Pearl Millet Amylases. I. Properties of Partially Purified Alpha-Amylase[1]

ADELAIDE BELEIA and E. VARRIANO-MARSTON,[2] Department of Grain Science and Industry, Kansas State University, Manhattan 66506

ABSTRACT

Cereal Chem. 58(5):433–437

The purification method affected the physicochemical characteristics of purified millet α-amylases. Polyacrylamide gel electrophoresis showed three protein bands for α-amylase purified by glycogen complex formation and seven for α-amylase purified by starch column procedures. All protein bands exhibited α-amylase activity. α-Amylase isozymes had molecular weights, determined by sodium dodecyl sulfate gel electrophoresis, ranging from 22,000 to 53,000 and isoelectric points ranging from 4.8 to 6.2. The pH optima were between 4.4 and 4.8; temperature optimum was 55°C. Many of the above characteristics are similar to those reported for amylases purified from immature cereal grains.

Pearl millet (*Pennisetum americanum* (L.) Leeke) is grown extensively for human consumption in India and Africa, especially in semiarid areas. Its resistance to drought often assures farmers an adequate yield under conditions considered unfavorable for other cereals and makes pearl millet an important component in the diet of individuals in many countries.

Many food uses of pearl millet involve a steeping or fermenting period before cooking, and fermented beverages made from millet are common (Vogel and Graham 1978). Although millet consumers subjectively distinguish "good" from "poor" quality grains for particular end-uses, little work has been done to determine what physical and chemical factors are related to grain quality.

Amylases are important enzymes for controlling the end-use quality of many food grains like wheat (Kulp 1975) and sorghum (Novellie 1962). Little work has been published on amylases in pearl millet. Based on current information on uses of pearl millet (Vogel and Graham 1978) and on suggestions that pearl millet has an active amylase system (Badi et al 1976), our objective was to isolate α-amylase from mature pearl millet grains and to determine some of its physical and chemical characteristics.

MATERIALS AND METHODS

Materials

Eleven random-mating bulk populations and nine F_1 hybrids of pearl millet were screened for α-amylase activity. Location, year of growth, and proximate analysis of the samples are presented in Table I. Hays millet populations (HMP) were developed at the Hays Branch Experiment Station. All pearl millets studied were from sound grains, ie, they showed no visible sprouting damage.

Isolation of Alpha-Amylase from Pearl Millet

α-Amylase was extracted from millet (HMP02) with $0.05M$ acetate buffer, pH 4.8, containing $0.01M$ $CaCl_2$. The ratio of buffer to meal was 2.5:1; extraction time was 2 hr. The slurry was centrifuged for 15 min at 2,000 rpm, and the precipitate was discarded.

The supernatant was brought to a concentration of 0.2% with calcium acetate, and the pH was adjusted to 6.0 with NaOH before the solution was divided into 10-ml fractions and heat treated at 70°C for 15 min to inactivate β-amylase. After heat treatment, the solution was rapidly cooled in an ice bath and centrifuged at 5,000 rpm for 15 min. The precipitate was discarded, and the supernatant was dialyzed overnight against 0.2% calcium acetate.

After the heat treatment, α-amylase was further purified by one of two methods: 1) the starch column procedure of Schwimmer and Balls (1949) and 2) organic solvent fractionation followed by glycogen complex formation (Loyter and Schram 1962).

Details of the isolation procedures are given in Fig. 1.

The procedure of Schwimmer and Balls (1949) includes an $(NH_4)_2SO_4$ precipitation followed by α-amylase adsorption on a starch column and elution with 0.5% Ca acetate. In this study, pearl millet starch, washed from the grain as previously described (Beleia et al 1980), was used as the adsorbent.

Before the glycogen complex was formed, the heat-treated sample was fractionated with acetone. Cold acetone (−10°C) was slowly added to the dialyzed solution, which was maintained at −10°C in an ice-salt bath, to a concentration of 20%. The mixture was centrifuged for 30 min at 10,000 rpm; then the precipitate was discarded. Additional cold acetone was added to the supernatant to a final concentration of 40%. The precipitate, containing the α-amylase, was recovered by centrifuging at 10,000 rpm for 30 min and dissolving in acetate buffer. Cold (−10°C) 80% ethanol containing 0.5% $CaCl_2$ was then added slowly to the enzyme solution to a final concentration of 40%. The solution was centrifuged (10,000 rpm, 20 min) and the precipitate was discarded. The α-amylase in the supernatant was complexed with purified glycogen in $0.2M$ phosphate buffer (pH 8.9) by the method of Loyter and Schramm (1962).

A portion of the heat-treated extract was also tested for β-amylase by first adjusting the pH to 3.2 with acetic acid and holding

TABLE I
Proximate Analyses (%) of Pearl Millets

Sample	Growth Location[a] and Year	Protein	Fat	Ash	Moisture
Bulk populations					
HMP559	M, 1978	10.3	5.2	1.5	7.6
HMP557A	M, 1978	9.5	5.6	1.6	8.4
HMP561	M, 1978	8.9	5.6	1.6	6.7
HMP02	H, 1978	13.4	5.2	1.5	8.9
HMP1700	M, 1978	10.7	6.2	1.6	6.7
HMP1700	H, 1977	14.7	6.9	1.6	10.4
HMP550	H, 1977	13.6	7.1	1.8	9.6
Serere 3A	H, 1977	13.5	6.2	1.6	9.5
RMPI(S)CI	A, 1978	15.7	6.8	2.0	10.4
Sudan Green	S, 1979	12.0	5.9	1.5	7.8
Sudan Yellow	S, 1979	14.5	7.8	2.0	7.4
F_1 hybrids					
2090 × 7101	M, 1978	11.6	5.7	1.7	7.3
2090 × 7101	T, 1978	14.5	6.0	1.6	7.6
2279 × 7101	M, 1978	10.4	5.6	1.6	7.0
1166 × 1700	M, 1978	11.0	6.0	1.6	6.8
1166 × 1700	J, 1978	9.0	6.6	1.7	7.5
2118 × 7024	M, 1978	10.3	4.9	1.8	7.1
2118 × 7024	H, 1978	12.9	6.1	1.7	10.0
2224 × 7024	M, 1978	12.1	5.2	1.6	7.2
2224 × 7024	J, 1978	9.1	5.3	1.7	8.8

[a]Location in Kansas: M = Mineola, H = Hays, A = Ashland, S = Sudan, T = Tribune, J = St. John.

[1]Contribution 81-28-J, Department of Grain Science and Industry, Kansas Agricultural Experiment Station, Manhattan 66506.
[2]Graduate research assistant and associate professor, respectively.

0009-0352/81/05043305/$03.00/0
©1981 American Association of Cereal Chemists, Inc.

for 1 hr at room temperature. After the acid treatment, the pH was adjusted to 4.6 with acetate buffer, and β-amylase activity was determined according to Robyt and Whelan (1968).

Alpha-Amylase Activity

Viscometric Methods. AACC methods were used to determine falling number and amylograph viscosity. Pearl millets were ground in a Quadrumat Jr., and the fractions were recombined before analysis.

Reducing Power Method. The activity of purified α-amylase preparations was determined by the method of Robyt and Whelan (1968) except that the temperature was maintained at 35°C instead of 25°C. Reducing sugars were determined by Nelson's colorimetric copper method (1944). Units of enzyme activity were expressed as micromoles of apparent maltose produced per milliliter of α-amylase solution per minute.

Specific Activity

Protein in the α-amylase solutions was determined by Miller's (1959) modification of the Lowry et al (1951) method. Specific activity was expressed as milliunits (mU) per milligram of protein.

Gel Electrophoresis

Electrophoresis of purified enzymes was done on polyacrylamide gels at pH 8.9 by the method of Davis (1964). Multiple forms of α-amylase were detected by incubating sliced electrophoresis gels, 2–3 mm sections, in a test tube containing 40 mg of Phadebas amylase substrate (a gift from Pharmacia, Piscataway, NJ) and 4 ml of $0.05M$ acetate buffer, pH 6.0 ($0.01M$ $CaCl_2$) at 50°C for 0.5 hr. The reaction was stopped by adding 1 ml of $0.5N$ NaOH. The mixture was filtered, and the absorbance was read at 620 nm.

Molecular weight determinations were done by sodium dodecyl sulfate (SDS)-gel electrophoresis according to the methods of Weber and Osborn (1969).

Isoelectric points were determined by the gel electrofocusing methods of Wrigley (1968). We used isoelectric focusing between pH 3 and 10 and dissolved purified, freeze-dried α-amylases in water before mixing them with the concentrated gel mixture and photopolymerizing. After electrophoresis, gels were stained overnight in 0.04% Coomassie brilliant blue G25 in 3.5% perchloric acid (Reisner et al 1975).

pH and Temperature

Optimum pH for activity of purified α-amylases from millet was determined with a soluble starch substrate and acetate or tris buffers in a pH range from 3.6 to 7.2. The enzyme was diluted with the buffer, and an aliquot was added to a 1:1 mixture of soluble starch and buffer. Reaction time was 10 min at 35°C, and reducing sugars were measured according to Nelson's method (1944).

RESULTS AND DISCUSSION

Alpha-Amylase Activity in Pearl Millet

Based on amylograph data from one pearl millet sample, Badi et al (1976) reported that millet flour contained an active α-amylase system. Whether high α-amylase activity was common to many pearl millet populations or hybrids was not known, so we used the falling number method to rapidly screen pearl millet samples for α-amylase activity.

Falling numbers of pearl millet bulk populations varied from 67 for HMP1700, harvested in 1977, to 383 for Sudan Yellow, harvested in 1979 (Table II). Falling numbers for F_1 hybrids varied from 185 for 1166 × 1700 to 319 for 2224 × 7024, both harvested in 1978. Falling numbers for sound pearl millets were, in general, lower than those reported for sound wheat.

To further confirm the viscosity data of Badi et al (1976), we also determined amylograph peak viscosities on millet meals with low, medium, and high falling numbers (Fig. 2). Amylograph pasting temperatures for millet meals varied from 64°C for HMP02 to 74°C for HMP559. Peak viscosities paralleled falling number data, ie, millets with low falling numbers also had low amylograph peak viscosities. For example, HMP02 had a falling number of 134 and a peak viscosity of 520 BU. Maximum amylograph viscosity was attained at temperatures from 80–85°C.

The falling number and amylograph viscosity data indicate that many sound pearl millets have active α-amylase systems. Because many traditional food uses of pearl millet involve natural fermentations (Vogel and Graham 1978), the level of amylolytic activity in the grain may be one important factor in grain quality. We therefore conducted studies to determine the characteristics of purified millet α-amylases.

Enzyme Activity in Purified Extracts

Enzyme activity determinations were recorded at each step in purifying α-amylase by the starch-column procedure and glycogen

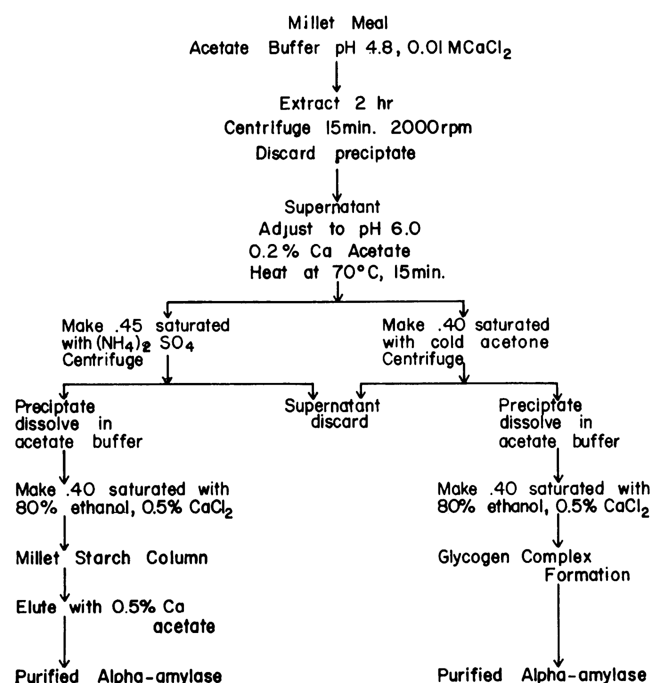

Fig. 1. α-Amylase isolation procedure.

TABLE II
Alpha-Amylase Activity in Sound Pearl Millets as Determined by Falling Number

Sample	Growth Location and Year	Falling Number[a] (sec)
Bulk populations		
HMP559	Mineola, 1978	248
HMP557A	Mineola, 1978	261
HMP561	Mineola, 1978	252
HMP02	Hays, 1978	134
HMP1700	Mineola, 1978	199
HMP1700	Hays, 1977	67
HMP550	Hays, 1977	230
Serere 3A	Hays, 1975	203
RMPI(S)CI	Ashland, 1978	259
Sudan Green	Sudan, 1979	346
Sudan Yellow	Sudan, 1979	383
F_1 hybrids		
2090 × 7101	Mineola, 1978	249
2090 × 7101	Tribune, 1978	243
2279 × 7101	Mineola, 1978	253
1166 × 1700	Mineola, 1978	185
1166 × 1700	St. John, 1978	324
2118 × 7024	Mineola, 1978	214
2118 × 7024	Hays, 1978	221
2224 × 7024	Mineola, 1978	247
2224 × 7024	St. John, 1978	319

[a] Least significant difference = 13.

complex formation; those data are presented in Tables III and IV, respectively. In both purification procedures, acetate buffer was used to extract α-amylase from millet meal; it was as efficient as 0.2% calcium acetate or 0.01M $CaCl_2$. The high enzyme activity of the initial extract resulted from both α-amylases and β-amylases. Heat-treating the crude extract increased specific enzyme activity but reduced amylase activity. Determination of α-amylase activity in buffer extracts before and after the heat treatment, using the Phadebas substrate (specific for α-amylase), indicated that 15–20% of the α-amylase activity was lost during heating. β-Amylase activity was found to be less than 30 mU/ml in all heat-treated extracts.

Controlling the pH before heat-treating the extract was crucial in obtaining an active enzyme preparation. If the pH of the buffer extract was not adjusted to 6.0 before the heat treatment, low recovery of the enzyme resulted. Even when the pH was adjusted, amounts of enzyme recovered varied. For example, heat treatment of the buffer extract that was later used in the starch column gave 58% recovery. An identical heat treatment of another buffer extract, used in glycogen complexing, gave 75% enzyme recovery. Variations in enzyme recovery could have been affected by the amount of protein solubilized during the extraction procedure; protein contents of dialyzed buffer extracts differed somewhat (Tables III and IV). Greenwood and Milne (1968a) also suggested that high protein concentrations in the enzyme extract during heat treatment were critical for removing β-amylase and recovering α-amylase.

Subsequent steps in both purification methods increased the specific activity to several times more than that of the heat-treated extracts (Tables III and IV). In general, glycogen complex formation increased specific enzyme activity more than starch column procedures did. In fact, the glycogen complex formation increased specific activity of the purified α-amylase 60-fold (Table IV) compared with a 21-fold increase with the starch column (Table III).

Greenwood and Milne (1968a) could not form the glycogen complex with α-amylases from mature oat, rye, or wheat, which they attributed to the low enzyme concentrations in the mature grains. Because they used a different method to measure α-amylase activity, we cannot directly compare our results with theirs to determine whether millet grain has a higher α-amylase content.

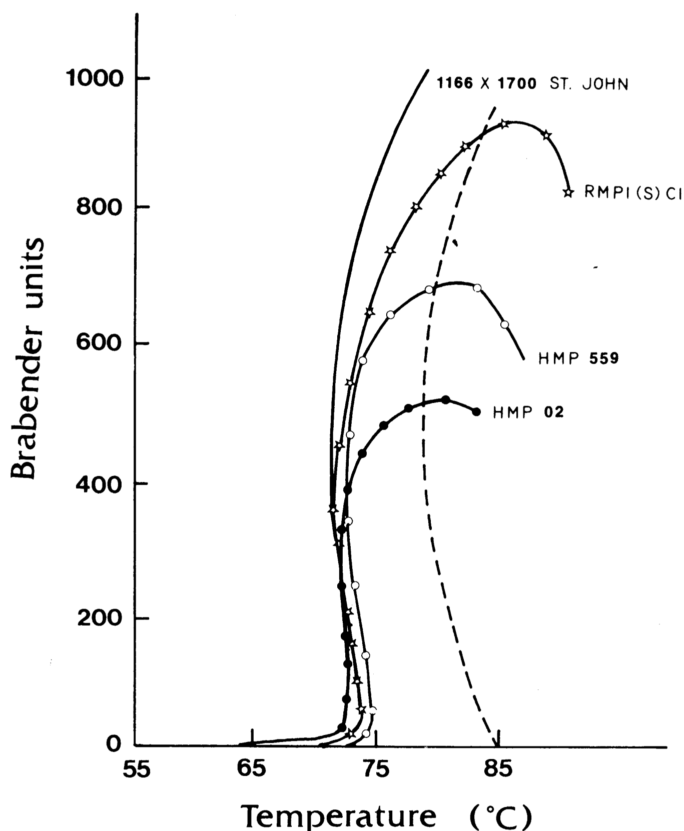

Fig. 2. Amylograms of millet meals.

Properties of Purified Millet Alpha-Amylases

Polyacrylamide Gel Electrophoresis. Polyacrylamide gel electrophoresis of pearl millet α-amylase purified by the starch column procedure revealed seven protein bands; α-amylase purified by glycogen complex formation exhibited three bands (Fig. 3). α-Amylase determinations on sliced gels, with the Phadebas substrate, indicated that all bands that stained for protein also showed α-amylase activity. The protein with the greatest mobility showed about two times greater α-amylase activity than did the other bands.

The difference in electrophoretic properties of millet α-amylase

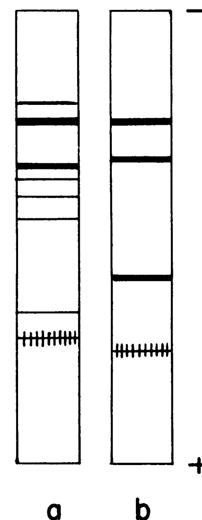

Fig. 3. Polyacrylamide gel electrophoresis of α-amylase purified by the starch column procedure (**a**) and by glycogen complex formation (**b**).

TABLE III
Purification of Millet Alpha-Amylase Using Salt Precipitation and a Starch Column

Fraction	Enzyme Activity (mU/ml)[a]	Protein (mg/ml)	Specific Activity (mU/mg protein)	Volume (ml)	Recovery[b] (%)
Buffer extract	1,780	5.9	302	140	100
Heat-treated extract	1,450	3.8	382	100	58
0.45-saturated $(NH_4)_2SO_4$	1,930	2.2	877	33	26
Starch column	2,010	0.3	6,700	14	11

[a] Units = [(μg maltose/ml/min) × 2]/342.
[b] [(mU/ml × volume) × 100]/total activity of buffer extract.

TABLE IV
Purification of Millet Alpha-Amylase Using Acetone Fractionation and Glycogen Complex Formation

Fraction	Enzyme Activity (mU/ml)[a]	Protein (mg/ml)	Specific Activity (mU/mg protein)	Volume (ml)	Recovery[b] (%)
Buffer extract	1,600	6.7	238	150	100
Heat-treated extract	1,200	4.5	266	150	75
40% acetone	2,500	2.4	1,042	50	52
Glycogen complex	4,300	0.3	14,333	8	14

[a] Units = [(μg maltose/ml/min) × 2]/342.
[b] [(mU/ml × volume) × 100]/total activity of buffer extract.

purified by adsorption on starch granules or glycogen complex formation is a result of the purification method. Adsorption of α-amylase on starch granules or formation of a glycogen amylase complex is dependent on temperature, pH, and concentration (Levitzki et al 1964, Walker and Hope 1963), so some forms of α-amylase may be preferentially adsorbed on starch granules or preferentially complexed with glycogen. Data reported by MacGregor (1977) lend some support to that hypothesis. He found that the elution pattern of a glycogen-purified α-amylase (malted barley) from a carboxymethyl cellulose column differed from the elution pattern obtained when the enzyme was purified without glycogen complex formation. Apparently, dextrins produced from the hydrolysis of the glycogen inhibited adsorption of one form of α-amylase onto the column.

SDS Gel Electrophoresis. SDS gels of α-amylases purified by glycogen complex formation showed three distinct protein bands, at molecular weights of 31,000, 46,000, and 53,000 (Fig. 4). The molecular weight of α-amylases purified from the same millet population by the starch-column procedure showed two bands in the SDS gels: one at 31,000 and one at 22,000. Lower molecular weights of α-amylases from starch column purifications than from glycogen complex formation supports the hypothesis that only certain α-amylase isozymes are preferentially adsorbed onto starch or complexed with glycogen.

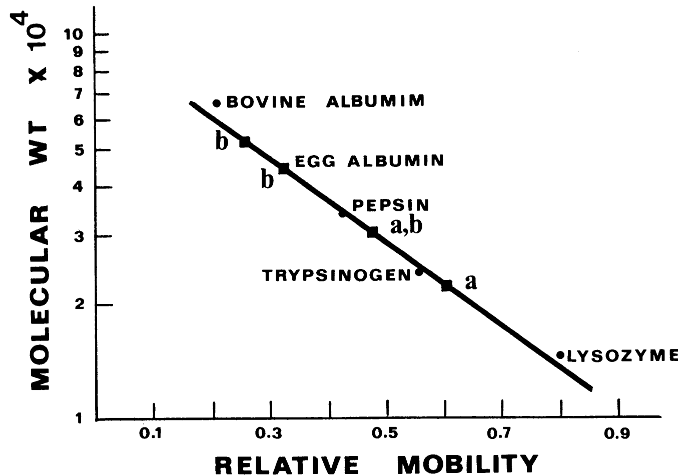

Fig. 4. Sodium dodecyl sulfate gel molecular weight determinations of α-amylase (■) purified by starch column procedures (**a**) and by glycogen complex formation (**b**). • = Examples of other enzymes.

Molecular weights of most cereal α-amylases range from 42,000 to 46,000 (Greenwood and Milne 1968b). But Tkachuk and Kruger (1974) reported molecular weights from 20,000 to 57,000 for α-amylases from malted wheat, and molecular weights have exceeded 50,000 for some α-amylase isozymes from malted barley (MacGregor 1978) and immature wheat (Marchylo et al 1976).

Isoelectric Focusing. In isoelectric focusing, a protein migrates in a gel support that has a pH gradient to the region of its isoelectric pH (Wrigley 1968). α-Amylases purified by either the starch column or glycogen complex showed three protein bands corresponding to isoelectric points of 4.8, 5.2, and 6.2. The isoelectric points of 4.8 and 5.2 agree with values reported by Marchylo et al (1976) for immature wheat α-amylase isozymes. They also reported isoelectric points from 6.05 to 6.20 for isozymes from malted wheat.

pH and Temperature Optima. Data on optimum pH of purified α-amylases, recorded as percentages of maximum activity, are presented in Fig. 5. Optimum pH for α-amylase purified on the starch column was between 4.4 and 4.8; optimum pH for the glycogen complex α-amylase was 4.4. An optimum pH from 4.4 to 4.8 is lower than that reported for other cereal α-amylases, especially for those from malted grain (Greenwood and Milne 1968b). However, α-amylase from malted sorghum had an optimum pH at 4.6 (Dube and Nordin 1961), and α-amylase from immature wheat showed maximum activity in that pH region (Marchylo et al 1976).

The activity of the glycogen complex α-amylase was not so drastically altered by changes in pH as was the activity of the α-amylase purified by the starch column procedure, particularly

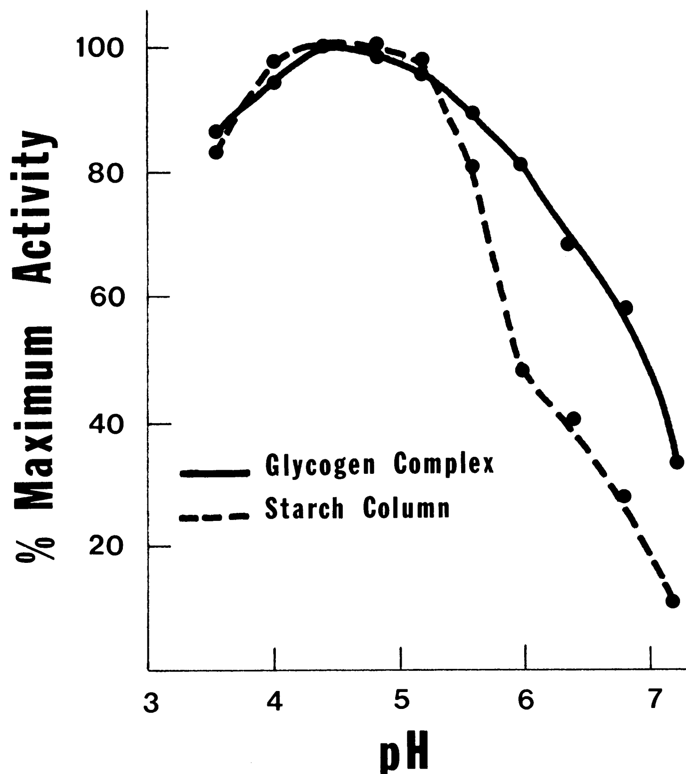

Fig. 5. pH optima for α-amylase purified by the starch column procedure (---) and by glycogen complex formation (—).

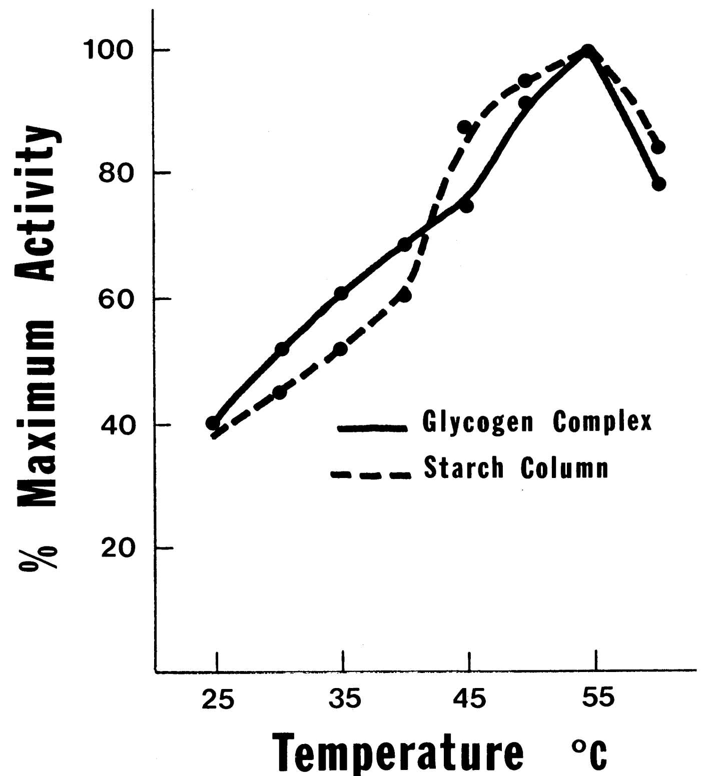

Fig. 6. Temperature optima for α-amylase purified by the starch column procedure (---) and by glycogen complex formation (—).

between pH values of 6.0 and 7.2. For example, at pH 6.0, the glycogen complex α-amylase still had 82% of its maximum activity, whereas the starch-column α-amylase had only 48%. The glycogen complex α-amylase exhibited 32% of its maximum activity at pH 7.2, but at that pH the activity of the starch-column α-amylase was only 12% of the maximum.

Temperature optima for purified millet α-amylases were determined with acetate buffer at the optimum pH determined above (Fig. 6). The optimum temperature for enzymes purified by either procedure was 55°C, which is similar to that reported for immature barley α-amylase (MacGregor et al 1974).

LITERATURE CITED

AMERICAN ASSOCIATION OF CEREAL CHEMISTS. 1972. Methods of the AACC. Method 56.81B, approved November 1972, and Method 22-10, approved April 1960. The Association: St. Paul, MN.

BADI, S. M., HOSENEY, R. C., and FINNEY, P. L. 1976. Pearl millet. II. Partial characterization of starch and use of millet flour in breadmaking. Cereal Chem. 53:718.

BELEIA, A., VARRIANO-MARSTON, E., and HOSENEY, R. C. 1980. Characterization of starch from pearl millets. Cereal Chem. 57:300.

DAVIS, B. J. 1964. Disc electrophoresis. II. Method and application to human serum proteins. Ann. N.Y. Acad. Sci. 121:404.

DUBE, S. K., and NORDIN, P. 1961. Isolation and properties of sorghum alpha-amylase. Arch. Biochem. Biophys. 94:121.

GREENWOOD, C. T., and MILNE, E. A. 1968a. Properties and action pattern of the alpha-amylase from barley, oats, rye and wheat. Staerke 20:101.

GREENWOOD, C. T., and MILNE, E. A. 1968b. Studies on starch degrading enzymes. VIII. A comparison of alpha-amylases from different sources: Their properties and action pattern. Staerke 20:139.

KULP, K. 1975. Carbohydrases. Page 54 in: Reed, G., ed. Enzymes in Food Processing. Academic Press: New York.

LEVITZKI, A., HELLER, J., and SCHRAMM, M. 1964. Specific precipitation of enzyme by its substrate: The alpha-amylase-macrodextrin complex. Biochem. Biophys. Acta 81:101.

LOWRY, O. H., ROSEBROUGH, N. J., and FARR, A. L. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265.

LOYTER, A., and SCHRAMM, M. 1962. The glycogen-amylase complex as means of obtaining highly purified alpha-amylases. Biochem. Biophys. Acta 65:200.

MacGREGOR, A. W. 1977. Isolation purification and electrophoretic properties of an alpha-amylase from malted barley. J. Inst. Brew. 83:100.

MacGREGOR, A. W. 1978. Alpha-amylase I from malted barley—Physical properties and action pattern on amylose. Cereal Chem. 55:754.

MacGREGOR, A. W., THOMPSON, R. G., and MEREDITH, W. O. S. 1974. Alpha-amylase from immature barley: Purification and properties. J. Inst. Brew. 80:181.

MARCHYLO, B., KRUGER, J. E., and IRVINE, G. N. 1976. Alpha-amylase from immature hard red spring wheat. I. Purification and some chemical and physical properties. Cereal Chem. 53:157.

MILLER, G. L. 1959. Protein determination for large numbers of samples. Anal. Chem. 31:964.

NELSON, N. 1944. A photometric adaptation of the Somogyi method for the determination of glucose. J. Biol. Chem. 153:375.

NOVELLIE, L. 1962. Kaffircorn malting and brewing studies. XI. Effect of malting conditions on the diastatic power of kaffircorn malt. J. Sci. Food Agric. 13:115.

REISNER, A. H., NEMES, P., and BUCHOLTZ, C. 1975. The use of Coomassie brilliant blue G250 perchloric acid solution for staining in electrophoresis and isoelectric focusing on polyacrylamide gels. Anal. Biochem. 64:509.

ROBYT, J. F., and WHELAN, W. J. 1968. Amylases and their actions on starch. Chap. 13 in: Radley, J. A., ed. Starch and Its Derivatives, 4th ed. Chapman and Hall, Ltd.: London.

SCHWIMMER, S., and BALLS, A. K. 1949. Isolation and properties of crystalline alpha-amylase from germinated barley. J. Biol. Chem. 179:1063.

TKACHUK, R., and KRUGER, J. E. 1974. Wheat amylases. II. Physical characterization. Cereal Chem. 51:508.

VOGEL, S., and GRAHAM, M. 1978. Sorghum and Millet: Food Production and Use. Intern. Dev. Research Center: Ottawa, Canada.

WALKER, G. J., and HOPE, P. M. 1963. The action of some alpha-amylases on starch granules. Biochem. J. 86:452.

WEBER, K., and OSBORN, M. 1969. The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. J. Biol. Chem. 244:4406.

WRIGLEY, C. 1968. Gel electrofocusing—A technique for analyzing multiple protein samples by isoelectric focusing. Instr. J. 15:17.

[Received September 15, 1980. Accepted March 5, 1981]