

Purification and structural analysis of an abundant thaumatin-like protein from ripe banana fruit

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Abstract. The pulp of ripe bananas (*Musa acuminata*) contains an abundant thaumatin-like protein (TLP). Characterization of the protein and molecular cloning of the corresponding gene from banana demonstrated that the native protein consists of a single polypeptide chain of 200 amino acid residues. Molecular modelling further revealed that the banana thaumatin-like protein (Ban-TLP) adopts an overall fold similar to that of thaumatin and thaumatin-like PR-5 proteins. Although the banana protein exhibits an electrostatically polarized surface, which is believed to be essential for the antifungal properties of TLPs, it is apparently devoid of antifungal activity towards pathogenic fungi. It exhibits a low but detectable in vitro endo- β -1,3-glucanase (EC 3.2.1.x) activity. As well as being present in fruits, Ban-TLP also occurs in root tips where its accumulation is enhanced by methyl jasmonate treatment of plants. Pulp of plantains (*Musa acuminata*) also contains a very similar TLP, which is even more abundant than its banana homologue. Our results demonstrate for the first time that fruit-specific (abundant) TLPs are not confined to dicots but occur also in fruits of monocot species. The possible role of the apparent widespread accumulation of fruit-specific TLPs is discussed.

Key words: Fruit (protein) – *Musa* (banana, plantain) – Pathogenesis-related protein – Protein structure – Thaumatin-like protein

Introduction

The hypersensitive response of plants is one of the most rapid and efficient mechanisms for conferring a significant protection towards a broad range of phytopathogens including fungi, bacteria and viruses. In addition to the local response, which includes local cell death at the site of infection and metabolic changes of the cells surrounding the necrotic zone, various so-called pathogenesis-related (PR) proteins are strongly induced both locally and systemically. Since these PR proteins or at least some of them exhibit antimicrobial properties, it is generally believed that they contribute to disease resistance (Fritig et al. 1998). At present, at least 12 different families of structurally and evolutionarily related PR proteins can be distinguished. Some of these PR families comprise proteins with a well-defined catalytic or biological activity. For example pathogenesis-related proteins of families 2 and 3 (PR-2 and PR-3) have been identified as β -glucanases and chitinases, respectively, whereas PR-4 proteins are chitin-binding proteins. Other PR protein families such as thionins and defensins definitely exhibit antimicrobial activity. However, the exact working mechanism(s) of these proteins remains to be elucidated. The same holds true for PR-5 proteins, which exhibit amino acid sequence (Cornelissen et al. 1986) and structural (Batalia et al. 1996; Koiwa et al. 1999) similarity to the sweet-tasting protein from *Thaumatococcus daniellii* fruits (called thaumatin) but lack any sweet property.

During the past decade numerous thaumatin-like PR-5 proteins have been isolated, cloned and assayed for antifungal activity. Although there is no doubt that some PR-5 proteins are potent antifungal proteins, other members of this PR family are apparently completely devoid of such activity. The reason for the obvious differences in antimicrobial properties is still poorly understood. According to the results obtained with zeamatin from maize (Roberts and Selitrennikoff 1990) and osmotin from tobacco (Abad et al. 1996), PR-5 proteins exert their antifungal effect through a very fast and dramatic increase in the permeability of the plasma

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Abbreviations: Ban-TLP = banana thaumatin-like protein; Plan-TLP = plantain thaumatin-like protein; PR = pathogenesis related; PR-X proteins = pathogenesis-related proteins of family X; TLP = thaumatin-like protein

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membrane. However, the mechanism by which PR-5 proteins disturb membrane permeability is not fully understood. According to some reports (Vigers et al. 1991) thaumatin-like proteins (TLPs) insert into the lipid bilayer thereby creating transmembrane pores and thus enhancing permeability. By virtue of their amphipathic character, PR-5 proteins are (like thionins) capable of disturbing the organization of the lipid bilayer causing rapid cell lysis.

Thaumatococcus-like proteins are not confined to vegetative tissues but have also been identified in fruits of different dicots. It has been demonstrated, for instance, that cherries, tomatoes and grapes accumulate large quantities of PR-5 proteins during ripening (Wu and Huynh 1994; Fils-Lycaon et al. 1996; Tattersall et al. 1997). Based on this information the hypothesis has been put forward that these fruit-specific PR-5 proteins play, in addition to their antifungal activity, a role in fruit ripening (Tattersall et al. 1997). In this report, we describe the isolation, characterization and cloning of a TLP which accumulates in large quantities in ripening banana (*Musa acuminata*) fruits together with other proteins involved in starch metabolism, senescence and defense (chitinase, β -1,3-glucanase, and lectin; Clendenen and May 1997). A very similar protein was also found in plantains. Since no antifungal or cytotoxic activity towards human cells could be observed, the physiological role of the banana (Ban-TLPs) and plantain (Plan-TLP) TLPs remains to be elucidated. However, the possible promotion of some allergenic responses (Inschlag et al. 1998) and elicitation of an IgE-mediated allergic reaction by TLPs may cause some health problems in sensitive individuals.

Materials and methods

Plant material

Bananas [*Musa* cv. Grande Naine (AAA)] and plantains (*Musa* spp.) were purchased from a local store and kept at 20–25 °C until the pulp was completely softened (i.e., beyond peel color index 7).

Extraction and purification of TLPs from bananas and plantains

Peeled ripe bananas (5 kg) were immersed in 50 mM acetic acid, soaked overnight at 2 °C and homogenized with a mixer in a total volume of 20 L of the same solution. The homogenate was kept at 2 °C for 24 h to allow the foam to separate from the liquid. After removal of the foam, the extract was poured through cheesecloth, adjusted to pH 3.0 with 1 M acetic acid and centrifuged at 9000 g for 15 min. The supernatant was filtered through Whatman 3MM paper and loaded onto a column (10 cm long, 5 cm i.d.; 100 mL bed volume) of S Fast Flow (Pharmacia) equilibrated with 20 mM acetic acid. The column was washed with 1 L of formate buffer (20 mM Na-formate, pH 3.8) and the bound proteins eluted in a single step with 200 mL of 1 M NaCl in formate buffer. The lectin was removed from the protein fraction desorbed from the column by affinity chromatography on immobilized mannose (Peumans et al. 2000b). The thaumatin-like protein was isolated from the lectin-depleted protein fraction by conventional protein purification techniques. The flow-through fraction and the first 50 mL of the wash fraction of the affinity chromatography step were pooled,

dialyzed against formate buffer and applied onto a column (15 cm long, 2.6 cm i.d. 75 mL bed volume) of S Fast Flow equilibrated with formate buffer. Proteins were eluted with a linear gradient of increasing NaCl concentration (500 mL; 0.0 to 0.5 M) in formate. Fractions were collected and kept at 2 °C until the proteins in the main peak were crystallized (after 3–4 d). Crystals were collected by centrifugation (3000 g for 10 min), dissolved in 0.1 M Tris-HCl (pH 8.7), dialyzed against formate buffer and reappplied onto the S Fast Flow column equilibrated with formate buffer. Chromatography was performed as described above and the proteins were allowed to crystallize for a second time. The crystals collected by centrifugation (3000 g for 10 min), were dissolved in 0.1 M Tris-HCl (pH 8.7) and dialyzed against appropriate buffers. Analysis by SDS-PAGE of this final protein preparation yielded a single polypeptide band of about 20 kDa, indicating that it was essentially pure. The total yield was about 50 mg of pure crystalline protein per kg of peeled bananas.

The TLP from plantains was isolated following the same procedure but, in this case, the total yield amounted to 250 mg pure crystalline protein per kg of peeled plantains.

Analytical methods

Proteins were analyzed by SDS-PAGE using 12.5–25% (w/v) acrylamide gradient gels as described by Laemmli (1970).

For N-terminal amino acid sequencing the proteins were separated by SDS-PAGE and electroblotted onto a polyvinylidene fluoride membrane. Polypeptides were excised from the blots and sequenced on an Applied Biosystems model 477A protein sequencer interfaced with an Applied Biosystems model 120A (PE Biosystems, Foster City, Calif., USA) on-line analyzer.

Total neutral sugar was determined by the phenol/H₂SO₄ method (Dubois et al. 1956), with D-glucose as standard.

The endo- β -1,3-glucanase activity of the Ban-TLP was assayed using the azurine-crosslinked 1,3- β -glucan substrate (AZCL-Pachyman tablets, Megazyme, Wicklow, Ireland) as substrate. Typically, a tablet of substrate was mixed with 1 mL of 50 mM sodium acetate buffer, 100 mM NaCl (pH 5.0) or 100 mM Hepes, 150 mM NaCl (pH 7.4) containing 100 μ g of Ban-TLP and the reaction stopped after 1, 2 or 3 d of incubation at 30 °C by addition of 10 ml of 2% (w/v) of Trizma base (Sigma). After stirring, the solutions were filtered through a Whatman No. 1 filter paper and their absorbance measured at 590 nm against a substrate blank devoid of Ban-TLP and similarly incubated at 30 °C for 1, 2 or 3 d. Results are expressed as the increase in absorbance at 590 nm.

The endo- β -1,3-glucanase activity of the Ban-TLP was also assayed using carboxy-methylated Pachyman (CM-Pachyman; Megazyme, Wicklow, Ireland) as a substrate. The Ban-TLP (50 μ g) was incubated at 37 °C for 3 d in 0.3 mL of 0.5% (w/v) CM-Pachyman in buffers at different pH values containing 0.05% (w/v) sodium azide. For pH 4.0 and 5.0, buffers containing 50 mM sodium acetate, 100 mM NaCl were used whereas 20 mM potassium phosphate, 100 mM NaCl buffers were used at pH 6.0 and 7.0. The enzymatic activity of the samples incubated at different pH values was measured against substrate blanks (devoid of Ban-TLP and similarly incubated) by adding 50 μ L of samples to 4.0 mL of 0.1% (w/v) of tetrazolium blue in 50 mM NaOH, 0.5 M sodium potassium tartrate and heating in a boiling-water bath for 3 min (Jue and Lipke 1985). After cooling for 3 min in running water, the quantity of liberated D-glucose equivalents was estimated by measuring the absorbance at 660 nm. Measurements were performed in triplicate. Results are expressed as nkat mg⁻¹ of protein (1 kat corresponds to the formation of 1 mol of D-glucose per second).

Sequencing of cDNA clones

The cDNA clone pBAN 3-28 encoding the Ban-TLP was isolated from a banana pulp cDNA library by differential screening

(Clendennen and May 1997). The complete sequence of the cDNA clone was determined and the open reading frame translated. The deduced amino acid sequence was analyzed using programmes from PC Gene (Intelligenetics, Mountain View, Calif., USA) and Genepro (Riverside Scientific, Seattle, Wash., USA).

Treatment of banana plants with methyl jasmonate

Young banana plants, each containing four leaves, were treated with methyl jasmonate for 3 d. Therefore plants were transferred to a closed container and 20 μ L of a 1% (w/v) solution of methyl jasmonate in ethanol was added daily. Controls were without methyl jasmonate. Samples of root tips and leaf material were collected and analyzed for induction of thaumatin transcripts using northern blot analysis of total RNA isolated from the plant tissue as described previously (Eggermont et al. 1996).

Antifungal activity

In the wells of a 96-well microplate, 20- μ L samples of a 2-fold dilution series of the test protein were mixed with 80 μ L of potato dextrose broth (12 gL⁻¹; Difco, Detroit, Mich., USA) containing 2×10^4 fungal spores mL⁻¹, with or without addition of extra salts (1 mM CaCl₂ and 50 mM KCl final concentration). *Neurospora crassa* strain FGSC 2489 (Fungal Genetics Stock Center, University of Kansas, Kansas, USA) and *Fusarium culmorum* strain IMI 180420 (BCCM/MUCL Agro-Industrial Fungi and Yeasts Collection, Université Catholique de Louvain-la-Neuve, Louvain-la-Neuve, Belgium) were used as test fungi. The plate was incubated at 25 °C in the dark and fungal growth was monitored by microspectrometry after 48 h. The IC₅₀ value (i.e. the concentration of the antifungal protein required to inhibit 50% of the fungal growth) was calculated as described by Cammue et al. (1992). The concentration of the test protein was calculated according to Waddell's method (Wolf 1983).

The activity of Ban-TLP on the growth of *Aspergillus niger*, *Botrytis cinerea*, *Candida albicans*, *Fusarium gramineum*, *Penicillium commune*, *Phytophthora infestans*, *Trichoderma viride* and *Verticillium albo-atrum* cultivated on potato dextrose agar (PDA) growth medium in Petri dishes was also studied. Hyphal growth inhibition was measured by placing sterile filter paper disks containing HBS (10 mM Hepes, 150 mM NaCl, pH 7.4) buffer (control) and Ban-TLP (50, 100 or 150 μ g in HBS) on the growth medium adjacent to the colony margin and allowing the fungus to grow for several days at room temperature. The absence of growth over the disks and/or the occurrence of an inhibition zone surrounding the disks were used as an assessment of growth inhibition.

Molecular modelling

The amino acid sequence alignments were carried on a MicroVAX 3100 (Digital, Evry, France) using the ialign program of PIR/NBRF (Nat'l Biomedical Research Foundation, Washington, DC, USA). The program SeqVu (J. Gardner, 1995, The Garvan Institute of Medical Research, Sydney, Australia) was used to compare the amino acid sequences of the Ban-TLP with thaumatin and PR-5 proteins. MacClade (Maddison and Maddison 1992) was run on a Macintosh 5400/180 to build a parsimony phylogenetic tree relating the Ban-TLP to other PR-5 proteins. Multiple amino acid sequence alignments based on Clustal W (Thompson et al. 1994) were carried out using SeqPup (D.G. Gilbert, Biology Dept., Indiana University, Bloomington, Ind., USA) and modified manually according to the Hydrophobic Cluster Analysis (HCA) results to build the phylogenetic tree.

The HCA (Gaboriaud et al. 1987; Lemesle-Varloot et al. 1990) was performed to delineate the structurally conserved regions along

the amino acid sequences of Ban-TLP and thaumatin used as a model. The HCA plots were generated using the programme HCA-Plot2 (Doriane, Paris, France).

Molecular modelling of the Ban-TLP was performed on a Silicon Graphics O2 R10000 workstation, using the programmes InsightII, Homology and Discover (Molecular Simulations, San Diego, Calif., USA). The atomic coordinates of thaumatin (code 1THV) were taken from the RCSB Protein Data Bank and used to build the three-dimensional model of the Ban-TLP. Steric conflicts resulting from the replacement or the deletion of some residues in Ban-TLP were corrected during the model-building procedure using the rotamer library (Ponder and Richards 1987) and the search algorithm implemented in the Homology programme (Mas et al. 1992) to maintain proper side-chain orientation. Energy minimization and relaxation of the loop regions were carried out by several cycles of steepest descent and conjugate gradient using the cvff forcefield of Discover. PROCHECK (Laskowski et al. 1993) was used to check the stereo-chemical assessment of the three-dimensional model. The program TurboFrodo (Bio-Graphics, Marseille, France) was run on an O2 workstation to draw the Ramachandran plots and perform the superimposition of the models. Cartoons were rendered using Molscrip (Kraulis 1991).

Electrostatic potentials were calculated and displayed with GRASP (Nicholls et al. 1991). The solvent probe radius used for molecular surfaces was 0.14 nm and a standard 0.2-nm Stern layer was used to exclude ions from the molecular surface (Gilson and Honing 1987). The inner and outer dielectric constants applied to the protein and the solvent were, respectively, fixed at 4.0 and 80.0 and the calculations were performed keeping a salt concentration of 0.145 M NaCl. No even distribution of the net negative charge of the carboxylic group of negatively charged residues was performed between their two oxygen atoms prior to the calculations. The surfaces occupied by hydrophobic (Leu, Ile, Val, Met, Phe, Trp, Tyr included) and positively charged (Arg, His, Lys) residues on the solvent-accessible surfaces of the modelled proteins were calculated using the GRASP facilities.

Results

Purification and partial characterization of the Ban-TLP

Previous work on the isolation and characterization of a lectin from ripe banana fruits (Peumans et al. 2000b), revealed the occurrence of an abundant fruit protein. This protein was purified to homogeneity using a combination of affinity chromatography (to remove the lectin) and conventional protein purification techniques including crystallization. According to the overall yield of about 50 mg pure crystalline protein per kg of peeled bananas, Ban-TLP is an abundant fruit protein. A very similar protein, plantain thaumatin-like protein, (Plan-TLP), was also isolated from ripe plantains which, according to the overall yield of 250 mg pure crystalline protein per kg of plantains is even more abundant than Ban-TLP. The molecular structure of Ban-TLP and Plan-TLP was determined by SDS-PAGE and gel filtration. Both the reduced and unreduced TLPs yielded a single polypeptide band of about 20 kDa (Fig. 1). According to Fig. 1, the Ban-TLP polypeptide is slightly larger than that of Plan-TLP. Native Ban-TLP and Plan-TLP eluted with an apparent molecular mass of 20 kDa, indicating that both proteins occur as monomers. No covalently bound carbohydrate could be detected on the purified proteins. N-Terminal sequencing

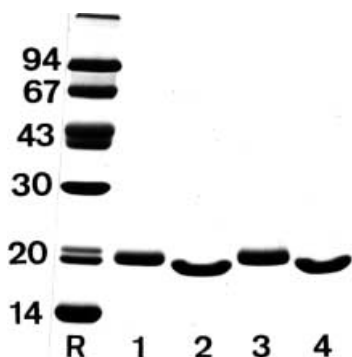


Fig. 1. SDS-PAGE of purified TLPs from banana and plantain fruits. Samples were loaded as follows: *lanes 1 and 3*, 25 µg Ban-TLP; *lanes 2 and 4*, 25 µg Plan-TLP. Samples 1 and 2 were reduced with 2-mercaptoethanol; samples 3 and 4 were unreduced. Molecular mass reference proteins (*lane R*) were lysozyme (14 kDa), soybean trypsin inhibitor (20 kDa), carbonic anhydrase (30 kDa), ovalbumin (43 kDa), bovine serum albumin (67 kDa) and phosphorylase b (94 kDa)

of both purified Ban-TLP and Plan-TLP yielded the amino acid sequence ATFEI VNRXS YTVWA AAVPG, which shows sequence similarity to thaumatin and TLPs (Fig. 2). Based on this sequence similarity the banana protein was tentatively identified as a TLP.

Molecular cloning of Ban-TLP

A study of differential gene expression during banana fruit ripening (Clendennen and May 1997) previously

revealed cDNA clones with sequence similarity to katemfe (*Thaumatococcus daniellii*) fruit thaumatin. The sequence of cDNA clone pBAN 3-28 was completed to determine the corresponding amino acid sequence. Sequence analysis of pBAN 3-28 revealed an open reading frame encoding a 233-amino-acid precursor of 23 873 Da (Fig. 2) in which the sequence ²⁴A-P⁴² almost perfectly matches the N-terminal sequence of mature Ban-TLP. Cleavage of a signal peptide between residues 23 and 24 of the precursor is in agreement with the rules for protein processing of Von Heijne (1986) and yields a mature protein of 21 233 Da, the size of which is in good agreement with the molecular mass estimated by SDS-PAGE. The calculated isoelectric point for this protein is 6.0. The sequence does not contain any putative N-glycosylation site.

Molecular modelling of Ban-TLP

Because of the high percentages of identity (69.7%) and homology (82.8%) between the amino acid sequences of thaumatin and Ban-TLP, the HCA plots of both proteins look very similar. Accordingly, an accurate delineation can be made of the secondary structural features (α -helices and strands of β -sheet) of Ban-TLP. The main difference between the sequences of Ban-TLP and thaumatin consists of a deletion of six amino acid residues located at the N-terminal end of Ban-TLP. The three-dimensional model of Ban-TLP built from the X-ray coordinates of thaumatin looks very similar to that of the template protein (Fig. 3). Like thaumatin (Ogata

A:	SSILSLSFFFFFSSLLVTLSHA	<u>ATFDIVNRCSYTVWAAAVPG</u>	-----	20
B:	MAATTCFFFLFPFLLLLTLSRA	ATFEIVNRCSYTVWAAASKGDAALDA		26
A:	GGRQLNQGSWTINVNAGTTGGRIWGRGTGCSFDGSGRGRCQTGDCGGVLSC			71
B:	GGRQLNSGESWTINVEPGTKGGKIWARTDCYFDDSGRGICRTGDCGGLLQC			77
A:	TAYGNPPNTLAEFALNQFNLDFFDISLVDGFNVPMDFSPTSGGCRGIRCA			122
B:	KRFGRPPTTLAEFSLNQYKG-DYIDISNIKGFNVPMDFSPTTRGCRGVRCA			127
A:	ADINGQCPGALKAPGG-CNNPCTVFKTDQYCCNSGSCSPTDYSQFFKRNC			172
B:	ADIVGQCPAKLKAPGGGCDNACTVFQTSEYCCTTGKCGPTEYSRFFKRLCP			178
A:	DAYSYPKDDQTTTFTCGGGTNYRVVFCP			200
B:	DAFSYVLDKP-TTVTCPGSSNYRVTFPCPTALELEDE			213

Fig. 2. Sequence comparison of the deduced amino acid sequence of Ban-TLP (A) and thaumatin from *Thaumatococcus daniellii* (B). The amino acid stretch corresponding to the N-terminal sequence of Ban-TLP is underlined

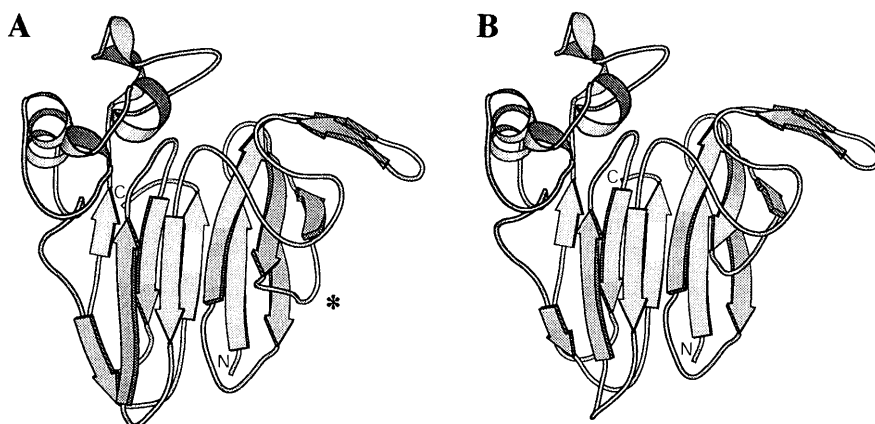


Fig. 3A,B. Comparison of the three-dimensional conformations of thaumatin (A) and the modelled Ban-TLP (B). The strands of β -sheet and stretches of α -helix are represented by arrows and ribbons, respectively. The N- and C-terminal ends of the polypeptide chains are indicated. The asterisk (*) indicates the exposed loop of thaumatin corresponding to a putative sweet-taste epitope which is absent from Ban-TLP. Cartoons rendered with Molscript (Kraulis 1991)

et al. 1992), the modelled protein consists of three distinct domains. The core domain (I), made of two bundles of antiparallel strands of β -sheets interconnected by loops to form a flattened β -sandwich, corresponds to the N-terminal part of the molecule. The second domain (II), which corresponds to the C-terminal part of the polypeptide, consists of a main α -helix associated to three shorter helical segments. The third domain (III) is made of two β -strands linked by a short loop associated with an extended loop. Domains I and II create a central cleft which mainly consists of hydrophilic residues. The eight disulfide bridges (C^9-C^{204} , $C^{56}-C^{66}$, $C^{71}-C^{77}$, $C^{121}-C^{193}$, $C^{126}-C^{177}$, $C^{134}-C^{145}$, $C^{148}-C^{158}$, $C^{159}-C^{164}$) which contribute to the stability of the thaumatin folding are strictly conserved in Ban-TLP (C^9-C^{199} , $C^{50}-C^{60}$, $C^{65}-C^{71}$, $C^{116}-C^{188}$, $C^{121}-C^{171}$, $C^{129}-C^{139}$, $C^{143}-C^{152}$, $C^{153}-C^{158}$). As an assessment for the validity of the three-dimensional model of Ban-TLP, all the modelled loops were shown to fall into the allowed regions of the Ramachandran plot and 73.2% of the residues occur in the core region of the plot (vs. 82% for thaumatin 1THV). A very similar three-dimensional fold was reported for zeamatin (Batalia et al. 1996) and PR-5d (Koiwa et al. 1999), two thaumatin-like PR-5 proteins from maize and tobacco, respectively.

Using the PEPSCAN-analysis technique, two stretches of amino acid residues $^{19}\text{KGDAALDAGGR}^{29}$ and $^{77}\text{CKRFGRRPP}^{84}$ corresponding to extended extruding loops were identified as possible epitope regions on the surface of thaumatin (Slootstra et al. 1995). The first stretch contains a sequence very similar to the peptide-sweeteners DAA (Asp-Ala-Ala) and DAG

(Asp-Ala-Gly) methyl esters, and both stretches create an aspartame-like site resulting from the confluence of D^{21} and F^{80} residues. Non-sweet thaumatin-like PR-5 proteins from tobacco and soybean lack the first stretch of amino acid residues due to a deletion of five residues (DAALD), whereas most of the amino acid residues forming the second exposed stretch are modified. A similar situation occurs in Ban-TLP: the first stretch exhibits a deletion of six residues ($^{19}\text{PG}.....\text{GGR}^{23}$ instead of $^{19}\text{KGDAALDAGGR}^{29}$) and the second stretch possesses a very different amino acid sequence ($^{71}\text{CTAYGNPP}^{78}$ instead of $^{77}\text{CKRFGRRPP}^{84}$). Due to these changes, neither a site for the peptide-sweeteners DAA and DAG methyl esters, nor an aspartame-like site DF can be formed on the surface of Ban-TLP which is accordingly devoid of any sweet taste. In this respect, no sweet taste was found by all the volunteers who did tasting trials with Ban-TLP whereas they constantly recognized the intense sweet taste of thaumatin under the same conditions.

Although both Ban-TLP and thaumatin exhibit very similar molecular surfaces with a deep cleft corresponding to the front face of the β -sandwich core domain I, Ban-TLP definitely differs from thaumatin when the electrostatic potentials are calculated and displayed on the molecular or solvent-exposed surfaces (Fig. 4). In thaumatin, the cleft contains only two conserved acidic residues (E^{89} , D^{101}) whereas the cleft of Ban-TLP contains four conserved residues (e.g. E^{83} , D^{96} , D^{101} and D^{181}). As a result, Ban-TLP exhibits a much more acidic cleft than thaumatin. In Ban-TLP, the other

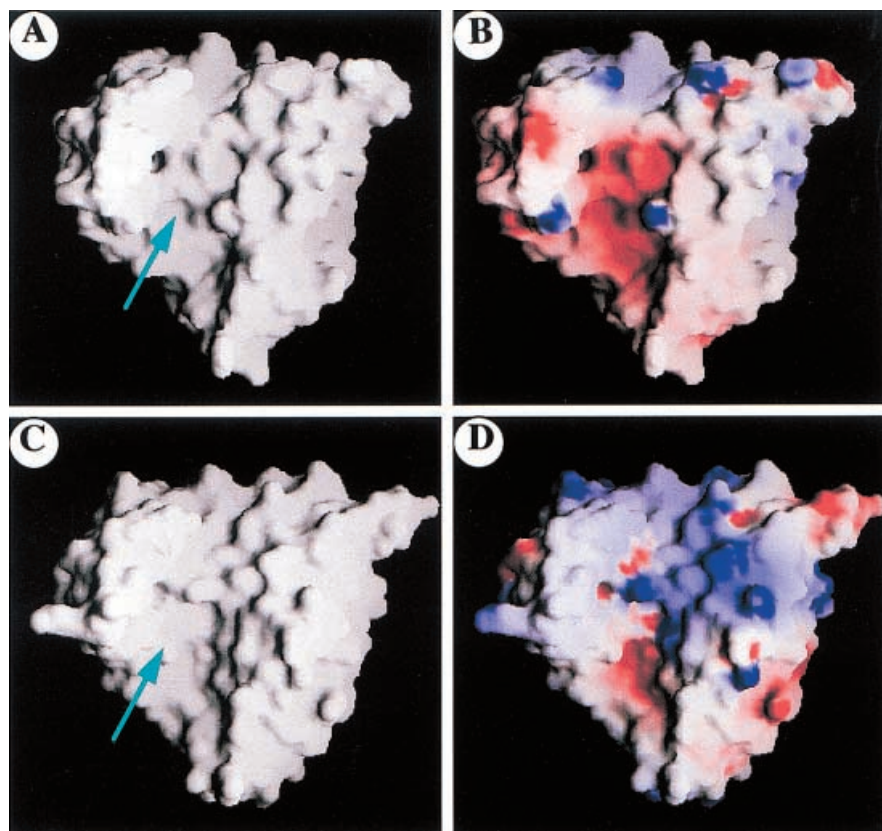


Fig. 4A–D. Representation of the molecular surfaces (A, C) and electrostatic surface potentials (B, D) of the front face of Ban-TLP (A, B) and thaumatin (C, D) by GRASP (Nicholls et al. 1991). The negative potential was colored red and displayed at -5 kT level, the positive potential was colored blue and displayed at $+5\text{ kT}$ level ($1\text{ kT} = 0.6\text{ kcal}$). Neutral surfaces are white. Arrows indicate the position of the central cleft

negatively (Asp and Glu) or positively (Arg, His and Lys) charged residues are randomly distributed on the surface of the back face of the molecule, whereas in thaumatin a semi-circle of a few positive residues neighbouring the cleft straddles the two faces of the molecule (Sloosstra et al. 1995). In addition, a few hydrophobic aromatic residues (e.g. F⁸⁴, F⁸⁹, F⁹⁴, F¹⁸⁶) occur in the vicinity of the negatively charged cleft of Ban-TLP. A very similar distribution of negatively charged residues in the central cleft has already been found in two other thaumatin-like PR-5 proteins, zeamatin (Batalia et al. 1996) and PR-5d from tobacco (Koiwa et al. 1999).

Relationships of Ban-TLP with other TLPs

Comparison of the amino acid sequences of Ban-TLP with thaumatin and other TLPs shows striking similarities (Fig. 5). The phylogenetic tree built from the amino acid sequences of thaumatin and TLPs consists of three main branches (Fig. 6). A first branch clusters the TLPs of the family Gramineae whereas the second branch groups most of the dicot TLPs as well as three monocot proteins, namely thaumatin (Marantaceae), Ban-TLP (Musaceae) and zeamatin (Gramineae). The TLPs E2 and E22 from tobacco form a separate small cluster. It should also be noted here that the tobacco proteins E2 and E22 are rather distantly located from the other TLPs (P23, M1M, NP24 and osmotin) from the same species. Some fruit- and flower-related TLPs (Prunus-TLP from *Prunus avium*, ARATH-TLP from *Arabidopsis thaliana*, Brassica-TLP from *Brassica campestris* and PsTL1, the stylar PR5-like protein from Japanese pear) are clustered into a third branch together with other TLPs such as the TLP-kinase receptor from *A. thaliana* (PR5K). A very similar classification of TLP was previously reported by Sassa and Hirano (1998).

Induction of Ban-TLP with methyl jasmonate

Young banana plants were treated with methyl jasmonate for 3 d and tissue samples of leaves and roots tips

were analyzed for the accumulation of mRNA encoding Ban-TLP. Northern blot analysis of total RNA revealed a clear increase in mRNA transcripts encoding Ban-TLP in roots tips but not in leaves after treatment with methyl jasmonate (Fig. 7).

Biological activity of Ban-TLP

The banana thaumatin-like protein was tested for antifungal activity in vitro. No antifungal effect could be observed towards *Neurospora crassa* and *Fusarium culmorum* even when the protein was tested at a concentration of 250 µg mL⁻¹. Similarly, no inhibition could be observed on the growth of *Aspergillus niger*, *Botrytis cinerea*, *Candida albicans*, *Fusarium gramineum*, *Penicillium commune*, *Phytophthora infestans*, *Trichoderma viride* and *Verticillium albo-atrum*, when Ban-TLP was tested at concentrations of 50, 100 and 150 µg mL⁻¹. Toxicity tests with human cells indicated that Ban-TLP exerts no cytotoxic effects on murine Leukemia cells (L1210), murine breast carcinoma cells (FM3A) and human CEM and Molt cells at concentrations of up to 1 mg mL⁻¹.

Enzyme activity of Ban-TLP

The banana thaumatin-like protein exhibited an in-vitro endo-β-1,3-glucanase activity with AZCL-Pachyman as a substrate (Fig. 8A). Though this activity, which is similar at pH 5.0 and pH 7.0, remained very weak, it was clearly detectable after 3 d of incubation at 30 °C. Using CM-pachyman as substrate, a very weak endo-β-1,3-glucanase activity could be measured after 3 d of incubation at four different pH values (4.0, 5.0, 6.0 and 7.0) with a maximum of activity at pH 4.0 (Fig. 8B). Under similar conditions, TLPs from cherry and tomato fruits exhibited a higher endo-β-1,3-glucanase activity (EC 3.2.1.x) whereas thaumatin did not exhibit any enzyme activity (Grenier et al. 1999).

Thaumat	1	-	A	T	F	E	I	V	N	R	C	S	Y	T	V	W	A	A	S	K	G	D	A	A	L	D	A	G	G	R	Q	L	N	S	G	E	S	W	T	I	N	V	E	P	43	
Zeamat	1	-	A	V	F	T	V	N	Q	C	P	E	T	V	W	A	A	S	V	P	V	-	-	-	-	-	-	-	G	G	R	Q	L	N	R	G	E	S	W	R	L	T	A	P	A	38
PR-5d	1	S	G	V	F	E	I	V	N	R	C	P	Y	T	V	W	A	A	T	P	V	-	-	-	-	-	-	-	G	G	R	Q	L	N	R	G	S	W	W	F	W	A	P	P	39	
Ban-TLP	1	-	A	T	F	E	I	V	N	R	C	S	Y	T	V	W	A	A	V	P	-	-	-	-	-	-	-	-	G	G	R	Q	L	N	R	G	S	W	T	I	N	V	N	A	37	
Thaumat	44	G	T	N	G	G	K	I	W	A	R	T	D	C	Y	F	D	S	G	S	G	I	C	K	T	G	D	C	G	G	V	L	R	C	K	R	F	G	R	P	P	T	T	L	87	
Zeamat	39	G	T	-	A	A	R	I	W	A	R	T	D	C	Y	F	D	A	S	G	R	G	S	C	R	T	G	D	C	G	G	V	L	Q	C	T	G	Y	G	R	A	P	N	T	L	81
PR-5d	40	G	T	K	M	A	R	I	W	G	R	T	N	C	N	F	D	G	A	G	R	G	C	Q	T	G	D	C	G	G	V	L	E	C	K	G	W	G	K	P	N	T	L	83		
Ban-TLP	38	G	T	T	G	R	I	W	G	R	T	G	C	S	F	D	S	G	R	G	R	C	Q	T	G	D	C	G	G	V	L	S	C	T	A	Y	G	N	P	P	N	T	L	81		
Thaumat	88	A	E	F	S	L	N	Q	Y	G	K	-	D	Y	I	D	S	N	I	K	G	F	N	V	P	M	N	F	S	P	T	T	R	G	-	-	C	-	R	G	V	R	C	126		
Zeamat	82	A	E	Y	A	L	K	Q	F	N	L	D	F	E	D	I	S	L	I	D	G	F	N	V	P	M	S	F	L	P	D	G	G	S	G	-	-	C	S	R	G	P	R	C	123	
PR-5d	84	A	E	Y	A	L	N	Q	F	S	N	L	D	F	W	D	I	S	V	I	D	G	F	N	V	P	M	S	F	G	P	T	K	P	G	P	G	K	-	H	G	I	Q	C	126	
Ban-TLP	82	A	E	F	A	L	N	Q	F	N	L	D	F	E	D	I	S	V	I	D	G	F	N	V	P	M	S	F	S	P	T	S	G	G	-	-	C	-	R	G	I	R	C	121		
Thaumat	127	A	A	D	I	V	G	Q	C	P	A	K	L	K	A	P	G	G	G	C	N	D	A	C	T	V	F	C	T	S	E	Y	C	C	T	T	G	-	-	K	C	G	P	T	167	
Zeamat	124	A	V	D	V	N	A	R	C	P	A	E	L	R	-	Q	D	G	V	C	N	N	A	C	P	V	F	K	K	D	E	Y	C	C	V	G	S	A	N	D	C	H	P	T	166	
PR-5d	127	T	A	N	I	N	G	E	C	P	G	S	L	R	V	P	-	G	G	C	N	N	P	C	T	T	F	G	G	Q	Y	C	C	T	T	G	-	-	P	C	G	P	T	166		
Ban-TLP	122	A	A	D	I	N	G	Q	C	P	A	L	K	A	P	-	G	G	C	N	N	P	C	T	V	F	K	T	D	Q	Y	C	C	N	S	G	-	-	S	C	S	P	T	161		
Thaumat	168	E	Y	S	R	F	E	K	R	L	C	P	D	A	F	S	Y	V	L	D	K	P	T	-	T	V	T	C	P	G	S	-	S	N	Y	R	V	I	F	C	P	T	A	207		
Zeamat	167	E	Y	S	R	F	E	K	R	L	C	P	D	A	F	S	Y	P	K	D	D	A	T	S	T	F	T	C	P	A	G	-	T	N	Y	K	V	V	F	C	P	T	205			
PR-5d	167	E	I	S	R	W	F	K	R	L	C	P	D	A	F	S	Y	P	K	D	D	A	T	S	T	F	T	C	T	S	W	T	-	D	Y	K	V	M	F	C	P	Y	G	208		
Ban-TLP	162	D	Y	S	Q	F	E	K	R	L	C	P	D	A	F	S	Y	P	K	D	D	A	T	S	T	F	T	C	P	G	G	-	T	N	Y	R	V	V	F	C	P	T	200			

Fig. 5. Multiple alignment of the amino acid sequences of thaumatin, zeamatin from *Zea mays*, PR-5d from *Nicotiana tabacum* and Ban-TLP. Identical residues are boxed. Dashes correspond to gaps introduced to maximize the homology

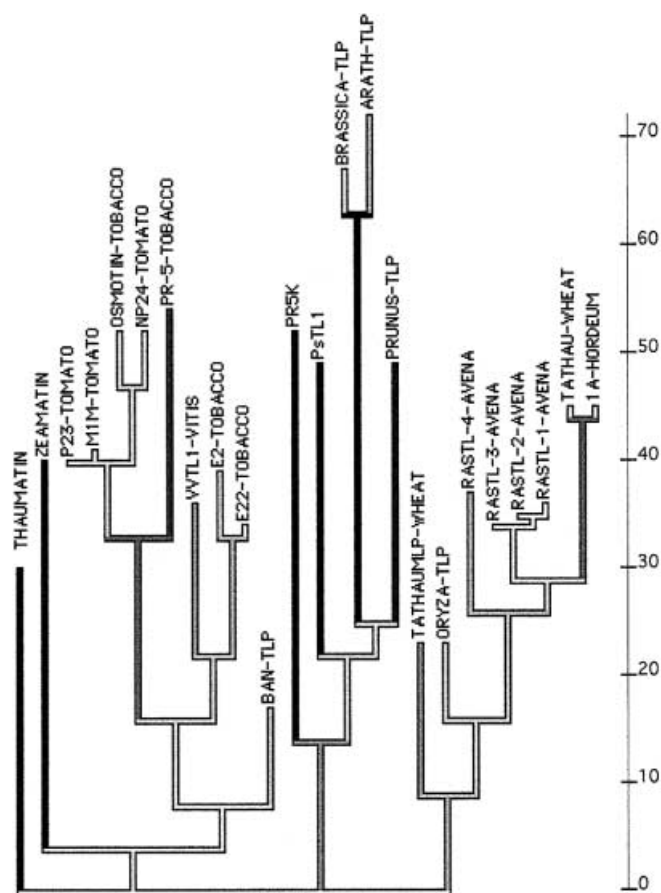


Fig. 6. Phylogenetic tree of thaumatin and TLPs. Sequences used to build the dendrogram include: thaumatin from *Thaumatococcus daniellii* (Genbank accession: P02884) and TLPs from *Arabidopsis thaliana* (ARATH-TLP: L34693; PR5K: U48698), *Avena sativa* (RASTL1-AVENA: L39774; RASTL2-AVENA: L39775; RASTL3-AVENA: L39776; RASTL4-AVENA: L39777), *Brassica campestris* (BRASSICA-TLP: U71244), *Hordeum vulgare* (1A-HORDEUM: S18033), *Lycopersicon esculentum* (P23-TOMATO: X70787; NP24-TOMATO: P12670; M1M-TOMATO: X66416), *Musa acuminata* (BAN-TLP, this work), *Nicotiana tabacum* (E2-TOBACCO: X15223; E22-TOBACCO: X15224; OSMOTIN-TOBACCO: AAB23375; PR-5-TOBACCO: 2981950), *Oryza sativa* (ORYZA-TLP: X68197), *Prunus avium* (PRUNUS-TLP: U32440), *Pyrus serotina* (PsTL1: AB006009), *Triticum aestivum* (TATHAU-WHEAT: X58394; TATHAUMLP-WHEAT: X97687), *Vitis vinifera* (VVTL1-VITIS: AF003007) and *Zea mays* (ZEAMATIN: AAA92882). Branches of the tree are shaded according to the amount of amino acid changes and the scale indicates the number of amino acid changes

Discussion

Ripe bananas and plantains are a rich source of TLPs, which can readily be purified in large quantities by combination of classical protein purification techniques, affinity chromatography and crystallization. Both Ban-TLP and Plan-TLP are monomeric proteins consisting of a single polypeptide of about 20 kDa. In this respect, the banana and plantain proteins closely resemble previously described PR-5 and PR-5-like proteins. Molecular cloning confirmed that Ban-TLP has a high sequence similarity with other TLPs and also allowed us to model the structure of the protein. According to our

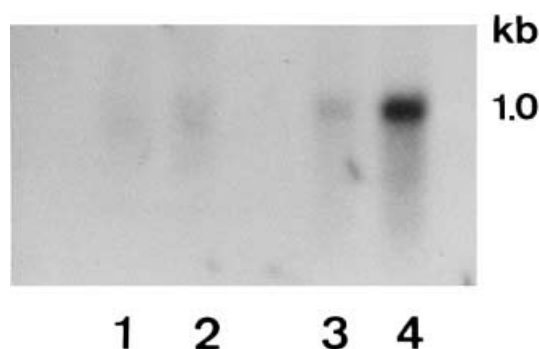


Fig. 7. Northern blot analysis of total RNA isolated from leaves and root tips of young banana plants before (lanes 1 and 3) and after treatment (lanes 2 and 4) with methyl jasmonate. Lanes 1 and 2 show RNA isolated from leaves. Lanes 3 and 4 show RNA isolated from root tips. The blot was hybridized using the labelled cDNA insert pBAN 3-28. Number on the right indicate the RNA size (kb)

modelling Ban-TLP, like other previously studied thaumatin-like PR-5 proteins, exhibits some structural features which are believed to account for the (putative) antifungal activity of some members of this protein family. Based on the extreme rapidity of the toxic effect provoked by, for example, zeamatin on some phytopathogenic fungi it has been proposed that TLPs exert their antifungal effect through a membrane-permeabilization mechanism (Roberts and Selitrennikoff 1990). Although the exact mechanism of the enhancement of permeability of the plasma membrane is still unclear, it has been suggested that the occurrence of polarized negative charges on the surface of PR-5 proteins enables these proteins to interact with positively charged membrane proteins, e.g. ion or water channels, resulting in a disturbance of the water flow across the plasma membrane (Batalia et al. 1996). It should be mentioned, however, that a direct effect of PR-5 proteins on the cell wall can not be excluded because it has been shown that different strains of *Saccharomyces cerevisiae* exhibit pronounced differences in sensitivity to osmotin from tobacco, which are apparently related to the expression of cell wall proteins encoded by a small gene family (Yun et al. 1997). The possible direct effect on the fungal cell wall is further supported by the observation that several TLPs from barley and maize (zeamatin) specifically bind to water-insoluble β -1,3-glucans (Trudel et al. 1998). Molecular modelling clearly indicates that the front face of the core domain of Ban-TLP contains the same highly negatively charged cleft (including strictly conserved Asp and Glu residues) as the corresponding domain of the fungitoxic zeamatin (Batalia et al. 1996) and PR-5d from tobacco (Koiwa et al. 1999). However, in contrast to zeamatin and tobacco PR-5d, Ban-TLP is apparently devoid of antifungal activity. It is questionable, therefore, whether the presence of the conserved negatively charged cleft is sufficient to confer antifungal activity to a TLP.

Although TLPs have already been identified in fruits of several dicot species [e.g. *Diospyros texana* (Wu and Huynh 1994), cherry (Fils-Lycaon et al. 1996), grape (Tattersall et al. 1997) and tomato (Pressey 1997)], no

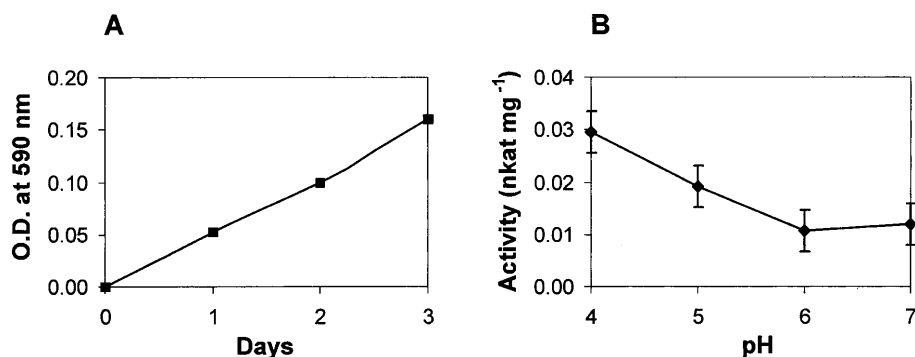


Fig. 8. **A** Endo- β -1,3-glucanase activity of Ban-TLP expressed as the increasing absorbance measured at 590 nm after incubation in the presence of AZCL-Pachyman at 30 °C for 1, 2 and 3 d. **B** Endo- β -1,3-glucanase activity measured at four different pH values with CM-Pachyman as a substrate, expressed in nkat mg⁻¹

evidence has been presented yet for the occurrence of such proteins in fruits of monocots. Therefore, the identification of Ban-TLP and Plan-TLP as major pulp proteins is important because it demonstrates that fruit-associated TLPs are widely distributed, which in turn suggests that these fruit proteins play an important role. At present, the possible physiological role(s) of the fruit-associated TLPs is still poorly understood. Although the thaumatin-like fruit proteins from *Dyospyros texana* (Wu and Huynh 1994), grapevine (Salzman et al. 1998) and tomato (Woloshuk et al. 1991; Rodrigo et al. 1993; Pressey 1997) inhibit the growth of several phytopathogenic fungi, this antifungal activity can not be extrapolated to all fruit-associated homologues because the TLP from cherry (Fils-Lycaon et al. 1996) and Ban-TLP (this work) are apparently devoid of antifungal activity, which makes a general role as an antifungal agent unlikely. Moreover, the timing of accumulation of fruit TLPs also argues against a role in defence against fungi. However, as previously reported for some TLPs, especially from cherry and tomato fruits (Grenier et al. 1999), Ban-TLP exhibited an endo- β -1,3-glucanase activity. Nevertheless, this enzyme activity measured in vitro after an incubation period of 3 d was extremely low since it corresponds approximately to the cleavage of 0.0004 glycosidic bond per protein molecule per minute. Accordingly, it seems unlikely that such an endo- β -1,3-glucanase activity plays a key role in the defence of the plant against pathogenic fungi. Moreover, a slight contamination of the Ban-TLP preparation with a genuine endo- β -1,3-glucanase present in the pulp extract (Peumans et al. 2000a) could possibly account for this very weak activity. It is difficult to explain, indeed, why these proteins are predominantly deposited during the final phase of fruit formation because ripe fruits are destined to perish in order to contribute to the dissemination of the seeds. Finally, the rather high expression level of most fruit-associated TLPs, which in several species (e.g. cherry, grape, banana, plantain) are the most abundant proteins in the fruit pulp, also does not argue for a specific role in defence against fungi.

Though these reflections do not preclude a role as antifungal agents they should stimulate the development of alternative hypotheses for the function of the fruit-associated TLPs. Considering the abundance and developmental regulation of most fruit TLPs, and the observation that hitherto they have been found exclu-

sively in species that depend for the dissemination of their seeds on fruit-eating organisms, it is tempting to speculate that these proteins are somehow beneficial for the spread of the seeds. Possibly, the typical fruit TLPs have a pleasant taste for some fructiferous animals and hence increase the attractiveness of the fruits, which in turn favors the dissemination of the seeds. Alternatively, TLPs may interact with some components of the surface of the digestive tract of the fructiferous animals. As a result, the transit time in the digestive tract is sufficiently reduced to prevent damage to the seeds. Such a mechanism can explain why, for example, birds suffer slight diarrhea when feeding on cherries. At present, no experimental evidence is available either in favor or against the above proposed hypotheses. However, since the procedures described in this paper enable the isolation of gram quantities of crystalline Ban-TLP and Plan-TLP, sufficient protein can be prepared for feeding trials with experimental animals.

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