2–3 ppm in the diet (Hooper and Scanlan, 1977; Jago and Peterson, 1979). Much lower intake rates would result from normal human consumption of *Echium* honey such as investigated here, and there is no evidence to suggest that these lower intake rates would produce adverse effects. However, a need for further investigation of the significance of the alkaloids in *Echium* honey, and of conditions which might increase or decrease their level, is emphasized by the carcinogenicity of some pyrrolizidine alkaloids and, particularly, of the plant *Symphytum officinale* (Hirono et al., 1978) which contains alkaloids very similar to those of *E. plantagineum.*

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Soybean Peroxidases: Purification and Some Properties

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Soybean peroxidases have been purified 628-fold with a yield of 4% by combined consecutive treatments consisting of ammonium sulfate fractionation from a pH 4.5 extract of partially defatted soybeans and column chromatographies by gel filtration on Bio-Gel P-60, gradient elutions from DEAE-Sephadex A-50, affinity chromatography on Con A–Sepharose 4B, and hydrophobic chromatography on phenyl-Sepharose CL-4B. Three anionic isozymes, detected by polyacrylamide disc gel electrophoresis, differed in charge rather than in molecular size. Soy peroxidases showed optimum activity at pH 5.5 with guaiacol as the hydrogen donor, a K_m for H_2O_2 of 0.58 mM at optimum guaiacol concentration, a K_m of 5.9 mM for guaiacol at optimum H_2O_2 concentration, and competitive cyanide inhibition with respect to guaiacol with K_i = 0.15 μM. A minimum molecular weight of 37 000 was estimated for the polypeptide chain by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. At 70 °C, it took 72 min to inactivate the peroxidase isozymes 97%, and at 80 °C, these enzymes were almost completely inactivated after 15 min.

Peroxidases can contribute to deteriorative changes in flavor, texture, color, and nutrition both in raw foods such as fruits and vegetables and in processed products (Burnette, 1977; Haard, 1977). In addition to the general peroxidatic reactions, peroxidases can also catalyze oxidasic, catalatic, and hydroxylation reactions (Haard, 1977). In the oxidasic reaction (Nicholls, 1962), molecular oxygen is used as an electron acceptor rather than hydrogen peroxide. We are interested in determining the oxidasic capacity of soybean peroxidase and its possible role in generating off-flavors from endogenous lipids and phenolic constituents.

Peroxidases have been implicated in fatty acid oxidation (Stumpf and Bradbeer, 1959). Rothe (1956) proposed that an antioxidant complex aided by a peroxidase caused breakdown of lipid hydroperoxides. Täufel and Rothe (1965) established that peroxidase in oats and wheat is involved in lipid oxidation. Meanwhile, Gini and Koch (1961) observed a rapid decrease in linoleic acid hydroperoxides incubated with soy flour extracts, which they attributed to a peroxidase action. Schormüller et al. (1969) separated a guaiacol–linoleic acid hydroperoxide oxidoreductase (GLO) in soybeans from the H_2O_2-converting peroxidase by chromatography on CM-Sephadex, but they could not separate GLO from lipoxygenase. Further investigations were made by Grosch et al. (1972) on the purification of the lipoxygenase–GLO complex from soybeans and its activity on linoleic acid hydroperoxide.

Sessa (1979) reviewed the literature on generation of bitter taste in legumes from oxidation of phosphatidylcholine. Bitterness of oxidized phosphatidylcholine has been attributed in part to an unsaturated trihydroxy fatty acid moiety that was generated via a lipoxygenase–peroxidase complex isolated from soybeans (Moll et al., 1979). The oxidasic capacity of the H_2O_2-converting soy peroxidase, as distinguished from the lipoxygenase–GLO complex, with action on endogenous lipid and phenolic

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substrates, has not been thoroughly investigated, particularly with regard to off-flavor development.

Not much information is known about soybean peroxidases. According to several literature reports (Larsen, 1967; Buttery and Buzzell, 1968; Seth and Pillay, 1971; Kamboj and Nainawatee, 1978), buffer extracts of soybean seeds when subjected to anionic polyacrylamide disc gel electrophoresis yield one main band and at least two minor bands with peroxidase activity. Flurkey et al. (1978) reported on the separation and partial purification of soybean lipoxygenase and peroxidase by hydrophobic chromatography. However, the peroxidase they obtained upon disc gel electrophoresis contained multiple protein contaminants. Also, peroxidase activity bands were not in high enough concentration to give corresponding protein bands. The objective of this study was to isolate for the first time a purified preparation of soy peroxidase and to study some of its basic peroxidatic properties in order to define the preparation that will ultimately be used to assess its oxidasic capacity.

MATERIALS AND METHODS

Sample Preparation. Amsoy "71" soybeans (100 g), frozen overnight in a deep freeze, were ground in liquid nitrogen with a Waring Blendor. The ground soybeans were partially defatted by regrinding with three consecutive 200-mL batches of chilled, glass-distilled petroleum ether in a Waring Blendor. Excess solvent was drawn off on a Buchner funnel through Whatman No. 1 filter paper. The resulting grits were then ground to a fine mesh flour by passing them through a Cyclone Sample Mill (Cyclotec by Tecator-Udy) equipped with 1.0 mm mesh screen.

Enzyme Extraction. Partially defatted soy flour was divided into four equal batches, and each batch was extracted with 10 volumes of chilled 0.2 M sodium acetate buffer (pH 4.5) by sonication in an ice bath for 5 min, applying 30-s bursts at maximum output with 15-s rest intervals with a Branson sonifier cell disruptor, Model 185. The resulting slurry was centrifuged at 18000*g* for 15 min at 4 °C. After the centrifugates were pooled, the remaining residues were consolidated and reextracted with 10 volumes of acetate buffer by sonicating for 1 min and centrifuging the dispersion as above.

All the centrifugates, pooled and assayed for peroxidase activity and protein content, were brought to 30% saturation with solid ammonium sulfate, allowed to stand 0.5 h at 4 °C, and then centrifuged 15 min at 18000*g*. The resulting centrifugate was brought to 80% saturation with ammonium sulfate and stirred for 3 h in a refrigerator. Peroxidase was concentrated in the resulting precipitate collected after centrifugation as described above.

Peroxidase Fractionation. The 30–80% ammonium sulfate precipitate was dispersed in 0.05 M Tris-HCl buffer (pH 6.8), dialyzed against the same buffer, and fractionated by passage through a Bio-Gel P-60 (50–150-mesh; Bio-Rad Laboratories, Richmond, CA) column bed (2.5 × 49 cm) equilibrated in the same buffer. Column eluates (10 mL/test tube) were monitored for protein absorbance at 280 nm by a Beckman DU spectrophotometer and also assayed for peroxidase activity (see below). Eluates with peroxidase activity were pooled and concentrated 10-fold by ultrafiltration on Amicon YM-10 membrane subjected to 40 psi of nitrogen.

The resulting concentrate was applied to a DEAE-Sephadex A-50 (Pharmacia Fine Chemicals, Piscataway, NJ) column (2.5 × 46.5 cm) with column material previously degassed and equilibrated in 0.05 M Tris-HCl buffer (pH 6.8). Components were separated by elution with a linear salt gradient from 0 to 1 M NaCl in 0.05 M Tris-HCl buffer. Tube eluates (8 mL/test tube) were monitored for conductivity, absorbance at 280 nm, and peroxidase activity. Eluates with peroxidase activity were pooled and dialyzed overnight at 4 °C against 2 L of 0.05 M Tris-HCl buffer (pH 6.8) containing 0.5 M NaCl.

The retentate was applied to a Con A–Sepharose 4B (Pharmacia Fine Chemicals) column (0.9 × 15.3 cm) equilibrated at 4 °C with 0.05 M Tris-HCl (pH 6.8) containing 0.5 M NaCl. As supplied by Pharmacia Fine Chemicals, Con A–Sepharose contained an excess of Mn^{2+} and Ca^{2+} so that under the near neutral conditions of our chromatography no further addition of these ions was warranted. Step elution (at 4 °C) was made with same buffer and then with buffer containing 2.5 M NaCl and 0.5 M methyl α-D-mannopyranoside (Pfanstiehl Laboratories, Inc., Waukegan, IL). Eluates monitored for protein at 280 nm, for Soret band at 403 nm, and for peroxidase activity, which showed both 403-nm absorbance and peroxidase activity, were pooled and then applied directly to a column containing phenyl-Sepharose CL-4B (Pharmacia Fine Chemicals) equilibrated in 0.05 M Tris-HCl (pH 6.8) with 2.5 M NaCl. The column (1.2 × 8.2 cm) was eluted at room temperature with 50 mL of the same buffer, 50 mL of buffer with 1.25 M NaCl, followed by 100 mL of buffer and 0.5 M NaCl, 50 mL of buffer (no NaCl), and finally 100 mL of 50% ethylene glycol. Eluates, monitored as in previous step, that contained peroxidase activity and 403-nm absorbance were pooled, dialyzed against 0.05 M Tris-HCl (pH 6.8), and rechromatographed on a DEAE-Sephadex column (2.5 × 46.5 cm) that was eluted with a linear salt gradient of 0–0.5 M NaCl. The eluates containing peroxidase activities were pooled, concentrated by ultrafiltration, and assessed for physical and chemical properties described below.

Peroxidase Assay. Peroxidase activity was assayed by measuring the slope from the initial increase in absorbance at 436 nm due to oxidation of guaiacol to tetraguaiacol in the presence of hydrogen peroxide. The assay mixture contained 2.95 mL of potassium phosphate–citric acid buffer, 0.1 M each at pH 5.5 (i.e., optimum pH), 0.10 mL of 0.2 M guaiacol (Aldrich Chemical Co., Milwaukee, WI), and 0.04 mL of 0.04 M H_2O_2 (30%, Fischer Scientific, Fairlawn, NJ), which dilution has an $A_{240nm} \cong 1.6$. The assay reactions, performed at 25 °C, were initiated by addition of 0.01 mL of diluted column effluents. Reference blank contained all reagents except enzyme. A Beckman Model B spectrophotometer equipped with thermostated cell compartment and a recorder was used to record absorbance change.

Protein Determination. Protein determination were performed by using the Lowry method (Lowry et al., 1951) with bovine serum albumin (Schwarz Mann, Orangeburg, NY), $E^{0.1\%}_{280nm} = 0.625$, as the standard; all samples and column effluents were dialyzed against 0.05 M KCl before assays were made.

Optimum pH. The pH optimum of soy peroxidase was assayed over the range 4.0–8.0 with 0.1 M potassium phosphate–citric acid buffer, pH 4.0–6.0, and 0.1 M potassium monobasic and dibasic phosphates, pH 6.2–8.0. A Beckman Model 4500 digital pH meter was used to measure buffer pH. Assay conditions were the same as those specified in the peroxidase assay.

Gel Electrophoresis. So that the purity of isolated peroxidase could be checked, disc gel electrophoresis was performed on 6% T polyacrylamide gels according to the method of Davis (1964) at 12 °C and 3 mA/gel. For determination of the nature of the peroxidase isozymes, disc gel electrophoresis was performed with different acryl-

amide gel concentrations (Hedrick and Smith, 1968) ranging from 6 to 9% T by keeping the acrylamide:bis monomer weight ratio constant at 32.3:1.

Thin-strand copper wire, inserted into gels at the bromophenol blue tracking dye band, permanently demarcated the leading ion. Peroxidase activity was located by incubating the gels either in benzidine dihydrochloride and H_2O_2 (Nagle and Haard, 1975) or in 40 mM guaiacol–40 mM H_2O_2. These gels were scanned immediately on a Gilford gel scanner at either 490 or 575 nm, respectively. Duplicate gels were stained for protein with Coomassie Brilliant Blue R-250 (Koenig et al., 1970), destained, and scanned at 590 nm. The R_M relative to the leading ion was determined from the apex of peaks on gel scans for comparative purposes.

The sulfate–borate discontinuous buffer system of Neville (1971) was used for molecular weight estimation in sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Samples containing 1.0% sodium dodecyl sulfate and 0.1 M dithioerythritol were heated in a boiling water bath for 15 min and then dialyzed against 0.04 M boric acid–0.10 M Tris containing 0.1% sodium dodecyl sulfate and 0.05% dithioerythritol. Molecular weight standards (Bio-Rad Laboratories) were phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme. Soy peroxidase molecular weight was obtained by comparison with the standards on log R_F vs. molecular weight plots after determination of Ferguson plots (Ferguson, 1964). Since all the $NaDodSO_4$–protein complexes had nearly identical relative free mobilities, these complexes possess similar surface charge density.

Kinetic Studies. So that the property of our isolated enzyme can be distinguished from that of a heme protein, the rate constants k_1 and k_4 were determined (Chance and Maehly, 1955). The calculated k_1/k_4 at pH 7.0 was compared to that of horseradish peroxidase.

The apparent K_m was determined from Lineweaver–Burk plots for soy peroxidase measured at optimum conditions of pH and concentration of H_2O_2 (see conditions under Peroxidase Assay) with guaiacol as the substrate. Likewise, the apparent K_m was determined at optimum guaiacol concentration with H_2O_2 as the substrate.

Potassium cyanide inhibition of soy peroxidase was determined at 0.01, 0.02, and 0.03 M H_2O_2 substrate concentrations and concentrations of CN^- in the final mixture over the range (1.64–164) × 10^{-8} M. The sequence for the inhibition study was injection of 0.05 mL of KCN into 3 mL of a mixture containing guaiacol (optimum concentration) and soy peroxidase in 0.1 M potassium phosphate–citric acid buffer, pH 5.5, and equilibration of this composite for 5 min before addition of 0.04 mL of H_2O_2. The reference blank consisted of all reagents except KCN and H_2O_2. The value for K_i was determined from a plot of $1/v$ vs. CN^- concentration (Dixon, 1953).

All regression lines in the kinetic studies were fitted by the method of least squares.

Heat Stability. Heat stability of soy peroxidase was performed at constant time, 15 min, over temperatures ranging from 25 to 85 °C and at constant temperature, 70 °C, over variable times from 0 to 90 min. Heat treatment was performed on an aluminum dryblock heater with buffer solutions (i.e., 0.1 M potassium phosphate–citric acid, pH 5.5) heated to a specified temperature before injection of diluted enzyme. After defined incubation periods were attained, solutions were rapidly cooled by immersing the test tubes in a salt–ice water bath. Samples were assayed immediately as described under Peroxidase Assay. Percent peroxidase activity remaining after treatment was calculated from initial activity.

Table I. Peroxidase Purification

procedure	total units	% yield	total protein, mg	sp act., units/mg of protein
crude extract	33 652	100	4982	6.8
0-30% $(NH_4)SO_4$ supernatant	32 200	96	3300	9.8
Bio-Gel P-60 column	25 560	76	1474	17.3
DEAE-Sephadex column	17 253	51	308	56.0
Con A–Sepharose column	4 614	14	5.9	782.0
phenyl-Sepharose CL-4B column	2 300	7	1.7	1352.9
rechromatography on DEAE-Sephadex column	1 410	4	0.33	4272.7

RESULTS AND DISCUSSION

Purification of Soy Peroxidase. Table I summarizes the results of purification procedures for soy peroxidase. Conventional methods for protein purification, including ammonium sulfate fractionation, gel filtration on Bio-Gel P-60, and ion exchange on DEAE-Sephadex, provided a partially purified preparation free of nonproteinaceous materials and low molecular weight peptides. The ultrafiltration step incorporated into the scheme to concentrate eluates from the Bio-Gel column resulted in a 1.6-fold increase in peroxidase activity with a 30% loss in protein. The YM10 filter retains proteins with molecular weights greater than 10 000; therefore, some low molecular weight peptides must have filtered through.

The bulk of peroxidase was eluted from the DEAE-Sephadex column with a NaCl gradient between 0.16 and 0.26 M. Since plant peroxidases have been reported to be carbohydrate-containing proteins, use of affinity adsorbents to adsorb soy peroxidases not only should demonstrate its glycoprotein nature but also should provide a highly effective purification step. From results shown in Figure 1, Con A–Sepharose 4B showed a remarkable affinity for soy peroxidase and was found to provide 115-fold purification (Table I). Virtually all the peroxidase was adsorbed onto this column, because none was evident in initial column eluates where the majority of protein, judged by 280-nm absorbance, eluted. A concentration of 0.5 M methyl α-D-mannopyanoside was required for elution of peroxidase; lesser amounts gave incomplete removal of peroxidase in much larger volumes of eluate with considerable dilution of the enzyme. The increased salt concentration from 0.5 to 2.5 M was included so that eluted enzyme would be in an optimal ionic environment for hydrophobic interaction chromatography. Soy peroxidase under these conditions was bound tightly to phenyl-Sepharose CL-4B and required 50% ethylene glycol for its removal. The RZ value (i.e., absorbance ratio 403:280 nm) of peroxidase from pooled column eluates was 2.1 vs. 3.0 for highly purified horseradish peroxidase (Brattain et al., 1976); our value indicated an impurity in our preparation. A 3-fold purification of peroxidase, based on specific activity, was effected by rechromatography of our enzyme preparation on a DEAE-Sephadex A-50 column eluted with a linear gradient of 0–0.5 M NaCl in Tris-HCl buffer. Our purified peroxidase had an RZ of 2.6.

The enzyme nature of our final product was clearly distinguishable from a heme protein by a k_1/k_4 of 12.0, which is similar in magnitude to the literature value of 27.3

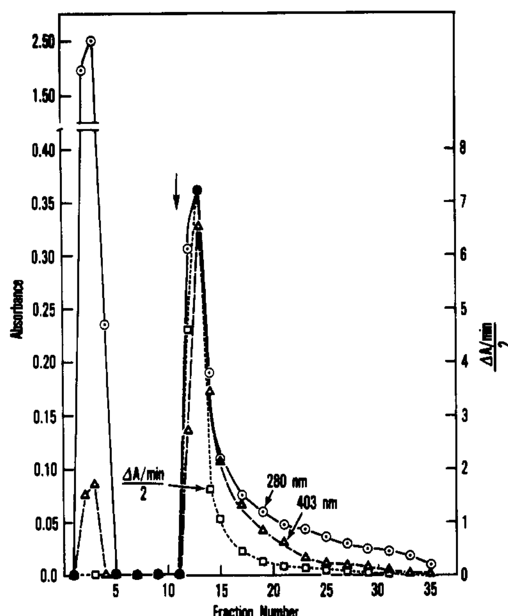

Figure 1. Elution profile of partially purified soybean peroxidase on Con A-Sepharose. The pooled enzyme fraction from the DEAE-Sephadex column described under Materials and Methods was applied to a 0.9 × 15.3 cm column equilibrated and eluted with 0.05M Tris-HCl buffer, pH 6.8 containing 0.5 M NaCl. At the point indicated by the arrow, elution was started with 0.5 M methyl α-D-mannopyranoside in the same buffer containing 2.5 M NaCl. Absorbance was measured at 280 (⊙) and 403 nm (Δ); peroxidase activity, $(\Delta A/\text{min})/2$, (□) was assayed as described in the text.

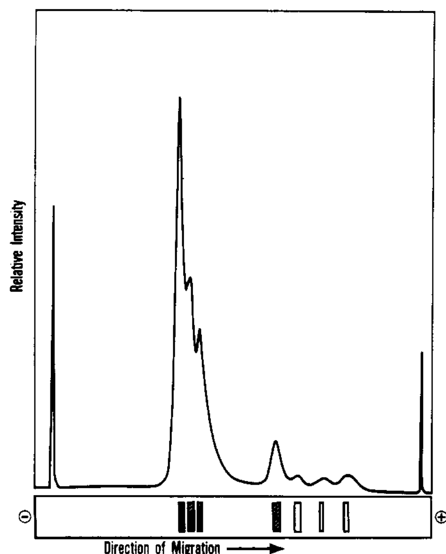

Figure 2. Disc polyacrylamide gel electrophoresis of soybean peroxidase (60 μg of protein) after rechromatography on the DEAE-Sephadex column; 6% T gel concentration; protein stain, Coomassie blue R-250; gel scan, 590 nm. Band designation: (■) major; (□) minor; (□) trace. The three bands that comprise the maximal amount of protein corresponded in R_M to the three peroxidases stained for activity with guaiacol and H_2O_2.

for horseradish peroxidase at pH 7.0 and 25 °C (Chance and Maehly, 1955). With guaiacol as the substrate, k_1/k_4 for Alaska pea peroxidases varied from 7.2 to 25 (Macnicol, 1966). Heme proteins, on the other hand, possess a k_1/k_4 many orders of magnitude less than these values (Kurozumi et al., 1961). Therefore, our enzyme preparation is not related to the pseudoperoxidase, leghaemoglobin, isolated from root nodules of soybean plants (Sievers and Rönnberg, 1978) but may be related to the peroxidase

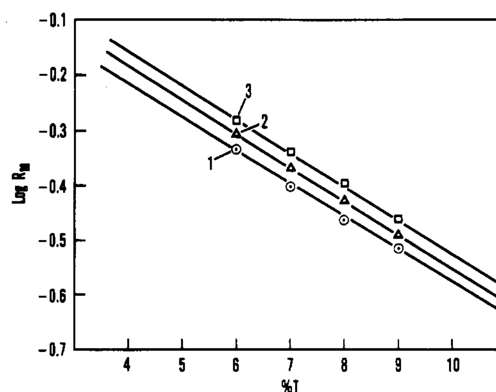

Figure 3. Separation of soybean peroxidase isozymes by disc polyacrylamide gel electrophoresis. Stain: Coomassie Brilliant Blue R-250. A plot of log protein mobility relative to the leading ion for each of three isozymes: 1 (⊙), 2 (Δ), and 3 (□) vs. gel concentration in % T.

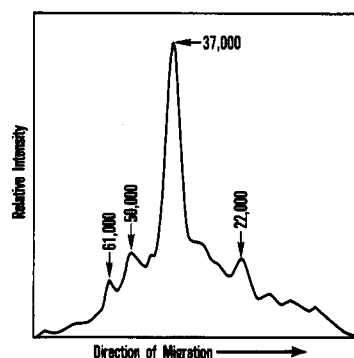

Figure 4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of soybean peroxidase. Conditions: 25.6 μg of protein; 12.0% gel concentration; 3.0% cross-linkage. Numerical values denote estimated molecular weights of polypeptides in protein bands.

isolated from these nodules (Puppo et al., 1980).

Minor amounts of nonperoxidase protein impurities were detected in our soy peroxidase preparation by polyacrylamide disc gel electrophoresis (Figure 2) at gel concentration 6% T. With a duplicate gel stained for peroxidase activity with guaiacol, we observed three isozyme bands which corresponded in R_M values to the three bands that comprise the maximal amount of protein upon staining with Coomassie blue (Figure 2). When the guaiacol-H_2O_2-developed 6% T gels were overstained with benzidine dihydrochloride, three additional satellite bands were noted that were not evident on gels stained for protein. Artifacts with greater net negative charge can arise from preexisting forms by incubation of peroxidases in solutions pH 7 or higher (Liu and Lamport, 1973). Since our enzyme preparations were stored at pH 6.8, some trace amounts of isozymes could have formed and then were made evident with the benzidine reagent, which is very sensitive to heme proteins.

Ferguson plot analysis (Ferguson, 1964) revealed a family of parallel lines (Figure 3) that clearly distinguish our peroxidase mixture as charge isomers rather than size isomers (Hedrick and Smith, 1968). Size isomers, based on sieving of protein molecules at different concentrations, according to Hedrick and Smith (1968) would yield a family of nonparallel lines extrapolating to a common point in the vicinity of 0% gel concentration. Peanut peroxidase isozymes likewise possesses similar molecular size but differ in charge (Srivastava and van Huystee, 1977).

Molecular Weight. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed several protein

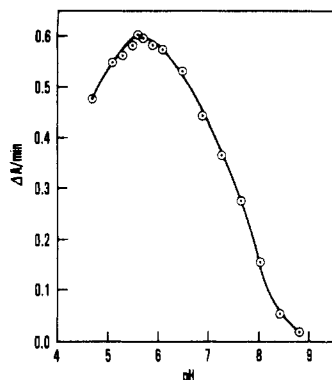

Figure 5. Soybean peroxidase activity as a function of pH. Reaction mixture: 2.95 mL of buffer, either 0.1 M potassium phosphate–citric acid, pH 4.0–6.0, or 0.1 M potassium phosphate, pH 6.2–8.0; 0.1 mL of 0.2 M guaiacol; 0.04 mL of 0.04 M H_2O_2; 0.01 mL of diluted enzyme. Equilibration at 25 °C and absorbance at 436 nm.

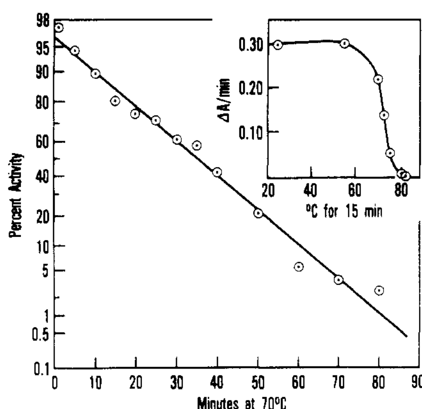

Figure 6. Effects of heat (at 70 °C) on the stability of soy peroxidase at pH 5.5 as a function of time. Assay conditions are described in the legend to Figure 5. The insert denotes effects of heat on soy peroxidase at constant time (15 min).

bands (Figure 4). The major band was estimated to have a molecular weight of 37000. According to Srivastava and van Huystee (1977), molecular weights of peroxidase enzymes from various sources ranged from 30000 to 60000.

Optimum pH. The pH optimum of soy peroxidase was found to be 5.5, with less than 80% of the maximal activity exhibited at pHs 4.6 and 6.7 (Figure 5). With guaiacol as the substrate, Jen et al. (1980) reported a similar pH optimum for tomato peroxidase. Mäder et al. (1977) found a pH optimum in range of 5.5–6.0 for peroxidase isozymes from tobacco.

Heat Inactivation. Plant peroxidases, in general, are very stable to thermal inactivation (Reed, 1975). The destruction time for peroxidase varies according to the substrate used (Nebesky et al., 1950), ionic strength of buffer (Wilder, 1962), and molecular species (Srivastava and van Huystee, 1977; Nessel and Mäder, 1977; Gordon and Alldridge, 1971). Heat inactivation was performed on a composite of soybean peroxidase isozymes. Therefore, any changes in relative amounts of isozymes (e.g., with different soybean varieties) may give results that differ from those shown in Figure 6. After 15 min of heating at 70 °C, ~15% of the enzyme was inactivated. It took ~35 min of heating at this temperature to inactivate the enzyme 50% and 72 min to inactivate 97%. With a constant time of 15 min and variable temperatures, a 3% increase in activity was noted around 40 °C, and the enzyme was stable until ~55 °C; however, activity decreased markedly (60%) between 70 and 75 °C, and at 80 °C the enzyme was 97% inactivated.

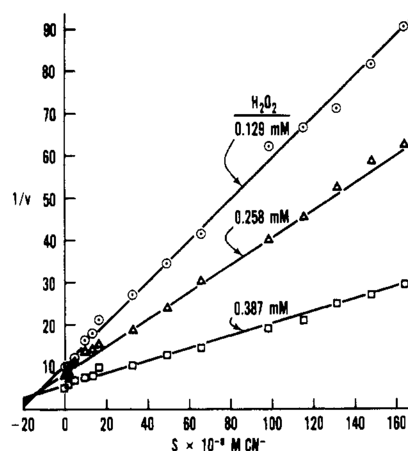

Figure 7. Effects of cyanide on soy peroxidase oxidation of guaiacol at ph 5.5 and three concentrations of hydrogen peroxide. Assay conditions are described under Materials and Methods. Concentrations of hydrogen peroxide in the cell: 0.129 (⊙); 0.258 (Δ); 0.387 mM (□). Regression lines have been fitted by the method of least squares.

Kinetics of the Peroxidatic Reaction. Classical kinetic analysis was performed on our isolated soy peroxidase fraction that contained three isozymes. Substrate concentration studies via Lineweaver–Burk plots showed an apparent K_m of 0.58 mM for hydrogen peroxide at 7 mM guaiacol concentration (optimum) and of 5.9 mM for guaiacol at 0.5 mM hydrogen peroxide concentration (optimum). It should be noted that K_m values for a composite of enzymes may differ from the K_m values for each species individually (Mäder et al., 1977).

The kinetic pattern depicted in Figure 7 via a Dixon plot (Dixon, 1953) indicates competitive inhibition of soybean peroxidase by cyanide. The inhibition constant, K_i, of cyanide was determined to be 0.15 μM at pH 5.5. Fridovich (1963) reported a K_i of 3.3 μM at pH 6 (0.025 M acetate) and 1.3 μM at pH 5 for horseradish peroxidase with *o*-dianisidine as the hydrogen donor. Because of its lower K_i compared to that of the horseradish peroxidase, soybean peroxidase is apparently more sensitive than horseradish peroxidase to inhibition with cyanide.

Implied Future Work. Future work should involve assessing the oxidasic capacity of the H_2O_2-converting soy peroxidase on unsaturated lipid and phenolic constituents endogenous to soy and its relationship to the development of off-flavors and distinguishing this enzyme from the lipoxygenase–GLO complex studied by Grosch et al. (1972). A free radical initiated oxidation of polyunsaturated lipids may occur from action of peroxidase on phenolic hydrogen donors. Another aspect to be considered is that heat denaturation of peroxidase can either expose heme groups or mobilize them so that they are more readily available to catalyze lipid oxidation (Eriksson and Vallentin, 1973). Recently, horseradish peroxidase was found to utilize malonaldehyde, formed during lipid peroxidation, as a substrate with emission of singlet oxygen which in turn can cause deterioration of lipid systems (Vidigal-Martinelli et al., 1979). These authors propose a dioxetane intermediate, which upon cleavage could give rise to a triplet species capable of transferring energy to oxygen to form singlet oxygen. In support of their proposed mechanism, Wilson and Wong (1976) isolated peroxidases from garbanzo, and in prior experiments from soybean seedlings, with oxidasic activity that converted 4,2′,4′-trihydroxychalcone to a 1,2-dioxetane derivative of the chalcone. Model systems will be developed with our

soy peroxidase to evaluate these observations.

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High-Performance Liquid Chromatography of Procyanidins in Developing Sorghum Grain

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The development of low molecular weight procyanidins was studied in bird-resistant sorghum grain during the reproductive developmental stages. It is the procyanidins which inhibit the amylase enzymes which are required during the brewing of sorghum beer. Grains were collected at various stages of maturity, and their procyanidins were monitored by using high-performance liquid chromatography (HPLC) on silica gel with anhydrous organic solvents. Sufficient material for chemical analysis was obtained from column chromatography of acetylated plant extracts. The melting point depression of camphor was used for molecular weight determination of the acetylated procyanidins. During the flowering stage, catechin was detected, but as the grain matured, several low molecular weight procyanidins were present. They reached a maximum concentration at the soft dough stage. As the grain matured, the low molecular weight procyanidins could no longer be detected, indicating the end of the period of their synthesis. By the melting point depression of camphor, one dimer and two trimer isomers of procyanidin were identified in immature sorghum grain.

Procyanidins, or condensed tannins, occur in the testa of bird-resistant sorghum grain [*Sorghum bicolor* (L.) Moench] (Rooney et al., 1979; Morrall et al., 1981). The chemical nature of procyanidins makes them difficult to isolate and purify. Although it is believed that simple mono-, di-, and trimeric forms are the basis for the synthesis of these high molecular weight polymers, they are not found in mature sorghum grain.

Amylase enzymes were found to be inhibited by the sorghum grain procyanidins (Strumeyer and Malin, 1969), and these enzymes are required during the brewing of sorghum beer (Daiber, 1975). When amylase inhibition by sorghum grain was studied over the 10-week preharvest

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