Enzymatic Activities in Proso Millets

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ABSTRACT

The β-amylase, protease, cellulase, and hemicellulase activities of eight cultivars of proso (*Panicum miliaceum*) millets were determined. Two of the cultivars were grown on different plots to permit study of the effects of different locations on enzyme levels. All of the cultivars showed β-amylase, protease, cellulase, and hemicellulase activities, with the exception of one sample that showed no hemicellulase activity. Optimum pH for β-amylase activity was approximately 5.0. Production of maltose per milliliter of extract ranged from 0.73 to 1.98 after 1 hr of incubation at pH 5.25. The pH optima for protease activity were near 3.0 and 5.0. Production of tyrosine per milliliter of extract ranged from 12.5 to 75.5 μg after 1 hr of incubation at pH 4.8. Cellulase activity produced decreases in viscosity of the solution, which ranged from 9 to 18% after 6-hr incubation at pH 5.0. Decreases in viscosity due to hemicellulase activity ranged from 0 to 11% after 6 hr of incubation at pH 5.0. Differences in enzymatic activity appeared to relate to the location where the millets were grown. Soil differences or slightly different levels of maturity were hypothesized as reasons for these differences.

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The term "millet" is used for several genera of small-seeded annual grasses that are of only minor importance in the Western world but a staple in the diet of some African and Asiatic people. Millet is consumed mostly in northern China, India, Africa, and southern Russia, where about 85% of the crop is used as human food. In the United States, millets are incorporated into feeds and birdseeds. At present, very little is used directly as human food (Schery 1963). Flours from millet can be used to partially replace wheat flour in breads, cookies, and pasta products (Badi et al 1976, Lorenz and Dilsaver 1979).

The five most common millets are: *Setaria italica, Pennisetum typhoideum, Eleusine coracana, Echinochloa frumentacea,* and *Panicum miliaceum.* Although the chemical composition and nutritive value of millets grown in different parts of the world have been reported, little information on enzyme activities of millets is available.

No reports have been published on enzyme activities in proso millets, and reports on other types of millet are few. Because the different types of millets extend over several genera, comparisons between types are not particularly meaningful. Much of the work has focused on the enzymatic activities of malts prepared from millets because of their importance in brewing.

Chandrasekhara and Swaminathan described the amylases (1953a), proteases (1953b), and pyrophosphatase and glycerophosphatase (1954) in ragi (*E. coracana*) and in ragi malt. They characterized amylase activity in ragi as very low; the activity in the germinated ragi was about two orders of magnitude higher. They found a pH maximum for the amylases between 4.6 and 5.0. Sheorain and Wagle (1973) and Jain and Date (1975) compared amylase activity in bajra millet (*P. typhoideum*) with that in barley. Amylase activity was higher in the millet.

Chandrasekhara and Swaminathan (1953b) found no protease activity in ungerminated ragi. Activity of ragi malt proteases ranged between pH 3.8 and 8.0 and reached a maximum at pH 4.4.

Relatively small amounts of both pyrophosphatase and glycerophosphatase were detected in the resting grain of ragi millet, but germination increased the activities of these enzymes approximately sixfold and fourfold, respectively. Pyrophosphatase showed maximum activity at pH 5.0 and glycerophosphatase at pH 5.6 (Chandrasekhara and Swaminathan 1954).

Féron and Bouquet (1948) studied the lipase activity in millet flour milled from an unspecified variety. The lipase activity showed a slight peak at pH 2.0, with a maximum around pH 8.0.

In this study, the levels of β-amylase, protease, cellulase, and hemicellulase were determined in eight cultivars of proso millets (*P. miliaceum*).

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MATERIALS AND METHODS

Samples

Eight different cultivars of proso (*P. miliaceum*) millets (Table I) were analyzed. All were grown in 1977 at the Colorado State University experimental farm in Springfield, CO on summer-fallowed land. Two of the cultivars were grown on different plots in the same area to permit study of the effects of different locations on enzymatic activity.

β-Amylase Activity

β-amylase activity was determined by Bernfeld's method (1955). Kernels of each cultivar were first ball-milled into a powder (100% through 200 mesh) at refrigeration temperatures using a Wig-L-Bug. Two separate extractions were made from each ground sample. One-gram samples of ground millet were incubated at 37°C for 30 min with 10 ml of distilled water (pH 6.8) and 0.1 *M* acetate buffer (pH 5.25), respectively. After 30 min of extraction, the tubes were removed from the water bath and immediately centrifuged at 12,000 × *g* for 10 min. The supernatants were poured into airtight bottles and refrigerated for β-amylase determinations the following day. β-Amylase activity was determined after 10, 20, 30, and 60 min of incubation at 37° C using 1 ml of extract and 1 ml of starch solution. Enzyme activity was stopped by immersion of the test tubes in boiling water for 5 min. The starch solution was prepared by adding 1 g of starch to 50 ml of boiling distilled water, stirring until clear, and then adding distilled water to 100 ml total volume. Blanks were made by boiling the extract for 5 min before the starch solution was added. Control tubes consisted of the starch solution in 1 ml of the acetate buffer. Spectrophotometric values measured at 530 nm were compared with a standard curve for maltose covering the range of values expected with the millet cultivars. β-Amylase activity is expressed as milligrams of maltose produced per milliliter of extract in a given length of time. Duplicate replications were done at each time. The pH optimum of β-amylase activity was determined in preliminary experiments.

Protease Activity

Protease activity was determined by a modification of the method described by Bushuk and Hwang (1971). Two separate extractions were made from each sample. The 1-g millet samples, ball-milled as described for β-amylase activity, were incubated at 37°C for 30 min with 10 ml of a pH 4.8 and a pH 3.8 acetate buffer, respectively. The supernatants were poured into airtight bottles and refrigerated for protease activity determinations the following day. The substrate solution was 1% hemoglobin in sodium acetate buffer. Extract (1 ml) was incubated with 2 ml of substrate solution for 10, 20, 30, and 60 min. Enzymatic activity was stopped by adding 2 ml of 5% trichloroacetic acid (TCA). The blank was made by adding 2 ml of 5% TCA to the extract, which stopped the

activity, before adding the substrate. The control tubes contained only substrate and buffer. The Lowry method (1951) was used to measure soluble proteins, and the spectrophotometric values measured at 500 nm were compared with a standard curve for tyrosine covering the range of values expected with the millet cultivars. Protease activity is expressed as micrograms of tyrosine produced per milliliter of extract in a given length of time. Duplicate determinations were done at each time. The pH optima of protease activity were determined in preliminary experiments.

Cellulase and Hemicellulase Activity

For cellulase and hemicellulase activity, 1 g of ball-milled millet was extracted with 20 ml of 0.6% NaCl solution for 30 min at 3°C. The solutions were then centrifuged at the same temperature at 8,000 × *g* and the supernatant refrigerated. A modification of the method of Schmitz et al (1974) was used to determined cellulase activity. Millet extract (2 ml) was incubated at 37°C with 2 ml of carboxymethyl cellulose solution (0.1 mg/ml in 0.02*M* sodium acetate buffer, pH 5.0, with 0.6% sodium chloride). Viscosities were determined in Ostwald flow-type viscometers. Measurements were taken every 30 min for the first hour and once an hour thereafter for a total of 6 hr. After 6 hr, enzymatic activity was stopped by immersion of the viscometer in boiling water for 5 min. Samples were then filtered and freeze-dried for later analysis.

Hemicellulase activity was determined similarly, except that the substrate solution was arabinogalactan (10 mg/ml in 0.02*M* sodium acetate buffer, pH 5.0, with 0.6% NaCl). Viscosity measurements were done as described for cellulase activity. After 6 hr of incubation, the solutions were heated to inactivate all enzyme activity and then freeze-dried for sugar determinations. Duplicate replications were done for each variety for both cellulase and hemicellulase activity.

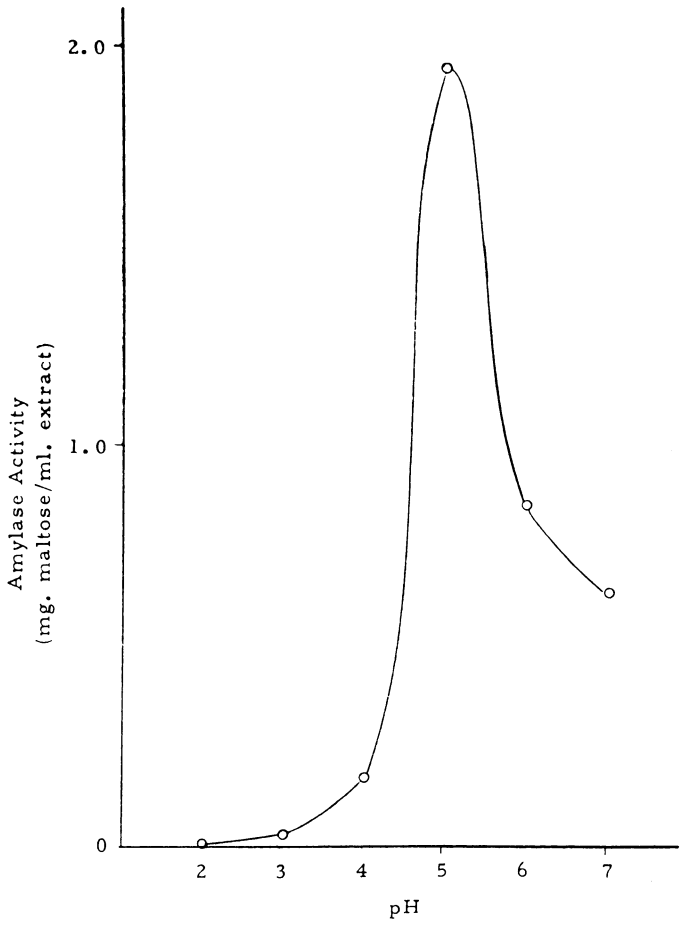

Fig. 1. Effect of pH on β-amylase activity in proso millets (Turghai I). Activity after 60-min incubation with substrate.

Sugar Determinations

The freeze-dried samples were put into solution with 1 ml of water, and thin-layer chromatography (silica gel) was conducted for qualitative and quantitative sugar determination. The solvent was 92:8 acetone/water. The R_f values of the control sugars (glucose and cellobiose for cellulase activity and arabinose for hemicellulase activity) were determined by spraying an anisaldehyde-sulfuric acid solution (9 ml of 95% ethanol, 0.5 ml of concentrated sulfuric acid, and 0.5 ml of anisaldehyde) on the sections of the plates containing the control sugars and then heating this area with a heat gun. Areas corresponding to the experimental samples were covered during the spraying and heating. When the control spots were visible, heating was discontinued. The corresponding areas over the experimental samples were scraped from the plate, and concentrations of glucose and cellobiose were determined by the phenol-sulfuric method (Dubois et al 1956). Standard curves covering the expected sugar concentrations in the millet cultivars were prepared for glucose, cellobiose, and arabinose, relating sugar concentration to spectrophotometric values measured at 490 nm.

RESULTS AND DISCUSSION

β-Amylase Activity

Three cultivars of millet (Turghai I, Leonard II, and Dawn) were incubated at different pH levels to determine the optimum pH for β-amylase activity. The results indicated a pH maximum of approximately 5.0. The data obtained with Turghai I are illustrated in Fig. 1. They agree with data previously reported for ragi millet, which showed a maximum between pH 4.6 and 5.0 (Chandrasekhara and Swaminathan 1953a).

β-amylase activities in water and in 0.1*M* buffer extracts for all cultivars are shown in Table II. With all cultivars, the amount of maltose liberated increased with length of incubation, as would be expected. Only the 60-min activity values are shown. There is a range of β-amylase activities between the different cultivars. Leonard II contained the least β-amylase activity (0.73 mg of maltose produced per milliliter of extract at pH 5.25), and Dawn had the highest activity (1.98 mg/ml of extract at pH 5.25). In all cultivars, the amount of maltose liberated after 60 min was greater in the 0.1*M* acetate buffer extract (pH 5.25) than in the water extract (pH 6.8) as would be expected from the pH optimum.

Because of differences in methods, meaningful comparisons are difficult to make between these values and the few previously reported for millets or cereals in general. Extraction times, buffers, and entire methods were usually different in previous studies. Lorenz and Saunders' study (1978) with rice used exactly the same method and reported 0.8–1.3 mg of maltose produced in 60 min per

TABLE I
Millet Varieties

Cultivar	Description	Protein[a] (%)	Crude Fat[a] (%)
Turghai I[b]	Standard red variety	13.4	9.4
Turghai II	Standard red variety	14.3	6.5
Akron	Colorado selection, red variety	14.7	6.1
Big Red	Experimental variety from Russia	13.3	9.0
Leonard I	Colorado selection, yellow variety	13.2	9.2
Leonard II	Colorado selection, yellow variety	12.9	9.3
Cope	Colorado selection from "common white"	13.2	9.5
Dawn	Nebraska white selection	14.5	8.1
Minco	White early maturing type from Minnesota	13.0	9.5
Abarr	Colorado selection from "common white"	13.6	9.3

[a] Expressed on 14% mb. Protein = N × 6.25.
[b] I and II indicate cultivars grown on different plots.

milliliter of extract (pH 4.75), compared with 0.73–1.98 mg of maltose produced in 60 min per milliliter of extract (pH 5.25) in the proso millets. Thus, at least some of these millets seem to have higher amylase activity then did the rice samples studied.

β-Amylase activity varied considerably within the same cultivars (Turghai I and II, Leonard I and II) because of location. This was surprising at first, but differences in proximate analysis between the same variety grown on different plots (Table I) showed that enzyme differences might be expected. On the other hand, enzymatic activities change as grain matures (Bushuk and Hwang, 1971); perhaps slightly different states of maturity between samples could help explain the differences.

These differences between single cultivars grown in slightly different locations emphasize the pitfalls of comparing enzymatic activities of different cultivars (and sometimes completely different grains) grown in different locations.

Protease Activity

Protease activity was determined at pH 2–7 for three different cultivars (Abarr, Big Red, Turghai II). Data for the cultivar Turghai II are shown in Fig. 2. As can be seen, peaks are present around pH 3.0 and 5.0, which would agree with the protease pH optima reported for rice (Tanaka et al 1975).

The results for protease activity of all cultivars at two pH values are given in Table II. In all cases, protease activity was higher in the pH 4.8 extract than in the pH 3.8 acetate buffer extract.

The samples showed a range of 12.5–75.5 μg of tyrosine produced in 60 min per milliliter of extract at pH 4.8. The cultivar with the lowest activity was Abarr; Turghai II showed the highest activity. Only Dawn, Turghai I, and Abarr produced less than 50 μg of tyrosine per milliliter of extract after 60-min incubation at pH 4.8. In the only study using the same method, Lorenz and Saunders (1978) found a range of activity in rice of 13–65 μg of tyrosine produced per milliliter of extract after 60-min incubation (pH 4.75). This activity and the activity of millet at pH 4.8 are comparable.

As with β-amylase activity, there seemed to be a trend indicating different protease activities in the same cultivar grown in different locations.

Cellulase

Cellulase activites for the samples are shown in Table III. After 6 hr the range of decrease in viscosity among cultivars was 9–18%. Turghai II, Leonard II, and Dawn showed the lowest celulase activity and Turghai and Cope the highest.

A 6-hr incubation was used for determination of both viscosity changes and sugar production. The decrease in viscosity was greatest in the first hour of incubation and changed little in the final 3 or 4 hr. This is probably because the initial breakdown of the very large cellulose chains caused the greatest change in viscosity. Further breakdown of their fragments after longer periods of incubaton did not cause a large change in viscosity.

TABLE II
β-Amylase and Protease Activities[a]

	β-Amylase Activity		Protease Activity	
Cultivar	pH of Extract	Maltose Produced (mg/ml Extract)	pH of Extract	Tyrosine Produced (μg/ml Extract)
Turghai I	6.8	1.42 ± 0.11	4.8	16.0 ± 0.6
	5.25	1.81 ± 0.05	3.8	12.5 ± 0.0
Turghai II	6.8	0.63 ± 0.07	4.8	75.5 ± 0.0
	5.25	1.31 ± 0.10	3.8	18.5 ± 0.0
Akron	6.8	0.92 ± 0.19	4.8	70.0 + 1.3
	5.25	0.93 ± 0.15	3.8	44.0 ± 1.0
Big Red	6.8	1.24 ± 0.04	4.8	53.0 ± 0.7
	5.25	1.75 ± 0.12	3.8	18.5 ± 0.0
Leonard II	6.8	0.64 ± 0.08	4.8	69.0 ± 0.0
	5.25	0.73 ± 0.05	3.8	13.0 ± 0.0
Leonard I	6.8	0.95 ± 0.06	4.8	53.5 ± 0.7
	5.25	1.63 ± 0.05	3.8	18.5 ± 0.0
Cope	6.8	0.70 ± 0.00	4.8	64.0 ± 0.0
	5.25	1.38 ± 0.21	3.8	44.0 ± 0.1
Dawn	6.8	1.19 ± 0.08	4.8	41.5 ± 0.7
	5.25	1.98 ± 0.03	3.8	6.5 ± 0.0
Abarr	6.8	0.88 ± 0.19	4.8	12.5 ± 0.0
	5.25	1.30 ± 0.22	3.8	12.5 ± 0.0
Minco	6.8	0.73 ± 0.02	4.8	50.5 ± 0.0
	5.25	1.80 ± 0.21	3.8	12.5 ± 0.0

[a]Results are averages of two determinations after 60-min incubation at 37°C.

TABLE III
Cellulase and Hemicellulase Activities[a]

	Cellulase Activity			Hemicellulase Activity	
Cultivar	% Decrease in Viscosity After 6 hr	μg of Glucose per 10 mg of Millet	μg of Cellobiose per 10 mg of Millet	% Decrease in Viscosity After 6 hr	μg of Arabinose per 10 mg of Millet
Turghai II	9.0 ± 2.5	7.6 ± 0.2	4.6 ± 0.2	3.0 ± 1.1	2.9 ± 0.2
Turghai I	18.0 ± 3.3	9.4 ± 0.5	4.1 ± 0.0	8.7 ± 0.1	6.4 ± 1.3
Akron	10.1 ± 1.1	6.8 ± 0.0	1.6 ± 0.4	11.2 ± 0.2	9.9 ± 0.4
Leonard II	9.2 ± 1.4	6.1 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Leonard I	11.1 ± 0.6	9.8 ± 0.0	5.1 ± 1.0	6.5 ± 1.1	4.9 ± 0.6
Dawn	9.2 ± 0.3	12.4 ± 1.2	0.0 ± 0.0	8.2 ± 1.6	3.6 ± 0.0
Cope	18.0 ± 0.2	12.7 ± 0.0	4.9 ± 0.5	8.6 ± 0.0	5.7 ± 0.3
Abarr	14.5 ± 1.1	13.1 ± 0.3	4.6 ± 0.8	5.7 ± 0.0	6.0 ± 0.7
Minco	14.3 ± 0.5	7.9 ± 0.2	1.6 ± 0.8	1.4 ± 0.1	0.0 ± 0.0

[a]Average of two determinations.

Lorenz and Saunders (1978) found that viscosity in rice decreases 5–13% over 6 hr. Thus, millet seems to have a higher cellulase content than rice.

A difference in cellulase activity due to location was indicated between the two Turghai samples, but very little difference was found between the Leonard samples.

Amounts of sugars in the incubating solution after 6 hr indicate exoenzymatic activity, and viscosity drops indicate endoenzymatic activity. High endoenzymatic activity will, of course, produce more substrate for the exoenzymes. Thus, sugars present in the final solution might be expected to correlate somewhat with the drop in viscosity over time. The correlation coefficient between total sugars and viscosity changes equals 0.56 ($P < 0.07$). The correlation is better than that of Schmitz et al (1974), who found little variation in the amount of endocellulases in six varieties of wheat but large variations in the exocellulase activities of the six varieties and thus a correlation coefficient close to zero.

Hemicellulase

Hemicellulase activities are presented in Table III. Decrease in viscosity ranged from 0 to 11.2% during the 6-hr incubation. Leonard II showed no activity; Akron showed the greatest activity. As with the cellulase determinations, the greatest change in viscosity occurred in the first hour of incubation, again probably because the initial breakdown of the large substrate molecules caused the greatest change in viscosity.

Lorenz and Saunders (1978) found 0–10% viscosity decreases in rice after 6 hr. Thus, rice and millet seem to have a very similar hemicellulase content.

Again hemicellulase activities seemed to differ between the same cultivars grown in different locations. Leonard II showed no activity, but Leonard I showed a fairly high activity. There were also apparent differences in hemicellulase activity in the Turghai samples.

Amounts of sugars in the final solution are presented in Table III. The correlation coefficient between final sugar values and viscosity changes was 0.91 ($P < 0.01$).

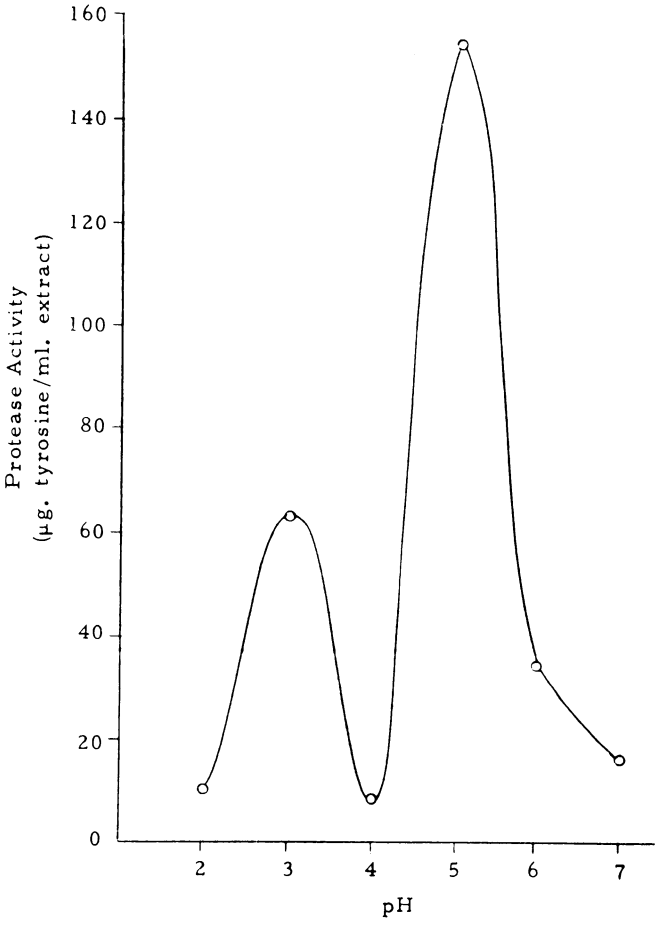

Fig. 2. Effect of pH on protease activity in proso millets (Turghai II). Activity after 60-min incubation with substrate.

CONCLUSIONS

All of the proso millet (*P. miliaceum*) cultivars showed β-amylase, protease, cellulase, and hemicellulase activities, with the exception of a few varieties that showed no hemicellulase activity.

Differences in location seemed to cause differences in enzymatic activities in the same cultivar. Soil differences or slightly different levels of maturity were hypothesized as reasons for these differences.

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