Vol. 187, No. 1, 1992 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 31, 1992 Pages 299-304

EXPRESSION OF TWO S-RIBONUCLEASES OF PETUNIA INFLATA USING BACULOVIRUS EXPRESSION SYSTEM

Jinghong Mu and Teh-hui Kao

Department of Molecular and Cell Biology, The Pennsylvania State University, University Park, PA 16802

Received June 29, 1992

Summary: We have previously shown that three Petunia inflata S-proteins, products of the multiallelic S-gene of the self-incompatibility system, are ribonucleases. Here we report the expression of cDNAs for two of these S-proteins using the baculovirus expression system. S2- and S3-proteins were found in both supernatants and lysates of Spodoptera frugiperda cells infected with recombinant baculoviruses. Both recombinant S-proteins contained glycosylated (25 kD) and nonglycosylated (23 kD) forms. Recombinant S2- and S3-proteins were purified from insect cell cultures, and the amino-terminal sequences determined from glycosylated S2- and S3-proteins indicated that the leader peptide encoded by each cDNA was correctly removed. Both glycosylated and nonglycosylated forms of S2- and S3-proteins exhibited ribonuclease activity.

Self-incompatibility is a major outbreeding mechanism in flowering plants. In the simplest case, it is controlled by a multiallelic locus - the S-locus. In the gametophytic self-incompatibility system, pollen bearing an S-allele identical to one of the two S-alleles carried by the pistil suffers growth arrest in the style. The tip of a self-pollen tube usually swells and bursts open. The S-allele-associated protein (S-protein) in the pistil has been identified and characterized in several solanaceous species, and cDNAs as well as genomic DNAs encoding some of these S-proteins have been isolated and sequenced (4, 5, 6, 8, 9). An interesting feature revealed in a comparison of the sequences of S-proteins is their similarity to RNase T2 of Aspergillus oryzae and RNase Rh of Rhizopus niveus (6, 8, 12). A number of S-proteins from Nicotiana alata, Petunia inflata, and Petunia hybrida have been shown to have ribonuclease activity (3, 12, 13). Thus, solanaceous S-proteins have also been referred to as S-ribonucleases or S-RNases (6).

One approach to addressing whether the inhibition of self pollen tubes in the style is caused by the cytotoxic effect of the ribonuclease activity of S-proteins is to use a heterologous expression system to produce "mutant" S-proteins lacking ribonuclease activity and then to examine their ability to inhibit self-pollen tube growth in an in vitro pollen germination system (3, 7). As a first step toward this end, we describe in this communication the use of a baculovirus expression system to produce catalytically functional S2- and S3-proteins of P. inflata.

Methods

Construction of recombinant transfer vectors. S2- and S3-cDNAs, each encoding a leader peptide of 22 amino acids plus a mature protein of 199 (S2) and 200 (S3) amino acids (1), were released

0006-291X/92 $4.00

from plasmid vector pUC19 by digestion with EcoR I. The staggered ends were filled in with Klenow enzyme and separately ligated to the BamH I site of the baculovirus transfer vector pAc373 (14) through the use of BamH I linkers. The clones containing S2- and S3-cDNA inserted in the correct orientation with respect to the polyhedrin promoter were designated pAcS2 and pAcS3, respectively.

Purification of recombinant baculoviruses. Cells of Sf9, an insect cell line derived from the ovarian tissue of Spodoptera frugiperda, were seeded in a 25 cm^2 T-25 flask (Corning) at a density of 2.0 X 10^6 cells per flask containing Sf-900 serum-free medium, and then cotransfected with 1 μg of pAcS2 or pAcS3 plasmid DNA, and 1 μg of wild-type AcNPV genomic DNA (Invitrogen) using the calcium phosphate precipitation procedure recommended by the manufacturer (Invitrogen). After five days of incubation at 27°C, the cotransfection mixture was harvested and subjected to dot blot hybridization (15), using S2- or S3-cDNA as a probe. Recombinant baculoviruses carrying S2- and S3-cDNA were purified by several screening runs, and were designated AcNPVS2 and AcNPVS3, respectively.

Western blot analysis of S2- and S3-proteins produced by Sf9 cells. Three days after Sf9 cells were infected with AcNPVS2 and AcNPVS3, supernatants were concentrated 30-fold and the infected cells were resuspended by sonication in a lysis buffer (50 mM Tris-HCl, pH8.0, 150 mM NaCl, 1% [v/v] Nonidet P-40) at a concentration of 1 X 10^7 cells/ml. An equal volume of the 2 X Laemmli sample buffer was added to aliquots of either supernatants or cell lysates, and the mixture was boiled for 5 min and then electrophoresed on a 12% SDS-polyacrylamide gel. The gels were either stained with silver-staining reagents, or electroblotted to nitrocellulose membranes. For Western blotting, membranes were first incubated with rabbit antisera containing polyclonal antibodies against S1- and S2-proteins purified from P. inflata pistils, and then incubated with goat anti-rabbit IgG conjugated with horseradish peroxidase. Proteins which cross-reacted with antibodies were visualized by using 3,3'-diaminobenzidine (Sigma) as a substrate. A laser densitometer (LKB model 2202) was used to scan negatives of Western blots to determine the relative amounts in the protein bands observed.

Purification of S2- and S3-proteins from supernatants of infected Sf9 cells. Sf9 cells were cultured at 27°C in a 500 ml slow-speed stirrer flask (Corning) with a stirring speed of 90 rpm. When cell density reached 2.0 X 10^6 cells/ml, they were infected with AcNPVS2 or AcNPVS3 at a multiplicity of infection of 0.1. Three days post infection, cells were pelleted at 4°C by centrifugation in a GS-3 rotor at 5,000 rpm for 10 min. PMSF and aprotinin (both from Boehringer Mannheim) were added to the supernatants to a final concentration of 1 μM/ml and 1 μg/ml, respectively. The supernatants were concentrated to 10 ml using Amicon stirred cells. A two-step procedure employing a BioGel P-60 (Bio-Rad) column (1.5 X 62 cm) and an FPLC Mono-S column (Pharmacia) was used to purify recombinant S2- and S3-proteins (13). The Mono-S column was equilibrated with 50 mM sodium phosphate and proteins were eluted with a linear gradient of 1 to 250 mM NaCl in the same buffer. The purified S2- and S3-proteins were electrophoresed on a 12% SDS-polyacrylamide gel, and transferred to a PVDF membrane. The bands corresponding to the glycosylated form of S2- and S3-proteins were subject to N-terminal sequencing carried out at the Macromolecular Core Facility of Penn State's Milton S. Hershey Medical Center.

Assay of ribonuclease activity. A ribonuclease-activity staining gel was used to examine ribonuclease activity of recombinant S2- and S3-proteins. The procedure was essentially that described by Blank et al. (2). Clear bands on a blue background of RNA stained by toluidine blue indicated the positions of proteins with ribonuclease activity.

Results and Discussion

As is true for all the other solanaceous S-proteins that have been characterized so far, S2- and S3-proteins of P. inflata are glycoproteins and are localized in the extracellular space of the transmitting tissue in the style. In addition, they contain 4 intramolecular disulfide bonds which

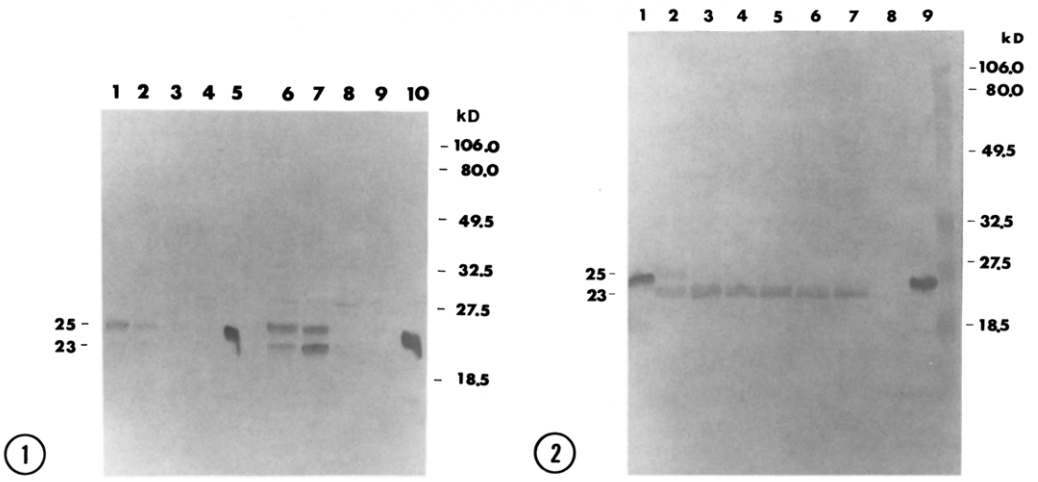

Figure 1. Western blot analysis of S2- and S3-proteins of P. inflata produced by Sf9 cells. Sf9 cells were infected with AcNPVS3 (lanes 1 and 6), AcNPVS2 (lanes 2 and 7), wild-type AcNPV (lanes 3 and 8), or remained uninfected (lanes 4 and 9). Lanes 1 to 4, total protein extracted from supernatants; lanes 6 to 9, total protein extracted from Sf9 cells; lanes 5 and 10, S2-protein (0.5 μg) purified from P. inflata pistils. Approximately 10% of the protein in supernatants and cell extracts were used for each sample.

Figure 2. Effect of tunicamycin on production of two forms of recombinant S2-protein by Sf9 cells. Lanes 1 and 9, S2-protein (0.5 μg) purified from P. inflata pistils; lanes 2 to 7, AcNPVS2-infected Sf9 cells treated with no tunicamycin (2), and treated with 0.1 μg/ml (3), 0.5 μg/ml (4), 1.0 μg/ml (5), 2.0 μg/ml (6), and 5.0 μg/ml (7) of tunicamycin; lane 8, uninfected Sf9 cells treated with 5.0 μg/ml of tunicamycin. Proteins were detected by Western blotting as in Fig.1.

are essential for their ribonuclease activity (unpublished results). These properties of S-proteins make it preferrable that a heterologous expression system be able to glycosylate the recombinant protein and to precisely process the leader peptide, and necessary that it form correct intramolecular disulfide bonds. With these factors in mind, the baculovirus expression system was chosen to express the S-alleles of P. inflata. This system has been shown to be able to post-translationally process recombinant eukaryotic proteins in a manner similar to that occurring in other eukaryotic organisms, including plants, to produce biologically active recombinant proteins (10, 11).

The recombinant transfer vectors pAcS2 and pAcS3 constructed would allow translation to start at the initiation codon of each cDNA to yield nonfused S2- and S3-proteins. When Sf9 cells were infected with the purified recombinant baculoviruses AcNPVS2 and AcNPVS3, occlusion body, typical of infection by the wild-type baculovirus, was not found. The presence of S-proteins in infected Sf9 cells and supernatants was revealed by Western blotting, shown in Figure 1. Both supernatants (lanes 1 and 2) and cell lysates (lanes 6 and 7) of AcNPVS3 and AcNPVS2 infected cells contained two protein bands, 25 kD and 23 kD, which cross-reacted with antisera against purified S1- and S2-proteins of P. inflata, while supernatants and cell lysates of the uninfected cells (lanes 4 and 9) and wild-type AcNPV infected cells (lanes 3 and 8) did not. Approximately one third of recombinant S2- and S3-proteins were found to be secreted.

The 25 kD band is identical in size to that of S2- and S3-proteins purified from P. inflata pistils (1), and the 23 kD band is identical in size to that of N-glycanase treated S2- and S3-

proteins (results not shown). To examine whether the former represents the glycosylated S-protein and the latter represents the nonglycosylated S-protein, tunicamycin was added to the medium at various concentrations 1 h after Sf9 cells were infected with AcNPVS2, and the pattern of S2-protein produced in Sf9 cells three days post infection was examined by Western blotting (Fig. 2). In the presence of 0.1 μg/mL or higher concentrations of tunicamycin, the 25 kD band of S2-protein completely disappeared and only the 23 kD band was observed (lanes 3 to 7 of Fig. 2). The same results were obtained for S3-protein. It was thus concluded that the 25 kD band is the N-glycosylated form of the S-protein, while the 23 kD band is the nonglycosylated form. For both S2- and S3-proteins, approximately half of the proteins produced were glycosylated (lanes 1, 2, 6, and 7 of Fig.1). Of the glycosylated S2- and S3-proteins, approximately 80% were found to be secreted into the medium, while less than 20% of the nonglycosylated S2- and S3-proteins were found in the medium (lanes 1 and 2 of Fig. 1).

A suspension culture was used to grow infected Sf9 cells for protein purification. As shown in Figure 3, the supernatant of AcNPVS3 infected Sf9 cells contained a large number of proteins (lane 4). These proteins were fractionated by a BioGel P-60 column and the fractions containing S3-protein, as revealed by Western blotting, were pooled (lane 3). These fractions were further chromatographed on a Mono-S column. The fraction eluted at 220 mM NaCl contained both glycosylated and nonglycosylated forms of S3-protein (lane 2), and the fraction eluted at 242 mM NaCl contained only the nonglycosylated form (lane 1). Recombinant S2-protein was similarly purified, but the two forms could not be separated by a mono-S column (results not shown).

The N-terminal 20 amino acids of the secreted glycosylated form of S2-proteins, and the N-terminal 6 amino acids of the secreted glycosylated S3-protein were determined. They were found to be identical to those of corresponding authentic S-proteins (1). These results indicate that the leader peptides of recombinant S2- and S3-proteins were proteolytically cleaved at precisely the same position as they are when subject to the processing machinery of P. inflata.

Both glycosylated and nonglycosylated forms of purified recombinant S3-protein (Panel A of Fig. 4) were found to have ribonuclease activity by an activity-staining gel assay (Panel B of Fig. 4). Similar results were obtained for recombinant S2-protein. Thus, the carbohydrate moiety appears not to be essential for the catalytic function. Consistent with these results is a recent finding that several P. hybrida S-proteins still retained ribonuclease activity after their N-glycan chains were either chemically or enzymatically removed (3).

Under the optimal conditions for the production of recombinant S-proteins, the amount of S2- and S3-proteins was estimated to be approximately 1 milligram each per liter of monolayer cell culture. This level of expression is at the low end of what has been obtained (1 to 500 milligrams per liter of insect culture) for expressing various eukaryotic genes in the baculovirus system (15). One possible reason for the low level expression of S-alleles might be that the ribonuclease activity of S-proteins has a cytotoxic effect on the insect cells. Since S-ribonucleases have no substrate specificity (12, 13), S-proteins produced in Sf9 cells might degrade the cellular RNA before they could be secreted. Proteases released after cell lysis could then further reduce the amount of recombinant proteins which had been secreted into the medium.

The ability to produce catalytically functional S-proteins by the baculovirus expression system will allow future use of this system to produce mutant S-proteins lacking ribonuclease activity to test whether or not ribonuclease activity is an integral part of the self incompatibility interaction. It will be of interest to see whether such mutant S-proteins can be produced in larger

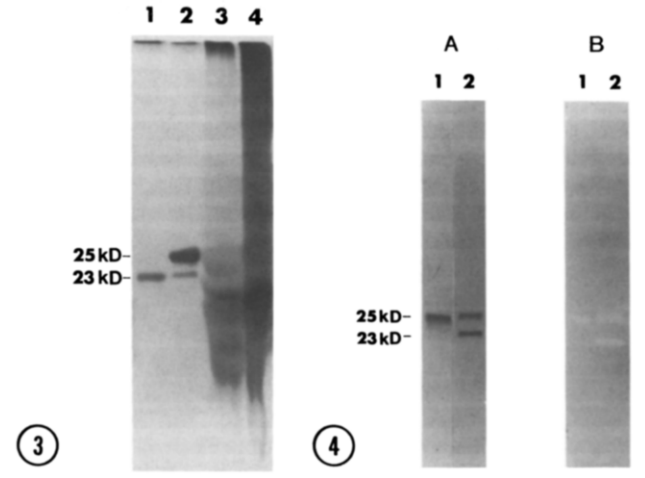

Figure 3. SDS-PAGE analysis of recombinant S3-protein at different stages of purification. Lane 4, supernatants from AcNPVS3-infected Sf9 cells; lane 3, pooled BioGel P-60 fractions containing recombinant S3-protein; lane 2, Mono-S fraction eluted at 220 mM NaCl; lane 1, Mono-S fraction eluted at 242 mM NaCl. Silver staining was used to detect proteins.

Figure 4. SDS-PAGE analysis (A) and ribonucleolytic analysis (B) of recombinant S3-protein. Lane 1 of A and B, S3-protein purified from P. inflata pistils; lane 2 of A and B, Mono-S fraction containing glycosylated (25 kD) and nonglycosylated (23 kD) forms of recombinant S3-protein. Silver staining was used to detect proteins in A; ribonuclease activity staining was used to detect proteins with ribonucleolytic activity in B.

amounts than catalytically functional S-proteins due to their lack of a cytotoxic effect on the insect cells. If the expression level of the S-gene can be improved, the system could also be used to produce other variant S-proteins for study of structure/function relationship, or to produce chimeric S-proteins for study of the domains responsible for allelic-specificity.

Acknowledgments

This work was supported by grants from the U.S. Department of Agriculture (90-37261-5560) and the National Science Foundation (DCB89-04008) to T.-h. K., and a graduate scholarship from Pioneer Hi-Bred International, Inc., Johnston, Iowa to J. M. We thank Dr. Donald Wojchowski for providing pAc373 and Sf9 cells, and for valuable advice on the baculovirus expression system; Drs. Richard Cyr, Anuradha Singh, and Yunjun Ai for help raising polyclonal antibodies; Dr. David Tu for use of FPLC system.

References

1. Ai, Y., Singh, A., Coleman, C. E., Ioerger, T. R., Kheyr-Pour, A. and Kao, T.-h. (1990) Sex. Plant Reprod. 3, 130-138.
2. Blank, A., Sugiyama, R. H. and Dekker, C. A. (1982) Anal. Biochem. 120, 267-275.
3. Broothaerts, W., Vanvinckenroye, P., Decock, B., Van Damme, J. and Vendrig, J. C. (1991) Sex. Plant Reprod. 4, 258-266.
4. Clark, K. R., Okuley, J. J., Collins, P. D. and Sims, T. L. (1990) Plant Cell 2, 815-826.
5. Coleman, C. E. and Kao, T.-h. (1992) Plant Mol. Biol. 18, 725-737.

6. Haring, V., Gray, J. E., McClure, B. A., Anderson, M. A. and Clarke, A. E. (1990) Science 220, 937-941.
7. Harris, P. L., Weinhandl, J. A. and Clarke, A. E. (1989) Plant Physiol. 89, 360-367.
8. Ioerger, T. R., Gohlke, J. R., Xu, B. and Kao, T.-h. (1991) Sex. Plant Reprod. 4, 81-87.
9. Kaufmann, H., Salamini, F. and Thompson, R. D. (1991) Mol. Gen. Genet. 226, 457-466.
10. Luckow, V. A. and Summers, M. D. (1988) Biotechnol. 6, 47-55.
11. Mauricio, M. B., Luckow, V. A., Griffing, L. R., Summers, M.D. and Hall, T. C. (1988) Plant Mol. Biol. 10, 475-488.
12. McClure, B. A., Haring, V., Ebert, P. R., Anderson, M. A., Simpson, R. J., Sakiyama, F. and Clarke, A. E. (1989) Nature 342, 955-957.
13. Singh, A., Ai, Y. and Kao, T.-h. (1991) Plant Physiol. 96, 61-68.
14. Smith, G. E., Summers, M. D. and Fraser, M. J. (1983) Mol. Cell. Biol. 3, 2156-2165.
15. Summers, M. D. and Smith, G. E. (1988) A manual of methods for baculovirus vectors and insect cell culture procedures. Texas Agricultural Experiment Station.