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Con A – Peroxidase Method: an Improved Procedure for Staining *S*-glycoproteins in Cellulose-acetate Electrofocusing in Crucifers

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Summary. In the analysis of stigma glycoproteins by cellulose acetate electrofocusing in self-incompatible crucifers, the staining method of the glycoproteins, described in the earlier report, has been improved by using Con A – peroxidase reactions to obtain a permanent profile of band patterns which are visible under day-light conditions. Identifying *S* alleles by the corresponding *S*-glycoproteins can be facilitated by the present *S*-glycoprotein analysis.

Key words: Self-incompatibility – Glycoprotein – Electrofocusing – Concanavalin A – Peroxidase

Introduction

A series of studies on stigma glycoproteins in crucifers has revealed that (1) there are several glycoproteins in the stigma, (2) some of the glycoproteins are heritable in correlation with *S* alleles; these are named *S*-glycoproteins, (3) the *S*-glycoproteins can be differentiated by their isoelectric points (pI), and (4) *S* alleles can be identified by the corresponding *S*-glycoproteins (Nishio and Hinata 1977, 1978, 1979; Hinata and Nishio 1978, 1980; Hinata 1981). Recently we have reported a rapid analytical method for detecting *S*-glycoproteins; cellulose-acetate electrofocusing followed by Con A – FITC staining (Concanavalin A labelled by fluorescein isothiocianate), and suggested that *S* genotypes of individual plants are easily inferable from the *S*-glycoprotein analysis without crossing experiments (Nishio and Hinata 1980). One of the drawbacks in this method is that the band profile of glycoproteins is not retainable for a long period because fluorescence fades within a few minutes.

In the present study we have attempted to obtain a permanent glycoprotein band pattern which is visible under day-light conditions by using peroxidase instead of FITC.

Materials and Methods

Flower buds a day before anthesis were sampled from the pre-determined *S*-homozygotes and -heterozygotes of *Brassica oleracea*, *B. campestris* and *Raphanus sativus*. Either fresh stigma or one stored at −20°C with silica gel was used: both gave the same results. Electrofocusing was carried out in the same manner as described in the earlier report (Nishio and Hinata 1980).

Glycoprotein staining: After the electrophoresis the gel sheets were immersed in a 12.5% trichloroacetic acid (TCA) solution for 15 min for protein fixation, and then washed with running water for another 15 min. The sheets were subsequently immersed in, or washed by, the following solutions in turn; a Con A solution (3% Con A was diluted six fold by 0.1 M phosphate buffer pH 7.4) for 15 min, running water for 15 min, a peroxidase solution (2 mg peroxidase from horseradish, crude; RZ : 0.39, Sigma Co., in 100 ml 0.1 M phosphate buffer pH 7.4) for 15 min, and running water for 15 min. For the detection of peroxidase activity, the method proposed by Yamamoto and Momotani (1971) was simplified as follows: 200 mg o-dianisidine was dissolved in 1.64 ml acetic acid; 140 ml water, 60 ml 0.2 M acetate buffer pH 6.5 and 0.4 ml 30% H_2O_2 were added in turn. The solution was made immediately before use and the sheets were dipped in the solution for a few min until band appearance. The sheets were once washed with water and dipped in glycerol, and then kept in between polyethylene films. Later, the Nadi reaction method defined by Endo (1972) for starch gel electrophoresis was applied, giving good results.

Results and Discussion

In the present staining method, the Con A reactive glycoproteins in stigma are detected by peroxidase activity, where the peroxidase is bound with glycoproteins through Con A. This is an application of an electron microscopic technique for detecting lectin reactive substances (Bernhardt and Avrameas 1971). Since *S*-glycoproteins are reactive with Con A (Nishio and Hinata 1980), they are involved in the stained bands. The sheet presented in Figure 1 is that stained by the present method (o-dianisidine) in the stigma analysis in *R. sativus* and *B. oleracea*.

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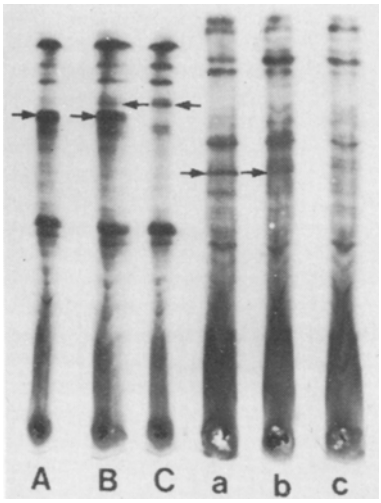

Fig. 1. An illustrative *S*-glycoprotein analysis in *R. sativus* (strain 33, A-C) and *B. oleracea* (strain K8, a-c). Two *S*-homozygotes (A and C, and a and c) and one *S*-heterozygotes (B and b) are taken from selfed progenies of an *S*-heterozygous plant in respective species. *S*-glycoproteins for respective *S* alleles are shown by arrows, but the *S*-glycoprotein for an *S* allele (c) in *B. oleracea* has not yet been determined

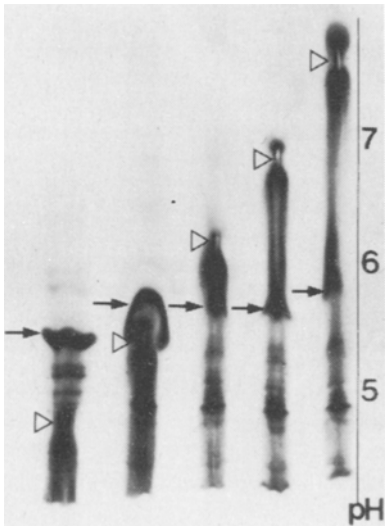

Fig. 2. A demonstration estimating an approximate pI of $S^{7(pI\,5.7)}$. Stigma homogenate was started from different positions (▷) on the sheet. The *S*-glycoprotein (arrows) moved towards the one position where the pH of the sheet was equivalent to the pI. The pI is estimated by measuring the pH gradient

The sheets after TCA fixation and after Con A treatment were directly immersed into the peroxidase detecting solution in order to determine substances other than the aiming bands would disturb the band analysis. No stained band appeared.

The merits of the present analysis over the earlier Con A – FITC method are (1) that glycoprotein band pattern is visible under day-light conditions without any special instruments and (2) that the pattern is retainable for a certain period. Further, the present method renders it unnecessary to do such chemical operations as conjugating Con A and FITC. Its sensitivity is as high or greater than the Con A – FITC method. It greatly depends upon the peroxidase activity, though a peroxidase solution kept in a refrigerator carefully is capable of being used repeatedly. Stained sheets have been preserved for one year without any discoloration. This enables us to compare the band patterns of materials at different flower seasons. If we store a quantity of stigma of an appropriate strain at low temperature with silica gel and run it in parallel with analyzing materials, comparison of band pattern can be facilitated.

Although for certain *S* alleles the corresponding *S*-glycoproteins have not yet been determined, we are able to analyze 8 *S*-glycoproteins in *B. oleracea*, 4 in *B. campestris* and 5 in *R. sativus*, for respective *S* alleles. We expect to register every *S*-allele by the pI of corresponding *S*-glycoproteins. The number of major *S* alleles may be less than hundred (Sampson 1967; Ockendon 1974). Hinata (1981) proposed the following description system: for example, when an S_{39} gene has a corresponding *S*-glycoprotein whose pI is 10.3, the gene can be described as $S^{39(pI\,10.3)}$. The pI value for the registration may be best estimated by a preparative electrofocusing. As discussed in the earlier report (Nishio and Hinata 1980), a two hour electrophoretic run is not long enough in the present method to obtain a final distribution of pH gradient.

We have attempted to estimate the pI of *S*-glycoproteins by cellulose-acetate electrofocusing. By using narrow ranged carrier ampholite, the material stigma homogenates were started from various positions on the gel slab. The *S*-glycoprotein moved towards the one position where the pH is equivalent to pI. By measuring the pH values at several different parts of the sheet, the pI values of given *S*-glycoproteins were approximately estimated. Figure 2 shows a demonstration for $S^{7(pI\,5.7)}$ where electrofocusing was made with a carrier ampholite pH 5-7 at 800 V for 4 hr. Sequential cut sections of the gel were immersed in distilled water and the pH of the solution was measured. When the pI of a given *S*-glycoprotein was acidic, this method gave good approximation. For the basic *S*-glycoproteins, however, problems remain which will be further studied. Taking $S^{39(pI\,10.3)}$ as a sample, we tried the analysis with carrier ampholite pH 9-11. However, the pH gradient at pH higher than pH 10 was not very good. This was partly improved when arginine was added to the carrier ampholite and NaOH granules were put into the electrophoretic apparatus in order to remove the CO_2 gas around the electrofocusing gels. It is a problem to measure the intact pH on the sheet. High pH solutions are variable for their pH

value due to ambient air CO_2. An application of pI markers to this analysis is now under investigation. For the analysis of *S*-glycoproteins in future, a set of standard *S*-glycoproteins may well be used as pI markers. In addition, carrier ampholites that cover pH ranges higher than pH 11 are necessary.

Although our present technique is not yet conclusive for determining the pI of every *S*-glycoprotein, it does provide us with *S*-glycoprotein profiles within one day. Tentatively, we are able to characterize *S* alleles by the band position of their corresponding *S*-glycoproteins, and the *S*-glycoproteins can be compared to each other by preserving the analyzed gels. This analysis may be a great help for inferring *S*-genotypes of individual plants without crossing experiments.

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