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High Resolution of Peroxidase-Indoleacetic Acid Oxidase Isoenzymes from Horseradish by Isoelectric Focusing

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ABSTRACT

Several improved techniques for isoelectric focusing of isoenzymes in polyacrylamide gel slabs were developed. Using these techniques, three commercial sources of horseradish peroxidase were each examined with three commercial sources of carrier ampholytes to determine their respective isoenzyme profiles.

A much higher degree of isoenzyme resolution was obtained than reported previously. A composite of all of the various tests gave a total of 42 peroxidase isoenzymes. Commercial sources of horseradish peroxidase showed many similarities in their isoenzyme patterns, but their differences were sufficient to recognize each source easily. Isoenzyme patterns for all three sources spanned the entire pH range with all three sources of wide range carrier ampholytes. Only a few isoenzymes stained darkly. Most isoenzymes stained moderately to lightly, but all were well resolved. Gels stained for indoleacetic acid oxidase activity showed the same pattern as gels stained for peroxidase activity. This was true for all three commerial sources of the enzyme. The isoenzyme patterns obtained in each run were entirely reproducible, and linear pH gradients were obtained in all cases. Limitations in the pH range of the wide range carrier ampholytes relative to the isoelectric points of the extreme anodic and cathodic isoenzymes led to the adoption of a modified definition of focusing time. In addition, the labeling of isoenzymes commonly used in electrophoresis was adapted for labeling isoenzymes resolved by isoelectric focusing.

The problem of determining the quantity and quality of peroxidase isoenzymes in plant tissues has persisted over several decades for many reasons important to plant physiologists. First, the phenomenon of isoenzymes itself is perplexing. Why are there so many different forms of peroxidase enzyme all possessing the same catalytic activity? What is the significance of some peroxidase isoenzymes being located in all parts of a cell while other peroxidase isoenzymes are located only in particular organelles, membranes, or walls? Peroxidase is thought to be involved in lignin formation (12). Are all peroxidase isoenzymes associated with this activity, or are just certain isoenzymes involved? Peroxidases from numerous plants are known to exhibit IAA oxidase activity (8). This is an important function in regulating growth in plants; but do all of the peroxidase isoenzymes have this capability to degrade IAA or do only a few possess it? Put another way—maybe some peroxidase isoenzymes are much more important in IAA oxidation than others. Total peroxidase activity generally increases with aging, wounding, pathogen invasion, and exposure to ethylene (6). Does the total complement of peroxidase isoenzymes respond to these factors or do only certain isoenzymes increase? More important, does the increased activity come from pre-existing isoenzymes or are new, specific isoenzymes formed *de novo?*

Our ability to answer these questions will parallel our ability to achieve reliable separations of the entire complement of peroxidase isoenzymes and to measure each one's kind and degree of catalytic activity.

Shannon's review (10) in 1968 of plant isoenzymes listed 36 studies to determine the number of peroxidase (EC 1.11.1.7) isoenzymes in numerous species. Of these, seven dealt with HRP.[1] Isoenzymes were separated by electrophoresis (most commonly) column (ion exchange) chromatography, and differential precipitation. Two to seven isoenzymes of HRP were listed with five isoenzymes reported most frequently.

The techniques of IEF in polyacrylamide gels (tubes or slabs) came into use about 1969 (9) with the commercial production of carrier ampholytes, which are needed to establish a pH gradient from about 3 to 10 in the medium used for separations. Ideally, amphoteric isoenzymes migrate (in an electric field) to a pH where their electric charge is zero and then stop or "focus." This pH at which an isoenzyme will focus is called its isoelectric point or pI. With the carrier ampholytes currently available, isoenzymes that differ in pI by 0.01 pH units can be separated (9). This degree of resolution is not normally obtained by conventional electrophoresis or column chromatography.

Recent studies of HRP by IEF have shown many more than seven isoenzymes. Delincée and Radola (3) performed IEF separations on plates covered with Sephadex[2] G-75 gel and detected 20 distinct isoenzymes of commercial HRP (Boehringer with RZ = 0.6) after staining with *o*-toluidine. Gove and Hoyle (5) did IEF with polyacrylamide gel in tubes, and also detected 20 isoenzymes of commercial HRP (Nutritional Biochemicals Co. with RZ = 1) when stained with either benzidine or scopoletin.

Continuing work in my laboratory indicated that further improvements in resolution, stability, and linearity of pH gradients, reproducibility, and staining of the isoenzymes could all be achieved. In this paper, I report on the development of an IEF technique in polyacrylamide gel slabs that shows more than twice the number of commercial HRP isoenzymes reported previously. Also, I present evidence that all of the isoenzymes exhibit both peroxidase and IAA oxidase activity to the same degree.

[1] Abbreviations: HRP: horseradish peroxidase; IEF: isoelectric focusing; RZ: a German abbreviation for the absorbance ratio $A_{403\ nm}/A_{275\ nm}$, which indicates the degree of purity of the enzyme; generally RZ = 3.0 is considered to be highly purified HRP; FBBB: Fast Blue Salt BB from Sigma Chemical Co., No. F-0125; bis: N,N'-methylene bis-acrylamide; TEMED: N,N,N',N'-tetramethylethyldiamine.

[2] Mention of a particular chemical or product should not be taken as endorsement by the Forest Service or the United States Department of Agriculture.

MATERIALS AND METHODS

Initially, the various problems with gel preparation, sample application, electrical parameters, run time, measuring pH gradients, staining isoenzymes, documenting results, and recording results were worked out. Then the general approach was to compare the zymograms for each of three commercial sources of HRP obtained with each of three commercial sources of carrier ampholytes.

Commercial Sources of HRP. Sources were: Sigma Chemical Co. Type II, RZ = 1.0 to 1.5; Worthington Biochemical Corp., RZ = 1.14; and Nutritional Biochemicals, RZ = 1.0. Stock solutions of 1 mg/ml were prepared fresh each day.

Commercial Sources of Carrier Ampholytes. A-l ampholytes were 40% (w/v) and wide range. All gel slabs were made up with 3% ampholytes. Trade names were used to distinguish among ampholyte sources: Ampholines, pH 3.5 to 10 (LKB, Rockville, Md.); pHisolytes, pH 2 to 10 (Brinkmann Instruments, Westbury, N.Y.); and Bio-Lytes, pH 3 to 10 (Bio-Rad Laboratories, Rockville Centre, N.Y.).

Preparation of Gel Slabs. Two glass plates (24 × 16 × 0.3 cm) were used for a gel former. One plate was coated lightly with silicone stopcock grease and set aside for 15 min. Then a portion of polyethylene tubing was laid (65 cm long and 1.57 mm outside diameter) in a straight line, 1 cm in from a long side, on the greased plate. A clean plate was placed directly over the tubing and the edges were clamped together with three small, binder paper clips (The Mattatuck Mfg. Co., Westbury, Conn.). Laying in the tubing was continued in this fashion along the bottom and up the remaining long side of the glass plates. Corners were made as square as possible without kinking the tubing. The upper end was left open. The gel slab former was placed upright and filled to 1 cm from the top with a 5% (w/v) polyacrylamide gel (ammonium persulfate catalyzed) containing 3% (w/v) carrier ampholytes. The gel was made with 23.3 ml of distilled, deionized H_2O, 8.9 ml of a 100-ml stock solution containing 20 g of acrylamide monomer, 0.3 g of bis, and 30 μl of TEMED (with a Hamilton syringe), 2.6 ml of 40% wide range carrier ampholytes, degassed, and then 0.6 ml of a 2% ammonium persulfate solution was added. Total volume was 35.4 ml. The gel was generally well polymerized after 0.5 hr. A thin layer of water formed above the top edge of the gel. After 1 hr of gelation, the water layer was poured off and the clamps, greased glass plate, and tubing were removed. Gel was about 1 mm thick.

Sample Application. HRP samples were injected into 1-cm slits cut into the gel, or injected directly without cutting slits using a Hamilton syringe. Sample size was usually 4 μl of HRP stock solutions (1 mg/ml). Application of samples on filter paper was totally unsatisfactory.

Set-up of IEF Chamber. Filter paper wicks (Whatman 3MM), 3.5 × 15 cm, were placed on each end of the gel so their inner edges were 16.5 cm apart. Enough anolyte solution (0.1 M HCl) was applied to just saturate one paper wick, and enough catholyte solution (0.5 M NaOH) was applied to just saturate the other paper wick. Carbon electrodes, 0.7 cm diameter and 16.4 cm long, were held 16.5 cm apart by gluing (epoxy) 20-cm glass stirring rods to the top side of the electrodes. This assembly formed a rectangle. The electrode assembly was placed on the gel so each carbon electrode rested on the inner edge of a paper wick. The glass rods were then taped to the bottom side of the glass plate to insure close contact between electrodes and gel. A positive lead was clipped to the anode with a battery clip, and a negative lead was clipped to the cathode. The entire unit—glass plate with gel and electrodes— was placed in a glass baking dish of slightly larger size, which in turn was placed on an aluminum cookie sheet. A plastic cover was placed over the baking dish and the electrode wires were brought through holes cut in the top. This unit was then placed in a cold room at 5 C and connected to a power supply.

Operation of Power Supplies. Use of power supplies giving constant voltage, constant current, or constant pulsed power did not give any better resolution than was obtained in previous IEF work (5). Given this experience and the arguments put forth by Allington *et al.* (1), I decided to use constant, continuous power for the IEF studies presented here. Initially, a nonregulated power supply (Buchler Instruments, Fort Lee, N.J.) was used. Constant, continuous power was obtained by continuously monitoring the power supply, reading the voltage and current, multiplying the two parameters to get the power level, and then manually readjusting the power supply to maintain a constant power level. In practice, readings, calculations, and readjustments were made every 15 min. This kept the power regulated between 1.5 to 3 w. Run times were 6.5 hr. This was determined by placing hemoglobin samples at opposite ends of the gel slab and stopping the run when the two samples were side by side.

In later work, a continuous constant-power supply was used (ISCO, model 492, Lincoln, Neb.). With such a power source, the desired wattage was dialed in and automatically maintained at a constant level. An applied constant power of 2.5 w for 6.5 hr gave comparable results to those obtained with the manually regulated power supply. Later in this work, an overnight run of 17 hr with continuous constant power of 1 w was found to give the sharpest resolution of all.

Measurement of pH Gradients. At the end of a run, the electrode assembly was removed from the gel slab. pH measurements were made directly on the gel at 1-cm intervals with a microelectrode (Ingold Electrodes, Inc., Lexington, Mass.).

Isoenzyme Staining for Peroxidase. Peroxidase activity was detected with 3-amino-9-ethyl carbazole. I used a modification of the method described by Shaw and Prasad (11). Buffer strength was increased from 0.05 M to 2 M at pH 5, the 0.1 M $CaCl_2$ was deleted, development was carried out at room temperature, and no glycerine was used as a fixative. The gel slab was simply rinsed with water after pouring off the reaction mixture.

Isoenzyme Staining for IAA Oxidase. The method used to visualize IAA oxidase activity on the IEF slabs was a modification of that reported by Endo (4). It was specifically designed to obtain the maximum IAA oxidase activity of HRP as reported in earlier work (7). Three solutions were prepared: A: FBBB, 4 mg/ml dissolved in ethanol; B: 2 μM *p*-coumaric acid (16.4 mg), 2 μM IAA (17.6 mg), each dissolved in a few ml of ethanol, and 2 μM H_2O_2 (2.4 ml of 0.1% solution) were mixed and made up to final volume of 50 ml with water; C: 2 M sodium acetate buffer (pH 4.2). Staining solution was 25 ml A plus 50 ml B plus 25 ml C. This solution was poured gently over the slab for about 0.5 hr at room temperature, then rinsed off with water.

Documenting Results. IEF gel slabs were photographed on Polaroid black and white positive/negative film type 105 (8.2 × 10.8 cm); Polacolor 2 Land film type 108, same dimensions; and 35-mm slides with Kodachrome 64, KR 135-20. Pictures and slides were taken on the day of development and again on the following day when very faint bands were more distinct.

Recording Results. Free-hand drawings of the isoenzyme banding patterns were made on graph paper with 1-mm lines to actual size. A clear plastic metric ruler laid along the edge of the gel insured the accuracy of band placement on the drawings. Six levels of staining intensity were subjectively determined and designated with different symbols.

RESULTS

Diagonal Runs. Maximum separation of isoenzymes was achieved by placing the enzyme samples in eight positions along a diagonal, from upper left to lower right, of the polyacrylamide

slab (Figs. 1 and 2). Hence there were eight replications of the enzyme sample/slab. Each commercial source of HRP was tested in this manner with each commercial source of carrier ampholyte. Anionic isoenzymes were best separated and resolved on the left half of the gel, and cationic isoenzymes were best separated and resolved on the right side of the gel (Figs. 1 and 2). Using this technique, the number of isoenzymes in each column was counted, and then the total number of isoenzymes was determined for a given combination of HRP and ampholyte source by making a composite of the eight separate analyses.

A large number of isoenzymes spanning the entire pH gradient was found for each combination of enzyme and ampholyte source; but there were distinct variations in the isoenzyme pattern of each HRP source so they could be easily distinguished one from the other. These differences in banding occurred mainly at the anionic (lower) end of the slab (Fig. 3A). Differences in banding patterns among ampholyte sources for a given HRP source were minimal. They occurred mainly in the minor (low intensity staining) bands.

However, there were differences among ampholyte sources in their effects on distance of isoenzyme migration—all other chemical and electrical parameters being equal. Essentially, Bio-Lytes and pHisolytes restricted all of the isoenzymes to the 16.5-cm length of the gels, whereas Ampholines usually exhibited greater spreading of the entire profile. As a result, extreme anionic and extreme cationic isoenzymes migrated off the ends of the gel and into the filter paper wick (compare Fig. 1 with Ampholines *versus* Fig. 2 with pHisolytes).

Side-by-Side Runs. In order to determine whether a particular isoenzyme was common among enzyme sources, dual samples of each enzyme source were placed at the same location across the gel (7.4 cm from anodic end) and run as before. Similarities and differences were easily seen by this technique (Fig. 3A). Most bands were visible on the day of staining, but light bands were much more visible the day after staining. This additional staining occurred while the gel slab was wrapped in clear plastic and kept overnight at 5 C. For this reason, pictures of the developed gel slabs were routinely taken on the day of development and again on the following day. The light staining bands occurred mostly in the anionic half of the gel and were considerable in number. These would not have been seen if the gels had been "fixed" to stop the enzyme reaction, or had been discarded at the end of the day of development.

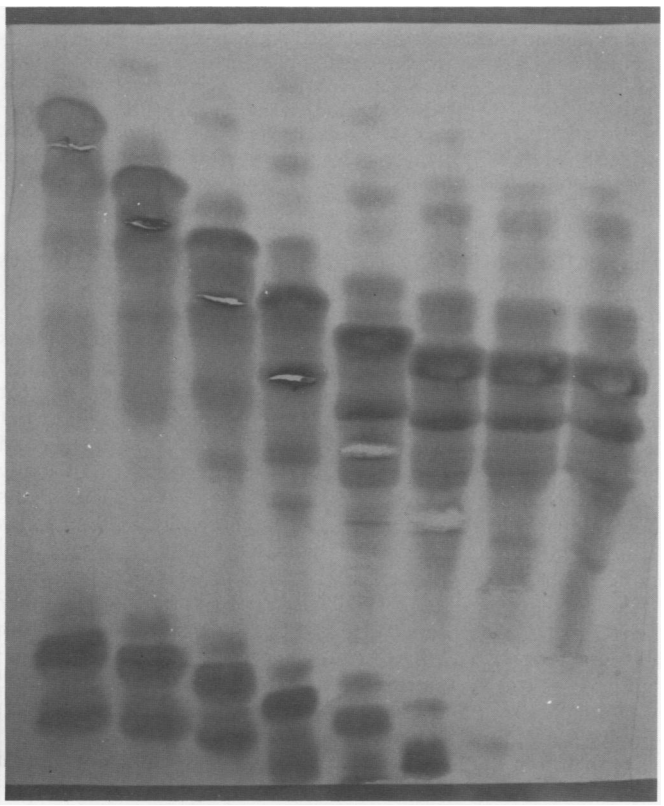

FIG. 1. Diagonal IEF of Sigma HRP with LKB Ampholines and constant power. Constant power maintained manually between 1.5 and 3 for a run time of 6.5 hr. Measured pH gradient was linear from 4.1 to 9.5. Cathode (−) at top of gel. Anode (+) at bottom of gel. Enzyme samples (see under "Materials and Methods") applied in 1-cm slits. Gel stained for peroxidase activity. Pictures taken on day of staining.

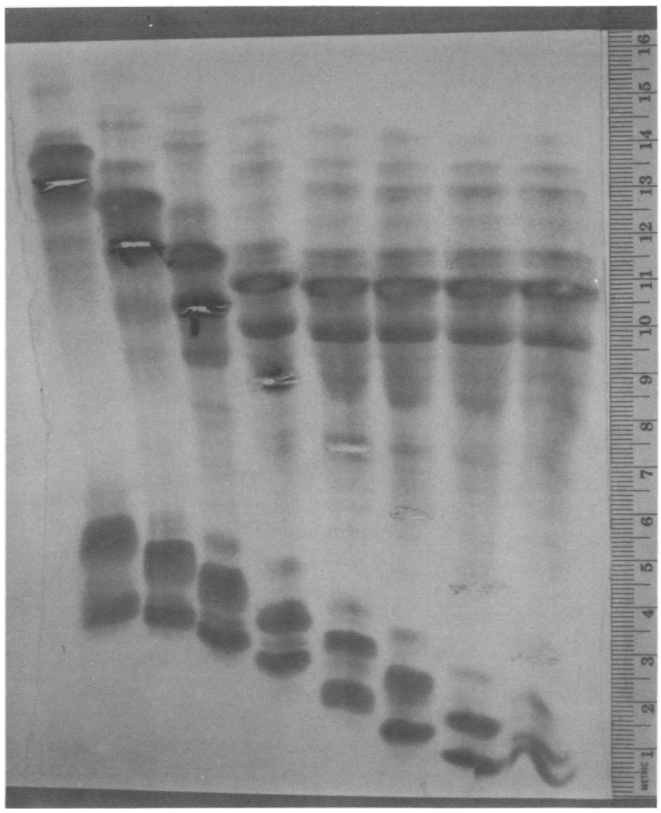

FIG. 2. Diagonal IEF of Sigma HRP with Brinkmann pHisolytes and constant power. Power and run time as in Figure 1. Measured pH gradient was linear from 3.2 to 9.5. Electrical orientation of gel, sample application, and gel staining as in Figure 1. Picture taken on day of staining.

Varying the Amount of Applied Enzyme. Two questions arose after observing many IEF gel slabs run with the same amount of enzyme/sample application: (*a*) were the dark bands actually single bands or were there several dark bands located very close to one another; and (*b*) was enough enzyme applied to visualize all of the very light bands? Sigma HRP samples from 0.5 to 16 μg were spaced in six positions across the gel at 7.4 cm from the anodic end and IEF run as usual (Fig. 4). The number of bands visualized increased from 18 at the lowest concentration to 37 at the highest concentration. The standard sample concentration (4 μg = 4 μl of 1 mg/ml stock) showed 36 bands. All of the dark staining bands appeared as a single band, but more light staining bands appeared at the higher concentrations. Again, this was especially true on the day after initial development.

IAA Oxidase Activity. In an earlier report (5), we tentatively concluded that all isoenzymes of HRP also contained equivalent IAA oxidase activity. The conclusion was dramatically substantiated in this work using the improved IEF technique with much greater resolution. All three sources of HRP showed banding

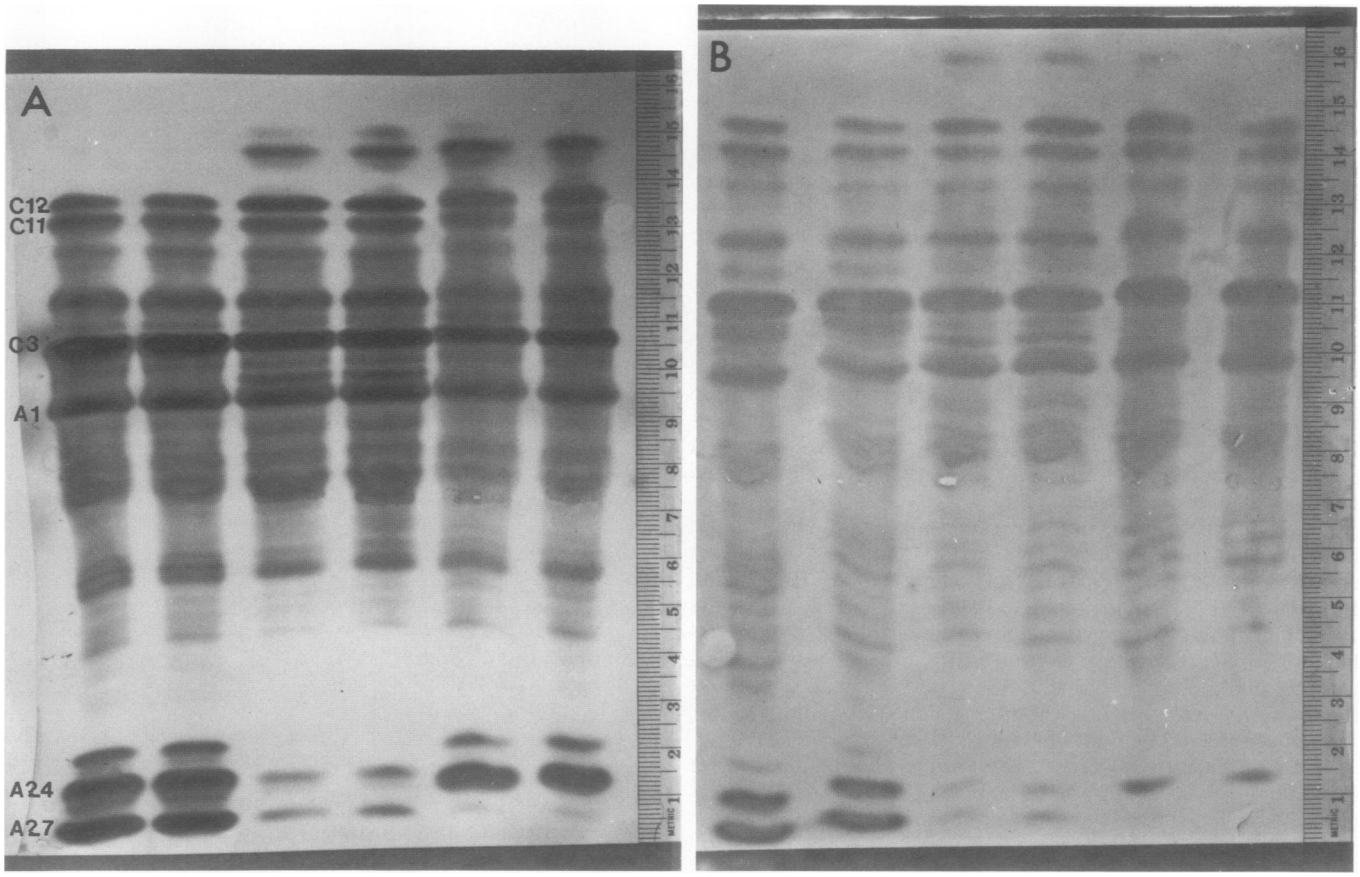

FIG. 3. A: Side-by-side IEF of Sigma HRP (left pair), Worthington HRP (middle pair), and Nutritional HRP (right pair) with Bio-Rad Bio-Lytes and continuous constant power maintained automatically at 1 w for a run time of 17.5 hr. Measured pH gradient was linear from 3.3 to 9. Electrical orientation and gel staining as in Figure 1. Enzyme samples injected directly into the gel at 7.4 cm above the anode. Picture taken 1 day after staining. Some key bands are labeled. B: Side-by-side IEF, stained for IAA oxidase activity, with Sigma HRP (left pair), Worthington HRP (middle pair), and Nutritional HRP (right pair) with Bio-Lytes and continuous constant power maintained automatically at 1 w for a run time of 17.5 hr. Measured pH gradient was linear from 3 to 9.6. Electrical orientation as in Figure 1. Enzyme samples injected diectly into gel at 7.4 cm above anode. Picture taken 1 day after staining. Uppermost (cathode) band has migrated into the paper wick and is not visible for Worthington and Nutritional HRP.

patterns of IAA oxidase activity that were essentially identical to banding patterns for peroxidase activity (compare Fig. 3, A and B), both in number of bands and degree of staining (*i.e.* that bands staining intensely for one type of activity also stain intensely for the other type of activity, etc.).

Equilibrium Focusing and Run Time. Theoretically the end of an IEF run comes when all of the isoenzymes have migrated to their respective isoelectric points (*i.e.* they have all reached a pH where their net charge is zero). Figures 1 to 4 in this paper show clearly that horseradish roots contain a large number of isoenzymes that cover or may exceed the limits of the various wide range carrier ampholytes used in this study.

A longer than usual run time was tested on Sigma HRP with wide range Ampholines to see if any of the isoenzymes could be brought to theoretical focusing at their true isoelectric points (Fig. 5). The measured pH gradient for this run spanned the range from 3.9 to 9.2 and was essentially linear throughout. Isoenzymes from about 3 to 9 cm (*i.e.* pH 5.2–6.9) were apparently focused. Those isoenzymes above and below this range were not focused, and those on each end had migrated out of the gel. This latter phenomenon meant that the extreme anionic isoenzymes had isoelectric points below pH 3.9 and the extreme cationic isoenzymes had isoelectric points above 9.2. With Bio-Lytes and pHisolytes, pH gradients down to (and sometimes below) pH 3 could be obtained. This lower pH level appeared to stop the migration of the extreme anionic isoenzymes at the bottom (*i.e.* positive end) of the gel, but this was for normal run times. The highest pH measured was usually around 9.5 regardless of ampholyte source. I tried flushing the chamber with N_2 to get a higher pH, but this approach was unsuccessful.

As a consequence of these results, I have adopted, for practical purposes, the definition of an end point for IEF as put forth by Chrambach *et al.* (2). He suggested defining the end point, ". . . as the time at which components are maximally resolved in a clearly recognizable pattern type. . . ." The results presented in this paper easily satisfy the requirements of this definition. The patterns are recognizable, highly resolved, and can be reproduced consistently with little or no deviation.

Number and Activity of HRP Isoenzymes. The total number of HRP isoenzymes composited for each enzyme source from the three runs with different sources of carrier ampholytes was: Sigma: 40; Worthington: 33; and Nutritional: 33. A composite of all enzyme sources gave a total of 42 HRP isoenzymes (Fig. 6).

Compared to the final composite, Sigma HRP was missing two cationic bands and no anionic bands. Worthington HRP exhibited all of the cationic bands, but was missing nine anionic bands. Nutritional HRP was missing two cationic bands (not the same two as Sigma) and seven anionic bands (Table I). Missing anionic bands common to both Worthington and Nutritional were A16, A19, A20, and A26.

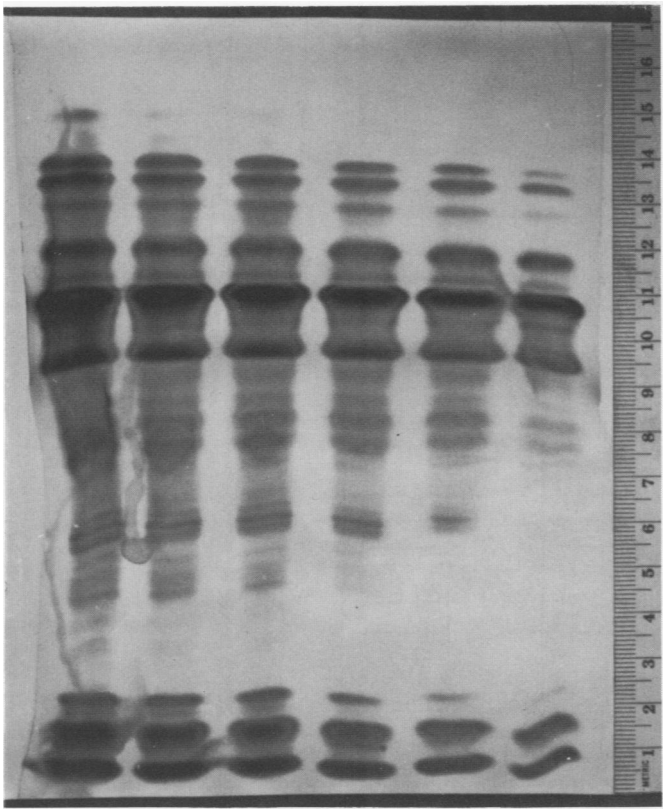

FIG. 4. Side-by-side IEF of Sigma HRP at various enzyme concentrations, with Bio-Rad Bio-Lytes and continuous constant power maintained automatically at 1 w for a run time of 17.5 hr. Measured pH gradient was linear from 2.5 to 9. Electrical orientation and gel staining as in Figure 1. Enzyme samples injected directly into the gel at 7.4 cm above the anode. Enzyme sample sizes were, from left to right, 16 μg, 8 μg, 4 μg (standard sample size used routinely), 2 μg, 1 μg, and 0.5 μg. Picture taken 1 day after staining.

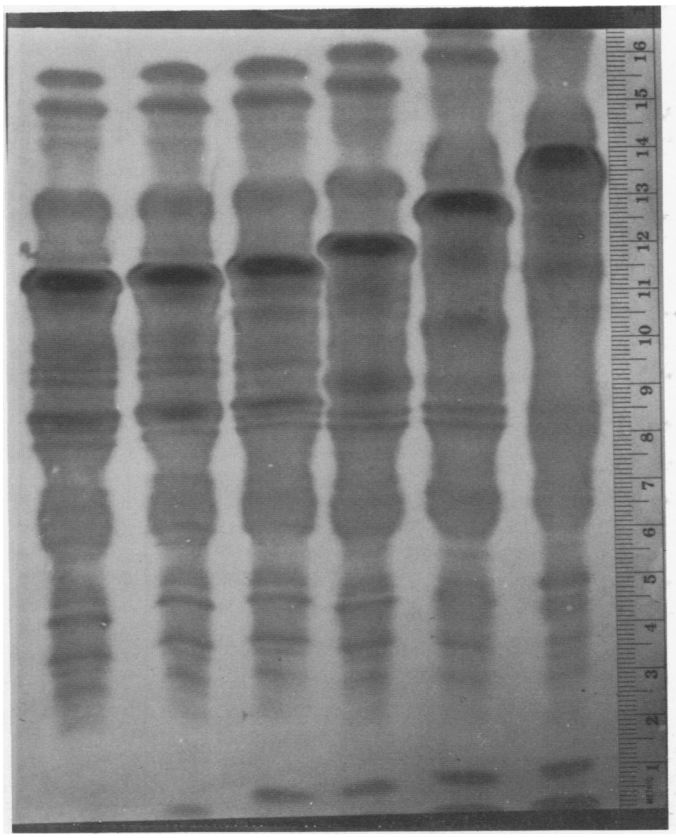

FIG. 5. Diagonal IEF of Sigma HRP with LKB Ampholines and continuous constant power for a longer run time to attempt focusing of middle range isoenzymes at their isoelectric points. Continuous constant power maintained automatically at 1 w for a run time of 21 hr. Measured pH gradient was linear from 3.9 to 9.2. Electrical orientation and gel staining as in Figure 1. Enzyme samples injected directly into the gel. Picture taken 1 day after staining.

Qualitatively, the final composite exhibited four major bands, 16 medium bands, and 22 minor bands (Fig. 6). Most of the minor bands were anionic.

The most distinguishing characteristic in banding patterns among the three commercial sources of HRP was the variable degree of staining in isoenzymes A24 and A27 (Figs. 3A and 6). In Sigma HRP, they were both darkly stained. In Worthington HRP, they were both moderately stained; and in Nutritional HRP, A24 was darkly stained while A27 was only faintly stained (Fig. 3, A and B).

Although cationic bands C13 and C14 were reported as missing in Sigma HRP run at the standard concentration of 4 μg (refer to Fig. 3A and Table I), there was evidence (see Fig. 4, left column) that they were present but at very low concentrations.

DISCUSSION AND CONCLUSIONS

Isoenzyme Designations for IEF. There are, at present, no standard methods for marking isoenzyme banding patterns obtained by IEF. Ideally, the isoenzymes would be labeled according to their respective isoelectric points; but there were several cationic and anionic isoenzymes of HRP whose isoelectric points exceeded the pH limits obtained with any of the wide range carrier ampholytes.

These problems were encountered in our earlier work (5). We arbitrarily labeled only the three darkest bands as A, B, and C. Their estimated isoelectric points were 6.1, 6.9, and 8.9, respectively. In this study, the isoenzymes corresponding to the A, B, and C of our earlier work were A6, A1, and C3, respectively (Fig. 6).

With the standard conditions adopted for this work, all of the HRP isoenzymes were retained on the gel, but only those in the lower middle portion of the profile were actually focused (Fig. 3A). The A1 band always stained darkly, and appeared at pH 6.9. This A1 band was used, therefore, as the major reference point for labeling all other isoenzymes in a manner that is commonly used in bidirectional electrophoresis. The A1 label means it was the first anionic band from neutrality. Accordingly, the C1 label means it was the first cationic band from neutrality. The rest were labeled in numerical sequence.

With this system, it was possible to label IEF IAA oxidase-peroxidase bands obtained from other plants relative to HRP bands (unpublished data). Sigma HRP was placed in the left column of the gel slab, and enzyme samples extracted from other plants were placed at the same level in the remaining five columns. After staining, the A1 band of Sigma HRP was located, and all other bands in the other five columns were labeled in reference to it as described above.

Important Parameters. A number of conditions were met in every run to achieve the high resolution of HRP isoenzymes presented in this paper. They were as follows. (*a*) Gels were made 5% in polyacrylamide concentration and polymerized with ammonium persulfate (refer to "Materials and Methods"). (*b*) A 3% concentration of carrier ampholytes in the gel slab gave a lower resistance than 2% or 1%, and always produced a linear pH gradient in the gel. No "plateau effect" (9) was ever detected around pH 7. Bio-Lytes and pHisolytes retained ex-

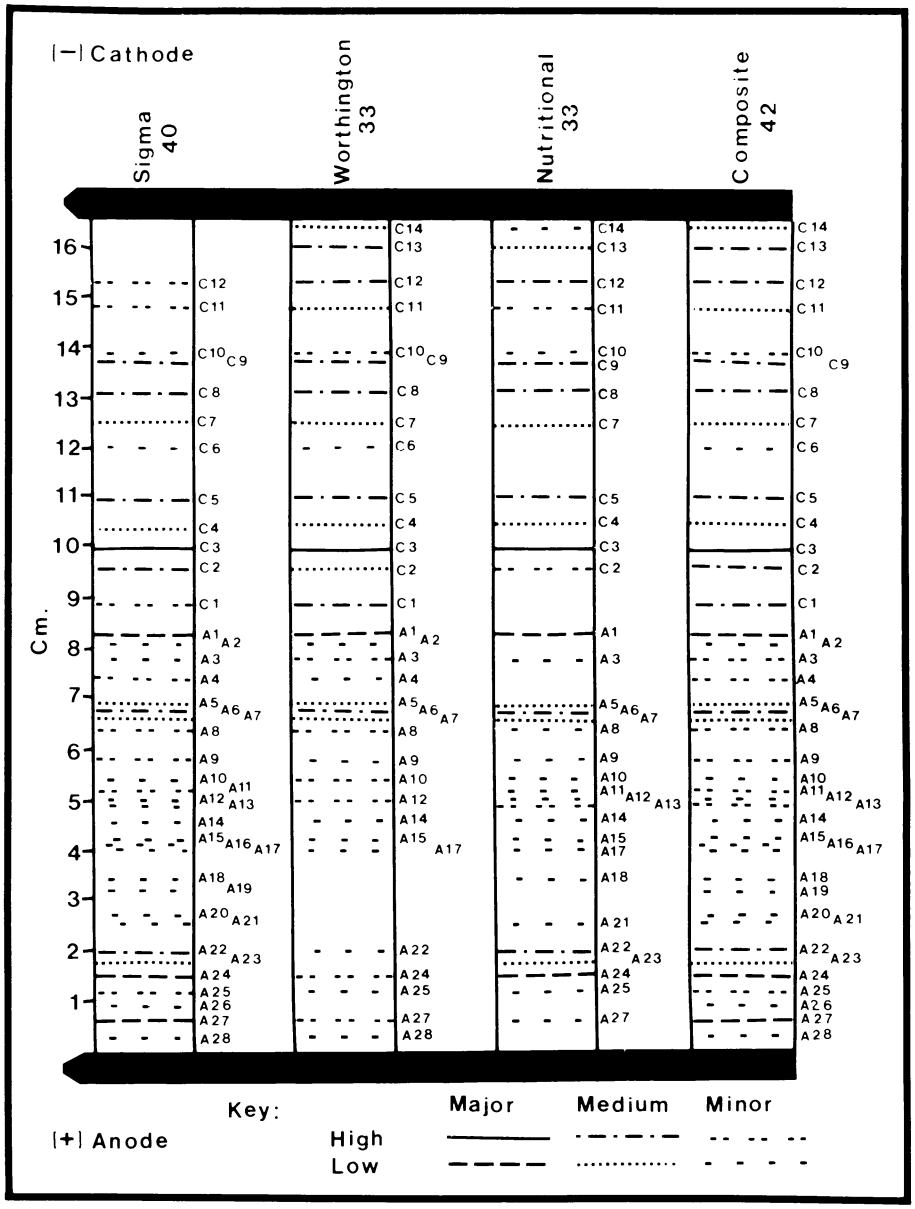

FIG. 6. Individual composite isozymograms for each commercial source of HRP summarized from tests with the three commercial sources of carrier ampholytes plus an over-all composite isozymogram of three commercial sources of HRP.

Table I. Differences in the Isoenzyme Banding Patterns Among the Three Commercial Sources of HRP.

Isoenzymes missing	Sigma	Worthington	Nutritional
Cationic	C13,C14	none	C1,C6
Anionic	none	A11,A13,A16, A18,A19,A20, A21,A25,A26	A2,A4,A16, A19,A20,A26, A28

treme anodic bands on the gel, and produced more compact bands than Ampholines. (*c*) Electrodes had to be in close contact with the gel slab. This technique also helped to prevent a "plateau effect" in the pH gradient (9). (*d*) The single most important condition to be met was the use of continuous constant power to insure high resolution of isoenzymes. Most of the results in this paper were achieved with manual or automatic control at 2.5 w for 6.5 hr, but the highest resolution was obtained with automatically regulated constant power of 1 w for 17 hr (*i.e.* overnight). (*e*) pH gradients were best measured directly on the gel with a microelectrode designed for this purpose (refer to "Materials and Methods"). (*f*) Best staining was obtained with a high enough concentration of buffer to insure that all isoenzymes were brought into the optimum pH for the peroxidase or IAA oxidase reaction. These were pH 5 and pH 4.2, respectively, in my experience (8). (*g*) For a sensitive peroxidase stain, 3-amino-9-ethyl carbazole was found to give as good results as benzidine (or any of its derivatives), and, to my knowledge, is noncarcinogenic. FBBB was also sensitive as an IAA oxidase stain and gave much better results than the dimethylaminocinnamaldehyde used in our earlier work (5). (*h*) A permanent record of the IEF results was easily obtained with Polaroid film. Black and white or color film gave equally good results, but the former also gave a usable negative. A clear plastic metric ruler laid along the edge of the gel slab before photographing insured accurate location of bands on the gel. (*i*) Gel slabs were superior to gel cylinders in my experience. Side-by-side comparisons were made easily, and by using thicker gels one could scale up to the preparative level. Sample application (in slits or by injection) was easier also.

Peroxidase Activity *versus* IAA Oxidase Activity. In earlier work (5, 7), evidence was presented which indicated (*a*) that both types of enzyme activity reside on the same molecule; (*b*) that every isoenzyme possesses both types of activity; and (*c*) that an isoenzyme having high peroxidase activity will also have high IAA oxidase activity and vice versa. These earlier conclusions were highly substantiated by the data given in this paper.

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