DURUM WHEAT α-AMYLASES: ISOLATION AND PURIFICATION[1]

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ABSTRACT Cereal Chem. 55(2): 146-156

α-Amylase was isolated in approximately 7% yields from germinated durum wheat. The optimum isolation conditions were: extraction for 4 hr with 0.001*M* calcium acetate at 4°C, heat treatment for 10 min at 70°C, fractionation with 35 to 50% acetone, glycogen complexing in 40% ethanol, and ultrafiltration followed by chromatography on DEAE-cellulose. The chromatography indicated that seven α-amylases were present. Polyacrylamide gel electrophoresis showed that germinated durum wheat has eight α-amylase components. The eight α-amylases that were found by electrophoresis in both germinated durum and germinated hard red spring wheat had similar mobilities.

Wheat α-amylases have an important effect on the rheological properties of wheat flour. Low levels of α-amylase can improve baking quality, while high levels cause excessively moist, sticky crumb (1,2). Thus, for effective wheat utilization, being able to measure and understand the properties of wheat α-amylases is essential.

Reports of previous studies from this laboratory have described the isolation and some characteristics of wheat α-amylases that were isolated from germinated Canadian hard red spring wheat (3,4). This article describes the isolation and some properties of α-amylases that are present in germinated durum wheat.

MATERIALS AND METHODS

Germinated Wheat

A sample of durum wheat (Wascana) grown during 1970 at the Glenlea Experimental Station was used for isolation of α-amylase. The wheat was soaked and germinated (95% relative humidity) in the dark at 17°C, and dried at 25°C (4).

During germination, the wheat was tumbled every 3 hr. The germinated wheat was dried by aeration at 25°C for two days. The germinated wheat had 15.5% protein (N × 5.7, 13.5% moisture basis), 7.9% moisture, and 1.5% ash (5).

A composite sample of hard red spring (HRS) wheat (Manitou) (14.4% protein [N × 5.7, 13.5% moisture basis], 11% moisture) that was grown during 1971 at five experiment stations (Morden, Brandon, and Winnipeg, Manitoba; Indian Head and Melfort, Saskatchewan) was germinated in a manner similar to that for the Wascana wheat. The germinated Manitou wheat was used to isolate α-amylase up to the glycogen complex stage of purity (4).

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α- and β-Amylase Activity

Combined α- and β-amylase activity was measured according to the method described by Bernfeld (6) and modified by Tkachuk and Kruger (4). A unit of amylase activity was defined as the amount of enzyme necessary to liberate dextrins or maltose equivalent to 1 μm of maltose in 1 min at 25°C from 0.9% soluble starch at pH 5.5. Substrate hydrolysis was not allowed to proceed beyond 15%. Specific activity was expressed as amylase activity per milligram of protein. Protein content in extracts was determined by the Lowry method (7).

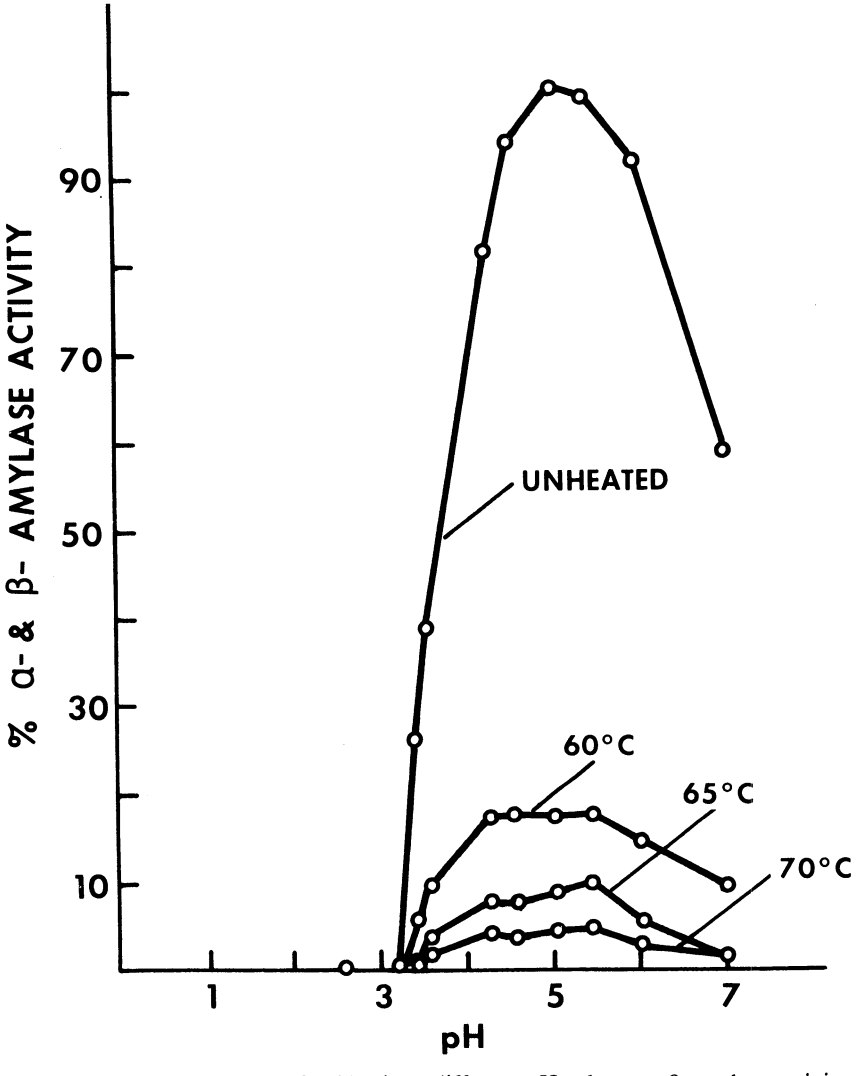

Fig. 1. Effect of heat treatment for 20 min at different pH values on β-amylase activity of crude extract of germinated wheat.

β-Amylase Activity

Activity was determined as described for the combined α- and β-amylase activity, except that the measurement was made at pH 3.4 (±0.01). At this pH, α-amylase is inactive (4) and β-amylase exhibits approximately 26% of the combined activity shown at its optimum pH of 5.5 (Fig. 1).

α-Amylase Activity

Activity was determined as described for the combined α- plus β-amylase activity, except that solutions to be analyzed were previously heated at 70° C for 10 min at pH 6.7 to destroy β-amylase (8,9) (Fig. 2).

Glycogen Purification

Insoluble colored materials, and much of the protein present in shellfish glycogen (Sigma Co., St. Louis, MO), were removed by successively treating an

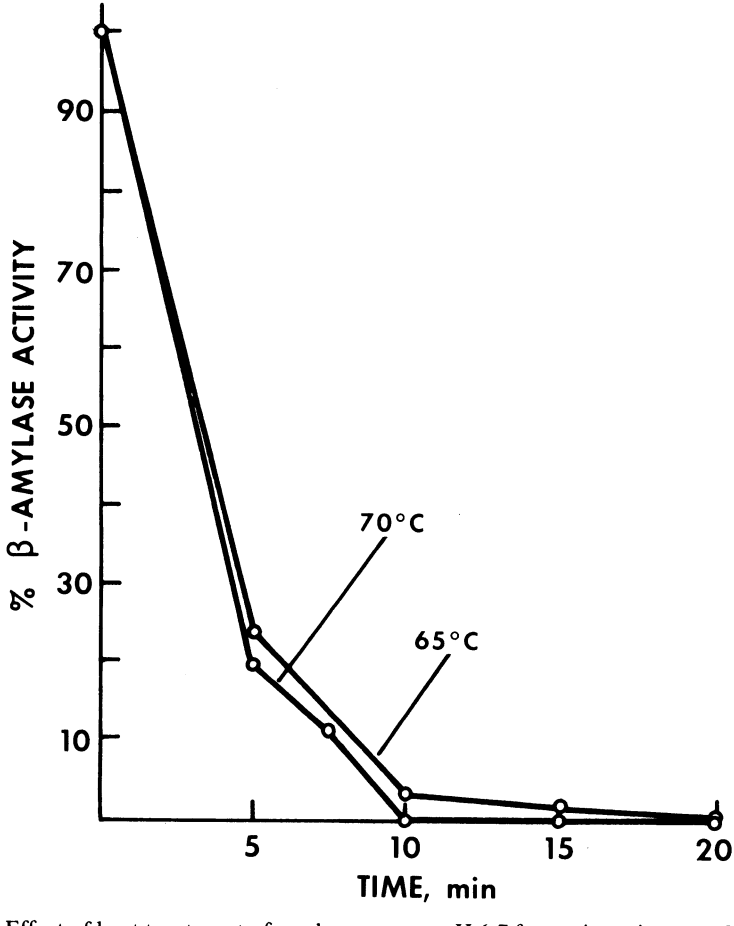

Fig. 2. Effect of heat treatment of crude extract at pH 6.7 for various times on β-amylase. Incubation was at pH 3.4.

aqueous 1% glycogen solution with 10 g of carboxymethylcellulose (COOH form) and 10 g of diethylaminoethyl-cellulose (DEAE-cellulose) (amine form) (Whatman ion exchange cellulose, H. Reeve Angel, Clifton, N.J.) and then freeze-drying. Treatment of 10 g of glycogen resulted in 6.1 g of purified material containing 0.06% nitrogen.

Chromatography

Whatman DE32 N,N-DEAE-cellulose was conditioned (10) by successive washing with 0.1*N* NaOH, water, 0.1*N* HCl, water, and 0.05*M* tris-HCl buffer pH 8.75 containing 0.0015*M* $CaCl_2$. The DEAE-cellulose fines were removed during washing by decantation. Columns of conditioned DEAE-cellulose were poured, and equilibrated with 0.003*M* tris-HCl buffer pH 8.75 containing 0.0015*M* $CaCl_2$. All buffers used for the preparation and equilibration of columns and for the chromatography were degassed by stirring them under vacuum for several minutes.

Electrophoresis Techniques

Electrophoresis. Slab gels to fit Ortec apparatus (11) were prepared as described by Davis (12) except that reagent F was not used. Gel polymerization was done by photoactivation. Electrophoresis was done for approximately 80 min at 4° C with a pulsed power supply operating at approximately 350 v and 110 ma.

α-Amylase detection on gel slabs. α-Amylase active bands were detected as described by Doane (13) and modified by MacGregor *et al.* (14). When electrophoresis was complete, the electrophoresis polyacrylamide gel slab was layered on top of a polyacrylamide-buffered slab at pH 5.5 containing 0.7% β-limit dextrin (15). After incubation for several minutes, the polyacrylamide slab was stained with 0.01% iodine in 1% potassium iodide. α-Amylase zones appeared as colorless areas against a pinkish-brown background.

Protein detection on electrophoresis gel slabs. After α-amylase detection was completed, the acrylamide gels were stained overnight with 1% Coomassie blue in ethanol diluted 20-fold with 12% trichloroacetic acid (TCA) to detect protein. The gel was destained by soaking the gels in fresh lots of 10% TCA for two days. Destained gels were stable for several weeks when stored in 10% TCA and when kept away from strong light.

EXPERIMENTAL

Isolation of α-Amylase

α-Amylase was isolated by following the five procedures described below. All operations were performed at 4° C unless described otherwise.

Crude extract. Germinated wheat (3000 g, db), was ground and dispersed for 3 min with 6000 ml of 0.001*M* calcium acetate in a Waring blender. The resulting slurry was gently stirred for 4 hr. The slurry was centrifuged at 8000 × *g* for 15 min, and the supernatants were collected.

Heat treatment. Initial experiments showed that treating 200- to 250-ml lots of crude extract at pH 6.7 for 10 min at 70° C destroyed all of the β-amylase (Fig. 2).

Accordingly, the crude extract was adjusted to pH 6.7 by slowly adding cold 4% ammonium hydroxide, and 200- to 250-ml stirred portions of the crude

extract were placed in boiling water. When the temperature of the crude extracts had risen to 70° C, they were transferred to a 70° C water bath for 10 min. After cooling in an ice-water bath and centrifuging at 8000 × *g* for 10 min, the supernatants were stored under nitrogen to minimize oxidation.

Acetone fractionation. Acetone at −60° C was added slowly to the stirred, heat-treated supernatant. Addition of −60° C acetone approximately counteracted the heat release caused by the mixing of acetone and water, and accordingly, the temperature of the resulting solution remained reasonably constant near 4° C. The precipitate that was saved was that which formed when the acetone concentration was increased from 35 to 50% (v/v), as this fraction contained most of the α-amylase activity (Fig. 3).

To the acetone precipitate was added 600 ml of 0.003*M* tris-HCl pH 8.0 buffer containing 0.0015*M* calcium acetate. The pH was adjusted to 8.0 by adding a few ml of 0.03*M* tris. The resulting mixture was gently stirred overnight and

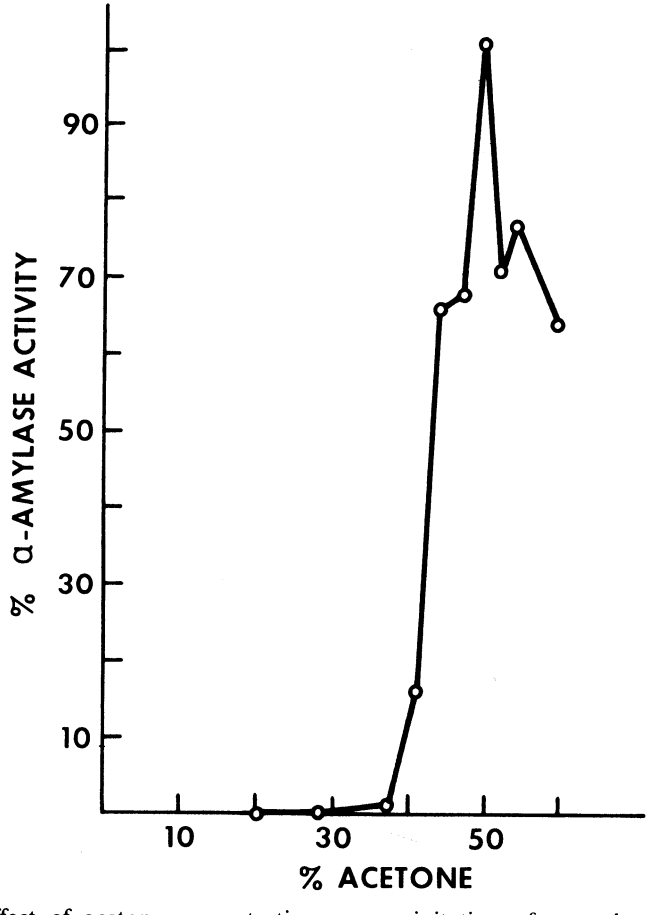

Fig. 3. Effect of acetone concentration on precipitation of α-amylase. Activity of precipitate is given as percentage of activity originally present in aqueous solution.

TABLE I
Complexing α-Amylase With Glycogen

Varying Ethanol Concentration[a]			Varying α-Amylase Concentration in 40% Ethanol		
Ethanol (%)	α-Amylase Recovered (%)	Protein Recovered (%)	α-Amylase Added[b] (units)	α-Amylase Recovered (%)	Protein Recovered (%)
10	0.85	1.6	25	28	5.2
20	23	4.9	50	49	5.8
30	38	5.4	75	38	5.8
40	49	5.8	100	34	5.9
			200	4	6.2

[a]α-Amylase (50 units) added per milligram of glycogen.
[b]Per milligram of glycogen.

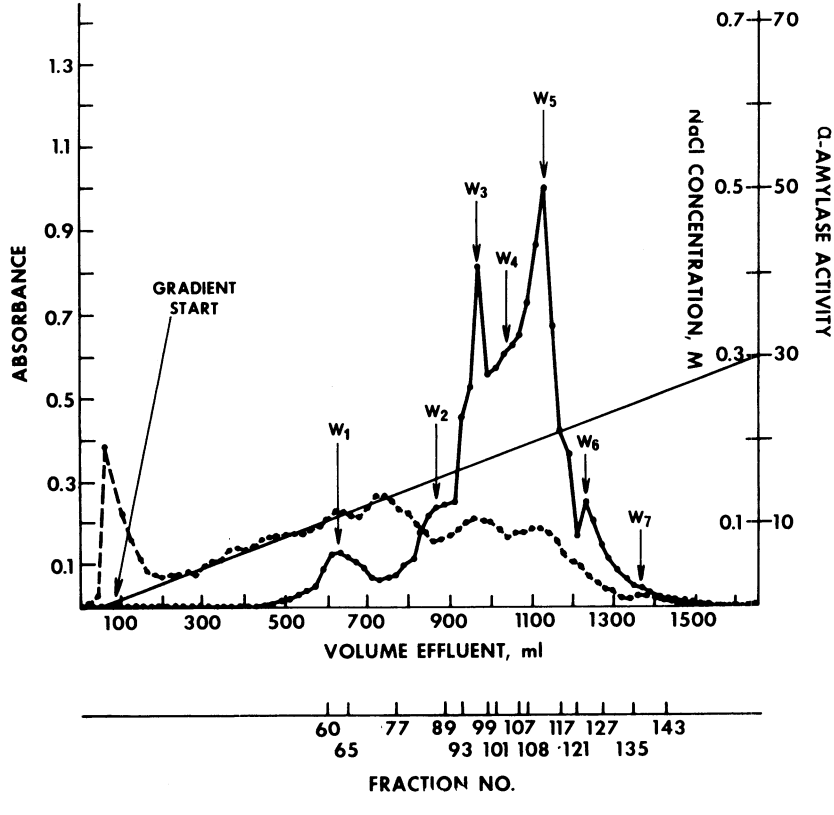

Fig. 4. DEAE-cellulose ion exchange chromatography of 99-mg α-amylases. •- -•- -•, α-Amylase activity (Bernfeld method); •——•——•, protein concentration (Lowry method).

centrifuged at 12,000 × *g* for 10 min at 0° C, and the supernatant collected.

Glycogen complexing. This procedure was done as described by Schramm and co-workers (16,17). Ethanol at −60° C was added slowly to the stirred solution of the acetone precipitate until the ethanol concentration reached 40% (v/v). Precipitate that formed during the ethanol addition was removed by centrifugation at 12,000 × *g* for 15 min at 0° C.

Shellfish glycogen solution in 0.003*M* tris-HCl buffer pH 8.0 containing 0.0015*M* calcium chloride was added dropwise to the clear stirred 40% ethanol solution at 4° C. Glycogen was added equivalent to 1 mg glycogen per 50 units of α-amylase, as this ratio of glycogen to α-amylase was found to give the highest recovery of α-amylase activity (Table I).

The mixture was stirred for 25 min, and then centrifuged at 12,000 × *g* for 15 min at 0° C. The drained precipitate was added to 300 ml of 0.003*M* tris-HCl buffer pH 5.5 containing 0.0015*M* $CaCl_2$, and the pH adjusted from 6.9 to 5.5 by adding a few drops of cold 1*N* HCl.

The resulting mixture was stirred overnight at 20° C to allow the α-amylase to digest the glycogen. The resulting solution was dialyzed overnight against several liters of 0.003*M* tris-HCl buffer pH 8.75 containing 0.0015*M* $CaCl_2$. The dialyzed solution was reduced in volume to approximately 150 ml by ultrafiltration through a XM50 Amicon membrane at 4° C.

Ion exchange chromatography. After equilibration by dialysis against 0.003*M* tris-HCl, 0.0015*M* $CaCl_2$ buffer pH 8.75, α-amylase solution was applied to a 2.5 × 30-cm DEAE-cellulose column. Elution for the first hour (50 ml) was with 0.003*M* tris-HCl, 0.0015*M* $CaCl_2$ buffer pH 8.75 (equilibration buffer) followed

TABLE II
α-Amylase Purification

Step	Protein Recovered		α-Amylase			
	(mg)	(%[a])	Units Recovered	Specific Activity (units/mg protein)	Recovery (%)	Purification
Crude extract	54,000	11	251,000	4.6	100	1.0
Heat-treated extract	30,000	5.9	202,000	6.7	80	1.4
Acetone fraction	3,970	0.78	158,000	39.7	63	8.6
Glycogen complex	228	0.05	42,300	186	17	40
Ultrafiltration of glycogen complex	167	0.03	38,300	229	15	49
Ion exchange chromatography fraction						
W_1			610	82.8	0.24	18
W_2		0.01	1,800	129	0.72	28
W_3			3,600	451	1.4	97
W_4			3,600	525	1.5 (6.7)	113
W_5			5,800	666	2.3	144
W_6			1,200	410	0.47	88
W_7			260	275	0.10	59

[a]Percentage of total protein present in 3000 g of germinated wheat.

by a 0 to 0.3*M* linear gradient of NaCl in the above tris buffer (Fig. 4). The elution gradient was formed by leading 800 ml of 0.3*M* NaCl in the equilibration buffer into 850 ml of stirred equilibration buffer, both solutions being in identically shaped vertical cylindrical containers. The chromatography was done at 20° C, with an approximate flow rate of 50 ml per hr, and 10-ml fractions were collected at 4° C. Effluent fractions that were examined by electrophoresis were first concentrated by ultrafiltration through XM50 Amicon membranes.

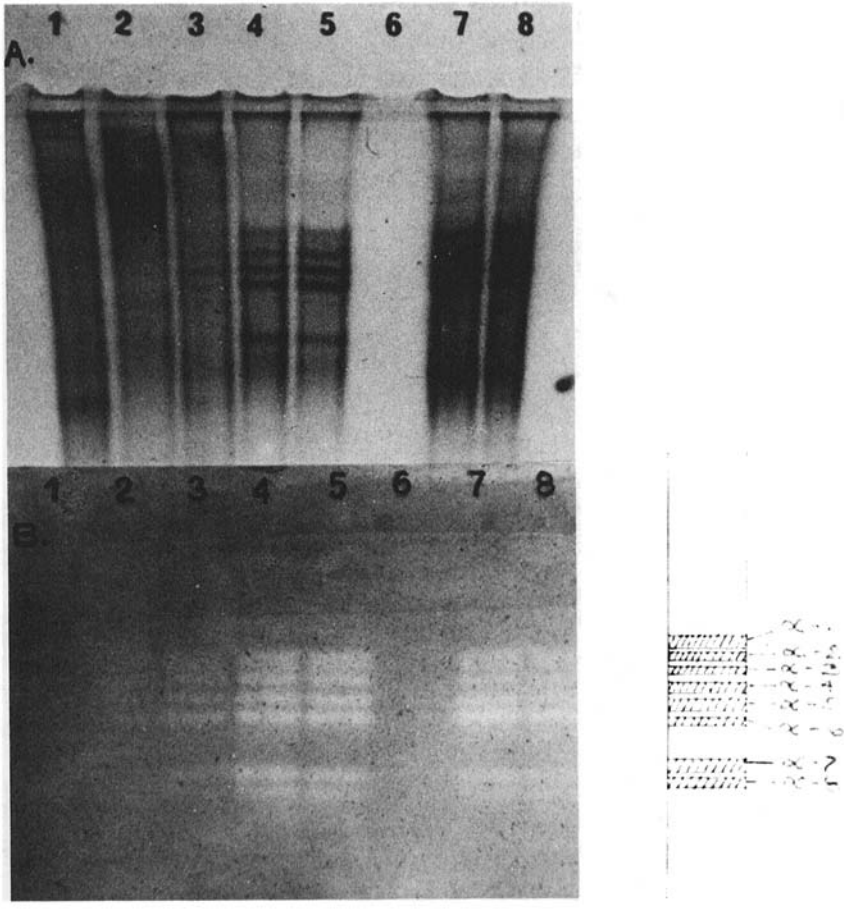

Fig. 5. Zymograms showing gel electrophoretic separation of α-amylases from malted durum wheat. Numbers refer to isolation stages: 1, crude extract; 2, heat-treated extract; 3, 35 to 50% acetone precipitate; 4, glycogen complex; 5, ultrafiltered glycogen complex; 7, ultrafiltered glycogen complex (repeat); and 8, glycogen complex washed with 40% ethanol. No sample was placed on position 6. A) Stained protein bands, B) α-amylase activity zymogram.

RESULTS AND DISCUSSION

Typical protein and enzymatic activity recoveries are given in Table II. About 7% of the α-amylase was recovered with an approximate 80-fold increase in specific activity. Electrophoresis showed that α-amylases that were originally present in the germinated wheat were present in all of the purification steps except for the final ion exchange chromatography step (Fig. 5).

The ion exchange chromatography effluent (Fig. 5) contains seven or more α-amylases. The latter number of α-amylases is not readily apparent from the ion exchange chromatography elution profile. Numerous electrophoretic separation experiments, however, such as the zymogram in Fig. 6 illustrates, indicate that eight α-amylases are present. This conclusion was reached by noting the intensity of the various stained protein bands and their corresponding α-amylase activity, and also the variation in electrophoretic mobility of the α-amylases. The seven components W_1 to W_7 possessed approximately 4, 11, 21, 22, 34, 7, and 2% of the activity eluted from the ion exchange column. Approximately 44% of the α-

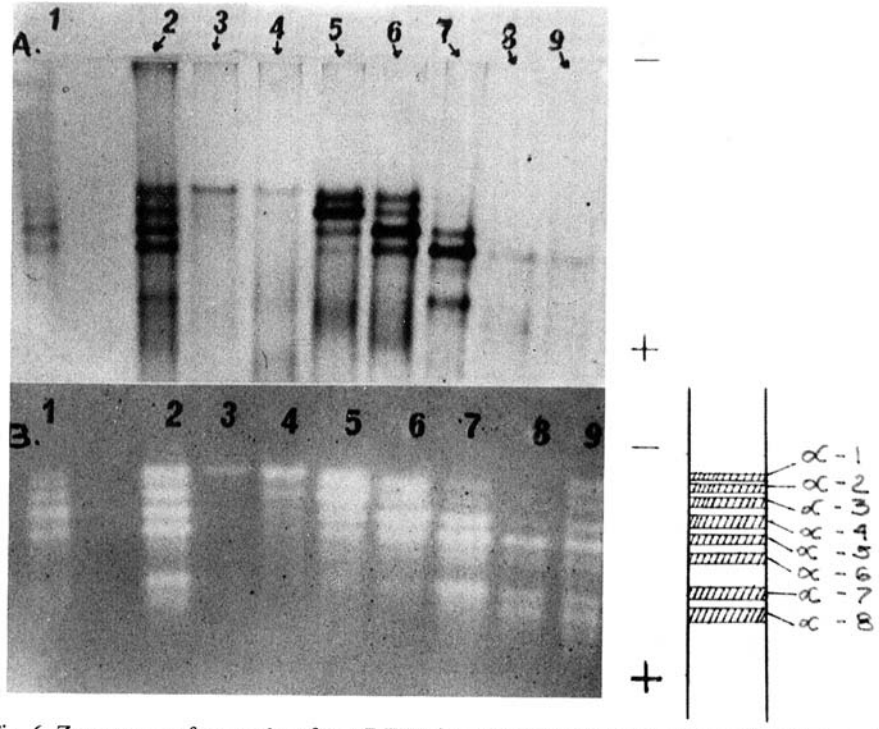

Fig. 6. Zymogram of α-amylase from DEAE ion exchange chromatography fractions and of HRS α-amylases (glycogen-complex stage). Numbers 1 and 2 refer to HRS wheat and durum ultrafiltered glycogen complex stage applied on the ion exchange column, respectively. Numbers 3 through 9 are, respectively, W_1, W_2, W_3, W_4, W_5, W_6, and W_7 ion exchange fractions as shown in Fig. 5. A) Stained protein bands, B) α-amylase activity zymogram.

amylase activity applied to the column was recovered in the column effluent.

Polyacrylamide gel electrophoresis of pooled fractions corresponding to the seven chromatographic fractions W_1 to W_7 (Fig. 6) showed that eight electrophoretic protein components were present that possessed α-amylase activity. Only the first chromatographic fraction (W_1) was homogenous; the remaining fractions were contaminated principally by adjacent α-amylase components except for the final fraction (W_7), which contained traces of all the other α-amylases. The number of α-amylases in germinated durum wheat is indicated by the number of bands in Fig. 4. Interpretation of the enzymatic activity in Fig. 6, which on first glance indicates extensive contamination of adjacent α-amylases, must be made with caution, since this monitoring technique is semiquantitative and tends to exaggerate the amount that is present of infrequently occurring components. This exaggeration is due to the limited amount of substrate that is present in the gel; thus, even α-amylases that occur in small amounts tend to produce clear bands in the gel. The apparent "reappearance" of α-1 in fraction 9 (Fig. 6) is an example of this phenomenon.

Examination of electrophoresis gels as illustrated in Fig. 4a showed that eight α-amylases are present in germinated durum wheat. Three of the α-amylases (α-3, α-4, α-5) account for approximately 65% of the enzymatic activity (Table II). Thus, germinated durum wheat contains three major and five minor α-amylases.

Polyacrylamide gel electrophoresis separations of durum and HRS wheat (Manitou) showed that each wheat possessed eight α-amylases (Fig. 6). The electrophoretic mobilities of the α-amylases were similar, if not identical, in each wheat. The amounts of the faster migrating α-amylases (α-6, α-7, α-8) were significantly lower in the HRS wheat. This finding agrees with the report by Kruger (18) that eight α-amylase components are present in HRS wheat.

The finding that HRS and durum wheats have identical or similar α-amylases agrees with the findings of Nishikawa (19), who found that durum wheat had 11 gel isoelectrofocusing α-amylase components that corresponded to 14 of those components found in an HRS wheat.

In summary, durum and HRS wheats have similar α-amylase components. The similarity of α-amylase in the two genetically different wheats supports the concept that the 42-chromosome hexaploid HRS wheat (*Triticum aestivum*) is derived from the 28-chromosome tetraploid durum wheat (*Triticum* durum) and a wheat related to *Aegilops squarrosa* (19). Additional studies, such as detailed enzymatic studies or protein sequence studies, would have to be done to determine the full extent of similarity of the α-amylases that are present in durum and HRS wheats.

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