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SHORT COMMUNICATION

CHROMATOGRAPHIC SEPARATION OF HORSERADISH PEROXIDASE ISOZYMES FROM COMMERCIAL PREPARATIONS

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Abstract—Chromatographic methods developed by Paul[4] and Shannon and co-workers[5] for separating individual isozymes of horseradish peroxidase (HRP) have been verified for highly purified but heterogeneous commercial preparations of the enzyme. Chromatography on carboxymethyl cellulose (pH 4·4) reveals that both the Worthington and Calbiochem purification procedures remove the more acidic HRP isozymes present in extracts of horseradish roots.

INTRODUCTION

IN 1958, PAUL[4] separated four to five peroxidase fractions from horseradish root extracts by chromatography on CM-cellulose, eluting with acetate buffer, pH 4·6. Recently, Shannon *et al.*[5,6] have purified seven molecular forms of peroxidase from horseradish roots by a combination of CM- and DEAE-cellulose chromatography.

The question arises whether four to seven peroxidase isozymes can also be prepared from commercial preparations of the enzyme. We had occasion to examine this question during the course of an investigation into the nature of the sugar-protein linkages in horseradish peroxidase (HRP). We found it necessary to separate and purify individual isozymes from highly purified but heterogeneous preparations of the enzyme. Using chromatographic methods similar to those of Paul[4] and Shannon and co-workers,[5] we have succeeded in purifying the more basic HRP isozymes present in several commercial preparations.

RESULTS AND DISCUSSION

Figure 1 (top and bottom) indicates that four to five distinct HRP isozymes can be separated from Worthington preparations by chromatography on CM-cellulose, pH 4·4. However, only several broad peaks can be discerned (Fig. 1, middle) in the Calbiochem preparation. Most likely, this preparation contains a greater number of isozymes closely related in charge than does the Worthington preparation.

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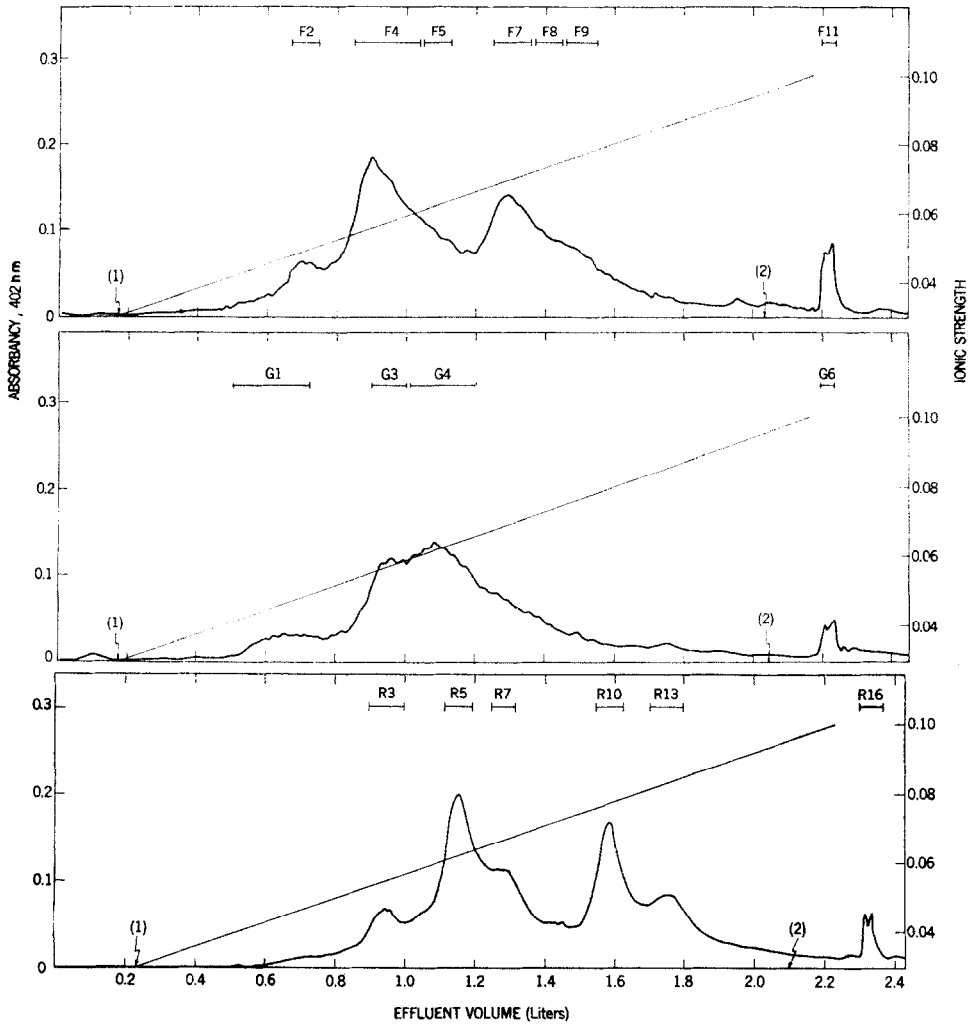

FIG. 1. COMPARISON OF THE ELUTION PROFILES OF WORTHINGTON ELECTROPHORETICALLY PURIFIED HRP AND CALBIOCHEM HRP ON CM-CELLULOSE.

Top: 50 mg of Worthington electrophoretically purified HRP (Batch No. HPOFF 6523S) were placed on a 33 × 2·6 cm column of *fibrous* CM-cellulose and eluted with a linear gradient of 0·03 to 0·1 M acetate buffer, pH 4·4, 1 l. of each buffer (Point 1).

Middle: 50 mg of Calbiochem crystalline HRP (Batch No. 54126) were chromatographed on *fibrous* CM-cellulose under the same conditions as above.

Bottom: 45 mg of Worthington electrophoretically purified HRP (Batch No. HPOFF 6520S) were placed on a 44 × 2·6 cm column of *microgranular* CM-cellulose and eluted with acetate buffer, pH 4·4, as above.

At Point (2), each of the above columns was eluted with 400 ml of 0·4 M acetate buffer, pH 4·4.

The Worthington preparation chromatographed in Fig. 1 (bottom) was prepared from horseradish roots by free-flowing electrophoresis. Chromatography on microgranular CM-cellulose instead of the fibrous form greatly improves the resolution and yields five distinct peaks. However, starch gel electrophoresis of the unfractionated preparation shows

only four cathodic bands. Worthington HRP samples prepared by ammonium sulfate fractionation and cellulose chromatography also show four cathodic peroxidase activity bands on starch gel electrophoresis.

Figure 1 also reveals that each of the HRP isozymes in both the Worthington and Calbiochem preparations adheres to CM-cellulose at pH 4·4. Furthermore, no isozymes are present in Worthington preparation No. CHPO 62502A which adhere to DEAE-cellulose at pH 8·5. These results contrast with those of Paul[4] and Shannon *et al.*,[5] each of whom observed a fraction from partially purified extracts of horseradish roots which passed through a CM-cellulose column at pH 4·4 to 4·6 without sticking to the resin. In fact, Shannon *et al.*[5] chromatographed this fraction on DEAE-cellulose and obtained three more fractions which they purified as distinct HRP isozymes after several recycles. These data indicate that both the Worthington and Calbiochem procedures must be removing at least three of the more acidic HRP isozymes present in horseradish roots. Thus, in all of the starch gel electrophoretograms of Worthington HRP we have examined, only in batch No. CHPO 6253 have we detected a faint band of peroxidatic activity moving slightly towards the anode. Klapper and Hackett[7] detected anodic HRP isozymes by starch gel electrophoresis in Calbiochem preparations Nos. 35470 and 35374. They reported, however, that the anodic bands, when present, were found in much lower concentration than the four main cathodic bands and were not detectable in all the preparations they examined.

After four passages through CM-cellulose, the major peaks in the Worthington electrophoretically purified HRP were still contaminated with small amounts of neighboring isozymes when examined by starch gel electrophoresis. A decrease in contaminating neighboring isozymes with each successive cycle indicated that true HRP isozymes were being purified from the preparation, not merely artifacts arising during the purification procedure. A similar conclusion was reached by Shannon *et al.*,[5] who obtained only a single band on polyacrylamide gel electrophoresis after their third rechromatography.

We therefore conclude that the chromatographic procedures of Paul[4] and Shannon *et al.*[5] for purifying HRP isozymes are applicable to commercial preparations of the enzyme. However, such preparations appear to lack the more acidic HRP isozymes. A greater number of chromatographic steps is necessary to purify completely the more basic isozymes in these preparations.

EXPERIMENTAL

The highly purified preparations of HRP used in these experiments were purchased from the Worthington Biochemical Corporation, Freehold, New Jersey, and the California Corporation for Biochemical Research (Calbiochem), Los Angeles, California. From Worthington we obtained twice-crystallized HRP, Batch No. CHPO 62502A. The enzyme purchased was obtained as highly purified crystals in 60 per cent ammonium sulfate (RZ (A_{402}/A_{280}) = 2·72), and was stored at 4°. The Worthington HRP was prepared from roots collected in an area around Bristol, Pennsylvania, or occasionally from St. Louis, Missouri. The purification process involved pressing the roots and subjecting the juice to ammonium sulfate fractionation as described by Theorell,[8] but modified for commercial feasibility. The Worthington procedure was outlined to the authors as follows.[9]

(1) The roots are pressed—no water added. (2) The extracted juice is brought to 0·50 saturation with ammonium sulfate and filtered. (3) The clear filtrate is then brought to 0·80 saturation with ammonium sulfate. (4) This precipitate is then refractionated with ammonium sulfate until maximum RZ is obtained. The number of refractionations varies with the batch of roots (9000 lb). To crystallize the enzyme we start

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with our regular product, RZ approximately 1, and recycle it on a cellulose (Avicel) column using 0·05 M phosphate buffer, pH 7·4. Crystallization is induced by addition of saturated ammonium sulfate to fractions containing the highest RZ (usually about 2·6). On preliminary studies of the purified enzymes we find four distinct bands using polyacrylamide gels according to Reisfeld *et al.*[10]

In addition, we obtained HRP samples from Worthington which were prepared using the Brinkman free-flowing electrophoretic separator (Batch Nos. HPOFF 6520S and HPOFF 6523S). These enzyme preparations were sold as a solution of HRP in 0·005 M phosphate buffer, pH 7, and had an RZ about 3. The enzyme preparation obtained from Calbiochem was Grade A crystalline HRP suspended in 2·8 M ammonium sulfate (Batch Nos. 54125 and 54126) having an RZ about 3. Calbiochem described its procedure for preparing crystalline HRP as follows:[11]

(1) Fresh roots are ground and extracted twice in 15 per cent ammonium sulfate. (2) The extract is refractionated twice with ammonium sulfate at 15 to 95 per cent saturation. (3) The 15 to 95 per cent precipitate is dialyzed exhaustively and refractionated 40 to 85 per cent with ammonium sulphate. (4) The 40 to 85 per cent precipitate is dialyzed vs. 0·003 M sodium hydroxide-glycine buffer, pH 9·6, until salt-free. (5) The above dialysate is fractionated on DEAE-cellulose and fractions having an RZ value greater than 1·0 are pooled. (6) These fractions are concentrated at 85 per cent saturation with ammonium sulphate. The precipitate is collected and redialyzed against the above buffer. (7) The salt-free solution is applied to another DEAE-cellulose column and the material eluted. Fractions with an RZ value in excess of 2·3 are collected and refractionated with ammonium sulphate 53 to 65 per cent. The aforementioned fractions, when suspended in ammonium sulphate, should have an RZ value of 2·8 to 3·0.

Commercial enzyme preparations were stored at 4°. For column chromatography, the enzyme was dialyzed at 4° against the starting buffer and placed directly on the column. The dialysis removed ammonium sulfate and brought the crystalline enzyme into solution.

Chromatography and rechromatography initially were performed on washed, fibrous CM-cellulose (Standard Type, 0·76 mEq/g) purchased from Carl Schleicher and Schuell Co., Keene, New Hampshire. The resin was washed successively with 0·5 M NaOH + 0·5 M NaCl and water, according to the method of Peterson and Sober[12] and columns packed with 2 to 10 lb/in^2 air pressure. In addition, several chromatographic runs were performed on the new Whatman carboxymethyl cellulose CM-32, microgranular form (H. Reeve Angel & Co., Clifton, New Jersey). This resin contains particles more uniform in size than does the fibrous resin, thus improving the resolution of individual isozymes. Whatman CM-32 was washed with 0·5 M NaOH and 0·5 M HCl as described in the company's technical bulletin.[13] After equilibration with starting buffer and removal of fines, the column was poured in one pass using a funnel on top. After washing, the pH of both the fibrous and microgranular CM-cellulose was adjusted to 4·4 by equilibrating the resin with the starting buffer (usually 0·03 M acetate buffer, pH 4·4). All chromatographic procedures were performed at 4°. The column was usually eluted with a linear gradient of 0·03 to 0·1 M acetate buffer, pH 4·4, followed by a washing with 0·4 M acetate buffer, pH 4·4. These conditions differed slightly from those of Shannon and coworkers[5] who used a linear gradient of 0·005 to 0·1 M acetate buffer, pH 4·4, to elute their HRP isozymes from CM-cellulose. The flow rate always was adjusted to 1 ml/min using a Minipump (Milton Roy Co., Model No. 196-31) and 5-ml fractions were collected.

The absorbancy of the eluted fractions was measured at 402 nm in order to monitor the haem moiety of the HRP isozymes emerging from the column. Peroxidatic activity, as measured by the *o*-dianisidine assay,[14] coincided with haem content.

Fractions were combined, dialyzed against water to remove salt and lyophilized. The dry sample was brought up in a small volume of water and stored at −10° for gel electrophoresis. Starch gel electrophoresis was performed at pH 8·1 in a discontinuous system of buffers as described by Klapper and Hackett.[7]

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