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# Heme Proteins. VI. Crystalline Pineapple Peroxidase B*

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ABSTRACT: The indoleacetic oxidase activity reported to be present in pineapple stem tissues has been shown to be due to the presence of peroxidases. Pineapple stem peroxidase B has been purified from both fresh pineapple stems and a commercial concentrate of pineapple stems and obtained in a crystalline form. From the enzymatic and physicochemical properties of the enzyme, it is shown to be an atypical peroxidase. The atypical properties include an acidic pH optimum, low specific activity, and anomalous Soret absorption. It is the first atypical peroxidase to be obtained in a crystalline form.

Plant peroxidases isolated thus far show great similarities in enzymatic and chemical properties and can be considered under the group called classical peroxidases (Theorell, 1951; Paul, 1963). Pineapple peroxidase B has been isolated in crystalline form and shown to be an atypical plant peroxidase. The atypical characteristics include low specific activity, a more acidic pH optimum, and anomalous Soret absorption. However, the other enzymatic and chemical properties of the enzyme are similar to the properties of the classical peroxidases.

## Methods and Materials

*Materials.* The enzyme was isolated from both fresh pineapple stem tissue and Dolzyme T-20, a pineapple stem juice concentrate which was generously provided by Dr. Ralph Heinicke of the Dole Pineapple Co. Hydroxylapatite was prepared according to the procedure of Tiselius *et al.* (1956). Carboxymethylcellulose was purchased from Brown and Co. Sulfoethyl-Sephadex was purchased from Pharmacia Fine Chemicals, Incorp. Guaiacol and indoleacetic acid[1] were purchased from the Eastman Chemical Co. *p*-Coumaric acid was obtained from the Mann Research Laboratories, Inc.

*Methods.* The peroxidase activity was measured by a modified procedure of Devlin (Chance and Maehly, 1955) based on the rate of formation of tetraguaiacol by observing the absorbancy at 470 mμ. For this assay, 1.8 ml of 0.2 M sodium acetate buffer, pH 4.4, 1 ml of 0.02 M guaiacol, and 0.1 ml of diluted enzyme were placed in a 10 × 75 mm cuvet. The reaction was started by adding 0.1 ml of 1.63 M (5%) hydrogen peroxide. Absorbancy readings were taken every 15 sec, and the reaction was run at room temperature (29–30°). Activity (1 unit) was defined as the amount of enzyme which catalyzed a change of 0.001 absorbance unit/min using this assay system. Specific activity was defined as units of activity per milligram of protein.

The indoleacetic acid oxidase activity was measured spectrophotometrically as suggested by Ray (1956).

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[1] Abbreviation used in this work: IAA, indole-3-acetic acid.

This method is based on the change of absorbance at 261 mμ as indoleacetic acid undergoes oxidation. The assays were run at *ca.* 37°. For the assay, 2.2 ml of 0.2 M sodium acetate buffer, pH 4.2, 0.1 ml of 0.001 M *p*-coumaric acid, 0.1 ml of diluted enzyme, 0.1 ml of 0.03 M $MnCl_2$, and 0.5 ml of 0.001 M neutralized indoleacetic acid were placed in 1-cm quartz cells. The reference cell contained 2.7 ml of 0.2 M acetate buffer, pH 4.2, 0.1 ml of the same dilution of enzyme, 0.1 ml of 0.001 M *p*-coumaric acid, and 0.1 ml of 0.03 M $MnCl_2$. The absorbancies at the various time intervals were recorded with the aid of a time-drive attachment on a Beckman DK-2 spectrophotometer. After a short lag period, a straight line increase in absorbancy was observed. Activities were calculated from this straight-line portion of the reaction curve. Activity (1 unit) was defined as the amount of enzyme which brought about a change of absorbance of 0.001/min under the standard assay condition. Specific activity was defined as the units of activity per milligram of protein.

Protein concentrations were determined by measuring the absorbance at 275 mμ. An $E_{1\,cm}^{1\%}$ value of 12.65 was experimentally determined at 275 mμ. The per cent nitrogen was determined by the micro Kjeldahl method (Hawk *et al.*, 1951).

All of the free boundary electrophoresis experiments were conducted in the Perkin-Elmer Model 38-A free boundary electrophoresis apparatus with a Land back camera attachment at 4°. The necessary buffers were prepared according to Miller and Golder (1958). Mobilities were calculated as described by Alberty (1948).

The zone electrophoresis experiment was run in the usual apparatus with a 3 × 24 cm bed containing Geon suspended in 0.02 M phosphate buffer, pH 6.0. A crude stem extract was prepared as described by Gortner and Kent (1953, 1958). It was centrifuged to remove particulate matter, the protein was precipitated by the addition of excess ammonium sulfate, and the dissolved precipitate was exhaustively dialyzed *vs.* 0.02 M phosphate buffer, pH 6.0. A 1-cm segment was removed from the middle of the block and replaced by a slurry of 1.2 ml of stem homogenate and about 2 g of Geon. The block was exposed to a 15-ma current for 6 hr (initial voltage 400 v at 5°). At the end of the experiment, the block was cut into 1-cm segments, each of which was placed in a centrifuge tube containing 5 ml of 0.02 M phosphate buffer, pH 6.0. After the suspensions were thoroughly stirred and centrifuged, the supernatant solution was analyzed.

ULTRACENTRIFUGATION. The sedimentation runs were carried out in 1-mm 4° sector cells in the Spinco Model E ultracentrifuge operating at 59,780 rpm at 24°. The Archibald run (Schachman, 1957) was made in the 1-mm 4° sector cell and the companion runs in the cup-type synthetic boundary cell. The speed setting was 8776 rpm and the temperature, 24°. Calculations were made as previously described (Yamada *et al.*, 1964).

AMINO ACID ANALYSES. The amino acid composition was determined by the Spinco Model 120 automatic amino acid analyzer (Spackman *et al.*, 1958). Samples (3.5 mg) were hydrolyzed for 12, 24, 48, and 72 hr in 6 N HCl at 110°. For the determination of cystine and cysteine content as cysteic acid, a sample was oxidized with performic acid (Hirs, 1956) and then hydrolyzed for 24 hr in 6 N HCl at 110°. A 7.5-mg sample was hydrolyzed in 1.5 ml of 4 N $Ba(OH)_2$ for 72 hr at 110° as described by Noltman *et al.* (1962) for the determination of tryptophan. The standardization of the instrument and the calculations of the results were performed as previously described (Matsubara *et al.*, 1962).

## Results

*Zone Electrophoresis of Crude Pineapple Stem Extract.* The experiment was conducted as described in the experimental section. The starch block was divided into 24 1-cm segments at the end of the run. The crude extract was applied at position 14 and the lower numbered fractions and the higher numbered fractions indicate anodic and cathodic movement, respectively. Peroxidase activity was detected chiefly in fractions no. 11 and 20. The specific activities of fractions no. 11 and 20, respectively, were 9230 and 2370 as determined by peroxidase assay, and 119 and 3 as determined by indoleacetic acid oxidase assay. However, 25% of the total peroxidase activity was found in fraction no. 11 and 75% in fraction no. 20. By definition, the slightly acidic fraction (fraction no. 11) is designated pineapple peroxidase A and the basic fraction, pineapple peroxidase B. The present study is concerned only with the purification of pineapple peroxidase B.

*Preparation of the Enzyme from Fresh Pineapple Stems.* Pineapple stems (100) in the flowering stage of development were obtained from the Pineapple Research Institute Field Station. The bulk of the leaf and root tissue was removed. The washed stumps (79 lb) were then cut into pieces of an appropriate size and ground in an electric meat grinder. The ground stem tissue was then pressed in a Carver press at 5000 psi. The resulting suspension was filtered through Whatman No. 1 filter paper. Sufficient solid ammonium sulfate was added to bring the solution to 0.45 saturation (277 g/l.) and the precipitate was separated by filtration. Sufficient solid ammonium sulfate (330 g/l.) was added to the filtrate to bring the solution to 0.90 saturation and the precipitate was collected by filtration.

The precipitate and filter paper were then placed in a Waring blender, covered with deionized water, and homogenized for 30 sec. The resulting suspension was filtered, the specific gravity of the filtrate was determined, and the concentration of ammonium sulfate was calculated. Additional solid ammonium sulfate was added to bring the solution to 0.55 saturation and the precipitate was removed by centrifugation. Sufficient ammonium sulfate (264 g/l.) was added to bring the solution to 0.90 saturation. The precipitate was collected by centrifugation and dialyzed *vs.* several 4-l. changes of 0.005 M sodium phosphate buffer, pH 6.0.

The dialyzed solution was placed on a 2.5 × 15 cm column of CM-cellulose which had been equilibrated with 0.005 M phosphate buffer, pH 6.0. The column was

TABLE I: Summary of the Purification of Enzyme from Fresh Pineapple Stems.

| Fraction | Procedure | Volume (ml) | Peroxidase Activity (units/ml) | IAA Oxidase Activity (units/ml) | Protein (mg/ml) | Specific Activity (units/mg) Peroxidase | Specific Activity (units/mg) IAA Oxidase | Total Protein (mg) | Yield (%) | Purification |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Filtered crude extract | 6965 | 24,800 | 301 | 102.4 | 242 | 2.94 | 71,322 | (100) | (1.0) |
| 2 | Supernatant from 0.45 ammonium sulfate satn | 7500 | 20,800 | 8 | 59.9 | 347 | 0.13 | 44,925 | | |
| | Fraction 2 dialyzed [a] | | | 36.4 | 5.3 | | 6.9 | | | 2.3 |
| 3 | Supernatant from 0.55 ammonium sulfate satn | 900 | 68,000 | 750 | 39.8 | 1,709 | 18.8 | 35,820 | 32 | 6.4 |
| 4 | After first carboxymethylcellulose chromatography | 45 | 900,000 | 2925 | 138.0 | 6,522 | 21.2 | 6,348 | 6.4 | 7.2 |
| 5 | After second carboxymethylcellulose chromatography | 11 | 880,000 | 2700 | 68.3 | 12,884 | 39.5 | 751 | 1.4 | 13.4 |
| 6 | After hydroxylapatite chromatography | 10 | 360,000 | 2275 | 13.2 | 27,273 | 172 | 132 | 1.1 | 59 |
| 7 | After gradient elution carboxymethylcellulose chromatography | 4.9 | 220,000 | 2725 | 5.65 | 38,938 | 482 | 27.8 | 0.64 | 164 |

[a] This was a sample (*ca.* 5 ml) of fraction 2 which was dialyzed against three 2-l. changes of deionized distilled water over a period of 24 hr. The results indicate the removal of an inhibitor whose effect was not overcome by the amount of cofactor (*p*-coumaric acid) added for the assay.

then washed with several hold-up volumes of the starting buffer, and eluted with 0.2 M phosphate buffer, pH 6.0. The enzyme at this point formed a dark brownish-red band on the column and its elution was followed visually.

The enzyme was then dialyzed *vs.* three 4-l. volumes of 0.005 M phosphate buffer, pH 6.0, and placed on a 1.5 × 10 cm column of CM-cellulose which had been equilibrated with starting buffer. After the column had been treated with several hold-up volumes of 0.005 M buffer, the protein was eluted with 0.2 M phosphate buffer, pH 6.0. The enzyme was collected as a single fraction by combining the brownish-red fractions.

The enzyme was then dialyzed against two 4-l. volumes of 0.005 M phosphate buffer, pH 6.0, and placed on a 2.0 × 3.5 cm column of hydroxylapatite which had been equilibrated with 0.005 M phosphate buffer, pH 6.0. The column was treated first with several hold-up volumes of 0.005 buffer. The protein was eluted with 0.2 M phosphate buffer, pH 6.0, and the enzyme was combined into a single fraction as described in the previous steps.

The enzyme solution was then dialyzed against two 4-l. volumes of 0.005 M phosphate buffer, pH 6.0. The enzyme solution was then absorbed on the top of a column (1 × 50 cm) of CM-cellulose which had been equilibrated with 0.005 M phosphate buffer, pH 6.0. A linear gradient was used, with 2 l. of 0.005 M phosphate buffer, pH 6.0, in the mixing chamber and 2 l. of 0.2 M phosphate buffer, pH 6.0, in the reservoir. Elution of the enzyme was accomplished in the region of 0.075 M buffer concentration. The *RZ* (reinheit zahl)[2] of the individual tubes (2.9-ml fractions) was determined and

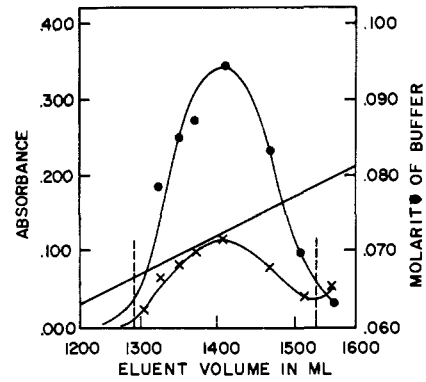


FIGURE 1: Gradient elution of enzyme on CM-cellulose. (See text for details.) The (×-×-×) indicates the absorbency at 275 mμ and the (•-•-•) indicates the absorbency at 407 mμ. The solid line indicates the molarity of the pH 6.0 phosphate buffer which was used. The fractions indicated by the dashed lines were combined to form the final fraction.

[2] The reinheit zahl (*RZ*) is the ratio obtained by dividing absorbancy at 407 mμ by the value at 275 mμ.

TABLE II: Summary of the Purification of the Enzyme Starting with Dolzyme T-20 Using the Peroxidase Assay.

| Fraction | Procedure | Volume (ml) | Activity (units/ml) | Protein (mg/ml) | Specific Activity (units/mg) | Total Units | Yield (%) |
|---|---|---|---|---|---|---|---|
| 1 | Dialyzed filtered crude | 5020 | 28,000 | 330 | 85 | $1406 \times 10^5$ | (100) |
| 2 | Supernatant from 0.40 ammonium sulfate satn | 4600 | 30,000 | 165 | 182 | $1380 \times 10^5$ | 98 |
| 3 | Dialyzed 0.55–0.90 ammonium sulfate fraction | 375 | 134,000 | 51.6 | 2,590 | $503 \times 10^5$ | 36 |
| 4 | After first carboxymethyl-cellulose chromatography | 75 | 504,000 | 37.2 | 13,550 | $378 \times 10^5$ | 27 |
| 5 | After hydroxylapatite chromatography | 93 | 74,400 | 3.6 | 20,600 | $69 \times 10^5$ | 4.9 |
| 6 | After second carboxymethyl-cellulose chromatography | 8.8 | 560,000 | 14.2 | 39,500 | $49 \times 10^5$ | 3.5 |

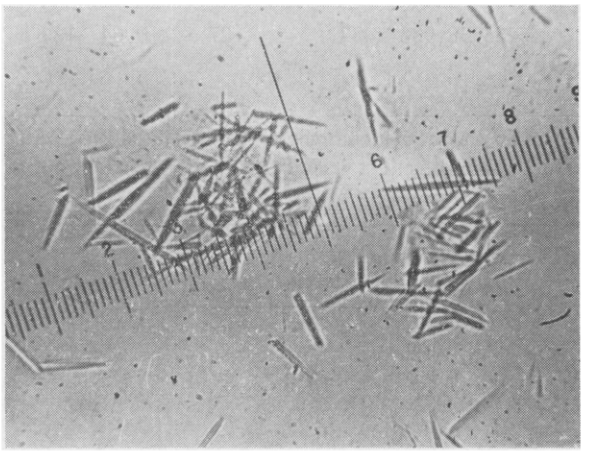

FIGURE 2: Photomicrograph of the crystalline pineapple peroxidase B. The *RZ* of the enzyme was 3.04 and the photograph magnified 675-fold.

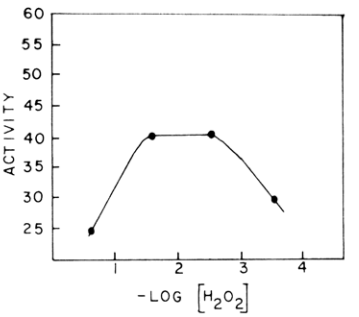


FIGURE 3: The effect of $H_2O_2$ concentration on the peroxidase activity of the crystalline enzyme. The standard peroxidase assay was used.

those tubes with *RZ* values greater than 2.5 were combined to give 236 ml of solution. The elution pattern of the enzyme is shown in Figure 1. The enzyme from this solution was precipitated by the addition of excess solid ammonium sulfate and was collected by centrifugation. Table I summarizes the purification procedure.

*Purification of Enzyme Using Dolzyme T-20.* Insufficient amounts of the purified enzyme were obtained from the fresh pineapple stem. Therefore, the possibility of using a commercially available form of the concentrated pineapple stem extract was explored.

Table II summarizes the results obtained from two 10-lb cans of Dolzyme T-20. One can (10 lb) (2.84 l. of solution) is equivalent to about 200 pineapple stems. The procedure was similar to that used for the purification of the enzyme from fresh stems but fewer steps were necessary since the enzyme was more amenable to purification. The spectra, specific activity, mobility, sedimentation, and electrophoretic properties of the enzyme from both starting materials were identical.

For the crystallization of the enzyme, the procedure was scaled to handle five 10-lb cans of Dolzyme. After step 6 of the purification procedure (see Table II), the enzyme was precipitated with ammonium sulfate, dissolved in water to form a 1% solution, and then dialyzed *vs.* distilled water. The procedure developed by Kenten and Mann (1954) was used to crystallize the enzyme. After fractionation with ammonium sulfate into the six fractions, the resulting precipitates were collected by centrifugation, dissolved in minimal amounts of deionized water, and centrifuged to remove any particulate matter present. Solid ammonium sulfate was added to each of the solutions at room temperature and a faint turbidity appeared. The solutions were refrigerated at 5°. After 2–5 days, crystals appeared in fractions II–IV which were originally 0.55–0.58, 0.58–0.61, and 0.61–0.64 saturated with respect to ammonium sulfate (Kenten and Mann, 1954). The enzyme was recrystallized by collecting the crystals by centrifugation, dis-

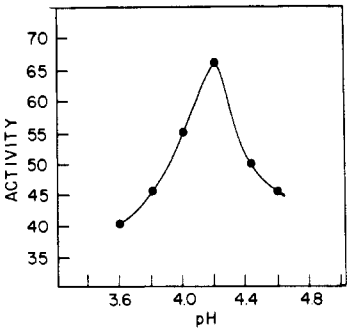


FIGURE 4: The effect of pH on the peroxidase activity of the crystalline enzyme utilizing 0.2 M buffers of different pH valves prepared according to Gomori (1955). Details of the assay are described in the experimental section.

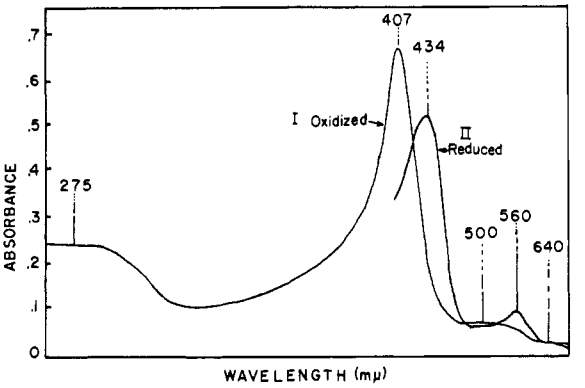


FIGURE 5: The ultraviolet and visible spectrum of the crystalline enzyme. The enzyme (0.2 mg/ml) in 0.05 M phosphate buffer, pH 7.0 (I), and the reduced enzyme (1 mg of sodium dithionite) (II) are shown in the figure.

solving them in a minimal amount of deionized water, and repeating the crystallization procedure. Crystalline enzyme (500 mg) was thus obtained from five 10-lb cans of starting material. Figure 2 shows a photomicrograph of the crystalline enzyme which resembles the crystals of horseradish peroxidase.

*Homogeneity of the Enzyme.* Analysis of the purified crystalline enzyme by ultracentrifugation in the pH range of 4.0–8.0 demonstrated the presence of only one component. Electrophoretic analyses of the enzyme at pH 4.0 showed a single component but at pH values above 8.0, a second hemeprotein component comprising 20–30% of the total protein became evident, but never completely separated from the main component. Rechromatography of the purified enzyme on carboxymethylcellulose, sulfoethyl-Sephadex, and hydroxylapatite all indicated the presence of only one component. When a sample which had been observed to show this shoulder at pH 10.0 was rerun at pH 4.0, a single peak was again observed. Because of the above and the fact that the enzyme is known to have a basic form, it is possible that (1) the system being examined contained a single enzyme component which existed in two states which were pH dependent, or (2) the system being examined contained a single enzyme but reacted with the media in a manner described by Cann and Goad (1965).

*Enzyme Properties.* There was a direct correlation between activity and protein concentration to a maximum of 800 enzyme units. The peroxidase activity was found to be independent of the buffer concentration between 0.1 and 2.0 M acetate buffer, pH 4.2. The effect of hydrogen peroxide concentration is shown in Figure 3, and it is evident that the enzyme is stable over a wide range of hydrogen peroxide concentration.

The pH optimum of the enzyme was determined using 0.2 M buffers of different pH values prepared according to Gomori (1955). The pH optimum was about 4.2 as shown in Figure 4.

The indoleacetic acid oxidase activity was proportional to enzyme concentration up to a maximum of 40 units. The pH optimum of the enzyme using this assay was about 4.4.

The cofactor requirements for the indoleacetic acid oxidase activity are summarized in Table III. The purified enzyme showed a requirement for $Mn^{2+}$ ions and its activity was enhanced by the addition of *p*-coumaric acid. Hydrogen peroxide could not substitute effectively for these components. Partially purified preparations, however, showed various degrees of activity in the absence of the cofactors but in all cases the activity of the enzyme was stimulated in their presence. The spectra

TABLE III: Summary of Indoleacetic Acid Oxidase Cofactor Requirements.[a]

| Expt No. | Conditions | Activity |
|---|---|---|
| I | Control (no enzyme) | 0 |
| II | Complete system | 2650 |
| III | No $Mn^{2+}$ | 100 |
| IV | No *p*-coumaric acid | 300 |
| V | Indoleacetic acid + $H_2O_2$ | 150 |
| VI | Pretreated with $H_2O_2$ and run with no *p*-coumaric acid | 100 |

[a] The reaction conditions were the same for all experiments except for the various changes in the addition of cofactors. The enzyme used for these experiments was from fresh stumps and had an *RZ* of 3.04 with an indoleacetic acid oxidase specific activity of 482 units/mg. The complete system contained 2.2 ml of 0.2 M sodium acetate buffer, pH 4.2, 0.1 ml of 0.001 M *p*-coumaric acid, 0.1 ml of diluted enzyme, 0.1 ml of 0.03 M $MnCl_2$, and 0.5 ml of 0.001 neutralized indoleacetic acid.

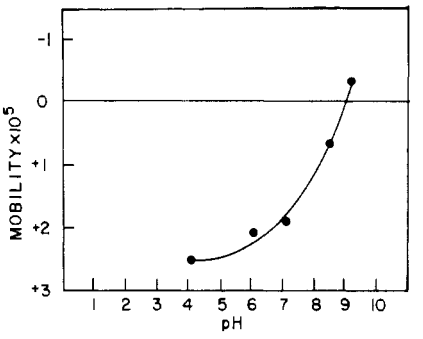


FIGURE 6: Determination of the isoelectric point of the crystalline enzyme. The protein concentration was 0.9% in each case. See text for details.

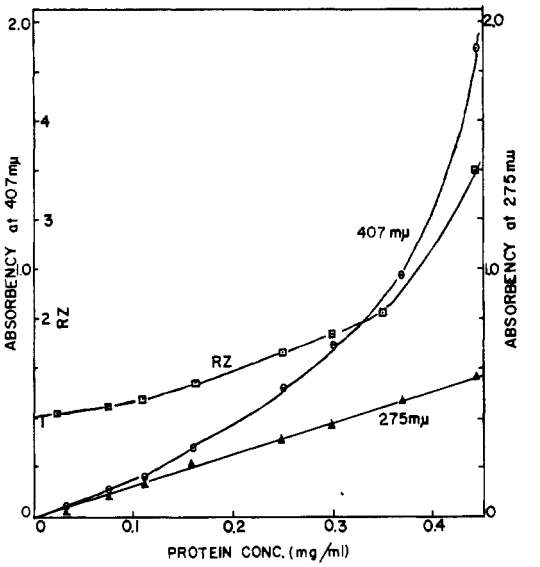


FIGURE 7: The variation of the absorbency at 407 mμ *vs.* protein concentration. The data were obtained by starting with the highest concentration and successively diluting the sample.

of the intermediates and products of the reaction were identical with those observed by Ray (1956) for the oxidation of indoleacetic acid by the *Omphalia flavida* enzyme.

A purified pineapple peroxidase B preparation (*RZ* 0.67) and a horseradish peroxidase preparation (Worthington Biochemical Co., *RZ* 0.81) were assayed for both peroxidase and indoleacetic acid oxidase activities. The specific activities for the horseradish peroxidase were 19,968 and 2,078,000 when determined by the indoleacetic acid oxidase and peroxidase assays, respectively, while the corresponding values were 172 and 27,273 for purified pineapple peroxidase B.

*Determination of Dry Weight, Absorbtivity Index, and Nitrogen Content.* Simultaneous determinations of the absorbancy and dry weights of three 3.25-mg samples yielded an $E_{1\,cm}^{1\%}$ value at 275 mμ of 12.75. The nitrogen content was 10.8% as determined by a modified micro Kjeldahl procedure (Hawk *et al.*, 1951).

*Spectral Properties of the Purified Enzyme.* The spectra of the oxidized and reduced enzymes are shown in Figure 5. The maxima observed in the visible wavelength region for the reduced, fluoride, azide, cyanide, and hydrogen peroxide derivatives are shown in Table IV. The compound exhibited the absorption spectrum of a typical plant peroxidase (Paul, 1963), strongly suggesting that hematin is the prosthetic group of the enzyme.

*Electrophoretic Mobility of the Purified Enzyme.* The isoelectric point of the enzyme was determined by recording electrophoretic patterns of the purified enzyme at various pH values in appropriate buffers of 0.1 ionic strength (Miller and Golder, 1958). The mobilities were calculated by the method described by Alberty (1948). A plot of mobility *vs.* pH revealed that the isoelectric point of the enzyme was close to 9.2 (Figure 6).

*Determination of the Molecular Weight.* The molecular weight of the enzyme was determined by the Archibald method (Schachman, 1957). The value obtained at a protein concentration of 1% and at an assumed partial specific volume of 0.72 was 35,200. The molecular weight of the enzyme obtained from the amino acid composition, on the basis of one residue of tryptophan per mole of enzyme, was 42,366. Estimation of the molecular weight on a Sephadex G-100 column (Whitaker, 1963) yielded a value of 42,000. The average of the three molecular weights yielded a value of 40,000.

TABLE IV: Summary of the Spectral Properties of Enzyme.[a]

| Derivative | Soret Band (mμ) | Visible Bands (mμ) | |
|---|---|---|---|
| Native | 407 | 500 | 640 |
| Reduced (hydrosulfite) | 434 | 560 | |
| Fluoride | 405 | 488 | 617 |
| Azide | 410 | 542 | |
| Cyanide | 422 | 550 | |
| Hydrogen peroxide | 418 | 551 | 584 |

[a] All forms of the crystalline enzyme were run at the same concentration of enzyme (0.25 mg/ml in 0.05 M phosphate buffer, pH 7.0). The various derivatives were formed in an excess of the appropriate reagent.

*Amino Acid Composition of the Enzyme.* Table V summarizes the amino acid composition of pineapple peroxidase B obtained by averaging the results from the analyses of the 12-, 24-, 48-, and 72-hr hydrolysates. Cysteine was determined as cysteic acid after performic acid oxidation. Tryptophan was determined on a 72-hr hydrolysate with barium hydroxide as the hydrolyzing agent (Noltman *et al.*, 1962). Values for threonine, serine, and ammonia were obtained by extrapolating

TABLE V: Amino Acid Composition of Various Peroxidases.

| Amino Acid | Pineapple Peroxidase B: Amino Acid Residues/100 g of Protein[c] | Pineapple Peroxidase B: Min Mol Wt | Pineapple Peroxidase B: Nearest Integral No. of Amino Acid Residues/ 40,000 g of Protein | JRP[a] Amino Acid Residues/ Mole | HRP I[b] Amino Acid Residues/ Mole |
|---|---|---|---|---|---|
| Lysine | 3.91 | 3,278 | 12 | 7 | 4 |
| Histidine | 1.00 | 13,715 | 3 | 4 | 2 |
| Ammonia[d] | 1.26 | 1,270 | 32 | | |
| Arginine | 3.26 | 4,791 | 8 | 11 | 13 |
| Tryptophan | 0.44 | 42,366 | 1 | 2 | 0 |
| Aspartic acid | 10.99 | 1,047 | 38 | 51 | 32 |
| Threonine[d] | 3.78 | 2,727 | 15 | | 16 |
| Serine[d] | 5.91 | 1,473 | 27 | 51 | 15 |
| Glutamic acid | 4.23 | 3,052 | 13 | 26 | 14 |
| Proline | 2.14 | 4,536 | 9 | 17 | 11 |
| Hydroxyproline | | | 0 | 12 | 0 |
| Glycine | 2.61 | 2,186 | 18 | 6 | 12 |
| Alanine | 2.61 | 2,723 | 15 | 64 | 16 |
| Half-cystine[e] | 1.16 | 8,891 | 4 | 10 | |
| Valine | 4.20 | 2,360 | 17 | 20 | 11 |
| Methionine | 1.34 | 9,791 | 4 | 4 | 2 |
| Isoleucine | 3.21 | 3,525 | 11 | 15 | 9 |
| Leucine | 5.12 | 2,210 | 18 | 36 | 23 |
| Tyrosine | 1.66 | 9,830 | 4 | 3 | 3 |
| Phenylalanine | 5.91 | 2,491 | 16 | 18 | 13 |

[a] Japanese radish peroxidase from Morita and Kameda (1959). [b] Horseradish peroxidase I from Klapper and Hackett (1965). [c] Average of 12-, 24-, 48-, and 72-hr hydrolysates. [d] Extrapolated values. [e] Determined as cysteic acid.

to 0 hydrolysis time (Hirs *et al.*, 1954). Noteworthy is the low tyrosine and tryptophan content in keeping with the low molar absorbtivity index at 280 mμ. The enzyme reacted strongly with the anthrone reagent but glucosamine and galactosamine were not detected in the amino acid chromatograms.

*Anomalous Soret Absorption.* The *RZ* value ($E_{407}/E_{275}$) has been considered a physical constant in establishing the purity of plant peroxidases (Maehly, 1955). The *RZ* values for the pure plant peroxidases range from 3.0 to 3.5. In the case of pineapple peroxidase B, a somewhat surprising phenomenon was observed when the *RZ* value was determined on a purified preparation at different protein concentrations. As shown in Figure 7, the *RZ* value varied from 1.0 to 3.5 for the same preparations when the concentration of the enzyme was varied, and the Soret absorption was not proportional to protein concentration. Therefore, in an attempt to standardize conditions for the *RZ* determination, the protein concentration was adjusted so that the absorbance at 407 mμ was about 0.8 for the *RZ* values reported in the present study.

## Discussion

Several peroxidase components were detected in the pineapple stem extracts which are responsible for the observed indoleacetic acid oxidase activity reported earlier (Gortner and Kent, 1953, 1958). One of the components, pineapple peroxidase B, has been obtained in crystalline form, the pineapple being the fifth plant source from which peroxidase has been crystallized (Paul, 1963).

The most noteworthy finding arising from this study is the demonstration of the existence of an atypical peroxidase. The atypical properties include: (1) the low specific activity of the enzyme; (2) the lower pH optimum; and (3) the anomalous Soret absorption. All three of the properties might be due to the fact: (1) that the enzyme is partially denatured; (2) that the enzyme is an atypical peroxidase; (3) that the true substrate of the enzyme is not being tested; (4) that the prosthetic group of the enzyme dissociates from the enzyme at low enzyme concentrations.

Concerning each of these points, it is very unlikely

that a grossly denatured enzyme would crystallize, and peroxidases are known to be very stable enzymes (Paul, 1963). The properties of the enzyme such as spectra, molecular weight, and substrate specificity all point toward the fact that the enzyme is a peroxidase. Furthermore, addition of hemin to the enzyme did not increase its activity, thus ruling out dissociation of the prosthetic group. Therefore, we are led to the tentative conclusion that the pineapple peroxidase is an atypical peroxidase. Since Ray (1960) has also observed a peroxidase with a pH optimum at about 4.2 rather than 5–7, other plants may contain this type of peroxidase. It should be noted that pineapple peroxidase B is not a paraperoxidase (horseradish peroxidase I) due to its high carbohydrate content and spectral properties (Paul, 1963; Theorell, 1951).

From the standpoint of comparative enzymology, the three plant peroxidases, horseradish peroxidase, Japanese radish peroxidase, and pineapple peroxidase, have quite different amino acid compositions. Pineapple and Japanese radish peroxidase contain tryptophan (Morita and Kameda, 1959) while horseradish peroxidase does not (Theorell, 1951; Klapper and Hackett, 1965). Japanese radish peroxidase A contains hydroxyproline (Morita and Kameda, 1959) while the other two enzymes do not. There are also numerous other differences in the quantity of specific amino acid residues. The carbohydrate content varies as well. Pineapple peroxidase B was shown to contain no hexosamines while Japanese radish peroxidase contains 15 residues of hexosamine/mole of enzyme (Morita and Kameda, 1958).

As a final note, pineapple peroxidase A has been purified to a point where the *RZ* was 1.49 and the specific activity was 47,000. The procedure involved fractionation of the fresh pineapple stem extract with ammonium sulfate, followed by chromatography on DEAE-cellulose, followed by a second ammonium sulfate fractionation step. Therefore, at best, the specific activity of pure pineapple peroxidase A (*RZ* 3) would be three times that of peroxidase B. Therefore, both forms have much lower specific activities than the other pure peroxidases isolated to date.

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