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Beta-Amylases from Alfalfa (*Medicago sativa* L.) Roots[1]

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ABSTRACT

Amylase was found in high activity (193 international units per milligram protein) in the tap root of alfalfa (*Medicago sativa* L. cv. Sonora). The activity was separated by gel filtration chromatography into two fractions with molecular weights of 65,700 (heavy amylase) and 41,700 (light amylase). Activity staining of electrophoretic gels indicated the presence of one isozyme in the heavy amylase fraction and two in the light amylase fraction. Three amylase isozymes with electrophoretic mobilities identical to those in the heavy and the light amylase fractions were the only amylases identified in crude root preparations. Both heavy and light amylases hydrolyzed amylopectin, soluble starch, and amylose but did not hydrolyze pullulan or β-limit dextrin. The ratio of viscosity change to reducing power production during starch hydrolysis was identical for both alfalfa amylase fractions and sweet potato β-amylase, while that of bacterial α-amylase was considerably higher. The identification of maltose and β-limit dextrin as hydrolytic end-products confirmed that these alfalfa root amylases are all β-amylases.

The pH optimum for both β-amylase fractions was 6.0. Both light and heavy β-amylases showed normal Michaelis-Menten kinetics, with soluble starch as substrate, and had respectively K_m values of 5.9 and 6.8 milligrams starch per milliliter and V_{max} of 640 and 130 international units per milligram protein. Arrhenius plots indicated that the energy of activation for the heavy β-amylase remained relatively unchanged (12.7 to 13.0 kilocalories per mole) from 0 to 30°C, whereas the energy of activation for the light amylase increased from 12.0 to about 28.0 kilocalories per mole at 8.7°C as temperature was lowered. The light amylase was shown to be inhibited by maltose.

Beta-amylase (α-1,4-glucan maltohydrolase, EC 3.2.1.2) is an exoamylase that attacks the nonreducing ends of starch molecules, producing β-maltose and β-limit dextrin as products (22). Beta-amylases are strictly plant enzymes that have been reported in ungerminated wheat and soybean seeds (11, 23); germinating barley, rice, sorghum, and wheat seeds (3, 6, 7, 15); sweet potato roots (1); broad bean leaves (5); and pea seedling roots (21).

Amylases are of particular interest in the perennial legume alfalfa (*Medicago sativa* L.), which stores large quantities of starch in its fleshy tap root. This starch serves as a carbohydrate reserve that is used for winter survival and shoot regeneration following harvest of the shoot as a forage (17, 19). Amylase has been reported in high activity in the tap root of several alfalfa cultivars (8), and activity staining of electrophoretically separated proteins on polyacrylamide gels has shown the presence of two or three isozymes of amylase in alfalfa tap roots (13). In this study, we describe the separation, identification, and characterization of isozymes of β-amylase from alfalfa root tissues.

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MATERIALS AND METHODS

Plant Material. Alfalfa (*Medicago sativa* L. cv. Sonora) was planted in the spring as described previously (8). In the autumn of the seeding year, plants were collected, shoots clipped to within 2.5 cm of crowns, and all secondary roots removed from tap roots. Trimmed plants were either potted and maintained in a greenhouse or washed, wrapped in wet cheesecloth and plastic, and stored at −2°C until use. Greenhouse-grown plants were inoculated with a mixture of *Rhizobium meliloti* strains (Nitragen Co., Milwaukee, WI) and irrigated with a nitrogen-free nutrient solution (9).

Separation of β-Amylase Isozymes. Whole tap roots were washed, chopped into small pieces with a razor blade, and then mechanically homogenized with a 'homogeniser' (Measuring and Scientific Equipment, London, UK), adding 10 ml extraction buffer (30 mM ethylenediamine (pH 7.0), 3 mM $CaCl_2$) per g tissue. Crude homogenates were filtered through a 150-μm Teflon mesh, and the filtrate was centrifuged at 15,000*g* for 20 min. Amylase activity was concentrated from the 15,000*g* supernatant by adding PEG to make 20% of the supernatant volume and then adding two volumes of ethanol and centrifuging at 15,000*g* for 20 min. This 15,000*g* pellet was resuspended in extraction buffer and centrifuged again at 15,000*g* for 20 min. The pellet was again resuspended in extraction buffer and centrifuged at 15,000*g* and the pellet was discarded. The supernatants from the final two centrifugations were pooled and applied to a 2.5 × 90-cm column containing Sephadex G-75 gel (Pharmacia) equilibrated with extraction buffer. Extraction buffer was used to elute 5-ml fractions which were assayed for amylase activity. Amylase activity peaks were identified, and fractions were pooled into either heavy or light amylase preparations. Amylase mol wts were determined by calibrating the Sephadex column with proteins of known mol wt (BSA, 67,000; ovalbumin, 43,000; chymotrypsinogen A, 25,000; lysozyme, 14,000).

Enzyme Assays. Amylase activity was routinely assayed by measuring the rate of generation of reducing sugars from starch. Appropriate dilutions of the enzyme preparations were made, and 0.2 ml of the diluted preparations was added to 0.5 ml of 100 mM succinate (pH 6.0). The reaction was initiated with 0.5 ml 5% (w/v) soluble starch (Sigma) and terminated after 10 min by adding 0.2 ml alEaline dinitrosalicyclic acid solution (2) and then placing immediately into a boiling water bath. Color was fully developed after 5 min, and tubes were removed from the bath, allowed to cool, diluted with 10 ml H_2O, and read at 540 nm against air as reference. Blank values (distilled H_2O replacing enzyme) were determined and subtracted from the values for the experimental samples and maltose standards used to construct a calibration curve. Assays were linear to about 10 mM maltose. Activity is defined as the amount of enzyme required to produce 1 μmol of maltose min^{-1} (IU).

A wide-range buffer containing 100 mM citrate, 100 mM ethylenediamine, and 100 mM Bicine (*N,N*-bis (2-hydroxyethyl) glycine) was substituted for succinate buffer in determining the pH

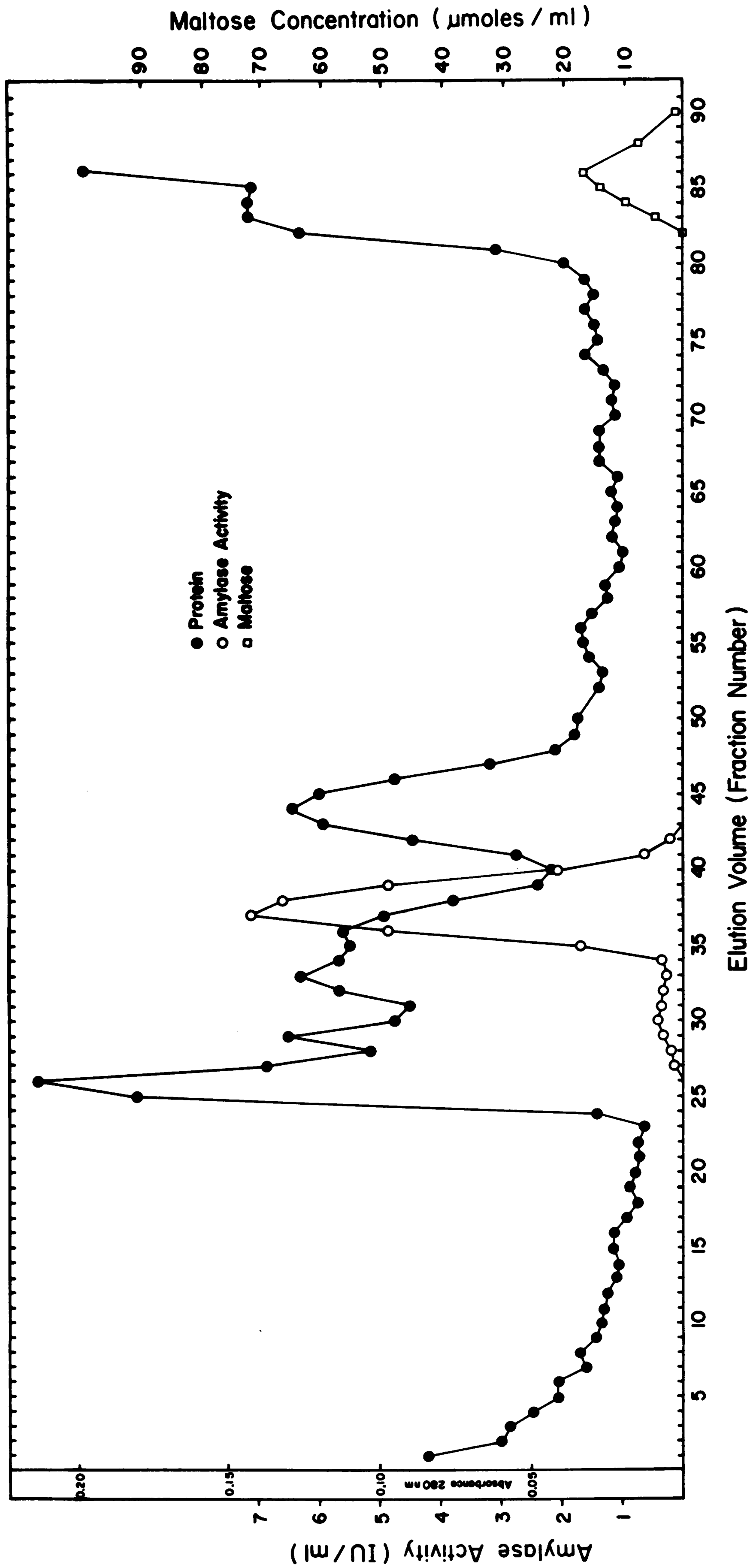

FIGURE 1. Elution profile of alfalfa root amylase activity from a 2.5- × 90-cm Sephadex G-75 column; 5-ml fractions were collected. The elution buffer was 30 mM ethylenediamine (pH 7.0) with 3 mM $CaCl_2$.

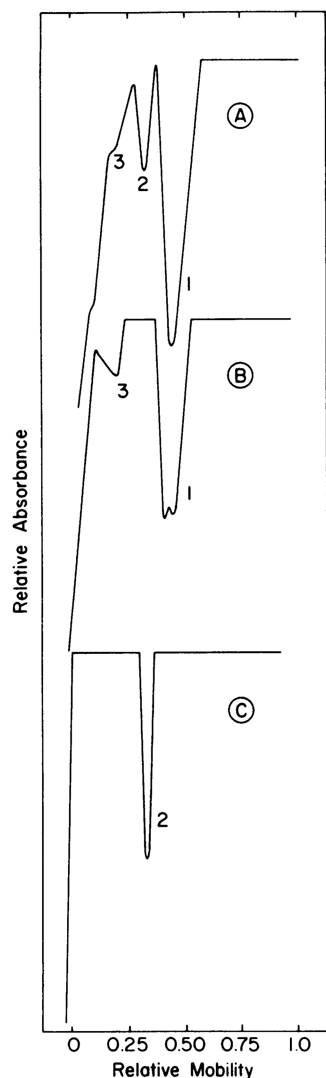

FIG. 2. Densitometric scans of polyacrylamide gels of electrophoretically separated proteins stained for amylase activity. Scans are crude root preparation (A), light amylase fraction (B), and heavy amylase fraction (C).

profile. For preparation of the Arrhenius plots, the routine assay was used, except that the enzyme and buffer mixture was allowed to incubate at the appropriate temperature for 10 min before initiating the assay. E_a[2] values were determined as previously outlined (10).

In the time course experiments, appropriate dilutions of enzyme preparations, sweet potato β-amylase (type I-B, Sigma), and bacterial α-amylase (from *Bacillus subtilis*, type II A, Sigma) were incubated in a solution containing 5% (w/v) polysaccharide, 100 mM succinate (pH 6.0), and 3 mM $CaCl_2$. Samples (0.5 ml) were taken from the reaction mixtures (50 ml) at appropriate time intervals, placed in tubes containing 1.2 ml alkaline dinitrosalicyclic acid (2), diluted with 0.7 ml distilled H_2O, and immediately placed in a boiling water bath. Amylopectin (Nutritional Biochemical Corp., Cleveland, OH), soluble starch (Sigma), amylose, (Sigma), pullulan (Sigma), and β-limit dextrin (prepared by the method of Swain and Dekker [20]) were used as substrates. The limit-dextrin experiment was performed similarly, except that only 1% amylopectin was used.

[2] Abbreviations: E_a, Arrhenius energy of activation; η_{sp}, specific viscosity; T_m, transition temperature.

Viscosity experiments were conducted using an Ostwald-type viscometer at 30°C, with treatments described by Hultin (12).

The maltose-inhibition experiment was performed, using the starch iodine color method, as described by Swain and Dekker (20), in which the change in starch-iodine color of a starch solution containing amylase and a predetermined concentration of maltose is measured over time. Starch-iodine color was assayed by measuring A_{660}.

Protein was assayed by the method of Bradford (4).

Electrophoresis. Amylase activity staining of electrophoretically separated protein was done by a method similar to that of Siepmann and Stegemann (18). Samples containing concentrated enzyme were applied to continuous 5% acrylamide gels containing 0.25% (w/v) soluble starch. Reservoir buffer containing 100 mM ethylenediamine (pH 8.3) with 3 mM $CaCl_2$ was used, and gels were run at 8 mamp/tube. Gels were removed when the tracking dye was within 1 cm of the tube bottoms and were then incubated in 100 mM succinate (pH 6.0) overnight. After incubation, the gels were stained for about 5 min in a solution containing 2.5 mM I_2 and 0.5 M KI. Gels were then washed in distilled H_2O, and activity peaks were measured with a scanning densitometer at 640 nm. The gels stained dark blue, except for regions containing amylase activity, in which the starch in the gel had been largely hydrolyzed and stained to a lesser extent.

Paper Chromatography. Identification of low mol wt products of starch hydrolysis was performed by descending paper chromatography, using a pyridine:1-butanol:H_2O (6:4:3 [v/v]) solvent system. Chromatograms were developed for about 40 h, and reducing sugars were visualized using the method of Trevelyan *et al.* (24), except that a final rinse of 200 mM sodium thiosulfate was used in place of the ammonium hydroxide rinse.

Replication and Repeatability. All experiments were conducted two or more times with nearly identical results. Each point in Figure 3 and 7 represents the mean of three replications.

RESULTS AND DISCUSSION

Separation of Amylase Isozymes. Amylase was found in high activity in alfalfa roots. In crude preparations, activities of about 193 IU mg protein^{-1} were found, corresponding to about 280 IU g·fresh wt^{-1} of root tissue (8). In contrast, activities of about 0.34 IU mg protein^{-1} were found in alfalfa leaf tissue.

Gel filtration chromatography on Sephadex G-75 produced two peaks of amylase activity (Fig. 1). The earlier peak corresponds to a mol wt of 65,700 and is referred to as the heavy amylase fraction, while the second, larger peak, corresponding to a mol wt of 41,700, is referred to as the light amylase fraction. These are similar to reported mol wt for germinating sorghum β-amylase (55,900) (3), soybean β-amylase (61,700) (11), wheat seed β-amylase (64,200) (14), and the monomer for sweet potato β-amylase (64,700), although sweet potato β-amylase is reportedly found as a tetramer with a mol wt of 197,000 (22).

A 6-fold increase in specific activity was obtained during the fractionation steps taken in the preparation of the light amylase, while a substantial decrease in specific activity was obtained following the separation of the heavy amylase. Protein staining of slab polyacrylamide electrophoresis gels of the heavy and light amylase fractions showed the presence of multiple protein bands, indicating that these are not homogenous preparations (data not shown).

Activity staining of electrophoretically separated proteins for amylase activity showed the presence of three isozymes in the crude preparation (Fig. 2). These are labeled 1, 2, and 3 by convention in order of decreasing mobility. Activity staining of the light amylase fraction shows the present of two amylase isozymes (bands 1 and 3). In that both isozymes appear to have identical mol wt, these data suggest that the light amylase isozymes are charge isomers. Gels of the heavy amylase fraction show only

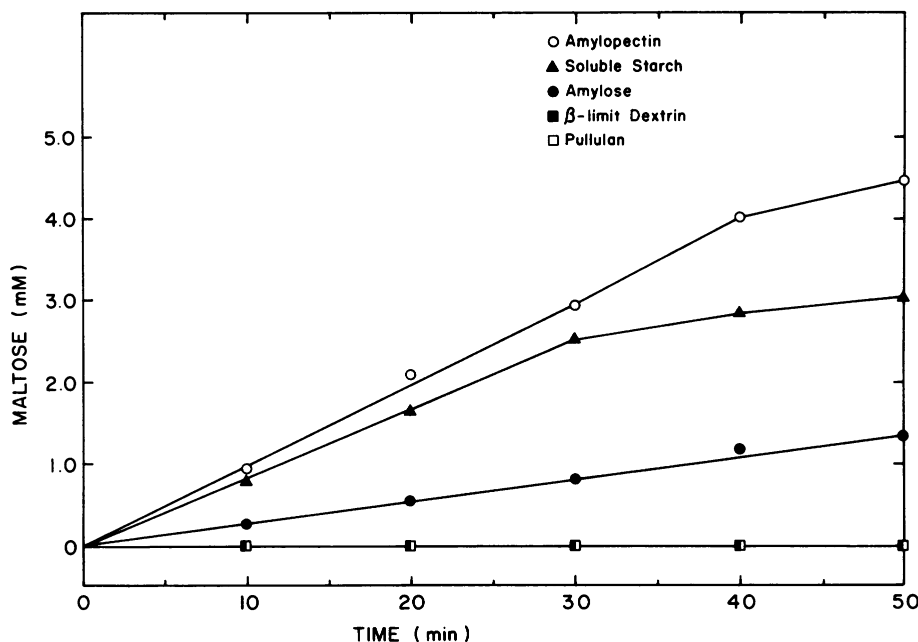

FIG. 3. Hydrolysis of 5% polysaccharide solutions by alfalfa root light amylase. Reactions were initiated with equal activities (1 IU in 7 ml) of light amylase.

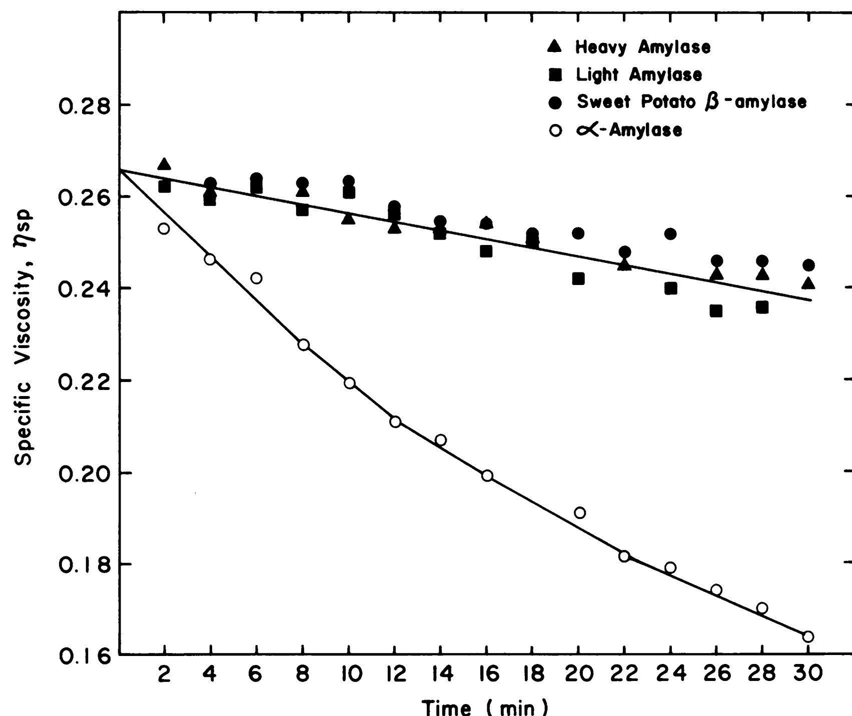

FIG. 4. Change in η_{sp} of a 1% starch solution by alfalfa heavy and light amylases, sweet potato β-amylase, and bacterial β-amylase.

one amylase isozyme (band 2). The intracellular localization of these isozymes is not known, although Okita *et al.* (16) have reported multiple intracellular localizations for amylases from spinach leaves.

Identification of β-Amylase Activity.

Substrate Specificity. Both the light and the heavy amylases hydrolyzed 5% solutions of amylopectin, soluble starch, and amylose, as shown in Figure 3 for the light amylase fraction. Similar curves were obtained with the heavy amylase. Highest rates were obtained using amylopectin as a substrate followed by soluble starch and amylose. Neither amylase preparation hydrolyzed pullulan, indicating the absence of debranching enzyme of these preparations. Also, neither preparation would hydrolyze β-limit dextrin, suggesting that these are not endoamylases.

Viscosity Experiment. One way of differentiating exo- and endoamylases is by a viscosity assay, since the ratio of change in η_{sp} to reducing power production is much higher for endoamylases than it is for exoamylases.

Preparations of the heavy and light amylases, sweet potato β-amylase, and bacterial α-amylase were appropriately diluted so that all contained about the same activity based on reducing power production. Their starch-liquifying power was measured in

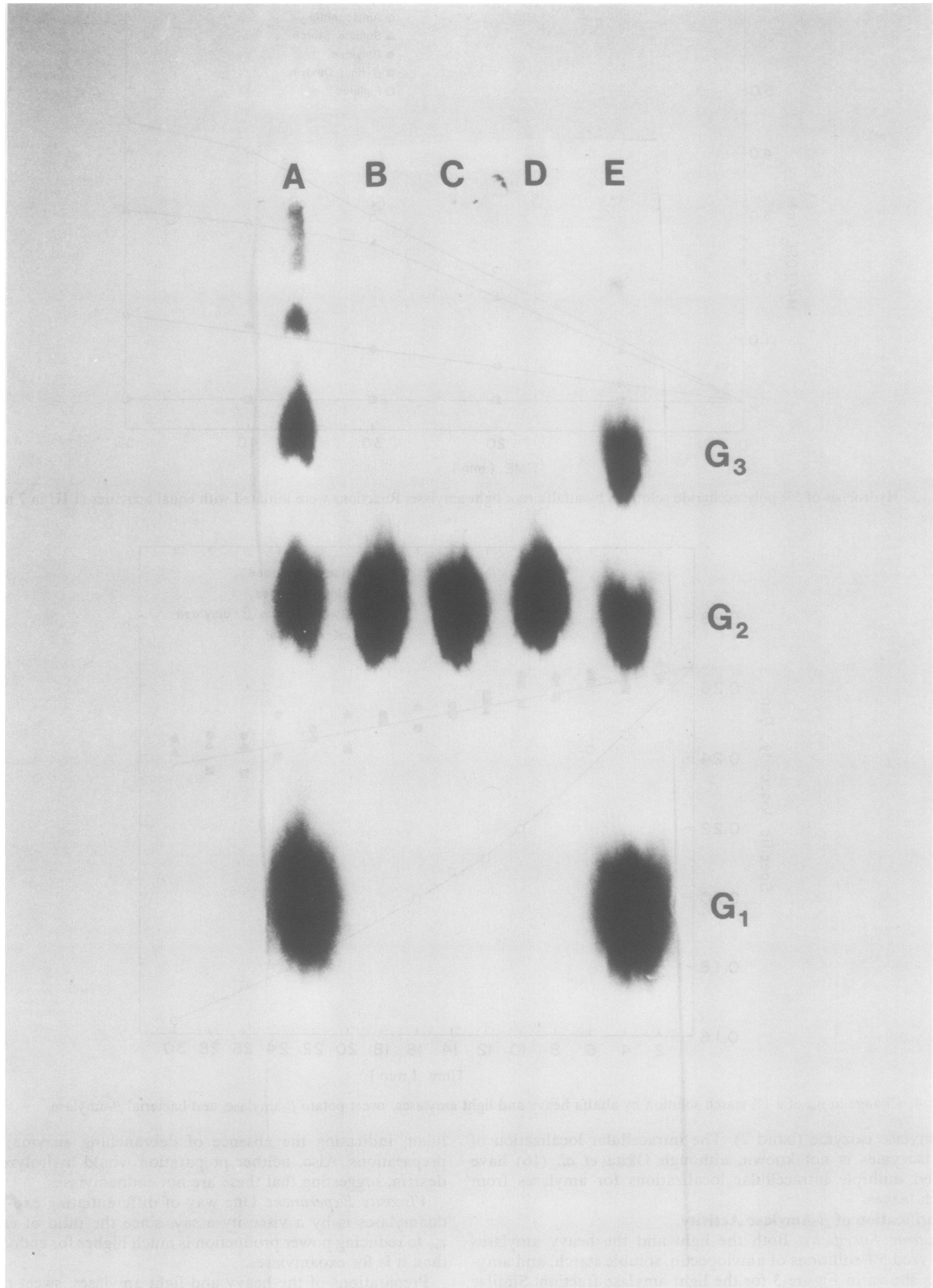

FIG. 5. Paper chromatography of low mol wt products of amylopectin hydrolysis by bacterial α-amylase (A), alfalfa crude extract (B), alfalfa light amylase (C), and alfalfa heavy amylase (D). The standard (E) contained glucose (G_1), maltose (G_2), and maltotriose (G_3). Enzymes were heat-killed after 24 h of hydrolysis.

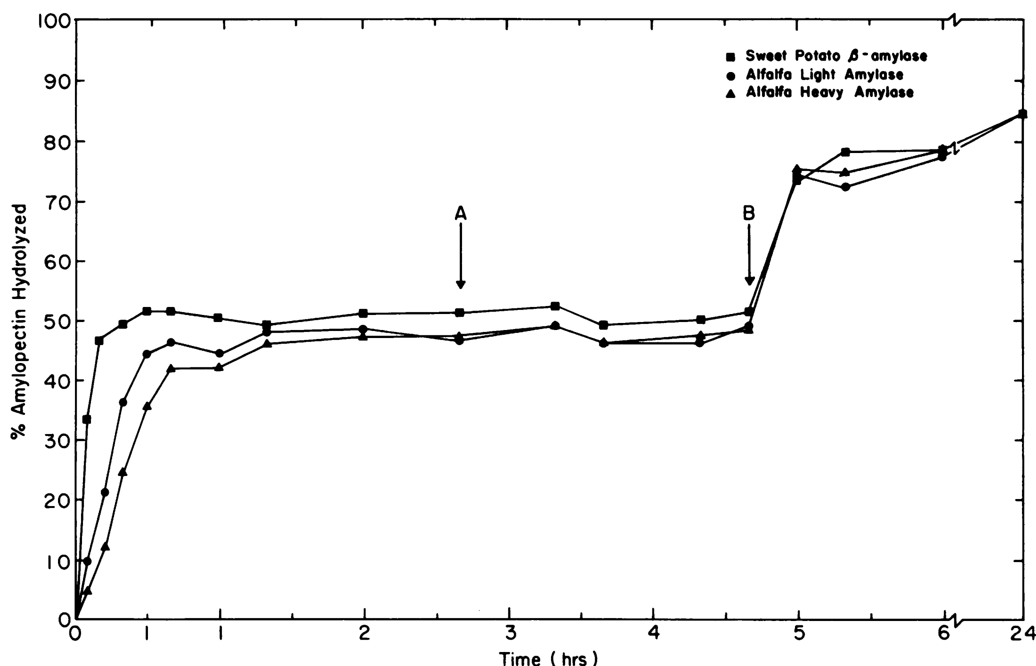

FIG. 6. Hydrolysis of amylopectin by alfalfa light and heavy amylases and sweet potato β-amylase. A, Additional enzyme added to each assay; B, bacterial α-amylase added to each assay.

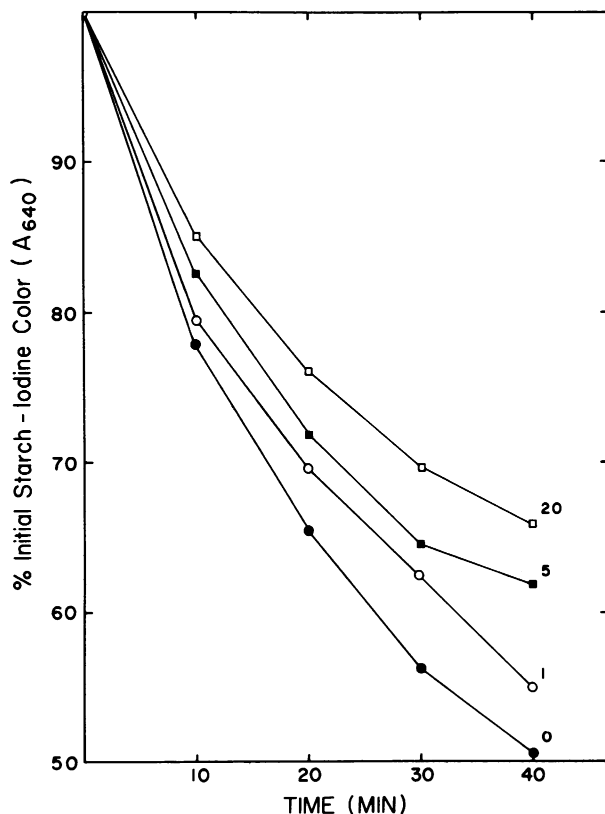

FIG. 7. Decrease in starch-iodine color of soluble starch solution containing light amylase and 0, 1, 5, and 0 mg/ml maltose.

an Ostwald viscometer (Fig. 4). The bacterial α-amylase rapidly decreased the viscosity of the starch and had a viscosity/reducing power ratio of 0.022 η_{sp}/μmol reducing power. The sweet potato β-amylase and both the light and heavy alfalfa amylases all reduced the viscosity of a starch solution similarly at a rate that was considerably slower than that of the α-amylase. The alfalfa and sweet potato amylases had identical viscosity-reducing power ratios of 0.003 η_{sp}/μmol reducing power. This indicates that the alfalfa root amylases, like sweet potato β-amylase, are exoamylases.

Product Analysis. Paper chromatography of the low mol wt products of amylopectin hydrolysis by the heavy and light amylase preparations; bacterial α-amylase; and standards containing glucose, maltose, and maltotriose was performed (Fig. 5). The bacterial α-amylase gave products having the same mobilities as the standards and also produced higher mol wt fragments. Both the light and the heavy alfalfa amylase preparations gave only maltose as an end product. Maltose production is a characteristic of β-amylase and strongly suggests that these are β-amylases. Product analysis was also performed on crude extracts. These also showed only maltose as a product.

Limit Dextrin Production. The production of a β-limit dextrin is also a property of β-amylases, so an experiment was performed to determine the extent of amylopectin hydrolysis by the alfalfa amylases (Fig. 6). Aliquots of light, heavy, and sweet potato β-amylases were incubated in solutions of 1% (w/v) amylopectin (with 100 mM succinate buffer [pH 6.0]), and samples were taken at regular intervals; the extent of amylopectin hydrolysis was derived from the reducing power of the solution, compared to the maltose. After about 2 h, all of the preparations had hydrolyzed about 45 to 50% of the amylopectin but would not further degrade it. Addition of fresh enzyme at 160 min (Fig. 6, arrow A) produced no further hydrolysis of the amylopectin; however, addition of about 20 units of bacterial α-amylase at 260 min (Fig. 6, arrow B) produced substantial additional hydrolysis in the first 20 min and resulted in a total of 84% hydrolysis in all preparations at 24 h.

Since the alfalfa amylases and sweet potato β-amylase hydrolyzed amylopectin to nearly equal extents, and this product was still susceptible to further hydrolysis by α-amylase, it appears that the alfalfa amylases also produce β-limit dextrin as a product. This evidence also suggests that the light and heavy amylases are all β-amylases. Together, these four experiments—substrate specificity, viscosity-reducing power ratio, product analysis, and limit dextrin production—substantially demonstrate that these amylases obtained from alfalfa tap roots are β-amylases.

Characterization of β-Amylases.

pH Profile. To eliminate buffer effects on the pH profile, a single, wide-range buffer was prepared (containing 100 mM citrate, 100 mM ethylenediamine, and 100 mM Bicine) that has buffering capacity over a pH range of 2.0 to 11.0. Over a pH range of 2.5 to 9.0, it was found that both light and heavy amylases have pH optima of 6.0, although they retained at least 80% of maximal activity over a pH range of 4.5 to 7.5 (data not shown).

Substrate Saturation Curves. The response of the light and heavy amylases to increasing concentration of soluble starch indicated that these enzymes follow normal Michaelis-Menten kinetics. Double reciprocal plots of the data (not shown) gave, for the light amylase, a V_{max} of 640 IU·mg protein^{-1} and a K_m of 5.9 mg soluble starch ml^{-1} and, for the heavy amylase, a V_{max} of 130 IU·mg protein^{-1} and a K_m of 6.8 mg soluble starch ml^{-1}.

Temperature Kinetics. Arrhenius plots (data not shown) for heavy amylase are linear from 0 to 30°C, indicating that there is little change in the E_a over this range (12.7 kcal/mol between 0 and 10°C; 13.0 kcal/mol between 20 and 30°C). In contrast, the Arrhenius plot for light amylase has an inflection at the apparent T_m of about 9°C, with an E_a of 28.3 kcal/mol below the T_m and 12.0 kcal/mol above the T_m. Wheat, barley malt, and sorghum malt β-amylases also have inflections in their Arrhenius plots, suggesting that these are not unusual for this enzyme (3, 14). Complete inactivation of both enzymes occurred above 67°C.

Maltose Inhibition. Inhibition of the light amylase by maltose was demonstrated by following starch degradation, as measured by starch-iodine coloration in the presence of increasing concentrations of maltose. The maltose slowed the rate of decrease of the starch-iodine color (Fig. 7). Although it is not possible to derive kinetic constants from these data, it does not appear that the inhibition is of physiological significance as a regulatory mechanism, because of the high concentration required.

CONCLUSIONS

From these data, it appears that there are very few differences between the isozymes of β-amylase from alfalfa roots other than the mol wt and electrophoretic mobilities. The functional role of these β-amylase is presumably in starch degradation. Alfalfa tap roots contain as much as 40% starch (dry weight), and the enzymes may function in the rapid breakdown of starch, as observed following shoot removal, where as much as one-half of this starch may be mobilized in a 7-d period (17, 19). A regulatory mechanism that can be suggested from these data is that β-amylase action is limited by the production of β-limit dextrin, in that only about 50% of an amylopectin molecule is directly susceptible to β-amylase hydrolysis. The presence of accompanying α-amylase or debranching enzyme would be necessary for further hydrolysis to occur.

Whereas β-amylase clearly represents the predominant amylase activity, subsequent experiments using a chromogenic substrate specific to α-amylase have detected α-amylase in crude alfalfa root preparations with activities of about 0.2% that of β-amylase, equivalent to about 0.40 IU·g fresh weight^{-1}. This is the subject of our current investigations.

Maltose produced by β-amylase is presumably metabolized toward sucrose synthesis. Since the enzyme maltose phosphorylase is found in alfalfa roots with activities (in the phosphorolytic direction) of about 0.01 to 0.20 μmol glucose 1-phosphate produced min^{-1} g fresh weight $^{-1}$, while no maltase activity is found in this tissue (D. C. Doehlert, S. H. Duke, unpublished data), it would appear that maltose phosphorylase is responsible for the further metabolism of maltose.

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