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Rapid Communication

A Salicylic Acid-Binding Activity and a Salicylic Acid-Inhibitable Catalase Activity Are Present in a Variety of Plant Species[1]

Paloma Sánchez-Casas[2] and Daniel F. Klessig*

Waksman Institute and the Department of Molecular Biology and Biochemistry, Rutgers, The State University of New Jersey, P.O. Box 759, Piscataway, New Jersey 08855

Recently, it has been demonstrated that the salicylic acid (SA)-binding protein (SABP) from tobacco (*Nicotiana tabacum*) is a SA-inhibitable catalase (Z. Chen, H. Silva, D.F. Klessig [1993] Science 262: 1883–1886). Here we report the presence of SABP and SA-inhibitable catalase activity in *Arabidopsis*, tomato, and cucumber. The cucumber SABP has properties similar to the tobacco SABP, including binding affinity and specificity for SA.

The mechanisms in plant disease resistance underlying the development of the hypersensitive response and systemic acquired resistance are poorly understood. One of the general responses to pathogen infection is the expression of genes encoding several families of PR proteins (for review, see Cutt and Klessig, 1992). However, the roles of PR genes in hypersensitive response and systemic acquired resistance remain to be elucidated. In the relatively well-characterized tobacco (*Nicotiana tabacum*)-TMV system, there is strong evidence that SA is an endogenous signal for local and systemic defense responses (White, 1979; Malamy et al., 1990, 1992; Yalpani et al., 1991; Ward et al., 1991; Gaffney et al., 1993).

To help elucidate SA's role in disease resistance, we have isolated and characterized a SABP and its encoding gene from tobacco (Chen and Klessig, 1991; Chen et al., 1993a, 1993b). SABP's binding affinity and specificity are consistent with it being a receptor for SA. Further analysis revealed that it is a catalase whose activity is inhibitable by SA.

Catalases belong to a group of enzymes involved in regulating the cellular levels of active oxygen species. They are present in all aerobic organisms and convert H_2O_2 to H_2O and O_2, thus protecting cells from the damaging effects of H_2O_2. Although highly elevated levels of H_2O_2 are toxic, at lower concentrations they appear to play important roles in signal transduction in both plants and animals (Devary et al., 1991; Schreck et al., 1991; Legendre et al., 1993; Prasad et al., 1994).

Since H_2O_2 production is an ongoing process in plants, inhibition of catalase activity, one of the main routes of H_2O_2 degradation, should result in H_2O_2 accumulation. As expected, we found that treatment of tobacco leaves with SA led to elevated H_2O_2 levels in vivo (Chen et al., 1993b). Moreover, artificially elevating H_2O_2 levels in tobacco leaves by injecting (a) H_2O_2, (b) 3-amino triazole, a known inhibitor of catalases, or (c) compounds that promote the generation of H_2O_2 in vivo induced expression of PR genes. Together these results suggest that SA's mode of action is to bind catalase and inhibit its activity. The resulting elevation of H_2O_2 or other active oxygen species derived from it then activates defense-related genes, such as the PR genes, perhaps by acting as a second messenger.

SA appears to be a defense-mediating signal not only in tobacco but other plant species (for review, see Klessig and Malamy, 1994), including cucumber (*Cucumis sativus*) (Métraux et al., 1990; Rasmussen et al., 1991) and *Arabidopsis* (Uknes et al., 1993; Summermatter et al., 1994). However, it is unclear whether its mode of action is to bind and inhibit the activity of catalases in these species. It is possible that SA has an additional mode(s) of action; however, the identification of catalases that bind and are inhibited by SA in other plant species that exhibit SA-mediated defense responses would support the mechanism proposed for SA action in tobacco. Here we have tested six other plant species for the presence of a SABP that exhibits SA-inhibitable catalase activity. Since there is considerable evidence implicating SA in disease resistance in cucumber, a more detailed characterization of its SABP is also presented.

MATERIALS AND METHODS

Materials

Plant material used included tobacco (*Nicotiana tabacum* cv Xanthi nc), cucumber (*Cucumis sativus* cv Wisconsin S.M.R.58), tomato (*Lycopersicon esculentum* var Craigella GCR number 237), *Arabidopsis thaliana* ecotype Dijon,

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[2] Present address: Department of Biochemistry and Biotechnology, School of Enology, University of Rovira and Virgili, 43005 Tarragona, Spain.

* Corresponding author; fax 1-908-445-5735.

Abbreviations: B_{max}, maximum concentration of bound ligand; K_d, dissociation constant; PR, pathogenesis-related; SA, salicylic acid; SABP, salicylic acid-binding protein; TMV, tobacco mosaic virus.

soybean (*Glycine max* cv Corsoy), maize (*Zea mays* inbred line A188), and rice (*Oryza sativa* cv M201).

Preparation of Soluble Protein

Leaves from different plant species were homogenized at 4°C with a Polytron in 4 volumes of homogenization buffer (20 mM citrate, pH 6.5, 5 mM $MgSO_4$, 1 mM EDTA, 0.2 mM PMSF, 1% [w/v] polyvinylpolypyrrolidone). The homogenate was filtered through four layers of cheesecloth and centrifuged for 30 min at 40,000*g*. The resulting supernatant was brought to 45% of $(NH_4)_2SO_4$ saturation. The precipitate was collected by centrifuging for 20 min at 10,000*g* and dissolved in binding buffer (20 mM citrate, pH 6.5, 5 mM $MgSO_4$, 1 mM EDTA). The protein solution was dialyzed against 4 L of binding buffer overnight and then centrifuged for 15 min at 3,500*g* to remove undissolved proteins prior to the binding assay. The supernatant from the 45% $(NH_4)_2SO_4$ cut was brought to 80% $(NH_4)_2SO_4$ saturation and the resulting precipitate was processed as described for the 45% $(NH_4)_2SO_4$ precipitate. All the preparations were done at 4°C.

[^{14}C]SA-Binding Assay

Soluble protein (15 mg mL^{-1} binding buffer) was incubated at 4°C for 3 h in the presence of 20 μM [^{14}C]SA (55 Ci/mol). Bound and free SA were separated by spin-column exclusion chromatography as described (Chen and Klessig, 1991), but using Sephadex G-25 in place of Bio-Gel P-6DG. Nonspecific binding was determined in the presence of 1 mM SA. K_d and B_{max} values were determined by Scatchard analysis performed with 5 μM [^{14}C]SA and concentrations of unlabeled SA ranging from 2.5 to 100 μM.

Catalase Activity Assay

Catalase activity was determined for the 0 to 45% and 45 to 80% $(NH_4)_2SO_4$ fractions by measuring the rate of H_2O_2 conversion to O_2 at room temperature, using an oxygen electrode, in a 10-mL solution containing 20 mM citrate, pH 6.5, 5 mM $MgSO_4$, 1 mM EDTA, 10 mM H_2O_2, and protein concentrations ranging from 5 to 100 μg depending on the plant species. Rate of the reaction was linear for at least 3 min.

RESULTS AND DISCUSSION

Presence of SABP in Various Plants

To determine if plants other than tobacco contain a SABP, crude soluble extracts were prepared from leaves of cucumber, tomato, *Arabidopsis*, soybean, maize, and rice in addition to tobacco. Since the majority of the SA-binding activity from tobacco is precipitated with $(NH_4)_2SO_4$ at 45% saturation (Chen and Klessig, 1991), the crude extracts were divided into 0 to 45% and 45 to 80% $(NH_4)_2SO_4$ fractions and then analyzed for binding of [^{14}C]SA in the presence or absence of excess unlabeled SA (Table I). As expected, there was little specific SA-binding activity in the 45 to 80% $(NH_4)_2SO_4$ fraction from any of the plant extracts. High levels of SA-binding activity were detected in the 0 to 45% $(NH_4)_2SO_4$ fractions from cucumber, tomato, *Arabidopsis*, and tobacco. Seventy percent to 90% of this binding was inhibited by excess unlabeled SA, indicating that the majority of the binding activity was specific. In contrast, very little specific SA-binding activity was present in the 0 to 45% $(NH_4)_2SO_4$ fraction from maize or rice, whereas soybean extracts contained much reduced but variable levels.

Table I. *Binding of [^{14}C]SA by soluble proteins from leaves of different plant species*

Species	[^{14}C]SA Bound[a]			Binding Relative to Tobacco[b]
	1 mM SA	0–45% $(NH_4)_2SO_4$ cut	45–80% $(NH_4)_2SO_4$ cut	0–45% $(NH_4)_2SO_4$ cut
		dpm/mg protein		%
Tobacco	−	1800 ± 329[c]	418 ± 10	100
	+	180 ± 34	381 ± 30	
Cucumber	−	1334 ± 263	252 ± 44	65
	+	276 ± 116	166 ± 20	
Tomato	−	1001 ± 239	418 ± 10	44
	+	288 ± 31	291 ± 32	
Arabidopsis	−	1217 ± 595	308 ± 78	64
	+	174 ± 21	228 ± 49	
Soybean	−	489 ± 46	430 ± 54	4[d]
	+	423 ± 43	462 ± 40	
Maize	−	399 ± 107	318 ± 66	7
	+	282 ± 81	297 ± 11	
Rice	−	250 ± 60	536 ± 34	4
	+	189 ± 44	342 ± 11	

[a] Values were obtained from three independent binding assays done in duplicate. SD values are given. All the data in this table are from a single experiment done on the same day so that the data could be readily compared among plants. Binding measurements were repeated with at least one independent extract for each species. Percentage binding relative to tobacco in other independent experiments were 55, 59, 57, 86, 67, 59, and 60% for cucumber; 50 and 62% for tomato; 40 and 85% for *Arabidopsis*; 14 and 17% for soybean; 2 and 8% for maize; and 3% for rice. [b] [^{14}C]SA binding in the presence of 1 mM unlabeled SA was considered nonspecific binding. This number was subtracted from the value obtained in the absence of unlabeled SA before calculating the percentage of tobacco SA-binding activity. [c] This value of 1620 dpm/mg protein equals 13.5 pmol of bound SA. [d] Two other extracts from soybean leaves had considerably more binding activity with levels between 14 and 17% of that found in tobacco leaves. Soybean seedlings exhibited levels similar to that detected in tobacco leaves.

Catalase Inhibition by SA

SA inhibits a substantial portion of the catalase activity in both crude extracts from tobacco leaves and in purified preparations of SABP (Chen et al., 1993b). To determine whether catalases from other plants are also sensitive to SA and whether this inhibition correlates with SA-binding activity, crude extracts were prepared from the various species. The amount of catalase activity and its sensitivity to SA were determined for both the 0 to 45% and 45 to 80% $(NH_4)_2SO_4$ fractions. Approximately 90% or more of the catalase activity was found in the 0 to 45% $(NH_4)_2SO_4$ fraction, consistent

Table II. *Inhibition by SA of catalase activity from different plant species*

	Catalase Activity[a]		Inhibition
	0 mM SA	1 mM SA	
	μmol O_2 mg^{-1} protein min^{-1}		%
Tobacco	3.21 ± 0.2	1.09 ± 0.2	66
Cucumber[b]	17.80 ± 3	8.71 ± 0.8	51
Tomato	0.23 ± 0.02	0.10 ± 0.02	57
Arabidopsis	13.50 ± 2	7.20 ± 2.4	47
Soybean	0.62 ± 0.4	0.42 ± 0.01	32
Maize	0.76 ± 0.3	0.76 ± 0.3	0
Rice	13.13 ± 3	13.10 ± 2	0

[a] The numbers presented are for the 0 to 45% $(NH_4)_2SO_4$ fraction. Assays were done at least three times. The same extracts were used for the data shown in Tables I and II. [b] Inhibition of catalase activity by SA in cucumber extracts required the presence of moderate concentrations of salt. No differences were found with salt concentrations in the range of 80 to 330 mM KCl. The presence of salt did not alter the sensitivity of catalase to SA in extracts from maize and rice, although inhibition by SA was increased slightly (about 10%) in tobacco extracts.

with the location of the SA-binding activity. Catalase activities from cucumber, tomato, *Arabidopsis*, and tobacco were substantially inhibited by SA, whereas those from maize and rice were very insensitive (Table II). Soybean catalase showed an intermediate level of sensitivity. Thus, there is a close correlation between SA-binding activity in the crude extracts from different plants and sensitivity of their catalases to SA.

There are several possible explanations for the lack, or at least near absence, of detectable SA-binding activity and SA-inhibitable catalase activity in the leaf extracts of certain plants. First, SA may not be a signal for defense responses in the leaves of these plants. A second possibility is that SA has a different mode of action in the leaves of these plants that does not involve catalase. Another explanation is that only certain catalase isozymes are SA inhibitable. Catalases are encoded by multiple genes that are developmentally and environmentally regulated (for review, see Scandalios, 1992, 1994). Thus, the leaves of plants with little SA-inhibitable catalase activity may contain predominantly SA-insensitive isozymes. Yet a fourth explanation for low levels of SA-binding activity and SA-sensitive catalase activity in certain extracts is that these activities are particularly labile and thus may require optimization of the extraction procedure.

Unexpectedly, the amount of total catalase activity from the leaves of different species varied from 0.2 to 20 μmol of O_2 evolution per mg protein per min and did not correlate with the amount of SA-binding activity in leaf extracts. For example, tobacco, cucumber, tomato, and *Arabidopsis* had high levels of SA-binding activity but only extracts of cucumber and *Arabidopsis* contained high catalase activity. A possible explanation for this discrepancy comes from the work of Chen et al. (1993a), who found that when tobacco protein extracts were prepared in the absence of antioxidants, the subunits of the tetrameric catalase complex were cross-linked together to form a SDS- and DTT-resistant complex of approximately 280 kD. We have found that these cross-linked complexes are readily formed in extracts of tomato and soybean as well as tobacco, but not in extracts of cucumber, *Arabidopsis*, or rice (data not shown). Since extracts from the first three plants contained only low levels of catalase activity and those from the latter three had high levels, these results suggest that cross-linking of the catalase subunits may destroy much of the catalase activity. However, cross-linking appears to have much less effect on SA binding, since both tobacco and tomato extracts contained high SA-binding activity and most of the remaining catalase activity was inhibitable by SA. Cross-linking is probably due to the activity of phenol oxidases, and the extent of cross-linking may reflect differences in the levels of phenol oxidases or their substrates (phenolic compounds) in the leaves of the various plants.

Further Characterization of the Cucumber SA-Binding Activity and Catalase Activity

The SABP and catalase activity from cucumber plants were analyzed in more detail, since there is substantial evidence that SA plays a role in mediating defense responses in this species. Similar to the tobacco SABP, the cucumber SA-binding activity was saturable (specific) and was found in the 0 to 45% $(NH_4)_2SO_4$ fraction (Table I). This activity was inhibited by 0.5% SDS and was pH dependent with an optimum between pH 5.5 and 6.5 (data not shown). The tobacco SABP exhibits a similar pH dependency (Z. Chen and D.F. Klessig, unpublished data). Scatchard analysis indicated that there was a single class of binding sites with an apparent K_d of 30 μM (Fig. 1) compared to 14 μM for the tobacco SABP (Chen and Klessig, 1991). This analysis also demonstrated that the amount of binding activity (5 pmol per mg of total soluble protein) was similar in both species (Fig. 1) (Chen and Klessig, 1991).

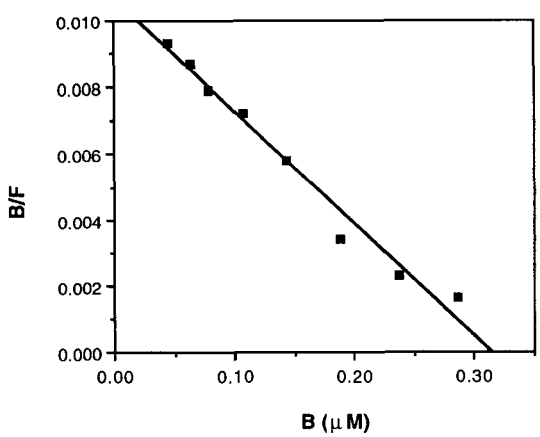

Figure 1. Scatchard plot of SA binding by soluble proteins from cucumber leaves. Values are averages from three independent protein preparations assayed in duplicate. K_d was 30 μM and B_{max} was 21 pmol per mg of soluble protein from the 0 to 45% $(NH_4)_2SO_4$ fraction. Since the protein from this $(NH_4)_2SO_4$ fraction represents approximately 25% of the total soluble protein, the actual B_{max} was approximately 5 pmol per mg of total soluble protein. B, Bound ligand; F, free ligand.

The ability of the cucumber SABP to selectively bind only biologically active SA analogs was also tested. The cucumber SABP exhibited highly specific ligand binding (Table III) similar to that observed with the tobacco SABP (Chen and Klessig, 1991; Chen et al., 1993a). A 10-fold molar excess of unlabeled SA or the very active analog 2,6-dihydroxybenzoic acid inhibited the cucumber SABP binding of [^{14}C]SA by 70 to 80%. Benzoic acid and 2,3-dihydroxybenzoic acid, which are much less biologically active in tobacco, were similarly less effective competitors for [^{14}C]SA binding to the cucumber SABP. In addition, six of the seven phenolic compounds that are inactive in tobacco did not compete. However, 2,4-dihydroxybenzoic acid, a compound that is biologically inactive in tobacco and is not bound by the tobacco SABP (Chen and Klessig, 1991; Chen et al., 1993a), exhibited a low level of binding to the cucumber SABP. This result suggests that there may be slight differences in the biological activity of the various SA analogs in different plant species.

Several of the SA analogs, with varying ability to induce PR gene expression and disease resistance in tobacco, were compared for their ability to inhibit the catalase activity from cucumber (Table IV). The biologically active analogs were 7- to 10-fold more effective at inhibiting catalase activity than the inactive analogs. Thus, in cucumber, as in tobacco, the ability of different SA analogs to inhibit catalase activity correlated with their biological activity and their ability to bind SABP. These results argue that the cucumber SABP, like that of tobacco, is a SA-inhibitable catalase. This conclusion is supported by the observation that monoclonal antibodies made against the tobacco SABP immunoprecipitated catalase activity as well as SA-binding activity from cucumber extracts (data not shown). The similarities between the SABP/catalase from these two species, where there is strong evidence for SA's involvement in defense responses, supports a general role for catalase in mediating the action of SA.

Table III. *Inhibition by phenolic compounds of [^{14}C]SA binding in cucumber leaf extracts*

Competitor	Biological Activity[a]	[^{14}C]SA Bound	Inhibition[b]
		dpm/1.5 mg protein	%
SA	++	629 ± 86	69
2,6-Dihydroxybenzoic acid	++	444 ± 192	78
Benzoic acid	+	1779 ± 301	12
2,3-Dihydroxybenzoic acid	+/−	1879 ± 312	7
3-Hydroxybenzoic acid	−	2009 ± 348	1
4-Hydroxybenzoic acid	−	1996 ± 252	1
2,4-Dihydroxybenzoic acid	−	1848 ± 276	9
2,5-Dihydroxybenzoic acid	−	2128 ± 379	−5
4-Aminosalicylic acid	−	2004 ± 282	1
5-Aminosalicylic acid	−	1960 ± 278	3
o-Coumaric acid	−	1942 ± 368	4
None	−	2021 ± 243	0

[a] Biological activities were determined in tobacco or tomato (see Chen et al., 1993a, and refs. therein). [b] 20 μM [^{14}C]SA binding was assayed in the presence of 200 μM unlabeled competitor. The assays were done in triplicate.

Table IV. *Inhibition by SA and its analogs of catalase activity in cucumber leaf extracts*

SA and Analogs[a]	Biological Activity[b]	Catalase Activity[c]	Inhibition
		μmol O_2 mg^{-1} protein min^{-1}	%
2-Hydroxybenzoic acid (SA)	++	8.3 ± 1	50
2,6-Dihydroxybenzoic acid	++	7.1 ± 0.6	57
4-Hydroxybenzoic acid	−	15.5 ± 0.2	7
2,5-Dihydroxybenzoic acid	−	15.7 ± 0.5	6
None	−	16.7 ± 0.3	0

[a] The concentration of SA and its analogs was 1 mM. [b] See footnote a of Table III. [c] The assays were done in triplicate.

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