JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Activity, Cloning, and Expression of an Isoamylase-Type Starch-Debranching Enzyme from Banana Fruit

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Unripe bananas have a high content of starch (almost 20%) that is metabolized during fruit ripening with a concomitant synthesis of soluble sugars. Since starch granules are composed of amylose and amylopectin, several enzymes have to be involved in its mobilization during banana ripening, with a necessary participation of one starch-debranching enzyme (DBE) to hydrolyze the α-1,6-branches of amylopectin. Banana DBE seems to be an isoamylase-type enzyme, as indicated by substrate specificity and the cloning of a 1575 bp cDNA, similar to the isoamylase sequences from potato, *Arabdopsis,* and maize. The assays for DBE indicated only minor changes in activity during ripening, and the results of the northern and western blots with antiserum against the recombinant banana isoamylase were in agreement with the steady-state level of activity, since no significant changes in gene expression were observed. The high activity on β-limit dextrin and the similarity to the potato isoform 3 suggest that during banana ripening the hydrolysis of α-1,6-linkage of amylopectin results from the activity of a pre-existing isoamylase-type debranching enzyme in coordination with other amylolitic enzymes. To the best of our knowledge, this is the first evaluation of activity and expression of a DBE from a fruit.

KEYWORDS: Banana; fruit ripening; isoamylase; starch; starch-debranching enzyme

INTRODUCTION

Starch represents almost 20% of banana pulp fresh weight, and it provides the carbon reserve for soluble sugars accumulation during ripening. Since starch granules are composed of amylose (linear α-1,4-linked glucan polymer) and amylopectin (α-1,4-linked glucan polymer with a high proportion of α-1,6-branch points), several enzymes have to be involved in its mobilization during ripening. Besides the α-amylases, β-amylases, starch phosphorylases, and α-1,4-glucosidases, which are responsible for the depolymerization of the linear chains, α-1,6-glucan-hydrolizing activity is essential for the complete starch mobilization and to remove the α-1,6-branch points of amylopectin, which correspond to almost 75% of the total banana starch content. In fact, the activity of an enzyme that used isomaltose as substrate was detected in crude extracts prepared from bananas at ripening (*1*). In plants, the α-1,6-debranching action can result from the activity of two classes of enzymes: pullulanases (EC 3.2.1.41) and isoamylases (EC 3.2.1.68). The pullulanases can act on the limit dextrin of amylopectin and in the yeast glucan pullulan, while isoamylases can use limit dextrin and glycogen as substrates but cannot attack the pullulan. It has been argued that the pullulanase-type debranching enzyme is responsible for the hydrolysis of α-1,6-linkages during starch mobilization, while the isoamylases have been considered to play an important role during the period of starch accumulation (*2, 3*). The role of isoamylases in amylopectin synthesis was inferred from the phenotypes of several mutants with reduced or no isoamylase activity. The *sugary1* mutations in maize (*4*−*6*), rice (*7, 8*), *Arabdopsis* (*2*), and *Chlamydomonas reinhardtii* cells (*9*) resulted in reduction of debranching enzyme activity and starch synthesis. As a consequence of these mutations there is accumulation of a highly branched, soluble polysaccharide termed phytoglycogen. On the basis of the studies on the mutants, several models were proposed to explain the role of isoamylase in amylopectin synthesis (*2, 10*−*12*).

Although it is likely that debranching enzymes such as isoamylases are necessary in starch synthesis during banana development, whatever the model to explain its role in granule formation, it is obvious that debranching activity is essential during starch breakdown, when it is needed during ripening. However, even though it is generally assumed that the debranching enzymes would have a role in starch degradation in bananas, knowledge of its activity and expression is limited. There is no information on the type of debranching activity involved in starch mobilization during fruit ripening, whether a pullulanase or isoamylase, or how its activity and expression correlates, if so, to the main changes in starch content at the

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10.1021/jf049300g CCC: $27.50
Published on Web 11/02/2004

climacteric raise. This article reports direct evidence that a DBE present during banana starch degradation is isoamylase-type enzyme as using different substrates or by cloning of an isoamylase-type cDNA expressed in the pulp.

MATERIALS AND METHODS

Plant Material. Mature green bananas (*Musa acuminata* AAA cv. Nanicão) were obtained at the local market within 1 day after harvest. After cleaning with sodium hypochlorite solution (0.1%), the fruits were maintained under controlled conditions of temperature (18 °C) and humidity (75%) to ripen both spontaneously (control group) or after exposure to ethylene (10 ppm) for 12 h (treated group). Fruits of both groups were sampled on a daily basis, and after ethylene and CO_2 quantification they were peeled, frozen in liquid N_2, and stored at −80 °C.

Ethylene and CO_2 Production. For the evaluation of ethylene production and respiration, the fruits were closed in jars for 1 h and the resulting atmosphere was analyzed by gas chromatography, as previously described by Purgatto et al. (*13*).

Soluble Sugar, Starch, and Protein Determination. Soluble sugars from pulp tissue were extracted successively with three volumes of boiling 80% (v/v) aqueous ethanol. The supernatants were combined, and the ethanol was evaporated under vacuum. Sugars were reconstituted in water and analyzed by HPAE-PAD (Dionex, Sunnyvale, CA) using a Carbopac PA-1 column. Starch content was determined as previously described by Cordenunsi and Lajolo (*14*), and proteins in crude extracts were estimated by Bradford methodology (*15*). Protein content in extracts for the western blots was determined as described by Peterson et al. (*16*).

Starch-Debranching Enzyme Extraction and Activity Assay. Protein extractions from pulp tissue and enzyme activity assay were based on the procedure described by Fujita et al. (*17*). One gram of the tissue was Polytron homogenized with 4 mL of 50 mM imidazole−HCl buffer (pH 7,4) containing 8 mM $MgCl_2$, 20 mM cysteine, 1 mM benzamidine, and 1% polyvinilpirrolydone (M_w 40 000). The homogenate was cleared by centrifugation at 15 000*g* for 40 min and desalted by gel filtration chromatography on Sephadex G-25 Hytrap column (Amersham Biosciences). Enzyme assay was performed in 50 mM imidazole−HCl (pH 7.4) buffer containing 100 μL of the desalted extract, 1% glycogen or β-dextrin limit, and 8 mM $MgCl_2$ in a total volume of 200 μL. The reactions were incubated at 30 °C for 2 h when using glycogen or 30 min for β-dextrin limit. The released reducing sugars were determined by the dinitrosalicylic acid method as described by Bassinello et al. (*18*).

RNA Extraction and Cloning of Banana DBE. Total RNA was isolated from ripe banana pulp tissues as described previously (*19*). To produce the cDNA, an aliquot of 5 μg of the total RNA was oligo-dT primed and reverse-transcribed using the First Strand cDNA Synthesis Kit (Amersham Biosciences). The forward primer 5′-GTGATCATGGACGTTGTCTATAACCA-3′ and the reverse primer 5′-TCGAAGGTGAAATCTGAAGCCATCGACATG-3′, from the consensus of the plant DBE sequences, were combined to PCR amplify the banana DBE cDNA. The PCR conditions for amplification were 94 °C/5 min, 35 cycles at 94 °C/30 s, 50 °C/30 s, and 72 °C/2min, followed by incubation at 72 °C for 7 min in a reaction volume of 25 μL in 50 mM Tris−HCl (pH 9) buffer containing 50 mM KCl, 0.2 mM dNTP, 0.5 mM each primer, 1.5 mM $MgCl_2$, 1 μL of first-strand cDNA reaction, and 0.5 units of *Taq* polymerase. The amplified 252 bp fragment of banana DBE was gel purified and TA cloned in the pGEM-T vector (Promega) for automatic sequencing.

On the basis of the partial sequence of banana DBE, additional primers were designed to amplify the 5′- and 3′-ends by RACE using the GeneRacer Kit (Invitrogen). A 1500 bp fragment containing additional 1263 bp from the 3′-end of banana DBE cDNA was amplified using the sequence-specific sense primer (5′-CGTCTATAACCATACTAATGAAGCTAATGA-3′) and the reverse universal primer supplied in the kit. The combination of sequence information from the 252 bp clone and the additional 3′-end fragment allowed PCR amplification of a banana DBE fragment of 1575 bp using the sense primer 5′-AGTGCTGGTGGACCGTTGGTTGCTTCT-3′ and the reverse primer 5′-CAGAACATTATTTCTTATTATGTGTCAATTTTC-3′.

Northern Blot. Aliquots of total RNA (25 μg) were separated on denaturing agarose gels containing 2.2 M formaldehyde (*20*) and vacuum-transferred to nylon membranes (Hybond N^+, Amersham Biosciences). The membranes were prehybridized at 65 °C in 5 × SSC, 5 × Denhardt's solution, 1% SDS, and 20 μg/mL of denatured salmon sperm. After 2 h of blocking the α-[^{32}P]-dCTP-labeled 1575 bp fragment of banana DBE was added to the solution and the hybridization was let to proceed for 12 h at the same temperature. The membranes were washed two times with 2 × SSC/0.1% SDS for 15 min at room temperature, two times with 1 × SSC/0, 1% SDS for 15 min at 65 °C, and a final high-stringency wash with 0.1 × SSC/0.1% SDS at 65 °C for 25 min. The bands were revealed after exposure to X-ray films for 72 h.

Sequencing and Analysis of the Sequences. DNA sequencing was performed automatically in an ALF Express DNA Sequence Analyzer and using the Thermo Sequenase Cy5 Dye Terminator Cycle Sequence Kit (Amersham Pharmacia Biotech). The procedures for sequence analysis were run under the University of Wisconsin GCG Package (*21*), available at the Brazilian Bioresources Research Center (BBRC-Embrapa), the BLAST (Basic Local Alignment Search Tool), and the ClustalW Program.

Expression of Recombinant DBE in *Escherichia coli*. The fragment of DBE used to construct the expression plasmid was obtained by PCR amplification of 636 bp fragment of banana cDNA using the sense primer 3 (5′-CGCAAACCCTACCACAGTATCAATTT-3′) and the reverse primer CT2 (5′-TTAAGATTCCAGATTTGTGTCAACCAC-3′). Although several attempts with other primers were made, only this combination resulted in a fragment with good expression levels. The amplified fragment was T/A cloned in frame to the start codon of the expression vector pCR-T7-NT/TOPO-TA (Invitrogen), and after amplification in *E. coli* TOP10F′ cells, the expression construct was purified and fully sequenced to check the fidelity of DBE construction. To express the recombinant DBE, *E. coli* strain BL21-(DE3)pLysS was transformed with the resulting construct, designated pCR-T7NT-DBE. After cultivating the transformed cells overnight in LB medium supplemented with 100 μg/mL ampicillin and 34 μg/mL chloraphenicol at 37 °C, 1 mM IPTG was added to half of the culture and further incubated for 4 h at 37 °C.

After IPTG induction of recombinant protein expression, the cells were collected by centrifugation and immediately frozen. To isolate the recombinant DBE, the cells were resuspended in lysis buffer and the lysate was submitted to affinity chromatography on *ProBond* (Invitrogen) poly-hystidine column, according to the manufacturer instructions. After purification the protein was dialyzed against PBS containing 0.1% SDS and concentrated using Centricon filters (Amicon).

Polyclonal Antiserum and Western Blotting. About 300 μg of purified recombinant protein diluted in 1 mL of PBS was emulsified in Freund's complete adjuvant and injected subcutaneously in New Zealand white rabbits. Two subsequent inoculations with the same protein amount were done with 10-day intervals, and the antiserum was harvest at day 7 after the last injection. The sera collected before the inoculations were used as the negative controls of the immunizations.

For the western blots banana pulp samples were homogenized in SDS−PAGE sample buffer in a 1:3 proportion (w/v). The homogenates were boiled for 10 min, and after centrifugation at 10000*g* for 10 min, the amount of extracted protein was determined in the supernatant. Fifty micrograms of protein from each sample was separated on SDS−PAGE (10%) and electroblotted to nitrocellulose membranes. Incubation of the filters with a 1:100 dilution of the rabbit antiserum and color development using alkaline phosphatase were performed according to Sambrook et al. (*20*).

RESULTS

Starch Mobilization and DBE Activity during Banana Ripening. Fruits from the same bunch were treated with 10 ppm of ethylene to observe the effect of the hormone on the

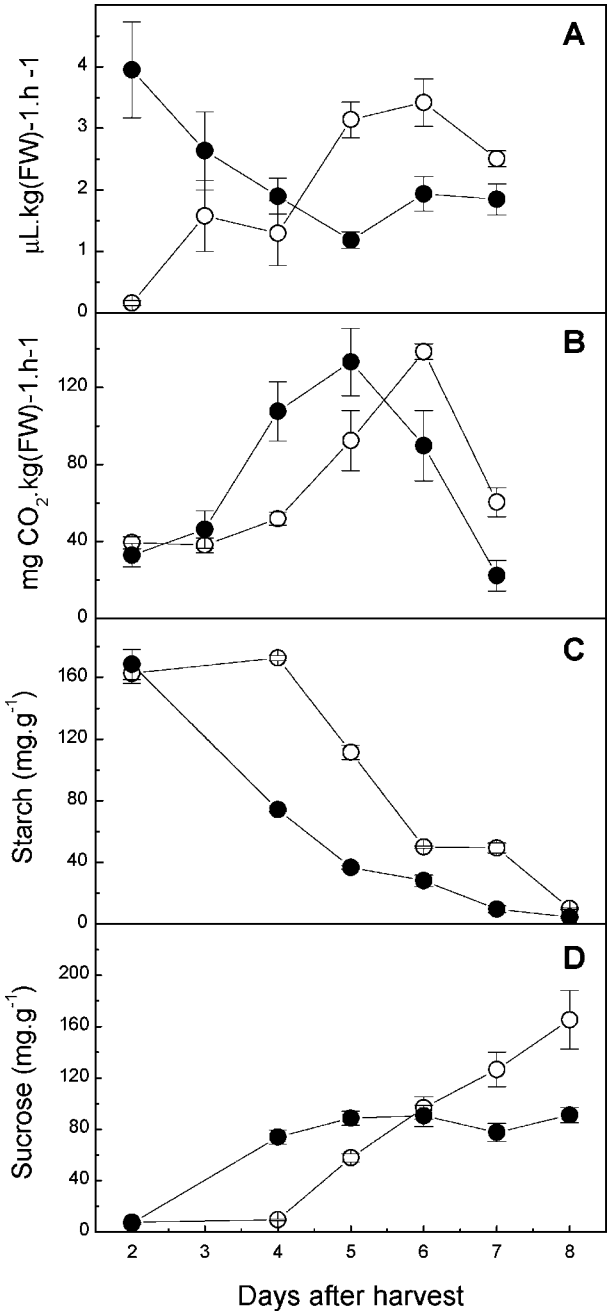

Figure 1. Changes in carbohydrate composition and physiological parameters during banana ripening. Banana fruits were ripened naturally (white circles) or after exposure to exogenous ethylene for 12 h (black circles). The ethylene production (**A**), respiration (**B**), starch (**C**), and sucrose (**D**) contents were monitored on a daily basis until the fruits were fully ripe. Vertical bars represent the standard error ($n = 3$ for starch and sucrose; $n = 6$ for ethylene and CO_2).

activity and expression of banana DBE. According to **Figure 1** both the ethylene-treated and nontreated (control) banana fruit had the expected changes in respiration and carbohydrate composition during the climacteric. As a consequence of exposure to exogenous ethylene (**Figure 1A**), the respiration burst (**Figure 1B**), starch degradation (**Figure 1C**), and sugar accumulation of treated fruits were anticipated in 4 days when compared to the bananas that were ripened naturally. Except for the sucrose content (**Figure 1D**), the achieved values for soluble sugars were very similar.

Since the starch-debranching enzymes can be classified as pullulanase- or isoamylase-type based on the substrate specific-

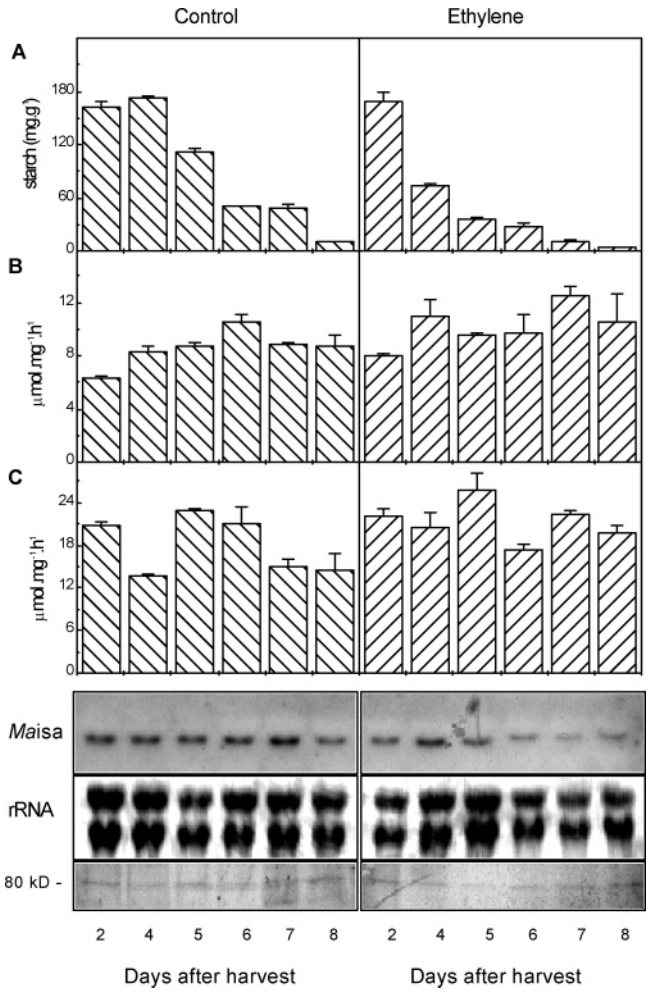

Figure 2. Starch-debranching enzyme activity and isoamylase expression during ripening. Control banana samples (columns on the left) and ethylene-treated samples (columns on the right) were submitted to determination of starch content (**A**) and assayed for debranching enzyme activity using glycogen (**B**) or β-limit dextrin (**C**) as substrates. Total RNA was extracted from the fruit samples, and northern blots were probed with the banana isoamylase cDNA (*Maisa*) after checking the filters for equal loading with methylene blue staining (rRNA). The detection of the 80 kDa isoamylase protein in banana extracts separated on SDS–PAGE was performed with the antiserum against the recombinant truncated isoamylase after transference to nitrocellulose membranes (boxes at the bottom). Vertical bars represent the standard error ($n = 3$).

ity, enzyme extracts from fruits at different ripening stages were tested using pullulan, glycogen, and β-dextrin limit as substrates. As can be seen in **Figure 2**, glycogen (**Figure 2B**) and β-limit dextrin (**Figure 2C**) substrates resulted in maltose production both in the ethylene-treated and control fruits, but no activity was detected if pullulan was the substrate.

When glycogen was used as the substrate, a discrete increase in the activity (6−10 μmol mg^{-1}) was observed through ripening of the control fruits, peaking at day 6 after harvest (**Figure 2B**). This increase in DBE activity was concomitant with the starch mobilization, and it seemed to be anticipated in 2 days when the fruits were treated with ethylene. Apparently the maximum DBE activity was observed when the starch content was around 5%, and the values were similar between groups.

When compared to the glycogen assays, those assays employing β-limit dextrin (**Figure 2C**) resulted in higher amounts of released maltose through the ripening period for both the control and treated groups. A correlation between the decrease in starch

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   1 AGTGCTGGTGGTGGACCGTTGGTTGCTTCTCATGAGTTCAAACAGATGGTGAAAACTTTG
     S  A  G  G  G  P  L  V  A  S  H  E  F  K  Q  M  V  K  T  L
  61 CACAATGCTGGCATTGAGGTGATCATGGACGTTGTCTATAACCATACTAATGAAGCTAAT
     H  N  A  G  I  E  V  I  M  D  V  V  Y  N  H  T  N  E  A  N
 121 GATCGTCATCCTTATACTTCTTCCTTTCGTGGTGTGGACAATAAGGTTTATTACATGTTG
     D  R  H  P  Y  T  S  S  F  R  G  V  D  N  K  V  Y  Y  M  L
 181 GATCCCGATAATGGTGCTAAGTACCTGAATTTTTCAGGCTGTGGAAATACATTAAACTGC
     D  P  D  N  G  A  K  Y  L  N  F  S  G  C  G  N  T  L  N  C
 241 AATCACCCTGTCGTCATGGAACTTATTCTTGACAGTTTAAGACATTGGGTTAACGAGTAC
     N  H  P  V  V  M  E  L  I  L  D  S  L  R  H  W  V  N  E  Y
 301 CATGTCGATGGCTTCAGATTTCACCTTGCAAGTATTCTTTGCCGAGGGACCGATGGTTCT
     H  V  D  G  F  R  F  H  L  A  S  I  L  C  R  G  T  D  G  S
 361 CCTCTTAATTCACCTCCATTAGCCAAGGCAATTGCTAAAGATGCTGTACTTTCAAGGTGT
     P  L  N  S  P  P  L  A  K  A  I  A  K  D  A  V  L  S  R  C
 421 AAGATAATTGCTGAACCATGGGACTGTGGAGGACTTTATCTTGTGGGAAATTTTCCAAAT
     K  I  I  A  E  P  W  D  C  G  G  L  Y  L  V  G  N  F  P  N
 481 TGGGACCGGTGGGCCGAATGGAATGGAAAGTACCGTGATGATATTCGAAGATTCATGAAG
     W  D  R  W  A  E  W  N  G  K  Y  R  D  D  I  R  R  F  M  K
 541 GGTGATTGTGGTATGAAAGGGACCTTTGCAACTCGTATATCTGGATCTGCTGACCTCTAC
     G  D  C  G  M  K  G  T  F  A  T  R  I  S  G  S  A  D  L  Y
 601 CAGGTGAACAAGCGCAAACCCTACCACAGTATCAATTTTGTGATAGCACATGATGGGTTT
     Q  V  N  K  R  K  P  Y  H  S  I  N  F  V  I  A  H  D  G  F
 661 ACACTGTGTGATCTTGTTTCTTACAATTTCAAGCATAATGATGCTAACGGGGAAGGTGGC
     T  L  C  D  L  V  S  Y  N  F  K  H  N  D  A  N  G  E  G  G
 721 AAAGATGGAAGCAATGACAATTTTAGCTGGAATTGTGGTGTTGAAGGAGAAACGGATGAT
     K  D  G  S  N  D  N  F  S  W  N  C  G  V  E  G  E  T  D  D
 781 GTTGATATTATAGGTCTTCGCTCACGGCAAATGAAGAACTTCCATTTGGCCTTAATGATC
     V  D  I  I  G  L  R  S  R  Q  M  K  N  F  H  L  A  L  M  I
 841 TCTCAGGGAACACCAATGATGCTAATGGGGGATGAATATGGCCATACACGTTATGGAAAC
     S  Q  G  T  P  M  M  L  M  G  D  E  Y  G  H  T  R  Y  G  N
 901 AATAATAGCTATGGACATGACACTTCCATAAAAATTTTCAGTGGAAACAGTTGGAAGAAA
     N  N  S  Y  G  H  D  T  S  I  K  I  F  S  G  N  S  W  K  K
 961 GAAGGGGCGGTCATTTTAGATTTTTCTGTGAGAATGATAAAATTTCGCTGCAAGCAATCC
     E  G  A  V  I  L  D  F  S  V  R  M  I  K  F  R  C  K  Q  S
1021 CATGTTTGCGACAAGACAGATTTCTTACCAAAAAATGATGTTGCATGGCATGAAGACAAC
     H  V  C  D  K  T  D  F  L  P  K  N  D  V  A  W  H  E  D  N
1081 TGGAGCAACCAGGAGAGCAAATTCTTAGCATTTACGCTTCATGAAAATCAGTTTGGAGGA
     W  S  N  Q  E  S  K  F  L  A  F  T  L  H  E  N  Q  F  G  G
1141 GACATCTATTTGGCTTTTAAGGCTCATGATTATTATGTCAAAGCTGCAGTACCTTCACCA
     D  I  Y  L  A  F  K  A  H  D  Y  Y  V  K  A  A  V  P  S  P
1201 CCACATAAGAAGAGATGGCCCCGAGTGGTTGACACAAATCTGGAATCTCCCAAAGATTTT
     P  H  K  K  R  W  P  R  V  V  D  T  N  L  E  S  P  K  D  F
1261 GTTCCCGAAGGTGTACCATTCAGCAACACCAGTTACAATATCGCTTCATATTCTGCAGTT
     V  P  E  G  V  P  F  S  N  T  S  Y  N  I  A  S  Y  S  A  V
1321 CTCCTTGAGGAAAGCCATGATCCTGTGACATTTGTTCTCCAGAATCATGATCAATAAagg
     L  L  E  E  S  H  D  P  V  T  F  V  L  Q  N  H  D  Q  *
1381 actcccactgtttgacaaattgctgcctaagggttagtcagtccatctgctgacattata
1441 taggaggtcggagagggagacgaaggagaattaaaaaggagagaggcaggggggttggatt
1501 gaagggaattatttgctataactatcccataaatataatttagaaaattgacacataata
1561 agaaataatgttctg (a)10
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Figure 3. Nucleotide and amino acid sequence of the *Musa acuminata* isoamylase (*Ma*isa) clone. The forward and reverse primers (boxed sequences) from conserved regions of plant debranching enzyme sequences were combined to PCR amplify a 252 bp fragment of banana cDNA. The additional information provided by the 5′- and 3′-RACE protocols allowed amplification of a 1575 bp cDNA encoding for 458 amino acids of the partial banana isoamylase. The underlined sequences indicate the sense and antisense primers that generated the DNA fragment successfully expressed in *E. coli.* Deduced amino acids are presented in one-letter code under the nucleotide sequence. The numbers on the left correspond to the position of the first nucleotide in each line.

content along ripening and an increase in activity of DBE would be expected to occur but did not happen. The overall profiles of DBE activity indicate that the activity is kept almost constant during ripening, regardless of the substrate employed in the assays.

Cloning and Expression of Banana DBE. Combination of the sequence information provided by amplification of the 252 bp fragment and the results obtained by the RACE protocol allowed the cloning of a partial banana DBE cDNA. As shown in **Figure 3**, the 1575 bp cDNA comprises a 1374 bp segment encoding for 458 amino acids of banana DBE and 224 bp from the 3′-untranslated region. When this sequence was submitted to BLAST analysis, the highest similarity scores were obtained for the available sequences of plant isoamylases. Using the ClustalW program, the deduced amino acid sequence from the *Musa acuminata* isoamylase (*Ma*isa) clone was compared to the isoforms 1, 2, and 3 of isoamylases from *Arabdopsis*, maize, and potato (data not shown), and the resulting phylogenetic tree generated from this alignment placed *Ma*isa together with the type 3 isoforms of isoamylase (**Figure 4**).

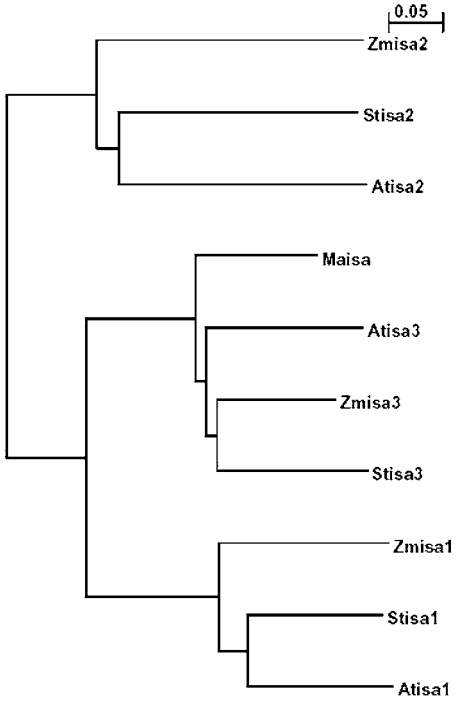

Figure 4. Phylogenetic tree of isoamylases from some plant species. Using the ClustalW Program the deduced amino acid sequence of banana isoamylase (*Ma*isa) was compared to those of the subgroups 1, 2, and 3 of isoamylases from *Arabdopsis thaliana* (*At*isa1, accession NM129551; *At*isa2, accession NM100213; *At*isa3, accession NM116971), potato tuber (*Solanum tuberosum*, *St*isa1 accession AY132996; *St*isa2, accession AY132997; *St*isa3, accession AY132998), and maize (*Zea mays*, *Zm*isa1 accession AF030882; *Zm*isa2, accession AY172633; *Zm*isa3, accession AY172634), and the resulting phylogenetic tree was presented.

When the *Ma*isa sequence is compared only to those from isoforms 3 from *Arabdopsis*, maize, and potato tuber, a high identity level can be observed (**Figure 5**). Although there are limitations of analysis employing a partial sequence, it is clear that besides the high identity at the amino acid level the size and composition of the characteristic domains that constitute the $(\alpha/\beta)_8$-barrel present in the amylase superfamily are almost identical and placed at similar positions to the other isoamylase 3 isoforms.

When the *Ma*isa clone was used as a probe in northern blots, a reactive band around 2600 bp was detected after washing at high stringent conditions. As can be seen in the **Figure 2**, there were no impressive changes in transcript amount in bananas undergoing natural ripening, only a marginal increase in band intensity at day 7 followed by a slight decrease on the next day. When ethylene was used to trigger banana ripening, these events seemed to be anticipated since higher amounts of transcripts were seen at day 4 after harvest, and it continuously decreased through the sampling period.

The expression of part of the *Ma*isa clone in *E. coli* cells resulted in a truncated peptide that was employed in the production of antiserum against banana DBE. As presented in **Figure 2**, a band around 80 kD appeared, which is close to the expected size of isoamylases from plants reacted with the antiserum raised against the recombinant protein. On the basis of the western blot performed with banana extracts, there was no significant change in the amount of reactive protein during banana ripening, although a discrete decrease in its amount could be seen after treatment with exogenous ethylene.

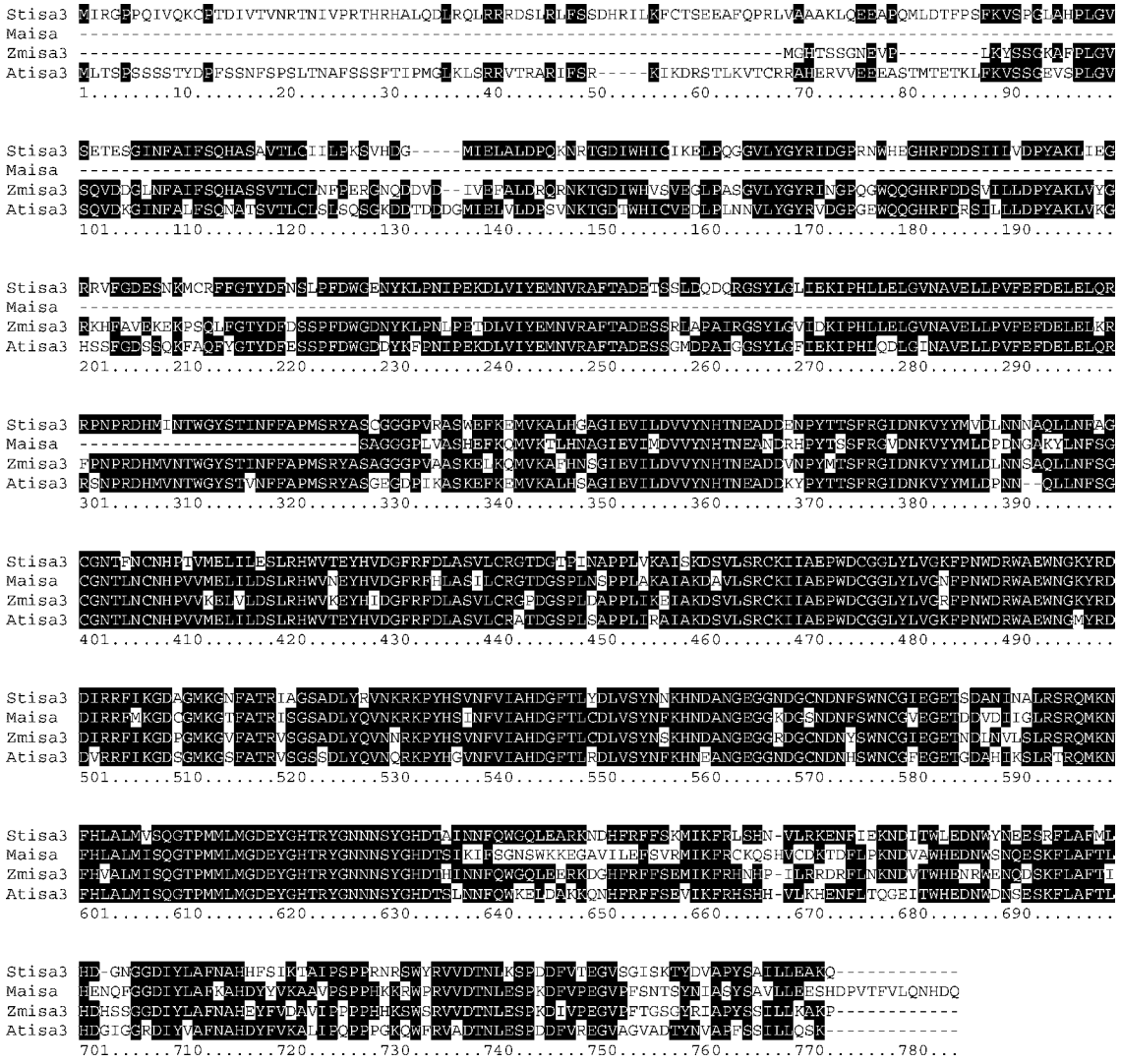

Figure 5. Alignment of amino acid sequences of isoamylases of subgroup 3. The deduced amino acid sequence of banana isoamylase (*Ma*isa) was compared to those of subgroup 3 from *Arabdopsis thaliana* (*At*isa3, accession NM116971), potato tuber (*Solanum tuberosum*; *St*isa3, accession AY132998), and maize (*Zea mays*; *Zm*isa3, accession AY172634) using the ClustalW program. Identical amino acids in at least two sequences are boxed in black. The numbers indicate the position of the characters in the lines. Deduced amino acids are presented according to the one-letter code.

DISCUSSION

The assays using glycogen and β-dextrin limit as substrates confirm the existence of a starch-debranching enzyme acting in the pulp tissue during ripening, and the observed rate of maltose production suggests that β-limit dextrin is more efficiently used than glycogen. On the basis of the presented results, the banana starch-debranching enzyme seems to be an isoamylase-type enzyme, not a pullulan-type one. Taking into account that there was no previous information on the type of debranching enzyme present in the banana pulp, the cDNA cloning strategy was based on the conserved regions among isoamylase and pullulan sequences, so the existent cDNA could be PCR-amplified whether it was an isoamylase or a pullulanase type. Therefore, the results from the sequence analysis of the cDNA isolated from the pulp of ripening bananas were in agreement with those predicted based on substrate usage because the cDNA clone was very similar to isoamylases from other plants and conservation at the characteristic domains of the $(\alpha/\beta)_8$-barrel site occurring in the amylases superfamily (*22*−*24*). To the best of our knowledge, this is the first isolation of a DBE cDNA clone from bananas, even though it was a partial sequence. Attempts to isolate the missing 5′-region of the *Ma*isa sequence by RACE were unsuccessful, probably because the banana sequence has a high G+C content at its 5′-end, similar to what was discussed for the sequences from rice endosperm (*17*) and wheat (*25*).

According to much biochemical and molecular evidence, plant isoamylases can exist as more than one isoform. In fact, as presented by Hussain et al. (*26*), three distinct isoform classes of isoamylase can be identified in potato tubers and other plant species. On the basis of the phylogenetic tree generated from the alignment of the *Maisa* sequence to those of isoforms 1, 2, and 3 from *Arabdopsis*, maize, and potato, it seems that the enzyme from banana fruit can be classified as an isoform 3 type of isoamylase, although the existence of other isoforms cannot be ruled out.

As discussed by Hussain et al. (*26*), isoamylase substrate specificity and efficiency are affected by the positioning of the β-strands and α-helix of the $(\alpha/\beta)_8$ barrel, which can originate different sites for binding of starch or its subproducts. According to the authors, the distance between loop 4 and 5 of potato *St*isa3 is similar to that of isoamylases from *E. coli* and *Chlamydia*, which efficiently use β- limit dextrin as substrate. Since the potato isoform 3 had the highest activity on β- limit dextrin, confirming the prediction based on the loop size, the authors proposed that the potato isoform 3 of isoamylase was probably

involved with the starch mobilization process but not with the amylopectin synthesis during tuber development. Considering that the predicted loop lengths of banana isoamylase are identical to the other isoamylase 3, except for the missing loops 1 and 2 which were not encompassed by the partial cDNA clone, it can be speculated that regarding substrate specificity, *Ma*isa has similar properties to *St*isa3.

Taking into account the activity assays presented, both glycogen and β-dextrin limit were converted in maltose by the banana isoamylase, but the enzyme extracts produced 50−100% more reducing sugar if β-limit dextrin was the substrate. Since β-limit dextrin may be produced during starch degradation by amylases and it is unlikely to be present at high levels during synthesis of starch in fruit development, the presence of an isoamylase in banana pulp with high affinity to this substrate is very plausible.

The differences in substrate efficiency were apparent by the amount of reducing sugar produced but not in relation to the resulting profile during ripening. As mentioned under Results, the activity profiles with these two substrates followed a constant pattern, indicating that their activity is not changed significantly during starch mobilization.

The overall steady-state activity level was compatible with the observed amounts of *Ma*isa transcript and protein during fruit ripening, suggesting that there was no stimulation in banana isoamylase gene expression. The marginal effect of ethylene treatment on transcript amount could be an indication that isoamylase transcription is downregulated at fruit senescence, when the fruit pulp would be depleted of most of the starch-derived substrates for isoamylases.

Since isoamylase can play a role both in amylopectin synthesis and debranching during banana ripening, it is likely that isoamylase activity would be maintained at an almost constant level during the entire life of the fruit. In pea embryos (*27*), bands in PAGE with isoamylase activity were detected from an early stage of development and changed little in intensity during embryo development, while those of pululanases increased significantly. Because banana isoamylase is not able to initiate the attack to the starch granules, the action on the α-1,6-branches during fruit ripening would be dependent on the previous action of other starch-degrading enzymes, like α- and β-amylases (*18*, *28*) and starch phosphorylases (*29*). In this way, additional DBE activity provided by a stimulation in gene expression or kinetic activation of the pre-existent enzyme would be not necessary during fruit ripening.

On the basis of the results presented, it can be concluded that during banana ripening hydrolysis of α-1,6-linkage of amylopectin is due to the activity of a pre-existent isoamylase-type debranching enzyme and not a pullulanase in a coordinated action with amylases and phosphorylases.

LITERATURE CITED

(1) Garcia, E.; Lajolo, F. M. Starch transformation during banana ripening: the amylase and glucosidase behavior. *J. Food Sci*. **1988**, *53*, 1181−1186.

(2) Zeeman, S. C.; Umemoto, T.; Lue, W.; Au-Yeng, P.; Martin, C.; Smith, A. M.; Chen, J. A mutant of Arabidopsis lacking a chloroplastic isoamylase accumulates both starch and phytoglycogen. *Plant Cell* **1998**, *10*, 1699−1711.

(3) Dinges, J. R.; Colleoni, C.; James, M. G.; Myers, A. M. Mutational analysis of the pullulanase-type debranching enzyme of maize indicates multiple functions in starch metabolism. *Plant Cell* **2003**, *15*, 666−680.

(4) Pan, D.; Nelson, O. E. A debranching enzyme deficiency in endosperms of the *sugary1* mutants of maize. *Plant Physiol*. **1984**, *74*, 324−328.

(5) Doehlert, D. C.; Kuo, T. M.; Juvik, J. A.; Beers, E. P.; Duke, S. H. Characteristics of carbohydrate metabolism in sweet corn (sugary-1) endosperms. *J. Am. Soc. Hortic. Sci*. **1993**, *118*, 661−666.

(6) James, M. G.; Robertson, D. S.; Myers, M. Characterization of the maize gene *sugary1*, a determinant of starch composition in kernels. *Plant Cell* **1995**, *7*, 417−429.

(7) Nakamura, Y.; Umemoto, T.; Takahara, Y.; Komae, K.; Amano, E.; Satoh, H. Changes in the structure of starch and enzyme activities affected by sugary mutations in developing rice endosperm: possible role of starch debranching enzyme (R-enzyme) in amylopectin biosynthesis. *Physiol. Plant*. **1997**, *97*, 491−498.

(8) Kubo, A.; Fujita, N.; Harada K., Matsuda, T.; Satih, H.; Nakamura, Y. The starch-debranching enzymes isoamylase and pullulanase are both involved in amylopectin biosynthesis in rice endosperm. *Plant Physiol*. **1999**, *121*, 399−409.

(9) Mouille, G.; Maddelein, M. L.; Libessard, N.; Talaga, P.; Decq, A.; Delrue, B.; Ball, S. G. Preamylopectin processing: a mandatory step for starch biosynthesis in plants. *Plant Cell* **1996**, *8*, 1353−1366.

(10) Ball, S.; Guan, H. P.; James, M.; Myers, A.; Keeling, P.; Mouille, G.; Buleon, A.; Colonna, P.; Preiss, J. From glycogen to amylopectin: A model for the biogenesis of the plant starch granule. *Cell* **1996**, *86*, 349−352.

(11) Sun, C. X.; Satish, P.; Ahlandsberg, S.; Jansson, C. Analyses of isoamylase gene activity in wild-type barley indicate its involvement in starch synthesis. *Plant Mol. Biol*. **1999**, *40*, 431−443.

(12) Burton, R.; Jenner, H.; Carrangis, L.; Fahy, B.; Fincher, G.; Hylton C.; Laurie D.; Parker M.; Waite D.; van Wegen S.; Verhoeven T.; Denyer K. Starch granule initiation and growth are altered in barley mutants that lack isoamylase activity. *Plant J*. **2002**, *31*, 97−112.

(13) Purgatto, E.; Nascimento, J. R. O.; Lajolo, F. M.; Cordenunsi, B. R. The onset of starch degradation during banana ripening is concomitant to changes in the content of free and conjugate forms of indole-3-acetic acid. *J. Plant Physiol*. **2002**, *159*, 1105−1111.

(14) Cordenunsi, B. R.; Lajolo, F. M. Starch breakdown during banana ripening: sucrose synthase and sucrose phosphate synthase. *J. Agric. Food Chem*. **1995**, *43*, 347−351.

(15) Bradford, M. M. Rapid and sensitive method for quantification of microgram quantities of protein utilizing principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248−254.

(16) Peterson, G. L. A simplification of the protein assay method of Lowry et al. which is more generally applicable. *Anal. Biochem*. **1977**, *83*, 346−356.

(17) Fujita, N.; Kubo, A.; Francisco, P.; Nakakita, M.; Harada, K.; Minaka, N.; Nakamura, Y. Purification, characterization, and cDNA structure of isoamylase from developing endosperm of rice. *Planta* **1999**, *208*, 283−293

(18) Bassinello, P. Z.; Cordenunsi, B. R.; Lajolo, F. M. Amylolitic activity in fruits: Comparison of different substrates and methods using banana as model. *J. Agric. Food Chem*. **2002**, *50*, 5781−5786.

(19) Nascimento, J. R. O.; Cordenunsi, B. R.; Lajolo, F. M.; Alcocer, M. J. C. Banana sucrose-phosphate synthase gene expression during fruit ripening. *Planta* **1997**, *203*, 283−288.

(20) Sambrook, J.; Fritsch, E. F.; Maniatis, T. *Molecular cloning: a laboratorial manual*, 2nd ed.; Cold Spring Harbor Laboratory: Cold Spring Harbor, NY, 1989; pp 1−3.

(21) Deveraux, J.; Haeberli, P.; Smithies, O. A comprehensive set of analysis programs for the wax. *Nucleic Acids Res*. **1984**, *12*, 387−395.

(22) Janecèk, S. α-Amylase family: molecular biology and evolution. *Prog. Biophys. Mol. Biol*. **1997**, *67*, 67−97.

(23) Jespersen, H.; MacGregor, E. A.; Sierks, M.; Svenson, B. Comparison of the domain-level organization of starch hydrolases and related enzymes. *Biochem. J*. **1991**, *280*, 51−55.

(24) MacGregor, E. A.; Janecek, S.; Svensson, B. Relationship of sequence and structure to specificty in the α-amylase family of enzymes. *Biochim. Biophys. Acta* **2001**, *1546*, 1-20.

(25) Genschel, U.; Abel, G.; Lörz, H.; Lütticke, S. The sugary-type isoamylase in wheat: tissue distribution and subcelular localization. *Planta* **2002**, *214*, 813−820.

(26) Hussain, H.; Mant, A.; Seale, R.; Zeeman, S.; Hinchliffe, E.; Edwards, A.; Hylton, C.; Bornemann, S.; Smith, A. L.; Martin, C.; Bustos, R. Three isoforms of isoamylase contribute different catalytic properties for the debranching of potato glucans. *Plant Cell* **2003**, *15*, 133−149.

(27) Zhu, Z. P.; Hylton, C. M.; Rössner, U.; Smith, A. M. Characterization of starch-debranching enzymes inpea embryos. *Plant Physiol.* **1998**, *118*, 581−590.

(28) Purgatto, E.; Lajolo, F. M.; Nascimento, J. R. O.; Cordenunsi, B. R. Inhibition of *β*-amylase activity, starch degradation and sucrose formation by IAA during banana ripening. *Planta* **2001**, *212*, 823−828.

(29) Mota, R. V.; Cordenunsi, B. R.; Nascimento, J. R. O.; Purgatto, E.; Rossetto, M. R. M.; Lajolo, F. M. Activity and expression of banana starch-phosphorylases during fruit development and ripening. *Planta* **2002**, *216*, 325−333.

Received for review May 3, 2004. Accepted September 14, 2004. The authors thank FAPESP for supporting this work.

JF049300G