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Isolation of a Plant Glycoprotein Involved with Control of Intercellular Recognition[1]

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ABSTRACT

A recognition molecule was isolated from stigmas of S-allele genotype S_2S_2 of *Brassica oleracea* var. capitata L. After Sephadex chromatography, it eluted as a single symmetrical peak during diethylaminoethane-cellulose chromatography. A high degree of purity was affirmed by: sedimentation as a single peak during ultracentrifugation through 5 to 20% sucrose gradients; elution as a single peak from Sephadex G-100; visualization as a single band which stains with Coomassie blue and periodic acid Schiff reagent after electrophoresis on polyacrylamide gels. Other criteria supporting the conclusion that it is a glycoprotein are: (*a*) the highly purified preparation is anthrone-positive and has a Lowry protein to anthrone-positive carbohydrate ratio of 1.3; (*b*) the preparation contains arabinose, galactose, glucose, and mannose, although it is not precipitated by concanavalin A; (*c*) the immunological properties of the molecule are lost following protease treatment, and it has a molecular weight of 90,000 by Sephadex gel-filtration analysis and 54,500 by velocity sedimentation analysis.

***In vitro* pretreatment of S_2S_2 pollen with the post-diethylaminoethane-purified S_2 glycoprotein prevented the S_2S_2 pollen from germinating on three classes of compatible stigmas: (*a*) mature stigmas of genotypes S_3S_3 and S_8S_8, which are non-self genotypes; (*b*) immature stigmas of genotype S_2S_2, where incompatibility is not expressed; and (*c*) mature stigmas with a recessive S_2 allele. Pretreatment of S_3S_3 and S_8S_8 pollen with the S_2 glycoprotein did not interfere with their germination.**

Intercellular communication includes processes by which cells interact with and respond to molecular signals from other cells. Little is known of how specific cells are recognized or how molecular signals which mediate cellular communications regulate cell metabolism. Intercellular communication is manifest on flower pistils during a genetically directed inhibition of pollen function (7). This self-, generative, or sexual incompatibility exists in nearly half of all plant species. Molecular components of the recognition system are coded by multiple alleles of the S gene. Pollen will germinate on the stigma, grow through the style, and fertilize ovaries of plants with different S allele(s). Fertilization is prevented following self-pollinations or crosses between plants carrying identical S allele(s), unless that allele is recessive or inactive (24).

With sexual incompatibility, recognition is a prerequisite to manifestation of the biochemical event(s) that prevent normal pollen function (7). We describe the isolation of an S-allele-specific, serologically identifiable glycoprotein from *Brassica* stigmas and demonstrate its involvement as an informational macromolecule in self-recognition and control of pollen incompatibility.

MATERIALS AND METHODS

ANTIBODY PREPARATION

Rabbit (New Zealand White) antiserum containing antibodies to genotype S_2S_2 stigmas (*Brassica oleracea* var. capitata) was prepared as reported (14, 15). Antiserum preparations were preadsorbed with saline extracts from S_1S_1 stigmas to remove antibodies common to both genotypes. The S_1S_1 extract was prepared as described for S_2S_2 stigmas, but with saline instead of buffer. S_2S_2 antiserum (20 ml) was incubated overnight at 0 C with the S_1S_1 extract and then centrifuged. The clarified supernatant fraction containing the S_2 antibody was frozen (−40 C) until used.

Stigmas for immunizing rabbits and extracting the S_2-allele-specific stigma antigen (S_2 antigen) had attached pollen as a contaminant. Thus, precipitation of non-S-allele-specific pollen antigens frequently occurred during immunoassay for S_2 antigen. Therefore, the S_2S_2 antibody preparation was preabsorbed with a S_2S_2 pollen extract. Dehisced S_2S_2 anthers (100 mg) were shaken in 1.0 ml saline at 0 C for 1 h, and (0.2 to 0.4 ml) unimbibed saline and suspended pollen were drawn off with a Pasteur pipette, then mixed with 2.0 ml preabsorbed S_2S_2 antibody, and clarified by centrifugation after overnight incubation at 0 C.

The S_2 antigen was assayed microimmunologically using the template-overlay method (2). Special agar-Noble (Difco) or ION-AGAR (Oxoid) (7 ml, 1.0%) containing 20 mM NaCl and 0.1% NaN_3 was hardened on a 5- × 7.5-cm glass slide. Samples (20-30 μl) were added to side wells of templates set on the agar. Equal volumes of preabsorbed S_2S_2 antiserum (see below) were added to center wells. After overnight incubation in humidity chambers at room temperature, a precipitation line indicated presence of the S_2 antigen. When necessary, the chambers were refrigerated (about 3 C) to sharpen precipitation lines.

The S_2 antigen was quantified by titrating (22) (30 μl) S_2S_2 antiserum against S_2 antigen (30, 20, 10, 5, 2 and 1 μl, in a total volume of 30 μl). The greatest dilution at which antigen precipitation was visible is inversely proportional to the amount of S_2 antigen in undiluted samples. After reduction to a common denominator of 1, the reciprocal of this value was used in the tables and figures.

Serum preabsorbed with S_1S_1 stigmas and S_2S_2 pollen produced a single precipitation line when tested against the crude S_2S_2-stigma wash; and no precipitation occurred against stigma washes from the genotypes S_3S_3, S_3S_5, S_5S_5, S_7S_7, S_8S_8, $S_{11}S_{11}$, and $S_{15}S_{15}$ or against pollen extracts from S_1S_1, S_2S_2, S_3S_3, S_5S_5, and S_8S_8 genotypes. Thus, preadsorbed S_2 antiserum was a specific diagnostic reagent for S_2-antigen identification (*e.g. cf.* Fig. 1).

EXTRACTION AND GEL FILTRATION OF S_2 ANTIGEN

Air-dried S_2S_2 stigmas were incubated overnight in buffer (2.0 ml/200 mg dry weight tissue) containing (10 mM) Tris-HCl (pH

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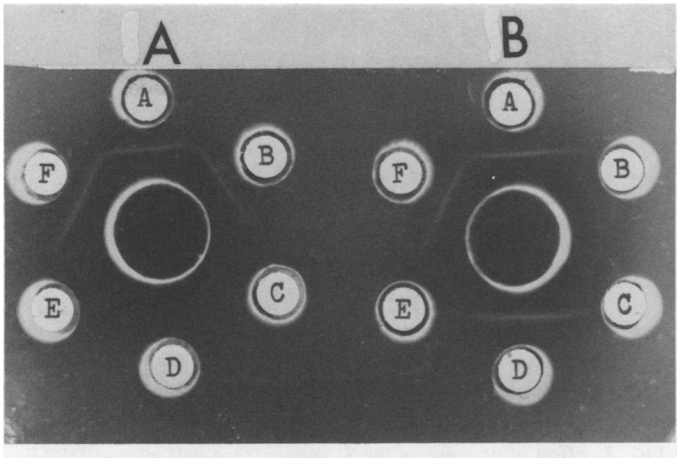

FIG. 1. Specificity of the S_2 antiserum and effect of mercaptoethanol on immunological detection of the S_2 antigen in the stigma-wash preparation. Each center well contained the absorbed S_2-antibody preparation (0.05 ml) and each outer well contained 0.05 ml of the designated treatments. In A: well A, control, S_2-stigma wash (S_2 antigen); well B, S_2 antigen + S_3S_3 pollen (20 mg); well C, S_3S_3 pollen; well D, S_3S_3-stigma wash + S_2S_2 pollen; well E, S_2S_2 pollen; well F, S_2S_2 pollen + S_2 antigen. In B: well A, S_2 antigen; well B, S_3S_3-stigma wash; well C, mercaptoethanol (0.5%); well D, S_2 antigen + mercaptoethanol; well E, S_3S_3-stigma wash + mercaptoethanol; well F, S_2 antigen + S_2S_2 pollen.

7.4), (1 mM) $CaCl_2$, and 0.02% NaN_3. Unimbibed liquid (about 0.5 ml) was collected, stigmas were rinsed three to five times in additional buffer to give a pooled volume of 2 to 3 ml, and the stigma wash was clarified by centrifugation (0-3 C, 10 min, 1000*g*). A shorter extraction time (1.25 *versus* 22 h) and different extraction media (distilled H_2O, 0.4 M sucrose, 0.15 M NaCl) were also tried.

The stigma wash was layered onto a column (2 × 34-cm) of Sephadex G-150-40 and eluted at room temperature (18-21 C) with buffer minus $CaCl_2$. Absorbance of the column eluant was monitored continuously at 220 nm using a Beckman DB spectrophotometer equipped with a linear-log recorder. NaN_3 was omitted from extraction and elution buffers when bioassays were performed on separated fractions. For mol wt determination, a 1- × 23-cm analytical Sephadex column was calibrated using ribonuclease A, chymotrypsinogen A, ovalbumin, aldolase, and blue dextran (Pharmacia).

ION-EXCHANGE CHROMATOGRAPHY

After Sephadex chromatography, fractions with peak content of S_2 antigen were pooled, concentrated by freeze-drying to about one-fifth their original volume (6–9 ml) as losses of antigenicity occurred if taken to dryness, and then eluted through a 1- × 30-cm DEAE-cellulose column with 10 mM Tris-HCl buffer (pH 7.7) and (30 mM) NaCl. The A_{220} and S_2-antigen contents of each fraction were determined. Probit analyses (8) were used to test for symmetry and coincidence of A_{220} and S_2-antigen elution profiles. Protein (12) and carbohydrate (4) content of the S_2-antigen preparation were determined using BSA and galactose as standards.

ELECTROPHORESIS

Samples containing a minimum of 50 μg protein were electrophoresed on polyacrylamide gels (21) after dialysis against two changes of 1 mM $CaCl_2$. Separating gels were prerun at less than 100 v for several hours to remove the persulfate used to catalyze polymerization. Electrophoresis at 2 mamp/gel was toward the cathode for about 2.5 h at 0 to 3 C. Pyronin Y (from Eastman Chemicals) was the tracking dye.

Gels were placed in 0.1 M Tris-HCl (pH 7.4) for 30 min prior to immunoassay for S_2 antigen. Successive transverse gel slices 1.6 mm thick were placed on a glass slide, in parallel rows 10 mm apart, and covered with agar. A trough, cut in the agar between the rows and sealed with a drop of hot agar, was filled with 0.1 to 0.2 ml antiserum.

To test for protein, gels were stained for 45 to 60 min with 0.2% Coomassie brilliant blue R-250 in ethanol-H_2O-acetic acid (45:45:10). Excess stain was removed by washing with 7% acetic acid in a mechanically circulated bath containing activated charcoal.

For confirmation, the post-DEAE fractions with peak amounts of S_2 antigen were electrophoresed in Drummond capillaries (1 μl) on density gradient (1–40%) polyacrylamide gels (17). Electrophoresis at 120 mamp was for 2 to 3 h, until the tracking dyes (bromphenol blue, xylenon cyanol) migrated off the gels. Ovalbumin was used to test the technique's resolution capability.

S_2-ANTIGEN CHARACTERIZATION

The quantity of S_2 antigen was estimated by titration after enzymic hydrolyses and chemical or heat denaturation. Hydrolases [10 mg/ml in 10 mM Tris-HCl (pH 7.4)] included protease (type 5), pectinase, ribonuclease A (Sigma), lysozyme (Nutritional Biochemicals Corp.), and Cellulysin (Calbiochem). Enzymes (10 μl) added to 40 μl S_2-stigma wash were incubated 18 to 22 h at 28 C. Denaturing treatments included: heating at 60 C for 90 min; incubation with 12.5, 20, and 50 μg pea agglutinin (Calbiochem) or con A[2] plus or minus $CaCl_2$, $MgCl_2$, and $MnCl_2$ (1 mM each); incubation for 40 min with 0.05, 0.1, 0.2, 0.5, or 1.0% mercaptoethanol.

AMINO ACID ANALYSIS

Two post-DEAE/S_2 antigen (15 μg) preparations were hydrolyzed in constant boiling (100 C) 6 M HCl for 24 h. Amino acids in the total hydrolysate were determined using a single-column, Beckman 119-CL analyzer; total quantities were adjusted based on recovery (87%) of a norleucine internal standard.

SUGAR ANALYSIS

Sugar content of the S_2 antigen was determined after formation of trimethylsilyl-*O*-methylglycosides (1, 10) and using a Perkin-Elmer model 910 gas chromatograph containing a 3% SP-2100 column (30.5 cm × 0.32 cm), programmed from 120 to 185 C at 0.5 degree/min with an initial hold of 4 min. The chromatograph was coupled to a SP-4100 computing integrator. The following standards (20 μg) were included in a separate analysis: glucose, mannose, arabinose, rhamnose, fucose, xylose, galactose, inositol, and mannitol (at 100 ng). Mannitol (100 ng) was added to samples as an internal standard.

BIOLOGICAL ASSAYS

Protein Synthesis. Pollen protein synthesis was monitored (5) to assay for biological activity by molecules purified from stigma washes. Following Sephadex and DEAE-cellulose chromatographies, 0.4- to 0.6-ml aliquots of each fraction or of pooled fractions were freeze-dried and resuspended in 0.1 ml freshly prepared 0.4 M sucrose, 20 mM $CaCl_2$, 2 mM H_3BO_3, and 0.5 μCi [^{14}C]leucine (354 mCi/mol). After mixing thoroughly, half the sample was removed to a duplicate tube. To start the assay, self-pollen (4 μl settled volume/0.05 ml, prepared as described below) was added to one tube and cross-pollen was added to its duplicate. Protein synthesis was terminated after 90 min at 28 C by adding a 30-fold

[2] Abbreviations: PAS, periodic acid Schiff reagent; con A, concanavalin A.

excess methanol-chloroform-H_2O (12:5:3, v/v) mixture containing 0.1 mM [^{12}C]leucine (5).

Pollen was prepared by suspending air-dried anthers (100 mg/ml) in 0.4 M sucrose, 20 mM $CaCl_2$, and 2 mM H_3BO_3. After shaking, pollen was removed with a pipette, and the anthers were similarly washed three more times. The pollen, collected by centrifugation (500*g*, 1–2 min) and washed twice with 2 to 3 ml medium, was resuspended in sufficient medium (usually 2–3 ml) to give a density equal to 4 to 6 μl settled volume/0.05 ml, as measured with a Bauer-Schenck graduated tube.

Isotope incorporation was not due to bacteria because: (*a*) incorporation was unaffected by (2 mM) puromycin; (*b*) incorporation was not detected in the absence of pollen or in the presence of pollen plus 0.1 mM cycloheximide; and (*c*) moreover, most bacteria would not sediment at the low centrifugation speed used to collect and wash the pollen: they would be discarded with the supernatant.

***In Situ* Pollination.** Pollen was treated in a "dry" state (6) with the post-DEAE/S_2 antigen preparation after removal of salts by dialysis (8–16 h; 3-6 C; at least one change of distilled H_2O). Samples (0.05 ml) of the dialyzed, S_2-antigen preparation contained about 5 μg protein (12). Distilled H_2O was used for a control treatment.

Pollinations were performed in the greenhouse in February and March. After 24 h, pollen-tube penetration into stigma and conducting tissue was assayed by means of the stigma squash fluorescence technique (23). Incompatible pollinations did not have more than three tubes present in stylar tissues. Compatible pollinations resulted in more than 50 tubes.

RESULTS

EXTRACTION OF S_2 ANTIGEN

From excised but otherwise intact stigmas, 2 to 5 times more S_2 antigen was extracted by distilled H_2O or (0.4 M) sucrose solution than by (0.15 M) NaCl medium. Much less S_2 antigen was released after washing stigmas for 1.25 than for 22 h, in each extraction media. Stigma deterioration occurred in all media, as evidenced by change of tissue color from yellow-green to brownish green, but not when buffer 10 mM [Tris-HCl (pH 7.4)] was included.

After further extraction by tissue homogenization, 2 to 10 times more S_2 antigen was released than from the stigma wash. However, nearly 10 times more 260-nm-absorbing contaminating molecules were released in the homogenate, and some of these eluted in regions of the Sephadex chromatograph which partially overlapped the S_2-antigen peak. Chromatography of fractions with S_2 antigen on DEAE-cellulose further indicated a shoulder on the S_2-antigen peak. Therefore washing, as compared to homogenizing stigmas, provided a preliminary purification step. The S_2 antigen was present only in extracts of stigmas with an S_2 allele, as previously demonstrated (see Fig. 1, for example, and "Materials and Methods") (14, 15).

CHARACTERIZATION

Incubation with pectinase, ribonuclease, lysozyme, cellulysin, pea agglutinin, con A (± salts) and mercaptoethanol had no effect on immunological detection of the S_2 antigen present in the stigma wash. Heat (60 C, 90 min) caused 50% reduction in the titer of S_2 antigen.

Serological identification of the S_2 antigen was abolished by preincubating it with protease, and BSA protected it from hydrolysis (Fig. 2). Separate preincubations of the S_2 antigen and protease, followed by combining them just prior to immunoassay (in the presence or absence of BSA), did not alter immunoprecipitation of the S_2 antigen (Fig. 2). These controls indicate that the loss of antibody-antigen precipitation caused by preincubation with protease alone was not due to a protease-mediated inactivation of the S_2-specific antibody during the subsequent immunoassay.

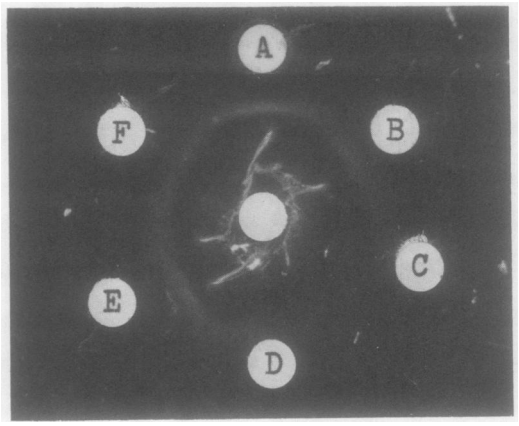

FIG. 2. Effect of protease on the S_2 antigen. One-half ml post-DEAE-purified S_2 antigen (50 μg/ml, final concentration) was added to: (A) 0.5 ml buffer [5 mM Tris (pH 7.4)], (B) protease (0.5 mg/ml) + BSA, (50 mg/ml), each suspended in buffer, or (C) protease alone. For immunoassay of S_2 antigen, 15 μl of each treatment was added to outer wells of templates along with 15 μl buffer. For control purposes, to test for protease effects on the S_2-specific antibody, 15 μl treatment A were mixed immediately prior to the time of immunoassay with 15 μl (D) BSA (50 mg/ml, final concentration), (E) BSA + protease (0.5 mg/ml), or (F) protease alone.

CHROMATOGRAPHY

After Sephadex (G-150-40) chromatography of washes from S_2S_2 stigmas, each fraction was assayed with S_2-specific antiserum. The S_2 antigen eluted between ovalbumin and aldolase and occurred consistently in fractions closely following the void volume, corresponding to apparent mol wt of 88,000, 90,000, and 94,000 (values for three experiments). The S_2 antigen extracted from stigmas of the S_2S_8 heterozygote also had mol wt of 88,000 (one determination).

Post-Sephadex-purified S_2 antigen could be stored in buffer at room temperature for 7 days, stored up to 4 weeks at 4 C, or kept as a freeze-dried powder with only slight (up to 20%) loss of antigenic activity. Measuring absorption of the eluant during Sephadex chromatography at 220 nm, rather than at 260 or 280 nm, improved detection of substances (small arrows) in fractions that flanked the S_2-antigen peak (large arrow) (Fig. 3). Peak fractions containing about 75% of the total S_2 antigen were pooled and concentrated by freeze-drying. Polyacrylamide gel electrophoresis of samples and assay with unabsorbed S_2 antiserum indicated the presence of at least two contaminating proteins (data not shown). Elution of the crude stigma wash preparation from DEAE-cellulose using a linear salt gradient indicated the S_2 antigen was separated from the bulk of 280-nm-absorbing materials (Fig. 4).

Five separately extracted S_2-antigen preparations were eluted through Sephadex and then through separate, freshly prepared DEAE-cellulose columns (elution medium included 30 mM NaCl). For each, the S_2 antigen eluted in four 1-ml fractions (Fig. 5; data for only one preparation are presented). Probit analysis (8) of the S_2-antigen elution profiles and the A_{220} profiles of the same region yielded linear regression lines, evincing molecular homogeneity and peak symmetry. With each preparation, a shoulder appeared in the A_{220} profile for fractions closely following the S_2 antigen. To minimize contamination by this substance, and to minimize dilution by buffer, only the two center fractions containing peak amounts of S_2 antigen were pooled for additional analyses. Rechromatography of the post-DEAE/S_2 antigen preparation on Sephadex G-100-40 indicated the presence of a single, symmetrical

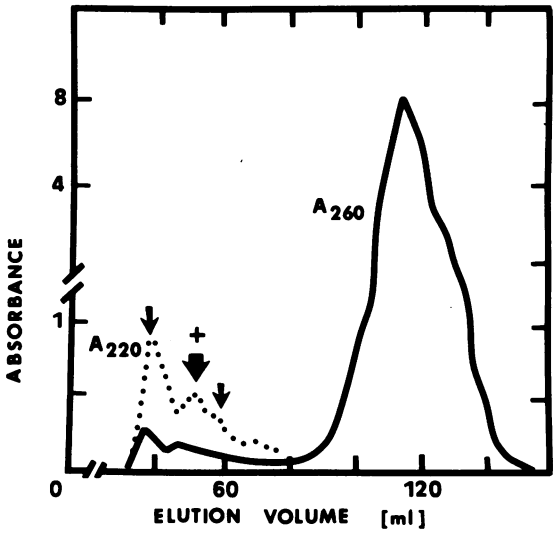

FIG. 3. Sephadex chromatography of the stigma-wash preparation. After 22 h wash in distilled H_2O, unabsorbed medium was removed and the intact stigmas were washed up to eight additional times with buffer containing 0.02% NaN_3. All washes were pooled, and the supernatant fraction was chromatographed through a column (35 × 2 cm) of Sephadex G-150-40. +, fraction with the most S_2 antigen.

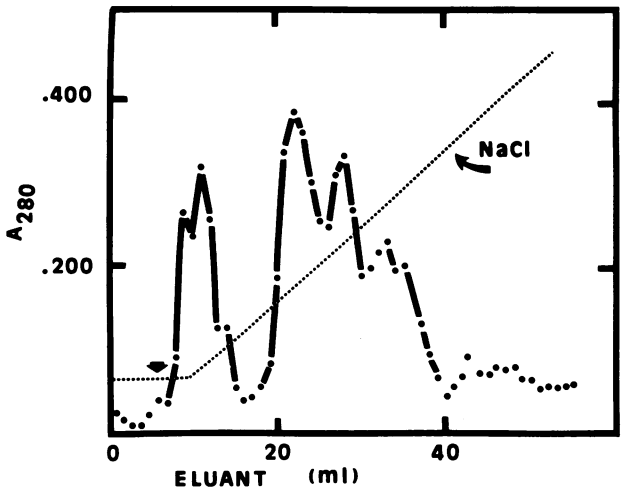

FIG. 4. DEAE-cellulose chromatography of the stigma wash S_2 antigen preparation. The S_2 antigen (0.2-0.4 ml) was layered onto a 1- × 30-cm DEAE-cellulose column and eluted with 10 ml 10 mM Tris-HCl buffer (pH 7.7) + 30 mM NaCl. ····, start and ending points for the 40 to 180 mM NaCl linear gradient.

peak (V_e/V_0 = 1.40) (data not shown).

CENTRIFUGATION

A single A_{220}-absorbing peak was obtained when the post-DEAE/S_2 antigen preparation was centrifuged through 5 to 20% sucrose gradients. The A_{220} peak, coincident with the peak for S_2 antigen (Fig. 6), sedimented just ahead of ovalbumin (mol wt, 45,000), giving, by comparison, an approximate mol wt of 54,000.

ELECTROPHORESIS

The concentrated, post-DEAE-purified S_2-antigen preparation was electrophoresed on linear (7.5%) or gradient (1–40%) polyacrylamide gels. In both types of gels, a single, sometimes diffuse, band was detected after staining with Coomassie blue (Fig. 7A;

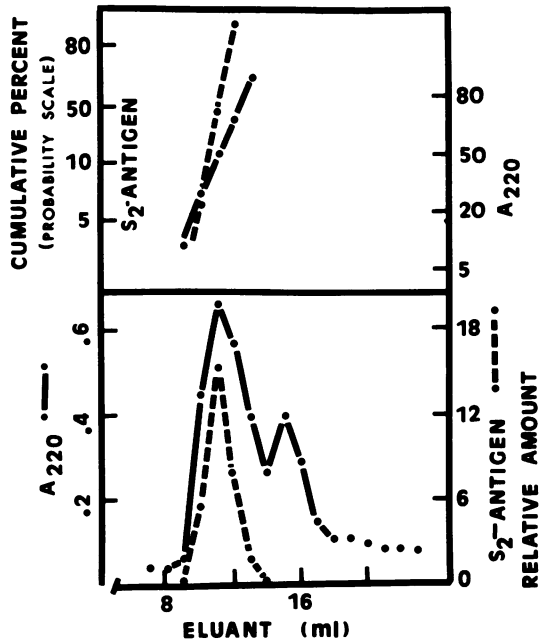

FIG. 5. DEAE-cellulose chromatography of post-Sephadex purified S_2-antigen. lower A, DEAE-elution profile; upper B, probit analysis of elution profiles. Data are for A_{220} (●——●) and S_2-antigen activity (●- - -●).

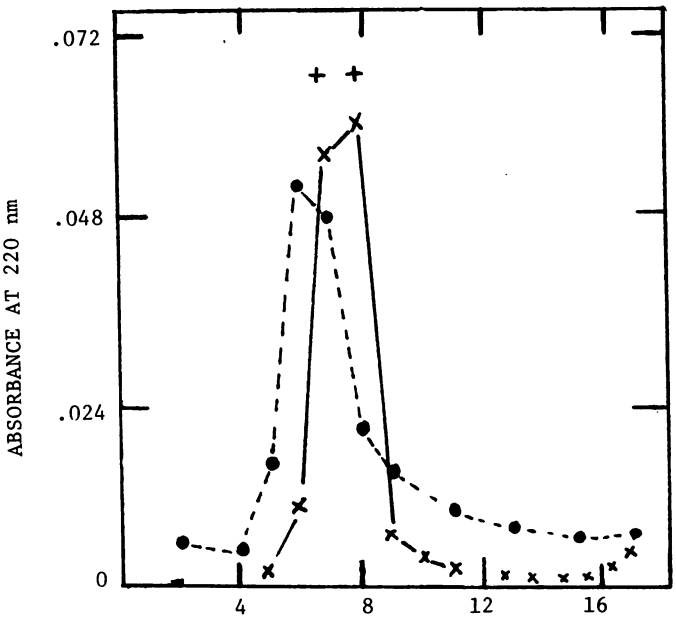

FIG. 6. Sucrose gradient centrifugation. The post-DEAE-purified S_2-antigen preparation (2 ml) was freeze-dried to 0.5-ml and 0.2-ml aliquots were layered onto 5 to 20% sucrose gradients (12 ml) containing 30 mM NaCl and 10 mM Tris (pH 7.7). After centrifugation (4 C; 40,000 rpm; 24 h) in a Beckman ultracentrifuge (model L-265B, Sw41 head), 0.55-ml fractions were collected and the S_2-antigen activity and A_{220} of each were determined. (×——×), S_2 antigen; (●- - -●), ovalbumin standard; +, fractions with S_2-antigen activity (fractions 7 and 8).

data are presented for linear gels only). On linear gels, a single band in the same location was also detected after staining with PAS reagent (Fig. 7B). No bands were detected when the direction of electrophoresis was reversed. As detected by immunoprecipi-

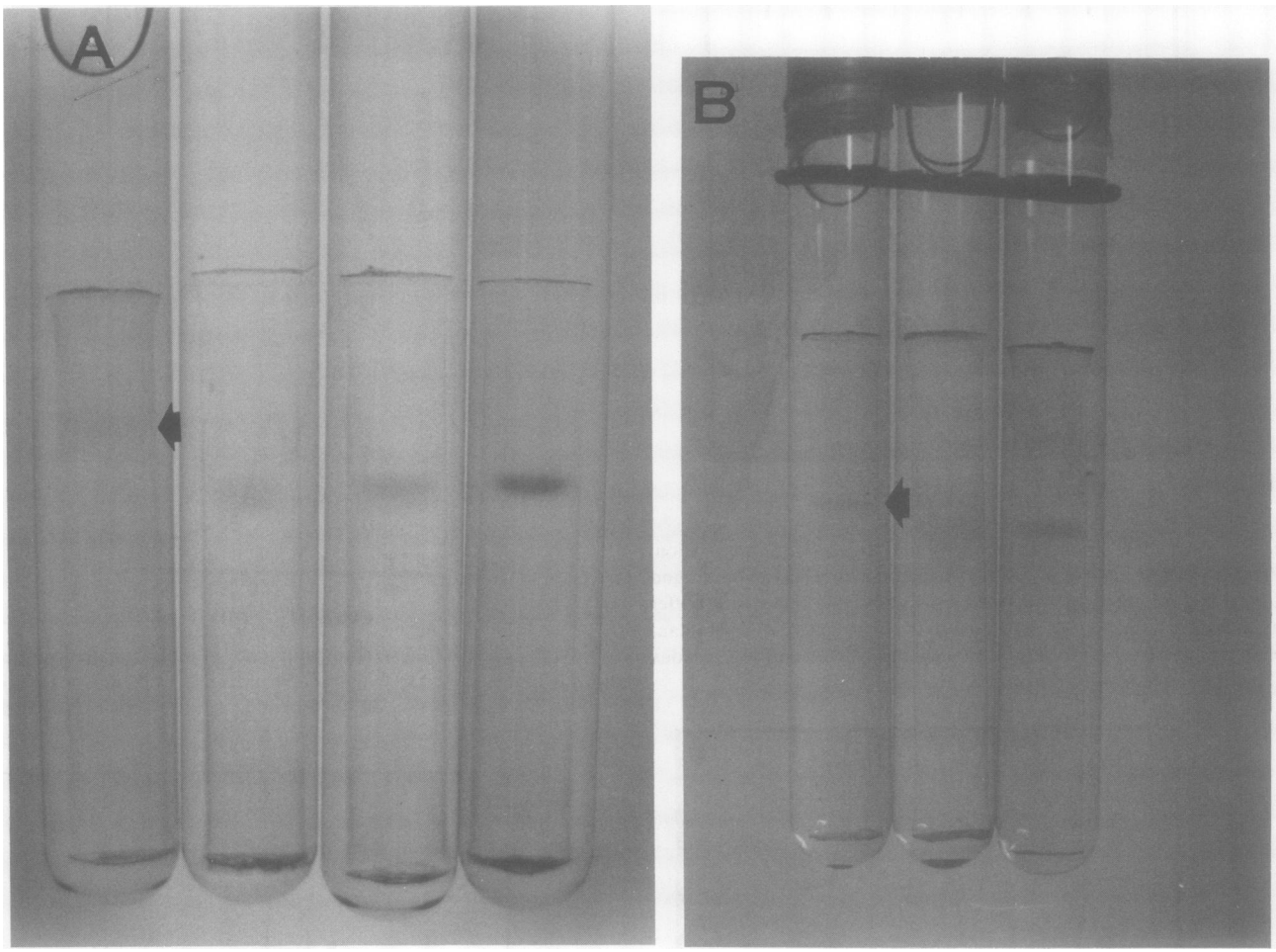

FIG. 7. Staining of the post-DEAE S_2 antigen preparation and ovalbumin on polyacrylamide gels after electrophoresis. A, gels were stained with Coomassie blue and contained, from left to right, S_2 antigen (70 μg protein) and 0.5, 1.0, and 2.0 μg (by weight) ovalbumin. B, gels were stained with PAS reagent and contained, from left to right, S_2 antigen (70 μg anthrone-positive carbohydrate) and 5.0 and 20 μg (by weight) ovalbumin. Photos A and B are from different experiments and are not directly comparable. They are included to show the relative stainability of the S_2 antigen compared to the ovalbumin standard (3–4% carbohydrate) and the lack of other contaminating Coomassie blue- and PAS-positive substances on the gels. Two other experiments, using other post-DEAE preparations, revealed that the S_2 antigen (determined by immunodiffusion) and the Coomassie blue- and PAS-positive bands were at the same location on the gel. Arrows indicate position of S_2-antigen band.

tation in replicate gels, the S_2 antigen was in the same location as the PAS and Coomassie blue-positive bands (data not shown).

S_2-ANTIGEN PROPERTIES

The post-DEAE/S_2 antigen preparation was anthrone-positive (4) and Lowry-positive (12) with a carbohydrate to protein ratio of 1.3. Asparagine/aspartic acid, glutamine/glutamic acid, serine, and glycine accounted for nearly 60% of the total amino acid content (Table 1). Principal sugar constituents were arabinose (56 mol %), glucose (18%), galactose (18%), and mannose (8%).

The post-DEAE/S_2 antigen preparation from S_2S_2 or S_2S_8 stigmas produced a single precipitation line when tested against pre- or unabsorbed S_2-antisera (Fig. 8). A single, continuous precipitation line indicates the S_2 antigen isolated from S_2S_2 and S_2S_8 stigmas are serologically indistinguishable.

The post-DEAE purified S_2 antigen (100 μg/ml) had absorption maxima at 275 to 280 nm and 194 to 198 nm, a minimum at 244 to 248 nm, and a shoulder at 220 nm.

Greater than 70% loss of antigenic activity occurred when post-DEAE- or post-Sephadex-purified S_2-antigen preparations were dialyzed 18 to 24 h against two or more changes of distilled, deionized H_2O. No losses occurred with 1 mM $CaCl_2$ or 0.5 M glucose in the dialysis medium.

EFFECT OF S_2 ANTIGEN

On *in Situ* Pollen Germination. Pollens of four different genotypes pretreated with the post-DEAE-purified S_2 antigen were applied to stigmas *in situ* of five different genotypes (Table II). S_2S_2 pollen, treated or untreated, failed to penetrate mature S_2S_2 stigmas, the normal incompatible response. S_2S_2 pollen pretreated with S_2 antigen failed to penetrate four otherwise compatible stigmas: bud (S_2S_2), cross-(S_3S_3, S_8S_8), and recessive compatible (S_2S_8). Induction of incompatibility by the S_2 antigen was specific to pollen containing an S_2 allele; compatibility was not changed to incompatibility for S_3S_3, S_8S_8, or S_2S_8 pollen genotypes.

On Protein Synthesis. The stigma-wash, post-Sephadex-, and post-DEAE-cellulose/S_2 antigen preparations were tested for effects on pollen-protein synthesis. Crude stigma-wash preparations inhibited [^{14}C]leucine incorporation into pollen proteins by 53%, the semipurified post-Sephadex preparation inhibited it by 23%, and post-DEAE-cellulose gave no inhibition. This suggested that the inhibition of protein synthesis by crude extracts was probably due to substance(s) other than the S_2 antigen.

Each fraction of the Sephadex chromatogram was therefore tested for its ability to inhibit protein synthesis. Fractions containing the S_2 antigen partially inhibited [^{14}C]leucine incorporation into proteins, with greater inhibition observed in two experiments

Table I. *Amino Acid Composition of S_2 Antigen*

Amino Acid	Amount[a]
	μg/mg protein
Asn/Asp	96
Thr	46
Ser	122
Gln/Glu	191
Pro	41
Hyl	+[b]
Gly	123
Ala	57
Val	38
Met	+[b]
Ile	27
Leu	56
Tyr	27
Phe	30
His	26
Lys	57
Arg	37
Trp	ND[b]
Cystine/2	+[b]

[a] Protein content of S_2 antigen was based on BSA standard in Lowry test.

[b] +, detectable, but the amount was low and uncertain; ND, not determined.

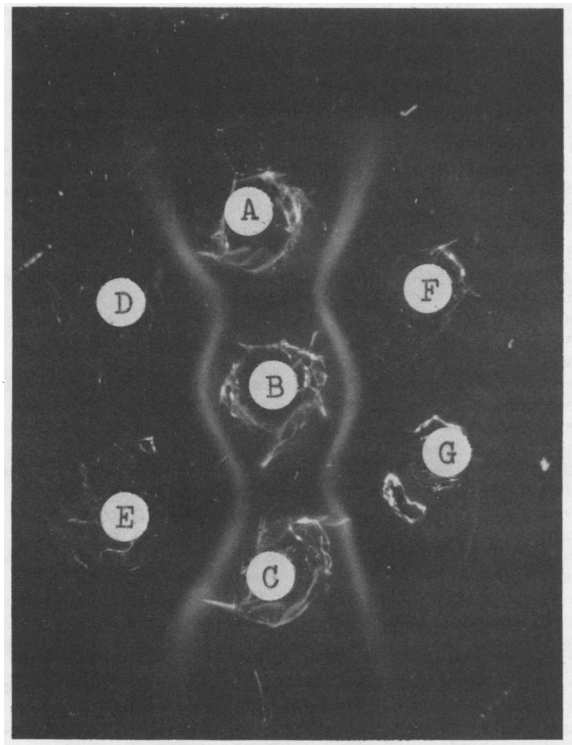

FIG. 8. Immunological detection of post-DEAE-purified S_2 antigen. Wells contained 30 μg preabsorbed S_2-antiserum (wells A and B), unabsorbed S_2 antiserum (well C), S_2 antigen from S_2S_2 stigmas (wells D and G), and S_2 antigen from S_2S_8 stigmas (wells E and F).

with self-pollen (22 to 44% and 34 to 54% for S_2S_2) than with cross-pollen (5 to 22% and 11 to 22% for S_8S_8). A light brown substance(s) from the low molecular weight (less than about 1,500 daltons) fractions inhibited pollen protein synthesis by 90% (average of four experiments). Its effect was not genotype-specific;

Table II. *Effect of Purified S_2 Antigen on (In)Compatibility Relationships of Pollen*

Pollen was treated in the dry state with purified S_2 antigen and then applied to stigmas of the indicated S-allele genotypes. Twenty to 24 h later, pistils were collected and pollen tube penetration through the stigma was determined. Incompatible pollinations did not have more than three tubes present in stylar tissues; compatible pollinations resulted in more than 50 tubes.

Pollen Genotype	± S_2 Antigen	(In)Compatibility Relationship with Stigma Genotype[a]					
		S_2S_2	S_3S_3	S_5S_5	S_8S_8	S_2S_8[b]	S_2S_2(bud)[c]
S_2S_2	−	I	C	—	C	C	C
	+	I	I	—	I	I	I
S_3S_3	−	C	I	C	C	—	C
	+	C	I	C	C	—	C
S_8S_8	−	C	—	—	I	—	—
	+	C	—	—	I	—	—
S_2S_8	−	I	—	—	—	I	—
	+	I	—	—	—	I	—

[a] —, pollination not performed; C, compatible pollination; I, incompatible pollination.

[b] S_8 is dominant over S_2 only in the stigma of this genotype, *i.e.* activity of the S_2 allele is not expressed (recessive) in the stigma. S_2 and S_8 are both expressed (codominant) in the pollen.

[c] Immature flower buds are self-compatible. The stigma was exposed for pollination by surgically removing sepals, petals, and immature anthers. Stigmas not from buds were from mature flowers with strong self-incompatibility.

self-pollen (S_2S_2) and cross-pollen (S_8S_8) were inhibited similarly.

DISCUSSION

Cell-cell recognition in plants has been hypothesized to involve binding of an external ligand produced by one cell, with a complementary molecule on another (11). Such extracellular molecules include antigenic determinants, lectins, arabinogalactan proteins, arabinoxylans, and allergens (3). Evidence indicates that S-allele-specific molecules from *Brassica oleracea* stigmas are glycoproteins involved with recognition.

S-allele-specific antigens are correlated with self- and cross-incompatibility (9, 14, 16, 22). The S_2 antigen segregated in absolute correlation with self- and cross-incompatibility phenotypes of F_2 plants (15, 16). Low quantities of S-allele-specific molecules occur on stigmas of immature flower buds where self-pollen is compatible (18, 21), and quantities increased as flowers matured and as incompatibility simultaneously increased (13, 18). A suppressor mutant that lowers the quantity of S_2 antigen was self-compatible (13). Stigmas homozygous for an S allele generally contain twice the amount of specific molecules as heterozygotes (9, 19, 22) and these molecules were precipitated by con A, were stained PAS-positive (18, 19) and were not detected in pollen or other plant tissues (18, 19, 22).

The evidence that S-allele-specific glycoproteins function in recognition and consequent control of incompatibility has been circumstantial since a purified molecule had not been correlated with a genotype-specific biochemical event or physiological response. *In situ* control of incompatibility by a highly purified S_2-allele-coded glycoprotein from *Brassica* stigmas is herein demonstrated. S_2S_2 pollen, pretreated with the post-DEAE-cellulose/S_2 glycoprotein (S_2 antigen) preparation failed to germinate on stigmas with which they are normally compatible (Table II). Pretreated pollens of other S-allele genotypes were not prevented from germinating.

Criteria are consistent with the S_2-antigen being a highly purified glycoprotein. The post-DEAE/S_2 antigen preparation (*a*) sedimented as a single peak following sucrose density-gradient ultracentrifugation, (*b*) eluted as a single peak following Sephadex gel filtration chromatography, (*c*) produced a single, weakly staining Coomassie blue- or PAS-positive band following electrophoresis on linear or gradient polyacrylamide gels, (*d*) produced a single, continuous precipitation line when tested serologically against absorbed or unabsorbed sera, and (*e*) showed coincident and symmetrical elution profiles through "Probit Analysis" (8) of DEAE-cellulose, ion-exchange chromatograms for light absorption and amount of S_2 antigen (evidence for molecular identity and homogeneity). All criteria indicate absence of significant amounts of contaminating proteins or glycoproteins.

Serological detection of the S_2 antigen was reduced by heating and eliminated by protease treatment. It also contains typical amino acids, is Lowry-positive, and has a typical protein absorption spectrum, yet stains only very weakly with Coomassie blue following polyacrylamide gel electrophoresis. With linear and density gradient gels, the resolving capabilities using ovalbumin as a standard were 5×10^{-7} and 8×10^{-10} g, respectively. When from 140 to 1,000 times these amounts of S_2 antigen were electrophoresed, only a single, sometimes diffuse, and weakly staining Coomassie blue-positive band was detected. (We feel the fuzziness of the bands is due to overloading the gels with protein, rather than to protein heterogeneity). Fast green and amido black were even less effective as stains. Maize "X" protein (25), some enzymically active pollen proteins (D. Mulcahy, personal communication), and hydroxyproline-rich proteins (D. T. A. Lamport, personal communication) stain either poorly or not at all with Coomassie blue. For some *Brassica* genotypes, S-allele-specific bands were not detected after polyacrylamide gel electrophoresis (9). Our results indicate this might be due to the poor stainability of some S-allele glycoproteins on polyacrylamide gels.

The post-DEAE/S_2 antigen preparation was anthrone-positive, contained arabinose, glucose, galactose, and mannose, yet was only weakly PAS-positive on polyacrylamide gels. Further, the S_2 antigen was not precipitated by con A, nor did pretreatment with con A interfere with its immunological detection. Conditions were suitable for con A binding because serum glycoproteins were precipitated (con A binds to α-D-mannopyranosyl, α-D glucopyranosyl, and α-D-*N*-acetylglucosamine residues). The possibilities cannot be excluded that reactive sugar groups are located in a part of the glycoprotein inaccessible to these agents or that they are sterically hindered from reacting. We speculate that lack of denaturation under assay conditions may cause the S_2 antigen to perform poorly with the Coomassie blue, PAS, and con A tests. Some genotype-specific glycoproteins from *Brassica* stigmas were precipitated with con A; however, con A-precipitated proteins were also present on self-compatible stigmas (18).

Purification of another glycoprotein from *Brassica campestris* stigmas specific for the S_7S_7 genotype utilized Sephadex (G-200) followed by con A-Sepharose chromatography (20). The S_2 antigen and the *B. campestris* glycoprotein (mol wt, 57,000) have similar mol wt. The higher estimate for the S_2 antigen obtained using Sephadex chromatography (mol wt, 90,000), compared to that from ultracentrifugation (mol wt, 54,500), is consistent with the frequent observation that this method overestimates the molecular weights of carbohydrate-rich glycoproteins.

The *B. campestris* glycoprotein had a ratio of carbohydrate to protein of 1.2 (glucose standard), compared to 1.3 (galactose standard) for the S_2 antigen. The four most predominant amino acids for both glycoproteins were serine, glutamine/glutamate, glycine, and asparagine/aspartate; the five least frequent were cystine (half), methionine, phenylalanine, tyrosine and isoleucine. The most frequent for the *B. campestris* glycoprotein was serine and that for the S_2 antigen was glutamine/glutamate; otherwise, the order of most frequent and least frequent amino acids were similar. Both glycoproteins had an unknown ninhydrin-positive material adjacent to the peak for histidine. These similarities suggest that different S-allele-specific glycoprotein recognition molecules might have similar molecular structures. Because of the approximately 50 S alleles and also because of the *B. oleracea versus B. campestris* species difference, there is slight probability that the same molecule was isolated.

Earlier results suggested that pollen-protein synthesis was involved in expression of incompatibility: pollen pretreated with cycloheximide germinated on, and penetrated into, self-stigmas (6); and stigmas released a water-soluble substance(s) which inhibited *in vitro* pollen germination and [^{14}C]leucine incorporation into proteins of self-pollen (5). Separation of components from the crude stigma wash by Sephadex chromatography indicated that the major inhibitor of protein synthesis was a genotype nonspecific, low molecular weight substance(s) of less than about 1,500 daltons, not the S_2 antigen. Ability of this substance(s) to block protein synthesis explains why crude stigma extracts mimicked the effects of cycloheximide and cordycepin on pollen germination (5). Protein synthesis of S_2 pollen was inhibited slightly more than cross-pollen by post-Sephadex/S_2 antigen preparations. This apparent specificity might be due to a greater sensitivity of the protein synthetic apparatus to inhibition by contaminants, after the pollen is recognized as self.

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