Wheat α-Amylases. II. Physical Characterization[1]

R. TKACHUK and J. E. KRUGER, Canadian Grain Commission, Grain Research Laboratory, Winnipeg, Manitoba, Canada R3C 3G9

ABSTRACT

Four α-amylase isoenzymes were isolated from malted hard red spring wheat. Isoenzyme purity was established by rechromatography on DEAE-cellulose, gel and sodium dodecyl sulfate (SDS) electrophoresis, and sedimentation velocity studies. The α-amylases were similar except for electrophoretic mobility and chromatography elution times. The purified isoenzymes have average sedimentation and diffusion coefficients of approximately 3.71*s* and 7.19×10^{-7} cm.^{2} sec.$^{-1}$. Molecular weights from sedimentation velocity/diffusion and SDS gel-electrophoresis data were 45,100 and 42,000. The α-amylases are acidic proteins with relatively large amounts of glutamyl, aspartyl, and glycyl residues and have isoelectric points between pH 6.05 and 6.20. Maximum enzymatic activity occurs at pH 5.5 to 5.6. Wheat α-amylase does not contain sulfhydryl groups but does have two disulfide bonds per residue.

α-Amylases are of great interest in cereal chemistry because of their significant effect on properties of bread. Since the details of this effect are not fully understood, our laboratory has undertaken a study of wheat α-amylase. Initial studies dealt with its isolation, and it was shown that four homogeneous α-amylases could be isolated from malted wheat by ion-exchange chromatography (1). The

[1]Paper No. 337 of the Canadian Grain Commission, Grain Research Laboratory, Winnipeg, Manitoba, Canada R3C 3G9.

present paper describes some of the physical characteristics of α-amylases isolated from a malted hard red spring (HRS) wheat.

MATERIALS

A sample of hard red spring wheat, variety Manitou, was used for the isolation of of the α-amylases. After all extraneous contaminants were removed by machine and manual cleaning, the wheat was soaked for 24 hr., and then malted for 5 days at 15° to 17°C. at 95% r.h. During malting the wheat was tumbled every 3 hr. The wheat was then dried by drawing air at 20° to 22°C. through it for 2 days. The malted wheat had 16.0% protein (N X 5.7, 14% moisture basis), 10.6% moisture, and 0.72% ash (dry basis).

EXPERIMENTAL AND RESULTS

Isolation

The isolation and purification of the α-amylases were carried out as described previously (1), with the following modifications.

The initial wheat extract was made with 0.01M calcium acetate or calcium chloride. Heat treatment of the initial extract at 70°C. was extended to 20 min. to ensure that all β-amylase was destroyed. *N,N*-Diethylaminoethyl (DEAE) cellulose chromatography was carried out on smaller columns, using a linear salt gradient (Table I and Fig. 1).

Final Purification

It was found that the previously described procedure (1) for isolating wheat α-amylase gives a product containing approximately 87% protein, the contaminants being sodium and calcium salts and sometimes traces of a yellowish phenolic pigment. Both contaminants can be removed either by gel filtration or rechromatography on DEAE-cellulose.

Enzymatically active α-amylases free from adjacent isoenzymes (see Fig. 1) were obtained by saving the peak portions of the elution profile obtained during DEAE-cellulose chromatography. In a typical preparation, α-amylase isoenzymes I,

TABLE I. DISTRIBUTION OF α-AMYLASES IN MALTED HARD RED SPRING WHEAT

α-Amylase	Amount Present, %	
	Previous study	Present study
Green[a]		
1	n.d.	
2	n.d.	0.1 - 0.5
3	n.d.	
Malted		
Ia	—[b]	5
I	9	18
II	34	22
III	21	29
IV	36	25

[a]Green α-amylases are those found in immature wheat (6).
[b]In our previous study, α-amylase Ia did not seem to be present.

II, III, and IV were isolated by pooling fractions represented by elution volumes of 1,154 to 1,322, 1,416 to 1,604, 1,723 to 1,852, and 1,911 to 2,153 ml. in Fig. 1. The pooled fractions were concentrated by ultrafiltration through Amicon P-10 or UM20E membranes. Rechromatography of the concentrated isoenzymes showed that contamination by adjacent isoenzymes was neglible, a finding verified by polyacrylamide gel electrophoresis. Buffer salts were removed by gel filtration on Sephadex G-10 columns; however, unstable, enzymatically active preparations are obtained when all of the calcium is removed. To obtain enzymatically active preparations of maximum stability, approximately 0.001M calcium ion must be present (Table II).

Solutions of α-amylase in 0.01M calcium containing 0.5 to 5 mg. protein per ml. are stable for at least 6 months when kept at −20°C. Similar solutions are also quite stable at 2°C., providing bacterial growth is prevented.

For amino acid analyses and spectrophotometric data, the α-amylases were desalted by Sephadex G-10 gel filtration in 1N acetic acid, and dried under vacuum over phosphorus pentaoxide. Such α-amylase preparations do not possess enzymatic activity, and are very hygroscopic, picking up approximately 8 to 9.5% water at relative humidities of 30 to 40% at 22°C. Once the α-amylases are dried, it is very difficult to redissolve them in buffer solutions unless the solutions are made quite acidic or basic.

Enzymatic Properties

α-Amylase Activity. α-Amylase activity was determined using 3,5-dinitrosalicylic acid. α-Amylase activity was measured by incubating for 5 min. at 25°C., 0.1 ml. enzyme solution with 1.0 ml. 1% soluble starch (starch, soluble according to Lintner, Fisher Scientific Co.) in 0.1M acetate buffer, pH 5.5, containing 0.01M calcium chloride. A unit of α-amylase activity is that amount of enzyme which produces starch hydrolysis products equivalent to the reducing capacity of 1 μmole maltose per min. Specific activity is expressed as units per mg. protein. Reduced 3,5-dinitrosalicylic acid was monitored at 497 nm.

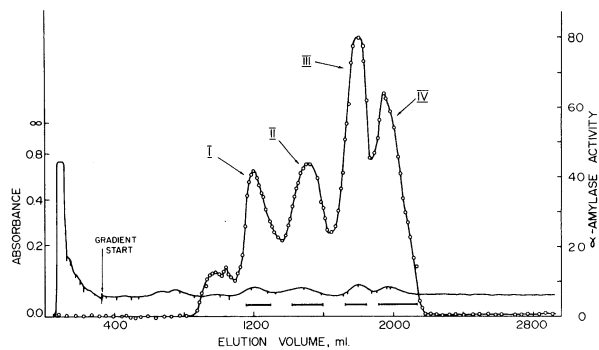

Fig. 1. Ion-exchange chromatogram of wheat α-amylase on a 16 × 300-mm. DEAE-cellulose column at 21° C. A linear gradient was used, made by leading 900 g. of 0.3M *tris* into 900 g. of 0.02M *tris*, both buffers adjusted to pH 8.6 with HCl. Both buffers contained 0.001M $CaCl_2$ and were in identical containers. Fractions of 8 ml. were collected at a flow rate of 68 ml. per hr. Circles represent activity measurements, and the solid line absorbance at 280 nm. Horizontal lines indicate the fractions pooled.

For the determination of pH optimum, effect of inhibitors, and thermal stability, studies were carried out by the automated ferricyanide method of Strumeyer and Romano (2) using 0.5% reduced starch as substrate.

pH Optimum. Michaelis's 0.01M barbital-sodium acetate buffers (3) were used to measure the α-amylase activities as a function of pH. The pH optimums for all the isoenzymes were found to be at 5.5 to 5.6 (Figs. 2 and 3). The isoenzymes produced nearly identical activity profiles with isoenzyme IV giving a slightly broader peak.

Contamination by Other Enzymes. The presence of transferase contamination was examined by incubating α-amylase with glucosse-^{14} C(U) and starch and

TABLE II. STABILITY OF α-AMYLASE AT VARIOUS CALCIUM CONCENTRATIONS

Conc. Calcium Acetate[a]	Recovery of Activity[b] %
0	9
0.0001	51
0.001	103

[a]Molarity of calcium acetate in 0.01M 3-dimethylamino-1-propanol buffer, pH 8.7; pH adjusted with acetic acid.

[b]Recovery of enzymatic activity measured after elution of the enzyme through a 20 X 1,450-mm. Sephadex G-10 column. In each case, a mixture of all the isoenzymes as isolated at the glycogen-complex stage (1) was used.

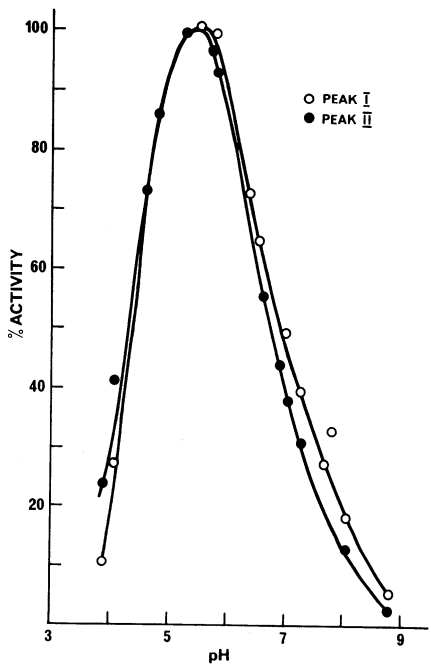

Fig. 2. Effect of pH on the activity of α-amylases I and II.

examining whether the glucose was being transferred to higher oligosaccharides (4). Paper chromatography with water:propanol:pyridine (4:6:4 v./v./v.) at 85°C. showed that no transfer of radioactivity occurred in 2 hr.

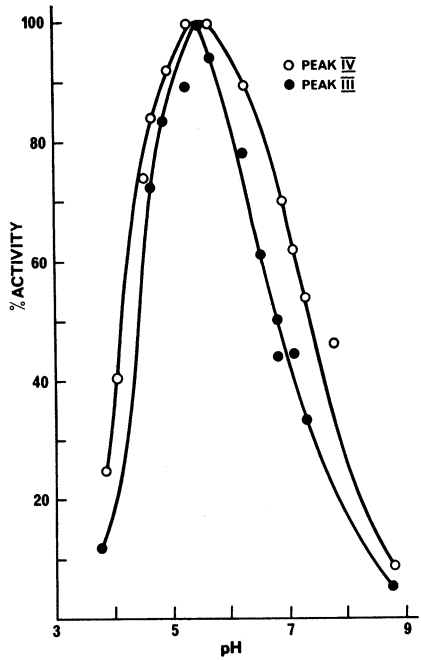

Fig. 3. Effect of pH on the activity of α-amylases III and IV.

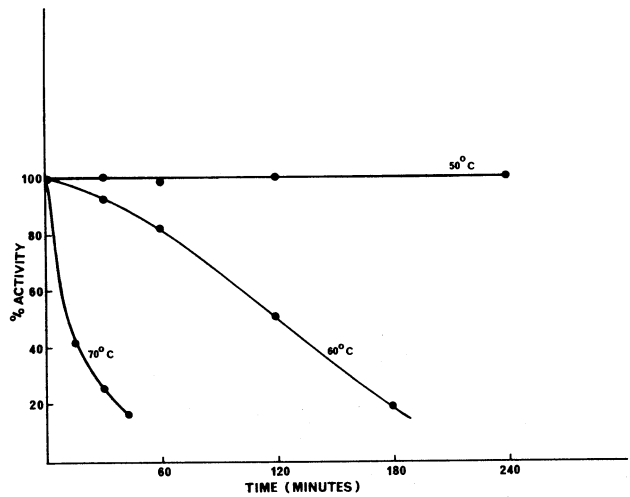

Fig. 4. Effect of temperature on α-amylase activity.

Reaction Order. Plots of reducing value at 25°C. vs. time gave straight lines up to 100 min., indicating zero-order kinetics.

Thermal Stability. The stability of the isoenzymes was determined for various durations in 0.1M acetate buffer and 0.001M Ca^{++}, pH 6.0, containing 0.1% gelatin at 50°, 60°, and 70°C. (Fig. 4). All of the isoenzymes behaved similarly.

Effect of Sulfhydryl (SH) Reagents. Incubation of the α-amylase isoenzymes with 1×10^{-4}M solutions of *p*-hydroxymercuribenzoate, mercuric chloride, iodoacetic acid, and *N*-ethylmaleimide for 10 min. at 20°C. resulted in only a slight decrease of enzymatic activities, as 76, 80, 90, and 95% of the activity of the control solution was obtained. The control solutions contained all the reagents except for the isoenzymes. The SH reagents were not removed before activity measurements were carried out, and at a concentration of 1×10^{-4}M in the enzyme solution which is equivalent to less than 1×10^{-5}M during the activity measurement, do not affect the activity measurement.

Physical Characterization

Polyacrylamide Gel Electrophoresis. Gel electrophoresis was carried out as described by Davis (5) using both flat-bed and cylindrical gels. Assay for α-amylase activity was usually carried out on the flat-bed gels (6), and staining for protein components on the cylindrical gels. The results (Fig. 5) showed that the purified α-amylase components were single, enzymatically active homogeneous components with traces of the adjacent components shown in Fig. 1.

Electrophoretic Mobility. Zone electrophoresis was carried out in 0.1-ionic strength buffers (7), using a Beckman-Spinco Model H electrophoresis-diffusion instrument as described for wheat β-amylase (8). Only α-amylase II was present in sufficient quantities to determine mobilities at various pH values to obtain an

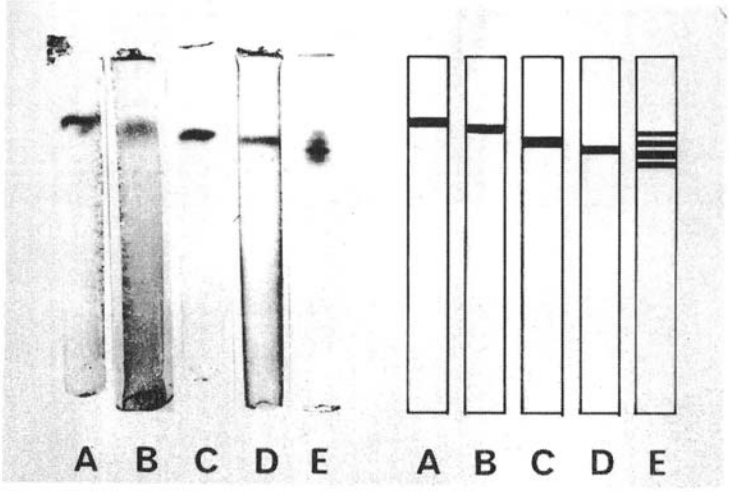

Fig. 5. Gel electrophoresis of α-amylases. Proteins stained with Coomassie Blue dye. A, I; B, II; C, III; D, IV; E, mixture obtained at glycogen complex isolation stage. The right-hand portion of the figure is a line drawing presentation of the photographs shown on the left.

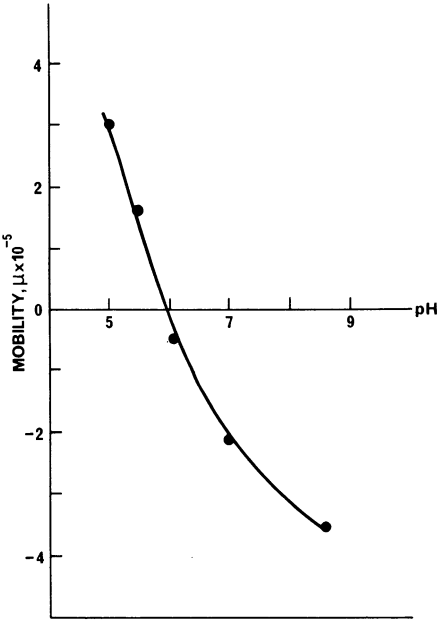

Fig. 6. Electrophoretic mobility of α-amylase II.

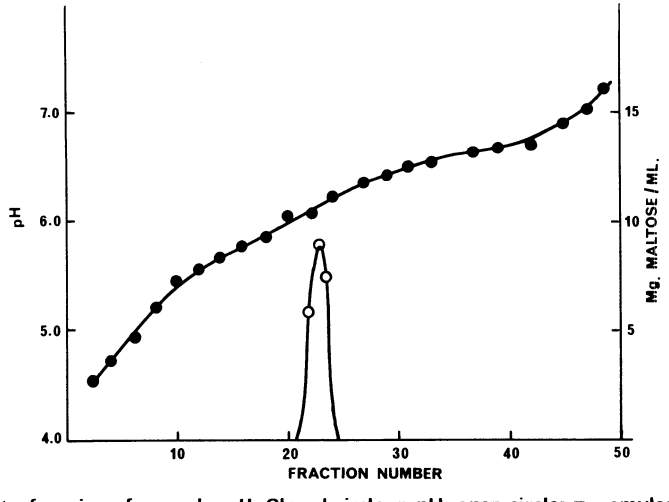

Fig. 7. Electrofocusing of α-amylase II. Closed circles = pH; open circles = α-amylase activity.

isoelectric point of pI 5.95 (Fig. 6). The mobilities of the other three isoenzymes were determined only at pH 7.0.

Electrofocusing. Electrofocusing of all α-amylase isoenzymes was carried out as described by Vesterberg and Svensson (9) with a 110-ml. column, ampholytes, power supply, and gradient mixer purchased from L.K.B. Products. The dialyzed

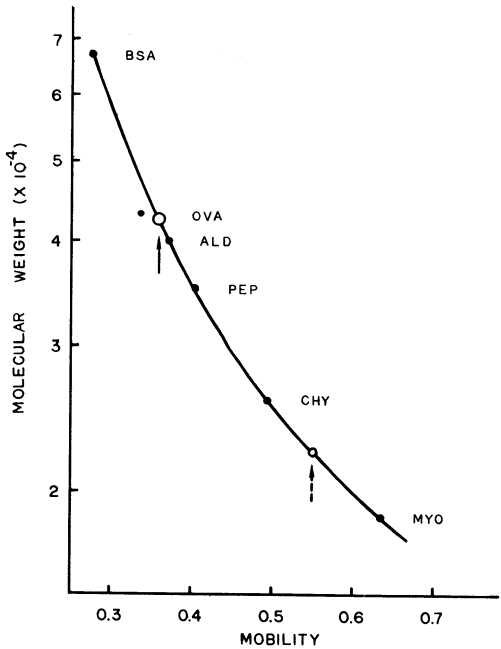

Fig. 8. SDS gel electrophoresis of α-amylases. BSA, OVA, ALD, PEP, CHY, and MYO are abbreviations for standard protein markers bovine serium albumin, ovalbumin, aldolase, pepsin, chymotrypsin, and myoglobin with molecular weights of 68,000, 43,000, 40,000, 35,000, 25,700, and 17,200, respectively. Solid arrow (——>) indicates mobility of major portion of all the isoenzymes (MW 41,500 to 42,500); dashed arrow (– – –>) indicates a minor component present in α-amylases I and II with a MW of 21,000 to 23,000.

sample (4 to 6 ml.) was added to the light solution used in forming the sucrose density gradient. Preliminary isoelectric focusing for 64 hr. at 300 v. at 11°C. in a pH 3 to 10 gradient indicated that the isoenzymes focused in the region of pH 5 to 7; and subsequent experiments were carried out at pH 5 to 7, 500 v. for 48 hr. Following electrofocusing 1.5-ml. fractions were collected and pH measurements were taken at 11°C. The various fractions were assayed for α-amylase activity by the ferricyanide method with reduced starch as substrate (2); it was necessary to run blanks with each fraction, as hydrolysis of sucrose near the acid side of the gradient caused increased blank values. Confirmation of the α-amylase activity was also made by incubating it with β-limit dextrin and noting the decrease in the dextrin-iodine color at 540 nm. Isoelectric points were obtained from plots of the electrofocusing experiments. Results for α-amylase II are shown in Fig. 7.

Sodium Dodecyl Sulfate (SDS) Electrophoresis. SDS electrophoresis was carried out as described by Weber and Osborn (10) using a flat polyacrylamide slab with 12 sample wells. A 10% acrylamide gel containing one-half of the amount of the *N,N'*-methylene bisacrylamide cross-linker was used. Samples were layered on the gels in a sucrose solution; and electrophoresis was carried out at 325 v., 200 cycles per sec. for 4 hr. at 0°C. (pulsed power supply, Ortec Inc., Oak Ridge). Standards used for calibration of the gel were: ovalbumin, myoglobin (Mann Research Lab., New York); bovine albumin, pepsin (Calbiochem, Los Angeles); and aldolase

(Pharmacia, Uppsala). The results (Fig. 8) showed that the α-amylase isoenzymes possessed an identical molecular weight (MW) of 41,500 to 42,500. Small amounts of proteins of lower MW (21,000) were found in components I and II.

Absorption Spectra. Absorption spectra were determined in water and 0.05N NaOH solutions. Typical protein absorption spectra were obtained with maximum absorption in the region of 279 nm. In alkaline solution (0.05N NaOH) a double absorption peak was obtained at 275 and 286 nm. Dry weight was determined by drying to constant weight in a high vacuum at 105°C. Depending on the humidity, samples equilibrated at room temperature (21° to 23°C.) contained 8 to 12% moisture. Purity (as % protein) of the samples was determined by amino acid analysis. Using the purity, dry weight, and absorption data, an average extinction coefficient, $E^{1\%}_{1\%\ cm}$, at 275 or 286 nm in 0.05M NaOH was found to be 24.3. In ordinary buffer solutions, $E^{1\%}_{1\%\ cm.}$ at 279 nm. was approximately 23.0.

Amino Acid Analysis. Vacuum-dried samples were hydrolyzed in constant-boiling HCl in evacuated sealed tubes for 24 hr. at 110°C. Hydrolysates were analyzed on a Beckman-Spinco Model 120 amino acid analyzer (11). Tryptophan was determined from chromatographic analysis of barium hydroxide hydrolysates (12) and by a spectrophotometric method on the intact protein (13); and cystine as cysteic acid on samples which were oxidized with performic acid

TABLE III. AMINO ACID COMPOSITIONS OF WHEAT α-AMYLASES
(μmoles per g)[a,b]

	Component				
	I	II	III	IV	Average
Tryptophan[c]	255	297	n.d.	213	255
Lysine	441	415	471	410	434
Histidine	276	292	327	290	296
Ammonia	795	853	824	789	815
Arginine	461	401	396	342	400
Aspartic acid	939	923	1,010	965	959
Threonine	464	451	495	472	471
Serine	469	477	467	475	472
Glutamic acid	943	970	986	893	948
Proline	589	586	596	642	603
Glycine	972	959	1,025	931	972
Alanine	795	714	798	743	763
Cystine[d]	44	48	61	49	51
Valine	582	584	590	535	573
Methionine	117	157	115	182	143
Isoleucine	436	464	492	446	460
Leucine	599	621	694	617	633
Tyrosine	272	280	316	302	293
Phenylalanine	289	309	359	367	331
Cysteine	0	0	0	0	0
Actual experimental amino acid nitrogen recovery, %	83	89	94	92	

[a]Calculated to 100% amino acid residue weight recoveries, e.g., values in column I were multiplied by 100/83.
[b]Threonine, serine, proline, valine, and isoleucine recoveries multiplied by 105, 109, 105, 108, and 107% to correct for incomplete hydrolysis and decomposition during hydrolysis.
[c]Determined by analyzing barium hydroxide hydrolysates.
[d]Determined as cysteic acid.

prior to acid hydrolysis (14). Tryptophan and cysteic acid were determined on the 8-cm. and 50-cm. columns of the amino acid analyzer. To correct for destruction during acid hydrolysis the values for threonine, serine, proline, valine, and isoleucine were multiplied by factors of 1.05, 1.09, 1.05, 1.08, and 1.07. The amino acid data are shown in Tables III, IV, and V.

Sulfhydryl (SH) Content. The absence of SH in the α-amylase was shown by reacting the isoenzymes in 8M urea solutions at pH 8.0 with iodoacetic acid. To increase the detection limits, iodoacetic acid-2-^{14}C was used. After 1 hr. at 20°C., the urea and unreacted iodoacetate were dialyzed away, the samples were hydrolyzed in the absence of traces of air, and analyzed on the amino acid analyzer equipped with a scintillation detector (15). The absence of *S*-carboxymethyl-2-^{14}C-cysteine [which is stable to acid hydrolysis (16)] in the hydrolysates indicated that the original isoenzymes did not contain any SH groups.

Carbohydrate Content. The absence of carbohydrate in purified α-amylase components was indicated by the absence of levulinic acid on the amino acid chromatograms (17), and by negative results on testing α-amylase solutions with phenol-sulfuric acid reagent (18).

TABLE IV. AMINO ACID COMPOSITION OF WHEAT α-AMYLASE

	Average[a] μm. per g.	Amino Acid Residue Weight (w.) mg. per 100 mg.	Residue Specific Volume[b] $\bar{v}$	$w\bar{v}$.	Minimal Molecular Weight[d]	Amino Acid[e]	Nearest Integral No. of Amino Acid Residues[e]
Tryptophan	255	4.748	0.74	3.514	3,926	11.0	11
Lysine	434	5.563	0.82	4.562	2,307	18.8	19
Histidine	296	4.059	0.67	2.720	3,383	12.8	13
Ammonia	815						
Arginine	400	6.247	0.70	4.373	2,503	17.3	17
Aspartic acid	552	6.352	0.60	3.811	1,814	23.9	24
Asparagine[c]	407	4.644	0.62	2.879	2,460	17.6	18
Threonine	471	4.762	0.70	3.333	2,125	20.4	20
Serine	472	4.110	0.63	2.589	2,121	20.4	20
Glutamic acid	540	6.972	0.66	4.602	1,854	23.4	23
Glutamine	408	5.228	0.67	3.503	2,454	17.6	18
Proline	603	5.856	0.76	4.451	1,660	26.1	26
Glycine	972	5.545	0.64	3.549	1,030	42.0	42
Alanine	763	5.421	0.74	4.012	1,312	33.0	33
Cystine	51	1.042	0.61	0.636	19,623	2.21	2
Valine	573	5.680	0.86	4.885	1,747	24.8	25
Methionine	143	1.876	0.75	1.407	7,001	6.2	6
Isoleucine	460	5.205	0.90	4.685	2,176	19.9	20
Leucine	633	7.162	0.90	6.446	1,582	27.4	27
Tyrosine	293	4.781	0.71	3.395	3,417	12.7	13
Phenylalanine	331	4.871	0.77	3.751	3,025	14.3	14

$$\text{Specific volume} = \frac{73.103}{100.114} = 0.730$$

[a]From Table III.
[b]From ref. 23.
[c]Amounts of asparagine and glutamine were each assumed to equal half of the ammonia content.
[d](Amino acid residue molecular weight X 100) [% amino acid residue (col. 2)].
[e]Per 43,300 g. α-amylase.

Sedimentation Velocity. Sedimentation velocities of the α-amylase isoenzymes were determined by a Beckman-Spinco Model E ultracentrifuge thermostatted at 20°C. A rotor speed of 60,000 r.p.m. was used; photographs were taken at 16-min. intervals; "zero-time" was calculated to be equivalent to the time when the rotor reached the speed of 40,000 r.p.m. (19). Solutions analyzed contained 0.2 to 0.5% protein in *tris*-HCl buffer solutions, pH 7.0 and pH 8.2, with an ionic strength of 0.1, the adjusting salt being NaCl. The photographs were evaluated with a Gaertner microcomparator, and sedimentation coefficients were calculated from slopes obtained by plotting the logarithm of the distance of the protein boundary to the axis of rotation vs. time (19). The sedimentation coefficients (s) were reduced to standard conditions ($s_{20,w}$.) where s represents the Svedberg unit of 1×10^{-13} sec. (Table VI). Sufficient pure α-amylase was not available for determining the effect of concentration on s; however, as relatively dilute solutions were analyzed on the ultracentrifuge, the influence of concentration should be negligible. Some typical sedimentation-velocity schlieren patterns are shown in Fig. 9.

Activation Energies. The effect of temperature on hydrolysis rates by wheat α-amylase isoenzymes, and the mixture obtained at the glycogen-complex isolation stage were measured over a temperature range of 20° to 74°C. Arrhenius plots of the results (Fig. 10) allowed activation energies to be calculated (Table VII).

Free Diffusion. Free diffusion studies were carried out on a Beckman-Spinco Model H electrophoresis-diffusion instrument using the supplied modified 11-ml. Tiselius electrophoresis cell. The runs were conducted at 2.18°C., in pH 8.2 or 7.0 *tris*-HCl buffer of 0.1 ionic strength, the ionic strength adjusted with NaCl (7). Sequence photography was used to record the Rayleigh patterns. The Rayleigh patterns were analyzed with a Gaertner microcomparator as described by Longsworth (20) and Schachman (21) with the aid of probability tables (22) to obtain the diffusion coefficients $D^{\circ}_{20,w}$. (Table VIII).

TABLE V. AMINO ACID COMPOSITIONS OF AMYLASES FROM DIFFERENT PLANT SOURCES

	Wheat Malt α-Amylase[a] μm./g.	Wheat β-Amylase μm./g.	Sorghum Malt α-Amylase μm./g.
Tryptophan	255	214	n.d.
Lysine	434	320	365
Histidine	296	307	324
Ammonia	815	843	714
Arginine	400	459	333
Aspartic acid	959	932	1,180
Threonine	471	264	351
Serine	472	334	325
Glutamic acid	948	936	675
Proline	603	518	388
Glycine	972	745	931
Alanine	763	677	737
Cysteine	Absent	59	n.d.
Cystine	51	34	17
Valine	573	623	461
Methionine	143	196	94
Isoleucine	460	329	514
Leucine	633	741	605
Tyrosine	293	378	330
Phenylalanine	331	372	377

[a]Average composition from Table III.

Specific Volume. The specific volumes ($\bar{v}$) were calculated from the averaged amino acid compositions of the α-amylase isoenzymes using the specific volumes of the amino acid residues given by Cohn and Edsal (23). The calculation gave a value ($\bar{v}$) of 0.730 cc. per g. for the specific volume.

Molecular Weight. Sedimentation and diffusion coefficients, specific volume, and buffer density (ρ) values were used to calculate the MWs of the α-amylase isoenzymes (Table IX) by use of the Svedberg equation,

$$MW = \frac{RTs}{D(1 - \bar{v}\rho)}$$

MW estimates were also obtained by gel filtration experiments. A 2.5 × 100-cm. column of Sephadex G-100 was calibrated with apoferritin, bovine albumin, ovalbumin, chymotrypsinogen A, and myoglobin as MW standards of 545,000, 67,000, 45,000, 25,000, and 17,500, respectively. Solutions of the α-amylase isoenzymes were then eluted through the column. The standards and α-amylases

TABLE VI. DETERMINATION OF SEDIMENTATION COEFFICIENTS

Component	$s_{20, w.}$
I	4.22
II	4.66,[a] 3.72
III	3.39, 3.42, 4.70[a]
IV	3.45, 3.89,[b] 3.48, 3.39, 3.57

[a]Values not used to obtain average values listed in Table VIII.
[b]Same preparation used for approach-to-equilibrium (Archibald method) experiment.

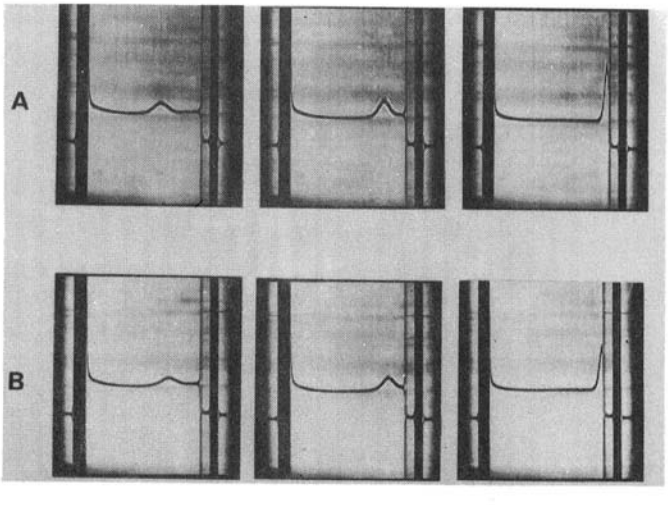

Fig. 9. Sedimentation velocity schlieren patterns obtained after 16, 48, and 80 min. of ultracentrifuging—the 32- and 64-min. patterns are not shown in this illustration. A, α-amylase I; B, α-amylase IV.

were eluted at 4°C. with 0.05M sodium acetate-0.01M calcium chloride buffer, pH 5.5, at 20.2 ml. per hr. to obtain elution volume data.

Studies with Bio-Gel P-150 were carried out on a 2.5 × 100-cm. column calibrated with thyroglobulin, aldolase, bovine albumin, ovalbumin, chymotrypsinogen A, myoglobin, and ribonuclease as MW standards of 640,000, 158,000, 67,000, 45,000, 25,000, 17,800, and 13,700, respectively. Elution volumes were obtained by eluting upwards through the column the α-amylase isoenzymes and MW standards using 0.05M phosphate-0.01M sodium chloride-0.001M calcium chloride-0.001M thioglycerol buffer, pH 6.0, at a flow rate of 14.7 ml. at 4°C.

MW estimates from both types of gel filtration experiments were obtained (Table IX) by noting the position of the elution volumes of the α-amylase isoenzymes on plots of the elution volumes versus the logarithm of the MW of the standards used to calibrate the columns.

DISCUSSION

Four α-amylase components were isolated from a malted sample of HRS wheat, variety Manitou. A summary of the α-amylase properties is given in Table VIII.

β-Amylase and transferase activity were absent from all the components, as paper and thin-layer chromatography showed that a normal amount of maltose was present (24) when starch was degraded by the enzymes, and incubation of starch with glucose-^{14} C(U) and α-amylase showed no transfer of radioactivity to higher oligosaccharides after incubation for 2 hr.

Electrophoresis experiments showed that all the isoenzymes isolated were single, relatively pure homogeneous components. The impurities observed were usually traces of the other isoenzymes. Whereas the isoenzymes possess different isoelectric

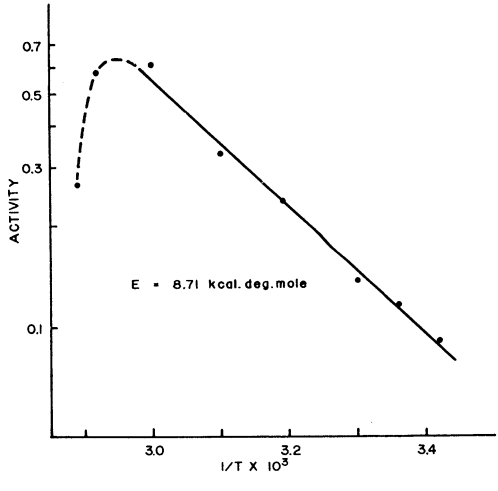

Fig. 10. Arrhenius plot for wheat α-amylase. Assay for α-amylase activity as described in text; curve shown is for the mixture of all isoenzymes obtained at the glycogen-complex isolation stage.

TABLE VII. ACTIVATION ENERGIES FOR α-AMYLASES

α-Amylase	E_a kcal. per mole	Temperature °C.
Wheat:		
Glycogen complex[a] (this study)	8.71	20-60
I	8.47	20-45
II	7.01	20-45
III	9.28	20-45
IV	7.01	20-45
Glycogen complex[a] (24)	11	25
	14	9
Barley:		
Malted (34)	7.05	
Malted and mature (35)	13	10-40
Pea, germinated (36)	7.6	25-51
Sorghum, malted (31)	12.0	15-40
Broad bean, mature (37)	0	45
	5	25
	14	9

[a]Glycogen complex refers to the mixture of α-amylases obtained at the glycogen complex isolation stage.

TABLE VIII. SUMMARY OF WHEAT α-AMYLASE PROPERTIES

	I	II	III	V	Average
Nitrogen, % (Amino acid analysis)	17.3	17.2	17.1	16.8	17.1
Activity per mg. enzyme, μm. maltose, min.$^{-1}$ 25° C., pH 5.5	1,480	1,300	1,510	1,570	1,465
Sedimentation coefficient, $s_{20,w.}$	4.22	3.72	3.41	3.56 ± 0.22	3.71[a]
Diffusion constant, $D^{\circ}_{20,w.}$ ($\times 10^7$) cm.^{2} sec.$^{-1}$	6.77	n.d.	7.24	7.57	7.19
Partial specific volume, ml. per g.	---	---	---	---	0.730[c]
Molecular weight, g. per mole sedimentation-diffusion	56,740	n.d.	42,900	42,800	45,100[b]
SDS electrophoresis	42,500	42,200	42,000	41,500	42,000
Isoelectric point, pI					
Electrofocusing	6.16	6.20	6.05	6.17	
Zone electrophoresis	n.d.	6.0	n.d.	n.d.	
Mobility, pH 7.0, cm^2/v. sec.	+196	+209	+278	+333	
pH optimum	5.5	5.7	5.5	5.5	5.5
$E^{1\%}_{1\%\ cm.}$ at 275 nm., 0.1N NaOH	23.8	25.6	n.d.	23.4	24.3
SH content	0	0	0	0	0

[a]Average values from Table VI. Values for IV most reliable.
[b]Using values of 3.56 and 7.19 for $s_{20,w.}$ and $D^{\circ}_{20,w.}$, respectively.

TABLE IX. MOLECULAR-WEIGHT ESTIMATES OF HRS MALTED WHEAT α-AMYLASES

Method	
Sedimentation velocity – diffusion	45,100
SDS gel electrophoresis[a]	41,500-42,500
Archibald method[b]	86,000
Gel filtration[c]	
Sephadex G-100	20,000[c]
Bio-Gel P-100	23,000[c]
Ultrafiltration[d]	30,000

[a]A smaller component with a MW of 21,000 representing approximately 10% of the total protein was present in components I and II.
[b]Single experiment.
[c]Average values for the four isoenzymes.
[d]In two experiments, about 30% of the enzyme passed through a P-30 membrane.

points, Table VIII shows that all of these occur between pH 6.05 and 6.20. Four isoenzyme II, it was also possible to determine a pI of 6.0 by zone electrophoresis. Although this value is lower than the value of 6.2 obtained by electrofocusing, such differences, and in the same direction, have been observed for other proteins. Zone electrophoresis experiments at pH 7.0 showed that the various isoenzymes had mobilities of +196 to +333 cm.^{2} per v. sec. It is seen that component IV is the most acidic, since it has the highest positive mobility which is in agreement with chromatographic properties as it is the last component to elute during chromatography on DEAE-cellulose. Homogeneity of the four α-amylase components was also indicated by sedimentation velocity experiments, SDS-electrophoresis evidence, and rechromatography experiments on DEAE-cellulose.

The number of α-amylase components seen in a malted wheat depends on whether a qualitative or quantitative experimental technique is used. A qualitative electrophoresis approach reveals that there are seven α-amylases definitely present, with the possible presence of an eighth component. These consist of three "green" α-amylases which originate during kernel development and remain in the malted wheat, four major α-amylases, and sometimes a fifth minor α-amylase component arising during malting (6).

A quantitative examination (Table I) indicates that approximately 95 to 99% of the α-amylases present in malted wheat is caused by four major α-amylase components synthesized during germination. Sometimes a fifth minor component accounting for 5% of the total α-amylase activity is present also. Attempts to show the presence of the fifth minor malt α-amylase component are not always successful; its presence may be due to varietal differences or perhaps an artifact arising out of the isolation procedure (proteolysis?). Also present in malted wheat are the three "green" α-amylase components, probably due to the presence of immature kernels, but these form only approximately 0.1 to 0.5% of the total α-amylase activity present. Of course the quantitative interpretation presented in the present study is confined within the limitations imposed by the substrate used to assay for α-amylase activity. The use of another substrate could possibly reveal a different quantitative distribution of α-amylase activities.

Our finding of four major and one possible minor α-amylase components in malted HRS wheat can be compared with other estimates of two or three

components by Olered and Jönsson (25), and two by Daussant and Corvazier (26) and Alexandrescu and Mihailescu (27).

Ultraviolet absorption spectra of the amylases in water or buffers showed normal protein spectra with maximum absorption at 279 nm. An average extinction coefficient, $E^{1\%}_{1\%\ cm.}$ at 275 or 286 nm. in 0.05N NaOH was found to be 24.3.

Analytical ultracentrifuge data indicated that the average $s_{20,\ w.}$ value was equal to 3.71. It was not possible to obtain a vlaue for $s^{\circ}_{20,w.}$ due to the lack of material that would be necessary to examine the effect of concentration on s. However, since very dilute solutions of 0.18 and 0.4% protein were used for the sedimentation studies, the values obtained in the present study should be virtually equivalent to $s^{\circ}_{20,\ w.}$.

Several techniques were used to obtain MW estimates. Sedimentation velocity (Table IX) and diffusion data indicate a weight-average MW value of 45,100 for wheat α-amylase, using the most reliable value of 3.56 for $s^{\circ}_{20,\ w.}$ (Table VI), and the average value of 7.19 for $D^{\circ}_{20,\ w.}$ This value is in excellent agreement with the MW of 41,500 to 42,500 obtained by SDS electrophoresis (Fig. 8). SDS electrophoresis was carried out under the strongly reducing conditions recommended by Robyt et al. (28) so that SS dimer formation would be reduced.[2]

MW estimates were also carried out by some other techniques, the results from which in at least one case are in apparent conflict with our conclusion that the MW of malted wheat α-amylase is approximately 43,000. Thus, gel permeation studies on Sephadex G-100 and Bio-Gel P-150 gave MW estimates of 20,000 and 23,000; in several instances approximately one-third of the α-amylase passed through ultrafiltration membranes which are supposed to retain proteins with a MW of 30,000 or larger; and a single approach-to-equilibrium experiment (Archibald method) gave a MW estimate of 86,000 for an α-amylase IV solution which the previous day gave a $s_{20,\ w.}$ value of 3.89 (a $s_{20,\ w.}$ value of 3.89 is approximately equivalent to a MW of 46,800).

Satisfactory explanations can be arrived at for the apparent low MW estimates in two of the cases. Gel filtration studies on Sephadex can be expected to give low-MW estimates because an enzyme-substrate type of interaction would be expected to occur between α-amylase and a carbohydrate material like Sephadex; for example, it has been noted by Manners and Marshall (30) that low anomalous MWs of 7,000 and 15,000 were obtained by Sephadex G-100 data for rye α-amylase isoenzymes. Ultrafiltration evidence can be suspect since larger pores can be present in a filtering membrane. However, it is difficult to interpret the results from the Bio-Gel experiments since α-amylase should not complex with a polyacrylamide material; and also, Greenwood and Milne (24) using Bio-Gel P-100 gel chromatography data obtained a MW of 45,000 ± 5,000 for malted wheat α-amylase purified up to the glycogen-complex stage. The high MW value of 86,000 obtained during one of the ultracentrifuge experiments probably indicates that wheat α-amylase can dimerize on standing.

Amino acid analysis showed that *S*-carboxymethyl cysteine was absent in 6N HCl

[2]The claim by Robyt et al. (28) that the MW of porcine pancreatic α-amylase is 24,000 to 25,000 when determined by SDS electrophoresis in the presence of the strongly reducing reagent dithiothreitol instead of the usually accepted value of approximately 50,000 could not not be duplicated in our laboratory. Our experiments indicated that porcine pancreatic α-amylase has a MW of 54,000 when examined by SDS electrophoresis as described by Robyt et al. (28), which is in good agreement with the usually accepted value of 52,500 (29).

hydrolysates of α-amylase isoenzymes previously treated with iodoacetic acid, indicating that wheat α-amylases do not seem to contain sulfhydryl (SH) groups. This view was verified by inhibition studies which showed that the isoenzymes were only slightly inhibited in the presence of 1×10^{-4} M solutions of *p*-hydroxymercuribenzoate, mercuric chloride, iodoacetic acid, and N-ethylmaleimide.

Amino acid analyses indicated also that all four wheat α-amylases possessed similar amino acid compositions, except for varying arginine content, with component IV having the smallest amount. Apparent significant variations in cystine and tryptophan contents were also obtained; however, these variations should not be considered to be as significant as the variation in arginine content because of the small amounts of cystine present and the difficulties associated in the analysis for cystine and tryptophan content. The lower arginine content in component IV indicates that it is the most acidic α-amylase component, an observation consistent with its chromatographic behavior on a DEAE-cellulose column, as it is the last component to elute. Component IV also has a higher methionine content than the other components. Values for tryptophan and tyrosine content measured by the spectrophotometric techniques were in good agreement with the chromatographic values: e.g., for α-amylase I the tryptophan values were 276 and 255, and the tyrosine values 286 and 293, by spectrophotometric and chromatographic techniques, respectively.

It is seen (Table V) that wheat α-amylase differs in its amino acid composition from wheat β-amylase (5), and sorghum malt α-amylase—the only other plant α-amylase for which an amino acid composition has been reported (31). As previously mentioned, amino acid analysis of carboxymethylated wheat α-amylases showed that SH groups were not present, a finding supported by noting that SH inhibitors had only a slight effect on the enzymatic activity. As there are known to be α-amylases containing SH groups (32,33) it is seen that α-amylases form a very large family of different enzymes not only varying in amino acid composition but also having or lacking SH groups.

From the amino acid composition the average specific volume of α-amylase was calculated to be 0.730 cc. per g. (Table IV). The latter value is similar to values usually obtained for proteins having an albumin-type of amino acid composition.

It perhaps should be emphasized that the data in Table IV are based on the averages of data for the four isoenzymes in Table III. The averaged data are convenient to use in calculating the specific volume of the α-amylase since the minor differences in the amino acid compositions do not result in significant changes in the specific volume, and the "average" amino acid composition possesses sufficient accuracy to serve as useful comparison with amino acid compositions of other α- or β-amylases, and to give an estimate of the integral number of amino acid residues. Table IV also is useful in estimating the amino acid composition differences of the individual isoenzymes. The most obvious differences are in the number of arginine residues and perhaps in methionine residues. The differences in cysteine content are probably not significant—a view which will need to be verified when larger amounts of the pure isoenzymes are available for additional amino acid studies. The true amino acid composition of the individual isoenzymes can probably only be obtained from total amino acid sequence studies, which only will be possible when very large amounts (approximately 100 to 1,000 mg.) of the individual, pure isoenzymes are available.

The integral number of amino acid residues for wheat α-amylase with an "average" amino acid composition is given in Table IV. The data show that there are 47 residues of aspartic and glutamic acid compared to 49 residues of lysine, histidine, and arginine, giving an excess of two cationic groups. As wheat α-amylase behaves like a slightly acidic protein, with an isoelectric point of approximately 6.1 to 6.2, perhaps several cationic residues are buried in the interior of the molecule.

The four α-amylase components gave similar pH-activity profiles, with pH maximums at 5.5 to 5.6. Compared with wheat β-amylases (8), the α-amylases have a higher pH optimum by one pH unit, and a narrower pH-activity profile. Thus, by measuring the activities at pH 3.4 where α-amylase has virtually no activity while β-amylase retains about 30% of its activity, it is possible to distinguish between wheat α- and β-amylase activities. The optimum activity of wheat α-amylase at pH 5.5 to 5.6 is in reasonable agreement with the value of 5.0 by Greenwood and Milne (24) for partially purified solutions of wheat α-amylases, particularly considering that different buffers were used, as sometimes enzymes show specific ion effects (Greenwood and Milne used phosphate-acetate, while we used barbital-acetate buffers).

The effect of temperature on rate of hydrolysis by wheat α-amylase was measured over a range of 20° to 74°C. An Arrhenius plot of the results obtained from the glycogen-complex mixture of α-amylases showed a linear relationship from 20° to 60°C., with an E_a value of 8.71 kcal. $mole^{-1} deg.^{-1}$ (Fig. 10). Experiments with individual components gave E_a values of 7.01 to 9.28, for the 20°-to-45°C. temperature range.

It was noticed that the activity rates of the individual α-amylases started to decrease at approximately 45° to 50°C., while the glycogen-complex mixture rate increased linearly to 60° to 62°C. This finding indicates that the glycogen-complex mixture may be more stable than the individual components. Greenwood and Milne (24) report values of 11 and 14 for E_a at 25° and 9°C. With some exceptions, most literature reports for plant α-amylase E_a values are in the range of 7.1 to 8.6 (Table VII).

Enzymatic activity measurements carried out at various temperatures (Fig. 4) indicate that purified wheat α-amylase is stable up to 60°C. and starts denaturing at 70°C. However, even at approximately 70°C. about 50% of the activity is still retained after 15 min. Thus in bread where there are higher concentrations of other proteins present to stabilize the α-amylase, it is apparent that before most of the enzyme is inactivated it will have caused considerable degradation of starch in the time taken for the temperature of a dough to rise in the oven. Accordingly it is expected, as claimed by Walden (38), that the presence of α-amylase in a dough should have a significant effect on its bread-baking properties.

The specific activity of wheat α-amylase components I, II, III, and IV was found to be equivalent to 1,480, 1,300, 1,510, and 1,570 μmole of maltose produced every min. at 25°C. per mg. enzyme. When one tries to estimate the weight of an anhydrous, salt-free protein in dealing with 1- to 2-mg. amounts of Ca^{++}-containing enzyme, accurate specific activities are difficult to obtain; and it is not known whether the differences in specific activity are real, or merely reflect experimental error.

The method used to determine the enzymatic activity was different from that described in our previously published study on the isolation of wheat α-amylase (1). In the previous study, the starch concentration was 0.5% at pH 4.6 (the publication

states that a 1% starch solution was used which is misleading since actually 1 ml. of enzyme solution was added to 1 ml. of 1% starch); the incubation temperature was 20°C.; and a monitoring wavelength of 525 nm. was used (1). In the present study, activity was determined using 0.91% starch at pH 5.5 (0.1 ml. enzyme solution added to 1 ml. 1% starch) containing 0.1% gelatin or bovine serum albumin, with an incubation temperature of 25°C. The amounts of reducing sugars were measured by monitoring the reduced product of 2,4-dinitrosalicylic acid at 497 nm. In the present work, also, the incubation pH was changed to the optimum pH for enzymatic activity of wheat α-amylase; an incubation temperature of 25°C. was chosen due to the convenience of maintaining water baths at this temperature in laboratories which are rarely cooler than 21° to 23°C.; the monitoring wavelength was changed to 497 nm. because this is the maximum absorption of reduced 2,4-dinitrosalicylic acid in agreement with the findings of Hostettler et al. (39) and Clayton and Meredith (40). For this reason literature recommendations for wavelengths of 505 nm. (41), 525 nm. (40), and 540 nm. (42), or the use of broad-band-width filters (43), were not followed. The addition of 0.1% inert protein was found necessary to maintain stability during activity measurements of extremely dilute solutions of highly purified enzyme.

It should be noted that in the use of 3,5-dinitrosalicylate to measure α-amylase activity it is necessary to understand its properties and behavior. This reagent measures the reducing capacity of the various oligosaccharides formed by an α-amylase action on starch (44); and the capacity varies with the various oligosaccharides (45) as has also been noted for a related reagent 3,4-dinitrobenzoic acid (46). The reagent is quite thermochromatic (47), so for accurate measurements the temperature of the test solutions must be kept constant. The maximum absorption wavelength of the reduced reagent lies fairly close to the absorption of the reagent itself, so a high-quality spectrophotometer will give a more sensitive calibration curve. Considering the above factors it is clear that 3,5-dinitrosalicylate is not an ideal reagent to measure α-amylase activity; while the use of it appears to be quite simple, careful attention to detail is necessary in its use.

Taking into consideration the factors described above plus the influence of substrate concentration, it was calculated that the average specific activity of 1,465 units (μmole maltose per min. per mg. protein at 25°C.) obtained in the present study is approximately equivalent to 1,080 units used in the previous study. As previously an average specific activity of 618 was obtained, it is seen that there is an apparent increase in specific activity of 75%. As higher α-amylase purity can only account for 1 to 4% of this increase, part of the increase is due to carrying out the activity measurements under more stable conditions (addition of 0.1% of bovine serum albumin), and possibly, a larger portion due to the larger amount or different type of amylopectin present in the current lot of soluble starch used as a substrate, since preliminary studies indicate a great dependence of specific activity on the amount of amylopectin present in the substrate.

Sorghum malt α-amylase appears to be the only other plant α-amylase which has been highly purified and had some of its properties determined (31). The wheat and sorghum α-amylases are similar in that four isoenzymes appear to be present; both have a MW of approximately 50,000; both have similar amino acid compositions (cysteine and tryptophan content was not determined for the sorghum enzyme). The reported specific activity of 10,304 μm. maltose per min. at 30° per mg. N of

the sorghum α-amylase using 2% starch as substrate (31) is approximately equivalent to 1,185 of the units used in the present study. Thus, the specific activity of the sorghum α-amylase is also quite similar to the average value of 1,465 units obtained for wheat α-amylases. However, wheat α-amylase does not seem to contain any carbohydrate, whereas the sorghum enzyme has 2% carbohydrate; and the N content, pH optimums, specific volume, and energy of activation are 16.8 to 17.2%, pH 5.5 to 5.6, 0.73 ml. per g., and 8.71 kcal. $mole^{-1}$ $deg.^{-1}$ for the wheat enzyme, whereas the sorghum values are 14.9 to 15.0%, pH 4.5 to 5.0, 0.68 ml. per g.[3], and 12.0 kcal. $mole^{-1}$ $deg.^{-1}$.

The four α-amylase isoenzymes found in malted wheat will need to be further examined for their enzymatic properties, substrate specificity, heat stability, and behavior towards various reagents. It will be of much interest to see whether the isoenzymes possess different enzymatic properties, and also to determine whether these differences, if found, are of any practical significance to dough and bread properties. In this examination, consideration should also be given to the report by Meredith and Jenkins (48) that field-sprouted New Zealand wheat can contain approximately 2% of a starch-thinning enzyme with a higher MW than that found for the α-amylases in malted wheat.

Acknowledgments

The authors thank W. Bushuk, Department of Plant Science, University of Manitoba, for use of a Beckman Model E ultracentrifuge; W. O. S. Meredith, A. W. MacGregor, and D. E. LaBerge of this laboratory, for malting wheat samples, carrying out SDS gel electrophoresis, and Bio-Gel chromatography; and W. MacRae and N. Turriff for technical assistance.

[3]The specific volume of 0.68 seems very low. The low value is perhaps due to a calculation error in the original paper (31) as the amino acid composition (31) indicates a similar value to the one calculated in the present paper.

Literature Cited

1. KRUGER, J. E., and TKACHUK, R. Wheat alpha-amylases. I. Isolation. Cereal Chem. 46: 219 (1969).
2. STRUMEYER, D. H., and ROMANO, A. T. Automated assay of submicrogram levels of amylase by a reducing sugar procedure. In: Automation in analytical chemistry; Vol. I, p. 469. Mediad: New York (1967).
3. MICHAELIS, L. Der Acetat-Veronal-Puffer. Biochem. Z. 234: 139 (1931).
4. FRENCH, D., and ABDULLAH, M. Transferase activity in malt amylase preparations. Cereal Chem. 43: 555 (1966).
5. DAVIS, B. J. Disc electrophoresis. II. Methods and applications to human serum proteins. Ann. N.Y. Acad. Sci. 121: 404 (1964).
6. KRUGER, J. E. Changes in the amylases of hard red spring wheat during germination. Cereal Chem. 49: 391 (1972).
7. MILLER, G. L., and GOLDER, R. H. Buffers of pH 2 to 12 for use in electrophoresis. Arch. Biochem. 29: 420 (1950).
8. TKACHUK, R., and TIPPLES, K. H. Wheat beta-amylases. II. Characterization. Cereal Chem. 43: 62 (1966).
9. VESTERBERG, O., and SVENSSON, H. Isoelectric fractionation, analysis and characterization of ampholytes in natural pH gradients. Further studies on resolving power in connection with separation of myoglobins. Acta Chem. Scand. 20: 820 (1966).
10. WEBER, K., and OSBORN, M. The reliability of molecular weight determinations by dodecyl sulphate-polyacrylamide gel electrophoresis. J. Biol. Chem. 244: 4406 (1969).
11. SPACKMAN, D. H., STEIN, W. H., and MOORE, S. Automatic recording apparatus for use in the chromatography of amino acids. Anal. Chem. 30: 1190 (1958).

12. MILLER, E. L. Determination of the tryptophan content of feeding-stuffs with particular reference to cereals. J. Sci. Food Agr. 18: 381 (1967).
13. BENCZE, W. L., and SCHMID, K. Determination of tyrosine and tryptophan in proteins. Anal. Chem. 29: 1193 (1957).
14. SCHRAM, E., MOORE, S., and BIGWOOD, E. J. Chromatographic determination of cystine as cysteic acid. Biochem. J. 57: 33 (1954).
15. TKACHUK, R. A continuous flow scintillation detector for C^{14}-containing aqueous solutions. Can. J. Chem. 40: 2348 (1962).
16. NOLTMANN, E. A., MAHOWALD, T. A., and KUBY, S. A. Studies on adenosine triphosphate transphosphorylases. II. Amino acid composition of adenosine triphosphate-creatine trans-phosphorylase. J. Biol. Chem. 237: 1146 (1962).
17. SCHILLING, E. D., BURCHILL, P. I., COFFMAN, J. R., and CLAYTON, R. A. Reaction of ninhydrin with keto acids. Biochem. Biophys. Res. Commun. 5: 217 (1961).
18. DUBOIS, M., GILLES, K. A., HAMILTON, J. K., REBERS, P. A., and SMITH, F. Colorimetric method for determination of sugars and related substances. Anal. Chem. 28: 350 (1956).
19. CHERVENKA, C. H. In: A manual of methods for the analytical ultracentrifuge, p. 23. Beckman Instruments, Inc., Spinco Division: Palo Alto, Calif. (1969).
20. LONGSWORTH, L. G. Diffusion measurements, at 1°, of aqueous solutions of amino acids, peptides and sugars. J. Amer. Chem. Soc. 74: 4155 (1952).
21. SCHACHMAN, H. K. Ultracentrifugation, diffusion, and viscosity. In: Methods in enzymology, ed. by S. O. Colowick and N. O. Kaplan; Vol. IV, p. 32. Academic Press: New York (1957).
22. U. S. DEPARTMENT OF COMMERCE, NATIONAL BUREAU OF STANDARDS. Tables of the error function and its derivative. Applied Mathematics Series 41 (1943).
23. COHN, E. J., and EDSALL, J. T. Density and apparent specific volume of proteins. In: Proteins, amino acids, and peptides, ed. by E. J. Cohn and J. T. Edsall; p. 370. Reinhold: New York (1943).
24. GREENWOOD, C. T., and MILNE, E. A. Studies on starch-degrading enzymes. Part VII. Properties and action-pattern of the α-amylases from barley, oats, rye and wheat. Staerke 20: 101 (1968).
25. OLERED, R., and JÖNSSON, G. Electrophoretic studies of α-amylases in wheat. II. J. Sci. Food Agr. 21: 385 (1970).
26. DAUSSANT, J., and CORVAZIER, P. Biosynthesis and modification of α- and β-amylases in germinating wheat seeds. FEBS Letters 7: 191 (1970).
27. ALEXANDRESCU, V., and MIHAILESCU, F. Electrophoretic study of wheat amylases during the first growing stages. Rev. Roum. Biochim. 7: 191 (1970).
28. ROBYT, J. F., CHITTENDEN, C. G., and LEE, C. T. Structure and function of amylases. I. The subunit structure of porcine pancreatic α-amylase. Arch. Biochem. Biophys. 144: 160 (1971).
29. COZZONE, P., PASERO, L., BEAUPOIL, B., and MARCHIS-MOUREN, G. Characterization of porcine pancreatic isoamylases. Chemical and physical studies. Biochim. Biophys. Acta 207: 490 (1970).
30. MANNERS, D. J., and MARSHALL, J. J. Studies on carbohydrate metabolising enzymes. XXVIII. Preparation and some properties of α-amylase isoenzymes from malted rye. Staerke 24: 3 (1972).
31. BOTES, D. P., JOUBERT, F. J., and NOVELLIE, L. Kaffircorn malting and brewing studies. XVII–Purification and properties of sorghum malt α-amylase. J. Sci. Food Agr. 18: 409 (1967).
32. THOMA, J. A., SPRADLIN, J. E., and DYGERT, S. Plant and animal amylases. In: The enzymes, ed. by P. D. Boyer; Vol. V, p. 115. Academic Press: New York (1971).
33. TAKAGI, T., TODA, H., and ISEMURA, T. Bacterial and mold amylases. In: The enzymes, ed. by P. D. Boyer; Vol. V, p. 235. Academic Press: New York (1971).
34. FISCHER, E. H., and STEIN, E. A. α-Amylases. In: The enzymes, ed. by P. D. Boyer, H. Lardy, and K. Myrback; Vol. 4, p. 313. Academic Press: New York (1960).
35. GREENWOOD, C. T., and MacGREGOR, A. W. The isolation of α-amylase from barley and malted barley, and a study of the properties and action-patterns of the enzymes. J. Inst. Brew. (London) 71: 405 (1965).
36. SWAIN, R. R., and DEKKER, E. E. Seed germination studies. I. Purification and properties

of an α-amylase from the cotyledons of germinating peas. Biochim. Biophys. Acta 122: 75 (1966).
37. GREENWOOD, C. T., MacGREGOR, A. W., and MILNE, E. A. The α-amylase from broad beans. Purification and properties. Arch. Biochem. Biophys. 112: 459 (1965).
38. WALDEN, C. C. The action of wheat amylases on starch under conditions of time and temperature as they exist during baking. Cereal Chem. 32: 421 (1955).
39. HOSTETTLER, F., BOREL, E., and DEUEL, H. Ueber die Reduktion der 3,5-Dinitrosalicylsaeure durch Zucker. Helv. Chim. Acta 34: 2132 (1959).
40. CLAYTON, J. W., and MEREDITH, W. O. S. The effect of thiols on the dinitrosalicylic acid test for reducing sugars. J. Inst. Brew. (London) 72: 537 (1966).
41. BENDELOW, V. M. Modified procedure for the determination of diastatic activity and α-amylase activity. J. Inst. Brew. (London) 69: 467 (1963).
42. NIKU-PAAVOLA, M.-L., and NUMMI, M. A new amylolytic enzyme. Acta Chem. Scand. 25: 1492 (1971).
43. BERNFELD, P. Amylases, alpha and beta. In: Methods in enzymology, ed. by S. P. Colowick and N. O. Kaplan; Vol. I, p. 149. Academic Press: New York (1955).
44. SUMNER, J. B. The estimation of sugar in diabetic urine, using dinitrosalicylic acid. J. Biol. Chem. 62: 287 (1924).
45. ROBYT, J. F., and WHELAN, W. J. Anomalous reduction of alkaline 3,5-dinitrosalicylate by oligosaccharides and its bearing on amylase studies. Biochem. J. 95: 10P (1965).
46. BOREL, E. von, and DEUEL, H. Quantitative Zuckerbestimmung mit 3,4-Dinitrobenzoesaeure. Helv. Chim. Acta 36: 801 (1953).
47. BOTTLE, R. T., and GILBERT, G. A. Thermochromism, a possible source of error in colorimetric analysis. Experiments with 3:5-dinitrosalicylic acid. Chem. Ind. (London) 575 (1956).
48. MEREDITH, P., and JENKINS, L. D. Amylase activity of sprout-damaged, malted, and immature wheat. Cereal Chem. 50: 240 (1973).

[Received April 17, 1973. Accepted December 20, 1973]