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A Simple Method of Identifying Peroxidase Isoenzymes from Crude Pea Seed Extracts

Helen C. Lee

Department of Food and Nutrition, California State University, Long Beach, PO Box 15474, Long Beach, California 90815, USA

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Barbara P. Klein

Department of Foods and Nutrition, University of Illinois, 905 S. Goodwin, Urbana, Illinois 61801, USA

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ABSTRACT

Using analytical thin-layer isoelectric focusing (IEF) on polyacrylamide gel (PAG), high resolution of peroxidase isoenzymes was obtained from crude extracts of pea seeds. All of the 18 detected isoenzymes appeared consistently in the three cultivars of pea seeds tested. The pIs ranged from 3·5 to 9·1. Two visualization methods, using o*-dianisidine: H_2O_2 or 3-amino-9-ethyl carbazole: H_2O_2 as substrates were sensitive and specific. Analytical IEF on PAG proved to be a simple and rapid method of detecting multiple forms of pea isoenzymes, and may be applicable to other plant systems.*

INTRODUCTION

The existence of multiple forms of peroxidase (donor:hydrogen peroxide oxidoreductase, EC 1.11.1.7) is well established in various vegetables and fruits (Sequi *et al.*, 1972; Cherry & Ory, 1973; Bugbee, 1975; Kobrehel & Gautier, 1974; Heidrich *et al.*, 1983; Wang & Luh, 1983). Although extensive purification and isolation of peroxidase isoenzymes can be achieved by repeated ion-exchange column chromatography, this procedure is time

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consuming. Application of starch and polyacrylamide gel electrophoresis for studies of isoenzymes patterns has been widely used, but in most cases, sample load was limited.

Preparative isoelectric focusing technique in granulated gels has been available as a powerful tool for protein fraction and partial purification of enzymes (Winter, 1976; Radola, 1977). Delincée & Radola (1970) detected 20 isoenzymes in commercial horseradish peroxidase preparations using preparative gel-stabilized thin-layer isoelectric focusing. Hoyle (1977) obtained a much higher degree of resolution than previously reported; 42 peroxidase isoenzymes were identified from three different commercial horseradish peroxidases using analytical thin-layer isoelectric focusing on polyacrylamide gels.

The isoelectric focusing (IEF) technique using polyacrylamide gels came into use in the late 1960s (Righetti & Drysdale, 1974) with the development of synthetic carrier ampholytes. The carrier ampholytes are needed to establish a pH gradient. Amphoteric isoenzymes migrate in an electric field to a pH where their electric charge is zero (Isoelectric Point or pI), and then focus into a narrow zone at their pI positions. Due to the focusing effect at each pI, the final resolution is, to a great extent, independent of sample volume, sample application, and running time.

The purpose of this experiment was to develop a simple method of identifying peroxidase isoenzymes directly from crude extracts, without any purification, for further studies of peroxidases in food materials, particularly in the application of thermal processing.

MATERIALS AND METHODS

Materials and reagents

Acrylamide, *N,N'*-methylene-bisacrylamide (BIS), *N,N,N,N'*,-tetra-methyl-ethylene-diamine (TEMED), Coomassie Blue R-250, ammonium persulfate, dimethyl formamide, and hydrogen peroxide (30%) were obtained from Eastman Kodak Co., Rochester, New York. Bovine serum albumin, hemoglobin (horse), and *o*-dianisidine were purchased from Sigma Chemical Co., St. Louis, Missouri. Sephadex G-25 columns (PD-10) were purchased from Pharmacia Fine Chemicals, Piscataway, New Jersey. Ampholine (carrier ampholyte solutions), and pre-cast Ampholine PAG plates, pH 3·5–9·5 [2·4% Ampholine (w/v), T-5%, C-3%, 110 × 245 × 1 mm] were the products of LKB-Produkter AB, Bromma, Sweden. All other chemicals were reagent grade and deionized distilled water was used in all preparation steps.

Three pea seed cultivars (*Pisum sativum*, cv. Little Marvel, Progress No. 9, and Wando) were purchased locally and kept at 4°C until used.

Sample preparation

Pea seed powders were prepared by grinding the seeds with dry ice in a Waring Blender for 8 min and extracting for 1 h at 4°C with 4 volumes of 50 mM sodium phosphate (pH 7·0). The slurry was centrifuged at 10 000 g for 1 h in a Sorvall RC-2B centrifuge at 4°C. The supernatant was filtered through 4 layers of cheesecloth and applied to a Sephadex G-25 column for desalting. The protein was eluted with 5 mM sodium phosphate buffer (pH 7·0) and the eluate used directly for isoelectric focusing.

The protein concentration of the eluate was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard. Further dilutions were then made with 5 mM sodium phosphate buffer (pH 7·0) so that the protein concentration of the final sample solution was in the range of 1 to 5 mg ml^{-1}.

Analytical electrofocusing in thin-layers of polyacrylamide gel (PAG)

Preparation of PAG plates

Both pre-cast polyacrylamide (PAG) plates (Ampholine PAG plate) and laboratory prepared PAG plates were used. PAG plates were made by a slight modification of the methods used by Righetti & Drysdale (1976) and Davies (1975). To facilitate polymerization of the gel solution, a combination of all three conventional catalysts, riboflavin, ammonium persulfate, and TEMED, were used, and after the gel was polymerized, it was stored in a cold room (4°C) overnight before being used. The gel plate, 110 × 245 × 2 mm, was composed of 5% total acrylamide (T), 3% cross-linkage (C), and 2·5% (w/v) Ampholine.

Sample application

Before the sample was applied, the gel plate was prefocused for 15 min at 1000 V, 15 W and 20 mA, with cooling water temperature of 8°C, using an LKB 2117 Multiphor system with an LKB 2103 constant power supply.

Sample application was performed by dipping 5 × 10 mm Whatman 3 MM filter paper applicators directly into the sample supernatant, allowing them to absorb to their maximum capacity, and the excess removed by gently touching a clean filter paper surface. Guided by an underlying template to form a straight line at a given distance from the anode electrode strip, the applicators were applied to the surface of the gel, leaving 0·5 cm of free gel space between them. On both types of gel plates, up to 24 samples

could be applied using the applicators. In this experiment, 20 samples were applied side by side, 4 cm away from the anode, and a marker protein, hemoglobin, was applied on both cathode and anode ends of the gel. Duplicates of extracts from each pea cultivar at two different protein concentrations (1·5 mg ml^{-1} and 3 mg ml^{-1}) were applied.

Electrofocusing parameters

For a pre-cast gel plate, the electrical parameters were set at 22 W, 1300 V and 50 mA, and for laboratory prepared plates, the settings were 18 W, 1200 V and 50 mA, using a constant power supply. During the runs, the voltage increased from approximately 280 to 1050 V, while the current dropped from about 50 to 18 mA. As proteins migrated to their pIs, the current stabilized, indicating completion of the focusing, which was also checked by noting the merging of the marker protein samples of hemoglobin in a single brown colored band at its isoelectric point. To ensure that all of the peroxidase isoenzymes were focused at their respective pIs, IEF was run for an additional 30 min. Total focusing time was 2 h with pre-cast gels and 2·5 h with laboratory made gels.

Measurement of pH gradient and peroxidase staining

The pH gradient was measured directly on the surface of the focused gel while it still rested on the cooling plate of the Multiphor apparatus by using a surface electrode (Markson Science Inc., Model 1888). The measured pH gradient was linear from 3·5 to 9·5.

Staining procedures. Isoenzyme patterns were detected by staining half of the gel with 3-amino-9-ethyl carbazole:H_2O_2 or *o*-dianisidine:H_2O_2. The *o*-dianisidine:H_2O_2 visualization method was similar to the method of Cullis & Kolodynska (1975). Fifty milligrams of *o*-dianisidine, wet with a few drops of 95% methanol, were dissolved in 76 ml 1 M citrate phosphate buffer (pH 4·6). Then, 0·5 ml of 30% H_2O_2 were added just before staining. The gel was incubated with the substrates for 30 min at room temperature until the reddish brown peroxidase bands were visible. The gel was rinsed with water and preserved in a 20% (v/v) glycerine solution.

The 3-amino-9-ethyl carbazole method was a slight modification of the method of Shaw & Prasad (1970). Fifty milligrams of 3-amino-9-ethyl carbazole were dissolved in 5 ml dimethyl formamide, followed by the addition of 94·5 ml of 1 M sodium buffer (pH 5·0) and 0·5 ml of 30% H_2O_2. The gel was incubated in the solution at room temperature until the peroxidase bands were stained brownish red, approximately 30 min. The gel was rinsed with H_2O and preserved in a 20% (v/v) glycerine solution.

A portion of the gel was stained for protein with Coomassie Brilliant Blue

R-250. The gel was first soaked in 10% trichloroacetic acid for 1 h to remove the carrier ampholytes, with 2 to 3 changes of the solution. The proteins were then stained with 0·25% Coomassie Brilliant Blue R-250 dissolved in methanol/distilled water/acetic acid (5:5:1, v/v/v). After staining overnight, the gel was rinsed with deionized water and placed in destaining solution, ethanol/distilled water/acetic acid (3:10:1, v/v/v), with frequent changes of the solution.

Determination of pI of isoenzymes

After preserving in the 20% glycerine solution 2 h overnight, the gel was placed on a thin polyethylene sheet (125 × 260 mm) and allowed to dry at room temperature until the surface of the gel became sticky. Another polyethylene sheet was rolled over the sticky gel surface with a paint roller to preserve the gel plate. The pI of each isoenzyme was determined by superimposing the preserved gel plate on the pH gradient curve. The gels could be permanently preserved by keeping them in 20% glycerol solution or by covering with polyethylene sheets.

RESULTS AND DISCUSSION

Optimum protein concentration appears to be important in assessing the isoelectric isoenzyme patterns as can be seen in Fig. 1. At lower protein concentrations (Fig. 1B), the isoenzymes present in small quantities, such as those with pIs of 4·5 and 5·1, could not be clearly distinguished. Concentrations of protein higher than 5 mg ml^{-1} resulted in smearing or streaking of the patterns. In this experiment, crude extracts with protein concentrations of 2·5 to 3·0 mg ml^{-1} consistently produced distinct and stable bands.

Analytical thin-layer IEF on PAG plates resulted in the resolution of 18 peroxidase isoenzymes from the crude extracts of the pea seeds. These isoenzymes had pI values ranging from 3·5 to 9·1 (Fig. 1). Major bands were produced at pIs of 9·0, 8·1, 7·9, 7·7, 6·9, 6·6, 3·7 and 3·5. Minor bands were produced at pIs of 9·1, 8·8, 8·7, 8·4, 5·7, 5·5, 5·1, 4·5, 4·0 and 3·6. All 18 isoenzymes could be detected with either staining method (*o*-dianisidine:H_2O_2 or 3-amino-9-ethyl carbazole:H_2O_2). Results were similar on the pre-cast and the laboratory prepared gels. All three cultivars tested produced similar isoenzyme patterns.

The number of pea isoenzymes isolated by IEF in this experiment exceeds the number of pea peroxidase isoenzymes previously reported by other investigators who used other separation techniques. In 1966, Macnicol (1966) found that Alaska pea seeds had four isoenzymes, three cathodic and

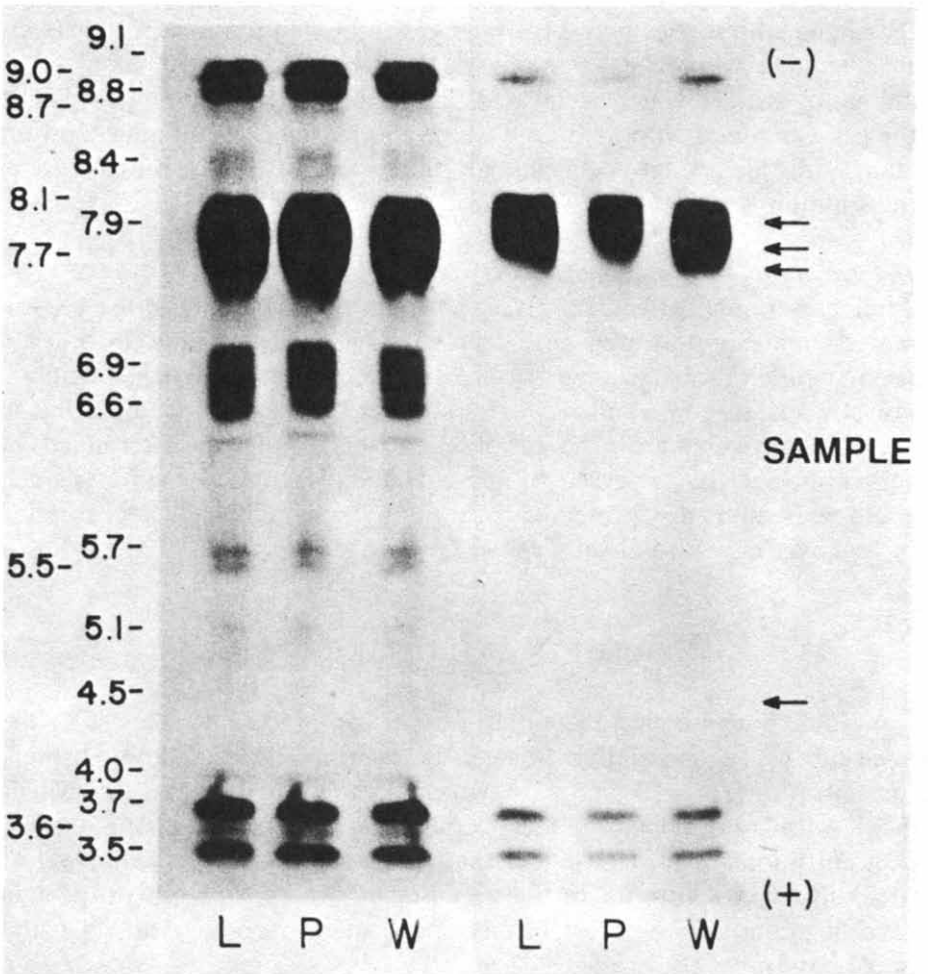

Fig. 1. pI values of pea peroxidase isoenzymes from crude extracts of three pea cultivars (Little Marvel, L; Progress No. 9, P; Wando, W) using 3-amino-9-ethyl carbazole as hydrogen donor. Arrows indicate bands which were not reproduced on photograph, but were visible on gels.

one neutral, by cation ion-exchange chromatography. Mills & Crowden (1968) found the presence of at least 17 different isoenzyme bands from six different stages of early development of *Pisum sativum* (pea plant) using polyacrylamide gel electrophoresis. However, all the detected 17 isoenzymes were not present at any one developmental stage; only five to ten different isoenzyme bands were identified at a single stage of development. Sano (1970) isolated two kinds of peroxidases from Alaska pea seedlings, Alaska pea peroxidase b and b′. They showed different absorption maxima and

electrophoretic mobilities. Peroxidase b′ was found to contain at least five isoenzyme components. Gadia (1977) detected a total of 21 different isoperoxidase bands in four pea cultivars at three different developmental stages and from the dry seeds. However, not more than four to ten isoenzymes were visualized in any one cultivar or in any one stage of development. Gadia (1977) examined the isoenzyme patterns of peroxidase using starch gel electrophoresis. From the crude extracts of pea seeds, two major bands and two minor bands were obtained using benzidine, while only two bands were visualized with guaiacol.

Previous workers indicated differences in peroxidase activity with different staining procedures (Burnette, 1977; Delincée & Radola, 1970). In this experiment, preliminary tests were performed with benzidine and guaiacol to detect pea peroxidase isoenzymes separated by IEF on PAG. The sensitivity, specificity, and stability were much inferior to the two staining methods used in this experiment. The two hydrogen donors used for detection of isoenzymes, *o*-dianisidine and 3-amino-9-ethyl carbazole, showed the same degree of sensitivity and stability.

Several investigators (Delincée & Radola, 1970; Hoyle, 1977) have identified multiple isoenzymes in commercial horseradish peroxidase preparations using isoelectric focusing. Previous workers had noted multiple forms of the enzyme, but the higher resolving power of the isoelectric focusing technique allows better separation. In the present study a large number of peroxidase isoenzymes, a total of 18, were identified from crude extracts of pea seeds without utilizing the conventional tedious methods of purification, such as salt precipitation, repeated ion exchange column chromatography and electrophoresis. The simultaneous application of many samples on a single IEF PAG plate enabled the detection of minute differences between isoenzyme patterns with a much higher resolution than previous electrophoretic methods. This thin-layer IEF method showed excellent resolution of of peroxidase isoenzymes and good reproducibility. Furthermore, the experimental procedure was simple and required a relatively short period of time. The use of either laboratory-made gels or pre-cast gels produced the same results. The two visualization methods used for detection of isoenzymes, *o*-dianisidine:H_2O_2 and 3-amino-ethyl carbazole:H_2O_2 showed equally high degrees of resolution and permanent patterns.

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