JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

*J. Agric. Food Chem.* **2002,** *50,* 5781–5786 **5781**

## ARTICLES

# Amylolytic Activity in Fruits: Comparison of Different Substrates and Methods Using Banana as Model

PRISCILA Z. BASSINELLO, BEATRIZ R. CORDENUNSI, AND FRANCO M. LAJOLO*

Laboratório de Química, Bioquímica e Biologia Molecular de Alimentos, Departamento de Alimentos e Nutrição Experimental-FCF, Universidade de São Paulo, Avenida Prof. Lineu Prestes 580, Bloco 14, 05508-900 São Paulo, SP, Brazil

Several methodologies have been developed for cereal amylolytic activity estimation, but there is lack of information about the application of these methods for fruits. Mature green banana fruit can achieve 20% of starch content which is degraded during the ripening period in a complex process involving α- and β-amylases and α-1,4 and α-1,6-glucosidases, besides phosphorylases that can compete for the same substrates. Methods used to determine total hydrolytic activity and individual activity of enzymes involved in starch breakdown were compared for banana extracts in several ripening stages. Total hydrolytic activity was measured by DNS and iodometric methods. Endoamylolytic activity on amylose−azure substrate was also evaluated. BPNPG7 and PNPG5 chromogenic substrates were used for α- and β-amylase activities, respectively. The results showed that methods that depend on the use of thermal treatment or on inhibitors to inactivate one of the enzymes were not adequate. The use of *p*-nitrophenol derivatives seemed to be the most specific, reproducible, and easiest method employed for single α- and β-amylases activities determination in complex tissues. The DNS and iodometric methods can be used only for initial screenings of total hydrolytic activity, because the nonspecific substrate used in these procedures allows the action of more than one enzyme simultaneously.

**KEYWORDS: Banana; fruit; ripening; amylases; enzyme activity; starch breakdown**

## INTRODUCTION

Unripe banana fruit has almost 20% starch which disappears during the ripening process concomitantly with the sucrose accumulation (*1*). The literature reports many enzymes and probably more than one pathway involved in this process. Amylases (*2, 3*), glucosidases (*2, 4*), phosphorylases (*5, 6*), sucrose-phosphate synthase (*7*), and sucrose synthase (*1, 8*) participate in the starch breakdown and sucrose synthesis during banana ripening. Amylases, glucosidases, and phosphorylases act differently on the same substrate, producing a mixture of oligosaccharides and soluble sugars that make the individual enzyme analysis inaccurate.

Literature on methods for amylolytic activity determination is extensive and dates back to past centuries. Most of the enzymatic parameters related to quantitative determination of amylase have been investigated, and many changes been suggested. Incubation times have been extended or shortened; substrate types and concentrations have varied; biological source and starch preparation have been investigated; attempts were made to stabilize the substrate; and hydrolysis-detecting methods have been altered and compared. This shows a general dissatisfaction among researchers concerning the techniques applied for existing amylase assays (*9*). This is particularly true in events such as ripening, germination, and fermentation, in which there is a need for determination of amylase and glucosidase activities, not only in the presence of each other but in the presence of inhibitors and interfering compounds as well.

The composition of the extraction medium should avoid compounds such as phenols and enzymes as proteases or poliphenoloxidases that are released when the plant cell is ruptured, and may lead to preparations with low or even no activity. The use of complex compounds such as PVP or enzymes inhibitors in the extraction medium can avoid this (*10*). One enzyme may be protected or inactivated depending on the choice of the buffer compound and pH.

Another important point to consider in the methods of enzyme activity determination is the substrate itself. Starch is a heterogeneous macromolecule with varying molecular weight and branching degrees, what can deeply affect its susceptibility concerning the amylase attack speed. In addition, once in solution, it can retrograde, rending a starch less susceptible to

* To whom correspondence should be addressed. Fax: (55 11) 38154410. Phone: (55 11) 30913647. E-mail: fmlajolo@usp.br.

10.1021/jf011370p CCC: $22.00 
Published on Web 09/05/2002

**Table 1.** Some Methods for Amylolytic Activity Determination, Chemical Reaction, and Method of Detection[a]

| method | substrate | reaction principle | detection |
|---|---|---|---|
| | | Saccharogenic Method | |
| DNS | native or soluble starch | alcalin 3,5-dinitrosalicylic acid reduced to 3-amino-5-nitrosalicylic | spectrophotometrically |
| | | Chromogenic Methods | |
| Ceralpha | BPNPG7[b] | endolytic attack followed by α-glucosidase/glucoamilase action on part of *p*-NP, releasing *p*-NP | spectrophotometrically |
| Betamyl | PNPG5[c] | exolytic attack and α-glucosidase action on part of *p*-NP, releasing *p*-NP | spectrophotometrically |
| dyed starch | amylose–azure[d] | liberation of the blue color due to amylose hydrolysis | visually or spectrophotometrically |
| | | Amyloclastic Method | |
| iodometric | starch or amylose | amylose–$I_3^-$ complex hydrolysis, reducing the complex and blue color | spectrophotometrically |

[a] Adapted from the source: Duedahl-Olesen et al. Suitability and limitations of methods for characterization of activity of malto-oligosaccharide-forming amylases. *Carbohydr. Res.* **2000**, *329*, 111. [b] Blocked *p*-nitrophenol-maltoheptaose. [c] *p*-Nitrophenol-maltopentaose. [d] Amylose cross-linked to the remazolbrilliant blue dye (RBB).

amylase hydrolysis (*11*). The reproducibility of methods that use starch as the substrate is reputed to be poor, with large differences being associated with distinct operators or slight protocol variations (*12*). It has been recognized that neither starch nor glycogen are the ideal substrates, and that there should be a better defined one.

A desirable method is one that gives a direct measure of the number of cleaved bonds in the substrate, which can be readily converted to International Units of enzymatic activity (*13*). However, the assays based on measures of released reducing-sugar equivalents from starch are tedious, varying according to the nature of the employed substrate and its storage conditions, and many enzymes can release reducing sugars if the substrate is not specific.

To overcome some of these deficiencies, a group of dye-linked amylaceous substrates was developed and has been used for α-amylase assays. These substrates include starch or starch-fractions directly dyed, or dyed and cross-linked starch, and dyed amylose chemically modified to increase the solubility. These useful substrates have been superseded by defined maltosaccharide substrates, chemically bonded through the reducing-end glucose unit to a chromophore, which is usually *p*-nitrophenol (*p*-NP) or 2-chloro-*p*-nitrophenol (*14*).

The more commonly used methods for amylase activity determination have been classified into three main groups: amyloclastic, saccharogenic, and chromogenic (*15*) (**Table 1**; *16*), but the information obtained from these methods cannot reveal the predominant product formed from starch hydrolysis.

The amyloclastic methods measure the starch degradation by viscometric, turbidimetric, or iodometric procedures (*17*). The saccharogenic methods are a direct measure of the hydrolysis, once they determine the number of formed reducing ends (*18*), and the chromogenic methods are based on releasing of soluble dye linked to the substrate (*13, 15*).

It is noteworthy that the information about enzymatic type and extent of activity profile in fruits is mostly based on methods of low specificity, such as reducing or amyloclastic, although recent works have used chromogenic substrates as starch azure, faced to the roll of enzymes present in plant extracts (*2, 19*). By investigating enzyme expression during ripening, it was observed that sometimes the appearance of RNA or protein shown by blotting did not have a correspondence to enzymatic activity, due to inadequacies of the method. Consequently, most of the results are dubious and hardly reproducible, which reinforces the necessity of using more accurate and specific methods.

As part of a project involving the investigation of banana starch breakdown, the aim of this work was to apply different methods of amylolytic activity determination in fruits and compare their efficiency of measuring the α- and β-amylase activities separately. Methodology details and elimination of interference present in complex tissues are also discussed.

## MATERIALS AND METHODS

**Plant Materials.** Banana fruit (*Musa acuminata* AAA, cv. Nanicão) was collected at pre-climacteric stage (110 days after anthesis) and stored at a controlled temperature (18 °C). It was sampled daily during this period until full ripening was achieved. Mango (*Mangifera indica* cv. Keitt; 123 days after anthesis) and pear (*Pyrus communis* cv. d'Anjou; ready to eat) fruits and grains of corn-cop (*Zea mays*) were acquired at central markets. Samples were immediately rinsed, peeled (except corn), sliced, or threshed in liquid nitrogen and stored at −80 °C. The stage of ripening of the fruits was characterized by the starch content and enzymatically determined as described by Cordenunsi and Lajolo (*1*).

**Enzyme Extraction and Assays.** Part of frozen samples was ground in 5 vol (w/v) of 50 mM Hepes−NaOH pH 7.0, containing 20 mM cysteine, 1% (w/v) PVP-40, and 1 mM benzamidine, centrifuged at 4 °C for 40 min at 10 500*g*. The supernatant constituted the enzymatic extract. When necessary, enzyme extracts were desalted by dialysis through a molecular porous dialysis membrane (Spectra/Por Membrane, MWCO 12−14.000 from Spectrum, Houston, TX) or by a Sephadex G-25 Hytrap (Pharmacia) desalting column. The extracts of mango and pear were prepared as described for banana, except for corn, where 50 mM sodium acetate buffer (pH 5.5) was used in the extraction medium (*15*). Total protein content was quantified by the Bradford method (*20*) using BSA as standard.

**Total Hydrolytic Activity.** *Modified DNS Assay (21).* Enzymatic extract was desalted by Sephadex G-25 Hytrap (Pharmacia) desalting columns previously equilibrated with dialysis buffer (20 mM Hepes−NaOH pH 7.0 containing 1 mM benzamidine). An aliquot (100 μL) of freshly desalted extract was incubated with 100 μL of a 1% potato starch solution, previously boiled for 10 min. The reaction was carried out at 37 °C for 30 min, stopped at 95 °C for 15 min with DNS solution (100 μL), and diluted with 900 μL of distilled water. The released reducing sugars were determined at 540 nm using maltose as standard. One unit of enzyme releases 1 mmol of maltose per 3 min at 37 °C.

*Iodometric Assay (22).* A 100-μL aliquot of enzyme extract was incubated with 1 mL of stable phosphate-buffered starch (pH 7.0) at 37 °C for 35 min. The reaction was stopped with 1 mL of 0.01 N iodine solution. The activity was estimated by measuring (at 660 nm) the blue color developed when the residual starch and iodine were linked. One unit of enzyme activity was arbitrarily defined as the enzyme present in 1 mL of sample that hydrolyzes 1 mg of potato starch solution at 37 °C for 15 min.

**α-Amylase Activity.** *Adapted Amylose−Azure Assay (23).* Amylose−azure (36 mg/mL) was suspended in 20 mM Hepes−NaOH buffer (pH 7.0) containing 0.3% NaCl (w/v). This substrate was incubated at 37 °C for 15 min before being added to 200 μL of enzyme extract (1:1). The reaction was carried out at 37 °C for 1 h and stopped with 160 μL of acetic acid (18%, v/v). Samples were centrifuged at 8000*g* for 15 min in order to pellet the remaining insoluble substrate. The supernatant absorbance was read at 595 nm. One unit of α-amylase activity releases 1 μg of soluble dye (RBB, remazolbrilliant blue) per gram of banana pulp per min. The calculated activity was based on a standard curve of RBB (Sigma Chemical Co.).

*BPNPG7 Assay (13).* The substrate mixture, BPNPG7 (benzylidene-blocked *p*-nitrophenyl maltoheptaoside reagent; Megazyme, Ireland), was previously equilibrated at 30 °C for 5 min. Enzyme extract (50 μL) was incubated with 50 μL of substrate at 30 °C for 60 min. The reaction was stopped by adding 1% (w/v) Trizma base (750 μL, pH >10) and the developed color was read at 410 nm. A calibration curve was prepared with *p*-nitrophenol in 1% Trizma base. The activity was expressed in μmol of *p*-nitrophenol released per min of incubation under the defined assay conditions.

***β*-Amylase Activity.** *PNPG5 Assay (24).* The same conditions applied for α-amylase assay (BPNPG7) were used in this case, except that PNPG5 (*p*-nitrophenyl-α-D-maltopentaoside; Megazyme, Ireland) was used as the substrate mixture instead.

## RESULTS AND DISCUSSION

**Extraction Medium.** High levels of phenolic compounds and correlated enzymes present in plant tissue make it difficult to proceed with the enzyme extraction with full activity. It demands an extraction medium with effective protective agents against them. Garcia and Lajolo (*2*) optimized a specific extraction medium for banana fruit that contains phosphate buffer, soluble PVP-40, and cysteine. In the present work, Hepes−NaOH buffer replaced the phosphate buffer, because the latter could be a source of phosphorus for an undesirable phosphorylase activity, and benzamidine was included as a protease inhibitor. Tris−HCl buffer was not considered here because it could inhibit the α-glucosidase present in the substrate mixtures used for the assay of α- and *β*-amylases using the chromogenic substrates BPGNP7 and BPNPG5, respectively. Extraction conditions were considered adequate to obtain full enzyme activity from all ripening stages covered by the experiment.

To detect the optimum pH for amylase activities using different methods, crude extracts were desalted in buffers covering a pH range from 6.0 to 7.5. The highest activity values were achieved with pH 6.5 for all methods.

**Desalting by Dialysis or Molecular Exclusion?** To use the DNS method (*21*) for total hydrolytic activity determination, samples were desalted through a molecular porous dialysis membrane or by a Sephadex G-25 desalting column (Hytrap-Pharmacia), and the results were compared. It was observed that the desalting process using Sephadex G-25 was more efficient than conventional dialysis, as almost no extract background and very low sugar concentration were detected. The responses obtained for the activity assay of the extracts desalted through molecular exclusion were clearly different as shown in **Figure 1**. Whereas the extracts desalted through Sephadex G-25 presented an increasing activity at 4 days after harvest, dialyzed extracts showed an almost flat profile. An explanation for this difference would be an inhibitory effect of the sugars accumulation during ripening or of other low-molecular-weight compounds on amylolytic activity, which could not have been efficiently removed by dialysis. Another possibility would be the time needed for cleaning samples. Molecular porous dialysis is a cheaper process, but it is time-consuming. Despite the use of protease inhibitors and of low temperature, a deleterious effect of proteases action on amylolytic activity cannot be discarded. Additionally, the desalting process through Sephadex G-25 column decreased the pectin interference, which is abundant in ripening banana.

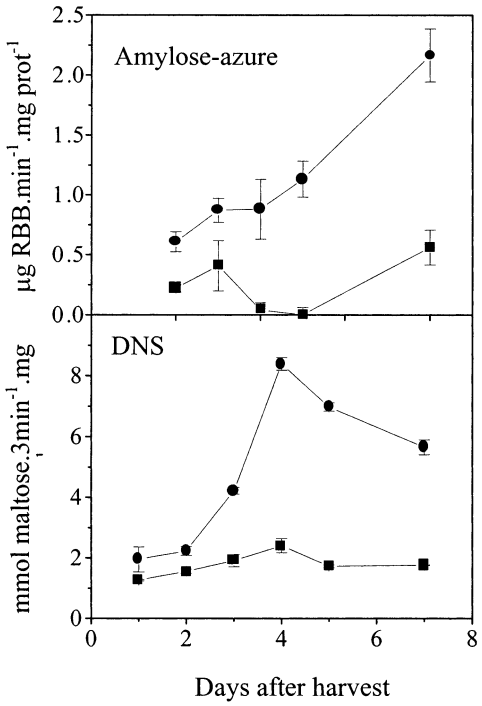


**Figure 1.** Total hydrolytic (DNS method) and endoamylolytic (amylose–azure) activities of banana extracts desalted by Sephadex G-25 Hytrap column (●) and by molecular porous dialysis membrane (■). Points represent the means ± SE of 3 replicates.

**Amylolytic Activity and the Interference of Other Enzymes.** Alpha and *β*-amylases have been detected and purified (*25*) in several plant species, mainly cereals. Unless there is a specific substrate to measure α- or *β*-amylase activity, it is necessary to inactivate one of the enzymes, especially in crude extracts. The commonly used procedures are based on selective inactivation of α-amylase by EDTA (*2, 26*) or inactivation of *β*-amylase by thermal treatment or $HgCl_2$ (*27, 28*). The specificity of the thermal treatment is questionable, because there are heat labile α-amylases from some plant species which could be inactivated by heat (*29, 30*). Another limitation could arise from the vegetal material being used. As fruits seem to have a significant *β*-amylase activity (*3*), any method for assay of α-amylase must be free from interference by *β*-amylase, if no specific substrates are used. In this way, any remaining activity of an enzyme originally present in excess would dramatically affect the results, leading to a unsuitable interpretation of the data. On the basis of this statement the efficiency of thermal treatment and inhibitors were tested for the evaluation of specific activity in bananas.

Thermal treatment (70 °C/15 min) was developed for cereal amylases, and its effect on α-amylase activity from other sources, such as fruits, is unknown. Literature reports indicate that α-amylase activity in bean (*26*), wheat (*31*), and corn (*32*) extracts were reduced by 80 to 100% after heat treatment. On the other hand, MacGregor et al. (*33*) and Doehlert and Duke (*34*) related a total inactivation of *β*-amylase through heat treatment in barley extracts, with no alteration of the α-amylase properties. In this way, to access the effect of heat treatment on some of the enzymes that can contribute to starch degradation in banana, total amylolytic and α-amylase activities were evaluated before and after incubation of the extracts at 70 °C for 15 min.

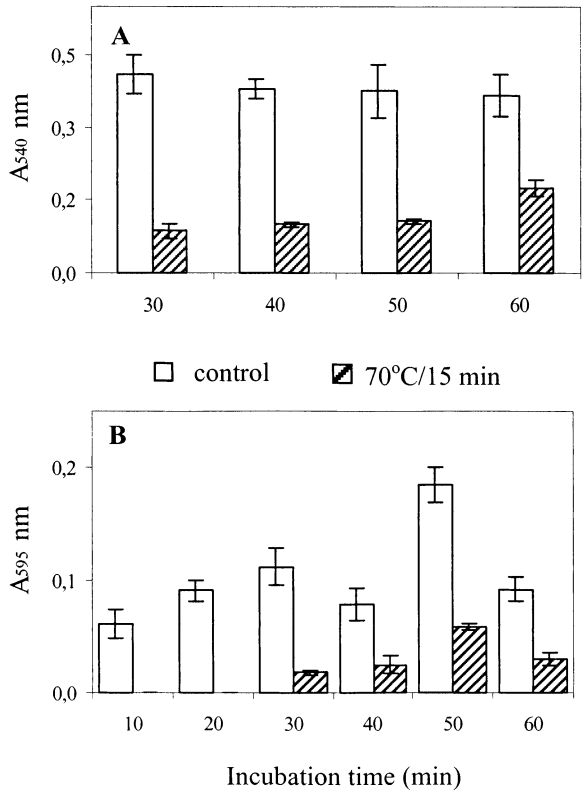


**Figure 2.** Effect of thermal treatment of crude banana extracts on (A) total hydrolytic activity (DNS method) and (B) α-amylase activity (amylose–azure), determined in different incubation times. Bars are means ± SE of 3 replicates.

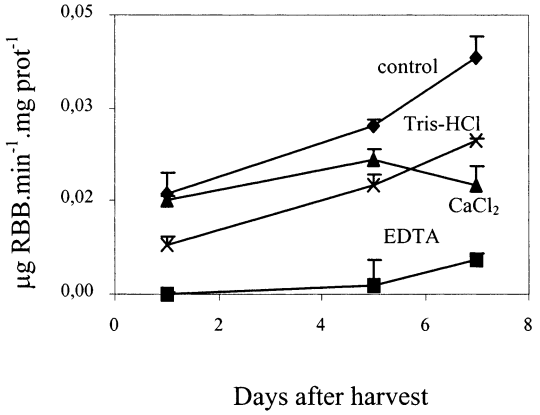


**Figure 3.** Effect of EDTA (30 mM), Tris–HCl (0.15 M), and $CaCl_2$ ($10^{-1}$ mM) on endoamylolitic activity (amylose–azure) in banana extracts. Points are means ± SE of 3 replicates.

**Figure 2** illustrates that thermal treatment affected both total hydrolytic (DNS method) and α-amylase (amylose−azure method) activities in banana extracts by decreasing their values significantly by 70% and 80%, respectively. This suggests that *β*-amylase might be responsible for almost 70% of total hydrolytic activity, or that other hydrolases including α-amylase have been inactivated. Use of the thermal treatment is not recommended for *β*-amylase inactivation in banana extracts, because α-amylase activity can be affected.

EDTA has been largely used as an α-amylase inhibitor by chelating the calcium present in the enzyme molecules altering their stability (*26, 30*), and $HgCl_2$ treatments have been used for *β*-amylase inactivation, but there are controversies about the specificity and efficiency of these inhibitors (*26, 35*).

**Figure 3** shows that endoamylolytic activity on amylose−azure increases during banana ripening at the same time most starch is degraded. The extracts treated with Tris−HCl had a profile similar to that of the controls, but a 35% activity reduction was observed. This result indicates that Tris−HCl, a probable inhibitor of α-glucosidases (*2*), partially inhibited either α-amylase activity or most α-glucosidase activity, or both. Because crude extracts have been used in the assays, one cannot ignore the presence of α-glucosidases. Therefore, conclusions can arise from this observation. Both Tris−HCl and amylose−azure might not be specific, the former as α-glucosidases inhibitor and the latter as α-amylase substrate. An assay in the presence of EDTA was performed to check the specificity of amylose−azure as an amylase substrate, which proved to be a potent inhibitor of α-amylase activity in banana extracts (*2*). Also shown in the same figure, the endoamylolytic activity was 87−100% reduced in the EDTA-treated extracts. On the basis of **Figure 3** one can conclude that most amylolytic activity observed in control extracts corresponds to α-amylase activity. The absence of calcium ions in the extraction medium probably contributed to the susceptibility of α-amylase to EDTA. The $CaCl_2$-treated extracts had almost the same activity as that of the control, but a greater reduction of endoamylolytic activity in the last ripening stages can be seen, when a high soluble pectin content is also released probably reacting with calcium ions and producing a gel consistence. It was also observed that this gel interferes with the activities determination, probably as a result of the combinatory effects of chelating of calcium ions, entrapment of substrate, or interactions with proteins. It can be considered that, in the absence of chelating agents, there are enough calcium ions linked to α-amylase to provide its full activity. Another important point is that α-amylase assay in pectin-rich tissue can be affected by its solubilization during development and ripening which addresses the importance of pectin removal on $Ca^{2+}$ supplementation.

Concerning the uncertainty and controversy observed in several related works, it is recommendable to use a general method for total hydrolytic activity determination, and to use more specific substrates for differentiation of α- and *β*-amylase activities which are better than any other kind of indirect determination.

**Enzymes Responses to BPNPG7 and PNPG5 Substrates and Comparison to Other Methods.** A compilation of results for amylase activity obtained by different methods in banana extracts is shown in **Figure 4**. Note that the expected increase in the hydrolytic activity during fruit ripening is confirmed in all tested methods and is parallel to the starch breakdown profile.

If the iodometric method is considered to measure mostly α-amylase activity, the decreasing slope found in its profile can be attributed mainly to α-amylase activity, because this behavior does not appear in the DNS profile where other hydrolytic enzymes also must be involved. Because α-amylase is the enzyme that hydrolyzes starch the fastest (*36*), the specificity of the iodometric method depends on the incubation time. The minimum incubation time applied would reflect mostly the α-amylase activity. As the profile obtained for hydrolytic activity by iodometric method was similar to that obtained with the BPNPG7 substrate, it can be implied that the incubation time used was adequate for most banana α-amylase activity measurement. It is important to reinforce that the adopted saccharogenic and iodometric methods were developed to suit human saliva, serum, and urine samples, where *β*-amylase and glucoamylase are not present. In this way, there should be a special concern about the specificity of interference when using fruit extracts.

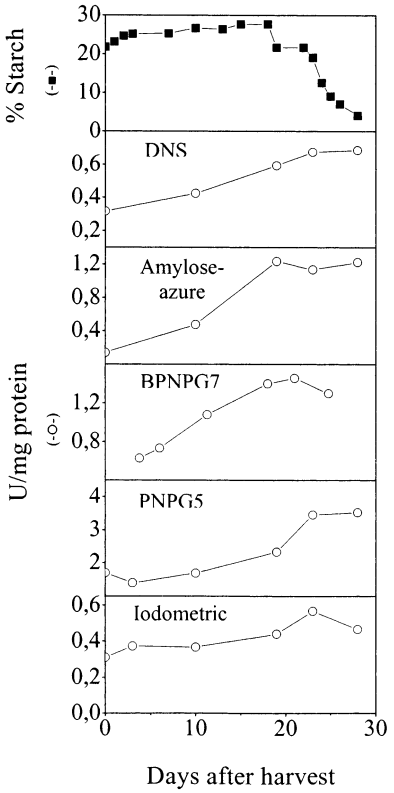


**Figure 4.** Amylolytic activity on different substrates versus starch breakdown during banana ripening. Points are means of 3 replicates of specific activity (U/mg protein). Total hydrolitic activity was analyzed by DNS (mmol maltose per 3 min per mg prot) and iodometric methods (mg hydrolyzed starch per mL per 15 min per mg prot); endoamylolitic activity by amylose–azure ($\mu$g RBB per min per mg prot); α-amylase activity by BPNPG7 ($\mu$mol *p*-nitrophenol per min per mg prot); and $\beta$-amylase activity by PNPG5 ($\mu$mol *p*-nitrophenol per min per mg prot).

Both α-amylase chromogenic assays (amylose−azure and BPNPG7) show increasing α-amylase activity **(Figure 4)**, although a better response was observed when *p*-nitrophenol derivative substrate was used. The BPNPG7 method is straightforward and can be indicated for routine tests.

The α-amylase activity profile on amylose−azure shows a more prominent peak when compared to that of BPNPG7 assay. As amylose−azure is a nonblocked substrate, it can suffer the action of other enzymes different from α-amylases, such as glucosidases. A commercial sample of α-glucosidase without α-amylase contamination was tested on amylose−azure substrate and showed a nonnegligible activity (data not shown). So, considering a possible interference of endogenous banana α-1,4 glucosidase activity on amylose−azure substrate, the method will not quantify only α-amylase activity.

The possibility of applying BPNPG7 and PNPG5 methods, which are considered specific methods for α- and $\beta$-amylases, respectively, is very attractive when dealing with complex extracts such as those obtained from banana fruit. **Figure 5** shows the results obtained when other fruits such as mango and pear, and seeds such as green corn, were analyzed. The presence of both α and $\beta$-amylases is revealed in each sample, and $\beta$-amylase activity seems to be higher than the α-amylase activity, in opposition to previous results that were obtained through nonspecific methods and suggesting the participation of this enzyme on starch degradation. It should be pointed out that the high $\beta$-amylase activity found for corn extracts justifies the difficulty in proceeding with the comparative analyses with

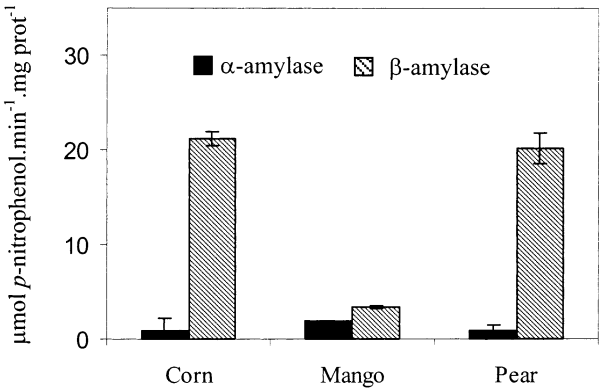


**Figure 5.** Alpha and $\beta$-amylases activities on BPNPG7 and PNPG5 substrates, respectively, in corn, mango, and pear extracts. Bars are means ± SD of 3 replicates.

other vegetal sources such as fruits, which have been submitted to methods previously developed for cereals.

However, before PNPG5 can be used as a specific substrate for $\beta$-amylase, a number of factors must be considered. In cereals, both α-glucosidase and α-amylase released *p*-nitrophenol from PNPG5, though at a very much slower rate (*24*). On the other hand, McCleary and Codd (*24*) concluded that the effect of the endogenous α-glucosidase on PNPG5 $\beta$-amylase assay was negligible. Although banana α-glucosidases have already been purified (*37*), there is not yet information available about their action on PNPG5.

The other factor to be considered when the PNPG5 reagent is being used is the possibility of interference by α-amylase, which can be overcome by a correction factor for $\beta$-amylase activity obtained with the activity of purified α-amylase measured on BPNPG7 (*24*). Once the purified banana α-amylase is obtained, it will be possible to evaluate its action rate on PNPG5 substrate and also consider some information about its different affinity for substrates of low molecular weight in order to draw conclusions about the specificity of PNPG5 when used to determine $\beta$-amylase activity. Not only banana α-amylase, but also endogenous α-glucosidases, activities on PNPG5 ought to be analyzed afterward. On the basis of our results and on other authors reports, it can be taken into account that PNPG5 is a reasonable substrate for $\beta$-amylase assays.

## CONCLUSIONS

The iodometric assay seems to be more sensitive when compared to the DNS method, detecting the hydrolytic activity even in nondesalted extracts. The chromogenic method using amylose−azure is a useful device to determine endoamylolitic activity in banana extracts and probably in other fruits, although the issue of α-1,4 glucosidase interference should not be disregarded.

The Sephadex G-25 desalting method proved to be more efficient to remove the low-molecular-weight compounds from banana extracts, favoring the total hydrolytic activity determination submitted to both DNS and iodometric procedures, besides showing more reliable results.

The use of EDTA and $HgCl_2$ or heat to inactivate α- and $\beta$-amylase activities, respectively, are not recommended for fruit extracts because of their low specificity. Therefore, more specific reagents such as *p*-NP derivative substrates can be a valuable alternative for the single analysis of α- or $\beta$-amylases.

Making the appropriate controls and considering the method limitations, the BPNPG7 and PNPG5 reagents can be used in the routine estimations of α- and $\beta$-amylases of banana extracts

and in tissues where there are changes due to synthesis or activation of enzymes during development or ripening.

In accordance with the previous discussion, when no specific substrates are available to measure α- and β-amylases activities, although other methods can be considered, care must be taken when interpreting the results.

## ACKNOWLEDGMENT

Appreciation is extended to Dr. João R. O. do Nascimento for helpful discussions.

## LITERATURE CITED

(1) Cordenunsi, B. R.; Lajolo, F. M. Starch breakdown during banana ripening: sucrose synthase and sucrose phosphate synthase. *J. Agric. Food Chem.* **1995**, *43* (2), 347−51.

(2) Garcia, E.; Lajolo, F. M. Starch transformation during banana ripening: the amylase and glucosidase behavior. *J. Food Sci.* **1998**, *53* (4), 1181−1186.

(3) 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.

(4) Konishi, y.; Harada, M.; D' Innocenzo, M.; Lajolo, F. M. Purification and characterization of soluble and cell wall-bound acid α-glucosidases of ripe yellow banana pulp. *J. Appl. Glycosci.* **2001**, *48* (1), 19−25.

(5) Arêas, J. A. G.; Lajolo, F. M. Starch transformation during banana ripening: I - The phosphorylase and phosphatase behavior in *Musa acuminata*. *J. Food Biochem.* **1981**, *5*, 19−37.

(6) Mota, R. V. da; Lajolo, F. M.; Ciacco, C.; Cordenunsi, B. R. Composition and Functional Properties of Banana Flour from Different Varieties. *Starch/Staerke* **2000**, *52*, 63−68.

(7) Nascimento, J. R. O.; Cordenunsi, B. R.; Lajolo, F. M.; Alcocer, M. J. C. Banana sucrose-phosphate synthase gene expression during fruit ripening. *Planta* **1987**, *203*, 283−288.

(8) Nascimento, J. R. O.; Cordenunsi, B. R.; Lajolo, F. M. Sucrose synthase activity and expression during development and ripening in bananas. *J. Plant Physiol.* **2000**, *156*, 605−611.

(9) Hall, F. F.; Culp, T. W.; Hayakawa, T.; Ratliff, C. R.; Hightower, N. C. An Improved Amylase Assay Using A New Starch Derivative. *Am. J. Clin. Pathol.* **1970**, *53* (5), 627−634.

(10) Rhodes, M. J. C. The extraction and purification of enzymes from plant tissues. In *Regulation of Enzyme Synthesis and Activity in Higher Plants*; Smith, H., Ed.; Academic Press: London, 1977; pp 245−267.

(11) Manners, D. J. Starch. In *Biochemistry of Storage Carbohydrates in Green Plants*; Dey, P. M., Dixon, R. A., Eds.; Academic Press: London, 1985; pp 149−203.

(12) Shephard, H. L.; Stuchbury, T.; Naylor, R. E. L. Locating sources of error in α-amylase assays and refining the methodology. *Seed Sci. Technol.* **1997**, *25*, 351−359.

(13) McCleary, B. V.; Sheehan, H. Measurement of cereal α-amylase: A new assay procedure. *J. Cereal Sci.* **1987**, *6*, 237−251.

(14) McCleary, B. V. New chromogenic substrates for the assay of α-amylase and (1−4)-beta-D-glucanase. *Carbohydr. Res.* **1980**, *86* (1), 97−104.

(15) Okuda, K.; Naka, K.-I.; Shimojo, N. In *Handbook of Amylases and Related Enzymes, Their Sources, Isolation Methods, Properties and Applications*; The Amylase Research Society of Japan, Ed.; Pergamon Press: Oxford, 1988; pp 178−185.

(16) Duedahl-Olesen, L.; Pedersen, L. H.; Larsen, K. L. Suitability and limitations of methods for characterization of activity of malto-oligosaccharide-forming amylases. *Carbohydr. Res.* **2000**, *329* (1), 109−119.

(17) Ohnishi, M.; Hiromi, K. In *Handbook of Amylases and Related Enzymes, Their Sources, Isolation Methods, Properties and Applications*; The Amylase Research Society of Japan, Ed.; Pergamon Press: Oxford, 1988; pp 10−12.

(18) Sinner, M.; Puls, J. Non-Corrosive Dye Reagent For Detection Of Reducing Sugars In Borate Complex. Ion-Exchange Chromatography. *J. Chromatogr.* **1978**, *156* (1), 197−204.

(19) Medina-Suárez, R.; Manning, K.; Flecher, J.; Aked, J.; Bird, C. R.; Seymour, G. B. Gene expression in the pulp of ripening bananas−Two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis of in vitro translation products and cDNA cloning of 25 different ripening mRNAs. *Plant Physiol.* **1997**, *115* (2), 453−461.

(20) Bradford, M. M. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248−254 DOI 10.1006/abio.1976.9999).

(21) Bernfeld, P. Amylases, α and β. *Methods Enzymol* **1955**, *1*, 149−158.

(22) Caraway, W. T. A stable starch substrate for the determination of amylase in serum and other body fluids. *Am. J. Clin. Pathol.* **1959**, *32* (1), 97−99.

(23) Hagenimana, V.; Vèzina, L.-P.; Simard, R. E. Distribution of amylases within sweet potato (*Ipomoea batatas* L.) root tissue. *J. Agric. Food Chem.* **1992**, *40*, 1777−1783.

(24) McCleary, B. V.; Codd, R. Measurement of β-amylase in cereal flours and commercial enzyme preparations. *J. Cereal Sci.* **1989**, *9*, 17−33.

(25) Silvanovich, M.; Hill, R. Affinity Chromatography Of Cereal α-Amylase. *Anal. Biochem.* **1976**, *71* (2), 430−433.

(26) de Morais, G. A.; Takaki, M. Determination of amylase activity in cotyledons of *Phaseolus vulgaris* L. cv. Carioca. *Braz. Arch. Biol. Technol.* **1998**, *41*, 17−25.

(27) Safwat, M. S. A.; Mahmoud, S. A. Z.; Attia, R. M.; Abd-Elnasser, M.; Ali, F. S. 1983; In Salieri et al. (below), *Anal. Chim. Acta* **1995**, *300*, 287−292.

(28) Salieri, G.; Vinci, G.; Antonelli, M. L. Microcalorimetric study of the enzymatic hydrolysis of starch: an α-amylase catalyzed reaction. *Anal. Chim. Acta* **1995**, *300*, 287−292.

(29) Ranwala, A. P.; Miller, W. B. Purification and characterization of an endoamylase from tulip (*Tulipa gesneriana*) bulbs. *Physiol. Plant.* **2000**, *109* (4), 388−395.

(30) Witt, W.; Sauter, J. J. Purification and properties of a starch granule-degrading α-amylase from potato tubers. *J. Exp. Bot.* **1996**, *47* (304), 1789−1795.

(31) Battershell, V. G.; Henry, R. J. High-performance liquid chromatography of α-amylases from germinating wheat and complexes with the α-amylase inhibitor from barley. *J. Cereal Sci.* **1990**, *12*, 73−81.

(32) Warner, D. A.; Grove, M. J.; Knutson, C. A. Isolation and characterization of α-amylases from endosperm of germinating maize. *Cereal Chem.* **1991**, *68*, 383−390.

(33) Macgregor, A. W.; Laberge, D. E.; Meredith, W. O. S. Separation of α-amylase and β-amylase enzymes from barley malt by ion-exchange chromatography. *Cereal Chem.* **1971**, *48*, 490−498.

(34) Doehlert, D. C.; Duke, S. H. Specific determination of α-amylase activity in crude plant extracts containing β-amylase. *Plant Physiol.* **1983**, *71*, 229−234.

(35) Robbins, D. J.; Egan, B. Failure of mercuric chloride to selectively inhibit β-amylase in sorghum malt. *J. Inst. Brew.* **1992**, *98*, 383−385.

(36) Whitaker, J. R. *Principles of Enzymology for the Food Sciences*, 2 ed.; Marcel Dekker: New York, 1994; 625 p.

(37) Konish, Y.; Kitazato, S.; Nakatani, N. Partial purification and characterization of acid and neutral α-glucosidases from preclimateric banana pulp tissues. *Biosci. Biotechnol. Biochem.* **1992**, *56* (12), 2046−2051.

**Received for review October 15, 2001. Revised manuscript received June 10, 2002. Accepted June 19, 2002. We thank FAPESP-SP-Brazil for the financial support and grant.**

JF011370P