JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Molecular Cloning and Characterization of an α-Amylase Occuring in the Pulp of Ripening Bananas and Its Expression in *Pichia pastoris*

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α-Amylases (EC 3.2.1.1) are glycosyl hydrolases with endoglycolytic activity on the α-1,4-D-glucosidic linkages in starch. In bananas, the mobilization of starch accounts for sugar accumulation during ripening, and among several hydrolytic enzymes, α-amylase is the only enzyme argued to be able to attack the intact granules, indicating a pivotal role for this enzyme. A 1953 bp full-length banana α-amylase cDNA (*MAmy*), encoded for a sequence of 416 amino acids, was cloned and used for heterologous expression in *Pichia pastoris*. The cloned *MAmy* presented the highly conserved motifs common to α-amylases, and the amylolytic activity of the extracts from yeast transformed with *MAmy* demonstrated that it encodes for a functional α-amylase, suggesting a putative role for this gene in starch degradation during fruit ripening.

KEYWORDS: ***Musa*** **spp; ethylene; gibberellins; starch metabolism; climacteric fruits; fruit ripening**

INTRODUCTION

The primary function of α-amylase (EC 3.2.1.1; 1,4-α-D-glucan glucanohydrolase) is the breakdown of starch in storage organs in plants. The endoglycolytic activity on the internal α-1,4-D-glucosidic linkages in raw and soluble starch produces dextrin and oligosaccharides, which are subject to further action of exoglucosidases (*1*, *2*). α-Amylases are members of glycoside hydrolase family 13 (GH-H), a classification based on the amino acid sequence similarity (*3*), and their structural and evolutionary relationship to the other glycosyl hydrolyze families has been widely reviewed (*1*, *4*).

The chemical and biochemical changes associated with the starch metabolism are highly coordinated, being related to the expression and suppression of specific genes. There are two current models for starch metabolism, one based on its degradation during cereal seed germination and the other based on the mobilization of the transitory starch stored in leaves during the night. These models have been extensively studied in the last few years, both in relation to the nature of the biochemical regulation and at the gene expression level (*5*, *6*). At the moment, the only clear model concerns the cereal seed germination requiring the induced de novo synthesis of α-amylase, which is secreted by the aleurone cells into the starch endosperm since the elucidation of the starch degrading pathway in leaves and the real contribution of α-amylase has been reappraised (*7*).

In addition to cereal seeds and leaves, fruits are plant organs that can have a significant amount of starch. In climacteric fruits, the mobilization of starch can account for most of the soluble sugars accumulated in the edible pulp, and the whole process usually takes place in a couple of days (*8*). Bananas (*Musa* spp.) are a globally important fruit crop, and the starch amount accumulated during development can be as high as 25% of the fresh weight, depending on the genomic group and the cultivar. These climacteric fruits can take only a few days to reach full maturity, while the stored starch is decreased to trace amounts, suggesting a high capability for starch mobilization (*8*−*10*).

On the basis of the composition of the starch granules, it is likely that the post-harvest mobilization of stored starch is dependent on the concerted action of several enzymes (*11*−*13*). However, α-amylase is the only enzyme that is argued to be able to attack the intact granules (*14*), indicating a pivotal role for this enzyme in the process of starch mobilization during banana ripening.

To obtain more information on the functionality of the α-amylase from a starch degradation model different from the cereal seed and chloroplast, this paper reports the cloning and isolation of a banana α-amylase cDNA from a banana pulp cDNA library. Along with the analysis of its genomic sequence, the clone was heterologously expressed in the methylotrophic yeast *Pichia pastoris*, a widely used eukaryotic model that drives the expression of recombinant protein by the *AOX* promoters (*15*).

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10.1021/jf060805b CCC: $33.50
Published on Web 09/26/2006

MATERIALS AND METHODS

Plant Materials. Preclimacteric bananas (*Musa* spp., AAA, cultivar nanicão) were obtained at the local market, rinsed with sodium hypochlorite solution, treated with 1000 ppm ethylene for 24 h, and kept at a controlled temperature and humidity until ripening was achieved. Fruit at the ripe stage, indicated by the peel color index 6, were used for total RNA extraction. For genomic DNA preparation, young leaves were collected, frozen in liquid nitrogen, and stored at −80 °C.

Extraction of Genomic DNA and Synthesis of Complementary DNA. Genomic DNA was extracted from young leaves as described in Ausubel et al. (*16*) and Clark (*17*) with the addition of PVP-40 1% and 2-ME 1% to the extraction buffers. For the synthesis of cDNA used in the cloning of α-amylase for heterologous expression in *P. pastoris*, total RNA from banana ripe pulp was isolated according to Nascimento et al. (*11*), and 5 μg was reverse transcribed using the SuperScript II reverse transcriptase (Invitrogen) and oligo(dT)$_{18}$ to prime the first strand cDNA.

cDNA Library Construction and Screening. Poly(A)+ RNA isolated from total RNA using a Poly(A) quick mRNA isolation kit (Stratagene) was used to construct a cDNA library in the lambda ZAP vector packaged in Gigapack III Gold packaging extract (Stratagene) according to the manufacturer's instructions. To obtain a full-length α-amylase clone, degenerated sense-1 (5′-GCCGACATYGTSATYAAC-CA-3′) and reverse-1 (5′-GCCGAGTAGCCCYTDGCRAAGTC-3) primers, which were designed based on the conserved calcium binding site and the active site of plant α-amylases deposited on GenBank, were used in PCR screening of a ripe banana pulp cDNA library on a lambda vector as described by Nascimento et al. (*18*). A pBK-CMV phagemid carrying the cloned full-length α-amylase was obtained by in vivo excision of a single clone from the lambda vector.

To obtain the intron sequences, a specific pair of primers upstream (5′-ATGTTTCTGCTTCTGTTTCTTGTCATT-3′) and downstream (5′-TCTCTTCTCCCACACGCAGTAGTCATT-3′) on the ORF of banana α-amylase cDNA was used to PCR amplify the genomic sequence of the banana α-amylase gene.

DNA Sequencing and Analysis of the Sequences. DNA sequencing of PCR products and DNA clones was determined in both orientations using the automated DNA sequencer ALF ExpressII with a Thermo Sequenase Cy5 Dye Terminator kit (Amersham Biosciences) with universal and custom-synthesized primers. Sequence analysis was performed using a ClustalX (*19*) GCG Package available at the Brazilian Bioresources Research Center (BBRC-Embrapa) and facilities of NCBI (*20*) and EXPASY (*21*) servers. The phylogenetic tree was calculated from the alignment of the plant α-amylases protein sequences by the neighbor-joining method with 1000 bootstrap replicates with the ClustalX program, and the phylogram was drawn with TreeView (*22*).

Southern Blot Analysis. For genomic Southern analysis, 10 μg of banana genomic DNA was digested with restriction enzymes (*Hind* III, *Pst* I, or *Sac* I) and separated on 0.8% agarose gel (*17*), and after depurination and transference to the HybondN$^+$ nylon membrane (Amersham Biosciences), the samples were permanently fixed by UV treatment. For probing, the ORF fragment of banana α-amylase cDNA was amplified by PCR, purified from agarose gel, and labeled using a random-primed MegaPrime labeling kit (Amersham Biosciences) with [α^{32}P]-dCTP. The membranes were prehybridized in 20 mL of standard hybridization buffer (5 × SSPE, 5 × Denhardt's solution, 0.5% SDS (w/v), 20 μg/mL salmon sperm DNA) at 65 °C for 1 h, and hybridization was performed at 65 °C overnight. The membranes were washed in 2 × SSPE, 0.1% SDS at room temperature for 15 min, followed by a medium-stringency wash using 1 × SSPE and 0.1% SDS at 65 °C for 15 min. Finally, a high stringency wash was performed in 0.1 × SSPE and 0.1% SDS at 65 °C for 10 min, and the membranes were exposed to radiographic films for visualization of the bands.

Expression of Recombinant Protein in *P. pastoris*. The heterologous expression of the banana α-amylase in the methylotrophic yeast *P. pastoris* host strain GS115 (*his4*), as well as strains and media manipulation, was done according to the instructions of EasySelect *Pichia* expression kit (Invitrogen).

For the cloning in the expression vector, banana α-amylase cDNA was amplified directly from the pulp cDNA using Platinum *Taq* DNA Polymerase HiFi (Invitrogen) and the upstream (5′-GAGAGA**TTC-GAA**ACGATGTTTCTGCTTCTGTTTCT-3′) and downstream (5′-CTCTCTC**TCTAGA**AATTATCTCTTCTCCCACACGC-3′) mutagenic primers to introduce a *Sfu* I (upstream) and *Xba* I (downstream) restriction sites for directional cloning of the ORF fragment. The amplified PCR product was isolated from a 1% agarose gel, double digested with *Sfu* I and *Xba* I, and ligated into a pPICZB vector previously digested with the same enzymes. The resulting plasmid construct, *pPICZB*::*MAmy*, was transformed into *Escherichia coli* TOP10F cells and propagated using standard techniques. Correct plasmid construction was verified by automated DNA sequencing.

The chemically competent *P. pastoris* (*Pichia* EasyComp kit, Invitrogen) was transformed using 3 μg of the *pPICZB*::*MAmy* construction linearized with *Pme* I. Positive transformants were selected by incubation at 30 °C for 4 days on YPDS agar plates containing 100 μg/mL Zeocin, and the clones were tested for the integration of the expression construction and vector by PCR amplification using the 5′ *AOX1* and 3′ *AOX1* primers (Invitrogen). Selected *P. pastoris* clones were inoculated into 30 mL of BMGY medium, and after incubation at 30 °C for 36 h (OD_{600} = 3), they were divided in two 15 mL aliquots, and both were harvested by centrifugation at 2000*g* for 5 min at room temperature, resuspended in 45 mL of either BMGY or BMMY, and incubated at 30 °C for 4 days; BMMY was supplemented with 0.5% methanol every 24 h to maintain induction. After growth, each expression culture was subjected to centrifugation at 3000*g* for 10 min at 4 °C, the cell pellets were resuspended in 4 mL of breaking buffer (20 mM Hepes-NaOH, pH 7.0, 10 mM cysteine, 1 mM $CaCl_2$, 1 mM benzamidine, and 5% glycerol), and cell lysates were obtained by vortexing with 500 mg of acid-washed glass beads followed by centrifugation at 8000*g* for 20 min at 4 °C. Recovered culture media were subjected to new centrifugation at 10 000*g* for 15 min at 4 °C followed by ammonium sulfate precipitation (0−30, 30−70, and 70−100% saturation fraction). Each pellet was resuspended in 2 mL of breaking buffer without glycerol and dialyzed against the same buffer.

Enzyme Functional Activity Assay. Starch-hydrolyzing activity on native discontinuous PAGE was conduced according to Zeeman et al. (*23*). Proteins separated on 10% polyacrylamide resolving gels at a constant voltage of 100 V per gel and at a low temperature (4 °C) were electroblotted (30 V for 45 min) onto gels containing 0.2% soluble starch potato in a cold room. The incubation for development of the amylolitic activity was done at 37 °C, and starch-hydrolyzing activities were revealed by iodine staining. For substrate SDS-PAGE (*24*), samples were separated on a discontinuous SDS-PAGE containing 0.2% of copolymerized starch in the 10% polyacrylamide resolving gel. Runs were carried out at 100 V and 4 °C, and the activity staining on substrate SDS-PAGE was as described for the native PAGE.

RESULTS

The PCR-based strategy for the cDNA library screening allowed the isolation of a full-length banana α-amylase cDNA, which was named *MAmy* (GenBank accession no. AF533648). The 1953 bp full-length cDNA clone presented an open reading frame of 1248 bp (**Figure 1**), encoding for a sequence of 416 amino acids, and 559 bp of nontranslated sequence at the 5′ end and 143 bp of non-translated sequence at the 3′ end. The typical polyadenylation signal AATAAA was not found, although a modified polyadenylation signal AAATAT had been located at position 1884−1889, and a putative pyrimidine box YCTTTTY was present at 57−63 in the complementary (inverted orientation) of the 5′ noncoding region.

Figure 1 summarizes the results on the analysis of the protein encoded by *MAmy*. The protein deduced from *MAmy* ORF was subject to a prediction of subcellular localization using a TargetP 1.1 Server (*25*), and the result suggested a secretory pathway. The sequence presented a putative signal peptide predicted by SignalP 3.0 (*26*) with the most likely cleavage site positioned

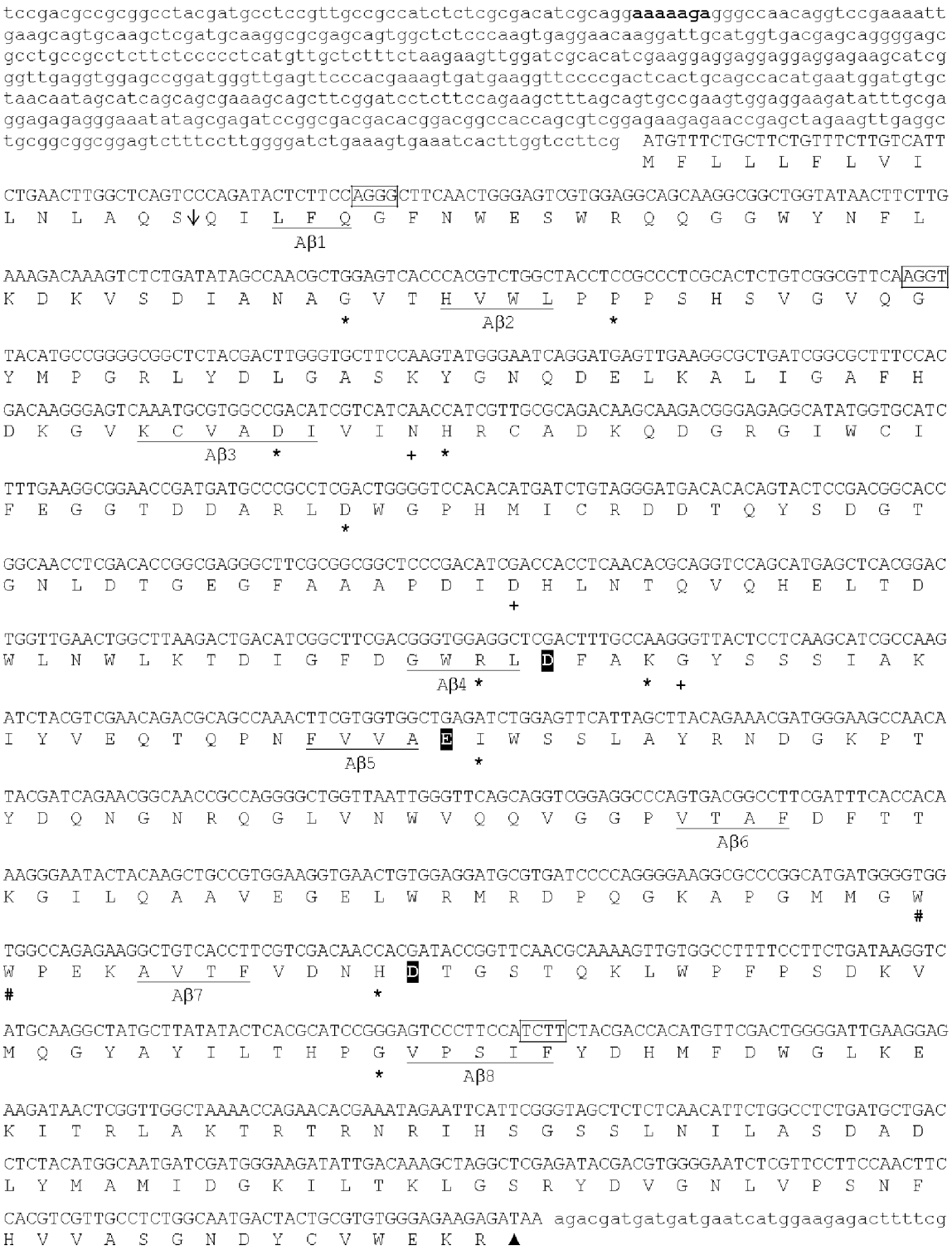

Figure 1. Nucleotide and deduced amino acid sequence of *MAmy* (GenBank accession no. AF533648). The open reading frame is shown in uppercase letters, while the noncoding regions are in lowercase. The predicted protein encoded is given by the one-letter code below the first nucleotide of each codon. The STOP codon is indicated by the black triangle, and the arrow points to the probable signal peptide cleavage site. The solid lines underlining the amino acid sequence denote the regions of the β-strand members from the $(\alpha/\beta)_8$ barrel. Signature regions and other common features to the α-amylases, such as the catalytic residues (boxed in black), amino acids related to calcium binding site (+), amino acids involved in the active site and binding of substrate (*), and the tryptophan residues from the noncatalytic raw starch binding site (#) are indicated. The seven nucleotides of the putative pyrimidine box placed in an inverted orientation at the 5′ noncoding region are in bold. The modified polyadenylation signal is indicated by a dotted line under the nucleotide sequence at the 3′ noncoding region. Nucleotides flanking the splicing junctions are boxed.

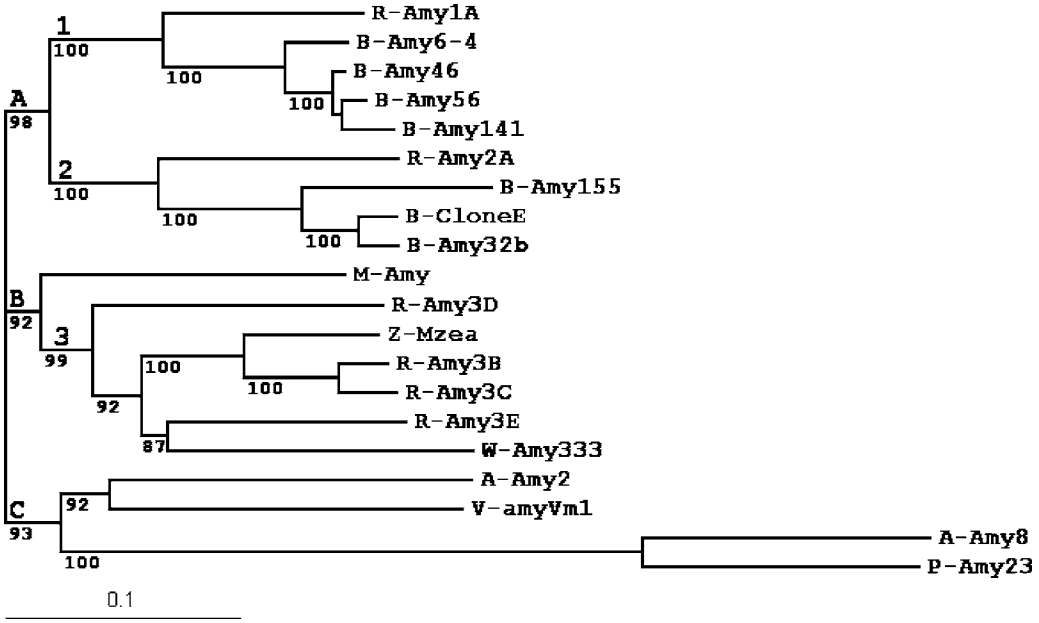

Figure 2. Unrooted phylogram showing relationships between *MAmy* and other plant α-amylases. The phylogenetic tree was calculated using Neighbor Joining method with a total of 1000 bootstrap replicates based on the alignment of deduced amino acids sequences, and bootstrap values are indicated below the branches. The branch lengths are proportional to divergence, with the scale bar meaning 0.1 residue substitutions per site. Sequences used are from barley (*Hordeum vulgare*): B-Amy141 (M17125), B-Amy46 (J04202), B-Amy6-4 (K02637), B-Amy56 (X15227), B-CloneE (M17128), B-Amy155 (M17127), and B-Amy32b (X05166); -ice (*Oryza sativa*): R-Amy1A (X16509), R-Amy2A (M74177), R-Amy3B (X56337), R-Amy3C (X56338), R-Amy3D (M59351), and R-Amy3E (M59352); wheat (*Triticum aestivum*): W-Amy333 (M16991); maize (*Zea mays*): Z-Mzea (L25805); apple (*Malus x domestica*): A-Amy8 (AF153828) and A-Amy2 (AY939870); potato (*Solanum tuberosum*): P-Amy23 (M79328); bean (*Vigna mungo*): V-amyVm1 (X73301); and banana (*Musa* spp.): *MAmy* (AF533648).

between Ser15 and Gln16. The mature polypeptide would have a predicted molecular mass of 45.1 kDa and a pI of 5.84.

According to the alignment of the amino acids deduced from the plant α-amylase DNA sequences deposited on GenBank, banana α-amylase would present the secondary structure elements and conserved parts of the $(\alpha/\beta)_8$-barrel, as well as the three proposed residues involved with catalysis (Asp179, Glu204, and Asp287) and two of a raw starch binding sites (Trp274−Trp275) and the others related to calcium and substrate binding (*27*). Prediction of tertiary structure based on the SWISS-MODEL (*21*) allowed the identification of the part of the $(\alpha/\beta)_8$-barrel, called domain A, and the two other domains, B and C, present in the α-amylase family (*1*).

A phylogenetic tree was calculated from the alignment of the plant α-amylases, and the resulting phylogram (**Figure 2**) indicated the divergence of three main classes, two (*AmyA* and *AmyB*) of monocot (grasses and banana), joining three subfamilies (*Amy1*, *Amy2*, and *Amy3*) of cereal α-amylases (*28*), and another one (*AmyC*) of dicot (bean, potato, and apple) plants. As can be seen in the tree, the deduced banana α-amylase was included in the *AmyB* class, surrounding the *Amy3* subfamily.

On the basis of the comparison of *MAmy* cDNA and the genomic clone (GenBank accession no. AY171068) (**Figure 3**A), the *MAmy* gene has four exons and three introns. Southern analysis of banana genomic DNA using the *MAmy* ORF as a probe revealed a great number of hybridization bands (**Figure 3B**). Besides the two stronger bands at 2.0 and 2.3 kb for *Hind* III and at 3.0 and 3.5 kb for *Sac* I and the band at 2.5 kb for *Pst* I digestion, several hybridized bands could be seen in the film, indicating the occurrence of other α-amylase-related sequences.

The identity of *MAmy* as a banana α-amylase coding gene was confirmed by its expression in *P. pastoris*. To produce the recombinant enzyme, the *MAmy* ORF was full cloned into pPICZB vector and used to transform the yeast. The amylolytic activity of the extracts from the cultured yeast was checked by iodine staining of native PAGE (**Figure 4A**) and substrate SDS-PAGE (**Figure 4B**) with copolymerized starch. As can be observed in **Figure 4A**, a fast migrating band was seen only in the 30−70% ammonium sulfate fraction of the supernatant from the GS115/*pPICZB*::*MAmy* yeast cultured with methanol induction on BMMY but not in extracts from *P. pastoris* transformed with the expression plasmid only (pPICZB without insert). The occurrence of the amylolytic activity in the culture media, although the vector did not include the α-factor responsible for extracellular expression, suggested that recombinant α-amylase was secreted by the yeast cells. Since the cDNA used for protein expression included a putative signal sequence, it is likely that the signal sequence of recombinant *MAmy* (*rMAmy*) was correctly processed by the yeast, driving the protein to the secretory pathway. Minor amylolitic activity in the cell lysate (**Figure 4A**, line 1) was also observed, probably caused by the absence of the correct modification or incomplete post-translation processing of *rMAmy*.

According to the substrate SDS-PAGE gel shown in **Figure 4B**, only a 46 kD band appearing in the 30−70% fraction of the supernatant from the GS115/*pPICZB*::*MAmy* culture supplemented with methanol presented the amylolytic activity. This protein size is in agreement with the value predicted by the banana α-amylase cDNA sequence and indicated that the recombinant enzyme is SDS-resistant, a property of only a few amylases (*24*).

DISCUSSION

The dramatic decrease in the starch amount during ripening indicates that the occurrence of high levels of hydrolytic activity in the pulp of bananas and α-amylase is supposed to be one of the main enzymes accounting for starch mobilization. Since the

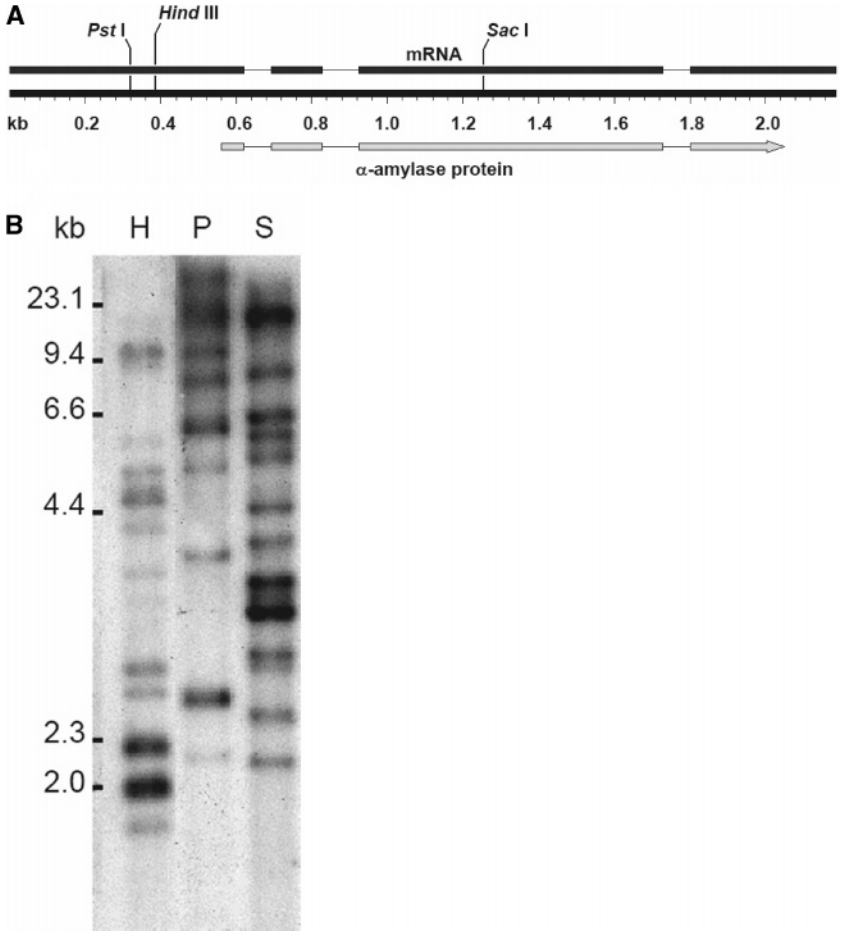

Figure 3. (**A**) Schematic representation of the *MAmy* gene, mRNA, and translated protein. The sites of the restriction enzymes that cut inside the clones are indicated. Exons are represented as black boxes in mRNA and protein; introns are denoted as solid lines. (**B**) Southern blot analysis of banana DNA. Ten micrograms of genomic DNA was digested with *Hind* III (H), *Pst* I (P), or *Sac* I (S), separated on 0.8% agarose gel, blotted, and hybridized with the ^{32}P-labeled *MAmy* ORF probe. Molecular weights are indicated on the left.

banana α-amylase clone was isolated from a cDNA library obtained from fruit induced to ripe by exogenous ethylene, it is likely that this clone represents a functional α-amylase gene that could play a role in starch mobilization during banana ripening. The cloned *MAmy*, its genomic clone, and the deduced protein presented most of the highly conserved α-amylases motifs, which are related to the function and regulation of α-amylase genes and specificity of the enzymes from this family (*27*).

The Southern analysis indicated the occurrence of several genomic sequences related to the α-amylase gene along with the main reacting bands. Since it was demonstrated that α-amylases from rice, wheat, and barley are members of multigenic families (*28*), it is not possible to exclude the existence of other α-amylase genes in bananas. If these other hybridizing bands correspond to functional α-amylase genes or pseudogenes, or even to sequences expressed in the pulp fruit or vegetative parts of the plant, it remains to be established. Similar to other monocot α-amylases, the banana α-amylase gene has the splicing sites of two introns located inside Aβ1 and Aβ8 of the $(\alpha/\beta)_8$-barrel, and the DNA sequences and amino acid residues around these splicing sites are highly conserved (**Figure 1**). However, since most members of the Amy3 subfamily have only two introns, a unique feature of *MAmy* gene was a third intron, placed between the other two. As the splicing site of the third intron was located outside of the barrel, it is likely that it was lost during the evolution of some genes from the *Amy3* subfamily. Additionally, the arrangement of the *MAmy* was in agreement to the α-amylase DNA sequence polymorphisms proposed by Huang et al. (*28*) in relation to the so-called α-amylase signature regions.

On the basis of the barley α-amylase model, the banana α-amylase protein would have putative calcium and substrate binding sites, as well as similar spatial localizations for the active centre. A similar comparison using the sequence of α-amylase from apple fruit (*29*) revealed significant differences from the barley α-amylase, which could be explained by the evolutionary distance between the dicot apple enzyme and the monocot barley model. Thus, since the protein modeling revealed a good agreement to the structure of α-amylases from barley, one could expect a similar behavior for the banana enzyme. However, the occurrence of a well-conserved sequence upstream of the coding region of the gene from banana, designated the pyrimidine box, could indicate that, although catalyzing a similar reaction, the expression of the enzyme from fruit would be regulated in a different way. The pyrimidine box can be found in either orientation in gibberellin-inducible genes such as several cereal α-amylases, which are secreted by cells of the aleurone layer during cereal grain germination. Previous studies with banana slices infiltrated with GA_3 (*12*, *30*) showed that gibberellins can delay or inhibit carbohydrate metabolizing enzymes related to starch degradation and sucrose synthesis during banana ripening. Thus, the presence of a putative pyrimidine box in the complementary 5′ noncoding region of *MAmy* could be an indication of responsiveness to the GA_3, but in a negative way.

The yeast expression of the cloned cDNA demonstrated that

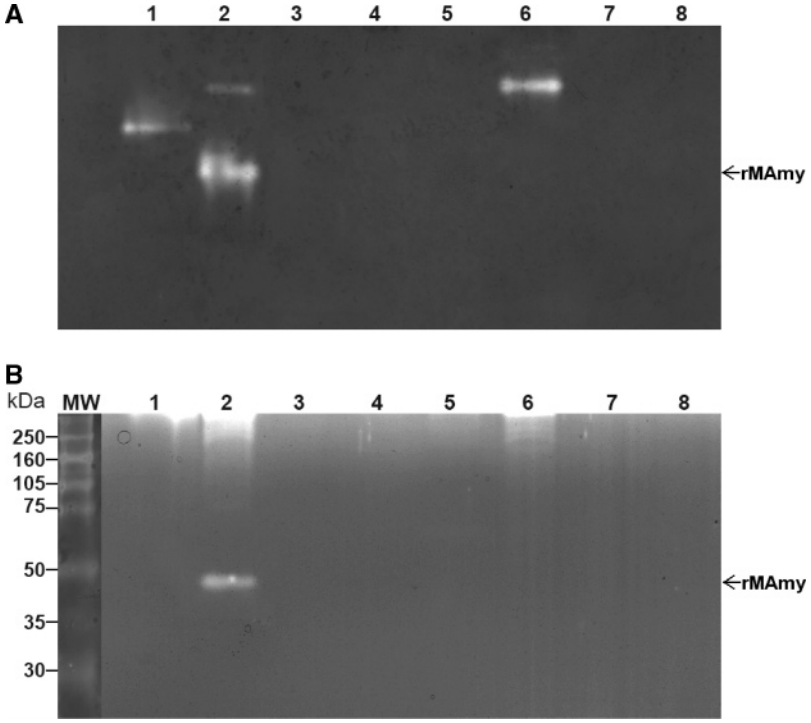

Figure 4. Analysis of amylolitic activity from the recombinant α-amylase on (**A**) native discontinuous PAGE and (**B**) substrate SDS-PAGE. Amylolitic activity is seen as clear zones in a background in stained gels. Cells and media from *P. pastoris* transformed with *pPICZB*::*MAmy* or pPICZB were harvested 4 days after post-induction with methanol. Equal aliquots (30 μL) from the cell lysates and media fractions were loaded on the gels followed by enzyme assay. MW indicates molecular weight marker. The arrow indicates the position of the *rMAmy*. Samples: lane 1, cell lysate from GS115/*pPICZB*::*MAmy* grown in BMMY; lane 2, 30–70% BMMY fraction from GS115/*pPICZB*::*MAmy*; lane 3, cell lysate from GS115/*pPICZB*::*MAmy* grown in BMGY; lane 4, 30–70% BMGY fraction from GS115/*pPICZB*::*MAmy*; lane 5, cell lysate from GS115/pPICZB grown in BMMY; lane 6, 30–70% BMMY fraction from GS115/pPICZB; lane 7, cell lysate from GS115/pPICZB grown in BMGY; and lane 8, 30–70% BMGY fraction from GS115/pPICZB.

it encodes for a functional banana α-amylase and that the enzyme probably has a functional signal peptide, which targets the expression to the secretory pathway. The correct processing of the *rMAmy* signal peptide by *P. pastoris* suggests that the α-amylase sequence under study could also be translocated in banana pulp cells. Translocation of banana α-amylase could involve secretion to the extracellular media or transportation to the plastids inside the pulp cells. The occurrence of extracellular α-amylase would suggest that the enzyme and starch are placed in different cells and that the access to the substrate would be dependent on the decompartmentalization of stored starch during ripening. However, since there is no evidence of a loss of integrity of banana pulp cells during ripening, it is more likely that the signal peptide would drive banana α-amylase to the amyloplasts. The processing by *P. pastoris* could be an indication that *rMAmy* would have a bifunctional signal peptide, as observed for rice α-amylase (*31*), which could be targeted both to the extracellular media and to plastids, depending on the physiological situation.

In summary, this gene sequence isolated from banana encodes for a functional α-amylase expressed in the pulp of the fruit during ripening. Although a physiological role for this sequence cannot yet be unequivocally attributed, it seems plausible that it could play a role in starch mobilization during fruit ripening. Evaluations on the changes in activity and protein abundance of this α-amylase during ripening, along with studies on the subcellular destination and activity over starch granules isolated from fruit, are under way, and they would provide a better understanding of the physiological role of this gene.

ABBREVIATIONS USED

PVP-40, polyvinylpyrrolidone 40 000; 2-ME, 2-mercaptoethanol; ORF, open reading frame; *mamy*, *musa* α-amylase.

ACKNOWLEDGMENT

This work was supported by CAPES and by FAPESP (Doctoral fellowship 01/09621-0 and Projeto Temático 02/12452-9).

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Received for review March 22, 2006. Revised manuscript received August 1, 2006. Accepted August 17, 2006.

JF060805B