SHORT COMMUNICATION

PURIFICATION OF AN S SPECIFIC GLYCOPROTEIN IN SELF-INCOMPATIBLE *BRASSICA CAMPESTRIS* L.

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A glycoprotein which is found specifically in the stigma of S_7 homozygous plants in self-incompatible *Brassica campestris* was well purified using ConA Sepharose chromatography and density gradient isoelectric focusing. Its isoelectric point, molecular weight, carbohydrate content and amino acid composition were described.

Self-incompatibility in higher plants is the manifestation of the recognition between pollen and pistils. Substances which may intermediate this recognition has been looked for to understand intercellular relationships. In Cruciferae, the genetic system of self-incompatibility is controlled by the sporophytic action of S alleles; stigmas disturb the penetration of pollen tubes when their phenotypes are determined by the same S allele (Bateman 1955). In the previous investigations on the stigma proteins of *Brassica* species, we have pointed out that certain glycoprotein bands analyzable by isoelectric focusing were inherited in correlation with S alleles, without exceptions so far, in the S -segregating populations and further, presumed that the S -correlated glycoproteins could be the specific products of S alleles (Nishio and Hinata 1977, 1978; Hinata and Nishio 1978). The present paper describes the purification technique and several properties of one S_7 specific glycoprotein in *B. campestris*.

Materials: S_7 homozygotes of *B. campestris* were the same that described as S_6S_6 of C-470-30 in the previous report, where the band B_8 was demonstrated to behave in correlate with the $S_7(=S_6)$ allele (Hinata and Nishio, 1978). Flower buds one day before anthesis were stood on agar gel in a box, which was kept with a lid to maintain high humidity. On the following day, stigmas were collected from open flowers before anther dehiscence. Collected stigmas were stored at -20°C together with silica gel until use.

Extraction: All the purification procedures were carried out at 5°C . Ten thousand stigmas (dw. 0.3886 g) were homogenized in 5 ml of 0.1 M phosphate buffer, pH 7.0, using motor and pestle, and the homogenate was centrifuged at $9,600\times g$ for 20 min. The precipitate was extracted again with 3 ml of the phosphate buffer and centrifuged. The supernatants were combined and centrifuged again at $31,000\times g$ for 1 h. The

Fig. 1. Sephadex G-200 column chromatography of crude extract. S_7 specific glycoprotein was obtained in fractions indicated by a solid line.

Fig. 2. Elution profile of ConA Sepharose chromatography. Explanation as in Fig. 1.

yellow supernatant (crude extract) was applied to the following purification.

Sephadex G-200 column chromatography: The crude extract (5.5 ml) was layered on a Sephadex G-200 column (2.2×90 cm) equilibrated with 0.1 M phosphate buffer, pH 7.0. Fractions (2.5 ml) were collected at a flow rate of 5 ml/h. Three peaks of absorption at 280 nm were obtained (Fig. 1). Presence of S_7 specific glycoprotein was tested by acrylamide gel isoelectric focusing as described previously (Hinata and Nishio 1978). The S_7 glycoprotein was not found in the first peak, which corresponds to the void volume of column, nor in the third peak, which corresponds to the bed volume. As the second peak was confirmed to contain the S_7 glycoprotein, fractions of this peak were combined together and concentrated to 2 ml using a collodion bag (Sartorius SM13200).

ConA Sepharose chromatography: The second peak fraction of Sephadex chromatography was applied to a column (1×15 cm) of concanavalin A (ConA) Sepharose (Pharmacia Co.) equilibrated with phosphate buffered saline (0.15 M NaCl in 0.05 M phosphate buffer, pH 7.0). Fractions (1 ml) were collected at a flow rate of 2 ml/h. After eluting the proteins that did not bind to ConA (first peak), glycoproteins captured by ConA were eluted with 0.1 M α -methyl glucoside in the phosphate buffered saline (second peak, Fig. 2). Since the second peak contained S_7 glycoprotein, the fractions were combined and dialyzed against water.

Density gradient isoelectric focusing: (1) Sucrose density gradient isoelectric focusing was carried out at pH range of 3.5–10 in 110 ml column (LKB Co.) with a constant voltage of 300 V for three days. Each fraction (1.5 ml) was measured on the pH value and the absorbance at 280 nm (Fig. 3). S_7 glycoprotein was detected at pH 5.8 with a little contaminant.

(2) The fraction was again separated at pH range of 5–7 with a constant voltage of 600 V for 36 h (Fig. 4). The peak of S_7 glycoprotein was found in the fractions of pH 5.7. Fractions in which only S_7 glycoprotein was detected by an acrylamide gel

Fig. 3. Sucrose density gradient isoelectric focusing at pH range of 3.5-10. The S_7 specific glycoprotein was obtained at pH 5.8. Broken line: pH value and solid line: absorbance at 280 nm.

Fig. 4. Sucrose density gradient isoelectric focusing at pH range of 5-7. The S_7 specific glycoprotein was obtained at pH 5.7. Explanation as in Fig. 3.

Fig. 5. Gel isoelectric focusing of stigma proteins of S_7 homozygotes (A) and finally purified S_7 specific glycoprotein (B), at pH range of 3.5-10. Sample gel was faced to the anode side (left).

Fig. 6. SDS polyacrylamide gel electrophoresis. The mobility of the purified S_7 specific glycoprotein is indicated by an arrow and its molecular weight was estimated to be 57,000.

isoelectric focusing (Fig. 5), were combined and chromatographed on a Sephadex G-50 to remove ampholite and sucrose. After dialysis against water, the glycoprotein was freeze-dried. Finally, 230 μ g protein was obtained as purified S_7 specific glycoprotein. Its isoelectric point thus determined was pH 5.7.

SDS polyacrylamide gel electrophoresis: Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis was carried out according to Weber and Osborn (1969) with cytochrome C, aldolase, ovalbumin, bovine serum albumin and γ -globulin as the standards of molecular weight. The purified S_7 specific glycoprotein gave a single band, though we preliminarily mentioned elsewhere that S specific glycoprotein preparation of *B. oleracea* gave two or more bands on the SDS gel electrophoresis (Hinata and Nishio 1978). Its apparent molecular weight was estimated to be 57,000 from the mobility (Fig. 6).

Protein and carbohydrate content: The contents of protein and of carbohydrate of the purified S_7 specific glycoprotein were estimated using bovine serum albumin and glucose as the standards, respectively (Lowry *et al.* 1951; Dubois *et al.* 1951). Ratio

Table 1. Amino acid composition of S_7 specific glycoprotein

Amino acid	Mole per cent	Amino acid	Mole per cent
Lys	2.5	Aly	7.4
His+unknown	9.7	Cys (half)	0
Arg	3.4	Val	3.4
Asp	7.2	Met	0.4
Thr	3.9	Ile	2.3
Ser	17.6	Leu	3.9
Glu	17.0	Tyr	2.0
Pro	2.6	Phe	1.8
Gly	15.0		

of carbohydrate to protein was estimated to be 1.2 (w/w).

Amino acid analysis: The amino acid composition of the purified S_7 specific glycoprotein was determined with a Hitachi KLA-3B amino acid analyzer after hydrolysis in 6 N HCl at 110°C for 24 h in an evacuated tube. The results are presented in Table 1. Serine, glutamic acid, and glycine were predominant amino acids. An unknown ninhydrin positive peak was detected. Histidine was observed at the shoulder of the unknown peak and the content of histidine plus the unknown substance was calculated using histidine equivalent value.

It has been pointed out that a glycoprotein band is heritable in correlate with S_7 allele in our previous report, where S_7 was denoted as S_6 of C-470-30 family and the specific glycoprotein band was B_8 (Hinata and Nishio 1978). The purified glycoprotein, being B_8 , is postulated to be the specific product of S_7 allele. Since the mechanism of self-incompatibility is yet unknown, two strategies of the approaching steps may be considered; one is to find first the function of a substance and then the substance is to be purified, and another is to purify a probable substance at first and then to investigate its functions. Because the genetic behaviour of self-incompatibility is understood by S multiple alleles, we are now on the latter. Purification of the S specific glycoproteins may make it possible to investigate their function and localization in stigmas, and further to compare their molecular structure, which may provide good informations to the understanding the molecular events responsible for the intercellular relationships.

In the present communication, we have pointed out that the S specific glycoproteins are able to be purified taking an example of S_7 specific glycoprotein. Properties of the S_7 specific glycoprotein was presented preliminarily, though further studies are needed for the conclusive determination of these values by several different techniques. Purification of the S specific glycoproteins of the other S alleles is in progress.

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LITERATURE CITED

- Bateman, A. J., 1955 Self-incompatibility systems in Angiosperms. III. Cruciferae. *Heredity* **9**: 53-68.
- Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith, 1951 A colorimetric method for the determination of sugars. *Nature* **168**: 167.
- Hinata, K., and T. Nishio, 1978 S-allele specificity of stigma proteins in *Brassica oleracea* and *B. campestris*. *Heredity* **41**: 93-100.
- Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall, 1951 Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**: 265-275.
- Nishio, T., and K. Hinata, 1977 Analysis of S-specific proteins in stigma of *Brassica oleracea* L. by isoelectric focusing. *Heredity* **38**: 391-396.
- Nishio, T., and K. Hinata, 1978 Stigma proteins in self-incompatible *Brassica campestris* L. and self-compatible relatives, with special reference to S-allele specificity. *Japan. J. Genetics* **53**: 27-33.
- Weber, K., and M. Osborn, 1969 The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. *J. Biol. Chem.* **244**: 4406-4412.