

Purification, characterization and structural analysis of an abundant β -1,3-glucanase from banana fruit

Willy J. Peumans¹, Annick Barre², Veerle Derycke³, Pierre Rougé², Wenling Zhang¹, Gregory D. May⁴, Jan A. Delcour³, Fred Van Leuven⁵ and Els J. M. Van Damme¹

¹Laboratory for Phytopathology and Plant Protection, Katholieke Universiteit Leuven, Belgium; ²Institut de Pharmacologie et Biologie Structurale, UPR CNRS 9062, Toulouse, France; ³Laboratory of Food Chemistry, Katholieke Universiteit Leuven, Belgium;

⁴Plant Biology Division, The Samuel Roberts Noble Foundation Inc., Ardmore, OK, USA; ⁵Center for Human Genetics, Katholieke Universiteit Leuven, Belgium,

An abundant, catalytically active β -1,3-endoglucanase (EC 3.2.1.39) has been isolated from the pulp of ripe bananas. Biochemical analysis of the purified protein, molecular modelling, and molecular cloning of the corresponding gene indicate that this banana enzyme closely resembles previously characterized plant β -glucanases with respect to its amino-acid sequence, structure and biological activity. The results described in this paper demonstrate both the occurrence of an abundant active β -1,3-endoglucanases in fruits and also readdress the question of the possible involvement of these enzymes in the ripening and/or softening process.

Keywords: banana; β -1, 3-glucanase; *Musa*; PR protein.

Plant β -1,3-endoglucanases (EC 3.2.1.39) are a ubiquitous family of evolutionarily related proteins that are able to catalyze the endo-type hydrolytic cleavage of β -1,3-D-glucosidic linkages in β -1,3-glucans [1]. At present, it is generally believed that β -1,3-endoglucanases fulfill different biological functions. Based on the spatial and temporal regulation of their expression, the role of β -glucanases has been associated with plant reproductive biology and pathogenesis-related defense. Although the major interest in β -glucanases is still focussed on their presumed role in defense mechanisms against fungal attack and the possible exploitation of these properties in plant protection, increasing efforts are being undertaken to unravel nondefense-associated roles in uninfected or uninduced plants. Strong evidence has already been obtained that β -glucanases are involved in diverse processes like cell division, pollen development and germination, fertilization and embryogenesis, seed germination, mobilization of storage polysaccharides in the endosperm of cereal grains, bud dormancy, response to wounding and abiotic stress [1]. In addition, it has also been proposed that β -glucanases play a role in fruit ripening [2]. Although there is convincing evidence that β -1,4-glucanases (EC 3.2.1.4; cellulases) are highly expressed in fruits, such as strawberry (*Fragaria* $\times$ *ananassa*) [3,4] and peach (*Prunus persica*) [5], and play an important role in fruit softening, the possible involvement of β -1,3-glucanases in fruit ripening is still unclear. Moreover, no clear evidence has yet been presented that catalytically active β -1,3-glucanases accumulate in fruits. In this report, we describe the isolation and cloning of

a catalytically active β -1,3-endoglucanase from the pulp of ripe bananas. Our results demonstrate the occurrence of an abundant active β -1,3-endoglucanase in fruits and also readdress the question of the possible involvement of these enzymes in the ripening and/or softening processes.

MATERIALS AND METHODS

Plant material

Ripe bananas (*Musa acuminata* 'Cavendish' cv. Grand nain) were purchased from a local store.

Extraction and purification of banana fruit pulp β -glucanase

Peeled bananas (5 kg) were immersed in a solution of 50 mM acetic acid, incubated overnight at 2 °C and gently homogenized with a mixer in the same solution. The total extraction volume amounted to 4 L per kg fruit. After manual 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 filter paper (Whatmann 3MM) and loaded onto a column (5 $\times$ 10 cm; 100-mL bed volume) of S Fast Flow (a strong cation exchanger from Pharmacia) equilibrated with 20 mM acetic acid. After loading the extract, the column was washed with formate buffer (20 mM sodium formate, pH 3.8) until the A_{280} fell below 0.01. Bound proteins were eluted with 200 mL of 1 M NaCl in formate buffer and passed through a column (2.6 $\times$ 10 cm; 50-mL bed volume) of mannose-Sepharose 4B (using formate buffer containing 0.1 M NaCl as running buffer) to remove the banana fruit lectin. The pass-through fraction and first 50 mL of the wash fraction of the affinity chromatography step were pooled, dialyzed against formate buffer and applied onto a column (2.6 $\times$ 15 cm; 75-mL bed volume) of S Fast Flow equilibrated with formate buffer. Elution of the proteins with a linear gradient of increasing NaCl concentration (500 mL, 0.0–0.5 M)

Correspondence to W. Peumans, Katholieke Universiteit Leuven, Laboratory for Phytopathology and Plant Protection, Willem de Croylaan 42, 3001 Leuven, Belgium. E-mail: Willy.Peumans@agr.kuleuven.ac.be
Abbreviation: HCA, hydrophobic cluster analysis.

Enzymes: β -1,3-glucanases (EC 3.2.1.39); β -1,3:1,4-glucanases (EC 3.2.1.73); β -1,4-glucanases (cellulase) (EC 3.2.1.4).

Note: the nucleotide sequence reported in this paper has been submitted to the GenbankTM/EMBL Data library under the accession number AF001523. (Received 2 November 1999, revised 16 December 1999, accepted 21 December 1999)

in formate buffer yielded two major peaks. Enzymatic assays indicated that all β -glucanase activity eluted in the first peak (around 0.15 M NaCl), which yielded almost exclusively a single polypeptide band of about 30 kDa upon SDS/PAGE. Peak fractions were pooled, diluted with 4 vol. of formate buffer and rechromatographed on the same column. Proteins were eluted with a linear gradient of increasing NaCl concentration (500 mL, 0.0–0.25 M) in formate buffer. Fractions containing β -glucanase activity were pooled, diluted with 4 vol. of formate buffer and applied onto a small column (1.6 $\times$ 5 cm; 10-mL bed volume) of S Fast Flow. Proteins were desorbed in a single step with 10 mL of 0.5 M NaCl in 0.1 M phosphate buffer (pH 7.5), applied onto a column (70 $\times$ 2.6 cm; 350-mL bed volume) of Sephacryl 100 and chromatographed using 0.2 M NaCl in phosphate buffer as running buffer. Virtually all the protein eluted in a single symmetrical peak, which contained all the β -glucanase activity and yielded a single polypeptide of about 30 kDa upon SDS/PAGE. Peak fractions were pooled and used as a source of banana β -glucanase. The total yield was about 40 mg pure β -glucanase with an overall recovery of about 70% of the original enzymatic activity.

Analytical methods

Purified proteins were analyzed by SDS/PAGE using 12.5–25% (w/v) acrylamide gradient gels as described by Laemmli [6]. For N-terminal amino-acid sequencing the proteins were separated by SDS/PAGE and electroblotted on a poly(vinylidene difluoride) membrane. Polypeptides were excised from the blots and sequenced on an Applied Biosystems model 477A protein sequencer interfaced with an Applied Biosystems model 120 A on-line analyzer.

Total neutral sugar was determined by the phenol/H₂SO₄ method [7], with D-glucose as standard.

Sequencing of cDNA clones

The cDNA clone pBAN 1-3 encoding the banana glucanase was isolated from a banana pulp cDNA library by differential screening [8]. The complete sequence of the cDNA clone was determined on an ABI 373A sequencer (Applied Biosystems) and the open reading frame translated. The deduced amino-acid sequence was analyzed using programs from PC Gene (Intelligenetics, Mountain View, CA, USA) and Genepro (Riverside Scientific, Seattle, USA).

Glucanase assay

The β -glucanase activity of the banana extracts and purified enzyme was assayed with an azurine-crosslinked β -1,3-glucan substrate (AZCL-Pachyman tablets from Megazyme, Wicklow, Ireland). To 1-mL aliquots of diluted extracts or purified enzyme solutions prepared in 0.2 M sodium acetate buffer (pH 4.5), an AZCL-Pachyman tablet was added and the mixture incubated for 10 min at 30 °C. The reaction was stopped by the addition of 10 mL of 1% Tris with vigorous stirring. The slurry was left at room temperature for about 5 min, stirred again and filtered through filter paper. Aliquots of the filtrate were transferred to a cuvette and the absorbance measured at 590 nm against a substrate blank.

The β -glucanase was also tested with azurine-crosslinked barley β -1,3:1,4-glucan substrate as well as azurine-crosslinked cellulose (AZCL-barley- β -glucan tablets and AZCL-hydroxyethyl(HE)-cellulose tablets, respectively, from Megazyme,

Wicklow, Ireland). The assay was performed as described by the manufacturer, except that the incubation was performed for several hours in order to make sure that the test was negative.

To check the presence of an endogenous substrate the purified glucanase was assayed with a crude polysaccharide fraction from bananas. One milliliter of purified enzyme (1 mg·mL⁻¹) was mixed with 5 mL of a suspension of the crude polysaccharide fraction. The mixtures were incubated at 30 °C. Samples (0.5 mL) were taken at regular intervals for activity measurements. To measure the activity, the release of ethanol soluble sugars was estimated. Two volumes of ethanol were added to the samples. After standing for 1 h on ice, the precipitate was removed by centrifugation. Aliquots of 0.1 mL of the supernatant were diluted with 0.3 vol. of distilled water and used for the determination of sugars following the phenol/H₂SO₄ acid method [7]. A blank without enzyme was included to check the possible presence of any remaining active glycosidase.

Preparation of a crude polysaccharide fraction from bananas

Five grams of pulp tissue from unripe (i.e. completely green collected 3 months post-anthesis) and ripe (i.e. yellow and soft) bananas were homogenized with mortar and pestle in 50 mL of a 0.1% solution (w/v) of ascorbic acid. Starch granules were allowed to settle and the supernatant transferred to a dialysis bag. Low molecular mass compounds were removed by dialysis against distilled water for 24 h at 2 °C. To inactivate the enzymes present in the crude extract, the dialyzed fraction was adjusted to 50 mM NaOH and incubated for 30 min at room temperature. After this step, the dialysate was adjusted to pH 4.5 with acetic acid and brought to 0.2 M NaCl (using solid salt).

Antifungal activity

In the wells of a 96-well microplate, 20 μ L samples of a twofold dilution series of the test protein were mixed with 80 μ L of potato dextrose broth (12 g·L⁻¹; Difco, Detroit, 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 and *Fusarium culmorum* strain IMI 180420 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 anti-fungal protein required to inhibit 50% of the fungal growth) was calculated as described by Cammue *et al.* [9].

Molecular modelling

The amino-acid sequence alignments were carried out on a Macintosh 5400/180 using the program SEQVU (J. Gardner, The Garvan Institute of Medical Research, Sydney, Australia). Multiple amino-acid sequence alignments based on CLUSTAL W [10] were carried out using SEQPUP (D. G. Gilbert, Biology Department, Indiana University, Bloomington, IN, USA) and modified manually according to the hydrophobic cluster analysis (HCA) data. The HCA [11,12] was performed to delineate the structurally conserved regions along the amino-acid sequences of the banana β -glucanase using barley β -1,3-glucanase as a model. HCA plots were generated using the program HCAPLOT2 (Doriane, Paris, France). Homology modelling of the banana β -glucanase was carried out on a Silicon Graphics O2 R10000 workstation, using the programs

INSIGHT II, HOMOLOGY and DISCOVER (Molecular Simulations, San Diego, CA, USA). The atomic coordinates of barley β -1,3-glucanase (accession number IGHS) were taken from the RCSB Protein Data Bank [13] and used to build the 3D model of the banana β -glucanase. Steric conflicts resulting from the replacement or the deletion of some residues in the banana β -glucanase were corrected during the model-building procedure using the rotamer library [14] and the search algorithm implemented in the HOMOLOGY program [15] to maintain proper side-chain orientation. Energy minimization and relaxation of the loop regions was carried out by several cycles of steepest descent and conjugate gradient using the cvff forcefield of DISCOVER. PROCHECK [16] was used to check the stereochemical assessment of the 3D model. The program TURBOFRDO (Bio-Graphics, Marseille, France) was run on the Indy R4600 workstation to draw the Ramachandran plots and perform the superimposition of the models. Cartoons were rendered using MOLSCRIPT [17]. The molecular surfaces and electrostatic potentials were calculated and displayed with GRASP [18]. The solvent probe radius used for molecular surfaces was 1.4 Å and a standard 2.0 Å-Stern layer was used to exclude ions from the molecular surface [19]. The inner and outer dielectric constants applied to the protein and the solvent were fixed at 4.0 and 80.0, respectively, and the salt concentration was set to 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.

RESULTS

Purification and partial characterization of the β -glucanase from banana fruit

Pulp of ripe bananas contains relatively large quantities of β -glucanase, which (because of the presence of only a few fruit proteins) can readily be isolated. Using the isolation procedure described in the Materials and methods, the overall yield of β -glucanase amounted to about 10 mg·kg⁻¹.

The molecular structure of the β -glucanases was determined by gel filtration and SDS/PAGE. Gel electrophoresis of both the reduced and unreduced banana β -glucanase yielded a single polypeptide band of 30 kDa (Fig. 1) and eluted with an apparent molecular mass of about 30 kDa upon gel filtration suggesting that the native protein occurs as a monomer. No covalently bound carbohydrate could be detected on the purified β -glucanase indicating that the polypeptide is not glycosylated. N-terminal sequencing of the banana polypeptide yielded the single sequence IGVCYGLGNNLPPPSEVVS, which clearly shows sequence similarity to previously described plant β -glucanases. The identity of the presumed β -glucanase was confirmed by its enzymatic activity (see below).

The unreduced protein yielded besides the major 30-kDa polypeptide also a faint band of 60 kDa upon SDS/PAGE (Fig. 1, lane b). N-terminal sequencing yielded the same sequence as the 30-kDa polypeptide indicating that the 60-kDa band corresponds to a dimeric form of the β -glucanase polypeptide.

Molecular cloning of the banana β -glucanase

A study of differential gene expression during banana fruit ripening previously revealed cDNA clones with sequence similarity to soybean β -1,3-glucanase [8]. The sequence of

this cDNA clone pBAN 1-3 was completed and the translated sequence analyzed. Analysis of the open reading frame revealed a precursor sequence of 348 amino acids with one putative initiation codon at position 9 of the deduced amino-acid sequence. Translation starting with this methionine residue results in a protein of 340 amino acids with a calculated molecular mass of 36 320 Da (Fig. 2). A comparison of the deduced amino-acid sequence of pBAN 1-3 and the N-terminal sequence of the purified banana glucanase demonstrates a perfect match between residues I29–S48 of the cDNA and the N-terminus of the mature enzyme. Cleavage of a signal peptide between amino acids 28 and 29 of the deduced amino-acid sequence is in accordance with the rules of von Heijne [20] and yields a protein of 33 451 Da with a calculated isoelectric point of 7.15.

A search in the database revealed that the banana β -1,3-glucan endohydrolase is closely related to other β -glucanases from higher plants (Fig. 2).

Biological activity of β -glucanase from banana

The enzymatic activity of the presumed banana β -glucanase was checked by measuring the release of water soluble fragments from an azurine-crosslinked β -1,3-glucan substrate (AZCL-Pachyman). Using AZCL-Pachyman as a substrate the enzyme had a pH optimum around 4.5 and was heat-stable up to 70 °C (Fig. 3). To check the substrate specificity of the banana β -glucanase, its catalytic activity was compared with AZCL-Pachyman, an azurine-crosslinked barley β -1,3:1,4-glucan substrate (AZCL-barley- β -glucan) and a β 1,4 glucan substrate (AZCL-HE-cellulose). The banana enzyme was about 100-fold less active with AZCL-barley- β -glucan than with AZCL-Pachyman. No activity could be detected with AZCL-HE-cellulose. As the activity with AZCL-barley- β -glucan was clearly detectable, and in addition was pH-dependent (with an optimum near pH 4.5), there is no doubt that the banana β -glucanase can use barley β -1,3:1,4-glucan as a substrate. These results are a little puzzling because other plant

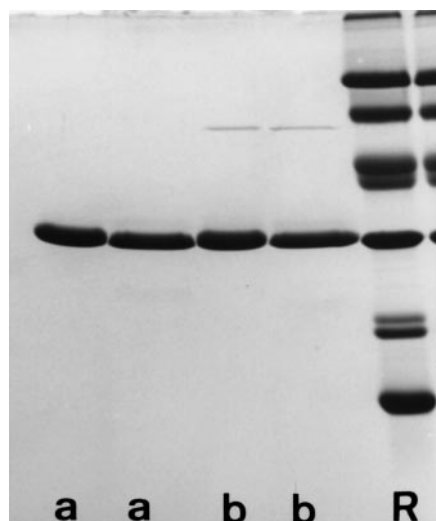


Fig. 1. SDS/PAGE of purified banana β -glucanase. Lanes a and b were loaded with 25 μ g reduced [with 1.25% (v/v) 2-mercaptoethanol] and unreduced β -glucanase, respectively. Molecular mass reference proteins (lane R) were lysozyme (14 kDa), soybean trypsin inhibitor (20 kDa), carbonic anhydrase (30 kDa), ovalbumin (43 kDa), bovine serum albumin (67 kDa) and phosphorylase b (94 kDa).

Hvu	1	- - - - - MARKDVASMFAAAL	F I G A F A A V P T S V Q S	I G V C Y G V I	G N N L P S
Ath	1	- - - - - M L R D A R R Y L A K S	I G R C G S L Q T T K H P A		
Nta	1	- - - - - M S T S S H K H N T P Q M A A I T L L G	L L L V A S S I D I A G A Q S	I G V C Y G M L	G N N L P N
Psa	1	- - - - - M A S F F A R T R R F S L V S L F L	L L E L F T I N L I P T T D A Q I	G I C Y G M M G N N L P P	
Bca	1	- - - - - M L A S S P M L L F L L S L L M A Y N F D T T A G Q	I G V C F G Q M G N N I P N		
Ghi	1	- - - - - M R N A I M H S T M L L L L S G L L L A I F K T T G A E V	G V C Y G M K A N D L P P		
Ppe	1	M T K S N S S S V G R L L S L I S I V L L L G Q L	V V G S L A T K Q H T G A P I	I G V C N G M V G D D L P P	
Mac	1	- - - - - M A T K A S L S I K G F A L	L V S V L V A V P T R V Q S	I G V C Y G M L G N N L P P	
Hvu	43	R S D V V Q L Y R S K G I N G M R I Y	F A D G Q A L S A L R N S G I	I G L I L D I G N D Q L A N I	A A S T S
Ath	27	N - - - - - A L Y G P D P G A L A A L R G S D I	E L I L D V P S S D L E R L A S S Q T		
Nta	50	H W E V I Q L Y K S R N I G R L R L Y	D P N H G A L Q A L K G S N I E V M L	G L P N S D V K H I A S G M E	
Psa	48	A N E V I A L Y K A N N I K R M R L Y	D P N Q P A L N A L R D S G I E L I	L G I P N S D L Q T L A T N Q D	
Bca	41	P S E V V A M F K Q Y S I P R M R M Y	G P N P D A L N A L R G S N I E F I L	D V P N G D L K R L A D S Q A	
Ghi	43	A A E V I D L F K Q K G I K R M R L Y	D P N P D A L Q A L G G T N I E L L	L D L P S A N L E S V A A S Q A	
Ppe	54	Q A E V V A L Y K T N N I P R M R L Y	D P N P A A L E A L R G S N I K L L L	G V P N E N L Q Y I A L S Q A	
Mac	43	P S E V V S L Y K S N N I A R M R L Y	D P N Q A A L Q A L R N S N I Q V L L	D V P R S D V Q S L A S N P S	
Hvu	96	N A A S W V Q N N V R P Y Y P A V N I K Y I A A	G N E V Q G G A - - - - T Q S I L P A M R N L N A A L S		
Ath	65	E A D K W V Q E N V Q S Y R D G V R F R Y I	N V G N E V K P S V - - - - G G F L L Q A M Q N I E N A V S		
Nta	103	H A R W W V Q K N V K D F W P D V K I K Y I A V G N E	L S P V T G T S Y L T S F L T P A M V N I Y K A I G		
Psa	101	S A R Q W V Q R N V L N E Y P S V K I K Y I A V G N E	V S P V G G S S W L A Q Y V L P A T Q N V Y Q A I R		
Bca	94	E A N T W V R D N V Q K Y - N D V R F K Y I	S V G N E V K P G E P G - - A A A L I Q A M Q N I D R A L S		
Ghi	96	N A D Q W V E D N L K K Y - N T V N F R Y I A V G N E	V K P T D S - - F A Q S L F P A M Q N I R T A I V		
Ppe	107	N A N A W V Q N N V R N Y - A N V K F K Y I A V G N E	V K P S D S - - F A Q F L V P A M R N I Q E A I S		
Mac	96	A A G D W I R R N V V A Y W P S V S F R Y I A V G N E	L I P G S D - - L A Q Y I L P A M R N I Y N A L S		
Hvu	144	A A G L G A - I K V S T S - I R F D E V A N S F P P S A	G V F K N A Y - - M T D V A R I L A S T G A P L		
Ath	113	G A G L E - - V K V S T A - I A T D T T T D T S P P S Q	G R F R D E Y K S F L E P V I G F L A S K Q S P L		
Nta	156	E A G L G N N I K V S T S - V D M T L I G N S Y P P S Q	G S F R N D A R W F T D P I V G F L R D T R A P L		
Psa	154	A Q G L H D Q I K V T T A - I D M T L I G N S F P P S K	G S F R S D V R S Y L D P F I G Y L V Y A G A P L		
Bca	143	A A G L S N - I K V S T T T F - M G P S R N T Y P P S R	G R F K D E Y R N F L Q P V I G F L V N K R S P L		
Ghi	145	N A G L G D Q I K V S T A T F F A A I D K S S F P P S K	G S L D P E Y Q K L L G Q V I T F L R D N Q A P L		
Ppe	156	L A G L A K K I K V S T A - I D T G V L G E T F P P S I	G S F K S E Y N A L L Y P I I R F L V S H Q S P L		
Mac	146	S A G L Q N Q I K V S T A - V D T G V L G T S Y P P S A	G A F S S A A Q A Y L S P I V Q F L A S N G A P L		
Hvu	192	L A N V Y P Y F A Y R D N P G S I S L N Y A L F Q P G T	T V R D Q N N G L T Y T S L F D A M V D A V Y A A		
Ath	163	L V N I Y P Y F S Y M G D T A N I H L D Y A L F T A Q S	T V - D N D P G Y S Y Q N L F D A M L D S V Y A A		
Nta	208	L V N I Y P Y F S Y S G N P G Q I S L P Y A L F T A P N	V V - V Q D G S R Q Y R N L F D A M L D S V Y A A		
Psa	206	L V N V Y P Y F S H I G N P R D I S L P Y A L F T S P G	V M - V Q D G P N G Y Q N L F D A M L D S V H A A		
Bca	194	L V N I Y T Y F G Y M N R D V S L Q F A L L Q P N S N	N E F T D P N N Q L R Y L N F F D A M L D S V Y A A		
Ghi	198	L V N T Y P Y F S H I G D P E H V P L D Y A L F T A P S	A V - V Q D G S L Q Y Q Y L F D A M L D T F Y S A		
Ppe	208	L V N L Y P Y F A Y S G N T Q D I R L D Y A L F T A P	S V V - V Q D G N F G Y R N L F D A M L D G V Y A A		
Mac	198	L V N V Y P Y F S Y T G N P G Q I S L P Y A L F T A S	G V V - V Q D G R F S Y Q N L F D A I V D A V F A A		
Hvu	245	L E K A G A P A V K V V V S E S G W P S A G G F A A S A	- G N A R T Y N Q G L I N H V G G - - G T P K K R		
Ath	215	L E K S G G G S L E I V V S E T G W P T E G A V G T S V	- E N A K T Y V N N L I Q H V K N - G S P R R P		
Nta	260	L E R S G G A S V G I V V S E S G W P S A G A F G A T Y	- D N A I T Y L R N L I Q H A K E - - G S P R K P		
Psa	258	L D N T G I G W V N V V V S E S G W P S D G G S A T S Y	- D N A R I Y L D N L I R H V K - - G T P R R P		
Bca	247	L E K S G G S L D V V V S E S G W P T Q G G P G A S V	- P N A E A Y V N N L R L H V N K N - G S P K R -		
Ghi	250	L E K A G G V S V D I V V S E T G W P S D G G Q A T S V	- D N A M T Y N T K L V Q H V N Q G K G T P K K P		
Ppe	260	L E K A G G G S L K V V I S E T G W P S A A G T A T T I	- D N A R T E I S N L I Q H V K E - - G T P R R P		
Mac	250	L E R V G G A N V A V V V S E S G W P S A G G G A E A S T S	- N A R T Y N Q N L I R H V G G - - G T P R R P		
Hvu	295	- E A L E T Y I F A M F N E N Q K T G - - D A T E R S	F G L F N P D - K S P A Y N I Q F		
Ath	265	G K A I E T Y I F A M F D E N K K E P - - - T Y E K	F W G L F H P D - R Q S K Y E V N F N		
Nta	310	- G P I E T Y I F A M F D E N N K N P - - - E L E K H	F G L F S P N - K Q P K Y N I N F G V S G G V W D		
Psa	308	- W A T E A Y I F A M F D E N Q K S P - - - E L E K H	F G V F Y P N - K Q K K Y P F G F G E R R D G E		
Bca	297	Q E A I E T Y I F A M F D E A P R Q T S P N D E Y E K Y	W G M F S P T T B Q L K Y G V K F N		
Ghi	302	E K A I V A Y I F A M F D E N E K E P - - - A Y E K	H W G L F F P K - K Q E K Y S I S F N		
Ppe	310	G R P I E T Y I F A M F D E N R K T P - - - E L E K H	W G L F S P T - K Q P K Y Q I S F N		
Mac	301	G K E I E A Y I F E M F N E N Q K A G - - - G I E	Q N F G L F Y P N - K Q P V Y Q I S F		
Nta	357	S S V E T N A T A S L V S E M			
Psa	355	I V E G D F N G T V S L K S D M			

Fig. 2. Sequence comparison of the deduced amino-acid sequence of pBAN 1–3 from banana (Mac, this work) and sequences encoding β-glucanases from *Hordeum vulgare* (Hvu, Swiss-Prot code P15737), *Arabidopsis thaliana* (Ath, Swiss-Prot code P33157), *Brassica campestris* (Bca, Swiss-Prot code P49236), *Gossypium hirsutum* (Ghi, TrEMBL code P93153), *Nicotiana tabacum* (Nta, Swiss-Prot code P15797), *Pisum sativum* (Psa, Swiss-Prot code Q03467) and *Prunus persicae* (Ppe, Swiss-Prot code P52408). Identical residues are boxed. Dashes correspond to gaps introduced to maximize the homology.

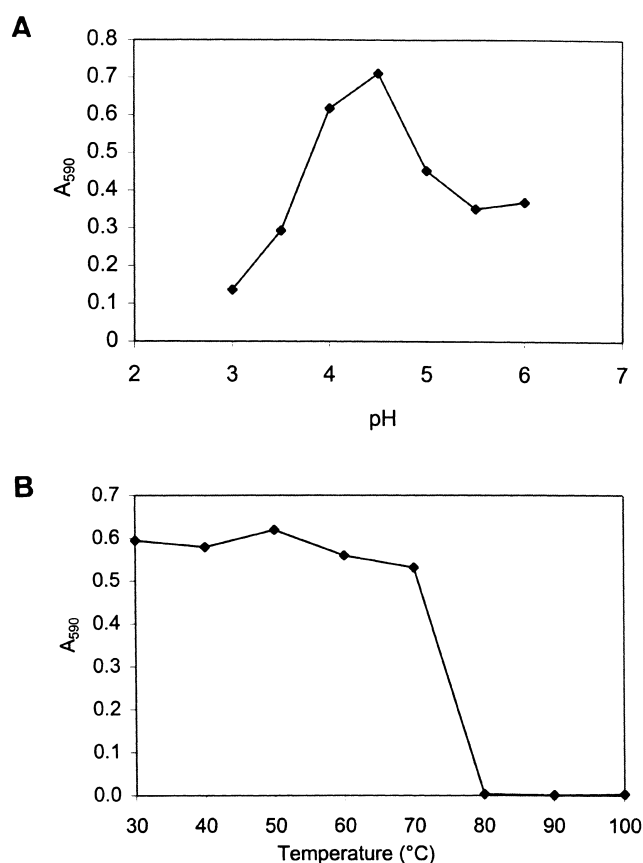


Fig. 3. pH-dependence (A) and thermal stability (B) of the banana β -1,3-endoglucanase. (A) The activity of the enzyme ($50 \mu\text{g}\cdot\text{mL}^{-1}$ final concentration) was tested (using AZCL-Pachyman tablets as a substrate) at different pH values as indicated. Buffers used were sodium acetate in the pH range between 3 and 6, and sodium phosphate in the pH range between 5 and 8. For each pH condition, a substrate blank was included. (B) Aliquots of the enzyme solution ($50 \mu\text{g}\cdot\text{mL}^{-1}$ final concentration in 0.2 M sodium acetate buffer pH 4.5) were heated for 10 min at the temperatures indicated, quenched cold in ice, and assayed as in (A). Activity is expressed as increase in the absorbance at 590 nm.

β -1,3-glucanases cannot use barley β -1,3:1,4-glucan as a substrate (due to the presumed absence of contiguous β -1,3 links, which are normally required for the activity of plant β -1,3-glucanases). Several explanations can be given for the observed activity of the banana enzyme on barley β -1,3:1,4-glucan. First, there may be some contiguous 1,3 links in the AZCL-barley β -glucan used in the experiments, and the banana enzyme has the same substrate specificity as other plant β -1,3 glucanases. Secondly, the banana enzyme may be able, albeit at a low rate, to hydrolyze single β -1,3-links, which are present in AZCL-barley β -glucan. Third, the preparation of the banana β -1,3-glucanase may be contaminated with low levels of a β -1,3:1,4-glucanase.

To check the presence of an endogenous substrate for the glucanase the purified enzyme was added to crude polysaccharide fractions prepared from unripe and ripe bananas. When a polysaccharide fraction from unripe bananas was used as a substrate the purified enzyme generated ethanol-soluble sugars in a time-dependent manner. After incubation for 6 h, approximately $2 \mu\text{g}$ of sugars soluble in 66% ethanol was generated per μg enzyme. No free sugars were formed in the absence of the purified enzyme (demonstrating that the polysaccharide fraction was devoid of glycosidase activity). When a similarly prepared polysaccharide fraction from ripe banana was used as a substrate, no activity could be detected with the purified enzyme. These results indicate that unripe bananas contain a substrate for the β -glucanase that apparently disappears during the ripening process.

As several β -glucanases have been shown to exhibit antifungal activity *in vitro*, the possible growth inhibitory effect of the banana β -glucanase was tested on *Neurospora crassa* and *Fusarium culmorum*. No anti-fungal activity could be observed for these two fungi even when the concentration of the banana β -glucanase was increased up to $0.5 \text{ mg}\cdot\text{mL}^{-1}$.

Molecular modelling of the β -glucanase sequence from banana

Despite a few insertions or deletions, the HCA plots of the barley and banana β -1,3-glucan endohydrolases are very similar (results not shown) suggesting a common 3D fold for both proteins. The 3D model built for the banana β -1,3-glucan endohydrolase resembles that of the barley β -glucanase. It

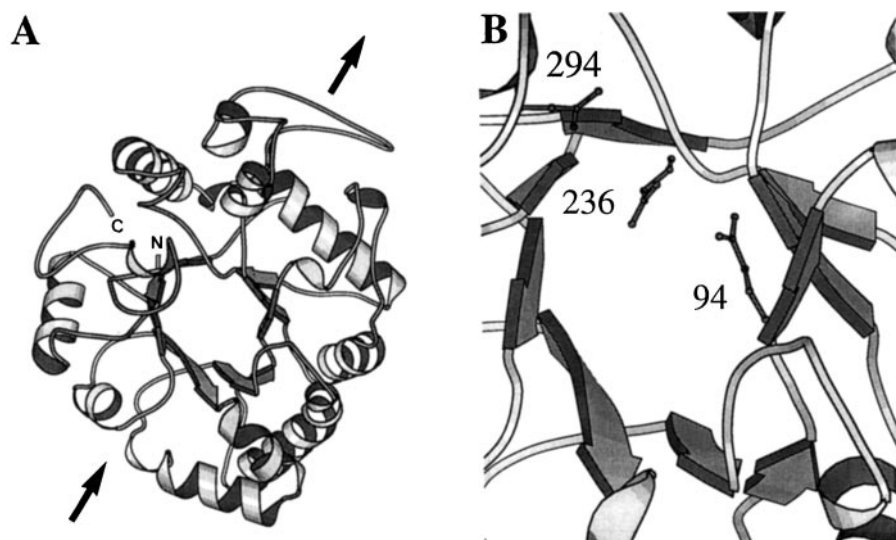


Fig. 4. 3D model of banana β -1,3-glucan endohydrolase. (A) Schematic representation of the 3D model of banana β -1,3-glucan endohydrolase built from the X-ray coordinates of barley β -1,3-glucan endohydrolase. The strands of β -sheet (indicated by gray arrows) form an inner crown surrounded by an outer crown of α -helices to create the $(\beta/\alpha)_8$ TIM-barrel. Arrows indicate the orientation of the catalytic groove located at the surface of the $(\beta/\alpha)_8$ TIM-barrel. (B) Localization of the three conserved glutamate residues Glu94, Glu236 and Glu294 protruding into the catalytic groove located at the surface of the $(\beta/\alpha)_8$ TIM-barrel. Figures were generated with MOLSCRIPT [17].

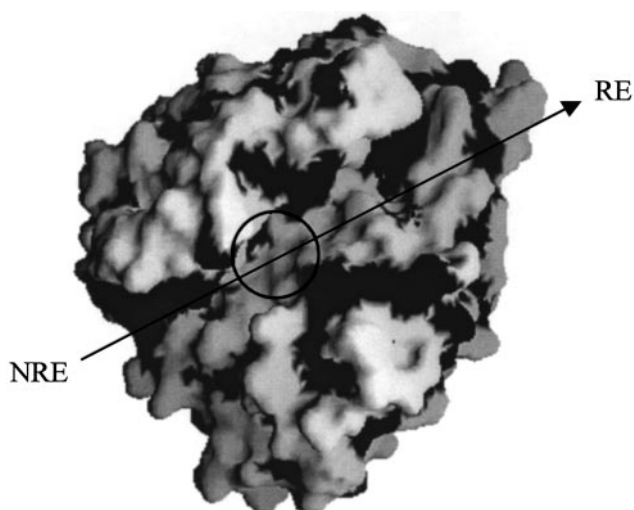


Fig. 5. Molecular surface of the modelled banana β -1,3-glucan endohydrolase showing the surface area occupied by apolar residues in black. The location of glutamate residues Glu94, Glu236 and Glu294 protruding on the edges of the catalytic groove of the $(\beta/\alpha)_8$ TIM-barrel corresponds to the open circle. The model is similarly oriented as in Fig. 4A and arrows indicate the orientation of the catalytic groove. All the calculations and displays were performed with Grasp [18]. The orientation expected for the reducing (RE) and non reducing end (NRE) of the substrate into the groove is indicated by the arrow.

consists of a typical $(\beta/\alpha)_8$ TIM-barrel motif corresponding to a central crown made of eight strands of β -sheet connected by more or less extended loops to eight surrounding α -helices which form an external crown (Fig. 4A). An extended loop located at the C-terminal part of the polypeptide chain protrudes out of the external crown of α -helices.

The substrate-binding site corresponds to a catalytic groove of approximately 35 Å long that occupies the center of the inner crown of β -sheet and lies between the α -helices $\alpha 2$ and $\alpha 3b$ at the N-terminus of the polypeptide chain and between the α -helices $\alpha 5$ and $\alpha 6$ at the C-terminus of the chain. As in barley β -1,3:1,4-glucan endohydrolase [21], one side (helices $\alpha 2$ and $\alpha 6$) of the catalytic groove is covered with apolar residues whereas the other side (helices $\alpha 3b$ and $\alpha 5$), containing the glutamate residues forming the active site, contains predominantly polar residues (Fig. 5). Banana β -1,3-glucan endohydrolase belongs to family 17 following the classification of glucosyl hydrolases [22,23] and to the superfamily of enzymes with eightfold β/α architecture [24]. The active site of these enzymes consists of two glutamate residues acting as proton donor and nucleophile residues, respectively, to cleave β -linked substrates at equatorial bonds yielding products that retain the β -anomeric configuration. According to Jenkins *et al.* [24] and Henrissat *et al.* [25], the proton donor residue occurs just after the C-terminal end of strand $\beta 4$ whereas the nucleophile residue is located on a bulge in strand $\beta 7$. These glutamate residues correspond to Glu94 (proton donor residue) and Glu236 (nucleophile residue) of banana β -1,3-glucan endohydrolase (Fig. 4B). However, there is some uncertainty concerning the exact location of the proton donor residue in β -glucanases and Glu294 (homologous to Glu288 of barley β -glucanase) of banana β -glucanase could also fulfill this function as proposed for barley β -1,3:1,4-glucan endohydrolase (EC 3.2.1.73) [21].

DISCUSSION

The pulp of ripe bananas contains an abundant β -glucanase, that can easily be purified in reasonable quantities. Analysis of the purified protein and molecular cloning of the corresponding gene indicate that the banana β -glucanase closely resembles other plant β -glucanases with respect to its molecular structure and amino-acid sequence. The banana enzyme readily hydrolyzes β -1,3-glucans but has a low activity towards β -1,3:1,4-glucans, suggesting that it is a typical β -1,3-glucan endohydrolase. Like many other plant β -glucanases, the banana enzyme is most active at low pH with an optimum around pH 4.5. The banana β -glucanase also withstands heating up to 70 °C without any loss of activity, which demonstrates that it is a reasonably heat-stable protein.

Molecular modelling indicates that the banana β -1,3-glucan endohydrolase exhibits a 3D fold very similar to that found in β -glucanases belonging to family 17 of glycosyl hydrolases [22,23] and a superfamily of other structurally related glycosidases [24]. This 3D fold displays a $(\beta/\alpha)_8$ TIM-barrel architecture made of an inner crown of β -sheet surrounded by an outer crown of α -helices. The active site of the enzyme consists of a catalytic groove that occupies the center of the $(\beta/\alpha)_8$ barrel and appears as a narrow channel running horizontally along the surface of the flattened barrel.

Three glutamate residues protruding from the surface of the catalytic groove of banana β -1,3-glucan endohydrolase, i.e. Glu94, Glu236 and Glu294 (homologous to Glu94, Glu231 and Glu288 of barley β -1,3-glucan endohydrolase) are most likely to be involved in the retention mechanism of substrate hydrolysis displayed by this enzyme. These three residues form an electro-negative area in the central part of the catalytic groove (Fig. 5). However, the exact location of the two glutamates acting as proton donor residue and nucleophile residue, respectively, is still unclear. As highlighted by Jenkins *et al.* [24], the distances separating O ϵ atoms of Glu94–Glu231 of barley β -1,3-glucan endohydrolase and Glu93–Glu232 of barley β -1,3:1,4-glucan endohydrolase are close to 5.0 Å; this agrees with the role of Glu94/93 as a proton donor in a retention mechanism of substrate hydrolysis [26]. Conversely, the distances > 8.0 Å [13] measured between O ϵ 2 atoms of Glu231–Glu288 and Glu232–Glu288 in both barley β -glucanases are more consistent with an inversion mechanism of substrate hydrolysis similar to that occurring in inverting enzymes like β -amylases [26]. Accordingly, Glu94 and Glu236 could represent the proton donor and nucleophile residues, respectively, of banana β -1,3-glucan endohydrolase, as a distance of 5.59 Å was measured between O ϵ 2 atoms of both residues. The distance of 9.93 Å occurring between O ϵ 2 atoms of Glu236 and Glu294 seems too high to assign Glu294 as the proton donor residue. However, site-directed mutagenesis experiments [27] and molecular dynamics simulations using a model β -glucan as substrate [21] suggest that Glu288 of barley β -1,3:1,4-glucan endohydrolase corresponds to the proton donor residue. Consistent with this, no hydrogen bond occurs between the carboxylate group of Glu288 and any other atom of the protein whereas Glu94 is hydrogen bonded to other residues [13]. A similar situation was observed in banana β -1,3-glucan endohydrolase, where Glu294 (homologous to Glu288 of barley glucanase) is not hydrogen bonded to other residues whereas Glu94 (homologous to Glu94 of barley glucanase) makes a weak hydrogen bond with Asn172.

Our results demonstrate the presence of high concentrations of enzymatically active β -1,3-endoglucanase in a typical fruit tissue. The high concentration of a typical β -1,3-endoglucanase

in ripe banana fruits raises the question of the physiological role of this protein. As it is questionable whether the banana enzyme has anti-fungal activity *in vitro* and, in addition, the pulp tissue is well protected from invading fungi by the peel, a role in defence is rather unlikely. It is tempting to speculate, therefore, that the banana β -1,3-endoglucanase contributes to the degradation of some yet unidentified cell-wall glucans and the subsequent pulp softening. This hypothesis is supported by the fact that unripe bananas contain a substrate for the β -glucanase that apparently disappears during the ripening process. In addition, the observation that the pulp of ripe plantains, which contain about 10 times less β -1,3-endoglucanase than the pulp of bananas remains much firmer during the ripening process is also consistent with this hypothesis (W. J. Peumans, unpublished results).

The identification of an abundant catalytically active β -1,3-endoglucanase in bananas is also important from a practical point of view. It is now possible to isolate by relatively simple methods large quantities of highly purified β -1,3-endoglucanase from a cheap commercial source. Such preparations will be useful for further studies of the catalytic properties of the enzyme and for practical applications in research, development and food industries.

Although the banana β -glucanase is the first abundant fruit specific glucanohydrolase of family 17 to be identified, it is certainly not the first example of an abundant fruit associated pathogenesis-related protein. Evidence has been presented that cherries, tomatoes and grapes accumulate relatively large quantities of PR-5 proteins (thaumatin-like proteins) during ripening [28–30]. The role of these fruit PR-5 proteins is still unclear. It has been proposed that at least some of them have anti-fungal properties. However, a defensive role against phytopathogenic fungi cannot be extrapolated to all fruit-associated PR-5 proteins because the thaumatin-like protein from, for example, cherry [29] is apparently devoid of anti-fungal activity. Interestingly, according to a recent report some thaumatin-like proteins (including the abundant thaumatin-like protein from cherry fruits) exhibit β -glucanase activity [31]. This observation puts the physiological role of thaumatin-like proteins in another perspective and also further emphasizes the importance of glucanases in the process of fruit ripening.

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