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MOLECULAR WEIGHT DIFFERENCES AMONG POTATO PEROXIDASES

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Key Word Index—*Solanum tuberosum*; Solanaceae; potato; peroxidase; isoenzyme; MWs.

Abstract—The isoenzyme of potato peroxidase A5 has MW 105 000; C3—94 000; C4 and C5—56 500 and C6—48 500. The isoenzymes retain activity on SDS-gels thereby allowing direct measurement of monomeric MW, even in crude extracts. One of the isoperoxidases showed anomalous behaviour on SDS-electrophoresis.

INTRODUCTION

During the past thirty years the scientific literature has been replete with studies of plant peroxidases (EC 1.11.1.7) from various sources [1, 2 and refs therein]. For the many plant peroxidases studied three factors seem to be common: multiple forms of the enzyme are produced (isoperoxidases), peroxidase activity generally is increased in response to wounding or environmental stress, and carbohydrates form an integral part of the structure of the enzyme (i.e., peroxidases are glycoproteins) [1]. The findings are most confused with respect to the isoenzyme studies. As pointed out by Gasper *et al.* [1] one can find reports ranging from a small number (3 to 4) of isoenzymes, to a large number (16 to 22) of forms of the enzyme, depending on the separation techniques used. These authors suggest, that in the cases of large numbers of isoenzymes, the multiple forms are probably not physiologically significant, but rather some artifact of sample preparation and/or separation.

Several workers report MWs for a variety of plant peroxidases. Horseradish peroxidase (HRP), the most thoroughly studied peroxidase, has seven principal isoenzymes [3] (although Hoyle [4] identifies up to 40 isoenzymes by electrofocusing of commercial HRP preparations) all of which have an apparent MW of *ca* 40 000 to 45 000. Cultured tobacco cells produce five major isoperoxidases which range in MW from 103 000 to 28 000 [5, 6]. The MW 103 000 species is reported to be a dimer [5].

In this communication we report the apparent MWs of the four main peroxidase isoenzymes from potato. In addition, we report the finding that potato peroxidase remains partially active under certain conditions of SDS gel electrophoresis. This observation makes possible the determination of the MWs of active monomers in crude preparations.

RESULTS

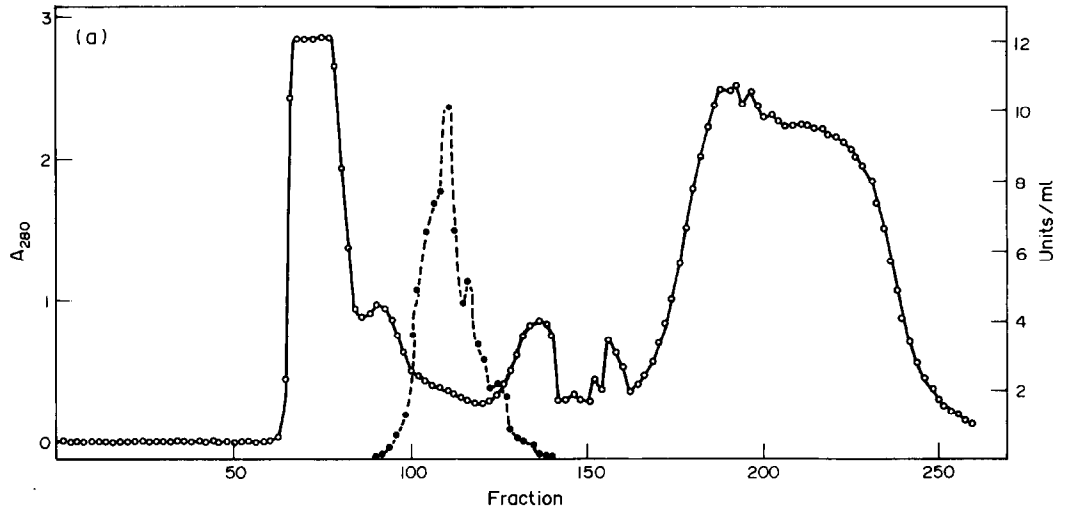
It has been reported for several plant peroxidases that gel filtration chromatography of crude extracts results in elution of a significant amount of enzyme with the void volume suggesting quite large molecules (see ref. [1]). Altering the pH or ionic strength of the eluting buffer causes dissociation of these large molecules to active peroxidases of much smaller MW. It was concluded that the enzyme was adhering to membrane structures resulting in artifically large apparent MWs. We have observed the same phenomenon with potato peroxidase (data not shown).

To prevent adhesion of isoperoxidases to large molecules, a crude extract of 4-day-old potato slices was chromatographed through Sephadex G-100 using buffer containing 0.5 M NaCl (Fig. 1). Under these conditions no peroxidase activity was found in the void volume. All of the activity (70–90% of applied activity) eluted later in an area covering the MW range from *ca* 140 000 to 40 000. Aliquots of every third fraction in this range were concentrated 10-fold by lyophilization (after dialysis vs. 5 mM potassium phosphate pH 7 to remove NaCl) and subsequently analysed for isoenzyme content by gel electrophoresis as described earlier [7]. The results (Fig. 1b) indicate that the leading edge of the peroxidase peak was predominantly the anodic isoperoxidase (A5 in our nomenclature). The trailing edge consisted mainly of the most cathodic isoenzyme (C6). Intermediate fractions contained the remaining isoenzymes, with C3 being found toward the front of the peroxidase peak and C4 and C5 toward the rear.

The staining pattern of the gel suggests that each isoperoxidase chromatographed as a symmetrical peak, overlapping its neighbours, to result in the large, non-symmetrical peak observed in Fig. 1a. The peak fraction for each isoperoxidase was identified in densitometer scans of the stained polyacrylamide gel. Using the elution volume V_e of the peak fractions an apparent MW was calculated for each isoenzyme by comparison to a standard curve. The results of these calculations show that isoperoxidase A5 has a MW of *ca* 105 000. Isoperoxidase C3 is somewhat smaller at MW 94 000. Isoperoxidases C4 and C5 (C5 is poorly resolved from C6 on the gel shown in Fig. 1b) each have an apparent MW of 56 500, and C6 is the smallest isoenzyme at 48 500.

SDS gel electrophoresis

When a crude extract of wounded potato tissue was



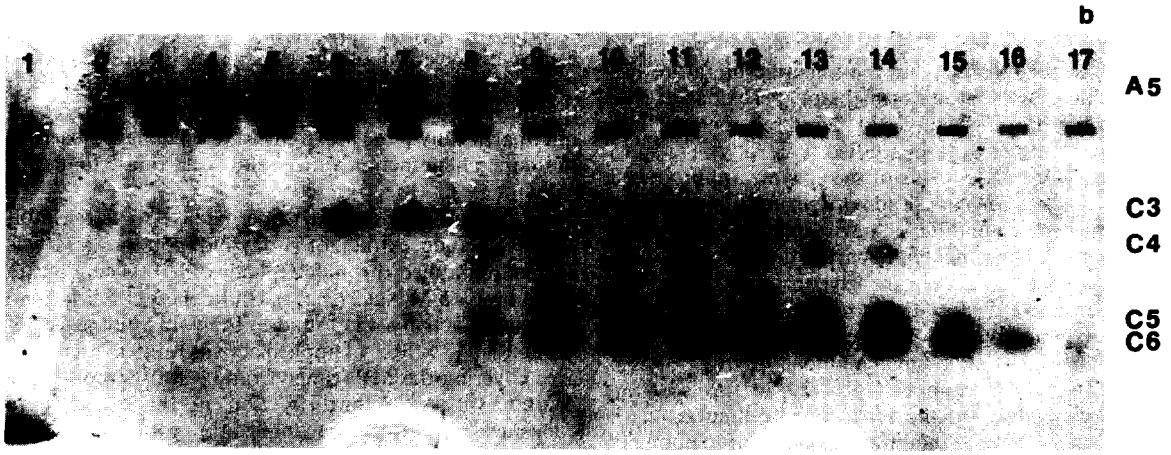


Fig. 1. (a) Sephadex G-100 chromatograph of potato crude extract. (○) A280, (●) peroxidase units. See Experimental for chromatography conditions. (b) Electrophoretogram of peroxidase containing fractions from Sephadex G-100. Active fractions were dialysed, lyophilized and redissolved in 0.1 vols deionized water and 9 μl was applied to each well. Anode is at top of gel. The completed gel was stained for peroxidase activity as described in ref. [8]. Well no. 1 contains cytochrome C. Well nos. 2–17 contain respectively fractions 90, 93, 96, 99, 102, 105, 108, 111, 114, 117, 120, 123, 126, 129, 132 and 135. See Experimental for conditions of electrophoresis.

denatured by incubating for 2 hr at 37° in 1% SDS, 0.1% β-mercaptoethanol, a significant proportion of the enzyme activity remained, allowing visualization on gels by activity stain. After SDS-electrophoresis the gels were stained for activity as described by Graham *et al.* [8]. Stained bands appeared within 5–20 min. By contrast, if the protein samples were denatured by heating to 100° for 2 min (also in 1% SDS, 0.1% β-mercaptoethanol) no activity bands were seen even after several hr of staining. Both denaturing conditions gave identical standard curves when proteins of known MW were used as markers.

Figure 2(J) shows an SDS gel of crude wounded potato extract stained for peroxidase activity. Two major bands and one minor band are clearly discernible. In addition the very top of the gel is stained suggesting the existence in crude extracts of a form of peroxidase unable to penetrate the gel under these conditions. Based on comparison with calibration curves, the three species which penetrate the gel correspond to MWs of *ca* 170 000, 125 000 and 48 500 from largest to smallest.

Certain other plant species known to produce peroxidases were examined by this same technique to determine if stability to SDS is unique to potato peroxidase. Purified HRP was not active in SDS gels, however, kohlrabi (*Brassica oleracea* var. *caulorapa*) had at least one activity band. In addition, cytochrome C had multiple activity bands.

We examined the active fractions from the Sephadex G-100 column, shown in Fig. 1, by SDS gel electrophoresis and staining for peroxidase activity. The results (Fig. 2, A–I) generally agree with the previous assignment of MWs, i.e. the leading fraction having large MW and the trailing fractions small MWs. Fractions corresponding to isoperoxidase A5 (see Fig. 1) have an apparent monomeric MW of *ca* 125 000 which is in general agreement with the

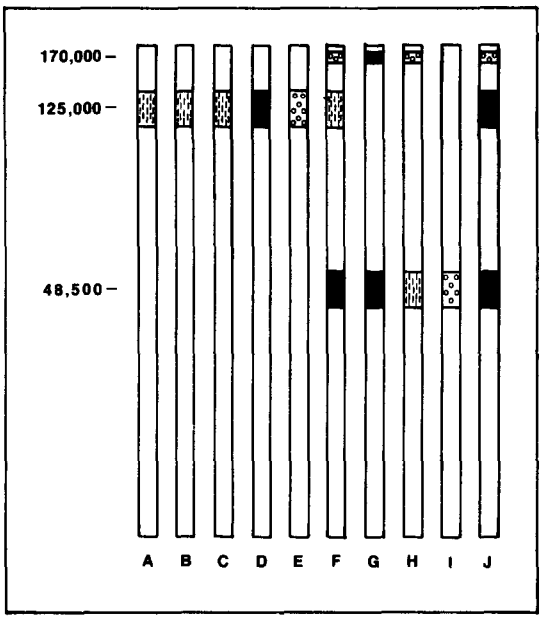


Fig. 2. SDS gels of peroxidase-containing fractions from Sephadex G-100. Concentrated fractions used for Fig. 1b were denatured and electrophoresed as described in text. The gels were stained for peroxidase activity as described in ref. [8]. Gels A–I contain respectively fractions 102, 105, 108, 111, 114, 117, 123, 126, 129. Gel J contains crude extract.

native MW determined by gel filtration. Furthermore, SDS-gels of partially purified isoperoxidase A5 (i.e. free of all other isoperoxidases) have a single activity band at the same relative migration (data not shown), thus confirming the identity of this band.

The trailing edge of the Sephadex G-100 peak, corresponding to isoperoxidase C6, had an activity band of apparent monomeric MW equal to *ca* 48 500. This value matches precisely the MW determined for C6 by gel filtration. The SDS-gels of the intermediate fractions are shown in Fig. 2 (D–H). Gels D and E (corresponding to the fractions shown in Fig. 1B, lanes 8 and 9) appear to contain peroxidases of monomeric MW equal to 125 000 and 48 500. Gels F and G (corresponding to the fractions shown in Fig. 1B, lanes 10 and 12) contain three activity bands, the 125 000 and 48 500 bands seen in the previous gels plus a very slow migrating band corresponding to a molecule of MW *ca* 170 000 as observed in crude extracts (Fig. 2, J). Obviously this is an anomalous result as a protein of this size would be expected to elute in the void volume of Sephadex G-100. Instead, it eluted in an area where one would expect to find proteins of MW *ca* 55 000.

The final two SDS-gels (Fig. 2, H and I) contain respectively the MW 170 000 and 48 500 peroxidases and the MW 48 500 peroxidase. Although activity bands can be seen for the fractions electrophoresed in slots 2, 3, 4 and 15, 16, 17 in Fig. 1, these bands are rather faint as the fractions contain little enzyme. Attempts to run SDS-gels on these fractions were unsuccessful because the recovery of activity on SDS-gels is insufficient for testing very low levels of activity. These data are summarized in Table 1 and compared to tobacco peroxidases.

DISCUSSION

The observed retention of peroxidase activity in SDS-gels enabled us to determine the MW of different wound-induced potato isoperoxidases, identified earlier by polyacrylamide electrophoresis [7], by two different methods, gel filtration and SDS gel electrophoresis, in crude extracts.

The results presented above strongly suggest that the isoperoxidases of potato are of different MWs. The evidence from both gel filtration and SDS gel electrophoresis indicates the existence of multiple size classes. It appears that the crude extract of potato slices contains at least four MW species which overlap on chromatography on Sephadex G-100. The same material has three distinct size classes on SDS gels.

The correlation between the native MWs determined by gel filtration and the monomeric MWs determined by SDS gel electrophoresis is not exact; nevertheless, it is

Table 1. Size classes of peroxidases

Potato			Tobacco*		
Isoenzyme	Molecular weight		Isoenzyme	Molecular weight	
	Gel filtration	SDS-gel electrophoresis		Gel filtration	SDS-gel electrophoresis
A5	105 000	125 000	A1	103 000	49 000
C3	94 000	125 000	A2	90 000	89 000
C4	56 500	170 000	C3	67 000	68 000
		125 000			
		48 500			
C5	56 500	170 000			
		125 000			
		48 500			
C6	48 500	48 500	C4	46 000	44 000

*Data from ref. [5].

possible to assign MWs to some of the isoperoxidases. Only isoperoxidase C6 shows an exact match, having a MW of 48 500 by both techniques.

The MW (presumably monomeric) observed on SDS gel electrophoresis for isoperoxidase A5 is about 20% larger than the native MW determined by gel filtration. This discrepancy may be a result of the fact that potato peroxidase, like other peroxidases, is a glycoprotein. It is well known that glycoproteins do not always give true migration patterns in SDS gels [9, 10]. Glycosylated proteins frequently have a lower ratio of SDS binding than do non-glycosylated proteins. The lower SDS binding results in a lower charge-to-mass ratio and a correspondingly slower migration [9, 11, 12]. The net effect is artificially larger apparent MWs for glycosylated proteins.

This phenomenon may explain the otherwise unaccounted for 170 000 species found on SDS gels. This very large isoperoxidase was found in those fractions of the Sephadex G-100 column which would be expected to have MWs about one-third that size (i.e. roughly MW 50 000 to 55 000). It is possible that this species represents an extreme manifestation of the problem of running glycoproteins on SDS gels. From the polyacrylamide gel we know these fractions to be richest in isoperoxidases C4 and C5 and fairly rich in C6 as well. Because the later fractions, which contain only C6, seem to account for the MW 48 500 species it is likely that either C4 or C5 (or perhaps both) is the isoperoxidase responsible for the anomalous band at MW 170 000.

The final potato isoperoxidase to be considered is C3. Gel filtration data indicates a MW of *ca* 94 000 yet on SDS gels the only activity band is found in the area corresponding to MW 125 000. It is possible that C3 is another glycosylated species which fortuitously migrates in SDS gels at exactly the same rate as A5 (MW 125 000 species) thereby giving the same apparent monomeric MW. It is perhaps more likely that C3, unlike the other potato isoperoxidases is inactivated under the conditions used for denaturation prior to SDS gel electrophoresis. The result would be the appearance in SDS gels solely of the band produced by isoperoxidase A5 which is a contaminant of the Sephadex fractions containing C3. At present we are unable to distinguish between these possibilities.

The MW differences reported here suggest that the isoperoxidases of potato are more than the artifacts of a separation technique or an aggregation phenomenon as described by Gaspar *et al.* [1]. Such factors must certainly be considered for potato peroxidase, as shown by the fact that large aggregates containing peroxidase are observed upon gel filtration at low ionic strength. In addition, electrophoresis in starch gels as opposed to polyacrylamide gels results in many more peroxidase staining bands [14]. Similarly, Mäder [14] observed that a partially purified anodic isoenzyme fraction of tobacco was separated into several bands of peroxidase activity by disc electrophoresis, but ran as a single protein band in SDS-gels. Nonetheless, the conditions used in this study (i.e. high ionic strength or SDS/β-mercaptoethanol denaturation) increase the likelihood that the observed differences reflect actual physical differences.

One cannot determine from studies such as these if the different isoperoxidases are unique gene products or the result of post-translational modification of a single gene product. The following indirect evidence suggests that potato isoperoxidases A5 and C3 might be true isoenzymes controlled by different gene loci: (i) C3 is the only isoperoxidase present in appreciable amounts in resting, intact potato tuber tissue [7]. Upon wounding, A5 is induced and increases in activity for several days exclusively in the outermost, suberizing cell layer of the wounded tissue [7] while C3 is found only in the lower, non-suberizing cell layers; (ii) C3 is also the major peroxidase fraction in callus cultures from potato tuber tissue [15]. On addition of abscisic acid, suberization and a 30-fold increase in A5 and other enzymes involved in suberin biosynthesis is observed [15]. (iii) It is possible that a single peroxidase gene produces a single polypeptide which is subsequently glycosylated to different extents or at different residues thereby producing the various isoenzymes which have been observed. However, it appears very unlikely that post-translational modification by glycosylation of the small (MW 48 000) C3 should yield A5, a molecule more than twice the size of C3, as determined by gel filtration (Table 1).

It is interesting to compare these results with studies of other peroxidases. HRP, the most extensively studied, has seven major isoenzymes all of which have MWs of *ca* 45 000 [3, 6], i.e. in contrast to potato isoperoxidases, HRPs appear to be charge isomers. The peroxidases produced by cultured tobacco cells are quite similar to those produced by potato. The tobacco peroxidases have a range of MWs from 103 000 to 46 000 with intermediate species of 90 000 and 67 000 [5]. The MW 103 000 peroxidase is reported to be a dimer with MW 49 000 subunits. Except for this dimeric species the similarity between tobacco and potato isoperoxidases is striking (see Table 1). Similarly, in several studies, electrophoretic separation of tobacco isoperoxidases yielded four groups of multiple peroxidase bands [16]. These observations suggest that the similarity of isoperoxidases in potato and tobacco, both Solanaceae, reflects genetic similarity between these genera.

The finding that potato peroxidase activity can be monitored directly on SDS gels should be useful. The techniques can be used to detect isoperoxidase species in crude extracts in much the same fashion that polyacrylamide gels are used. When used in conjunction with polyacrylamide gels much useful information can be obtained without the need for protein purification. It is anticipated that activity stains of SDS gels will be helpful in monitoring the purification of the various potato isoperoxidases.

EXPERIMENTAL

Potato tubers (*Solanum tuberosum* L., cv. Kennebec) were grown locally and stored at 4° until used. Sephadex G-100, SDS gel MW marker proteins (Dalton Mark VI), HRP, SDS, EDTA, cytochrome c, and *o*-dianisidine were purchased from Sigma (St. Louis). Gel filtration MW markers (aldolase, ovalbumin, chymotypsinogen and ribonuclease) were purchased from Pharmacia (Sweden). All other chemicals were reagent grade.

Peroxidase assay, slab gel procedure and preparation of crude extract of wounded tubers were as described earlier [7]. The activity stain is that of ref. [8]. After fixing in MeOH–H_2O–HOAc (20:17:3) the gels were soaked in EtOH–H_2O (19:6). This mixture has the effect of further shrinking the gels and enhancing the contrast by turning the background white while leaving the brown stained activity bands intact.

SDS gel electrophoresis. Gels were prepared and electropho-

resed as described in ref. [17] except that polyacrylamide was used at 6% instead of 7.5%.

Chromatography. Sephadex G-100 gel filtration was performed in a 2.5 × 160 cm column. The buffer was 5 mM NaPi pH 7, containing 0.5 M NaCl, sample size was 10 ml, and chromatography was performed at room temp.

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