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Overexpression of *Nicotiana tabacum* Acetolactate Synthase as an Inducible Fusion Protein in *Escherichia coli*: Production of a Polyclonal Antibody to *Nicotiana tabacum* Acetolactate Synthase

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Abstract: Acetolactate synthase (ALS, EC 4.1.3.18) is the first common enzyme in the biosynthesis of leucine, isoleucine, and valine. It is the target enzyme for several classes of herbicides, including the sulfonylureas, the imidazolinones, the triazolopyrimidines, the pyrimidyl-oxy-benzoates, the pyrimidyl-thio-benzens, and the 4,6-dimethoxypyrimidines. An amino-terminal fragment of the sulfonylurea-resistant ALS gene (*SurB*) from *Nicotiana tabaccum* was cloned into the bacterial expression vector pGEX-2T. The resulting recombinant plasmid pGEX-ALS1 was used to transform *Escherichia coli* strain BL21, and the tobacco ALS was expressed in the bacteria as a protein fused with glutathione S-transferase (GST). Polyclonal antibodies against the fusion product (GST-ALS) were produced, and the sensitivity of GST-ALS with the rabbit anti-GST-ALS IgG was up to 50 ng. This antibody was used for Western blot analysis of the partially purified ALS from barley shoots. The results suggest that the polyclonal antibody produced in this study can be used to detect plant ALS.
Key words: acetolactate synthase, antibody, fusion protein, glutathione S-transferase, tobacco.

Acetolactate synthase (ALS, EC 4.13.18) is an enzyme catalyzing the first common step in the biosynthesis of branched chain amino acids in bacteria, yeast, and higher plants. It is the target of several classes of structurally unrelated herbicides, including the sulfonylureas (Chaleff and Mauvis, 1984; LaRossa and Schloss, 1984; Ray, 1984), the imidazolinones (Shaner *et al.*, 1984), the triazolopyrimidines (Gerwick *et al.*, 1990), the pyrimidyl-oxy-benzoate (Babczinski and Zelinski, 1991; Choi *et al.*, 1993), the pyrimidylthiobenzenes (Choi *et al.*, 1993), and the 4,6-dimethoxypyrimidines (Shim *et al.*, 1995). In bacteria and yeast, ALS has been purified to homogeneity and is well characterized with respect to its isozyme pattern and subunit structure. In *Escherichia coli* and *Salmonella typhimurium*, ALS exists in three different forms, isozymes I, II and III, and ALS is a tetramer composed of two large 59~60 kDa and two small 9~17 kDa subunits (Schloss *et al.*, 1985). In yeast, ALS is a dimer of two large 75 kDa subunits (Poulsen *et al.*, 1989). In contrast, none of the plant ALS enzymes has been purified yet since in plants ALS is present in low abundance and is unstable when isolated from plants. Accordingly, the isozyme pattern and subunit strucuture of ALS from higher plants are not well characterized. As a useful alternative, expression of the plant ALS gene in bacteria could be used to purify plant ALS, which is in low amounts.

In plants, serveral structurally conserved genomic sequences encoding the enzyme ALS have been isolated from *Arabidopsis thaliana*, *Nicotiana tabacum* or tobacco (Mazur *et al.*, 1987), *Brassica napus* (Wiersma *et al.*, 1989), *Pisum sativum* (Sin *et al.*, 1994), and *Xanthium* sp. (Bernasconi *et al.*, 1995). Tobacco mutants that are resistant to sulfonylurea herbicides have been isolated using a seed mutagenesis protocol, and two herbicide-resistant forms of ALS genes (*SurA* and *SurB*) were isolated (Lee *et al.*, 1988). A single and a double amino acid change in *SurA* and *SurB* ALS genes, respectively, resulted in the production of an enzyme ALS resistant to the herbicide. The mutant *N. tabacum* gene (*SurB*) has been functionally expressed in *Escherichia coli* (Kim and Chang, 1995). In addition,

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the mutant *Arabidopsis* ALS genes have been functionally expresed in *Escherichia coli* (Smith *et al.*, 1989; Singh *et al.*, 1992), and *Brassica napus* ALS has been expressed in *Salmonella typhimurium* (Wiersma *et al.*, 1990). None of these plant ALS enzymes expressed in bacteria, however, have yet been purified to homogeneity. The problems could probably be due to i) lengthy purification steps with low recovery, ii) inefficient expression in heterogous system and iii) lack of a rapid and easy immunological assay system for detecting ALS.

The purpose of this study is to prepare an antibody to *Nicotiana tabacum* ALS, which will be in turn a valuable tool for purification and analysis of the plant ALS enzyme. In order to prepare the antibody, large amounts of protein and, more importantly, purified ALS are needed. We report here the overexpression of the sulfonylurea-resisitant ALS gene (*SurB*) from *Nicotiana tabacum* as a fusion protein with glutathione S-transferase (GST) in *E. coli*. An amino-terminal fragment of the mutant *SurB* gene was cloned in the bacterial expression vector pGEX-2T, in which expression of the tobacco ALS gene is under the control of the tac promoter (Smith and Johnson, 1988). The fusion product (GST-ALS) was purified and a polyclonal antibody against the protein was prepared. We show that the anti-ALS antibody reacted with the partially purified ALS from barley shoots.

Materials and Methods

Chemicals, enzymes, oligonucleotides, and plasmids

Bacto-tryptone, yeast extract and bactor-agar were purchased from Difco Laboratories (Detroit, USA). Restriction and modifying enzymes were from Boehringer Mannheim (Mannheim, Germany) and Amersham Corporation (Arlington Heights, USA). Oligonucleotides were obtained from Korea Biotechnology (Taejon, Korea). An immuno-blot assay kit and prestained molecular weight standards were obtained from Bio-Rad Laboratories (Richmond, USA). An enhanced chemiluminescence Western blot analysis kit (RPN 2108) and biotinylated molecular weight markers (RPN 2107) were from Amersham Corporation (Arlington Heights, USA). Freund's adjuvants (complete and incomplete) were from Sigma Chemical Co. (St. Louis, USA). The plasmid pAGS148 was obtained from Dr. B. J. Mazur (E. I. Du Pont de Nemours & Co., Wilmington, USA). The expression vector pGEX-2T was obtained from Dr. Sang-Ki Paik (Chungnam National University, Taejon, Korea).

Construction of the expression vector for GST-ALS, pGEX-ALS1

A 900 bp *Nco*I and *Bam*HI fragment of the *Nicotiana tabacum* ALS gene (*SurB*) which is resistant to sulfonylurea herbicides was cloned into the expression vector pGEX-2T as follows: First, the vector pBals was constructed by transfering the ALS gene into pBluescript SK(−) by simultaneously amplifying the gene from the plasmid pAGS148 and introducing both *Bam*HI and *Nde*I and *Bam*HI restriction sites at the 5′ and 3′ ends, respectively, of the coding strand by polymer chain reaction (PCR) with the oligonucleotide primers 1 and 2:

1: 5′-ATTGGATCCAACACATATGGCGGCGGCTGCGG-3′
*Bam*HI *Nde*I

2: 5′-ATGCGGATCCTCAAAGTCAATAGG-3′
*Bam*HI

The PCR was carried out as described elsewhere (Saiki *et al.*, 1988). Each reaction contained 10 ng of template DNA, 100 pmol of the primers 1 and 2, 200 μM dNTPs in 50 mM KCl, 10 mM Tris (pH 8.3), and 1.5 mM $MgCl_2$ in 100 μl. The reactions were overlayed with 100 μl of mineral oil and subjected to 30 cycles of the following program: 94℃, 1 min 30 sec; 55℃, 2 min; 72℃, 3 min. PCR products were subcloned by ethanol precipitation, restriction digestion with *Bam*HI, and ligation with *Bam*HI-treated pBals (Kim and Chang, 1995). An amino-terminal fragment of the ALS gene was excised from the pBals with *Bam*HI and *Eco*RI, isolated from 1% agarose gel, and cloned into the *Bam*HI and *Eco*RI sites of pGEX-2T. The resulting vector pGEX-2TA was then digested with *Nde*I and *Bam*HI and treated with the Klenow fragment. Finally, the vector pGEXT-2TB was digested with *Nco*I and *Bam*HI and treated with the Klenow fragment. The resulting expression vector pGEXT-ALS1 was used to transform the *E. coli* strain BL21 cells. Plasmid DNA was isolated according to the alkaline lysis method, and transformation of BL21 cells was achieved using a standard $CaCl_2$ transformation protocol (Sambrook *et al.*, 1989).

Expression and purfication of GST-ALS

The plasmid pGEX-ALS1 was transformed into the strain BL21, and the cells were grown at 37℃ in Luria-Bertani medium in a 2-L round-bottomed flask to an A_{600} of 0.5~0.8. GST-ALS was induced with 0.1~1.0 mM IPTG (Isopropyl-thio-β-D-galactopyranoside). Cells were grown for an additional 4~5 h at 37℃ and harvested by centrifugation at 5,000×*g* for 10 min. The cell paste was stored at −20℃.

For the purification of GST-ALS, the cell paste was thawed and suspended in PBS (1.4 mM NaCl, 27 mM KCl, 101 mM Na_2HPO_4, 18 mM KH_2PO_4, pH 7.3). The suspension was lysed by sonication at 4℃ and centrifuged at 20,000×*g* for 15 min. The pellet was

suspended in extraction buffer (8 M urea in PBS, pH 7.3) and kept at room temperature for 12 h. Preparative SDS-PAGE was performed using 10% acrylamide, and gels were stained with Coomassie blue. The GST-ALS containing band at 60 kDa was cut out, and GST-ALS was eluted from the slices according to the manufacturer's protocol (Bio-Rad Laboratories, Richmond, USA). Protein quatifications were determined by the method of Lowry *et al.* (1951).

Preparation of antibodies

Rabbits were injected subcutaneously with 150 μg of GST-ALS emulsified in Freund's complete adjuvant. Booster injections of GST-ALS (150 μg) in Freund's incomplete adjuvant were given at 10-day intervals. After the third immunization, blood was collected and the antiserum was isolated. The IgG fraction of anti-GST-ALS antisera was purified by protein A-Sepharose chromatography.

Western blot analysis

The cell paste was resuspended in cracking buffer (60 mM Tris-HCl, pH 6.8, 1% 2-mercartoethanol, 1% SDS, 10% glycerol, 0.001% bromophenol blue), and then SDS-PAGE was performed by using 10% gels as described by Laemmli (1970). Proteins were transferred electophoretically to 0.45 μm nitrocellulose membranes by using a Hoeffer TE22 mini transphor electrophoresis unit at a constant current of 200 mA for 2 h. Following transfer, sheets were incubated for 1 h with 20 mM Tris, 0.5 M NaCl, pH 7.5 (TBS) containing 3% gelatin to prevent nonspecific adsorption. Sheets were then washed with TBS containing 0.05% Tween 20 (TBS/Tween) and incubated overnight at 4℃ with goat anti-GST IgG (dilution 1/100 in TBS containing 1% gelatin) or rabbit anti-GST-ALS IgG diluted to 3.4 μg/ml. After being washed with TBS/Tween, sheets were incubated for 2 h at room temperature with horseradish peroxidase-conjugated donkey anti-rabbit IgG (diluted 1/300 in TBS containing 1% gelatin) or horseradish peroxidase-conjugated rabbit anti-goat IgG (diluted 1/300 in TBS containing 1% gelatin). The sheets were washed with TBS/Tween and later with TBS, and then probed with the ECL substrate (Amersham, RPN 2106), according to the manufacturer's protocol (Amersham Corporation, Arlington Heights, USA). ECL was detected with Kodak X-Omat K film at variable exposure times.

Results and Discussion

We wished to express tobacco acetolactate synthase (ALS) in *E. coli* as a fusion protein with glutathione S-transferase (GST) and prepare antibodies against the glutathione S-transferase-acetolactate synthase (GST-ALS) to be used for detection and quantification of plant acetolactate synthase. In this study, we cloned an amino-terminal fragment of the sulfonylurea resistant tobacco *SurB* gene into the bacterial expression plasmid pGEX-2T as described in Fig. 1. Induction of GST-ALS by IPTG was established by analyzing crude bacterial extracts on SDS-PAGE as shown in Fig. 2A. A 60 kDa band corresponding to the fusion protein composed of GST (26 kDa) and the amino-terminal fragment of ALS (34 kDa) was detected in pGEX-ALS

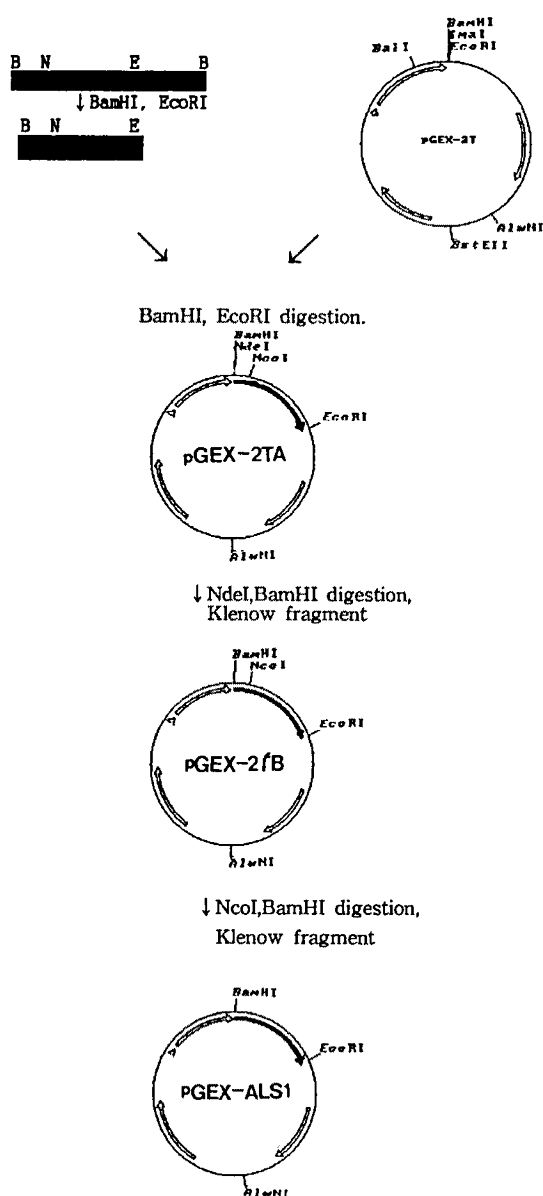

Fig. 1. Construction of pGEX-ALS1 gene fusion. A 900 bp *Nco*I and *Bam*HI fragment of tobacco ALS (Kim and Chang, 1995) was inserted into the pGEX-2T vector, resulting in pGEX-ALS1. B: *Bam*HI; N: *Nde*I; E: *Eco*RI.

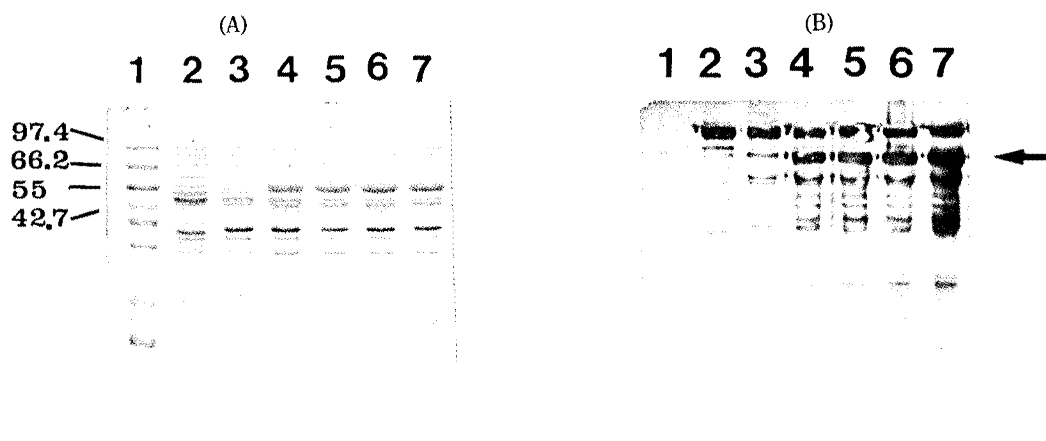

Fig. 2. Expression of GST-ALS fusion protein in *E. coli* cells transformed with plasmid pGEX-ALS1. After 4 h induction of the *tac* promoter by 0.1~0.8 mM IPTG, *E. coli* (strain BL21) harbouring the plasmid pGEX-ALS1 (see Fig. 1) were harvested and lysed with craking buffer (60 mM Tris-HCl pH 6.8, 1% 2-mercarptoethanol, 1% SDS, 10% glycerol, 0.01% bromophenol blue). After clarification by high speed centrifugation, the supernatant was electrophoresed on 10% polyacrylamide gels containing SDS followed by transfer to nitrocellulose sheets. Sheets were then reacted with goat anti-GST IgG, followed by treatment with horseradish peroxidase conjugated rabbit anti-goat IgG and the ECL substrate (Amersham). ECL was detected for 5 sec. (A) SDS-PAGE stained with coomassie blue. (B) Western blot analysis of the same gel. Lane 1: molecular weight markers; lane 2: BL21 (DE3); lanes 3~7: BL21 (DE3)/pGEX-ALS1 with 0, 0.1, 0.2, 0.5, 0.8 mM IPTG, respectively. The arrow indicates GST-ALS fusion protein.

1 after IPTG induction. In order to find the optimum conditions for the expression of GST-ALS, several parameters were adjusted. We found that the optimum growth temperature for the recombinant *E. coli* was determined to be 37℃ (data not shown), and that the optimum level of IPTG for GST-ALS induction was 0.1 mM, added when the culture reached an A_{600} of 0.5 (Fig. 2). Western blot anaysis of the crude extract indicated that the fusion protein reacted with antibodies against GST (Fig. 2B). In a similar manner, we cloned a full coding region of the *SurB* gene, a 2000 bp *Bam*HI/*Bam*HI fragment of pBals (Kim and Chang, 1995), into pGEX-2T as described in Materials and Methods, but no correct product was detected upon induction by IPTG (data not shown).

We next examined whether GST-ALS is soluble or not in extracts prepared by sonication. The fusion protein was insoluble and was found in the pellet of the extract after centrifugation (Fig. 3, lane 4). Purification of the fusion protein was carried out to near homogeneity by electroelution as shown in Fig. 3, lane 5, and resulted in the recovery of 200 mg GST-ALS per liter of bacterial culture.

Purified GST-ALS was subjected to 6 h digestion at 4℃ in the presence of thrombin. When aliquots of the reaction mixture were analyzed by SDS-PAGE, a band at 26 kDa, corresponding to GST, and a band at 34 kDa, corresponding to ALS, were obtained (Fig. 4).

The GST fusion system used in this study allowed us to prepare large amounts of GST-ALS for production of antibodies against the fusion product. Purified GST-ALS was electrophoresed and probed with GST-ALS antibodies produced as described in Materials and Methods. Fig. 5 shows Western blot analysis of GST-ALS at various concentrations. The antibodies recog-

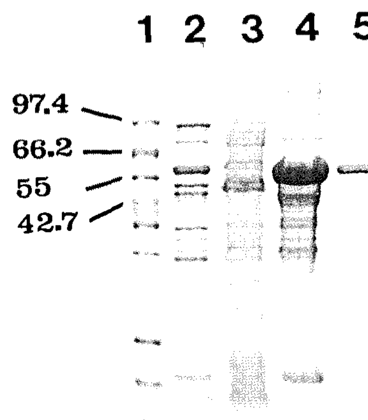

Fig. 3. Purification of GST-ALS fusion protein obtained after transformation of *E. coli* cell transformed with plasmid pGEX-ALS1. After 4 h induction of the tac promoter by 0.1 mM IPTG, *E. coli* (strain BL21) harbouring the plasmid pGEX-ALS1 (see Fig. 1) were harvested and lysed with PBS. After clarification by high speed centrifugation, the insoluble pellet and supernatant were electrophoresed on 10% polyacrylamide gels containing SDS. GST-ALS fusion protein was purified as described in Materials and Methods. Lane 1: molecular weight markers; lane 2: total cell extract after GST-ALS induction; lane 3: supernatant after centrifugation; lane 4: insoluble pellet after centrifugation; lane 5: GST-ALS purified by the method described in Materials and Methods.

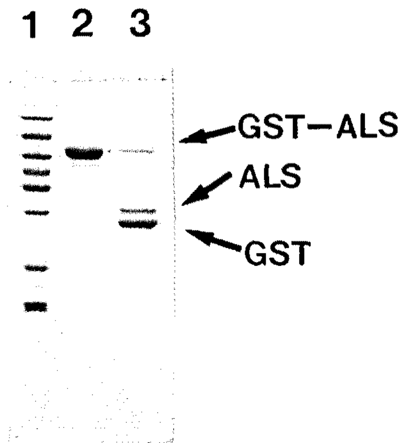

Fig. 4. Thrombin cleavage of purified GST-ALS fusion protein. GST-ALS fusion protein, which was purified by the method described in Materials and Methods, was incubated with thrombin at 4℃ for 6 h. Lane 1: molecular weight markers; lane 2: 30 μg of GST-ALS, no thrombin; lane 3: 30 μg of GST-ALS, which was incubated with 1 unit of thrombin. The molecular weight of GST-ALS fusion protein is 60 kDa. After thrombin cleavage, GST and ALS are 26 and 34 kDa, respectively.

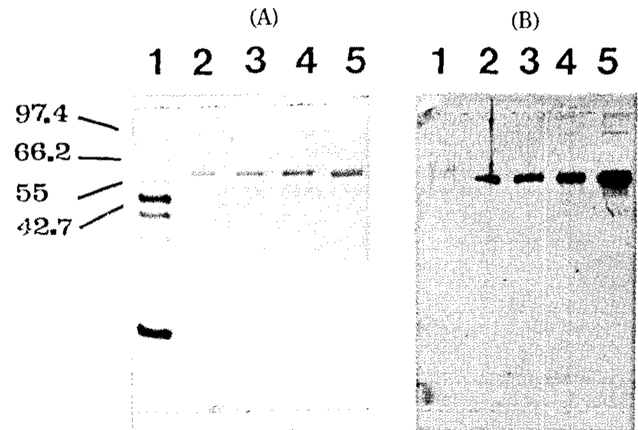

Fig. 5. Western blot analysis of purified GST-ALS fusion protein using rabbit anti-GST-ALS IgG. Purifed GST-ALS fusion protein (0.05∼5 μg) was electrophoresed on 10% polyacrylamide containing SDS followed by transfer to nitrocellulose sheets. Sheets were then reacted with rabbit anti-GST-ALS IgG diluted to 3.4 μg/ml, followed by treatment with horseradish peroxidase conjugated donkey anti-rabbit IgG and the ECL substrate (Amersham). ECL was detected for 5 sec. (A) SDS-PAGE stained with silver nitrate. (B) Western blot analysis of the same gel. Lane 1: molecular weight markers; lanes 2∼5: 0.05, 0.1, 1, 5 μg of purified GST-ALS fusion protein, respectively.

nized a band at 60 kDa, corresponding to GST-ALS. The sensitivity of GST-ALS with the rabbit anti-GST-ALS IgG was up to 50 ng.

Since ALS is quite conserved among plant species (Mazur *et al.*, 1987), antibody against GST-ALS can be used to detect and quantify ALS in plants and a recombinant plant ALS expressed in *E. coli*. Western blot analysis of the partially purified ALS from barley shoots detected a 65 kDa protein (Fig. 6B, lane 1). These results are consistent with that of Singh *et al.* (1991), in which a 65 kDa protein was detected in the crude extracts of all plant species tested on a Western blot using polyclonal antibodies to carboxyl-terminal fragments of *Arabidopsis* ALS. In addition, Bekkaoui *et al.* (1993) suggested that *B. napus* ALS is functional as a dimer of 65 and/or 66 kDa subunits. Thus, the results in this study suggest that a molecular weight of 65 kDa protein in extracts of barley shoots represents barley ALS, and only one isozyme exists in the partially purified barley ALS.

In contrast, when the partially purified barley ALS was stored at 4℃ for several weeks, concentrated with Centricon-30 microconcentrators (Amicon, Beverly, USA) and then analyzed by Western blot, a 58 kDa protein was found to have reacted with the rabbit anti-GST-ALS IgG (Fig. 6C). Since plant ALS is unstable when isolated from plants, the 58 kDa protein could be a breakdown product of the barley ALS. It is worthy to note that Durner and Boger (1988) reported a molecular weight of 58 kDa for the purified barley ALS enzyme, and the results in this study may provide a

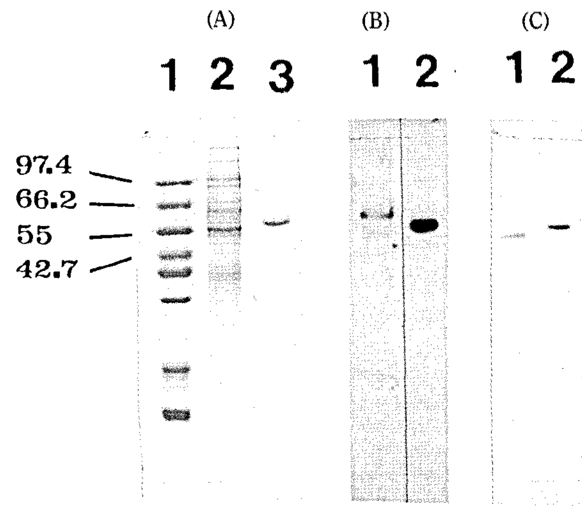

Fig. 6. Western blot analysis of partially purified ALS from barley shoots using rabbit anti-GST-ALS IgG. Partially purified ALS from barley shoots and purifed GST-ALS fusion protein were electrophoresed, transfered, and blotted as described in Fig. 5. (A) SDS-PAGE stained with coomassie blue. Lane 1: molecular weight markers; lane 2: partially purified ALS from barley shoots; lane 3: 0.5 μg of purified GST-ALS fusion protein. (B) Western blot analysis of the same gel. Lane 1: partially purified ALS from barley shoots; lane 2: 0.5 μg of purified GST-ALS fusion protein. (C) Western blot analysis of the partially purified barley ALS which was stored at 4℃ for several weeks and concentrated with Centricon-30 microconcentrators (Amicon). Lane 1: partially purified ALS from barley shoots; lane 2: 0.5 μg of purified GST-ALS fusion protein.

possible explanation for the conflicting reports from Singh *et al.* (1991) and Durner and Boger (1988).

In summary, we demonstrated here that a large amount of GST-ALS fusion protein was produced, and that antibodies against the fusion protein were produced to be used to detect plant ALS. Antibodies produced in this study could be used to possibly determine the isozyme pattern in plants and to purify plant ALS that occurs in low abundance and extreme lability.

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