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Specific Determination of α-Amylase Activity in Crude Plant Extracts Containing β-Amylase[1]

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ABSTRACT

The specific measurement of α-amylase activity in crude plant extracts is difficult because of the presence of β-amylases which directly interfere with most assay methods. Methods compared in this study include heat treatment at 70°C for 20 min, $HgCl_2$ treatment, and the use of the α-amylase specific substrate starch azure. In comparing alfalfa (*Medicago sativa* L.), soybeans (*Glycine max* [L.] Merr.), and malted barley (*Hordeum vulgare* L.), the starch azure assay was the only satisfactory method for all tissues. While β-amylase can liberate no color alone, over 10 International units per milliliter β-amylase activity has a stimulatory effect on the rate of color release. This stimulation becomes constant (about 4-fold) at β-amylase activities over 1,000 International units per milliliter. Two starch azure procedures were developed to eliminate β-amylase interference: (a) the dilution procedure, the serial dilution of samples until β-amylase levels are below levels that interfere; (b) the β-amylase saturation procedure, addition of exogenous β-amylase to increase endogenous β-amylase activity to saturating levels. Both procedures yield linear calibrations up to 0.3 International units per milliliter. These two procedures produced statistically identical results with most tissues, but not for all tissues. Differences between the two methods with some plant tissues was attributed to inaccuracy with the dilution procedure in tissues high in β-amylase activity or inhibitory effects of the commercial β-amylase. The β-amylase saturation procedure was found to be preferable with most species. The heat treatment was satisfactory only for malted barley, as α-amylases in alfalfa and soybeans are heat labile. Whereas $HgCl_2$ proved to be a potent inhibitor of β-amylase activity at concentrations of 10 to 100 micromolar, these concentrations also partially inhibited α-amylase in barley malt. The reported α-amylase activities in crude enzyme extracts from a number of plant species are apparently the first specific measurements reported for any plant tissues other than germinating cereals.

The specific determination of α-amylase activity in crude plant extracts is difficult because of the presence of β-amylase activity in these tissues that directly interferes with most assay methods. The most commonly used procedure involves the selective inactivation of β-amylase by heating. This procedure is described by Briggs (4) and is based on the original observations of Schwarzer (23) which were elaborated on by Ohlsson (16) and Olson *et al.* (19). The procedure was developed for the determination of α-amylase activity in barley malt. β-Amylases are selectively inactivated by heating the malt to 70°C for 20 min in the presence of Ca^{2+}. α-Amylase, which is heat stable in barley malt, is then assayed by reducing power production or starch-iodine color disappearance. Whereas this procedure is used routinely in the malting industry, it has also been applied to tissues and plant species where the heat stabilities of the constitutive α- and β-amylases are unknown (1, 5, 12, 24). In fact, several investigators have shown that in several plant species, α-amylases are heat labile under these conditions (1, 17, 18).

Another procedure involves the putative selective inactivation of β-amylases by the addition of low concentrations of $HgCl_2$, (10–12). This method is clearly dependent on the selective $HgCl_2$ inhibition of β-amylase, a premise that has not been adequately tested.

A clinical method for the determination of α-amylase was developed by Rinderknecht *et al.* (21), using a chromogenic substrate specific to α-amylase. This substrate is potato starch derivatized with RBB[4] commercially available as starch azure or amylopectin azure. This insoluble substrate is suspended in buffer and α-amylase action results in the solubilization of colored fragments of the starch azure. After the assay is terminated, the unreacted substrate is removed by filtering or by centrifugation and the color in solution is used as an estimation of the α-amylase activity. This method was originally developed for use in health sciences, and because animals have no β-amylase, interference by β-amylase was not considered. The use of this and similar chromogenic substrates for the determination of α-amylase activity (25) has received widespread use in clinical applications; however, this method has received only occasional use in the plant sciences (6, 13, 14, 20). Previous plant studies using this assay have generally assumed that this substrate is specific for α-amylase and that β-amylase is not reactive. Bilderbach (3) demonstrated that the β-limit amylopectin azure, generated by the digestion of amylopectin azure with β-amylase, would release no color by further treatment with β-amylase, but would release color upon treatment with α-amylase. He also found that the presence of β-amylase had an interfering effect on the release of color from this substrate by α-amylase, but did not characterize this in detail. He presented the procedure as a qualitative rather than quantitive procedure for α-amylase determination. Apparently, no further characterization of this assay with plant samples has appeared in print, although the suggestion for the use of a similar chromogenic substrate in the automated analysis of α-amylase activity in barley malt has been made (15), without demonstrating the absence of β-amylase interference.

A method of commercial value is described in the American Society of Brewing Chemists, Manual of Methods (2) for use with malt, where excess amounts of β-amylase are added to an amylopectin solution generating the β-limit dextrin. An unknown sample containing α-amylase is then added to the mixture of β-

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[4] Abbreviation: RBB, Remazol Brilliant Blue.

amylase and β-limit dextrin. Any endogenous β-amylase is masked by the excess exogenous β-amylase and α-amylase is determined by the rate of change of starch-iodine color in the solution. This procedure was developed and validated by Sandstedt *et al.* (22) with barley malt. Although this nondestructive method is suitable for the specific α-amylase determination in any plant species, its use has apparently been limited to industrial applications.

This investigation was initiated by an effort to quantitate α-amylase activity in alfalfa tap roots, which contain high β-amylase activity (7). In the process, the aforementioned methods were compared with emphasis on the use of starch azure. Two new procedures with the starch azure were developed to nondestructively eliminate β-amylase interference.

MATERIALS AND METHODS

Plant Material. Alfalfa (*Medicago sativa* L., cv Vernal) roots, leaves, and nodules were obtained from greenhouse-grown plants maintained by procedures previously described (8). Soybeans (*Glycine max* [L.] Merr., cv Wells) were imbibed and germinated for 5 d in a manner similar to that previously described (9). Green barley malt (*Hordeum vulgare* L. cv Kluger) was supplied by the United States Department of Agriculture Barley and Malt Laboratory (Madison, WI) and immediately frozen. Sweet potato (*Ipomea batatas* [L.] Lam. var Puerto Rico) storage roots, tomato (*Lycopersicon esculentum* Mill.) fruits, spinach (*Spinacia oleracea* L.) leaves, and alfalfa seedling cotyledons were obtained at a local market. Potato (*Solanum tuberosum* L. var Russet Burbank) leaves and tubers, carrot (*Daucus carota* subsp. *sativus* [Hoffm.] Arcang. var Imperator) tap roots and leaves, sweet corn (*Zea mays* L. var Golden Wonder) leaves and developing kernels, garden bean (*Phaseolus vulgaris* L.), and white pine (*Pinus strobus* L.) needles were obtained from field-grown plants.

Preparation of Enzyme Extracts. Plant tissues were homogenized in a MSE 'homogeniser' (Measuring and Scientific Equipment, London, UK), adding 10 ml extraction buffer (30 mM ethylenediamine dihydrochloride KCl [pH 7.0], 3 mM $CaCl_2$, 3 mM β-mercaptoethanol, 20% v/v glycerol) per g of tissue fresh weight. The crude homogenate was filtered through 150-μm Teflon mesh to remove gross particulate matter. α-Amylase from *Bacillus subtilis* and β-amylase from sweet potato (both from Sigma Chemical Co.) in the extraction buffer were used for the characterization of the starch azure assay. The commercial β-amylase was dialyzed before use to eliminate $(NH_4)_2SO_4$ toxicity.

Enzyme Assays. Measurement of reducing power production by α- and β-amylases were performed using the alkaline dinitrosalicylic acid reagent as described by Doehlert *et al.* (7). An IU for α- and β-amylase activity is defined as the amount of enzyme necessary to generate 1 μmol maltose reducing power equivalent min^{-1}.

The starch azure assay of α-amylase activity was carried out by a modification of procedures described by Rinderknecht *et al.* (21) and Wahlefeld (25). A suspension was prepared by adding 2% (w/v) starch azure (Sigma) to a solution of 100 mM potassium succinate (pH 6.0) and 3 mM $CaCl_2$. This solution was heated slowly to boiling while stirring continuously. After boiling, the starch azure suspension was stirred continously until use. The starch azure suspension may be stored covered in a refrigerator and reheated with stirring before use. For the α-amylase assay, 5 ml of the starch azure suspension are placed in a small test tube in a constant temperature bath at 30°C. One ml of enzyme solution is added to the starch azure suspension and the solution mixed thoroughly. At a designated zero time, the assay mixture is mixed thoroughly and a 1-ml aliquot is removed and put into a sample tube containing 1 ml 20% (w/v) TCA. Additional aliquots are taken at 5, 10, 20, and 30 min from time zero. At the end of the 30-min assay, the tubes are centrifuged at about 2,800*g* for 20 min to pellet the unreacted starch azure and the A_{595} of the supernatant is determined on a spectrophotometer. We used a spectrophotometer with a spectrophotometric accuracy of ± 0.5% from 2 to 3 A_{595} (Pye Unicam SP8-100 double beam spectrophotometer) in our experimental assays. If additional time points are needed, 10 ml of starch azure is used, adding 2 ml of enzyme at zero time and again taking 1-ml aliquots at appropriate intervals. Enzyme activities are calculated by taking the change in A_{595} in the interval of 5 to 30 min and dividing by that time interval to obtain the average change in $A_{595} \cdot min^{-1}$. Inasmuch as the increase in A_{595} is nonlinear, it is necessary to use a uniform time interval although at higher activities, it is possible to use a shorter interval. The activity in $A_{595} \cdot min^{-1}$ can be converted into reducing power units (IU/ml) by multiplying by the appropriate calibration factor (Fig. 5).

In procedures involving mixed α- and β-amylase, enzyme activities were determined by reducing power production before mixing. In procedures involving the serial dilution of samples, samples were diluted with extraction buffer before assaying, and the data were normalized by multiplying the rates ($A_{595} \cdot min^{-1}$) by the dilution factors. In procedures involving the addition of excess β-amylase, a stock solution containing about 10,000 IU/ml β-amylase was prepared and a mixture of 50% sample and 50% (v/v) stock β-amylase prepared and assayed with starch azure, taking the β-amylase saturation and dilution into account during calculations.

Heat treatment of enzyme samples involved placing enzyme samples in a constant temperature water bath at 70°C for exactly 20 min as suggested by Briggs (4). Mercuric chloride studies were conducted by adding the specified concentration of $HgCl_2$ to all solutions including enzyme extracts for at least 20 min before assay.

RESULTS AND DISCUSSION

α-Amylase Production of Soluble Color from Starch Azure in the Presence of β-Amylase. Initial results using the starch azure assay indicated that no color was released by hydrolysis of starch azure by 586 IU/ml sweet potato β-amylase, while color was released by 0.082 IU/ml bacterial α-amylase (Fig. 1). Although

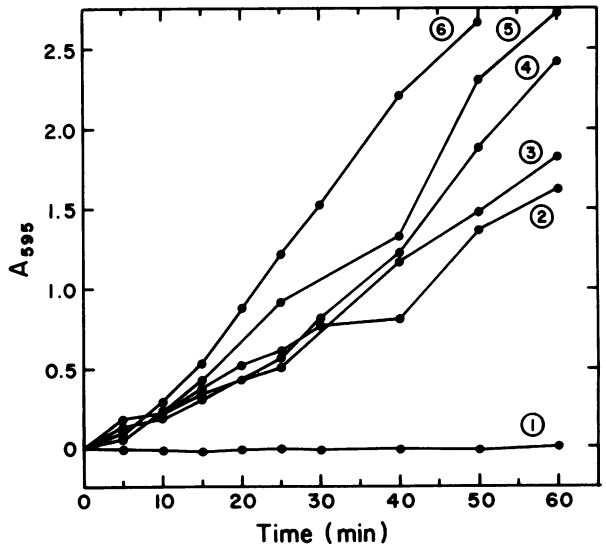

FIG. 1. The effects of α- and β-amylase on color solubilization from starch azure: (1), contains 586 IU/ml sweet potato β-amylase only; (2), contains 0.082 IU/ml bacterial α-amylase only; (3), contains 0.082 IU/ml β-amylase and 0.586 IU/ml α-amylase; (4), contains 0.082 IU/ml α-amylase and 5.86 IU/ml β-amylase; (5), contains 0.082 IU/ml α-amylase and 58.6 IU/ml β-amylase; and (6), contains 0.082 IU/ml α-amylase and 586 IU/ml β-amylase.

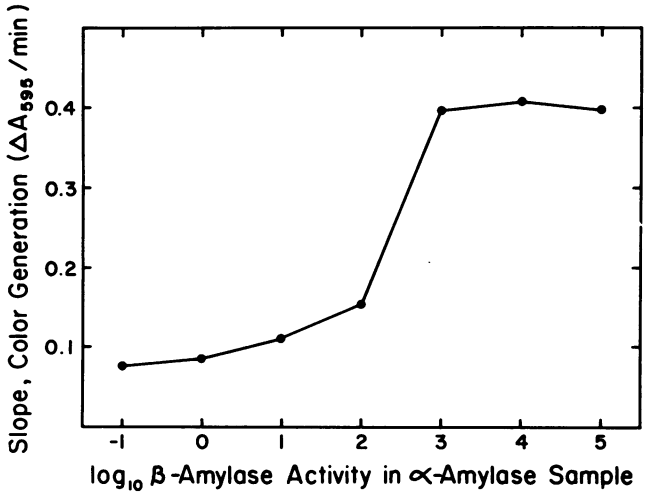

FIG. 2. Effect of increasing sweet potato β-amylase activity (IU/ml) on slope of color generation from starch azure by α-amylase. All assays contained 0.30 IU/ml bacterial α-amylase.

the addition of 0.586 and 5.86 IU/ml sweet potato β-amylase to 0.082 IU/ml α-amylase did not significantly change the rate of color appearance (based on *t* test of regressions of the interval 5 to 30 min), the addition of 58.6 and 586 IU/ml β-amylase did significantly increase the rate of color release (Fig. 1). The change in the slope of the progress curve for α-amylase release of color from starch azure is minimal with the addition of up to 10 IU/ml β-amylase (Fig. 2). Plotted on a logarithmic scale, the addition of increasing β-amylase caused a sigmoidal increase in the rate of color generation by α-amylase, reaching a maximal rate with the addition of about 1,000 IU/ml β-amylase. Additions of up to 100,000 IU/ml β-amylase produced no further increase in the rate of color generation. This is similar to the phenomenon observed by Sandstedt *et al.* (22) who found that β-amylase interference of the starch-iodine color disappearance assay reaches a maximum when adding increasing wheat endosperm β-amylase preparation to a barley malt α-amylase assay.

This information has led us to develop two procedures for the elimination of β-amylase interference on the starch azure assay. These involve either the dilution of a sample to reduce the endogenous β-amylase activity to below interfering levels, or the addition of exogenous β-amylase to increase endogenous β-amylase activity over saturation levels.

Dilution Procedure for Measuring α-Amylase Activity. This procedure involves the serial dilution of a sample to reduce the β-amylase activity to below interfering levels and then normalizing the rate to the original concentration. This procedure was tested by mixing a sample containing 0.30 IU/ml bacterial α-amylase with 400 IU/ml sweet potato β-amylase. Serial dilutions of this sample were made up to 10X dilution and the starch azure assay was run on each of these (Fig. 3A). These responses were then normalized by multiplying by the dilution factor (Fig. 3B). When the calculated α-amylase rates from these normalized slopes are plotted *versus* dilution factor, it is evident that a mixture of α- and β-amylase does not yield a linear response to dilution by the starch azure method. With increasing dilution, the β-amylase interference is reduced and the calculated starch azure α-amylase activity asymptotically approaches the actual α-amylase content of the sample, although in this case, even at 10X dilution the actual α-amylase content is still overestimated by 20% (Fig. 4).

This procedure has been applied to several plant tissues. Alfalfa tap roots have been shown to contain high β-amylase activity (7) and extracts produced a similar response to serial dilution as the standard (Fig. 4), as did alfalfa leaf and soybean cotyledon. Malted barley extract (diluted 100X) gave a linear response to dilution indicating that β-amylase interference is not significant in this tissue.

When using the dilution procedure for α-amylase determination it is important to establish a dilution curve as in Figure 4 to determine what dilution is necessary to obtain a reliable β-amylase determination. Although dilution will reduce β-amylase interference, with some tissues it may not eliminate it, resulting in an overestimation of α-amylase activity. Another disadvantage of this procedure is that in tissues containing very low α-amylase activity (*e.g.* soybean cotyledon) a substantial sample dilution may reduce the β-amylase activity to levels below those accurately measured by the starch azure method.

β-Amylase Saturation Procedure for Measuring α-Amylase Activity. This procedure involves the addition of excess β-amylase to a sample to increase endogenous β-amylase activities over levels which saturate β-amylase interference of starch azure hydrolysis by α-amylase. This procedure was originally developed by Sandstedt *et al.* (22) for use with the starch-iodine color disappearance assay and is probably also applicable to the viscosity assay (13). The addition of 1,000 to 10,000 IU/ml β-amylase is considered

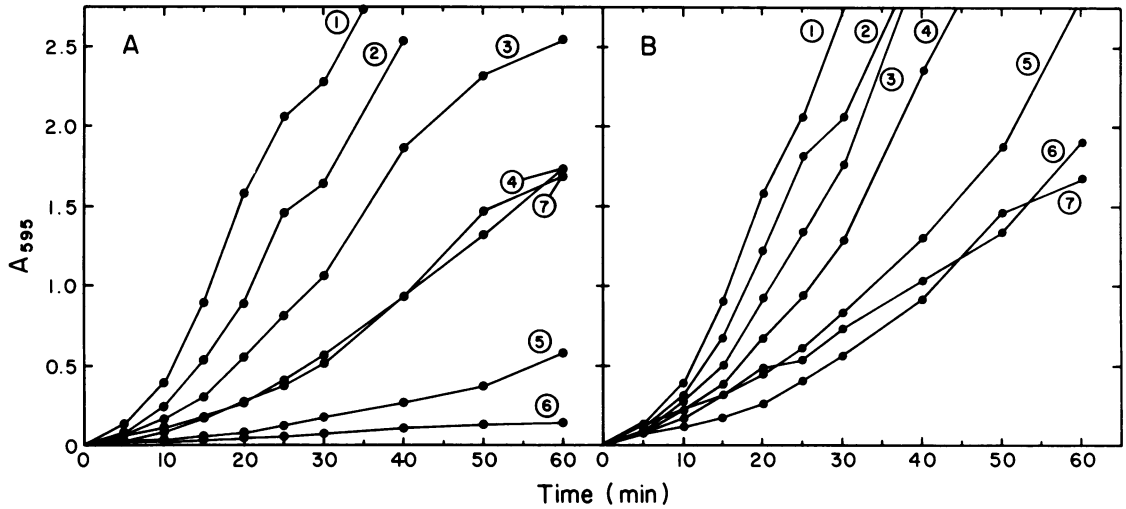

FIG. 3. Effects of serial dilution on color generation of a sample containing a mixture of bacterial α-and sweet potato β-amylase. A, Color generation of a serial dilution from a sample containing 0.066 IU/ml α-amylase and 400 IU/ml β-amylase: (1), full strength; (2), 1.25X dilution; (3), 1.65X dilution; (4), 2.5X dilution; (5), 5X dilution; (6), 10X dilution; (7), α-amylase only, 0.066 IU/ml, full strength. B, Data from A normalized to full strength.

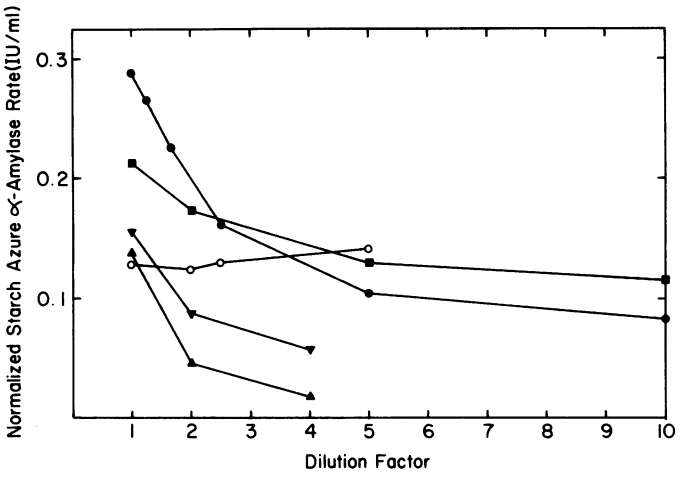

FIG. 4. Effect of dilution on calculated rate of α-amylase in enzyme samples. (●), Standard containing 0.066 IU/ml α-amylase and 400 IU/ml β-amylase; (■), alfalfa tap root preparation; (▼), alfalfa leaf preparation; (▲), soybean cotyledon preparation, rates shown multiplied by 10 to match scale, (○), malted barley preparation, rates and dilution factor multiplied by 0.01 to match scale.

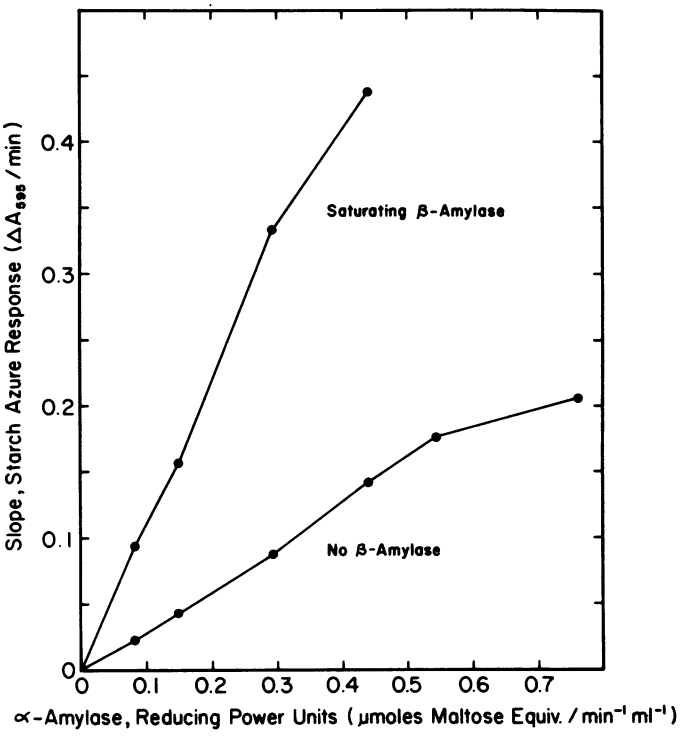

FIG. 5. Calibration curves for the conversion of rate of starch azure color solubilization to reducing power units with either no β-amylase or with saturating sweet potato β-amylase (about 1,400 IU/ml). Conversion factors of 0.298 A_{595} (no β-amylase) and 1.212 A_{595} (saturating β-amylase) per IU α-amylase activity were derived from 0 to 0.3 reducing power units.

sufficient for the saturation of β-amylase in this system (Fig. 2). Calibration curves were constructed to translate rates of color liberation from starch azure by α-amylase activity to conventional reducing power units (Fig. 5). Two calibrations were made, one with no β-amylase, one with saturating β-amylase. Both procedures yielded linear plots up to 0.3 IU/ml with slopes of 0.298 and 1.212 A_{595}/IU, respectively.

The dilution and β-amylase saturation procedures were compared with various plant samples (Table I). After the preparation

Table I. *Comparison of α-Amylase Activity in Crude Plant Extracts as Measured by the Starch Azure Method Using the Dilution Procedure and the β-Amylase Saturation Procedure*

Dilutions are given in parentheses. Each value represents the mean ± SD for three assays.

Sample	Dilution Procedure	β-Amylase Saturation Procedure
	IU/ml	
Alfalfa tap root	(10X) 0.0330 ± 0.0020	0.0562 ± 0.0031
Alfalfa leaf	(5X) 0.0220 ± 0.0028	0.0253 ± 0.0029
Soybean seedling cotyledon	(4X) 0.0084 ± 0.0032	0.0079 ± 0.0006
Soybean seedling root	(1X) 0.0029 ± 0.0007	0.0027 ± 0.0006
Malted barley	(200X) 13.07 ± 1.88	1.427 ± 0.219

Table II. *Comparison of Total and α-Amylase Activities in Crude Plant Samples before and after Heat Treatment at 70°C for 20 Minutes*

α-Amylase was determined by starch azure β-amylase saturation procedure in all samples except malted barley where the dilution procedure was used.

Sample	Before Heat Treatment		After Heat Treatment	
	Total amylase	α-Amylase	Total amylase	α-Amylase
	IU/g fresh wt			
Alfalfa tap root	1490.0	1.110	3.21	0.043
Alfalfa leaf	15.0	0.183	0.913	0.063
Alfalfa nodule	10.5	0.134	ND[a]	ND
Soybean seedling cotyledon	431.0	0.038	0.091	0.015
Soybean seedling root	6.38	0.013	0.051	0.006
Malted barley	953.0	125.0	120.0	113.0

[a] Not determined.

of crude plant extracts, appropriate dilutions were made according to the dilution considered necessary from the responses in Figure 4 to produce a maximal reduction in β-amylase interference. α-Amylase activities measured in soybean cotyledons and roots, and alfalfa leaves were not significantly different as measured by the two procedures. With malted barley, the rate estimated by the β-amylase saturation procedure was substantially lower than that from the dilution procedure. Addition of exogenous β-amylase preparation actually decreased the rate of color release in malted barley samples. This is attributed to inhibitory effects of the β-amylase preparation on the malted barley α-amylase. This inhibition was not eliminated by dialysis of the β-amylase preparation, nor was it observed with α-amylase preparations from any other tissue. Rates obtained with the two methods for alfalfa root differed by about 40%. Because of the high endogenous β-amylase in alfalfa root, the dilution procedure does not appear satisfactory and the β-amylase saturation rate is considered more reliable. Determinations of α-amylase by either of these procedures produced consistent results for most tissues, with SD of about 10%. In tissues with very low α-amylase activity, the reduction in accuracy of the assay is evident from the increase in SD. In these cases, it is probably more reliable to use the β-amylase saturation procedure, where the amplified rates provide more reliable estimations. Differences in α-amylase activity among various preparations of the same tissues from the same species were observed. These differences were due to plant to plant variation and differences in plant stage of growth (Fig. 4; Tables I, and II). It was not possible to determine whether debranching enzyme has an interfering effect on the starch azure assay. It is possible that this enzyme may also

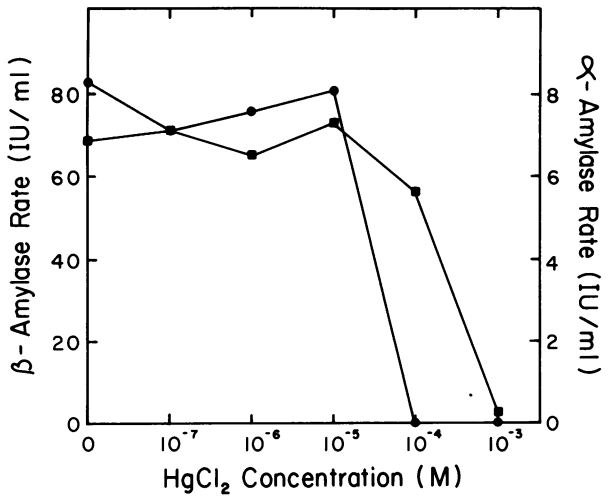

FIG. 6. Effect of $HgCl_2$ on: (●), β-amylase activity (as determined by reducing power production); (■), α-amylase activity in barley malt as determined by starch azure method.

Table III. *A Survey of Total Amylase Activity (as Determined by Reducing Power Production) and α-Amylase Activity (as Determined by the Starch Azure, β-Amylase Saturation Procedure) in Selected Organs of Some Economically Important Plant Species*

Sample	Total Amylase	α-Amylase
	IU/g fresh wt	
Alfalfa seedling cotyledon	14.0	0.061
Bush bean pod	2.77	0.726
Bush bean leaf	15.65	0.109
Bush bean stem	37.47	0.239
Sweet potato storage root	211.0	0.130
White pine leaf	0.766	0.134
Sweet corn kernels	2.34	0.039
Sweet corn leaf	0.328	0.023
Sweet corn stem	2.19	0.016
Potato leaf	0.219	0.189
Potato tuber	0.608	0.324
Spinach leaf	0.183	0.025
Carrot leaf	0.943	0.011
Carrot root	1.58	0.009
Tomato fruit	ND[a]	0.009

[a] Not detected.

have a stimulatory affect on color solubilization.

Heat and $HgCl_2$ Procedures for Measuring α-Amylase Activity. The effects of heat treatment on total amylase activity as determined by reducing power production and α-amylase activity as determined by the starch azure method in plant extracts is shown in Table II. These data clearly show that the heat treatment method is valid only for barley malt α-amylase determination. All other tissues tested have heat labile α-amylases and the total amylase activity remaining in these samples after heat treatment has little correlation with the actual α-amylase in the sample before or after the heat treatment. These data indicate that the use of this method for tissues other than germinating cereal is unsatisfactory.

Although 10 to 100 μM $HgCl_2$ is shown to be potent inhibitor of barley malt β-amylase, these Hg^{2+} concentrations also partially inhibit α-amylase in these preparations (Fig. 6). Because of this and because $HgCl_2$ is a dangerous poison, we consider the $HgCl_2$ method to be unsatisfactory.

Brief Amylase Activity Survey. A survey of total and α-amylase activities in selected organs of some economically important plant species was conducted (Table III). A wide range of activities of both α- and β-amylase were found. In addition to alfalfa root and soybean cotyledon (Table II), sweet potato root appears to be a rich source of β-amylase activity, while potato tuber is surprisingly low in β-amylase activity. Bush beans and potatos are rich sources of α-amylase activity. All non-legume leaf samples (white pine, sweet corn, potato, spinach, and carrot) have similar amylase activities except potato leaf which appears to be low in β-amylase, and potato and white pine which have higher α-amylase activities. The wide range of amylase activities in different plant organs and species suggest that the importance and roles of these hydrolytic enzymes may vary greatly from species to species.

CONCLUSIONS

Both the dilution procedure and the β-amylase saturation procedure of the starch azure assay appear satisfactory for determining α-amylase activity in plant tissue granted that certain validation procedures have been met. Although both procedures often produce nearly identical values, the dilution procedure is most appropriate for tissues having a relatively high proportion of α-amylase activity, while the β-amylase saturation procedure is most satisfactory in tissues containing very high endogenous β-amylase activity. Precautions must be taken to avoid possible inhibitory affects of exogenous β-amylase preparations. Plant debranching enzyme may also interfere with both assays. Experimental trial is necessary to determine which procedure is best for a tissue in question.

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