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SIMILARITIES BETWEEN SWEET PROTEIN THAUMATIN AND A PATHOGENESIS-RELATED PROTEIN FROM TOBACCO

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Key Word Index— *Thaumatococcus daniellii*; Marantaceae; *Nicotiana tabacum*; Solanaceae; tobacco mosaic virus; thaumatin; pathogenesis-related proteins; hypersensitive reaction; immunological relationships.

Abstract—A pathogenesis-related protein, PR-R from virus-infected tobacco leaves, has been compared in immunological, electrophoretic and chromatographic properties with the sweet protein thaumatin with which it is believed to have ~65% homology of amino acid sequence. PR-R runs faster in PAGE after it has been reduced, and both proteins run slower in SDS PAGE when reduced. PR-R and thaumatin eluted from Superose 12 FPLC columns with apparent M_rs of 14 500 and 12 000 respectively, i.e. 68 and 55% of expected values: following reduction and *S*-carboxymethylation thaumatin eluted with an apparent M_r of 48 000. Neither protein in the native state reacts with polyclonal antisera produced against the other protein. Following reduction, cross-reactions can be demonstrated in immunoblots. The results implicate the intramolecular disulphide bridges of both proteins in conferring a compact configuration on the native proteins, rendering some common epitopes inaccessible.

INTRODUCTION

An examination of new mRNA species induced during the hypersensitive infection of tobacco plants with tobacco mosaic virus (TMV), led to the prediction of the existence of a virus-induced protein with a striking structural resemblance to the sweet tasting protein, thaumatin that occurs in the fruit of the West African shrub *Thaumatococcus daniellii* (Benth.) [1]. This protein was identified with a pathogenesis-related (PR) protein called PR-R in the terminology of Pierpoint [2] which had previously been isolated from TMV-infected tobacco leaves [3]. PR-R contains, like thaumatin, 16 cysteine residues per molecule of estimated M_r 23 000. It exists in two isoforms; one with an *N*-terminal sequence of 54 amino acid residues that is identical to that predicted from the induced mRNA, and the other with only two amino acid replacements in this sequence.

The circular dichroism spectra of PR-R resembles that of thaumatin [2] and there is a high (65%) degree of amino acid sequence homology with thaumatin [1]. However, PR-R differs from thaumatin in a number of respects. PR-R is a neutral (p*I* between 6.2 and 6.8) rather than a basic protein and it has no marked sweet taste. Moreover it did not react in preliminary immunodiffusion tests with a polyclonal antibody produced against thaumatin. This lack of reaction was surprising, even though a major antigenic epitope of thaumatin seems to be associated with its sweetness-conferring structure [4]. However, we recently observed that the reaction of thaumatin with the rabbit antibody could be markedly increased in conditions where the disulphide bonds were reduced. This increase in reaction was examined more closely in an investigation of the immunological relationship between thaumatin and PR-R. We have also detected similarities in the anomalous chromatographic behaviour of the two proteins which probably reflect structural similarities dependent on their high content of intramolecular disulphide bonds.

RESULTS AND DISCUSSION

Behaviour of the proteins on permeation chromatography

Thaumatin and PR-R are single polypeptide chains of 207 and 203 amino acids and calculated M_r's of 22 209 [5] and 21 596 [1] respectively; all the cysteine residues of thaumatin are cross-linked and the cd spectrum of PR-R suggests that most, if not all of its cysteine residues are cross-linked. The high degree of disulphide 'scaffolding' confers a compact configuration on the molecules. PR-R and thaumatin eluted from a Superose 12 gel permeation FPLC column with apparent M_r's of 14 500 and 12 000 respectively (14 598 ± 639; 11 900 ± 737). These values are 68 and 55% of expected values. Evidence that the retarded elution of thaumatin from the gel permeation column depends on the configuration conferred by the disulphide bonds was obtained from the effect of reducing and carboxymethylating the SH groups with iodoacetate. The main product of this treatment migrated as if its molecular weight was four times larger (47 738 ± 1841) than the apparent M_r of unmodified thaumatin. It is presumably, a looser and partly uncoiled structure that now gives this material a M_r higher than expected. A minor (~18%) product of the carboxymethylation eluted as if it were over a million (1 368 225 ± 17 370); it is probably polymeric material in which incompletely alkylated thaumatin molecules are linked by intermolecular disulphide bonds.

Seven other PR-proteins exhibit large discrepancies between apparent M_r's derived from SDS PAGE and

from gel permeation behaviour [6]. SDS PAGE behaviour was considered to be anomalous and HPLC derived values were preferred. However, the values derived from SDS PAGE for the molecular weights of PR-Ia, b and c are in better agreement with the values measured from sequence data [7] and centrifugation measurements [8]. In our experience, with PR-R, M_r values closer than deduced from cDNA studies (21 596) [1] could also be derived from SDS PAGE,both in the system described here and in SDS urea gels (20 885), than from FPLC. By analogy with thaumatin, it is likely that anomalous FPLC behaviour is due to PR-R having an unusually compact structure maintained by disulphide bonds. It is possible that the other PR-proteins like PR-R also have compact structures: the properties that they have in common, including secretion and acumulation in intercellular spaces and resistance to proteolytic enzymes [9] make it likely that they also have similar structural features.

Behaviour of PR-R on non-denaturing PAGE

The conformation of PR-R as judged by mobility on native PAGE, was affected by reduction. The changes have been described briefly [2], but are shown in more detail in Fig. 1. Addition of an excess of dithiothreitol at pH 8.1 converts it to a diffuser faster running band presumably consisting of reduced, less tightly-coiled molecules. On incubation in aerobic conditions, the amount of this material decreases, and the original form is partly regenerated, but the final product is mainly material which is too large or insoluble to enter the resolving gel and remains in the stacking gel; this probably consists of PR-R polymerized by intermolecular disulphide bonds. Thaumatin is too basic (p$I \sim 12$) to be examined in this PAGE system.

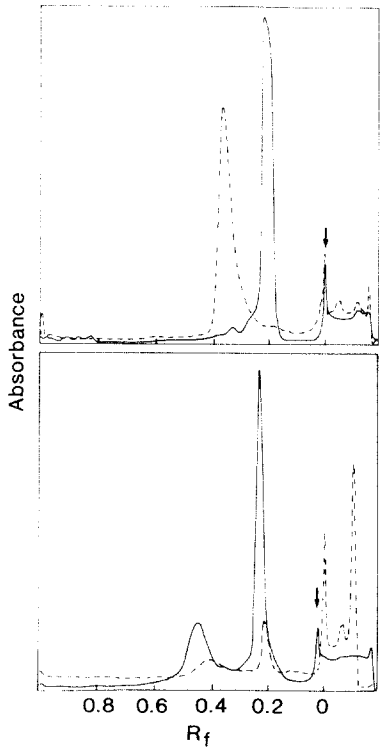

Fig. 1. Effect of reduction on the electrophoretic mobility of PR-R. PR-R (25 μg in 50 μl Tris–glycine buffer, pH 8.1) was incubated for 1 hr at 37°, diluted three-fold with buffer and electrophoresed directly (upper graph; solid line) or immediately after the addition of dithiothreitol to 14.3 mM (upper graph; dashed line). Other samples of R had the DTT present during 10 min (lower graph; solid line) or 60 min (lower graph; dashed line) of their incubation. After staining, the gels complete with stacking gels were scanned photometrically. The arrows mark the junction of stacking and resolving gels.

Behaviour of PR-R and thaumatin in SDS PAGE

The apparent M_r of thaumatin in SDS PAGE is 20 500 [10]. That of PR-R is similar, i.e. 23 000 [3]. Both proteins show a decrease in mobility in SDS PAGE when the disulphide bonds are broken by reduction with mercaptoethanol (Fig. 2). Presumably, the uncoiled structure has less favourable dynamics in SDS PAGE than the compact configuration of the non-reduced proteins. This phenomenon is more marked in the case of PR-R. In both cases, a more diffuse protein band is observed on reduction.

Immunodiffusion of PR-R and thaumatin

In immunodiffusion tests, thaumatin and PR-R gave obvious precipitin lines with their respective antibody, but neither gave a discernable reaction with the reciprocal antibody. Figure 3 shows this absence of cross reaction using undiluted antisera, but the same results were obtained when four and 16-fold dilutions were tested

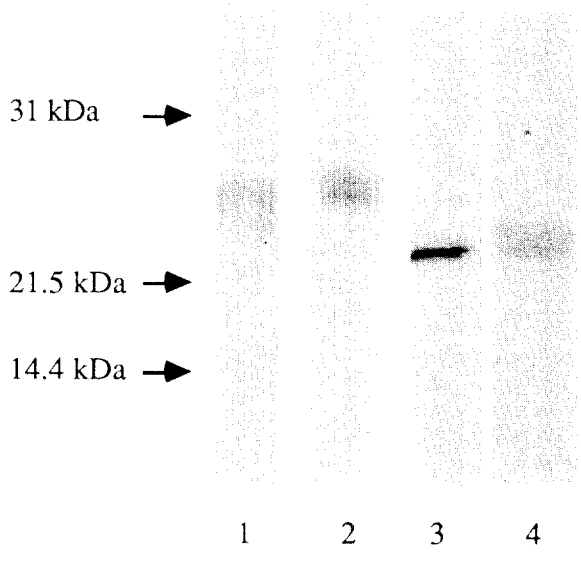

Fig. 2. SDS PAGE on 15% acrylamide of PR-R and thaumatin. Thaumatin and PR-R were boiled for four min in sample buffer [final concentration of 500 mM Tris, pH 8.6, 20% glycerol, 1% (w/v) SDS reducing sample buffer contains a final concentration of 200 mM mercaptoethanol]. Samples containing 10 μg protein were examined in a discontinuous 15% polyacrylamide gel system. Fast Green stain was used to detect all of the proteins in the gel. Lanes 1 and 3 contain thaumatin treated with reducing and non-reducing sample buffer respectively. Lanes 2 and 4 represent PR-R treated with reducing and non-reducing sample buffer.

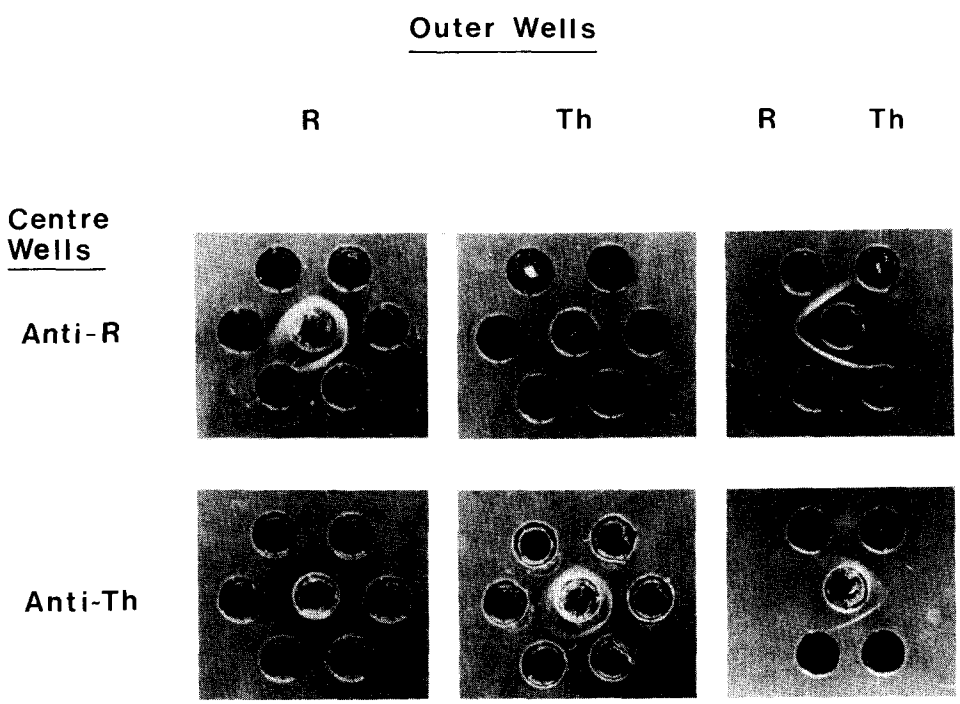

Fig. 3. Antigenic dissimilarity of PR-R and thaumatin (Th) in diffusion tests. The proteins were tested against both corresponding undiluted antisera which were in the centre wells in each test. Protein concentrations (mg per ml) in the outer wells of the four left-hand tests were, clockwise from the top left-hand wells, 1, 0.25, 0.062, 0.031, 0.0156, 0.0039. For the top right-hand test they were R, 0.031; Th, 1; Th, 0.25; R, 0.062. For the bottom right-hand test they were R, 0.6; Th, 0.25; Th, 0.125; and R, 0.25 mg per ml.

against the proteins in a range of concentrations (4–0.004 mg/ml). Neither protein gave a reaction with pre-immune serum taken from the rabbit used for the production of antibody to PR-R.

Effect of reduction on the recognition of PR-R and thaumatin by each antisera

Aliquots of the samples which were examined in SDS PAGE (Fig. 2) were applied to nitrocellulose in dot blots and then probed with antisera against both proteins (Fig. 4). No heterologous reaction occurs when the proteins are not reduced. Reduction of both proteins with mercaptoethanol to break the disulphide bonds enhances the homologous reaction and exposes a heterologous reaction in both cases. Cleavage of disulphide bonds reveals epitopes that are common to both proteins.

The availability of PR-R, so similar to thaumatin, but without the sweet taste, should make it a useful adjunct in protein engineering studies designed to explore either of these proteins. Two more proteins which may be equally useful were recently described. The first is a maize storage protein which is an inhibitor of trypsin and α-amylase. It has 52 and 57% amino acid sequence homology with thaumatin and PR-R respectively, including the 16 cysteine residues [11]. The second is a protein(s), osmotin(s), which occurs in cultured tobacco cells adapted to high osmotic stress, and whose size and amino acid composition are again strikingly similar to those of thaumatin [12]. Although serving apparently unrelated functions, it seems unlikely that these common features have arisen by chance. Presently, one can only speculate over the possible function of this common structure. One possibility is that this compact conformation protects much of the molecule from damage, e.g. from virus-induced necrosis, allowing the proteins to withstand conditions normally deemed unfavourable.

EXPERIMENTAL

Proteins. PR-R was extracted from the intercellular fluid (IF) of TMV-infected leaves of *Nicotiana tabacum* var Xanthi nc as previously described [2]. The IF extracts were freed from some glycoproteins by treatment with immobilized concanavalin A, and the PR-R separated from the other PR-proteins by chromatofocussing on a column of the Pharmacia ion-exchanger PBE 94. The product was judged by PAGE to contain only small amounts (about 1.5%) of other proteins. The sample used for antibody production was further purified by passage through the Superose 12 column of the Pharmacia FPLC system in phosphate buffered saline as described below. Fractions of the eluate containing the main peak were separated from small amounts of faster and slower running material and had a UV spectrum with a maximum ($A = 1.0$) near 280 nm suggesting [13] a protein content of *ca* 1 mg/ml. The thaumatin used was thaumatin I purified by ion exchange chromatography from a commercial sample, (Talin), kindly provided by Tate and Lyle.

Protein modification. Disulphide bonds of thaumatin (10 mg Talin) were reduced and *S*-carboxymethylated with iodoacetate [14]. After reaction, the mixture was dialysed for 20 hr against Pi buffer (0.05 M, pH 7.2) containing NaCl (0.15 M), centrifuged, filtered and analysed directly by FPLC.

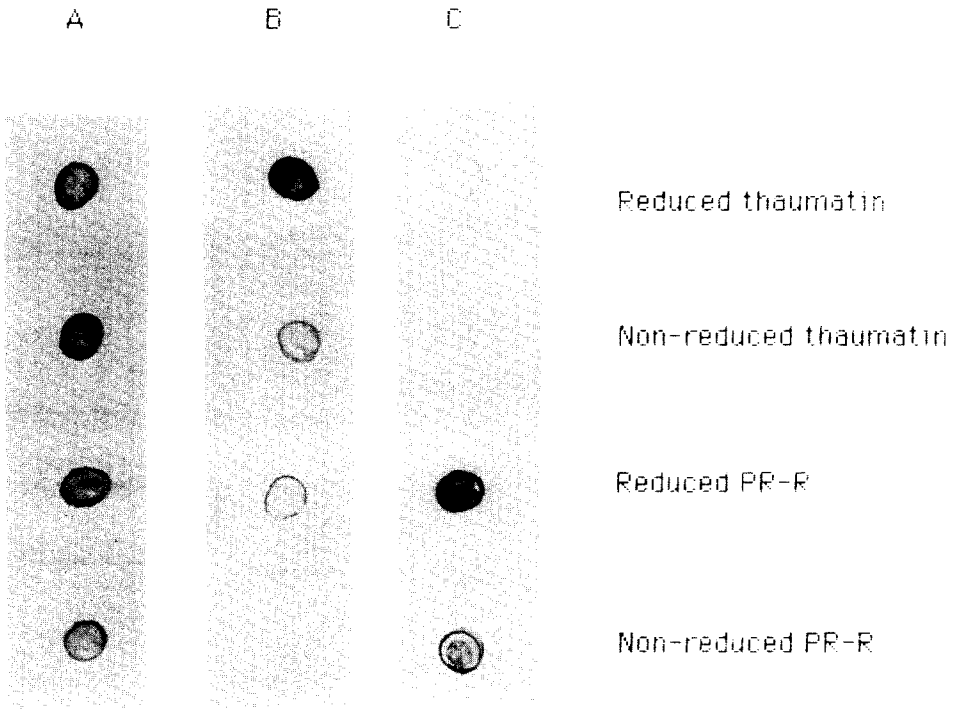

Fig. 4. Immunoblot of reduced and non-reduced denatured PR-R and thaumatin. Samples of PR-R and thaumatin were denatured with or without mercaptoethanol as for SDS PAGE analysis (Fig. 2). Triplicate dot blots were prepared using 2 μg of each protein spotted in a volume of 6 μl. A. Fast Green stain revealing the total protein present. Strips B and C were incubated for 1 hr with a 1:5000 dilution of anti-thaumatin and anti-PR-R antisera respectively. The incubations were in phosphate buffered saline (10 mM Na_2 PO_4 and 150 mM NaCl) containing 0.2% Tween 20. B and C were then incubated for a further hour with a 1:3000 dilution of peroxidase conjugated goat anti-rabbit IgG in PBS/0.2% Tween. Recognition of the proteins by the antisera was visualized by the addition of diaminobenzidine (20 mg) and hydrogen peroxide (200 μl) in PBS/0.2% Tween (50 ml).

Reduction of PR-R with dithiothreitol (DTT). Samples of PR-R (25 μg), dissolved in 50 μl of Tris (33 mM)–glycine (25 mM) buffer, pH 8.1, were incubated either at 0° or 37° for 1 hr. DTT was added in 20 μl of buffer to bring the concentration to 14.3 mM when required. Samples were diluted with 150 μl of PAGE sample buffer (Tris 5 mM; glycine 38.5 mM; glycerol 12.5 v/v%, pH 8.3), before aliquots were taken for electrophoresis. When samples were reduced after dilution and immediately before electrophoresis, 60 μl of DTT was added to ensure a concentration of 14.3 mM.

Protein chromatography. Proteins were chromatographed on a column (1 × 30 cm) of Superose 12 (HR 10/30: Pharmacia) attached to a Pharmacia Fast Protein Liquid Chromatography (FPLC) system. They were dissolved in Na_2HPO_4 buffer (0.05 M, pH 7.2) containing 0.15 M NaCl, and after passage through a Millipore membrane (0.22 μm, Millex-GV), samples (200 μl, usually 0.1–1.0 mg protein) were chromatographed in the same de-gassed buffer at the rate of 0.2 ml/min.

Standards were bovine serum albumin (67 000; dimer 134 000), ovalbumin (43 000), chymotrypsinogen A (25 000), ribonuclease A (13 700), cytochrome C (12 400), and aprotinin (6500). In the conditions used cytochrome *c* was consistently eluted a little earlier than ribonuclease. The elution volume of each standard protein in any experiment was obtained by averaging all the values obtained in that experiment. The correlation between elution vol. and log M_r (correlation coefficient −0.9866) was established by further averaging the values from all the experiments.

Immunodiffusion. Tests were performed in agarose gels (1.5% w/v) as previously described [2]. Proteins were dissolved in 50 mM Na_2HPO_4 buffer, pH7, containing 0.07 m NaCl and were diluted, as were antisera, in this solution, usually in 4-fold steps. The wells cut into the agar accommodated 20 μl samples. In the conditions of this test, the undiluted antiserum to PR-R gave precipitin lines with the protein down to a concn of 0.015 mg/ml. Conversely, at a concentration of 1 mg/ml, PR-R gave a reaction with serum down to a 16-fold dilution. The reaction of thaumatin with its antisera was observable over a similar range of concentration.

Preparation of antibodies. 0.6 ml of purified PR-R solution, containing *ca* 0.6 mg of protein in Na_2HPO_4 (0.05 M) buffer, with 0.075 M NaCl, pH 7.2 was mixed with Freunds complete adjuvant (FCA) and injected intramuscularly into a New Zealand white rabbit (2.5–3 kg). A second injection using the same amount of PR-R in incomplete adjuvant (FIA) was injected a month later. The rabbit was subsequently bled fortnightly, and the serum clarified by centrifugation (*ca* 7000 *g* for 10 min) before being stored at −20°. In the experiments reported, only the material from the first two bleedings was used. Pure thaumatin I (~200 μg/ 1 ml) in PBS (10 mM Na_2HPO_4, 0.15 M NaCl, pH 7.4) was emulsified with FCA and injected sub-cutaneously into a New Zealand White rabbit. An injection was given three weeks later, this time FIA was used. Two weeks later the rabbit was bled, the serum clarified and stored as for anti-PR-R.

Polyacrylamide gel electrohoresis (PAGE). PAGE was performed in rod gels containing 10% (w/v) polyacrylamide cross-

linked with 0.2% *N,N*-methylene-bisacrylamide (MEB) which was overlaid with a stacking gel (2.5% w/v polyacrylamide 0.23% w/v MEB) as described previously [3, 14]. They were stained with Coomassie Brilliant Blue R250 for 1 hr, destained in 5% (v/v) HOAc, and scanned at 610 nm. In order to illustrate the production, during the reduction of PR-R, of polymeric protein too large to enter the resolving gels, the stacking gels were left *in situ* and also scanned; their slight opacity gave a higher base line than that of the resolving gels (Fig. 1).

SDS polyacrylamide gel electrophoresis (SDS PAGE). SDS PAGE was performed in slab gels containing 15% polyacrylamide cross-linked with 0.08% MEB, with a stacking gel (5% w/v polyacrylamide, 0.15% MEB). Resolving and stacking gels both contained 0.1% SDS (w/v). The gels were stained in Fast Green for 30 min and then destained with an aqueous solution of MeOH, (45% v/v) and AcOH, (7% v/v).

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