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STARCH DEGRADING ENZYMES OF TEMPERATE AND TROPICAL SPECIES*

J. L. CARTER, L. A. GARRARD and S. H. WEST†

Department of Agronomy, Agricultural Experiment Station, University of Florida, Gainesville, FL 32601, U.S.A.

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Key Word Index—*Dactylis glomerata; Digitaria decumbens; Pennisetum glaucum; Saccharum officinarum* Gramineae; starch degradation; amylase; isoenzymes.

Abstract—The starch degrading enzymes of the leaves of one temperate species, orchardgrass (*Dactylis glomerata* L.), and three tropical species, 'Pangola' digitgrass (*Digitaria decumbens* Stent.), pearlmillet (*Pennisetum glaucum* L.), and sugarcane (*Saccharum officinarum* L.), were separated by polyacrylamide gel disc electrophoresis, and some of their properties were examined. Densitometer tracings of gels negatively stained by starch-iodine showed distinctive patterns for each species. Incubation of gels at 10° as opposed to 30° reduced the activities of the enzymes of a temperate and tropical species; however, the enzymes of the tropical species were affected more than those of the temperate species. Tracings of orchard grass gels varied with type of incubation buffer. Relative activity of starch degrading enzymes varied with pH and time of incubation. α-Amylase is probably the major starch degrading enzyme of the leaves of these grasses.

INTRODUCTION

RECENT reports suggested that growth reduction in a tropical grass, 'Pangola' digitgrass (*Digitaria decumbens* Stent.), caused by 10° night temperature could be attributed to a depression in the activities of starch degrading enzymes and the concomitant immobilization of assimilate starch in leaf mesophyll chloroplasts;[1,2] in turn, accumulated starch could reduce the photosynthetic capacity of chloroplasts.[3,4] A temperate species, orchardgrass (*Dactylis glomerata* L.), was not similarly affected by the 10° treatment.[1] Starch degrading enzymes are generally considered to include α- and β-amylases and phosphorylase.[5] Inasmuch as these enzymes appear to have roles in growth, we attempted to separate them (without rigorous isolation and purification procedures) in order that their individual properties and activities might be compared. This study included separation of the starch degrading enzymes of some tropical and temperate species on starch-impregnated polyacrylamide gels and a measure of the relative activities of the enzymes following incubation of the gel columns under a variety of conditions.

RESULTS AND DISCUSSION

We have used comparatively simple methods to investigate the occurrence and properties of some starch degrading enzymes of three tropical plants, 'Pangola' digitgrass (*Digitaria decumbens* Stent.), sugarcane (*Saccharum officinarum* L.), and pearlmillet (*Pennisetum*

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† Plant Science Research Division, Agricultural Research Service, U.S. Department of Agriculture.

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[2] J. H. HILLIARD and S. H. WEST, *Science* **168**, 494 (1970).

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[4] S. H. WEST, *Proc. 11th Int. Grassland Conf. Austr.* 514 (1970).

[5] T. AKAZAWA, in *Plant Biochemistry* (edited by J. BONNER and J. E. VARNER), pp. 258–297, Academic Press, New York (1965).

glaucum L.), and one temperate plant, orchardgrass (*Dactylis glomerata* L.). We have accomplished the following: (1) provided composite profiles from densitometer tracings of polyacrylamide gel columns containing separated enzymes characteristic of each species; (2) estimated relative activities of starch degrading enzymes of a temperate and tropical species under two incubation temperatures; (3) in two species, demonstrated an effect of buffer pH on relative enzyme activity; and (4) investigated variations in the gel profiles of starch degrading enzymes after incubation with two different buffers at the same pH.

After separation of the enzymes on gels, the gel columns were placed in test tubes of buffer for predetermined periods to observe enzyme activity. During this incubation period, various treatments were imposed. In each case, the effect of the variable on relative activity of the enzymes was determined by the absence of iodine-stained starch in bands along individual gels. The areas of portions of densitometer tracings corresponding to each gel band were used as rough measurements of enzyme activity, referred to as 'relative activity'. The 'total' relative starch degrading activity for extracts of each species and each treatment was taken to be the sum of the areas of all cleared bands on the gel. This term does not imply the measurement of specific activity, or, for that matter, true total activity, but rather combines measurements of both the specific activity of the enzyme and the concentration of the enzyme for the purpose of comparing similarly treated gels bearing separated enzymes of different plants.

Appearance of the Gels

After electrophoresis gels were incubated for 15 min in 200 mM acetate buffer (pH 4·8) at 30°. On staining, the uppermost band found near the origin in orchardgrass extracts developed into an irregular pattern which, while consistently observed at this pH and temperature after short incubation periods, increased in width and lost this configuration with longer incubation. The 'tailing' ends of the centre bands of both gels were characterized by brownish-red to purple colour, fading into the blue background of the gel. This pattern of colour change is like that which occurs during α-amylolysis of amylose,[5] and its occurrence here may be considered evidence that the centre bands of these gels contain α-amylase.

Generally, there were two bands for pearlmillet, three for 'Pangola' and sugarcane, and four for orchardgrass (variations in the number in orchardgrass will be discussed later) when these gels were incubated at 30° in 200 mM acetate buffer (pH 4·8). Although areas of the bands varied with temperature and time of incubation, the profiles (i.e. the mobility and number of bands present) did not vary. In the case of 'Pangola', however, relative activity of the uppermost band was so reduced at 10° that when averages of measurements of replicates were compiled, the value was very small and did not appear in the profile in Fig. 1. Hereafter, for descriptive purposes, reference will be made to the 'centre bands' and the 'origin bands'.

Incubation Time Series

Gels bearing separated enzymes from extracts of 'Pangola' and orchardgrass were incubated at 30° in 200 mM acetate buffer (pH 4·8) for intervals of 15, 30, 45, 60, 90, 120, 150 and 180 min, after which they were immediately placed in cold 10% trichloroacetic acid (TCA) for 1 hr, then iodine stained. Origin bands did not develop any consistent pattern on a time scale in this particular experiment; however, these bands did not contribute greatly to total area under these conditions. Activity for both extracts was nearly proportional to time over the 3 hr period, though the rate of relative activity was at all times lower

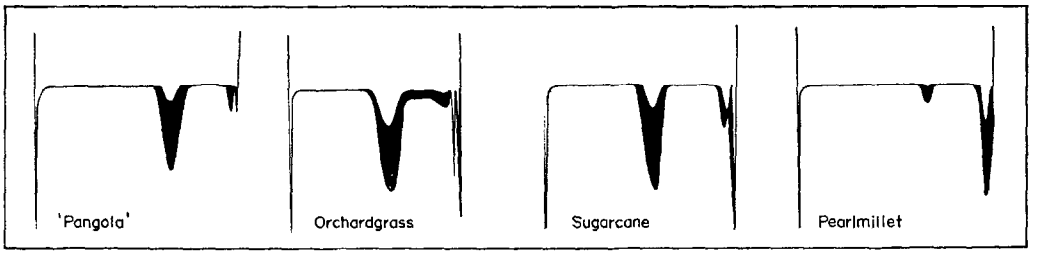

FIG. 1. EFFECT OF TEMPERATURE ON ACTIVITIES OF STARCH DEGRADING ENZYMES OF FOUR SPECIES. Each profile represents a composite of three to six densitometer tracings of replicate gels incubated at either 10° or 30°. The areas in black represent the differences between profiles from gels incubated at the two temperatures. Since negative staining techniques were employed, areas of highest activity are indicated by the deepest depressions of the base lines. Origin of the gel is to the right.

in 'Pangola' than in orchardgrass. This was consistent with findings reported in this paper under effects of incubation temperature.

Effect of pH

Gels containing separated enzymes of extract equivalent to 0·6 g fr. wt. of pearlmillet and 0·3 g fr. wt of orchard grass were incubated for 3 hr at 30° in 200 mM citrate–phosphate buffer at pH values of 4·2, 4·6, 5·0, 5·6, and 6·0. Total relative activities at the various incubation pH values are shown in Table 1. Pearlmillet had a peak of starch degrading activity at pH 5·0 and showed some decline in relative activity as the pH was either raised or lowered. However, the relative activity of this tropical species appeared to be less affected by changes in pH than did the relative activity of orchardgrass, a temperate species. On the other hand, starch degrading activity of orchardgrass extracts was high at pH 4·2, declined somewhat, and rose again to nearly the same level at pH 5·0. Above this point, activity declined rapidly. Again, the temperate species, orchardgrass, showed considerably more starch degrading activity than did the tropical pearlmillet, this being shown over a comparatively wide pH range. Subsequent incubations were conducted using buffers at pH 4·8.

TABLE 1. TOTAL STARCH DEGRADING ACTIVITY AT DIFFERENT INCUBATION pH VALUES

Plant	pH	Total relative activity* (cm^2)	Plant	pH	Total relative activity* (cm^2)
Orchardgrass	4·2	17·1 a	Pearlmillet	4·2	6·0 d, e
	4·6	12·9 b		4·6	6·7 d
	5·0	15·9 a		5·0	6·9 c, d
	5·6	8·8 c		5·6	6·3 d
	6·0	4·7 e, f		6·0	2·8 f

* Estimated area (cm^2) under the curves of the densitometer tracings of the gels. The area values (total relative activity) are averages of measurements made from at least three densitometer tracings of replicate gels. Means having the same letter are not significantly different at $P = 0{\cdot}01$ according to Duncan's New Multiple Range Test, $S_{\bar{x}} = 0{\cdot}66$.

Temperature of Incubation

Gels from extracts of 'Pangola', orchardgrass, sugarcane, and pearlmillet were incubated in 200 mM acetate buffer (pH 4·8) at either 10° or 30° for 2 hr. Composite densitometer tracings (prepared from tracings of three to six replicates) of each species at each temperature are shown in Fig. 1. The activities of enzymes of the centre bands were reduced to a greater extent by low temperature incubation than those of enzymes found near the origin. Both concentration of enzyme and concentration of starch in the gel were maintained at levels at which substrate was non-limiting and at which the concentration of enzyme gave clear bands with minimal tailing. Tracings of unstained gels and of stained gels without enzymes gave straight lines at the lowest and highest limits, respectively, represented in Fig. 1.

TABLE 2. INCUBATION TEMPERATURE OF GELS AND TOTAL STARCH DEGRADING ENZYME ACTIVITY*

Plant	Incubation Temp.	Total relative activity† (cm^2)	Reduction (%)
Orchardgrass	30	18·1	67·4
	10	5·9	
'Pangola'	30	11·6	89·0
	10	1·3	

* Area in cm^2 under the curves of the densitometer tracings. The area values (total relative activity) are the means from at least three replicate gel densitometer tracings.

† S_d values determined by analysis of variance are as follows: for comparing plant means, $S_d = 0{\cdot}77$; for comparing total relative activities of plants at the same temperature, $S_d = 1{\cdot}06$; for comparing total relative activities of either plant at the two temperatures, $S_d = 1{\cdot}06$. Error $d.f. = 4$. All of the above comparisons are significant at the $P = 0{\cdot}01$ level.

Reduction in total relative activity on lowering the temperature of incubation was found to be greater in the tropical species ('Pangola') than in the temperate species (orchardgrass) (Table 2). This is in accord with findings of Karbassi *et al.*,[1] who determined starch degrading activity with enzymes in solutions rather than separated on gels. However, our work showed that enzymes forming origin bands on gels with extracts of some species (sugarcane and pearlmillet in particular) were not as responsive to reduction in temperature during incubation as were the enzymes forming centre bands of the gels. Because bands near the origin were comparatively narrow at any temperature, they would be expected to represent only a small fraction of the total activity of a crude extract.

Of the four species tested, the level of activity of starch degrading enzymes during 30° incubation was highest in orchardgrass, followed closely by sugarcane; the level of enzyme activity was lowest in pearlmillet, while the activity level in 'Pangola' was between pearlmillet and sugarcane (Fig. 1, Table 2). This observation is in agreement, in the cases of orchardgrass and 'Pangola', with the time course data of relative activity.

Buffer Effects

Gels from extracts of orchardgrass, 'Pangola', and pearlmillet were incubated at 30° with either acetate or citrate-phosphate buffers, both 200 mM at pH 4·8. In the profiles from tracings of three gels, 'Pangola' and pearlmillet showed no apparent differences attributable to incubation in the different buffers, either in the number of bands present or in total relative activity. However, in the case of orchardgrass, two distinct peaks developed in the area of the centre band of gels incubated in citrate-phosphate where only one was seen in acetate buffer-incubated gels. One of the peaks may have been formed by a phosphorylase activated by the inorganic phosphate of the incubation buffer. This enzyme has been postulated to have a role in starch degradation in leaves;[6,7] however, Karbassi[1] found only a very low level of phosphorylase activity in 'Pangola'. If phosphorylase activity is indeed higher in temperate species, resistance to growth retardation at low temperatures in temperate species might be partially explained. If the other peak were α-amylase, as is suggested in this discussion, it would apparently be the leading band, as α-amylase has been found to migrate faster than either β-amylase or phosphorylase.[7] Another possible explanation of the appearance of the double peak would be the presence of an isozyme.

Total relative activities of starch degrading enzymes forming centre bands were reduced by incubation in citrate-phosphate buffer, as compared to incubation in acetate buffer. Reduction of relative activity of the centre band(s) was 35%, while reduction in the total relative activity (bands combined) was only 12·6%. By contrast, relative activities of enzymes near the origin increased in citrate-phosphate buffer. The reduction in relative activity of the centre band(s) may be explained if we assume the centre band to be formed (all or partially) by α-amylase. If this were the case, it could be postulated that the reduction in relative activity was due, totally or in part, to the competition for the Ca^{2+} between the enzyme and the phosphate of the incubation buffer, the phosphate precipitating some of the Ca^{2+} required for α-amylase activity. The fact that no such similar reductions occurred in total relative activities of the starch degrading enzymes of the two tropical grasses tested may indicate that Ca^{2+} is bound more tightly to the α-amylase of these tropical grasses.

On the basis of the evidence presented in this discussion, the double band in the centre of the orchardgrass gel incubated in citrate–phosphate buffer was tentatively designated as either α-amylase or α-amylase plus phosphorylase, and the chief starch degrading enzyme of these grasses is probably α-amylase. Data supporting this include colour changes in the gel which would be typical of α-amylase activity, the probable Ca^{2+} dependence of the major enzyme, and the greater mobility of the major peak. The temperate species, orchardgrass, known to grow better at low temperatures than the tropical species, had more bands with starch degrading activity on electrophoresis and these enzymes were less sensitive to change in temperature than those of tropical species. Moreover, the temperate species had a higher total relative activity of starch degrading enzymes than the tropical species. The temperate plant appeared to have an active phosphorylase which was not found in tropical species. All of these factors would contribute to a higher level of soluble sugars in the temperate species than in the tropical species at reduced temperatures, thus enhancing subsequent growth and cold resistance in the former.

EXPERIMENTAL

Plant material. Sugarcane, 'Pangola', pearlmillet, and orchardgrass plants were grown in plastic pots containing steam-treated potting soil, in a greenhouse between 27° and 33°. Plants were watered every other

[6] M. A. R. DeFekete, *Planta* **79**, 208 (1968).

[7] B. O. Juliano and J. E. Varner, *Plant Physiol.* **44**, 886 (1969).

day and received nutrients each week. 'Pangola' plants used in these studies were of 5-weeks regrowth after topping; pearlmillet plants were 4–5-weeks-old from seed; orchardgrass plants were from 4 to 5 weeks from propagation by division, and sugarcane was 3–6 months regrowth from cuttings.

The centre 5–10 cm of the second fully expanded leaves of each species were harvested and the midribs removed.

Preparation of extracts. Samples (0·3 g) were immediately ground for 10–15 min in a glass homogenizer cooled in ice containing 5 ml ice-cold 10^{-4} M $CaCl_2$. The homogenate was washed with an additional 5 ml 10^{-4} M $CaCl_2$ into a polypropylene centrifuge tube and placed for 1 hr at 0° on a shaker to permit lysis of cells and leakage of enzymes into solution. Approximately 95% of the total starch degrading enzymes present in the leaf sample was found in the supernatant fraction after centrifugation. The homogenate was then centrifuged in the cold at 10^5 *g* for 2 hr. The centrifugate was then decanted and sucrose added to 20%.

Electrophoresis. Polyacrylamide gels were made by a modification of the method of Davis.[8] No sample gels or spacer gels were used. Amylose solution (1 g amylose/100 ml deionized distilled water, with boiling and filtration, cleared before each gel preparation by briefly heating) was substituted for the water phase of the small pore (separating) gel. Davis' Tris-glycine buffer, pH 8·3, was used in both upper and lower reservoirs of the electrophoresis apparatus which was a vertical disc type with glass tubes 175 × 5 mm (i.d.). The entire apparatus was pre-cooled and used in a cold box (2–4°). Samples (50 μl) of the extracts were applied to each gel, and the current was 1 mA/tube for the first 15 min and 2 mA/tube thereafter. The period of electrophoresis varied from 2–3 hr and was terminated when the natural pigment in the extract was 3–5 mm from the base of the gel.

Band development and staining. Gels were removed from the glass tubes with a jet of water from a hypodermic needle and rinsed in distilled water. They were then placed in the selected buffer in glass culture tubes. At the completion of incubation, the gels were removed from the buffer and washed again with distilled water, then placed immediately in cold 10% TCA and refrigerated for at least 1 hr. They were then stained in an iodine-K acetate buffer solution (4×10^{-3}% I_2 and 1·5% KI in 200 mM acetate buffer, pH 4·8). Maximum staining was achieved within 1 hr.

Densitometer tracings. The optimum wavelength was determined by a series of scans of several gels at different wavelengths and by scanning the amylose-iodine complex (in the gel) throughout the visible range The wavelength giving maximum absorbance was 575 nm.

[8] B. J. DAVIS, *Annals N.Y. Acad. Sci.* **404**, 121 (1964).