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PARTIAL CHARACTERIZATION OF PEROXIDASE ISOENZYMES FROM RUST-AFFECTED WHEAT LEAVES*

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Key Word Index—*Triticum vulgare*; Gramineae; wheat; peroxidase; peroxisozymes; isoenzymes; *Puccinia*; fungi; wheat rust.

Abstract—Four anodic peroxidase isoenzymes from wheat leaves were purified by column chromatography and their kinetic behavior with common substrates were examined. One isoenzyme is more active in wheat resistant to stem rust fungi and differed from the others in carbohydrate content and also by a specific activity 2–4-fold higher with non-physiological electron donors. As a substrate, eugenol exhibited kinetic behavior different from *p*-phenylenediamine, guaiacol or *o*-dianisidine with all isoenzymes. All four isoenzymes showed similar pH and temperature optima and kinetic behavior and apparent K_m values for both H_2O_2 and non-physiological electron donors.

INTRODUCTION

In previous studies [1,2] evidence for at least 14 bands of peroxidase activity was obtained by disc gel electrophoresis of homogenates prepared from normal primary leaves of wheat and leaves infected with the stem rust organism, *Puccinia graminis tritici*. The much higher peroxidase activity of homogenates prepared from infected resistant leaves appeared to be the result of a change of only one of the anodic isoenzymes, designated number 9. Certain observations, however, suggested caution in attempting to assign to particular isoenzymes a quantitative role in the total activity of crude homogenates [2]. Potential inhibitors or stimulators most likely are removed by electrophoresis, for example. More critical are the kinetic uncertainties arising from the choice of experimental conditions and substrates, such as benzidine, which provide stable gel stains but are not directly comparable with the conventional rapid spectrophotometric assay of homogenates [2].

Resolution of the uncertainty can only be accomplished by studies of isolated individual isoenzymes. This also is a requisite if the biological significance of changes in peroxidase activity of diseased plants is to be evaluated. Alterations of isoenzyme patterns occur during development [3], application of growth regulators [4,5], environmental stress, wounding and disease [6], but none of the changes have been linked conclusively with any metabolic processes in which peroxidase has been implicated: IAA degradation [7–9], aromatic oxidation [10,11], lignin formation [12], ethylene biosynthesis [13], oxidation of fatty acids [14] or pyridine nucleotides [15].

Seven horseradish peroxidase isoenzymes were purified by Shannon *et al.* and some physical properties were described [16]. Studies of their catalytic properties [17] were restricted to two substrates, one involved in peroxidative reactions and the second in oxidative reactions. The present paper describes methods for the purification of 4 isoenzymes from infected wheat leaves and examines some catalytic properties with several non-physiological substrates routinely used for peroxidase assay.

RESULTS AND DISCUSSION

Two separate fractionations of extracts from 40 g fr. wt each of primary leaves of wheat were carried out with only minor differences. All the data given below are from the second fractionation. The numbering of isoenzymes was the same as in previous work [1,2] which showed

Table 1. Specific activity of fractions obtained during isolation of peroxidase isoenzymes from rust-affected wheat leaves

	Total activity* (ΔA/min)	Total protein (mg)	Specific activity†
Crude extract	70760	3300	21.4
0–50% $(NH_4)_2SO_4$ fraction	20410	520	39.5
50–95% $(NH_4)_2SO_4$ fraction	45600	1340	33.9
CM-Fraction I	15840	950	16.7
CM-Fraction II	2660	27.7	96.0
CM-Fraction III	80	2.5	32
DEAE-Group 1	11730	7.9	1482

* Phenylenediamine substrate. † ΔA/min/mg protein.

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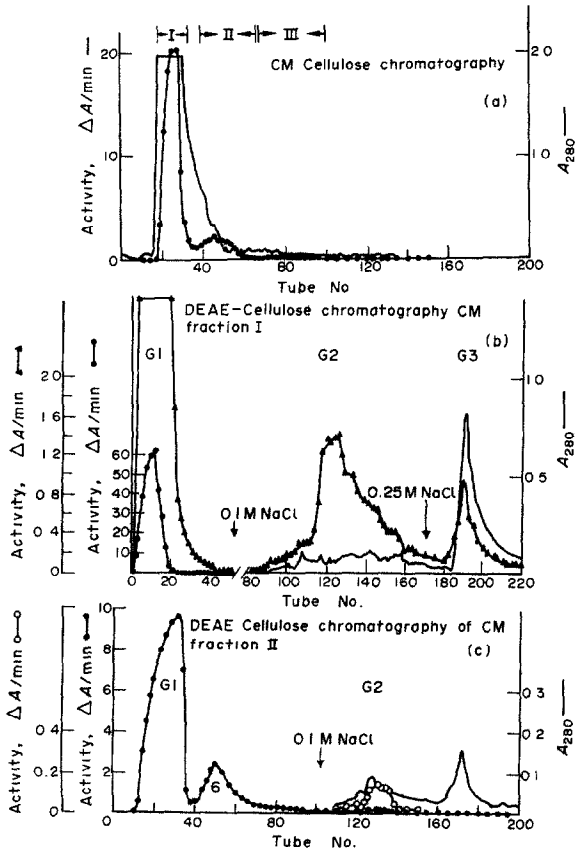

Fig. 1a. Peroxidase elution profile upon CM-cellulose chromatography of 50–95% ammonium sulfate fraction. Column size: 5 × 76 cm; elution buffer: 10 mM Pi, pH 7; fraction volume, 2 ml (·——), ΔA/min tube with phenylenediamine substrate, (——), A_{280}. (See text for description of I, II, III.)

Fig. 1b. DEAE-cellulose chromatography of CM-fraction I. Fractions comprising CM-fraction I were applied to a DEAE-cellulose column (2.5 × 63 cm), equilibrated in 5 mM Tris–HCl buffer, pH 8. Elution systems were: 5 mM Tris–HCl, pH 8, 1 l.; 5 mM Tris–HCl—0.1 M NaCl, pH 8, 500 ml; 5 mM Tris–HCl—0.25 M NaCl, pH 8, 500 ml, in that order. Fraction volume, 5–10 ml. (●——●) and (▲——▲), ΔA/min tube with phenylenediamine substrate (note scale difference); (——), A_{280}.

Fig. 1c. DEAE-cellulose chromatography of CM-fraction II. Similar column and separation conditions were used as in Fig. 4 except that fractions comprising CM-fraction II were applied to the column. (●——●), (○——○), ΔA/min tube with phenylenediamine substrate (note scale difference); (——), A_{280}.

isoenzymes 9 and 10 to be the most significantly affected by disease. Purification procedures were directed toward obtaining good yields of these two isoenzymes.

About 60% of the total activity was recovered in the 50–95% ammonium sulfate precipitate (Table 1). This consisted primarily of anodic isoenzymes. The 0–50% precipitate was mainly cathodic enzymes or else aggregates which did not penetrate the gel at pH 9.3 and it was not studied further.

Nearly all of the activity of the 50–95% ammonium sulfate precipitate was eluted in the first peak (CM-I) from CM-cellulose columns (Fig. 1a). CM-I contained all the anodic isoenzymes except for isoenzyme 6, which was first detected in tube 40 and peaked in tube 50 (CM-II, Fig. 1a). There was some overlap with other isoenzymes, particularly 9, 10 and 11. Although activity in each tube was low, tubes 60 to 100 contained only isoenzyme 6 and these pooled tubes constituted fraction CM-III.

Elution of CM-I from DEAE-cellulose with buffer of increasing ionic strength yielded three groups of isoenzymes: G1, isoenzymes 8, 9, 10, 11; G2, isoenzymes 4, 5, 6, 7; G3, isoenzymes 1, 2, 3 (Fig. 1b). G1 had much more activity than the total activity of G2 and G3 isoenzymes. When fraction CM-II was subjected to the same procedure, G3 isoenzymes were not detected, but additional G1 and G2 isoenzymes were resolved with isoenzyme 6 cleanly separated (Fig. 1c). A small amount of isoenzyme 6 was also detected in G3 separated from fraction CM-I (Fig. 2b), but the question as to whether this represented a separate isoenzyme with the same electrophoretic mobility as the major isoenzyme 6 fraction (Figs 1a and 1c) was not examined. Isoenzyme 5 exhibited similar behavior. Isoenzyme 6 isolated from either fraction CM-III (Fig. 2b) or by chromatography of G2 isoenzymes (Fig. 1c) was used in further work.

Sephadex G-200 chromatography. G1 isoenzymes from both CM-I and CM-II were pooled and concentrated before passage over Sephadex G-200. Peaks corresponding to 10, 9 and a mixture of 8, 9 and 10 were obtained. Rechromatography on Sephadex another 2 or 3 times yielded electrophoretically homogeneous preparations of isoenzymes 6, 8, 9 and 10 with reproducible mobilities of 2.3, 1.5, 1.3 and 0.8 cm from a standard development time of 80 min at 3 mA/tube. Several other isoenzymes were also isolated but in such small amounts that characterization was not feasible. Table 1 summarizes the enzyme activities found at the major steps of isolation.

Diffuse peaks I and II. Figure 2 shows two additional peaks of peroxidase activity (D1, D2) which as yet are unexplained. Although fairly sharp peaks were eluted from Sephadex, when the components were examined in gels each gave diffuse zones of activity from 0.2 to 1.7 cm. This area overlaps the region to which isoenzymes 7–11 migrate. It is possible that the diffuse bands are artifacts produced during purification procedures. It should be noted, however, that in the examination of many gels in several studies [1,2,18] it was not possible to obtain gel absorbancy tracings in which the base lines returned to zero absorbance in the region corresponding to the two diffuse bands. Previously, we had thought this phenomenon to be due to the overlapping of isoenzymes and diffusion of reaction products but, as discussed later, the diffuse bands may be components, perhaps natural, of extracts.

Isoelectric focusing. Several separate determinations provided average isoelectric pH values of 7.8, 7.4 and 6.0 for isoenzymes 6, 9 and 10, respectively. The small amount of isoenzyme 8 available permitted only one determination which resulted in a pH value corresponding to 4.8. Diffuse bands I and II surprisingly gave sharp focusing bands similar to those of the isolated isoenzymes but with nearly identical values of 9.4 and 9.5, respectively.

General properties of isolated isoenzymes. The individual isoenzymes were highly active and thus were easily monitored during purification. Only a very small amount (less than 0.2 mg) of each protein was obtained, however, and considerable concentration was required for protein

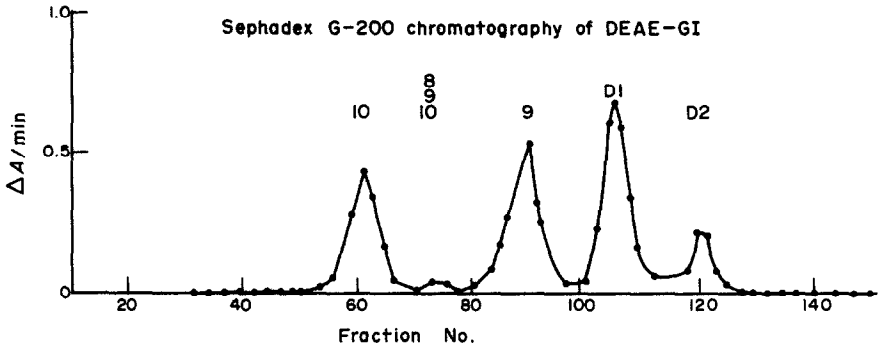

Fig. 2. Elution profile upon Sephadex G-200 chromatography of DEAE Group 1 isoenzymes. The first peak (G1) from the DEAE-cellulose chromatography of both CM-fractions I and II was passed through a Sephadex G-200 column (2 × 150 cm), and 2 ml fractions were collected and assayed for peroxidase activity (●——●).

estimation. Because of the possible importance of carbohydrate in determining such properties as electrophoretic mobility, heat stability and potential linkages to cell walls [20], a modified anthrone assay was conducted on separate samples prepared from two different sets of plants. Only isoenzyme 9 and diffuse bands I and II had significant carbohydrate components. Shannon *et al.* [16] reported about 13–16% carbohydrate for each of the horseradish peroxidases for which carbohydrate was determined and showed that the nature of the carbohydrate was different between the anodic and cathodic species, but characteristic for each isoenzyme of each group. Because the limit of the anthrone method was equivalent only to 1 μg per ml of sample, it cannot be concluded that isoenzymes 6, 8 and 10 are devoid of carbohydrate. It is clear that isoenzyme 8 must have a lower percentage of carbohydrate than isoenzyme 9, even though they are both anodic [16]. In one preparation of isoenzyme 10 a positive anthrone reading was observed at the lower limit of sensitivity (Table 2), but the second preparation did not give a positive test.

The time course of reaction with four routinely used substrates was the same for each isoenzyme, when enzyme concentrations were adjusted to give equivalent rates. Phenylenediamine and dianisidine yielded linear rates, but eugenol and guaiacol exhibited a lag phase of 1–3 min which was identical for all isoenzymes.

There is variation among isoenzymes in their specific activities on various substrates (Table 3). Although not shown because of insufficient replication, dianisidine appeared to have the same general relationship with individual isoenzymes. Isoenzyme 9 had the highest and isoenzyme 6 the lowest specific activity with dianisidine.

Table 2. Protein and carbohydrate contents of isolated isoenzymes

Isoenzyme	Protein* (μg/ml)	Carbohydrate† (μg glucose/ml)	Maximum carbohydrate (%)
6	5	n.d.†	<20
8	11	n.d.	<9
9	16	3.0	19
10	10	1(?)	<10
Diffuse band I‡	37	4.4	12
Diffuse band II	35	4.3	12

* Average of three or more determinations. † n.d. = not detectable. ‡ Contained minor amount of isoenzyme 9.

Of particular note is the very high specific activities of diffuse band I and II with *p*-PDA as substrate. It seems unlikely that a component of such high activity could arise as an artifact by aggregation of isoenzymes of lower specific activity. On gel electrophoresis of isolated diffuse bands I and II, relatively high concentrations were necessary in order to stain adequately. With dilutions of crude homogenates as previously described [2], showing good resolution between isoenzymes 9 and 10, there was also some general background stain in the same region to which isoenzymes 9 and 10 migrate. The background might be due to peroxidase activity of the diffuse bands present in the homogenate.

The specific activities in Table 3 support previously tentative conclusions about the characteristic association of isoenzyme 9 with infection of resistant wheats by rust fungi [2]. In resistant infections, isoenzyme 9 showed 100–300% increases in activity over healthy tissue in gels with benzidine as substrate [1,2]. Isoenzyme 10 increased 50 to 200%, but in both resistant and susceptible infected leaves at all stages of infection. On the basis of the peroxidase patterns in gels, much larger increases in total peroxidase should have been observed for homogenates from susceptible infected tissue than the 30–50% increases actually found. On a protein basis, however, isoenzyme 10 produces a more intense stain than isoenzyme 9 and its contribution to activity in gels would be more pronounced. The higher specific activity of isoenzyme 9 on substrates used in spectrophotometric assays (Table 3) indicates the reverse for the relative contributions of these two isoenzymes in homogenates. Con-

Table 3. Specific activities of some peroxidase isoenzymes with different substrates. Assays at 25° using 0.1 M Pi buffer, pH 6

Isoenzyme	Specific activity* Guaiacol	Phenylenediamine	Eugenol
6	44	136	70
8	510	640	510
9	1840	1400	1780
10	650	120	230
Diffuse band I	—	3130	—
Diffuse band II	—	3000	—

* ΔA/min/mg protein.

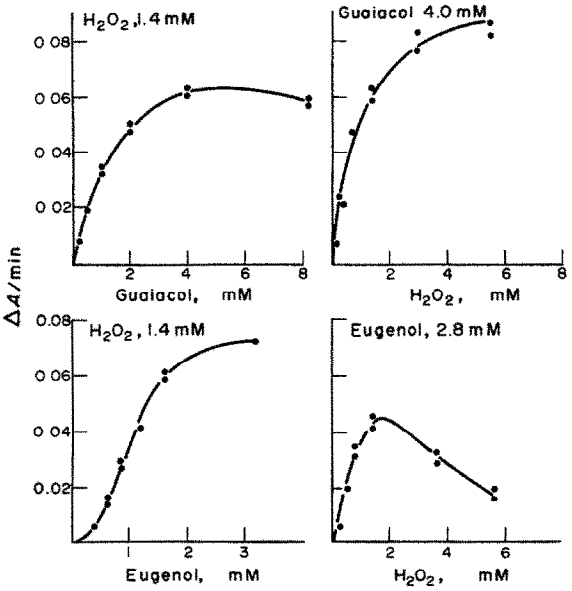

Fig. 3. Rate versus substrate curves for H_2O_2 and reductant with isoenzyme 10. *p*-PDA and *o*-dianisidine showed effects similar to those obtained with guaiacol. All four purified isoenzymes behaved similarly, including the sigmoidal behavior exhibited by eugenol.

sequently, the observed change in isoenzyme 9 in gels can satisfactorily account for much, if not all, of the increases in peroxidase activity initially reported for homogenates from resistant infected plants [1]. As pointed out elsewhere, however, the increase appears to be a consequence of, not a causal factor in, biochemical events in resistance [18].

The effect of pH from 3 to 9 on activity was determined with acetate, citrate, phosphate and Tris buffers, with duplication at common pH values for buffers with usable overlapping points. The range for activity was from pH 4.0 to 8.5, with a relatively narrow optimum at pH 6.0. With guaiacol and eugenol, the optimum was independent of buffer, except for isoenzyme 10 which had an optimum of pH 5.5 in phosphate and pH 6.0 with acetate. The same phenomenon was observed for all isoenzymes when *p*-PDA was substrate. In agreement with the observations of Kay *et al.* [17], each isoenzyme showed typical Michaelis–Menten kinetics when guaiacol and *p*-PDA concentrations were varied at pH 6 at 25° with optimal H_2O_2. With eugenol as a substrate, the activity curve was somewhat sigmoidal for all isoenzymes. When the H_2O_2 concentration was varied, the activity curves were hyperbolic, again with the exception of eugenol. With this substrate, concentrations of H_2O_2 above 3 mM were inhibitory, as reported for aniline as a substrate [21]. Representative effects of substrate concentrations are shown for isoenzyme 10 in Fig. 3. All isoenzymes had similar apparent K_m values of H_2O_2 (Table 4) which are similar to other values of K_{app} reported [17]. For each isoenzyme and reductant the apparent K_m values vary slightly, but not significantly, among isoenzymes (Table 6). As with H_2O_2, the K_m values are similar to other peroxidase substrates [21,22].

A final parameter tested which might be important in physiological processes in plants was temperature response. Again, however, there were no distinctive differences among isoenzymes. With guaiacol, *p*-PDA and dianisidine as substrates, the temperature optimum was broad, from 35–55°. The optimum with eugenol, however, was fairly narrow at 25° and rates above the optimum fell more sharply than with other substrates. Shannon *et al.* reported that anodic isoenzyme A_3 of horseradish peroxidase appeared more temperature sensitive than A_1 or A_2 and suggested it was due to interaction with the substrate or reaction products rather than an effect on native protein conformation. The different behavior of eugenol in several kinetic parameters, when compared with the other substrates, reinforces this idea. Free radicals which may be intermediates of the reaction with eugenol can play a significant role in stability of the enzyme. A test of temperature stability in the absence of substrate indicated that all isoenzymes behave similarly. When held at 60° for 15 min 70% of activity was retained, but at 75° for the same time nearly 85% was lost.

Although this survey has not been exhaustive, it appears that the four isoenzymes under comparison are not strikingly different in their characteristics on some commonly used, non-physiological substrates. The exception may be isoenzyme 9 because of its somewhat greater specific activity. The occurrence of multiple forms of peroxidase may be related to conformational or compositional features associated with specific tissue or subcellular location, for example cell walls. We have attempted cell fractionations in order to determine if this was the case, but found that all the isoenzymes of Fig. 1 could be obtained in solutions infiltrated gently under vacuum into leaf sections. Association of anodic isoenzymes with cellular structures, such as walls [20,23], must be loose and not involve covalent linkages, if it occurs. Functions for individual isoenzymes may involve natural substrates with a greater specificity among isoenzymes than that found for non-physiological compounds.

EXPERIMENTAL

Growth and inoculation of resistant wheat at 20° was as described [1]. Between 6–8 days after infection, when peroxidase activity was maximum, primary leaves were blended at high speed for 1 min in a Waring blender with 0.1 M Tris buffer, pH 8, containing 0.1% ascorbic acid, 0.1% cysteine–HCl and 17% sucrose (5 ml per g of fr. wt). The effectiveness of the extraction medium has been compared with other media [2]. After filtering through cheesecloth, the extract was centrifuged at 20000 *g* for 20 min and the supernatant brought to 50% saturation with $(NH_4)_2SO_4$ at 0° and held at that temp. for 8 to 14 hr. After centrifugation at 20000 *g* for 20 min, the supernatant liquid was brought to 95% saturation with $(NH_4)_2SO_4$ and held at 0° for an additional 8–14 hr. The ppt.

Table 4. Apparent K_m values of the peroxidase isoenzymes for different substrates and H_2O_2

Isoenzyme	K_m, mM Guaiacol		K_m, mM Phenylenediamine		K_m, mM Eugenol	
	Guaiacol	H_2O_2	PDA	H_2O_2	Eugenol	H_2O_2
6	0.6	0.4	1.3	0.7	3.9	0.7
8	1.0	—	1.6	1.2	1.4	1.7
9	1.0	0.8	3.9	0.5	1.4	0.4
10	0.2	0.6	1.5	0.3	2.5	0.5

obtained by centrifugation at 20000 *g* for 20 min was dissolved in, and dialyzed 2× against 10 mM Pi buffer, pH 7. All succeeding steps were conducted at 2°.

CM-Cellulose chromatography. The 50–95% $(NH_4)_2SO_4$ fraction was passed through a CM-cellulose column (5 × 76 cm) equilibrated with 10 mM Pi buffer, pH 7, and eluted with the same buffer. Appropriate tubes were pooled and concentrated by a Diaflo apparatus after which they were dialyzed twice against 5 mM Tris–HCl buffer, pH 8.

DEAE-Cellulose chromatography. Pooled fractions from CM-cellulose chromatography was passed through a DEAE-cellulose column (2.5 × 63.5 cm) equilibrated in 5 mM Tris–HCl buffer, pH 8, using this buffer as the initial eluant. After the first group of isoenzymes were eluted, gradients of 0–0.1 M NaCl and 0.1–0.25 M NaCl in the same buffer were applied to the column. Appropriate fractions were pooled and concentrated.

Sephadex G-200 chromatography. Isoenzymes were further separated by passing through a Sephadex G-200 column (2 × 150 cm) using H_2O as the eluant. To obtain purified isoenzymes, a second and sometimes a third passage through Sephadex G-200 column was necessary, applying only pooled fractions rich in the desired component.

Isoelectric focusing. A 0–50% sucrose gradient of 30 ml, containing 1% ampholine, was prepared by layering 0, 10, 20, 30, 40, and 50% sucrose soln (with the sample in the 20% layer) and allowed to develop 18 hr. The gradient was pumped into an isoelectric focusing apparatus with 60% sucrose. The anode compartment contained H_3PO_4 in 40% sucrose and the cathode compartment 5% ethanolamine. Electrophoresis was carried out at constant V (usually 800), for at least 36 hr with pH 3–10 ampholine and for 60 hr with narrower pH ranges. After electrophoresis, the gradient was pumped out at 0.5 ml/min with the aid of 60% sucrose. Enzyme activity and pH were assayed in alternate 0.5 ml fractions. pH was measured by diluting the fractions 1:10 with H_2O. A 0.02–0.03 pH change was observed in doing this; however, it facilitated pH measurements at high sucrose concentrations. Fractions eluted from columns were monitored for protein by $A_{280\,nm}$. Enzyme activity in such cases was measured at 25° by the phenylenediamine (*p*-PDA) assay [2] with Pi buffer at pH 6. For substrates other than *p*-PDA, some minor variations from previous procedures [2] were made: guaiacol, 0.4 ml at 20 mM plus 1.6 ml of 0.1 M NaPi buffer, pH 6: eugenol; 0.2 ml at 25 mM in 50% EtOH plus 1.8 ml NaPi buffer, pH 6: *o*-dianisidine 0.05 ml of 1% soln of MeOH plus 1.95 ml NaPi buffer, pH 6. In all cases, 0.1 ml of 0.1% H_2O_2 and 0.1 ml of enzyme preparation were added immediately before spectral readings were started. The selected wavelengths (nm) were: *p*-PDA, 485; guaiacol, 470; eugenol, 425; *o*-dianisidine, 470. The identity of individual isoenzymes was checked routinely during purification by gel electrophoresis carried out as described by Seevers *et al.* [2]. To quantitatively estimate the activity, the recorded peaks were traced on bond paper and weighed or the output of the Gilford spectrophotometer was fed to a signal integrator.

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