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Specific Enzymatic Microassays of α-Amylase and β-Amylase in Cereals

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Two colorimetric assays for specific determination in cereals of α- and β-amylase, respectively, were adapted for use in microtiter plates. The two micromethods allow easy and reliable determinations of α- and β-amylase in a large number of samples. Application is provided with K_m determination of barley, maize, sorghum, and rice α- and β-amylases, respectively.

Two methods using commercially available reagents have been recently developed for specific determination of α-amylase (McCleary and Sheehan, 1987) and β-amylase (Mathewson and Seabourn, 1983; Sopanen and Laurière, 1989) in cereals. Both are based on mixtures of maltosaccharide substrates and ancillary enzymes normally intended for α-amylase assay in human serum and urine. These substrates are chemically bonded to *p*-nitrophenol (PNP) through the reducing glucosyl group. Hydrolysis by the amylase, in conjunction with an α-glucosidase acting on short maltosaccharides, leads to PNP liberation, and *p*-nitrophenoxide formation is read at 405 nm.

The α-amylase assay substrate is *p*-nitrophenyl maltoheptaoside blocked at its nonreducing end (BPNPG7). β-Amylase can act only after the cleavage of BPNPG7 by α-amylase, in conjunction with α-glucosidase present in the reagent. This avoids any interference by β-amylase (McCleary and Sheehan, 1987) and renders the test fully specific for α-amylase.

The β-amylase assay substrate consists of a mixture of *p*-nitrophenyl maltopentoside (PNPG5) and *p*-nitrophenyl maltohexoside (PNPG6). Compared to β-amylase, cereal α-amylase cleaves these substrates very slowly (Mathewson and Seabourn, 1983), and it was shown on germinating barley, by selective inhibition studies, that α-amylase action on the test can be neglected (Sopanen and Laurière, 1989).

These assays were compared to the previous standard methods (Mathewson and Seabourn, 1983; McCleary and Sheehan, 1987). The new methods were found more specific and easily adaptable to microtitration. We report here the development of these two assays to a microplate format, maximizing the number of samples to be tested.

MATERIALS AND METHODS

An automatic BioTek enzyme immunoassay reader was used for absorbances in the microplate wells. The Enzyline PNP Unitaire kit used for α-amylase was from BioMérieux (Marcy l'Etoile, France). It is similar to the one used by McCleary and Sheehan (1987), except that the α-glucosidase relative amount is higher. The Testomar kit used for β-amylase was from Behring Diagnostics (La Jolla, CA). Chemicals were analytical grade from Serva (Heidelberg, FRG).

A barley malt (*Hordeum vulgare* L., cv. Conquest) was used for the assays developments. Grains of maize (*Zea mays* L., cv. Dea), barley (*Hordeum vulgare* L., cv. Menuet), sorghum (*Sorghum bicolor* L., cv. Oasis), and rice (*Oryza sativa* L., cv. Cigalon) germinated for 6 days were used for the determination of K_m values. Extracts were prepared by stirring 1 g of malt flour or germinated grains in 5 mL of 50 mM sodium malate, 50 mM NaCl, and 2 mM $CaCl_2$, adjusted to pH 5.2 (McCleary and Sheehan, 1987), for 30 min, centrifuging, and filtering the supernatant through 0.22-μm pore membranes (Millex GV, Millipore, Bedford, MA).

All determinations were made in triplicate. Linear regressions were computed and printed by means of the RS/1 graphic software (BBN Software Products Corp., Baltimore, MD).

α-Amylase Assay. The lyophilized substrate was dissolved in 150 mM malate, pH 5.0, to buffer it at pH 5.2, in the optimal pH range for cereal α-amylases activity.

For the standard assay, 1 mL of this buffer/vial was used, which gave concentrations in the test of 1.3 mmol/L for BPNPG7 and $\geq$15 U/mL for α-glucosidase. A 30-μL portion of Enzyline reagent was pipetted into individual wells of a microtiter 96-well plate (Greiner Labortechnik). A 30-μL portion of extract diluted in extraction buffer was added, and the reaction was allowed to proceed for 20 min at 30 °C. A 1% Tris solution (150 μL), was then added, the pH rise stopping the reaction and turning PNP to its phenoxide colored form. Absorbance at 405 nm was then read.

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For saturation assays and K_m determinations, the vial content was reconstituted in different volumes of 150 mM malate, pH 5.0.

β-Amylase Assay. The Testomar reagent was reconstituted by dissolving the lyophilized powder in 47 mM NaH_2PO_4 to give a final pH of 6 in standard conditions (Mathewson and Seabourn, 1983). The reagent is available in different sizes of sealed vials, and for the standard assay, the volume recommended by the manufacturer is used for reconstitution. The standard assay of β-amylase previously described (Sopanen and Laurière, 1989) was modified as follows: 30 μL of reagent solution was incubated with 30 μL of extract diluted in 50 mM sodium phosphate buffer, pH 6, for 20 min at 30 °C. Final reagent concentrations were 0.405 mmol/L for PNPG5, 0.310 mmol/L for PNPG6, and ≥400 U/L for α-glucosidase. Afterward, 125 μL of 150 mM Na_2CO_3 was added and the absorbance was read at 405 nm.

For assays with increasing substrate concentrations, the reagent was reconstituted in a 5-fold lower volume and then diluted to prepare the different substrate concentrations. Under these nonstandard conditions, it was checked that the pH during incubation was maintained to 6.

Conversion of Absorbances to Enzymatic Units. One unit of activity was defined as the amount of enzyme required to provide 1 μmol/min of PNP under test conditions. Conversion of A_{405} to units was made considering the ϵ of PNP is 18 800 mol^{-1} L^{-1} cm^{-1} for pH >10 (Mathewson and Seabourn, 1983).

In the diluted extract, the activity was (U/mL)

$$c = \frac{10^6 A_{405} V}{\epsilon l t v} = \frac{10^3 A_{405} \pi D^2}{4 \epsilon t v}$$

D being the well diameter (cm), l the optical length (cm), t the time of reaction (min), V the total final volume (L), and v the volume of enzyme added (mL). By multiplication with the dilution factor, the activity in the extract was obtained.

Thus, the activity in the tested dilution was $34A_{405}$ (mU/mL) and in the start extract $3.4 \times 10^{-2} \times A_{405} \times$ dilution (U/mL).

RESULTS AND DISCUSSION

Reagent Concentration. Near saturated substrate concentrations, sensitivity is maximum and small changes in concentration do not affect the measure.

Kinetic curves of substrate hydrolysis by malt extract in the α-amylase and β-amylase assays are shown on Figure 1, parts a and b, respectively. Substrate concentrations of 1.3 mM for Enzyline and 0.715 mM for Testomar were retained for following studies.

Assay Linearity. The linearity of both tests is shown in Figure 2. The reactions are stoichiometric, and the reaction curves pass through the origin. The linear dynamic range of a series of dilutions reaches 28 mU/mL for α-amylase and 33 mU/mL for β-amylase (Figure 3), after conversion of A_{405} to enzymatic units. It can be noted that, for barley malt, high dilutions (between 500 and 10 000) are used, leading to a protein concentration of less than the microgram per milliliter. At these high dilutions, a coating phenomenon was observed in the tubes used for dilution: Adsorption of proteins with time reduced the amount of amylases available in the tube. High accuracy, with the microassays as well as with the previously described methods (McCleary and Sheehan, 1987; Mathewson and Seabourn, 1983; Sopanen and Laurière, 1989), implies performance of the dilution extemporaneously or use of protein-precoated tubes for dilution.

Reproducibility. As very small volumes were used (only 30 μL of both extract and substrate), reproducibility was analyzed to check the reliability of the assays. Two dilutions of two extracts were assayed to determine the within-run precision of each method, giving a CV of about 2% for both assays.

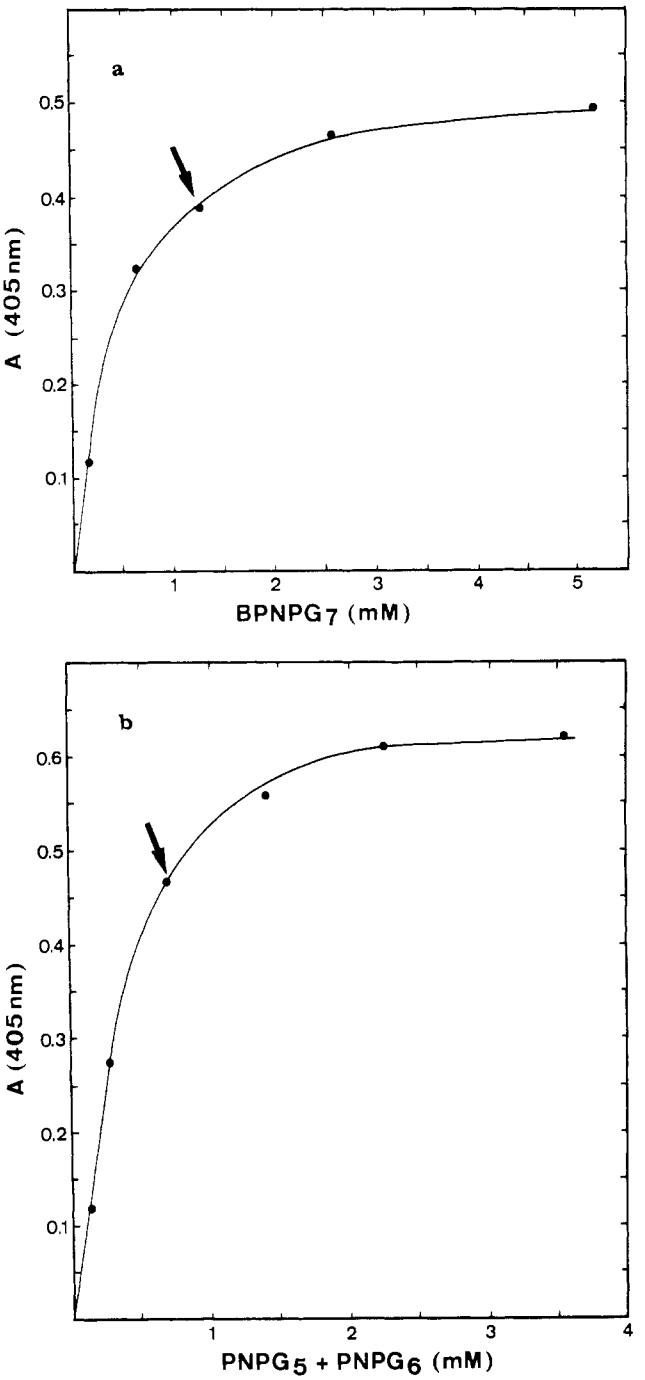


Figure 1. Kinetic curves of substrate hydrolysis by malt extract in Enzyline α-amylase (a) and Testomar β-amylase (b), respectively. Arrows indicate substrate concentrations used in the standard assays.

Determination of α-Amylase K_m toward BPNPG7. A K_m of 607 μM BPNPG7 was previously reported for wheat α-amylase (McCleary and Sheehan, 1987). The values obtained here (Figure 4a) are in the same range but differ significantly from one cereal to another. Rice α-amylase has a much lower K_m (273 μM) than the others, suggesting a higher affinity for BPNPG7. Barley and maize α-amylases have a very similar value (715 μM for maize, 730 μM for barley). Sorghum α-amylase K_m is higher (992 μM).

Determination of β-Amylase K_m toward PNPG5 + PNPG6. K_m values of β-amylases are very similar for maize (370 μM), rice (381 μM), and sorghum (386 μM) and just slightly higher for barley (466 μM), suggesting a similar affinity (Figure 4b).

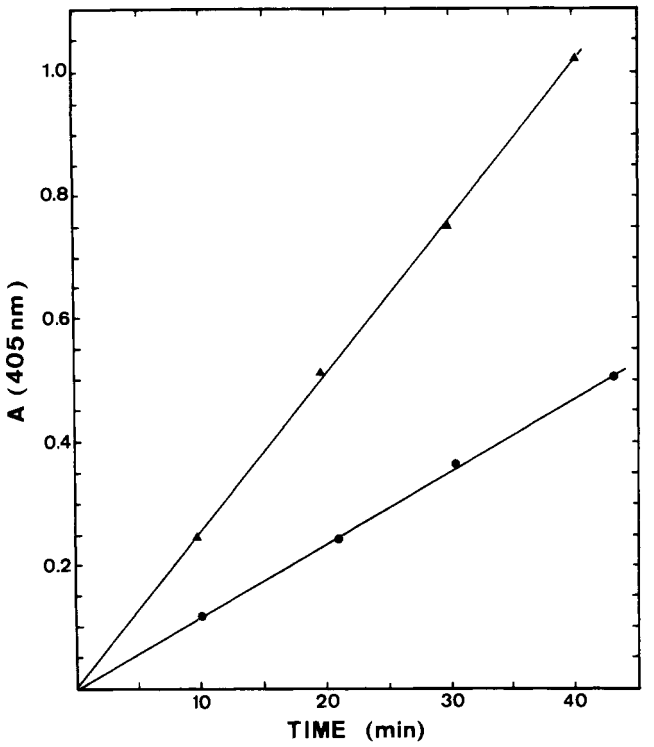


Figure 2. Linearity of the BPNPG7 α-amylase microassay (●) and the PNPG5 + PNPG6 β-amylase microassay (▲) with incubation time of a 1/1600 dilution of the extract.

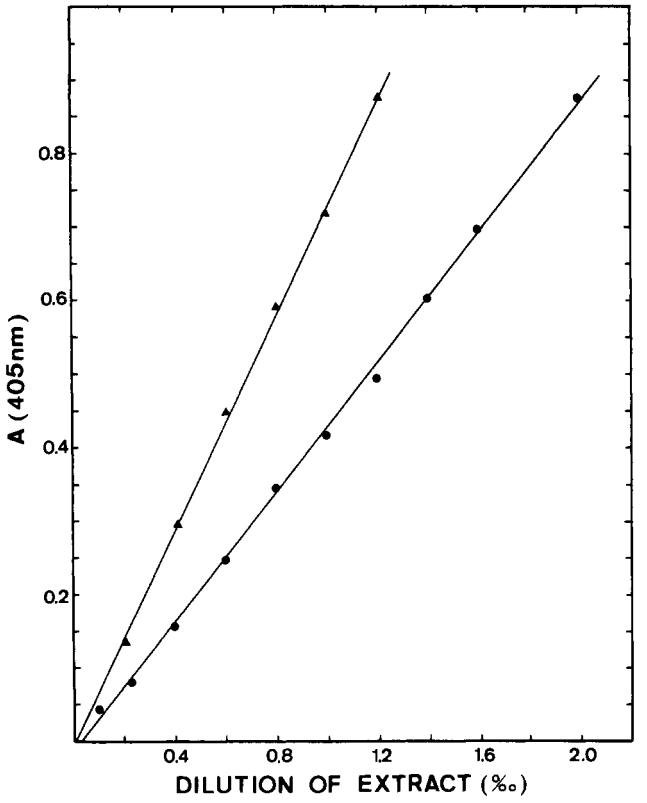
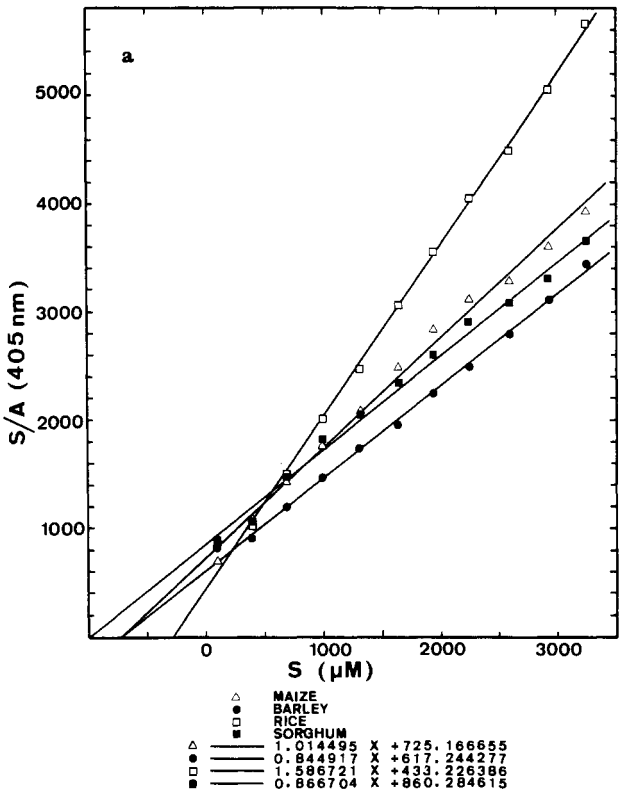


Figure 3. Linearity of the BPNPG7 α-amylase microassay (●) and the PNPG5+PNPG6 β-amylase microassay (▲) with malt extract amount.

CONCLUSION

Microassays of α-amylase and β-amylase in cereals described here allow economical determinations in a large number of samples in a short time. Adaptation of α- and β-amylase assays to microtiter plates was made possible by the recent introduction of PNP-maltosides as substrates. Enzyline and Testomar reagents are the most specific substrates available so far for cereal α- and β-amylases, respectively. Their use can be extended to α- and β-amylases from other sources, provided each assay is buffered to optimal pH conditions of the enzyme and the ratio of α- to β-amylases as well as the affinity of α-amylase for Testomar is not too high, allowing absence of interference from α-amylase in this assay.



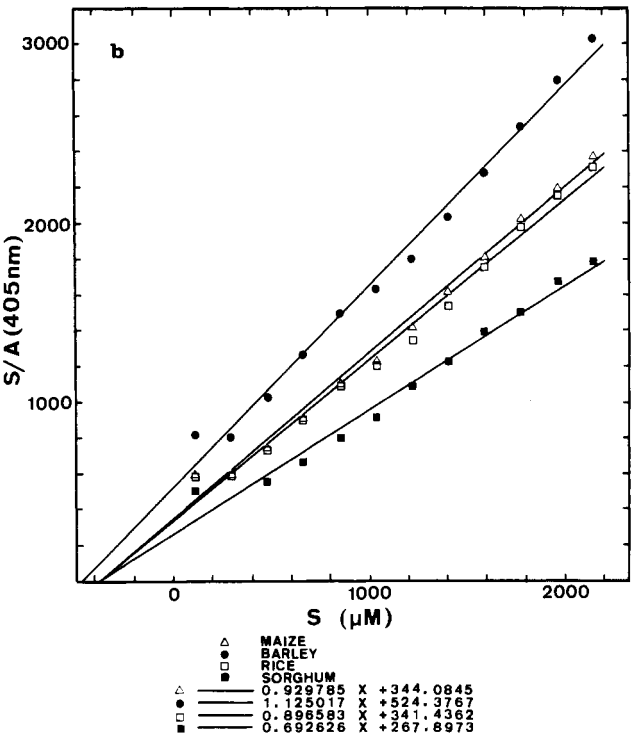


Figure 4. Hanes plots of enzyme kinetics toward BPNPG7 α-amylase (a) and PNPG5+PNPG6 β-amylase (b) microassays. Grains of barley, maize, sorghum, and rice germinated for 6 days were used.

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Registry No. BPNPG7, 74173-31-2; PNPG5, 66068-38-0; PNPG6, 74173-30-1; α-amylase, 9000-90-2; β-amylase, 9000-91-3.

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Evaluation and Confirmation of Acetylation—Gas–Liquid Chromatographic Method for the Determination of Triadimenol in Foods

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An acetylation technique using acetic anhydride followed by gas–liquid chromatography is evaluated for a triadimenol fungicide. Identity of the derivative was confirmed by nuclear magnetic resonance and infrared spectral data. Recoveries of the acetylated analogue of triadimenol from foods studied at fortification levels of 0.1–0.2 mg/kg were usually >80%.

Triadimenol [1-(4-chlorophenoxy)-3,3-dimethyl-1-(1*H*-1,2,4-triazol-1-yl)-2-butanol] and bitertanol [1-(biphenyl-4-yloxy)-3,3-dimethyl-1-(1*H*-1,2,4-triazol-1-yl)-2-butanol] (Figure 1) are fungicides with activity on a wide variety of crops. Both chemicals have similar structures, with a functional group (–OH) in the secondary carbon, a phenoxy group, and a triazolyl ring present.

The first and usual method of residue determination for both fungicides is that from Specht (1977), in which the fungicides are extracted from the matrix with methylene chloride and determined after Florisil column cleanup by gas chromatography with nitrogen-selective detection.

Recently, a new method was reported for the determination of bitertanol in foods (Mendes, 1985), which consists of acetylation with acetic anhydride to convert bitertanol to the bitertanol acetate, which is measured quantitatively by gas chromatography with a nitrogen–phosphorus thermionic detector.

This paper includes evaluation of the acetylation method and the feasibility of using acetylation as a general reaction for the confirmation of the identity of other triazoid fungicides with structures similar to that of bitertanol.

EXPERIMENTAL SECTION

Equipment. NMR were obtained in $CDCl_3$ solution with Me_4Si as internal standard on a Bruker AC 300 spectrometer. IR spectra were obtained in KBr disks on a Bruker IFS 85. GC–MS were obtained on an HP 5970 and GC 5880A. A Varian Model 3700 gas chromatograph equipped with a thermionic detector (TSD) was used with a 1.80 m × 2 mm glass column.

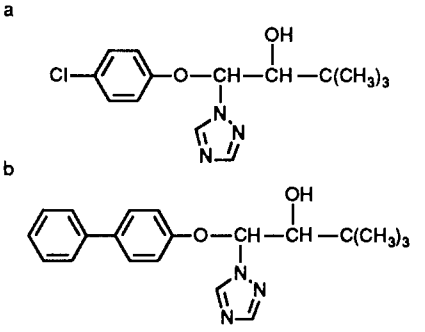

Figure 1. Structures of triadimenol (a) and bitertanol (b).

Reagents. Acetic anhydride was obtained from Merck and was used as received. Bitertanol and triadimenol were products from Bayer and were stated to be 99.0% pure. Pyridine from Grupo Química was also used without purification. Anhydrous sodium sulfate, petroleum ether, and acetone were pesticide-grade materials. All other solvents were analytical grade and were used as obtained from the suppliers. Silica gel GF (Merck, Art. 7730) and Celite 545 (Grupo Química) were used as received.

Thin-Layer Chromatography (TLC). This technique was used to confirm the product of esterification of triadimenol. The standard of fungicide and the product of derivatization were spotted onto silica gel GF plates. Blue spots with different R_f values appeared when visualized by ultraviolet after the plates were developed in a 4:1 benzene–ethyl acetate solvent system.

Derivative and Standard Preparation. Pyridine solution (2 mL) of triadimenol (50 mg/mL) and acetic anhydride (2 mL) were placed in a 25-mL screw-capped vial with a Teflon-lined cap. The vial was wrapped in aluminum foil to protect the solution from light and allowed to stand at room tempera-

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