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α-Amylase Assay and Action Pattern Determination Using Radioactive Substrate, HPLC, and a Radioactive Flow Detector

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A new assay system is presented for the analysis of α-amylase. The disappearance of ^{14}C-labeled starch substrate and the appearance of its radioactive degradation products were monitored by HPLC and a radioactive flow detector/integrator. The hydrolysis of radioactive substrate was proportional to enzyme concentration when two commercially available α-amylase preparations of *Bacillus subtilis* origin were studied. The method demonstrated an average recovery of 101.7 ± 6.5% when modified food starch was spiked with amylase and analyzed. In addition, the method was shown to be useful for predicting detailed action patterns of various types of amylases.

α-Amylases (1,4-α-D-glucan glucanohydrolase) hydrolyze starch, glycogen, and their degradation products by splitting central 1,4-α-glucosidic linkages, causing rapid liquefaction and slower saccharification. α-Amylases have been detected in plants, in microorganisms, and in the pancreas, blood, urine, and saliva of animals. The presence of α-amylases has been confirmed in milk (Guy and Jenness, 1958), whey protein powder (Thomas et al., 1984), fungal protease preparations that are commonly used as substitutes for calf rennets in cheese making (Thomas et al., 1984), and other food ingredients.

Adding α-amylase-contaminated ingredients to food products containing starch-thickening agents can cause spoilage in the form of a dramatic loss of viscosity or thinning. For example, in some instances ultrahigh-temperature- (UHT-) sterilized puddings have undergone dramatic viscosity losses within a few days to several months after manufacture (Anderson et al., 1983). Although they were not able to identify the exact source of the enzyme, Barefoot and Adams (1980) concluded that the cause of thinning of sterile UHT-treated pudding was an extremely heat-stable amylase—probably a bacterial α-amylase that tends to be more heat stable than those from other sources. They recommended that manufacturers of puddings and similar products containing starch as their primary thickening agent take steps to control α-amylase—either by keeping the enzyme out of the product or by inactivating the enzyme in the product.

Numerous assay procedures have been applied to monitor α-amylase activity (Allen and Spradlin, 1971). Most of these methods measure either the increase in reducing groups liberated as starch hydrolysis proceeds or the decrease in the starch iodine color that occurs as the starch substrate is degraded.

Malacinski (1971) developed a microassay for α-amylase based on the solubilization of ^{14}C-labeled starch. With this assay technique, [^{14}C]starch was incubated with enzyme for 30 min at 37 °C. An aliquot of the reaction mixture was then precipitated with 95% ethanol and carrier nonradioactive starch. The rate at which radioactive starch was hydrolyzed was determined by measuring the accumulation of radioactivity in the supernatant fluid. Malacinski demonstrated that this radioactive substrate approach offered at least two significant advantages over commonly used colorimetric procedures: It was more sensitive and applicable to more complex sample matrices (e.g., crude tissue extracts). Malacinski's radiochemical procedure was approximately 15-fold more sensitive than the starch–iodine assay and 2.5-fold more sensitive than the dinitrosalicylate assay.

Rather than using alcohol precipitation as a means of fractionating [^{14}C]starch from its hydrolysis products, Thomas et al. (1984) used paper chromatography and resolved ^{14}C-labeled maltotriose (dp 3), maltose (dp 2), and glucose (dp 1) degradation products. By using more so-

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phisticated chromatography techniques, the radioactive starch assay could be extended for the examination of α-amylase action patterns.

The present work was conducted to refine the methodologies of Malacinski and Thomas. By combining the resolving power of HPLC with the specificity characteristics of a radioactive flow detector, it was possible to develop a simple assay procedure that was both sensitive and capable of providing detailed information about amylase action patterns. The characteristics of this assay system, standard calibration curves with two different commercial enzyme preparations, and typical action patterns for several different types of amylases are presented.

MATERIALS AND METHODS

Amylase Preparations. The following commercial (liquid) amylase preparations were obtained from NOVO Laboratories, Inc. (Danbury, CT): Ban 120L, an α-amylase produced by submerged fermentation of *Bacillus subtilis* [activity 120 KNU/g (KNU = kilo novo unit, the amount of enzyme that hydrolyzes 5.26 g of starch/h at 37 °C and pH 5.6 in the presence of 0.0043 M calcium)]; Termamyl 120L, a bacterial α-amylase produced from *Bacillus licheniformis* (activity 120 KNU/g); Fungamyl 800L, a fungal α-amylase produced from *Aspergillus oryzae* [activity 800 FAU/g (FAU = fungal amylase unit, the amount of enzyme that breaks down 5.26 g of starch/h at 37 °C and pH 4.7)]; AMG 200L, an exo-amylase (glucoamylase) produced by submerged fermentation of *Aspergillus niger* [activity 200 AGU/mL (AGU = amyloglucosidic unit, the amount of enzyme that hydrolyzes 1 μmol of maltose/min at 25 °C and pH 4.3)].

In addition, a powdered α-amylase mixture, Tenase-1200, was obtained from Miles Laboratories, Inc. Tenase-1200, a bacterial α-amylase *B. subtilis* origin, had an activity of 1 200 000 MWU/g, where MWU = modified wohlgemuth unit, that amount of activity that dextrinizes 1 mL of a 2% (w/v) soluble starch solution to a defined blue value at 70 °C and pH 6.0 in the presence of 0.1% (w/v) calcium chloride.

Radioactive Starch Substrate. The radioactive starch substrate was obtained from New England Nuclear, Boston, MA (Catalog No. NEC-730 starch, *Nicotiana tobacum* L.). The radioactive starch, which comes solubilized in 0.5 mL water/ethanol (97:3) solvent, had an average molecular weight of 8000 Da (as determined by New England Nuclear with HPLC size exclusion columns) and an activity of 1.9 mCi/mg. The activity of the radioactive starch solution was 0.05 mCi/0.5 mL.

Buffer/Activator Solution. The buffer/activator solution was prepared as follows: 0.08 mol of glacial acetic acid was diluted with about 500 mL of distilled water, and 2.2 g of $CaCl_2$ was added. The acetic acid/calcium chloride solution was adjusted to pH 6.5 with 50% NaOH and diluted to 1 L with distilled water.

HPLC Equipment and Conditions. Chromatography was performed with a Varian 5000 HPLC using a Bio-Rad HPLC oligosaccharide analysis column (Aminex HPX-42A, Catalog No. 125-0097). The column temperature was 65 °C. The mobile phase was degassed distilled water, and the flow rate was 0.6 mL/min. A 50-μL loop was used.

A FLO-ONE/Beta Model IC radioactive HPLC flow detector was used (Radiomatic Instruments & Chemical Co., Inc., Tampa, FL). The radioactive flow detector, which is a microprocessor/computer-controlled instrument that both detects and quantitates radioactivity in a flowing system, uses the principle of liquid scintillation counting. The HPLC effluent entering the system was mixed with nongelling scintillation cocktail (Flow-Scint III, Catalog No. 9002040, Radiomatic) automatically supplied by a pump that comes as part of the detector system. The flow rate of the scintillator was 2.4 mL/min. After being mixed in a specially designed chamber, the homogeneous mixture of HPLC effluent and scintillator was pumped through a 0.5-mL flow cell (liquid type) positioned between two photomultiplier tubes. Other detector settings/conditions were as follows: signal update time, 6 s; mode, integrate (in cpm); minimum integrated peak area, 20 cpm; discriminator settings (channel 2), 20–90; background subtract, 60 cpm.

Assay Procedure. Ten microliters of radioactive starch substrate (equivalent to 1 μCi of activity and 0.5263 μg of starch), 2.5 mL of buffer/activator solution, and the desired type and amount of amylase spike (as indicated in Results and Discussion) were added to a 6-mL glass vial, sealed, and incubated for 1 h at 55 °C. The vial was then opened, and 0.6 g of Amberlite MB-1 ion-exchange resin was added. After the mixture was shaken vigorously for 1 min and the resin allowed to settle, a portion of the sample was withdrawn with a syringe. The syringe was fitted with a 0.45-μm Acrodisc CR (Gelman Sciences), and the sample was injected into the HPLC.

Quantitation was based on the internal normalization method (Yost et al., 1980). Peak integration was performed by the radioactive flow detector. The instrument's software integration package separates two peaks that are not base-line-resolved by dropping perpendiculars at the valley between the unresolved peaks to the base line and integrating the area under the peaks. This integration technique is recommended over tangent-skim techniques by the column manufacturer for this application; it is commonly empolyed in the industry to measure oligosaccharides in corn syrups with a refractive index detector.

The area percent for the initial [^{14}C]starch peak (the first major peak in the chromatograms) was determined for three blank samples (i.e., samples that contained buffer/activator solution [^{14}C]starch but no added amylase). The average area percent value was calculated for the starch peak of the three blank samples and subtracted from 100% to give the percent [^{14}C]starch hydrolyzed in the blank; this value was 27.2 ± 0.7%.

Calculation of percent [^{14}C]starch hydrolyzed in the samples was made as follows: The area percent for the unhydrolyzed starch peak in the sample chromatograms was subtracted from 100% to give the percent [^{14}C]starch hydrolyzed by the amylase, and the average percent [^{14}C]starch hydrolyzed in the blank samples (27.2%) was subtracted from this value to correct for the hydrolysis products in the blank.

The internal normalization method provides accurate quantitation because it is based on measurement of substrate concentration as well as the concentrations of all of its degradation products.

RESULTS AND DISCUSSION

Time Course Study. Reaction mixtures containing 5 μg of Ban 120L amylase solution and 2.5 mL of buffer/activator solution were incubated with [^{14}C]starch as described in Materials and Methods. The precent [^{14}C]starch hydrolyzed was observed to be a linear function of incubation time for approximately 60–70 min (Figure 1).

Standard Calibration Curves. Standard calibration curves were prepared for two different enzymes—Ban 120L and Tenase-1200. The data, plotted in Figure 2, showed that both calibration curves were nonlinear in the range from 0 to 50 μg of added amylase. Inspection of the radiochromatograms for these samples revealed that at high enzyme concentrations—when the hydrolysis of radioactive

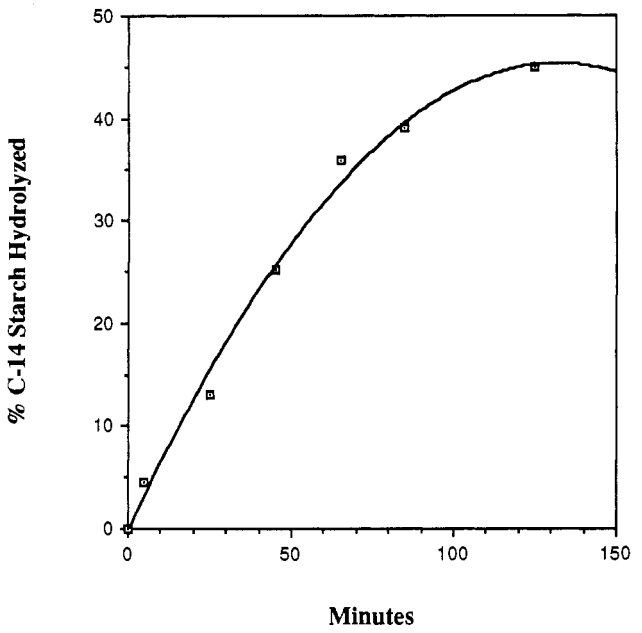

Figure 1. Time course study (all samples contained 2.5 mL of buffer/activator solution, 10 μL of [^{14}C]starch, and 5 μg of Ban 120L amylase).

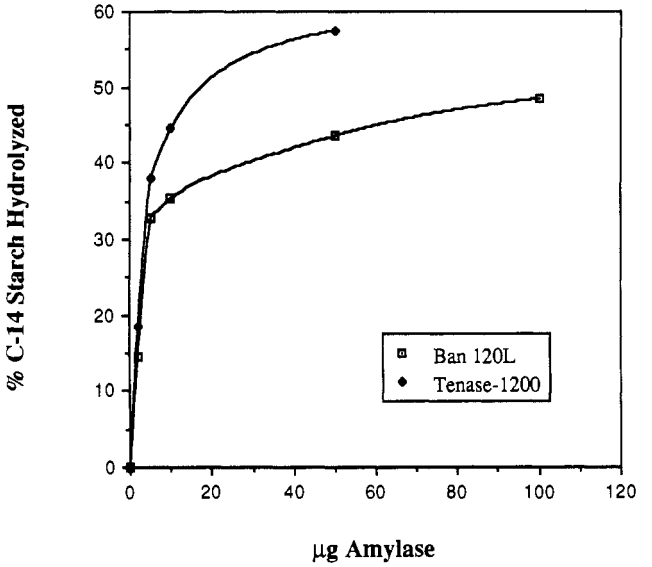

Figure 2. Standard calibration curves for Ban 120L (0–100 μg) and Tenase-1200 (0–50 μg). In addition to the amylase, all samples contained 2.5 mL of buffer/activator solution and 10 μL of [^{14}C]starch.

starch substrate exceeds 35–40%—the enzymes preferentially hydrolyze the high molecular weight oligosaccharide degradation products, which have increased substantially to that point. As a result of these competing reactions, calibration data of percent [^{14}C]starch micrograms of amylase were nonlinear at higher enzyme concentrations.

However, as indicated in Figure 3, calibration curves for both enzymes were linear in the range from 0 to 5 μg of added amylase. The linear least-squares regression equations and correlation coefficients were as follows: Ban 120L, μg amylase = −0.0977 + (0.1533)(% [^{14}C]starch hydrolyzed), R^2 = 1.000; Tenase-1200, μg amylase = −0.0812 + (0.1315)(% [^{14}C]starch hydrolyzed), R^2 = 0.980.

Radiochromatograms of a reagent blank sample (containing 2.5 mL of buffer/activator solution and 10 μL of [^{14}C]starch, incubated 1 h at 55 °C), a standard sample (containing the same amount of buffer/activator solution and radioactive starch and 5 μg of Ban 120L, incubated

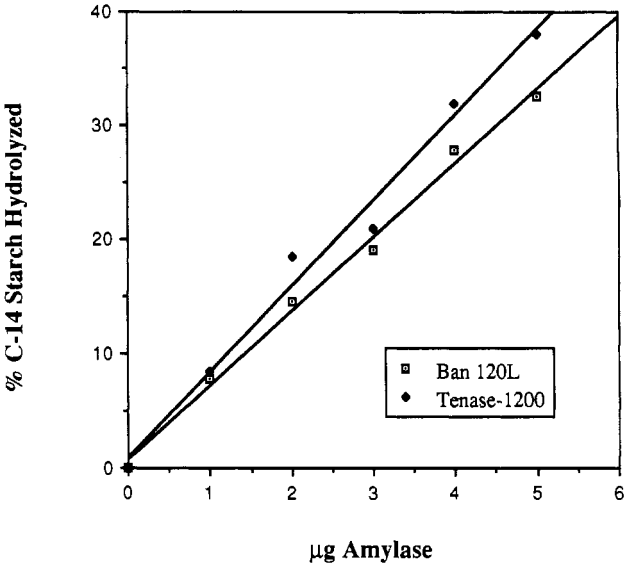

Figure 3. Standard calibration curves (linear range 0–5 μg of amylase) for Ban 120L and Tenase-1200. The substrate concentration was 0.526 μg of [^{14}C]starch/2.5 mL of solution.

Table I. Recovery of Ban 120L or Tenase-1200 Added to 2.0-g Samples of Modified Food Starch

enzyme	spike amt, μg	recovery, % trial 1	trial 2	trial 3	mean ± SD
Ban 120L	2.00	90.5	104.5	110.0	101.7 ± 9.9
	5.00	96.2	108.0	104.2	102.8 ± 6.0
Tenase-1200	2.00	92.5	99.5	97.7	96.6 ± 3.6
	5.00	103.8	91.3	94.3	96.5 ± 6.5

1 h at 55 °C), and a standard (containing the same amount of buffer/activator solution and radioactive starch and 0.5 μg of Ban 120L, incubated 24 h at 55 °C) appear in Figure 4. These radiochromatograms indicate that the sensitivity of the assay may be further extended by increasing incubation time.

Precision/Recovery Study. Modified food starch is one ingredient that could possibly be contaminated with α-amylase (Barefoot and Adams, 1980). To estimate the precision and recovery of the test procedure, several 2-g samples of a commercial modified food starch (Rezista starch, lot no. 139-M6, A. E. Staley Manufacturing Co., Decatur, IL) were spiked with either 0, 2, or 5 μg of Ban 120L and extracted with 5 mL of buffer/activator solution. After centrifugation (2500 rpm, 5 min), a 2.5-mL aliquot of clear centrifugate was withdrawn and spiked with 10 μL of [^{14}C]starch substrate. The samples were incubated for 1 h at 55 °C and analyzed as previously described. Three samples at each level of amylase spike were prepared and analyzed. Results appear in Table I. The average percent recovery was 101.6 ± 6.5%.

Amylase Action Patterns. Action patterns for various commercial amylase preparations and human saliva are presented in Figure 5. This figure demonstrates that the radioactive starch method is capable of differentiating action patterns of amylases from bacterial, fungal, and animal sources. The peaks have been tentatively identified by comparing retention times with the retention times of peaks obtained when nonradioactive saccharide standards were analyzed by HPLC using the same column, chromatographic conditions, and a refractive index detector.

Knowledge of the specific breakdown products can be important in determining whether or not an ingredient used in a food product will cause spoilage by degrading the starch thickener. For example, if the action pattern produces mostly glucose (as in Figure 5D) and no high mo-

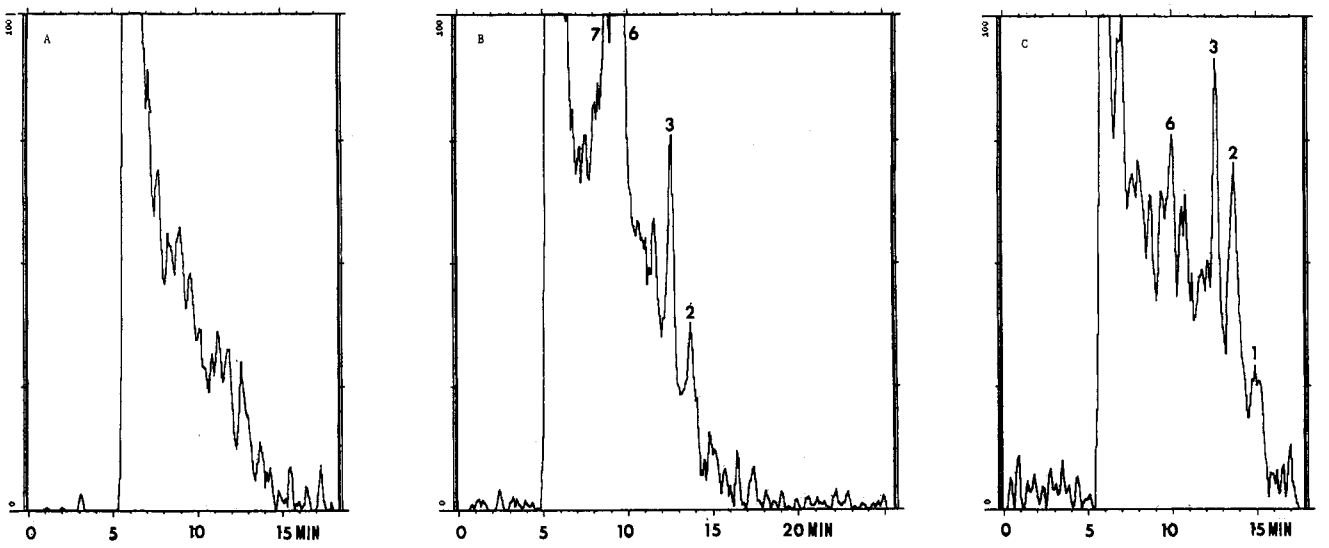

Figure 4. Radiochromatograms: (A) blank (no added amylase, 1-h incubation at 55 °C); (B) 5 μg of added Ban 120L (1-h incubation at 55 °C); (C) 0.5 μg of added Ban 120L (24-h incubation at 55 °C). All samples contained 2.5 mL of activator/buffer solution and 10 μL of [^{14}C]starch. Peak identification: 1 = dp 1; 2 = dp 2; 3 = dp 3; 4 = dp 4; 5 = dp 5; 6 = dp 6; 7 = dp 7.

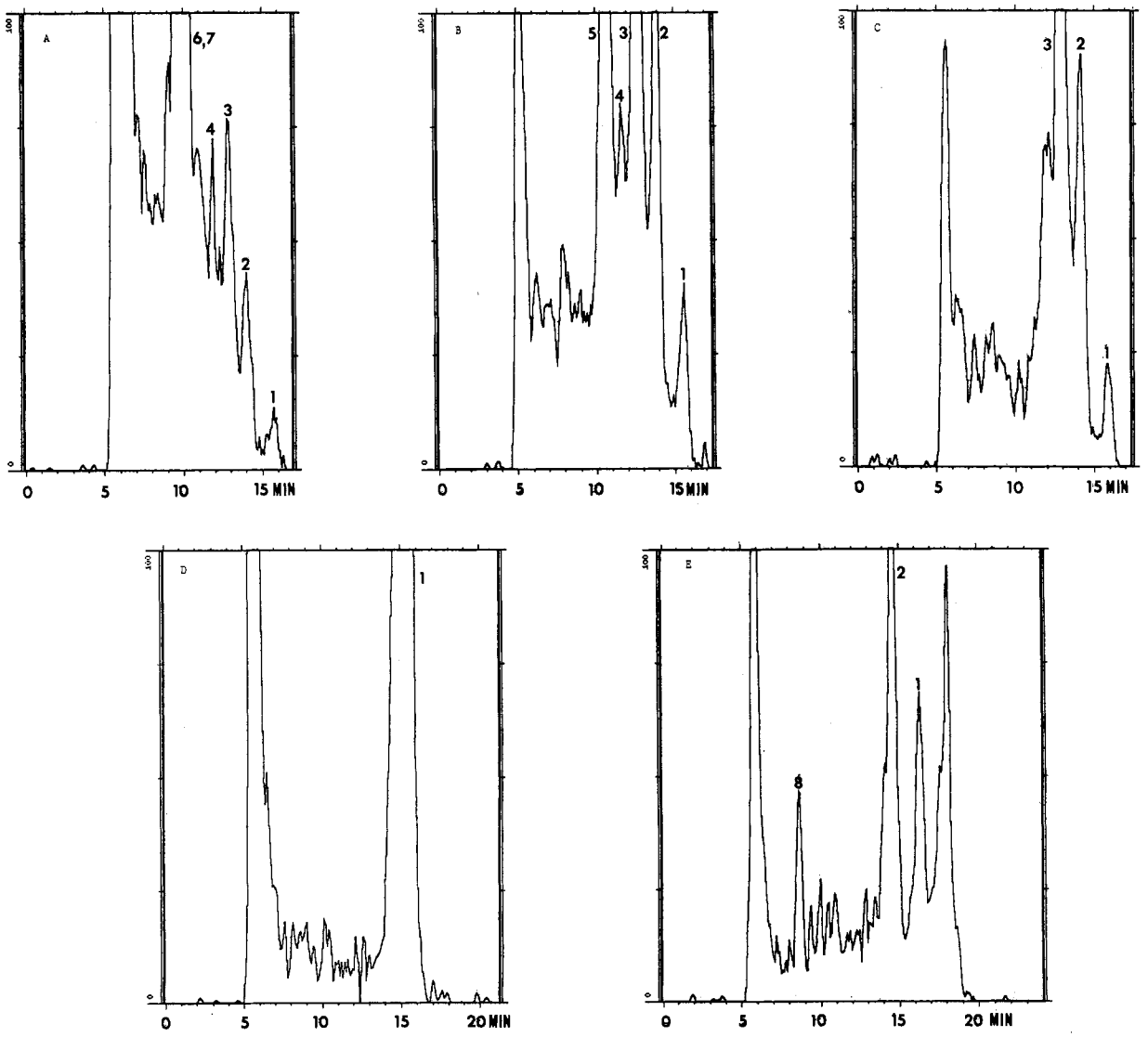

Figure 5. Enzyme action patterns: (A) 10 μg of Tenase-1200; (B) 100 μg of Termamyl 120L; (C) 100 μg of Fungamyl 800L; (D) 100 μg of AMG 200L; (E) 200 μg of human saliva. In addition, all samples contained 2.5 mL of buffer/activator solution and 10 μL of [^{14}C]starch. Peak identifications are as in Figure 4.

lecular weight oligosaccharides, glucoamylase contamination is likely. If maltose is the primary degradation product, then β-amylase contamination is suspected. These sacchrifying amylases attack the starch substrate in an "exo" fashion (i.e., β-amylases split off maltose and glucoamylases split off glucose in a stepwise manner from

one end of the starch polymer) and are unlikely to cause thinning at low levels of contamination.

Colorimetric assays, which merely measure the total reducing sugars liberated, are not able to provide this important type of detailed information. Furthermore, detailed action patterns might be useful in tracking down the source of the amylase contamination in food products.

Analytical Considerations. Bio-Rad product literature recommends operating the HPX-42A column at 85 °C. However, noticeable temperature-induced hydrolysis of the radioactive starch occurred when blank samples were analyzed at that temperature. The problem did not occur at temperatures below 70 °C.

HPX-42A columns are packed with a 4% cross-linked cation-exchange resin in the silver form. Bio-Rad recommends the use of deashing guard cartridges, which contain both cation- and anion-exchange resins, to protect the silver chloride salts. However, when in-line deashing precolumns were employed, there was a tendency for the amylases (especially the AMG 200L) to be retained on the column and to cause hydrolysis of radioactive starch in subsequent injections. As a result, the use of the precolumn was abandoned. Deashing was accomplished, instead, by the addition of Amberlite MB-1 ion-exchange resin to the samples prior to injection.

The HPX-42A column is capable of resolving oligosaccharides as large as dp 11. Combining two columns in series can separate oligosaccharides to dp 14, and by using several of these columns in series, separation of oligiosaccharides as large as dp 20 can be achieved.

Conclusion. The analytical approach using radioactive starch, HPLC, and a radioactive flow detector is sensitive and provides a more comprehensive picture of amylase-catalyzed hydrolysis than the commonly used colorimetric and viscometric amylase assay procedures. Futhermore, it is more useful than colorimetric assay methods for measuring small increases in α-amylase-induced starch degradation products in sample matrices that commonly contain high background levels of reducing sugars (e.g., corn syrups, milk, whey protein powders, and crude tissue extracts).

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Characterization of Trifluoroacetic Acid Hydrolyzed Subtropical Forage Grass Cell Walls[1]

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Hemicellulose determinations by trifluoroacetic acid (TFA) hydrolysis (2.0 N followed by 0.25 N TFA, 1 h at 121 °C each step) of forage grass [*Pennisetum flaccidum* Griseb. *Panicum amarum* (Elliot) var. *amarum*, *Panicum amarum* × var. *amarulum* (Hitchc. & Chase) P. G. Palmer, *Panicum virgatum* L.] cell walls were compared with acid (1 N H_2SO_4, 1 h at 121 °C), base (10% KOH, 90 min at 55 °C), and neutral detergent (ND) solubilization. Of these, TFA hydrolysis proved to be most useful for cell wall compositional analysis of subtropical grasses. Residue from TFA hydrolysis resembled a pectin-free cellulose fraction, and the hydrolysate contained stable neutral sugar monomers derived from hemicellulose. Weak acid (1 N H_2SO_4, 1 h at 55 °C) and weak base (1% KOH, 45 min at 55 °C) pretreatment of *Panicum* cell wall preparations for TFA hydrolysis resulted in minor (3%) yield increases, not considered sufficient for a subsequent modification of the TFA hydrolysis procedure.

Analytical estimation of structural polysaccharides in forages has been based on the differential solubilities of the various chemical components in acid or base. However, difficulties can occur in the chemical separation of plant cell walls into structural entities such as xylans (hemicellulose), β-glucan, cellulose, and pectin as some cleavage of covalent bonds may occur (Bailey et al., 1976). The hemicellulosic fraction of plant tissue remains the most difficult to quantify with many methods proposed (Blake and Richards, 1971). Traditionally, hemicellulose is the fraction insoluble in water and ammonium oxalate solution but soluble in acid or base under mild conditions. These methods include acidic fractionation after reflux with 0.5% ammonium oxalate (depectination) followed by reflux with 1 N H_2SO_4 (Bailey et al., 1978). Alternatively, hemicellulose is determined by alkaline fractionation in 5–10%

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