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Functional Expression of *Arabidopsis thaliana* Anthranilate Synthase Subunit I in *Escherichia coli*

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Anthranilate synthase is involved in tryptophan (Trp) biosynthesis. Functional expression of subunit I from *Arabidopsis* (ASA1) was achieved in bacteria as a protein fused with glutathione *S*-transferase (GST). The active product was purified in a single step on a glutathione-Sepharose column. The V_{max} (45 nmol min^{-1} mg^{-1}), the apparent K_M for chorismate (180 μM), and the feedback inhibition by Trp (complete inhibition by 10 μM Trp) of the purified fusion product (GST-ASA1) were comparable to anthranilate synthase purified from plants. Polyclonal antibodies raised against the fusion protein product and purified by affinity chromatography on a GST-ASA1-Sepharose column cross-reacted with a 61.5-kD protein in a partially purified anthranilate synthase preparation from corn seedlings. GST-ASA1 cleavage by thrombin, as well as site-directed mutagenesis modifications of the Trp allosteric site, inactivated the recombinant protein.

AS (EC 4.1.3.27) catalyzes the amination of chorismate to anthranilate. This is the first step at the branch point of the shikimate pathway, leading to Trp biosynthesis in plants and microorganisms (Zalkin, 1973; Bauerle et al., 1987; Bentley, 1990). AS has been purified to homogeneity from a number of microorganisms and is well characterized with respect to catalysis (Robinson and Gani, 1985) and feedback regulation (Caligiuri and Bauerle, 1991; Graf et al., 1993). The holoenzyme is composed of two nonidentical protomers, components I (also referred to as the α subunit) and II (β subunit). Both components are required for the Gln-dependent formation of anthranilate. Component II catalyzes the transfer of the amido group from Gln to component I, which in turn catalyzes the amination of chorismate. The same reaction also occurs in the presence of high concentrations of ammonium chloride and only component I. Both ammonium-dependent and Gln-dependent reactions require magnesium as a cofactor. In microorganisms, AS is synthesized as both an aggregate with downstream enzymes of the pathway and in a nonassociated form. The flux of carbon into the Trp pathway is controlled by the end product by negative modulation of component I (Belser et al., 1971; Miozzari et al., 1978).

Low levels of AS activity have been reported in the crude extract or after one purification step from several plant cultures as well as whole plant sources (Poulsen and Verpoorte, 1991). AS from plants has not been purified to homogeneity. The only extensively purified AS is from the cell cultures of *Catharanthus roseus* (Poulsen et al., 1993). After five purification steps, including four involving column chromatography, 54 μg of AS was obtained from 1 kg of cells, with a specific activity of 164 nmol min^{-1} mg^{-1}. Like the enzyme from microorganisms, this purified AS is composed of two subunits of molecular masses of 25 and 67 kD. Although not as extensively studied, it is apparent that the plant AS shares several similarities with its microbial counterparts: (a) the ability to utilize both Gln and ammonium as an amino group donor, (b) feedback inhibition by Trp and Trp analogs; and (c) the aggregation into higher molecular mass forms.

It has been suggested that two aromatic pathways are present in plants: a highly regulated plastidic pathway and an unregulated cytosolic pathway (Jensen, 1986). Consistent with this proposal, Carlson and Widholm (1978) partially separated two isozymes of AS, one sensitive to inhibition by Trp and the other less sensitive. The sensitive form was found to be predominant in normal cells, and the insensitive form was found in 5-methyltryptophan-resistant lines. In contrast, Poulsen et al. (1993) found no evidence for a Trp-insensitive form of AS in *C. roseus*. However, they separated two sensitive forms of AS from the crude extract in an anion-exchange column. It was theorized that one of the two forms was an artifact of the isolation procedure. The only report describing AS genes from a plant also contradicts the existence of regulated and unregulated forms of AS in plants (Niyogi and Fink, 1992). Two AS genes, termed *ASA1* and *ASA2*, were found in *Arabidopsis thaliana*. Both coded for component I and both had a chloroplast-targeting sequence at the N-terminal end. *ASA1* was found to be about 10 times more abundant than *ASA2*. The two genes were found to be functional by complementation in yeast and *Escherichia coli*. However, the enzymatic activity of the products of these genes was not characterized or quantified.

In the present paper, we report the functional expression in *E. coli* of *Arabidopsis* ASA1 as a fusion protein with GST. This expression technique facilitated the purification of the fusion product to homogeneity in a single step by GSH-Sepharose affinity chromatography. The fused protein generally retained the specific activity and kinetic and regulatory properties of the native protein isolated from whole plants.

Abbreviations: AS, anthranilate synthase; EPPS, *N*-(2-hydroxyethyl)piperazine-*N*′-3-propanesulfonic acid; GST, *Schistosoma japonicum* glutathione *S*-transferase; GST-ASA1, GST fused to *Arabidopsis thaliana* anthranilate synthase subunit ASA1; IPTG, isopropyl-β-D-thiogalactoside; TPBS, phosphate-buffered saline and 0.1% Tween-20.

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Also, polyclonal antibodies raised against the fusion protein cross-reacted with a partially purified AS from corn.

MATERIALS AND METHODS

Construction of GST-ASA1

The PCR primers (Keystone Laboratories, Menlo Park, CA) for the amplification of the mature form of *Arabidopsis* ASA1 (Val_{55} to Ser_{590}; Niyogi and Fink, 1992) were: CGATTGGATCCGTCTCTGTTTCTCCC and GACGGATCCAGATTCAGCCAAGTCGATGGC. *Arabidopsis* cDNA (2 ng; Clontech Laboratories, Palo Alto, CA) were subjected to 25 cycles of PCR amplification (2 min at 94°C, 1 min at 63°C, 2 min at 72°C) using the Ampli-Taq kit (Perkin Elmer-Cetus, Emeryville, CA) according to the manufacturer's instructions. The amplification product was digested with *Bam*HI and cloned (Ausubel et al., 1993) into the expression vector pGEX_2T (Pharmacia-LKB, Piscataway, NJ) digested with *Bam*HI. Both sense (pSCI674) and anti-sense (pSCI675) constructs were obtained and cloned into *Escherichia coli* MC1061. ASA1 was sequenced (Ausubel et al., 1993) using custom primers (Keystone Laboratories). JM109 cells (Clontech Laboratories) were transformed with the constructs and used for expression.

Purification of GST-ASA1

Unless stated otherwise, 500 mL of fresh Luria-Bertani (Ausubel et al., 1993) medium in a 2-L Erlenmeyer flask were inoculated with bacteria expressing pSCI674 and allowed to grow at 30°C at 125 rpm to an A_{600} of 0.5. The fusion protein was induced by the addition of IPTG (0.1 mM final concentration), and cells were harvested at the end of the growing phase by centrifugation (10 min at 25,000*g*). Enzyme purification was carried out at 4°C. The cell pellet was resuspended in 50 mL of extraction buffer (50 mM Tris-HCl at pH 8.0, 5 mM $MgCl_2$, 2.5 mM DTT, 100 mM NH_4Cl, 20% glycerol) and subjected to two passages in the French press at 20,000 psi (SLM-Aminco Instruments Inc., Urbana, IL). The resulting extract was centrifuged for 10 min at 30,000*g*. The supernatant was applied to a 5-mL GSH-Sepharose 4B column (Pharmacia-LKB). The retained fusion protein, GST-ASA1, was eluted in 25 mL of extraction buffer containing 5 mM GSH and kept on ice until used.

Partial Purification of AS from Corn

Corn (Pioneer 3379) was grown in the dark for 3 d to approximately 10 cm. The shoots were homogenized in liquid nitrogen with a mortar and pestle. They were resuspended in ice-cold extraction buffer (50 mM EPPS at pH 7.5, 5 mM $MgCl_2$, 5 mM DTT, 1 mM EDTA, 5 mM $NaHSO_3$, 0.1 mM PMSF, 20% ethylene glycol). The extract was filtered through cheesecloth and Miracloth and brought to 0.3% (w/w) with protamine sulfate. After 30 min of precipitation, the mixture was centrifuged for 30 min at 45,000*g*. The supernatant was mixed with an equal amount of a 40% PEG-3350 solution in 75 mM EPPS buffer at pH 7.5 containing 7.5 mM $MgCl_2$, 7.5 mM DTT, and 1.5 mM EDTA. After 3 h on ice, the mixture was centrifuged for 30 min at 45,000*g*, and the resulting pellet was resuspended in 50 mM EPPS buffer at pH 7.5 containing 5 mM DTT, 5 mM $MgCl_2$, 1 mM EDTA, and 20% ethylene glycol. The sample was desalted on a PD10 column (Pharmacia-LKB) using the same buffer but substituting glycerol for ethylene glycol.

Assay of AS Activity

Anthranilate formation was monitored by recording the increase over 3 min of the fluorescence emission at 400 nm in a Perkin Elmer LS-5 spectrofluorometer (Perkin Elmer Corp., Norwalk, CT). Excitation was set at 308 nm and slit width was set at 5 nm. The enzymatic reaction was performed in the presence of 0.5 mM chorismate in a total volume of 400 μL in 50 mM Tris-HCl buffer at pH 7.8 containing 1 mM EDTA, 5 mM $MgCl_2$, 100 mM NH_4Cl, and 20% glycerol. A standard curve of anthranilic acid was prepared to quantify the amount of product formed, and specific activity is expressed as nmol anthranilate formed min^{-1} mg^{-1} protein.

SDS-PAGE and Protein Measurement

SDS-PAGE was performed using 10% acrylamide according to published protocols (Garfin, 1990), and gels were stained with Coomassie blue. Protein was determined with the Bradford reagent (Bio-Rad, Hercules, CA) according to manufacturer specifications using BSA as a standard.

Antibody Preparation and Purification

SDS-PAGE slices containing a total of 0.33 mg of GST-ASA1 were used for the production of rabbit anti-GST-ASA1 serum (Josman Laboratories, Napa, CA). Twelve days before the final bleeding, a final boost was done by injecting SDS-PAGE slices containing 45 μg of ASA1 without GST. Igs were prepared by ammonium sulfate precipitation (40% saturation) and a 48-h dialysis against distilled water. The dialyzed Ig fraction was resuspended in PBS buffer and stored at 4°C. The affinity matrix GST-ASA1-Sepharose 4B was prepared by incubating 3 mg of purified GST-ASA1 in the presence of 0.3 g of cyanogen bromide-activated Sepharose 4B (Pharmacia-LKB) in 3 mL of 50 mM borate buffer at pH 8.3. The incubation was performed overnight at 4°C on an end-to-end mixer. The excess GST-ASA1 was removed by five washes with borate buffer, and the unreacted groups were blocked by incubating the resin in 0.2 M Gly at pH 8.3 for 2 h at room temperature. The resin was then washed as above and the success of the coupling was confirmed by the presence of Bradford reacting groups on the resin. The resin was packed in a disposable column and equilibrated with PBS. The Ig fraction was loaded onto the column, the retained fraction was eluted by 50 mM Gly buffer at pH 2.5, and 900-μL fractions were collected in tubes containing 100 μL of 1 M phosphate buffer at pH 7.0.

Western Blot

SDS-PAGE was performed using 10% acrylamide according to published protocols (Garfin, 1990) and stained with a modified silver-staining method using a microwave oven (Stone et al., 1994). The blotting onto nitrocellulose paper

(Schleicher & Schull, Keene, NH) was performed for 20 min at 100 V using the Polyblot semidry blotting apparatus (American Bionetics, Hayward, CA) and the three-buffer system described by Kyhse-Andersen (1984) as follows: first-anode buffer: 0.3 M Tris-HCl at pH 10.4, 20% methanol; second-anode buffer: 25 mM Tris-HCl at pH 10.4, 20% methanol; and cathode buffer: 25 mM Tris-HCl at pH 9.4, 40 mM 6-aminohexanoic acid, 20% methanol. Efficiency of transfer was assessed by a 3-min staining of the nitrocellulose blot with Ponceau S red solution (0.1% Ponceau in 1% acetic acid). The blot was blocked for 2 h in 5% nonfat dry milk in TPBS. Primary antibody incubation was performed overnight in TPBS at 4°C using 0.5 μg/mL of the affinity purified antibody. The secondary antibody incubation was performed for 1 h at room temperature in TPBS containing a 1:2000 dilution of goat horseradish peroxidase-anti-rabbit IgG (Amersham, Arlington Heights, IL). Detection was performed using the ECL detection kit (Amersham) and XAR-5 autoradiography film (Eastman Kodak, New Haven, CT).

Site-Directed Mutagenesis

GST-ASA1 site-directed mutagenesis experiments were conducted using the U.S.E. kit from Pharmacia-LKB. Plasmid pSCI674 was digested with *Bam*HI to yield the cDNA fragment coding for the mature ASA1. This cDNA was cloned into pBluescript (Stratagene) and mutagenized according to the manufacturer's instructions. The mutation was confirmed by restriction enzyme mapping. The mutated fragment was reinserted into pGEX_2T and sequenced. Preparation of the mutated protein was performed as described for the native protein.

RESULTS AND DISCUSSION

Expression and Purification of GST-ASA1

The PCR amplification yielded a 1.6-kb fragment. Its sequence revealed a perfect match with the published ASA1 sequence (Niyogi and Fink, 1992). A time course for IPTG induction was established by analyzing crude bacterial extracts on SDS-PAGE as shown in Figure 1. An 86-kD band corresponding to the fusion protein composed of GST (25.5 kD) and the mature form of ASA1 (60.5 kD) was detected in pSCI674 after IPTG induction. The corresponding 86-kD band was absent in pSCI675, indicating that this band is specific for the sense construct. The efficiency of the GST-ASA1 isolation protocol was demonstrated by analysis of the purified extract on SDS-PAGE (Fig. 3a, top, first lane). An 86-kD GST-ASA1 band was obtained for pSCI674 with only some low molecular mass contaminants.

Optimization of Active GST-ASA1 Isolation

Since the GST fusion system has been developed for the preparation of large amounts of protein or peptide for immunological purposes, little attention has been paid in the literature to the determination of the conditions for maintaining the activity of the recombinant protein. The preparation of the active enzyme was achieved by adjusting several parameters for the growth of the recombinant bacteria and

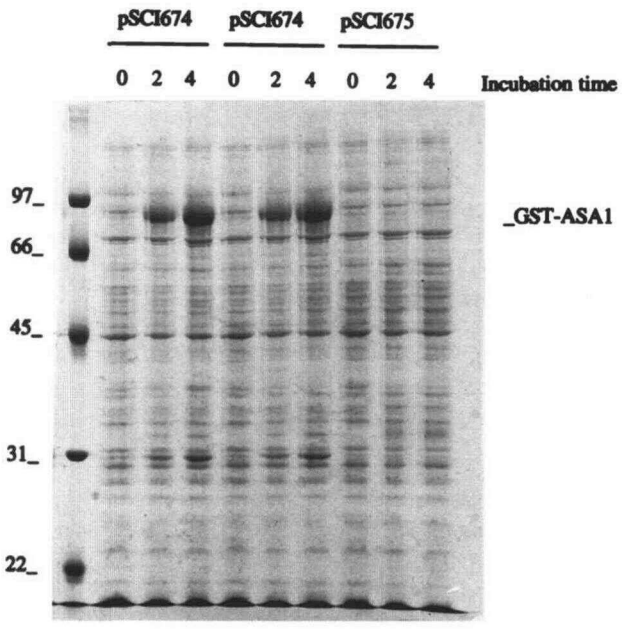

Figure 1. Time-course induction of GST-ASA1 in two independent clones of pSCI674. Coomassie blue-stained 10% SDS-PAGE analysis of bacterial crude extracts prepared after 0, 2, and 4 h of incubation at 37°C of *E. coli* expressing pSCI674. Bacteria expressing pSCI675 were used as negative controls. GST-ASA1 expression was induced after 1 h by addition of 1 mM IPTG (final concentration). Left lane, Molecular mass.

the purification of GST-ASA1. The optimum growth temperature for the recombinant *E. coli* was determined to be 30°C. An active enzyme was obtained at 22°C, but the overall activity yield was 50% lower. A totally inactive protein was obtained from cultures grown at 37°C. The optimum level of IPTG for GST-ASA1 induction was 0.1 mM, added when the culture reached an A_{600} of 0.5 (Fig. 2). When growth and induction conditions were not optimal, such as with a high temperature of growth or a high IPTG concentration, most of the recombinant protein was expressed in a denatured, inactive form. The denatured fusion product was insoluble and was found mostly in the pellet of the French press extract (data not shown). The composition of the extraction buffer was probably the single most important component. Inclusion of 20% glycerol in the extraction buffer was essential to retain activity of the fused protein but could be replaced by ethylene glycol. Since the cloned subunit lacks the Gln amidotransferase activity, ammonium chloride must be provided for activity and must be included in the extraction buffer to maintain the protein in an active conformation. The pH of the buffer was also important since the enzyme was rapidly inactivated at pH 7.5 or less.

It has been reported (Cull and McHenry, 1990) that the use of a French press as opposed to sonication is a gentler way to disrupt cells and, therefore, increases the possibility of obtaining an active protein. This has proven to be true in our study since the yield of active protein was much lower in extracts prepared by sonication (data not presented). Three

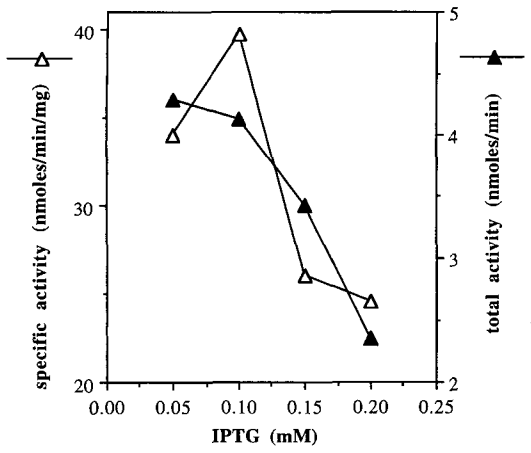

Figure 2. Effect of IPTG concentration on the yield of GST-ASA1 in 25-mL culture grown at 30°C at 125 rpm. IPTG at the stated final concentrations was added at a culture density corresponding to an A_{600} of 0.5. Cells were harvested at the end of the growth phase.

bacterial strains (MC1061, JM109, and XLI-Blue) were used to study whether the bacterial genetic background affected the active protein yield. The size of the culture did not affect the yield, and 15 mg of active protein were consistently recovered from a 1-L culture.

The purified GST-ASA1 exhibited enzymatic properties similar to those observed for AS purified from *C. roseus* (Poulsen et al., 1993). The apparent K_M for chorismate was about 180 μM in the presence of 100 mM ammonium chloride, in accordance with reported values (Table I). The calculated V_{max} was comparable to the V_{max} for the purified *C. roseus* AS. The relatively high value for the fused protein may have been due to a hysteretic lag in reaching full activity that was observed following the addition of chorismate. It is possible that both substrates must be present for the fusion protein to adopt the optimal conformation or aggregation state.

The allosteric site for Trp feedback inhibition has been described in detail for the *Salmonella typhimurium* (Caligiuri and Bauerle, 1991) and the yeast enzyme (Graf et al., 1993). For the latter, the sequence Leu_{62}-Leu_{63}-Glu_{64}-Ser_{65} has been implicated in the feedback inhibition. The published ASA1 sequence predicts its presence on this subunit as well (Leu_{112}-Phe_{113}-Glu_{114}-Ser_{115}; Niyogi and Fink, 1992). GST-ASA1 was found to be very sensitive to inhibition by Trp. The activity was completely inhibited by 10 μM Trp in the presence of 500 μM chorismate. Similar results were obtained in the presence of 10 μM 4-methyltryptophan and 5-methyltryptophan, which are known inhibitors of plant anthranilate synthase (Widholm, 1972b).

Two site-directed mutagenesis attempts were made to modify the Trp allosteric site to yield a Trp-insensitive recombinant enzyme. In the first case, the putative allosteric site was completely mutated to -Gly^{112}-Gly^{113}-Thr^{114}-Gly^{115}-. In the second case, the residue Glu^{114} was modified to Gly^{114}. Mutation of the corresponding Glu residue in *S. typhimurium* (Glu to Lys) led to a 5-methyltryptophan-insensitive AS (Caligiuri and Bauerle, 1991). In both cases, SDS-PAGE analysis showed that the apparent molecular mass of the mutated recombinant protein was correct, but both mutated proteins were completely inactive in the AS fluorescent assay. This finding could indicate that a modification of the allosteric site destabilizes the fusion product.

Although we were successful in obtaining a clean, active fusion product, it was interesting to investigate the activity of the recombinant AS separated from GST. The design of the expression vector pGEX_2T allows the cleavage of the fusion protein by thrombin (Smith and Johnson, 1988). Purified GST-ASA1 was subjected to overnight digestion at 4°C in the presence of increasing amounts of thrombin. An aliquot was analyzed by SDS-PAGE, and another was assayed for activity (Fig. 3). When 20 μg of GST-ASA1 were incubated overnight at 4°C in the presence of 1 μg of thrombin (0.05 unit) in 20 μL and analyzed on SDS-PAGE, a band at 25 kD, corresponding to GST, and a second band at 60 kD, corresponding to ASA1, were obtained. Lower amounts of thrombin (0.1 μg) gave partial cleavage, and higher amounts (10 μg) hydrolyzed ASA1 without affecting GST. Although partially cleaved GST-ASA1 retained activity, completely cleaved enzyme was surprisingly inactive. One can speculate

Table I. *Comparison of kinetic parameters of AS from various sources*

Ten micrograms of fusion protein were used in reactions containing 34 μM to 2 mM chorismate. Five micrograms of partially purified corn AS were assayed in the presence of 5 μM to 1 mM chorismate. In both cases, the change in fluorescence was recorded over 3 min.

Enzyme Source	Apparent K_M	V_{max}; Assay Temp.	Ref.
	μM	*nmol* min^{-1} mg^{-1}	
GST-ASA1	180	45; 22°C	Present study
Whole enzyme purified from *C. roseus*	67	164; 30°C	Poulsen et al., 1993
Whole enzyme partially purified from corn	20		Present study
Whole enzyme isolated from tobacco	160		Belser et al., 1971
Whole enzyme isolated from carrot	46		Widholm, 1972a

that the GST portion was retaining ASA1 in the active conformation in the absence of component II.

Another application of the GST-ASA1 purified protein is the preparation of specific antibody. If we assume that AS is quite conserved among plant species, this antibody can in turn be used to detect AS in plants. The specificity of such an antibody was demonstrated in Figure 4, where partially purified AS from corn seedlings was analyzed by western blot. A 61.5-kD band in these extracts cross-reacted with the affinity-purified antibody. The molecular mass of the corn enzyme is well in accord with values reported for other plants. This analysis suggested the presence of only one isozyme in these extracts.

This study shows that GST-fused protein expression can provide a useful alternative to purification of plant enzymes that are in low abundance or cumbersome to purify. This protocol allows a rapid and efficient one-step purification of the enzyme, thus avoiding lengthy protocols with low recovery. In the case of AS, kinetic studies are difficult to perform in a crude extract unless chorismate mutase, which competes with AS for the substrate, is separated (Poulsen et al., 1993). The present technique eliminated this problem. Furthermore, the antibody against the recombinant protein could be used to determine the isozyme pattern in plants and help resolve the question of whether Trp-insensitive enzymes occur naturally or whether they are artifacts of purification protocols. A possible explanation for this artifact may be a partial proteolysis of AS during purification with the loss of its N-terminal part where the Trp allosteric site is located.

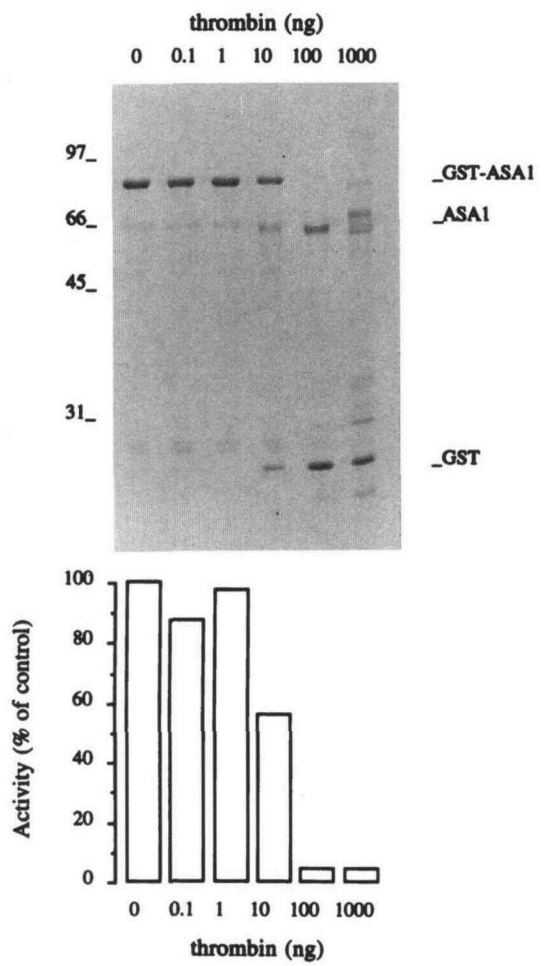

Figure 3. Thrombin digestion of GST-ASA1. GST-ASA1 (20 μg) was incubated overnight at 4°C in the presence of increasing amounts of thrombin. Top, Two micrograms were analyzed on SDS-PAGE and stained with Coomassie blue. Left lane, Molecular mass. Bottom, Ten micrograms were assayed for AS activity.

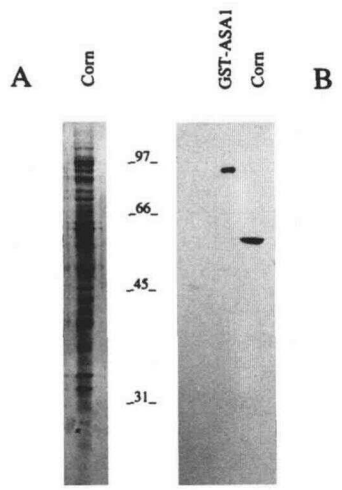

Figure 4. Western blot analysis using affinity-purified antibody. A, Silver stain of 2.5 μg of partially purified corn AS (corn). B, Western blot of 50 ng of GST-ASA1 (GST-ASA1) and 25 μg of partially purified corn AS (corn). Center lane, Molecular mass.

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