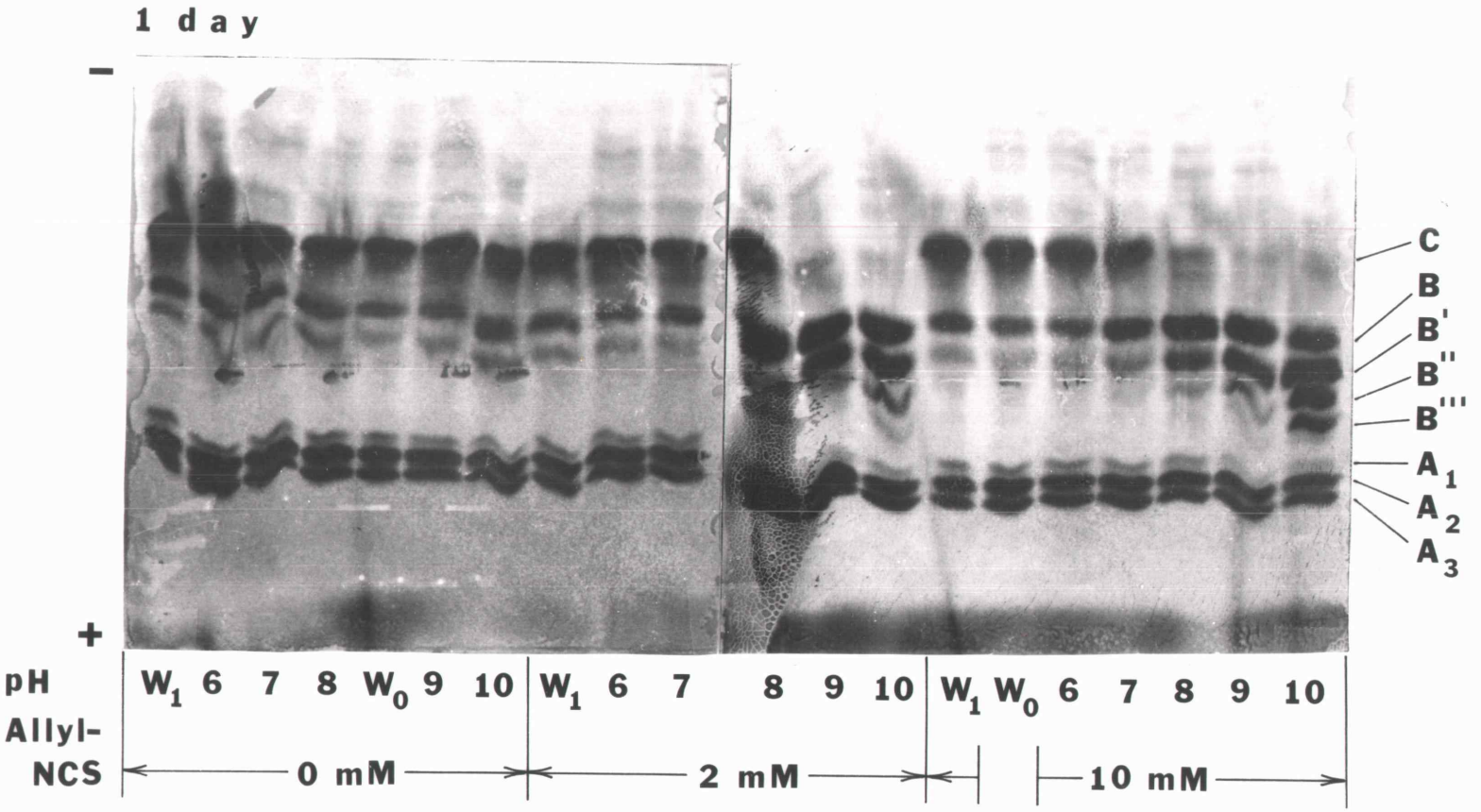
aValues given as percent of original enzyme activity. The original activity determined at day 0 on the freshly-prepared, untreated H_2O solution. Original activity, 55 units/mg.

in most treatments little, if any, enzyme activity was lost. The values of 49 % and 38 % after 30 days at pH 10 and in water respectively may well be real, as Sandstrom and Loomis (unpublished) found that 10 µl of allylNCS per ml caused extensive loss of activity when incubated with 0.1 or 0.01 mg HRP per ml. Liu and Lamport (1973) found that alteration of HRP isozyme pattern by incubating the enzyme in an alkaline solution did not change the total peroxidase activity. Ugarova et al. (1979) modified $-NH_2$ lysine residues of isozyme C with acetic anhydride and found no change in the total peroxidase activity. These data suggest that lysine $-NH_2$ groups are not involved in enzyme catalytic activity, and that the positions of the lysine residues are far enough from the active site of the enzyme that the modified lysines do not have any effect on the catalytic activity.

Figure 6 shows the changes of the HRP isozyme patterns after the allylNCS treatments. Some of the bands at the application sites appear to be artifacts due to artifactual binding or to blockage of focusing. These bands have unusually sharp edges, and their appearance does not seem to follow a regular pattern. We will assume that they are artifacts of application. AllylNCS treatments at pH 7 or higher initially caused loss of C band activity, which correlated with increases in activity of preexisting bands of lower pI. With time,

Figure 6. Isoelectric focusing of Sigma Type I HRP after allylNCS treatments.

Sigma Type I HRP RZ 0.66 (1 mg/ml; 0.025 mM), was treated with 0.2 and 1.0 µl/ml allylNCS at 4°C for 1, 7 and 30 days. IEF was done in pH 3 - 10 Bio-Lyte on a 20 x 20 x 0.06 cm Sephadex G-75-40 layer, and focused for 4 hrs at 200 V, followed by another 3 hrs at 800 V.



W_0 : freshly made Sigma Type I solution in water

W_1 : Sigma Type I HRP solution in water, incubated for 1 day

Figure 6

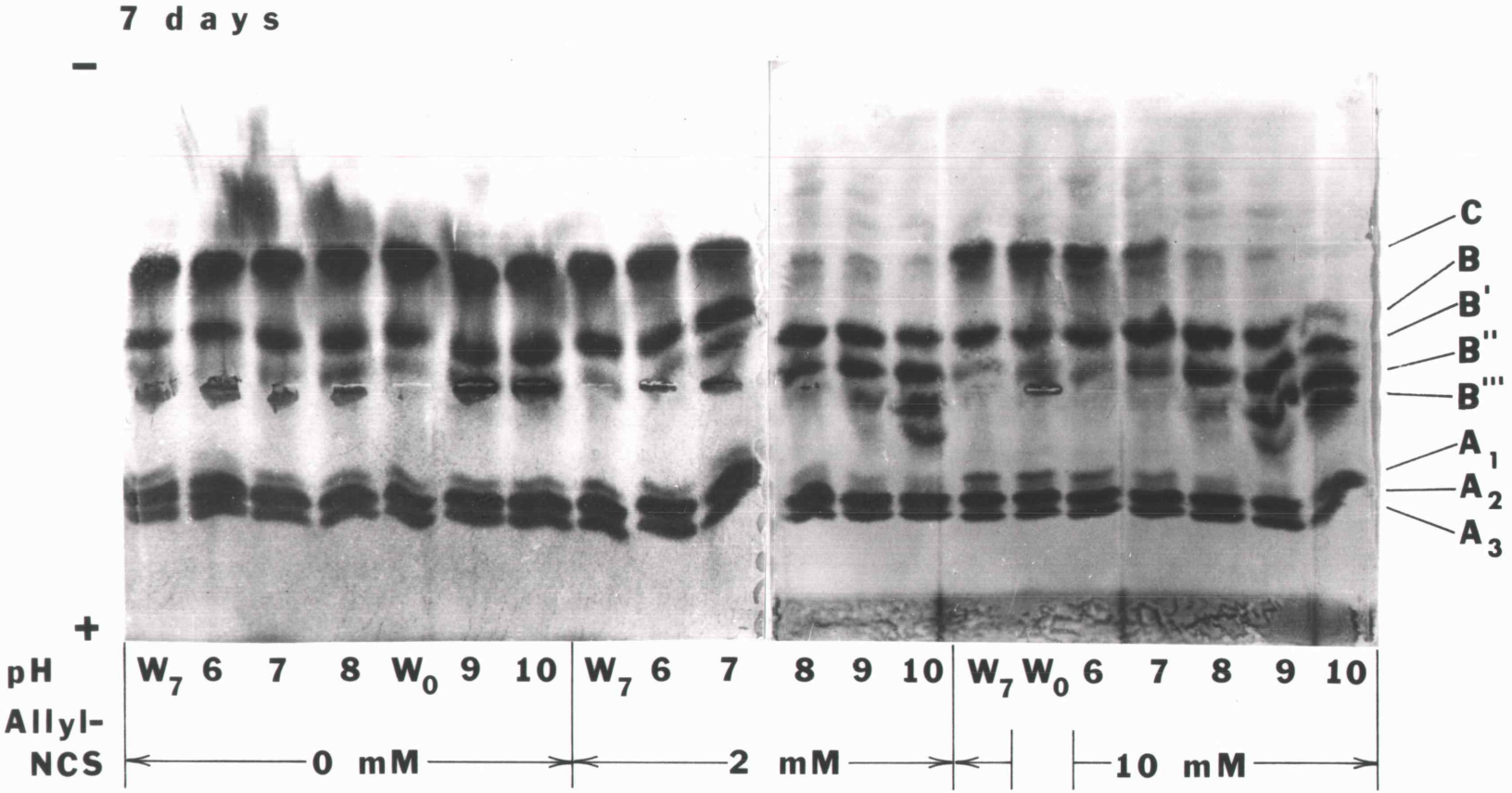


Figure 6 (Continued)

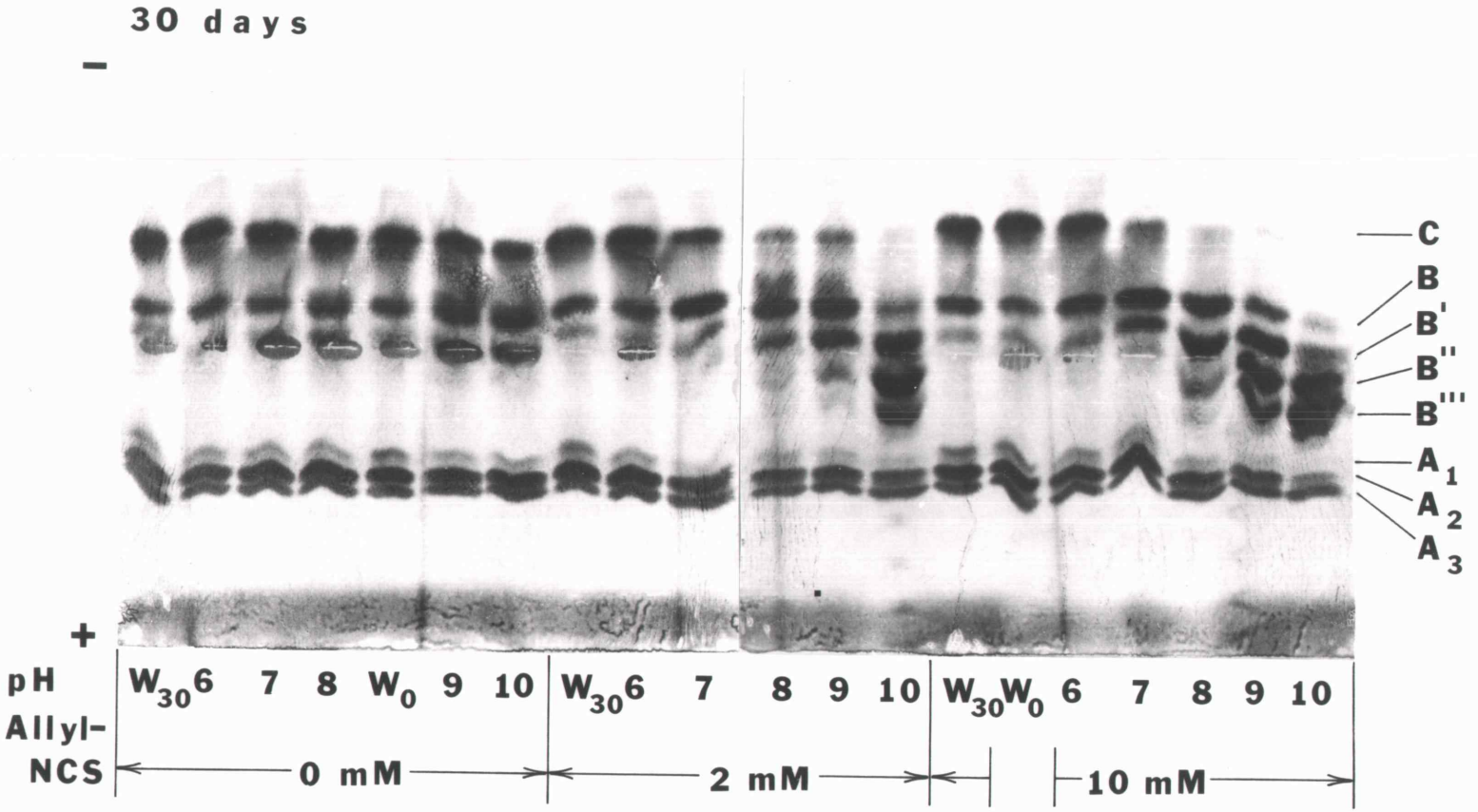


Figure 6 (Continued)

new bands of still lower pI appeared. Eventually, the C band disappeared completely, to be replaced by bands of enhanced peroxidase activity at lower pIs, some of them preexisting B and B' bands, and some of them new (B" and B"' bands). Again these data suggest that conversion of C band to the preexisting and new bands occurs sequentially. Band A_1 also disappeared after incubating with allylNCS at pH 8 or higher.

Figure 6 also shows that incubation of the enzyme with buffer alone did not alter the HRP isozyme pattern. Liu and Lamport (1973) reported that incubation of freshly made horseradish root homogenate at alkaline pH altered the HRP isozyme pattern. This suggests that their system contained isothiocyanates which were produced and adsorbed during root homogenization.

This experiment showed that allylNCS treatments at pH 7 or higher altered the "isozyme" pattern of HRP by eliminating the original C and A_1. The reaction between allylNCS and HRP was dependent on pH, allylNCS concentration and incubation time. These data supported our initial hypothesis, that some of the commercial HRP isozymes may arise from HRP modification by isothiocyanates (mustard oils) during extraction and purification.

Modification of Horseradish Peroxidase with Phenyl(^{14}C)isothiocyanate

In an initial experiment, 0.06 ml phenyl(^{14}C)NCS in n-heptane solution (0.016 mg; 6 μCi) was added directly to 1 ml of Sigma Type I HRP (0.30 mg/ml; 7.5 μM) at pH 6.0, 8.0 and 10.0. The reaction mixtures containing 7.5 μM HRP and 120 μM phenyl(^{14}C)NCS (13.5 x 10^6 dpm), were incubated at 4°C for 1, 3 and 7 days. It was found that the enzyme was coagulated, and the precipitate floated on the surface of the solution. This indicated that n-heptane binds hydrophobically to the protein.

The experiment was repeated, but phenyl(^{14}C)NCS was transferred to methanol solution before using, to avoid coagulation. The labeled phenylNCS (0.40 ml) containing 9 x 10^7 dpm, was adsorbed on a Silica gel G plate, and then dried. The silica gel was scraped and transferred into a small column. The column was then washed with methanol (3 ml). The effluent was collected, and then evaporated to a final volume of 0.06 ml. The percent recovery of this technique was very low (23 %). Unlabeled phenylNCS was added to this methanol solution in order to provide 1.5 μmoles total phenylNCS and 2 x 10^7 dpm (determined by liquid scintillation counting) in the 0.06 ml methanol solution (specific activity 6.06 μCi/μmole). Then 5 μl of this solution containing 0.12

μmoles phenylNCS (1.7×10^6 dpm) was added to 0.3 ml peroxidase solution (0.30 mg/ml; 7.5 μM) with pH 6.0, 8.0 and 10.0, and mixtures were then incubated at 4°C for 1, 3 and 7 days. After incubation, the mixtures were treated with powdered Amberlite XAD-4 as described above, the amount of protein-bound radioactivity in the mixtures was counted, and the average number of molecules of labeled phenylNCS bound per molecule of HRP was calculated. This number does not represent the amount of ^{14}C per molecule of isozyme or band, but rather per molecule of total HRP, using a molecular weight of 40,000. The results are shown in Table VII. The amount of label bound per molecule of enzyme at pH 6.0 was very low and remained constant with increasing incubation time. In contrast, on incubation of the enzyme at pH 8.0 and 10.0, the incorporation of phenylNCS per molecule of enzyme increased with increasing incubation time and increasing pH.

The isozyme patterns of HRP (same HRP as shown in Table VII) were followed by IEF. In doing IEF on a Sephadex layer or polyacrylamide gel, 6 μl of sample was applied on the gel. Figure 7 shows IEF patterns of peroxidase activity of the same HRP as shown in Table VII. The total ^{14}C activity in each sample is shown in Figure 7. The isozyme patterns of HRP treated with phenylNCS were identical to those of the enzyme treated

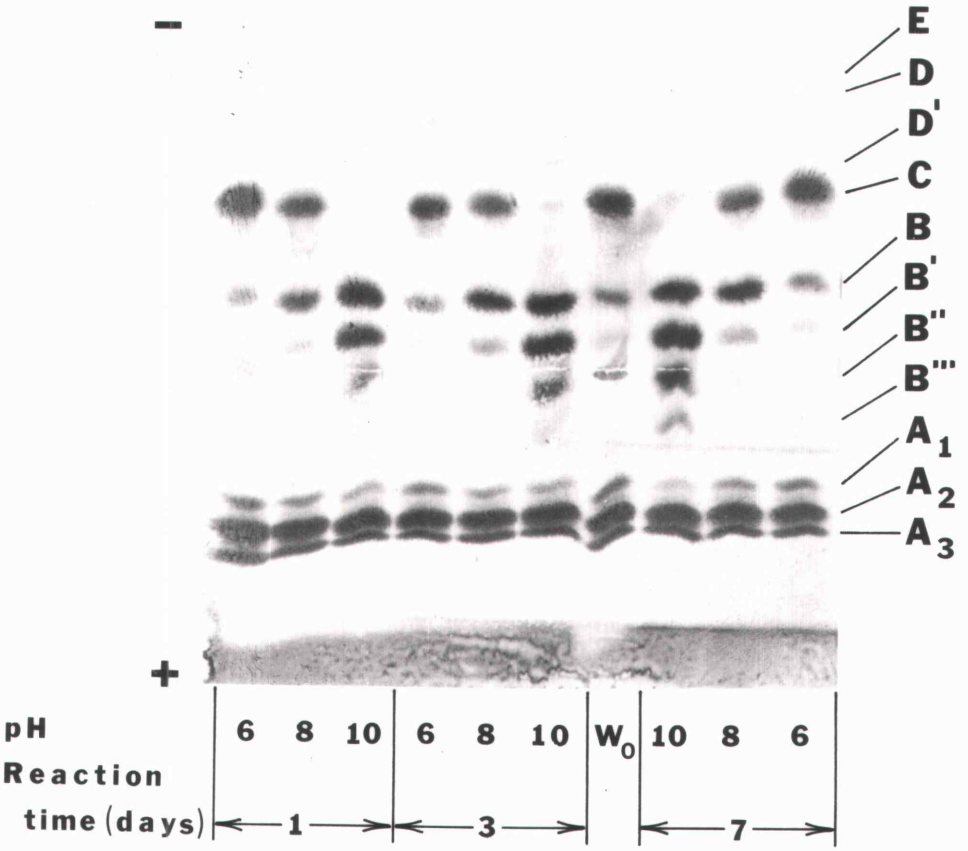
Table VII. Phenyl (^{14}C)NCS Labeling of Sigma Type I HRP.

pH	Total Incorporation of ^{14}C (%)			Molecules of Phenyl(^{14}C)NCS Incorporated per Molecule Protein		
	1 Day	3 Days	7 Days	1 Day	3 Days	7 Days
6	0.44	0.33	0.33	0.23	0.23	0.22
8	1.24	1.67	1.77	0.60	0.90	1.00
10	3.71	6.18	9.94	1.80	3.62	4.93

Figure 7. Isoelectric focusing of HRP activity on Sephadex layer after treatment with phenyl(^{14}C)NCS.

Sigma Type I HRP, RZ 0.66, was incubated with phenyl(^{14}C)NCS as explained in the text. The IEF was performed in pH 3 - 10 Bio-Lyte on a 20 x 20 x 0.06 cm Sephadex G-75-40 layer, and focused at 200 V for 4 hrs, and followed by another 3 hrs at 800 V. 6 µl of each sample was applied on the layer. This would give approximate total radioactivity value as follows:

pH 6.0, 1 day	:	150 dpm
8.0, 1 day	:	415 dpm
10.0, 1 day	:	1250 dpm
6.0, 3 days	:	110 dpm
8.0, 3 days	:	560 dpm
10.0, 3 days	:	2080 dpm
6.0, 7 days	:	110 dpm
8.0, 7 days	:	430 dpm
10.0, 7 days	:	3350 dpm.



W_0: freshly made Sigma Type I HRP solution in water

Figure 7

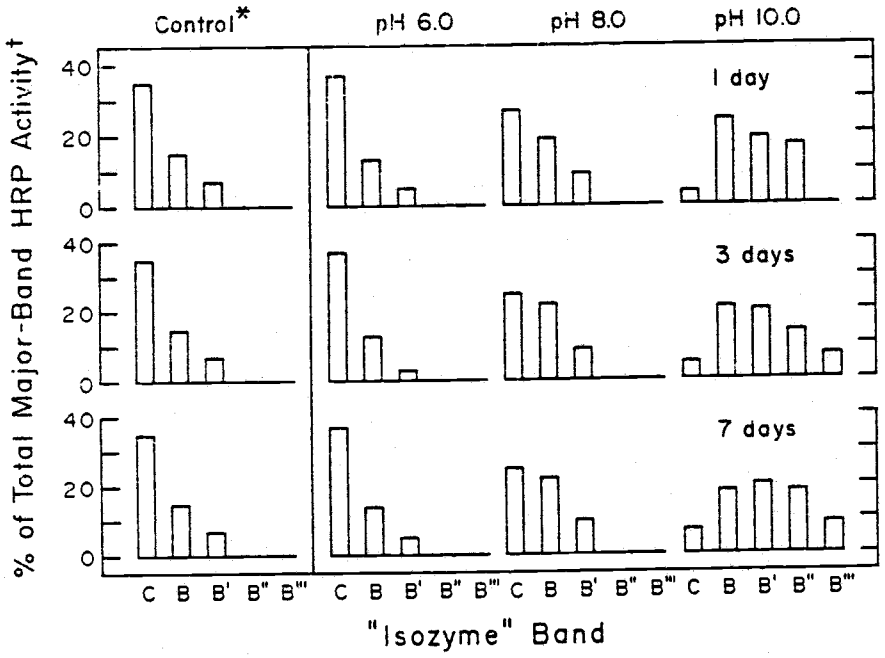
with allylNCS (Figure 6). There was no apparent change in isozyme pattern when the enzyme was incubated at pH 6.0, while incubation at higher pH showed alteration of the isozyme pattern. It was shown that C band activity was shifted to bands of lower pI. The C band finally almost disappeared, while peroxidase activities of preexisting bands (B and B') increased, and two new bands (B" and B"') were generated.

The changes of isozyme pattern by phenylNCS treatments are shown quantitatively in Figure 8 (from densitometry of a positive transparency of Figure 7). The activity of C band was decreased by increasing pH and increasing reaction time. The C band activity was shifted to B, B', B" and B"' bands. Incubation at pH 8.0 showed conversion of some C band activity to B and B' bands. This was not shown clearly in Figure 7. By following the shifting of C band activity to the other bands, it appears that the reactions between HRP and phenylNCS occur sequentially. Autoradiography was attempted, using X-ray film (Kodak X-Omat AR), by placing the film directly on the surface of the Sephadex layer, for 60 days at -80°C, and no result was obtained due to low activity.

Because of difficulties in doing autoradiography of the fragile Sephadex layer, the 3-day and 7-day samples were focused on a polyacrylamide gel slab (the 1-day

Figure 8. Distribution of peroxidase activity among B and C "isozymes" of HRP after phenyl(^{14}C)isothiocyanate treatments. Densitometric quantitation of Figure 7.

Sigma Type I HRP was treated with phenyl(^{14}C)NCS as explained in the text. The percent peroxidase activities were based on densitometry of Figure 7. A bands were included in the "100 % activity" but are not graphed. The A bands showed very little change.



*Control : Freshly made H_2O solution of Sigma Type I HRP (W^0 of Figure 7). For convenience it is repeated with each set of treatments

†The activities of E, D, D', A_1, A_2 and A_3 (Figure 4) bands were not shown.

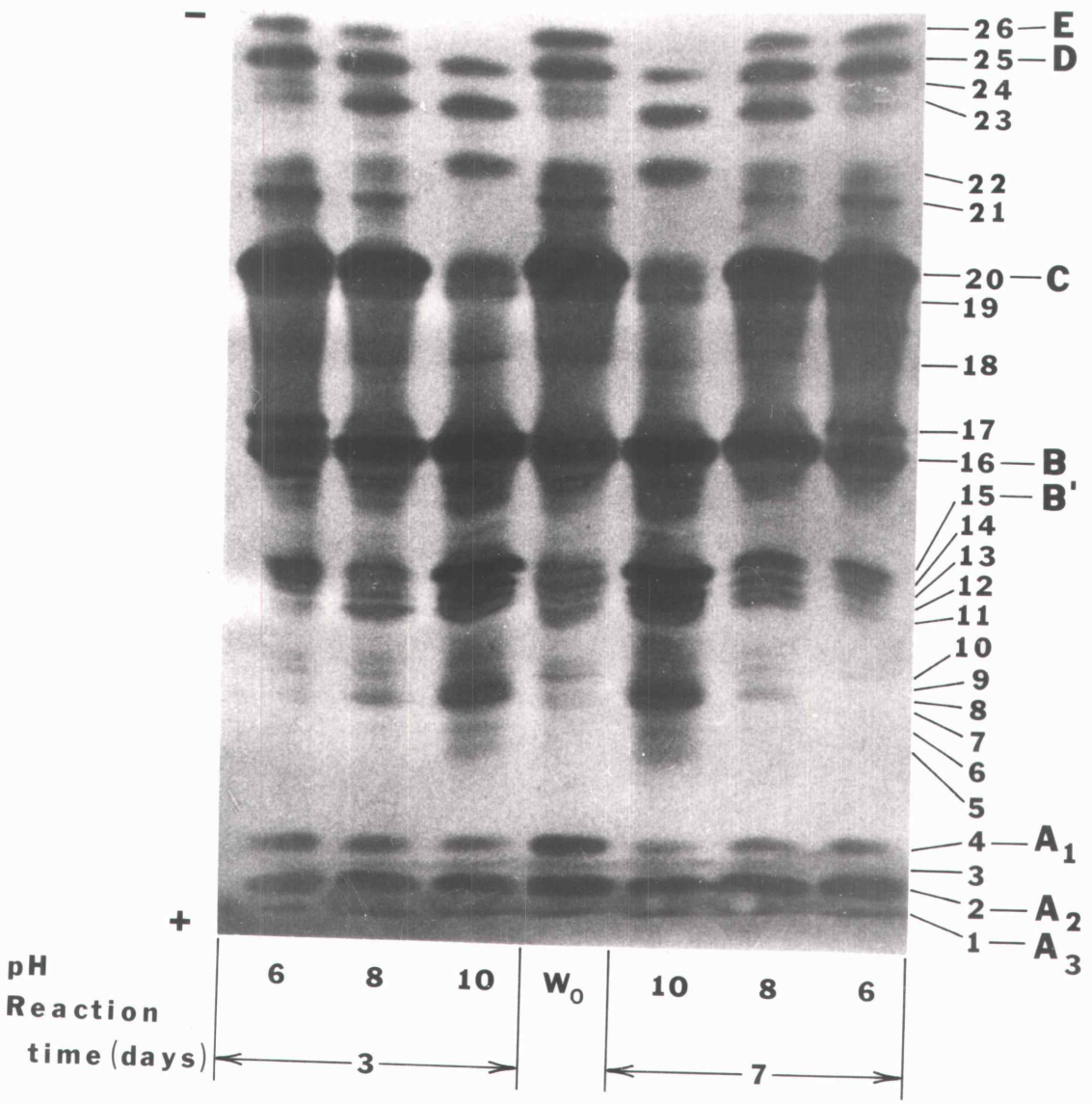
Figure 8

sample was lost). Figure 9 shows the IEF patterns of peroxidase activity on the polyacrylamide slab. The total activity (dpm) in each sample is the same as in Figure 7. In agreement with Hoyle (1977) the number of bands shown by this procedure is substantially greater than seen on Sephadex layers. The changes in isozyme patterns on treatment with phenylNCS are similar to those seen on Sephadex gel (Figure 7). Loss of activity of bands with higher pI correlated with increasing activity in bands of lower pI. Autoradiography was done on the dried polyacrylamide gel with a scintillation fluor (EN^3HANCE) added, by exposing the gel to the X-ray film for 30 days at -80°C. Figure 10 shows the distribution of ^{14}C in the modified HRP bands. It can be seen that isozyme C is not labeled, while "isozyme" B becomes labeled at pH 8.0 and 10.0, and bands Nos. 15 (B') and 14 become labeled at pH 10.0. Close examination of the autoradiogram suggests that bands of still-lower pI (Nos. 5, 6, 7, 8, 9, 10, 11, 12, 13) formed at pH 10 are very slightly labeled. It may be that the film exposure time was simply not adequate. However, one should also consider that there may be protein modifications due to isothiocyanate but without permanent binding of the R-group.

Addition of isothiocyanates (RNCS) to unprotonated amino groups of proteins produces a symmetrical linkage,

Figure 9. Isoelectric focusing of HRP activity on polyacrylamide slab gel after phenyl(^{14}C)NCS treatment.

The IEF was performed in pH 3 - 10 Bio-Lyte on a 16.5 x 13 x 0.1 cm polyacrylamide gel. The IEF was run at constant power (1 W) for 14 hrs. 6 µl of each sample was applied, as in Figure 7. Radioactivity values would be identical to Figure 7.

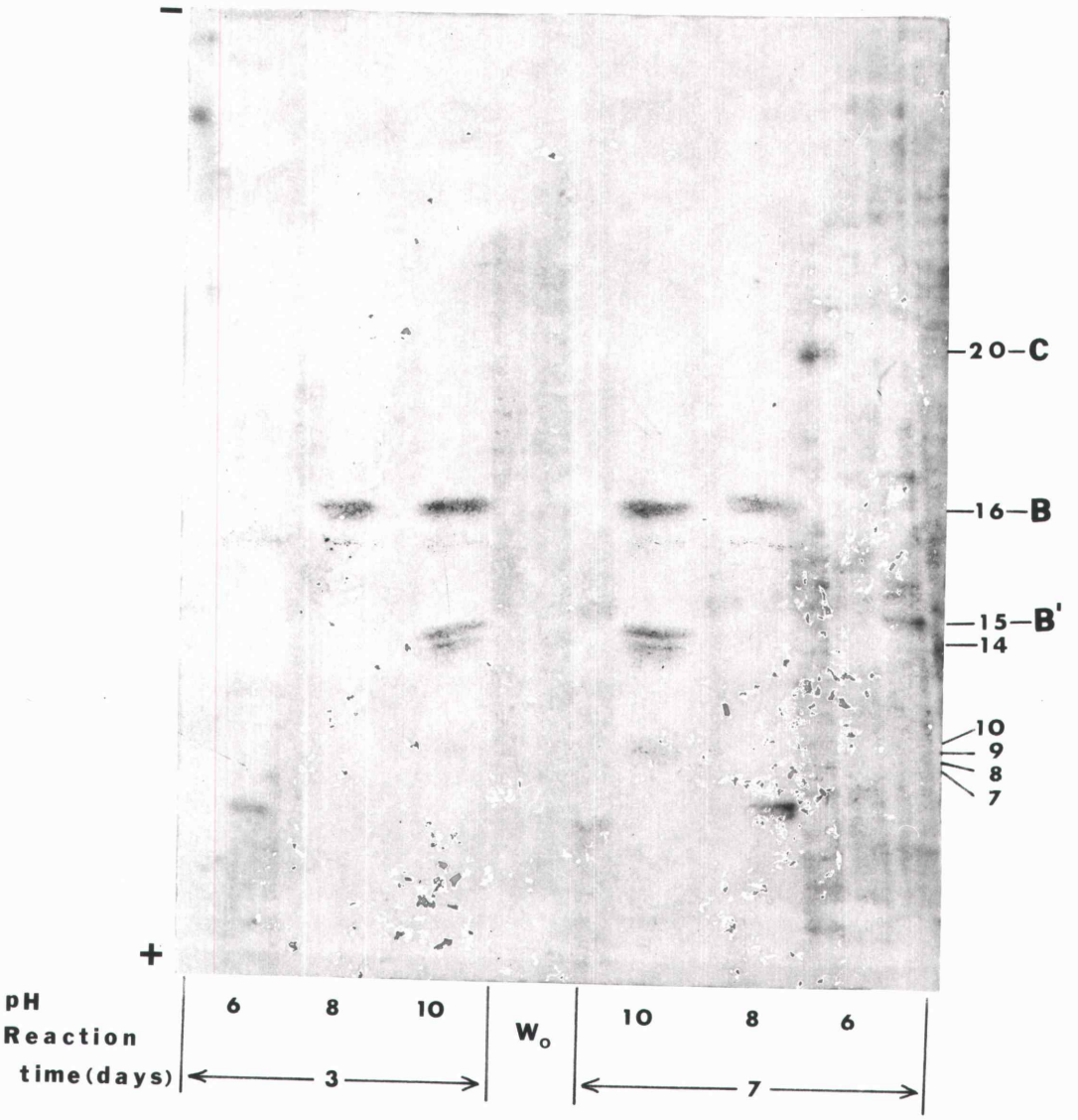


W_0: freshly made Sigma Type I HRP solution in water

Figure 9

Figure 10. Autoradiogram of ^{14}C-phenylNCS-modified HRP. Same IEF slab as Figure 9.

Sigma Type I HRP, incubated with phenyl(^{14}C)NCS at various pH values. The autoradiogram resulted from exposing (30 days at -80°C) the polyacrylamide gel of Figure 9 to Kodak X-Omat AR X-ray film. (Extraneous spots and bands are due to use of a film drier that was apparently contaminated. Radioactivity in bands 7-10, 14, 15 and 16 of the pH 8 and 10 samples coincide exactly with the corresponding bands of enzyme activity in Figure 9.)



W_o: freshly made Sigma Type I HRP solution in water

Figure 10

so that the reverse reaction can yield either the starting materials or R-NH_2 plus a protein-isothiocyanate. This could produce a modified protein which does not contain the R-group of the isothiocyanate. The phenyl(^{14}C)NCS which was used in this experiment was labeled in the benzene ring. Therefore, the reverse reaction could yield an unlabeled modified protein. Another possibility is that products of phenyl(^{14}C)NCS decomposition, such as SCN^- (Kawakishi and Muramatsu, 1966; Kawakishi et al., 1967; Kawakishi and Namiki, 1969), could also produce an unlabeled modified-protein.

The loss of enzyme activity in bands Nos. 26 (E) and 25 (D) at pH 10.0 correlated with increasing activity in bands Nos. 22 and 23 (Figure 9). Band 21 also disappeared at pH 10. Perhaps these bands are members of the E group that was reported by Aibara et al. (1981) (see p. 4 and Table III above).

Reaction of Peroxidase with Allylisothiocyanate in the Presence of L-lysine

Since ε-NH_2 groups of L-lysine residues react with isothiocyanates, it was of interest to see if L-lysine has any effect on the reaction. Sigma Type I HRP, RZ 0.46, (1 mg/ml; 0.025 mM) was incubated with allylNCS (1 µl/ml; 10 mM) in the presence of L-lysine (0, 0.075,

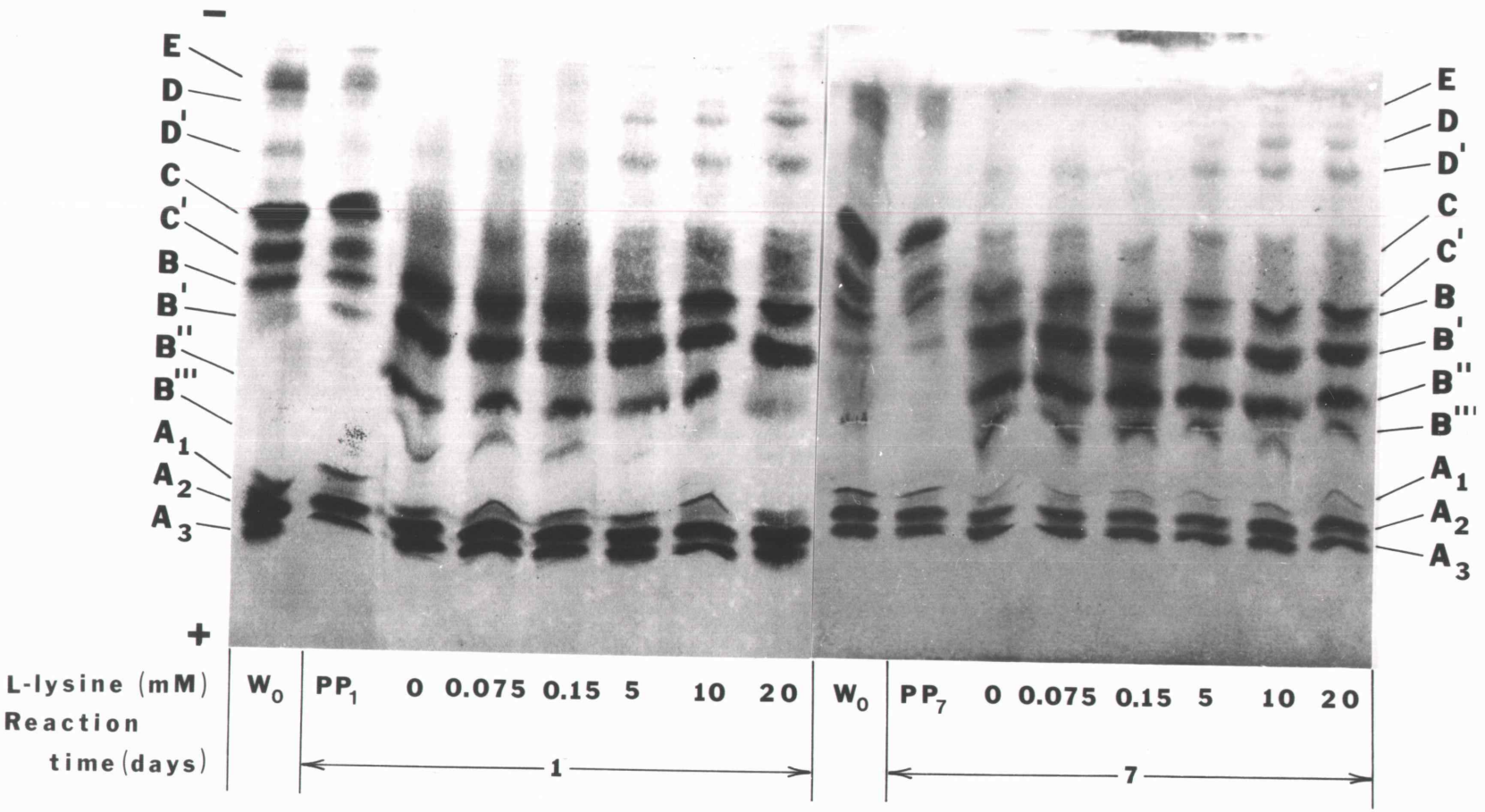
0.15, 5, 10 and 20 mM) at pH 10.0 and 4°C for 1 and 7 days. This concentration of HRP is equivalent to approximately a 0.15 mM concentration of lysine residues in the enzyme (6 lysine residues per molecule of isozyme C, 4 per molecule of A isozymes).

Figure 11 shows the isozyme patterns after allylNCS treatments in the presence and absence of L-lysine. Incubation of the enzyme without L-lysine altered the isozyme pattern as shown in previous experiments. It was shown that C and C' bands were present in the control, but the peroxidase activity of these bands were largely shifted to bands of lower pI by allylNCS treatments leaving a smear of activity in the C - C' region. This might indicate that the C' band was intermediate in the conversion of the C band to bands of lower pI. Lysine seems to have little protective effect against modification of the HRP C band. At concentrations of 5 mM or higher, L-lysine does definitely slow down the formation of the lower pI B" and B"' bands (Table VIII). At 5 mM, the concentration of L-lysine was 200 times the enzyme concentration or about 30 times the concentration of lysine residues. Even when L-lysine concentration was increased to 20 mM (800 times HRP concentration or two times allylNCS concentration), the modification of HRP bands still took place.

Isothiocyanates require unprotonated amino groups to

Figure 11. Isoelectric focusing of HRP after incubating at pH 10.0 with allylNCS in the presence of L-lysine.

Sigma Type I(1 mg/ml; 0.025mM), RZ 0.46, was incubated with allylNCS (1 µl/ml; 10 mM) in the presence of L-lysine. The mixtures were incubated at 4°C for 1 and 7 days. The IEF was performed in pH 3 - 10 ampholyte on a 20 x 20 x 0.06 cm Sephadex G-75-40 layer.



W_0 : freshly made Sigma Type I HRP solution in water

PP_1 PP_7 : Sigma Type I HRP solution in pyrophosphate buffer (pH 10.0), incubated without allylNCS or L-lysine

Figure 11

Table VIII. Distribution of Peroxidase Activity among HRP "Isozyme" Bands after Allylisothiocyanate Treatments in the Presence and Absence of L-lysine.

Percent peroxidase activities were derived from densitometry of a positive transparency of Figure 11. Sample designation are as in Figure 11.

Table VIII

1 Day

Band	W_0[b]	PP_1[c]	Percent of Total Peroxidase Activity[a]					
			L-lysine added to Reaction Mixture (mM)					
			0	0.075	0.15	5	10	20
E	3.0	3.0	--	--	--	--	--	1.0
D	5.5	5.2	--	--	--	2.7	1.1	3.1
D'	2.6	8.2	--	--	--	4.5	2.3	4.6
C	17.5	21.1	6.4	5.8	4.9	5.6	4.4	7.4
C'	12.5	13.3	--	--	--	--	--	--
B	11.3	12.0	23.4	22.6	16.1	16.2	18.7	17.7
B'	5.1	8.2	21.2	20.8	23.7	23.9	19.5	17.8
B"	--	--	12.4	11.8	13.1	13.2	17.7	12.4
B'''	--	--	7.1	7.7	8.5	--	--	--
A_1	8.6	10.4	6.6	8.0	8.2	8.3	11.3	8.9
A_2	18.9	15.0	9.1	10.3	12.3	12.4	14.2	13.5
A_3	15.1	11.8	13.7	13.0	13.2	13.2	10.7	13.7

[a]Peroxidase activity was calculated from densitometric data of IEF photograph in Figure 11. Total peroxidase activity : 45 units/mg

[b]W_0 : freshly-made Sigma Type I HRP (RZ 0.46) solution (1 mg/ml) in water

[c]PP_1 :Sigma Type I HRP solution in pyrophosphate buffer (pH 10.0), incubated for 1 day without allylNCS or L-lysine

B" and B''': new bands generated by allylNCS treatment.

Table VIII (continued)

7 Days

Band	Percent of Total Peroxidase Activity							
	W_0	PP_7[d]	L-lysine added to Reaction Mixture (mM)					
			0	0.075	0.15	5	10	20
E	3.7	3.9	--	--	--	--	--	--
D	3.7	4.0	--	--	--	--	1.8	0.1
D'	5.4	--	--	--	--	2.0	3.3	2.7
C	22.7	21.4	8.0	6.0	4.1	7.7	4.8	3.2
C'	10.2	8.2	--	--	--	--	--	--
B	15.1	16.6	17.6	16.0	17.3	14.7	16.9	19.6
B'	6.8	10.5	21.1	21.3	17.3	16.2	19.5	18.1
B"	--	--	17.9	18.6	22.6	20.1	18.5	20.1
B'''	--	--	9.1	11.1	13.4	10.8	9.3	6.1
A	9.2	9.0	6.6	5.5	5.5	4.7	2.4	3.0
A	10.6	12.4	8.3	8.3	9.1	11.3	10.2	14.9
A	12.5	14.0	11.5	11.0	10.8	12.6	13.3	11.9

[d]PP_7 : Sigma Type I HRP solution in pyrophosphate buffer (pH 10.0), incubated for 7 days without allylNCS or L-lysine.

react with proteins, therefore besides pH of the solution, the pK_a of ε-NH_2 of lysine residues will be a factor. Edsall (1943) reported that the pK_a values for ε-NH_2 of lysine residues in proteins range from 9.4 to 10.6. This range corresponds to 80 % to 20 % of unprotonated $-NH_2$ groups in solution at pH 10.0. On the other hand, free L-lysine has α-NH_2 and ε-NH_2 groups with pK_a values of about 8.95 and 10.53 respectively (Lehniger, 1982). In a solution with pH of 10.0, 92 % of α-NH_2 and 23 % of ε-NH_2 L-lysine will be unprotonated. Therefore, based on pK_a values of the amino groups, L-lysine would be expected to strongly inhibit, by competition, the addition of isothiocyanates to protein.

Babson (1980) suggested that the ability of chymotrypsin to compete with L-lysine in reaction with cyclohexylisocyanate was due to a high degree of noncovalent binding of the cyclohexyl side chain to the hydrophobic pocket of chymotrypsin. In the native tertiary structure of proteins, the hydrophobic parts of the molecule are largely kept in the interior of folded structures, with grooves in the surface. These grooves act as hydrophobic binding sites for small lipophilic molecules such as isocyanates or isothiocyanates (Mohammadzadeh _et al_., 1969[a] and 1969[b] ; Loomis _et al_., 1979). As a result, isothiocyanates can be expected to

be locally concentrated in these grooves. Lysine residues near the grooves would react rapidly with the "concentrated" isothiocyanates. This presumably gives HRP the ability to compete with L-lysine in reacting with isothiocyanates.

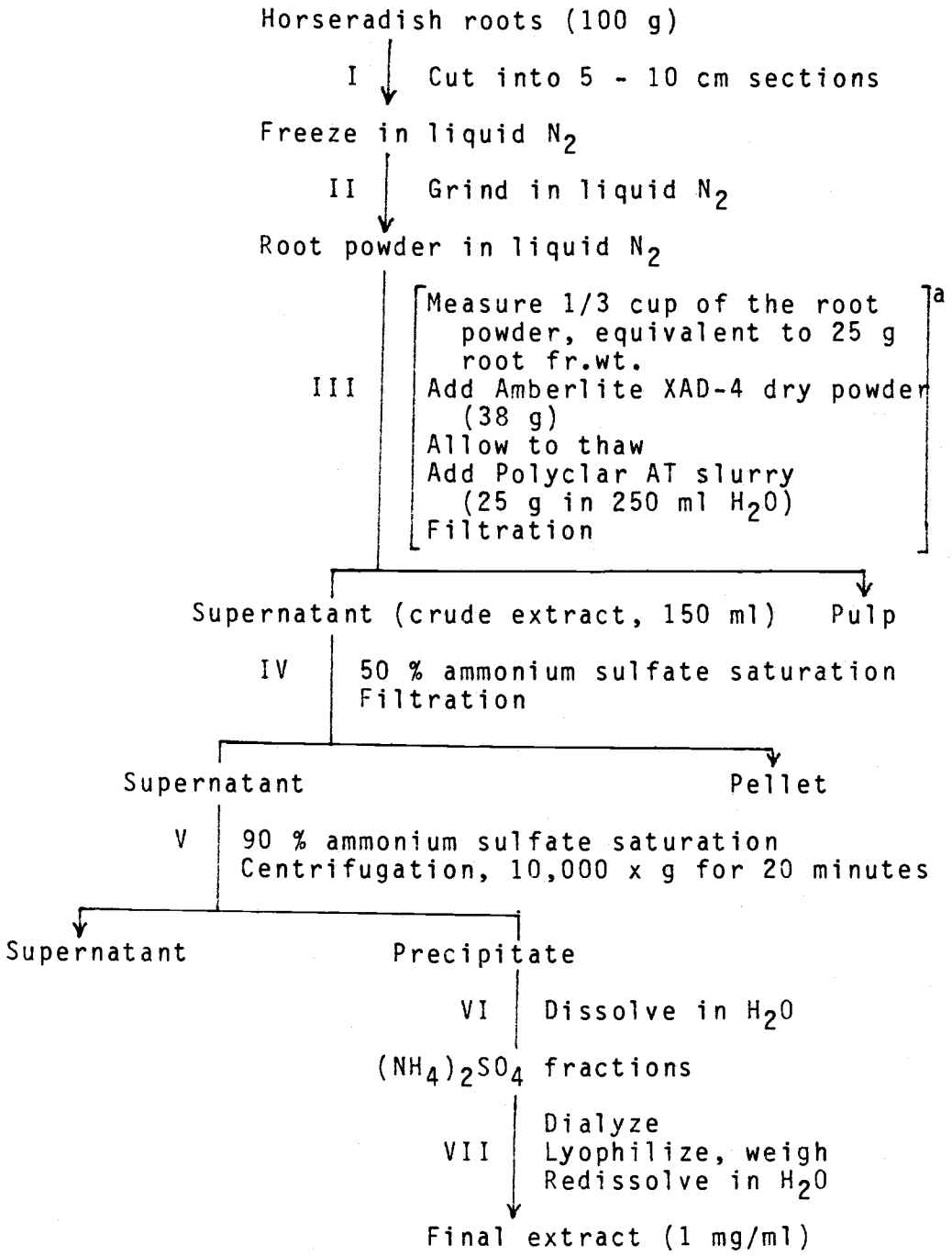
Maehly (1955) reported that during HRP extraction, he found that ammonium sulfate precipitates float. We have made the same observation. In the present study it was found that mixing phenyl(^{14}C)NCS in n-heptane directly into HRP solution, caused HRP precipitation and that the precipitate floated. These observations suggest that low molecular weight lipophilic compounds can bind noncovalently to HRP, and thus be held in positions for covalent reactions, which may be immediate or delayed.

Isolation of Horseradish Peroxidase in the Presence of Adsorbents Amberlite XAD-4 and Polyclar AT

Horseradish roots were extracted with and without adsorbent polystyrene (Amberlite XAD-4) and Polyclar AT. The extraction procedure is schematically presented in Figure 12. Horseradish root tissue was homogenized in liquid N_2, and homogeneous replicate samples were obtained by measuring with a measuring cup (1/3 US cup =

79 ml; 25 g fresh weight). These tissue samples were extracted with glass-distilled water as shown in Figure 12 with and without adsorbents. As reported by Loomis et al. (1979) the presence of Amberlite XAD-4 and Polyclar AT during extraction resulted in a superior enzyme extract. Their results were confirmed in this work. Extraction of HRP with distilled water without the adsorbents resulted in an extract with very strong isothiocyanate odor, brownish yellow in color, and with very strong UV absorbance. In comparison, extraction of the enzyme in the presence of the adsorbents resulted in a clear extract with no isothiocyanate odor. The UV absorbance was still high, but a distinct shoulder was shown at about 400 nm (Soret band of heme). The untreated extract could be stored for at least three months without showing any growth of microorganisms. In contrast, the treated extract showed spontaneous growth of microorganisms in three weeks. The microorganisms were whitish, non-mycelial, and settled readily by gravity. This behaviour is similar to what one would expect of a yeast, but no further examination was made. Loomis (personal communication) made a similar observation with crude horseradish extracts prepared in New Zealand, but the microorganisms appeared within one week and were bright red. Apparently isothiocyanates prevented the growth of microorganisms in the extract.

Figure 12. Flowsheet for HRP extraction.



[a]Adsorbents omitted in control preparations.
1/3 cup = 79 ml.

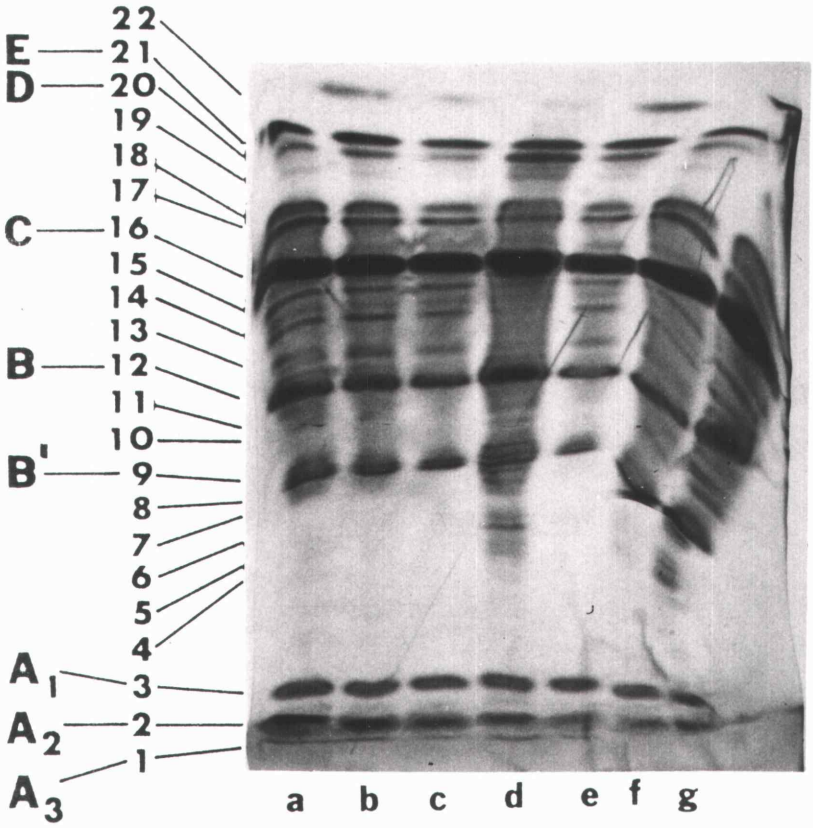
Figure 12

Isothiocyanates have been reported to have antibiotic and antifungal properties (Virtanen, 1962).

The 50 % ammonium sulfate saturation fractions ("pellet") were assayed for peroxidase activity by a spot test (Worthington, 1977) and showed a light color only after about 5 minutes. They were considered not to contain true peroxidase and were not examined further. The 90 % saturation ammonium sulfate precipitates obtained from treated and untreated extracts were redissolved in glass-distilled water (2 ml). These solutions were designated as ammonium sulfate fractions. Ammonium sulfate fractions were dialyzed against distilled water, then lyophilized in order to allow weighing the product. The brownish HRP precipitate was redissolved (1 mg/ml) in glass-distilled water. This solution was designated as final extract. The water homogenates (6 µl), final extract (6 µl), and ammonium sulfate fractions (3 µl) were applied to a polyacrylamide gel slab for IEF. The peroxidase activity in each sample was not determined. However, the activities in a pair of samples (treated and untreated) probably are the same, since they were extracted from the same amount of tissue. Figure 13 shows that the isozyme patterns in the crude water extracts and final extracts, treated and untreated, were not distinctly different (except for the untreated ammonium sulfate fraction; see below). The untreated

Figure 13. Isoelectric focusing of crude water extract of horseradish root, ammonium sufate fractions, and final extracts.

Horseradish roots were homogenized in water and extracted in the presence and absence of adsorbents Amberlite XAD-4 and Polyclar AT. Extraction in the presence of adsorbents was designated as "treated", and in the absence of adsorbents was designated "untreated". Proteins were precipitated with $(NH_4)_2SO_4$, and the precipitate was redissolved in glass-distilled water; this solution was designated as $(NH_4)_2SO_4$ fraction. The $(NH_4)_2SO_4$ fraction was dialyzed against glass-distilled water, then lyophilized, and the enzyme was redissolved (1 mg/ml) in glass-distilled water. This solution was designated as final extract. IEF was performed in pH 3 - 10 Bio-Lyte on a 16 x 14 x 0.1 cm polyacrylamide slab gel, and run at constant power (1 W) for 14 hrs.



a. final extract, treated, 1 mg/ml, 6 μl

b. final extract, untreated, 1 mg/ml, 6 μl

c. crude water extract, treated, 6 μl

d. Sigma Type I HRP, 1 mg/ml, 6 μl

e. crude water extract, untreated, 6 μl

f. $(NH_4)_2SO_4$ fraction, treated, 3 μl

g. $(NH_4)_2SO_4$ fraction, untreated, 3 μl

Figure 13

crude water extract and ammonium sulfate fraction contained basic isozyme band No. 22 while the treated extracts did not. Aibara et al. (1981) reported that four of the six basic HRP isozymes had pI higher than 12. Thus they would very likely be lost during IEF in pH 3 - 10 ampholytes, unless isothiocyanates modified them to bands of lower pI. The isozyme No. 22 that occured in untreated homogenate, was perhaps a product of isothiocyanate reaction with a more basic isozyme. Figure 13 also shows that the ammonium sulfate fraction from untreated water extract contained more bands of low pI than any of the other extracts. These bands correspond to bands 4 - 7 of the Sigma Type I control, and we suggest that they may be artifacts generated by isothiocyanate reactions during ammonium sulfate precipitation and processing. The ammonium sulfate precipitate of untreated extracts floated. This is an indication that lipid materials (isothiocyanates) bind to the protein. It was shown in experiments described above that activity in these bands is increased by isothiocyanate treatments. It has also been shown that B (No. 12) and B' (No. 9) bands were, at least in part, products of isothiocyanate addition, presumably to the C isozyme. These bands (B and B') are present in all samples. These data suggest that the HRP has been modified by isothiocyanates during extraction , and

probably Amberlite XAD-4 could not completely adsorb isothiocyanates that are produced during extraction.

The experiment was repeated, comparing glass-distilled water, 0.3 M potassium acetate buffer, pH 4.4, and 0.1 M K_2HPO_4 solution were used as extractants. K_2HPO_4 solution is the extractant that was used by Shannon et al. (1966). Horseradish roots (25 g) were extracted with the solvent (250 ml), with and without Amberlite XAD-4 and Polyclar AT. The final volume of each extract was adjusted to 150 ml. The extracts are described in Table IX. Again, it was shown that extraction of HRP in the presence of adsorbents resulted in a cleaner extract, and with no isothiocyanate odor. The spectra of extracts are shown in Figure 14. The UV absorbance of untreated homogenates was very high in comparison to treated extracts. Extraction of HRP in the presence of adsorbents resulted in much lower UV absorbance, and a distinct shoulder at about 400 nm.

Extracts (6 µl; equivalent to 1 mg root) and ammonium sulfate fractions (3 µl; equivalent to 19 mg root) were applied on polyacrylamide gel for IEF. Figure 15 shows the isozyme pattern of HRP in extracts and ammonium sulfate fractions. Since the amount of horseradish root tissue that was used in each extraction was the same, and the final volume of each extract was also adjusted to 150 ml, we could assume the

Table IX. Comparison of some Properties of Horseradish Root Homogenates.

Liquid-N_2 powder of horseradish roots was extracted with glass-distilled water, potassium acetate buffer (pH 4.4), K_2HPO_4 solution (0.1 M, pH 7.8), with and without adsorbents (Amberlite XAD-4 and Polyclar AT). The final volume of each extract was ajusted to 150 ml.

Table IX

	Acetate Buffer Extract		K_2HPO_4 Extract		Water Extract	
	Treated[a]	Untreated[b]	Treated	Untreated	Treated	Untreated
Color	colorless	brown	colorless	brown	colorless	brown
Pungent isothiocyanate odor	none	strong	none	strong	none	strong
pH	4.4	4.3	7.7	7.6	5.1	5.0
Peroxidase activity[c]:						
(units/ml)	70	66	124	92	96	60
(units/g fr.wt.tissue)	404	380	715	530	438	346
HRP concentration:						
(μg/ml)[d]	0.72	0.85	0.97	1.2	0.94	0.68
(μg/g tissue)[d]	4.3	5.1	5.82	7.2	5.6	4.1
(μg/ml)[e]	2.3	--	1.6	--	2.5	--
(μg/g tissue)[e]	13.8	--	9.6	--	15.0	--

[a]Roots were extracted in the presence of adsorbents Amberlite XAD-4 and Polyclar AT

[b]Roots were extracted without adsorbents

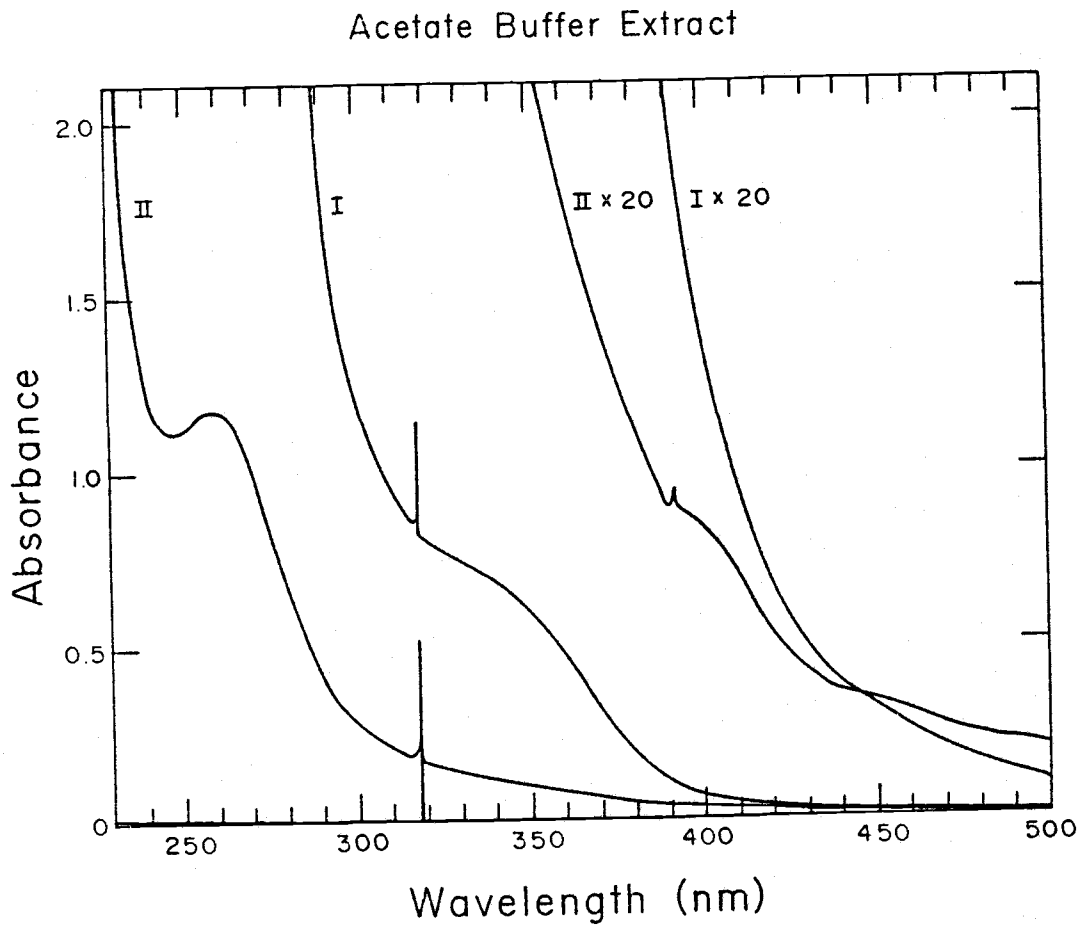
[c]Peroxidase activity determied with o-dianisidine as hydrogen donor according to Liu (1971)

[d]HRP concentration determined by the guaiacol method of Maehly and Chance (1954)

[e]HRP concentration calculated from the size of shoulder at 401 nm (ΔA_{401} as compared to the estimated baseline), $\varepsilon = 9.5 \times 10^5\ M^{-1}\ cm^{-1}$ (Shannon *et al.*, 1966), MW = 40,000. No values were calculated for the untreated extracts as no Soret band could be discerned in their spectra.

Figure 14. Absorption spectra of horseradish root homogenates.

Horseradish root homogenates in Table IX were scanned to obtain absorbance spectra in UV-VIS Varian Spectrophotometer, Model 635. Two different sensitivites were used.



I extracted without adsorbents (untreated)
II extracted with adsorbents (treated)

Figure 14

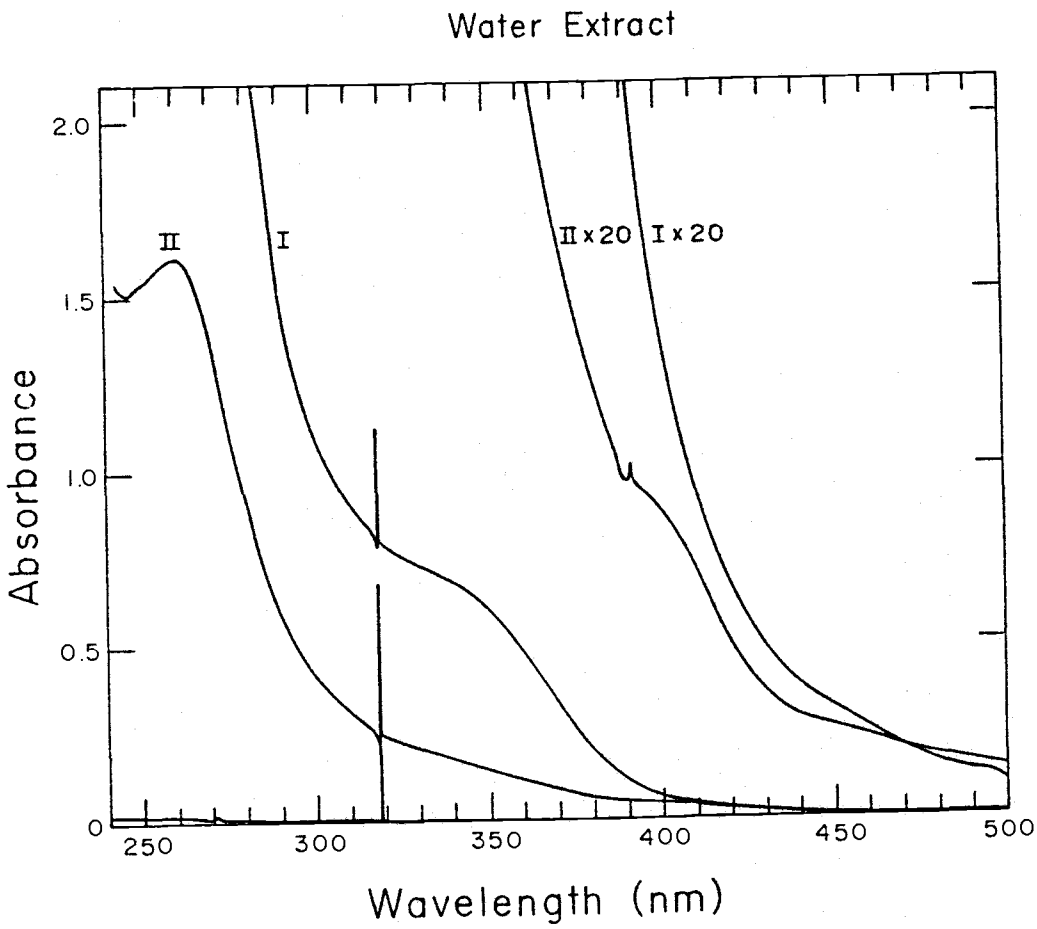


Figure 14 (continued)

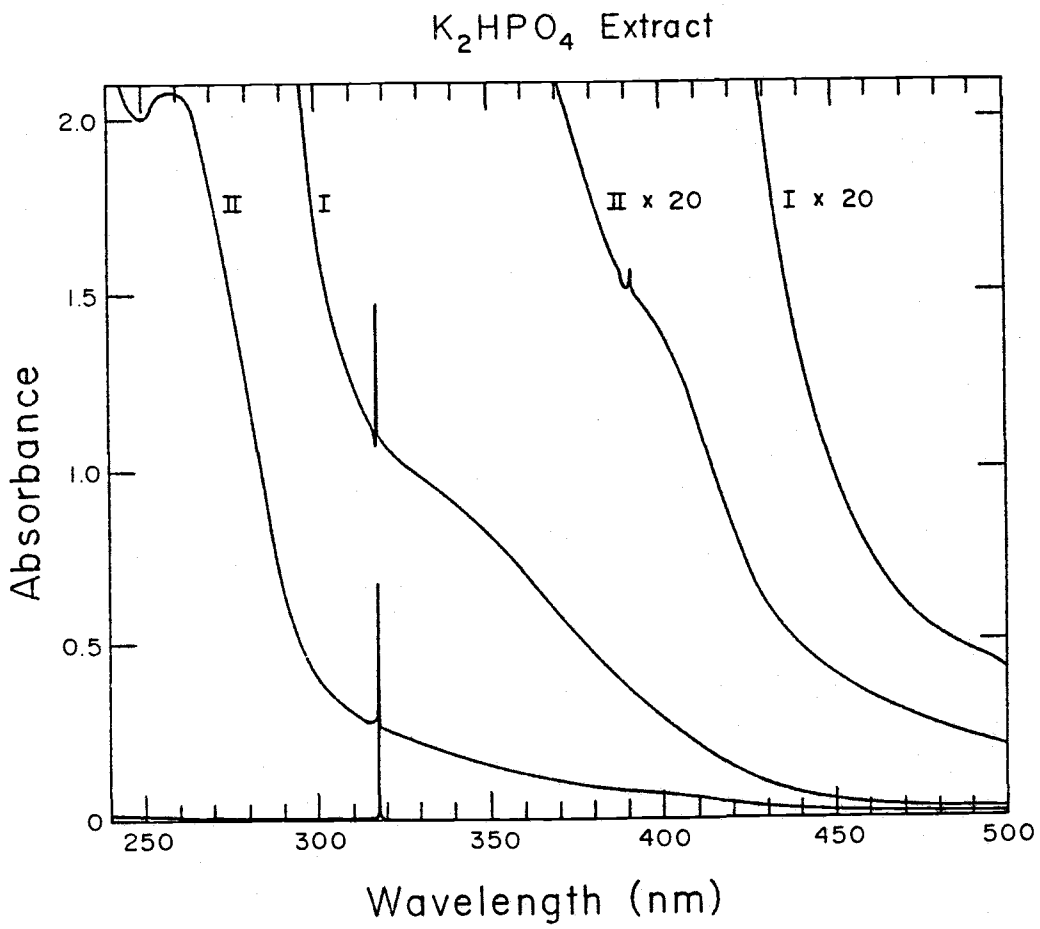
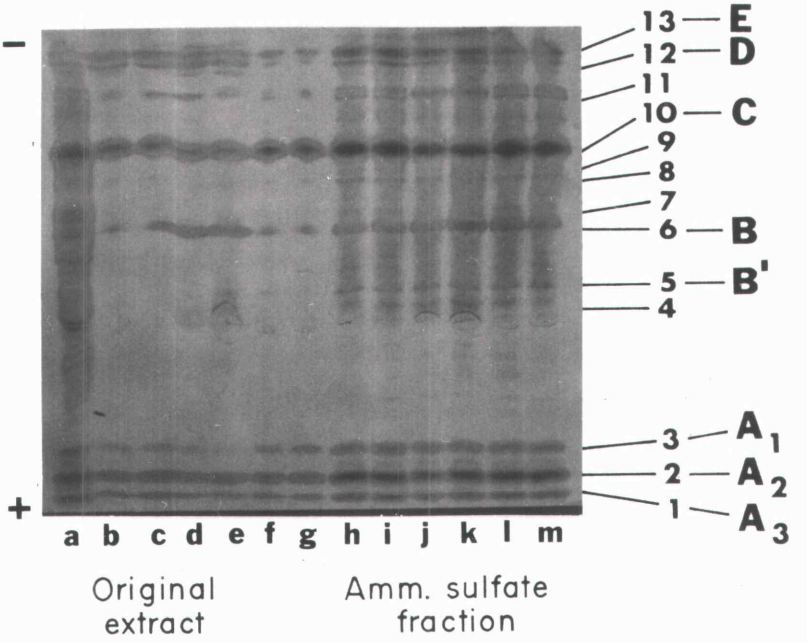


Figure 14 (continued)

Figure 15. Isoelectric focusing of horseradish root homogenates and ammonium sulfate fractions.

IEF was performed in pH 3 - 10 Bio-Lyte on a 20 x 16 x 0.1 cm polyacrylamide slab gel, run at constant power of 2 W for 14 hrs.



a. Sigma Type I HRP, RZ 0.66

Original extracts:

b. acetate buffer extract, treated
c. acetate buffer extract, untreated
d. K_2HPO_4 extract, treated
e. K_2HPO_4 extract, untreated
f. water extract, treated
g. water extract, untreated

Ammonium sulfate fractions:

h. from acetate buffer extract, treated
i. from acetate buffer extract, untreated
j. from K_2HPO_4 extract, treated
k. from K_2HPO_4 extract, untreated
l. from water extract, treated
m. from water extract, untreated

Figure 15

concentration of HRP and total peroxidase activity in each extract were the same. Table IX shows that the various extracts had similar values for peroxidase activity and A_{401} (The values for K_2HPO_4 extracts appeared to be somewhat higher than for the other extracts). Bearing in mind this possibly greater activity in the alkaline K_2HPO_4 extracts, it is reasonable to compare IEF patterns based on equal sample volumes (6 µl; equivalent to 1 mg root for the original extracts) of each extract. However, isozyme patterns in the original extracts (b-g) cannot be compared directly to isozyme patterns of the ammonium sulfate fractions (h-m), because the 3 µl of ammonium sulfate fraction applied to the polyacrylamide gel was equivalent to 19 mg root. Therefore, b-g and h-m can each be compared directly, but comparisons between the two groups must allow for the 19-fold difference in the amount of tissue represented. Figure 15 shows that HRP isozyme patterns in every extract were comparable, but peroxidase activity of the B band in untreated acetate buffer extract was higher than in the treated one. B-band activity relative to C-band activity appeared to be greater in the K_2HPO_4 extracts than in the other extracts. This result is consistent with an artifactual conversion of HRP-C to HRP-B, since reaction between HRP and isothiocyanates would be favoured at alkaline pH. As shown in Table IX,

the pH of the 0.1 M K_2HPO_4 extract (7.6) was much higher than the pH of the other extracts. Figure 15 also shows that all ammonium sulfate fractions had similar isozyme pattern, and contained B and B' bands. Sigma Type I HRP contained more bands of lower pI than extracts and ammonium sulfate fractions.

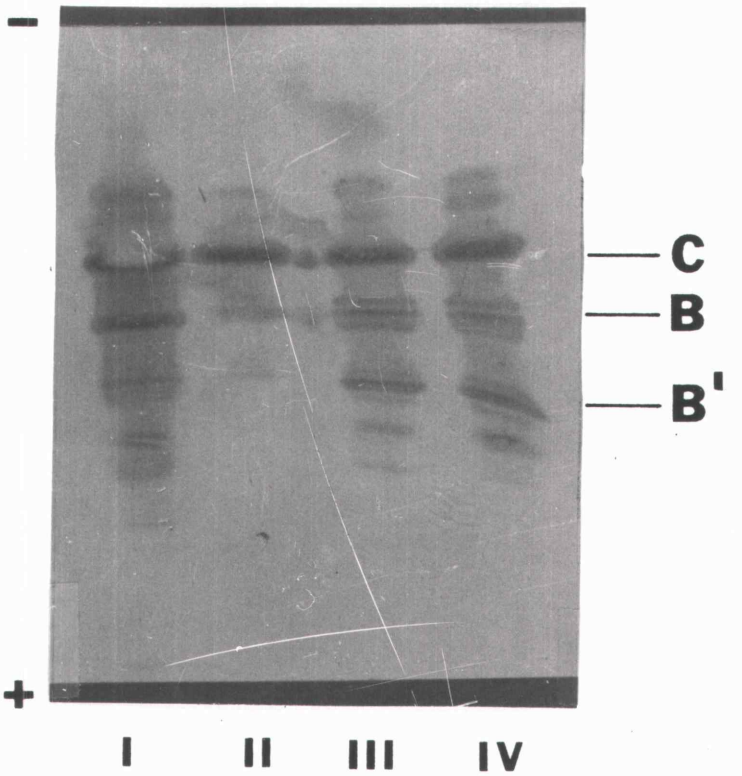
Isolation of Isozyme C

Fraction (B + C), RZ 3.1, that was obtained by fractionation of Sigma Type I HRP (RZ 0.46) on a CM-cellulose column in a previous experiment (see page 42) was lyophilized, and redissolved in 1 ml glass-distilled water (15 mg/ml). This solution was used for isozyme C separation by IEF and polyacrylamide gel electrophoresis (PAGE). 0.5 ml of this solution was applied to a polyacrylamide slab gel along a line for preparative IEF. Preparative IEF was performed as described in Materials and Methods. The C band was cut off the gel, and electroeluted from the gel pieces. The C protein was separated from ampholytes in a mixed bed ion exchange column [AG 501-X8(D)]. Separation of isozyme C with PAGE was also attempted in a vertical slab electrophoresis apparatus under acidic (pH 4.5) and neutral (pH 7.0) conditions. 0.25 ml of the fraction (B + C) solution was transferred into wells of the

polyacrylamide slab gel. PAGE was performed as described in Materials and Methods. Part of the gel was cut and stained with peroxidase activity staining. It was found that only one broad band was shown, about 2 cm from the boundary of running and stacking gels after 16 hrs run. The band was cut from the gel and electroeluted from the gel pieces. The homogeneity of HRP C resulting from IEF and PAGE was tested by IEF on polyacrylamide gel. The result is shown in Figure 16. It is shown that the C band isolated by IEF was not homogeneous when refocused but was contaminated by a small amount of B band. It was also found in this work (using IEF, data not shown) that the relative amount of B band increased during storage. The same results were found when C band was separated from Sigma Type IX HRP or our crude HRP (extracted in the presence of adsorbents Amberlite XAD-4 and Polyclar AT). Perhaps isozyme C is unstable, and exposing the isozyme to ampholyte (pH range 3 - 10) during IEF process might cause the isozyme C modification. Separation of C band with PAGE under these conditions (at pH 4.5 and 7.0) was unsuccessful. It is shown (Figure 16) that there was no separation between C band and other bands. The C band obtained from PAGE had almost the same band patterns as the (B + C) fraction.

Figure 16. Isoelectric focusing of "isozyme C" fractions.

IEF was performed in pH 3 - 10 Bio-Lyte on a 9 x 8 x 0.1 cm polyacrylamide slab gel, run at constant power of 0.75 W for 4 hrs. The isozymes were stained by peroxidase activity staining according to Hoyle (1978).



I : Fraction (B + C) of CM-cellulose column
II : Band C isolated from Fraction (B + C) by preparative IEF
III : Band C isolated from Fraction (B + C) by preparative PAGE at pH 4.5
IV : Band C isolated from Fraction (B + C) by preparative PAGE at pH 7.0

Figure 16

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