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SEROLOGICAL STUDIES ON PHENOLASE FROM SPINACH LEAVES

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Key Word Index—*Spinacia oleracea*; Chenopodiaceae; spinach; phenolase; antibodies; multiple forms; inactive form.

Abstract—Antibodies were prepared against phenolase Form X, one of the electrophoretically fast-moving forms (VII–X) which are spontaneously liberated from thylakoid membranes after leaf homogenization, phenolase Form VIa, one of the slow-moving forms (III–VI) which are successively liberated from washed thylakoid membranes, and a preparation containing phenolase Forms IV–VI. Although these forms were not interconvertible *in vitro* by artificial treatments, they were immunologically related as was shown by double diffusion and by the precipitin technique. We, therefore, concluded that they were multiple forms rather than true isoenzymes. Form II, which gives a brown band on electrophoresis gels after incubation with phenolase substrates, did not react with Anti X, but all phenolase activity was precipitated by Anti X from homogenates containing Form II besides phenolases. This indicated that Form II was not an isoenzyme and that the colour produced in gels was due to a non-specific browning reaction. One other protein which had no phenolase activity showed cross-reaction with phenolase antibody. A fast-moving phenolase form was purified to homogeneity. The MW of SDS-treated purified enzyme was determined as 43000.

INTRODUCTION

Phenolases (EC 1.14.18.1) are usually reported to occur as multiple forms or isoenzymes [1–7]. In some cases multiplicity can be explained by association–dissociation processes, leading to *in-vitro* interconversion of active phenolase forms [1, 2], or by occurrence of true phenolase isoenzymes, differing in primary structure [3]. In addition, it can be due to partial enzyme denaturation, tanning or proteolysis during the isolation and purification processes, as clearly outlined in some recent reviews [6, 7].

From spinach chloroplasts 10 proteins which exhibit phenolase activity independent of proteolytic activation or inhibitors can be separated by electrophoresis [5]. While the slowest-moving forms (I and II) do not possess real phenolase properties [5] (Table 1), Forms III–X are true phenolases [5]. The latter are divisible into 'membrane' forms (III–VI), which can be extracted from washed chloroplast membranes, and 'stromatic' forms (VII–X), which are found only in the stroma-containing supernatants [4, 5, 8, 9]. All active phenolases result from liberation of membrane-bound latent phenolase during homogenization and isolation procedures [4, 9]. This is true also for 'stromatic' forms, which in contrast to membrane forms are released spontaneously [5]. None of these forms are interconvertible *in vitro* by urea, pH shock, repeated washings or extraction with acetone or ether of membrane fractions [5]. *In vivo*, however, during onset of leaf senescence, the membrane forms decrease and the stromatic forms increase significantly as does a broad electrophoretic band of phenolase activity (VIa, Table 1) between Forms VI and IX. It has been shown by tracer studies, application of detergents, and washing experiments that there is no *de novo* synthesis during the onset of senescence [10]. From these investigations, we have concluded that several latent phenolase forms occur in the chloroplast membrane and these can be extracted and activated without interconversion *in vitro*, but during senescence they show interconversion as a consequence of changes in membrane composition, so that spontaneous release of 'stromatic' forms is facilitated [5]. An alternative explanation is that different isoenzymes exist in a latent, membrane-bound state and are specially activated during different developmental states of the leaves.

Immunological methods can be used to determine heterogeneity in primary protein structure and have been used successfully to prove the existence of isoenzymes [11, 12].

RESULTS

Purification of antigens and specificity of antibodies

Of the three antigens used for immunization two were partially purified on a semipreparative scale by slab gel electrophoresis. Areas containing three membrane forms (IV–VI) or Form VIa (Table 1) were cut off the gel, eluted in phosphate buffer and concentrated by ultrafiltration. These preparations contained other proteins besides phenolase proteins but were pure in terms of the phenolases. Form X, one of the stromatic forms, was purified to homogeneity by a combination of gel filtration, ion exchange chromatography and electrophoresis (Table 2, Fig. 1). Antiserum and purified antibody (Anti X) produced against purified Form X revealed only one precipitation line when tested by the Ouchterlony double diffusion technique (Fig. 2) and by immunoelectrophoretic methods, indicating that only antibodies against one protein had been produced. Purified antibodies against the single-step-purified phenolase forms, called Anti VIa and Anti M (for Form VIa and for membrane

Table 1. Multiple forms of spinach leaf phenolases

Forms	I	II*	Group of slow-moving 'membrane' forms (III–VI)	Group of fast-moving 'stromatic' forms (VII–X)†	VIa [5]	XI‡ [5]
Relative mobility	0.01–018	0.18–0.31	0.32–0.48	0.50–0.7	Ranging from 0.44 to 0.61	0.7
Typical sources	Stromatic fractions	Thylakoid extracts	Thylakoid extracts	Stromatic fractions	Pellets of leaf homogenates after onset of senescence	Only in totally yellow leaves
Conversion of						
monophenols	+	+	–	–	–	–
o-diphenols	+	+	+	+	+	+
Inhibition by						
CN^-	–	–	+	+	+	+
DIECA	–	–	+	+	+	+

The table shows all those forms which revealed browning after separation of proteins on PAGE and incubation of gels in phenolase substrates such as 5 mM DOPA or 10 mM 4-MC in 0.067 M NaPi buffer, pH 6.0. As substrates for monophenolase activity tyrosine, cresol and *p*-coumaric acid (each 10 mM) in NaPi buffer were used. Browning zones after DOPA incubation of gels do not develop as distinct sharp lines but as zones of about 1–2 mm width, the borders of the browning zones were measured and used in the calculation of rM. Stroma fractions were all those samples which contain soluble matrix (stroma) of chloroplasts. Thylakoid extracts were hypertonic or hypotonic washings of thylakoid membranes after separation from stroma; for details see ref. [9]. For a schematic diagram of distribution of browning zones I–X after DOPA incubation of gels and relative abundance of forms see ref. [5, 8].

*This form is not a phenolase, as is shown in this publication.

†Form VII was rarely found.

‡This form is only to be found in yellow, totally senescent leaves without any photosynthetic activity. It cannot be excluded that this form has been modified by lytic processes in the late stages of senescence.

Table 2. Purification of phenolase Form X from spinach leaves

Step	Purification procedure	Activity (μmol O_2/hr × sample)	Protein (mg/sample)	Sp. act. (μmol O_2/hr × mg protein)	Comments
1	Supernatant of 30000-*g* hypertonic leaf homogenate, concentrated by ultrafiltration	5328	180	29.6	Aged leaves with high Form X activity were chosen
2	Sepharose 6 B gel filtration, eluting with Tris–HCl, pH 7.2:				Elution volume (V_e) of phenolase activity corresponded exactly
	fractions 19–23, 31–38	503	49.9	10.1	with V_e of catalase
	fractions 24–30	4154	30.2	137.5	from beef liver (MW 240000)
3	Chromatography of combined fractions 24–30 on DE 52 Cellulose anion exchanger (Fig. 1):				
	0.0–0.2 M NaCl-eluate	91			Five protein bands by
	0.2–0.3 M NaCl-eluate	3351	0.80	4189	alkaline disc electrophoresis
4	Chromatography on Sephadex G 100 eluted with Tris–HCl, pH 7.2; 0.2–0.3 M NaCl eluate	2285	0.32	7140	Phenolase activity is eluted in the void volume
5	Preparative electrophoresis	n.d.	n.d.	n.d.	Elution of Form X protein with Pi buffer from gel

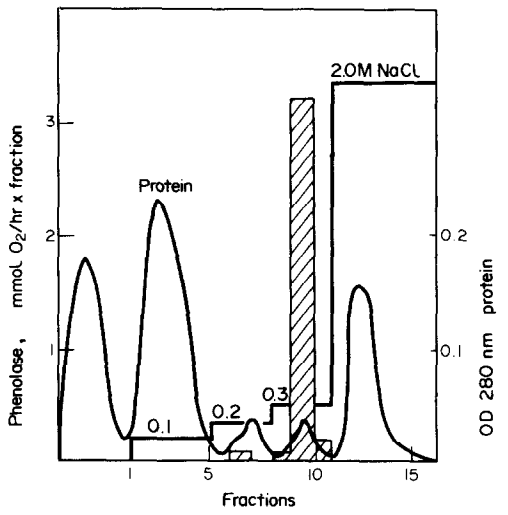

Fig. 1. Elution pattern of phenolase activity from anion exchange column DE 52. Sample applied to the column (4 × 30 cm) in 0.05 M in Tris–HCl, pH 7.2. A discontinuous NaCl gradient was applied after intensive washing with starting buffer. Flow rate ~20 ml/hr; fraction volume, 60 ml; shaded area indicates where phenolase activity was measured polarographically. Activity eluted with 0.2 M NaCl showed exactly the same phenolase forms after electrophoretic separation as did that eluted in 0.3 M NaCl.

Forms IV–VI respectively), revealed two and three precipitation lines, respectively, when tested with their antigenic samples or with a total spinach leaf homogenate. In every case, only one of the precipitation lines was specific for active phenolase forms, because after incubation of the gels with DOPA only one of the lines revealed enzyme activity and browning.

Precipitin reaction

Three samples derived from hypertonic leaf homogenates were used for antibody Anti X titration experiments: (1) 30000-*g* supernatants containing phenolase Forms I, II and VI–X, (2) washings of thylakoid membranes containing Forms III–VI, and (3) extracts of aged, frozen and thawed chloroplast samples containing the highly active Form VIa. In all cases, activities could be completely precipitated from the supernatants (Fig. 3) regardless of the combination of antigens and Anti X. No phenolase activity (polarographic assay) was found in the supernatant after the precipitin reaction with 4-methylcatechol as substrate. Very small amounts of Anti X led to slightly increased activities of the membrane extracts and of the Form VIa activity (Sample 3). Such an effect was not observed for hypertonic leaf homogenate or purified Form X. The slopes of the precipitation experiments with different antigens showed only slight differences. In all cases, less than 100 µl of antibody solution per ml of assay mixture led to total precipitation of enzyme activities.

Cross-reactivity of forms

Using the Ouchterlony double-diffusion method it was shown that all phenolases tested were serologically related. All precipitation lines were fused in all combinations tested (Fig. 2, Table 3). No single or double spur reaction occurred. Since during precipitation not more than 2.6% of the activity was lost, as found in the precipitin assay, the precipitation lines could be developed by protein staining and by the zymogram technique. Incubation of the gels in DOPA solutions as used in electrophoretic analysis [8], gave rise to sharp lines. Even after 10 days of washing at room temperature in PBS, pH 7.2, containing 0.02% sodium azide, the enzymatic activity of immune precipitates was preserved.

Immunoelectrophoretic analysis of Form II

When electrophoresis gels were incubated in substrate solutions, Forms III–X did not reveal any mono-

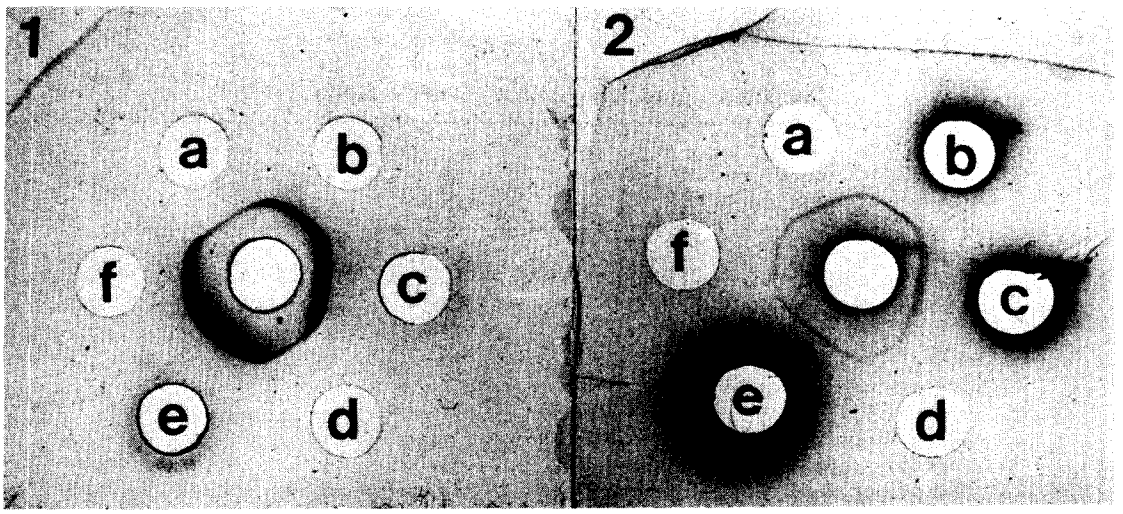

Fig. 2. Double diffusion of different phenolases with antiserum Anti X. Centre well: 50 µl Anti X; a, d, purified Form X; b, membrane washings containing phenolase Forms III–VI; c, extracts of aged, frozen and thawed chloroplast samples containing phenolase Form VIa; e, hypertonic leaf homogenate containing phenolase Forms I, II, VI–X; f, single-step purified Form V. Diffusion, 4 hr at room temp. or 20 hr at 3°. The gels were washed in PBS, pH 7.2, containing 0.02% NaN_3 (×6). Gel 1 was incubated in 5 mM DOPA solution in PBS, pH 6.0, for 2 hr, gel 2 was stained with Coomassie blue.

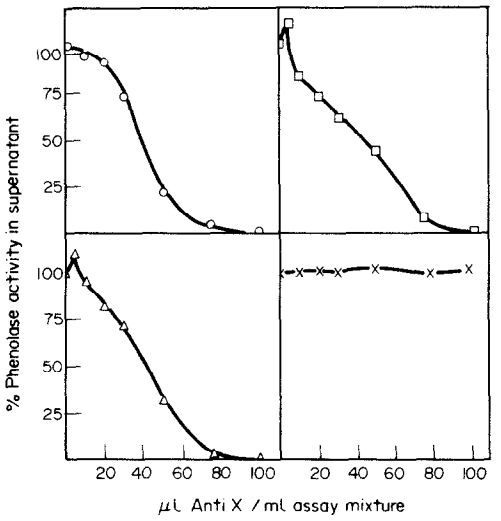

Fig. 3. Precipitin reaction of antibody preparation Anti X with samples containing different phenolase forms: ○——○, hypertonic leaf homogenate containing phenolase Forms I, II, VI–X; □——□, membrane washings containing phenolase Forms III–VI; △——△, extracts of aged, frozen membranes containing Form VIa; × —— ×, control with serum of rabbits before immunization showed no precipitations, regardless of the phenolase fractions tested. Each assay mixture contained the same amount of enzyme activity (20.1 μmol O_2/hr/ml assay mixture, when tested with 7.5 mmol 4-methylcatechol as substrate). The phenolase samples were made up to 0.9 ml with PBS, pH 7.2, to which 0.1 ml of a serial dilution of Anti X in PBS, pH 7.2, was added. After 1 hr at room temp., samples were kept for 24 hr at 3°, then centrifuged at 15000 g for 15 min and the activity was measured in supernatants and resuspended sediments.

phenolase or laccase activities and were inhibited by cyanide and diethyl-dithio-carbamate (DIECA), whereas Forms I and II were not inhibited by cyanide or DIECA and formed brown products from monophenols and *p*-diphenols [8] (Table 1). Tests were made to determine whether Form II reacts with Anti X. By using a combination of PAGE, line and rocket immunoelectrophoresis, it was shown that Form II did not react with Anti X but binds the brown reaction products of the phenol oxidation reaction (Fig. 4, arrow 1). Although Form II did not react with Anti X, the total phenolase activity of samples containing Forms I and II, besides other phenolases (Sample 1 in section on precipitin reaction), was precipitated by all antibody samples used in this study, including Anti X. So Form II, which must have been present in a soluble state in the 30000-g supernatants of the precipitin assays (Fig. 3) did not react with 4-methylcatechol when tested polarographically. This indicated that the browning reaction shown by Form II in polyacrylamide (PAA) gels after substrate incubation (DOPA, 4-methylcatechol) was not catalysed by a phenolase. Therefore, Form II was not a catecholase. The same experiment also revealed a protein without phenolase activity which reacted with Anti X (Fig. 4, arrow 2). Form I, in contrast, cannot be tested by this method because of its very slow electrophoretic migration.

Antibody-affinity chromatography

To study the antibody binding of Form I, an Anti X affinity column was produced by coupling Anti X to CNBr-activated Sepharose according to Livingstone [13]. A concentrated 30000-g supernatant of hypertonic leaf homogenate, which revealed phenolase Forms I, II and X after DOPA incubation of PAA gels, was diluted 5-fold by phosphate-buffered saline (PBS), pH 6.8, and was applied to the column. After washing the column with PBS until all non-bound protein was removed, elution of protein bound to the coupled antibodies was performed. Neither elution with 15 mM acetic acid nor with 10 mM NH_4OH resulted in desorption of more than 5% of the phenolase activity which had been applied and bound to

Table 3. Cross-reaction of phenolase and related antibodies

Phenolase-containing samples	Antibodies		
	Anti M	Anti VIa	Anti X
Purified Form X	+	+	+
30000-g supernatant containing Forms I, II, VI–X	+	+	+
Membrane washings (Forms III–VI)	+	+	+
VIa	+	+	+
Single-step purified Form V	n.t.	n.t.†	+
Single-step purified Form VI	+	+	+

All incubations were tested at least 4 times. All lines were stained with Coomassie blue and in a second experiment with the zymogram technique. No spurred reactions were seen. Protein staining of precipitates of Anti M and Anti VIa with 30000-g supernatants led to more than one precipitation line, but one of each combination could easily be identified as specific for phenolase by producing a confluent line without spurs with the reaction products of monospecific Anti X and the supernatant fraction. Single-step purified Forms V and VI have been cut off slab gels after electrophoretic separation of concentrated membrane washings.

*+, Precipitation lines after 24 hr of diffusion (4 hr at room temp., 20 hr at 3°).

†n.t., not tested.

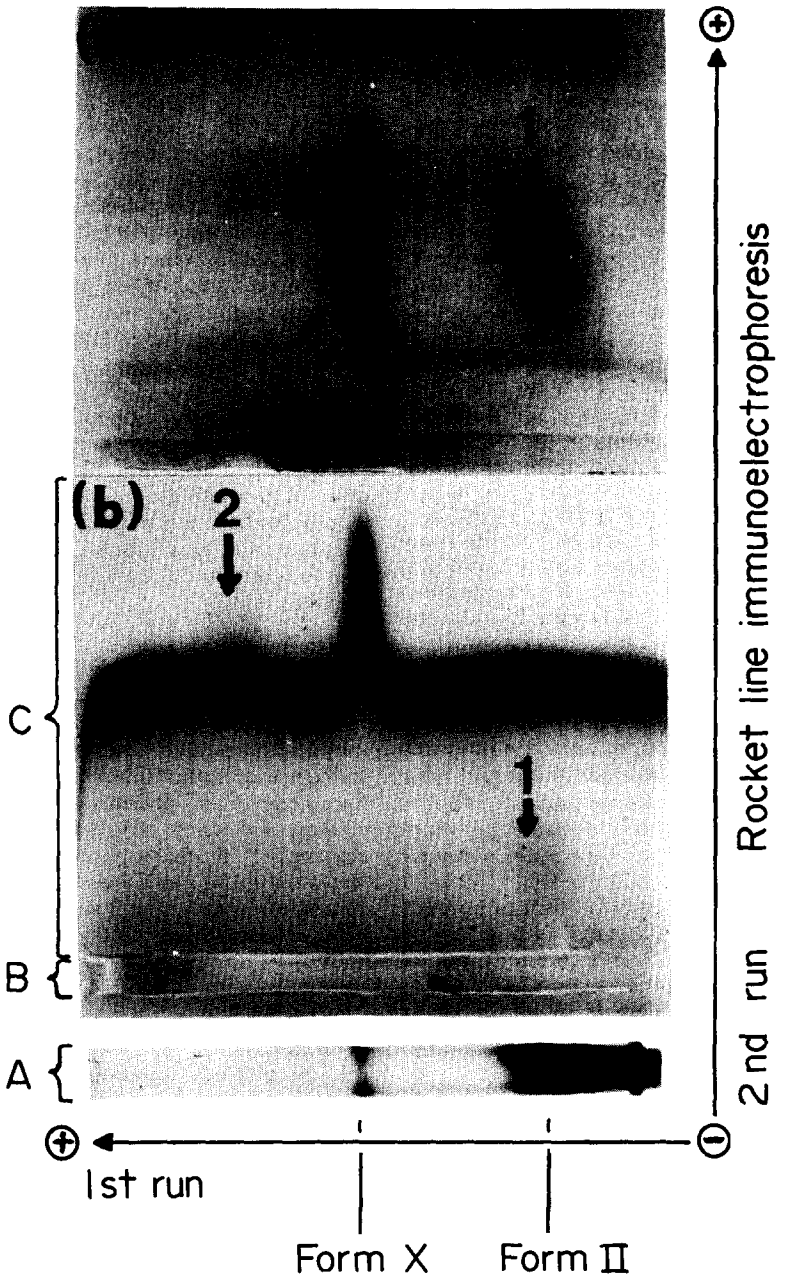

Fig. 4. Rocket line immunoelectrophoretic analysis of phenolase Forms II and X. 200 μg protein of $(NH_4)_2SO_4$ (70% saturation) precipitated and dialysed sample from hypertonic leaf homogenate was applied to PAA disc electrophoresis (first run). After the first run Forms I, II and X only were detected by DOPA incubation of the gel (A). Gels without DOPA incubation were embedded in Agarose gels [1% (w/v) in 0.05 M Veronal buffer, pH 8.6, containing 2.5% (v/v) Anti X]. A strip of Agarose gel without Anti X, containing a known amount of active enzyme (B) was placed parallel to the PAA gel. By the second electrophoresis the enzymes in the Agarose (strip B) migrated into the gel containing Anti X (C) and formed non-migrating antigen–antibody complexes. A line perpendicular to the migration axis is built, which can be stained by incubation with DOPA solution. Migration distance and staining intensity are correlated to the amount of enzyme in strip B. (a) 15 μl of concentrated hypertonic leaf homogenate in 400 μl of Agarose (1%) was used; (b) 60 μl of enzyme solution in 400 μl Agarose was used. Antigenically active phenolase forms, present in the embedded first run PAA gel, also migrated during the second electrophoresis and interfered with the enzyme of strip B, resulting in deformation of the precipitation line. Arrow 1 shows Form II which does not react with antibody Anti X; arrow 2 shows an inactive phenolase form. The rocket formed in (a) and (b) corresponds to Form X.

the column. However, elution with 3 M sodium thiocyanate in PBS, pH 6.8, according to [14] gave a fraction which contained more than 20% of the applied activity after removal of SCN^- by ultrafiltration or by gel filtration on Sephadex G 50. Electrophoretic analysis of the eluate (1–15 μg protein/gel) revealed three protein bands after staining with Coomassie blue, corresponding to phenolase Forms I, V and X, and a faintly stained zone in the region of the inactive protein (Fig. 4). Only Form X still exhibited phenolase activity.

Though there was no activity with DOPA it was shown by its binding to Anti X that Form I was immunologically related to phenolase Forms III–X. Furthermore, these findings underlined the fact that Form II does not react with Anti X. Whether the protein band corresponding to phenolase Form V was due to inactive phenolase Form V which was present in the sample applied to the column, or whether this protein was an artefact obtained by SCN^- treatment of active phenolases, awaits further elucidation.

Detergent treatments

(a) *Non-ionic detergents.* When purified Form X or samples containing membrane Forms III–VI were incubated in PBS pH 6.8, containing Triton X 100 (1% w/v) or Tween 60 (1%), respectively, for 1 hr at room temperature, and were tested by the Ouchterlony double-diffusion technique, the precipitation patterns were no different from those in Fig. 2. Such samples revealed confluent, fused lines compared with control samples. The distances from precipitation lines to the antibody-containing well were identical to those of the untreated control samples. Therefore, it was concluded that these forms are neither dissociated into subunits nor do they undergo drastic changes of their conformation which would result in the loss of antigenic capacity by non-ionic detergents.

(b) *SDS as an anionic detergent.* Purified Form X incubated in SDS (for further details see Fig. 5) still reacted with antiserum Anti X (Ouchterlony double diffusion) to give two precipitation lines: the inner one, nearest to the well containing antiserum, was a diffuse, broad line of about 1 mm, whereas the outer one was a sharp line which fused with the precipitation line of the untreated control (Fig. 5). To test whether the inner line was specific for a phenolase/antiphenolase reaction or whether it was due to the presence of protein–SDS complexes, BSA was incubated with SDS in the same manner as the phenolase protein. This sample reacted with Anti X to form a broad precipitation zone which fused with the inner line of the phenolase Form X precipitation pattern (Fig. 5). As Anti X did not react with BSA or SDS 0.1% alone, this broad precipitation line was due to a non-specific effect of SDS–protein complexes on antisera Anti X. When the same experiment was run in gels containing 1% (w/v) Triton X 100, the precipitation patterns were indistinguishable from the patterns given by samples without SDS (Fig. 2). Furthermore, in the presence of Triton no precipitation lines were produced by BSA incubated in SDS. Therefore, there was no loss of enzyme due to formation of non-specific precipitates.

After incubation of these gels in DOPA solution, only precipitates of the untreated controls showed browning. After SDS treatment in the presence of 2-mercaptoethanol and heating for 3 min at 90°, phenolases lose their enzymatic activities but they do not lose their antigenic characteristics and can easily be analysed by reaction with antibody Anti X.

Molecular weight

Form X activity (Table 2), as well as Form VIa activity, was eluted in the void volume on Sephadex G 100 when 50 mM Tris–HCl, pH 7.2 or 10 mM NaPi, pH 6.8, were used as buffers. On Sepharose 4 B both activities were eluted in exactly the same elution volume as catalase (beef liver), one of the proteins used for calibration of the column, indicating a very high MW of 240000. In contrast, SDS electrophoresis of purified Form X incubated in 0.1% SDS and 1.5% mercaptoethanol in 10 mM NaPi, pH 7.2, for 3 min at 90° gave a single protein band of MW 43000 ± 3000.

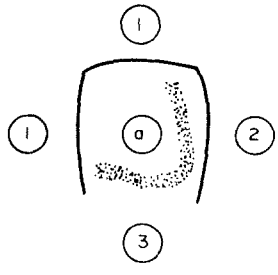

Fig. 5. Precipitation pattern of SDS-treated purified phenolase Form X. a, Antiserum Anti X; 1, purified Form X, diluted with 10 mM NaPi buffer, pH 7.2, to the same dilution as Sample 2; 2, purified Form X, incubated in 10 mM NaPi buffer, pH 7.2, containing 0.1% SDS and 1.5% mercaptoethanol; 3, BSA (0.25 mg/ml) incubated in the same way as purified Form X (No. 2). Diffusion was performed in moist chambers for 4 hr at room temp. or 20 hr at 3°. The broad SDS-containing precipitates, seen in front of Samples 2 and 3, disappeared in the course of washing the gels.

DISCUSSION

The serological data do not provide any evidence for the occurrence of spinach phenolase isoenzymes with different primary structures. All the observed fused precipitation lines and even the precipitin curves have almost identical slopes. These findings suggest Forms III–X are multiple forms of one enzyme which are present in the intact chloroplast. They are different with respect to electrophoretic mobility but not in antigenic characteristics. These different forms do not seem to be extraction artefacts because they are not only found with good reproducibility in different developmental stages of spinach leaves [5, 9], but they are also typically distributed in stromatic and thylakoid fractions, respectively. They differ in their extractability from the membranes [8].

SDS treatment of highly purified phenolase Form X revealed a single protein band in SDS electrophoresis. All other analysed phenolase forms of spinach contain at least this antigenic component with which Anti X is reacting. Membrane forms or Form I possibly contain additional proteinaceous or non-proteinaceous components which influence their activity and their electrophoretic mobility.

Activation of membrane forms (Forms III–VI) or Form VIa of phenolase by very low concentrations of Anti X, which is not seen in the case of stromatic Form X,

underlines the differences between stromatic and membrane forms. Activation of enzymes by specific antibodies has been found with other enzymes, too. Bovine pancreatic ribonuclease A [15] or bacterial β-lactamase [16] activity is enhanced by addition of antibodies. This activation was interpreted as due to conformational changes of the enzyme during interaction with antibody. However, if phenolases also undergo conformational changes during interaction with antibody, this should not only take place with membrane forms but with stroma forms as well, as they all contain the same antigenic components. As mentioned in the Introduction, other experiments have suggested that differences of electrophoretic mobility and extractability from the chloroplast membranes of the various multiple phenolase forms are due to the differential binding of additional components derived from the chloroplast membranes. Form X may be free of such components but the membrane forms possibly are not. Antibody binding might alter these complexes. Meyer and Biehl [5] showed that treatment of phenolases with acetone or ether does not alter the electrophoretic pattern, so it is unlikely that membrane lipids form complexes with these forms.

Sato and Hasegawa [17] reported interconversion during thin layer gel filtration of two phenolase forms from spinach leaves after treatment with Triton X 100 and acetone, which suggested association–dissociation of active subunits. This could not be shown by electrophoresis. The electrophoretic spectrum of the forms even remained unchanged after such drastic artificial treatments as 4 M urea, acid shock (pH 3.5), alkali shock (pH 11.5) or delipidization with acetone or ether. Double diffusion of membrane forms or Form X treated with Triton X 100 or Tween 60 did not reveal any differences in precipitation by Anti X compared to untreated controls which could be interpreted as a dissociation of polymeric forms into identical antigenic subunits. These findings are in contrast to the assumption of Sato [18] that phenolases may dissociate from a dimeric state into two identical subunits. A possible explanation for the conversion of forms reported by these authors [17, 18] is that the phenolase with a high MW is a complex with other, presumably proteinaceous components. The difference of molecular weights as estimated by gel filtration on Sepharose 4 B (240 000) or by SDS electrophoresis (43 000) does not necessarily indicate that active phenolase Form X is composed of six subunits. There are two other possible explanations: (1) The elution pattern of proteins often changes with different buffers used for gel filtration, due to alterations of Stoke's radius of proteins [19]. (2) Phenolases are membrane-extracted proteins and do not necessarily behave like soluble globular proteins, for which the estimation of MW by gel filtration was developed and which are used as calibration proteins.

Combination of preciptin reaction and line and rocket immunoelectrophoresis clearly showed that Form II is not a catecholase. The browning reaction of this protein, which formerly led to its classification as a phenolase [9], can be attributed to the protein either binding with brown reaction products or to enhanced auto-oxidation of *o*-diphenols in gel regions containing this protein. Autoxidation rates of *o*-diphenols are generally enhanced with alkaline pH or with metallo-protein complexes.

Up to now it has not been possible to determine the total amount of phenolase enzyme protein by activity measurements due to the activation of phenolases during liberation from thylakoid membranes and the inactivation of solubilized phenolases [9]. Monospecific antibody Anti X reacts with all active phenolases present in spinach chloroplasts as well as inactive forms, if these still possess antigenic determinants. So this provides a means of analysing the actual amounts of soluble phenolase in chloroplasts, even after SDS treatment of membranes, and for elucidating the interrelation of different forms.

EXPERIMENTAL

Preparation of chloroplast fractions was carried out as in ref. [14]. Grinding medium for hypertonic leaf homogenates contained 0.6 M sucrose in 0.01 M NaPi–HCl buffer, pH 7.8. Homogenates were centrifuged at 30 000 *g* for 1 hr. Supernatants were concentrated in a Millipore Ultrafiltration cell using Pellicon membranes PTCG. Concentrated samples were centrifuged at 30 000 *g* for 1 hr and stored at −18°. Phenolase activity was determined polarographically at 25° in a YSI oxygen electrode 5331. The air-saturated reaction mixture (3 ml) contained 7.5 mM 4-methylcatechol in 0.067 M Pi buffer (according to Sörensen). PAGE was performed at pH 8.9 (disc electrophoresis system No 1a of Maurer [20] in a preparative apparatus of Stegemann [21]. SDS electrophoresis was done in a 5% polyacrylamide gel with 0.1% SDS at pH 7.2 according to refs. [22, 23]. The samples were heated (3 min, 90°) in NaPi buffer, pH 7.2, containing 0.1% SDS and 1.5% mercaptoethanol. Trypsin inhibitor (soya bean), BSA, RNA-polymerase (*Escherichia coli*) and α, β, and β′ subunits of the Boehringer (Mannheim) combithek were used as calibration proteins.

For immunization, adult rabbits were injected intraperitoneally and subcutaneously at multiple sites of the back with a suspension of 1 ml protein solution in PBS, pH 6.8, and 1 ml of Freund's complete adjuvant (Behring). The first injection was performed using 10–20 μg of purified phenolase followed by a second application of 50–100 μg of protein 1 week later. Booster injections were performed intraperitoneally with 50–100 μg of protein about 30 days after the first injection. Seven days after the booster injection the animals were exsanguinated and sera prepared in PBS, pH 7.2, containing 0.02% NaN_3. The NaN_3 did antibodies was done according to ref. [11]. The resulting antibody fraction, Anti X, contained 1.73 mg protein/ml.

Ouchterlony diffusion tests were run in 1% agarose gel prepared in PBS, pH 7.2, containing 0.02% NaN_3. The NaN_3 did not inhibit phenolase activity at this concentration. Rocket line immunoelectrophoresis [24, 25] was combined with the zymogram technique according to the method of ref. [26] and was performed in 1% Pharmacia C Agarose (very low electroendosmosis, $m_r = -0.02$) in 0.05 M veronal buffer, pH 8.6, using 3 V/cm. The gel contained 2.5% of Anti X.

Precipitates in gels were stained with Coomassie blue after washing the gel in PBS, pH 7.2, or were used for the zymogram technique by incubating unwashed gels in air-saturated PBS, pH 6.0, containing 5 mM dihydroxyphenylalanine. Protein was determined according to ref. [27] or, if very low concentrations had to be determined, the method of ref. [28]. Prep. gel filtration with Sepharose 6 B was performed in a 5 × 60 column with a flow of 140 ml/hr. Coupling the antibody to CNBr–Sepharose (Pharmacia) was performed as in ref. [13]. 98.5% of the applied protein was coupled to the Sepharose with a loss of about 30% of the antibody-binding capacity as estimated with a sample containing active Form X.

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