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De Novo Synthesis of Peroxidase Isozymes in Sweet Potato Slices[1,2]

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ABSTRACT

The peroxidase content of sweet potato slices (*Ipomoea batatas* Lam.) increased nearly 100-fold following 84 hours incubation in an air atmosphere containing ethylene, 1 microliter per liter. The object of experiments reported here is to determine if this increase in peroxidase activity results from synthesis *de novo* of the enzyme or from activation of a preexisting inactive form of the enzyme.

The enzymatic activity of each peroxidase isozyme increased during the incubation period, and each peroxidase isozyme appeared to incorporate ^{14}C-leucine. Polyacrylamide gel electrophoresis of the neutral peroxidase fraction showed that all peroxidase activity and essentially all radioactivity migrated as a single superimposable band. The other peroxidase fractions were less pure. Treatment of fresh slices, or slices collected midway in the time course with the inhibitor of protein synthesis, blasticidin S, (1 microgram per milliliter for one minute) caused an abrupt cessation of peroxidase formation and simultaneously an abrupt cessation of incorporation of ^{14}C-leucine into peroxidase isozymes. These observations indicate that the rapid increase in peroxidase activity in sweet potato slices results from synthesis *de novo* of the enzyme.

Numerous investigators have shown that when storage tissues, such as the sweet potato, are cut into slices, many metabolic activities begin to rise (1, 3, 4, 9, 18, 20–22). The rise usually begins after a lag of several hours and continues to increase over a period of several days, at which time the activity may be many times larger than in fresh tissue. Included among the metabolic changes is the increase in the activities of several enzymes, including peroxidase (E.C.1.-11.1.7) (8, 15).

Kawashima and Uritani (15) showed that peroxidase activity increased even more rapidly when the slices were infected with the black rot fungus, *Ceratocystis fimbriata*. Stahmann *et al.* (24) reported that sweet potato slices infected with the black rot fungus produced low concentrations of ethylene and that ethylene *per se* stimulated peroxidase formation, even in uninoculated tissue. Imaseki *et al.* (10, 12) also showed that the peroxidase content of sweet potato slices was significantly enhanced by a low concentration of ethylene exogenously supplied to the slices. It was noted by Imaseki *et al.* (11) that even though ethylene caused a marked increase in peroxidase activity in sweet potato slices, the ethylene did not alter the qualitative complement isozymes. Kawashima and Uritani (16) showed that the physico-chemical properties of peroxidase isozymes isolated from cut tissue and from tissue infected with the black rot fungus were essentially identical.

In order to understand the physiological significance of the rapid increase in peroxidase, it is necessary to ascertain if the increased activity results from synthesis *de novo* of the enzyme or from activation of a previously inactive form of the enzyme. Early work by Kanazawa *et al.* (14) noted that sweet potato slices treated with inhibitors of protein synthesis contained less peroxidase than untreated tissue, thus suggesting that the increment in peroxidase content resulted from synthesis *de novo*. Using deuterium oxide as a density label, Anstine *et al.* (2) recently showed the newly appearing peroxidases in germinating barley embryos arise by synthesis *de novo*. Longo (19) presented evidence for the synthesis *de novo* of isocitratase and malate synthetase in cotyledons of germinating peanuts. Jacobsen *et al.* (13) reported that the four α-amylases produced by barley aleurone tissues in response to gibberellic acid arise by synthesis, *de novo*, whereas the four β-amylases seemed to arise by activation of preexisting zymogens.

The present report describes the increase in peroxidase activity in sweet potato slices and shows the effect of the protein synthesis inhibitor, blasticidin S, on the increase in peroxidase activity and on the incorporation of uniformly labeled ^{14}C-leucine into peroxidase isozymes. The slices were incubated in an air atmosphere containing ethylene (1 μl/liter) to enhance peroxidase formation. The results indicate that the rapid increase in peroxidase activity results from synthesis *de novo* of the enzyme.

MATERIALS AND METHODS

Sweet potatoes (*Ipomoea batatas* Lam. cv. Norin No. 1) were obtained commercially and stored at 10 C until used. The roots were washed, the outer cortex tissues removed, and cross sectional slices 2 mm thick and 20 mm in diameter were obtained from the inner parenchymatous tissues. The slices were thoroughly washed with water, blotted, counted, and weighed.

Incubation Conditions. Fifty slices were carefully positioned in 11-cm Petri dishes to allow air circulation over all cut

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surfaces. The Petri dishes were transferred to 12-liter desiccators which were maintained at 25 C. High humidity was maintained by lining the desiccator with moistened filter paper. The desiccator was lightly evacuated and the appropriate quantity of ethylene introduced to yield an atmosphere containing 1 μl/liter. Ethylene-free air (26) was then freely introduced to return the atmosphere within the desiccator to ambient atmospheric pressure. A beaker containing 20% KOH was placed in the desiccator to remove CO_2. In experiments involving the protein synthesis inhibitor, blasticidin S, sweet potato slices were removed at specified times, dipped 1 min in blasticidin S, (1 μg/ml), blotted, returned to the desiccator, and the desiccator recharged with ethylene.

Enzyme Preparation. After specified lengths of incubation, a given number of sweet potato slices were removed from the desiccator and cut into small pieces with a razor blade. The diced tissue was transferred to an all glass Potter-Elvehjem tissue homogenizer and thoroughly ground with 2.5 volumes of cold grinding solution per gram initial fresh weight. The grinding solution was 0.05 M tris-HCl, pH 7.0, and also contained 1% (v/v) β-mercaptoethanol. The homogenate was passed through four layers of cheesecloth and centrifuged under refrigeration at 12,000g for 20 min. The supernatant solution was collected and passed through a 2.5- × 30-cm column containing Sephadex G-25 previously equilibrated with 0.005 M tris-HCl, pH 8.5. The protein fraction was collected and designated "crude homogenate."

The crude homogenate fraction was passed through a 2.5- × 20-cm column containing DEAE[5]-cellulose previously equilibrated with 0.005 M tris-HCl, pH 8.5. That fraction of peroxidase which was adsorbed to the DEAE-cellulose column was eluted batchwise with 0.025 M tris-HCl, pH 8.5 which also contained 0.25 M NaCl. The eluted fraction was designated "anionic peroxidases."

The fraction of peroxidase which was not adsorbed to DEAE-cellulose was dialyzed for 48 hr against 0.005 M sodium acetate, pH 5.0, with four changes of dialyzing solution and passed through a 2.5- × 40-cm column containing CM-cellulose previously equilibrated with 0.005 M sodium acetate, pH 5.0. That fraction of peroxidase which was adsorbed to the CM-cellulose column was eluted batchwise with 0.05 M sodium acetate, pH 5.0, which also contained 0.5 M NaCl. The eluted fraction was designated "cationic peroxidases."

The fraction of peroxidase which was not adsorbed to CM-cellulose was collected and designated "neutral peroxidases."

Resolution of Anionic Peroxidase Isozymes. The fraction designated anionic peroxidases was dialyzed for 48 hr against 0.005 M tris-HCl, pH 8.5, with four changes of dialyzing solution. The anionic peroxidase fraction was then passed through a 2.5- × 20-cm column containing DEAE-cellulose previously equilibrated with 0.005 M tris-HCl, pH 8.5. The anionic peroxidases were eluted from the DEAE-cellulose using a linear gradient consisting of 1000 ml of 0.005 M tris-HCl, pH 8.5, as the initial buffer and 1000 ml of 0.025 M tris-HCl, pH 8.5, which also contained 0.25 M NaCl as the limit buffer. Ten-milliliter fractions were collected and assayed for peroxidase activity. This elution scheme resolved the anionic peroxidases into four individual peroxidase isozymes which were designated I, II, III, and IV after Kawashima and Uritani (16), Figure 3.

Peroxidase Assay. Peroxidase was assayed as described previously with slight modification (23). The dye, 3-3 dimethoxybenzidine (*o*-dianisidine) was recrystallized from ethanol, dried, and a 0.5% solution (w/v) prepared with 1 M HCl. A stock solution of *o*-dianisidine and buffer was prepared by adding in the following sequence: 16 ml of 0.5% (w/v) *o*-dianisidine, 48 ml of 0.6 M sodium acetate, pH 5.4, and 416 ml of H_2O. When stored in the refrigerator this stock solution was stable for months. The reaction cuvette contained 2.8 ml of *o*-dianisidine-buffer solution, 0.1 ml of 0.1 M H_2O_2, and 0.1 ml of enzyme. The reaction was started by addition of peroxide and the rate of *o*-dianisidine oxidation measured at 460 nm on a Shimadzu QV-50 recording spectrophotometer. The rate of reaction was calculated from the linear portion of the curve during the first 2 min. One enzyme unit is defined as that quantity of enzyme which will cause an increase in absorbance of 1 A per min per g fresh weight.

^{14}C-Leucine Incorporation. Sweet potato slices were prepared as described above, placed in a beaker, and soaked for 3 hr with frequent stirring in a solution containing uniformly labeled ^{14}C-leucine, 1 μc/ml (305 mc/mmole); streptomycin, 20 μg/ml; and penicillin, 20 μg/ml. The slices were then removed from the ^{14}C-leucine solution, placed in Petri dishes, and transferred to the incubation desiccator.

Radioactivity Determination. Aliquots not exceeding 1 ml were placed in a vial and dissolved in 15 ml of Bray's scintillation fluid (5). The radioactivity was measured using a Packard Tri-Carb liquid scintillation spectrometer, Model 314E.

Semi-Preparative Polyacrylamide Gel Electrophoresis. Polyacrylamide gel electrophoresis was carried out at pH 8.5 according to the method of Davis *et al.* (6) with minor modifications. The 7.5% running gel was 1.5 × 8 cm and was polymerized with ammonium persulfate. The large pore spacer gel was omitted. The sample was mixed with sucrose and added under buffer to the top of the running gel. Electrophoresis was conducted for 3.5 hr. The buffer temperature was maintained at 5 to 10 C by refrigerated cooling coils. Following electrophoresis, the gels were removed from the glass columns by gently reaming under water. Peroxidase activity was detected by streaking the gel with a cotton swab which had been dipped in a 1:1 (v/v) mixture of 3% hydrogen peroxide-benzidine solution. The benzidine solution was prepared by dissolving 1 g of benzidine in 9 ml of glacial acetic acid and then adding 36 ml of water. The gel was then cut into segments, and the segments forced through the orifice of a syringe to break the gel into small particles. The resulting gel particles were placed in a scintillation counting vial and soaked for 24 hr with 1 ml of 0.05 M tris-HCl, pH 7.0. The vial then received 15 ml of Bray's scintillation fluid, and the radioactivity was measured. Protein bands were detected by staining with amido black and then destaining electrophoretically (6).

RESULTS AND DISCUSSION

Time Course of Peroxidase Activity. Slices of sweet potato roots were placed in an atmosphere containing ethylene (1 μl/liter) and collected after 0, 16, 40, 64, and 88 hr of incubation. A crude homogenate was prepared from the slices and assayed for peroxidase activity. The solid line in Figure 1 shows the increase in peroxidase activity. It may be noted that peroxidase activity increased from 30 units in fresh slices to over 2800 units after 88 hr of incubation. This amounts to nearly a 100-fold increase in peroxidase content during an 88-hr period. The object of experiments reported here is to establish if this phenomenal increase in peroxidase activity results from synthesis *de novo* of the enzyme or from activation of a preexisting inactive form of the enzyme.

Influence of Blasticidin S on Time Course of Peroxidase Activity. After 0, 16, 40, and 64 hr of incubation, samples of

[5] Abbreviations: CM: carboxymethyl; DEAE: diethylaminoethyl.

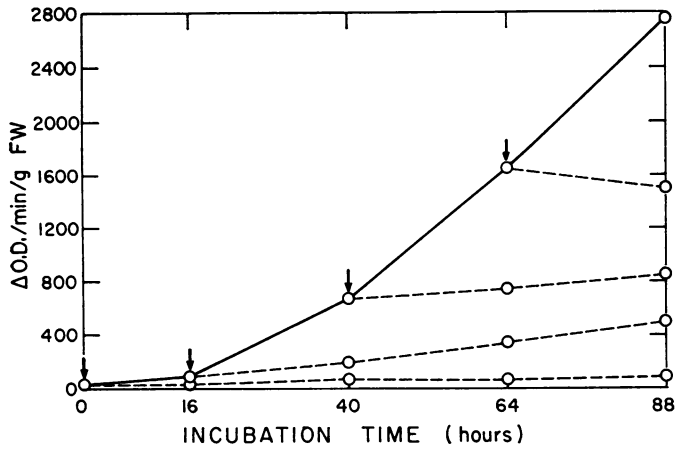

FIG. 1. Effect of blasticidin S on the time course of peroxidase activity. Slices of sweet potato roots (20 mm diameter × 2 mm thick) were incubated in a moist atmosphere containing ethylene (1 μl/liter). Some of the slices were treated with blasticidin S, 1 μg/ml for 1 min, at points in the time course denoted by the arrows (↓). Crude homogenates were prepared and peroxidase activity was measured in the control (O——O) and in the blasticidin S treated slices (O- - -O) at 0, 16, 40, 64, and 88 hr.

root slices were collected, dipped for 1 min in a solution containing blasticidin S (1 μg/ml), blotted, and returned to the incubation chamber. The arrows in Figure 1 indicate the points in the time course of the experiment that slices were treated with blasticidin S. Crude homogenates were prepared, and peroxidase activity was measured in the blasticidin S-treated slices at each subsequent collection period. The broken lines in Figure 1 show the peroxidase activity in slices following treatment with blasticidin S. The experiment was terminated after 88 hr.

It is clearly evident from Figure 1 that treatment of sweet potato slices with blasticidin S prevented the phenomenal increase in peroxidase activity. When fresh slices were treated with blasticidin S and then incubated for 88 hr, they contained only 100 units of peroxidase, compared with 2800 units in the untreated tissue. The increase in peroxidase content in tissue treated after 16, 40, and 64 hr of incubation was also markedly reduced when compared with the untreated tissue.

Blasticidin S (25) possesses an amino acyl-cytosine nucleosidic structure and behaves as an analog of amino acyl-sRNA. Yamaguchi and Tanaka (25) suggest that blasticidin S acts at the step of peptide transfer from peptidyl-sRNA, without effect on preceding steps of protein biosynthesis.

It is well established that plant peroxidases possess a protoporphyrin IX heme prosthetic group and a covalently linked polysaccharide prosthetic group (16, 17, 23). The two prosthetic groups are believed to be attached after the completed polypeptide chain is released from the polysome. Hence, it is possible that the inhibition of peroxidase formation by blasticidin S might result from a deficiency of an enzyme required in synthesis or attachment of the heme or polysaccharide prosthetic groups.

It is significant that upon treatment with blasticidin S midway in the time course, the increase in peroxidase content appeared to cease immediately. If blasticidin S prevented formation of an enzyme responsible for activating an inactive precursor, one would expect peroxidase formation to continue at about the same rate for a period, with perhaps a decrease at a later time. One would not expect an immediate cessation of peroxidase formation. The immediate cessation of peroxidase formation following treatment with blasticidin S suggests the inhibitor is preventing formation of peroxidase *per se*.

Influence of Blasticidin S on the Time Course of Peroxidase Activity and ^{14}C-Leucine Incorporation. Slices of sweet potato roots were soaked in uniformly labeled ^{14}C-leucine and incubated in an atmosphere of ethylene (1 μl/liter). After 28 and 48 hr of incubation, samples of root slices were collected, dipped for 1 min in blasticidin S (1 μg/ml), blotted, and returned to the incubation desiccator.

Crude homogenates were prepared from control and blasticidin S treated slices harvested at 28, 48, and 68 hr. Fractions containing anionic, cationic, and neutral isozymes of peroxidase were then isolated from the crude homogenate. Figure 2 presents the peroxidase activity (left column of figures) and radioactivity (right column of figures) in the different fractions of peroxidase. The solid lines show the time course of peroxidase activity and radioactivity and represent control treatments. The arrows in Figure 2 denote the times that slices were treated with blasticidin S. The broken lines represent the peroxidase activity and radioactivity following treatment with blasticidin S. The experiment was terminated at 68 hr.

Peroxidase activity in the crude homogenate and in the anionic, cationic, and neutral fractions increased dramatically during the 68-hr time course, Figure 2 (left column, solid lines). It may also be noted (broken lines) that upon treatment with blasticidin S there was an immediate cessation of peroxi-

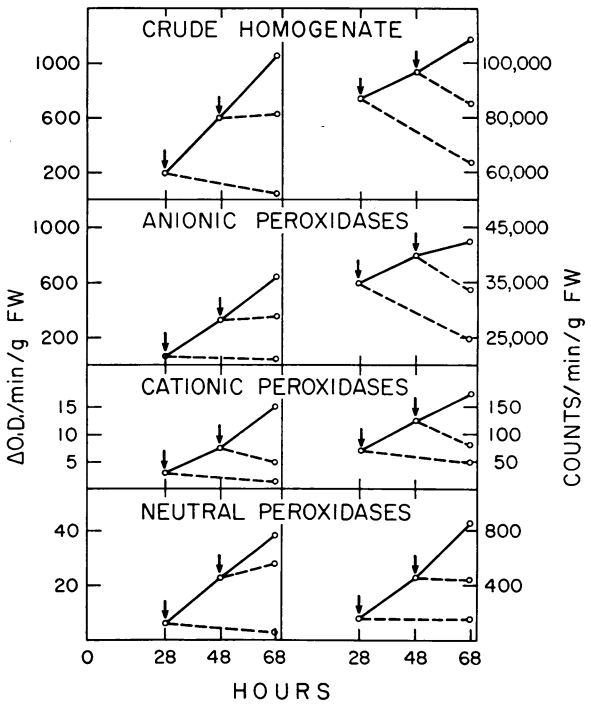

FIG. 2. Effect of blasticidin S on the time course of peroxidase activity (left column of figures) and ^{14}C-leucine incorporation (right column of figures). Slices of sweet potato roots (20 mm diameter × 2 mm thick) were soaked in uniformly labeled ^{14}C-leucine (305 mc/mmole) for 3 hr and then incubated in an atmosphere containing ethylene (1 μl/liter). Some of the slices were treated with blasticidin S (1 μg/ml) for 1 min at points in the time course denoted by the arrows (↓). Crude homogenates were prepared from control (O——O) and blasticidin treated slices (O- - -O) at 0, 28, 48, and 60 hr. The anionic, cationic, and neutral peroxidases were isolated from each crude homogenate and the time course plotted for peroxidase activity (left column of figures) and radioactivity (right column of figures).

dase formation in each fraction of peroxidase. These observations are in precise agreement with the previous time course experiment.

It is readily apparent from radioactivity measurements in Figure 2 that ^{14}C-leucine was incorporated into protein isolated in the crude homogenate and in the anionic, cationic, and neutral peroxidases. The amount of radioactivity incorporated (solid lines) increased markedly during the 68-hr time course. It is also apparent that upon treatment with blasticidin S after 28 and 48 hr of incubation, there was an abrupt cessation of incorporation of radioactivity in each peroxidase fraction (broken lines). These experiments with radioactive leucine indicate that amino acids were incorporated into the partially purified peroxidase fractions during the period of rapid peroxidase formation and that blasticidin S inhibited amino acid incorporation.

Multiple forms of peroxidase have been detected in many species of plants, including sweet potato. Kawashima and Uritani (15) reported that sweet potato slices possessed four major anionic peroxidases and several minor forms of the enzyme. The purification scheme employed in the previous experiment resolved the peroxidase isozymes into anionic, cationic, and neutral fractions. Upon subjecting the anionic fraction to additional DEAE-chromatography, it was possible to resolve the anionic fraction into the four major peroxidase isozymes.

Each anionic fraction collected from the ^{14}C-leucine incorporation experiment was subjected to additional DEAE-chromatography and separated into the four major peroxidase isozymes. Figure 3 presents the elution profile of peroxidase activity obtained from the anionic fractions collected after 28, 48, and 68 hr of incubation. The activity of peroxidase isozymes isolated from fresh tissue was too low to resolve. While the quantity of each peroxidase isozyme increased during the time course, the relative increase in fractions I, III, and IV was much larger than that in fraction II. This observation indicates an unequal rate of synthesis among the individual isozymes. Using the zymogram technique, Imaseki *et al.* (11) showed that certain isozymes appeared earlier in the time course than others, again indicating an unequal rate of synthesis.

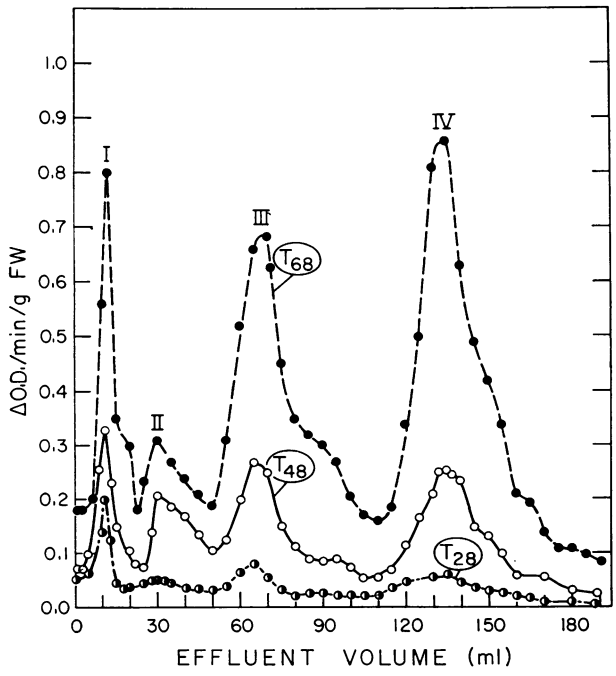

FIG. 3. Elution profiles showing peroxidase activity in the four major isozymes collected after 28 (◑——◑), 48 (○——○), and 68 (●——●) hr of incubation. The peroxidase isozymes were designated I, II, III, and IV after Kawashima and Uritani (14).

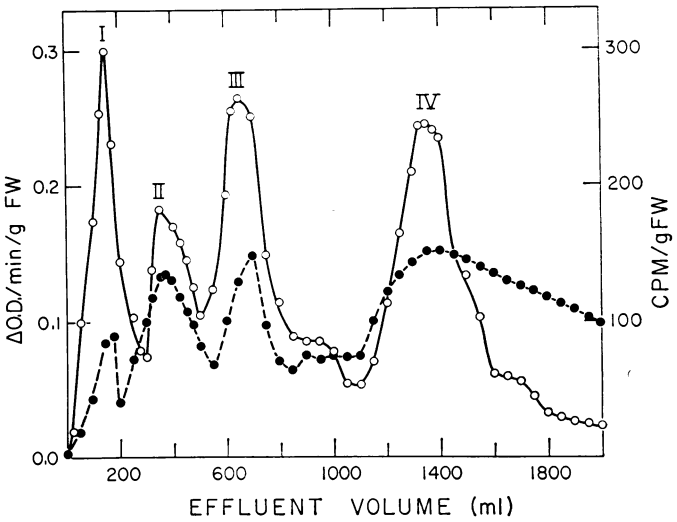

FIG. 4. Elution profiles showing peroxidase activity (○——○) and radioactivity (●——●) in the four major isozymes collected after 48 hr of incubation. The peroxidase isozymes were designated I, II, III, and IV after Kawashima and Uritani (14).

The elution profile (not presented) of peroxidase activity from slices treated with blasticidin S after 28 and 48 hr was nearly identical, qualitatively and quantitatively, with the elution profile of isozymes from slices collected after 28 and 48 hr, respectively, in Figure 3. This observation indicates that treatment with blasticidin S midway in the time course prevented further synthesis of all four peroxidase isozymes.

Figure 4 presents the elution profile of peroxidase activity and radioactivity obtained from the anionic fraction of tissue collected after 48 hr of incubation. These superimposed elution profiles indicate that each of the four major isozymes incorporated ^{14}C-leucine into the enzyme.

Polyacrylamide Gel Electrophoresis of Peroxidase Isozymes. Slices of sweet potato roots were soaked in uniformly labeled ^{14}C-leucine for 3 hr and incubated 114 hr in an atmosphere of ethylene (1 μl/liter). A crude homogenate was prepared and fractions containing anionic, cationic, and neutral peroxidases were isolated. Each fraction was concentrated with Carbowax 6000 and dialyzed 48 hr against 0.005 M tris-HCl, pH 8.5.

An aliquot of the neutral peroxidases was subjected to polyacrylamide gel electrophoresis. Following electrophoresis the gel was stained to detect peroxidase, cut into segments, and the radioactive proteins eluted and counted. Figure 5 presents a schematic drawing of the gel following electrophoresis and shows the distribution of peroxidase activity and radioactivity. All peroxidase activity was detected in a 3-mm segment of the gel slightly removed from the origin. Essentially all the radioactivity was also present in the same segment. Another gel prepared in the same manner was stained with amido black to detect protein. The only protein detected was localized in the same 3-mm segment. These observations indicate a high degree of homogeneity in the neutral peroxidase fraction and also indicate that radioactive leucine was incorporated into the neutral peroxidases.

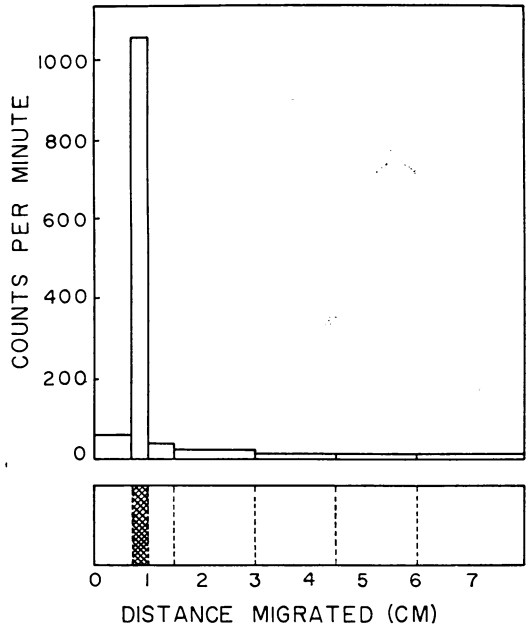

FIG. 5. Distribution of peroxidase activity and radioactivity in polyacrylamide gel following electrophoresis of neutral peroxidase fraction. The lower figure is a schematic drawing of a polyacrylamide gel following electrophoresis and shows the distribution of peroxidase activity (crosshatched area). The broken lines represent the locations where the gel was cut into segments. The top of the gel (left side) was connected to the cathode. The upper figure presents a bar graph showing the distribution of radioactivity eluted from corresponding segments of the electrophoresis gel.

The anionic peroxidases were subjected to DEAE-chromatography and peroxidase isozymes I, II, III, and IV were isolated. An aliquot of each isozyme was then subjected to polyacrylamide gel electrophoresis. Following electrophoresis the gel was stained to detect peroxidase. In peroxidase isozymes I, II, III, and IV the most intense peroxidase activity band migrated 9, 17, 23, and 30 mm, respectively. The gel was then cut into segments, and the radioactive proteins were eluted and counted. In each isozyme, that segment of gel possessing the most intense peroxidase activity stain also possessed the most radioactivity. Contrary to the neutral peroxidase fraction, however, other segments of the gels contained appreciable radioactivity. These observations indicate a lack of homogeneity in anionic peroxidase isoenzymes I, II, III, and IV. Consequently, even though in Figure 4 the peaks of radioactivity and peroxidase activity in isozymes I, II, III, and IV coincide, a portion of the radioactivity in each peak is contributed by radioactive proteins other than peroxidase.

In summary, the observation that the quantity of each peroxidase isozyme increased during the time course (Fig. 3); the observation that four partially purified peroxidase isozymes became radioactively labeled with ^{14}C-leucine, (Fig. 4); the observation that following polyacrylamide gel electrophoresis of the neutral peroxidase fraction, all peroxidase activity and essentially all radioactivity migrated together, (Fig. 5); and the observation that the inhibitor of protein synthesis, blasticidin S, inhibited both the increase in peroxidase activity and the incorporation of ^{14}C-leucine into partially purified peroxidase fractions, (Figs. 1 and 2), suggest very strongly that the rapid increase in peroxidase activity results from synthesis *de novo* of the enzyme.

It has been suggested that endogenous ethylene may regulate enzyme formation in injured plant tissues (10). Information concerning the mechanism by which ethylene initiates the induction of peroxidase isozymes, however, is lacking. Since the level of peroxidase remains approximately constant following blasticidin S treatment midway in the time course, it would appear that peroxidase is quite stable and that there is little enzyme inactivation or turnover. This observation implies that the induction factor involves enzyme synthesis only. This is in contrast to the complex induction phenomena involved in phenylalanine ammonia-lyase (E.C.4.3.1.5.) in which both enzyme synthesis and enzyme inactivation are involved (7, 27).

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