Communication

Production of an Antibody Specific for the Propeptide of Wheat Germ Agglutinin

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ABSTRACT

Wheat germ agglutinin (WGA) is synthesized as a proprotein with a glycosylated, 15 amino acid, carboxyl-terminal propeptide. This glycopeptide is cleaved from pro-WGA to produce the mature lectin during the transport of WGA to the protein bodies/vacuoles. To study the posttranslational modification of WGA, it would be useful to be able to differentiate between pro-WGA and mature WGA. Therefore, a peptide corresponding to the propeptide of WGA was synthesized (WGA-B 172–186), and an antiserum was raised in rabbits (anti-WGA-B 172–186). Anti-WGA-B 172–186 reacted with pure WGA-B 172–186 and pro-WGA in ELISA. Anti-WGA-B 172–186 was also specific for and readily differentiated between pro-WGA and mature WGA on Western blots. This provided an assay to monitor pro-WGA on Western blots before and after endo-β-N-acetylgalcosaminidase H digestion. Using this assay, direct evidence was obtained that the oligosaccharide of pro-WGA is of the high mannose type.

WGA and their relevance to the proper targeting of WGA to protein bodies/vacuoles, it would be useful to be able to differentiate between pro-WGA and WGA, two closely related products of posttranslational modification. Toward this end, a peptide representing the propeptide of WGA was synthesized (WGA-B 172–186) and used to generate a polyclonal antiserum in rabbits (anti-WGA-B 172–186). Anti-WGA-B 172–186 reacted with pure WGA-B 172–186 and pro-WGA in ELISA. In addition, anti-WGA-B 172–186 was specific for pro-WGA on Western blots of WGA affinity-purified from developing embryos. Finally, the usefulness of these antibodies was demonstrated by monitoring pro-WGA on Western blots before and after endo-H digestion. These results provide direct evidence that the oligosaccharide of pro-WGA is of the high mannose type.

MATERIALS AND METHODS

Plant Material

Wheat (Triticum aestivum, L. cv Marshall) was obtained and grown as described previously (5).

Peptide Synthesis and Antigen Production

The solid phase synthesis of the hexadecapeptide, WGA-B 172–186, was according to Houghten (3). WGA-B 172–186 consists of amino acids 172–186 of the WGA isoelectric B (isolectin 3) proprotein (sequence derived from cDNA [9]) (Fig. 1). Cysteine was incorporated at the amino-terminus for coupling to the carrier protein, KLH. WGA-B 172–186 was coupled to KLH using succinimidyl bromoacetate as described in (1). WGA-B 172–186, insoluble in aqueous solvents, was dissolved in 6 M guanidine HCl for coupling. Peptide synthesis and coupling to KLH were performed by Applied Biosystems (Foster City, CA).

Antibody Production

Rabbits were immunized according to the following schedule: 200 μg KLH-coupled WGA-B 172–186 in complete Freund’s adjuvant (1:1) subcutaneously on d 0; 200 μg in incomplete Freund’s adjuvant (1:1) subcutaneously on d 14; 200 μg with 4 mg alum intraperitoneally on d 21. Rabbits were bled from the marginal ear vein on d 28 and, thereafter, at 1 to 2 week intervals. Antisera were separated from red blood cells by clotting red cells overnight at 4°C followed by

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2 Abbreviations: WGA, wheat germ agglutinin; WGA-B 172–186, a synthetic hexadecamer composed of the 15 carboxyl-terminal amino acids (amino acids 172–186) of pro-WGA isoelectric B (isolectin 3) with an added cysteine at the amino terminus; endo H, endo-β-N-acetylgalcosaminidase H; KLH, keyhole limpet hemocyanin; PBS, 10 mM sodium phosphate, 150 mM NaCl (pH 7.2); PBS-Tween, PBS containing 0.3% (v/v) Tween 20; dpa, days postanesthesia.
centrifugation at 10,000g in an IEC clinical centrifuge. Antisera were concentrated by addition of (NH₄)₂SO₄ to 60% saturation and resuspension in PBS to half the original volume. The rabbit polyclonal antiserum to denatured WGA was described previously (5).

**Standard WGA**

Standard WGA, prepared by the method of Nagata and Burger (7), was from Sigma.

**Affinity-Purified WGA**

Isolated wheat embryos were homogenized in a ground glass homogenizer with 1 mL of 50 mM HCl containing 1 mM PMSF (added from a 600 mM stock in DMSO) and 0.5% (v/v) Triton X-100 (Research Products International, Elk Grove Village, IL). Triton X-100 was included to maximize the yield of organelle-associated lectin (11). The homogenate was centrifuged at 10,000g for 10 min to remove debris, and solid (NH₄)₂SO₄ was added to 60% saturation. After precipitation for 2 hr at 4°C, the mixture was centrifuged at 10,000g for 10 min and the precipitate was resuspended in 200 μL of 50 mM Tris-acetate (pH 5), 0.1 M NaCl, 1 mM PMSF. WGA was isolated by affinity chromatography on immobilized GlcNAc as described in (5) with the exception that the GlcNAc, used to elute WGA, was dissolved in 50 mM Tris-acetate (pH 5), 0.1 M NaCl, 1 mM PMSF.

**SDS-PAGE and Western Blots**

Proteins for SDS-PAGE were carboxymethylated using a modification of the method in (8). Affinity-purified proteins were dissolved in loading buffer (12.5 mM Tris [pH 6.8], 4% [w/v] SDS, 20% [v/v] glycerol, 10% [v/v] 2-mercaptoethanol, and 0.005% [w/v] bromophenol blue). Iodoacetamide (final concentration of 0.24 M) and Tris base (final concentration of 0.47 M) were added to the samples which were then heated for 30 to 60 min at 37°C. Reduced and carboxymethylated proteins were separated by SDS-PAGE on 12.5% polyacrylamide gels (4).

After SDS-PAGE, Western blots were carried out according to Towbin and Gordon (12). Blots were developed using either anti-WGA-B 172–186 (1:1000 dilution) or anti-WGA (1:2000 dilution) as primary antibody, alkaline-phosphatase conjugated Protein A (Sigma P9650) as secondary antibody, and nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate as substrate. Blots were stained for total protein by incubation in 0.1% (v/v) India ink in PBS-Tween.

**ELISA**

ELISA was essentially as described by Voller et al. (14) using polystyrene microtiter plates (Falcon 3910). Proteins and peptides were coated overnight at 4°C in carbonate buffer (15 mM Na₂CO₃, 35 mM NaHCO₃ [pH 9.6], 0.2% [w/v] NaN₃). Adsorbed proteins and peptides were detected with either anti-WGA-B 172–186 (1:1000 dilution) or anti-WGA (1:2500 dilution) as primary antibody and alkaline-phosphatase conjugated goat anti-rabbit immunoglobulin (Sigma A8025) as secondary antibody. The primary and secondary antibodies were diluted in PBS-Tween containing 100 mM GlcNAc to block the GlcNAc binding sites of WGA and prevent nonspecific antibody binding (8). After incubation with the p-nitrophenyl phosphate substrate (Sigma S104-105), the absorbance of each well was read at 405 nm using a BioTek Industries model EL370 EIA Reader. WGA and the WGA-B 172–186 epitope were estimated in affinity-purified WGA samples from developing wheat embryos by constructing standard curves of standard WGA detected by anti-WGA and WGA-B 172–186 detected by anti-WGA-B 172–186. Absorbance values which were within the linear ranges of the standard curves were then translated into ng values.

**Endo H Digestion**

Affinity-purified WGA was digested with endo H using a modification of the procedure in Trimble and Maley (13). Ten minutes of endo H (Sigma) were dissolved in 10 μL of 50 mM Tris-acetate (pH 5.2), 0.1 M NaCl, and added to affinity-purified WGA dissolved in 150 μL of the same buffer containing 100 mM GlcNAc (from affinity chromatography) and 1 mM PMSF. The resulting solution was incubated for 16 h at 37°C. The reaction was stopped by lyophilization. Inclusion of 1 mM PMSF in the endo H reaction mixture was imperative to prevent nonspecific degradation of pro-WGA.

**RESULTS AND DISCUSSION**

**Reactivity of Anti-WGA-B 172–186 with WGA-B 172–186 and Pro-WGA: ELISA**

The reactivities of anti-WGA-B 172–186 with WGA-B 172–186 and standard WGA were determined by ELISA (Fig. 2). These reactivities were specific. Strong signals were obtained when 100 or 10 ng of pure WGA-B 172–186 were assayed with anti-WGA-B 172–186, while 1 ng gave a weak, but reproducibly above background, signal. Anti-WGA-B 172–186 did not react with standard WGA. On the other
which transferred were from extracts (23 kD) by treated and therefore the antigenicity detected and anti-WGA reacted with developing wheat embryos. Western blots of standard WGA and WGA affinity-purified WGA-B 172-186 for Pro-WGA: (Fig. 3, lane 3). Since anti-WGA-B 172-186 did not react with mature WGA in standard WGA, we concluded that these two bands did not arise from binding of anti-WGA-B 172-186 to the mature monomer, but from binding to either processing intermediates or degradation products of pro-WGA containing the WGA-B 172-186 epitope.

**Digestion of Pro-WGA with Endo H**

Mansfield et al. (5) showed that pro-WGA is glycosylated. To determine the nature of the oligosaccharide portion of pro-WGA, we incubated pro-WGA with endo H, which specifically cleaves high-mannose oligosaccharides from glycoproteins (13). Western blots probed with anti-WGA-B 172-186 provided a simple, nonradioactive way to detect endo H cleavage. When WGA, affinity-purified from developing wheat embryos, was incubated without endo H, no change in the apparent Mr of pro-WGA (23 kD) was observed (Fig. 4, lane 2). When affinity-purified WGA was incubated in the presence of endo H, the Mr of pro-WGA (23 kD) was reduced to 20 kD (Fig. 4, lane 3). These results provide direct evidence that the oligosaccharide portion of pro-WGA is of the high-mannose type.
Figure 4. Endo H digestion of pro-WGA. Pro-WGA and WGA were affinity-purified from developing wheat embryos (16 dpa) and digested with endo H. Proteins were separated by SDS-PAGE, blotted to nitrocellulose, and either stained with India ink for total protein or probed with anti-WGA-B 172–186. Lane 1, affinity-purified WGA, without endo H digestion, stained with India ink; lane 2, as lane 1 except probed with anti-WGA-B 172–186; lane 3, affinity-purified WGA, as in lane 2, probed with anti-WGA-B 172–186 after endo H digestion. Lanes 2 and 3 contain nine times more protein than lane 1, which was included to indicate the position of mature WGA (Mr = 18 kD).

LITERATURE CITED