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CROSS-REACTIVITIES OF POLYCLONAL ANTIBODIES AGAINST EXTENSIN PRECURSORS DETERMINED VIA ELISA TECHNIQUES

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Key Word Index—*Lycopersicon esculentum*; Solanaceae; tomato; extensin precursor; antibodies; ELISA.

Abstract—We raised four sets of rabbit polyclonal antibodies against two highly glycosylated extensin precursors, P1 and P2, before and after hydrogen fluoride-deglycosylation. Use of an indirect non-competitive sandwich ELISA technique to determine antibody-antigen cross-reactivities revealed three epitope classes: 1. Glycosylated; 2. Nonglycosylated in the intact glycoprotein; 3. Exposed only after deglycosylation. Thus polyclonals raised against *glycosylated* P1 or P2 cross-reacted highly (> 50%) with the heterologous glycosylated antigen, i.e. antibody–antigen pairs P1/P2 and P2/P1, but much less with the deglycosylated antigens dP1 and dP2 (< 25%), implying that the major epitopes are glycosylated; these probably correspond to hydroxyproline oligoarabinosides. The free sugars D-glucose, D-galactose and L-arabinose did not inhibit antibody–antigen binding, in contrast to free hydroxyproline arabinooligosaccharides which did compete at high levels (20–50 mM). Cross-reactivities towards other related macromolecules were low but positive for the following antibody/antigen pairs: P1/potato lectin, P2/AGP, but negative towards larch arabinogalactan. Polyclonals dP1 and dP2 (raised against the deglycosylated precursors dP1 and dP2) cross-reacted significantly with their homologous glycosylated antigen (reactions dP1/P1 and dP2/P2), but only slightly with their heterologous antigen (reactions dP1/P2 and dP2/P1). These results imply that nonglycosylated epitopes of the glycosylated antigens P1 and P2 differ markedly from one another, and therefore corroborate primary structure information suggesting Val-Lys-Pro-Tyr-His-Pro as the major nonglycosylated epitope of P1 and Val-Tyr-Lys-Tyr-Lys as the major nonglycosylated epitope of P2.

INTRODUCTION

Hydroxyproline-rich glycoproteins (HRGPs) are integral structural components in the extracellular matrix of multicellular eukaryotes. The primary cell wall of higher plants contains extensin, a firmly-bound structural [1] HRGP implicated in the control of extension growth [2] and disease resistance [3].

For some years the insolubility of extensin prevented its structural elucidation. Even reagents such as anhydrous hydrogen fluoride failed to solubilize extensin [4], implying its existence *in muro* as a highly crosslinked network [5]. Recent data support that hypothesis: first, the isolation of extensin tryptides containing the crosslinked amino acid isodityrosine [6], and second, the extraction of monomeric extensin precursors from cell walls of rapidly growing intact cells [7] and from the isolated cell wall fraction where a time-dependent insolubilization of precursor occurred [8]. These data indicate that assembly of the extensin network occurs *in muro*.

Polyclonal antibodies raised against cell wall fractions from higher plants [9] and a related synthetic peptide [10], and monoclonals raised against *Chlamydomonas* wall HRGPs [11] and *Fucus* cell wall carbohydrates [12], demonstrate the feasibility of immunological approaches [13] to cell wall structure. Thus availability of purified extensin monomers not only facilitates determination of their primary structure [14], but also opens up a whole arsenal of sensitive immunological techniques for detection, assay, cytochemical localization and manipulation of extensin. Enzyme-linked immunosorbant assays (ELISA) are especially sensitive and " . . . useful for the detection of antigenic determinants on molecules in solution or on cell surfaces, or for the investigation of antibody specificities or affinities" [15]. Especially relevant are ELISAs developed recently for other soluble *precursor* glycoproteins which, like extensin, also form cell-surface networks: elastin [16] and fibronectin [17].

Here we describe the properties and cross-reactivities of polyclonal rabbit antibodies raised against two extensin precursors P1 and P2 of tomato and their HF-deglycosylated polypeptides dP1 and dP2. Cross-reactivities determined by an 'indirect non-competitive sandwich' [18] type of ELISA technique helped us identify three types of antigenic determinants (epitopes) in each antigenic precursor: (1) glycosylated epitopes; (2) nonglycosylated epitopes of the intact glycoprotein; (3) epitopes exposed only after deglycosylation.

Abbreviations: ABTS, 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid); BSA, bovine serum albumin; HAs, hydroxyproline arabinosides; IgG and IgM, immunoglobulin G or M; P1 and P2, glycosylated precursors to the extensin network; dP1 and dP2, HF-deglycosylated extensin precursors; PBS, phosphate buffered saline.

RESULTS AND DISCUSSION

Four weeks after challenging rabbits with 100–500 μg of the appropriate antigen (P1, P2, dP1 or dP2) typical

immunogenic response was apparent (Fig. 1) as determined by micro ELISA assays of diluted immune serum compared with NRS controls (normal rabbit preimmune serum). Antigen purity was established by the highly reproducible tryptic peptide maps obtained after HF-deglycosylation of the antigen [14]. (Note however that P1 refers to a microheterogenous mixture of P1a and P1b variants giving very similar tryptic peptide maps [14].) Thus antibody titres peaked first at five weeks (probably corresponding to IgMs [15]) and then again at about eight to ten weeks (Fig. 1), this second peak probably corresponding to the more specific IgG antibodies. Titres declined slowly with time reaching a low plateau after several months, but a single booster injection restored the high titre within a week.

As antibody specificity tends to increase with time after inoculation we used serum from later rather than earlier bleeds for determination of cross-reactivities. Although P1 and P2 were clearly antigenic, P2 titres were significantly greater than P1 (Fig. 1). Thus P2 was more antigenic than P1. Similarly the antigenicity of HF-deglycosylated precursor P2 was greater than that of HF-deglycosylated P1 (Fig. 1).

The extent of antibody cross-reactivity depends on the number of common epitopes in the antigens used to raise antibodies. Therefore we determined the cross-reactivities of polyclonals raised against P1, P2, dP1 and dP2 to measure similarities between the antigens both before and after their deglycosylation. Polyclonals raised against *glycosylated* P1 or P2 cross-reacted very highly (Fig. 2) with their heterologous *glycosylated* antigen (i.e. antibody/antigen pairs: P1/P2 and P2/P1), but much less with the deglycosylated antigens. Indeed, the P1/dP2 cross-reactivity was essentially zero (Fig. 2). These data imply that P1 and P2 antibodies cross-react mainly with glycosylated rather than nonglycosylated epitopes of the intact glycoprotein. This assumes that the nonglycosylated domains of a flexible rodlike glycoprotein [19] are exposed and available for antibody binding. As the major P1, P2 glycosylated domains consist of arabinosylated hydroxyproline residues [7] these are most likely to be major epitopes of the glycosylated extensin precursors.

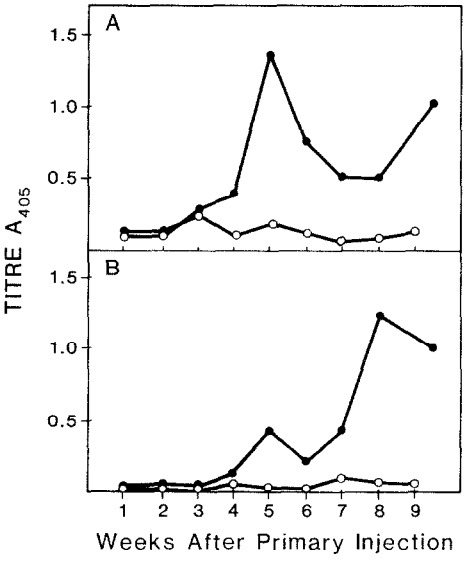

Fig. 1. Rabbit immune response to antigen challenge: (A) P1; (B) P2. Time course of immune response following 200 μg primary injection. We assayed sera diluted × 200 for P1, and × 800 for P2, using the non-competitive sandwich micro-ELISA described in methods. Assays were triplicated three times. ●——●, Immune rabbit serum. ○——○, Normal (preimmune) rabbit serum control.

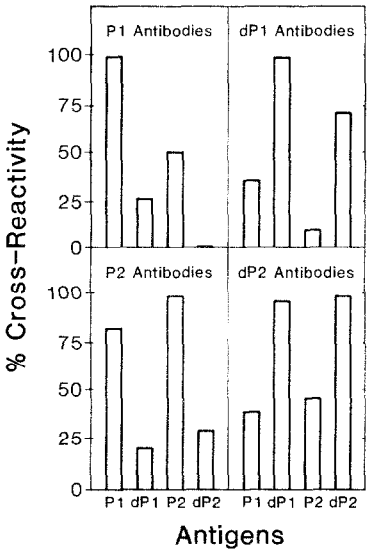

Fig. 2. Cross-reactivity of antibodies raised against antigens P1, P2, dP1 and dP2. After coating micro-ELISA wells with 0.2 μg of the appropriate antigen, we added 25 μl of diluted sera (× 200 for P1 and dP1; × 800 for P2 and dP2) expressing cross-reactivity of a given antibody with each antigen, as a percentage of the appropriate homologous antibody/antigen reaction (e.g. P1/P1, P2/P2, dP1/dP1 or dP2/dP2).

Such arabinosylation could conceivably lead to the formation of relatively non-specific antibodies recognizing other highly arabinosylated macromolecules [20]. We therefore tested this possibility by assaying the cross-reactivity of P1 and P2 antibodies against a range of small M_r saccharides (both related and unrelated to L-arabinose) and a range of arabinosylated macromolecules. Results are as follows: Preincubation of P1 and P2 antisera with the single sugar monomers D-glucose, D-galactose or L-arabinose, even at high sugar concentrations (100 mM) did not inhibit antibody–antigen binding. However preincubation of P1 or P2 antisera with mixed hydroxyproline tri- and tetraarabinosides did reduce antibody–antigen binding significantly though at rather high HA levels: 20–50 mM HAs reduced the binding to 50% under our assay conditions (Fig. 3). Thus the epitope bound at the antibody recognition site probably consists of an oligosaccharide profile rather than a single sugar determinant reported by others [20]. Therefore we examined P1 and P2 antibody cross-reactivities towards other HRGPs such as arabinogalactan proteins (AGPs), potato lectin and a polysaccharide such as larch arabinogalactan (which contains no protein). P1 antibodies did not cross-react with an AGP isolated from sycamore cells, whereas P2 did show 27–29% cross-reactivity ascribable to a common glycosylated epitope (i.e. arabinosylated hydroxyproline), as antibodies raised against the *deglycosylated* antigens dP1 and dP2 did not cross-react significantly with the AGP (Fig. 4). Interestingly P1

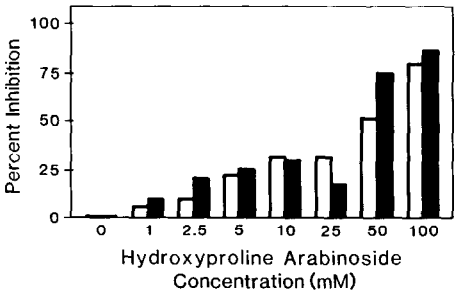

Fig. 3. Competitive inhibition of P1 and P2 antibodies by hydroxyproline oligoarabinosides. Shows % inhibition of antibody/antigen binding as a function of increasing hydroxyproline arabinoside concentrations, preincubated with antibody as described in the Experimental.

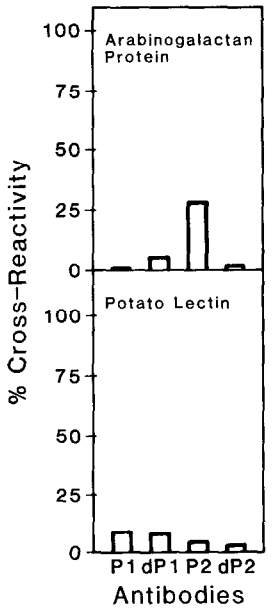

Fig. 4. Cross-reactivity of P1, P2, dP1 and dP2 antibodies with arabinogalactan protein and potato lectin. After coating micro-ELISA wells with 0.2 μg of AGP, lectin, or appropriate antigen for comparison, we added 25 μl of diluted serum (as described for Fig. 2 in Experimental) and expressed cross-reactivity, shown by a given antibody towards AGP or lectin, as a percentage of the P1/P1, P2/P2, dP1/dP1 or dP2/dP2.

cross-reacted slightly with potato lectin (which contains some arabinosylated hydroxyproline) [21, 22], although the P2 cross-reaction was hardly significant (Fig. 4). As AGPs and potato lectin both contain arabinosylated hydroxyproline, we suggest that different P1, P2 cross-reactivities simply reflect topographically different hydroxyproline arabinoside profiles of the extensin precursors themselves, and of course, AGP and potato lectin. However larch arabinogalactan did not react significantly with P1, P2, dP1 or dP2 antibodies even at an arabinogalactan concentration 1000 times greater than that of the antigen (data not shown). Significantly arabinosyl residues are alpha-linked in the arabinogalactan [23], but mainly beta-linked in the hydroxyproline arabinosides; the exception is the single alpha-linked terminal arabinofuranoside of HA_4 [24].

As extensin precursors are not 100% glycosylated we tested for nonglycosylated epitopes (of *glycosylated* antigens) by determining the cross-reactivity of polyclonals dP1 and dP2 (i.e. raised against the deglycosylated antigen) with *glycosylated* antigens P1 and P2 (i.e. reactions dP1/P1, dP1/P2, dP2/P1 and dP2/P2). Absence of cross-reactivity would indicate absence of nonglycosylated epitopes in the glycosylated antigen. Figure 2 shows however significant cross-reactivity for the homologous reactions, dP1/P1 and dP2/P2, implying the presence of nonglycosylated epitopes in the glycosylated antigens. However cross-reactivities for the heterologous reactions dP1/P2 and dP2/P1 were remarkably low (Fig. 2), implying marked differences between the nonglycosylated epitopes of P1 and P2. These data corroborate our reported [14] primary structures: P2 has a single major nonglycosylated domain, Val-Tyr-Lys-Tyr-Lys, which is absent from P1, where the major repeating nonglycosylated domain is: Val-Lys-Pro-Tyr-His-Pro, with a possible second being Thr-Hyp-Val-Tyr-Lys if the internal Hyp is nonglycosylated. This small number of nonglycosylated epitopes in the intact glycoprotein makes the facile preparation of monospecific antibodies ("poor-man's monoclonals" [25]), via immunoaffinity chromatography feasible; indeed polyclonal antibodies raised against the closely related HRGP 'bacterial agglutinin' from potato [26], have already been used for immunofluorescent detection after antibody purification on an immobilized-antigen column [27].

We interpret the data reported here, in conjunction with known P1 and P2 primary structure [14], in the following general terms: extensin precursors are flexible rods consisting of rigid domains separated by more-or-less flexible spacers as represented schematically in Fig. 5. Ser-Hyp-Hyp-Hyp-Hyp pentapeptides stabilized by oligoarabinoside substituents [19, 28, 29] and galactosylated serine residues [30] comprise the rigid domains (glycosylated epitopes), while the flexible spacers correspond to the nonglycosylated epitopes of the intact glycoprotein. Each glycoprotein precursor has its own characteristic nonglycosylated epitope(s); we suggest a dual significance for these flexible domains. First they determine the overall flexibility of the macromolecule, and second they are prime candidates for (IDT?) crosslink sites necessary for creation of an extensin network [31–33].

EXPERIMENTAL

Animals. Four 5-1b female New Zealand white rabbits were from the Small Animal Care Facility, Michigan State University.

Antigens. We prepared extensin precursors P1 and P2 by elution of intact tomato cell suspension cultures as previously described [7], and prepared the deglycosylated polypeptides (dP1 and dP2) by solvolysis in anhydrous liquid HF with 10% v/v anhydrous MeOH as a scavenger for the reactive sugar fluorides, in a micro-apparatus as detailed previously [34].

Antigen challenge. The primary injection of glycosylated antigens was a 500 μl aliquot of a water-in-oil emulsion [prepared by vigorously mixing 500 μl 2 mg/ml P1 or P2 in 0.85% sterile saline, with 500 μl Freund's complete adjuvant (Cappel Laboratories)] injected subcutaneously into each shoulder and hip of the rabbit. The primary injection of HF-deglycosylated antigen (dP1 or dP2) was also a 500 μl water-in-oil emulsion of

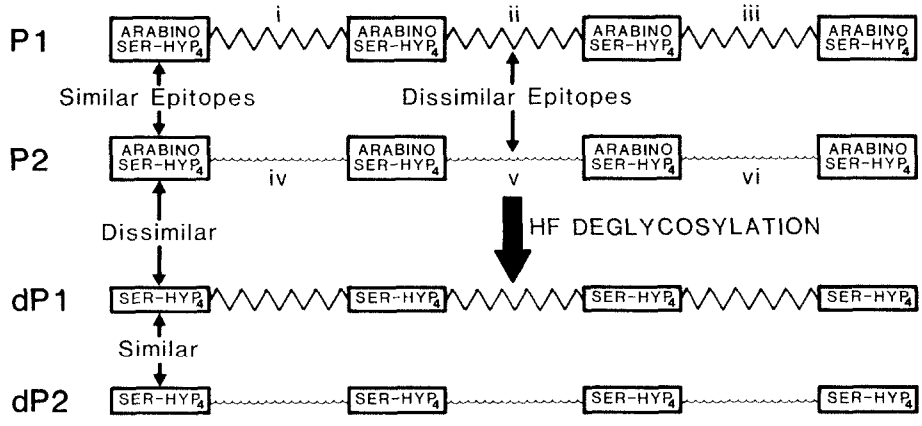

Fig. 5. Schematic representation of extensin precursors before (P1 and P2) and after HF-deglycosylation (dP1 and dP2). Rigid blocks consisting of oligoarabinosylated tetrapeptides of hydroxyproline and single galactosylated serine residues represent glycosylated epitopes, while flexible regions 1–6 represent nonglycosylated epitopes of the intact glycoprotein. Note that in P1 $1 \neq 2 \neq 3$ while in P2 probably $4 = 5 = 6$. After HF-deglycosylation new epitopes appear represented by the Ser-tetra Hyp pentapeptides.

antigen in Freund's complete adjuvant but contained only 100 μg dP1 or dP2, and then injected subcutaneously as above. Booster injections of antigen contained 100 μg precursor in 500 μl water-in-oil emulsion of Freund's incomplete adjuvant, injected subcutaneously over each hip.

Enzyme-linked immunoassay (ELISA). Essentially this is based on that of ref. [15]. We coated each test well of 96 well polystyrene plates (Nunc, Thomas Scientific) with 0.2 μg antigen in 200 μl pH 9.6, 50 mM $NaHCO_3$ buffer, for 15 hr at 4°; washed the plate once in H_2O and briefly dried it before blocking all remaining protein binding sites by addition of 200 μl 1% BSA in PBS (final pH 7.5), for 30 min. at 37°, followed by washing twice with H_2O and then drying.

We diluted the control (pre-immune) and test sera as follows: × 200 for P1 dP1 and pre-immune control, and × 800 for P2, dP2 and pre-immune control, in pH 7.5 PBS, and then added 25 μl of the diluted sera to the antigen-coated wells containing 25 μl 1% BSA-Tween-20 (1 μl/ml)/PBS at pH 7.5. After 1 hr at 37° we washed the plate twice in H_2O, added 50 μl diluted (× 2000) goat-anti-rabbit serum coupled to peroxidase (Cappel Laboratories) in BSA/Tween-20/PBS to each well, incubated at 37° for 30 min, washed the plate × 5 with H_2O, again briefly dried the plate, and then added 100 μl substrate to each well (11 mg ABTS and 15 μl 30% H_2O_2 in 50 ml pH 4, 50 mM citrate buffer). After 30 min incubation at 23° we added 100 μl NaF/EDTA stopping reagent (prepared by adding 50 μl 40% w/v tetrasodium EDTA to 50 ml 6 mM NaF in 2.5 mM HF) to each well, and then determined the absorbance at 405 nm. We assayed extensin antibody cross-reactivities to potato lectin (Sigma) and sycamore arabinogalactan protein via ELISA, adding 0.2 μg AGP or lectin in pH 9.6 $NaHCO_3$ buffer to each well, and then assaying as described.

To test for possible antibody binding of free monomeric sugars, hydroxyproline oligoarabinosides or arabinogalactan polysaccharide (from Larch via Sigma), we used a competitive ELISA by pre-incubating antibodies with suspected competitor (2 hr at 23°) at concentrations ranging from 0 to 100 mM (except for the polysaccharide whose concentrations was purely on a weight basis), and then adding aliquots to antigen-coated wells and completing the binding assay as described above for the noncompetitive ELISA. Controls consisted of antigen-coated wells plus (a) P1 or P2 antisera, (b) pre-immune serum and (c) pre-immune serum pre-incubated with suspected competitor.

Arabinogalactan protein preparation. After sonic disruption of sycamore cell suspensions we centrifuged the homogenate (9000 rpm, 20 min), added TCA (10% w/v final concn) to the supernatant and, after 18 hr at 4°, again centrifuged (9000 rpm, 20 min), dialysed the supernatant and freeze dried it to yield crude AGP. As the second purification step we fractionated the crude AGP on Sephadex CL-6B (4.5 × 100 cm) eluted with 0.1 M NH_4OH, collected the HRGP fractions and freeze dried them, yielding partially purified AGPs containing *ca* 26 mol% Hyp based on amino acid composition.

Hydroxyproline arabinoside 3 and 4 preparation. We refluxed 3 g tomato cell walls in 420 ml 0.2 M $Ba(OH)_2$ for 24 hr, then neutralized the cold hydrolysate with conc. H_2SO_4, carefully adjusting the final pH to 7.5–8.0, centrifuged to remove $BaSO_4$, rotary evaporated (to 50 ml) and freeze dried the neutral soln. Further purification of the HAs involved absorption of the water soluble fraction on Dowex-50 (H-form), and, after washing the resin first with water, eluting with 6 M NH_4OH, and then further fractionating the eluate on Sephadex G-25 (2 × 100 cm × 1.25 cm i.d.) eluted with water at 10 ml/hr. We assayed the hydroxyproline-rich fractions corresponding to HA3 and HA4 using a hydroxyproline analyser as previously described [35, 36].

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