SHORT COMMUNICATION

RAPID SEPARATION AND DETECTION OF CONCANAVALIN A REACTING GLYCOPROTEINS: APPLICATION TO STORAGE PROTEINS OF A LEGUME SEED

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Detection of carbohydrate covalently linked to proteins is important considering the wide occurrence of glycoproteins and the roles that bound sugar may play.

Concanavalin A has been used for detecting glycosylated polypeptides separated by SDS-PAGE (Hawkes 1982). This denaturing technique, however, does not allow proteins to separate particularly oligomeric proteins, in their native state; a disturbing limitation in the detection and studies of intact molecules. This situation, among others, does not allow use of assays based on enzymic or biological activity.

In the present communication we describe a method for the rapid separation and detection of native glycoproteins in protein mixtures in microgram amounts. The procedure is applied to identify bound carbohydrate in the storage globulins of lupin seed, a controversial issue since one class of these proteins, namely the legumin-like proteins, are not glycosylated in most legumes (Duranti *et al.* 1987).

Chemicals were the purest available from Sigma and Merck. Cellulose acetate (CA) membranes were from ELVI: Milano, Italy; nitrocellulose (NC) membranes were from BioRad; Concanavalin A, horseradish peroxidase, and alkaline phosphatase linked goat-antirabbit antibody were from Sigma.

Proteins on CA and NC membranes were stained by soaking for 5 min in a 1% solution of Coomassie brilliant blue R250 in methanol, acetic acid and water (5:1:4 v/v), followed by destaining in the solvent without Coomassie blue.

Glycoproteins were detected by binding Concanavalin A (50 μg/mL, in 50 mM Tris-HCl, pH 7.4, containing 200 mM NaCl (TBS)) and assayed according to Hawkes (1982) with peroxidase (50 μg/mL in TBS), 0.01% (v/v) hydrogen peroxide and 0.03% (w/v) *p*-chloronaphthol.

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Storage globulins were extracted at 4 °C from one mature dry seed of *Lupinus albus*, var. Multitalia and desalted according to Duranti *et al.* (1987), with some modifications due to the small amount of material. About 1–2 micrograms of globulin mixture were submitted to cellulose acetate electrophoresis (CAE) in 50 mM Na phosphate butter, pH 7.5, at 150 Volt for 30 min at room temperature, according to Blagrove and Gillespie (1975). CAE was preferred to nondenaturing polyacrylamide gel electrophoresis (PAGE) because it is more rapid, easy to perform and, as shown in what follows, it also allows efficient and fast blotting of high M_r proteins.

The separated globulins were blotted onto NC membranes by semi-dry-electroblotting in a Sartorius semi-dry blotting apparatus. The "sandwich" was assembled as follows: three layers of Whatman 3M filter paper soaked in 25 mM Tris buffer pH 10.4, laid on the anode, were carefully packed with the NC and the CAE membranes and with three layers of the same filter paper soaked in a similar buffer at pH 9.4 containing 6 amino-n-esanoic acid. A constant current of 30 mA was applied for 30 min at room temperature.

Blotting efficiency was estimated as follows: two cellulose acetate membranes (2 cm × 2 cm) received equal amounts of protein (from 6 to 18 μg); one was submitted to electroblotting. Proteins were then stained in test tubes with Coomassie blue by the micro Bradford method (Bradford 1976); the difference between optical densities at 590 nm measured the amount of protein transferred. With 30 min blotting, about 75% of total protein applied onto CA membranes was transferred. This is a satisfactory yield considering the size of proteins used (see below). Longer blotting times did not improve transfer. The efficiency of transfer was not quantified for each separated protein band; however all bands separated by CAE had an apparent similar decrease in staining after blotting.

The procedures were applied to lupin storage proteins as follows. Fractionation of lupin storage proteins by CAE is shown in Fig. 1A. The three main fractions on the Coomassie blue stained strip (Fig. 1A) are conglutin γ (Mr 200,000), vicilin-like protein (conglutin β) and legumin-like protein (conglutin α). The vicilins, oligomers with Mr from 150,000 to 250,000 and the legumins, oligomers of Mr 180,000 and 320,000 (Duranti *et al.* 1981, 1987), give more diffuse bands than conglutin γ, because of their mass and charge heretogeneity (Duranti *et al.* 1981).

The separated proteins were blotted onto NC membrane and were immunodetected by reaction with specific antibodies purified by immunoaffinity chromatography (Duranti and Cerletti 1990). Immunoreactivity was not lost during CAE and blotting, similarly to that observed on transfer from a polyacrylamide gel to a NC sheet (Towbin *et al.* 1979). Immunodetection of conglutin α is shown in Fig. 1B.

Since carbohydrate covalently linked to the protein cannot be assayed directly on immunodetected proteins because of the carbohydrate bound to the im-

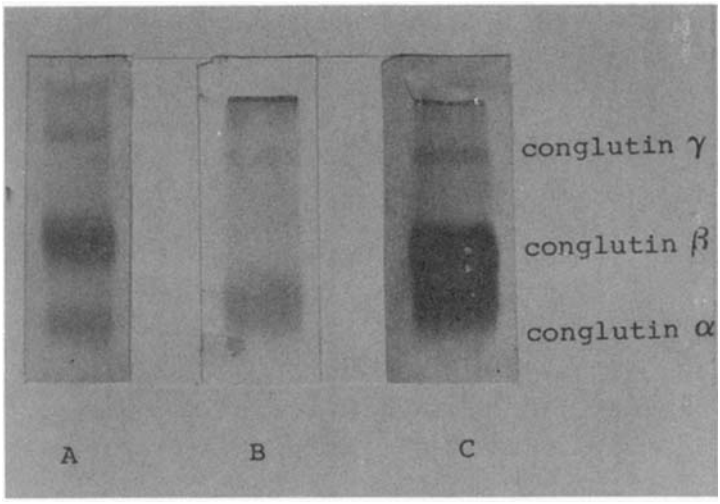

FIG. 1. ASSAY AND CHARACTERIZATION OF LUPIN GLOBULINS SEPARATED BY CELLULOSE ACETATE ELECTROPHORESIS

A: Coomassie blue staining.
B: Immunodetection of conglutin α with specific antibodies after blotting the separated globulins on a nitrocellulose membrane. The immuno complex as evidenced with alkaline phosphatase goat antirabbit antiserum, naphthyl-phosphate and Fast Red as described by Towbin *et al*. (1979).
C: Concavalin A binding and peroxidase staining of the globulins blotted on a nitrocellulose membrane. For experimental details, see text.

munoglobulins, the sugar assay was applied to samples run on parallel lanes. Glycoprotein detection on the NC sheets is shown in Fig. 1C. Concanavalin A stained all conglutin bands. Low color intensities in conglutin γ, which has highest bound sugar (4.3 mg/100 mg protein) is attributed to its low mannose content (10.7%), and high galactose content (6.2%) (Duranti *et al.* 1981), which does not react with concanavalin A (Poretz and Goldstein 1970). In the other proteins mannose represents 95 to 70% of sugar; glucose is negligible in all conglutins (Duranti *et al.* 1981). In previous work we had shown that conglutin α binds to Concanavalin A Sepharose 4B (Duranti *et al.* 1987).

The present results indicate that the procedure outlined can be applied for the separation and assay of glycosylated proteins in their native state, using commonly available laboratory equipment and easy procedures. Other important features of the native protein, such as immunoreactivity and catalytic activity (Ohlsson *et al.* 1978) are preserved and can be separately assayed.

Evidence is also provided on the intact, separated molecules, that legumin-like proteins, i.e., conglutin α, are indeeed glycosylated in lupin seed and differ in this aspect from most other legumins, which do not contain bound sugar.

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