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Purification and characterization of a soluble salicylic acid-binding protein from tobacco

(monoclonal antibody/pathogenesis-related proteins/plant defense mechanism/plant signal transduction/systemic acquired resistance)

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ABSTRACT **Previously, we identified a soluble salicylic acid (SA)-binding protein (SABP) in tobacco whose properties suggest that it may play a role in transmitting the SA signal during plant defense responses. This SA-binding activity has been purified 250-fold by conventional chromatography and was found to copurify with a 280-kDa protein. Monoclonal antibodies capable of immunoprecipitating the SA-binding activity also immunoprecipitated the 280-kDa protein, indicating that it was responsible for binding SA. These antibodies also recognized the 280-kDa protein in immunoblots of the partially purified SABP fraction or the crude extract. However, when the crude extract was prepared in the presence of antioxidants, only a 57-kDa protein was recognized. Since the SABP has a native molecular mass of 240 kDa, it appears that the SABP is a complex which contains a 57-kDa subunit and perhaps one or more additional proteins which are covalently crosslinked in the absence of antioxidants. The ability of a variety of phenolic compounds to compete with SA for binding to the SABP was both qualitatively and quantitatively correlated with their biological activity in inducing defense-related genes. Moreover, the inducibility of the pathogenesis-related (PR)-1 genes by SA was proportional to the abundance of the SABP in different organs. These correlations are consistent with a role for the SABP in perceiving and transducing the SA signal in plant defense.**

Plants, like other organisms, use environmental signals to make appropriate adaptive responses. An excellent example of this is the induction of defense responses by plants upon infection by microbial pathogens (1, 2). In the well-characterized tobacco/tobacco mosaic virus system, the resistance response consists of both local and systemic components. The local response, referred to as the hypersensitive response, involves the formation of necrotic lesions and the restriction of virus proliferation to small zones around the sites of infection (3). In addition, plant defense genes, including five or more families of unrelated pathogenesis-related (PR) genes, are activated in both the inoculated and the uninoculated portions of the plant (4). The systemic expression of PR genes correlates with the establishment of systemic acquired resistance (SAR) (5), in which the entire plant exhibits elevated levels of resistance to secondary infection by the same or unrelated pathogens (3).

A growing body of evidence has suggested that salicylic acid (SA) is a natural signal in SAR. More than a decade ago, application of exogenous SA or its derivative acetylsalicylic acid was shown to induce PR genes and, at least, partial resistance to plant diseases (6). More recently, increases in the levels of endogenous SA were observed to correlate with the expression of defense-related genes and the development of SAR (7–11). The strongest evidence for the involvement of SA in the induction of defense responses is provided by the transgenic tobacco plants which contain and constitutively express the *nahG* gene encoding salicylate hydroxylase from *Pseudomonas putida* (21). In these transgenic plants, induction of SAR by inoculation with tobacco mosaic virus was blocked, presumably due to the destruction of the SA signal by the hydroxylase.

We have been interested in identifying cellular component(s) which directly interact with SA, as a first step to elucidate the mechanism(s) of action of SA in plant signal transduction. We have detected and partially characterized a soluble SA-binding protein (SABP) in tobacco leaves (12). Here, we report the successful purification of SABP by using conventional chromatography and monoclonal antibodies (mAbs). The isolated SABP has also been further characterized to assess its functional relevance to plant defense mechanisms induced by SA.

MATERIALS AND METHODS

Materials. [7-^{14}C]SA (55 Ci/mol; 1 Ci = 37 GBq) was from New England Nuclear. SA and other phenolic compounds were from Sigma or Aldrich. DEAE-Sephacel, Sephacryl S-300, and protein A-Sepharose were from Pharmacia. Blue dextran-agarose and other general chemicals were from Sigma.

Assays. [^{14}C]SA binding was assayed with spin-column exclusion chromatography (12). Protein concentrations were determined according to Bradford (13) with the Bio-Rad protein assay kit.

Purification Procedure. Tobacco (*Nicotiana tabacum* cv. Xanthi nc) leaves (200 g) were sliced and homogenized with a Polytron homogenizer (Brinkmann) in 1 liter of binding buffer [20 mM citrate (pH 6.5)/5 mM $MgSO_4$/1 mM EDTA/10% (vol/vol) glycerol/phenylmethylsulfonyl fluoride at 30 μg/ml] with 2% (wt/vol) polyvinylpolypyrrolidone. The homogenate was filtered through four layers of cheesecloth and then clarified by centrifugation at 40,000 × *g* for 40 min. The resulting supernatant was loaded onto a DEAE-Sephacel column (2.5 × 15 cm) that had been equilibrated with binding buffer. After loading, the column was washed with binding buffer and a 400-ml gradient from 0 to 1 M KCl in the binding buffer was applied at a flow rate of 0.5 ml/min. Fractions with peak binding activity were pooled and loaded onto a Sephacryl S-300 gel filtration column (2.5 × 100 cm) equilibrated with binding buffer at a flow rate of 0.5 ml/min. The peak fractions from the gel filtration column were pooled and loaded onto a blue dextran-agarose column (0.5 × 15 cm) equilibrated with binding buffer. After extensive washing with the binding buffer, the SA-binding activity was step eluted at a flow rate of 0.1 ml/min with binding buffer

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Abbreviations: PR, pathogenesis-related; SA, salicylic acid; SABP, SA-binding protein; SAR, systemic acquired resistance; mAb, monoclonal antibody.

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containing 0.7 M KCl. The peak fractions were again combined and injected (0.5 ml per run) into a Superose 6 HR 10/30 column connected to an FPLC system (Pharmacia). Proteins were eluted with binding buffer at a flow rate of 0.35 ml/min, and peak fractions were pooled for further characterization.

mAb Production. The Superose 6 HR 10/30 peak fractions were pooled and submitted for mAb production (14) to the Hybridoma Laboratory, Department of Molecular Biology, Princeton University.

ELISA. ELISA was performed essentially as described by Walker and Huber (15) except that all steps were carried out at room temperature and 0.2% bovine serum album was added to the solution containing goat anti-mouse–alkaline phosphatase-conjugated antibodies.

Immunoprecipitation. In standard assays, 100–500 μl of hybridoma culture medium was incubated with 40 μl of protein A-Sepharose (50% slurry) at 4°C for 2 hr. The antibody–protein A-Sepharose complexes were pelleted and washed three times with RIPA buffer (150 mM NaCl/5 mM EDTA/1% sodium deoxycholate/0.1% SDS/10 mM Tris, pH 7.4), and once with binding buffer containing 150 mM KCl and 0.1% Nonidet P-40. The complexes were incubated at 4°C for 2 hr with 100 μl of the partially purified SABP obtained after blue dextran-agarose chromatography. Antigen–antibody–protein A complexes were pelleted and supernatants were assayed for residual SA-binding activity. The pellets were washed three times with RIPA buffer before SDS/PAGE.

Immunoblot Analysis. Protein samples were fractionated by SDS/PAGE and the separated proteins were electrophoretically transferred to a nitrocellulose filter. The filter was blocked by a 1-hr incubation at room temperature in PBST buffer (100 mM phosphate, pH 7.5/100 mM NaCl/0.1% Tween 20) containing 5% nonfat milk and was washed three times with PBST buffer. Blots were incubated for 1 hr with diluted hybridoma medium (1:100) in PBST buffer containing 0.2% bovine serum albumin and washed three times with PBST buffer. The antigen–antibody complexes were detected with a 1:10,000 dilution of horseradish peroxidase-conjugated sheep anti-mouse IgG antibodies and the ECL (enhanced chemiluminescence) detection kit from Amersham.

Analysis of PR-1 Induction. To determine the biological activity of SA and its analogues for inducing PR-1 gene expression, three leaf discs (1 cm in diameter) were floated on 30 ml of solution containing 10 μM to 1 mM inducer. To analyze the inducibility of PR-1 genes by SA in different organs, 6-week-old plants were watered with 1 mM SA. Tissue was harvested 48 hr after treatment and homogenized in 50 mM Tris, pH 8.0/1 mM EDTA/12 mM 2-mercaptoethanol/phenylmethylsulfonyl fluoride at 10 μg/ml. After clarification by centrifugation, the homogenate was fractionated by SDS/PAGE, and immunoblot analysis was performed with a 1:1,000 dilution of mAb 33G1, which specifically recognizes PR-1 proteins (16).

RESULTS

Purification of SABP. During the initial characterization of the soluble SABP, we found that the protein exhibited a very high molecular mass (>600 kDa) when analyzed by gel filtration chromatography after 0–35% ammonium sulfate precipitation (12). Subsequent fractionation steps resulted in very inefficient purification because SABP was eluted with the majority of the other proteins present. These results suggested that there might be substantial aggregation of

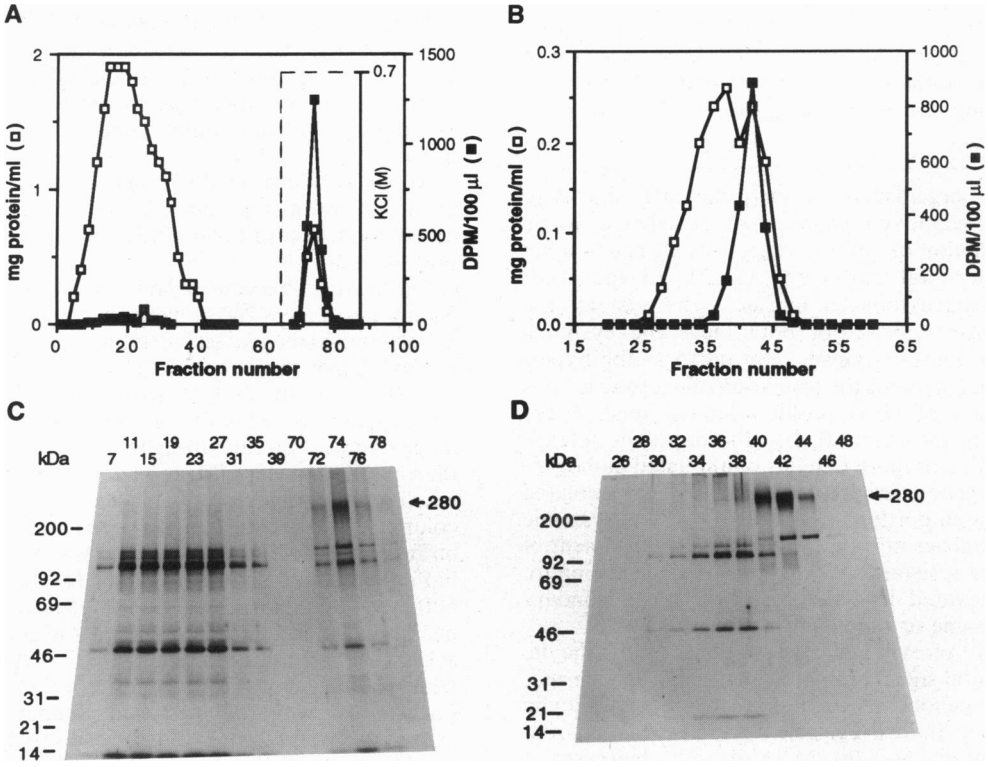

FIG. 1. Elution profiles of proteins and SA-binding activity on blue dextran-agarose (*A* and *C*) and Superose 6 HR 10/30 (*B* and *D*) columns. Active fractions from the Sephacryl S-300 column were chromatographed on a blue dextran-agarose column. After extensive washing, the bound SA-binding activity was eluted with the starting buffer containing 0.7 M KCl (*A*). SDS/7.5–15% PAGE analysis indicated that a 280-kDa protein was substantially enriched in the eluted fractions (*C*) in correlation with increased specific SA-binding activity. The eluted, active fractions from the blue dextran-agarose column were pooled and applied to a superose 6 HR 10/30 column. The eluted fractions were again subjected to SDS/PAGE (*D*) and the 280-kDa protein was found to be coeluted with the SA-binding activity (*B*). Fraction numbers from the columns are indicated above the gels (*C* and *D*), which were silver stained. Molecular masses of marker proteins are shown at left.

SABP with other proteins in the 0–35% ammonium sulfate fraction. While many conditions can cause protein aggregation, we suspected that the ammonium sulfate precipitation was responsible.

To overcome this potential problem, the tissue homogenate was clarified by centrifugation and directly fractionated on DEAE-Sephacel. After loading and washing, the retained SA-binding activity was eluted as a single broad peak between 0.3 and 0.6 M KCl. Peak fractions of the binding activity were pooled and chromatographed on a Sephacryl S-300 column. The majority of the SA-binding activity (>70%) was eluted from this gel filtration column with an apparent molecular mass of 240 kDa, while a minor portion of the activity was eluted at >600 kDa (data not shown). However, when ammonium sulfate precipitation was performed prior to DEAE-Sephacel and gel filtration chromatography, the >600-kDa peak was greatly enhanced whereas the 240-kDa peak essentially disappeared. Thus, the 240-kDa peak appears to represent an unaggregated or less aggregated form of SABP, which can readily aggregate into large complexes (>600 kDa) upon ammonium sulfate precipitation.

Further purification of SABP from the Sephacryl S-300 fractions containing the 240-kDa species was accomplished by chromatography on a blue dextran-agarose column. Fractions from this purification step were analyzed for SA-binding activity (Fig. 1*A*) and protein composition by SDS/PAGE (Fig. 1*C*). In the lanes corresponding to peak binding activity (fractions 72–76), five to six major and several minor proteins were detected. Among these proteins, only a 280-kDa protein was substantially enriched. In addition, the intensity of the 280-kDa band correlated with the level of SA-binding activity found in these fractions.

Subsequent purification of pooled blue dextran-agarose fractions was performed by chromatography on a Superose 6 column. The eluted fractions were analyzed for binding activity (Fig. 1*B*) and protein composition by SDS/PAGE (Fig. 1*D*). In the lanes corresponding to fractions 40–45, which contained the most binding activity, two major polypeptides of 150 kDa and 280 kDa were observed. The level of SA-binding activity correlated best with the intensity of the 280-kDa protein. These four chromatography steps resulted in a 1570-fold reduction of total proteins and a 250-fold increase in specific binding activity (Fig. 2 and Table 1).

Identification of SABP by Immunoprecipitation. Positive clones of mAbs raised against the Superose 6 fractions were identified by ELISA and further screened for their ability to immunoprecipitate SA-binding activity. Four clones exhibiting the strongest recognition of the SA-binding activity (3B6, 1F5, 2C11, and 7F10) were chosen for further analysis. mAbs from these four clones immunoprecipitated the SA-binding activity in a concentration-dependent manner (Fig. 3*A*). In contrast, mAb 5A8 failed to remove any significant amount of SA-binding activity. Most importantly, analysis of the immunoprecipitated complexes by SDS/PAGE demonstrated that mAbs which were able to immunodeplete SA-binding activity recognized only the 280-kDa protein (Fig. 3*B*). In contrast, mAb 5A8 immunoprecipitated only the 150-kDa protein present in the highly purified fraction (Fig. 3*B*). From these results, we conclude that the 280-kDa protein is responsible for the SA-binding activity. The size of this protein is in close agreement with the native molecular mass of the SA-binding activity determined by gel filtration (240 kDa).

Immunoblot Analysis of SABP. The four SABP-specific mAbs were used to detect the SABP by immunoblot analysis. As controls, three additional mAbs (5A8, 6E10, and 33G1) which did not recognize SABP were also included in the immunoblot analysis. mAbs 5A8 and 6E10 were obtained from the same fusions from which the four SABP-specific mAbs were obtained. mAb 33G1 specifically recognizes the

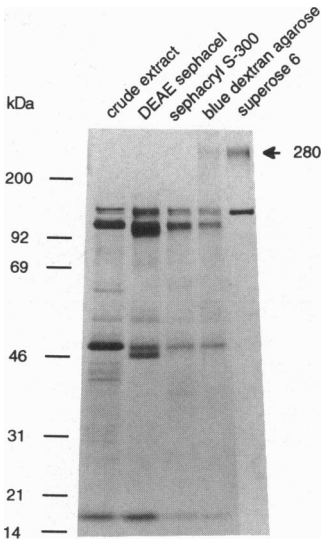

FIG. 2. Purification of a soluble SABP from tobacco leaves. SDS/7.5–15% PAGE of protein samples from crude extract, DEAE-Sephacel, Sephacryl S-300, blue dextran-agarose, and Superose 6 HR 10/30. Molecular masses of marker proteins are shown at left. The 280-kDa protein that was coeluted with SA-binding activity (Fig. 1) is indicated at right. The gel was silver stained.

16-kDa PR-1 proteins of tobacco (16). The PR-1 proteins are not synthesized in uninfected plants such as those used for SABP purification (4, 16). The four SABP-specific mAbs, but not the three control mAbs, each recognized the 280-kDa protein in the partially purified preparation of SABP (Fig. 4). Thus, the 280-kDa protein was the only protein in the partially purified fraction which could be specifically recognized by the SABP-specific mAbs, further indicating that the 280-kDa protein was SABP.

In contrast, the four SABP-specific mAbs recognized only a 57-kDa protein in tobacco leaf homogenates freshly prepared in the presence of thiol-reducing antioxidant (15 mM 2-mercaptoethanol; Fig. 4) or other antioxidants (e.g., dithiothreitol or ascorbic acid; data not shown). Inclusion of reducing agents or other antioxidants in the preparation should repress the activity of phenol oxidases that could cause crosslinking of proteins in the presence of phenolic compounds (17). This result suggested that the 280-kDa protein might be a covalently crosslinked complex, one of whose components was the 57-kDa protein. To verify this, immunoblot analysis was also performed with a crude extract prepared in the absence of any antioxidant. Under these conditions, the SABP-specific antibodies detected only the 280-kDa protein (data not shown). Therefore, the 280-kDa protein appears to be a homomeric or heteromeric complex whose components, including the 57-kDa protein, are crosslinked during homogenization.

Binding Affinity and Specificity. The highly purified SABP had a K_d of 15.5 μM for SA as compared to the K_d of 14 μM found with the crude extract (12). To determine the binding specificity of the SABP, 23 phenolic compounds were tested for their ability to compete with [^{14}C]SA for binding to the

Table 1. Summary of SABP purification

Step	Protein, mg	Activity, %	Purification, fold
Crude extract	2350	100	1
DEAE-Sephacel	650	70	2.5
Sephacryl S-300	70	35	12
Blue dextran-agarose	4	26	152
Superose 6 HR 10/30	1.5	16	250

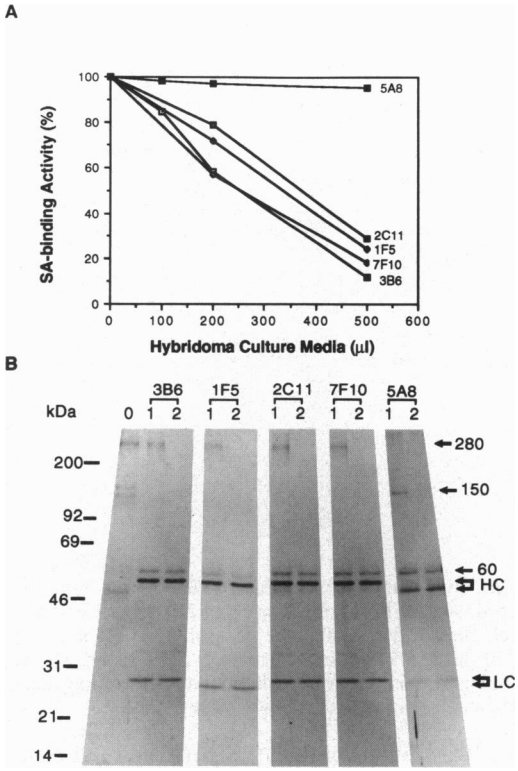

FIG. 3. Coimmunoprecipitation of the SA-binding activity and the 280-kDa protein. mAbs 3B6, 1F5, 2C11, and 7F10, but not 5A8, immunoprecipitated SA-binding activity in an antibody-concentration-dependent manner (*A*). Complexes immunoprecipitated by these mAbs were analyzed by SDS/PAGE (*B*). Immunoprecipitation was performed with either the partially purified SABP obtained after blue dextran-agarose chromatography (lanes 1) or buffer only (lanes 2). The protein composition of the partially purified SABP is shown in lane 0. The 280-kDa polypeptide was the only protein precipitated by mAbs 3B6, 1F5, 2C11, and 7F10. The 150-kDa protein was immunoprecipitated by mAb 5A8. The heavy chains (HC) and light chains (LC) of mAbs are also indicated. The 60-kDa protein seen in both lanes 1 and 2 is a contaminant from hybridoma media, in which it is present at high levels.

highly purified SABP. Among these 23 phenolic compounds, only those derivatives with biological activity in inducing disease resistance and PR genes (SA, 2,6-dihydroxybenzoic acid, acetylsalicylic acid, benzoic acid, and 2,3-dihydroxybenzoic acid; refs. 6 and 18–20) could compete with [^{14}C]SA for binding to the highly purified SABP (Table 2). In contrast, biologically inactive phenolic compounds did not compete (Table 2). Furthermore, for those biologically active phenolic compounds, their potency in inducing plant defense responses was directly correlated with their effectiveness in competing with [^{14}C]SA for binding to SABP (i.e., SA and 2,6-dihydroxylbenzoic acid > acetylsalicylic acid > benzoic acid > 2,3-dihydroxybenzoic acid; Table 2). Thus, the biological activity of these phenolic compounds was both qualitatively and quantitatively correlated with the ability to bind to SABP.

Correlation Between SABP Abundance and Inducibility of PR-1 Genes by SA in Different Organs. Immunoblot analysis indicated that the 57-kDa subunit of SABP was present at relatively high levels in leaves, at low levels in stems, and at barely detectable levels in roots (Fig. 5*A*). In leaf tissues, a slightly higher molecular weight band was occasionally observed, probably due to posttranslational modification of the protein. If SABP is a receptor for SA in plant defense responses, it is likely that the abundance of SABP would correlate with the responsiveness of defense-related genes to SA in different organs. To test this possibility, the inducibility

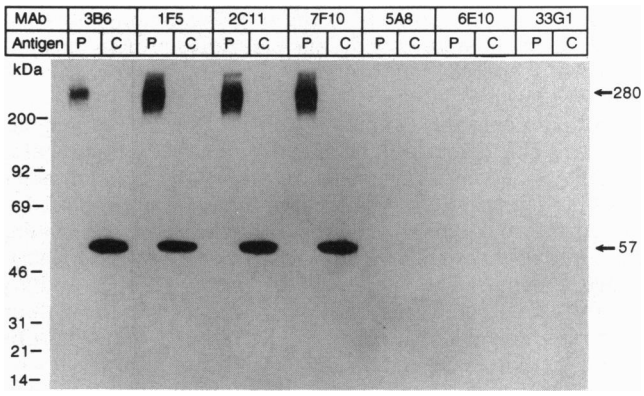

FIG. 4. Immunoblot analysis of partially purified SABP (P) and crude extract (C) freshly prepared in the presence of 15 mM 2-mercaptoethanol. The SABP-specific mAbs (3B6, 1F5, 2C11, and 7F10) recognized the 280-kDa protein in the partially purified SABP obtained after three chromatographic steps. In contrast, they recognized a 57-kDa protein in the crude extract prepared in the presence of 2-mercaptoethanol. Control mAbs 5A8, 6E10, and 33G1 did not recognize any protein in the partially purified fraction or crude extract. mAb 5A8 appeared to recognize only the native form of the 150-kDa protein (see Fig. 3*B*).

of PR-1 genes by SA was analyzed. While SA induced PR-1 protein synthesis to high levels in leaf tissue, induction in stem was much lower (Fig. 5*B*). No accumulation of PR-1 protein was detected in roots after SA treatment. Thus, the inducibility of PR-1 genes by SA in these organs was directly proportional to the abundance of SABP.

DISCUSSION

Since the initial identification of SABP, we have been focusing on its purification, which will enable us to further analyze

Table 2. Inhibition of [^{14}C]SA binding by phenolic compounds

Inhibitor	Biological activity*	Inhibition, %† 2×	10×
2-Hydroxybenzoic acid (SA)	+	48	89
2,6-Dihydroxybenzoic acid	+	52	92
Acetylsalicylic acid	+	21	48
Benzoic acid	+	15	27
2,3-Dihydroxybenzoic acid	±	2	9
3-Hydroxybenzoic acid	−	−2	1
4-Hydroxybenzoic acid	−	1	−2
2,4-Dihydroxybenzoic acid	−	1	−4
2,5-Dihydroxybenzoic acid	−	−2	1
3,4-Dihydroxybenzoic acid	−	3	0
3,5-Dihydroxybenzoic acid	−	1	−7
2,3,4-Trihydroxybenzoic acid	−	0	2
2,4,6-Trihydroxybenzoic acid	−	5	3
3,4,6-Trihydroxybenzoic acid	−	1	−5
o-Coumaric acid	−	0	1
3-Aminosalicylic acid	−	2	4
4-Aminosalicylic acid	−	1	−4
5-Aminosalicylic acid	−	5	2
Thiobenzoic acid	−	1	0
Thiosalicylic acid	−	2	−4
2-Chlorobenzoic acid	−	0	−5
2-Ethoxybenzoic acid	−	0	1
Catechol	−	2	−6

*Biological activity is based on results of White (6), Van Loon (18), Abad *et al.* (19), and Doherty *et al.* (20) and on data on PR-1 gene expression obtained in the present study.

†[^{14}C]SA (20 μM) binding was assayed with highly purified SABP obtained after four steps of chromatography (see Fig. 2 and Table 1). Assays were done in the presence of 40 μM (2×) or 200 μM (10×) unlabeled competitor.

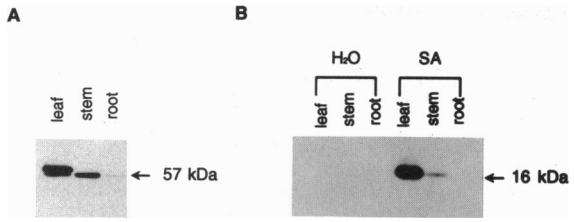

FIG. 5. Abundance of SABP (*A*) and inducibility of PR-1 genes by SA (*B*) in tobacco leaves, stems, and roots. SABP levels were determined by immunoblot analysis of the crude extracts freshly prepared from these organs in the presence of 15 mM 2-mercaptoethanol. Immunoblots were probed with a combination of the four SABP-specific mAbs. Induction of the 16-kDa PR-1 proteins by SA was determined by immunoblot analysis with the PR-1-specific mAb 33G1. SA treatment of tobacco plants did not enhance the levels of the SABP or SA-binding activity (Z.C. and D.F.K., unpublished results).

its functional relevance in plant defense responses. By employing four chromatographic steps, a 250-fold purification was achieved (Fig. 2 and Table 1). Analysis of the highly purified SABP fractions showed that a 280-kDa protein copurified with the SA-binding activity (Fig. 1). Furthermore, four mAbs which were able to deplete the SA-binding activity (Fig. 3*A*) also immunoprecipitated the 280-kDa protein from the partially purified SABP preparation (Fig. 3*B*). Thus, the 280-kDa protein was responsible for SA binding.

Surprisingly, immunoblot analysis suggested that the 280-kDa protein observed on SDS/PAGE was not a single polypeptide but rather a covalently crosslinked complex which contained a 57-kDa protein. Since the native molecular mass of SABP was ≈240 kDa as determined by gel filtration, and as the four SABP-specific mAbs all recognized the 57-kDa protein (Fig. 4), the native SABP complex might well be a homotetramer of the 57-kDa polypeptide. Analysis of the specific SA-binding activity of the highly purified SABP indicated that there were approximately two binding sites per 280-kDa molecule of SABP. The actual number of binding sites per 280-kDa SABP might be higher, since some of the proteins may have been inactivated during the purification. This stoichiometry is consistent with a homomeric complex or a simple heteromeric complex containing the 57-kDa polypeptide and one or a few additional proteins.

The crosslinking of SABP occurred rapidly and efficiently during homogenization of tobacco leaf tissue. Antioxidants such as 2-mercaptoethanol, dithiothreitol, and ascorbic acid effectively inhibited the crosslinking process (Fig. 4 and data not shown) and reduced the development of brown pigments in the homogenate. This coloration is believed to result from the polymerization of phenolic compounds (17). These results suggest that the phenolic compounds or reactive quinones produced by phenol oxidases might be the cause of the crosslinking (17). We have tested several antioxidants or phenol oxidase inhibitors (e.g., thiourea) for their ability to reduce crosslinking during the purification. However, those compounds either greatly inhibit the SA-binding activity in an irreversible manner or make the binding activity very unstable during the subsequent purification. Thus, SABP appears to be a very unstable protein, and the crosslinking process may stabilize its structure and consequently its SA-binding activity.

A large number of phenolic compounds were tested in the present study for their ability to compete with SA for binding to the highly purified SABP. Among the 23 phenolic compounds tested, only SA, 2,6-dihydroxybenzoic acid, and acetylsalicylic acid were able to compete effectively with [^{14}C]SA for binding to SABP (Table 2). Benzoic acid competed less effectively, and 2,3-dihydroxybenzoic acid competed only marginally. The level of competition directly correlated with the ability of these compounds to induce plant defense-related genes. Thus, the biological activity of SA analogues was not only qualitatively, but also quantitatively, correlated with their ability to bind to SABP.

The data suggest that SABP plays a role in signal transduction during defense responses. It may function in a manner similar to a typical receptor—i.e., perceiving and transducing a specific signal to other appropriate factors and ultimately activating cellular responses. Alternatively, SABP may be a cellular target of SA which when associated with SA exhibits altered properties/activities. That might lead to changes of certain biochemical/physiolocal states of plant cells which are directly or indirectly related to the activation of plant defense responses. Purification of SABP and subsequent cloning of its gene will make it possible to carry out further functional analysis and investigate these possibilities.

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